Down-regulation of miR-1/miR-133 Contributes to Re-expression of Pacemaker Channel Genes HCN2 and HCN4 in Hypertrophic Heart

* This work was supported in part by the Canadian Institute of Health Research, Fonds de la Recherche de l’Institut de Cardiologie de Montreal (to Z. W.) and by the National Basic Research Program of China (973 Program; 2007CB512000/2007CB512006) (to B. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence may be addressed: Research Center, Montreal Heart Institute, 5000 Belanger E., Montreal, PQ H1T 1C8, Canada. Tel.: 514-376-3330; Fax: 514-376-4452; E-mail: wz.email@gmail.com.

Xiaobin Luo‡§, Huixian Lin†, Zhengwei Pan*, Jiening Xiao†, Yong Zhang†, Yanjie Lu†, Baofeng Yang*†, and Zhiguo Wang*‡§

From the ‡Research Center, Montreal Heart Institute, Montreal, PQ H1T 1C8 Canada, the §Department of Medicine, University of Montreal, Montreal, PQ H3C 3J7, Canada, and the ¶Department of Pharmacology (the State-Provence Key Laboratories of Biomedicine-Pharmaceutics of China) and Institute of Cardiovascular Research, Harbin Medical University, Harbin, Heilongjiang 150086, P.R. China

Cardiac hypertrophy is characterized by electrical remodeling with increased risk of arrhythmogenesis. Enhanced abnormal automaticity of ventricular cells contributes critically to hypertrophic arrhythmias. The pacemaker current, carried by the hyperpolarization-activated channels encoded mainly by the HCN2 and HCN4 genes in the heart, plays an important role in determining cardiac automaticity. Their expressions reportedly increase in hypertrophic and failing hearts, contributing to arrhythmogenesis under these conditions. We performed a study on post-transcriptional regulation of expression of miR-1/miR-133 and HCN2/HCN4 genes in the heart, plays an important role in determining cardiac automaticity. Their expressions reportedly increase in hypertrophic and failing hearts, contributing to arrhythmogenesis under these conditions. We performed a study on post-transcriptional regulation of expression of miR-1/miR-133 and HCN2/HCN4 genes by microRNAs. We experimentally establish miR-1/miR-133 and HCN2 as a target for repression by the muscle-specific microRNAs miR-1 and miR-133 and established miR-1 only. We unraveled robust increases in miR-1/miR-133 protein levels in a rat model of left ventricular hypertrophy in angiotensin II-induced hypertrophic cardiomyopathy. The up-regulation of miR-1/miR-133 by transfection prevented overexpression of HCN2/HCN4 in hypertrophic cardiomyocytes. The serum-reconstituted left ventricles, termed hyperpolarization-activated current (Ih), plays a critical role in determining ventricular automaticity. This results in a loss of the capacity of adult ventricular cells to generate spontaneous activity. Strikingly, substantial up-regulation of Ih expression has been observed in a variety of animal models of cardiac hypertrophy and heart failure and in human failing hearts as well (1–3, 9–16). In such circumstances, Ih is at least doubled in left ventricular (LV) cells, depending upon severity of conditions (3, 11, 14) and reaches values comparable with those observed in the neonatal stage. Consistently, prominent re-expression of the Ih-encoding genes, belonging to the hyperpolarization-activated cyclic nucleotide-gated channel (HCN) gene family in ventricular myocytes of hypertrophic heart has also been documented (17–19). The HCN family is composed of at least four members (HCN1–HCN4), of which HCN4 is believed to be the major molecular component for sinus Ih and HCN2 is believed to be the dominant isoform for ventricular Ih. However, mechanisms underlying the pathological re-expression of Ih and HCN2/HCN4 remained yet to be elucidated.

The adult heart is susceptible to stress (such as hemodynamic alterations associated with myocardial infarction, hypertension, etc.) by undergoing remodeling process and hypertrophic growth to adapt to altered workloads and impaired cardiac function. The remodeling process in hypertrophic hearts includes electrical remodeling (1), characterized by a reprogrammed electrical activity, and the reactivation of genes characteristic of arrhythmogenesis.
Expression Regulation of HCN2 and HCN4 by miRNAs

Discovery of microRNAs (miRNAs) has revolutionized our understanding of the mechanisms that regulate gene expression (20–22). miRNAs are endogenous ∼22-nucleotide non-coding RNAs that anneal to inexact complementary sequences in the 3′-UTRs of target mRNAs of protein-coding genes to specify translational repression or/mRNA cleavage. Among >500 mammalian miRNAs identified thus far, miR-1 and miR-133 are believed to specifically express in adult cardiac and skeletal muscle tissues where miR-1 and miR-133 importantly regulate myogenesis (21, 23, 24). Recent studies demonstrated that both miR-1 and miR-133 are significantly down-regulated in hypertrophic and failing hearts (25–27), and down-regulation of either miR-1 or miR-133 can determine the pathogenesis of cardiac hypertrophy. More strikingly, correction of the down-regulation by forced expression of these miRNAs reversed the pathological process. This study was designed to shed light on the underlying mechanisms for post-transcriptional repression of HCN2 and HCN4 genes.

EXPERIMENTAL PROCEDURES

Synthesis of miRNAs and Anti-miRNA Antisense Inhibitors—miR-1 and miR-133, their respective mutant constructs, and their antisense inhibitor oligonucleotides (AMOs) (see Fig. 1C) were synthesized by Integrated DNA Technologies, Inc., as described previously (28–31).

Construction of Luciferase Reporter Vector Carrying miRNA Target Site—To construct reporter vectors to verify the exact target sites for miR-1 and miR-133, 3′-UTR mutations by PCR and antisense AMOs were designed to verify the exact target sites for miR-1 and miR-133. All of the luciferase gene vectors used in this study, as previously described in detail (28–31). miRNA expression reporter vectors were manufactured from Ambion, Inc.

Mutagenesis—Deletion mutations were created by direct PCR-based methods. Mutations were introduced into HCN2 and HCN4 genes (see Fig. 1D) (28–31).

Cell Culture—HEK293 (human embryonic kidney) cells used in this study was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium.

Hypertrophic Model—LV hypertrophy was induced by an aortic stenosis technique, similar to that described by Fernández-Velasco et al. (3, 12). Briefly, adult male Sprague-Dawley rats weighing 180–200 g were used. The animals were anesthetized via an intraperitoneal injection of sodium pentobarbital (50 mg/kg). A silver clip was placed around (0.3-mm aperture) the abdominal aorta above the left renal artery. The LV pressure was progressively developed as the animals grew, and LV hypertrophy was established at 8 weeks after surgery. Sham-operated and age-matched rats were used as control. Hypertrophied hearts were defined as the ratio of heart weight (wet) to body weight >25% of control hearts. The use of animals was in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Harbin Medical University.

Myocyte Isolation and Primary Cell Culture—Neonatal rat ventricular cardiomyocytes were isolated and cultured as described previously (28).

Angiotensin II-induced Hypertrophy and Cell Surface Area Analysis of Cardiomyocytes—Isolated neonatal rat ventricular cells were cultured for 24 h in serum-containing medium after which they were serum-starved for 24 h before treatment. To induce hypertrophy, cells were stimulated by angiotensin II (AngII, 1 μM) for 36 h in serum-free culture medium (32, 33). Cell surface area was analyzed using a Leica inverted microscope equipped with a Polaroid digital camera at ×200 magnification.

Small Interference RNAs (siRNAs) Synthesis and Treatment—An siRNA directed against serum-response factor (SRF) was designed to target the coding region of the SRF mRNA (position 1864, TTGGCACAGTGAGATCCCTGTTTCA): 5′-UGAAAACAGGAUCUGACUGCCCA-3′ (SRF-siRNA). A negative control siRNA (NC siRNA) was also designed by replacing eight nucleotides as indicated below by the bold and italic letters: 5′-UTCCGTAGGGAUCUTAAAC-3′ (NC-siRNA). These siRNAs were synthesized by Invitrogen and transfected into cultured neonatal rat ventricular cardiomyocytes (Invitrogen). Transfection for Western blot and real-time RT-PCR miRNA expression regulation was performed using the luciferase activities of cells with 420 A-microgram/siRNA assayed by the Promega dual luciferase assay kit (Promega). Statistical comparisons (performed using analysis of variance followed by Dunnett’s method) were carried out using Microsoft Excel. A two-tailed p < 0.05 was taken to indicate a statistically significant difference.

RESULTS

Post-transcriptional Repression of HCN2 and HCN4 Expression by miR-1 and miR-133—Neither HCN2 nor HCN4 is listed as candidate targets for miR-1 and miR-133, according to the prediction by TargetScan hosted by Wellcome Trust Sanger Institute (34). However, by detailed analysis of the 3′-UTR of HCN2, we identified one putative target site for miR-1 and one for miR-133, based on complementarity to the 5′-ends of the
Expression Regulation of HCN2 and HCN4 by miRNAs

The sequences of miR-1/miR-133 and the AMOs are shown because our experiments were conducted with rat cells, and the GenBank accession numbers are included in the table. miR-1 and miR-133 and the complementaris with the mutated target sites in the 3'-UTRs of HCN2/HCN4, however, it does not produce any effects on luciferase expression. Co-application of miR-1 with AMO-1 (anti-miR-1 antisense) or AMO-133 (anti-miR-133 antisense) eliminated the silencing effects on luciferase reporter activities. miR-1 elicited significant repression on luciferase activity with the 3'-UTR of HCN4 (Fig. 2B) despite the fact that the 3'-UTR of HCN4 matches the center portion but not the 5' end of miR-1. As expected, miR-133 did not produce any effects on luciferase expression.

We then observed that the mutated target sequences of HCN2 fused to the 3'-UTR of luciferase were not responsive to transfection of the chimeric plasmid alone. As expected, miR-1 did not affect the luciferase activity when the putative site for miR-1 in the 3'-UTR of HCN4 was mutated, but the mutant miR-1 that matches the mutant HCN4 (Fig. 1D) elicited remarkable reduction of the luciferase activity (Fig. 2C).

To see whether HCN2/HCN4 repression by the miRNAs reported by luciferase assays has any functional implications,
Expression Regulation of HCN2 and HCN4 by miRNAs

we determined the effects of the miRNAs on endogenous expression of HCN2/HCN4 at the protein level in neonatal rat ventricular myocytes by Western blot analysis. Transfection of either AMO-1 or AMO-133 at 100 nM did not cause significant changes of HCN2 protein level (Fig. 2D). Co-transfection of AMO-1 and AMO-133, however, was able to increase the level by ~87 ± 6.5% (p < 0.05). By comparison, application of AMO-1 alone increased HCN4 protein level by 46 ± 3.7%, and AMO-133 did not produce any effects (Fig. 2E). Co-transfection of AMO-1 and AMO-133 produced a similar effect as AMO-1 alone. Transfection of mir-1 or mir-133 alone produced remarkable depression of HCN2 (Fig. 2D), but only mir-1 and not mir-133 decreased HCN4 level (Fig. 2E). On the other hand, AMO-1 and AMO-133, transfected alone or together, failed to produce any significant effects on HCN2 and HCN4 mRNA levels (Fig. 2, F and G), indicating that the miRNAs do not affect HCN2/HCN4 mRNA stability.

Finally, to confirm that the observed effects of AMO-1 and AMO-133 were ascribed to their anti-miR-1 or anti-miR-133 actions, we analyzed the changes of endogenous mir-1 and mir-133 upon transfection of the AMOs since AMOs have been shown to degrade their target miRNAs (28–31, 35). Our data indeed support this notion; as shown in Fig. 2H, AMO-1 and AMO-133 significantly knocked down the levels of mir-1 and mir-133, respectively. For cross comparisons, AMO-1 did not affect mir-133 level, and neither did AMO-1 affect mir-1 level. Moreover, to ensure the specificity of the probes (primers) for quantifying mir-1 and mir-133 levels, we transfected the mutant mir-1 and mir-133 constructs (Fig. 1A) into neonatal cells. Our data showed that the mutants did not cause any significant alteration in the levels of mir-133 and miR-133 levels relative to non-transfected.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.

Role of Decreased SRF in Overexpression of f-Channel Gene—Because the hypertrophic left ventricle (HLV)—HCN2/HCN4 overexpression is mediated by 3-fold and HCN4 overexpression is caused by AngII (Fig. 3A). To determine if AngII decreases miR-133 in hypertrophy, we quantified the miR-133 levels in HLV was significantly decreased in hypertrophic neonatal rat ventricular myocytes. AngII markedly increased the expression of both HCN2/HCN4 mRNA stability.
comitantly, SRF-siRNA treatment robustly increased HCN2 and HCN4 protein levels (Fig. 5C). The efficacy of SRF-siRNA to silence SRF expression was verified at both mRNA and protein levels in rat cells (Fig. 5D). The negative control siRNA (NC siRNA) failed to alter miR-1/miR-133, HCN2/HCN4, and SRF expressions (Fig. 5, B–D). As an additional control for the SRF-siRNA actions, we performed experiments testing the effects of the SRF-siRNA on the mRNA levels of two housekeeping genes GAPDH and Sp1 (stimulating protein) (36). As shown in Fig. 5E, the SRF-siRNA failed to alter the mRNA levels of these genes. In addition, as a positive control, we also assessed the effects of the SRF-siRNA on the mRNA level of α-MHC since it has been shown that the promoter region of α-MHC gene contains an SRF binding site and that SRF can activate α-MHC transcription (37). The data in Fig. 5F confirm that the SRF-siRNA indeed reduced the transcript level of α-MHC, whereas the NC siRNA failed to do so. These results are consistent with the long recognized down-regulation of α-MHC in hypertrophic hearts in light of the fact that SRF is also down-regulated in hypertrophic heart as shown in this study (Fig. 5A).

Finally, to confirm that the myocytes treated with angiotensin II indeed developed hypertrophic conditions, we measured the changes of cell size and expression of several biomarkers for cardiac hypertrophy including β-MHC, MLC-2, and ANF (38). As depicted in Fig. 6, A and B, the cell surface area was significantly enlarged, and β-MHC, MLC-2, and ANF were all up-reg...
Expression Regulation of HCN2 and HCN4 by miRNAs

A

Western Blot

SRF
GAPDH

68 kDa
35 kDa

HLV

Ctl

B

miRNAs

miR-1
miR-133

Relative Level

0.8
1.2

0.8
1.2

C

HCNs

miR-1
miR-133

Relative Level

0.8
1.2

0.8
1.2

D

Western Blot

SRF
GAPDH

68 kDa
35 kDa

AP

Ctl

SRF-siRNA

E

GAPDH

Relative Level

1.5
1.2

1.5
1.2

F

Relative Level

0.8
1.2

0.8
1.2

FIGURE 5. Role of decreased SRF expression in down-regulation of HCN2 and HCN4 levels in hypertrophic cardiomyocytes, when compared with the untreated control cells (Ctl). Values are mean ± S.E. (n = 6 for each group). A, surface area of hypertrophic cardiomyocytes, as determined by Western blotting analysis. The values were calculated by first normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for internal control and then by further normalizing to non-hyper trophy control mRNA levels. *, p < 0.05 versus Ctl; †, p < 0.05 versus AngII; n = 5 for each group. The effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. B, the effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. *, p < 0.05 versus Ctl; †, p < 0.05 versus AngII; n = 5 for each group. The effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. C, the effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. *, p < 0.05 versus Ctl; †, p < 0.05 versus AngII; n = 5 for each group. The effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. D, the effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. *, p < 0.05 versus Ctl; †, p < 0.05 versus AngII; n = 5 for each group. The effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. E, the effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. *, p < 0.05 versus Ctl; †, p < 0.05 versus AngII; n = 5 for each group. The effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. F, the effects of SRF siRNA on expression of α-MHC gene at the mRNA level were quantified by real-time RT-PCR methods. *, p < 0.05 versus Ctl; †, p < 0.05 versus AngII; n = 5 for each group.

One of the characteristic alterations of hypertrophic hearts is the up-regulation of HCN2 and HCN4 expression or overexpression of pacemaker channel genes, and thereby I_{f} current, which likely contributes importantly to the enhanced abnormality and the risk of arrhythmias under such pathological conditions (1–3, 9–16). However, the mechanisms remained poorly understood. In this study, we demonstrated significant overexpression of HCN2 and HCN4 in LV hypertrophy induced by angiotensin II. We experimentally established HCN2 and HCN4 genes as targets for post-transcriptional repression by the muscle-specific miRNAs miR-1 and miR-133. The up-regulation of HCN2 and HCN4 was accompanied by substantial decreases in miR-1/miR-133 mRNA levels. Moreover, correction of miR-1/miR-133 down-regulation prevented overexpression of HCN2/HCN4 in hypertrophy myocytes. We conclude that down-regulation of miR-1 and miR-133 expression contributes to the re-expression of HCN2/HCN4, and thereby the electrical remodeling process in hypertrophic hearts. Our study also sheds new light on the cellular function of miR-1 and miR-133 in the heart and on the regulation of ion channel genes by these miRNAs.

ulated in their expression. To exclude the possibility of repression of β-MHC, MLC-2, and ANF by miR-1/miR-133 underlying the hypertrophic changes of HCN2/HCN4 expression, we demonstrated the lack of ability of miR-1/miR-133 to alter the protein levels of β-MHC, MLC-2, and ANF in cultured rat myocytes (Fig. 6C). In agreement, bioinformatics analysis using miRBase and miRanda websites revealed the absence of miR-1 or miR-133 binding sites in the whole sequences of β-MHC, MLC-2, and ANF genes.
miR-1 and miR-133 are expressed in a chamber-specific manner during cardiogenesis and are activated during the period of differentiation (23, 24). Increasing expression of miR-1 and miR-133 has been found in neonatal hearts, and substantially higher levels are maintained in adult cardiac tissues (24). This may at least partially account for the decreased I_\text{f} density in adult ventricular cells (1–3) for enhanced miR-1/miR-133 expression limits HCN2/HCN4 expression. When this limiting factor becomes weaker as in hypertrophic myocytes where miR1-/miR-133 levels drop, HCN2/HCN4 re-express. The inhibitory effects of miR-1/miR-133 on HCN2/HCN4 expression are primarily ascribed to post-transcriptional inhibition of translation of HCN2/HCN4 mRNAs into their respective protein products. Decreases in miR-1 and miR-133 hypertrophic hearts found in this study are consistent with the recent studies (25–27). The present study represents a novel insight into the pathophysiological role of miR-1/miR-133 by regulating HCNs in the heart.

Our results indicate that as suggested previously, complementarity with six consecutive nucleotides matching the 5'-end of a miRNA is adequate for a target mRNA to interact with the miRNA leading to translational repression (22). This form of complementarity has been described as “seed site.” Notably, the 3'-UTR of HCN4 does not contain such complementary sequence; instead, it has a stretch of nucleotides complementary to the middle portion of miR-1, and some of base pairing obviously is sufficient for miR-1/miR-133 to target HCN4 and to elicit the consequence. This form of complementarity confers similar degrees of repression with the seed site strongly suggested by a previous study. Noticeably, to obtain a substantial decrease in the HCN2 protein level, co-transfection of AMO-1 and AMO-133 is required, and either of the two AMOs alone failed to affect HCN2 level. This is likely a simultaneous repression by the two miRNAs, as the expression of either of the miRNAs is not sufficient to relieve the post-transcriptional repression of HCN2 and to elicit the consequence. The complementarity confers similar degrees of repression with the seed site strongly suggested by a previous study. Noticeably, to obtain a substantial decrease in the HCN2 protein level, co-transfection of AMO-1 and AMO-133 is required, and either of the two AMOs alone failed to affect HCN2 level. This is likely a simultaneous repression by the two miRNAs, as the expression of either of the miRNAs is not sufficient to relieve the post-transcriptional repression of HCN2 and to elicit the consequence.

The present study aimed at understanding the mechanisms for the re-expression of HCN2 and HCN4 in hypertrophic conditions, and our findings indicate the altered expression of miR-1/miR-133 as one of the important factors. The lack of effects of miR-1 and miR-133 on expression of ANF, \( \beta \)-MHC, and MLC-2 indicates that the link between down-regulation of miR-1/miR-133 and up-regulation of HCN2/HCN4 is likely direct and that other hypertrophic factors may not be directly involved in the relationships. It should be emphasized, however, that regulation of HCN2/HCN4 expression must be a complex process, and our study merely suggests the potential contributions of miR-1/miR-133 to this process but does not at all exclude other regulatory factors.

We have also quantified the changes of HCN2 and HCN4 expression at the mRNA level in hypertrophic hearts. Our data showed that the mRNA levels of HCN2 and HCN4 were also elevated but to much lesser extents relative to the increases at the protein level, with 172 ± 21 and 50 ± 13% increases in HCN2 and HCN4 mRNA levels, respectively, when compared with 300 ± 26 and 173 ± 12% increases in their respective protein levels. This discrepancy can be easily explained by a relief of the post-transcriptional repression of HCN2 and HCN4 expression due to down-regulation of miR-1 and miR-133. However, our study does not provide the mechanisms for the up-regulation of HCN2 and HCN4 at the transcriptional level.

Our study also revealed down-regulation of SRF expression as a potential mechanism for the up-regulation of HCN2 and HCN4 expression via down-regulation of miR-1/miR-133 in hypertrophic myocytes. Expression of miR-1/miR-133 has previously been shown to critically depend upon SRF binding to their promoter regions (23, 24). In agreement with the changes of SRF expression in hypertrophic heart, in failing hearts of both humans and rabbits, a substantial decrease in expression of full-length SRF accompanied by robust expression of a low molecular-mass domain of the full-length SRF isoform has been documented (39, 40).

Our findings also suggest that expression of miR-1 and miR-133 can directly inhibit the expression of SRF by suppressing overexpression of its mRNA. We have developed experimentally designed non-base-paired nucleotides complementary to the target genes to investigate the efficacy of miRNAs targeting f-channel expression in a chamber-specific manner.

Acknowledgements. We thank Drs. Nestor F. DiCiolla, F., Davoli, G., Sani, G., and Mugelli, A. (2001) Am. J. Physiol. 280, H142–H150
2. Cerbai, E., Sartiani, L., DePaoli, P., Pino, R., Maccherini, M., Bizzarri, F., DiCiolla, F., Davoli, G., Sani, G., and Mugelli, A. (2001) J. Mol. Cell. Cardiol. 3, 441–448
3. Fernandez-Velasco, M., Goren, N., Benito, G., Blanco-Rivero, J., Bosca, L., Delgado, C. (2003) J. Physiol. (Lond.) 553, 395–405
4. Cerbai, E., Pino, R., Sartiani, L., and Mugelli, A. (1999) Cardiovasc. Res. 42, 416–423
5. Yasui, K., Liu, W., Opthof, T., Kada, K., Lee, J. K., Kamiya, K., and Kodama, I. (2001) Circ. Res. 88, 536–542
6. Robinson, R. B., Yu, H., Chang, F., and Cohen, I. S. (1997) Pfluegers Arch. Eur. J. Physiol. 433, 533–535
7. Abi-Gerges, N., Ji, G. J., Lu, Z. J., Fischmeister, R., Hescheler, J., and Fleischmann, B. K. (2000) J. Physiol. (Lond.) 523, 377–389
8. Thuringer, D., Lauribe, P., and Escande, D. (1992) J. Mol. Cell. Cardiol. 24, 451–455
9. Cerbai, E., Pino, R., Porciatti, F., Sani, G., Toscano, M., Maccherini, M., Giunti, G., and Mugelli, A. (1997) Circulation 95, 568–571
10. Hoppe, U. C., Jansen, E., Sudkamp, M., and Beuckelmann, D. J. (1998) Circulation 97, 55–65
11. Lonardo, G., Stillitano, F., Zicha, S., Cerbai, E., Mugelli, A., and Nattel, S. (2004) Circulation 110, (suppl.) III–129
12. Fernandez-Velasco, M., Ruiz-Hurtado, G., and Delgado, C. (2005) Pfluegers Arch. Eur. J. Physiol. 452, 146–154

Withdrawn.
Expression Regulation of HCN2 and HCN4 by miRNAs

13. Cerbai, E., Barbieri, M., and Mugelli, A. (1994) J. Physiol. (Lond.) 481, 585–591
14. Sridhar, A., Dech, S. J., Lacombe, V. A., Elton, T. S., McCune, S. A., Altschuld, R. A., and Carnes, C. A. (2006) Am. J. Physiol. 291, H2192–H2198
15. Han, W., Bao, W., Wang, Z., and Nattel, S. (2002) Circ. Res. 91, 790–797
16. Baruscotti, M., and DiFrancesco, D. (2004) Ann. N. Y. Acad. Sci. 1015, 111–121
17. Qu, J., Barbuti, A., Protas, L., Santoro, B., Cohen, I. S., and Robinson, R. B. (2001) Circ. Res. 89, E8–E14
18. Cohen, I. S., and Robinson, R. B. (2006) Handb. Exp. Pharmacol. 171, 41–71
19. Ambros, V. (2004) Nature 431, 350–355
20. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003) Cell 115, 787–798
21. Kazemi, E., Catalucci, D., and Condorelli, G. (2007) Circ. Res. 101, 1225–1236
22. Griffiths-Jones, S. (2004) Nucleic Acids Res. 32, D109–D111
23. Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005) Nature 438, 685–689
24. Lin, H., Xiao, J., Luo, X., Xu, C., Gao, H., Wang, H., Yang, B., and Wang, Z. (2007) J. Cell. Physiol. 212, 285–292
25. Xiao, J., Yang, B., Lin, H., Lu, Y., Luo, X., and Wang, Z. (2007) J. Biol. Chem. 282, 18255–18260
26. de Virginy, D. R. (2006) Heart Fail. Rev. 11, 333–334
27. Tian, R. (2003) Curr. Hypertens. Rep. 5, 454–458
28. Davis, F. J., Gupta, M., Pogwizd, S. M., Bacha, E., Jeevanandam, V., and Gupta, M. P. (2002) Am. J. Physiol. 282, H1521–H1533

WITHDRAWN

July 1, 2011