miRNAs Regulate hERG

JIANFANG LIAN, PH.D.,* JIAN GUO, M.D.,* XIAOYAN HUANG, M.D.,* XI YANG, M.D.,* GUOCHANG HUANG, PH.D.,* HAIYAN MAO, M.D.,* HUAN HUAN SUN, M.D.,† YANNA BA, M.D.,* and JIANQING ZHOU, M.D.*

From the *Li Hui Li Hospital, Medical School of NingBo University, NingBo, China; and †Department of Surgery, University of Rochester Medical Center, Rochester, New York, USA

miRNAs Regulate hERG. Background: The human ether-a-go-go-related gene (hERG) is the major molecular component of the rapidly activating delayed rectifier K⁺ current (I₉). Impairment of hERG function is believed to be a mechanism causing long-QT syndromes (LQTS). Growing evidences have shown that microRNAs (miRNAs) are involved in functional modulation of the hERG pathway. The purpose of this study was to screen and validate miRNAs that regulate the hERG pathway. The miRNAs identified in this study will provide new tools to assess the mechanism of LQTS.

Methods: Six miRNAs were selected by algorithm predictions based on potential interaction with hERG. The effects of each miRNA on hERG were assessed by use of the Dual-Luciferase Reporter assay system, qRT-PCR, Western blotting, and confocal fluorescence microscopy. Furthermore, whole-cell patch clamp technique was used to validate the effect of miR-103a-1 on the electrophysiological characteristic of the IKr of the hERG protein channel.

Results: miR-134, miR-103a-1, miR-143, and miR-3619 significantly downregulated luciferase activity (P < 0.05) in a reporter test system. These 4 miRNAs significantly suppressed expression of hERG mRNA and protein in U2OS cells (P < 0.05). Corresponding AMOs rescued expression of hERG mRNA and protein. Confocal microscopy showed that all 4 miRNAs reduced the expression of both immature and mature hERG protein. miR-103a-1 decreased the maximum current and tail current amplitudes of hERG channel.

Conclusions: Expression and functions of hERG are regulated by specific miRNAs. (J Cardiovasc Electrophysiol, Vol. 27, pp. 1472-1482, December 2016)

AMOs, hERG, long-QT sydrome, microRNA, rapidly activating delayed rectifier potassium current

Introduction

Human ether-à-go-go-related gene (HERG) encodes the rapid component of the cardiac delayed rectifier K⁺ current, which plays an important role in the repolarization phase of the cardiac action potential. Reduced repolarizing currents result in a prolongation of the ventricular action potential, which increases the risk for life-threatening arrhythmias, such as torsades de pointes or ventricular fibrillation. The HERG potassium channel has been widely used to counter ischemic arrhythmogenesis. Dysfunction of HERG caused LQT2. It has been confirmed that up-regulation of miR-21 and miR-23a contributes to As_{2}O_{3}-induced HERG deficiency. Therefore, a better understanding of the molecular mechanisms underlying HERG-related LQT2 will be critical for LQTS patients.

MicroRNAs (miRNAs) are a class of 21–25 noncoding RNA nucleotides, which are important in many major eukaryotic biological processes through post-transcriptional regulation of the target genes. miRNAs cause mRNA degradation or translational inhibition through imperfect hybridization to 3'- untranslated region (3'-UTR), 5'-UTR region or mRNA coding sequences of their target mRNAs. They target genes involved in numerous biological processes, such as proliferation, differentiation, development, and cell death. Recently, studies demonstrated that the expression patterns of miRNAs are substantially altered in a number of cardiac diseases, including cardiac arrhythmia. Altered expression of miRNAs can cause dysregulation of ion channel genes and lead to channelopathies. For example, previous study demonstrated that miR-1 targeted GJA1 (gap junction channel protein connexin 43) and KCNJ2 (Kir2.1 K⁺ channel subunit) to slow down cardiac conduction and thereby led to ischemic arrhythmogenesis. Clinically, miRNAs can be potentially used as diagnostic biomarkers as well as therapeutic targets. Inhibition of miRNA expressions can be achieved by using anti-miRNA oligonucleotides (AMO). AMOs are oligonucleotides, which are fully complementary to their target miRNAs. For instance, over-expression of miRNA-1 has been associated with arrhythmogenesis under several pathological conditions. Previous studies showed that AMO-1 was able to reverse the deleterious effect of miR-1, suggesting that AMO-1 is a potential therapeutic target for treatment of ischemic arrhythmias.

We undertook bioinformatics and molecular approach to identify miRNAs that target HERG. 4 miRNAs (miR-134,
miR-143, miR-103a-1, and miR-3619 consistently downregulated the expression level of hERG. This provides a better understanding of the molecular basis of dysfunctional hERG protein channel.

Material and Methods

hERG miRNA Predication

The miRNA target sites on the hERG (NM_172056) were predicted using bioinformatics website RegRNA (http://regrna.mbc.nctu.edu.tw) according to the instruction. This resulted in 6 miRNAs, including miR-134, miR-143, miR-103a-1, miR-147, miR-185, and miR-3619.

cDNA Cloning, Cell Culture, and Transfection

The hERG gene was cloned into pcDNA3 vector (Invitrogen, Carlsbad, CA, USA), as described previously.19 Human embryonic kidney (HEK) 293T or U2OS cells were transiently co-transfected with 2.5 μg of hERG and 0.65 μg of pRK5-GFP plasmids using Lipofectamine 2000 (Invitrogen). The pRK5-GFP was used to monitor transfection efficacy. For miRNA experiment, cells were first transfected with 2.5 μg of hERG for 24 hours and followed by another transfection with 10 mmol/L miRNA or miRNA plus corresponding anti-miRNA antisense inhibitor sequence (AMO) for another 24 hours. HEK293T or U2OS cells were cultured in Dulbecco’s Minimum Essential Medium (DMEM) (Invitrogen, Burlington, ON, Canada), supplemented with 10% fetal bovine serum, maintained in a humidified 5% CO₂ incubator at 37 °C.

Luciferase Reporter Assay

To construct hERG-related dual luciferase reporter plasmids, the corresponding miRNA target sites of hERG were prepared with forward and reverse primer pairs shown in Table 1. These sites were then cloned into the pmirGLO vector (Promega) using DraI and XhoI restriction sites. These plasmids were named pmirGLO-134, pmirGLO-143, pmirGLO103a-1, pmirGLO147, pmirGLO-185, and pmirGLO-3619, respectively. For the luciferase reporter assays, cells were co-transfected with the hERG dual-luciferase plasmids and miRNA mimics or negative control (NC). Cells were collected 48 hours after transfection and analyzed using Dual-Luciferase Reporter Assay System. Relative luciferase activity was normalized to renilla luciferase activity. Transfections were done in duplicates and repeated 3 times independently.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was isolated from transfected U2OS cells with Trizol reagent (Invitrogen). The RNA was subsequently treated with RNase-free DNase I. mRNA levels of hERG were assessed with qRT-PCR using Taqman probe. Primers and probe are from ABI Biosystem with Cat. #4331182 (ID: Hs00234270-g1). The ABI Prism 7900 sequence detector (Applied Biosystems Life Technologies) was set with an initial step of 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. The real-time PCR reactions were performed in triplicate and included appropriate controls. Results were analyzed using the 2⁻ΔΔCT relative quantification method.20

Western Blotting

Western blot analysis of hERG protein was performed as previously described.21 Briefly, U2OS cells were transfected with pcDNA-hERG. Cells that expressed hERG were further transfected with respective miRNA or miRNA plus corresponding AMOs. Cells were harvested 48 hours after transfection. Equal amounts of protein were separated on 7.5% SDS polyacrylamide gels and then transferred onto nitrocellulose membranes (Pierce Biotechnology, Woburn, MA, USA). Membranes were blocked with 5% nonfat dry milk, followed by an overnight incubation at 4 °C with rabbit polyclonal anti-hERG antibody (Alomone Labs, Jerusalem, Israel). The membrane was washed 3 times in TBST and then incubated with a goat anti-rabbit IgG (ZSGB-BIO, Beijing, China) for 1 hour at room temperature. Blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, MA, USA) and developed using Syngene Chemi-Genius imaging system (Syn-Gene, Cambridge, London, UK).

Confocal Laser Scanning Microscopy

U2OS cells were seeded on coverslips in 6-well plate. The U2OS cells were transfected with pcDNA-hERG. Cells that expressed hERG were further transfected with respective miRNA or miRNA plus corresponding AMOs. At 48 hours post-transfection, cells were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS for 3 times, and permeabilized with 0.1% Triton X-100 and blocked with 5%
goat serum at room temperature. Cells were labeled with rabbit polyclonal anti-hERG at 4°C overnight, washed with PBS for 3 times, and incubated with Rhodamine-conjugated goat anti-rabbit IgG secondary antibody for 30 minutes. DAPI, a nuclear marker, was used as counterstaining. Images were visualized using a Leica TCS SP2 confocal laser scanning microscope.

**Electrophysiology**

Electrophysiological recordings were performed in HEK293T cells, a cell line that is amenable to patch clamp analysis. To assess the effect of miRNAs on hERG by the whole-cell patch-clamp technique, HEK293T cells were harvested at 24 hours (hERG plasmid only), 48 hours (hERG followed by miRNA), or 72 hours (hERG plasmid followed by microRNA and corresponding AMOs) after transfection. The transfected cells were transferred to a bath mounted on the stage of an inverted microscope (Nikon, Japan). Pipettes were heat polished and resistances were obtained ranging from 2 to 5 MΩ when filled with a solution containing (in mM): 140 KCl, 1 MgCl₂, 5 EGTA, 10 HEPES, 5 MgATP (pH 7.2 with KOH). The extracellular bath solution contained (in mM): 140 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 5 HEPES (pH 7.4 with NaOH). The final resistance of the electrode was 3–5 MΩ when filled with pipette solution. Membrane currents were amplified (Axopatch 200B amplifier; Axon Instruments, Union City, CA, USA) and digitized (Digidata 1440; Axon Instruments) before storage on a computer. Series resistance compensation was typically 80%. The exact voltage protocols are indicated in respective figures. Currents were recorded at 23–25 °C within 2–3 hours after cells were removed from their culture conditions.

**Statistical Analysis**

The pCLAMP Ver. 10.3 software (Axon Instruments) was used to generate voltage clamp protocols, acquire data and analyze current traces. Voltage dependence of activation was determined by fitting the peak tail current (Iₜa₁) with Boltzmann function: \[ I/I_{max} = 1 - \{ 1 + \exp(\frac{V - V_{1/2}}{k}) \}^{-1} \]. \( V_{1/2} \) represents the half-maximal voltage and \( k \) is the slope factor. Time constants of inactivation or recovery from inactivation were fitted by single exponential standard. Results are expressed as mean ± S.E.M. Statistical analyses were performed using Student’s t-test. Results were considered statistically significant at \( p < 0.05 \).

**Results**

**Reverse Screening for the hERG-Related miRNAs**

To identify miRNAs that interact with hERG, we applied RegRNA, which is an integrated web server for identifying regulatory RNA motifs and elements. Six miRNAs, miR-134, miR-143, miR-103a-1, miR-147, miR-185, and miR-3619, were identified by queried target prediction programs and were selected for further experiments.

To validate the relevance of identified miRNAs, we used luciferase reporter assay. The hERG-related miRNA target sequences were cloned into the pmirGLO plasmid and sequenced. These plasmids are pmirGLO-134, pmirGLO-143, pmirGLO-103a-1, pmirGLO-147, pmirGLO-185, and pmirGLO-3619 (Fig. 1). The firefly and renilla dual luciferase reporter assays were performed in the HEK293T cells, which were co-transfected with pmirGLO-134, 143, 103a-1, 147, 185, or 3619 and corresponding miR-134, miR-143, miR-103a-1 miR-147, miR-185, or miR-3619, respectively. The results showed that miR-134, miR-103a-1, miR-143 and miR-3619 significantly decreased the luciferase activity to 20.58%, 34.71%, 27.12%, and 23.67%, respectively, compared to cells transfected with luciferase plasmid alone (\( p < 0.05 \)) (Fig. 2A). Therefore, miR-134, miR-143, miR-103a-1, and miR-3619 potentially regulate the expression of hERG.

Next, we used qRT-PCR and Western blot analysis to determine the effects of miR-134, miR-143, miR-103a-1, miR-147, miR-185, and miR-3619 on the expression of hERG in U2OS cells. U2OS cells with stable expression of hERG have the typical hERG signature of 2 single protein bands at approximately 135 and 155 kDa (Fig. 2C). The 135-kDa
miRNAs Regulate hERG

Figure 2. hERG is one of the targets of miR-134, miR-143, miR-103a-1, and miR-3619. A: Initial screening of hERG-related miRNAs. Dual luciferase assays were performed in HEK293T cells transfected with the hERG-related luciferase report and respective miRNA mimics. The result showed that miR-134, miR-143, miR-103a-1, and miR-3619 significantly suppressed hERG-related luciferase activity but miR-147 and miR-185 had no significant effect on hERG-related luciferase activity. Experiments were done in triplicate. *P < 0.05. B: Real-time RT-PCR showed that miR-134, miR-143, miR-103a-1, and miR-3619 suppressed hERG mRNA expression. miR-134, miR-143, miR-103a-1, and miR-3619 plus corresponding AMO-134, AMO-143, AMO-103a-1, and AMO-3619 restored hERG mRNA expression. Experiments were done in triplicate. *P < 0.05. C: Western blot assay showed that miR-134, miR-143, miR-103a-1, and miR-3619 suppressed hERG protein expression and addition of corresponding AMO-134, AMO-143, AMO-103a-1, and AMO-3619 rescued hERG protein expression. Tubulin was used as an internal quantitative control. Experiments were repeated 3 times.

band represents the core-glycosylated, immature Kv11.1 protein retained in the endoplasmic reticulum, whereas the 155-kDa band represents the fully glycosylated, mature form of hERG protein on cell surface membrane.\(^\text{21,22}\) The appearance of the 155-kDa band is often used as an indicator of channel rescue.\(^\text{23-25}\) As a result, overexpression of miR-134, miR-143, miR-103a-1, or miR-3619 decreased the expression of hERG at both mRNA and protein levels (the 135-kDa protein band and 155-kDa protein band were either faint or absent). On the other hand, overexpression of miR-147 or miR-185 did not affect hERG at either mRNA or protein level (Fig. 2B,C). Furthermore, co-expression of miR-134, miR-143, miR-103a-1, or miR-3619 and corresponding AMOs restored expression of hERG at both mRNA and protein levels in the U2OS cells.

These results were confirmed by confocal imaging using U2OS cells. Fluorescence microscopy showed hERG protein localized both on the cell membrane and in the cytoplasm when it was expressed alone in U2OS cells (Fig. 3, first row, first image from the left). In contrast, hERG signal was suppressed by expression of miR-134, miR-143, miR-103a-1, and miR-3619. Interestingly, the loss of hERG signal in cells transfected with miR-134, miR-143, miR-103a-1, or miR-3619 was rescued by co-transfection of corresponding AMOs. This suggests that AMO-134, AMO-143, AMO-103a-1, and AMO-3619 can counteract the effects of corresponding miRNAs and potentially restore hERG function by restoring its localization to the plasma membrane and cytoplasm. The results from protein localization studies were consistent with the results from the Western blotting experiments, which further indicate that hERG is a direct target of miR-134, miR-143, miR-103a-1, and miR-3619.

Effect of miR-103a-1 on hERG Current Amplitudes

Cells with stable expression of hERG were further transfected with cel-miR-67, miR-103a-1 or miR-103a-1 plus AMO-103a-1. Cel-miR-67 was taken as random negative controls. The hERG currents were elicited by voltage clamp protocol as previously described.\(^\text{26,27}\) Cells were depolarized to test voltage between –60 and +60 mV in −10 mV increments for 3 seconds from a holding potential of −80 mV, followed by −40 mV for 4 seconds to elicit tail currents.
Figure 3. Confocal imaging of hERG channel in U2OS cells. U2OS cells with transient expression of hERG were transfected with respective miRNA or miRNA plus corresponding AMO. The hERG localized to both the plasma membrane and cytoplasm, whereas membrane localization of hERG was lost when cells were treated with miR-134, miR-143, miR-103a-1, or miR-3619. This loss was rescued by addition of corresponding AMOs. For a high quality, full color version of this figure, please see Journal of Cardiovascular Electrophysiology’s website: www.wileyonlinelibrary.com/journal/jce

(Fig. 4, inset). Representative evoked current traces are shown in Figure 4A,D, and the corresponding current–voltage (I–V) relationships for both depolarization and tail currents are plotted in Figure 4E,F. As shown in Figure 4A, the maximal current amplitude in hERG-expressing cells is $660.67 \pm 159.55$ pA ($n=6$), the tail current is $981.28 \pm 5.94$ pA. To further assess the effect of miR-103a-1 on hERG channel activation, we treated HEK293T cells expressing hERG channel with either miR-103a-1 or cel-miR-67 and compared current amplitudes to untreated controls. Figure 4B and C show the typical tracings of cells treated with miR-103a-1 or cel-miR-67. In cells with stable expression of hERG that were transfected with miR-103a-1, the maximal current amplitude was $253.72 \pm 47.35$ pA ($n=6$). The maximal current amplitude decreased by $61.60\%$ ($P<0.05$) compared with untreated cells expressing hERG alone. Similarly, the peak $I_{\text{tail}}$ amplitude was $376.23 \pm 2.84$ pA for cells transfected with miR-103a-1. The $I_{\text{tail}}$ amplitude also decreased by $61.66\%$ ($P<0.05$) compared with untreated cells expressing hERG alone.

In contrast, the negative control, cel-miR-67, failed to affect $I_{\text{Kr}}$ current amplitude. The addition of cel-miR-67 had minimal effect on the maximal current amplitudes ($691.30 \pm 143.57$ pA with cel-miR-67 vs $660.67 \pm 159.55$ pA without cel-miR-67; 4.6\% [$n=6$]) and tail currents ($985.79 \pm 3.96$ pA with cel-miR-67 $981.28 \pm 5.94$ pA without cel-miR-67; 0.45\% [$n=6$]). As expected, co-transfection of AMO-103a-1 nearly abolished the effects of miR-103a-1. In hERG expressing cells, cells transfected with miR-103a-1 and AMO-103a-1 restored the maximal current amplitude to $595.75 \pm 99.08$ pA ($n=6$) ($P<0.05$), from $253.72 \pm 47.35$ pA ($n=6$) in cells treated with miR-103a-1 alone. Similarly, the tail current amplitude was also restored from $376.23 \pm 2.84$ pA in cells treated with miR-103a-1 alone, to $984.58 \pm 6.49$ pA ($n=6$) ($P<0.05$) in cells transfected with miR-103a-1 and AMO-103a-1.
Figure 4. Effect of miR-103a-1 on voltage-dependent activation of hERG channel. Inset shows the voltage-clamp protocol. A to D show representative current traces in HEK293T cells transfected with hERG, in the presence or absence of miR-103a-1, cel-miR-67, or miR-103a-1 plus AMO-103a-1. E, F: Current–voltage relationships for peak currents and tail currents amplitudes of hERG transfected cells in the presence or absence of miR-103a-1, cel-miR-67, or miR-103a-1 plus AMO-103a-1. G: Amplitudes of tail currents plotted as a function of test potential and fitted to a Boltzmann function (n = 6). For a high quality, full color version of this figure, please see Journal of Cardiovascular Electrophysiology’s website: www.wileyonlinelibrary.com/journal/jce

Compared with hERG expressing cells, the cells co-transfected with miR-103a-1 and AMO-103a-1 showed minimal differences in maximal current amplitudes (595.75 ± 99.08 pA vs. 660.67 ± 159.55 pA, P > 0.05) and tail currents (984.58 ± 6.49 pA vs. 981.28 ± 5.94 pA, P > 0.05). These findings indicate that hERG is one of the targets of miR-103a-1.

Effect of miR-103a-1 on Gating Properties of hERG Protein Channel

To assess the effect of miR-103a-1 on the voltage dependence of hERG protein channel activation, kinetics of inactivation, recovery, and deactivation, normalized Itail values of hERG were plotted against the test potentials and fitted to the Boltzmann function. As shown in Figure 4G, in hERG-expressing cells, miR-103a-1 treatment resulted in a significant shift of the activation curve in a negative direction. The half-maximal activation voltage (V1/2) for hERG-expressing cells (V1/2 = –8.66 ± 0.38 mV; n = 6) was statistically different from the corresponding value in cells treated with miR-103a-1 (V1/2 = –25.31 ± 0.52 mV; n = 6; P < 0.05). Similarly, statistically significant difference was seen in the slope factor k values of cells expressing hERG (6.88 ± 0.44 mV with miR-103a-1 vs. 9.99 ± 0.34 mV without miR-103a-1; n = 6; P < 0.05). Interestingly, cel-miR-67 treatment
resulted in a significant change in activation voltage in hERG expressing cells ($V_{1/2} = -20.07 \pm 3.30$ mV vs. $-8.66 \pm 0.38$ mV control; $n = 6$, $P < 0.05$) but no change in slope factor ($k = 9.49 \pm 0.27$ mV vs. $9.99 \pm 0.34$ mV control; $n = 6$). Between cells treated only with miR-103a-1 and cells treated with miR-103a-1 plus AMO-103a-1, there was no significant change in either the activation voltage ($V_{1/2} = -25.31 \pm 0.52$ mV with miR-103a-1 vs. $-18.64 \pm 0.45$ mV with miR-103a-1 plus AMO-103a-1; $n = 6$, $P > 0.05$; Fig. 4G) or the slope factor ($k = 6.88 \pm 0.44$ mV with miR-103a-1 vs. $8.29 \pm 0.39$ mV with miR-103a-1 plus AMO-103a-1; $n = 6$, $P > 0.05$; Fig. 4G). The half-maximal activation voltage was $-8.66 \pm 0.38$ mV for cells expressing hERG, compared with $-18.64 \pm 0.45$ mV for cells treated with miR-103a-1 plus AMO-103a-1 ($n = 6$, $P < 0.05$). On the other hand, the slope factor was $9.99 \pm 0.34$ mV for hERG expressing cells and $8.29 \pm 0.39$ mV for cells treated with miR-103a-1 plus AMO-103a-1 ($n = 6$, $P > 0.05$). These results indicate that miR-103a-1 inhibits the properties of hERG protein channel in the activation phase.

To measure channel steady-state inactivation, test potentials between $-130$ and $20$ mV in $10$ mV increments for $20$ milliseconds were applied after a depolarizing pulse back to $20$ mV for $4$ seconds. Next, a test pulse back to $20$ mV for $500$ milliseconds was performed, followed by a final return of the voltage to the holding potential of $-80$ mV (Fig. 5, inset). Figure 5A-D shows the typical steady-state inactivation current traces and Figure 5E depicts the normalized steady-state inactivation curves. The half-maximal inactivation voltage ($V_{1/2}$) for cells expressing hERG and cells treated with miR-103a-1 were $-39.07 \pm 3.13$ mV and $-46.88 \pm 4.35$ mV ($n = 6$), respectively ($P > 0.05$). The slope factors for cells expressing hERG and cells treated with miR-103a-1 were $14.96 \pm 2.88$ mV and $11.30 \pm 3.90$ mV, respectively ($n = 6$, $P > 0.05$). Compared to untreated control ($V_{1/2} = -39.07 \pm 3.13$ mV, $k = 14.96 \pm 2.88$ mV, $n = 6$, $P > 0.05$), the hERG protein channels were largely unaffected by treatment with either cel-miR-67 ($V_{1/2} = -40.07 \pm 2.03$ mV, $k = 17.32 \pm 1.94$ mV) or miR-103a-1 plus AMO-103a-1 ($V_{1/2} = -31.44 \pm 1.75$ mV, $k = 19.00 \pm 1.54$ mV). Lastly, there was also no difference in the $k$ or $V_{1/2}$ value among cells expressing hERG, cells treated with miR-103a-1, and cells treated with miR-103a-1 plus AMO-103a-1. Our results demonstrate that miR-103a-1 treatment do not alter the steady-state inactivation kinetics of hERG protein channels.

To study the inactivation time course of hERG protein channel, cells were depolarized from a holding potential of $-80$ mV for $4$ seconds. A test pulse back to $20$ mV for $500$ milliseconds was performed, followed by a final return of the voltage to the holding potential of $-80$ mV (Fig. 5, inset). Figure 5A-D shows the typical steady-state inactivation current traces and Figure 5E depicts the normalized steady-state inactivation curves. The half-maximal inactivation voltage ($V_{1/2}$) for cells expressing hERG and cells treated with miR-103a-1 were $-39.07 \pm 3.13$ mV and $-46.88 \pm 4.35$ mV ($n = 6$), respectively ($P > 0.05$). The slope factors for cells expressing hERG and cells treated with miR-103a-1 were $14.96 \pm 2.88$ mV and $11.30 \pm 3.90$ mV, respectively ($n = 6$, $P > 0.05$). Compared to untreated control ($V_{1/2} = -39.07 \pm 3.13$ mV, $k = 14.96 \pm 2.88$ mV, $n = 6$, $P > 0.05$), the hERG protein channels were largely unaffected by treatment with either cel-miR-67 ($V_{1/2} = -40.07 \pm 2.03$ mV, $k = 17.32 \pm 1.94$ mV) or miR-103a-1 plus AMO-103a-1 ($V_{1/2} = -31.44 \pm 1.75$ mV, $k = 19.00 \pm 1.54$ mV). Lastly, there was also no difference in the $k$ or $V_{1/2}$ value among cells expressing hERG, cells treated with miR-103a-1, and cells treated with miR-103a-1 plus AMO-103a-1. Our results demonstrate that miR-103a-1 treatment do not alter the steady-state inactivation kinetics of hERG protein channels.

To study the inactivation time course of hERG protein channel, cells were depolarized from a holding potential of...
miRNAs Regulate hERG

Figure 6. Effect of miR-103a-1 on time courses of inactivation of hERG channel. Inset shows the voltage-clamp protocol. A to D show representative current traces of time courses of inactivation time constant (tau) values for currents in HEK293T transfected with hERG, in the presence or absence of miR-103a-1, cel-miR-67, or miR-103a-1 plus AMO-103a-1. E: The time constant value was measured by fitting inactivating currents during test pulses at each potential with a single exponential function (n = 6).

For a high quality, full color version of this figure, please see Journal of Cardiovascular Electrophysiology’s website: www.wileyonlinelibrary.com/journal/jce

Figure S1E, the recovery from inactivation properties of cells expressing hERG were not affected by miR-103a-1 or cel-miR-67, and there was no statistically significant difference between hERG with miR-103a-1 treatment and hERG with miR-103a-1 plus AMO-103a-1 treatment.

Deactivation was measured by using the same pulse protocol as recovery from inactivation (Fig. S2). Representative current tracings are shown in Figure S2A-D. The mean fast and slow deactivation time courses were assessed by fitting deactivation tail currents during test pulses to a double exponential function. As shown in Figure S2E, no significant differences were found in the fast and slow time constants of deactivation for hERG channel treated with miR-103a-1 or cel-miR-67, compared to control (n = 6, P > 0.05). In addition, there were also no statistically significant differences between cells treated with miR-103a-1 alone and cells treated with with miR-103a-1 plus AMO-103a-1 (n = 6).

Discussion

Cardiovascular diseases remain the major cause of morbidity and mortality in developed countries. The cardiac-specific hERG protein channel carrying rapid delayed rectifier K⁺ current is responsible for phase 3 repolarization. Functional studies have revealed multiple mechanisms by which hERG dysfunction leads to LQTS, including abnormalities in Kv11.1 synthesis (class 1 mechanism), intracellular transport (protein trafficking) of hERG to the cell membrane (class 2 mechanism), channel gating (class
3 mechanism), and permeation (class 4 mechanism)\textsuperscript{19,28}.

The pathophysiology of cardiac arrhythmias is associated with altered expression profile of genes that are important for cardiac function. miRNAs have been reported to regulate cardiac arrhythmogenesis by targeting genes encoding ion channels.\textsuperscript{15,29-33} To date, it is estimated that approximately 30\% of protein genes could serve as miRNA targets.\textsuperscript{32,33}

Previous studies have demonstrated that aberrant miRNAs expression could result in deregulation of ion channel gene expressions, leading to channelopathy-arrhythmogenesis. In previous study, Dana et al. validated that miR-212 could down-regulate K\textsubscript{i},2.1 (KCNJ2) and significantly alter K\textsubscript{i},1 density.\textsuperscript{32} MiRNA-mediated gene regulation operates a fundamental layer of genetic programs. However, identifying target genes and clarifying their functions are 2 major challenges to miRNA research. This study provides novel insights into the role of miRNA in the pathophysiology of hERG. Much is known about how hERG transcripts are regulated at the transcriptional and posttranscriptional level, but regulation of hERG expression by miRNAs has not yet been described.

Here, we provide several lines of evidence showing that hERG is regulated by miR-134, miR-103a-1, miR-143, and miR-3619. First, hERG is a putative target gene, and we screened 6 miRNAs according to the corresponding website score. Second, miR-134, miR-103a-1, miR-143, and miR-3619 repressed luciferase reporter gene containing coding DNA sequence of hERG through its binding site. However, miR-147 and miR-185 did not significantly repress luciferase reporter gene. There are multiple studies regarding the requirement for miRNA binding sites to be located in the 3’-UTR of protein-coding genes.\textsuperscript{5} Moreover, recent reports have shown that functional miRNA binding sites have occasionally been reported in 5’-UTR region\textsuperscript{6} and, more frequently, in the mRNA coding sequences.\textsuperscript{5,7} Third, overexpression of miR-134, miR-103a-1, miR-143 and miR-3619 downregulated the expression of hERG at both mRNA and protein levels. Most animal miRNAs are imperfectly complementary to their mRNA targets and they inhibit protein synthesis without interfering on the mRNA level. However, we found that the change in hERG mRNA was parallel with that of hERG protein, which might be due to that the higher level of complementarity leads to degradation of mRNA while lower level of complementarity results in translation inhibition.\textsuperscript{35,36}

Inhibition of miR-134, miR-103a-1, miR-143, and miR-3619 with their corresponding specific inhibitor almost abolished the suppressive effect caused by these 4 miRNAs.miR-147 and miR-185 overexpression did not affect expression of hERG at either the mRNA or protein level. Consequently, these results clearly indicate that hERG is a novel functional target of 4 miRNAs (miR-134, miR-103a-1, miR-143, and miR-3619). In addition, the fact that corresponding AMOs are able to reverse the effects of these 4 miRNAs on the expression of hERG suggests that these 4 miRNAs could be as potential therapeutic targets.

Currently, patch-clamp techniques are considered the “gold standard” for assessing Kv11.1 functions. In our study, we performed detailed analysis of miRNA-hERG channel interactions. We chose miR-103a-1 as the representative miRNA to further confirm the relationship between hERG and miR-103a-1 on an electrophysiological level. We investigated the effect of miR-103a-1 on the current amplitude and gating properties of hERG. First, our results showed that miR-103a-1 decreased the activation current and tail current amplitude of hERG channel. Second, miR-103a-1 accelerated the inactivation process of hERG protein channel. These findings indicate that miR-103a-1 has an effect on the activation and inactivation kinetics of hERG protein channel. miR-103a-1 was also shown to significantly decrease both the maximum amplitude and tail current amplitude of hERG cells. Furthermore, miR-103a-1 decreased hERG current magnitude by attenuating activation, as well as accelerating hERG channel inactivation. According to our expectations, the current amplitude of I\textsubscript{k} is lower, the main reason should be the decrease of membrane protein expression. Any impairment in the synthetic machinery leads to suppression of the membrane protein expression. We demonstrated miRNA-mediated reduction in hERG protein level and a reduction of the hERG current amplitude. Interestingly, Part of the hERG gating kinetics have changed. hERG channels are modulated by several intracellular signaling pathways. Studies have founded that over-expression of the miR-17-5p caused impaired trafficking of hERG by modulating multiple endoplasmic reticulum stress-related chaperones.\textsuperscript{37}

We supposed about miR-103a-1 may affect other (native) proteins expressed in HEK293 cells that can modulate hERG channel gating. Last, AMO-103a-1 rescued the effect of miRNA-103a-1 on the electrophysiological properties of hERG channel.

It has been reported that a number of modulators up-regulate hERG channels, including phosphatidylinositol 4,5-bisphosphate,\textsuperscript{38} PKC,\textsuperscript{39} and direct binding of cAMP.\textsuperscript{40} Conversely, PKA-dependent phosphorylation has been shown to decrease current amplitude.\textsuperscript{41,42} and direct phosphorylation by protein kinase B (PKB) plays an important role in the downregulation of hERG, in which a PKB site is generated with the K897T polymorphism.\textsuperscript{43} The acute effects of PKC activation have been shown to reduce hERG current in HEK293 cells.\textsuperscript{44-46} Furthermore, Zhang et al. also confirmed that hERG current amplitude was decreased following inhibition of EGFR kinase. Recently, Lin et al. demonstrated that the length of a TG repeat polymorphism upstream of the KCNH2 transcription start site correlate negatively with the level of hERG transcription and QT prolongation. Given that hERG has a notable effect on cardiac electrical activity, further study of the mechanism of KCNH2 electrophysiology is clearly warranted.

As hERG gene could be regulated by miR-134, miR-143, and miR-103a-1. The detailed molecular mechanism by which miRNAs regulate cardiovascular arrhythmia is unclear and the underlying basis of the unusual kinetic properties of Kv11.1 channels continue to fascinate physiologists. Although there is no literature reports about endogenous levels of miR-103a-1 in the myocardium, there is the website showing miR-103a-1 is expressed natively in the human and mouse heart (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/organ.php). Thus, we are conducting experiments on isolated neonatal mouse myocardium cells to measure the effect on endogenous hERG currents.

**Conclusions**

Multiple miRNAs, including miR-134, miR-103a-1, miR-143, and miR-3619, were identified to regulate the expression of hERG. Corresponding AMOs may silence the effects of those miRNAs. Our results provided new insights to
better understand the functional mechanism of hERG protein channel and further build the foundation for gene therapy as alternative treatment for LQTS. However, these results need to be further validated in animal models.

Acknowledgements: The research was supported by the grants from the National Natural Science Foundation of China (81370207, 81370165, 31271541), Fund of Ningbo Science and Technology Innovation team (2011B28015), Natural Science Foundation of the Zhejiang Province (LY13H020008, LY13H020009). Natural Science Foundation of Ningbo (2013A610231, 2014A610276).

References

1. Sanguinetti MC; Tristani-Firouzi M: hERG potassium channels and cardiac arrhythmia. Nature 2006; 440:463-469.

2. Vandenberg JI, Perry MD, Perrin MJ, Mann SA, Ke Y, Hill AP: hERG K+ channels: Structure, function, and clinical significance. Physiol Rev 2012;92:1393-1478.

3. Raschi E, Vasina V, Poluzzi E, De Ponti F: The hERG K+ channel: Target and antitarget strategies in drug development. Pharmacol Rev 2008;57:181-195.

4. Zhao X, Shi YQ, Yan CC, Feng PF, Wang X, Zhang R, Zhang X, Li BX: Up-regulation of miR-21 and miR-23a contributes to As2O3-induced hERG channel deficiency. Basic Clin Pharmacol Toxicol 2015;116:516-523.

5. Reczko M, Maragkakis M, Alexiou P, Grosse I, Hatzigeorgiou AG: Functional microRNA targets in protein coding sequences. Bioinformatics 2012;28:771-776.

6. Grey F, Tirabassi R, Meyers H, Wu G, McWeeney S, Hook L, Nelson JA: A viral microRNA down-regulates multiple cell cycle genes through mRNA 5’UTRs. PLoS Pathog 2010;6:e1000967.

7. Hafner M, Landthaler M, Burger L, Khorshid M, Hauser J, Berninger P, Rothballer A, Ascano M Jr, Jungkamp AC, Munschauer M, Ulrich M, Wardle GS, Dewell S, Zavolan M, Tischler T: Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 2010;141:129-141.

8. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004;116:281-297.

9. Plasterk RH: Micro RNAs in animal development. Cell 2002;128:877-881.

10. Ambros V: The functions of animal micro RNAs. Nature 2004;431:350-355.

11. da Costa Martins PA, Leptidis S, Salic K, De Windt LJ: MicroRNA regulation in cardiovascular disease.Curr Drug Targets 2010;11:900-906.

12. Ikeda S, Pu WT: Expression and function of microRNAs in heart disease. Curr Drug Targets 2010;11:913-925.

13. Jazbutyte V, Thum T: MicroRNA-21: From cancer to cardiovascular disease. Curr Drug Targets 2010;11:926-935.

14. Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z: The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 2007;13:486-491.

15. Terentyev D, Belevych AE, Terentyeva R, Martin MM, Malana GE, Kuhn DE, Abdellatif M, Feldman DS, Elton TS, Gyorke S: miR-1 down-regulation contributes to re-expression of cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 2007;13:486-491.

16. Xiao L, Xiao J, Luo X, Lin H, Wang Z, Nattel S: Feedback remodeling of cardiac potassium current expression: A novel potential mechanism for control of repolarization reserve. Circulation 2008;118:983-992.

17. Luo X, Xiao J, Lin H, Li B, Lu Y, Yang B, Wang Z: Transcriptional activation by stimulating protein 1 and post-transcriptional repression by muscle-specific microRNAs of Kβs-encoding genes and potential implications in regional heterogeneity of their expressions. J Cell Physiol 2007;212:358-367.

18. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tischk T: Identification of tissue-specific microRNAs from mouse. Curr Biol 2002;12:735-739.

19. Zhou Z, Gong Q, Epstein ML: January CT: hERG channel dysfunction in human long QT syndrome. Intracellular transport and functional defects. J Biol Chem 1998;273:21061-21066.

20. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2^-delta delta C(T) method. Methods 2001;25:402-408.

21. Anderson CL, Delisle BP, Anson BD, Kilby JA, Will ML, Tester DJ, Gong Q, Zhou Z, Ackerman MJ: January CT: Most LQT2 mutations reduce Kv11.1 (hERG) current by aclass 2 (transfiguring-deficient) mechanism. Circulation 2006;113:365-373.

22. McPate MJ, Duncan RS, Milnes JT, Witchel HJ, Hancox JC: The N858K-hERG K+ channel mutation in the ‘short QT syndrome’: Mechanism of gain-in-function determined at 37 degrees C. Biochem Biophys Res Commun 2005;334:441-449.

23. Paulussen A, Raes A, Mathijs G, Snijders DJ, Cohen N, Aarsens J: A novel mutation (T65P) in the PAS domain of the human potassium channel HERG results in the long QT syndrome by trafficking deficiency. J Biol Chem 2002;277:48610-48616.

24. Ficker E, Objebero-Paz CA, Zhao S, Brown AM: The binding site for channel blockers that rescue misprocessed human long QT syndrome type 2 ether-a-go-go-related gene (HERG) mutations. J Biol Chem 2002;277:4989-4998.

25. Zhou Z, Gong Q, January CT: Correction of defective protein trafficking of a mutant hERG potassium channel in human long QT syndrome. Pharmacological and temperature effects. J Biol Chem 1999;274:31123-31126.

26. Huang N, Liang JF, He YH, Huang CP, Huang YL, Li N, Zhang X, Zhu QJ, Li ZF, Song TS, Huang C: The EGFP-hERG fusion protein alter the electrophysiological properties of hERG channels in HEK293 cells. Cell Biol Int 2011;35:193-199.

27. Lian J, Huang N, Zhou J, Ge S, Huang X, Huo J, Liu L, Xu W, Zhang S, Yang X, Huang C: Novel characteristics of a trafficking-defective G572R-hERG channel linked to hereditary long QT syndrome. Can J Cardiol 2010;26:417-422.

28. Delisle BP, Anson BD, Rajamani S, January CT: Biology of cardiac arrhythmias: Ion channel protein trafficking. Circ Res 2004;94:1418-1428.

29. Lu Y, Zhang Y, Wang N, Pan Z, Gao X, Zhang F, Han L, Xue Y, Bai Y, Sun L, Song W, Xu C, Wang Z, Yang B: MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. Circulation 2010;122:2378-2387.

30. Wang Z: The role of microRNA in cardiac excitability. J Cardiovasc Pharmacol 2010;56:460-470.

31. Luo X, Lin H, Pan Z, Xiao J, Zhang Y, Lu Y, Yang B, Wang Z: Down-regulation of miR-1/miR-133 contributes to re-expression of pacemaker channel genes HCN2 and HCN4 in hypertrophic heart. J Biol Chem 2008;283:20045-20052.

32. Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B: Rigoutsos I: A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. Cell 2006;126:1203-1217.

33. Lewis BP, Burge CB, Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15-20.

34. Goldoni D, Yarham JM, McGahon MK, O’Connor A, Guduric-Fuchs J, Edgar K, McDonald DM, Simpson DA, Collins A: A novel dual-fluorescence strategy for functionally validating microRNA targets in 3’ untranslated regions: Regulation of the inward rectifier potassium channel Kir2.1 by miR-212. Biochem J 2012;448:103-113.

35. Sun W, Julie Li YS, Huang HD, Shyy JY, Chien S: microRNA: A master regulator of cellular processes for bioengineering systems. Annu Rev Biomed Eng 2010;12:1-27.

36. Huttugnagir G, Zamore PD: A microRNA in a multiple-turnover RNAi enzyme complex. Science 2002;297:2056-2060.

37. Wang Q, Hu W, Lei M, Wang Y, Yan B, Liu J, Zhang R, Jin Y: MiR-17-5p impairs trafficking of h-ERG K+ channel protein by targeting multiple ER stress-related chaperones during chronic oxidative stress. PLoS One 2013;8:e84984.

38. Bian J, Cui J, McDonald TV: HERG K+ channel activity is regulated by changes in phosphatidyl inositol 4,5-bisphosphate. J Biol Chem 1999;274:31123-31126.
40. Cui J, Melman Y, Palma E, Fishman GI, McDonald TV: Cyclic AMP regulates the HERG K(+) channel by dual pathways. Curr Biol 2000;10:671-674.
41. Afrasiabi E, Hietamaki M, Viitanen T, Sukumaran P, Bergelin N, Tornquist K: Expression and significance of HERG (KCNH2) potassium channels in the regulation of MDA-MB-435S melanoma cell proliferation and migration. Cell Signal 2010;22:57-64.
42. Aomine M, Tatsukawa Y, Yamato T, Yamasaki S: Antiarrhythmic effects of magnesium on rat papillary muscle and guinea pig ventricular myocytes. Gen Pharmacol 1999;32:107-114.
43. Gentile S, Martin N, Scappini E, Williams J, Erxleben C, Armstrong DL: The human ERG1 channel polymorphism, K897T, creates a phosphorylation site that inhibits channel activity. Proc Natl Acad Sci U S A 2008;105:14704-14708.
44. Ramstrom C, Chapman H, Viitanen T, Afrasiabi E, Fox H, Kivela J, Soini S, Korhonen L, Lindholm D, Pasternack M, Tornquist K: Regulation of HERG (KCNH2) potassium channel surface expression by diacylglycerol. Cell Mol Life Sci 2010;67:157-169.
45. Wang YH, Shi CX, Dong F, Sheng JW, Xu YF: Inhibition of the rapid component of the delayed rectifier potassium current in ventricular myocytes by angiotensin II via the AT1 receptor. Br J Pharmacol 2008;154:429-439.
46. Cockerill SL, Tobin AB, Torrecilla I, Willars GB, Standen NB, Mitcheson JS: Modulation of hERG potassium currents in HEK-293 cells by protein kinase C. Evidence for direct phosphorylation of pore forming subunits. J Physiol 2007;581:479-493.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s website:

**Figure S1.** Effect of miR-103a-1 on recovery from inactivation of hERG channel. Inset shows the voltage-clamp protocol. A to D show representative current traces showing recovery from inactivation as reflected in the initial phase of the tail currents (arrow) in HEK293T cells transfected with hERG, in the presence or absence of miR-103a-1, cel-miR-67 or miR-103a-1 plus AMO-103a-1. E: The time constant value for channel recovery from inactivation was plotted as a function of test voltage (n = 6).

**Figure S2.** Effect of miR-103a-1 on deactivation of hERG channel. Inset shows the voltage-clamp protocol. A to D show representative deactivation in HEK293T cells transfected with hERG, in the presence or absence of miR-103a-1, cel-miR-67 or miR-103a-1 plus AMO-103a-1. The phase of deactivation is marked with an arrow. E: Fast and slow components of deactivation time constants (tau) were plotted as a function of the test potentials (n = 6).