Abstract. MicroRNA (miRNA/miR) dysregulation has been reported to be fundamental in the development and progression of cardiac hypertrophy and fibrosis. In the present study, miR-327 levels in fibroblasts were increased in response to cardiac hypertrophy induced by transverse aortic constriction with prominent cardiac fibrosis, particularly when compared with the levels in unstressed cardiomyocytes. In neonatal rat cardiac fibroblasts, induced expression of miR-327 upregulated fibrosis-associated gene expression and activated angiotensin II-induced differentiation into myofibroblasts, as assessed via α-smooth muscle actin staining. By contrast, miR-327 knockdown mitigated angiotensin II-induced differentiation. Cardiac fibroblast proliferation was not affected under either condition. In a mouse model subjected to transverse aortic constriction, miR-327 knockdown through tail-vein injection reduced the development of cardiac fibrosis and ventricular dysfunction. miR-327 was demonstrated to target integrin β3 and diminish the activation of cardiac fibroblasts. Thus, the present study supports the use of miR-327 as a therapeutic target in the reduction of cardiac fibrosis.

Introduction

Mechanical and chemical stimuli, including changes in pressure and changes in the levels of cytokines, hormones or growth factors, trigger cardiac hypertrophy, which subsequently leads to cardiomyopathy and heart failure (1,2). Despite improvements in treatment strategies and the current understanding of the pathology, heart failure continues to be the leading cause of mortality worldwide. Pathological hypertrophy, as well as other types of cardiac injury, may trigger cardiac fibrosis, which in turn leads to adverse ventricular remodeling and heart failure (3-5). Therefore, identifying therapeutics that prevent the onset of pathological cardiac hypertrophy is of great interest.

Aberrant expression of microRNAs (miRNAs/miRs), small noncoding RNAs that regulate gene expression at the post-transcriptional level (6-8), serves a distinct role in disease pathology by disrupting signaling cascades and gene-regulatory networks. For instance, miRNA has been linked to adverse cardiac remodeling and fibrosis (4,9-11). The current understanding of the exact role of miRNA in cardiac hypertrophy has been increased by studies using mouse models coupled with gain- and loss-of-function strategies (12,13).

In a previous study, through the use of miRNA arrays, it was demonstrated that miR-327 levels were enhanced in mouse left ventricle (LV) tissues following myocardial infarction (MI), suggesting that miR-327 may be involved in cardiac hypertrophy and fibrosis induced by pressure overload (3). In the present study, it was demonstrated that miR-327 expression was increased in response to transverse aortic constriction (TAC) in the LV of mice. Angiotensin II-induced differentiation of cardiac fibroblasts into myofibroblasts was increased by miR-327 overexpression. By contrast, miR-327 knockdown inhibited angiotensin II-induced differentiation. In the mouse model, downregulation of miR-327 using antagoniR prevented pressure overload-induced hypertrophy and fibrosis. In addition, integrin β3 (ITGB3) was identified as a target gene of miR-327. The present study suggests that cardiac fibrosis is promoted by miR-327; therefore, therapeutics aimed towards inhibiting miR-327 may be effective treatments for cardiac fibrosis.
Materials and methods

Antibodies and reagents. Antibodies targeting GAPDH (cat. no. 5174), phosphorylated extracellular signal-regulated protein kinases 1/2 (p-ERK1/2, cat. no. 4370), p-p38 (cat. no. 4511), p-c-Jun N-terminal protein kinase (p-JNK, cat. no. 9251), matrix metalloproteinase-9 (MMP-9, cat. no. 13667) and proliferating cell nuclear antigen (PCNA, cat. no. 13110) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Collagen type I α1 (Coll1a1, cat. no. ab34710) and α-smooth muscle actin (α-SMA, cat. no. ab32575) were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies targeting rabbit and mouse were from Cell Signaling Technology, Inc. Alexa Fluor 488-conjugated streptavidin antibody (cat. no. 11545-003) was from Jackson ImmunoResearch (West Grove, PA, USA) and angiotensin II was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). miR-327 agomiR (cat. no. miR4000561), antagoniR (cat. no. miR3000561) and scrambled controls (for control of agomiR, cat. no. miR4000561; for control of antagoniR, cat. no. miR4000561) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Ethics statement. The Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China) approved all animal protocols. As described below, a cardiac hypertrophy and heart failure model was developed using TAC surgery (14). All procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (no. 85-23; revised 1996) and the study protocol was approved by The Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University (Nanjing, China; nos. IACUC-1701020 and IACUC-14030149). The mice were used for TAC surgery, while the rats were subjected to isolation of cardiac fibroblasts for in vitro experiments.

Animals and TAC surgery. A total of 72 male C57BL/6 mice (age, 8 weeks; weight, 22-24 g) obtained from the model animal research center of Nanjing University (Nanjing, China) were stratified into groups of 6 animals each: Sham NC antagoniR, TAC NC antagoniR, Sham miR-327 antagoniR and TAC miR-327 antagoniR. Mice were maintained under appropriate barrier conditions under a 12-h light/dark cycle, constant temperature range from 20-22°C, humidity range from 50-60%, and received food and water ad libitum. TAC is the primary method for inducing cardiac hypertrophy in mice and rats (15). In brief, mice were deeply anesthetized with 2% isoflurane, maintained under 1.5-2.0% isoflurane and constricted using a 0.4-mm diameter constrictor. The sham surgery mice were subjected to the same procedure except for the tightening around the transverse aorta.

Animal treatment. To determine whether miR-327 inhibition prevents cardiac fibrosis in vivo, antagoniR, a 2’-O-methyl-5’-cholesterol-modified miR-327 inhibitor, or a scrambled control (aforementioned; Guangzhou RiboBio Co., Ltd.) was administered to the mice at 80 mg/kg body weight via tail-vein injection, daily for 3 days prior to TAC surgery (n=6 per group).

Cardiac imaging. Transthoracic, two-dimensional M-mode echocardiography was performed using a Vevo 770 high-resolution in vivo imaging system with a 30-MHz transducer (VisualSonics, Inc., Toronto, ON, Canada). Echocardiographic studies were performed at 28 h following TAC surgery. The LV mass, LV wall thickness, ejection fraction (EF) and percent of fractional shortening were calculated as previously described (16).

Isolation of neonatal rat cardiomyocytes and fibroblasts. Sprague Dawley rats (age, 1-3 days; weight, 5-6 g) obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were used to isolate cardiac fibroblasts. The ventricular tissue was finely minced and digested in buffer (37°C, with agitation, 30 min) containing trypsin (6 mg/ml, Sigma-Aldrich; Merck KGaA) and collagenase type II (4 mg/ml, Worthington Biochemical Corporation, Lakewood, NJ, USA) at a ratio of 3:2. The resultant cell suspensions were pelleted by centrifugation (room temperature, 120 g, 3 min), resuspended in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS; Sciencell Research Laboratories, Inc., San Diego, CA, USA), 5% horse serum (Hyclone; GE Healthcare, Little Chalfont, UK) and seeded into culture plates. Fibroblasts were allowed to attach for 2 h at 37°C and 5% CO2. The unattached cardiomyocytes were then plated in culture plates coated with 10 mg/ml gelatin (Sigma-Aldrich; Merck KGaA).

Cell transfection. Cardiac fibroblast experiments were performed in low-serum (1% FBS) medium. At passage 3, cardiac fibroblasts (5x104/ml) were transfected with miRNA agomiR (100 nM), antagoniR (200 nM) or the scrambled controls (100 or 200 nM) for 48 h at 37°C. Subsequently, cells were treated with 1 µM angiotensin II (Sigma-Aldrich; Merck KGaA) for 24 h at 37°C.

Immunohistochemistry. At 4 weeks following TAC surgery, mice were deeply anesthetized with 2% isoflurane, maintained at 1.5-2% isoflurane and intubated using a volume-cycled ventilator. The mice were perfused with PBS, followed by 4% buffered paraformaldehyde. The hearts were isolated, subjected to PBS perfusion and formalin-fixed overnight. The head and apex of the heart were divided, tissues were embedded in paraffin blocks and tissue sections (n=20-25 per fragment was aligned parallel to the transverse aorta, and the suture was loosely tightened around the transverse aorta twice. The two knots were tied against the needle in rapid succession. Following removal of the needle, a 0.4-mm diameter constriction remained. The sham surgery mice were subjected to the same procedure except for the tightening around the transverse aorta.
Neonatal rat cardiac fibroblasts (NRCF) were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 10 min. NRCF were blocked with 5% normal antibody and incubated with α-SMA primary antibody (1:500 dilution) overnight at 4°C, followed by fluorochrome-conjugated secondary antibodies (1:500 dilution) for 1 h at room temperature. DAPI was used to counterstain nuclei. Images were captured with a Carl Zeiss Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany).

Cell proliferation assay. Cell proliferation was measured in real-time using the xCELLigence system (Roche Applied Science, Penzberg, Germany) (18). This system measures electrical impedance caused by adherent cells to evaluate proliferation in real time (19). Cells were seeded at 2,000 cells per well and allowed to attach for 12 h. The cell number, and therefore cell proliferation, was correlated with the relative change in impedance when measurements were made with and without cells through a unit-less parameter referred to as the Cell Index (20). The Cell Index at each time-point (0, 1, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60 and 66 h) was normalized to the value recorded at time-point 0/baseline, which represented as a fold-change relative to the control. Primers used for the amplification were as follows: Rat COL1 forward, 5′-CCC AAGGAAAAGAACGACGT-3′ and reverse, 5′-AGGTCA GCTTGATAGGGCATC-3′; rat α-SMA forward, 5′-GTC CCAGACATCATGGGAT-3′ and reverse, 5′-TCGGAT ACTTCCAGCTCAAGG-3′; rat MMP9 forward, 5′-CCTCTG CATGAAGACCAT-3′ and reverse, 5′-GAGGGTCAAGTG GACACATA-3′; rat GAPDH forward, 5′-GGCCAGTCAAG GTCAAGATG-3′ and reverse, 5′-GGAAGTCAGTCAA GCCAAGT-3′; mouse COL1 forward, 5′-GCTCTCCTTATGGGCCC AG-3′ and reverse, 5′-ATGGGACCCCTTGGCAAGCT-3′; mouse COL3 forward, 5′-CTGTAACATCGGAACCTGGAAGAA-3′ and reverse, 5′-CCATGCTGAACTCAGGAGA-3′; mouse GAPDH forward, 5′-TTGGTGTTATTCCTCCCCTCTGGG-3′ and reverse, 5′-ATGGCGTGTGACCCATGGG-3′; mouse GAPDH forward, 5′-TTGGTGTTATTCCTCCCCTCTGGG-3′ and reverse, 5′-ATGGCGTGTGACCCATGGG-3′.

For quantitative miRNA analysis, the Bulge-Loop™ miRNA qPCR Primer Set (Guangzhou RiboBio, Guangzhou, Guangdong, China) was used with the Takara SYBR Premix Ex Taq™ (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) on an ABI-7900 Real-Time PCR Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). A cDNA library was generated via RT using 20 µl reaction system: 4 µl 5′ reaction buffer, 1 µl RNA sample, 8 µl RNase-free water, 2 µl dNTPs, 2 µl enzyme inhibitor, and 0.5 µl MMLV reverse transcriptase (Takara Bio, Inc.) and 2.5 µl specific RT primers of miRNA (Guangzhou RiboBio Co., Ltd.). The RT conditions were as follows: 42°C for 60 min followed by 95°C for 5 min, then immediate cooling to 4°C. Primers used for the amplification of miR-327 were as follows: mmu-miR-327-5′-ACUUUG GGGGCAUGGGAU-3′; rno-miR-327-5′-CCUUAGGG GCAUAGGGGU-3′; U6 (cat. no. ssD0904071008, Guangzhou RiboBio Co., Ltd.) was used as an internal control for miRNA template normalization. The PCR conditions were as follows:
95°C for 20 sec, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 70°C for 10 sec. The ΔΔCq method was used for analysis (21).

Statistical analysis. Values are expressed as the mean ± standard error. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was determined using Student’s t-test or one-way analysis of variance with Bonferroni’s post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-327 expression is upregulated in fibrotic cardiac tissues. By using RT-qPCR analysis, a series of candidate miRNAs in the heart tissue of mice at 4 weeks were measured following TAC surgery. These candidate genes were the top 14 dysregulated miRNAs identified by miRNA arrays of post-MI ventricles with prominent fibrosis performed in a previous study (3). In the present study, miR-327 was identified as the highest upregulated miRNA in fibrotic heart tissue (Fig. 1). To confirm the cellular expression of miR-327, its expression was...
examined in isolated neonatal rat cardiomyocytes and cardiac fibroblasts. The expression of miR-327 in cardiac fibroblasts was higher compared with that in the unstressed cardiomyocytes (Fig. 2A).

Differentiation of cardiac fibroblasts in vitro is attenuated by miR-327 inhibition. The development of myofibroblasts from fibroblasts is a critical step in the pathogenesis of cardiac fibrosis. In vitro experiments were used to improve the current...
understanding of the role that miR-327 serves in regulating fibrosis. AgomiR-stimulated overexpression of miR-327 promoted angiotensin II-induced cardiac fibroblast differentiation, as indicated by the increase in α-SMA expression and staining (Fig. 2B-D). The protein expression of MMP-9 and Col1a1 was increased (Fig. 2E and F); however, cardiac fibroblast proliferation was not affected (Fig. 2G). By contrast, antagomiR-induced downregulation of miR-327 decreased cardiac fibroblast differentiation, although the proliferation remained unaffected (Fig. 2G-J). These results suggested that, in vitro, cardiac fibroblast differentiation into myofibroblasts was attenuated by miR-327 inhibition.

Cardiac hypertrophy and pressure overload-induced fibrosis are prevented by miR-327 inhibition. To investigate the effect of miR-327 on stressed hearts, miR-327 antagomiR was administered daily by tail-vein injection to mice for 3 days prior to TAC surgery. At 4 weeks following surgery, the loss of miR-327 in the heart was confirmed by RT-qPCR (Fig. 3A). Examination of the appearance of the hearts and the heart weight normalized to either body weight or tibia length demonstrated that miR-327 antagomiR administration prevented TAC-induced increases in LV volume and mass (Fig. 3B and C). Echocardiography revealed significantly improved EF, LV mass and LV posterior wall thickness in miR-327 antagomiR-treated mice compared with those in the control mice (Fig. 3D and E). miR-327 antagomiR-treated mice also possessed decreased cardiac chamber sizes and cardiomyocyte cross-sectional areas (Fig. 3F). The mitogen-activated protein kinase (MAPK) cascade is a central signaling cascade known to promote the development of cardiac hypertrophy (22). To investigate the antihypertrophic effects of miR-327 antagoniR, the protein levels of activated MAPK members, including p-JNK, p-p38, and p-ERK1/2 were analyzed. Downregulation of miR-327 by antagoniR prevented phosphorylation of the MAPK proteins, which is typically caused by TAC (Fig. 3G and H). Finally, the mRNA levels of known pathological cardiac fibrosis markers, including TGF-β, Col1a1 and Col3a1, were observed to be decreased in miR-327 antagoniR-treated mice (Fig. 3I).

Discussion

Myocardial fibrosis is a frequent hallmark of cardiomyopathy (4). Although its original cause is known to be part of a physiological process, sustained stress induces pathological hypertrophy. In addition, interstitial fibrosis increases the stiffness of the myocardium. Subsequently, diastolic dysfunction-induced global cardiac remodeling leads to dilated cardiomyopathy and heart failure (23,24). Despite the devastating nature of cardiac fibrosis, effective treatment strategies currently are not available. Thus, with regard to the treatment of cardiac dysfunction and heart failure, identification of therapeutic targets is of importance. Aberrant miRNA expression has been linked to cardiac fibrosis and heart failure (9,25); Therefore, miRNAs have emerged as novel therapeutic targets for the treatment of fibrotic changes (6,26-28).

miRNAs are endogenous, non-coding small RNA molecules. miRs are recruited to the RNA-induced silencing complex and regulate target genes through diverse mechanisms (29). The importance of miRNAs in numerous cellular processes has been well documented. However, the significance of miRNAs in heart function, particularly with regard to cardiac fibrosis, has remained elusive. miR-122, miR-145, miR-134, miR-133a and miR-370 were reported to be associated with coronary artery disease, even following adjustment for other...

Figure 4. (A) Predicted miR-327 seed sequence matches the potential target gene mRNA. (B and C) miR-433 negatively regulated ITGB3 in neonatal cardiac fibroblasts (n=6). **P<0.01, ***P<0.001 vs. respective controls. ITGB3, integrin B3; miR, microRNA; UTR, untranslated region.
cardiovascular risk factors (30-33). Dysregulated miRNAs, particularly miR-21 and miR-29b, were previously indicated to contribute to cardiac fibrosis (34,35). Increased expression of miR-21 in fibroblasts promotes their proliferation, suggesting the important role of miR-21 in cardiac remodeling (36,37). By miRNA arrays, it was previously demonstrated that high levels miR-327 are associated with hypertrophic cardiac tissues (3).

In the present study investigated whether miR-327 serves a role in the development of pathological cardiac hypertrophy and fibrosis. Using in vivo and in vitro models, it was demonstrated that miR-327 antagoniR inhibited cardiac hypertrophy and fibrosis. Cardiac fibrosis occurs in numerous types of heart disease, including diabetic cardiomyopathy, MI, dilated cardiomyopathy, aortic stenosis and hypertrophic cardiomyopathy (38,39). In cardiac fibrosis, cardiac fibroblasts proliferate and transform into myofibroblasts (40). The present study demonstrated that cardiac fibroblast differentiation, which was enhanced by miR-327 overexpression, was attenuated by miR-327 inhibition. In addition, miR-327 levels were enhanced in cardiac fibroblasts compared with those in cardiomyocytes. Esposito et al (41) indicated that TAC-induced pressure overload is associated with the activation of MAPks (ERK1/2, p38 MAPK and JNK) in mice. Consistent with numerous animal studies, clinical studies reported that in failing human hypertrophic hearts, ERK1/2, JNK and p38 MAPK were significantly activated (42). In line with these results, MAPK activation has been demonstrated to be a key element directly enhancing cardiac remodeling (43,44). Through a bioinformatics analysis performed in the present study, ITGB3 was identified as a target gene for miR-327 in cardiac fibroblasts. ITGB3, which is an ITGB-chain subunit encoded by a serotonin-associated gene on chromosome 17, was reported to be linked with the risk of cancer, including colorectal cancer and acute myeloid leukemia (45,46). The ITGB3 family includes the subtypes αβ3 and αvβ3. The ITGB3 gene encodes the integrin β3 subunit and has been consistently identified as a quantitative locus for regulating serum levels of 5-hydroxytryptamin levels (47). In the present study, ITGB3 was inhibited by miR-327 antagoniR, suggesting that ITGB3 may be a target gene of miR-327 inducing differentiation of cardiac fibroblasts. However, the direct association between ITGB3 and MAPK and their functional roles in the regulation of cardiac fibrosis require further confirmation.

In summary, the present study suggested that cardiac fibrosis may increase miR-327 expression and that miR-327 antagoniR improves LV mass and attenuates cardiac fibrosis during cardiac hypertrophy. The protective effect of miR-327 inhibition against cardiac fibrosis was mediated through inhibition of TAC-induced increases in ERK1/2, p38 and JNK phosphorylation. ITGB3 may be a target gene of miR-327 that induces differentiation of cardiac fibroblasts. Thus, a previously unknown role for miR-327 in cardiac hypertrophy and fibrosis was identified in the present study, but this requires to be confirmed in future studies.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant nos. 81627802 and 81570247), the Natural Science Foundation of Jiangsu Province for Youth (grant no. BK20141024), the Six Talent Peaks project in Jiangsu Province (grant no. 2015-WSN-29) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Competing interests

The authors declare that they have no competing interests.

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