Original Paper

MiR-223-3p as a Novel MicroRNA Regulator of Expression of Voltage-Gated K⁺ Channel Kv4.2 in Acute Myocardial Infarction

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Key Words

MicroRNA • miR-223-3p • Acute myocardial infarction • Arrhythmia • KCND2 • Kv4.2 • I_{to}

Abstract

Background/Aims: Acute myocardial infarction (AMI) is a devastating cardiovascular disease with a high rate of morbidity and mortality, partly due to enhanced arrhythmogenicity. MicroRNAs (miRNAs) have been shown to participate in the regulation of cardiac ion channels and the associated arrhythmias. The purpose of this study was to test our hypothesis that miR-223-3p contributes to the electrical disorders in AMI via modulating KCND2, the gene encoding voltage-gated channel Kv4.2 that carries transient outward K⁺ current I_{to}. **Methods:** AMI model was established in male Sprague-Dawley (SD) rats by left anterior descending artery (LAD) ligation. Evans blue and TTC staining was used to measure infarct area. I_{to} was recorded in isolated ventricular cardiomyocytes or cultured neonatal rat ventricular cells (NRVCs) by whole-cell patch-clamp techniques. Western blot analysis was employed to detect the protein level of Kv4.2 and real-time RT-PCR to determine the transcript level of miR-223-3p. Luciferase assay was used to examine the interaction between miR-223-3p and KCND2 in cultured NRVCs. **Results:** Expression of miR-223-3p was remarkably upregulated in AMI relative to sham control rats. On the contrary, the protein level of Kv4.2 and I_{to} density were significantly decreased in AMI. Consistently, transfection of miR-223-3p mimic markedly reduced Kv4.2 protein level and I_{to} current in cultured NRVCs. Co-transfection of AMO-223-3p (an antisense inhibitor of miR-223-3p) reversed the repressive effect of miR-223-3p. Luciferase assay showed that miR-223-3p, but not the negative control, substantially suppressed the luciferase activity, confirming the direct binding of miR-223-3p to the seed site within the KCND2 sequence. Finally, direct intramuscular injection of AMO-223-3p into the ischemic

myocardium to knockdown endogenous miR-223-3p decreased the propensity of ischemic arrhythmias. **Conclusions:** Upregulation of miR-223-3p in AMI repressed the expression of KCND2/Kv4.2 resulting in reduction of I_{to} density that can cause APD prolongation and promote arrhythmias in AMI, and therefore knockdown of endogenous miR-223-3p might be considered a new approach for antiarrhythmic therapy of ischemic arrhythmias.

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Introduction

Acute myocardial infarction (AMI) is a major cause of morbidity and mortality worldwide, and electrophysiology disorder is the main cause of lethal cardiac arrhythmias when the heart suffered ischemic attack [1-6]. A hallmark of the electrophysiological remodeling process in AMI is action potential (AP) prolongation along with reduction of transient outward K^+ current (I_{to}) [4, 5, 7]. AP is a key cellular determinant of cardiac electrical activity, which is shaped by multiple underlying ionic currents. Due to its early activation, I_{to} primarily governs the early re-polarization (phase 1) and determines the amplitudes and duration of AP in cardiomyocytes [8, 9]. Moreover, I_{to} can indirectly impact on later phases of membrane repolarization by setting the voltage-time trajectory for the activation of the subsequent ion currents such as I_{Ca} , I_{Kr} , and I_{Ks} [10]. In addition, blocking I_{to} has been reported as an ionic mechanism for the therapeutic efficacy of many anti-arrhythmic drugs [11-13].

MicroRNAs (miRNAs), a class of 22-26nt small endogenous single-stranded non-coding RNA, are post-transcriptional modulators that cause translational depression or target degradation of protein-coding genes by hybridizing to complementary sequences on the three prime untranslated regions (3'UTR) of the target messenger RNA transcripts (mRNAs) [14-17]. However, some recent papers have shown that miRNAs also target the coding sequences (CDS) of mammalian genes [18-20]. Several miRNAs have been reported to play prominent regulatory roles in cardiac excitability and heart pathological processes, including AMI, cardiac hypertrophy, and diabetic cardiomyopathy, through targeting the relevant ion channels encoding genes [21-27]. For instance, cardiac enriched microRNA-1 (miR-1) participates in regulating pathophysiological processes of coronary artery disease, cardiac hypertrophy [28] and ischemic arrhythmia [21, 29]. The proarrhythmic property of miR-1 has been verified not only in ischemic hearts but also in normal hearts [21]. Moreover, van Rooij et al. reported that 17 miRNAs are upregulated and 23 miRNAs downregulated in the heart 3-days post-MI [30]. This finding strongly indicates that the deregulated miRNAs might contribute to the pathogenesis of cardiac electrical and mechanical dysfunction in AMI. However, the potential participation of these deregulated miRNAs in AMI, particularly in the AMI-induced electrical disturbances, remains mostly uninvestigated.

Our initial computational analysis of the deregulated miRNAs identified by van Rooij et al. [30] using the BiBiServ database revealed that miR-223-3p has the potential to bind the CDS region of KCND2 (the gene encoding Kv4.2, the α -subunit of voltage-gated transient outward K^+ channel carrying I_{to}) [13]. Given the critical role of Kv4.2 in cardiac repolarization and the fact that miR-223-3p is upregulated in AMI [30], we anticipated that this miRNA might contribute to the regulation of arrhythmogenicity in AMI. This thought prompted us to conduct a series of experiments with both *in vivo* and *in vitro* approaches and combined molecular biology methods and electrophysiology techniques to establish the role of miR-223-3p in regulating the expression of KCND2/Kv4.2/ I_{to} and propensity of arrhythmias in ischemic hearts.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats of about 300g were provided by the Experimental Animal Center of the Harbin Medical University (Harbin, China). Our surgical and test procedures were in accordance with

the Guiding Principles of Laboratory Animal Care and Protocols, and were approved by the Animal Care Committee of the Harbin Medical University.

Rat model of acute myocardial infarction (AMI)

Male SD rats were randomly divided into two groups: sham group and AMI group. The procedure for establishing AMI model was similar to that previously described in detail [21]. Briefly, experimental animals were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). The animals were fixed on the table and their thorax were opened by blunt dissecting muscle with the heart exposed. The LAD or sham procedure was performed using a 6-0 silk suture, and then the thorax was closed and sutured. Standard lead II ECG recording was initiated 1 hour after ligation to monitor the electrical activities and arrhythmias. After surgery, survival animals were housed at about 20°C environmental temperature for 12 hours until the next experimental step.

Scoring of arrhythmias

Arrhythmic scores were calculated according to Curtis and Walker [31] scoring system: 0=no arrhythmia; 1>10s premature ventricular contraction (PVC) and/or ventricular tachycardia (VT); 2=11-30s PVC and/or VT; 3=31-90s PVC and/or VT; 4=91-180s PVC and/or VT, or reversible ventricular fibrillation (VF) of <10s; 5>180s PVC and/or VT>10s reversible VF; 6=irreversible VF.

Evans blue and TTC staining

We retrogradely injected 5% Evans blue dye (Sigma-Aldrich, USA) into vena cava to delineate myocardial perfusion region. After washing out remaining blood and Evans blue dye, the heart was cut into 2mm thick slices and stained with 1% triphenyltetrazolium chloride (TTC, Sigma Chemical Co, St Louis, MO, USA) at 37°C for 15min. The infarct area was expected to be stainless, the border zone to be stained in red, and the normal tissue to be stained in blue. For further study, the tissues in border zone of the hearts were collected and stored at -80°C.

Isolation of rat cardiomyocytes

The procedure for isolating rat ventricular cardiomyocytes has been described in detail elsewhere [32]. Briefly, the hearts were quickly removed from anesthetized rats and retrograde perfused with oxygen saturated Ca²⁺-containing Tyrode solution via aorta in the Langendorff perfusion apparatus. The Ca²⁺-containing Tyrode solution composition was as follows (in mM): NaCl 136.0, KCl 4.7, hydroxyethyl piperazine ethanesulfonic acid (HEPES) 10.0, MgCl₂ 1.0, CaCl₂ 1.0, and glucose 10.0, with pH balanced to 7.4 with NaOH at 37°C. The heart was then perfused with oxygen saturated Ca²⁺-free Tyrode solution (in mM): NaCl 136.0, KCl 4.7, HEPES 5.0, NaH₂PO₄ 0.33, MgCl₂ 1.0, and glucose 10.0, with pH 7.4 adjusted with NaOH at 37°C to remove Ca²⁺. At last, the heart was digested with the same solution containing 0.05% collagenase type II and 0.1% bovine serum albumin by perfusion. The whole perfusion process was kept warm at 37°C. Enzyme digestion was terminated by perfusion with Kraftbrune stock solution containing (in mM) potassium glutamate 70.0, β-hydroxybutyric 10.0, taurine 20.0, KCl 20.0, KH₂PO₄ 10.0, egtazic acid (EGTA) 10.0, glucose 25.0, 0.1% albumin, and mannitol 40.0 (pH 7.4 adjusted with KOH). Left ventricular peri-infarct tissues were cut off and minced into pieces in Kraftbrune stock solution. Cells were dispersed in suspension by blowing and the dispersed cells were stored at 4°C for patch.

Whole-cell patch-clamp recording of I_{to}

We recorded I_{to} current in isolated rat cardiomyocytes and cultured neonatal rat ventricular cells (NRVCs). The patch electrodes had tip resistance of 2-3 MΩ when filled with the pipette solution (in mM): KCl 20.0, potassium aspartate 110.0, HEPES 10.0, MgCl₂ 1.0, GTP 0.1, EGTA 10.0, Mg-ATP 5.0, and phosphocreatine 5.0 (pH 7.3 adjusted with KOH). The cells were dropped into the recording chamber placed on an inverted microscope (IX-70, Olympus) and perfused with Ca²⁺-containing Tyrode solution. Whole-cell recording was performed using a patch-clamp amplifier (Axopatch 200B, Axon instrument, USA). Signals were filtered at 1 KHz and data were obtained by an A/D converter (Digidata 1320, Axon instrument). Data were analyzed using Clampfit software 10.2. To record pure I_{to} with minimal contamination from other currents, 0.2 mM Cd²⁺ and 0.2 μM Ba²⁺ were used to block L-type Ca²⁺ current (I_{Ca,L}) and inward rectifier K⁺

current (I_{K1}), respectively. I_{to} was elicited by 300-ms depolarizing pulses to varying test potentials from a holding potential of -80 mV. Amplitude of I_{to} was measured as the difference between peak outward current and current at the end of the test pulse. The data were analyzed from 14 myocytes in each group. And these myocytes were collected from 6 rats for each group.

Isolation of primary neonatal rat ventricular cells (NRVCs)

To obtain the neonatal rat ventricular cells (NRVCs) for primary culture, hearts were removed from neonatal SD rats and minced in DMEM medium. The tissue trunks were then digested by 0.25% pancreatin at 37°C for 1-2 min, and cell suspension were transferred into DMEM containing 10% fetal bovine serum to terminate the digestion. Such a procedure was repeated several times until the tissue trunks disappeared. The collected suspensions were filtered and centrifuged at 1500 rpm for 5 min. And the cells were re-suspended in DMEM containing 10% fetal bovine serum and 100 µg/ml penicillin/streptomycin. The cells were cultured for 1.5-2 h for fibroblasts to attach to the bottom of culture dish, the cardiomyocytes were transferred into a new culture dish. After 48 h culturing, NRVCs that had grown with adherence to the bottom were used for experiments.

Synthesis of miR-223-3p mimic and its antisense inhibitor

rno-miR-223-3p (MIMAT0000892; sense strand: 5'-UGUCAGUUUGUCAAUACCCC-3'; anti-sense strand: 5'-ACAGUCAACAGUUUAUGGGG-3') and its exact antisense inhibitor AMO-223-3p (5'-ACAGTCAAACAGTTTATGGGG-3') were synthesized by GenePharma (Shanghai, China). A scrambled RNA mimic was synthesized and used as a negative control (miR-NC; sense strand: 5'-UUCUCCGAACGUGUCACGUTT-3'; anti-sense strand: 5'-ACGUGACACGUUCGGAGAATT-3') for miR-223-3p, which we labeled miR-NC. In addition, a separate scrambled RNA as a control for AMO-223-3p (AMO-NC: 5'-CAGUACUUUUGUGUAGUACAA-3') was also synthesized.

Western blot

Total protein was extracted from the peri-infarct zone of the rat left ventricular wall with the procedures essentially the same as described previously by our laboratory [21]. Heart tissue was lysated with lysis buffer on ice for 30 min and centrifuged at 13500 r/min for 15 min. Total protein concentration was quantified by BCA (bicinchoninic acid) Protein Assay Kit (Bio-Rad). One hundred micrograms total protein per sample was boiled for 5 min with SDS-loading buffer to denature the protein before loading. Then the denatured protein was separated by SDS-PAGE (10% polyacrylamide gels) and transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with blocking buffer (5% nonfat milk in PBS) at room temperature for 1 h, followed by incubation with primary antibodies to Kv4.2 (alomone), β -actin (an internal control, Kangcheng Inc.), Kv1.4 (proteintech) and Kv4.3 (alomone) at 4°C overnight. After wash, the membrane was then incubated with a secondary polyclonal antibody (Alexa Fluor, purchased from Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive bands were captured and quantified with Odyssey v3.0 software. For whole animal studies, five independent experiments were performed for each group (AMI and Sham), and for cultured NRVCs, three batches of cells for each group were measured.

Real-time reverse-transcription polymerase chain reaction (RT-PCR)

For quantification of miR-223-3p mRNA, real-time RT-PCR was carried out with total RNA samples extracted from rats left ventricle. The mirVana™ qRT-PCR microRNA Detection Kit (Ambion) was used according the procedures detailed elsewhere [21]. The RT primer sequence for miR-223-3p was 5'-GTCGTATCCAGTGGCTGTCTGCTGGAGTCGGCAATTGCACTGGATACGACGGGTA-3' and the PCR primer pairs for miR-223-3p were 5'-TGTCAGTTTGTCAAA-3' (forward) and 5'-CAGTGGCTGTCTGCTGGAGT-3' (reverse). U6 rRNA was used as an internal control. The RT primer sequence for U6 was 5'-CGCTTCACGAATTTGCGTGTCTAT-3' and the PCR primers for U6 were 5'-GCTTCGGCAGCACATATACTAAAAT-3' (forward) and 5'-CGCTTCACGAATTTGCGTGTCTAT-3' (reverse). The RT primer sequence for miR-1 was 5'-GTCGTATCCAGTGGCTGTCTGCTGGAGTCGGCAATTGCACTGGATACGACATACATAC-3' and the PCR primer pairs for miR-1 were 5'-GGCGTGGAATGTAAAGAA-3' (forward) and 5'-CGGCAATTGCACTGGATA-3' (reverse). We performed 5 independent experiments for each group.

In vivo gene transfection

Rats were randomly designed into two groups (AMO group and NC group). The animals were anesthetized with sodium pentobarbital (40 mg/kg i.p.). Then, the chest was opened under sterile conditions and the heart was exposed. AMO-223-3p (80 mg) and negative control (NC, 80 mg) were pretreated with X-treme (Roche, Germany) and then were injected into myocardium in about 10 sites with a 26-gauge needle, as previously described [21]. The standard lead II electrocardiogram was recorded after transfection.

Luciferase reporter gene activity assay

We amplified the CDS region of KCND2 containing the predicated target sites for miR-223-3p by PCR. The PCR fragment was inserted into the multiple cloning sites (Hind III and Sac I sites) in the pMIR-REPORT luciferase miRNA expression vector (Ambion, USA). HEK293T cells (1×10^5 /well) were co-transfected with 0.1 µg of the luciferase-KCND2 chimeric vector and miR-223-3p mimic (miR-NC, AMO-223-3p or AMO-NC) by lipofectamine 2000 (Invitrogen, USA). We collected the cell lysis after 24h transfection and measured luciferase activities with a dual luciferase reporter assay kit (Promega, USA).

Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test was used for two-group comparisons. Comparisons of incidence of arrhythmia and mortality between groups were performed using χ^2 -test. $P < 0.05$ was considered to indicate statistical significance.

Results

Enhanced arrhythmogenesis and mortalities in a rat model of AMI

The myocardial infarct size was assessed by Evans blue and TTC staining (Fig. 1A). Infarct regions were obviously visible in the cardiac apex sections and extended to the anterolateral free wall to varying extents during the early stage of AMI (1 h after LED ligation). Besides, significant elevation of ST segment was observed in the AMI group, which was not seen in the sham control mice. Moreover, three typical types of ventricular arrhythmias occurred in AMI rats: premature ventricular contraction (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF) (Fig. 1B).

Electrophysiological disorders occurred frequently during the first hour of AMI, but were hardly observed in the sham group (Fig. 1C). Cumulative arrhythmic duration was significantly longer in the AMI rats than in the sham animals (Fig. 1D). Arrhythmic score was enormously higher in the AMI group than in the sham group (Fig. 1E). Consistent with the enhanced arrhythmogenicity in AMI rats, the mortality rate reached as high as 40% (Fig. 1F).

Up-regulation of miR-223-3p and down-regulation of $K_v4.2$ and I_{to} in AMI

The level of miR-223-3p was found 7.2 ± 2.2 fold higher in the ischemic border zone (peri-infarct area) of AMI rats than that of sham control animals (Fig. 2A). As a positive control, we observed remarkable up-regulation of miR-1 in AMI (Fig. 2B). On the other hand, the protein level of $K_v4.2$ was decreased by 30.8% in AMI compared to sham (Fig. 2C). In accordance with the down-regulation of $K_v4.2$, I_{to} density was reduced in ventricular cardiomyocytes from AMI rats relative to the sham counterparts (Fig. 2D & 2E).

$K_v4.2$ as a target gene of miR-223-3p

According to our bioinformatics analysis, the coding region of KCND2 mRNA carries a sequence motif at the position from 938 to 964 complementary to the seed site of miR-223-3p with minimal free energy of -22.5 kcal/mol (Fig. 3A). To experimentally verify $K_v4.2$ as a target of miR-223-3p, we first looked at the effect of miR-223-3p on the expression of $K_v4.2$ at the protein level in primary cultured neonatal rat ventricular cardiomyocytes (NRVCs). As illustrated in Fig. 3B, transfection with miR-223-3p mimic markedly reduced $K_v4.2$ protein level, and this repressive effect was abrogated by co-transfection with AMO-223-3p, the antisense inhibitor of miR-223-3p. The scrambled RNA as a negative control for miR-223-3p

Fig. 1. Cardiac damages in a rat model of acute myocardial infarction (AMI) established after ligation of the left anterior descending artery. (A) Representative images of cardiac slices with Evans blue and triphenyltetrazolium chloride (TTC) staining showing the infarcted area. (B) Typical examples of ECG traces showing the ischemic ventricular arrhythmias: premature ventricular contraction (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF). (C) The statistics of the incidences of arrhythmias. (D) Duration (in second) of arrhythmias. (E) Arrhythmia scores. (F) The mortality of animals between sham groups and AMI groups. * $P < 0.05$ vs. sham control; $n = 12$ rats.

(miR-NC) failed to affect Kv4.2 expression. As further controls for the specificity of action of miR-223-3p, the protein levels of Kv1.4 and Kv4.3, the other two α -subunits of I_{to} [13], were unaffected by miR-223-3p (Fig. 4), nor by miR-1 that has no putative binding sites in the mRNAs of Kv1.4, Kv4.2 and Kv4.3 (Fig. 5).

Furthermore, whole-cell patch clamp studies of I_{to} was used to verify the functional significance of Kv4.2 regulation by exogenous miR-223-3p over-expression in NRVCs. I_{to} density in NRVCs transfected with miR-223-3p was severely diminished by 76.8%. And this depression induced by exogenous miR-223-3p was abolished by AMO-223-3p (Fig. 3C & 3D).

We then went on to perform luciferase reporter gene assay to further verify the targeting relationship between miR-223-3p and Kv4.2. To this end, we subcloned the CDS of Kv4.2 into a luciferase plasmid to construct a chimeric vector, and then transfected this vector into NRVCs with or without co-transfection of miR-223-3p or miR-NC. As depicted in Fig. 3E, miR-223-3p, but not miR-NC, substantially suppressed the luciferase activity.

As the expression of miR-223-3p is extremely high when the heart suffered AMI, we

Fig. 2. Deregulation of miR-223-3p expression causes down-regulation of Kv4.2 and reduction of I_{to} density in AMI. (A, B) Up-regulation of miR-223-3p (A) and miR-1 (B) expression in AMI relative to sham hearts. miR-223-3p was quantified by real-time RT-PCR. * P <0.05 vs. sham control; n =5 for each group. (C) Reduction of Kv4.2 protein (72 kDa) level in the peri-infarct zone of the AMI hearts, as determined by Western blot analysis. * P <0.05 vs. sham control; n =6 for each group. (D) Representative traces of I_{to} recorded by whole-cell patch-clamp techniques showing the decrease of the current amplitude in ventricular myocytes isolated from AMI hearts as compared with those from sham control hearts. I_{to} was elicited by the voltage protocols shown in the inset. (E) Comparison of current-voltage relationship (I-V curve) of I_{to} between the AMI and sham groups. * P <0.05 vs. sham control; n =14 cells from 6 hearts for each group.

transfected its inhibitor AMO-223-3p into ventricular cardiomyocytes to test whether it could alleviate the incidences of arrhythmias caused by AMI. Our results in Fig. 3F showed that AMO-223-3p significantly reduced the incidence of VT and VF.

Discussion

A number of miRNAs have been identified for their ability to target ion channel encoding genes to control arrhythmogenicity in the heart. For example, miR-1 has been documented to repress KCNJ2 (encoding Kir2.1 inward rectifier K^+ channel α -subunit) and GJA1 (encoding connexin-43 gap junction channel) so as to slow cardiac conduction in the setting of AMI

Fig. 3. Experimental verification of KCND2/Kv4.2 as a target for miR-223-3p and the role of miR-223-3p in ischemic arrhythmias. (A) Complementarity between rno-miR-223-3p (green) and the seed site in the KCND2 mRNA sequence (red) with sequence alignment between miR-223-3p and the CDS of KCND2 of rat. Watson-Crick complementarity is connected by "I", and the G:U/U:G wobble is connected by "I". (B) Repressive effect of miR-223-3p on Kv4.2 protein expression in neonatal rat ventricular cells (NRVCs). Left panel: the representative Western blot

band images; right panel: averaged band densities. Note that the negative control (miR-NC) failed to alter Kv4.2 protein level. * $P < 0.05$ vs. Ctl & # $P < 0.05$ vs. miR-223-3p; $n = 3$ for each group. (C) Representative traces of I_{to} recorded by whole-cell patch-clamp techniques showed that miR-223-3p decreased the current amplitude of I_{to} in neonatal rat ventricular cells (NRVCs). (D) Comparison of current-voltage relationship (I-V curve) of I_{to} between the miR-NC, miR-223-3p and +AMO-223-3p. * $P < 0.05$ vs. miR-NC; $n = 5$ cells in miR-NC and miR-223-3p group. # $P < 0.05$ vs. miR-223-3p; $n = 7$ cells in +AMO-223-3p group. (E) Luciferase assay for the interactions between miR-223-3p and the CDS region of KCND2. * $P < 0.05$ vs. Ctl; $n = 3$ for each group. (F) Knockdown of endogenous miR-223-3p by its antisense inhibitor AMO-223-3p, but not by the negative control (AMO-NC) decreased the incidence of VT and VF induced by AMI. * $P < 0.05$ vs. Ctl & # $P < 0.05$ vs. AMO-NC; $n = 6$ rats for AMO-NC group, $n = 10$ rats for AMO-223-3p group.

[21]. miR-1 has also been found to cause atrioventricular block in rodents by repressing $I_{Ca,L}$, as well as I_{K1} and connexin-43 [33]. In addition, miR-1 has also been demonstrated to downregulate HCN2 and HCN4 protein levels in ischemic rat myocardium [34]. SCN5A that codes for cardiac Nav1.5 fast-activating and inactivating Na^+ channel was reported to be directly regulated by miR-98, miR-106, miR-200, and miR-219, and indirectly by miR-125

Fig. 4. Effect of miR-223-3p on the expression of Kv1.4 and Kv4.3. No effect of miR-223-3p on Kv1.4 (A) or Kv4.3 (B) protein expression in neonatal rat ventricular cells (NRVCs). Left panel: the representative Western blot band images; right panel: averaged band densities. Note that after transfecting miR-NC, miR-223-3p or AMO-223-3p in NRVCs, the protein expression of Kv1.4 and Kv4.3 has no change. n=3 for each group.

Fig. 5. Effect of miR-1 on the expression of Ito components. There is no effect of miR-1 on Kv1.4 (A), Kv4.2 (B) or Kv4.3 (C) protein expression in neonatal rat ventricular cells (NRVCs). Left panel: the representative Western blot band images; right panel: averaged band densities. Note that after transfecting miR-NC, miR-1 or AMO-1 in NRVCs, the protein expression of Kv1.4, Kv4.2 and Kv4.3 has no change. n=3 for each group.

and miR-153 [35]. It was found that both miR-21 and miR-23a are involved in As₂O₃-induced abnormal QT prolongation as cardiotoxicity by causing deficiency of hERG (rapid delayed rectifier K⁺ channel) at transcriptional and transportational/trafficking levels [36]. miR-

21 has also been shown to decrease $I_{Ca,L}$ density by downregulating Ca^{2+} channel subunits expression in human atrial myocytes [37]. Similarly, another cardiac-enriched miRNA miR-133a negatively regulates the expression of L-type calcium $\alpha 1C$ subunit, resulting in the decrease of intracellular Ca^{2+} content and the attenuation of ISO-induced cardiomyocyte hypertrophy [38]. Probably the most relevant to the present study is the finding reported by Panguluri et al. [39] who showed that miR-301a inhibits Kv4.2 expression in diabetic myocardium, which might contribute to the excessive prolongation of AP under such a pathological condition. However, miR-301a has not been shown to be deregulated in its expression in AMI in any published studies, indicating its minimal contribution to Kv4.2/ I_{to} suppression observed in AMI. Inhibition of I_{to} is a common phenomenon in a wide variety of cardiac pathological processes [40]. The exact consequence of this inhibition, as well as the molecular mechanisms for this inhibition, remained incompletely understood. To date, there have not been any published studies on whether miRNAs might play a role in regulating Kv4.2 in the setting of AMI. Our finding that miR-223-3p targeted directly on Kv4.2/ I_{to} therefore represents the first of such an effort.

Our finding together with those from the published studies suggests that miRNAs are indeed involved in the regulation of Kv4.2/ I_{to} and it is possible that under different cardiac conditions, different miRNAs predominate in terms of the regulation of Kv4.2/ I_{to} . For instance, miR-301a plays a role in defining the level of Kv4.2/ I_{to} in diabetic heart [39] and miR-223-3p might be more important in ischemic heart, as revealed by the present study. It is further likely that multiple miRNAs participate in the overall down-regulation/inhibition of Kv4.2/ I_{to} in AMI, which remains yet to be elucidated.

miR-223-3p is a hematopoietic specific miRNA, which plays an important role in myeloid lineage development and granulocytic differentiation [41-43]. Down-regulation of miR-223-3p has been associated with higher tumor burden, disease aggressiveness, and poor prognostic factors [44]. It has also been correlated with other diseases, such as rheumatoid arthritis, sepsis, type 2 diabetes and hepatic ischemia [45-48]. Most interestingly, elevated serum miR-223-3p was identified as one of the miRNA biomarkers for AMI and angina pectoris diagnosis [49]. The present study showed that miR-223-3p was elevated in its expression level in ischemic myocardium, consistent with the published study [30]. It is not clear what the relationship is between the circulating and myocardial miR-223-3p. Is myocardial miR-223-3p a source of circulating miR-223-3p? If yes, how is myocardial miR-223-3p released into the bloodstream? Future studies are absolutely needed to clarify this issue. Nonetheless, our study unraveling the participation of miR-223-3p in regulating KCND2/Kv4.2/ I_{to} in AMI adds to our understanding of the molecular mechanisms underlying the electrical alterations in ischemic myocardium.

It is commonly accepted that I_{to} plays a crucial role in controlling phase 1 of repolarization of the cardiac action potential and, as a result, is key to modulating excitation-contraction coupling and propensity for arrhythmia [50]. Post-MI myocytes have significantly prolonged action potential duration (APD) with marked heterogeneity of the time course of repolarization. The prolongation of APD could be explained by the significant decrease of the density of both I_{to} [51-53]. Hence, it is conceivable that up-regulation of miR-223-3p in AMI represses KCND2/Kv4.2 to reduce I_{to} that in turn lengths APD leading to enhanced susceptibility to arrhythmia induction.

Perhaps the most important finding of this study was that knockdown of endogenous miR-223-3p by its antisense inhibitor suppressed arrhythmogenesis induced by AMI, indicating this miRNA as a potential new molecular target for antiarrhythmic therapy of ischemic arrhythmias. Our Blast search results indicate that the rat version of KCND2 cDNA (*Oryctolagus cuniculus*; NM_001082118.1) shares 91% identity to the human homolog (*Homo sapiens*, NM_012281.2) and the 24 nucleotides containing the seed site for miR-223-3p are 100% homologous between the two species. This indicates that the targeting relationship established in rat heart in the present study might be applicable to humans.

Yet, it should be noted that like other miRNAs, miR-223-3p likely act on multiple target genes that are involved in the regulation of the pathophysiological phenotypes of

AMI. Moreover, multiple miRNAs or even some other types of RNA molecules can also have regulatory roles in KCND2/Kv4.2/ I_{to} . The present study does not exclude any of the additional possible mechanisms. Our study has merely touched on the topic and future investigations are warranted to gain comprehensive understanding of the nature of cardiac injuries in AMI and the regulation of genes relevant to these aspects.

Conclusion

On the basis of our results, we concluded that up-regulation of miR-223-3p in AMI repressed the expression of KCND2/Kv4.2 resulting in reduction of I_{to} density that can cause APD prolongation and promote ischemic arrhythmias. Our finding that knockdown of endogenous miR-223-3p by its antisense inhibitor suppressed arrhythmogenesis induced by AMI suggest this miRNA as a potential new molecular target for antiarrhythmic therapy of ischemic arrhythmias.

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Disclosure Statement

The authors report that they have no conflicts of interest.

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