The H19 long noncoding RNA is a novel negative regulator of cardiomyocyte hypertrophy

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1. Introduction
Cardiac hypertrophy is an adaptive reaction of the heart against various stresses to maintain cardiac function at the early stage. However, sustained cardiac hypertrophy with maladaptive cardiac remodelling often leads to increased risk for heart failure and cardiac death. Although a variety of specific peptide hormones, growth factors, and miRNAs have been identified as the regulators of cardiac hypertrophy, the underlying molecular mechanisms of cardiac hypertrophy are still not fully understood.

Long non-coding RNAs (lncRNAs) are transcribed RNA molecules >200 nucleotides in length, but have no potential of protein-coding. LncRNAs have been shown to play important roles in various physiological processes, such as RNA processing, modulation of apoptosis and invasion, chromatin modification, and as a competing endogenous RNA (ceRNA). Recent studies have indicated that lncRNAs also function in heart development and diseases, while only a limited number of lncRNAs have been identified as the regulators of cardiac hypertrophy. For example, cardiac hypertrophy related factor is shown to regulate cardiac hypertrophy by targeting mir-489. Myosin heavy chain-associated RNA transcript (Mhrt), a cardiac-specific lncRNA, has been demonstrated to protect the heart from pathological cardiac hypertrophy. Therefore, further investigation on function of lncRNAs in cardiac hypertrophy is necessary for better understanding the regulation of cardiac homeostasis.
As one of the first identified imprinted genes, the lncRNA H19 gene has been verified as an important regulator in mammalian development and diseases.15,16 Exon 1 of H19 carries a miRNA containing hairpin, which has been found to act as the template for miR-675, and it has been shown that miR-675 can confer functionality on H19.17–19 A series of RNA-seq data from pathological cardiac remodelling have indicated that H19 is always up-regulated in hypertrophic hearts,20–22 suggesting its possible role in cardiac hypertrophy. Until now, the exact function of H19 in the heart is rarely known. In this study, we provided the first evidence to show that H19 could inhibit the hypertrophic growth of cardiomyocytes, suggesting a novel function of H19 as a negative regulator of cardiac hypertrophy.

2. Methods

2.1 Transverse aortic constriction

Transverse aortic constriction (TAC) surgeries were performed on male C57BL/6 mice (8 weeks old). The mice were anaesthetized with 2.0% iso-flurane (v/v) in 45 mL/min oxygen flow. After thoracotomy, the transverse thoracic aorta was dissected, and a 6-0 silk suture was tied around the aorta against a 26-gauge needle. The sham groups underwent a sham operation involving thoracotomy and aortic dissection without constriction of the aorta. Animals were sacrificed by cervical dislocation, and the hearts were harvested for analysis. The experiments were performed according to the protocols approved by the Animal Experiment Committee of the Institute of Biotechnology and conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Isolation, culture, and treatment of cardiomyocytes

Neonatal ventricular myocytes were isolated from 2-day-old pups, infected, and stained as described.23 In brief, a central thoracotomy was performed using a Nikon laser microscope (Eclipse E600, Nikon Instruments Inc., Japan). For each sample, more than five fields covering the whole slide were picked and counted.

2.4 Generation of adenovirus expressing wild type of H19 and H19 with deleted or mutant pre-miR-675 sequence

H19 cDNA was PCR-amplified from cDNA fragments of mouse hearts and inserted into the adenoviral vectors. The primers were as following: H19 sense: 5′-TTCTTCCGAGGCGGTGTCGGAAGGGGGGTT-3′; H19 antisense: 5′-TTTTCTGAAGCTATGAACTTATGTTGAGA-3′.

To generate truncation of H19 without pre-miR-675, the two fragments were amplified separately using the primers as following: H19-True sense: 5′-TTCTTACGTCGTGCTACTGATGGTGTG-3′; H19-True mid-antisense: 5′-TTTTCTGAAGCTATGAACTTATGTTGAGA-3′; and H19-True mid-sense: 5′-TTTTCTGAAGCTATGAACTTATGTTGAGA-3′, and then were ligated and inserted into the adenoviral vectors.

To generate H19 with mutant pre-miR-675, the sequences were changed as follows: TGTTGCGGAAAGGGCCACAGT was changed to CCAACTTCTAAAGGGGGCAGT, and CTGTATCGCCTTACGGCT CAGT was changed to CCAACCTTCAAGGGGGGCTAGT.

2.5 Cloning of CaMKIIb 3′-UTR and luciferase reporter assay

For target assay, we performed luciferase reporter experiments in the HEK293 cells. The 3′-UTR fragment of CaMKIIb was amplified using primers: 5′-TTTCTCAGACGGTACACTTCCACCA-3′ and 5′-TTTCTCAGG TATACAGACGCTCAAG-3′, and cloned into pGL3-CM luciferase reporter vector. The construct (Luc-CaMKIIb mutant) containing a mutated CaMKIIb-UTR (GCATAA was mutated to GAATTC) was also constructed. The luciferase activity was measured with a dual luciferase reporter assay kit (Promega, USA) that expresses a renilla luciferase used for normalizing transfection efficiency. After transfection, luciferase activities were measured with a dual luciferase reporter assay kit (Promega, USA), Lot No. E1910) using LB 960 Centro X53 luminometer (Berthold Technologies, GmbH & Co. KG, Germany).

2.6 Western blot

Western blots were carried out on myocardial extracts as described.28 Proteins (30 μg) were electrophoresed on SDS-PAGE and transferred onto PVDF membranes. Immunoblotting was performed according to manufacturer’s instructions using the following antibodies: CaMKIIb (Abcam, UK, Lot No. EPR13095), GAPDH (ZhongShanJinQiao, China, Lot No. TA-08), HDAC4 (Cell Signaling, USA, Lot No. #5392), and p-HDAC4 (Cell Signaling, USA, Lot No. #3424).

2.7 Real-time PCR

Total RNA was isolated from heart tissues and neonatal cardiomyocytes using TRIzol Reagent (Invitrogen, USA). cDNA was synthesized using SuperRT One Step RT–PCR Kit (CWBio, China) and subjected to real-time PCR using SYBR Green Real-time PCR Master Mix (TOYOBO, Japan) with 7500 Fast Real-Time PCR System (Applied Biosystems, USA), and GAPDH was used as a reference gene. The following primers were used: GAPDH, 5′-TGCCCAAGGACATCATCCCTCCT-3′ and 5′-GGTCTCTAGTTTGGAGGTG-3′; ANF, 5′-GCCGGTGAAGAATGATGGTCA-3′ and 5′-GGGGCTGAACTCTGTCAC-3′; Nppb, 5′-CTGAGGCGGTTGGCCTTAT-3′ and 5′-GGGCGGCTTATGATTT-3′; and MHC, 5′-GGGCGGCTTATTGGAC-3′ and 5′-GGGCGGCTTATTGGAC-3′.

miRNA real-time PCR was performed with miR-specific primers from the TaqMan miR assays (Applied Biosystems, USA) in the 7500 Fast Real-time PCR System (Applied Biosystems, USA) according to manufacturer’s protocol, and U6 was used as a reference gene.
2.8 Northern blot
Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed by 1% agarose gel electrophoresis in the presence of ethidium bromide. Northern blot analysis was performed as described using 30 μg total RNA from each sample.25 Probes were synthesized in Invitrogen Biotechnology Co., Ltd. (Beijing, China) as following: miR-675-3p, 5′-ACTGAGCGGTATGCGCATACAG-3′; and U6 probe, 5′-CGAATTTCGTTGCATCCTTGC-3′, was served as a loading control. Probes were labelled with 32P g-ATP using T4 polynucleotide kinase (New England Biolabs, Inc., Ipswich, MA, USA).

2.9 Antagomir application on TAC models
TAC surgeries were performed on male C57BL/6 mice (8 weeks old) as described. Chemically modified antisense oligonucleotides (antagomir) were synthesized by GenePharma Co. Ltd. (Suzhou, China). Treatments started 1 week after TAC, and animals received 0.2 mL saline, antagomir-675 (3 injections, 30 mg per kg body weight) via tail vein injections.

2.10 Histology and immunohistology
Heart tissues were fixed in 4% PFA at 4 °C overnight, embedded in paraffin, and sectioned at 5 μm. Sections were stained with haematoxylin and eosin (H&E), Masson trichrome, and laminin antibody (ZhongshanJinQiao, China, Lot No. ZA-0351) as standard protocols.

2.11 Statistical analysis
All statistical analyses were performed using SPSS software. Results are means ± SEM. Statistical differences between two groups were determined by Student’s t-test, and statistical differences among more than two groups were determined by ANOVA followed by SNK-q method. P-values of <0.05 were considered significant.

3. Results
3.1 H19 and its encoded miR-675 are up-regulated in pathological cardiac hypertrophy
To identify whether H19 was altered in cardiac diseases, we first detected H19 RNA level in a mouse model of cardiac hypertrophy induced by TAC. Consistent with the previously reported RNA-seq data,20-22 we found H19 RNA level was significantly up-regulated in hypertrophic hearts (Figure 1A; see Supplementary material online, (H&E), Masson trichrome, and laminin antibody (ZhongshanJinQiao, China, Lot No. ZA-0351) as standard protocols.

Figure 1 H19 and its encoded miR-675 were up-regulated in pathological cardiac hypertrophy. (A) Detection of H19 and miR-675 levels in heart samples from sham- and TAC-treated mice by real-time PCR. n = 3 independent experiments, *P < 0.05 vs. sham group. (B) Detection of H19 and miR-675 levels in PE-treated cardiomyocytes by real-time PCR. n = 5 independent experiments, *P < 0.05 vs. control group. (C) The expression levels of H19 and miR-675 during heart maturation after birth were detected by real-time PCR. n = 5 independent experiments, *P < 0.05. (D) Expression levels of H19 in cardiomyocytes and cardiac fibroblasts were detected by real-time PCR. Data represent means ± SEM. n = 3 independent experiments, *P < 0.05.
Phenylephrine (PE) has been well documented to induce cardiac hypertrophy. The H19 expression level was also found to be up-regulated in PE-treated cardiomyocytes (Figure 1B). Since H19 encodes miR-675-3p and miR-675-5p, and miR-675-3p is more abundant than miR-675-5p as seen from miRBase, we detected the expression of miR-675-3p (hereinafter referred to as miR-675) and found that it was also up-regulated under these pathological conditions (Figure 1A and B; see Supplementary material online, Figures S1A and B and S5A and F). Moreover, H19 and miR-675 were found to be up-regulated in human heart failure samples (see Supplementary material online, Figure S1C) but decreased in mouse model of physiological cardiac hypertrophy induced by tread-mill training (see Supplementary material online, Figure S1D). The expressions of H19 and miR-675 during postnatal heart maturation were found to be gradually down-regulated with ages after birth (Figure 1C). Detection of H19 in different cardiac cells showed that H19 was more abundant in myocytes than fibroblasts (Figure 1D). All these data suggested that H19 and its encoded miR-675 may participate in the development of pathological cardiac hypertrophy.

3.2 H19 inhibits the hypertrophic growth of cardiomyocytes

To determine the role of H19 in cardiac hypertrophy, we overexpressed H19 in neonatal cardiomyocytes using an adenovirus that contained mouse H19 (Ad-H19). Infection of Ad-H19 in cardiomyocytes successfully increased the expressions of H19 and miR-675 (Figure 2A; see Supplementary material online, Figure S2A and B). The cardiomyocytes infected with adenoviruses for 48 h were immunostained for α-actinin to study the morphological changes. Immunostaining revealed...
that PE treatment increased the size of cardiomyocytes, while overexpression of H19 reduced the cell size both at basal condition and in response to PE (Figure 2B and C). Similarly, the foetal genes such as atrial natriuretic factor (ANF), skeletal muscle and cardiac actin (SKA), β-myosin heavy chain (β-MHC), and brain natriuretic peptide (BNP) were also significantly down-regulated by H19 overexpression both at baseline and in response to PE (Figure 2D–G). To further examine the function of endogenous H19, a siRNA for mouse H19 was administered in mouse neonatal cardiomyocytes. Transfection of cardiomyocytes with siRNA-H19 significantly reduced the expressions of endogenous H19 and miR-675 (Figure 2H; see Supplementary material online, Figure S2C) and increased the cell size (Figure 2I and J) and mRNA expression of foetal genes at baseline (Figure 2K). These data indicated that H19 overexpression is sufficient to inhibit the hypertrophic growth of cardiomyocytes.

3.3 miR-675 inhibits the hypertrophic growth of cardiomyocytes

To test whether miR-675 also played an important role in cardiomyocyte hypertrophy, miR-675 mimics were administered in neonatal cardiomyocytes to induce miR-675 overexpression (see Supplementary material online, Figure S2D). The exogenous miR-675 reduced the cell size at baseline and attenuated PE-induced hypertrophic growth of cardiomyocytes, as seen by the morphological analysis and quantification of relative cell surface area (Figure 3A and B). Consistently, expression levels of ANF, SKA, and β-MHC were also repressed by miR-675 both at baseline and in response to PE (Figure 3C–E). We also knocked down endogenous miR-675 in cardiomyocytes using antiMiR-675 (see Supplementary material online, Figure S2E) and found that inhibition of miR-675 increased the cell size (Figure 3F and G) and mRNA expression levels of foetal genes at baseline (Figure 3H). These data demonstrated that miR-675 could negatively regulate the cardiomyocyte hypertrophy.

3.4 CaMKIIδ is a direct target of miR-675 in cardiomyocytes

Because miR-675 inhibited hypertrophic growth of cardiomyocytes, we anticipated that its target genes would include genes that induced cardiac hypertrophy. Target prediction led to the identification of a pro-hypertrophic factor, Ca/calmodulin-dependent protein kinase IIδ (CaMKIIδ), whose mRNA 3′-UTR region comprised the seed sequences and flanking nucleotides matching miR-675, which was highly

Figure 3 miR-675 inhibited the hypertrophic growth of cardiomyocytes. (A) Isolated neonatal cardiomyocytes were transfected with control or miR-675 mimics and treated with or without PE. Cardiomyocytes were stained for α-actinin (green) and Hoechst (blue). The scale bar represents 40 μm. (B) Fold change in mean cell surface area of α-actinin-immunostained cardiomyocytes (213 cells per condition; n = 4). *P < 0.05 vs. respective control group. (C–E) ANF, SKA, and β-MHC transcripts were detected by real-time PCR. n = 5 independent experiments, *P < 0.05 relative to respective control. (F) Isolated neonatal cardiomyocytes were transfected with antiMiR-ctrl or antiMiR-675. Cardiomyocytes were stained for α-actinin (green) and Hoechst (blue). The scale bar represents 40 μm. (G) Fold change in mean cell surface area of α-actinin-immunostained cardiomyocytes (215 cells per condition; n = 4). *P < 0.05. (H) Levels of ANF, SKA, and β-MHC were measured by real-time PCR. n = 5 independent experiments, *P < 0.05.
conserved among different species (Figure 4A). To confirm whether CaMKII\(\text{d}\) was a direct target of miR-675, we constructed luciferase reporter gene harbouring the normal or mutant type of 3′-UTR of CaMKII\(\text{d}\) and performed luciferase reporter assays in HEK293 cells. Co-transfection of miR-675 with the luciferase reporter gene linked to the wild-type 3′-UTR of CaMKII\(\text{d}\) strongly inhibited the luciferase activity, while no effect was observed with the construct harbouring a mutant segment of CaMKII\(\text{d}\) 3′-UTR (Figure 4B). Consistent with the luciferase results, the CaMKII\(\text{d}\) mRNA and protein levels were all down-regulated by transfection of cardiomyocytes with miR-675 (Figure 4C–E). In a reciprocal experiment, we inhibited the endogenous miR-675 in cardiomyocytes and observed the increased CaMKII\(\text{d}\) expression both at mRNA and protein levels (Figure 4C–E). These results suggested that miR-675 might inhibit cardiomyocyte hypertrophy by targeting CaMKII\(\text{d}\).

### 3.5 miR-675 mediates the anti-hypertrophic effect of H19 on cardiomyocytes

Since both H19 and miR-675 could inhibit the hypertrophic growth of cardiomyocytes, we hypothesized that miR-675 might mediate the anti-hypertrophic effect of H19. To test this hypothesis, we first knocked down miR-675 in H19-overexpressing cardiomyocytes by treating the cardiomyocytes with Ad-H19 together with either anti-miR-675 or anti-miR-ctrl. The results showed that inhibition of miR-675 could reverse the reduction of cardiomyocyte size (Figure 5A and B) and foetal genes expression induced by H19 overexpression (Figure 5C–F). We further detected the expression of CaMKII\(\text{d}\) and HDAC4, whose phosphorylation was induced by CaMKII\(\text{d}\),\(^{29}–^{31}\) and found that miR-675 inhibition could also reverse the reduction of CaMKII\(\text{d}\) and phosphorylation of HDAC4 induced by H19 overexpression (Figure 5G).

To further test this result, we also generated two adenoviruses containing the fragment of H19 without pre-miR-675 (Ad-H19-Tru) and the mutant type of H19 with mutant sequences of pre-miR-675 (Ad-H19-Mut) to infect neonatal cardiomyocytes. H19 but not miR-675 was successfully overexpressed in cardiomyocytes infected with Ad-H19-Tru or Ad-H19-Mut (see Supplementary material online, Figure S3A and B). Ectopic expression of these two types of H19 fragments lost the ability to reduce cardiomyocyte size and foetal genes expression (see Supplementary material online, Figure S3C–H). All these results suggested that H19-induced suppression of cardiomyocyte hypertrophy was mainly mediated by miR-675.

### 3.6 CaMKII\(\text{d}\) partially reverses the anti-hypertrophic action of H19 in cardiomyocytes

Since miR-675 mainly mediated the effect of H19 on cardiomyocyte hypertrophy, we then determined whether CaMKII\(\text{d}\) could reverse the inhibitory effect of H19 on cardiomyocyte hypertrophy. We found that overexpression of H19 reduced and inhibition of endogenous H19 induced the expression of CaMKII\(\text{d}\) (Figure 6A and B), while expression of mutant type of H19 did not alter the expression of CaMKII\(\text{d}\) in other treatments.
When simultaneously knocking down CaMKII\textsubscript{d} and H19 in cardiomyocytes, we found that CaMKII\textsubscript{d} down-regulation partially overcame the enhanced cardiomyocyte hypertrophy due to H19 inhibition, as measured by the morphological analysis of cardiomyocytes and foetal genes expression (Figure 6C–G). These results suggested that CaMKII\textsubscript{d} partially reversed the anti-hypertrophic action of H19 in cardiomyocytes.

**3.7 Inhibition of miR-675 exacerbates cardiac hypertrophy in a TAC mouse model**

To test the function of H19-miR-675 in vivo, we injected antagomiR-675 into mice subjected to pressure overload of the left ventricle by TAC and followed the disease for additional 3 weeks (Figure 7A). H19 and miR-675 were all found to be up-regulated in ventricles from TAC-operated mice (see Supplementary material online, Figure 5A–D). Considering the fundamental roles of cardiomyocytes and fibroblasts in the heart response against stress, we also detected the expression of H19 and miR-675 in the set of cells isolated from sham- or TAC-operated mice, and found that H19 and miR-675 were up-regulated in both cells under the condition of TAC. Although the extent of up-regulation was much more prominent in cardiomyocytes than in fibroblasts (see Supplementary material online, Figure 5E and F), it could not preclude the possibility that the up-regulation of these two genes in fibroblasts also played important roles in cardiac hypertrophy, and they need to be further investigated in future. TAC-induced miR-675 up-regulation was effectively abolished by antagomiR-675 treatments (Figure 7B). Significantly, inhibition of miR-675 enhanced the expression of CaMKII\textsubscript{d} (Figure 7C). TAC-operated mice demonstrated significant cardiac hypertrophy, characterized by enlarged heart, higher ratio of LV mass to the body weight (LVM/BW), increased...
cell cross-sectional area, and increased foetal genes expression, while these effects were accentuated in TAC-operated mice treated with antagomiR-675 (Figure 7D–G). All these results indicated that inhibition of miR-675 in vivo exacerbates cardiac hypertrophy in a TAC mouse model.

4. Discussion

Recent researches on lncRNAs have renovated our understanding about the regulation of cardiac diseases, but little is known about the function of lncRNAs in cardiac hypertrophy. H19, a non-protein-coding imprinted and maternally expressed lncRNA, is abundant in embryonic tissues of endodermal and mesodermal origin, but is thought to be expressed mainly in skeletal muscle and heart in adults. In contrast to increasing recognition of the role of H19 in tumour genesis and myoblast differentiation, how H19 functions in the heart remains unknown. In this study, we investigated the function of H19 in cardiomyocyte hypertrophy and the related molecular mechanisms. The important novel findings of our study are as follows: (i) H19 is up-regulated under stimulation of pathological stresses and inhibits cardiomyocyte hypertrophy both at baseline and in response to PE. (ii) H19-encoded miR-675 inhibits cardiomyocyte hypertrophy. (iii) miR-675 mediates the anti-hypertrophic effect of H19 on cardiomyocytes. (iv) CaMKII\(_d\) is a downstream target of H19-miR-675 axis in inhibiting hypertrophic growth of cardiomyocytes. (v) CaMKII\(_d\) partially mediated the effect of H19 on cardiomyocyte hypertrophy. (A and B) Detection of CaMKII\(_d\) mRNA level in cardiomyocytes by real-time PCR. \(n = 5\) independent experiments, *\(P < 0.05\). (C) Isolated neonatal cardiomyocytes were infected with si-H19 together with NC or si-CaMKII\(_d\). Cardiomyocytes were stained for \(\alpha\)-actinin (red) and Hoechst (blue). The scale bar represents 40 \(\mu\)m. (D) Fold change in mean cell surface area of \(\alpha\)-actinin-immunostained cardiomyocytes (236 cells per condition; \(n = 4\)). *\(P < 0.05\). (E–G) The levels of ANF, SKA, and \(\beta\)-MHC were measured by real-time PCR. Data represent means \(\pm\) SEM. \(n = 5\) independent experiments, *\(P < 0.05\).

Figure 6 CaMKII\(_d\) partially mediated the effect of H19 on cardiomyocyte hypertrophy. (A and B) Detection of CaMKII\(_d\) mRNA level in cardiomyocytes by real-time PCR. \(n = 5\) independent experiments, *\(P < 0.05\). (C) Isolated neonatal cardiomyocytes were infected with si-H19 together with NC or si-CaMKII\(_d\). Cardiomyocytes were stained for \(\alpha\)-actinin (red) and Hoechst (blue). The scale bar represents 40 \(\mu\)m. (D) Fold change in mean cell surface area of \(\alpha\)-actinin-immunostained cardiomyocytes (236 cells per condition; \(n = 4\)). *\(P < 0.05\). (E–G) The levels of ANF, SKA, and \(\beta\)-MHC were measured by real-time PCR. Data represent means \(\pm\) SEM. \(n = 5\) independent experiments, *\(P < 0.05\).
vitr experiments (Figure 1A; see Supplementary material online, Figure S2D), which might lead to insufficient inhibition of CaMKII d in TAC mouse model. On the other hand, CaMKII d can be activated by many other molecules, which might conquer the inhibitory effect of miR-675 on CaMKII d in response to pressure overload. In vivo inhibition of miR-675 in TAC mice could enhance the up-regulation of CaMKII d and accentuate the cardiac hypertrophy, verifying that miR-675 might inhibit cardiomyocyte hypertrophy by targeting CaMKII d. Supportively, CaMKII d was down-regulated by H19 overexpression, and inhibition of CaMKII d could partially rescue the cardiomyocyte phenotype caused by H19 inhibition, indicating that miR-675-regulated CaMKII d might mediate H19-induced inhibition of cardiomyocyte hypertrophy. In this context, our studies provided an important insight into how H19's function in cardiac hypertrophy was mediated by the miRNAs embedded within.

Altogether, our results revealed a novel function of H19-miR-675 axis targeting CaMKII d as a negative regulator of cardiomyocyte hypertrophy, providing new insights for understanding the function of IncRNAs in pathogenesis of cardiac hypertrophy.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**

Function of H19 in cardiac hypertrophy


