ORIGINAL RESEARCH

Deficiency of MicroRNA miR-1954 Promotes Cardiac Remodeling and Fibrosis

Valorie Chiasson, BS;* Ana Paula Cremasco Takano, PhD;* Rakeshwar S. Guleria, PhD; Sudhiranjan Gupta, PhD

***Background*—**Cardiac fibrosis occurs because of disruption of the extracellular matrix network leading to myocardial dysfunction. Angiotensin II (AngII) has been implicated in the development of cardiac fibrosis. Recently, microRNAs have been identified as an attractive target for therapeutic intervention in cardiac pathologies; however, the underlying mechanism of microRNAs in cardiac fibrosis remains unclear. Next-generation sequencing analysis identified a novel characterized microRNA, miR-1954, that was significantly reduced in AngII-infused mice. The finding led us to hypothesize that deficiency of miR-1954 triggers cardiac fibrosis.

***Methods and Results*—**A transgenic mouse was created using α-MHC (α-myosin heavy chain) promoter and was challenged with AngII infusion. AngII induced cardiac hypertrophy and remodeling. The in vivo overexpression of miR-1954 showed significant reduction in cardiac mass and blood pressure in AngII-infused mice. Further analysis showed significant reduction in cardiac fibrotic genes, hypertrophy marker genes, and an inflammatory gene and restoration of a calcium-regulated gene (Atp2a2 [ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2]; also known as SERCA2), but no changes were observed in apoptotic genes. THBS1 (thrombospondin 1) is indicated as a target gene for miR-1954.

***Conclusions*—**Our findings provide evidence, for the first time, that miR-1954 plays a critical role in cardiac fibrosis by targeting THBS1. We conclude that promoting the level of miR-1954 would be a promising strategy for the treatment of cardiac fibrosis. **(*J Am Heart Assoc*. 2019;8:e012880. DOI: 10.1161/JAHA.119.012880.)**

Key Words: cardiac remodeling • fibrosis • microRNA • transgenic mice

Cardiac fibrosis is defined as an imbalance in extracellular matrix protein turnover. This highly debilitating process results in the alteration of ventricular geometry, which ultimately leads to myocardial dysfunction.[1,2] It is a severe pathologic manifestation resulting from an injury to the myocardium due to pressure overload, ischemic insult, or metabolic stress.[3] The underlying molecular and morphological correlate of cardiac fibrosis is disruption of myocardial structure via uncontrolled deposition of extracellular matrix proteins, which include collagens and matrix metalloproteinases, in the interstitium and perivascular region of the heart.[4–6] As a result, myocardial stiffness occurs and alters the mechanics of the heart and impairs the function. It is known that angiotensin II (AngII) and TGFβ1 (transforming growth factor β1) induce profibrogenic cascades that include collagens (types I and III) and extracellular matrix deposition in cardiac fibroblasts.[7,8] Despite the progress delineating the underlying molecular mechanisms of adverse cardiac remodeling, therapies are limited.

Recently, microRNAs (miRNAs) have emerged as a new class of posttranscriptional regulators of various cardiac diseases. A single miRNA may influence several signaling pathways associated with cardiac fibrosis and heart failure.[9–12] MiRNAs are 22 nucleotides in length and act as a negative or positive regulator of gene expression by inhibiting mRNA translation or promoting mRNA degradation.[13,14] Contributions of miRNAs in cardiac fibrosis are well documented in either experimentally induced rodents or knockout rodent models using miRNA array analysis.[15–23] Although microarray technologies are used extensively to identify novel candidates, the procedure cannot provide adequate sequence coverage. This study used next-generation sequencing (NGS) approaches to analyze myocardial miRNAs comprehensively and quantitatively and to detect novel miRNAs in AngII-infused cardiac remodeling and fibrosis. We identified a novel miRNA,

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Clinical Perspective

What Is New?

- Using next-generation sequencing, we identified a novel microRNA, miR-1954.
- This report is the first to demonstrate that deficiency of miR-1954 triggers adverse cardiac remodeling by targeting THBS1 (thrombospondin 1).

What Are the Clinical Implications?

- Our study highlights that overexpression of miR-1954 in the heart is protective under angiotensin II infusion and offers a possible therapeutic benefit in future intervention.
- Future studies are warranted to evaluate the effect of miR-1954 knockout mice in cardiac remodeling.

miR-1954, that was significantly reduced in AngII-infused mice. The finding led us to formulate a testable hypothesis that deficiency of miR-1954 triggers adverse cardiac remodeling leading to fibrosis.

In this study, we investigated the role of miR-1954 in AngII-mediated cardiac remodeling and fibrosis in a mouse model and compared the effect with a miR-1954 transgenic mouse model in vivo. Results indicated that miR-1954 was significantly reduced in AngII-infused mouse heart. Overexpression of precursor miR-1954 in the heart showed significant reduction of cardiac fibrosis in an AngII-infusion setting. A possible target for miR-1954 was THBS1 (thrombospondin 1). Our results suggested that miR-1954 plays a critical role in AngII-induced cardiac fibrosis.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Generation of miR-1954 Transgenic Mice

A murine precursor of miR-1954 was inserted downstream of the cardiac-specific α-MHC (α-myosin heavy chain) promoter (Figure 1A). This DNA vector was submitted to Cyagen Biosciences Inc to generate a transgenic mouse model. Routine genotyping was performed by polymerase chain reaction (PCR) with the use of an upper primer from the α-MHC promoter (5-CACATAGAAGCCTAGCCCACAC-3) and a lower primer from the miR-1954 DNA (5-GTAAGCTTCC-TACTCTGATGTTC-3) to amplify a 400-base pair fragment spanning the junction between the α-MHC and miR-1954 DNA. Quantitative and semiquantitative reverse transcription PCR (RT-PCR) were carried out to determine the expression levels of miR-1954 transgenic hearts. Relative fold change of target gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. U6 (small nuclear RNA 6) was used as a RT-PCR loading control.

Infusion of AngII in Mice and Evaluation of Cardiovascular Parameters

The animal experiments were done at the Department of Medical Physiology, Texas A&M University, Health Science Center (Temple, TX). Animal care and all experimental procedures were conducted with the approval of the institutional animal care and use committee at the Texas A&M Health Science Center and Baylor Scott & White Healthcare. The approved animal protocol number is 2012-29R. For AngII infusion, male c57Bl6/j mice aged 8 to 10 weeks were implanted with Alzet mini-osmotic pumps (Alza Pharmaceutics) containing either normal saline or AngII (1.5 mg/kg per day for 14 days) under sterile conditions, as described previously.[21] Systolic blood pressure was measured in conscious mice by the noninvasive tail-cuff method using the CODA blood pressure system (Kent Scientific), as described previously.[21] The hearts were excised for biochemical, histological, and molecular analysis. Cardiac mass was quantified using the heart weight: tibia length ratio.

RNA Isolation and Quantitative RT-PCR Analysis

To detect mRNA and miRNA from left ventricles and cardiac fibroblasts, total RNA was isolated using the RNEasy Kit and miRNEasy Mini Kit (Qiagen) separately, according to the manufacturer's protocol. Total cDNA was generated with a high-capacity cDNA synthesis kit (Applied Biosystems). The miRNA-specific cDNA was generated with the RT miRNA First Strand Kit from Qiagen. Mouse gene-specific primers were used to perform quantitative RT-PCR (qRT-PCR) for miR-1954: α-MHC, β-MHC, ANP (atrial natriuretic peptide)/NppA (natriuretic peptide A), BNP (brain natriuretic peptide)/NppB (natriurectic peptide B, Col1a1 (collagen type I, α1), Col3a1 (collagen type III, α1), Col4a1 (collagen type IV, α1), CTGF (connective tissue growth factor), FSP-1 (fibroblast activating protein 1), Acta2 (α-smooth muscle actin), THBS1, TGFβ1, Il6 (interleukin 6), and Atp2a2 (ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2; also known as SERCA2), Bak1 (BCL2 antagonist/killer 1), and Casp3 (caspase 3). The procedure for qRT-PCR was performed as described previously.[24,25]

Western Blot Analysis

Myocardial tissues were lysed using RIPA lysis buffer containing protease inhibitors, and Western blotting along with quantification of images were performed, as described previously.[21] The antibodies for THBS1 and GAPDH were purchased from Cell Signaling Technology. The Col1a1 antibody was purchased from Abcam.

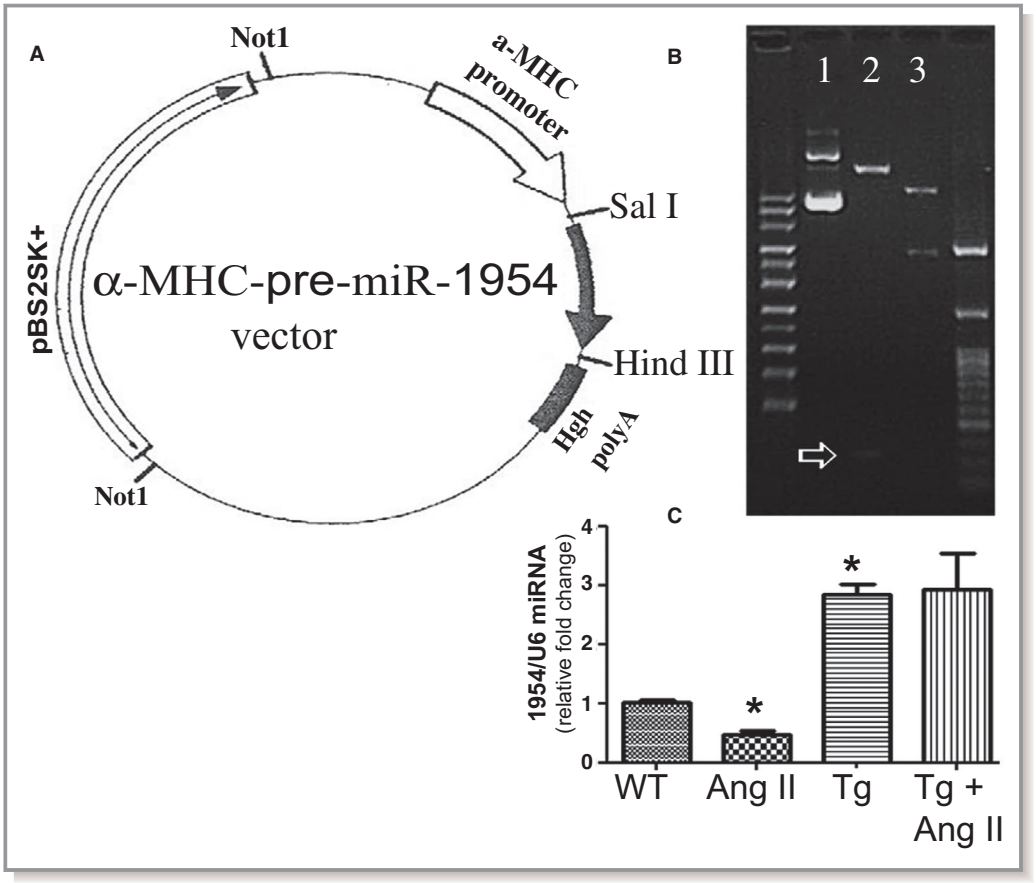

Figure 1. **A**, Schematic presentation of construction of cardiac specific pre–miR-1954 transgene. **B**, Enzyme digestion result of cloning of pre–miR-1954. Lane 1 (L1) is the uncut plasmid; in L2, plasmid digested with Sal I and Hind III resulted in 2 expected band (9042/~228 base pairs); and in L3, plasmid digested by Not I resulted in 2 expected bands (~6386/288 base pairs). **C**, Wild-type (WT) and miR-1954 transgenic mice were treated with angiotensin II (AngII; 1.5 mg/kg per day) for a period of 2 weeks. Expression of mature miR-1954 in WT, AngII-treated WT, miR-1954, and miR-1954 treated with AngII, 6 to 8 independent mice per group. *P<0.05 compared with WT. Tg indicates transgenic. Sal I: Type II restriction enzyme from Streptomyces albus G; Hind III: Type II restriction enzyme from Haemophilus influenzae Rd; Not I: NotI restriction endonuclease from Nocardia otitidiscaviarum. PolyA: polyadenylation.

Morphological Examination of Left Ventricle

Masson's trichrome staining and microscopy image analysis in left ventricular sections were performed, as described previously.[21]

Preparation of Neonatal Rat Cardiac Fibroblasts and AngII Stimulation

Primary rat neonatal cardiac fibroblasts were prepared as described.[25] In brief, left ventricles were minced and digested with collagenase type II (Worthington Biochemical Corp). Finally, cells were plated on 6-well plates containing 10% FBS in DMEM and incubated at 37°C with 5% CO_2. For transfection and stimulation, passages 2 and 3 were used. For AngII stimulation, cells were serum starved overnight and treated with 1-μmol/L AngII for 48 hours.

Transfection with miR-1954 Mimic and Inhibitor

The miRNA mimic and inhibitor of miR-1954 were purchased from Qiagen. We used miRCURY mimic mouse mmu-miR-1954 (YM00471699) and miR-1954 inhibitor (YI04101555). Both mimic and inhibitor of miR-1954 target the sequence ACUGCAGAGUGAGACCCUGUU. Cardiac fibroblasts were transfected with a final concentration of 10 nmol/L for miR-1954 mimic and inhibitor using DharmaFECT Duo Transfection Reagent, according to the manufacturer's instruction (Thermo Scientific).

Reporters Containing THBS1 3′ Untranslated Region Sequences

The miRNA 3′ untranslated region (UTR) target expression clone for mouse THBS1 (NM_001313914.1) in pEZX-FR02

Table 1. Twenty Most Highly Differentially Expressed MicroRNA Names and Annotation Based on Counts

Name	S1	S2	ΔCt(s1−s2)
mmu-miR-1a-3p+1	74610.2	99297.3	−24687.1
mmu-miR-378a-3p	14818.4	36181.7	−21363.3
mmu-miR-30d-5p	12422.6	23997.9	−11575.3
mmu-miR-143-3p	31392.4	40343.5	−8951.1
mmu-miR-30a-5p	7199.7	12250.4	−5050.7
mmu-miR-21a-5p	8036.4	3471.0	4565.4
mmu-miR-148a-3p	6888.2	11404.6	−4516.4
mmu-miR-133a-3p+1	11572.6	15715.3	−4142.8
mmu-miR-3107-5p	2402.6	5292.4	−2889.8
mmu-miR-486-5p	2279.8	4958.3	−2678.5
mmu-miR-126a-3p	28471.3	31100.4	−2629.2
mmu-miR-10a-5p	2379.8	3777.4	−1397.6
mmu-miR-99a-5p	16468.8	15133.2	1335.6
mmu-miR-26a-5p+1	5296.2	6621.2	−1325.0
mmu-miR-30e-5p	3632.9	4838.4	−1205.5
mmu-let-7g-5p	5697.8	6800.6	−1102.9
mmu-miR-145a-5p	1332.1	2406.4	−1074.3
mmu-miR-125b-5p+1	1713.5	2681.2	−967.8
mmu-miR-149-5p	1025.2	1983.4	−958.2
mmu-let-7c-5p+1	2054.9	3007.1	−952.3

Comparison of experimental groups S1 (wild-type mouse heart) and S2 (angiotensin II–treated mouse heart). The samples were normalized with the "tags per million" (TPM) method, and the difference between the 2 samples is presented as a TPM difference.

and the corresponding control vector firefly luciferase and Renilla luciferase were made from GeneCopoeia. Cells (neonatal cardiac fibroblasts) were cotransfected with miR-1954 mimic and inhibitor separately, and luciferase assays were performed as described previously.[21,25] The firefly luciferase activity was normalized to Renilla luciferase activity for each sample. All experiments were performed at least 3 times.

Statistical Analysis

Data are expressed as mean±SE from 7 to 12 independent mice. Data were analyzed using Prism 7.0 GraphPad software. Once data from the animal experiments passed the Shapiro-Wilk normality test, they were analyzed with 1-way ANOVA. Data form cell experiments were compared by Student *t* test for 2 groups, and 1-way ANOVA for multiple groups followed by Tukey–Kramer post tests. $P<0.05$ was considered statistically significant.

Results

MiRNA NGS Analysis in Wild-Type and AngII-Infused Mice

The NGS analysis for miRNA was performed by Exiqon using wild-type (WT) and AngII-treated mice. The differential expression analysis of read counts identified a subset of miRNAs that had present differences in the "tag per million" normalized read counts between the experimental groups. The arrays are based on Sanger miR Base release 20.0 databases. The dysregulated miRNAs are shown in Tables 1 and 2. We identified 7 predicted miRNA sequences. Prediction is based on counting mapped reads in the vicinity of potential precursor hairpins. Prediction scores and miRNAs were estimated based on structural features of the hairpin. For miRNA prediction, the miRPara software (Exiqon) was used to predict the most probable mature miRNA coding regions from genome scale sequences in a species-specific manner. One of the sequences, UCUCUCA-CUCUGCAUGGUUA, was identified as miR-1954 in TargetScan, RNA22, DIANA, miRBase, miRDB, and miRNA.org databases. The read count data showed that miR-1954 was significantly

Table 2. Twenty MicroRNAs Showing the Highest Fold Change

Name	S1	S2	Fold Change
mmu-miR-29b-1-5p	−1.8	1.4	−3.2
mmu-miR-679-5p	0.5	−2.6	3.1
mmu-miR-6956-3p	−2.8	0.2	−3.1
mmu-miR-155-3p	−2.8	0.2	−3.1
mmu-miR-208b-3p	5.5	2.7	2.9
mmu-miR-7235-3p	−2.8	0.0	−2.8
mmu-miR-1247-5p	−2.8	0.0	−2.8
mmu-miR-8120	−2.8	0.0	−2.8
mmu-miR-122-5p	7.1	4.6	2.4
mmu-miR-488-3p	−1.2	0.7	−2.0
mmu-miR-101c	1.0	−1.0	2.0
mmu-miR-298-5p	0.3	−1.6	1.9
mmu-miR-8103	−0.5	1.3	−1.8
mmu-miR-540-3p	0.8	−1.0	1.8
mmu-miR-6952-3p	−1.2	0.4	−1.7
mmu-miR-3963	−1.2	0.4	−1.7
mmu-miR-1943-5p	1.3	2.9	−1.7
mmu-miR-667-3p	0.6	−1.0	1.6
mmu-miR-376b-5p	0.6	−1.0	1.6
mmu-miR-34c-3p	0.6	−1.0	1.6

Comparison of experimental groups S1 (wild-type mouse heart) and S2 (angiotensin II–treated mouse heart). Samples were normalized with "tags per million" method and converted to log2 scale and the fold change taken (linear scale).

reduced by 2.26-fold (44%) in AngII-treated mice compared with the WT mice. Our data indicate that reduction of miR-1954 may lead to the development of cardiac fibrosis.

Generation of miR-1954 Transgenic Mice

A cardiac-specific miR-1954 transgenic mouse was generated using 228 base pairs of pre–miR-1954 and cloned downstream of mouse cardiac α-MHC promoter. The detailed map and restriction digestion profile of the vector are shown in Figure 1A and 1B. A purified Not I (Not I restriction endonuclease from Nocardia otitidiscaviarum) digested fragment was used for pronuclear injection. To determine the effect of AngII in miR-1954 transgenic mice, the expression of mature miR-1954 was measured in the heart (Figure 1C). The transgenic mice expressed 2.85±0.16-fold (P<0.05) mature miR-1954 compared with the WT counterparts (Figure 1C, bar 3). However, AngII infusion did not show any significant change in miR-1954 expression (Figure 1C, bar 4). WT mice challenged with AngII reduced miR-1954 expression to 0.76±0.05 (P<0.05) compared with untreated WT mice (Figure 1C, bar 2). Together, data indicated that miR-1954 transgenic mice maintain an elevated level of mature miR-1954 in the heart and that AngII treatment did not reduce expression compared with the WT mice.

Cardiac-Specific Overexpression of miR-1954 Rescues AngII-Induced Cardiac Remodeling

We investigated whether overexpression of miR-1954 exerts any protective effect in in vivo conditions. Both WT and miR-1954 transgenic mice were infused with AngII for a period of 2 weeks and then euthanized. Typical heart sizes of WT, AngII-infused WT, and AngII–miR-1954 groups are shown in Figure 2A. Data in Figure 2B show a significant increase in heart weight:tibia length ratio in AngII-infused WT mice (8.49±0.44 versus 5.87±0.21, P<0.001). Untreated miR-1954 did not show any change (5.84±0.28). However, AngII-treated miR-1954 transgenic mice showed reduced heart weight:tibia length ratio (7.50±0.25, P<0.05) compared with AngII-infused mice (8.49±0.44). The data suggest that cardiac overexpression of miR-1954 attenuates AngII-induced cardiac remodeling.

Overexpression of miR-1954 Reduces AngII-Induced Blood Pressure

AngII is a potent stimulus for increasing blood pressure level. In this study, we determined the influence of overexpression of miR-1954 in controlling blood pressure (Figure 2C). The WT mice with AngII infusion for 2 weeks had a significant increase

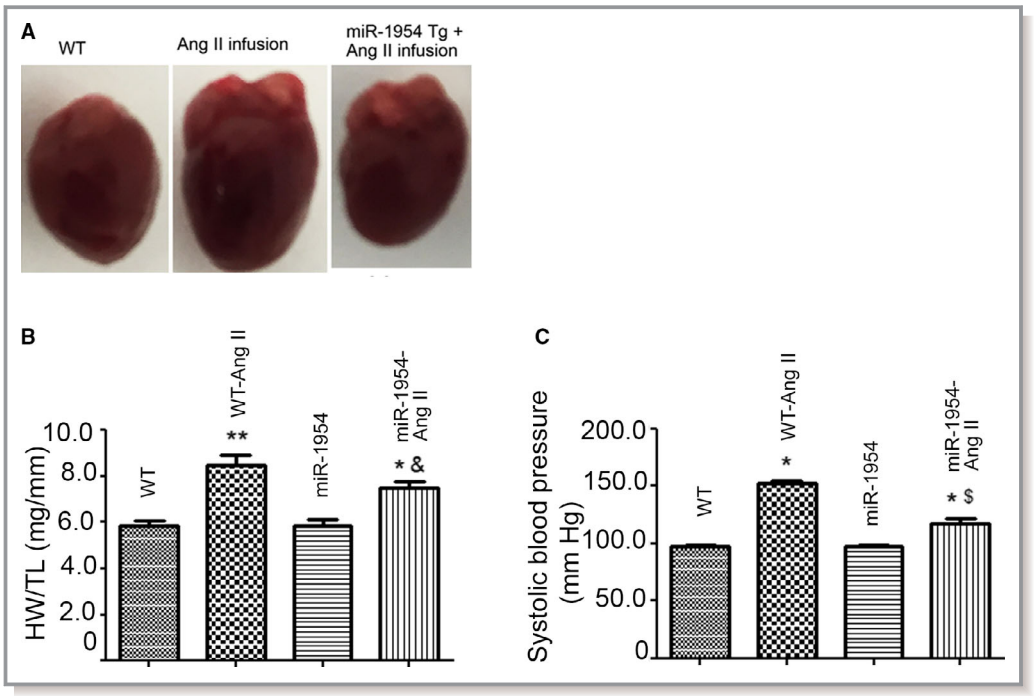

Figure 2. Characterization of miR-1954 transgenic (Tg) mice. Wild-type (WT) and miR-1954 Tg mice were treated with angiotensin II (AngII; 1.5 mg/kg per day) for 2 weeks and were euthanized. **A**, Typical heart sizes of WT, WT-AngII, and miR-1954 Tg+AngII. **B**, Determination of heart weight:tibia length (HW/TL) ratio in WT, WT-AngII, miR-1954 Tg, and miR-1954 Tg+AngII groups. *P<0.05 vs WT; **P<0.001 vs WT; $^{\&}P$<0.05 vs miR-1954 Tg; 7 to 11 per group. **C**, Measurement of systolic blood pressure of WT, WT-AngII, miR-1954 Tg, and miR-1954 Tg+AngII groups. *P<0.001 vs WT; $^{\$}P$<0.001 vs WT+AngII; 7 to 12 per group.

in systolic blood pressure to 152.1±1.18 mmHg (P<0.001) compared with the control (97.17±1.14 mm Hg). However, the blood pressure level of AngII-treated miR-1954 transgenic mice reduced significantly to 116.9±5.36 mmHg (P<0.001) compared with AngII-infused WT mice, thus proving a role for miR-1954 in blood pressure regulation.

Cardiac-Specific Overexpression of miR-1954 Attenuates AngII-Induced Cardiac Hypertrophy Marker Genes

It is known that AngII infusion increases cardiac hypertrophy marker gene expression (eg, NppA, NppB, and β-MHC) and decreases α-MHC. Data showed increased expression of NppA (3.48±0.58, P=0.0008), NppB (2.58±0.18, P<0.001), and β-MHC (4.80±0.92, P<0.001) in AngII-treated mice compared with WT mice (Figure 3). Interestingly, data showed significant reduction of NppA (1.79±0.14, P<0.05), NppB (1.37 ±0.14, P<0.05), and β-MHC (3.63±0.43, P<0.05) in miR-1954 transgenic mice treated with AngII compared with AngII-infused WT mice. The expression of α-MHC showed restoration in miR-1954 mice treated with AngII (0.72±0.03 vs. 1.13±0.05, P=0.0023). Together, data suggest that miR-1954 regulates cardiac hypertrophy.

Cardiac-Specific Overexpression of miR-1954 Attenuates AngII-Induced Cardiac Fibrotic Genes

Gene expression of Col1a1, Col3a1, and Col4a1 were increased to 2.59±0.36-, 2.37±0.27-, and 1.41±0.12-fold, respectively (P<0.001, P=0.0002, and P=0.0122) in AngII-treated mouse hearts compared with untreated mice (Figure 4A through 4C). Conversely, the gene expression of Col1a1, Col3a1, and Col4a1 were significantly reduced in AngII-infused miR-1954 mice compared with WT mice treated with AngII (2.06±0.24, 1.81±0.15, and 1.11±0.05; P<0.05). Upregulation of Col1a1 protein level in the hearts of AngII-infused mice was reduced in miR-1954 transgenic mice treated with AngII infusion (2.65±0.35 versus 2.03±0.14,

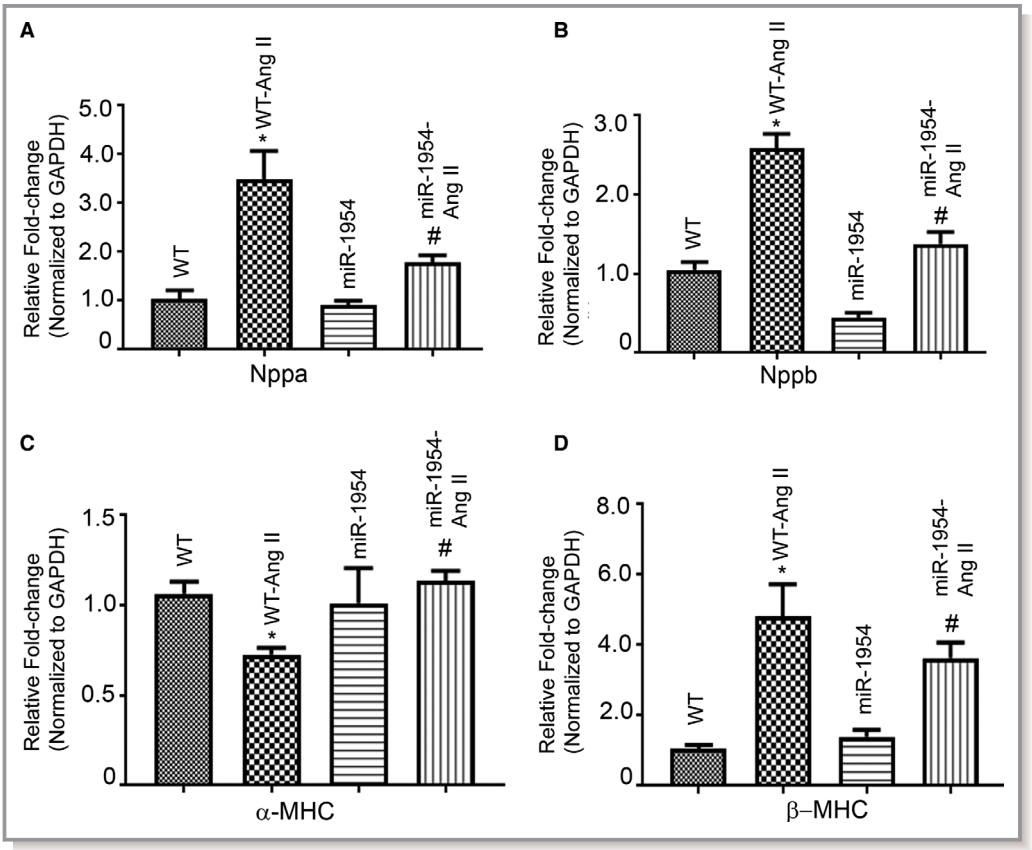

Figure 3. Overexpression of miR-1954 reduces cardiac hypertrophy genes in mouse model with angiotensin II (AngII) infusion in vivo. Cardiac hypertrophy gene expression of NppA (**A**), NppB (**B**), α-MHC (**C**), and β-MHC (**D**) were determined by quantitative reverse transcription polymerase chain reaction. GAPDH was used as an internal control. Data are expressed as mean±SE from 7 to 12 independent mice. *P<0.05 vs wild type (WT); #P<0.05 vs WT+AngII. MHC indicates myosin heavy chain; NppA, natriuretic peptide A; NppB, natriurectic peptide B.

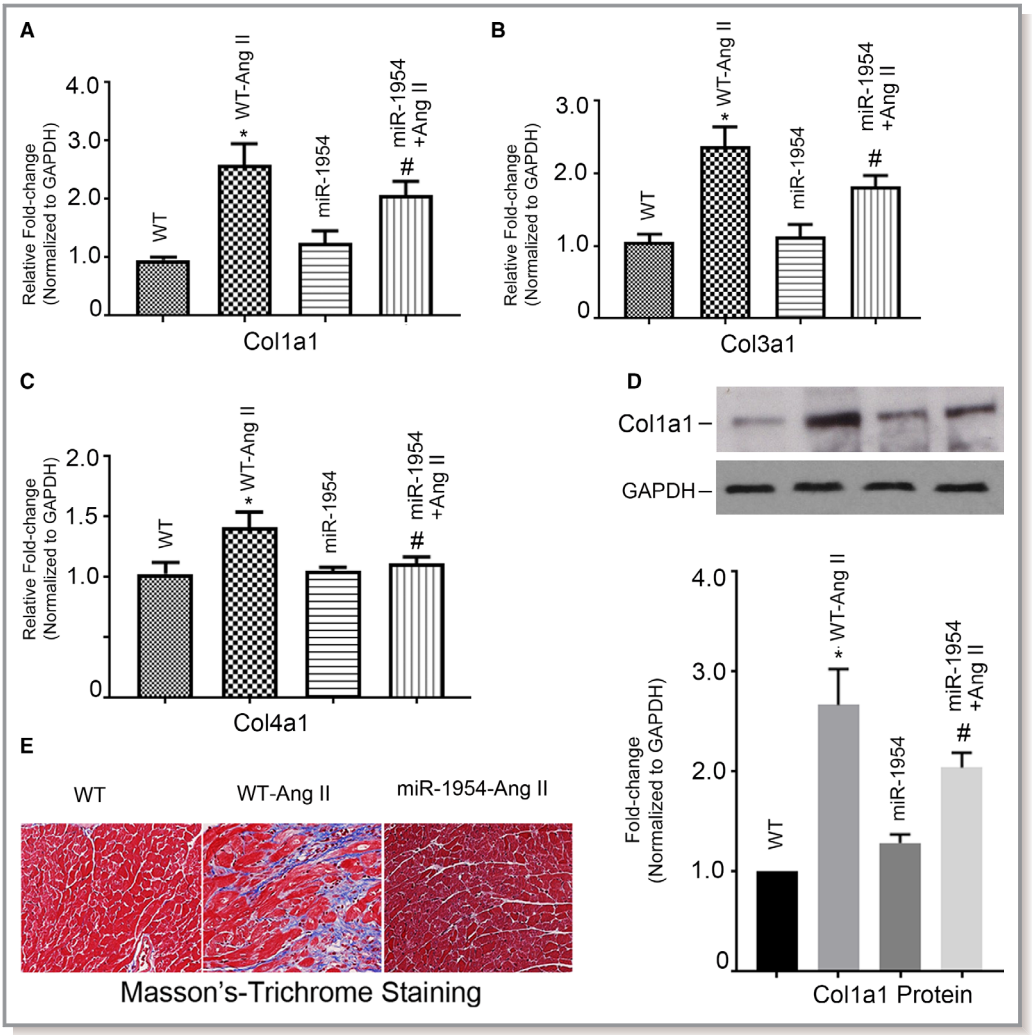

Figure 4. Overexpression of miR-1954 reduces cardiac fibrosis in a mouse model with angiotensin II (AngII) infusion in vivo. Cardiac gene expression of Col1a1 (**A**), Col3a1 (**B**), and Col4a1 (**C**) were determined by quantitative reverse transcription polymerase chain reaction. GAPDH was used as an internal control. Data are expressed as mean±SE from 7 to 12 independent mice. *P<0.05 vs wild type (WT); #P<0.01 vs WT+AngII. **D**, Representative image of cardiac Col1a1 protein level determined by Western blot analysis. Quantification is shown at the bottom. *P<0.05 vs WT; #P<0.05 vs WT+AngII. **E**, Representative image of Masson's trichrome staining of WT, WT+AngII, and miR-1954+AngII groups. Col1a1 indicates collagen type I, α1; Col3a1, collagen type III, α1; Col4a1, collagen type IV, α1.

P<0.05), as demonstrated by Western blotting (Figure 4D). Compared with the control group, the AngII-treated mice showed significant cardiac fibrosis, as determined by Masson's trichrome staining (Figure 4E). However, miR-1954 transgenic mice treated with AngII showed rare collagen deposition. Data indicate that miR-1954 regulates cardiac fibrosis.

We also checked the expression of additional genes associated with tissue remodeling response (Figure 5). The AngII-treated mice showed significant upregulation of CTGF (2.03±0.17, P=0.0014), TGFβ1 (1.35±0.08, P=0.0003), FSP-1 (1.27±0.14, P<0.05), and Acta2 (1.29±0.12, P=0.06) in comparison to the control group. The CTGF expression was significantly attenuated in AngII-treated miR-1954 transgenic mice (1.37±0.10, P<0.05) compared with WT mice treated with AngII. There was marginal reduction in TGFβ1 (1.16±0.06), FSP-1 (1.00±0.12), and Acta2 (1.20±0.12) expression compared with WT mice treated with AngII.

Cardiac-Specific Overexpression of miR-1954 Attenuates AngII-Induced Inflammation and Restored a Ca^{++} Regulated Gene

To gain mechanistic insight, mRNA levels of inflammatory cytokine were examined by qRT-PCR in heart tissues. Expression of Il6 was increased in AngII-infused mice to

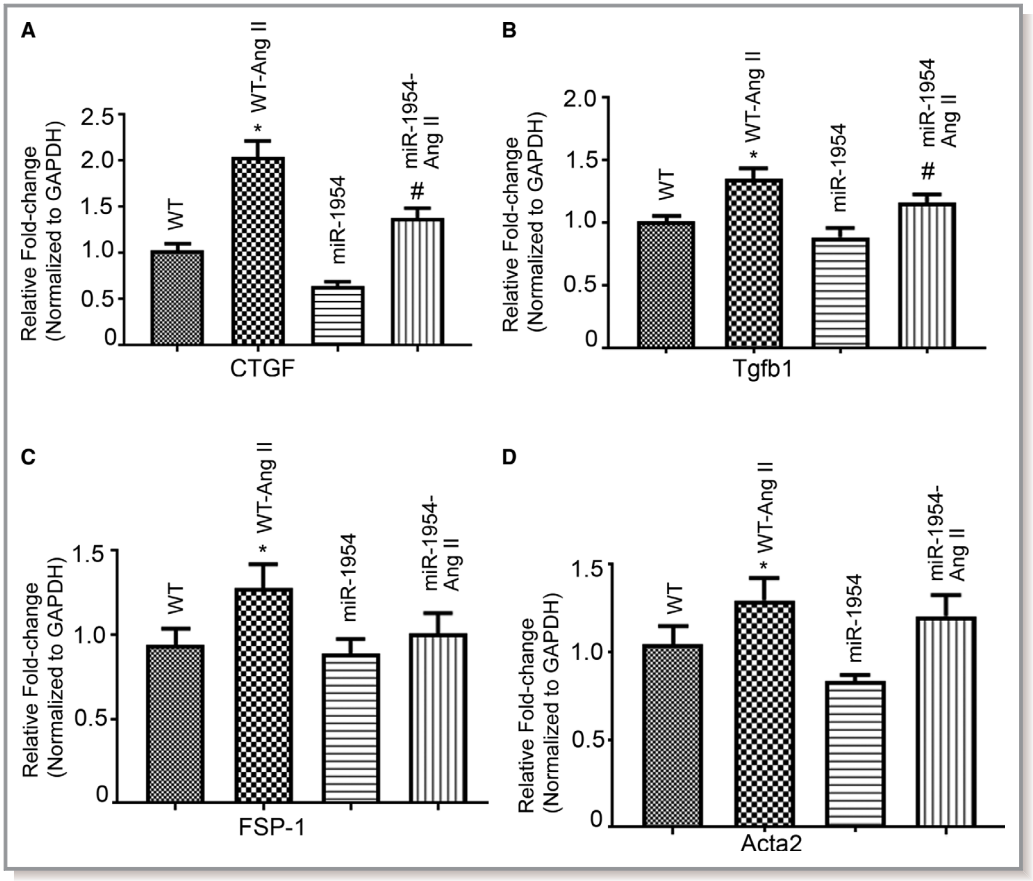

Figure 5. Overexpression of miR-1954 reduces profibrotic gene in mouse model with angiotensin II (AngII) infusion in vivo. Cardiac profibrotic gene expression of CTGF (**A**), TGFβ1 (**B**), FSP-1 (**C**), and Acta2 (**D**) were determined by quantitative reverse transcription polymerase chain reaction. GAPDH was used as an internal control. Data are expressed as mean±SE from 7 to 12 independent mice. *P<0.05 vs wild type (WT); #P<0.05 vs WT+AngII. Acta2 indicates α-smooth muscle actin; CTGF, connective tissue growth factor; FSP-1, fibroblast activating protein 1; TGFβ1, transforming growth factor β1.

2.69±0.42-fold (P=0.0004) compared with untreated WT mice. Conversely, gene expression of Il6 was significantly reduced in AngII-infused miR-1954 mice compared with WT mice treated with AngII (1.31±0.20-fold, P<0.05; Figure 6A). In addition, expression of Atp2a2 (SERCA2) was reduced in AngII-treated WT mice to 0.77±0.05-fold compared with untreated WT mice, whereas AngII-infused miR-1954 mice restored Atp2a2 expression to 0.91±0.06 (P=0.06; Figure 6B). Interestingly, our data did not show any changes in apoptotic genes Bak1 and Casp3 (Figure 6C and 6D). The changes are not significant. Data suggest that miR-1954 may have a role in Atp2a2 regulation and inflammation.

Cardiac-Specific Overexpression of miR-1954 Attenuates AngII-Induced THBS1

Bioinformatics databases (TargetScan, miRNA.org, miRBase) showed THBS1 as a potential target for miR-1954 (Figure 7A). We evaluated the THBS1 levels in WT and miR-1954 transgenic mice treated with AngII. Data showed that THBS1 mRNA expression was upregulated to 3.69±0.47-fold (P=0.0465) compared with untreated WT mice. Conversely, gene expression of THBS1 was significantly reduced in AngII-infused miR-1954 mice compared with WT mice treated with AngII (2.09±0.42-fold, P=0.0465; Figure 7B). The THBS1 protein level is also significantly reduced in AngII-treated miR-1954 transgenic mice compared with AngII-treated WT mice (1.71±0.21 versus 3.65±0.17-fold, P<0.0001; Figure 7C and 7D). Data suggest that THBS1 may be a potential target for miR-1954.

THBS1 Appears to Be a Potential Target for miR-1954

We determined the expression of mature miR-1954 in both cardiac myocytes and fibroblasts. The ΔCt values from both myocytes and fibroblasts showed no significant differences, but fibroblasts showed slightly more (Figure 8A). To corroborate the in vivo data, expression of mature miR-1954 was

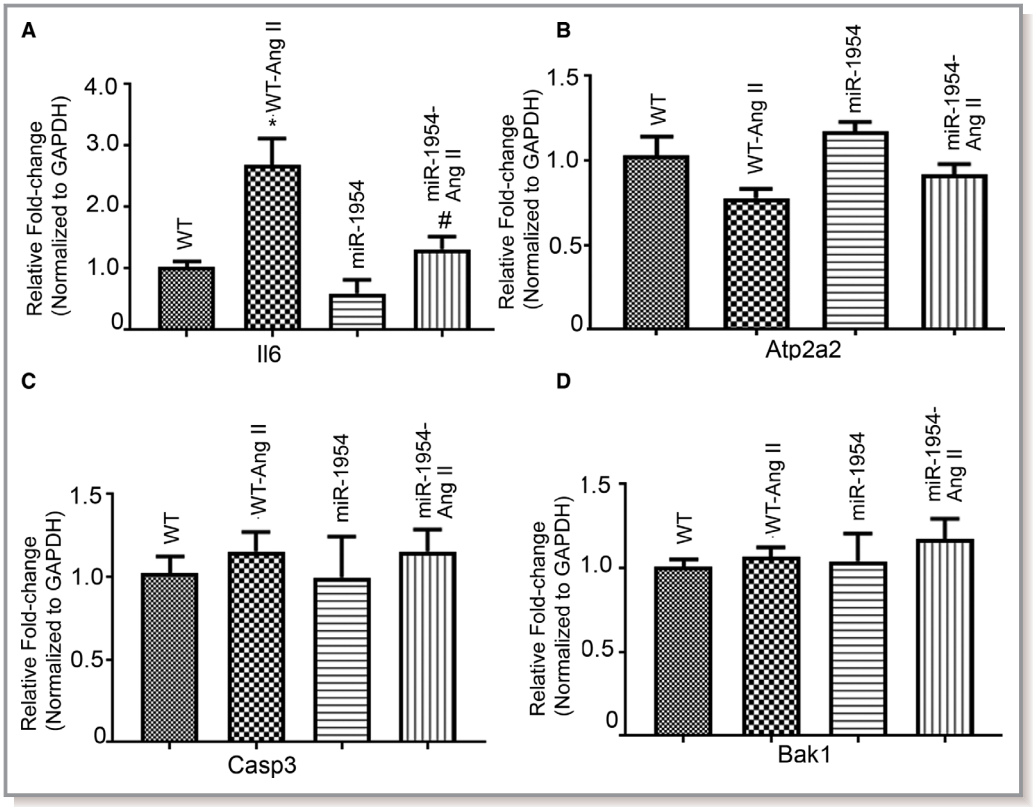

Figure 6. Overexpression of miR-1954 attenuates inflammatory gene and restores Ca^{++} gene in mouse model with angiotensin II (AngII) infusion in vivo. The mRNA expression of Il6 (**A**), Atp2a2 (**B**), Casp3 (**C**), and Bak1 (**D**) was determined by quantitative reverse transcription polymerase chain reaction. GAPDH was used as an internal control. Data are expressed as mean±SE from 7 to 12 independent mice. *P<0.05 vs wild type (WT); #P<0.05 vs WT+AngII. Atp2a2 indicates ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2 (also known as SERCA2); Bak1, BCL2 antagonist/killer 1; Casp3, caspase 3; Il6, interleukin 6.

determined in AngII-stimulated cardiac fibroblasts. Treatment of cardiac fibroblasts with AngII for 48 hours reduced miR-1954 to 0.54±0.05 (P<0.001; Figure 8B). THBS1 mRNA expression was increased to 2.67±0.16-fold (P<0.001) compared with untreated cardiac fibroblasts (Figure 8C). Furthermore, the miR-1954 mimic transfection reduced THBS1 expression to 0.68±0.06-fold, and AngII stimulation slightly increased to 1.37±0.26-fold (P<0.001) compared with AngII-stimulated cardiac fibroblasts. Cardiac fibroblasts transfected with miR-1954 inhibitor significantly increased THBS1 mRNA expression to 2.68±0.17-fold (P<0.001), and AngII stimulation further enhanced THBS1 expression to 3.66±0.22-fold (P<0.0001). To identify putative binding sites of miR-1954 in the 3′ UTR of THBS1, we used the miRNA target prediction search engine TargetScan 7.1 (Figure 7A). Bioinformatics databases like miRNA.org and miRBase further showed that THBS1 is a potential target gene for miR-1954. Based on the analysis, cells were transiently transfected with an empty vector, a THBS1 3′-UTR luciferase construct, a THBS1 3′-UTR luciferase construct with miR-1954 mimic, and THBS1 3′-UTR luciferase construct with a miR-1954 inhibitor. The miR-1954 mimic potently reduced luciferase activity, whereas miR-1954 inhibitor increased luciferase activity (Figure 8D). The results indicate that miR-1954 targets 3′ UTR of THBS1 mRNA.

Together, the data provided evidence that THBS1 is a possible target for miR-1954.

Discussion

Our study demonstrates that miR-1954, a newly identified miRNA, is reduced in AngII-infused mouse hearts and plays a key role in cardiac fibrosis. Using a transgenic approach, the study shows for the first time that cardiac-specific overexpression of pre–miR-1954 is protective under AngII infusion. Overexpression of mature miR-1954 into the heart reduces systolic blood pressure and regulates cardiac remodeling by modulating fibrotic genes and possibly targeting THBS1. Cardiac-specific overexpression of miR-1954 attenuated expression of several fibrotic genes, including collagens,

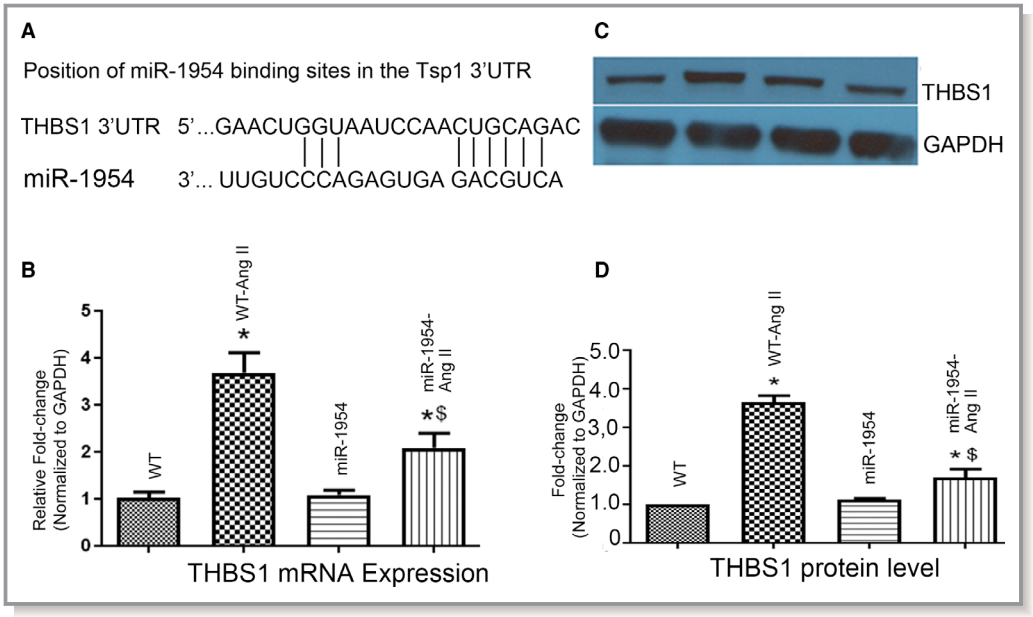

Figure 7. Overexpression of miR-1954 reduces THBS1 (thrombospondin 1) in mouse model with angiotensin II (AngII) infusion in vivo. **A**, Alignment between THBS1 and miR-1954 as predicted by TargetScan 7.1 and miRbase analyses, as shown by vertical bars. The sequence alignment of putative miR-1954 and its targeting site on 3′ untranslated region of THBS1 shows a high level of complementarity. **B**, Determination of THBS1 expression and level in AngII-treated wild-type (WT) and miR-1954 mice. The mRNA expression of THBS1 was determined by quantitative reverse transcription polymerase chain reaction (5–8 per group). GAPDH was used as an internal control. **C**, THBS1 protein level was determined by THBS1-specific antibody (3–6 per group). GAPDH was used as an internal protein loading control. **D**, Quantification of Western blotting. Data are expressed as mean±SE. **P*<0.0001 vs WT; $^{\$}$*P*<0.0001 vs WT+AngII.

TGFβ1, FSP1, Acta2, and CTGF; hypertrophy genes, including ANP, BNP, α-MHC, and β-MHC; inflammatory gene (Il6); calcium-regulated gene Atp2a2 (SERCA2); and THBS1, compared with AngII-infused WT mice. We also elucidated that miR-1954 plays a key role in myofibroblast differentiation by reducing Acta2 level. Overexpression of miR-1954 in cardiac fibroblasts decreases expression of THBS1, and inhibition of miR-1954 increases THBS1 expression. These results underscore that miR-1954 plays an important role in AngII-induced cardiac remodeling and fibrosis. Consequently, miR-1954 may be considered as a therapeutic molecule to treat cardiac fibrosis. Further studies using explanted human tissues are necessary to corroborate our experimental findings.

This study was intended to discover an uncharacterized miRNA in cardiac remodeling using miRNA NGS. The NGS analysis identified 7 uncharacterized miRNAs using miRPara, a support vector machine–based software tool for prediction of miRNA coding regions in genome scale sequences.[26] The miRPara tool identified miR-1954 as a novel miRNA in AngII-induced cardiac remodeling that was significantly reduced compared with the WT mice. We speculate that reduction of miR-1954 under AngII infusion triggered cardiac remodeling. We hypothesized that overexpression of miR-1954 may protect cardiac remodeling in an AngII infusion setting. Results showed that AngII infusion of miR-1954 for 2 weeks significantly reduced cardiac mass, systolic blood pressure, and several hypertrophy and fibrotic genes compared with AngII-infused WT mice. These data indicate, for the first time, that miR-1954 mice are protected under AngII infusion.

Excessive deposition of extracellular matrix proteins is thought to be produced by myofibroblasts.[27] However, downregulation of a miRNA that causes myofibroblast activation—the specialized cardiac fibroblasts formed by irreversible acquisition of expression of Acta2[6]—in fibrosis remains unknown. FSP-1 has been suggested as a fibroblast-specific marker in pressure overload–induced cardiac hypertrophy[28,29]; however, the role of miRNA-mediated FSP-1 in AngII-induced cardiac fibrosis is unknown. Our study showed that AngII infusion significantly reduced miR-1954 expression and upregulated Acta2 and FSP-1 expression in the heart compared with untreated WT mice. The study demonstrated that cardiac-specific overexpression of miR-1954 significantly attenuated AngII-induced Acta2α, and FSP-1 expressions indicates that phenotypic conversion of activated cardiac fibroblast is regulated by miR-1954. It is known that excessive deposition of collagens is the primary determinant of fibrotic myocardium[30] and is a highly debilitating process that leads to cardiac dysfunction. Data showed that overexpression of miR-1954 significantly reduced AngII-induced

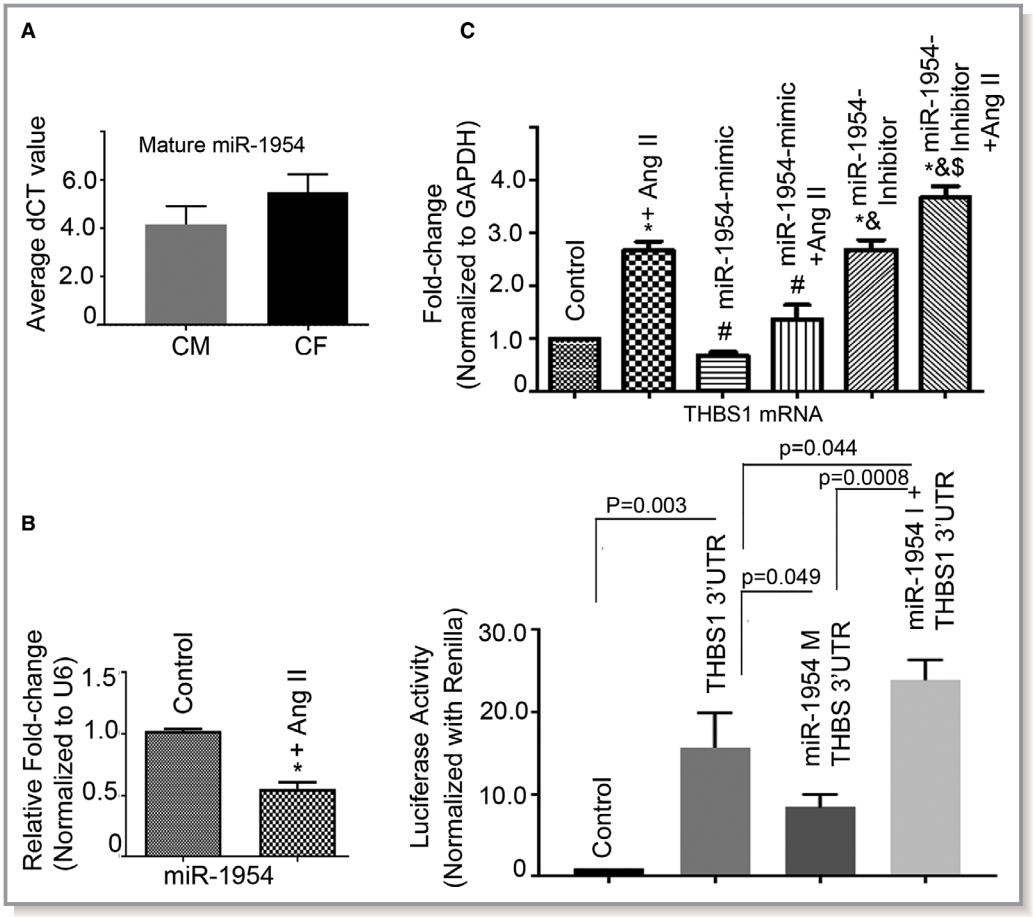

Figure 8. Angiotensin II (AngII) stimulation downregulates miR-1954 expression and upregulates THBS1 (thrombospondin 1) expression in cardiac fibroblasts. **A**, Determination of miR-1954 expression in cardiac myocytes (CM) and cardiac fibroblast (CF). **B**, CFs were treated with 1 μmol/L AngII for 48 hours. Mature miR-1954 expression was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). U6 was used as an internal control. **C**, Gene expression of THBS1 is a likely a target for miR-1954. CFs were transfected with scramble sequence, miR-1954 mimic, and miR-1954 inhibitor for 24 hours, followed by AngII treatment for another 48 hours. Gene expression of THBS1 was determined by qRT-PCR. GAPDH was used as an internal control. Data are expressed as mean±SE from 3 to 5 independent experiments. *$P<0.0001$ vs control; $^{\&}P<0.001$ vs mimic+AngII; $^{\$}P<0.05$ vs inhibitor. Student *t* test was used in panel B; 1-way ANOVA with Tukey post test was used in panel C. **D**, Neonatal CFs were transfected with an empty vector, THBS1 3′ untranslated region (UTR) luciferase construct, THBS1 3′ UTR luciferase construct with miR-1954 mimic (miR-1954 M) and THBS1 3′ UTR luciferase construct with miR-1954 inhibitor (miR-1954 I). After 48 hours of transfection, cells were collected, and firefly luciferase activities were estimated and normalized to Renilla luciferase activity. Data are expressed as mean±SE from 3 to 5 independent experiments. *P* values for comparisons of columns are demonstrated in the figure.

collagens, and CTGF expression indicates its pivotal role in cardiac fibrosis. Mechanistically, data revealed that overexpression of miR-1954 restored the THBS11 level that was upregulated in AngII infusion, suggesting a direct link between THBS1 and miR-1954. Disruption of the THBS1 gene resulted adverse cardiac remodeling in response to pressure overload.[31] It has been reported that in pressure-overloaded heart, THBS1 modulates fibrotic responses by activating TGFβ.[32] Our data corroborating that AngII infusion increased THBS1 expression in the heart and are associated with collagen deposition and TGFβ1 upregulation during fibrotic remodeling. Bioinformatic analysis indicated that THBS1 is a target for miR-1954. Therefore, reduction of miR-1954 and upregulation of THBS1 corroborated our findings. AngII-infused miR-1954 transgenic mice rescued the heart from fibrosis by restoring the THBS1 level, indicating a novel mechanism. The data further support in vitro analysis. Cardiac fibroblast transfected with miR-1954 mimic reduced THBS1 mRNA expression, whereas inhibition of miR-1954 increased expression. Our study suggested THBS1 as a target; however, other potential targets cannot be ignored. The ability of a single miRNA to modulate multiple genes and influence several signaling

pathways is suggested.[33] Bioinformatic analysis also indicated Col1a1 as another target, and in vivo data indicate that overexpression of miR-1954 significantly reduced Col1a1 mRNA expression and protein levels. The observation is significant, and future studies will investigate the potential association between miR-1954 and the collagen network. The current study is focused primarily on in vivo analysis, and in vitro studies are ongoing. Therefore, further studies are warranted to delineate the detail and mechanism and other possible interactions and are currently under way.

It is evident that AngII is an important mediator in cardiac fibrosis, inflammation, and cardiac dysfunction.[34,35] AngII-induced cardiac remodeling is reflected by hypertrophy, fibrosis, and inflammation through numbers of signaling pathways, including TGFβ/Smad signaling pathways.[34,35] Our study showed that AngII-induced Il expression was significantly reduced in miR-1954 transgenic mice, indicating a potential link between inflammation and cardiac fibrosis. Furthermore, data show restoration of TGFβ1 expression, corroborating a link between inflammation and TGFβ signaling. Together, data suggest that loss of miR-1954 triggers an inflammatory pathway and possibly a key mechanism for the development of cardiac remodeling and fibrosis. Furthermore, data show significant downregulation of the Atp2a2 gene in AngII-infused mice, but miR-1954 transgenic mice are rescued. It is reported that AngII infusion induces Ca^{++} release from intracellular calcium store.[36] Atp2a2, a well-characterized Ca^{++}-activated ATPase that pumps Ca^{++} into the sarcoplasmic reticulum lumen, has been shown to be depressed during the myocardial relaxation phase.[37] The 2 critical genes, NCX1 and Atp2a2, play major roles in Ca^{++} modulation during systole and diastole.[38] This phenomenon may demonstrate that AngII-stimulated cardiac hypertrophy induced by Ca^{++} overload is related to the altered function of Atp2a2. Future studies are warranted for Ca^{++}-regulated gene interplay in miR-1954 transgenic mice.

In conclusion, we identified a novel miRNA, miR-1954, which is involved in AngII-induced cardiac remodeling and fibrosis. Our study demonstrated that depletion of miR-1954 in the heart triggers cardiac remodeling and fibrosis. Overexpression of pre–miR-1954 in the heart rescued the heart from fibrosis and provided a protective role in AngII-infused cardiac remodeling. The study showed that THBS1 appears to be a target gene for miR-1954 in AngII-induced cardiac fibrosis.

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Disclosure

None.

References

1. Creemers EE, Pinto YM. Molecular mechanisms that control interstitial fibrosis in the pressure-overloaded heart. *Cardiovasc Res*. 2011;89:265–272.
2. Segura AM, Frazier OH, Buja LM. Fibrosis and heart failure. *Heart Fail Rev*. 2014;19:173–185.
3. Edgley AJ, Krum H, Kelly DJ. Targeting fibrosis for the treatment of heart failure: a role for transforming growth factor-β. *Cardiovasc Ther*. 2012;30:e30–40.
4. Espira L, Czubryt MP. Emerging concepts in cardiac matrix biology. *Can J Physiol Pharmacol*. 2009;87:996–1008.
5. Fan D, Takawale A, Lee J, Kassiri Z. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis Tissue Repair*. 2012;5:15.
6. Krenning G, Zeisberg EM, Kalluri R. The origin of fibroblasts and mechanism of cardiac fibrosis. *J Cell Physiol*. 2010;225:631–637.
7. Ju H, Dixon IM. Effect of angiotensin II on myocardial collagen gene expression. *Mol Cell Biochem*. 1996;163–164:231–237.
8. Schellings MW, Vanhoutte D, van Almen GC, Swinnen M, Leenders JJ, Kubben N, van Leeuwen RE, Hofstra L, Heymans S, Pinto YM. Syndecan-1 amplifies angiotensin II-induced cardiac fibrosis. *Hypertension*. 2010;55:249–256.
9. Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol*. 2010;11:264–275.
10. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of miRNAs on protein output. *Nature*. 2008;455:64–71.
11. Latronico MV, Catalucci D, Condorelli G. Emerging role of microRNAs in cardiovascular biology. *Circ Res*. 2007;101:1225–1236.
12. Control of stress-dependent cardiac growth and gene expression by a microRNA Science. 2017;316:575–579.
13. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–297.
14. Ambros V. The function of animal miRNAs. *Nature*. 2004;431:350–355.
15. Nishiga M, Horie T, Kuwabara Y, Nagao K, Baba O, Nakao T, Nishino T, Hakuno D, Nakashima Y, Nishi H, Nakazeki F, Ide Y, Koyama S, Kimura M, Hanada R, Nakamura T, Inada T, Hasegawa K, Conway SJ, Kita T, Kimura T, Ono K. MicroRNA-33 controls adaptive fibrotic response in the remodeling heart by preserving lipid raft cholesterol. *Circ Res*. 2017;120:835–847.
16. Li J, Cai SX, He Q, Zhang H, Friedberg D, Wang F, Redington AN. Intravenous miR-144 reduces left ventricular remodeling after myocardial infarction. *Basic Res Cardiol*. 2018;113:36.
17. Ji Y, Qiu M, Shen Y, Gao L, Wang Y, Sun W, Li X, Lu Y, Kong X. MicroRNA-327 regulates cardiac hypertrophy and fibrosis induced by pressure overload. *Int J Mol Med*. 2018;41:1909–1916.
18. Wei Y, Yan X, Yan L, Hu F, Ma W, Wang Y, Lu S, Zeng Q, Wang Z. Inhibition of microRNA-155 ameliorates cardiac fibrosis in the process of angiotensin II-induced cardiac remodeling. *Mol Med Rep*. 2017;16:7287–7296.
19. Gupta SK, Itagaki R, Zheng X, Batkai S, Thum S, Ahmad F, Van Aelst LN, Sharma A, Piccoli MT, Weinberger F, Fiedler J, Heuser M, Heymans S, Falk CS, Förster R, Schrepfer S, Thum T. miR-21 promotes fibrosis in an acute cardiac allograft transplantation model. *Cardiovasc Res*. 2016;110:215–226.

20. Nagpal V, Rai R, Place AT, Murphy SB, Verma SK, Ghosh AK, Vaughan DE. MiR-125b is critical for fibroblast-to-myofibroblast transition and cardiac fibrosis. *Circulation*. 2016;133:291–301.
21. Li L, Bounds KR, Chatterjee P, Gupta S. MicroRNA-130a, a potential antifibrotic target in cardiac fibrosis. *J Am Heart Assoc*. 2017; 6:pii:e006763.
22. Thum T, Catalucci D, Bauersachs J. MicroRNAs: novel regulators in cardiac development and disease. *Cardiovasc Res*. 2008;79:562–570.
23. Thum T, Condorelli G. Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. *Circ Res*. 2015;116:751–762.
24. Kumar S, Gupta S. Thymosin beta 4 prevents oxidative stress by targeting antioxidant and anti-apoptotic genes in cardiac fibroblasts. *PLoS One*. 2011;6: e26912.
25. Wei C, Kim IK, Kumar S, Jayasinghe S, Hong N, Castoldi G, Catalucci D, Jones WK, Gupta S. NF-kappaB mediated miR-26a regulation in cardiac fibrosis. *J Cell Physiol*. 2013;228:1433–1442.
26. Wu Y, Wei B, Liu H, Li T, Rayner S. MiRPara: a SVM-based software tool for prediction of most probable microRNA coding regions in genome scale sequences *BMC Bioinfor*. 2011;12:107.
27. Weber KT, Sun Y, Bhattacharya SK, Ahokas RA, Gerling IC. Myofibroblast-mediated mechanisms of pathological remodelling of the heart. *Nat Rev Cardiol*. 2013;10:15–26.
28. Balasubramanian S, Quinones L, Kasiganesan H, Zhang Y, Pleasant DL, Sundararaj KP, Zile MR, Bradshaw AD, Kuppuswamy D. β3 integrin in cardiac fibroblast is critical for extracellular matrix accumulation during pressure overload hypertrophy in mouse. *PLoS One*. 2012;7:e45076.
29. Schneider M, Kostin S, Strom CC, Aplin M, Lyngbaek S, Theilade J, Grigorian M, Andersen CB, Lukanidin E, Lerche Hansen J, Sheikh SP. S100A4 is upregulated in injured myocardium and promotes growth and survival of cardiac myocytes. *Cardiovasc Res*. 2007;75:40–50.
30. Gyöngyösi M, Winkler J, Ramos I, Do QT, Firat H, McDonald K, González A, Thum T, Díez J, Jaisser F, Pizard A, Zannad F. Myocardial fibrosis: biomedical research from bench to bedside. *Eur J Heart Fail*. 2017;19:177–191.
31. Xia Y, Dobaczewski M, Gonzalez-Quesada C, Chen W, Biernacka A, Li N, Lee DW, Frangogiannis NG. Endogenous thrombospondin 1 protects the pressure-overloaded myocardium by modulating fibroblast phenotype and matrix metabolism. *Hypertension*. 2011;58:902–911.
32. Frangogiannis NG, Ren G, Dewald O, Zymek P, Haudek S, Koerting A, Winkelmann K, Michael LH, Lawler J, Entman ML. Critical role of endogenous thrombospondin-1 in preventing expansion of healing myocardial infarcts. *Circulation*. 2005;111:2935–2942.
33. Tarang S, Weston MD. Macros in microrna target identification: a comparative analysis of in silico, in vitro, and in vivo approaches to microrna target identification. *RNA Biol*. 2014;11:324–333.
34. Jia L, Li Y, Xiao C, Du J. Angiotensin II induces inflammation leading to cardiac remodeling. *Front Biosci*. 2012;17:221–231.
35. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82–C97.
36. Xu H, Zhang Y, Sun J, Wei J, Sun L, Zhang J. Effect of distinct sources of Ca(2+) on cardiac hypertrophy in cardiomyocytes. *Exp Biol Med (Maywood)*. 2012;237:271–278.
37. Phillips RM, Narayan P, Gomez AM, Dilly K, Jones LR, Lederer WJ, Altschuld RA. Sarcoplasmic reticulum in heart failure: central player or bystander? *Cardiovasc Res*. 1998;37:346–351.
38. Shao Q, Ren B, Saini HK, Netticadan T, Takeda N, Dhalla NS. Sarcoplasmic reticulum Ca2+ transport and gene expression in congestive heart failure are modified by imidapril treatment. *Am J Physiol Heart Circ Physiol*. 2005;288: H1674–H1682.