Overexpression of microRNA-1 impairs cardiac contractile function by damaging sarcomere assembly

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Aims
The purpose of the present study was to evaluate the effects of overexpression of microRNA-1 (miR-1) on cardiac contractile function and the potential molecular mechanisms.

Methods and results
Transgenic (Tg) mice (C57BL/6) for cardiac-specific overexpression of miR-1 driven by the α-mysin heavy chain promoter were generated and identified by real-time reverse-transcription polymerase chain reaction with left ventricular samples. We found an age-dependent decrease in the heart function in Tg mice by pressure–volume loop analysis. Histological analysis and electron microscopy displayed short sarcomeres with the loss of the clear zone and H-zone as well as myofibril fragmentation and deliquescence in Tg mice. Further studies demonstrated miR-1 post-transcriptionally down-regulated the expression of calmodulin (CaM) and cardiac myosin light chain kinase (cMLCK) proteins by targeting the 3′ UTRs of MYLK3, CALM1, and CALM2 genes, leading to decreased phosphorylations of myosin light chain 2v (MLC2v) and cardiac myosin binding protein-C (cMyBP-C). Knockdown of miR-1 by locked nucleic acid-modified anti-miR-1 antisense (LNA-antimiR-1) mitigated the adverse changes of cardiac function associated with overexpression of miR-1.

Conclusion
miR-1 induces adverse structural remodelling to impair cardiac contractile function. Targeting cMLCK and CaM likely underlies the detrimental effects of miR-1 on structural components of muscles related to the contractile machinery. Our study provides the first evidence that miRNAs cause adverse structural remodelling of the heart.

Keywords
miR-1 • Transgenic mice • Heart failure • CaM • cMLCK

1. Introduction
Progressive impairment of the cardiac contractile function is an indicator of heart failure as a result of adverse structural remodelling. However, how the structural remodelling is triggered and sustained is still poorly understood. Evidence from recent studies has been rapidly evolving for the critical roles of microRNAs (miRNAs) in regulating cardiac development and function.1–3 MicroRNA-1 (miR-1) stands out as a most prominent one, not only because it is a muscle-specific, cardiac-enriched miRNA, but also because it has diverse functions in the heart. The pioneer studies on miRNAs and heart showed that miR-1 was related to myoblast differentiation during embryonic development of the heart.4,5 Down-regulation of miR-1 has been observed in certain circumstances such as cardiac hypertrophy and chronic myocardial infarction (MI).6–8 These properties of miR-1 are ascribed to relief of repression of some growth-related and hypertrophy-associated genes. On the other hand, up-regulation of miR-1 has also been frequently seen in a variety of cardiac conditions, such as acute myocardial infarction (AMI),9–11 ischaemia/reperfusion injuries,12,13 ischaemic preconditioning,14 diabetic cardiomyopathy,15 oxidative stress,12 heat-shock insult,13 and hyperglycaemia.15 Coinciding with the up-regulation of miR-1 in cardiac tissues of AMI, the level of miR-1 was related to myoblast differentiation during embryonic development of the heart.4,5 Down-regulation of miR-1 has been observed in certain circumstances such as cardiac hypertrophy and chronic myocardial infarction (MI).6–8 These properties of miR-1 are ascribed to relief of repression of some growth-related and hypertrophy-associated genes. On the other hand, up-regulation of miR-1 has also been frequently seen in a variety of cardiac conditions, such as acute myocardial infarction (AMI),9–11 ischaemia/reperfusion injuries,12,13 ischaemic preconditioning,14 diabetic cardiomyopathy,15 oxidative stress,12 heat-shock insult,13 and hyperglycaemia.15 Coinciding with the up-regulation of miR-1 in cardiac tissues of AMI, the level...
of miR-1 is also markedly elevated in the plasma of MI patients as revealed by several research groups.\textsuperscript{12–20} Also, pharmacological down-regulation of miR-1 appears to produce cardioprotective outcomes.\textsuperscript{21–23} These studies suggested the importance to elucidate the effects of up-regulation of miR-1 on adult heart. However, despite the fact that miR-1 has been found up-regulated in diverse cardiac conditions, including those associated with prominent changes of cardiac contractility, the consequences of up-regulation are poorly studied with our current understanding limited to only the pro-arrhythmic and pro-apoptotic actions of miR-1.\textsuperscript{9,11,12} The purpose of this study was to evaluate whether overexpression of miR-1 could induce an impairment of the cardiac contractile function by having a direct effect on the sarcomeric function.

2. Methods

2.1 Animals

Adult male and female C57BL/6 mice and male Wistar rats were housed on a regular chow under controlled temperature of 23 ± 1°C and humidity of 55 ± 5%. Animals were maintained on 12 h dark–light artificial cycle (lights on at 07:00 A.M.) with food and water available ad libitum. Female mice used for microinjection of eggs and male mice used for heart functional detection and tail vein injection were anaesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal) and maintained by administering 0.5–1.0% isoflurane. The depth of anaesthesia was monitored by detecting reflexes, heart rate, and respiratory rate. Samples for morphological detection, real-time reverse-transcription polymerase chain reaction (qRT–PCR), and western blot assay were obtained from the left ventricles of mice after anaesthesia with sodium pentobarbital (100 mg/kg) and confirmation of death by exsanguination. Hearts for isolating ventricular myocytes were from neonatal Wistar rats after administration with 20% isoflurane and confirmation of death by cervical dislocation. All animal procedures were approved by the Institutional Animal Care and Use Committee at Harbin Medical University (No. HMUIRB-2008-06) and the Institute of Laboratory Animal Science of China (A5655-01). All procedures conformed to the Directive 2010/63/EU of the European Parliament.

2.2 Generation of miR-1 transgenic (Tg) mice

miR-1 transgenic (Tg) mice were generated as previously reported.\textsuperscript{24} Sexually immature female C57BL/6 mice (4–5 weeks of age) were used to obtain sufficient quantity of eggs (>250) for microinjection. The Tg mice used in this study were the fifth generation or later. Two-, 4-, and 6-month-old mice were investigated separately.

2.3 Detection of heart function

Heart functional parameters were measured in C57BL/6 male wild-type (WT) littermate mice at 2, 4, and 6 months of age and age-matched miR-1 Tg mice in the anaesthetized state, using a pressure–volume (PV) loops (Scisense, Ontario, Canada).\textsuperscript{25}

2.4 Synthesis of various oligonucleotides

miR-1, anti-miR-1 oligonucleotide (AMO-1), and the miRNA-masking antisense oligodeoxynucleotides (ODNs) were synthesized by Shanghai GenePharma Co., Ltd. Locked-nucleic-acid (LNA)-modified oligonucleotides (methylene bridge between the 2′-O and the 4′-C atoms) were synthesized by Exiqon (Denmark).

2.5 Injection of LNA-modified miR-1 antisense oligonucleotide (LNA-antimiR-1) into mice

LNA-antimiR-1 was delivered into control mice at 4 months of age and age-matched miR-1 Tg mice (8 mice for each group) through tail vein injection at a dose of 1 mg/kg once every 2 weeks for 8 weeks (i.e. four injections in 2 months). Measurements were made 15 days after the last (or the forth) injection when the mice were at an age of 6 months.

2.6 Preparation of cardiac myosin light chain kinase antibody (ARM-Mylk3 (150-164))

Polyclonal antibody was prepared. ‘CPGRKKSLADGPSPEENK’ was selected as the antigen for immunization.

2.7 Evaluation of morphological remodelling

Transmission electron microscopy (TEM) detection and hematoxylin and eosin (H&E) staining were performed as routing methods. TEM images were examined by a Hitachi H-7650 electron microscope. The heart-to-body-weight ratio (HW/BW) was calculated for each group. The length of sarcomeres was evaluated from 30 sarcomeres per heart sample (n = 6 animals) by Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA, USA).

2.8 Culture of neonatal rat ventricular cardiomyocytes (NRVCs)

The enzymatic dispersion technique was used to isolate single ventricle myocyte from neonatal Wistar rats. The cultures with 90 ± 5% myocytes, as assessed by microscopic observation of cell beating, were used for experiments.

2.9 Transfection procedures

Neonatal rat ventricular cardiomyocyte (NRVC) cells (2 × 10^6/well) were transfected with 2.5 μg miR-1, AMO-1, ODNs, or NC siRNAs with X-treme GENE siRNA transfection reagent (Cat.#04476093001, Roche). Forty-eight hours after transfection, cells were used for total RNA or protein purification.

2.10 RNA isolation and qRT–PCR for mRNAs

Total RNA samples were isolated from the left ventricles of mice using phenol/chloroform. The SYBR Green PCR Master Mix Kit was used in qRT–PCR to quantify target genes. GAPDH was used as an internal control. For ODNs and LNA-antimiR-1 experiments, total RNA samples were extracted using Trizol from NRVCs or the left ventricles of mice. The miR-1 level was quantified by the TaqMan® MicroRNA Reverse Transcription Kit and the TaqMan® MicroRNA Assay, and U6 was used as an internal control. The qRT–PCR was performed on 7500 FAST Real-Time PCR System (Applied Biosystems) for 40 cycles.

2.11 Western blot analysis

Protein samples extracted from the left ventricles of mice or cultured NRVCs were used for the immunoblotting analysis. GAPDH was used as an internal control. The final results were expressed as fold changes compared with the control values.

2.12 Luciferase reporter assay

The 3′UTRs of mouse MYLK3, CALM1, and CALM2 genes were obtained by PCR amplification. These fragments were cloned separately into the multiple cloning sites in the psi-CHECK™-2 luciferase miRNA expression reporter vector. HEK293T cells were transfected with 20 μmol/L miR-1, AMO-1, or negative control siRNAs (NC) as well as 0.5 μg psi-CHECK™-2-target DNA (firefly luciferase vector) and 1 μL blank plasmid, using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase activities were
measured with a dual luciferase reporter assay kit. Nucleotide-substitution mutations were carried out using direct oligomer synthesis for the 3′UTRs of MYLK3, CALM1, and CALM2. All constructs were sequence verified.

2.13 Data analysis
Data are presented as mean ± SEM. The ANOVA test was performed for statistical comparisons among multiple groups. Two-tailed Student’s t-test was applied for comparisons between the two groups. P < 0.05 was considered significant. SPSS13.0 was used for all statistical analyses.

For more detailed information, see Supplementary material online.

3. Results

3.1 Impairment of cardiac contractile and diastolic functions in miR-1 Tg mice

The miR-1 subfamily consists of miRNAs encoded by distinct genes that share nearly the same sequence and is designated miR-1-1 and miR-1-2. Since mature or functional miR-1-1 and miR-1-2 have an identical sequence and miR-1-2 has been used to generate both overexpression and knockout transgenic (Tg) mice in previous studies,5,26 we generated the Tg mouse line for cardiac-specific overexpression of miR-1-2 driven by the α-myosin heavy chain (α-MHC) promoter (see Supplementary material online, Figure S1A). The cardiac-specific overexpression of miR-1 in our Tg mice was verified (Figure 1A; see Supplementary material online, Figure S1B–E). The cardiac-specific overexpression of miR-1 in our Tg mice was verified (Figure 1A; see Supplementary material online, Figure S1B–E).

Compared with WT mice, ejection fraction (EF) was reduced by approximately 25.8 and 35.6% in Tg mice at 4 and 6 months of age, respectively (Figure 1B). Both of the impaired cardiac contractile and diastolic functions were indicated by decreased end-systolic pressure (ESP) and ± dp/dt max, as well as increased end-diastolic pressure (EDP) (Figure 1C–F; see Supplementary material online, Table S1). In addition, preload recruitable stroke work (PRSW) was also decreased in the Tg mice at 4 and 6 months of age, indicating depressed LV contractility (Figure 1G; see Supplementary material online, Table S1). Furthermore, the monoexponential time constant of relaxation (τ, Weiss), an index of LV relaxation, was found prolonged in the Tg mice, implying reduced diastolic function (Figure 1H). Qualitatively similar changes were observed in the 2-month-old Tg mice over the age-matched WT mice, although statistical significance of these changes was not attained.

3.2 Cardiac structural remodelling in miR-1 Tg mice

Although the miR-1 Tg mice survived well before 6 months of age, decreases in body weight and changes in the HW/BW ratios were observed compared with age-matched WT mice (Figure 2A and B). H&E staining showed increased heart size in the Tg mice at 4 and 6 months of age (Figure 2C). We did not find increases in cell surface area and myocardial fibrosis in the Tg mice compared with control (Figure 2D; see Supplementary material online, Figure S2). TEM images displayed shortened and uneven sarcomeric length as well as loss of clear zone and H-zone in the Tg mouse hearts (Figure 2E).

This observation was confirmed by quantification of the length and variation of sarcomeres (Figure 2F), suggesting a participation of remodelled sarcomeric assembly in the detrimental alterations of heart function in the Tg mice. In addition, severe myofibrillar fragmentation and deliquescence of cardiomyocytes were found in 6-month-old Tg mice hearts but not in 2-month- or 4-month-old Tg mice (Figure 2E), suggesting age-dependent damages of cardiac structure in miR-1 Tg mice.

3.3 Repression of MYLK3, CALM1, and CALM2 genes by miR-1

To understand how miR-1 could damage cardiac contractile function, we searched the potential target genes relevant to the issue by computational prediction from miRNA database. We found that miR-1 has
A binding site in the 3′ UTRs of MYLK3, CALM1, and CALM2 mRNAs containing highly conserved miR-1 seed-matching sequences (see Supplementary material online, Figure S3). MYLK3 encodes the cardiac myosin light chain kinase (cMLCK), and CALM1 and CALM2 encode the cardiac calmodulin (CaM) protein. We then cloned the miR-1-binding sites from the 3′ UTRs of MYLK3, CALM1, and CALM2 into the luciferase reporter plasmid separately, which contains a constitutively active promoter to determine the effects of miR-1 on reporter activities in HEK293T cells (see Supplementary material online, Figure S4). Co-transfection of miR-1 with the plasmid into HEK293T cells consistently produced less luciferase activities than transfection of the plasmid alone, whereas miR-1 failed to reduce the luciferase activity of the mutant 3′ UTRs of MYLK3, CALM1, and CALM2. Transfection of AMO-1 into the cells eliminated the silencing effects of miR-1 on the activities of the luciferase-wild-type 3′ UTR chimeric vectors (Figure 3A).

The repressive effects of miR-1 on cMLCK (Figure 3B) and CaM (Figure 3C) protein levels were confirmed by immunoblotting analyses in cultured NRVCs. Since the anti-cMLCK-specific antibody was generated in our lab, we also verified the specificity of the antibody (see Supplementary material online, Figures S5–S8). In all cases, AMO-1 rescued the down-regulation of proteins elicited by miR-1 transfection and scrambled negative control (NC) miRNA failed to affect the protein levels, indicating that the observed changes were specifically ascribed to miR-1 actions. The successful uptake of miR-1 into NRVCs after transfection was verified (see Supplementary material online, Figure S9A).

Consistently, we found that both mRNA and protein levels of cMLCK in the Tg mice were significantly decreased, relative to the control values from age-matched WT mice (Figure 3D; see Supplementary material online, Figure S9B). Similarly, the protein level of CaM was also decreased in the Tg mice (Figure 3E), but mRNA levels of CALM1 and CALM2, both encoding CaM, were similar in the Tg mice and age-matched WT mice (see Supplementary material online, Figure S9C).

In order to further confirm that the observed changes of CaM and cMLCK in both Tg mice and NRVCs were the direct results of miR-1 actions, we employed the miRNA-masking antisense ODN (miR-Mask) techniques previously developed.27 The anti-miRNA action of a miR-Mask is gene-specific because it is designed to be fully complementary to the target mRNA sequence of a miRNA (Figure 3F). It is important to note that this miR-Mask only acts on this gene but minimally affects the effects of the miRNA on other target genes.27 In our study, we designed three miR-1-Masks that can base pair the miR-1-binding sites in the 3′ UTRs of MYLK3, CALM1, or CALM2 genes labelled MYLK3-ODN, CALM1-ODN, and
CALM2-ODN. As expected, these miR-Masks, unlike AMO-1, failed to affect the miR-1 level when co-transfected with miR-1 (Figure 3F). MYLK3-ODN blocked the repressive effects of miR-1 on cMLCK but did not affect the effects on CaM (Figure 3G). On the other hand, when cells were co-transfected with miR-1 alone, miR-1, and AMO-1 together or NC, n = 8. (D) An age-dependent decrease in the cMLCK protein levels in Tg mice. n = 6. (E) Examples of western blot bands and mean values of band densities for CaM. n = 6. (F) Schematic of miR-1 silencing using the miRNA-masking antisense ODNs. Gene-specific ODNs (designed as 22-oligonucleotides fully complementary to the complete sequence of miR-1 target sites in the 3′ UTRs of target mRNAs) with high binding affinity completely mask the target sites of miR-1 in the 3′UTRs of target mRNAs, which block the repression of target mRNAs. ORF, open reading frame; AGO, argonaute. And lack of effects of gene-specific ODNs on miR-1 expression in NRVCs. The miR-1 level was quantified by qPCR. (G and H) Derepression of target genes of miR-1 by MYLK3-ODN, CALM1-ODN, or CALM2-ODN, respectively, determined by western blot analysis. n = 6. *P < 0.05 vs. Ctl or NC; #P < 0.05 vs. miR-1; mean ± SEM.

3.4 Reduction in p-MLC2v, p-CaMKIIδ, and cardiac myosin binding protein-C in miR-1 Tg mice

cMLCK is known to be critical to the phosphorylation of myosin light chain 2v (MLC2v),28 which has a unique function in the maintenance of cardiac contractility and sarcomeric assembly.29 Decreased phosphorylation of MLC2v can result in reduced cardiac diastolic function by defected assembly of the myosin thick filaments.30 We thought that MLC2v might be involved in this process. As displayed in Figure 4A, the protein levels of MLC2v were in the same range in Tg and WT mice at younger ages, and became lower in the Tg mice after 6 months of age, relative to the age-matched WT animals. However, the phosphorylation status of MLC2v in hearts from the Tg mice was reduced to 83.6 (2 months), 65 (4 months), and 57% (6 months) of the values from control mice, for all age groups tested. These results suggested that miR-1 directly affects MLC2v function via a mechanism independent of gene expression regulation.

CaM is an upstream activator of Ca²⁺-dependent CaM-activated protein kinase II (CaMKII)31 which is responsible for phosphorylation of cardiac myosin binding protein-C (cMyBP-C),32 a sarcomeric thick filament protein that interacts with titin, myosin, and actin to regulate sarcomeric assembly, structure, and function.33,34

Figure 3 Validation of miR-1 target genes. (A) Luciferase reporter gene assay for measuring interactions between miR-1 and its binding sites in the 3′UTRs of the MYLK3, CALM1, or CALM2 mRNAs in HEK293T cells. Cells were transfected with luciferase-target motif chimeric vector alone (Ctl) or with miR-1 or NC using lipofectamine 2000. n = 3. (B and C) Effects of miR-1 on protein levels of cMLCK and CaM, respectively, in primary cultured neonatal rat ventricular cells (NRVCs), using western blot analysis. Cells were transfected with miR-1 alone, miR-1, and AMO-1 together or NC. n = 8.
Reduced phosphorylation accompanied by increased cleavage of cMyBP-C has been reported to induce malformation of actomyosin cross-bridges so as to initiate changes in myofibril thick filament structure and, thereafter depart the interaction of myosin heads with actin thin filaments. In the present study, the phosphorylation level of CaMKIIα was significantly decreased in the Tg mice at 4 and 6 months compared with age-matched WT mice (Figure 4B), although the level of the CaMKIIa total protein was not changed at younger ages. Consistent with the reduced phosphorylation of CaMKII, the phosphorylated protein levels of cMyBP-C were decreased in hearts of Tg mice, compared with age-matched WT mice (Figure 4C). Although the total protein levels of cMyBP-C were also decreased, the changes were smaller than the phosphorylated cMyBP-C levels (Figure 4C).

3.5 Knockdown of miR-1 reverses structural remodelling and improves contractile function

To verify the specificity and the reversibility of miR-1 actions on cardiac contractile function, we treated the Tg mice with LNA-modified miR-1 antisense oligonucleotide (LNA-anti-miR-1), a technique that has been shown to efficiently knockdown target miRNAs with long-lasting efficacy under in vivo condition. The results showed that all aforementioned adverse changes were abolished in the Tg mice after tail vein injection with LNA-anti-miR-1 (Figure 5A–D; see Supplementary material online, Figure S10A–I). The inhibitory effect of LNA-anti-miR-1 on expression of miR-1 in Tg mice was verified and displayed in Supplementary material online, Figure S10f.

4. Discussion

In the present study, we used the Tg mouse line to achieve overexpression of miR-1 under the control of cardiac-specific α-MHC promoter for studying the possible remodelling process in adult hearts. We found that overexpression of miR-1 in Tg mice induces reduction in the heart function. The molecular mechanism may be that miR-1 post-transcriptionally inhibits the expression of cMLCK and CaM, which are structural components of muscles related to the contractile machinery.

It has been shown that overexpression of miR-1 in a Tg mouse model resulted in a phenotype characterized by thin-walled ventricles, attributable to premature differentiation and early withdrawal of cardiomyocytes from the cell cycle. This overexpression was under the control of the β-MHC which is dominant in the late foetal life but is almost completely replaced after birth by α-MHC and the influences of miR-1 on the cardiac function in the postnatal life to adulthood cannot be investigated with this model. In another study, adenoviral-mediated overexpression of miR-1 suppressed sarcomeric α-actin organization that is normally observed in serum-deprived cultured neonatal cardiac myocytes in vitro. However, these studies were not performed in adult hearts. Because, in mouse hearts, α-MHC is the dominant isoform over β-MHC in the adult, we performed the Tg mouse line to achieve overexpression of miR-1 under the control of cardiac-specific α-MHC promoter for studying the possible remodelling process in adult hearts. With this model, we demonstrated that overexpression of miR-1 in mouse hearts caused age-dependent decline in the cardiac function, as indicated by decreases in EF%, ESP, +dp/dtmax, and PRSW, as well as increases in EDP and τ. Notably, the decreased PRSW, an index of modification of the Frank–Starling law for left ventricular contractile function, suggested that miR-1 repressed heart function by damaging sarcomeric assembly. Indeed, our data also showed that miR-1 induced adverse structural remodelling of the heart, as revealed by electron microscopic examination showing shortened and uneven sarcomeric length, loss of clear zone and H-zone, and severe myofibrillar fragmentation and deliquescence of cardiac muscles in the Tg mice heart. Our findings suggest that, in addition to its recognized role in cardiac development, overexpression of miR-1 can cause adverse structural remodelling to impair cardiac contractile function directly by damaging sarcomere assembly.

Previous studies reported that fine tuning of phosphorylation of MLC2v was necessary for maintaining the normal structure of the sarcomere in vivo. The reduced phosphorylation of MLC2v directly

![Figure 4 Regulation of expression of proteins key to cardiac contractile function by miR-1 overexpression, determined by western blot analysis.](https://academic.oup.com/cardiovascres/article-abstract/95/3/385/378000/figure-4)
led to unstable, short myofilaments following defective assembly of the myosin thick filaments, which interrupted the basic structure for the Frank–Starling law and resulted in impairment of diastolic and systolic functions. Our study showed that phosphorylation of MLC2v was substantially abrogated in the miR-1 Tg mice. cMyBP-C was also reported to play a critical role in sarcomeric structure and function and in protecting the heart from ischaemia/reperfusion injury. The decreases in expression or dephosphorylation of cMyBP-C either could induce loss of H-zone in sarcomeres. In the present study, both decrease in dephosphorylation of cMyBP-C and loss of the sarcomeric H-zone were observed in the Tg mice.

cMLCK has been demonstrated to regulate sarcomeric structure in adult mice by phosphorylating MLC2v and activation of cMLCK is controlled by CaM. Moreover, CaMKII, which is automatically activated by CaM, phosphorylates MyBP-C. These proteins together form a network to finely regulate cardiac function. Dysfunction of these proteins can lead to the reduction in cardiac contraction. Thus, cMLCK serves as a specific kinase for MLC2v in a Ca\(^{2+}/\)CaM-dependent manner and regulates sarcomere assembly through the phosphorylation of MLC2v. Here, we found that the levels of both cMLCK and CaM were decreased in the miR-1 Tg mice and verified MYLK3 and CALM1/CALM2 coding for cMLCK and CaM, respectively, as targets for miR-1. It is conceivable that down-regulation of cMLCK and CaM may underlie the observed decreases in phosphorylations of MLC2v and cMyBP-C with overexpression of miR-1. Of particular, it has been reported that overexpression of miR-1 attenuated cardiomyocyte hypertrophy in vitro and in vivo by negatively regulating CaM and Ca\(^{2+}/\)CaM-CN-NFAT signalling. This result provided positive evidence not only for the post-transcription of miR-1 on CaM expression, but also for the absence of hypertrophic remodelling of the heart in miR-1 Tg mice displayed in the present study.

Our results allow us to establish a novel signalling mechanism by which overexpression of miR-1 impairs cardiac contractile function, as illustrated in Figure 6. Overexpression of miR-1 produces post-transcriptional repression of cMLCK and CaM, which attenuates phosphorylations of MLC2v, CaMKII, and cMyBP-C leading to destruction of sarcomeric assembly. These changes trigger an adverse structural remodelling process causing impairment of heart function. This is, to our knowledge, the first to study the regulation of the cardiac contractile function by miRNAs in the context of structural remodelling in adult hearts.

Our results provided evidence that chronic overexpression of miR-1 can produce progressive increases in damages of heart function over time. Intriguingly, overexpression of miR-1 has been identified in a number of cardiac conditions associated with the pathogenesis of the heart. In patients with AMI, the miR-1 level was found upregulated by 2.8-fold in one study and 3.8-fold in another with the samples of infarcted tissue and remote myocardium from 24 patients with AMI. In diabetic cardiomyopathy of a rat model of type I diabetes mellitus, miR-1 transcript was increased up to six times. In our Tg mice, the miR-1 level was ~4-fold higher...
than that in WT animals. This amount of overexpression falls well within the range of up-regulation of miR-1 under various diseased states described above. This coincidence points to a possibility that the structural remodelling mechanisms leading to impairment of cardiac function observed in our miR-1 Tg mouse hearts may well operate in the real pathological conditions mentioned above. A recent study displayed that down-regulation of miR-1 can induce heart failure via sorcin-dependent calcium homeostasis by the tamoxifen-inducible cardiac-specific Dicer knockdown model in adult mice. These results suggested that both up- and down-regulation of miR-1 might result in abnormal heart function, although the molecular mechanisms were different. Moreover, the fact that LNA-antimiR-1 is able to rescue the damaging actions of miR-1 in this study suggests knockdown of miR-1 using the miRNA-loss-of-functional approach to be a rational strategy to reverse the adverse changes of cardiac function associated with aberrant up-regulation of miR-1. The findings in the present study also provide mechanistic explanations for the beneficial effects of pharmacological inhibition of miR-1 expression.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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