Long Noncoding RNA–MicroRNA–mRNA
A Novel Tripartite Axis in the Regulation of Cardiac Hypertrophy

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Study Hypothesis

A large fraction of mammalian genomes is transcribed into noncoding RNAs.¹ Despite the prevalence in the genome, up until recently, noncoding RNAs were regarded as junk RNAs that do not code for protein and, thereby, have no biological purpose. However, over the past decade, it has become increasingly evident that noncoding RNAs can and do serve important regulatory functions in pathophysiological processes.² Short noncoding RNAs called microRNAs, typically 20 to 24 nucleotides in length, have been widely reported to post-transcriptionally regulate gene expression by binding to partially complementary sequences in target mRNAs or promoting mRNA degradation and translational repression.² Another class of noncoding RNAs, typically >200 nucleotides in length, called long noncoding RNAs (IncRNAs) have been recently discovered to regulate gene expression not only at the post-transcriptional level, but also at transcriptional and epigenetic levels.³ Notably, IncRNAs have also been reported to function as microRNA sponges, altering microRNA expression levels by binding to and sequestering microRNAs.⁴⁵ In the present study, Wang et al⁶ present evidence that a IncRNA modulates the ability of a microRNA to target an mRNA, which plays a role in the development of cardiac hypertrophy.

How Was the Hypothesis Tested?

To identify microRNAs whose expression is altered by hypertrophic stimulation, the authors⁶ performed microRNA microarray analysis and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) on neonatal mouse cardiomyocytes treated with or without angiotensin II (Ang-II). In particular, microRNA-489 (miR-489) was found to be most downregulated microRNA in response to Ang-II treatment. The authors used several methods to study the role of miR-489 in cardiac hypertrophy. First, they infected cardiomyocytes with adenoviruses encoding miR-489 to overexpress miR-489 and subsequently treated the cardiomyocytes with Ang-II. Second, they transfected cardiomyocytes with miR-489 antagonors (anta-489) to knockdown the expression of miR-489, followed by treatment of the cardiomyocytes with Ang-II. Third, they generated transgenic C57BL/6 mice overexpressing miR-489 in a cardiac-specific manner (miR-489 Tg mice) and induced a prohypertrophic condition in these mice by implanting osmotic Ang-II–containing minipumps. The authors assessed cardiac hypertrophy by measuring cell surface area, determining protein/DNA ratio, and using qRT-PCR to measure mRNA levels of the hypertrophic markers, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC).

To determine the target gene of miR-489, the authors performed luciferase 3′ untranslated region (3′UTR) reporter assays in human embryonic kidney 293 (HEK293) cells to screen the several genes associated with cardiac hypertrophy and identified myeloid differentiation primary response gene 88 (Myd88). The authors verified the ability of miR-489 to regulate the expression of Myd88 by infecting cardiomyocytes with adenoviruses encoding miR-489 or transfecting cardiomyocytes with anta-489 and performing immunoblot to measure the protein levels of Myd88. To confirm the interaction between miR-489 and the 3′UTR of Myd88, the authors utilized a biotin-labeled miR-489 pull-down assay to pull-down endogenous Myd88 as well as Argonaute 2 immunoprecipitation followed by affinity purification with biotinylated DNA probes complementary to the Myd88 3′UTR to pull-down endogenous miR-489. Moreover, the authors investigated the role of Myd88 in cardiac hypertrophy by knocking down Myd88 with adenviral Myd88 small interfering RNA (siRNA) in cardiomyocytes, which were subsequently treated with Ang-II. They also used C57BL/6 mice deficient of Myd88 (Myd88−/− mice) to determine the importance of Myd88 in promoting a hypertrophic phenotype in response to Ang-II treatment.

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To test whether lncRNAs may be involved in regulating the expression of miR-489, the authors used qRT-PCR to analyze the levels of 10 lncRNAs (shown to be expressed in the heart based on results from an Affymetrix lncRNA array) in cardiomyocytes treated with or without Ang-II. This analysis revealed that the lncRNA, AK048451, which the authors named CHRF, was the most upregulated lncRNA on Ang-II treatment. The authors investigated the effect of knocking down CHRF with adenoviral CHRF siRNA or overexpressing CHRF with adenoviral CHRF on miR-489 levels in cardiomyocytes. The authors also generated an miR-489 sensor construct containing a perfect miR-489 target cloned downstream of a firefly luciferase gene to assess the effect of knocking down or overexpressing CHRF on miR-489 activity. Next, the authors constructed a CHRF RNA luciferase construct (Luc-CHRF-wt) containing the site in CHRF complementary to miR-489 cloned downstream of a firefly luciferase gene and a construct (Luc-CHRF-mut) in which the predicted miR-489-binding site in CHRF was mutated to demonstrate the ability of CHRF to interact with miR-489. The authors further confirmed the interaction between CHRF and miR-489 by performing a pull-down assay using either biotin-labeled miR-489 or a biotin-labeled DNA probe complementary to CHRF RNA. Moreover, the authors investigated the effect of adenoviral knockdown or overexpression of CHRF on Myd88 protein levels in cardiomyocytes and the ability of the overexpression of CHRF to counteract the inhibitory effect of miR-489 on Myd88. Finally, to determine the role of CHRF in the regulation of hypertrophy, the authors assessed hypertrophic responses like cell surface area, protein/DNA ratio, and so on in response to adenoviral overexpression or knockdown of CHRF in cardiomyocytes as well as in mice treated with or without Ang-II.

**Principal Findings**

MicroRNA microarray analysis on neonatal mouse cardiomyocytes treated with or without Ang-II revealed that the levels of 9 microRNAs were significantly decreased in response to Ang-II treatment, with miR-489 showing the greatest downregulation. The authors confirmed the decrease in the levels of miR-489 in Ang-II–treated cardiomyocytes by qRT-PCR. Of note, downregulation of miR-489 levels was also observed in mice subjected to transverse aortic constriction and in human heart failure samples. The authors showed that adenoviral overexpression of miR-489 in cardiomyocytes attenuated the Ang-II–mediated induction of cell surface area, protein/DNA ratio, and mRNA levels of ANF, BNP, and β-MHC. On the other hand, transfection of cardiomyocytes with anta-489 to knockdown miR-489 increased cell surface area and protein/DNA ratio in the presence and absence of Ang-II treatment. The authors also found that miR-489 Tg mice infused with Ang-II exhibited an attenuated hypertrophic response (such as decreased cardiomyocyte size, reduced heart weight/body weight ratio, and decreased ANF, BNP, and β-MHC mRNA levels) and reduced cardiac fibrosis compared with wild-type mice infused with Ang-II. Conversely, administration of anta-miR-489 to wild-type mice infused with Ang-II resulted in a further increase in heart weight/body weight ratio, cardiomyocyte size, and BNP and β-MHC mRNA levels compared with wild-type mice infused with Ang-II treated with an antagonist negative control. Taken together, these findings suggest that miR-489 exhibits antihypertrophic properties and that inhibition of miR-489 promotes cardiac hypertrophy.

By conducting luciferase 3′ UTR reporter assays to screen genes associated with cardiac hypertrophy, the authors identified Myd88 as a candidate target of miR-489. Adenoviral overexpression of miR-489 reduced luciferase activity in HEK293 cells containing a luciferase construct in which the wild-type 3′ UTR of Myd88 was cloned downstream of the firefly luciferase gene. However, this decrease in luciferase activity was abrogated in HEK293 cells containing a luciferase construct in which the predicted miR-489–binding site in the Myd88 3′ UTR was mutated to disrupt base-pairing with miR-489. These results suggest that the 3′ UTR of Myd88 is a direct target of miR-489. The authors demonstrated that adenoviral overexpression of miR-489 in cardiomyocytes decreased the protein levels of endogenous Myd88, whereas the opposite was seen when cardiomyocytes were transfected with anta-489 to knockdown miR-489. Consistent with these findings, miR-489 Tg mice also showed reduced protein levels of Myd88 compared with wild-type controls. In a biotin-based pull-down assay, in which cardiomyocytes were transfected with biotinylated wild-type miR-489 or its mutated form that disrupted base-pairing with the Myd88 3′ UTR, the authors found that bound levels of Myd88 were much higher in the presence of the wild-type miR-489 than in the presence of the mutated form. In addition, Argonaute 2 immunoprecipitation followed by affinity purification with biotinylated DNA probes complementary to the Myd88 3′ UTR detected the presence of miR-489, providing further evidence for the interaction between miR-489 and Myd88 in vivo.

The authors observed that Myd88 protein levels were increased in cardiomyocytes treated with Ang-II compared with untreated cardiomyocytes. Protein levels of Myd88 were similarly upregulated in the hearts of mice subjected to transverse aortic constriction and in human heart failure samples. The authors found that cardiomyocytes infected with adenoviral Myd88 siRNA and subsequently treated with Ang-II displayed reduced cell surface area, protein/DNA ratio, and mRNA levels of ANF, BNP, and β-MHC compared with Ang-II–treated cardiomyocytes infected with a scrambled siRNA. Myd88−/− mice infused with Ang-II also showed an attenuated hypertrophic phenotype, reduced heart weight/body weight ratio, decreased cardiomyocyte size, and decreased ANF, BNP, and β-MHC mRNA levels compared with wild-type controls infused with Ang-II. Preserved cardiac function and reduction of cardiac fibrosis were also seen in Myd88−/− mice infused with Ang-II compared to wild-type controls infused with Ang-II. Moreover, administration of either miR-489 mimic or anta-489 to Myd88−/− mice infused with Ang-II had no effect on the hypertrophic phenotype, heart weight/body weight ratio, or cardiomyocyte size compared with Ang-II–treated Myd88−/− mice. The authors also demonstrated that miR-489 exerts its antihypertrophic effects by targeting the 3′UTR of Myd88 and downregulating the expression of Myd88.
Interestingly, by performing qRT-PCR to measure the levels of 10 lncRNAs known to be expressed in the heart, the authors observed that the lncRNA, AK048451 or CHRF, was the most upregulated lncRNA in cardiomyocytes treated with Ang-II. Levels of CHRF were also found to be increased in the hearts of mice subjected to transverse aortic constriction and in human heart failure samples. Knockdown of CHRF in cardiomyocytes resulted in elevated levels of miR-489 and reduced luciferase activity of the miR-489 sensor construct, whereas the opposite was seen when cardiomyocytes were infected with adenoviral CHRF. Although the overexpression of miR-489 in cardiomyocytes transfected with the miR-489 sensor construct decreased the luciferase activity of the construct, this decrease was abrogated when both CHRF and miR-489 were overexpressed, suggesting that CHRF can modulate the activity of miR-489, probably by binding to and sequestering miR-489. The authors demonstrated that adenoviral overexpression of miR-489 decreased the luciferase activity in HEK293 cells transfected with the Luc-CHRF-wt construct, but this decrease was attenuated in HEK293 cells transfected with the Luc-CHRF-mut construct. These results suggest that CHRF can directly interact with miR-489. Using biotin-based pull-down assays, in which cardiomyocytes were transfected with biotinylated wild-type miR-489 or its mutated form that disrupted base-pairing with CHRF, the authors showed that bound levels of CHRF were much higher in the presence of the wild-type miR-489 than in the presence of the mutated form. The authors were also able to pull-down miR-489 when they transfected cardiomyocytes with a biotin-labeled DNA probe complementary to CHRF RNA, presenting additional evidence for the interaction between CHRF and miR-489 in vivo.

Knockdown of CHRF by infecting cardiomyocytes with adenoviral CHRF siRNA resulted in decreased Myd88 protein levels, whereas adenoviral overexpression of CHRF increased the protein levels of Myd88 in cardiomyocytes. Overexpression of miR-489 together with CHRF in cardiomyocytes counteracted the ability of miR-489 to decrease Myd88 protein levels and its ability to reduce the luciferase activity of the Myd88 3′UTR luciferase construct mentioned earlier. Moreover, overexpression of CHRF in cardiomyocytes increased the cell surface area and protein/DNA ratio. In vivo, overexpression of CHRF increased the percentage of cardiomyocytes that were apoptotic. Mice injected with adenoviral CHRF siRNA exhibited increased miR-489 levels. In addition, knockdown of CHRF in mice attenuated the Ang-II–mediated induction in ANF and β-MHC mRNA levels, as well as cardiomyocyte size. Furthermore, overexpression of CHRF together with miR-489 in cardiomyocytes counteracted the ability of CHRF to increase cell surface area. Similarly, the knockdown of Myd88 in cardiomyocytes overexpressing CHRF counteracted the ability of CHRF to increase the protein/DNA ratio. Taken together, these findings suggest that CHRF promotes cardiac hypertrophy by interacting with its downstream target, miR-489, which in turn modulates the expression of Myd88 to affect hypertrophic responses.

**Implications**

This study uncovers a novel tripartite mechanism by which an lncRNA, CHRF, sequesters a microRNA, miR-489, inhibiting its ability to repress the expression of its target mRNA, Myd88, thereby promoting cardiac hypertrophy. These findings provide the first evidence that lncRNAs are key players in regulating cardiac hypertrophy and further highlights the important functions of noncoding RNAs, specifically microRNAs and lncRNAs, in cardiovascular biology. In addition to designing strategies to modulate the levels and activity of microRNAs, developing methods to manipulate the expression of lncRNAs could represent an alternative therapeutic approach for the treatment of cardiac hypertrophy and heart failure.

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**Disclosures**

None.

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