

# RNAs that make a heart beat

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*Comment on:* Liu X, Zhang Y, Du W, *et al.* MiR-223-3p as a Novel MicroRNA Regulator of Expression of Voltage-Gated K<sup>+</sup> Channel Kv4.2 in Acute Myocardial Infarction. *Cell Physiol Biochem* 2016;39:102-14.

**Abstract:** An increase in stress-associated microRNAs has been observed in the heart after an induced myocardial infarction. Liu and colleagues now demonstrate that one of these stress-associated microRNAs, *miR-223-3p*, can regulate a component of the voltage-gated channel that mediates rapid outward efflux of potassium during an action potential. Aberrations in the potassium current have been associated with ventricular arrhythmia and heart disease. Strikingly, introducing a small RNA antagonist directed against *miR-223-3p* into rat hearts, while also inducing a myocardial infarction, resulted in a reduction in arrhythmias. We place these studies in the larger context of the field and discuss the potential of anti-*miR-223-3p* molecules as new therapeutics for myocardial infarction.

**Keywords:** Action potential; potassium channel; voltage-gated channel; microRNA; arrhythmia; myocardial infarction

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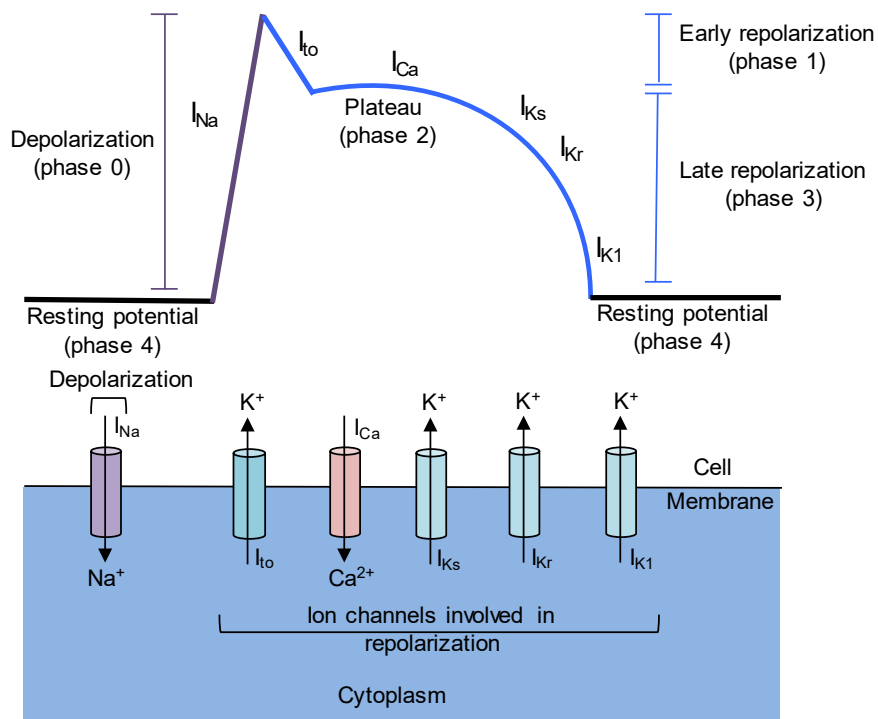
## Action potentials drive heart beat

The beating of a heart is a symbol of life and vitality. The ability of the heart to pump blood throughout the body is essential for bringing oxygen and nutrients to peripheral tissues and removing carbon dioxide and other wastes. Heart beats power this flow of blood. Each time the heart beats, arteries expand and fill with blood; the pauses between the beats allow for constriction followed by a re-expansion with the next heart beat.

The ability of the heart to beat relies on the conversion of electrical to mechanical energy (1). The electrical basis for heart beats are action potentials that start in the specialized pacemaker cells of the heart, and are then transmitted to the atrial and ventricular heart muscle cells, cardiac myocytes, via the passage of ions between cells through gap junctions. Action potentials involve the flow of ions into and out of cardiac myocytes through voltage-gated channels. These channels open and close depending on the

membrane potential, the difference in electrical potential on the inside of the cell compared with the outside (*Figure 1*). During an action potential, the opening and closing of one set of channels leads to a change in membrane potential, thereby allowing a different set of channels to open. These changes lead to entrance or exit of different ions, and subsequent changes in transmembrane potential (3-7).

During cardiac action potentials, there is a flux of sodium (Na<sup>+</sup>) into the cell, along with an inward flux of calcium (Ca<sup>+</sup>), that leads to the depolarization, followed by outward potassium (K<sup>+</sup>) currents that repolarize the cell (1,8-11). An action potential is divided into several phases (1,9-12). In phase 4, the resting phase, the membrane potential of a ventricular cardiomyocyte is about -90 mV because, like most cells, ventricular cardiomyocytes have a more negative charge inside than outside of the cell. The membrane potential is established largely by potassium channels that allow for a loss of positively charged potassium ions from the inside of the cell resulting in a negative membrane



**Figure 1** Example of a ventricular action potential. The membrane potential is plotted over time on the y axis. Different currents active at different phases of the action potential are labeled. Below, the different ion channels that open and close to produce an action potential are shown. Adapted with permission from Kim 2013 (2).

potential. In addition, sodium-potassium pumps actively transport three sodium ions out of the cell and two potassium ions into the cell, both against a concentration and electrical gradient, thus maintaining the concentrations of both ions and preserving the voltage polarization.

Phase 0 is the depolarization phase that initiates the action potential. An action potential can result from an electrical stimulation, or, in the case of the pacemaker cells of the heart, from spontaneous automaticity of the pacemaker cells due to a slowly depolarizing Na channel that generates a pacemaker current called the funny current (10). In ventricular myocytes, rapidly activating, phase 0, voltage-gated channels specific for sodium ions open. Sodium ions are more abundant outside than inside the cell, and opening these channels results in a rush of positively charged sodium ions into the cell ( $I_{Na}$ ). The flooding of sodium ions changes the transmembrane potential from negative to positive, resulting in depolarization of the membrane to about +20 mV.

The subsequent phases involve a repolarization of the membrane as positive ions flow outward from the cell. In the next phase, phase 1, the sodium channels close and

voltage-gated potassium ( $K_v$ ) channels open, creating an outward potassium current  $I_{to}$ . The rapid efflux of positively charged potassium ions results in a sharp decrease in the membrane potential from  $\sim +20$  mV to  $\sim +10$  mV at the end of phase 1. Phase 1 of an action potential can influence the height and duration of the ensuing phases (12,13).

The membrane repolarization of phase 1 activates voltage-gated calcium currents ( $I_{Ca}$ ). In phase 2, a plateau phase, there is a balance between a slow inward flux of calcium ions and a reduced outward flux of potassium ions through a different set of potassium channels, the slow delayed rectifier potassium channels ( $I_{Ks}$ ). The membrane potential in phase 2 ends at  $\sim -20$  mV. The calcium ions that enter the cell in phase 2 act as triggers for contraction of the myofilaments of the heart muscle.

In phase 3, the calcium channels close. The rapid delayed rectifier potassium current ( $I_{Kr}$ ) contributes to the rapid potassium efflux that creates phase 3 of repolarization. Finally, an inward delayed rectifier potassium current ( $I_{K1}$ ) helps establish and maintain the cell transmembrane potential (phase 4). The  $I_{K1}$  current represents the current that must be overcome to establish the next action potential.

A cardiac action potential lasts ~200 msec. After an action potential, there is a refractory period of ~250 msec in which another action potential is unable or less likely to occur. After the refractory period, the cell returns to the resting phase and can respond to signals that evoke a subsequent action potential.

### Voltage-gated potassium channels are tightly regulated to control myocyte function and arrhythmia

Voltage-gated channels allow fast and selective ion permeation that is regulated by opening and closing of a pore through a mechanism that senses transmembrane voltage (14). Voltage-gated channels are composed of alpha pore-forming subunits and accessory subunits (1,15). The pore-forming alpha subunits contain multiple transmembrane domains. The channels can be homotetrameric or heterotetrameric with four voltage-sensitive alpha subunits arranged surrounding a common center that, when open, serves as a pore for ions (15).

The  $K_v$  channel has six transmembrane domains, with both amino and carboxy termini localized within the cell (14). The two transmembrane domains at the carboxy terminus of the protein form the pore for potassium ion flux. The transmembrane domain adjacent to the pore-forming domains contains positively charged arginine and lysine residues and moves in response to membrane depolarization, thereby mediating the voltage-dependent opening of the channel (15).

Through extensive gene duplication events, the human genome encodes 40 voltage-gated potassium channels (12). The expression and regulation of these potassium channels, for instance, in different positions within the heart, can lead to changes in action potential amplitude, duration, waveforms, and rhythmicity (8,12). The transient voltage-gated potassium current ( $I_{to}$ ) in phase one is sometimes divided into  $I_{to,fast}$  and  $I_{to,slow}$  based on the rate of recovery (20–100 ms for the fast current and seconds for the slow current) (11). The pore-forming subunits of  $I_{to,fast}$  are  $K_v4.2$  and  $K_v4.3$ , which are encoded by the *KCND2* and *KCND3* genes. These proteins can likely form pores as homomers or heteromers (16,17).

Heart beats that deviate from the normal rhythm are called arrhythmias (11). Heart failure, a common cause of death worldwide, is frequently associated with arrhythmias and electrical instability. Abnormalities in the repolarization phase of action potentials due to heart failure can contribute

to arrhythmias (11,18). Dramatic changes in the levels and properties of myocardial potassium currents have been observed with cardiac disease (8), including myocardial infarction, the death and destruction of heart muscle as a result of lack of oxygen. In canine models of myocardial infarction, potassium currents are down-regulated in cells in the infarcted zone (19–21), that is, the portion of tissue that is dying or dead due to lack of blood supply. The down-regulation is most pronounced within days following the infarct and returns to normal over the course of two months. These findings highlight the physiological importance of the regulation of potassium channels in the heart.

### miRNAs regulate cardiac function

The regulation of potassium channels in normal physiology or heart disease can occur through multiple mechanisms. Transcriptional regulation plays an important role in the proper expression of different potassium channel components (8). Other mechanisms that regulate potassium channel activity include splicing, RNA editing, and post-translational modifications such as phosphorylation (8,11). In addition, microRNAs have been demonstrated as post-transcriptional regulators of potassium channel expression (2).

Mature microRNAs are small 22–26 nucleotide single-stranded endogenously encoded RNAs (22,23). Originally transcribed as pri-miRNAs, they are processed by the RNase III *Drosha*, *Dgcr8*, and other factors to form a hairpin of ~70 nucleotides in the nucleus. The hairpins are then processed in the cytoplasm by *Dicer* to give rise to mature miRNAs (24–26). These miRNAs can associate with Argonaute proteins in complexes called RNA-induced silencing complexes (RISC). miRNAs in the RISC complex can anneal to mRNA transcripts with similar base pair sequences in their 3'UTRs or coding regions (27,28). In most cases, miRNA targeting leads to the degradation of transcripts or inhibition of their translation (29,30).

Families of miRNAs including *miR-1*, *miR-29*, *miR-15* and *miR-208* have been demonstrated to respond to cardiac stress and play a role in controlling heart function (2,8,11). Van Rooij and colleagues found that microRNAs are up- and down-regulated in cardiac tissue from mice undergoing cardiac stress (31), and in response to myocardial infarction (32). Downregulation of *miR-29*, a microRNA that targets extracellular matrix proteins (33–35), was found to contribute to the fibrotic response post myocardial infarction (32). Ikeda and colleagues extended these studies to humans, and reported reproducible changes in miRNA levels

in human patients with heart failure (36). The functional importance of miRNAs for maintaining healthy hearts has been demonstrated by cardiomyocyte-specific deletion of either *Dicer* or *Dgcr8*. Deletion of *Dicer* in 3-week-old mouse cardiomyocytes resulted in arrhythmias and lethality, while deletion of *Dicer* in the hearts of adult mice led to severe heart failure (37). Similarly, perinatal deletion of *Dgcr8* in mice resulted in severe and lethal heart failure (38).

Overexpression and loss-of function studies have revealed roles for individual miRNAs in heart disease (31,39,40). For example, *miR-208* is expressed from the intron of myosin heavy chain 6 and is expressed specifically in cardiac and slow skeletal muscle (41). *miR-208*-knockout mice respond to cardiac stress with reduced fibrosis and hypertrophy (heart cell enlargement) (42). Inhibition of *miR-208a* in rats fed a high salt diet resulted in improved survival and reduced cardiac fibrosis (43). As another example, *miR-15* is induced in the infarcted region of the heart in response to ischemia-reperfusion injury in mice and pigs (39). Systemic delivery of inhibitors of *miR-15* family members in mice with myocardial infarctions reduced the size of the infarcted region and enhanced cardiac function (39).

In addition to miRNAs that affect the survival of myocardial cells, there are also examples in which miRNAs specifically target mRNAs encoding ion channels. *miR-1* is expressed in heart and skeletal muscle and is overexpressed in individuals with coronary artery disease (44). Homozygous deletion of one of two *miR-1* genes (*miR-1-2*) in mice resulted in mortality of 50% of the offspring by developmental abnormalities (45). Most of the survivors died from sudden cardiac death caused by arrhythmias (45). Several targets of *miR-1* could explain these findings. In addition to the notch ligand delta (46) and the Rho GTPase *Cdc42* (47), *miR-1* also regulates Iroquois related homeobox 5 (*Irx5*), a transcriptional repressor of the  $I_{to}$  component potassium voltage-gated channel, Shal-related family, member 2 (*Kcnd2*) (45). *miR-1* also post-transcriptionally represses potassium voltage-gated channel subfamily J member 2 (*Kcnj2*), which encodes the potassium channel subunit Kir2.1, a component of the inwardly rectifying potassium channel (44), and gap junction protein alpha 1 (*Gja*), which encodes connexin 43, a connexin important for cardiac gap junctions (44). Consistent with an important role for *miR-1* in regulating action potentials, introduction of *miR-1* was found to exacerbate arrhythmias, while introduction of *miR-1* antisense inhibitors in rat hearts undergoing myocardial infarction reduced arrhythmias (44).

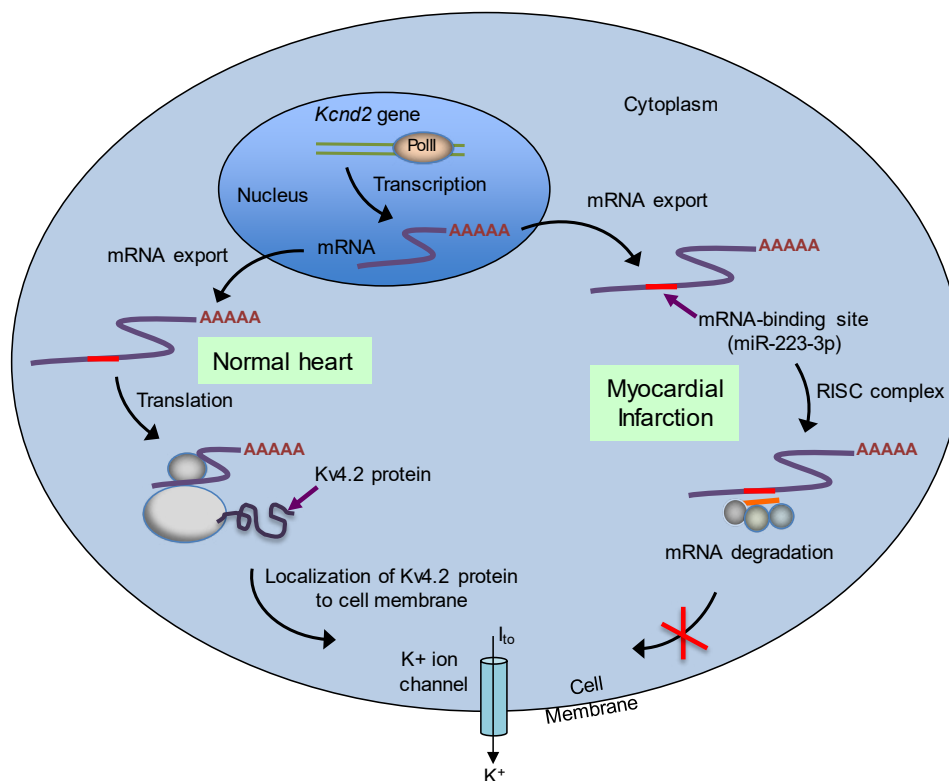
### **miR-223-3p is induced with myocardial infarction, targets Kv4.2 in its coding region, and promotes arrhythmia**

In a recent issue of *Annals of Translational Medicine: Cellular Physiology and Biochemistry*, Liu and colleagues now report that *miR-223-3p* regulates cardiac function (48). Inspired by previous studies showing that *miR-223-3p* is upregulated in hearts after myocardial infarction (32), Liu and colleagues induced myocardial infarction in rats by tying off the artery that supplies the heart with blood (a method called left anterior descending artery ligation). This procedure resulted in arrhythmias in the rats and mortality in 40% of animals. Heart tissue surrounding the infarct area contained strongly elevated levels of *miR-223-3p* and *miR-1*.

Liu and co-authors recognized that *miR-223-3p* is complementary to, and therefore has the potential to bind to, the coding sequence of the *Kcnd2* transcript. *Kcnd2* encodes  $K_v4.2$ , the alpha subunit of the voltage-gated transient outward potassium channel that carries  $I_{to}$  in the rat during phase 1 of an action potential (48) (Figure 2). Consistent with this hypothesis, Liu and colleagues found that  $K_v4.2$  protein levels were lower in the area around the rat's infarcted heart tissue. Further, down-regulation of  $K_v4.2$  was found to be associated with reduced  $I_{to}$  flux. To determine whether  $K_v4.2$  is a bonafide target of the *miR-223-3p* miRNA, the authors subcloned the coding sequence of  $K_v4.2$  into a luciferase-expressing plasmid to generate a luciferase-*Kcnd2* chimeric vector, and transfected the vector into neonatal rat ventricular cardiomyocytes. Co-transfection of *miR-223-3p*, but not a negative control, substantially suppressed luciferase activity. Finally, Liu and colleagues transfected an inhibitor of *miR-223-3p* into the ventricular cardiomyocytes of rats and found that the inhibitor significantly reduced the incidence of arrhythmias after acute myocardial infarction. The findings, taken together, support an important role for *miR-223-3p* as a regulator of cardiac action potentials after a myocardial infarction in rats.

### **miR-223-3p inhibition as a therapy for myocardial infarction-induced arrhythmia**

The findings suggest that inhibiting the induction of *miR-223-3p* during a myocardial infarction could normalize action potentials and benefit patients. Indeed, potassium channels are being recognized as important therapeutic targets and strategies to inhibit potassium channels are being



**Figure 2** Schematic of the research findings of Liu *et al.* (48). In the normal heart, the *Kcnd2* gene encodes expression of the K<sub>v</sub>4.2 protein that localizes to the cell membrane and forms voltage-gated potassium channels that contribute to the I<sub>to</sub> current. Under conditions of myocardial infarction, *miR-223-3p* is induced and targets K<sub>v</sub>4.2 for degradation through a recognition site in the coding region. Reduced levels of the voltage-gated potassium channels contribute to altered action potentials and arrhythmia after a myocardial infarction.

explored as treatment for arrhythmias and other conditions including seizures, pain, and Alzheimer's disease (49). Among the strategies to inhibit potassium channels, miRNA inhibitors are particularly appealing because they recognize specific mRNA sequence (50). Indeed, preclinical models are being developed by miRagen/Servier and other companies for the inhibition of miRNAs associated with cardiovascular disease (50,51).

The possibility of a new therapeutic strategy for myocardial infarction raises several issues. First, upregulation of *miR-223-3p* in human hearts undergoing myocardial infarction and *miR-223-3p* targeting of human *KCND2* would need to be established.

Next, *miR-223*, the same miRNA discovered to be associated with arrhythmia, has also been associated with cardioprotection. In one study, both arms of *miR-223*, 3p and 5p, were discovered to be induced after ischemia reperfusion in mouse hearts (52). But in this study, as opposed to Liu *et al.*, overexpressing a precursor miRNA

containing both strands (5' and 3') of *miR-223* was associated with better contractility and reduced necrosis after myocardial ischemia (52). Transgenic mice with a knockout of the *miR-223* locus exhibited aggravated ischemia-reperfusion-induced cardiac dysfunction and more cell death (52). This study identified two cell death receptors, *Tnfr1* and *Dr6*, as *miR-223* targets in mouse hearts (52). In another study, downregulation of *miR-223* occurred in the hearts of mice with severe sepsis (53). In this study, sepsis-induced mortality, inflammation and cardiac dysfunction were exacerbated in mice with knockout of *miR-223-3p*. Semaphorin 3A was identified as a candidate gene mediating this effect. These studies raise concerns that inhibition of *miR-223-3p* in hearts would have negative consequences.

Another issue is that Liu and colleagues focus on *Kcnd2*, which is well-established as a mediator of outward potassium current in rodent heart left ventricle apex cells. However, heart rates and action potential duration differ

between species. While the phases of a heart beat are similar in all mammals, human hearts beat ~60 times per minute while rodent hearts beat ~600 times each minute (11). Until recently, the prevailing paradigm was that larger mammals such as dogs and humans rely on *KCND3*-encoded channels, while rodents use both *Kcnd2* and *Kcnd3*-encoded channels and thereby achieve more rapid depolarization required for their fast heart beats (11,54,55). However, a recent discovery of a gain-of-function point mutation in *KCND2* in a human patient with sudden cardiac arrest suggests *KCND2*-encoded proteins may be important for proper action potentials in humans as well (54). Thus, additional studies will be needed to determine the importance of  $K_{v4.2}$  in human action potentials.

In addition, achieving the correct level of  $K_{v4.2}$  may be challenging. Adding inhibitors of *miR-223-3p* would be expected to increase  $K_{v4.2}$  and thus  $I_{to}$ . Established anti-arrhythmia drugs inhibit  $I_{to}$  (56), supporting the importance of  $I_{to}$  in establishing proper action potentials. However, inhibition of *miR-223-3p* could result in excess  $K_{v4.2}$ , elevated  $I_{to}$ , and arrhythmias, raising concern that miRNAs targeting  $K_{v4.2}$  may increase rather than reduce arrhythmia.

Other concerns relate to the characteristics of miRNAs. miRNAs tend to have modest effects on the target gene, off-target effects, and more pleiotropic effects than expected. For example, an anti-miR against the cardiac-specific miR-208a was unexpectedly discovered to prevent systemic phenotypes of obesity and metabolic syndrome (57). In addition, because miRNA and miRNA inhibitors are not expected to affect proteins that exist, but rather affect the levels and translation of mRNA transcripts that code for new protein, determining the pharmacokinetics of any proposed therapy will be important. Finally, developing anti-miRNAs that are not rapidly degraded and will accumulate to therapeutic levels in the heart will also be necessary (43).

In summary, proper regulation of the ion channels that control action potentials is critical for normal heart beat. Dysregulation of these channels can contribute to arrhythmia-induced mortality. miRNAs represent an emerging mechanism for regulating the expression levels of ion channel components, and are being developed as novel emerging therapeutic targets for heart disease.

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## Footnote

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

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