Circulation: Arrhythmia and Electrophysiology

ORIGINAL ARTICLE

# MicroRNA-1 Deficiency Is a Primary Etiological Factor Disrupting Cardiac Contractility and Electrophysiological Homeostasis

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**BACKGROUND:** MicroRNA-1 (miR1), encoded by the genes *miR1-1* and *miR1-2*, is the most abundant microRNA in the heart and plays a critical role in heart development and physiology. Dysregulation of miR1 has been associated with various heart diseases, where a significant reduction (>75%) in miR1 expression has been observed in patient hearts with atrial fibrillation or acute myocardial infarction. However, it remains uncertain whether miR1-deficiency acts as a primary etiological factor of cardiac remodeling.

**METHODS:** *miR1-1* or *miR1-2* knockout mice were crossbred to produce 75%-miR1-knockdown (75%KD; *miR1-1*$^{+/-}$:*miR1-2*$^{-/-}$ or *miR1-1*$^{-/-}$:*miR1-2*$^{+/-}$) mice. Cardiac pathology of 75%KD cardiomyocytes/hearts was investigated by ECG, patch clamping, optical mapping, transcriptomic, and proteomic assays.

**RESULTS:** In adult 75%KD hearts, the overall miR1 expression was reduced to ≈25% of the normal wild-type level. These adult 75%KD hearts displayed decreased ejection fraction and fractional shortening, prolonged QRS and QT intervals, and high susceptibility to arrhythmias. Adult 75%KD cardiomyocytes exhibited prolonged action potentials with impaired repolarization and excitation-contraction coupling. Comparatively, 75%KD cardiomyocytes showcased reduced $Na^+$ current and transient outward potassium current, coupled with elevated L-type $Ca^{2+}$ current, as opposed to wild-type cells. RNA sequencing and proteomics assays indicated negative regulation of cardiac muscle contraction and ion channel activities, along with a positive enrichment of smooth muscle contraction genes in 75%KD cardiomyocytes/hearts. miR1 deficiency led to dysregulation of a wide gene network, with miR1's RNA interference–direct targets influencing many indirectly regulated genes. Furthermore, after 6 weeks of bi-weekly intravenous tail-vein injection of miR1 mimics, the ejection fraction and fractional shortening of 75%KD hearts showed significant improvement but remained susceptible to arrhythmias.

**CONCLUSIONS:** miR1 deficiency acts as a primary etiological factor in inducing cardiac remodeling via disrupting heart regulatory homeostasis. Achieving stable and appropriate microRNA expression levels in the heart is critical for effective microRNA-based therapy in cardiovascular diseases.

**GRAPHIC ABSTRACT:** A graphic abstract is available for this article.

**Key Words:** arrhythmia ■ heart contractility ■ microRNAs ■ pathogenesis ■ventricle remodeling

Heart disease remains the leading cause of death, imposing a significant health and economic burden on modern society.[1] When faced with environmental and genetic changes, the heart undergoes pathophysiological remodeling adapting to new circulatory demands. Cardiac remodeling encompasses changes in gene expression, molecular, cellular, and interstitial alterations, and clinically presents as modifications

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Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCEP.123.012150.
For Sources of Funding and Disclosures, see page 47.

*Circulation: Arrhythmia and Electrophysiology* is available at www.ahajournals.org/journal/circep

## WHAT IS KNOWN?

- MicroRNA-1 (miR1) is the most abundant microRNA in the heart.
- miR1 expression is reduced in hearts of patients with atrial fibrillation or acute myocardial infarction.

## WHAT THE STUDY ADDS

- Our extensive assessments unveil compromised hemodynamic function and elevated arrhythmogenesis risk in miR1-75%-knockdown hearts.
- miR1 deficiency disrupts the homeostasis of molecular regulatory gene networks, serving as a primary etiological factor in cardiac remodeling.
- miR1 treatment notably improves the contractility of miR1-deficient hearts; however, it fails to alleviate their heightened susceptibility to arrhythmias.

## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **50%KD** | 50%-miR1-knockdown |
| **75%KD** | 75%-miR1-knockdown |
| **AP** | action potential |
| **APD** | action potential duration |
| **Cx43** | connexin-43 |
| **GSEA** | gene set enrichment analysis |
| $I_{to}$ | transient outward potassium current |
| **miR1** | microRNA-1 |
| **Mtpn** | myotrophin |
| **TnnT2** | troponin T |
| **Twf1** | twinfilin-1 |
| **Wdr1** | WD-repeat protein 1 |
| **WT** | wild type |

in heart size, shape, and function following injury.[2] Cardiomyocytes are the primary cardiac cells involved in this remodeling, alongside the interstitium, fibroblasts, collagen, and coronary vasculature. Patients experiencing major pathological remodeling exhibit a progressive decline in cardiac function, leading to heart failure and malignant ventricular arrhythmias. The molecular mechanisms underlying cardiac remodeling are intricate and involve various genetic and epigenetic regulatory molecules,[3,4] including microRNAs.[5] Those regulatory pathways form interconnected molecular networks, making it challenging to elucidate the singular role of any individual factor in the development of cardiac remodeling.

MicroRNAs, evolutionarily conserved small noncoding RNA molecules (≈22 nucleotides), exert pivotal roles in diverse cellular processes. They finely tune the expression of targeted genes at the posttranscriptional level, and the precise spatiotemporal control of gene expression is critical for maintaining normal heart function. Typically, each microRNA can directly target hundreds of genes, leading to changes in the expression of thousands of indirectly targeted genes.[6] For instance, microRNA-1 (miR1), the most abundant microRNA in the heart, is encoded by 2 distinct genes, *miR1-1* and *miR1-2*, accounting for ≈40% of total microRNA transcripts in the heart.[7] miR1 regulates a wide range of target genes involved in cardiogenesis, intracellular trafficking, cardiac cell cycle, metabolism, cell communication, cardiac excitability, and contractility.[8–12] Studies on *miR1-1* knockout or *miR1-2* knockout mice with 50% downregulation of miR1 revealed increased susceptibility to perinatal heart failure[8,10]; while complete deletion of miR1 (*miR1-1*$^{-/-}$:*miR1-2*$^{-/-}$) resulted in postnatally lethal due to severe cardiac dysfunction.[10,11] The dysregulation of miR1 is implicated in cardiac remodeling in various heart diseases. miR1 is downregulated in murine heart failure model[13,14] and significantly decreased (>75%) in hearts of patients with acute myocardial infarction[15] or atrial fibrillation.[16] Conversely, miR1 upregulation in the heart is associated with various types of cardiac arrhythmias as well.[17–20] Notably, upregulation or downregulation of miR1 showed a negative correlation with the expression of cardiac genes directly targeted by miR1, including ion channels, regulatory proteins, and hypertrophy-associated genes. However, it remains unclear whether miR1 deficiency is a primary etiological factor, inducing structure and functional cardiac remodeling and leading to heart failure and malignant arrhythmias, rather than merely being a result of cardiac remodeling.

To investigate the potential etiological role of miR1 deficiency, we generated 75%-miR1-knockdown (75%KD) mice by crossbreeding *miR1-1* knockout or *miR1-2* knockout transgenic animals. We performed comprehensive assessments of the hemodynamics and electrophysiology of 75%KD hearts. Utilizing a combination of transcriptomic and proteomic analyses alongside functional assays, we examined cardiac cellular function and electrophysiology of 75%KD cardiomyocytes, while also exploring the dysregulation of molecular regulatory networks. Furthermore, we sought to assess if the abnormal functions observed in 75%KD hearts could be rectified by direct in vivo administration of miR1. This comprehensive study aimed to shed light on the potential causal role of miR1 deficiency in cardiac dysfunction and to explore possible therapeutic strategies for its modulation.

## METHODS

Animal models and all experimental procedures have been thoroughly described in the Supplemental Material. To ensure ethical and regulatory compliance, the study received institutional review board approval, and all animal studies were conducted following the established institutional guidelines.

### Quantification and Statistical Analysis

All biochemistry experiments were independently replicated at least 3×. Data are presented as mean±SEM with n indicating the number of distinct biological samples. Statistical significance of mean differences was performed using unpaired 2-tailed Student *t* test, log-rank (Mantel-Cox) test, or 1-sided Fisher exact test, using GraphPad Prism, version 9.4.1, for Windows (GraphPad Software, www.graphpad.com). A $P<0.05$ indicates statistical significance.

### Data Availability

The data, methods, and study materials that support the findings of this study are available from the corresponding author upon reasonable request.

## RESULTS

### miR1-Deficient Heart Exhibits Decreased Contractile Function and a Heightened Susceptibility to Arrhythmias

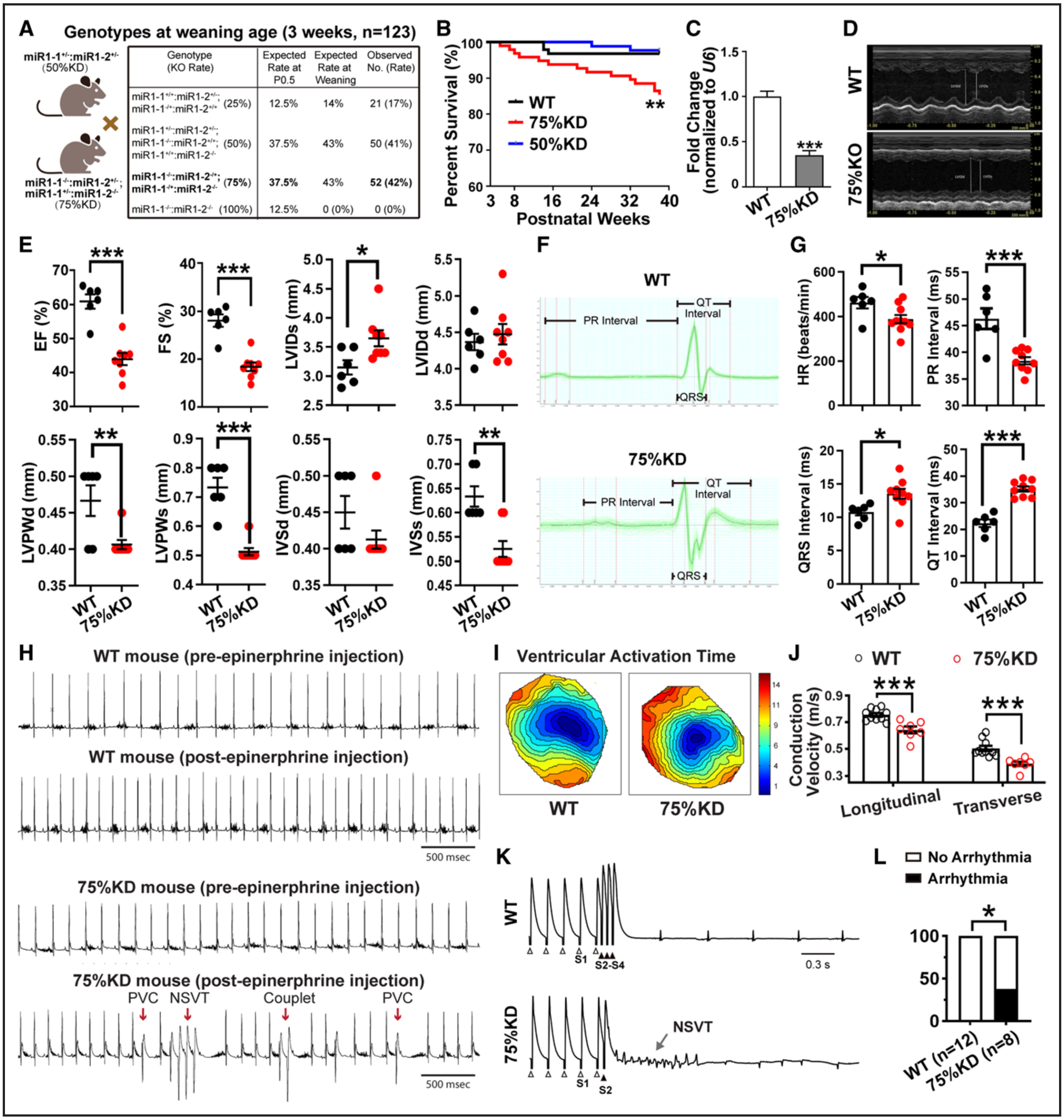
The intercrossing *miR1-1* knockout and *miR1-2* knockout mice resulted in various genotypes, with 75%KD mice having genotypes of *miR1-1*$^{+/-}$:*miR1-2*$^{-/-}$ or *miR1-1*$^{-/-}$:*miR1-2*$^{+/-}$. These mice were used to investigate the functional deterioration of adult hearts. We analyzed the genotyping results of special intercrosses in which 50%-miR1-knockdown (50%KD) heterozygous (*miR1-1*$^{+/-}$: *miR1-2*$^{+/-}$) mice were mated with single-allele 75%KD mice (Figure 1A). A noticeable mortality rate (≈10%) was observed among newborns, while no animals with 100% knockout genotype survive at the weaning age. However, 50%KD and 75%KD mice survived at the expected Mendelian ratios. Compared with wild-type (WT) animals and 50%KD heterozygous mice, 75%KD mice exhibited a significantly lower survival rate, with unexpected death frequently observed (Figure 1B). We measured the expression level of miR1 in the heart by quantitative polymerase chain reaction and found that the expression of miR1 in the hearts of 75%KD mice was significantly reduced, reaching ≈25% of the expected level in WT hearts (Figure 1C). Neither the miR1 expression level (Figure S1A) nor the survival rate showed any significant differences between 75%KD mice with 2 different genotypes or between male and female animals.

Echocardiography of adult hearts (Figure 1D) showed that 75%KD mice had significantly reduced ejection fraction (43.946±0.625% [n=8] versus 60.907±0.858% for WT [n=6]; $P<0.001$) and fractional shortening (18.409±0.319% versus 28.050±0.523% for WT; $P<0.001$; Figure 1E), indicating severe impairment in hemodynamic function. The left ventricular internal diameter end systole was significantly higher in 75%KD mice (3.650±0.0486 versus 3.150±0.050 mm for WT; $P=0.023$), while no significant change was observed in the left ventricular internal diameter end diastole, suggesting lower ventricular contractility without notable dilation. 75%KD hearts displayed thinner left ventricular posterior walls at both systole (0.513±0.004 versus 0.733±0.014 mm for WT; $P<0.001$) and diastole (0.406±0.002 versus 0.467±0.009 mm for WT; $P=0.003$), as well as a thinner interventricular septum at the end of systole (0.525±0.006 versus 0.633±0.009 mm for WT; $P=0.0014$). Surface ECG measurements (Figure 1F and 1G) revealed that, under isoflurane anesthesia, the heart rate of 75%KD mice (385±6 bpm; n=9; $P=0.0436$) was significantly slower than that of WT animals (463±11 bpm; n=6). 75%KD mice had significantly prolonged QRS (13.543±0.245 ms; $P=0.0147$ versus 10.753±0.199 ms for WT) and QT intervals (35.183±0.334 ms; $P<0.001$ versus 22.295±0.576 ms for WT) and a shortened PR interval (38.409±0.228 ms; $P=0.0006$ versus 46.310±0.790 ms for WT). Our surface ECG recording revealed that epinephrine induced premature ventricular contractions, couplets, and nonsustained ventricular tachycardia in 75%KD hearts (2 of 7 animals) but had no such effect in WT hearts (0 of 6 mice; Figure 1H). Consistently, optical mapping assays of ex vivo hearts (Figure 1I and 1J) demonstrated slower propagation of electrical action potential (AP) signals in the ventricle and significantly slower conduction velocity in 75%KD hearts (longitudinal: 0.642±0.010 m/s [n=7], $P<0.001$ versus 0.755±0.004 m/s [n=10] for WT; transversal: 0.389±0.006 m/s; $P<0.001$ versus 0.504±0.006 m/s for WT). Additionally, programmed S1 to S4 pacing stimulations of ex vivo hearts induced nonsustained ventricular tachycardia frequently in 75%KD hearts (3 of 8 animals; $P=0.0491$ versus 0 of 12 WT animals; Figure 1K and 1L). Notably, Masson Trichrome staining did not reveal significant fibrosis in 75%KD hearts compared with WT tissue (Figure S1B and S1C).

In summary, 75%KD mice exhibited cardiac contractile defects and a heightened susceptibility to arrhythmias, suggesting that miR1 deficiency plays an etiological role in inducing significant pathological remodeling in the heart.

### miR1 Deficiency Leads to a Broad Dysregulation of Cardiomyocyte Transcriptome

A large number of genes targeted by miR1 have been reported to be associated with various functions of the heart. Thus, cardiac remodeling resulting from miR1 deficiency can be attributed to the dysregulation of miR1-centered molecular regulatory cascades. To depict the gene regulatory network of miR1, we isolated cardiomyocytes from adult 75%KD and littermate WT mice and performed deep RNA sequencing (Figure S2A). Our analysis revealed that 1766 genes were differentially expressed in 75%KD cardiomyocytes (fold change >1.5 compared with the WT group; FDR, <0.05; Figure 2A), including validated miR1 direct targets (eg, *Slc8a1*, *Irx5*, *Kcne1*, *Mylk*).



**A** Genotypes at weaning age (3 weeks, n=123)

| Genotype (KO Rate) | Expected Rate at P0.5 | Expected Rate at Weaning | Observed No. (Rate) |
|---|---|---|---|
| miR1-1$^{+/+}$:miR1-2$^{+/-}$;<br>miR1-1$^{-/+}$:miR1-2$^{+/+}$ (25%) | 12.5% | 14% | 21 (17%) |
| miR1-1$^{+/-}$:miR1-2$^{+/-}$;<br>miR1-1$^{-/-}$:miR1-2$^{+/+}$; (50%)<br>miR1-1$^{+/+}$:miR1-2$^{-/-}$ | 37.5% | 43% | 50 (41%) |
| **miR1-1$^{-/-}$:miR1-2$^{-/+}$;<br>miR1-1$^{-/+}$:miR1-2$^{-/-}$ (75%)** | **37.5%** | 43% | **52 (42%)** |
| miR1-1$^{-/-}$:miR1-2$^{-/-}$ (100%) | 12.5% | 0 (0%) | 0 (0%) |

**Figure 1. Cardiac dysfunction and increased ventricular arrhythmia susceptibility in microRNA-1 (miR1)–deficient mice.**
**A**, Genotypes of offspring generated from 50%-miR1-knockdown (50%KD) heterozygous (*miR1-1*$^{+/-}$:*miR1-2*$^{+/-}$) mice and single-allele 75%-miR1-knockdown (75%KD; *miR1-1*$^{+/-}$:*miR1-2*$^{-/-}$ or *miR1-1*$^{-/-}$:*miR1-2*$^{+/-}$) mice intercrosses. Expected genotype ratios were calculated at postnatal day 0.5 (P0.5) and weaning age; animals were genotyped at weaning age. **B**, Kaplan-Meier survival curve of 50%KD (n=86), 75%KD (n=134), and wild-type (WT, n=84) littermates. **C**, Quantitative polymerase chain reaction analysis showing ≈70% lower expression level of microRNA in the heart of adult 75%KD mice (n=10; $P<0.001$) compared with that in wild-type (WT) mice (n=7). The expression level was normalized to *U6* RNA. **D** and **E**, Representative echocardiography images (M mode, **D**) and analysis (**E**) indicating significantly decreased cardiac function in adult 75%KD mice (n=9) compared with WT mice (n=6). **F** and **G**, Representative traces of surface electrocardiography (**F**) and analysis (**G**) showing reduced heart rate (HR), shortened PR interval, and prolonged QRS and QT intervals in 75%KD mice (n=8) compared with WT mice (n=6). **H**, Surface electrocardiography revealed a high vulnerability of 75%KD mice to arrhythmias, indicated by premature ventricular contractions (PVCs), couplets, and nonsustained ventricular tachycardia (NSVT) induced by epinephrine. **I** and **J**, Representative contour map of ventricular activation time (**I**) in 75%KD and WT hearts during pacing. A summarized bar graph of conduction velocity (**J**) indicates isochrone crowding and slower action potential propagation in 75%KD hearts (n=7) compared with WT hearts (n=10). **K** and **L**, Representative traces of programmed electric stimulation assays of ex vivo hearts (**K**) and analysis (**L**) revealing higher arrhythmia inducibility of 75%KD hearts (n=8; $P$=0.049) compared with WT hearts (n=12). Δ and ▲ indicate S1 and S2 to S4 stimulations, (*Continued*)

**Figure 1 Continued.** respectively. The statistical significance of differences estimated using unpaired 2-tailed Student *t* tests (**C**, **E**, **G**, and **J**), log-rank (Mantel-Cox) test (**B**), and 1-sided Fisher exact test (**J**). EF indicates ejection fraction; FS, fractional shortening; IVSd, interventricular septal thickness at the end of diastole; IVSs interventricular septal thickness at the end of systole; KO, knockout; LVIDd, left ventricular internal diameter end diastole; LVIDs, left ventricular internal diameter end systole; LVPWs, left ventricular posterior wall thickness at the end of systole; and LVPWd, left ventricular posterior wall thickness at the end of diastole. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs WT group.

To understand the biological processes primarily affected by miR1 deregulation, we conducted gene ontology enrichment analysis using Clue gene ontology plugged-in Cytoscape. The enriched biological processes of the differentially expressed genes were related to cardiac system development, cell-to-cell communication, protein phosphorylation, and ion transports (Figure 2B), aligning with the diverse roles of miR1 in regulating heart development and function maintenance. Further, gene set enrichment analysis (GSEA) of the genome-wide RNA expression revealed a significantly negative normalized enrichment score for the terms voltage-gated channel activity (normalized enrichment score, −2.00; FDR, <0.001; Figure 2C). Analysis of voltage-gated calcium, potassium, and sodium channel genes displayed negative normalized enrichment scores (Figure 2D and 2E; Figure S2B and S2C). Quantitative polymerase chain reaction analysis validated the significant upregulation of many of miR1's directly targeted genes, including *Kcne1*, *Irx5*, *Slc8a1*, and *B56α*, at the mRNA transcript level in 75%KD myocytes (Figure 2F).

GSEA analysis also revealed a positive involvement of gene expression pattern in smooth muscle contraction in 75%KD myocytes (Figure S2D), with smooth muscle-specific genes (eg, *Acta2*, *Myh11*, *Cnn1*) and miR1-targeted smooth muscle-cell fate controlling factors (eg, *Myocd, Mylk*, *Dlg1*) being significantly increased. This dysregulation of muscle contractile protein genes may partially explain the decreased hemodynamic function observed in 75%KD hearts (Figure 1). Additionally, gene sets associated with cell fate decision, cell body membrane, and microtube bundle formation were negatively enriched in 75%KD groups, consistent with the positive role of miR1 in regulating cardiogenesis.

In summary, our whole-transcriptome assay demonstrated that miR1 deficiency results in a broad transcriptomic dysregulation of cardiomyocytes. Interestingly, GSEA detected a significant alteration in gene sets involved in cytoplasmic translation, suggesting a disturbance of protein translation in miR1-deficient cells.

## Proteomic Profile of 75%KD Hearts Uncovered a Complex miR1-Driven Regulatory Network Involving Multiple Interconnected Factors

To gain deeper insights into the cellular and molecular dysfunction in 75%KD hearts at the protein level, we conducted a fractionation mass spectrometry assay on ventricle heart tissues (Figure S3A). This analysis quantified 3650 proteins from mouse heart tissues, among which the expression of 849 proteins was significantly altered between the 2 groups. Specifically, 520 proteins were upregulated, while 329 were downregulated in 75%KD mice compared with WT (Figure 3A).

Gene ontology analysis of the differentially expressed proteins revealed their involvement in broad categories related to heart contraction, cardiac metabolism, cell organization, muscle-cell development, and ion channel activities (Figure S3B), largely overlapping with the terms obtained in the RNA-sequencing analysis. Moreover, GSEA analysis identified that ion channels/transports and cardiac muscle contraction (Figure 3B; Figure S3C) were negatively involved, while smooth muscle contraction and actin filament organization and length regulation were positively involved in 75%KD mice (Figure 3B and 3C). These findings align with the observed decreased contractile capacity of 75%KD mice. Notably, some miR1 direct targets in cytoskeletal proteins, such as Twf1 (twinfilin-1), Mtpn (myotrophin), and Cx43 (connexin-43), were significantly increased in 75%KD hearts compared with the WT group (Figure 3A). Additionally, cell-to-cell adhesion was positively enriched, and metabolic and mitochondria-related pathways were negatively enriched in 75%KD hearts (Figure S3D and S3E). These results consistently demonstrated that cardiac remodeling in 75%KD hearts was induced by miR1 deficiency. The proteomic profile provided valuable insights into the molecular changes contributing to the observed dysfunction in miR1-deficient hearts.

To explore how miR1 deficiency leads to cardiac remodeling, we examined all miR1 direct targets (729 genes), as predicted by TargetScan,[21] in our transcriptome and proteome assays of 75%KD hearts (Figure S4A). Among these direct targets, we identified 183 miR1 direct targets that were significantly upregulated in 75%KD cardiomyocytes. Specifically, 126 genes were upregulated at the mRNA transcriptional level, 27 at the protein translational level, and 30 at both mRNA and protein levels. Functional annotation revealed that miR1, through these 183 direct targets, is involved in various biological processes, regulating cardiac electrophysiology, cell morphology, metabolism, and stress responses in the heart (Figure S4B). Further analysis with those 57 upregulated miR1 targeted proteins unveiled a cluster of core genes forming miR1-centered regulatory cascades, which play crucial roles in heart development, electrophysiology, and contraction (Figure 3D). Additionally, using Human Phenotype Ontology, we found that these upregulated miR1 direct target genes are closely associated with a wide range of cardiac diseases (Figure 3E).

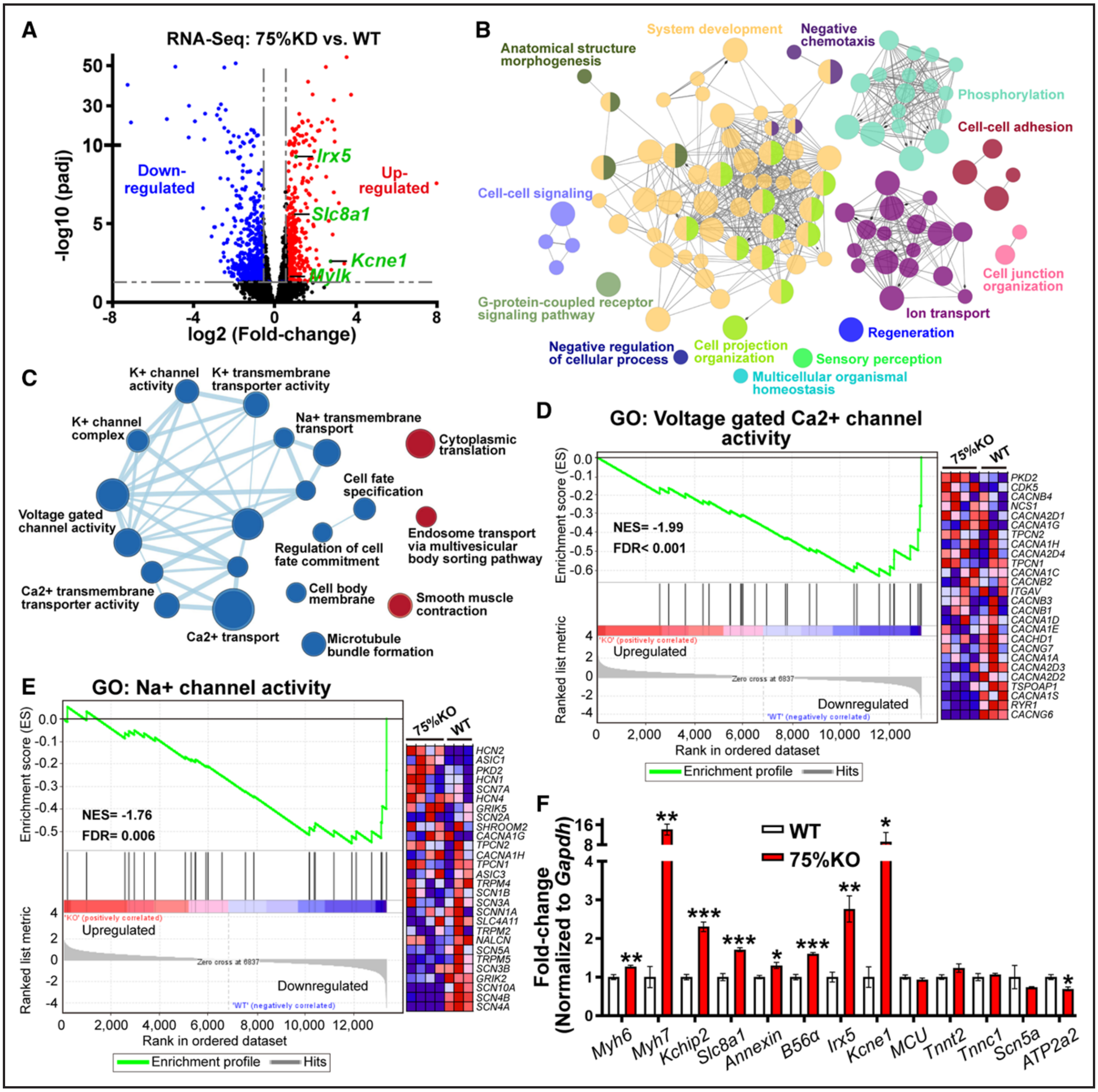


**Figure 2. Reshaping of cardiomyocyte transcription profile by microRNA-1 (miR1) deficiency.**

**A**, Volcano plot displaying expressed RNAs with transcript per million (TPM) >1 in both 75%-miR1-knockdown (75%KD) and wild-type (WT) cardiomyocytes. A false discovery rate (FDR) of <0.05 is indicative of significant enrichment. Transcripts with log2-fold change ≥1.5 or ≤−1.5 (FDR <0.05) are highlighted in red or blue, respectively. Previously validated miR1 direct targets are highlighted in green. **B**, Clue gene ontology (GO) plugged-in Cytoscape analysis presenting differentially expressed genes between 75%KD and WT myocytes, organized by GO terms. Each bubble (network node) represents a specific biological process or pathway, and lines (network edges) denote relationships between different terms. Nodes of the same color indicate functionally related terms or pathways. Node size represents term enrichment significance ($P<0.0005$ to $P<0.05$). **C**, Gene set enrichment analysis of transcriptome comparison between 75%KD and WT groups. Normalized enrichment score indicates negative or positive enrichment in 75%KD cardiomyocytes. An enrichment map of Cytoscape summarizes gene sets related to cardiac electrophysiology. Node size reflects the number of associated genes, and network edges display similarity between different gene sets. Filled node colors indicate positive (red) or negative (blue) enrichment in 75%KD groups. **D** and **E**, Assessment of the regulation of the GO terms showing positive regulation of calcium transport into the cytosol (**D**) and sodium channel activity (**E**), accompanied by heat maps depicting low (blue) to high (red) expression of individual component genes. **F**, Quantitative polymerase chain reaction analysis validating that many of miR1's RNAi–direct targets were upregulated at the transcriptional level in 75%KD groups compared with WT groups. Gene expressions were normalized to the *Gapdh* housekeeping gene. Statistical significance of differences estimated using ANOVA followed by unpaired 2-tailed Student *t* tests with multiple testing adjustments. KO indicates knockout. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs WT group.

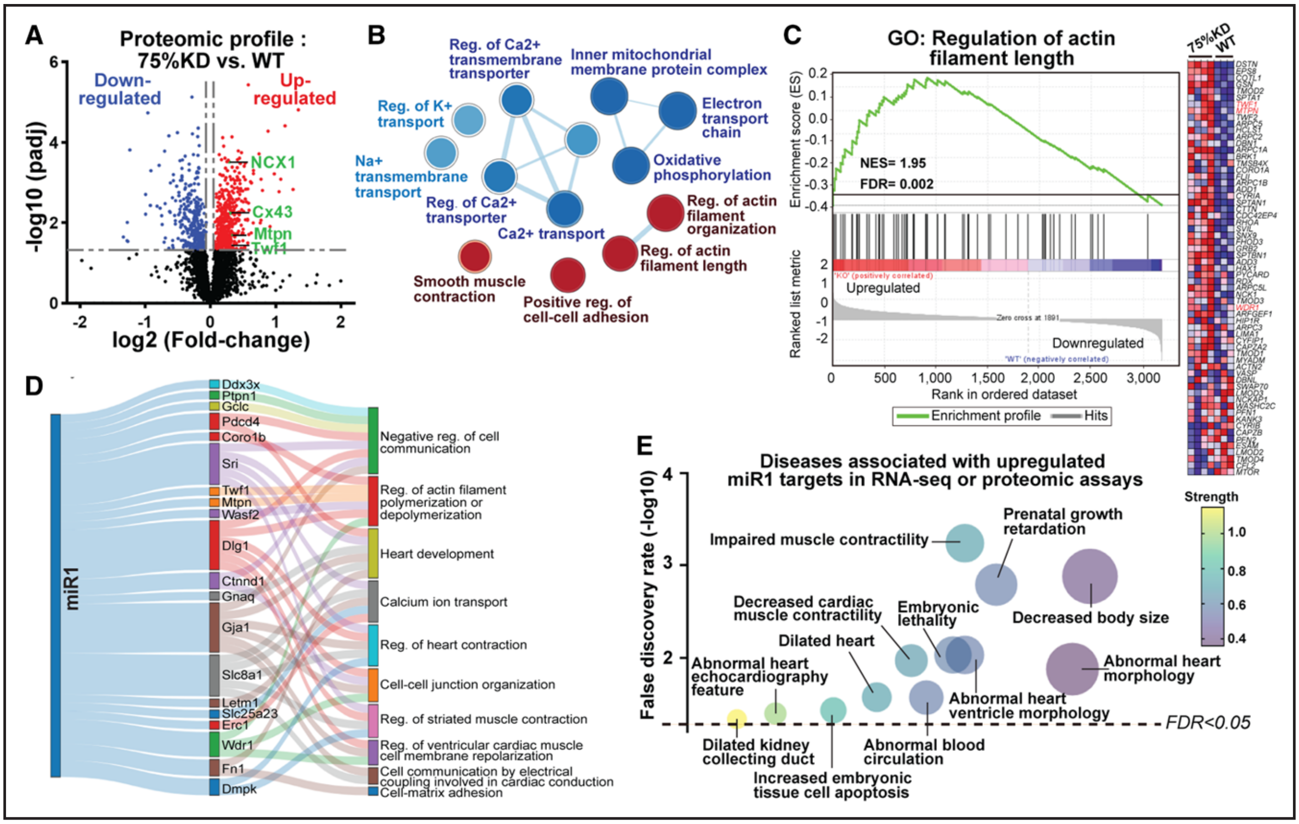


**Figure 3. Revealing the combinatorial regulatory network of microRNA-1 (miR1) in heart homeostasis.**
**A**, Volcano plot displaying the log2-fold change of expressed proteins in 75%-miR1-knockdown (75%KD) and wild-type (WT) ventricular tissues, detected by mass spectrometry. Significantly upregulated or downregulated proteins in 75%KD hearts are highlighted in red and blue, respectively. Direct miR1 target proteins are denoted by green text. **B**, Gene set enrichment analysis (GSEA) proteomic comparison of 75%KD and WT mouse hearts revealed a positive enrichment of cardiomyocyte contractile. Normalized enrichment score denotes negative or positive enrichments in 75%KD cardiomyocytes. A false discovery rate (FDR) <0.05 is considered a significant enrichment. Node size corresponds to the number of genes, and network edges represent similarity between different gene sets. Filled node colors indicate positively (red) or negatively (blue) enriched gene terms in 75%KD groups. **C**, Evaluation of the regulation of the gene ontology (GO) term revealed positive regulation of actin filament length, alongside heat maps illustrating low (blue) to high (red) expression of each gene. Cytoskeletal protein names among miR1's direct targets are highlighted in red. **D**, Sankey diagram based on the GO Biological Process analyses, depicting biological processes, including cardiac electrophysiology and contractile, associated with miR1 target proteins upregulated in 75%KD hearts. **E**, Human Phenotype Ontology analysis of upregulated miR1 direct targets revealed a diverse array of cardiovascular diseases listed in the OMIM database. Node size represents the number of associated genes. KO indicates knockout.

Typically, microRNA dysregulation initiates changes in a small fraction of genes through the RNA interference (RNAi) mechanism, and those microRNA direct targets could subsequently regulate the expression or activities of large numbers of downstream genes.[22] To better understand the miR1-centered regulatory cascade, we delved into the protein-protein association networks of those 183 miR1 direct targets. This analysis involved in incorporating the proteomic data of proteins that physically or functionally interact with these 183 miR1 targets. Our analysis results in a concise protein-protein interaction network of numerous genes directly or indirectly regulated by miR1 (Figure S5). The upregulated (red frame) or downregulated (blue frame) component genes of this network illustrate how miR1 deficiency induces remodeling of cardiovascular biological processes via direct targets or indirect protein-protein associations.

Considering that miRs can propagate their regulatory effect through transcription factors,[22–24] we also studied transcription factors that are miR1-targeted genes and were changed in our proteomic study. A transcription factor specificity protein 1 (SP1) was significantly increased in 75%KD hearts, along with significant changes of a large set of SP1-regulated genes (Figure S6). Mapping those SP1-targeted genes, which were upregulated/downregulated in 75%KD hearts, to the functional biological processes, we found their involvement in the regulation of cell communication, transmembrane transport, and cell junction organization. Notably, *Kncd2* is both a miR1 direct target and potentially regulated by SP1, indicating a multilayer regulatory mechanism of miR1.

Overall, our transcriptomic and proteomic data demonstrated that miR1 deficiency leads to changes in a cluster of directly targeted genes and triggers a large secondary regulatory network, resulting in cardiac remodeling.

## Repolarization of AP Was Impaired in miR1-Deficient Cardiomyocytes

To assess the functional consequence of miR1 deficiency at the cellular level, we conducted single-cell patch clamping on isolated adult ventricular cardiomyocytes. In 75%KD cardiomyocytes, we observed a significant hyperpolarization of the resting membrane potential (−86.729±1.407 mV, n=12; $P$=0.0438 versus −77.871±0.480 mV for WT, n=7). The amplitude of APs showed no significant change between 75%KD and WT cells, but the AP duration (APD) was significantly prolonged (Figure 4A). Specifically, the APD at 50% repolarization of 75%KD cells was 20±0.616 ms ($P$=0.0003 versus 6.857±0.373 ms for WT) and APD at 90% repolarization was 65.333±2.647 ms ($P$=0.0402 versus 36.714±2.166 ms for WT; Figure 4B). Previous reports have shown that miR1 directly targets multiple voltage-gated ion channels and their accessory proteins.[12] In our voltage clamping experiments, we measured $Na^+$ current,

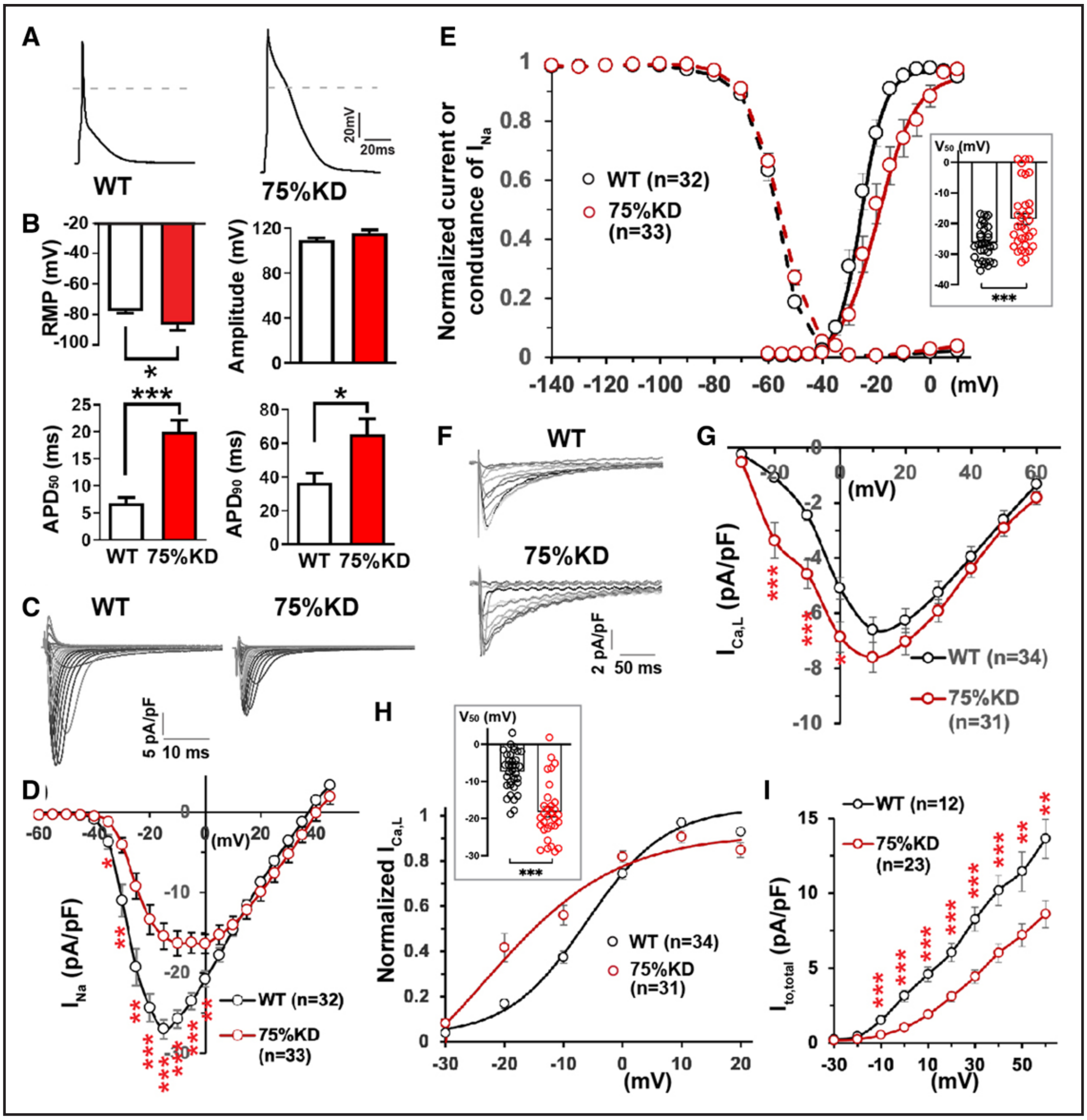


**Figure 4. Altered cellular electrophysiology in microRNA-1 (miR1)–deficient cardiomyocytes.**
**A**, Representative action potential (AP) traces from both adult 75%-miR1-knockdown (75%KD) and WT ventricular cardiomyocytes. **B**, Statistical analyses portraying resting membrane potential (RMP), amplitude, and AP duration at 50% ($APD_{50}$) and 90% ($APD_{90}$) repolarization of 75%KD (n=12) and WT (n=7) ventricular cardiomyocytes. **C** through **E**, Representative traces (**C**) and I/V curves (**D**) of $Na^+$ current ($I_{Na}$), showcasing diminished $I_{Na}$ in 75%KD cardiomyocytes relative to WT cells. Activation curve (**E**, solid lines) highlights a right-shifted voltage dependence of $Na^+$-channel activation in 75%KD cardiomyocytes, while inactivation curves (dash lines) show no significant difference between 75%KD and WT cells. Inserted box in **E** provides the summarized half-activation voltage ($V_{50}$). **F** through **H**, Representative traces (**F**), I/V curves (**G**), and activation curves (**H**) of L-type $Ca^{2+}$ current ($I_{Ca,L}$), indicating earlier activation of $I_{Ca,L}$ in 75%KD cardiomyocytes compared with WT cells. Inserted box of **H** presents the summarized $V_{50}$ of $I_{Ca,L}$. **I**, I/V curves of total transient outward potassium current ($I_{to,total}$) revealing significantly declined $I_{to,total}$ in 75%KD cardiomyocytes relative to WT cells. The statistical significance of differences was assessed by unpaired 2-tailed Student $t$ tests. *$P$<0.05, **$P$<0.01, ***$P$<0.001 vs WT group.

L-type $Ca^{2+}$ currents, and transient outward potassium current ($I_{to}$). Compared with WT cells, 75%KD cardiomyocytes exhibited significantly decreased $Na^+$ current with a right-shifted voltage dependence of $Na^+$-channel activation (Figure 4C through 4E). The half-activation voltage of $Na^+$ channels was −18.47±1.82 mV in 75%KD cardiomyocytes (n=33; *P*=0.0004 versus WT −26.25±0.99 mV, n=32), while the inactivation curves were the same.

75%KD cardiomyocytes showed a significant increase in L-type $Ca^{2+}$ currents particularly at more negative membrane potential voltages (Figure 4F through 4H). The voltage dependence of $Ca^{2+}$ channels was shifted to the left with a −18.20±1.40 mV half-activation voltage in 75%KD cells (n=31; *P*<0.0001 versus WT −7.33±0.91 mV, n=34). Although the mRNA expression of the L-type calcium channel α subunit (encoded by *Cacna1c*) was not altered in miR1-deficient hearts, the protein level of β subunit (encoded by *Cacnb2*), known to facilitate the trafficking and enhance the current density of the α subunit,[25–28] was significantly upregulated in 75%KD cardiomyocytes compared with that in WT cells (Figure S3C). Additionally, the $I_{to}$ was significantly smaller in 75%KD cardiomyocytes (4.501±0.100 pA/pF at +30 mV, n=22; *P*=0.0001 versus WT 8.258±0.232 pA/pF, n=12; Figure 4I). Our whole-transcriptome analysis also revealed a specific set of genes correlated with membrane repolarization that were significantly negatively enriched in 75%KD hearts (Figure S7), including downregulation of *Kcnd2* (Figure S6B).

Taken together, our cellular electrophysiological studies demonstrated that miR1 deficiency induces cardiac electrical remodeling, resulting in slower repolarization of APs in 75%KD cardiomyocytes.

## Excitation-Contraction Coupling Was Impaired in miR1-Deficient Cardiomyocytes

Cardiomyocytes contract in response to electric excitation of the sarcolemma membrane potential depolarization via excitation-contraction coupling. To investigate if miR1 deficiency could alter $Ca^{2+}$ handling, we examined the dynamics of intracellular $Ca^{2+}$ transients in 75%KD cardiomyocytes compared with WT cells under basal conditions and in response to isoproterenol (100 mol/L) stress (Figure 5A). Under both basal and isoproterenol-stress conditions, the baseline levels of $Ca^{2+}$ transients in 75%KD cardiomyocytes were significantly higher (1.2±0.003; n=59; *P*<0.0001) than that in WT cells (0.935±0.002; n=50, Figure 5B), indicating a $Ca^{2+}$ overload in miR1-deficient cells. However, the amplitudes of $Ca^{2+}$ transients were similar between 75%KD and WT cells (Figure 5C). At the basal state, the upstroke velocity of 75%KD cardiomyocytes was significantly faster (35.212±0.258 versus 25.48±0.195 for WT; *P*<0.0001) with a reduced time to the peak (Figure 5D and 5E). The decay time of $Ca^{2+}$ transients was slightly but significantly prolonged in 75%KD myocytes (389.879±1.025 versus 354.327±1.094 in WT; *P*=0.002; Figure 5G), while the decay rate was similar between the 2 groups (Figure 5F). Notably, isoproterenol dramatically increased the $Ca^{2+}$ release rate to a similar level in both 75%KD and WT cells, suggesting that the maximum $Ca^{2+}$-handling capacity of 75%KD cardiomyocytes is comparable to that of WT cells.

The $Ca^{2+}$ store in the sarcoplasmic reticulum was found to be unchanged, as indicated by the comparable amplitude of caffeine-induced $Ca^{2+}$ release (Figure 5H through 5K). However, the upstroke velocity of caffeine-induced $Ca^{2+}$ release was significantly faster in 75%KD cells compared with WT cells, indicating faster $Ca^{2+}$ release from the sarcoplasmic reticulum. Indeed, the impaired intracellular $Ca^{2+}$ homeostasis is consistent with the discoveries from the omics assays, where changes in many $Ca^{2+}$-handling genes were detected at the mRNA and protein levels (Figure S8).

To further understand the contractile function of 75%KD cardiomyocytes, we studied sarcomere shortening/relaxation with different pacing rates (Figure 6A). At 1 and 3 Hz stimulations, both 75%KD and WT cardiomyocytes demonstrated similar sarcomere shortening with no significant changes in amplitude or percentage shortening rate between these 2 groups (Figure 6B and 6C). At 1 Hz pacing, 75%KD myocytes exhibited longer systole and diastole time than WT cells (Figure 6D and 6E). At the fast 5 Hz pacing rate that is close to mouse fast heart rate (≈500–700 bpm), sarcomere shortening was significantly decreased in 75%KD cells compared with WT cells, with significantly decreased systolic and diastolic velocities (Figure 6F and 6G), indicating a defect in contractility at higher heart rates.

Transmission electron microscopy of ventricular tissues revealed structure differences in 75%KD cardiomyocytes compared with WT cells. The myofibrils of 75%KD cardiomyocytes were not aligned in parallel, and mitochondria were unevenly positioned among the myofibrils (Figure 6H). Adherens junctions between neighboring 75%KD cardiomyocytes were also more abundant, consistent with the transcriptomic and proteomic findings of positive enrichments in genes associated with cell-cell adhesion/communication in miR1-deficient myocytes.

In summary, miR1 deficiency was found to impair intracellular $Ca^{2+}$ homeostasis and excitation-contraction coupling in cardiomyocytes, leading to decreased contractility, particularly at higher pacing rates.

## Restoration of miR1 Mitigates the Contractile Deterioration in miR1-Deficient Hearts

The comprehensive omics and functional studies conducted in this research revealed that miR1 deficiency plays a critical role as a primary factor in triggering

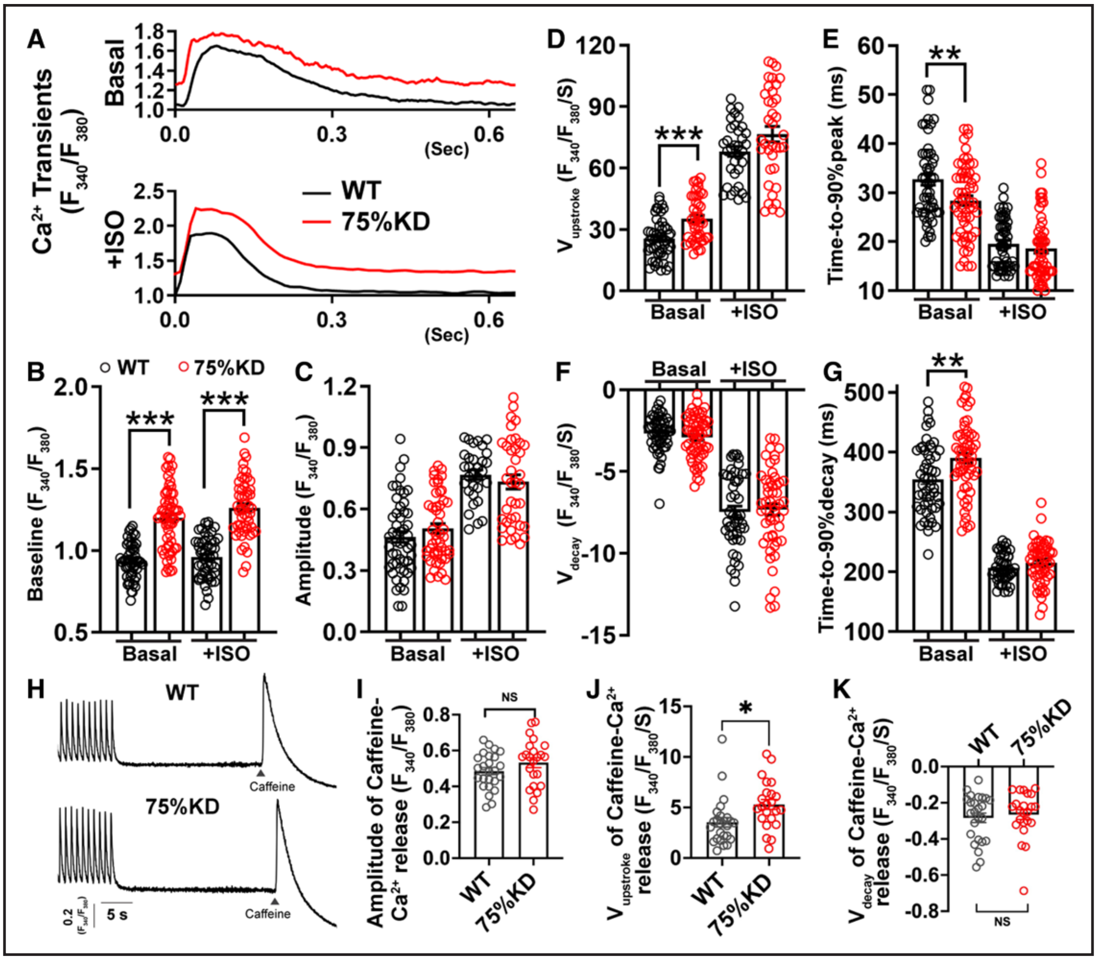


**Figure 5. Impaired intracellular Ca2+ handling in 75%-miR1-knockdown (75%KD) cardiomyocytes.**

**A**, Representative Ca$^{2+}$ transient traces observed in 75%KD and wild-type (WT) ventricular cardiomyocytes under normal (**top**) and stress induced by isoproterenol treatment (Iso, 100 nmol/L; **bottom**). **B** through **G**, Statistical analyses illustrating Ca$^{2+}$ transient parameters in 75%KD (n=59) and WT (n=50) cardiomyocytes, including intracellular Ca$^{2+}$ baseline level (**B**), amplitude (**C**), Ca$^{2+}$ upstroke velocity (V$_{upstroke}$, **D**), time to 90% of peak (**E**), Ca$^{2+}$ decay velocity (V$_{decay}$, **F**), and time to 90% of baseline (**G**). **H** through **K**, Representative traces (**H**) of caffeine-induced Ca$^{2+}$ release observed in WT (n=25) and 75%KD (n=23) cardiomyocytes, along with statistical analyses of amplitude (**I**), V$_{upstroke}$ (**J**), and V$_{decay}$ (**K**). Statistical significance of differences was assessed by unpaired 2-tailed Student *t* tests. KO indicates knockout. ***P*<0.01, ****P*<0.001 vs WT group.

dysfunctions of broad molecular networks in the heart. We next explored the therapeutic potential of restoring miR1 in mitigating the pathological phenotypes observed in miR1-deficient hearts. Paired adult 75%KD mice were selected at 3 months of age and subjected to bi-weekly tail-vein injections of polyethylenimine-encapsulated miR1 mimics or control mimics. We monitored the cardiac function by echocardiogram and evaluated the susceptibility to arrhythmia by optical mapping of ex vivo hearts (Figure 7A).

The quantitative real-time polymerase chain reaction assays demonstrated that the miR1 expression level in the heart significantly increased (≈2-fold) after 3 rounds of microRNA tail-vein injection (Figure 7B). However, at week 5 post-miR1 delivery, there was no noticeable functional improvement observed in 75%KD hearts. We then extended bi-weekly administration of miR1 and found that, compared with the control-mimic group, the hemodynamic function of the heart, including the ejection fraction and fractional shortening, significantly improved in miR1-administrated animals at weeks 7 and 11 post-miR1 delivery (Figure 7C and 7D). Relative to the control-mimic group, miR1-mimic treated hearts exhibited lower expression levels of *Myh7* and *Cacna1c*, with no significant changes observed in other examined genes (Figure S9). However, it is essential to note that this miR1 treatment did not improve the electrical conduction of the ventricle in 75%KD mice (Figure 7E). Despite the improvement in cardiac contractility, miR1-administrated animals still exhibited a high inducibility to arrhythmias (Figure 7F). In summary, these results suggest that miR1 restoration has the potential to ameliorate the contractility dysfunction in miR1-deficient hearts; however, it does not fully rescue the arrhythmic susceptibility associated with miR1 deficiency.

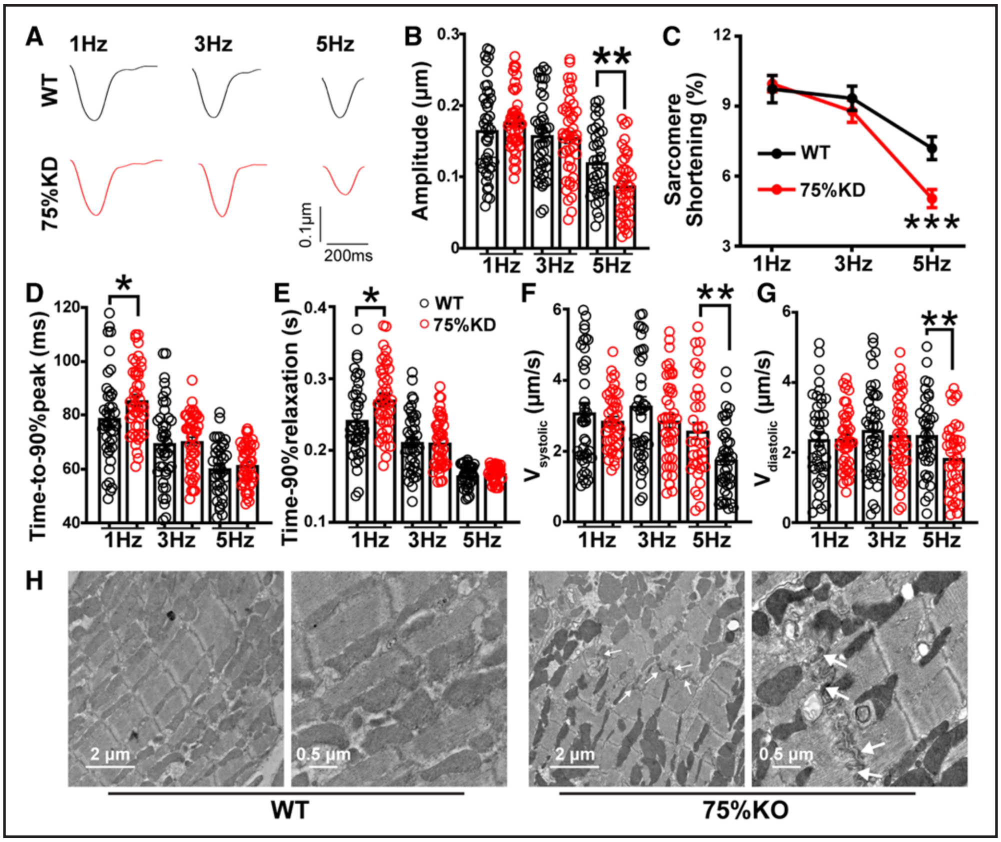


**Figure 6. Contractility defects in microRNA-1 (miR1)–deficient cardiomyocytes.**

**A**, Representative sarcomere shortening traces of 75%-miR1-knockdown (75%KD; n=49) and wild-type (WT; n=50) cardiomyocytes paced at rates of 1, 3, or 5 Hz. **B** through **G**, Statistical analyses of sarcomere shortening parameters, including amplitude (**B**), percentile of sarcomere shortening (**C**), time from baseline to 90% peak (time to 90% peak, **D**), time from peak to 90% relaxation (time to 90% relaxztion, **E**), velocity of systole ($V_{systolic}$, **F**), and velocity of diastole ($V_{diastolic}$, **G**). **H**, Transmission electron microscopy depicting intracellular sarcomere and mitochondrial structures in ventricular heart tissues from 75%KD and WT mice. Arrows highlight the adherens junction structures. Statistical significance of differences was assessed by unpaired 2-tailed Student *t* tests. *$P$<0.05, **$P$<0.01, ***$P$<0.001 vs WT group.

## DISCUSSION

In the present study, we leveraged miR1-75%KD mice to probe the etiological significance of miR1 in cardiac remodeling. Our findings illuminate how miR1 deficiency alleviates the restraint on its RNAi–direct target genes, subsequently perturbing a comprehensive regulatory gene network and ultimately leading to cardiac remodeling. miR1 deficiency triggers disruptions in cardiac repolarization and excitation-contraction coupling within cardiomyocytes, culminating in reduced heart contractility and an increased vulnerability to arrhythmia, ultimately contributing to sudden death in 75%KD mice. A reintroduction of miR1 via tail-vein administration partially reinstated cardiac hemodynamic function, but it proved ineffective in mitigating arrhythmic tendencies, suggesting the intricate balance required for precise miR1 expression within the heart.

### miR1 Intricately Modulates Cardiac Homeostasis

In both mice and humans, 2 discrete genes, *miR1-1* and *miR1-2*, encode mature miR1 with an identical sequence. The expression of *miR1* genes progressively increases in muscle cells during the development and maturation of muscle tissues.[29,30] miR1 stands as a predominant microRNA in the heart, exerting numerous functions crucial for heart homeostasis. Loss-of-function animal models displaying varying degrees of miR1 depletion highlight the sensitivity of miR1 functions to dosage. The survival of adult 75%KD mice indicates that a single allele of either *miR1-1* or *miR1-2* gene is adequate to maintain basal heart function, although an escalated risk of cardiac dysfunction was observed in proportion to the reduction in miR1 levels. While 25%KD mice exhibit no overt cardiac anomalies, those with 50% miR1 reduction generally survive, although some manifest diverse heart developmental irregularities.[8] This study aligns with prior research,[10,11] noting a preweaning lethality in miR1-full-knockout mice, while 50%KD and 75%KD mice reach adulthood. Although adult 50%KD heterozygous mice display survival rates like WT animals; sudden death emerges in 75%KD mice, implying an aberration in cardiac electrophysiology. Notably, 75%KD adult animals exhibit reduced heart rates, shortened PR intervals extended QT and QRS intervals, along with decreased ventricular conduction velocity (Figure 1).

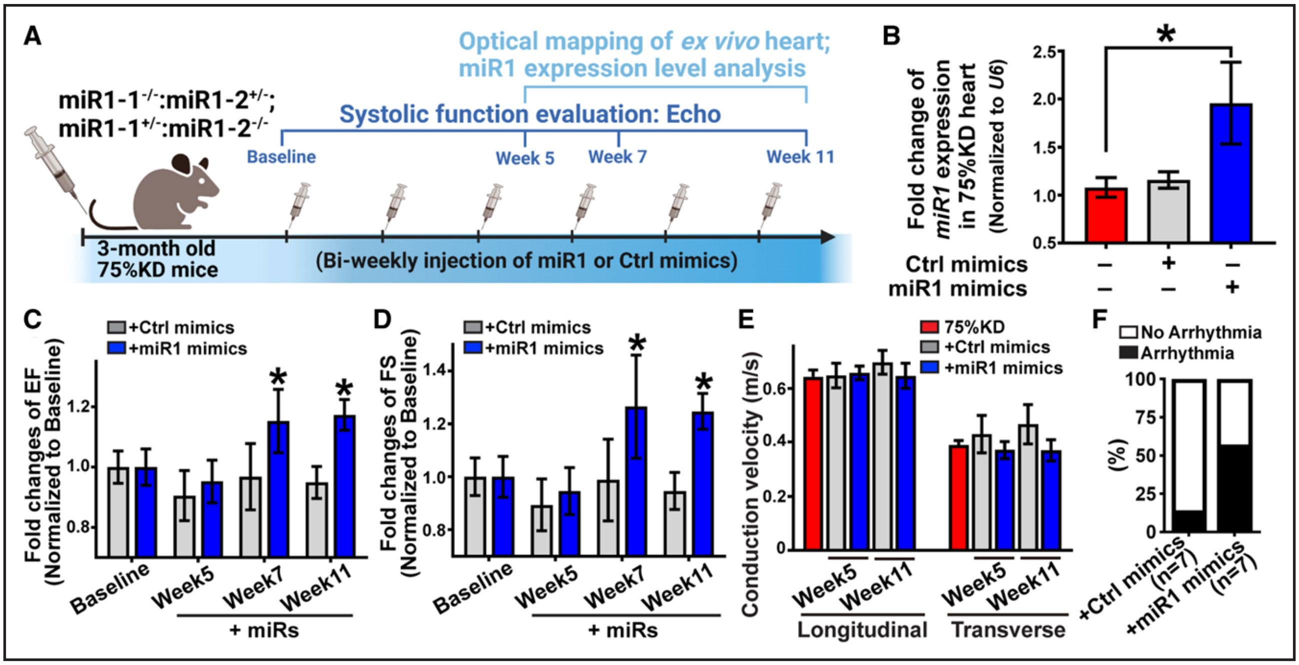


**Figure 7. Effects of microRNA-1 (miR1) restoration on 75%-miR1-knockdown (75%KD) hearts.**

**A**, Schematic representation of the rescue experiment for 75%KD heart through microRNA administration via intravenous tail-vein injections. **B**, Quantitative polymerase chain reaction (qPCR) analysis illustrating the significant increase of miR1 expression in 75%KD hearts (n=4), following tail-vein injection of miR1 mimics. Expression levels were normalized to *U6* RNA. **C** and **D**, Echocardiography analysis demonstrating the enhanced ejection fraction (EF; **C**) and fractional shortening (FS; **D**) of 75%KD mice at weeks 7 and 11 (n=4), compared with the control-mimic group (n=4). **E**, Optical mapping of ex vivo hearts showing no significant improvement in conduction velocity with miR1 administration in 75%KD animals at weeks 5 (n=3) and 11 (n=4). **F**, Programmed electric stimulation assays revealing no improvement in arrhythmia inducibility of 75%KD hearts following miR1 administration. Statistical significance of differences was assessed by unpaired 2-tailed Student *t* tests. *$P<0.05$ vs control mimics or indicated group.

miR1 orchestrates cardiac electrophysiology by targeting a spectrum of ion channels, transporters, and Ca$^{2+}$ handling proteins.[12] In miR1-deficient 75%KD cardiomyocytes, the expression of several ion channels, including inward rectifier potassium channel Kir2.1 and L-type Ca$^{2+}$ channel subunits, experiences significant upregulation. *Irx5*, known to suppress the expression of *Kcnd2* involved in the $I_{to}$ current, displays elevated levels in 75%KD mice, consistent with reduced $I_{to}$ observed in 75%KD cardiomyocytes. Altered $I_{to}$ and enhanced L-type Ca$^{2+}$ currents jointly contribute to prolonged APDs in miR1-deficient 75%KD cardiomyocytes. Coupled with the decreased Na$^{+}$ current and its right-shifted voltage dependence, this remodeled ion channel profile comprehensively explains the decelerated electrical signal propagation within the ventricle, accompanied by the elongation of both QRS and QT intervals. Beyond ion channel/transport modulation, transmission electron microscopy reveals structure remodeling in 75%KD cardiomyocytes, characterized by increased adherens junctions. Correspondingly, cardiac remodeling of 75%KD myocytes impacts Ca$^{2+}$ handling and cardiac contractility, evident through Ca$^{2+}$ overload, prolonged decay of Ca$^{2+}$ transients, and reduced cell shortening during fast pacing (5 Hz). At the basal slow pacing rate (1 Hz) and in response to caffeine, notably, 75%KD cardiomyocytes exhibit a faster upstroke velocity of Ca$^{2+}$ transient than WT cells; however, the time to peak of cell shortening is prolonged in 75%KD cells, indicating contractile protein remodeling. Our proteomic profiling reveals substantial alterations in the expression of pertinent Ca$^{2+}$ and phosphorylation handling proteins due to miR1 deficiency, encompassing downregulations of Ca$^{2+}$/calmodulin-dependent protein kinase II γ and δ (encoded by *camk2d* and *camk2g*, respectively), sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2), TnnT2 (troponin T), and upregulations of protein phosphatase 2A regulatory subunit B56α (encoded by *PPP2R5A*), sodium-calcium exchanger 1 (encoded by *Slc8a1*), β subunit of L-type inward Ca$^{2+}$ channel (Figure S8). Notably, miR1 downregulation has been observed in human heart tissue from acute myocardial infarction[15] and the heart of patients with atrial fibrillation.[16] Our study of 75%KD cells/hearts unveils the primary etiological significance of miR1 deficiency in driving cardiac remodeling, although the underlying cause of miR1 deficiency in heart diseases remains enigmatic.

## miR1 Deficiency Orchestrates a Comprehensive Gene Network Reshaping Cardiac Remodeling

As the predominant microRNA in the heart, miR1 exerts regulatory control over a diverse array of target genes spanning various biological functions, including cytoskeletal dynamics, cell cycle progression, metabolism, and cardiac electrophysiological properties. Our comprehensive omics analysis of 75%KD cardiomyocytes/hearts consistently illuminates the wide-reaching impact of numerous biological processes. miR1 along with its counterpart miR133 collaboratively suppresses smooth muscle gene expression in the heart. Full-knockout miR1 reactivates smooth muscle genes ectopically in miR1-full-knockout hearts.[10] Consistently, our findings also reveal that adult 75%KD hearts prominently express a repertoire of smooth muscle genes (Figure S2D), such as *Myh11* and *Mylk*, while exhibiting a decreased representation of cardiac muscle genes. The intricate landscape of metabolic and mitochondria-related pathways is also perturbed significantly in 75%KD hearts, as indicated by the negative enrichment of terms associated with inner mitochondrial membrane protein complex and oxidative phosphorylation. This comprehensive transcriptome blueprint of multiple miR1-targeted genes is consistent not only with the disrupted sarcomere organization and diminished cardiac contractility observed in 75%KD mouse cardiomyocytes and hearts (Figure 6), but also with the heightened susceptibility to arrhythmias (Figure 1H and 1J). GSEA analyses of our RNA sequencing and proteomic data unveil the downregulation of ion channel/transport terms, including voltage-gated $Na^+$/$Ca^{2+}$/$K^+$ channels, thereby providing mechanistic insights into the prolonged APD (Figure 4) and extended QRS/QT intervals, as well as the reduced conduction velocity of 75%KD hearts (Figure 1G and 1I). Furthermore, a subset of miR1 target proteins, including Twf1, Mtpn, Cx43, and Wdr1 (tryptophan-aspartic acid [WD]-repeat protein 1), is significantly elevated in 75%KD groups relative to WT. Notably, Twf1 participates as an actin-depolymerizing factor through sequestering actin monomer and capping barbed ends[31–33]; Mtpn is implicated in actin filament growth regulation and the F-actin-capping protein complex modulation,[34,35] while Wdr1 is known as actin-interacting protein 1 and strongly enhances actin filament disassembly.[36] The influence of miR1 deficiency extends to cytoskeletal regulatory proteins, fostering cardiac remodeling of cell adhesion. Indeed, 75%KD myocytes exhibit augmented adherens junctions in contrast to WT tissues, potentially contributing to increased arrhythmogenic susceptibility, given the pivotal role of gap junctions and adherens junctions in cardiac electromechanical function.[37,38]

At its core, cardiac remodeling in the context of miR1 deficiency is driven by the abrogation of miR1's RNAi actions. A substantial cluster of miR1's RNAi–direct target genes, spanning diverse biological themes, demonstrates significant upregulation in 75%KD mice (Figure S4). The cardiac anomalies observed in 75%KD heart result from a spatiotemporal convergence of perturbation across multiple biological domains. It is noteworthy that miR1-targeted transcription factor SP1 displays upregulation in 75%KD hearts, triggering alterations in genes harboring SP1 binding sites, with ensuing impacts on both upregulated or downregulated gene expression (Figure 3F), reflecting the dual regulatory role of transcription factors in gene modulation.[39] Additionally, proteins often execute their functions through interactions with other proteins or nucleotides (DNA, RNA).[40] miR1's potential to amplify its regulatory impact through protein-to-protein interactions is exemplified in Figure S5, where miR1's RNAi targets exhibit extensive associations with proteins, many of which undergo differential expression in 75%KD mice. Our annotation reveals the vast repertoire of functionally relevant genes influenced by miR1 deficiency, mirroring the concurrent dysregulation of associated biological processes. While further investigations are required to meticulously map these intricate molecular networks, our findings underscore the pivotal role of miR1 in orchestrating a finely tuned cardiac homeostasis.

## Challenges and Considerations in Harnessing miR1 for Therapeutic Interventions

Our investigation has underscored the pivotal etiological significance of miR1 deficiency in instigating cardiac remodeling and fostering hemodynamic anomalies and arrhythmias. Unveiling the therapeutic potential of miR1 administration, however, presents a complex landscape. While studies have indicated that miR1 overexpression can enhance the maturation of human stem cell-differentiated cardiomyocytes and hold promise for epigenetic-induced cardiomyocytes,[30,41,42] the multifaceted regulatory nature of miR1 in the heart poses challenges for translating this knowledge into effective cardiovascular therapies. In our bi-weekly administration of miR1-mimics to 75%KD mice via tail-vein injection, we observed limited functional improvement in heart performance only after several weeks of treatment. The restoration of miR1 indeed mitigated the contractile decline in adult miR1-deficient hearts, consistent with prior findings that miR1 reintroduction partially ameliorates sarcomere disruption of neonatal cardiomyocytes lacking miR1.[10] However, the arrhythmia susceptibility of 75%KD hearts remained unaltered despite miR1 administration. One limitation is that sustained VT was not induced in any of those ex vivo hearts; programed electrical stimulation performed in vivo[43,44] might be able to reveal more severe sustained VT and demonstrate miR1's therapeutic potential more comprehensively.

Several factors contribute to these observations. First, rectifying the accumulated cardiac defects spanning fetal to adult stages proves challenging. Additionally, the meticulous control of microRNA delivery dosage and efficacy presents a formidable hurdle in microRNA-based gene therapy. Our bi-weekly tail-vein injections achieved a modest 2-fold elevation of miR1 expression in 75%KD hearts, which still fell short of normal levels. Furthermore, an excess of miR1 may not necessarily confer greater benefits to the heart. Research has shown that miR1 upregulation postacute myocardial infarction can exacerbate arrhythmogenesis.[17,45,46] Recent insights have illuminated the multifaceted role of miR1 in regulating cardiac ion channels through a spectrum of mechanisms. Beyond its canonical RNAi pathway, miR1 engages in the modulation of cardiac ion channels by directly binding to ion channel proteins, thereby influencing their activities.[12,47] The administration of miR1-mimics via bi-weekly tail-vein injections may contribute to fluctuations in miR1 expression over time. Such dynamic dosing profiles could disrupt cardiac ion channel functions not only through RNAi-mediated effects but also via intricate biophysical interactions. These multifarious interactions between miR1 and ion channels could collectively contribute to the potential emergence of arrhythmic events. Considering these complexities, achieving a delicate balance in miR1 expression and activity becomes pivotal to the successful implementation of microRNA-based therapeutic strategies.

## ARTICLE INFORMATION

Received May 17, 2023; accepted November 22, 2023.

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### Acknowledgments

The authors are grateful to Dr Jill Dunham for editorial assistance and thank Dr Deepak Srivastava for generously sharing miR1-1 homozygous-null (miR1-$1^{-/-}$:miR1-$2^{+/+}$) and miR1-2 homozygous-null (miR1-$1^{+/+}$:miR1-$2^{-/-}$) mice.

### Sources of Funding

This research was supported by the start-up fund from the Ohio State University (OSU; to Dr Fu), funding provided by the OSU President's Research Excellence Accelerator grant (to Dr Fu), College of Medicine (COM) Office of Research Dean's Nationwide/COM Cross-Campus-Collaborative Pilot Program (to Dr Fu), grants from the National Institutes of Health (grants NIH-R01HL139006 to Drs Deschênes and Fu, R21OD031965 to Dr Fu, R01HL096962 and R01HL132520 to Dr Deschênes, R01HL142754 to Dr Laurita, R01HL139348 and R01AG057046 to Dr Wold, R01HL156652 to Dr Hund, and R01HL135754 to Dr Mohler), the Centers for Disease Control and Prevention/National Institute for Occupational Safety and Health (U01 OH012056 to Dr Wold), the Leducq Foundation (TNE FANTASY 19CV03 to Dr Mohler), and the American Heart Association (20YVNR35490079 to Drs Wold and Mohler). Dr Yang was funded by the Kenneth M. Rosen Fellowship from the Heart Rhythm Society.

### Disclosures

None.

### Supplemental Material

Supplemental Methods
Figures S1–S9
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