The Long Noncoding RNA CHRF Regulates Cardiac Hypertrophy by Targeting miR-489

Kun Wang,* Fang Liu,* Lu-Yu Zhou,* Bo Long, Shu-Min Yuan, Yin Wang, Cui-Yun Liu, Teng Sun, Xiao-Jie Zhang, Pei-Feng Li

Rationale: Sustained cardiac hypertrophy is often accompanied by maladaptive cardiac remodeling leading to decreased compliance and increased risk for heart failure. Maladaptive hypertrophy is considered to be a therapeutic target for heart failure. MicroRNAs (miRNAs) and long noncoding RNAs (lncRNAs) have various biological functions and have been extensively investigated in past years.

Objective: We identified miR-489 and lncRNAs (cardiac hypertrophy related factor, CHRF) from hypertrophic cardiomyocytes. Here, we tested the hypothesis that miR-489 and CHRF can participate in the regulation of cardiac hypertrophy in vivo and in vitro.

Methods and Results: A microarray was performed to analyze miRNAs in response to angiotensin II treatment, and we found miR-489 was substantially reduced. Enforced expression of miR-489 in cardiomyocytes and transgenic overexpression of miR-489 both exhibited reduced hypertrophic response on angiotensin II treatment. We identified myeloid differentiation primary response gene 88 (Myd88) as a miR-489 target to mediate the function of miR-489 in cardiac hypertrophy. Knockdown of Myd88 in cardiomyocytes and Myd88-knockout mice both showed attenuated hypertrophic responses. Furthermore, we explored the molecular mechanism by which miR-489 expression is regulated and found that an lncRNA that we named CHRF acts as an endogenous sponge of miR-489, which downregulates miR-489 expression levels. CHRF is able to directly bind to miR-489 and regulate Myd88 expression and hypertrophy.

Conclusions: Our present study reveals a novel cardiac hypertrophy regulating model that is composed of CHRF, miR-489, and Myd88. The modulation of their levels may provide a new approach for tackling cardiac hypertrophy. (Circ Res. 2014;114:1377-1388.)

Key Words: cardiomegaly ■ RNA, long noncoding

Cardiac hypertrophy is an adaptive reaction of the heart against cardiac overloading to maintain cardiac function at the early stage. However, sustained cardiac hypertrophy is often accompanied by maladaptive cardiac remodeling leading to decreased compliance and increased risk for heart failure and sudden death. Maladaptive hypertrophy is considered to be a therapeutic target for heart failure. Nevertheless, the underlying molecular mechanisms of cardiac hypertrophy are still poorly understood. To prevent heart failure, it is necessary to identify and characterize molecules that may regulate hypertrophy.

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MicroRNAs (miRNAs) are ≈22 nucleotides long and act as negative regulators of gene expression by inhibiting mRNA translation or promoting mRNA degradation.1,2 Growing evidence has demonstrated that miRNAs can play a significant role in the regulation of development, differentiation, proliferation, and apoptosis.3-6 miRNAs can regulate cardiac function including the conductance of electric signals, heart muscle contraction, heart growth, and morphogenesis. They also participate in the regulation of cardiac hypertrophy.7,8 Given the important role of miRNAs in the heart, it is necessary to identify those miRNAs that are able to regulate cardiac hypertrophy and to characterize their signal transduction pathways in hypertrophic cascades.

miRNAs themselves are not hypertrophic executioners, they exert their effect through targeting hypertrophic genes. Although it has been reported that a variety of miRNAs can be altered during cardiac hypertrophy,9,10 the molecular targets of miRNAs still remain to be identified. Growing evidence has

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Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANF</td>
<td>atrial natriuretic factor</td>
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<tr>
<td>Ang-II</td>
<td>angiotensin II</td>
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<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
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<tr>
<td>CHRF</td>
<td>cardiac hypertrophy related factor</td>
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<tr>
<td>IncRNA</td>
<td>long noncoding RNA</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>Myd88</td>
<td>myeloid differentiation primary response gene 88</td>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
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demonstrated that myeloid differentiation primary response gene 88 (Myd88) has an impact on cardiac pathology. Myd88 is related to myocardial infarction induced by ischemia/reperfusion, and knockout of Myd88 is able to reduce myocardial infarct sizes.11,12 There are a few reports showing that Myd88 is involved in hypertrophy. The blockade of Myd88 by adenosines expressing dominant negative Myd88 significantly reduces cardiomyocyte hypertrophy.13 Fibrinogen induces hypertrophic response of cardiomyocytes partially through a toll-like receptor 4–mediated, Myd88-dependent nuclear factor-κB (NF-κB) pathway.14 However, it is not yet clear whether Myd88 is a target of miRNAs in the hypertrophic machinery.

Long noncoding RNAs (lncRNAs) are transcribed RNA molecules >200 nucleotides in length but have no significant protein-coding potential. IncRNAs regulate the expression of genes at epigenetic, transcriptional, and post-transcriptional levels and play an important role in physiological processes. They have various functions such as RNA processing,15 structural scaffolds,16 modulation of apoptosis and invasion,17 marker of cell fate,18 reprogramming of induced pluripotent stem cells,19 and chromatin modification.20 In addition, IncRNAs can act as antisense transcripts or as decoys for splicing factors leading to splicing malfunctioning,21,22 and as a competing endogenous RNA in mouse and human myoblasts.23 However, it is not yet clear whether IncRNA is involved in the regulation of cardiac hypertrophy.

Our present work aims at finding out miRNAs and lncRNAs that are able to regulate cardiac hypertrophy. miR-489 was found to be altered substantially in response to hypertrophic stimulation. In searching for downstream targets of miR-489, we identified that Myd88 can be regulated by miR-489. miR-489 affects cardiac hypertrophy through targeting Myd88. In exploring the mechanism how miR-489 expression is regulated, we identified that CHRF may act as an endogenous sponge that represses miR-489 activity. CHRF regulates Myd88 expression and consequent cardiac hypertrophy through miR-489. Our results reveal a novel hypertrophic regulating model that is composed of CHRF, miR-489, and Myd88.

Methods

Cell culture, quantitative RT-PCR analyses, Western blot analyses, adenoviral constructions and infection, transverse aortic constriction, histological assessments, and immunochemistry were performed according to routine protocols. Details of materials and methods are provided in the Online Data Supplement.

Statistical Analysis

Results are expressed as mean±SEM. Statistical comparison among different groups was performed by 1-way ANOVA. Two groups were evaluated by Student t test. P<0.05 was considered statistically significant.

Results

miR-489 Is Able to Inhibit Hypertrophy in the Cellular Model

Angiotensin II (Ang-II) has been well documented to induce cardiac hypertrophy. However, its underlying molecular mechanisms, including a possible role for miRNAs, remain to be elucidated fully. miRNA microarray analysis on cardiomyocytes identified significant downregulation of 9 miRNAs in response to Ang-II, including the evolutionary conserved miR-489 (Figure 1A and 1B; Online Figure IA). Ang-II–induced miR-489 downregulation in cardiomyocytes was confirmed by qRT-PCR (Figure 1C). We also detected the expression of miR-489 in different heart cell types. The expression of miR-489 was no different among myocytes, fibroblasts, and endothelial cells (Online Figure IB). The expression of miR-489 was also significantly reduced in the transverse aortic constriction model in mice and human heart failure sample (Online Figure IC and ID). Because the cardiac function of miR-489 was unknown, we investigated its role in the heart under physiological and pathological conditions. In vitro miR-489 knockdown in cardiomyocytes using antagonirs (anta-489; Online Figure IIA) promoted cardiomyocyte hypertrophy both at baseline and after Ang-II stimulation, as evidenced by increased cell surface areas (Online Figure IIB) and protein/DNA ratios as a measure of protein synthesis (Online Figure IIC). In contrast, the enforced expression of miR-489 (Online Figure IID) resulted in a reduction of hypertrophic responses, including cell surface area (Figure 1D), protein/DNA ratio (Figure 1E), hypertrophic marker atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) (Figure 1F), and sarcomere organization (Figure 1G). These data suggest that miR-489 participates in antagonizing hypertrophy.

miR-489 Antagonizes Hypertrophy in the Animal Model

To better understand the function of miR-489 in the heart, we generated transgenic mice with cardiac-specific overexpressed miR-489. Five lines of miR-489 transgenic mice demonstrated a high level of miR-489 in the heart (Figure 2A). miR-489 transgenic mice of Line#1 were used for the studies. We analyzed the phenotype of these mice under physiological conditions. These mice developed normally to adulthood without significant alterations in terms of hypertrophy (Figure 2B) and apoptosis (Online Figure IIIA). Subsequently, we detected the hypertrophic responses of these mice in response to hypertrophic stimulation. miR-489 transgenic mice exhibited a reduced hypertrophic phenotype on Ang-II treatment (Online Figure IIIB; Figure 2C). We also observed attenuation of other hypertrophic responses including cross-sectional area (Figure 2D), heart weight/body weight ratio (Figure 2E), and ANF, BNP, as well as β-MHC (Figure 2F). Concomitantly, cardiac function was ameliorated (Online Table I). We also analyzed cardiac fibrosis and observed that it was reduced in miR-489 transgenic mice (Figure 2G). In addition, miR-489 mimic could increase...
the expression of miR-489 (Online Figure IIC) and reduce hypertrophic responses on Ang-II treatment in vivo (Online Figure IIID–IIIG).

To understand the role of endogenous miR-489 in vivo, we tested whether knockdown of miR-489 could influence hypertrophy. Knockdown of miR-489 using antagomirs potentiated hypertrophic responses as revealed by heart/body weight (Online Figure IV A), cardiomyocyte size (Online Figure IVB), BNP levels (Online Figure IVC), and β-MHC levels (Online Figure IVD). Echocardiographic assessment demonstrated an aggravated cardiac function in miR-489 antagomir-treated mice (Online Figure IVE). Thus, it seems that miR-489 exerts an antihypertrophic function in the animal model.

**Figure 1.** miR-489 is able to inhibit hypertrophy in the cellular model. **A**, Microarray results depicting the log-log scatter plot of intensity of microRNA (miRNA) expression from control vs angiotensin II (Ang-II) treatment. Neonatal mouse cardiomyocytes were untreated (control) or treated with Ang-II. Twenty-four hours later, miRNAs were detected by microarray. Red dots and green dots indicate 1.5-fold up- or downregulated genes, respectively. Arrow indicates miR-489. **B**, Downregulated miRNAs on Ang-II treatment. **C**, qRT-PCR analysis of miR-489. Cardiomyocytes were treated with Ang-II at the indicated time, and the expression of miR-489 was analyzed; *P*<0.05 vs control. **D** to **G**, Enforced expression of miR-489 reduces hypertrophic responses induced by Ang-II. Cardiomyocytes were infected with adenoviral miR-489 or β-gal at multiplicities of infection of 80. Twenty-four hours after infection, cells were treated with Ang-II. Hypertrophy was assessed by cell surface area measurement (**D**), protein/DNA ratio (**E**), and analysis of the transcripts for atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) by qRT-PCR (**F**); *P*<0.05 vs Ang-II alone. Photos show sarcomere organization (**G**). Bar, 20 μm.

**Myd88 Is a Downstream Target of miR-489**

miRNAs negatively regulate gene expression by inhibiting mRNA translation or promoting mRNA degradation. To find out the target gene of miR-489, we screened some hypertrophic associated genes (Cyclin T, GSK3β, PKGI, Ras, MYL2, CSRP3, MCIP1, Foxo3a, Calcineurin, NFAT, Myocardin, Myd88) by luciferase assay. We analyzed the 3′UTR of each mRNA sequence except Cyclin T (we analyzed the CDS region because of a lack of 3′UTR) by qRT-PCR (Figure 1F); *P*<0.05 vs Ang-II alone.
miR-489 inhibited the luciferase activity of Myd88 (Online Figure VA) and that it had no effect on other genes (data not shown). Myd88 has been reported to be involved in cardiomyocyte hypertrophy.13,14 However, it is not yet clear whether Myd88 is a target of miRNAs in the hypertrophic machinery. The inhibitory effect of miR-489 on Myd88 3’UTR led us to consider if they are related in the hypertrophic pathway. We analyzed the 3’UTR region of Myd88 by RNAhybrid and noticed that miR-489 has a complementary sequence with Myd88 3’UTR (Figure 3A). To find out the binding sites of miR-489 to the 3’UTR of Myd88, mutations were introduced to Myd88 3’UTR and a mutated (mut) form was obtained (Figure 3B).

Luciferase assay revealed that miR-489 was able to suppress the luciferase activity of wild-type Myd88. However, the mutated form of Myd88 3’UTR demonstrated lesser response to miR-489 (Figure 3C). We tested whether miR-489 can regulate Myd88 levels. Enforced expression of miR-489 led to a reduction of Myd88 on Ang-II treatment (Figure 3D). The knockdown of endogenous miR-489 induced an increase in Myd88 expression (Figure 3E). In contrast, enforced expression of miR-489 resulted in a reduction of endogenous Myd88 (Figure 3F). In human cell line HEK293, we also got similar results (Online Figure VB and VC). miR-489 transgenic mice exhibited a low level of Myd88 (Figure 3G).

Figure 2. miR-489 antagonizes hypertrophy in the animal model. A, Detection of miR-489 levels in miR-489 transgenic (Tg) mice. The expression of miR-489 was analyzed by qRT-PCR from wild type (WT) and different lines of miR-489 Tg mice, and the results were normalized to that of U6. B, miR-489 Tg mice developed normally without obvious phenotype alterations under basal conditions (bar, 20 μm). Heart weight to body weight ratios (n=16). Histological sections were stained with wheat germ agglutinin–FITC conjugate to determine cell size. Fractional shortening (n=10 per group). C to F, miR-489 Tg mice exhibit reduced hypertrophic responses to angiotensin II (Ang-II) infusion. WT and miR-489 Tg mice were infused with Ang-II. C, Gross hearts (top; bar, 2 mm); heart sections stained with hematoxylin and eosin (middle; bar=2 mm; bottom; bar, 20 μm). D, Cross-sectional areas analyzed by staining with TRITC-conjugated wheat germ agglutinin; *P<0.05. E, The ratios of heart weight to body weight; *P<0.05 vs Ang-II plus WT. F, Expression levels of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC); *P<0.05 vs Ang-II plus WT. G, miR-489 Tg mice exhibited reduced cardiac fibrosis in response to Ang-II infusion. WT and miR-489 Tg mice were infused with Ang-II. Masson trichrome staining for collagen; *P<0.05; bar, 20 μm.
To verify the interaction between miR-489 and Myd88 3'UTR in vivo, we performed biotin-labeled miR-489 pull-down assay to test whether miR-489 could pull down Myd88 in vivo. Cardiomyocytes were transfected with biotinylated wild-type miR-489 (Bio-489-wt) or its mutated form (Bio-489-Myd88-mut; Online Figure VIA) and then harvested for biotin-based pulldown assay. Myd88 was pulled down by wild-type miR-489. The mutated form that disrupts base-pairing between Myd88 3'UTR and miR-489 significantly reduced the ability to pull down Myd88 (Online Figure VIB), indicating that the interaction between miR-489 and Myd88 3'UTR actually exists in vivo. We further explored the relationship between endogenous miR-489 and Myd88 in RNA-induced silencing complex in vivo.
is the catalytic center of RNA-induced silencing complex and it associates with small regulatory RNAs that silence target RNAs through partial base-pairing. Also, Ago2 immunoprecipitation can pull down non-seed-pairing target as previously reported. And the nonseed target is also regulated by miRNA. Thus, we used Ago2 to explore the interaction between endogenous miRNAs and mRNAs in RNA-induced silencing complex in vivo. First, we conducted immunoprecipitation in mouse heart lysates with Ago2 antibody. Next, we performed further affinity purification using biotin-labeled probes that are complementary to the 3'UTR region of Myd88 and is also the binding site of miR-489 (P-BS489). The other 2 probes are located outside the 3'UTR region of Myd88 and is also the binding site of miR-489 (P-BS489). The other 2 probes are located outside the binding site of miR-489 (P1 and P2). A random probe that is not complementary to Myd88 was used as a negative control. Our results showed that probes outside the binding site of miR-489 could pull down miR-489, whereas the probe in the binding site could not (Online Figure VII). In addition, we also detected miR-489 from argonaute complexes after Ang-II treatment. The argonaute complexes were obtained by immunoprecipitation using the Ago2 antibody. As shown in Online Figure VIE, Ang-II treatment led to a reduction in the enrichment of miR-489 from argonaute complexes. Taken together, these results indicate that there is a direct interaction between endogenous miR-489 and Myd88 in RNA-induced silencing complex in vivo.

Furthermore, we attempted to investigate whether miR-489 and Myd88 are functionally related in hypertrophy. To this end, we used the target protector technology in which a target protector is able to disrupt the specific interaction of miRNA–mRNA pairs. The target protector of Myd88 was able to augment the expression levels of Myd88 in the presence of miR-489 (Figure 3H, upper panel). Concomitantly, miR-489 could not significantly suppress hypertrophic responses in the presence of Myd88 target protector (Figure 3H, lower panel). Together, these data suggest that miR-489 exerts its effect through Myd88.

**Myd88 Conveys Hypertrophic Signal**

Next, we tested Myd88 expression levels on Ang-II treatment and observed that there was an increase (Figure 4A). The expression levels of Myd88 were also significantly increased in the heart of transverse aortic constriction mouse model (Online Figure VIIA) and human heart failure sample (Online Figure VIIB). The knockdown of Myd88 attenuated hypertrophic responses revealed by cell surface area measurement (Figure 4B), analysis of protein/DNA ratio, and ANF, BNP, as well as β-MHC (Figure 4C). Myd88 often has NF-kB as downstream effector, and so we also detected the activity of NF-kB. Our results showed that Ang-II treatment can activate the NF-kB system (Figure 4A and 4B). To further explore the role of Myd88 in hypertrophy, we used Myd88-knockout mice. In response to Ang-II treatment, Myd88-knockout mice exhibited a reduced hypertrophic phenotype (Figure 4D) and heart weight/body weight ratio (Figure 4E). The cross-sectional area (Figure 4F) and ANF, BNP, as well as β-MHC (Figure 4G) were attenuated in Myd88-knockout mice. We observed a preserved cardiac function in Myd88-knockout mice (Figure 4H). Furthermore, cardiac fibrosis was reduced in Myd88-knockout mice (Figure 4I). Myd88 is an inflammatory mediator, and inflammation is a known player in pressure overload–driven cardiac hypertrophy. Our results showed that Myd88-knockout mice also exhibit attenuated inflammatory response to proinflammatory Ang-II (Online Figure VIIIC and VIIID). Taken together, the results suggest that Myd88 is a prohypertrophic factor. To further explore whether miR-489 exerts its effect through Myd88 in vivo, we tested the effects of miR-489 in Myd88-knockout mice. The administration of miR-489 mimic or antagonir was able to alter miR-489 levels on Ang-II treatment (Online Figure VIIIA). However, the hypertrophic phenotype (Online Figure VIIIB), heart weight/body weight ratio (Online Figure VIIIC), and cross-sectional area (Online Figure VIIID) were not influenced by altering miR-489 levels in Myd88-knockout mice.

**CHRF Is Able to Regulate miR-489 Expression and Activity**

How is miR-489 expression regulated under pathological conditions? Recent studies have suggested that lncRNAs may act as endogenous sponge RNA to interact with miRNAs and influence the expression of miRNA. To understand which lncRNA is involved in the hypertrophic pathway of Ang-II treatment, we screened 100 lncRNAs with moderate expression level in heart from a result of lncRNA array performed by Affymetrix Company. We performed real-time RT-PCR to detect lncRNA levels in response to Ang-II treatment. Among the lncRNAs, AK048451, which we named CHRF, was substantially elevated (Figure 5A). CHRF is located in the first intron of Dcc (deleted in colorectal carcinoma) in mouse, and the length is 1843 nt (Online Table II). The expression level of CHRF is more than one third of miR-489 in mouse heart (Online Figure IXA), and it is no different among myocytes, fibroblasts, and endothelial cells (Online Figure IXB). CHRF is also widely expressed in various tissues (Online Figure IXC), and it is conserved across species in the binding site of miR-489 (Online Figure XA). Ang-II treatment led to a time-dependent elevation of CHRF levels (Figure 5B). CHRF was also significantly increased in the heart of transverse aortic constriction mouse model (Online Figure XB) and human heart failure sample (Online Figure XC). miR-489 levels were elevated in the cells on knockdown of endogenous CHRF (Figure 5C and 5D). To know whether CHRF can affect miR-489 activity, we constructed a miR-489 sensor construct contains a perfect miR-489 target, and a reduced luciferase activity of the sensor indicates the induction of miR-489 activity. Our results showed that the luciferase activity of miR-489 sensor was decreased in cells treated with CHRF siRNA (Figure 5E, lower panel), suggesting the induction of miR-489 activity. Enforced expression of CHRF (Figure 5F) induced a reduction in miR-489 levels (Figure 5G) and miR-489 activity (Figure 5H). Furthermore, we wanted to know whether CHRF may act as a sponge of miR-489. Cardiomyocytes were transfected with the miR-489 sensor luciferase reporter, along with adenoviral miR-489, CHRF, or β-gal. The luciferase activity showed that CHRF counteracted the effect of miR-489.
suggesting that CHRF is a functional sponge for miR-489. Taken together, these data suggest that CHRF is able to regulate miR-489 levels and activity.

To understand the mechanism by which CHRF regulates the levels of mature miR-489, we tested whether CHRF can interact with miR-489. We compared the sequence of CHRF with that of miR-489 using RNAhybrid and noticed that CHRF contains a target site of miR-489 (Figure 6A). We produced a luciferase construct of CHRF RNA (Luc-CHRF-wt) and a mutated form (Luc-CHRF-mut). Luciferase assay revealed that miR-489 could suppress the luciferase activity of CHRF RNA, but it had less effect on the mutated form of CHRF RNA compared with the wild type (Figure 6B). These results reveal that CHRF may interact with miR-489 by this putative binding site.

Furthermore, we applied a biotin–avidin pulldown system to test whether miR-489 could pull down CHRF. Cardiomyocytes were transfected with biotinylated miR-489 and then harvested for biotin-based pulldown assay. CHRF was pulled down by miR-489 as analyzed by real-time RT-PCR, but the introduction of mutations that disrupt base-pairing between CHRF and miR-489 (Figure 6C) led to the inability of miR-489 to pull down CHRF (Figure 6D), indicating that the recognition

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of miR-489 to CHRF is in a sequence-specific manner. We also used inverse pulldown assay to test whether CHRF could pull down miR-489, using a biotin-labeled specific CHRF probe. miR-489 was precipitated as analyzed by the Northern blot (Figure 6E). Taken together, it seems that CHRF is able to directly bind to miR-489.

**CHRF Regulates Hypertrophy Through miR-489 and Myd88**

Because CHRF can interact with miR-489, we thus tested whether CHRF is able to regulate hypertrophy. Knockdown of CHRF reduced Myd88 levels (Figure 7A). The overexpression of CHRF resulted in the upregulation of Myd88 expression and the activation of NF-κB system (Figure 7B). CHRF counteracted the effect of miR-489 on Myd88 expression (Figure 7C). The luciferase reporter assay showed that CHRF counteracts the inhibitory effect of miR-489 on Myd88 (Figure 7D). These results indicated that CHRF may act as endogenous sponge antagonir of miR-489.

Enforced expression of CHRF induced hypertrophic responses, including sarcomere organization and increase in cell surface area and protein/DNA ratio (Figure 7E). In the animal model, enforced expression of CHRF increased the apoptosis of cardiomyocytes (Online Figure XIA). Knockdown of CHRF by siRNA (Online Figure XIB) significantly increased miR-489 levels (Online Figure XIC) and attenuated ANF and β-MHC levels (Online Figure XID) as well as cross-sectional areas (Online Figure XIE). These results indicate that CHRF is able to regulate hypertrophy.
We explored the downstream targets of CHRF in hypertrophy. The modulation of miR-489 (Figure 7F and 7G) or Myd88 levels (Figure 7H) affected hypertrophic responses induced by CHRF, suggesting that Myd88 is a downstream target of CHRF. The target protector of Myd88 attenuated the inhibitory effect of CHRF knockdown on hypertrophic responses (Figure 7I and 7J). Taken together, these data suggest that CHRF targets the miR-489/Myd88 axis in hypertrophic cascades.

Discussion
Cardiac hypertrophy is a common response to a variety of physiological as well as pathological stimuli and will eventually lead to heart failure. Heart failure is one of the leading causes of hospitalization and death worldwide. Maladaptive hypertrophy is considered to be a therapeutic target for heart failure. It is essential to discover impactful therapeutic targets suppressing maladaptive hypertrophy and the consequent heart failure. Our present work identified miR-489 to be an antihypertrophic molecule. We produced miR-489 transgenic mice, and these mice are resistant to hypertrophy. We found that Myd88 is a target of miR-489, and Myd88-deficient mice exhibit reduced hypertrophic responses. Our data further showed that miR-489 inhibits hypertrophy through repressing Myd88. Moreover, we identified that CHRF regulates hypertrophy through miR-489. Our results provide new insights into understanding the pathogenesis of cardiac hypertrophy.

Recent works about miRNAs renovate our understanding about the regulation of cardiac hypertrophy. It is of note that miR-489 transgenic mice exhibit no obvious alterations in phenotype under physiological conditions. However, these mice are resistant to hypertrophy under pathological stimulation with Ang-II. This is consistent with other reports showing that some miRNAs function only under pathological conditions.33 Because physiological cell growth and pathological hypertrophy can be regulated by the distinct cellular machinery, the underlying mechanism by which a miRNA regulates cell size under physiological condition is an interesting topic for future studies. We have identified through the present work that Myd88 is a target of miR-489. Although most miRNA-mediated suppression of target genes have a seed-match sequence, there is no perfect seed-match sequence between miR-489 and Myd88 3' UTR. Many reports also demonstrate that perfect seed-pairing is not mandatory for miRNA target recognition.34,35 The 3' pairing, especially the 3' core (positions 13–16), is also effective when there is at least a 6mer seed match.36 The 3' pairing can help compensate for imperfect seed-pairing.37 In the present study, enforced expression of miR-489 resulted in a reduction of Myd88 on both mouse cardiac myocytes and human cell line HEK293. We also verified the interaction of Myd88 and miR-489 by miR-489 pull-down assay and Ago-2 pulldown experiment. This indicated that the interaction between miR-489 and Myd88 3'UTR actually exists in vivo. Our present work uses Myd88-knockout mice to study its role in cardiac hypertrophy and demonstrates that Myd88 is a prerequisite for Ang-II to initiate hypertrophy.

It remains largely unknown as to the molecular mechanism by which Myd88 regulates cardiac hypertrophy. The activation of NF-kB has been documented to stimulate cardiac hypertrophy through miR-489/Myd88 axis in hypertrophic cascades.
Figure 7. Cardiac hypertrophy related factor (CHRF) regulates hypertrophy through targeting miR-489 and myeloid differentiation primary response gene 88 (Myd88). A, Knockdown of CHRF reduces the expression levels of Myd88. Cardiomyocytes were infected with adenoviral CHRF-siRNA or CHRF-sc. Twenty-four hours after infection, Myd88 levels were analyzed. B and C, Enforced expression of CHRF upregulates Myd88 levels. Cardiomyocytes were infected with adenoviral miR-489, CHRF, or β-gal. Twenty-four hours after infection, Myd88, p-IκBα, and p65 levels were analyzed. D, CHRF counteracts the inhibitory effect of miR-489 on Myd88. Cardiomyocytes were infected with adenoviral miR-489, CHRF, or β-gal and then transfected with Myd88 3′UTR luciferase construct. Luciferase activity was analyzed; *P<0.05. E, CHRF induces hypertrophic responses. Cardiomyocytes were infected with adenoviral CHRF or β-gal. Forty-eight hours after infection, hypertrophy was assessed by sarcomere organization (left; bar, 20 μm), cell surface area measurement (middle), and protein/DNA ratio analysis (right); *P<0.05 vs control. F, Cardiomyocytes were infected with adenoviral CHRF, miR-489, or β-gal. Expression of miR-489 was analyzed by Northern blot. G, miR-489 reduces hypertrophic responses induced by CHRF. Cardiomyocytes were infected with adenoviral CHRF, miR-489, or β-gal. Hypertrophy was assessed by cell surface area measurement; *P<0.05 vs CHRF alone. H, Knockdown of Myd88 reduces hypertrophic responses induced by CHRF. Cardiomyocytes were infected with adenoviral CHRF, Myd88-siRNA, or Myd88-sc. Hypertrophy was assessed by protein/DNA ratio analysis; *P<0.05 vs CHRF alone. I and J, Myd88 target protector attenuates the inhibitory effect of CHRF knockdown on hypertrophic responses induced by angiotensin II (Ang-II). Cardiomyocytes were infected with adenoviral CHRF-siRNA or CHRF-sc, transfected with the target protector (Myd88-TPmiR-489) or the control (Myd88-TPcontrol), and then exposed to Ang-II. Hypertrophy was assessed by cell surface area measurement (I) and protein/DNA ratio analysis (J); *P<0.05.
hypertrophy. For example, MAFbx can convey the signal of cardiac hypertrophy in response to pressure overload. The down-regulation of MAFbx inhibits cardiac hypertrophy through inactivation of NF-κB. The interleukin-1 receptor–mediated MyD88-dependent signaling pathway predominately activates NF-κB. Our present results also show that Ang-II treatment can activate the NF-κB system, and Myd88-knockout mice reduce the inflammatory response to Ang-II. Thus, it can be speculated that NF-κB may be a downstream target of Myd88 in hypertrophy.

Mammalian genomes encode numerous IncRNAs. In mammalian genomes, IncRNAs have been defined to have important functions in specific cell types, tissues, and developmental conditions such as chromatin modification, RNA processing, structural scaffolds, and modulation of apoptosis and invasion. Despite the biological importance of IncRNAs, it is not yet clear whether IncRNAs are involved in the regulation of hypertrophy. It has been shown that IncRNAs may act as endogenous sponge RNAs to interact with miRNAs and influence the expression of miRNA target genes. A recent report showed that a muscle-specific IncRNA, linc-MD1, governs the timing of muscle differentiation by acting as a competing endogenous RNA in mouse and human myoblasts. Highly upregulated liver cancer may act as an endogenous sponge, which downregulates miR-372 leading to reduced translational repression of its target gene, PRKACB. Transient knockdown and ectopic expression of HSUR 1 directs the degradation of mature miR-27 in a sequence-specific and binding-dependent manner. Our results show that the expression of miR-489 is reduced by the sponge CHRF, which is consistent with a previous report. We speculate that there may exist some mechanisms that can degrade part of the binding miRNA; it is similar to the function of antagonirs that promote miRNA degradation. But the exact mechanism is still unclear. It is also an interesting research topic and we will focus on it in future.

In general, IncRNAs lack strong conservation; many well-described IncRNAs, such as Xist, are poorly conserved. The poor conservation of IncRNAs may be the result of recent speciation and rapid adaptive selection. One report indicates that thousands of poor conservation sequences at the primary sequence level in the mammalian genome have shown evidence of conserved RNA secondary structures. Despite the low conservation of IncRNAs in general, it should be noted that many IncRNAs may have more plastic structure function constraints and conserve only short regions that are constrained by structure or sequence-specific interactions. Our present work reveals a novel function of IncRNA (CHRF) in regulating cardiac hypertrophy. CHRF serves as a sponge of miR-489 regulating the expression of Myd88, which activates hypertrophic responses. CHRF is located in the first intron of Dve in mouse, and the length is 1843 nt. The full length of CHRF is poorly conserved across species, but it is conserved between species in the binding site of miR-489 (Online Figure XA). In addition, we also observed an increased expression of CHRF in human heart failure tissue. This indicates that the CHRF may have similar function between the mouse and the human. Our results may provide a new clue for the understanding of IncRNAs-controlled cellular events. The discovery of an IncRNA in cardiac hypertrophy may shed new light on the understanding the complex molecular mechanisms of cardiac hypertrophy.

In summary, our present work identified miR-489 to be an antihypertrophic miRNA. miR-489 is able to influence hypertrophy in cellular and animal models. Our results further reveal that Myd88 is a target of miR-489 in the hypertrophic pathway. Moreover, we demonstrated that CHRF acts as an endogenous sponge RNA and inhibits miR-489 expression and activity. The modulation of miR-489 and CHRF may provide an intriguing approach for tackling cardiac hypertrophy.

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Disclosures

None.

References


25. Schirle NT, MacRae IJ. The crystal structure of human Argonaute2. Science. 2013;346:1037–1040.


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**Novelty and Significance**

**What Is Known?**

- MicroRNA participates in the regulation of cardiac hypertrophy.
- Knockdown of myeloid differentiation primary response gene 88 (Myd88) attenuates hypertrophic responses.
- Long noncoding RNAs (lncRNAs) act as antisense transcripts or competing endogenous RNAs and have various biological functions.

**What New Information Does This Article Contribute?**

- miR-489 is a key regulator of cardiac hypertrophy.
- Myd88 is a downstream target of miR-489.
- lncRNA cardiac hypertrophy related factor (CHRF) performs as an endogenous sponge RNA to interact with miR-489 and regulate cardiac hypertrophy.

Heart failure is one of the leading causes of mortality worldwide. The sustained cardiac hypertrophy usually leads to heart failure. Thus, figuring out the underlying mechanism of pathological hypertrophy is essential for discovering novel therapeutics to inhibit or reverse maladaptive hypertrophy and heart failure. The present work identifies miR-489 as a new modulator of cardiac hypertrophy. Knockdown of miR-489 stimulated cardiac hypertrophy. Conversely, transgenic overexpression of miR-489 reduced cardiac hypertrophy. We found that miR-489 directly inhibited Myd88 in cardiomyocytes. Furthermore, we also found an lncRNA, CHRF, that acts as an endogenous sponge of miR-489. CHRF is able to directly bind to miR-489 and regulate Myd88 expression and cardiac hypertrophy. These findings suggest that CHRF and miR-489 could be 2 potential therapeutic targets for cardiac hypertrophy and heart failure.
The Long Noncoding RNA CHRF Regulates Cardiac Hypertrophy by Targeting miR-489
Kun Wang, Fang Liu, Lu-Yu Zhou, Bo Long, Shu-Min Yuan, Yin Wang, Cui-Yun Liu, Teng Sun, Xiao-Jie Zhang and Pei-Feng Li

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Supplemental Material

Materials and Methods

Generation of transgenic mice with cardiac-specific overexpression of miR-489 and Myd88 knockout mice

For creating transgenic mice with cardiac-specific overexpressed miR-489, a 396 bp DNA fragment containing murine miR-489 was cloned to the vector, pαMHC-clone26 (kindly provided by Dr. Zhongzhou Yang), under the control of the α-myosin heavy chain (MHC) promoter. The primers used to generate miR-489 transgenic mice include, forward primer: 5′-TCTGGTAAACCAAGCAAAACTGAT-3′; reverse primer: 5′-TAAAGGTGACATGACACACAAACA-3′. Microinjection was performed following standard protocols. The primers for genotyping miR-489 transgenic mice include, forward primer in the α-MHC promoter, 5′-CAGAAATGACAGACAGATCCCTCC-3′; the reverse primer in the miR-489 DNA, 5′-ACTTGTTGTCCATGTAACAG-3′.

Conventional Myd88 knockout mice were purchased from Nanjing University Model Animal Research Center (MARC), China. Myd88+/− mice were interbred to give knockout mice (Myd88−/−), which were used for the studies. Mice were genotyped by multiplex PCR (primers and conditions are available from MARC). All experiments were performed on Myd88−/− mice and their wild type littermates (Myd88+/+), and were approved by government authorities.

Cardiomyocyte culture and treatment

Cardiomyocytes were isolated from 1-2 days old mice as we described. Briefly, after dissection hearts were washed, minced in HEPES-buffered saline solution. Tissues were then dispersed in a series of incubations at 37°C in HEPES-buffered saline solution containing 1.2 mg/ml pancreatin and 0.14 mg/ml collagenase. After centrifugation cells were re-suspended in Dulbecco’s modified Eagle medium/F-12 (GIBCO) containing 5% heat-inactivated horse serum, 0.1 mM ascorbate, insulin-transferring-sodium selenite media supplement (Sigma, St. Louis, MO), 100
U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM bromodeoxyuridine. The dissociated cells were pre-plated at 37°C for 1 h. The cells were then diluted to 1x10^6 cells/ml and plated in 10 μg/ml laminin-coated different culture dishes according to the specific experimental requirements. Cells were treated with angiotensin II (Ang-II, Sigma) at 150 nM for 24 h, except as otherwise indicated elsewhere.

Isolation of cardiac fibroblasts
Cardiac fibroblasts were isolated as described in previous reports. Briefly, the C57BL/6 mice (8-10 weeks) hearts were rapidly removed, rinsed, and mounted via the aorta onto a 27-gauge cannula attached to a Langendorff-type apparatus allowing retrograde perfusion of the coronary arteries. Hearts were perfused at 80 mmHg for 5 min with 37°C sterile calcium-free Krebs-Ringer bicarbonate buffer (KRB) containing (in mM) 110 NaCl, 2.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11 glucose. They were then perfused for 20–25 min with KRB enzyme solution containing 0.5 mg/ml type II collagenase (Worthington Biochemical), 2.5 mM CaCl₂, and 1 mg/ml fatty-acid free albumin. After digestion, the ventricles were trimmed free and minced in KRB enzyme solution containing 10 mg/ml albumin, filtered through sterile nylon mesh, and centrifuged at 25 g for 5 min to remove cardiomyocytes, red blood cells, and debris. The resultant supernatant was then centrifuged at 1,000 g for 8 min. The cell pellet was resuspended in 5 ml RPMI 1640 medium with 5 mM glucose, 10% heat-inactivated FBS, and antibiotics (pH 7.3) and plated into T25 tissue-culture flasks. Non-adherent cells were removed by aspiration after 4 h and discarded.

Cultured cardiac microvascular endothelial cells (CMVECs)
Primary MMVEC were isolated as previously described. Briefly, C57BL/6 mice (8-10 weeks) were anesthetized and heparinized. After thoracotomy, the heart and aorta were rapidly excised and washed in ice-cold D-Hanks solution. After removal of the epicardial and endocardial surfaces, the remaining myocardial tissue was cut into pieces of 1 mm³ without visible vessels.
Myocardial tissues were seeded on type I rat-tail tendon collagen-coated plastic disks (Corning). After 30 min attachment period in the incubator, the tissues were cultured in DMEM (1000 mg/L d-glucose) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin.

Cell Death Assays
Apoptosis was determined by the terminal deoxyribonucleotidyl transferase–mediated TUNEL using a kit from Roche. The detection procedures were in accordance with the kit instructions.

Determinations of cell surface areas, sarcomere organization and protein/DNA ratio
Cell surface area of F-actin stained cells or unstained cells was measured as we described 4. 100-200 cardiomyocytes in 30-50 fields were examined in each group. For staining of filamentous actin, the cardiomyocytes were fixed in 3.7% formaldehyde in PBS. Cells were dehydrated with acetone for 3 min and treated with 0.1% Triton X-100 for 20 min. They were then stained with a 50 μg/ml fluorescent Phalloidin-TRITC conjugate (Sigma, St. Louis, MO) for 45 min at room temperature, and visualized by a laser confocal microscopy (Zeiss LSM 510 META). To measure protein/DNA ratio, total protein and DNA contents were analyzed as we described 5.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)
Stem-loop qRT-PCR for mature miR-489 was performed as described 6 on an Applied Biosystems ABI Prism 7000 sequence detection system. Total RNA was extracted using Trizol reagent. After DNase I (Takara, Japan) treatment, RNA was reverse transcribed with reverse transcriptase (ReverTra Ace, Toyobo). The results of qRT-PCR were normalized to that of U6. The sequences of U6 primers were forward: 5’-GCTTCGGCAGCACATATACTAA-3’; reverse: 5’-AACGCTTCACGAATTTGCGT-3’. The relative levels of miRNA were normalized to the levels of U6 using the 2^{-ΔΔCt} method as previously described 7. The detailed calculating method
was as follows: The relative levels of miRNA were quantified according to the formula of $2^{-\Delta \Delta CT}$, where $\Delta \Delta CT = ((CT_{miRNA} - CT_{U6})_{Treatment \ group} - (CT_{miRNA} - CT_{U6})_{Control \ group})$.

qRT-PCR for ANF, $\beta$-MHC, IL-6, MCP1 and CHRF were performed as we described. The sequences of ANF primers were forward: 5’-CTCCGATAGATGCTGCCCTCTTGAA-3’; reverse: 5’-GGTACCGGAAGCTGTTGCAGCCTA-3’. BNP forward primer: 5’-GCTCTTGAAGGACCAAGGCCTCAC-3’; reverse: 5’-GATCCGATCCGTCTATCTTGTCG-3’. $\beta$-MHC forward primer: 5’- CAGACATAGAGACCTACCTTC-3’; reverse: 5’- CAGCATGTCTAGAAGCTCAGG-3’. Mouse CHRF forward primer: 5’-CAACCTTTACCCATCTCTTC-3’; reverse: 5’-CTGAATTACTTCAGGAAAG-3’. Human CHRF forward primer: 5’-AGATTCACATGGTATCCTGAAC’; reverse: 5’-TAGTCTGGCCACATTGTGTC-3’. The results were standardized to control values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH forward primer: 5’-TGTGTCCCGTCTAGGATCTGA-3’; reverse: 5’-CCTGCTTCACCACCTTCTGTC-3’. The specificity of the PCR amplification was confirmed by agarose gel electrophoresis. The relative levels of mRNA or CHRF were normalized to the levels of GAPDH. The formula is $2^{-\Delta \Delta CT}$, where $\Delta \Delta CT = ((CT_{mRNA} - CT_{GAPDH})_{Treatment \ group} - (CT_{mRNA} - CT_{GAPDH})_{Control \ group})$.

For Bio-labeled pulldown assay, the bound levels were normalized to the input of each group. The formula is $2^{-\Delta \Delta CT}$, where $\Delta \Delta CT = ((CT_{mRNA} - CT_{input})_{Treatment \ group} - (CT_{mRNA} - CT_{input})_{Control \ group})$. The values for the control group were set to 1.

**Absolute quantification Real time RT-PCR**

We measured the absolute copy number using the standard curve method. The cDNA of CHRF was cloned into pcDNA 3.1 vector. The vector was linearized and generated sense RNA transcript using in vitro T7 promoter transcription system (Promega). After digested with RNase-free DNAse and purification, the transcript was quantified using a spectrophotometer and converted to the number of copies as following formula: Copy number/μl
\[
\frac{A260 \times 40 \times 10^{-9} \times (6.02 \times 10^{23})}{(n \text{ of } A \times 329.2) + (n \text{ of } U \times 306.2) + (n \text{ of } C \times 305.2) + (n \text{ of } G \times 345.2) + 159}
\]

The quantified RNA was used as the standard of CHRF. Synthetic miR-489 RNA was used as the standard of miR-489. For detection of CHRF, the standard and total RNA were reverse transcribed with ReverTra Ace (Toyobo) using the reverse primer. The standard of miR-489 were reverse transcribed the same as endogenous miR-489. The standard cDNA was serially diluted in nuclease-free water. Serial dilutions from \(10^6\) to \(10^1\) copies were used for standard in a final volume of 20 μl alongside a negative control (RNA) and a non-template control. The SYBR® Premix Ex Taq™ II Kit (Takara) was used for amplification. Quantitative PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Absolute quantification determines the actual copy numbers of target genes by relating the Ct value to a standard curve. The data were analyzed by CFX96 software. The final data was expressed as the copy number per 10pg of total RNA.

Adenoviral constructions and infection

Mouse Myd88 cDNA was from Origene. The adenoviruses harboring Myd88 were constructed using the Adeno-X™ expression system (Clontech). Myd88 3’UTR mutants were generated using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The adenovirus containing β-galactosidase (β-gal) is as we described elsewhere. To construct adenoviruses encoding miR-489, mouse genomic sequence harboring the pre-miR-489 was amplified using the following primer sets: 5’-TCTGGTAACCCAAAAGCAAACTGAT-3’; 5’-TAAAGGTGACAATGACACACAAACA-3’, and then subcloned into the adenoviral system. To construct adenoviruses encoding CHRF, mouse genomic sequence harboring the CHRF was amplified using the following primer sets: 5’-GCTCTTCTAAATTCATAGCCCC-3’; 5’-ATATGAGATGCGAGATGCCATC-3’, and then subcloned into the adenoviral system.

Constructions of mouse Myd88 RNAi and mouse CHRF RNAi
The mouse Myd88 RNAi target sequence is 5′-CGATATCGAGTTTGTGCAG-3′. A nonrelated, scrambled RNAi without any other match in the mouse genomic sequence was used as a control (5′-TATTGCGGTACTGATGCAG-3′). The mouse CHRF RNAi target sequence is 5′-TGCTCTCTAGAGAGCAGC-3′. A nonrelated, scrambled RNAi without any other match in the mouse genomic sequence was used as a control (5′-CCGATCTGACATGACTGCG-3′). The adenoviruses harboring these RNAi constructs were generated using the pSilencer™ adeno 1.0-CMV System (Ambion) according to the Kit’s instructions. Adenoviruses were amplified in HEK293 cells. Adenoviral infection of cardiomyocytes was performed as we described previously.

Pull-down assay with biotinylated miRNA
Cardiomyocytes were transfected with biotinylated miRNA (50 nM), harvested 72h after transfection. The cells were washed with PBS followed by brief vortex, and incubated in a lysis buffer [20 mM Tris, pH 7.5, 200 mM NaCl, 2.5 mM MgCl₂, 0.05% Igepal, 60 U/mL Superase-In (Ambion), 1 mM DTT, protease inhibitors (Roche)] on ice for 10 min. The lysates were precleared by centrifugation, and 50μl of the samples were aliquoted for input. The remaining lysates were incubated with M-280 streptavidin magnetic beads (Sigma). To prevent non-specific binding of RNA and protein complexes, the beads were coated with RNase-free BSA and yeast tRNA (both from Sigma). The beads were incubated at 4°C for 3h, washed twice with ice-cold lysis buffer, three times with the low salt buffer (0.1%SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), and once with the high salt buffer (0.1%SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl). The bound RNAs were purified using Trizol for the analysis.

Pull-down assay with biotinylated DNA probe
The biotinylated DNA probe complementary to CHRF RNA was synthesized and dissolved in 500 μl of wash/binding buffer (0.5M NaCl, 20 mM Tris-HCl, pH 7.5, and 1 mM EDTA). The
probes were incubated with streptavidin-coated magnetic beads (Sigma) at 25 °C for 2 h to generate probe-coated magnetic beads. Cardiomyocyte lysates were incubated with probe-coated beads, and after washing with the wash/binding buffer, the RNA complexes bound to the beads were eluted and extracted for Northern blot analysis. The following primer sequences were used: CHRF pull-down probe, GTTGATGGGATGATACTAACTATG; and random pull-down probe, TGATGTCTAGCGCTTGCGCTTTG.

**Ago2 IP followed by biotin-labeled probe pulldown assay**

Adult C57BL/6 mouse hearts were cut into small pieces, and cross-linking with 1.5% formaldehyde at room temperature for 15 minutes. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M at room temperature for 5 minutes. After wash with PBS, the tissues were homogenated in lysis buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 2.5 mM MgCl₂, 0.05% Igepal, 60 U Superase-In/ml (Invitrogen), 1 mM DTT, 1×Pefabloc (Roche)).AGO2-specific antibody was added into the lysates. After incubation and rotation at 4°C overnight, the immune complexes were pulled down with protein A-agarose beads (Roche) and washed with the lysis buffer. Following the last wash, elute the beads with 0.2 M glycine pH 2.6 (1:1) by incubating the sample for 10 minutes with frequent agitation before gentle centrifugation. Pool the eluate and neutralize by adding equal volume of lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 2.5 mM MgCl₂, 0.05% Igepal, 60 U Superase-In/ml (Invitrogen), 1 mM DTT, 1×Pefabloc (Roche)). The eluate was ready for next biotin-labeled probe pulldown assay.

Three biotin-labeled DNA probes complementary to Myd88 3’UTR were synthesized, Probe-1 (GGCTGGGAGGAAAGGCAGTCCTAGT) and Probe-2 (GCAAAGGACACCCACTTTGTCAG) are outside the miR-489 binding site, and the Probe-BS₄⁸⁹ (CCAAAGGAAACACACATGACAGAT) targets to the binding site of miR-489. A random probe (TGATGTCTAGCGCTTGCGCTTTG) that is not complementary to Myd88 was used as a negative control. These probes were dissolved in 500 µl of wash/binding buffer (0.5M NaCl, 20 mM Tris-HCl, pH 7.5, 60 U Superase-In/ml and 1 mM EDTA). The probes were
separately incubated with streptavidin-coated magnetic beads (Sigma) at 25 °C for 2 h to generate probe-coated magnetic beads. The neutralized eluate after Ago2 IP was separately incubated with the probe-coated beads, and after washing with the wash/binding buffer, the RNA complexes bound to the beads were eluted and extracted for northern blot analysis.

Luciferase constructs and transfection of Myd88 3’UTR, CHRF and miR-489 sensor reporter
Myd88 3’UTR was amplified by PCR. The forward primer was 5’-GGAAGATGAGACTGATGCGGA-3’. The reverse primer was 5’-TCACTTTCTTGGGGACTCAGG-3’. To produce mutated 3’UTR, the mutations were generated using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The constructs were sequence verified. Wild type and mutated 3’UTRs were subcloned into the pGL3 vector (Promega) immediately downstream the coding region of luciferase gene. Mouse CHRF wild type (CHRF-wt) and the mutant derivative devoid of miR-489 binding site (CHRF-mut) were cloned downstream the coding region of luciferase gene. The forward primer was 5’-GCTCTTCTAAATTCATAGCCCC-3’; the reverse primer was 5’-CATATGAGATGGCAGATGCCATC-3’. miR-489 sensor reporter was constructed according to the method previously described 9. Briefly, mouse genomic sequence (200bp) flanking pre-miR-489 was reversely inserted into the pGL3 vector, downstream of the coding region of luciferase gene.

HEK293 cells (Fig. 4G and Fig. 6B) or cardiomyocytes (Fig. 5E, Fig. 5H, Fig. 5I and Fig. 7D) were infected with the indicated adenoviruses, then transfected with the indicated luciferase constructs as described in the corresponding figure legends. The transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The luciferase activity was analyzed as we described elsewhere 10.

Transfection of antagomir and mimic
miR-489-3p antagomir, antagomir negative control (antagomir-NC), miR-489-3p mimic and the mimic negative control (mimic-NC) were purchased from GenePharma Co. Ltd. All the bases were 2′-OMe modified, and the 3′-end was conjugated to cholesterol. Cells were transfected with the antagomir or mimic at 50 nM. The transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

**Immunoblot**
Immunoblot was performed as we described \(^{11}\). In brief, cells were lysed for 1 h at 4°C in a lysis buffer (20 mM Tris [pH 7.5], 2 mM EDTA, 3 mM EGTA, 2 mM DTT, 250 mM sucrose, 0.1 mM PMSF, 1% Triton X-100 and a protease inhibitor cocktail). Samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Equal-protein loading was controlled by Ponceau red staining of membranes. Blots were probed using antibodies. The anti-Myd88 antibody was from Abcam, the p-I\(\kappa\)B\(\alpha\) antibody and p65 antibody were from Cell Signaling technology, the Ago2 antibody was from Abcam and horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology. Western blot bands were quantified using the ImageJ software.

**Microarray analysis**
Total RNA was extracted from cardiomyocytes by using Trizol reagent. For the miRNA microarray experiments, low-molecular-weight RNA was isolated with the described method \(^{12}\), and then used for miRNA microarray by using the Affymetrix Arrays (CapitalBio Corp.). The miRNA microarray analysis identified 224 miRNAs, and the Significance Analysis of Microarrays (SAM) software was used for data analysis. 19 miRNAs were identified to have at least a 1.5 fold change [q-value < 5%] in expression.

**Northern blot analysis**
Northern blot was performed as described \(^{13}\). In brief, the samples were run on a 15%
polyacrylamide-urea gel, transferred to positively charged nylon membranes (Millipore) followed by cross-linking through UV irradiation. The membranes were subjected to hybridization with 100 pmol 3’-digoxigenin (DIG)-labeled probes for miR-489 overnight at 42°C. miR-489 probes were labeled with DIG using a 3’-End DIG Labeling Kit (Roche). The detection was performed using a DIG luminescent detection kit (MyLab) according to the manufacturer’s instructions. The probe sequence for miR-489 was 5’-GCTGCCATATATGTGGTGTCATT-3’. DIG-labeled U6 probe was used as an internal control, and its sequence was 5’-TGGAAACGCTTCACGAATTGTG-3’.

**Target protector preparation and transfection**

Target protector was designed and named as others and we described 10, 14. In brief, Myd88-TPmiR-489 sequence is 5’-CAAAGGAAACACATATGCAGATG-3’. Myd88-TPcontrol sequence is 5’-TGACAAATGAGACTCTCTCCTTCC-3’. They were synthesized by Gene Tools, and transfected into the cells using the Endo-Porter kit (Gene Tools) according to the kit’s instructions.

**Transverse aortic constriction**

For pressure-overload, transverse aortic constriction was carried out as described 15. Sham mice were subjected to a comparable operation without tightening of the suture encircling the aorta. Three weeks after the surgery, the hearts were harvested for real time RT-PCR analysis.

**Echocardiographic assessment of cardiac dimensions and function**

Transthoracic echocardiography was performed on lightly anesthetized mice by using a Vevo 770 high-resolution system (Visualsonics, Toronto, Canada) equipped with a 40-MHz RMV 704 scanhead. Two-dimensional guided M-mode tracings were recorded in both parasternal long and short axis views at the level of papillary muscles. End-diastolic interventricular septum thickness (IVSd), left ventricular internal diameters at end-diastole (LVIDd), left ventricular posterior wall
thickness at end-diastole (LVPWd), end-systolic interventricular septum thickness (IVSs), left ventricular internal diameters at end-systole (LVIDs), left ventricular posterior wall thickness at end-systole (LVPWs), and fractional shortening (FS) were calculated with the established standard equation. All the measurements were made from more than three beats and averaged.

**Animal experiments**

C57BL/6 mice (wild type mice) were purchased from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). The adult male mice (8-10 weeks old) were used in the study. Wild type mice, transgenic mice and knockout mice were infused with Ang-II (Sigma, 0.6mg/kg/day dissolved in 0.9% NaCl), saline-infused mice served as controls. All mice were infused with Ang-II for 2 weeks. The infusions were executed with implanted osmotic minipumps (Alzet model 1002, Alza Corp.) as we described 10.

We infused the adult male C57BL/6 mice or Myd88 knockout mice with Ang-II (0.6 mg/kg/day dissolved in 0.9% NaCl), along with miR-489 antagomir (30mg/kg/day), miR-489 mimic (30mg/kg/day) or their negative control (anta-NC or mimic-NC) for 2 weeks. All the bases of antagomir and mimic were 2′-OMe modified. The 5′ terminal two and 3′ terminal four nucleotides contained phosphorothioate modification and the 3’-end was conjugated to cholesterol. Saline-infused mice served as controls. We executed all of the infusions with implanted osmotic minipumps (Alzet model 1002; Alza Corp.).

Adenoviral CHRF siRNA (2X10^{11} moi) was administered by direct injection to the jugular vein before Ang II infusion using an implanted osmotic minipump. After 14 d, mice were subjected to hypertrophic analysis.

**Histological analysis**

Histological analysis of the hearts was carried out as we described 10. Briefly, hearts were excised, fixed in 10% formalin, embedded in paraffin and sectioned into 7 μm slices, and stained with hematoxyline-eosin (HE). To measure the cross-sectional area of the cardiomyocytes, the
sections were stained with FITC-conjugated or TRITC-conjugated wheat germ agglutinin (Sigma, St. Louis, MO) according to the method previously described. To determine cardiac fibrosis, we stained the heart sections with standard Masson trichrome staining according to manufacturer’s instructions (Sigma).

**Human heart samples**

Samples of human heart failure were collected from patients with end-stage heart failure. Control samples were obtained from the left ventricles of the patients who died without cardiac disease. Written informed consent was obtained from the family of prospective heart donors. The samples were obtained according to the regulations of the Institute of Zoology, Chinese Academy of Sciences.

**Supporting Information References:**


Online Figure I. The expression of miR-489. **A.** miR-489 conservation in various species and the position of miR-489 in the genome in various species. **B.** The expression of miR-489 in myocytes, fibroblast and endothelial cells in mouse heart detected by real time RT-PCR. **C.** The expression of miR-489 in the heart of TAC mouse modal. **D.** The expression of miR-489 in human normal and heart failure tissue.
Online Figure II. Knockdown of miR-489 induces hypertrophic responses in cardiomyocytes. A. Cardiomyocytes were transfected with antagomir (anta-489) or its negative control (anta-NC). 24h after transfection miR-489 expression levels were assessed by northern blot. B and C. Knockdown of miR-489 induces hypertrophic responses. Cardiomyocytes were transfected with antagomir (anta-489) or its negative control (anta-NC). 24h after transfection cells were treated with Ang-II (100nM). Hypertrophy was assessed by cell surface area measurement (B) and protein/DNA ratio analysis (C), *p<0.05. D. Overexpression of miR-489 by adenovirus harboring miR-489. Cardiomyocytes were infected with adenovirus harboring miR-489 or β-gal control. 24h after infection cells were harvested and analyzed by northern blot.
Online Figure III. miR-489 mimic attenuates Ang-II induced cardiac hypertrophy.
A. miR-489 transgenic mice developed normally without obvious apoptotic alterations under basal conditions. Apoptosis of cardiomyocytes were detected by TUNEL staining.
B. WT or miR-489 transgenic mice were infused with Ang-II. The expression levels of miR-489 were analyzed by northern blot.
C. Adult male C57BL/6 mice were infused with miR-489 mimic (mimic-489) or mimic negative control (mimic-NC) as described in the section of Material and Methods. The expression levels of miR-489 were analyzed by northern blot.
D-F. Adult male C57BL/6 mice were infused with Ang-II along with mimic-489 or mimic-NC as described in the section of Material and Methods. The ratios of heart/body weight (D), cross-sectional areas (E) and the expression levels of ANF and BNP (F) were analyzed. *p<0.05 vs Ang-II alone.
G. Echocardiographic analysis of cardiac function. Fractional shortening (FS) was analyzed. *p<0.05 vs Ang-II alone.
Online Figure IV. The mice upon knockdown of miR-489 are susceptible to undergoing hypertrophy. A-D. Adult male C57BL/6 mice were infused with or without Ang-II along with miR-489 antagomir (anta-489) or antagomir negative control (anta-NC) for 2 weeks. The ratios of heart/body weight (A), cross-sectional areas (B), the expression levels of BNP (C) and β-MHC (D) were analyzed. *p<0.05. E. Echocardiographic analysis of cardiac function. Fractional shortening (FS) was analyzed. *p<0.05.
Online Figure V. Myd88 is a downstream target of miR-489 in HEK293 cell line.

A. Luciferase assay. HEK293 cells were infected with adenoviral miR-489 or β-gal, then transfected with Myd88-3’UTR. The luciferase activity was analyzed. *p<0.05 vs Myd88-3’UTR alone. B. HEK293 cells were transfected with miR-489 antagonim (anta-489) or the antagonim control (anta-NC). Myd88 expression was analyzed by immunoblot 48h after transfection. C. HEK293 cells were infected with adenoviral miR-489 or β-gal at a moi of 80. Myd88 expression was analyzed by immunoblot 48h after infection.
Online Figure VI. The interaction between miR-489 and Myd88 in vivo. 

A. The wild type biotin labeled miR-489 (Bio-489-wt) and its mutated form (Bio-489-Myd88-mut) are shown. 

B. Biotin labeled miR-489 could pull down Myd88 in vivo. Cardiomyocytes were transfected with biotinylated wild-type miR-489 or its mutated form. Cells were harvested for biotin-based pull-down assay 24 hours after transfection. The bound levels of Myd88 were analyzed by real-time RT-PCR. *p<0.05. 

C. The diagram of Ago2 IP followed by biotin-labeled probe pulldown. 

D. Endogenous miR-489 can directly bind to Myd88 in RISC complex in vivo. Ago2 IP and biotin-labeled probe pulldown assay were conducted in C57BL/6 mouse heart lysates as described in the method. The levels of miR-489 were analyzed by northern blot. 

E. Ang-II reduces the levels of miR-489 in argonaute complexes. Wild type C57BL/6 mice were infused with Ang-II for 2 weeks. And then, mouse heart lysates were immunoprecipitated using the Ago2 antibody, and the levels of miR-489 were analyzed by northern blot. IgG was used as a negative control.
**Online Figure VII.**

**A.** Expression levels of Myd88 are increased in mouse TAC model. Mouse hearts were harvested after 3 weeks of TAC. The expression of Myd88 was analyzed by immunoblot.

**B.** The expression levels of Myd88 were analyzed by immunoblot in human heart failure tissue.

**C and D.** Myd88 knockout mice and wild type mice were infused with Ang-II for 2 weeks, hearts were removed and the inflammatory factors, IL-6 (C) and MCP-1 (D) were analyzed by real-time RT-PCR. *p<0.05 vs Ang-II plus WT.
Online Figure VIII. miR-489 exerts its effect through Myd88. Wild type (WT) and Myd88 knockout mice were infused with Ang-II along with miR-489 antagonir (anta-489), antagonir negative control (anta-NC), miR-489 mimic (mimic-489) or mimic negative control (mimic-NC) for 2 weeks. A. Administration of miR-489 mimic and antagonir affected miR-489 expression levels upon treatment with Ang-II, *p<0.05. B. Gross hearts (bar=2mm). C and D. The ratios of heart/body weight (C) and cross-sectional areas (D) were analyzed. *p<0.05.
**Online Figure IX. The expression of CHRF.** A. The copy numbers of CHRF and miR-489 in C57BL/6 mouse heart were determined by qRT-PCR using standard curve method. B. The expression levels of CHRF were analyzed by qRT-PCR in different heart cell types (myocytes, fibroblasts and endothelial cells). C. qRT-PCR analysis of CHRF expression levels in different organs or tissues isolated from wild type mice.
Online Figure X. A. Conservation of CHRF in the binding site of miR-489. This is a snapshot from mouse genome (2011 assembly) in UCSC Genome Browser. B. CHRF expression levels are increased in mouse TAC model. Mouse hearts were harvested after 3 weeks of TAC. The expression levels of CHRF were analyzed by qRT-PCR. C. The expression levels of CHRF were analyzed by qRT-PCR in human heart failure tissue.
Online Figure XI. Cardiac hypertrophy can be attenuated by knockdown of CHRF.

A. CHRF induces apoptotic responses. Adenoviral CHRF or β-gal was injected into mice. Apoptosis was detected by TUNEL staining. *p<0.05 vs saline. B and C. Adenoviral CHRF-siRNA or CHRF scramble form was injected into mice. The expression levels of CHRF (B) and miR-489 expression levels (C) were analyzed by qRT-PCR. *p<0.05 vs saline. D and E. Adenoviral CHRF-siRNA or CHRF scramble form was injected into mice with or without Ang-II for 2 weeks. The expression levels of ANF and BNP (D) and cross-sectional areas (E) were analyzed. *p<0.05 vs Ang-II alone.
Online Table I

Echocardiographic data in mice treated with saline or angiotensin II (Ang II)

<table>
<thead>
<tr>
<th>Parameters Analyzed</th>
<th>Saline</th>
<th>miR-489 Tg</th>
<th>Ang II</th>
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<td>WT</td>
<td>miR-489 Tg</td>
<td>WT</td>
<td>miR-489 Tg</td>
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<td>IVSd (mm)</td>
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<td>0.73±0.07</td>
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<td>LVIDd (mm)</td>
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<td>LVPWd (mm)</td>
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<td>IVSs (mm)</td>
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<td>LVIDs (mm)</td>
<td>2.03±0.17</td>
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<td>LVPWs (mm)</td>
<td>1.23±0.03</td>
<td>1.21±0.07</td>
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<td>FS (%)</td>
<td>45.1±2.6</td>
<td>47.3±3.1</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>436±12</td>
<td>423±10</td>
<td>447±13</td>
<td>438±17</td>
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IVSd, end-diastolic interventricular septum thickness; LVIDd, left ventricular internal diameters at end-diastole; LVPWd, left ventricular posterior wall thickness at end-diastole; IVSs, end-systolic interventricular septum thickness; LVIDs, left ventricular internal diameters at end-systole; LVPWs, left ventricular posterior wall thickness at end-systole; FS, fractional shortening. Data are expressed as mean±SEM, (n=8).

*P < 0.05 vs WT saline; #P <0.05 vs WT Ang II.
<table>
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