J Physiol Sci (2013) 63:31–38
DOI 10.1007/s12576-012-0230-y

ORIGINAL PAPER

Angiotensin II induced differentially expressed microRNAs in adult rat cardiac fibroblasts

Xiaoying Jiang · Qilan Ning · Juanli Wang

Received: 20 May 2012 / Accepted: 24 August 2012 / Published online: 25 September 2012

Abstract Angiotensin II (Ang II) plays a pivotal role in cardiac fibrosis, and microRNAs (miRNAs) have been shown to participate in diverse pathological processes. Our aim is to identify the Ang II-induced miRNAs in cardiac fibroblasts (CFs). The miRNA array was used to analyze the miRNA expression profile in CFs treated by Ang II and control cells. Stem-loop real-time PCR was performed to re-measure the levels of the differentially expressed miRNAs. Analysis of miRNA arrays showed that 33 miRNAs were differentially expressed (13 up- and 20 downregulated) in response to Ang II (100 nM) for 24 h as compared to control cells. Quantitative PCR revealed that Ang II upregulated the levels of miR-132, -125b-3p and miR-146b but downregulated the levels of miR-300-5p, -204* and miR-181b in CFs. The trend of miRNA change is consistent with microarray and qRT-PCR. Bioinformatic analysis revealed that MMP9 as the target of miR-132, MMP16 as the target of miR-146b and TIMP3 as the target of miR-181b have been listed in the miR database with experimentally validated targets, indicating the potential role of those miRNAs in cardiac fibrosis. Our results demonstrated that we did identify a subset of miRNAs that was differentially expressed in Ang II-treated CFs, which provide a starting point to explore their potential roles in cardiac fibrosis and hypertension.

X. Jiang (✉) · Q. Ning · J. Wang
Department of Genetics and Molecular Biology,
School of Medicine, Xi'an Jiaotong University,
76 Yanta West Road, Xi'an 710061, Shaanxi, China
e-mail: jiangxy@mail.xjtu.edu.cn

X. Jiang
NIHR Translational Research Facility in Respiratory Medicine Group, School of Translational Medicine, Stopford Building, University of Manchester, Manchester M13 9PT, UK

Keywords Angiotensin II · Cardiac fibroblasts · miRNA · Cardiac fibrosis

Introduction

Cardiac fibroblasts (CFs) are the most numerous cell type in the heart, accounting for approximately 70 % of the total cell number in the heart [1, 2]. Cardiac fibrosis is the excess accumulation of extracellular matrix (ECM) in the heart, which leads to the loss of normal cardiac function and is closely associated with numerous cardiovascular diseases, including hypertension, myocardial infarction and cardiomyopathy [3]. CFs play a pivotal role in the development of cardiac fibrosis through the synthesis of ECM proteins and the degradation of ECM by producing matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) [2, 3]. CFs also secrete a variety of cytokines that can regulate the function of CFs and cardiomyocytes and then regulate cardiac remodeling [4]. Angiotensin II (Ang II) is considered to be a major player in the pathogenesis of cardiac remodeling [5–7] and has been used to induce cardiac fibrosis through the stimulation of cell proliferation, ECM synthesis and cytokine secretion in CFs [8–10]. It is well known that most effects of Ang II are mainly mediated via Ang II receptor type 1 [11]. At the present time, the molecular mechanisms underlying the actions of Ang II upon cardiac fibrosis are still not completely understood.

MicroRNAs (miRNAs) are an endogenous conserved class of small non-coding RNAs of 18–25 nucleotides that are generally believed to either block the translation or induce the degradation of target mRNA by binding to the untranslated region (3′-UTR) of target genes [12]. miRNAs have been shown to play fundamental roles in diverse biological and pathological processes [12, 13]. Reports have

indicated that miRNAs are also involved in the pathological mechanism in cardiac fibrosis [13–22]. It was reported that miR-21, which was enriched in CFs, contributed to cardiac fibrosis by enhancing extracellular regulated kinase signaling and increasing fibroblast MMP 2 [14, 15]. The miR-29 family was implicated in cardiac fibrosis through targeting the collagen genes in the border zone of myocardial infarction [16]. The miR-133 and miR-30 decreased the expression of connective tissue growth factor in cultured rat cardiomyocytes and fibroblasts [17]. Decreased miR-133 and miR-590 increased atrial fibrosis in a canine model of nicotine-induced atrial remodeling [18]. The transfection of premiR-25 and pre-miR-29a into CFs decreased the expression of collagen I and III [19]. Decreased miR-18 and miR-19 were closely linked to the increase of ECM proteins in aging-associated heart failure [20]. High mobility group box-1 protein injection into chronically failing heart decreased cardiac fibrosis, which is associated with increased miR-206 targeting inhibition of TIMP 3 [21]. MiR-24 attenuated cardiac fibrosis via a furin-TGF-beta in the border zone of myocardial infarction [22].

Although several miRNAs have been reported to be involved in cardiac fibrosis, no evidence is available that those miRNAs are regulated by Ang II in CFs. We also do not know whether there are unknown miRNAs that are regulated by Ang II in CFs. For those reasons, we investigated the expression profile of miRNAs using miRCURY^{TM} lock nucleic acid (LNA) expression arrays (Exiqon) in adult rat CFs treated with Ang II and control cells. Stem-loop real-time PCR was then performed to re-measure the levels of the differentially expressed miRNAs. Using the two methods, we found that Ang II did induce a set of miRNAs that was differentially expressed in adult rat CFs, with bioinformatic analysis suggesting that these miRNAs might participate in the molecular mechanism of cardiac fibrosis.

Methods

Materials and animals

Collagenase, trypsin and Ang II were obtained from Sigma Chemical (St. Louis, USA). Dulbecco's modified Eagle's medium (DMEM) and TRIzol were obtained from Life Technologies (Invitrogen, Carlsbad, CA, USA). The miRCURY^{TM} LNA expression arrays (v.16.0) were purchased from Exiqon (Denmark). The First Strand cDNA Synthesis kit was purchased from Fermentas (Burlington, ON, Canada). SYBR Premix Ex Taq^{TM} II was purchased from TaKaRa (Ohtsu, Shiga, Japan). Sprague-Dawley (SD) rats were supplied from the Experimental Animal Center of Xian Jiaotong University (China). The animal experiments were approved by the University Committee of Laboratory Animal Care and Use and followed the guidelines of the National Animal Research Center.

Isolation and culture cardiac fibroblasts

Cardiac ventricular fibroblasts were obtained from hearts of adult male SD rats weighing 250–300 g after anesthesia with 3 % pentobarbital sodium as described previously [23]. In brief, following rapid excision of the hearts, fibroblasts were prepared by enzymatic digestion with a collagenase/trypsin solution and a selective plating technique. After a 2-h period of attachment to uncoated culture plates, the cells, which were weakly attached or unattached, were rinsed free and discarded, and attached cells (mostly fibroblasts) were washed and grown in the plating medium at a density of 1×10^4 cells/cm^2. After 2 days, when cells in culture reached 80–90 % confluence, the cells were digested by trypsin and amplified in DMEM with 10 % FBS. The purity of these cultures was >96 % CFs as determined by positive staining for vimentin and negative staining for factor VIII. The CFs (passages 3–5) were grown to 80–90 % confluence and serum starved for 24 h before treatment.

Preparation of RNA

Following 24 h serum starvation, adult rat CFs were treated with Ang II (100 nM) for 24 h, and then cells were harvested for RNA extraction using TRIzol according to manufacturer's instructions [24]. Briefly, cells were lysed in TRIzol (1 ml) prior to the addition of 200 μl chloroform. Following vigorously shaking for 10 s, samples were incubated on ice for 2–3 min, clarified by centrifugation (12,000*g* for 15 min at 4 °C) and the aqueous phase was transferred into a fresh tube. Then 1.5 volume of 100 % ethanol was added and mixed up and down. The mixture was transferred into an RNeasy Mini spin column and washed several times. The column was transferred to a new collection tube, and 25 μl RNase-free water was added to elute the RNA. The RNA quantity was determined spectrophotometrically on an A_{260} and A_{260}/A_{280} ratio using the NanoDrop 1000, and RNA quality was checked by electrophoresis on a 1.2 % agarose/formaldehyde gel. Isolated RNA was stored at −70 °C prior to gene array analysis and real-time polymerase chain reaction.

Microarray analysis of miRNA expression

To study the expression of miRNAs in CFs, we performed miRNA expression arrays in adult rat CFs as previously reported [25]. Briefly, RNAs from three pairs of control CFs and Ang II-treated CFs were extracted and mixed, respectively. RNA samples were labeled using the miRCURY^{TM} Hy3^{TM}/Hy5^{TM} Power Labeling kit (Exiqon) and hybridized on the miRCURY^{TM} LNA Array version.16.0 (Exiqon),

which contains more than 1,891 capture probes. Following the washing steps the slides were scanned using the Axon GenePix 4000B microarray scanner. Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged, and miRNAs with intensities $\geq$50 in all samples were chosen for calculating the normalization factor. Expressed data were normalized using the Median normalization. After normalization, differentially expressed miRNAs were identified through fold change filtering. Finally, hierarchical clustering was performed using MEV software (v4.6, TIGR) to show distinguishable miRNA expression profiling among samples.

Quantitative real-time PCR analysis of miRNA expression

To validate our finding from the miRNA arrays, we performed stem-loop real-time PCR to quantify the levels of several miRNAs (rno-miR-125b-3p, rno-miR-132, rno-miR-146b, rno-miR-300-5p, rno-miR-204*, rno-miR-181b). Briefly, total RNA from six pairs of control CFs and Ang II-treated CFs was extracted using TRIzol reagent. cDNAs were synthesized from total RNA by using the First Strand cDNA Synthesis kit with miRNA-specific primers (Table 1). The 20-μl reactions were incubated for 60 min at 42 °C, 5 min at 70 °C, and then stored at −20 °C. Quantitative PCR was performed using SYBR Premix Ex Taq™ II in the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and 2× PCR master mix (Superarray) in a 7900 Real-Time PCR machine (Applied Biosystems). PCR reactions were performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The specificity of PCR products was assessed by melting curve analysis. The primer sequences for qPCR are shown in Table 1. U6 small nuclear RNA was used as an internal control to normalize miRNA. Relative quantitation of miRNA expression was evaluated by the $2^{(-\Delta\Delta C_t)}$ methods for each miRNA compared with U6.

Bioinformatic analysis and target prediction

Four online software programs, TargetScan (http://www.targetscan.org), miRBase (http://www.mirbase.org/), miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/index.html) and miRWalk (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/), were used for bioinformatic analysis and target prediction of miRNAs. The miRWalk and miRTarBase databases, which target genes of miRNAs, have been experimentally validated.

Statistical analysis

Quantitative data were presented as the mean ± SEM. Student's t test was used to evaluate data between two groups. $P < 0.05$ was considered statistically significant.

Table 1 The sequences of miRNA primer

miRNA	Sequences
miR-125b-3p	RT primer: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTCC-3′
	PCR primer F: 5′-CGGCACGGGTTAGGCTCTTG-3′
	PCR primer R: 5′-GTGCAGGGTCCGAGGT-3′
miR-132	RT primer: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGACCA-3′
	PCR primer F: 5′-GCCGCTAACAGTCTACAGCCAT-3′
	PCR primer R: 5′-GTGCAGGGTCCGA GGT-3′
miR-146b	RT primer: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGCC-3′
	PCR primer F: 5′-CGGC TGAGAACTGAATTCCATAGG-3′
	PCR primer R: 5′-GTGCAGGGTCCGAGGT-3′
miR-300-5p	RT primer: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACAAAG-3′
	PCR primer F: 5′-CAGTGCGTGTCGTGGAG-3′
	PCR primer R: 5′-CAGTGCGTGTCGTGGAG-3′
miR-181b	RT primer: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACCCACCG-3′
	PCR primer F: 5′-GGGAACATTCATTGCTG-3′
	PCR primer R: 5′-TGCGTGTCGTGGAGTC-3′
miR-204*	RT primer: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAACGTC-3′
	PCR primer F: 5′-GCTGGGAAGGCAAAGG-3′
	PCR primer R: 5′-CAGTGCGTGTCGTGGAG-3′
U6	PCR primer F: 5′-CTCGCTTCGGCAGCACA-3′
	PCR primer R: 5′-AACGCTTCACGAATTTGCGT-3′

Table 2 Upregulated miRNAs in cardiac fibroblasts induced by angiotensin II

Probe ID	MicroRNA	Regulation	Fold change
10306	rno-miR-146b	Up	1.91
10937	rno-miR-132	Up	2.59
10986	rno-miR-193	Up	1.86
11053	rno-miR-32	Up	1.52
17822	rno-miR-490*	Up	1.58
31867	rno-miR-145*	Up	1.59
42566	rno-miR-224	Up	2.09
42630	rno-miR-140*	Up	1.62
145746	rno-let-7i*	Up	2.17
145838	rno-miR-125b-3p	Up	2.30
145852	rno-miR-210	Up	1.79
145859	rno-miR-33	Up	1.87
148389	rno-miR-3571	Up	1.68

Table 3 Downregulated miRNAs in cardiac fibroblasts induced by angiotensin II

Probe ID	MicroRNA	Regulation	Fold change
10972	rno-miR-181b	Down	1.94
11215	rno-miR-292-3p	Down	1.95
17427	rno-miR-200c	Down	2.41
17624	rno-miR-532-5p	Down	1.5
27536	rno-miR-190	Down	2.21
28547	rno-miR-675	Down	1.54
42462	rno-miR-883*	Down	1.57
42502	rno-miR-204*	Down	13.13
42694	rno-miR-485*	Down	2.36
42702	rno-miR-30c-1*	Down	2.83
42739	rno-miR-339-5p	Down	1.88
42826	rno-miR-300-5p	Down	2.87
42866	rno-miR-451	Down	1.83
42881	rno-miR-463	Down	1.54
42902	rno-miR-185	Down	1.52
46251	rno-miR-1193-3p	Down	2.54
145640	rno-miR-328a	Down	2.06
148261	rno-miR-208*	Down	2.15
148271	rno-miR-328b-3p	Down	1.98
148584	rno-miR-3572	Down	1.55

Results

Differential expression of miRNAs in Ang II-induced cardiac fibroblasts

To examine the Ang II-induced miRNAs in CFs, we analyzed the miRNA profile in Ang II-treated CFs and control

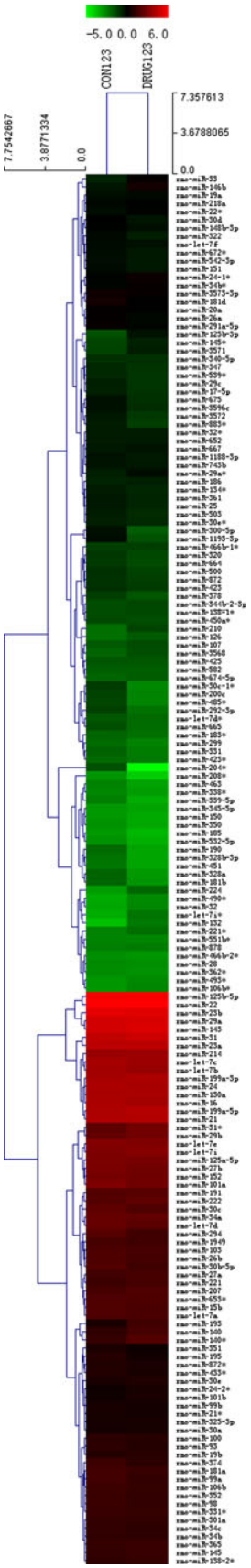

Fig. 1 Heat map presentation of the expression profile of miRNAs in angiotensin II-treated and control cardiac fibroblasts. "Red" indicates high relative expression, and "green" indicates low relative expression. *CON-123* indicates control cardiac fibroblasts. *DRUG-123* indicates cardiac fibroblasts treated by angiotensin II (100 nM) for 24-h (color figure online)

CFs with miRCURY^{TM} LNA Array version.16.0 (Exiqon, Denmark). These arrays contain more than 1,891 capture probes. The hybridization of the total RNA mixture from three pairs of Ang II-treated CFs and control CFs to miRNA arrays showed detectable expression of 678 mature rat miRNAs. The subsequent analysis of array data revealed that 33 miRNAs demonstrated ≥1.5-fold differential expression with 13 miRNAs being upregulated and 20 miRNAs being downregulated (Tables 2, 3). Remarkably, miR-125b-3p and miR-132 were upregulated 2.30- and 2.59-fold, respectively, in Ang II-treated CFs compared to control CFs. Conversely, miR-300-5p and miR-204* were downregulated 2.87- and 13.13-fold. The heat map and the alteration in the levels of specific miRNAs were shown in Fig. 1.

Quantitative real-time PCR analysis of miRNA expression

To confirm the finding obtained by analyzing the miRNA profiling, quantitative real-time PCR assay was performed on several miRNAs in six pairs of Ang II-treated and control CFs. Six miRNAs (rno-miR-132, -125b-3p, -146b, -300-5p, -204* and miR-181b) among 33 significantly altered miRNAs were chosen to be measured by qPCR. As shown in Fig. 2 and Tables 2 and 3, the results of qRT-PCR matched the microarray data closely. Quantitation by qRT-PCR revealed that the levels of miR-125b-3p and miR-132 in Ang II-treated cells were upregulated by 4.20-fold ($p < 0.0001$) and 3.15-fold ($p = 0.013$) (Fig. 2), respectively. The levels of these two miRNAs provided by array analysis were found to be 2.30- and 2.59-fold (Table 2) higher in Ang II-treated CFs as compared to control CFs. qPCR revealed a 2.28-fold ($p = 0.0454$) (Fig. 2) increase of miR-146b in Ang II-treated cells, while array analysis showed that miR-146b was increased 1.91-fold (Table 2) when stimulated by Ang II. While array analysis showed a 2.87- and 13.13-fold decrease (Table 3) in the levels of miR-300-5p and miR-204*, qPCR also revealed a 5.74-fold ($p < 0.0001$) and 3.94-fold ($p < 0.0001$) decrease (Fig. 2) in their levels. The analysis of miRNA arrays showed a 1.94-fold decrease of miR-181b (Table 3) in Ang II-treated cells, while qPCR revealed a 3.58-fold ($p < 0.0001$) decrease (Fig. 2). These results clearly demonstrate that the trend of miRNA changes was consistent with array hybridization and qPCR.

Bioinformatic analysis of the potential target genes

We searched for potential mRNA targets of the five miRNAs (miR-132, -125b-3p, -146b, -300-5p and miR-181b) differentially expressed in CFs that were confirmed by miRNA arrays and qPCR using the online software programs of four miRNA database, including TargetScan, miRase, miRWalk and miRTarBase. Among them, miRWalk and miRTar Base are the databases in which target genes of miRNAs have been experimentally validated in the published literature. Their predicted miRNA target genes are listed in Table 4. Most of these targets were involved in ECM regulation, cell cycle regulation, inflammation and apoptosis. For examples, the targets of miR-132 include MMP9, MMP14, MMP16, SPRY1, MAPK3, MAPK13 and CDC2a (Table 4). The targets of miR-146b include MMP16, TRAF6, IFAK1, KLF4 and KLF7 (Table 4). The targets of miR-181b include Col7a1, Col16a1, Integrin a2, a3, a6, b8, elastin, TIMP2, TIMP3, IL1a, IL6, TGFBR3, TGFa, MAP3K6 and MAP3K10 (Table 4). Importantly, MMP9 as the target of miR-132, MMP16 and TRAF6 as the targets of miR-146b, TGFBR1 as the target of miR-300-5p, and TIMP3 and IL1a as the targets of miR-181b have been listed in the miRNA database in which miRNA targets have been experimentally validated. This suggests that those miRNAs probably exert their roles via the tested target genes in CFs.

Discussion

Using miRNA arrays, we identified a number of differentially expressed miRNAs in Ang II-treated CFs. Six of these miRNAs (rno-miR-125b-3p, -132, -146b, -300-5p, -204* and rno-miR-181b) were confirmed by qRT-PCR in CFs. Most potential targets of the confirmed miRNAs were involved in ECM regulation (MMP and TIMP), cell cycle regulation (cyclin and MARK), inflammation (TRAF6, IRAK1) and apoptosis (caspase) according to the analysis using TargetScan and miRBase (Table 4). Importantly, MMP9 as the target of miR-132, MMP16 as the target of miR-146b and TIMP3 as the target of miR-181b have been listed in the miRTarBase and miRWalk with experimentally validated targets. Our results demonstrated that AngII did induce a new subset of differentially expressed miRNAs in CFs, which provide a starting point to explore their potential roles in cardiac fibrosis.

Currently, there has only been one report about miR-125b-3p, which was regulated by p53 in a transgenic mouse model of neuroblastoma [26]. Our results indicated that increased miR-125b-3p in Ang II-treated CFs probably negatively regulated the level of Ang II by targeting angiotensin-converting enzyme and regulated the matrix accumulation by MMP15 and cell proliferation by cell cycle targets (Table 4). Our results indicated that upregulated miR-132 in Ang II-treated CFs has potential targets including MMP9, MMP14, MMP16 and spry1 (Table 4). MMP9 as a miR-132 target has been confirmed in mammary stroma [27]. MiR-21 has been reported to regulate

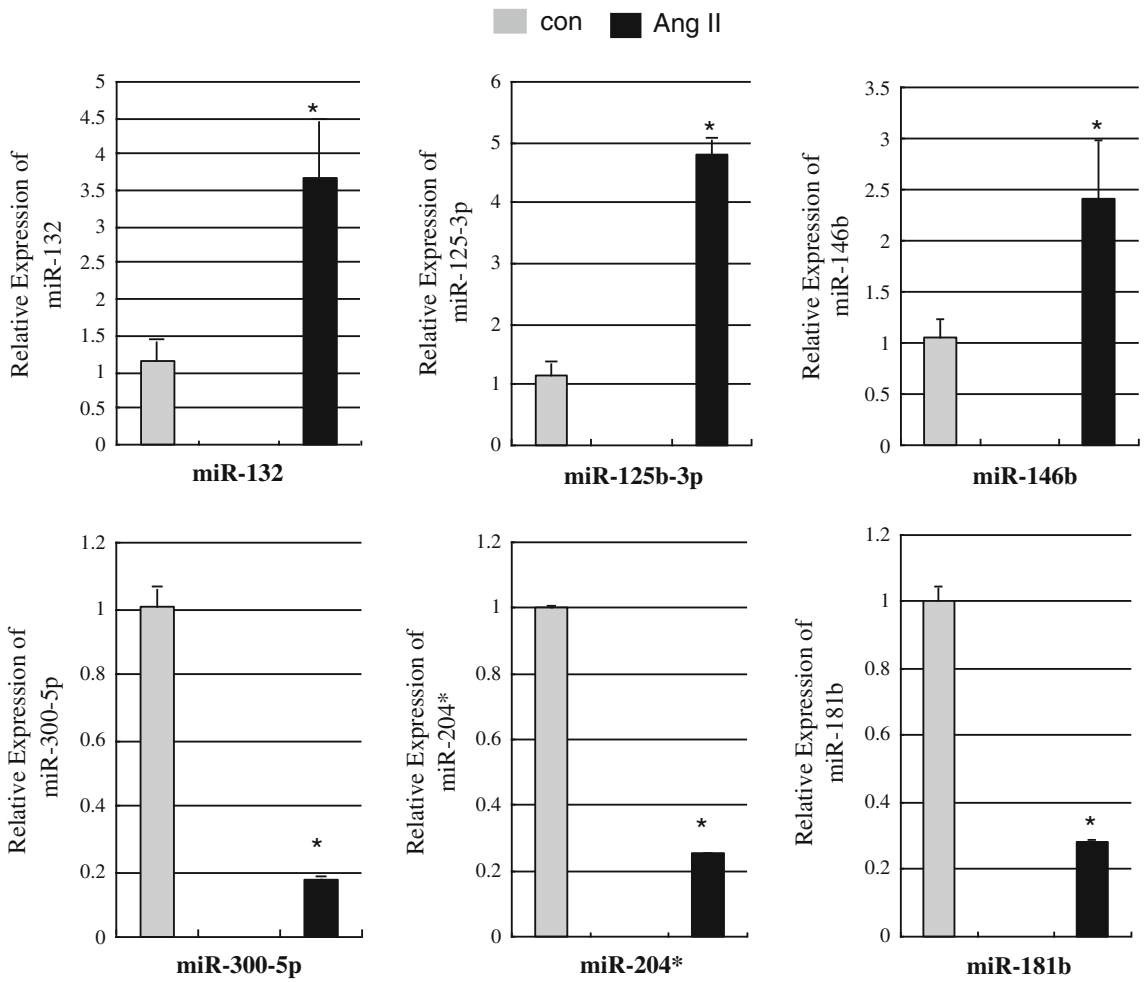

Fig. 2 Measurement of changes in microRNAs using quantitative real-time PCR. The expression levels of rno-miR-125b-3p, rno-miR-132, rno-miR-146b, rno-miR-300-5p, rno-miR-204* and rno-miR-181b in cardiac fibroblasts treated by angiotensin II-treated and non-treated cells were measured by qRT-PCR. Expression of miRNAs was normalized to endogenous U6 expression. Data are the mean ± SEM ($n = 6$). *$P < 0.05$ compared with the Ang II-non-treated group

Table 4 The potential target genes of differentially expressed miRNAs in cardiac fibroblasts

miRNA	Potential targets in TargetScan, miRBase	Experimentally validated targets in miRwalk, miRTarBase
miR-125b-3p	ACE, MMP15, MAPK1, MAP2K7, CCNM4, FGFR3, FGF15	
miR-132	MMP9, MMP14, MMP16, SPRY1, MAPK3, MAPK13, CDC2a	MMP9, MAP3K3
miR-146b	MMP16, TRAF6, IRAK1, KLF7, KLF4	MMP16, TRAF6, IRAK1
miR-300-5p	Col12a1, FSD1L, Integrin a3, caspase 8, CCND2, CCNB1, CCNK, CCNT2, CCNM4, CCNA2, CDK1	TGFBR1, caspase 8
miR-181b	Col7a1, Col16a1, Integrin a2, a3, a6, b8, elastin, TIMP2, TIMP3, IL1a, IL6, TGFBR3, TGFa, MAP3K6, MAP3K10	TIMP3, IL1a

ACE angiotensin-converting enzyme, *MMP* matrix metalloproteinases, *MAPK* mitogen-activated protein kinase, *FGFR3* fibroblast growth factor receptor 3, *FGF15* fibroblast growth factor 15, *SPRY1* sprouty homolog 1, *CDC2a* Cdk1(cyclin-dependent kinase 1), *TRAF6* TNF receptor-associated factor 6, *IRAK1* interleukin-1 receptor-associated kinase, *KLF7(4)* Kruppel-like factor 7 (4), *Col12a1* collagen, type XII, alpha 1, *CCND2* cyclin D2, *CCNB1* cyclin B1, *CCNK* cyclin K, *CCNT2* cyclin T2, *CCNM4* cyclin M4, *CCNA2* cyclin A2, *Col7a1* collagen, type VII, alpha 1, *Col16a1* collagen, type XVI, alpha 1, *IL1a* interleukin 1, alpha, *TGFBR3* transforming growth factor, beta receptor III, *TIMP* tissue inhibitors of matrix metalloproteinases

proliferation of CFs by spry1 [14]. This suggests that miR-132 might participate in cardiac fibrosis via its target MMPs and spry1. MiR-146b has been shown to be involved in glioma call migration and invasion by targeting MMP16 [28]. TRAF6 and IRAK1 as the targets of miR-146b have been reported [29]. Our results indicated that increased miR-146b has the potential targets of MMP16, TRAF6 and IRAK1 (Table 4). These suggest that miR-146b probably plays a role in cardiac fibrosis by MMP and inflammation cytokines. The potential targets of miR-300-5p include matrix molecules (Col12a1, FSD1L, Integrin a3), caspase 8 and several cyclins (CCND2, CCNB1, CCNK, etc.). This suggests that decreased miR-300-5p probably participates in CFs by increasing targets of matrix molecules to promote fibrosis and enhance cell proliferation by cyclins. TIMP3 has been reported to be a validated miR-181 target with luciferase reporter assay [30, 31]. In our study, miR-181b decreased in Ang II-treated CFs, and predicted potential targets include matrix molecules (Col7a1, Col16a1, integrin a2, a3, a6, b8, elastin), TIMP2, TIMP3, IL1a and IL6 (Table 4). This indicated that miR-181b might be an active miRNA in cardiac fibrosis.

Interestingly, a paper also reported several miRNAs induced by Ang II [32]. They found that five miRNAs (miR-29b, -129-3p, -132, -132* and -212) were upregulated in HEK293N cells overexpressing the AT1 receptor when treated by Ang II (100 nm) for 24 h. Moreover, the effects of Ang II on five miRNAs were confirmed in adult CFs [32]. Our studies showed that several miRNAs (miR-125b-3p, -132 and -146b) were upregulated, while several miRNAs (miR-300-5p, miR-204* and miR-181b) were downregulated in adult rat CFs when treated by Ang II (100 nm) for 24 h, which was verified by miRNA arrays and qRT-PCR. We speculated that the differences in the results perhaps come from the differences in cell types. After all, HEK293N cells overexpressing the AT1 receptor is not totally the same in adult rat CFs. Importantly, the increase of miR-132 is consistent in the two studies, strongly indicating the role of miR-132 in cardiac fibrosis.

Further experimental studies need to be carried out to better understand the biological function of these Ang II-induced miRNAs. Our current results demonstrated that we did identify a subset of miRNAs that were differentially expressed in Ang II-treated CFs, and these miRNAs might play a role in cardiac fibrosis by their potential targets. Our current studies provide a starting point to further explore their potential roles in cardiac fibrosis.

Acknowledgments This study was supported by the Fundamental Research Funds for the Central Universities (08143023) and National Science Foundation of China (31100834).

Conflict of interest None.

References

1. Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, Shindo T, Sano M, Otsu K, Snider P, Conway SJ, Nagai R (2010) Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. J Clin Investig 120(1):254–265
2. Porter KE, Turner NA (2009) Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther 123:255–278
3. Díez J (2009) Do microRNAs regulate myocardial fibrosis? Nat Clin Pract Cardiovasc Med 6(2):88–89
4. Jiang X, Tsitsiou E, Herrick SE, Lindsay MA (2010) MicroRNAs and the regulation of fibrosis. FEBS J 277(9):2015–2021
5. Iwata M, Cowling RT, Yeo SJ, Greenberg B (2011) Targeting the ACE2-Ang-(1–7) pathway in cardiac fibroblasts to treat cardiac remodeling and heart failure. J Mol Cell Cardiol 51(4):542–547
6. Ren J, Yang M, Qi G, Zheng J, Jia L, Cheng J, Tian C, Li H, Lin X, Du J (2011) Proinflammatory protein CARD9 is essential for infiltration of monocytic fibroblast precursors and cardiac fibrosis caused by angiotensin II infusion. Am J Hypertens 24(6):701–707
7. Huang XR, Chung AC, Yang F, Yue W, Deng C, Lau CP, Tse HF, Lan HY (2010) Smad3 mediates cardiac inflammation and fibrosis in angiotensin II-induced hypertensive cardiac remodeling. Hypertension 55(5):1165–1171
8. Olson ER, Shamhart PE, Naugle JE, Meszaros JG (2008) Angiotensin II-induced extracellular signal-regulated kinase 1/2 activation is mediated by protein kinase C and intracellular calcium in adult rat cardiac fibroblasts. Hypertension 51:704–711
9. Schellings MW, Vanhoutte D, van Almen GC, Swinnen M, Leenders JJ, Kubben N, van Leeuwen RE, Hofstra L, Heymans S, Pinto YM (2010) Syndecan-1 amplifies angiotensin II-induced cardiac fibrosis. Hypertension 55(2):249–256
10. Lijnen PJ, Van Pelt JF, Fagard RH (2012) Stimulation of reactive oxygen species and collagen synthesis by angiotensin II in cardiac fibroblasts. Cardiovasc Ther 30(1):e1–e8
11. Zhang P, Su J, King ME, Maldonado AE, Park C, Mende U (2011) Regulator of G protein signaling 2 is a functionally important negative regulator of angiotensin II-induced cardiac fibroblast responses. Am J Physiol Heart Circ Physiol 301(1):H147–H156
12. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215–233
13. Jiang X, Tsitsiou E, Herrick SE, Lindsay MA (2010) MicroRNAs and the regulation of fibrosis. FEBS J 277:2015–2021
14. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 456:980–984
15. Roy S, Khanna S, Hussain SR, Biswas S, Azad A, Rink C, Gnyawali S, Shilo S, Nuovo GJ, Sen CK (2009) MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue. Cardiovasc Res 82:21–29
16. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN (2008) Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci USA 105:13027–13032
17. Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, Van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P et al (2009) miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. Circ Res 104:170–178

18. Shan H, Zhang Y, Lu Y, Zhang Y, Pan Z, Cai B, Wang N, Li X, Feng T, Hong Y et al (2009) Downregulation of miR-133 and miR-590 contributes to nicotine-induced atrial remodelling in canines. Cardiovasc Res 83:465–472
19. Divakaran V, Adrogue J, Ishiyama M, Entman ML, Haudek S, Sivasubramanian N, Mann DL (2009) Adaptive and maladaptive effects of SMAD3 signaling in the adult heart after hemodynamic pressure overloading. Circ Heart Fail 2(6):633–642
20. van Almen GC, Verhesen W, van Leeuwen RE, van de Vrie M, Eurlings C, Schellings MW, Swinnen M, Cleutjens JP, van Zandvoort MA, Heymans S, Schroen B (2011) MicroRNA-18 and microRNA-19 regulate CTGF and TSP-1 expression in age-related heart failure. Aging Cell 10(5):769–779
21. Limana F, Esposito G, D'Arcangelo D, Di Carlo A, Romani S, Melillo G, Mangoni A, Bertolami C, Pompilio G, Germani A, Capogrossi MC (2011) HMGB1 attenuates cardiac remodelling in the failing heart via enhanced cardiac regeneration and miR-206-mediated inhibition of TIMP-3. PLoS ONE 6(6):e19845
22. Wang J, Huang W, Xu R, Nie Y, Cao X, Meng J, Xu X, Hu S, Zheng Z (2012) MicroRNA-24 regulates cardiac fibrosis after myocardial infarction. J Cell Mol Med. doi:10.1111/j.1582-4934.2012.01523.x
23. Dubey RK, Gillespie DG, Mi Z, Jackson EK (1997) Exogenous and endogenous adenosine inhibits fetal calf serum-induced growth of rat cardiac fibroblasts: role of A2B receptors. Circulation 96:2656–2666
24. Li S, Zhu J, Zhang W, Chen Y, Zhang K, Popescu LM, Ma X, Lau WB, Rong R, Yu X, Wang B, Li Y, Xiao C, Zhang M, Wang S, Yu L, Chen AF, Yang X, Cai J (2011) Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection. Circulation 124(2):175–184
25. Guo CJ, Pan Q, Li DG, Sun H, Liu BW (2009) miR-15b and miR-16 are implicated in activation of the rat hepatic stellate cell: an essential role for apoptosis. J Hepatol 50(4):766–778
26. Terrile M, Bryan K, Vaughan L, Hallsworth A, Webber H, Chesler L, Stallings RL (2011) miRNA expression profiling of the murine TH-MYCN neuroblastoma model reveals similarities with human tumors and identifies novel candidate miRNAs. PLoS ONE 6(12):e28356
27. Ucar A, Vafaizadeh V, Jarry H, Fiedler J, Klemmt PA, Thum T, Groner B, Chowdhury K (2010) miR-212 and miR-132 are required for epithelial stromal interactions necessary for mouse mammary gland development. Nat Genet 42(12):1101–1108
28. Xia H, Qi Y, Ng SS, Chen X, Li D, Chen S, Ge R, Jiang S, Li G, Chen Y, He ML, Kung HF, Lai L, Lin MC (2009) microRNA-146b inhibits glioma cell migration and invasion by targeting MMPs. Brain Res 1269:158–165
29. Zilahi E, Tarr T, Papp G, Griger Z, Sipka S, Zeher M (2012) Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjögren's syndrome. Immunol Lett 141(2):165–168
30. Wang B, Hsu SH, Majumder S, Kutay H, Huang W, Jacob ST (2010) Ghoshal K.TGFbeta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. Oncogene 29(12):1787–1797
31. Lu Y, Roy S, Nuovo G, Ramaswamy B, Miller T, Shapiro C, Jacob ST, Majumder S (2011) Anti-microRNA-222 (anti-miR-222) and -181B suppress growth of tamoxifen-resistant xenografts in mouse by targeting TIMP3 protein and modulating mitogenic signal. J Biol Chem 286(49):42292–42302
32. Jeppesen PL, Christensen GL, Schneider M, Nossent AY, Jensen HB, Andersen DC, Eskildsen T, Gammeltoft S, Hansen JL, Sheikh SP (2011) Angiotensin II type 1 receptor signaling regulates microRNA differentially in cardiac fibroblasts and myocytes. Br J Pharmacol 164(2):394–404