Feedback Mechanisms for Cardiac-Specific MicroRNAs and cAMP Signaling in Electrical Remodeling

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Background—Loss of transient outward K+ current (Ito) is well documented in cardiac hypertrophy and failure both in animal models and in humans. Electrical remodeling contributes to prolonged action potential duration and increased incidence of arrhythmias. Furthermore, there is a growing body of evidence linking microRNA (miR) dysregulation to the progression of both conditions. In this study, we examined the mechanistic basis underlying miR dysregulation in electrical remodeling and revealed a novel interaction with the adrenergic signaling pathway.

Methods and Results—We first used a tissue-specific knockout model of Dicer1 in cardiomyocytes to reveal the overall regulatory effect of miRs on the ionic currents and action potentials. We then validated the inducible cAMP early repressor as a target of miR-1 and took advantage of a clinically relevant model of post myocardial infarction and miR delivery to probe the mechanistic basis of miR dysregulation in electrical remodeling. These experiments revealed the role of inducible cAMP early repressor as a repressor of miR-1 and Ito, leading to prolonged action potential duration post myocardial infarction. In addition, delivery of miR-1 and miR-133a suppressed inducible cAMP early repressor expression and prevented both electrical remodeling and hypertrophy.

Conclusions—Taken together, our results illuminate the mechanistic links between miRs, adrenergic signaling, and electrical remodeling. They also serve as a proof-of-concept for the therapeutic potential of miR delivery post myocardial infarction. (Circ Arrhythm Electrophysiol. 2015;8:942-950. DOI: 10.1161/CIRCEP.114.002162.)

Key Words: electrical remodeling  ■  cAMP  ■  ion channel  ■  microRNA  ■  myocardial infarction

MicroRNAs (miRs) are endogenously expressed, noncoding RNAs of ≈22 nucleotides in length.1 When processed by the ribonuclease III (RNase III) enzyme, DICER1, and incorporated into the RNA-induced silencing complex, they target a variety of coding messenger RNAs for degradation and translational repression.2 In cardiac hypertrophy and failure, the cardiomyocyte-specific miR-1 and miR-133a are dramatically reduced.3,4 These miRs are cotranscribed from a common gene and together have been ascribed critical regulating roles in hypertrophy,3,5,6 apoptosis,7,8 fibrosis,9 and ion channel expression.10-14 MiR-1, in particular, has been shown to enhance the transient outward K+ current (Ito) by repressing iroquois homeobox domain 5 (IRX5), a transcriptional inhibitor of Kcnd2 (Kcnq2), the main pore-forming subunit for Ito, and loss of miR-1 may underlie the Ito remodeling seen post myocardial infarction (MI).12,15,16

In the acute phase of MI, levels of miR-1 and miR-133a increase and can be detected in the circulation.17 However, these miRs quickly decline,18,19 and lead to chronically depressed levels.20 Myocardia from patients with ischemic heart failure exhibited decreased levels of miR-1 and miR-133a, which were restored after implantation of a left ventricular assist device.21 In a separate study, loss of DICER1 reported in the end-stage heart failure patients was similarly rescued by left ventricular assist device implantation.22

A recent study has identified the presence of a cAMP response element (CRE) sequence in the miR-1/miR-133a promoter region,23 implicating a potential role for β-adrenergic signaling in the regulation of miR expression. In addition, CRE modulator (CREM) is a predicted target of miR-1. CREM acts as a regulator of CRE-binding protein (CREB) signaling.24 Both the proteins are activated by β-adrenergic signaling and compete for binding to the CRE in gene promoters.25,26 One isoform of CREM, termed the inducible cAMP early repressor (ICER), arises from an alternative internal promoter and is induced by β-adrenergic signaling.7 ICER contains only the CRE DNA-binding domain and acts as a powerful repressor of CREB signaling. Under physiological conditions, ICER acts in a negative feedback fashion to prevent over activation of CREB-dependent genes. Under chronic pathological conditions, excessive β-adrenergic signaling drives a progressive increase in ICER expression that may contribute to inhibition
WHAT IS KNOWN

- Electrical remodeling is well documented in cardiac hypertrophy and failure both in animal models and in humans, contributing to prolonged action potential duration and increased incidence of arrhythmias.
- Even though cardiomyocyte-specific miR-1 and miR-133a have been documented to be dramatically reduced in cardiac hypertrophy and failure, the underlying mechanisms for the miR dysregulation remain incompletely understood.

WHAT THE STUDY ADDS

- Expression of the inducible cAMP early repressor is significantly increased in animal models post myocardial infarction or chronic isoproterenol infusion leading to long-term suppression of miR-1 and miR-133a.
- Furthermore, both cAMP response element modulator and inducible cAMP early repressor are targets of miR-1 and delivery of miR-1 and miR-133a suppressed inducible cAMP early repressor expression and prevented both electrical remodeling and hypertrophy.
- Our study establishes a feedback mechanism between cardiac-specific miRs, adrenergic signaling, and electrical remodeling. They also serve as a proof-of-concept for the therapeutic potential of miR delivery post myocardial infarction.

of CREB-dependent gene expression and β-adrenergic desensitization. Recent studies have documented the beneficial effects of preserving β-adrenergic sensitivity after an MI and knockout of CREM was shown to be protective under chronic β-adrenergic signaling. In addition, cardiac-specific knockout of CREB led to electrical remodeling in cardiomyocytes similar to that seen post MI with a loss of I\textsubscript{kr} and prolonged action potential durations (APDs).

Electrical remodeling has been well documented in cardiac hypertrophy and failure with downregulation of K\textsuperscript{+} currents and APD prolongation. Moreover, recent studies have provided strong evidence for the critical roles of miRs in ion channel regulation. In addition, loss of miRs may underlie the well-documented electrical remodeling seen in pathological cardiac hypertrophy and failure. However, the mechanistic basis leading to miRs dysregulation with significant loss of miR-1/miR-133a in diseased conditions remains incompletely understood.

Because the promoter region of cardiac-specific miR-1/miR-133a contains the CRE sequence, we hypothesize that chronic cAMP signaling may underlie miRs dysregulation. Specifically, we hypothesize that under pathological conditions, chronic overexpression of ICER from excessive β-adrenergic signaling may repress miR-1 expression leading to the well-documented electrical remodeling. To test the hypothesis, we first examined the roles of miRs in the regulation of cardiac excitability by taking advantage of a knockout model of Dicer1 and cardiac delivery of Cre recombinase. This model allowed us to directly test the roles of miRs on ionic currents without the interference of organ level changes, such as hypertrophy or fibrosis. We then investigated the relationship between miR-1/miR-133a, cAMP signaling, and electrical remodeling in a clinically relevant mouse model of MI. Indeed, we documented that I\textsubscript{c} mRNAs is significantly increased in the MI model. We further demonstrated that chronic isoproterenol infusion results in similar upregulation of I\textsubscript{c} mRNAs. In vivo delivery of miRs was used to directly establish the relationship between miR-1/miR-133a and ICER. Expression of ICER is normalized after delivery of miRs in both the MI and the chronic isoproterenol infusion models. Moreover, we directly validate both CREM and ICER as the targets of miR-1 establishing a feedback mechanism between cardiac-specific miRs and cAMP signaling. Our study uncovers novel mechanisms of interactions between cardiac-specific miRs, pathological cAMP signaling post MI, and their possible roles in proarrhythmic electrical remodeling.

In vivo isoproterenol infusion at a dose of 30 mg/kg per day in C57 BL/6J mice (10–16 weeks) was performed as previously published. C57 BL/6J mice (10–16 weeks) were prepared and the heart exposed to reveal the left anterior descending coronary artery. The left anterior descending coronary artery was then ligated for 45 minutes and allowed to reperfuse.

In Vivo Mouse Model

Ischemia–reperfusion (IR) model of MI was performed as previously published. Mmu-miR-1 and miR-133a mimics (Dharmacon C-310377, C-310408) were packaged as using MaxSuppressor in vivo RNA-LANCEr II (Bioo scientific 3410-01) and delivered together via tail vein injections 1 week after left anterior descending coronary artery surgery at 30 μg per 28 g mouse. Nonsilencing MiR mimic transfection control with and without Dy547 (Dharmacon CP-004500-01) was injected in control animals.

In Vivo miR Injections

Mmu-miR-1 and miR-133a mimics (Dharmacon C-310377, C-310408) were packaged as using MaxSuppressor in vivo RNA-LANCER (Bioo scientific 3410-01) and delivered together via tail vein injections 1 week after left anterior descending coronary artery surgery at 30 μg per 28 g mouse. Nonsilencing MiR mimic transfection control with and without Dy547 was injected in control animals.

In Vivo Mouse Model of Isoproterenol Infusion

In vivo isoproterenol infusion at a dose of 30 mg/kg per day in C57 BL/6J mice (10–16 weeks) was performed using Alzet osmotic...
Sham littermate animals received osmotic mini-pumps loaded with saline alone.

Analysis of Cardiac Function by Echocardiography
Echocardiograms using M-mode and 2-dimensional measurements to assess systolic function were performed as described previously.37

Electrocardiographic Recordings
Electrocardiographic recordings were obtained at 33°C using Bioamplifier (BMA 831, CWE, Incorporated, Ardmore, PA) as previously described.39 A total of 100 beats were analyzed from each animal in a blinded fashion. The rate-corrected QT interval (QTc) was calculated using modified Bazett formula as reported by Mitchell et al39 for mouse models, whereby the R–R interval was first expressed as a unitless ratio (R–R in ms/100 ms). QTc interval was defined as (QT interval [in ms]/[RR/100]1/2).

Adult Mouse Cardiomyocyte Isolation
Single mouse left ventricular myocytes were isolated as previously described.41 The cells were then enriched for cardiomyocytes. Final cell populations were determined to be 95% myocytes as measured by flow cytometry (Figure I in the Data Supplement).

Patch-Clamp Recordings
Whole cell and perforated patch clamp recordings were performed as previously described.41,42

Flow Cytometric Analysis
Flow cytometric analysis of isolated mouse cells was performed as described.37

Quantitative Real-Time Polymerase Chain Reaction
RNA was isolated from adherent neonatal and dissociated adult cardiomyocytes. Quantitative real-time polymerase chain reaction was performed using RT² SYBR Green Master Mix (Qiagen 330520) and the Viia 7 Real-Time polymerase chain reaction System (ABI).

Immunofluorescence Confocal Microscopy
Immunofluorescence confocal microscopy was performed as previously described.43,44

miR-1 Target Validation Using Luciferase Assay and Western Blot Analysis
Luciferase assay was performed using the Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System (Life Technologies T1003). Additional validation was performed using Western blot analysis.

Statistical Analyses
Significant difference between groups was determined using Origin Pro 7 software by 1-way ANOVA and validated by Bonferroni, Sheffe, and Tukey tests with an n>3 at P<0.05 unless otherwise noted.

Results
A Loss of DICER1 Results in Electrical Remodeling Independent of Cardiac Hypertrophy and Failure
Mouse hearts subjected to IR displayed decreased levels of Dicer1 mRNA (Figure 1A). To directly determine if a loss of DICER1 alone without concomitant cardiac hypertrophy can result in electrical remodeling of cardiac ionic currents and action potentials,

![Figure 1. Dicer knockout leads to electrical remodeling in adult cardiomyocytes. A, Quantitative real-time polymerase chain reaction results normalized to GAPDH expression from sham and myocardial infarction (MI) mice for Dicer1 mRNA (n=3, *P<0.05). B, Paraffin sections of a Dicerflx/flx mouse heart after Ad-GFP-Cre injection showing green fluorescence protein (GFP) expression at the injection site at low magnification (top; scale, 100 μm) and high magnification (bottom; scale, 20 μm). C, Action potentials recorded from left ventricular cardiomyocytes isolated from wild-type (WT) compared with Dicerflx/flx mice using a stimulation frequency of 0.1 Hz. Cardiomyocytes which were transduced with Ad-GFP-Cre were identified using GFP fluorescence. Bottom, The summary data of action potential duration (APD) at 50% and 90% of repolarization (APD50 and APD90) n=8 for each group. *P<0.05. D, Examples of I\textsubscript{r} recorded from left ventricular myocytes isolated from WT and Dicerflx/flx mice. I\textsubscript{r} was elicited from a holding potential of −80 mV using a family of voltage steps from −70 to +60 mV with 10-mV increment and 2.5 s in duration. The interpulse interval was 10 s. Bottom, The summary data for the current–voltage relationships for the peak and steady-state currents (n=7 for each group, *P<0.05). E, Examples of I\textsubscript{Ca} recorded from left ventricular myocytes isolated from adult WT and Dicerflx/flx mice. I\textsubscript{Ca} was elicited from a holding potential of −55 mV using a family of voltage steps from −40 to +60 mV with 10-mV increment and 500 ms in duration. The interpulse interval was 5 s. Bottom, The summary data for the current–voltage relationships (n=5 for WT and 10 for Dicerflx/flx mice, respectively, *P<0.05).]
we delivered a Cre recombinase expressing adenovirus (Ad-GFP-Cre) to the left ventricles of Dicer1tm1Bdh/J mice (Figure 1B) that contain a floxed RNase III domain critical for DICER1 function.45 This model allowed us to directly test the roles of miRs on ionic currents without the interference of organ level changes, such as hypertrophy or fibrosis. Patch-clamp analyses were performed using isolated ventricular myocytes. Knockout of Dicer1 in adult mouse cardiomyocytes led to prolonged APDs (Figure 1C) resulting from decreased transient outward K+ current (Ito; Figure 1D) and increased Ca2+ currents (Icav; Figure 1E).

Parallel experiments were performed using cardiomyocytes isolated from neonatal Dicer1tm1Bdh/J mice. Cells were transduced 48 hours after isolation with Ad-GFP-Cre or Ad-GFP for experimental and control groups, respectively, at a multiplicity of infection of 10 to 50. Myocytes were cultured for a total of 7 days and subjected to patch-clamp recording of Ito currents and real-time polymerase chain reaction analyses. Figure 2A shows immunofluorescence confocal images of neonatal cardiomyocytes transduced with Ad-GFP-Cre (left) compared with cardiomyocytes transduced with Ad-GFP-Cre (right). Ito was elicited using the same protocol as in Figure 1. Bottom. The summary data for current–voltage relationships from peak and steady-state component comparing cardiomyocytes transduced with Ad-GFP or Ad-GFP-Cre (n=6 cells for each group, *P<0.05). DAPI indicates 4',6-diamidino-2-phenylindole; and TBP, TATA box binding protein.

**Figure 2. Dicer knockout leads to electrical remodeling in neonatal cardiomyocytes.** A, Immunofluorescence confocal images of neonatal cardiomyocytes transduced with Ad-GFP and Ad-GFP-Cre. Cre recombinase (red) is seen in the nuclei of Ad-GFP-Cre cells. Green fluorescence protein (GFP) expression (green) is seen in transfected cells (scale, 50 μm). B, Reverse transcription polymerase chain reaction products show Cre transcripts in Ad-GFP-Cre transfected cells. C, Quantitative polymerase chain reaction results normalized to GAPDH expression and relative to Ad-GFP–treated controls (n=3, *P<0.05). D, Ito currents recorded from neonatal cardiomyocytes transduced with Ad-GFP (left) compared with cardiomyocytes transduced with Ad-GFP-Cre (right). Ito was elicited using the same protocol as in Figure 1. Bottom, The summary data for current–voltage relationships from peak and steady-state component comparing cardiomyocytes transduced with Ad-GFP or Ad-GFP-Cre (n=6 cells for each group, *P<0.05). DAPI indicates 4',6-diamidino-2-phenylindole; and TBP, TATA box binding protein.

**Crem and Icer are Targets of miR-1**

Using microRNA.org and miRwalk databases, we identified CREM, as well as its 2 ICER isoforms, as potential targets of miR-1 with an mirSVR score of −1.2211. To directly validate CREM and ICER as miR-1 targets, we first identified the predicted miR-1 binding sequence to be present in the 3′ untranslated regions of all CREM isoforms except CREM7 and 8, which correspond to τ-like isoforms not found in the heart (Figure 3A). Importantly, the miR-1 binding sequence was found in the 2 ICER isoforms of CREM (Figure 3A). The conserved sequence (Crem) and scramble sequence (scramble) were subcloned into luciferase reporter vectors (Figure IIa in the Data Supplement). Luciferase assays were then performed using a miR-1 mimic compared with a nonsilencing control miR. A vector expressing β-galactosidase served as a loading control for normalization. MiR-1 treatment induced a dose-dependent decrease in luminescence in the Crem-transfected cells (Figure IIb and IIC in the Data Supplement). Crem-transfected cells displayed a 32% decrease in luminescence with miR-1 treatment (1 pmol) compared with the control miR (Figure 3B and 3C). No change from control was observed with miR-1 treatment in cells transfected with the scramble sequence or empty vector.

We further validated CREM and ICER as miR-1 targets at the protein level using Western blot analysis (Figure IIId in the Data Supplement). HEK 293 cells were transfected with a plasmid containing human Crem. Cells were then treated with miR-1 mimic and compared with a nonsilencing control miR. Western blot analysis was performed using anti-CREM and anti-β-tubulin antibodies demonstrating a significant reduction in the level of
CREM protein in cells treated with miR-1 mimic compared with nonsilencing control (Figure IIId in the Data Supplement).

**Delivery of miR-1/133a Preserves Left Ventricular Function and Prevents Hypertrophy After MI**

We took advantage of in vivo delivery of miR-1 and the co-transcribed miR-133a to directly test the roles of miR-1 and miR-133 on the long-term electrical remodeling after an MI. The optimization of miR-1/miR-133a delivery was first performed in HEK 293 cells (Figure III in the Data Supplement). We used a clinically relevant model of IR in wild-type C57 mice where the left ascending coronary artery was ligated for 45 minutes and then allowed to reperfuse (Figure IVa in the Data Supplement). We reason that this model accurately represents the clinical situation where occluded coronary arteries are rapidly revascularized. In both the IR model and in patients, the long-term effects of MI include hypertrophy, fibrosis, electrical remodeling, and progression toward heart failure.

Echocardiographic recordings 1 week after IR surgery revealed decreased fractional shortening and increased left ventricular end-systolic diameter (Figures 4C; Figure IVb in the Data Supplement). Mice were then randomized to receive miR-1 and miR-133a or control nonsilencing miR packaged in a neutral lipid emulsion by tail vein injections (Figure 4A and 4B). Three weeks after IR surgery, fractional shortening continued to decline in MI mice receiving the control injection (MI control), whereas MI mice that received the miR-1/miR-133a injection (MI+miRs) displayed a significant improvement in fractional shortening (*P<0.05; Figures 4C; Figure IV in the Data Supplement). MI control mice also exhibited increased heart weight/body weight ratio (Figure 4D) and demonstrated increased transcripts of atrial natriuretic peptide.

![Figure 3](http://circep.ahajournals.org/content/10/2/466/F3)

**Figure 3.** Validation of Icer and Crem as miR-1 targets. **A**, Alignment of miR-1 sequence with the predicted target sequences in Crem isoforms. **B**, Time course of luciferase signals in cells transfected with Crem or scramble inserts and treated with either miR-1 or control miR mimics. Cells transfected with the original vector served as a positive control and untransfected cells served as a negative control. Luciferase intensity was normalized by β-galactoside signal. **C**, Fold change between miR-1 and control miR treatment in cells transfected with Crem, scramble, or vector constructs. Values were normalized to control miR intensity and made relative to vector values for comparison (n=4, *P<0.05). Overlaps over time were also calculated against values at time=0 s (t/P<0.05). These changes reflected the decay of the luciferase signals with time and were consistent among all groups including cells transfected with Crem, scramble, or vector constructs and treated with miR-1 or control miR.
(Nppa) and angiotensinogen (Agt; Figure 4D). In all cases, hypertrophy was prevented in the MI miR mice (Figure 4D, heart weight/body weight ratio) and levels for Nppa and Agt returned to the sham levels (Figure 4D). Angiotensinogen is a predicted target of miR-133a and local release of angiotensinogen has been described as pathological in MI.47 Nppa and skeletal α-actin (Acta1) are not predicted targets of miR-1 nor miR-133a but their expression has been linked to both miRs through indirect mechanisms.6,9

Delivery of miR-1/miR-133a Prevents Electrical Remodeling After MI

To directly test the roles of miR-1 and miR-133a on electrical remodeling, we performed patch-clamp recordings of I to, ICa, and APD on isolated cardiomyocytes. In MI mice, peak I to current was significantly reduced (Figure 5A), leading to prolonged APD (Figure 5B). Moreover, delivery of miR-1 and miR-133a resulted in the recovery of peak I to density toward the sham levels (Figure 5A) together with normalization of the
ICER is Increased in MI and Chronic Isoproterenol Infusion Models and the Expression of ICER is Normalized After the Delivery of miRs in Both Models

Using a primer specific to ICER isoforms, we found that Icer mRNA increased >2-fold in MI control mice, consistent with previous studies, which demonstrate that Icer can be activated by β-adrenergic signaling. More importantly, the level of Icer returned to the sham levels with miR delivery (Figure 6B). Using a primer that recognizes all isoforms of Crem except for Icer, we found that non-Icer Crem mRNA did not increase in MI control but that miR delivery decreased the expression by 60% in both sham+miR and MI+miR mice. Creb expression decreased slightly with MI but was not affected by miR delivery.

To directly test the roles of β-adrenergic signaling on the expression of ICER and miRs in the heart, we took advantage of the second mouse model with chronic isoproterenol infusion. Transcript levels of Icer, Creb, Kcnq2, miR-1, and miR-133 were assessed from cardiac myocytes (Figure VIIa in the Data Supplement). Chronic isoproterenol challenge resulted in a significant increase in Icer mRNA similar to the post MI model with a corresponding decrease in Kcnq2 and miR-1. The effects of isoproterenol on the upregulation of Icer mRNA occurred as early as 6 hours and lasted up to 2 weeks in our study (Figure VIIa in the Data Supplement). More importantly, similar to the MI model, treatment with miR-1/133a by tail vein injections normalized the levels of Icer (Figure VIIb in the Data Supplement). We directly documented a significant increase in heart rate as measured using R–R intervals (Figure VIIc in the Data Supplement) in the chronic isoproterenol infusion model.

Discussion

Even though significant loss of miRs has previously been documented in pathological cardiac hypertrophy and failure, the underlying mechanisms for the miR dysregulation remain incompletely understood. This study directly tests the hypothesis that chronic overexpression of ICER from excessive β-adrenergic signaling may repress miR-1 expression leading to the well-documented electrical remodeling. First, by taking advantage of a knockout model of Dicer1 in the heart, we demonstrated the direct regulation of cardiac excitability by miRs. We further confirmed that miR-1 directly targets and represses the expression of CREM and ICER. The level of ICER expression is increased during pathological conditions, such as post MI. Moreover, chronic isoproterenol stimulation induces a similar increase in ICER expression supporting the roles of β-adrenergic stimulation in the upregulation of ICER. By using in vivo delivery of miRs, we directly establish the relationship between miR-1/133a and the expression of ICER. Finally, our study provides a tantalizing proof-of-concept for the therapeutic potential of miR delivery post MI.

Possible Feedback Mechanism Between miR-1/133a, CREs, and Electrical Remodeling

In the post MI model, chronic β-adrenergic stimulation is predicted to result in the overexpression of ICER, which leads to long-term suppression of miR-1 and miR-133a. Indeed, using a chronic isoproterenol infusion, we directly document a significant increase in ICER expression by β-adrenergic stimulation. A decrease in miR-1 leads to a further increase in ICER level. Moreover, exogenous delivery of miR-1 results in the normalization of ICER levels in both models (Figure 6B; Figure VIIb in the Data Supplement).

Loss of miR-1 leads to overexpression of IRX5, loss of K, 4.2 and I, prolonged APDs. Loss of miR-1/133a leads to increased angiotensin expression and hypertrophy. Indeed, the delivery of cardiac-specific miRs can restore the

![Figure 6. miR-1/133a Delivery prevents remodeling of K, 4.2 and ICER after myocardial infarction (MI). A. Quantitative real-time polymerase chain reaction qPCR results normalized to GAPDH expression and relative to sham controls for Kcnq2, Irx5, and Kcnq3 (n=3, *P<0.05). B. qPCR results for Icer, Crem, and Creb expression in mice (n=3, *P<0.05). C. Schematic representation of the role of inducible cAMP early repressor (ICER) signaling on miR-1/133a during MI. AFA indicates atrial natriuretic factor; AGT, angiotensinogen; APD, action potential duration; CRE, CRE-binding protein; CREM, CRE modulator; and IRX5, iroquois homeobox domain 5.](https://circep.ahajournals.org/doi/10.1161/CIRCEP.117.005937)
level of ICER toward the control. Taken together, the results in our study suggest a feedback mechanism between miR-1/133a, CREs, and electrical remodeling in pathological conditions with heightened βAR stimulation (Figure 6C).

Roles of CREM and ICER in the Heart
Consistent with our hypothesis, recent studies have shown that prevention of β-adrenergic desensitization after an MI can improve ejection fraction and survivability while decreasing cardiac hypertrophy and apoptosis.30-33 It has been demonstrated that cardiac-specific knockout of CREM prevented hypertrophy, fibrosis, and LV dysfunction associated with chronic β-adrenergic signaling.32 Conversely, cardiac-driven ICER and CREM overexpression led to increased apoptosis and progressive mortality.44 Moreover, cardiac-specific knockout of CREB led to a 50% reduction in Kv4.2 expression and remodeling of gap junction proteins.12,22 The knockout of Dicer1 driven by the α–myosin heavy chain promoter has been shown to result in increased mortality, hypertrophy, dilated cardiomyopathy, increased fibrosis and promoter has been shown to result in increased mortality, hypertrophy, dilated cardiomyopathy, increased fibrosis and apoptosis, and remodeling of gap junction proteins.12,22 The mechanism of DICER1 loss seen in heart failure and post MI remains elusive, although evidence suggests it may be a mechanism of DICER1 loss seen in heart failure and post MI.12

Clinical Implications and Future Studies
Electrical remodeling in cardiac hypertrophy and failure can predispose patients to malignant arrhythmias and sudden cardiac death. Our study reveals novel mechanisms underlying electrical remodeling via the dysregulation of miRs and implicates ICER as a potential therapeutic target. At the translational level, our study provides a molecular basis for the potential roles for miR-1 and miR-133a therapy post MI and offers a proof-of-concept for miR intervention. Future studies are required to further dissect and quantify the beneficial effects of miR delivery on cardiac remodeling, including the degree of cardiac fibrosis and apoptosis.

In this study, miR delivery was performed 1 week post MI. However, at 3 weeks of follow-up, ICER level remains elevated in the MI control group (Figure 6B). Therefore, we expect miR delivery to reduce ICER level and possibly prevent further remodeling and provide beneficial outcomes. Nonetheless, additional studies are required to test whether this strategy may be applicable to pre-existing MI with substantial remodeling as well as the long-term beneficial effects of miR intervention.

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Disclosures
None.

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METHODS

All animal care and procedures were approved by the University of California, Davis, Institutional Animal Care and Use Committee. Animal care and use was in accordance with National Institutes of Health and institutional guidelines.

Neonatal Mouse Cardiomyocyte Culture

Cardiomyocytes from 1-3 days old neonatal Dicer1<sup>tm1Bdh/J</sup> were isolated by enzymatic digestion using trypsin and preplated for 45 min to reduce fibroblast contamination. Cells were then plated on fibronectin-coated coverslips and maintained in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Twenty four hours after isolation, adherent cells were washed thoroughly with media to remove debris. Cells were transduced 48 hours after isolation with Ad-GFP-Cre or Ad-GFP for experimental and control groups, respectively at a multiplicity of infection (MOI) of 10-50 (Vector Biolabs, 1700 and 1060). Myocytes were cultured for a total of 7 days, with media changed daily, and subjected to either patch-clamp recording of Ik<sub>a</sub> currents or RT-PCR analysis.

Intramyocardial Adenovirus Injection

C57 BL/6J and Dicer1<sup>tm1Bdh/J</sup> mice (8-12 weeks) were anesthetized with a solution of ketamine and xylazine, intubated, and further anesthetized with vaporized isoflurane. The thoracic wall was opened and ribs retracted to reveal the heart. Adenovirus (50-100 μl) was delivered by intramyocardial injection to the left ventricular free wall. Mice were monitored for 2 weeks and then the hearts isolated for digestion to single cardiomyocytes by Langendorff Perfusion. Transduced cells were identified based on GFP fluorescence. Dicer1<sup>tm1Bdh/J</sup> mice
injected with Ad-GFP-Cre served as the experimental group. Control groups were \( Dicer_1^{tm1Bdh/J} \) mice injected with Ad-GFP and WT C57 BL/6J mice injected with Ad-GFP-Cre.

**Myocardial Infarction Mouse Model**

Ischemia-reperfusion model of myocardial infarction was performed as previously published.\(^1\)\(^-\)\(^3\) C57 BL/6J mice (10-16 weeks) were prepped as described above and the heart exposed to reveal the left anterior descending coronary artery (LAD). The LAD was then ligated for 45 minutes and allowed to reperfuse. One week after LAD ligation, mice were treated with a cocktail of mmu-miR-1 and miR-133a mimics (\( \text{Dharmacon C-310377, C-310408} \)) packaged with MaxSuppressor \textit{in vivo RNA-LANCEr II} (\textit{Biooscientific 3410-01}) via tail vein injection. MiR Mimic Transfection Control with and without Dy547 (\textit{Dharmacon CP-004500-01}) was injected in control animals. Mice were monitored by echocardiography and the hearts isolated 3 weeks after LAD ligation.

**Analysis of cardiac function by echocardiography**

Echocardiograms using M-mode and two-dimensional (2D) measurements to assess systolic function were performed as described previously in conscious animals.\(^3\)\(^,\)\(^4\) The measurements represented the average of six selected cardiac cycles from at least two separate scans performed in random-blind fashion with papillary muscles used as a point of reference for consistency in the level of the scan. End diastole was defined as the maximal left ventricular (LV) diastolic dimension and end systole was defined as the peak of posterior wall motion. Fractional shortening (FS), a surrogate of systolic function, was calculated from LV dimensions as follows: \( FS = \left(\frac{\text{EDD}-\text{ESD}}{\text{EDD}}\right) \times 100\% \), where EDD and ESD are LV end diastolic and end systolic dimension, respectively.
**In Vivo miR Injections**

Mmu-miR-1 and miR-133a mimics (*Dharmacon C-310377, C-310408*) were packaged as using MaxSuppressor *in vivo* RNA-LANCEr II (*Biooscientific 3410-01*) and delivered together via tail vein injections 1 week after LAD surgery at 30ug per 28g mouse. Non-silencing MiR Mimic Transfection Control with and without Dy547 (*Dharmacon CP-004500-01*) was injected in control animals. Mice were monitored by echocardiography and the hearts isolated 3 weeks after LAD ligation.

**In Vivo Mouse Model of Isoproterenol Infusion**

*In vivo* isoproterenol (ISO) infusion at a dose of 30 mg/kg/day in C57 BL/6J mice (10-16 weeks) was performed using Alzet osmotic mini-pumps for 6 hours, 2, 4, and 14 days.\(^5\) Sham littermate animals received osmotic mini-pumps loaded with saline alone.

**Electrocardiographic (ECG) Recordings**

ECG recordings were obtained at 33ºC using Bioamplifier (BMA 831, CWE, Incorporated, Ardmore, PA) as previously described.\(^6\) The animals were placed on a temperature-controlled warming blanket at 37ºC. Four consecutive two-minute epochs of ECG data were obtained from each animal. Signals were low-pass filtered at 0.2 kHz and digitized using Digidata 1200 (Axon Instrument, CA). A total of 100 beats were analyzed from each animal in a blinded fashion. The Q-T interval was determined manually by placing cursors on the beginning of the QRS and the end of the T wave. The rate-corrected QT interval (*QTc*) was calculated using modified Bazett's formula as reported by Mitchell et al for mouse models, whereby the RR interval was first expressed as a unitless ratio (RR in ms/100 ms). *QTc* interval was defined as \((QT \text{ interval (in ms)} / (RR/100))^{1/2}\).\(^7\)
Adult Mouse Cardiomyocyte Isolation

Single mouse left ventricular (LV) myocytes were isolated as previously described. The procedure was performed according to the approved UC Davis Animal Care and Use protocol. Due to the known electrophysiologic heterogeneity in various regions of the heart, we used only LV free wall cells for our electrophysiologic recordings. Briefly, mice were injected with 0.1 ml heparin (1000 units ml$^{-1}$) 10 min prior to heart excision, then anesthetized with pentobarbital intraperitoneally (80 mg kg$^{-1}$). Hearts were removed and placed in Tyrode’s solution (mmol l$^{-1}$: NaCl 140, KCl 5.4, MgCl$_2$ 1.2, N-2-hydroxyethylpiperazine-N$'$-2-ethanesulphonic acid (HEPES) 5 and glucose 5, pH 7.4). The aorta was cannulated under a dissecting microscope and mounted on the Langendorff apparatus. The coronary arteries were retrogradely perfused with Tyrode’s solution gassed with O$_2$ at 37°C for 3 min at a flow rate of ~3 ml min$^{-1}$. The perfusion pressure was monitored and the flow rate was adjusted to maintain perfusion pressure at ~80 mmHg. The solution was switched to Tyrode’s solution containing collagenase type 2 (1 mg ml$^{-1}$, 330 units mg$^{-1}$, Worthington Biochemical Corporation). After ~12 min of enzyme perfusion, hearts were removed from the perfusion apparatus and gently teased in high-K$^+$ solution (mmol l$^{-1}$: potassium glutamate 120, KCl 20, MgCl$_2$ 1, EGTA 0.3, glucose 10 and HEPES 10, pH 7.4 with KOH). All chemicals were obtained from Sigma Chemicals (St. Louis, MO) unless stated otherwise. Cells were allowed to rest for 2 hours before use for electrophysiological recording. The isolation procedure yielded ~85% of rod-shaped myocytes with clear striation. The solution was then enriched for cardiomyocytes by allowing larger cells to settle and then removing the supematant. The pellet was then resuspended in high-K$^+$ solution and enrichment repeated twice more. Final cell populations were determined to be 95% myocytes as measured by flow cytometry (FigS1). The enriched cardiomyocytes were used
for electrophysiologic recordings, qRT-PCR, immunofluorescence staining, and western blot analysis.

**Patch-Clamp Recordings**

Whole-cell and perforated patch-clamp recordings performed as previous published.\(^9,10\) All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless stated otherwise. All experiments were performed using 3 M KCl agar bridges. Cell capacitance was also calculated as the ratio of total charge (the integrated area under the current transient) to the magnitude of the pulse (20 mV). Currents were normalized to cell capacity to obtain the current density. The series resistance was compensated electronically. In all experiments, a series resistance compensation of \(\geq 90\%\) was obtained. Currents were recorded using Axopatch 200A amplifier (Axon Instrument), filtered at 1 kHz using a 4-pole Bessel filter and digitized at sampling frequency of 2 kHz. Data analysis was carried out using Clampfit 10 software and commercially available PC-based spreadsheet and graphics software (MicroCal Origin Pro version 7.0).

For \(\text{Ca}^{2+}\) current recordings, the external solution contained (in mM) \(N\)-methylglucamine (NMG) 140, CsCl 5, MgCl\(_2\) 0.5, CaCl\(_2\) 2, 4-AP 2, glucose 10, and \(N\)-2-hydroxyethylpiperazine-\(N\)'-2-ethanesulphonic acid (HEPES) 10, pH 7.4 with methanesulfonic acid (MSA). The pipette solution contained (in mM) CsOH 100, aspartic acid 100, CsCl 20, MgCl\(_2\) 1, ATP 2, GTP 0.5, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; Life Technologies) 10, and HEPES 5, pH 7.4 with HCl.

For \(\text{K}^+\) current recording, the external solution contained (in mM) NMG 130, KCl 5, CaCl\(_2\) 1, MgCl\(_2\) 1, Nimodipine 1 \(\mu\)M, glucose 10, HEPES 10, pH 7.4 with HCl. The pipette solution contained (mM) KCl 140, Mg-ATP 4, MgCl\(_2\) 1, EGTA 10, HEPES 10, pH 7.4 with KOH.
For AP recordings, the patch-pipettes were backfilled with amphotericin (200 µg/ml). The pipette solution contained (mM) K-glutamate 120, KCl 25, MgCl\(_2\) 1, CaCl\(_2\) 1, HEPES 10, pH 7.4 with KOH. The external solution contained NaCl 138, KCl 4, MgCl\(_2\) 1, CaCl\(_2\) 2, NaH\(_2\)PO\(_4\) 0.33, glucose 10, HEPES 10, pH 7.4 with NaOH.

**Flow cytometric analysis of mouse cells**

Flow cytometric analyses was performed as previously described.\(^3\) Cells from mouse hearts were fixed with 0.4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), treated with 0.1% Triton-X 100 and stained with anti-troponin T antibody (Thermo Scientific) in PBS with 5% donkey serum and 20 µg ml\(^{-1}\) DNAse-free RNAse (Sigma) overnight at 4°C.\(^3\) Cells were washed with PBS stained with 40 µg ml\(^{-1}\) 7-amino-actinomycin D (7AAD, BD Bioscience, San Jose, CA). Data was collected using a standard FACScan cytometer (BD Biosciences, San Jose, CA) upgraded to a dual laser system with the addition of a blue laser (15mW at 488nm) and a red laser (25mW at 637nm Cytek Development, Inc, Fremont, CA). Data was acquired using CellQuest software (BD Bioscience). Cells stained with isotype-matched IgG antibodies were used as controls to determine the positive cell population. Data was analyzed using FlowJo software (ver9.7.2 Treestar Inc., San Carlos).

**Quantitative Real-Time PCR**

RNA was isolated from adherent neonatal and dissociated adult cardiomyocytes using RNeasy Plus Mini Kit (Qiagen 74134) and cDNA generated using RT\(^2\) First Strand Kit (Qiagen 330401). cDNA was combined with RT\(^2\) SYBR Green Master Mix (Qiagen 330520) and specific qRT-PCR primers and qRT-PCR analysis run using the Viia™ 7 Real Time PCR System (ABI). Samples were run in triplicate and average Ct values normalized to GAPDH expression. TATA-binding protein (TBP) and β-actin primers were also tested as housekeeping genes but rejected
due to a higher standard deviation between samples. ΔΔCt values were determined as relative to sham controls. Fold change was determined as $2^{\Delta\Delta Ct}$ and graphed with Origin Pro 7 software.

Primers for Cre Recombinase$^{11}$ and angiotensinogen (agt)$^{12}$ were previously published. Primers for CREM and CREB isoforms:

- **icer** (Forward: 5'-TGAAACTGATGAGGAGACTGAC, Reverse: 5'-CAGCCATCACCACACCTTG),
- **crem** (Forward: 5'-CAGACTAGCACGGGCAATAC, Reverse: 5'-AGCCACAGATTITCAAGACAT),
- **creb** (Forward 5'-TTGTTTGTTCAAGCTGCTCTG, Reverse: 5'-CAGCAGGCTGTTAGGAAATG).

Commercial primer catalogue numbers (SA Biosciences, Qiagen):

- **dicer** (PPM26135E),
- **kcnd2** (PPM04097A),
- **irx5** (PPM30519A),
- **cacna1c** (PPM04040E),
- **gapdh** (PPM02946E),
- **nppa** (PPM04489A), and
- **acta1** (PPM05064E).

**Immunofluorescence Confocal Microscopy**

Immunofluorescence confocal microscopy was performed as previously described.$^{13,14}$ Cells were affixed to poly-L-lysine coated coverslips and fixed with 4% paraformaldehyde for 15 minutes. Cells were then blocked with 5% goat serum and 0.1% triton X-100 in phosphate buffered saline (PBS) for 1 hour at room temperature. Coverslips were incubated with primary antibody overnight at 4 °C and then washed with PBS 7x5mins. Secondary antibodies were incubated for 1 hour at room temperature and then washed again with PBS 7x5mins. Coverslips were mounted on slides using VectaShield hard set mounting medium w/DAPI (Vector Biolabs H-1500). Slides were visualized using a LSM 700 confocal microscope (Zeiss) and imaged using the Zeiss Zen software.

Primary antibodies used were anti-Cre (1:1000, Novagen 69050-3) and anti-α-actinin (1:4000, Sigma A7811) antibodies. Secondary antibodies used were Alexa Fluor 555 goat anti-
rabbit IgG (1:500, Invitrogen A-21429) and Alexa Fluor 488 F(ab’)2 goat anti-mouse IgG (1:500, Invitrogen A-11017) antibodies.

miR-1 Target Validation using Luciferase Assay and Western Blot Analysis

A potential miR-1 target region was identified on the 3’UTR of a CREM mRNA sequence using microRNA.org and miRWalk (www.umm.uni-heidelberg.de) online software. The predicted target sequence (5’-ACTTTCTAAAACACATTCCCT) matched miR-1 with a mirSVR score of -1.2211 and a PhastCons score of 0.6879. The target sequence was identified in the 3’UTRs of all 15 NCBI CREM isoforms except isoforms 7 and 8. Inserts were designed from the target region to include the target sequence and conserved flanking sequencing. Scramble sequences had identical flanking sequences but a scrambled target sequence.

Inserts:

CREM: 5’-CTAGTTTTGTATCATCTCATACTTTCTAAAAACTAACATTCCTAAGATGCTTTA
SCRAM: 5’-CTAGTTTTGTATCATCATTCTCTAGGTCTAAGTAACCGCGGTGCAAGATGCTTTA

These inserts were ligated into the pMIR-REPORT™ miRNA Expression Reporter Vector System (Life Technologies) using traditional cloning techniques and transfected into HEK293 cells using Lipofectamine 2000 (Life Technologies). Cells transfected with the original pMIR-REPORT™ luciferase vector served as a positive control and cells not transfected with a luciferase construct served as negative controls. A second construct expressing β-galactosidase was transfected into all cells and served as a loading control for normalization. Cell were then transfected with either mmu-miR-1a-3p mimics (Life Technologies MC10617) or miRIDIAN microRNA Mimic Negative Control #1 (Thermo Scientific CN-001000-01-05) using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies 13778030).
Luciferase signal was measured 48 hours later using the Dual-Light® Luciferase & β-Galactosidase Reporter Gene Assay System (Life Technologies T1003). Luciferase signal intensity was normalized to β-galactosidase signal. Samples (n=5) were analyzed as a time course of signal intensity over 80 seconds with a reading performed every 20 seconds. Fold change due to miR-1 suppression was assessed by further normalizing miR-1 transfected cells to their control miR counterparts. Values were then compared relative to vector (positive control) samples.

We further validate miR-1 targets at the protein level using western blot analysis. HEK 293 cells were transfected with 2000 ng per well of plasmid containing human CREM (Transomic pCS6-BC090051) using Lipofectamine® 2000 (Life Technologies). Cells were then transfected with either mmu-miR-1a-3p mimics (3 pmol) or miRIDIAN microRNA Mimic Negative Control #1 (3 pmol) using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies). Western blot analysis was performed using 15 μg of total protein per sample and anti-CREM and anti-β-tubulin antibodies from Abcam.

**Statistical Analyses**

Significant difference between groups was determined using Origin Pro 7 software by One-Way ANOVA and validated by Bonferroni, Sheffe, and Tukey tests with an n ≥ 3 at p < 0.05 unless otherwise noted. For qRT-PCR, each sample was run in triplicate and data were normalized to GAPDH expression. For luciferase assay, signal intensity was normalized to β-galactosidase signal. A total of 5 samples were used.
REFERENCES


**FIGURE LEGENDS**

**Figure S1: Enrichment for Cardiomyocytes**

Flow cytometric analyses showing fluorescence intensity for troponin T (cTnT) against side-scatter before myocyte enrichment, in the supernatant, and after myocyte enrichment. Myocyte (MC) and non-myocyte (NMC) populations are labeled for clarity.

**Figure S2: Optimization of Crem Inserts for Luciferase Assay and Validation of CREM as a miR-1 target.**

(a) Nucleotide sequences for insert of the targeted region (Crem) and a negative control insert (scramble). (b) Optimization of luciferase plasmid transfection for detection of luciferase signal and time course of signal decay. (c) Dose-response curve of miR-1 suppression on luciferase signal in HEK 293 cells transfected with crem inserts. Values were made relative to nontransfected controls (n=4, \( *p<0.05 \)). (d) Western blot analysis of HEK 293 cells transfected with 2000 ng per well of plasmid containing human CREM (Transomic pCS6-BC090051). Cells were then transfected with either 0 pmol (-) or 3 pmol of miRIDIAN miR Mimic Negative Control #1 (SC) or 3 pmol of miR-1 mimics (miR-1). Western blot analysis was performed using 15 \( \mu \)g of total protein per sample and anti-CREM and anti-β-tubulin antibodies from Abcam. Experiments were repeated 3 times using 3 different sets of cells.

**Figure S3: Optimization of miR-1/133a NLE Packaging and Delivery in HEK293 Cells**

(a) Confocal images of HEK 293 cells transfected with NLE packaged miR mimics labeled with Dy547 (red). Nuclei are labeled with DAPI (blue). (b) qRT-PCR results showing a dose-
dependent increase in miR-1 and miR133a levels with increased transfection (n=3, *p<0.05). (c) qRT-PCR products run on an agarose gel for comparison.

**Figure S4: Ischemia-Reperfusion (IR) Mouse Model of MI**

(a) Photographs (scale = 200mm) and paraffin sections stained with Sirius Red (scale = 200μm) of sham and MI mouse hearts after treatment with miR-1 and miR-133a or control miRs. (b) Representative M-mode echocardiographs of sham and MI mice at 1 week and 3 weeks after surgery treated with miR-1 and miR-133a or control miRs.

**Figure S5: Steady-State Voltage-Dependent Activation of Ito**

Steady-state voltage-dependent activation of Ito in sham and MI mice administered either miR-1/133 (miRs) or a control miR (control). Currents were elicited using voltage-clamp protocols as described in Figure 1. G/GMax refers to whole-cell conductance normalized to maximal conductance.

**Figure S6: Ca²⁺ Current (ICa) and Ca²⁺ Channel Expression in MI animals after in vivo miR Delivery**

(a) Representative ICa traces recorded from left ventricular myocytes isolated from sham and MI mice after tail vein injection of miR-1/133 mimics or control miR. The corresponding current-voltage relations are shown in the lower panel (*p<0.05). (b) qRT-PCR results for Cacna1c expression (n=3, p=NS).
Figure S7: Chronic Isoproterenol Infusion Results in the Significant Up-regulation of Icer mRNA and Down-regulation of miR-1/133a and Kcnd2 mRNA. Delivery of miR-1/133a Prevents Remodeling of Icer after Chronic Isoproteronal Infusion

(a) qRT-PCR results normalized to GAPDH expression comparing cardiomyocytes isolated from sham controls (Saline) and chronic isoproterenol infusion (ISO) at different time points (6, 48, 96 hours, and 2 weeks) for Icer, Creb, Crem, Kcnd2, miR-1 and miR-133a. Experiments were performed in triplicate (n=3 animals for each group (saline vs. ISO), *p<0.05). (b) qRT-PCR results for Icer expression shown in (a) compared to a third group of mice receiving miR-1/133a via tail vein injection (n=3 for each group, *p<0.05). (c) Electrocardiogram (ECG) obtained from mice receiving chronic ISO infusion at two weeks comparing to sham animals (Saline). Left panel shows examples of ECG tracings while the right panel shows the summary data for RR, PR, and QTc intervals (n=3 for each group, *p<0.05).
Figure I
**Figure II**

- **a** Inserts:
  - **crem**
  ```
  5' - CTAGTTTTGTATTCCATCTTCTAATTCTCTAAACCTGAGATGCTTTA
  AAAACATAGTARGGATTGAAGGATTTCGGTATTCTACGAAATTCA
  ```
  - **scramble**
  ```
  5' - CTAGTTTTGTATTCCATCTTCTAATTCTCTAAACCTGAGATGCTTTA
  AAAACATAGTARGGATTGAAGGATTTCGGTATTCTACGAAATTCA
  ```
  - miR-1 region
  - miR-1 direct binding sequence

- **b**
  Normalized Luminescence (a.u.)
  - DNA (ng)
  - 0
  - 50
  - 100

- **c**
  Relative Luminescence (a.u.)
  - miR-1 (pmol)

- **d**
  kD: 55
  - SC
  - miR-1
  - β-Tubulin
  - CREM
Figure III
Figure IV
Figure V

The graph shows the relationship between G/Gmax and V (mV) for different conditions: Sham control, Sham + miRs, MI control, and MI + miRs.
Figure VI
Figure VII