MicroRNA miR-133 Represses HERG K⁺ Channel Expression Contributing to QT Prolongation in Diabetic Hearts

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We have previously found that the ether-a-go-go related gene (ERG), a long QT syndrome gene encoding a key K⁺ channel (I_{Kr}) in cardiac cells, is severely depressed in its expression at the protein level but not at the mRNA level in diabetic subjects. The mechanisms underlying the disparate alterations of ERG protein and mRNA, however, remained unknown. We now report a remarkable overexpression of miR-133 in hearts from a rabbit model of diabetes, and in parallel the transcriptional repression of ERG, down-regulating ERG protein and mRNA, however, remained unknown. We report here the remarkable overexpression of miR-133 in hearts from a rabbit model of diabetes, and in parallel the transcriptional repression of ERG, down-regulating ERG protein and mRNA, however, remained unknown. We have now found that the muscle-specific miR-133 has been found in in vitro systems to be significantly higher levels are maintained in adult cardiac tissues (13, 14), suggesting that in addition to regulating myogenesis the function of these miRNAs is still limited to differential regulations of HERG expression at protein and transcript levels. However, our current understanding of the function of these miRNAs is still limited to differential regulation and their possible roles in other cellular processes have not yet been explored.

We propose that the muscle-specific miRNAs miR-1/miR-133 are able to repress HERG translation while keeping its mRNA unaffected and their levels are up-regulated in diabetic hearts, which causes the disparate changes of HERG protein and mRNA levels. This study was designed to test this hypothesis.

EXPERIMENTAL PROCEDURES

Preparation of Rabbit Model of DM—Male New Zealand White rabbits weighing 1.6–2.0 kg (Charles River Canada Inc.) were used and the procedures for development of alloxan-induced DM model were the same as previously described in

Abnormal QT interval prolongation is a prominent electrical disorder and has been proposed a predictor of mortality in patients with diabetes mellitus (DM), presumably because it is associated with an increased risk of sudden cardiac death consequent to lethal ventricular arrhythmias (1–8). Our recent study revealed that the long QT syndrome gene, human ether-a-go-go-related gene (HERG) encoding the channel responsible for rapid delayed rectifier K⁺ current (I_{Kr}), is significantly down-regulated in its expression in diabetic hearts and this down-regulation contributes critically to diabetic repolarization slowing and QT prolongation (9, 10). Strikingly, HERG expressions at transcriptional and post-transcriptional levels diverge in diabetic hearts, with its protein levels being reduced by some 60% while the mRNA levels remaining essentially unaltered (10). These disparate changes indicate that HERG expression is impaired mainly at the post-transcriptional level; however, it remained unclear what are the determinants for the differential regulation of HERG expression at protein and transcript levels.

MATERIALS AND METHODS

MiR-133 and miR-1 were cloned into the pSilencer2.1-U6 vector system (Ambion, Austin, TX) and used together with the CMV promoter. The constructs were transfected into the rabbit myocytes and cell lines produced post-transfection, to overexpress miRNAs. To silence miRNAs, we used the miR-133+miR-1 lentivirus system (Ambion, Austin, TX) and functional inhibition or gene silencing of miR-133 by anti-miRNA oligonucleotides specific for the muscle-specific miRNAs was achieved by the introduction of the oligonucleotide, antisense inhibitor. Functional inhibition or gene silencing of miRNA expression was confirmed by the introduction of the oligonucleotide, antisense inhibitor. Functional inhibition or gene silencing of miRNA expression was confirmed by examining RNA against SRF; RT, reverse transcription; qRT, quantitative RT.

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3 The abbreviations used are: DM, diabetes mellitus; AMO-1 and AMO-133, anti-miRNA oligonucleotides specific for the muscle-specific miRNAs; miR-1 and miR-133, respectively; ERG, ether-a-go-go related gene; rbERG, rabbit ERG; HERG, human ERG; I_{Kr}, rapid delayed rectifier K⁺ current; miRNA, microRNA; SRF, serum response factor; SRF-siRNA, small interference RNA against SRF; RT, reverse transcription; qRT, quantitative RT.
detail (9, 10). The QT measurements and simultaneously recorded RR intervals were used to derive heart rate corrected QT intervals. Incidences of ventricular tachycardia and ventricular fibrillation were determined. All procedures are in accordance with the guidelines set by the Animal Ethics Committee of the Montreal Heart Institute and of Harbin Medical University. Isolation of Rabbit Ventricular Myocytes and Cell Culture—Myocytes were isolated from rabbit left ventricular endocardium via enzymatic digestion of the whole heart on a Langendorff apparatus with the procedures similar to previously described (9, 10). The freshly isolated myocytes were stored either in the extracellular solution for patch clamp recordings or in 199 Medium as detailed elsewhere (9, 15).

Whole-cell Patch Clamp Recording—Patch clamp recording of I_{Kr} currents has been described in detail elsewhere (9, 10).

Synthesis of miRNAs and Anti-miRNA Antisense Inhibitors and Their Mutant Constructs—miR-1 and miR-133 and their respective mutant constructs were synthesized by Integrated DNA Technologies, Inc. as detailed elsewhere (16) (also see supplemental Fig. 1). The mutant miRNAs each had eight nucleotides mismatches at the 5’-end, which disrupts their binding to the target sites and thus turns the miRNAs into negative controls (11–14, 16).

Construction of Chimeric miRNA-Target Site-Luciferase Reporter Vectors—To construct reporter vectors containing the exact target sites for miRNA-target sites, we synthesized (by Invitrogen) fragments reporter vectors Vectors.

miR-1 and miR-133 have some 2.2- and 3-fold higher, respectively, in the diabetic ventricular RNA samples from rabbits with diabetes than in diabetic and healthy control animals. Up-regulation of miR-133 was also observed in the ventricular samples from DM patients (Fig. 1). We also reproduced the observations reported in our previous study (9, 10), i.e. the protein level of the rabbit ERG (rERG) was significantly lower in diabetic hearts than in healthy hearts despite that the transcript level remained unchanged. We further demonstrated the same disparity between HERG protein and mRNA expression levels in the hearts from DM patients (Fig. 1). Note that the molecular masses of ERG in rabbit (155 and 135 kDa) and human (140 and 120 kDa) were somewhat different presumably due to different glycosylations in different species; the larger band represents the mature glycosylated form and the smaller band represents the non-glycosylated form of ERG protein (16). The size rERG is consistent with our previous finding (9, 10) and that of HERG is identical to the results reported by Jones et al. (18).

Post-transcriptional Repression of HERG Expression by miR-133—HERG and rERG share 91% homology in their sequences. We identified multiple putative target sites for miR-133 in rERG and in HERG based on complementarity: at least six nucleotides exactly matching the 2–10 nucleotides from the 5’-end of miR-133 (supplemental Fig. 1). These sites may cooperate to confer the regulation by miR-133. Neither HERG nor
Role of microRNA-133 in Diabetic QT Prolongation

The uptake and activities of transfected miRNAs was confirmed by using miR-1 and miR-133 standards in which the miR-133 (10 and 100 nM), specific antisense inhibitor oligo (AMO-133, 100 nM). Control (Ctl), age-matched and sham-operated control; +, p < 0.05 versus miR-133 (100 nM) alone; *, p < 0.05 versus miR-1 (100 nM) alone. "AP+" represents pretreatment of the antibody with its antigenic peptide. AMO-133 (100 nM) was co-transfected with miR-133 (100 nM) and AMO-133 (100 nM); AMO-133 (100 nM) alone, respectively. +, p < 0.05 versus control; +, p < 0.05 versus miR-133 (100 nM); n = 5 for each group. C, Western blot analysis of HERG protein levels with membrane protein samples isolated from SKBr3 cells. Cells were co-transfected with the chimeric vector and miR-133 (10 and 100 nM), M-miR-133, AMO-133, or miR-1 (100 nM). "AP+" represents pretreatment of the antibody with its antigenic peptide. AMO-133 (100 nM) was co-transfected with miR-133 (100 nM), * p < 0.05 versus control; n = 4 for each group. D, failure of miR-133 and miR-1 used for these experiments was 100 nM. AMO-133 was co-transfected with miR-133 (100 nM); n = 5 for each group. E, whole-cell patch clamp recordings of rapid delayed rectifier K⁺ current (Iₑ₆) encoded by rERG in left ventricular myocytes isolated from diabetic (DM) and healthy rabbits (Ctl). Current recording was made 24 h after transfection. Iₑ₆ in display was elicited by a 2-s depolarizing voltage step to a test potential of +10 mV from a holding potential of −60 mV (see supplemental Fig. 3 for the full range of voltages tested). AMO-133, control or DM cells treated with AMO-133 (100 nM) alone; +AMO-133, control cells co-transfected with miR-133 (100 nM) and AMO-133; M-miR-133 and miR-1, control cells treated with miR-133 (100 nM) and miR-1 (100 nM) alone, respectively. * p < 0.05 versus control or DM; +, p < 0.05 versus miR-133 (100 nM) alone; n = 8 cells for each group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

miR-133 eliminated the silencing effect on luciferase reporter activity (16, 19, 20). As an additional negative control, application of miR-1 failed to affect luciferase reporter activity.

The uptake and activities of transfected miRNAs was confirmed by using miR-1 and miR-133 standards in which the complementary sequences of miR-1 and miR-133 were cloned downstream of luciferase gene in the pMIR-REPORT plasmid (Fig. 2B).

We determined the effects of miR-133 on endogenous expression of HERG at the protein level by Western blot with SKBr3 membrane protein samples. SKBr3 was used because it is a human cell line that expresses endogenous HERG (17) but does not express the muscle-specific miR-1 or miR-133 (supplemental Fig. 2). Our data showed that transfection of miR-133 reduced HERG protein level down to ~10% of control value, and as a negative control the mutant miR-133 did not cause any appreciable changes (Fig. 2C). Co-application of AMO-133 nearly abolished the effects of miR-133, verifying the specificity of the miR-133 action. Moreover, transfection of miR-1 failed to
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Potential Role of SRF in miR-133 Overexpression—It has been shown that expression of miR1/miR-133 is dependent upon binding of SRF to their promoter regions (13, 14), an important transcriptional factor in cardiac cells (21–24). SRF protein level was found significantly increased in diabetic hearts relative to healthy hearts and so was SRF transcript level (Fig. 3A). Incubation of the DM myocytes in primary culture with distamycin A, which has been shown to selectively inhibit binding of SRF to its cis-element (25), largely reversed the increases in miR-1/miR-133 expression (Fig. 3B). This effect was further confirmed by silencing of SRF using the siRNA directed against SRF (SRF-siRNA) (Fig. 3C). Moreover, in cells isolated from DM hearts, we demonstrated that endogenous miR-133 levels or HERG protein levels were markedly diminished, being the major factor for QT prolongation in diabetic rabbits, while ERG mRNA level was unaffected (9). Reduction of \( I_{\text{Kr}} \) due to expression repression of HERG by miR-133 is expected to result in repolarization slowing thereby QT prolongation. In our recent study, we found that miR-133 repressed KCNQ1 (16), a channel protein responsible for the slow delayed rectifier \( K^+ \) current \( (I_{\text{Kr}}) \) in cardiac cells. However, whether \( I_{\text{Kr}} \) has significant contribution to diabetic QT prolongation is still an open question and our previous studies suggest a minimal role of miR-133 in abnormal QT prolongation in diabetes and maybe in other pathological conditions as well. Our data also indicate that the cardiac-specific miR-1 is not responsible for the down-regulation of \( I_{\text{Kr}} \) in DM heart.

It is important to note here that the phenomenon of disparate changes of ERG expression at protein and mRNA levels

ffect HERG protein level. By comparison, miR-133 produced virtually no effects on HERG mRNA level (Fig. 2D), indicating that miR-133 does not affect HERG mRNA stability.

The functional significance of ERG regulation by miR-133 was explored by whole-cell patch clamp studies of \( I_{\text{Kr}} \) in isolated ventricular myocytes in primary culture. \( I_{\text{Kr}} \) density in the myocytes from DM hearts or in the myocytes from healthy control heart transfected with miR-133 was severely diminished (Fig. 2E). The depression induced by DM was partially reversed by AMO-133 and that induced by exogenous miR-133 was abolished by AMO-133. AMO-133 slightly enhanced \( I_{\text{Kr}} \) in control cells, presumably by eliminating the repressive effects of basal endogenous miR-133. As a negative control, miR-1 failed to affect \( I_{\text{Kr}} \).

**DISCUSSION**

miRNA-mediated gene regulation is a fundamental layer of genetic program control at the posttranscriptional level. However, the current knowledge about miRNAs, regulatory targets have been limited and unpredictably for any of the miRNAs, which has hampered progress toward elucidation of functions of miRNAs. Our current understanding of the functions of miRNAs primarily relies on their tissue-specific or developmental stage-specific expression patterns as well as their evolutionary conservation and is thus largely limited to biogenesis and oncosuppression. Target finding and function discovery are two major challenges to researchers in miRNA research. The present study revealed the ability of a miRNA to regulate ion channel expression and the possible role in electrical remodeling in diabetic myocardium. It thus expanded our understanding of the cellular function and pathophysiological roles of miRNAs in a whole, reconsolidating the view that miRNAs likely have widespread functions in the cells.

Our study provides an explanation for the observed discrepancy between changes of HERG/rbHERG expression at protein and mRNA levels. In our recent study on QT prolongation of diabetic hearts, we demonstrated that \( I_{\text{Kr}} \) density and ERG protein level were remarkably diminished, being the major factor for QT prolongation in diabetic rabbits, while ERG mRNA level was unaffected (9). Reduction of \( I_{\text{Kr}} \) due to expression repression of HERG by miR-133 is expected to result in repolarization slowing thereby QT prolongation. In our recent study, we found that miR-133 repressed KCNQ1 (16), a channel protein responsible for the slow delayed rectifier \( K^+ \) current \( (I_{\text{Kr}}) \) in cardiac cells. However, whether \( I_{\text{Kr}} \) has significant contribution to diabetic QT prolongation is still an open question and our previous studies suggest a minimal role of miR-133 in abnormal QT prolongation in diabetes and maybe in other pathological conditions as well. Our data also indicate that the cardiac-specific miR-1 is not responsible for the down-regulation of \( I_{\text{Kr}} \) in DM heart.

It is important to note here that the phenomenon of disparate changes of ERG expression at protein and mRNA levels
have also been observed in failing heart and ischemic myocardium. For example, several studies found that $I_{Kr}$ current density was significantly diminished in myocytes from failing hearts that is also electrophysiologically characterized by repolarization slowing and QT prolongation similar to diabetic hearts, despite that the mRNA level of HERG was barely altered under these conditions (24–29). Whether these disparate changes of ERG protein and mRNA in failing hearts and ischemic myocardium are consequent to up-regulation of miR-133 expression is worthy of detailed studies.

Our study also provides evidence for the potential role of SRF in miR-133 overexpression in DM myocytes. The SRF-siRNA not only nullifies the increase in miR-133 but also rescues depressed $I_{Kr}$ in DM. Whether SRF inhibition or knockdown could have beneficial effects on diabetic QT prolongation merits future investigations.

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