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MicroRNA Regulation of Cardiac Conduction and Arrhythmias

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Abstract

MicroRNAs are now recognized as important regulators of cardiovascular genes with critical roles in normal development and physiology, as well as disease development. MicroRNAs (miRNAs) are small non-coding RNAs approximately 22 nucleotides in length that regulate expression of target genes through sequence-specific hybridization to the 3' untranslated region of messenger RNAs and either block translation or direct degradation of their target messenger RNA. They have been shown to participate in cardiovascular disease pathogenesis including atherosclerosis, coronary artery disease, myocardial infarction, heart failure and cardiac arrhythmias. Broadly defined, cardiac arrhythmias are a variation from the normal heart rate or rhythm. Arrhythmias are common and result in significant morbidity and mortality. Ventricular arrhythmias constitute a major cause for cardiac death, particularly sudden cardiac death in the setting of myocardial infarction and heart failure. As advances in pharmacologic, device, and ablative therapy continue to evolve, the molecular insights into the basis of arrhythmia is growing with the ambition of providing additional therapeutic options. Electrical remodeling and structural remodeling are identified mechanisms underlying arrhythmia generation; however, published studies focusing on miRNAs and cardiac conduction are sparse. Recent studies have highlighted the role of microRNAs in cardiac rhythm through regulation of key ion channels, transporters, and cellular proteins in arrhythmogenic conditions. This article aims to review the studies linking miRNAs to cardiac excitability and other processes pertinent to arrhythmia.

Keywords

microRNAs; cardiovascular; arrhythmia; atrial fibrillation; conduction; ventricular fibrillation; ventricular tachycardia; electrical remodeling

Introduction

Cardiac arrhythmias have classically been divided into supraventricular or ventricular depending on the origin of the arrhythmic focus. Aside from location, arrhythmias are also classified as bradyarrhythmias, and tachyarrhythmias depending on the rate of the arrhythmic focus. These arrhythmias can be the result of a variety of structural changes or ion channel dysfunction. Regardless of the mechanism, the manifestation of these arrhythmias continues to be a major contributor to human morbidity and mortality. Unstable

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ventricular arrhythmias secondary to channelopathies significantly increase the risk of sudden cardiac death (1). Atrial fibrillation (AF) is the most common arrhythmia, with an estimated prevalence of AF is 0.4% to 1% in the general population, increasing with age to approximately 8% in those >80 years (2). Heart failure is a syndrome caused by significant impairments in cardiac function. Sudden death, generally due to arrhythmic causes, is responsible for a significant proportion of deaths among patients with heart failure. Patients with heart failure experience a variety of other significant rhythm abnormalities beyond ventricular tachyarrhythmias. Atrial arrhythmias, particularly atrial fibrillation, are very common in heart failure and can contribute substantially to morbidity and mortality (3). These arrhythmias are a manifestation of diverse abnormalities (electrical, structural, metabolic, neurohormonal, or molecular alterations) in multiple pathological conditions (heart failure, hypertension, diabetes mellitus, hyperthyroidism, aging, etc). Dysregulation or functional impairment of ion channels, transporters, intracellular Ca^{2+} -handling proteins, and other relevant proteins can create structural and metabolic substrates, which have the potential to render electrical disturbances predisposing to conduction abnormalities and arrhythmias (4). Although published studies focusing on miRNAs and cardiac conduction are still sparse, available results have demonstrated the importance of miRNAs in arrhythmia.

Basics of MicroRNA Biology

MicroRNAs are ~22 nucleotide single-stranded RNAs that inhibit the expression of specific mRNA targets through Watson–Crick base pairing between the miRNA and sequences typically located in the 3' untranslated regions (UTRs) of mRNAs. Although they do not encode proteins, miRNAs are transcribed by RNA polymerase II as part of longer molecules called pri-miRs (approximately 2 kb in length). Pri-miRs are cleaved in the nucleus by the RNase III-type enzyme Drosha to yield a pre-miR, which has a short stem-loop structure consisting of approximately 70 nucleotides (5). This premiR is exported to the cytoplasm, via exportin-5, and further processed by the ribonuclease Dicer to form a double-stranded RNA molecule.

The “passenger strand” of the miRNA duplex is eliminated to leave a single-stranded miRNA, which interact with Argonaute to form the RNA-induced silencing complex (RISC) and then guide the RISC to their target mRNAs (6). Target recognition is dependent on the specific interaction between the miR and miR recognition elements (seeds) located in the 3'UTR of target messenger RNAs. In order for a miRNA to elicit functional consequences, the nucleotides at the 2-8 position at the 5'-end must have exact complementarity to the target mRNA, the so-called ‘seed’ region, and partial complementarity to the rest of its sequence. Finally, translational inhibition or, in some cases, mRNA degradation, contributes to the downregulation of the corresponding target protein (7, 8).

The human genome is estimated to encode more than 1,000 miRNAs, which are either transcribed as stand alone transcripts, often encoding several miRNAs, or generated by the processing of introns of protein-coding genes. MicroRNAs typically exert inhibitory effects on several mRNAs, which often encode proteins that govern the same biological process or several components of a molecular pathway (9, 10). The multiplicity of miRNA targets may also promote combinatorial regulation by miRNAs that individually target various mRNAs whose protein products contribute to one particular regulatory axis. In this model, a biological response would be expected only after co-expression of several miRNAs that cooperatively target various components of a functional network or are all required to sufficiently repress a single target (11, 12). Clearly, miRNA biology is a complex and highly orchestrated mode of gene regulation, potentially impinging on nearly all biological processes in mammals and having particularly important roles in disease states (13). Cardiac

specific miRNAs have been shown to regulate a number of critical cellular functions in the developing and normal adult heart (14). Aberrant expression of these miRNAs have also been shown to have dramatic impacts in the development of cardiac hypertrophy, fibrosis, and angiogenesis (15). Not surprisingly, miRNAs are being increasingly recognized as important regulators of cardiac conduction and the development of arrhythmias.

Therapeutic Applications of MicroRNAs

The importance of miRNAs in the maintenance of cardiac function and the development of dysfunction creates opportunities for therapeutic applications of miRNA biology in heart disease. In principle, miRNA mimics can be used to elevate the expression of beneficial miRNAs in disease settings, whereas miRNA inhibitors can be delivered to block the activity of miRNAs that drive disease progression. Thus, identification and validation of miRNA targets is especially important to the development of miRNA-based therapeutics.

Currently, the most widely used approach to regulate miRNA levels *in vivo* is by using antisense oligonucleotides harboring the full or partial complementary reverse sequence of a mature miRNA that can reduce the levels of a microRNA. Single-stranded oligonucleotides have been shown to be effective in inactivating miRNAs *in vivo* (16, 17). A novel class of chemically engineered oligonucleotides termed “antagomirs” have been shown to effectively silence miRNA in mice (18). Antagomirs are cholesterol-conjugated single-stranded RNA molecules 21–23 nucleotides in length and complementary to the mature target miRNA. Interestingly, antagomirs are very stable *in vivo* and, after one intravenous injection, have the ability to silence target miRNA in the liver, lung, intestine, heart, skin, bone marrow for more than a week.

In addition to modulating miRNA levels by using chemically modified antisense oligonucleotides, modulation of miRNAs could potentially also be established by preventing the miRNA from doing its job by “soaking up” the miRNA. This technique has been named miRNA erasers, sponges, or decoys (19). A vector expressing miRNA target sites can be used to scavenge a miRNA and prevent it from regulating its natural targets. The vectors can harbor multiple miRNA binding sites downstream of a reporter such as green fluorescent protein or luciferase, expressed from a strong promoter, whereby the reporter can indicate whether the miRNA is effectively scavenged away by the decoy. Because the interaction between microRNA and target is largely dependent on base-pairing in the seed region (positions 2–8 of the microRNA), a decoy target should interact with all members of a microRNA family. In so doing, it provides a way to inhibit functional classes of microRNAs as opposed to single microRNA sequences (20). As demonstrated by Care *et al*, an adenoviral system can be used to deliver to miRNA decoys in cardiac tissue to suppress specific miRNA function (21). As such, current decoy technology can also be used to validate target predictions and assay microRNA loss-of-function phenotypes.

Basics of Cardiac conduction

Cardiomyocytes require strict coordination of excitation, impulse propagation, and repolarization in order to maintain a normal rhythm in sync with the mechanical actions of the heart. Automaticity is a measure of the ability for cells to generate spontaneous action potentials. Conduction refers to the propagation of excitation within a cell and between cells and the velocity by which this occurs is determined by the rate of membrane depolarization and the intercellular conductance. Repolarization refers to the return of membrane resting potential in order to generate another depolarization and the rate of membrane repolarization determines the length of action potential duration (APD) and effective refractory period. This directly impacts the ability to generate the next excitation in a cardiac cell.

These electrical activities of the heart are orchestrated by multiple categories of ion channels. Four major categories of ion channels exist in cardiac cells, including Na^+ channels, Ca^{2+} channels, K^+ channels, and Cl_2 channels. The sum of the actions orchestrated by the variety of ion channels and transporters result in a characteristic action potential (AP) (Figure 1). A cardiac AP can be divided into 4 phases beginning with initial rapid depolarization (phase 0) and ending with the return to the resting state (phase 4). A typical cardiac AP contains a long plateau phase (phase 2) distinct from APs in noncardiac cells, but AP from different regions of the heart possesses different morphologies (22, 23). Intricate interplays of all these ion channels maintain the normal heart rhythm. Channelopathies, or dysfunction of the ion channels, which may result from genetic alterations in ion channel genes or aberrant expression of these genes, can render electrical disturbances predisposing to cardiac arrhythmias (4).

Transmembrane proteins control the movement of ions across the cytoplasmic membrane of cardiomyocytes such as sodium (Na^+) channels, which determine the rate of membrane depolarization. Pacemaker channels carry the non-selective cation currents and are critical in generating sinus rhythm and ectopic heart beats as well. Gap junction proteins such as connexins are important for inter-cell conductance of excitation. Calcium channels (L-type Ca^{2+}) account for excitation–contraction coupling and contribute to pacemaker activities in the sinoatrial and atrioventricular nodal cells. Potassium (K^+) channels govern the membrane potential and the rate of membrane repolarization (22) (Figure 2).

Regulation of cardiac automaticity

Cardiac pacemaker activity sets the rhythm and rate of cardiac chamber contraction. Automaticity is the property of cardiac cells to generate spontaneous action potentials. This spontaneous activity is the result of diastolic depolarization caused by a net inward current during phase 4 of the action potential, which gradually brings the membrane potential to threshold. Pacemaker channels carry the hyperpolarization-activated pacemaker current, I_f , which is a mixed cationic current carried by Na^+ and K^+ . Almost all spontaneously active cells coming from heart rhythm centers express f-channels (24).

The sinoatrial (SA) node normally displays the highest intrinsic rate. Cells of the SA node express abundant hyperpolarization-activated cation channel HCN genes and T-type Ca^{2+} channel as well. HCN4 is the predominant f-channel isoform expressed in the SA node and AV node. The HCN1 and HCN2 isoforms are also expressed in these rhythmogenic centers and therefore represent important gene for rhythm generation (25). When the membrane potential reaches certain threshold, T-type Ca^{2+} channels activate to elicit a spontaneous excitation (24, 25).

Regulation of Cardiac Depolarization

At rest, cardiomyocytes transmembrane potential is -90 mV. Upon activation, depolarization occurs by rapid entry of Na^+ through Na^+ channels to generate a large inward depolarizing current (I_{Na}). Ca^{2+} entry into the cell through the L-type Ca^{2+} current (I_{CaL}) also contributes to phase 0 depolarization (Figure 1). Voltage-gated Na^+ channels are activated by depolarization and are responsible for the rapid upstroke of the cardiac AP. Once threshold depolarization for an AP is reached, the opening of Na^+ channels leads to further depolarization, which in turn activates additional Na^+ channels and depolarization. The result is a very rapid depolarization toward the electrochemical potential of Na^+ . Depolarization stops, in part because Na^+ channels inactivate, the amplitude of I_{Na} decreases and switches off Na^+ conductance (26).

SCN5A encodes the α -subunit of the voltage-gated cardiac sodium channel (designated $\text{Na}_v1.5$), conducting the cardiac sodium current I_{Na} . $\text{Na}_v1.5$ is an integral membrane protein involved in the initiation and conduction of action potentials. Mutations in SCN5A, have been associated with a variety of arrhythmic disorders, including long QT, Brugada, and sick sinus syndromes as well as progressive cardiac conduction defect and atrial standstill (27). Moreover, alterations in the $\text{Na}_v1.5$ expression level and/or sodium current density have been frequently seen in acquired cardiac disorders, such as heart failure (28).

The $\text{Ca}_v1.2$ gene (CACNA1C) and $\text{Ca}_v\beta1$ (CACNB1) encode the cardiac L-type Ca^{2+} channel (I_{CaL}) $\alpha1c$ - and $\beta1$ subunits, in ventricular myocytes (29). Voltage-gated Ca_v1 channels ($\text{Ca}_v1.2$ and $\text{Ca}_v1.3$) mediate L-type Ca currents that play distinct roles in different cardiac cell-types. Expressed throughout the heart, $\text{Ca}_v1.2$ regulates action potential duration and excitation-contraction coupling in the ventricle, thus, changes in $\text{Ca}_v1.2$ channel function have important implications for excitability (30). $\text{Ca}_v1.3$ is largely absent from the ventricles but is highly expressed in atria, atrioventricular node, and sinoatrial node (31, 32). Ca^{2+} enters cardiomyocytes via a variety of Ca^{2+} conductances, of which that carrying L-type Ca^{2+} current is the most prominent. The membrane density of I_{CaL} channels has been shown to be reduced by cardiac failure (33). Increased Ca^{2+} influx via L-type Ca^{2+} channels has also been implicated in the development of hypertrophy and pathological remodeling of the heart with select stress stimuli. Interestingly, it has been shown that genetically-based reduction in L-type Ca^{2+} current in mice induced spontaneous cardiac hypertrophy or dramatically enhance hypertrophy after pressure overload stimulation (34).

An example of a human pathological condition associated with aberrant $\text{Ca}_v1.2$ channel gating is Timothy syndrome, a rare, autosomal dominant, multiorgan disorder caused by *de novo* gain-of-function missense mutations in exon 8 or an alternatively spliced exon 8A of $\text{Ca}_v1.2$. Timothy syndrome is characterized by a prolongation of the electrocardiogram QT interval, which is why it is also known as long QT syndrome 8 (LQT8). Timothy syndrome patients commonly suffer sudden cardiac death resulting from lethal cardiac arrhythmias. Accordingly, functional studies revealed that the mutated Ca^{2+} channel presented a “gain of function” state by impairing open-state voltage-dependent inactivation (35). This may lead to sustained Ca^{2+} influx, AP prolongation, and Ca^{2+} overload, which promotes early and delayed afterdepolarizations (36, 37).

A potential role for microRNA regulation of cardiac depolarization was identified when Lu *et al* utilized computational prediction algorithms to identify CACNA1C and CACNB1 as potential targets for miR-328. Subsequent microRNA microarray analysis and real-time reverse transcriptase–polymerase chain reaction demonstrated an approximate four-fold elevation of miR-328 level in left atrial samples from dogs with AF established by right atrial tachypacing for 8 weeks, and from human atrial samples from AF patients with rheumatic heart disease. Overexpression of miR-328 through adenovirus infection in canine atrium and transgenic approach in mice recapitulated the phenotypes of AF, exemplified by enhanced AF vulnerability, diminished L-type Ca^{2+} current, and shortened atrial action potential duration. Normalization of miR-328 level with antagomiR reversed the conditions, and genetic knockdown of endogenous miR-328 dampened AF vulnerability. CACNA1C and CACNB1 were established as the cognate target genes for miR-328 by western blot and luciferase activity assay showing the reciprocal relationship between the levels of miR-328 and I_{CaL} channel protein subunits (38).

The role of microRNA regulation of $\text{Ca}_v1.2$ in cardiac arrhythmias is highlighted in another genetic disorder, myotonic dystrophy, which is the most common muscular dystrophy in adults, is characterized by multiple symptoms including muscle weakness, myotonia, insulin resistance, neuropsychiatric impairment, and cardiac defects. Cardiac involvement in

affected individuals can include degeneration of the conduction system leading to fatal atrioventricular blocks. Furthermore, individuals with myotonic dystrophy are also characterized by atrial and ventricular tachyarrhythmias (39). Myotonic dystrophy is a RNA gain-of-function disease caused by expanded CUG or CCUG repeats, which sequester the RNA binding protein muscle blind-like protein 1 (MBNL1). In a study by Rau *et al*, the authors demonstrated an important role for MBNL1 as a cytoplasmic regulator of the biogenesis of pre-miR-1 resulting in pre-miR-1 uridylation thus blocking pre-miR-1 processing from Dicer. Consequently, loss of mature miR-1 increases levels of its targets. Analysis with miRanda predicted various targets of miR-1, including the cardiac L-type calcium channel gene CACNA1C (CAV1.2). The authors confirmed CACNA1C regulation by miR-1 through luciferase reporter assays. Expression of CACNA1C protein was upregulated in myotonic dystrophy heart samples compared to control, whereas CACNA1C mRNA levels were not altered.

Regulation of cardiac repolarization

After depolarization, the membrane potential begins to return toward the resting state. The inward flowing currents such as Na^+ and L-type Ca^{2+} currents tend to depolarize the membrane and delay repolarization to prolong APD, whereas the outward going K^+ currents, mainly including I_{K1} , I_{to} , I_{Kr} , and I_{Ks} , tend to repolarize the membrane to shorten APD (Figure 1). Cardiac repolarization is determined by an intricate interplay and a delicate balance between inward and outward ion currents. Membrane depolarization is immediately followed by a brief rapid repolarization phase (phase 1) due to K^+ efflux through a rapidly activating and inactivating transient outward K^+ current (I_{to}). During the characteristic plateau phase (phase 2), there is a balance between inward currents I_{CaL} and I_{Na} and outward K^+ currents. There are progressive time-dependent sequential activations of delayed rectifier K^+ currents, first the rapid delayed rectifier K^+ current (I_{Kr}) and then the slow delayed rectifier K^+ current (I_{Ks}). I_{Kr} acts to terminate the AP with an appropriate delay by producing rapid phase 3 repolarization, and I_{Ks} serves as a repolarization reserve to prevent excessive slowing of repolarization (40). Finally, inward rectifier K^+ current (I_{K1}) aids to complete the terminal phase of repolarization.

The rate of repolarization importantly affects the likelihood of developing arrhythmias. Slowly activating delayed rectifier K^+ current (I_{Ks}) along with its underlying channel proteins KCNQ1 (pore-forming α -subunit) and KCNE1 (auxiliary β -subunit) importantly affect cardiac action potential duration and arrhythmogenesis through two mechanisms. First, I_{Ks} acts as a powerful repolarization reserve or safety factor to restrict excessive cardiac action potential duration and QT prolongation caused by other factors (41, 42). Second, a varying density of repolarizing K^+ currents within the ventricular myocardium creates a spatial heterogeneity of cardiac repolarization (43). The distribution of I_{Ks} in the heart follows important spatial patterns across the myocardium from epicardium to endocardium, interventricular from RV to LV, across the interventricular septum, and an apex to base difference (44, 45). These intrinsic patterns of distribution are important in maintaining the sequential excitation of cardiac muscles. Disruption of the patterns and/or exaggeration of the regional heterogeneity can create substrates for arrhythmogenesis.

One of the earliest evidence for miRNA regulation of cardiac repolarization came from Zhou *et al* in 2007 with a targeted deletion of miR-1-2 in mice. This resulted in 50% lethality that was largely attributable to ventricular septal defects. However, approximately half of the surviving mutant animals died suddenly and revealed electrophysiological defects manifested by conduction block. Surface electrocardiography in mutant mice demonstrated a reduced average heart rate, accelerated atrioventricular conduction (reflected by the shortened PR interval), and slowed ventricular conduction (manifested by the widened QRS

complex) (46). The authors identified *Irx5* as a target for miR-1-2. *Irx5* belongs to the Iroquois family of homeodomain-containing transcription factors that regulate cardiac repolarization by repressing transcription of *KCND2*. *KCND2* encodes a K⁺ channel subunit (Kv4.2) responsible for transient outward K⁺ current (I_{to}) that is the major determinant for the transmural repolarization gradient in the ventricular wall (47). The increase in *Irx5* protein levels in miR-1-2 mutants corresponded with a decrease in *KCND2* expression. The authors concluded that the loss of *Irx5* disrupted ventricular repolarization with a predisposition to arrhythmias (46).

Additional evidence supporting a role for miR-1 in cardiac repolarization and arrhythmogenesis came from a rat model of myocardial infarction (MI) induced by occlusion of the left anterior descending coronary artery. An increase (approximately 2.6-fold) in miR-1 expression in the ischemic hearts was accompanied by exacerbated arrhythmogenesis. Elimination of miR-1 by an antisense inhibitor in the infarcted rat hearts relieved arrhythmogenesis. Overexpression of miR-1 resulted in slowed cardiac conduction, as indicated by the widening of QRS complex and measurement of cardiac velocity, and depolarizes the cytoplasmic membrane, which is likely the cellular mechanism for the arrhythmogenic potential of miR-1. MicroRNA-1 was found to target *KCNJ2*, which encodes the K⁺ channel subunit Kir2.1 (48). Kir2.1, which carries inward rectifier K⁺ current, is critical for setting and maintaining membrane potential. The resultant conduction slowing may be one mechanism for ventricular arrhythmias in the setting ischemia.

In contrast to ventricular arrhythmias, Girmatsion *et al* studied changes in miR-1 and Kir2 subunit expression in relation to I_{K1} alterations in the left atrial tissue samples from patients with persistent atrial fibrillation. I_{K1} density was increased in LA cells from patients with atrial fibrillation. There was a corresponding increase in Kir2.1 protein expression, but no change in other Kir protein levels. Expression of inhibitory miR-1 was reduced by approximately 86% in tissue samples of AF patients. *Ex vivo* tachy-stimulation of human atrial slices up-regulated Kir2.1 and down-regulated miR-1. The authors concluded that miR-1 levels are greatly reduced in human AF, possibly contributing to up-regulation of Kir2.1 subunits, thus leading to increased I_{K1} (49).

These studies together indicate that aberrant upregulation of miR-1 in ischemic myocardium contributes to arrhythmogenesis. The finding that an anti-miR-1 oligomer suppressed ischemic arrhythmias has implications as a potential therapeutic. An excessive decrease (as seen in the miR-1-2 knockout) or increase in miR-1 level can induce arrhythmia, which supports a central role for miR-1 for regulation of cardiac electrophysiology in pathological and normal conditions. Interestingly, in the setting of atrial fibrillation levels of miR-1 appear to be decreased in contrast to the increased miR-1 levels seen in ventricular arrhythmias (Table 1). To date, this differential regulation of miR-1 in atrial versus ventricular tissue has not been explored. Taken together, miR-1 levels within the myocardium must be kept within a proper concentration range to maintain normal cardiac conduction. Further study is warranted as this understanding could have profound implications for therapeutic applications.

Like miR-1, miR-133 is a muscle-specific miRNA preferentially expressed in cardiac and skeletal muscle. They represent the most abundant miRNAs expressed in the heart (50). In addition to miR-1, miR-133 has also been documented to be a miRNA regulator of cardiac ion channels. In a study by Matkovich *et al*, the authors described that increased miR-133a levels prolonged QT intervals in surface electrocardiographic recordings in mice and action potential duration in isolated ventricular myocytes, with a decrease in the fast component of the transient outward K⁺ current, $I_{to,f}$, at baseline (51). Interestingly, decreased mRNA and protein levels of Kv4-encoded $I_{to,f}$ (*Kcnip2*) were observed; however, *Kcnip2* has not been

previously reported as a target of miR-133a and is not suggested to be an miR-133a target. The authors concluded that regulation of Kcnp2 transcript levels in miR-133a transgenic hearts occurred by an indirect mechanism not yet identified.

Arsenic trioxide (As₂O₃) has been shown to induce abnormal cardiac QT prolongation (52, 53). To study this, a guinea pig model of As₂O₃-induced QT prolongation was established by intravenous injection with As₂O₃. The QT interval and QRS complex were significantly prolonged in a dose-dependent fashion after 7-day administration of As₂O₃. Interestingly, As₂O₃ induced a significant up-regulation of the muscle-specific miR-1 and miR-133. As₂O₃ depressed the protein levels of ether-a-go-go related gene (ERG) and Kir2.1, the K⁺ channel subunits responsible for delayed rectifier K⁺ current I_{Kr} and inward rectifier K⁺ current I_{K1}, respectively. *In vivo* transfer of miR-133 by direct intramuscular injection prolonged the QT interval and decreased the levels of ERG protein and I_{Kr} in guinea pig hearts. Similarly, forced expression of miR-1 increased the QT interval and QRS duration with a down-regulation of Kir2.1 protein and I_{K1}. Delivery of antisense inhibitors to knockdown miR-1 and miR-133 abolished the cardiac electrical disorders caused by As₂O₃ (54).

MicroRNA-212 has been found to be upregulated in both animal models of heart failure as well as in human heart failure (55, 56). Bioinformatic analysis revealed a potential target in the 3' UTR of Kir2.1 (KCNJ2) for miR-212. As mentioned previously, Kir2.1 carries an inward rectifier K⁺ current that is critical for setting and maintaining membrane potential and also a target for miR-1. Goldoni *et al* recently reported the targeting of Kir2.1 by miR-212 with functional down-regulation of inward rectifier K⁺ channels. Transfection of HeLa cells with a miR-212 overexpression construct reduced inward rectifier K⁺ current density, as demonstrated by whole-cell patch-clamp recordings, most likely due to reduced expression of Kir2.1 (57). The ability for miR-212 to result in cardiac arrhythmias was not studied in an animal model. However, miR-212 was found to be slightly up-regulated in patients with mitral stenosis and atrial fibrillation compared to those in sinus rhythm with mitral stenosis (58).

Regulation of Intercellular Conduction

Electrical activation of the heart requires transfer of current from one discrete cardiac myocyte to another, a process that occurs at gap junctions. Gap junction channels form the basis of intercellular communication in the heart. In the working myocardium, connexin43 (Cx43) is most abundantly found, whereas connexin40 (Cx40) is expressed in the atria and in the conduction system. Remodeling of gap junction organization and connexin expression is a common feature of human heart disease in which there is an arrhythmic tendency (61, 62). This remodeling may take the form of disturbances in the distribution of gap junctions and/or quantitative alterations in connexin expression, notably reduced ventricular connexin43 levels. In advanced stages of heart disease, connexin expression and intercellular coupling are diminished, and gap junction channels become redistributed. These changes have been strongly implicated in the pathogenesis of lethal ventricular arrhythmias (63).

Several studies have highlighted the role of miR-1 in the regulation of Connexin43. In a mouse model of viral myocarditis, the expression levels of miR-1 and its target Connexin43 were measured by real-time PCR and western blotting, respectively. The miR-1 expression levels were significantly increased in cardiac myocytes from the viral myocarditis cohort compared to control. In cultured myocardial cells, overexpression of miR-1 via transfection accompanied resulted in a reduced levels of Cx43 protein with a non-significant drop in

Cx43 mRNA levels (59). In the rat model of myocardial infarction discussed previously, miR-1 was found to regulate GJA1 in addition to KCNJ2 (48).

The multiplicity of miRNA targets allows miRNAs to target various mRNAs whose protein products contribute to several components of a pathway. In the study of myotonic dystrophy by Rao *et al*, the disrupted processing of pre-miR-1 resulted in the reduction of mature miR-1. In addition to Cav1.2, the expression of connexin43 was also upregulated in heart samples of affected individuals. These data demonstrated altered Dicer processing of miR-1 and the subsequent misregulation of miR-1 targets, CACNA1C and GJA1, likely contribute to the cardiac arrhythmias observed in affected persons.

Callis *et al* reported a role for miR-208a in regulation of connexin40 (64). MicroRNA-208a is encoded by an intron of the α -cardiac muscle myosin heavy chain gene (Myh6) and was initially found to regulate stress-dependent cardiac growth (65). The authors found miR-208a was required to maintain expression of cardiac transcription factors known to be important for development of the conduction system. In miR-208a knockout mice, approximately 80% developed an ECG consistent with atrial fibrillation. In the mice without atrial fibrillation, the PR intervals were significantly prolonged compared with wildtype animals. Overexpression of miR-208a was sufficient to induce prolonged PR intervals and occasional second-degree AV blocks suggesting that miR-208a regulates cardiac conduction system components. These results demonstrate the pathological consequences of perturbing the expression of a single miRNA in the heart and establish miR-208a as a regulator of hypertrophic growth and the cardiac conduction system (64).

Previous studies suggested that abnormal connexin protein expression might account for the cardiac conduction defects seen in the mouse models with altered miR-208a levels (66, 67). The authors evaluated the expression of Cx43 and Cx40 in hearts from 4-month-old miR-208a transgenic and miR-208a^{-/-} mice. Though Cx40 mRNA transcript levels were not affected in miR-208a transgenic hearts, they were markedly decreased in miR-208a^{-/-} hearts compared with wild-type, indicating that miR-208a is required for Cx40 expression. Western blot and immunohistological analyses of hearts from miR-208a^{-/-} mice showed decreased Cx40 protein levels compared with wild-type hearts. Consistent with the miR-208a^{-/-} phenotype, mice lacking Cx40 suffer cardiac conduction abnormalities, including first-degree AV block (66).

In the realm of therapeutics, inhibition of miR-208a has been investigated as a strategy to repress β MHC expression and remove some of the maladaptive features of hypertrophy. Given the findings of conduction abnormalities in the miR-208a^{-/-} mouse model, arrhythmogenesis induced by perturbing miR-208a levels warrants caution. To verify whether anti-miR-208a treatment resulted in cardiac conductance effects, Montgomery *et al* analyzed ECGs in both wild-type mice and diseased rats. Interestingly, both species showed proper cardiac electrophysiology after anti-miR-208a treatment for an extended period of time (68).

Calcium cycling and arrhythmogenesis

Calcium enters cardiomyocytes via a variety of mechanisms, most prominent of which is the L-type Ca²⁺ current (I_{CaL}). In a process commonly called Ca²⁺-induced Ca²⁺ release, voltage-dependent trans-sarcolemmal entry of Ca²⁺ triggers the release of additional Ca²⁺ from sarcoplasmic reticulum Ca²⁺ stores through closely coupled sarcoplasmic reticulum Ca²⁺ release channels. Ca²⁺ release channel function is regulated by key intracellular phosphorylating enzymes such as protein kinase A and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), as well as a variety of phosphatases, resulting in dephosphorylation. Sarcoplasmic reticulum Ca²⁺ content is dependant on several mechanisms such as cellular

Ca^{2+} entry (particularly via I_{CaL}), calcium removal from the cell (particularly via the sarcolemmal Ca^{2+} pump and forward-mode $\text{Na}^{+}\text{-Ca}^{2+}$ exchange) and Ca^{2+} pumping into the sarcoplasmic reticulum by the $\text{Ca}^{2+}\text{-ATPase}$ Ca^{2+} pump (the principal cardiac form of which is SERCA2a).

Heart failure has major effects on cellular Ca^{2+} handling, which has important implications not only for cardiac function but arrhythmogenesis as well. Triggered activity related to delayed afterdepolarizations (DAD) is caused by spontaneous diastolic Ca^{2+} release and is an important mechanism underlying ventricular tachyarrhythmias (69). Delayed afterdepolarizations occur in congestive heart failure despite reduced cell Ca^{2+} stores because of a number of features of cardiac failure-induced ion transport remodeling (70-72). First, hyperphosphorylated Ca^{2+} release channels are prone to spontaneous diastolic Ca^{2+} release. Second, for any given level of Ca^{2+} release, enhanced $\text{Na}^{+}\text{-Ca}^{2+}$ exchange function increases the depolarizing current resulting from electrogenic Ca^{2+} extrusion, with three Na^{+} transported into the cell for every Ca^{2+} ion transported out. Lastly, inward rectifier potassium current downregulation by elevated diastolic Ca^{2+} in heart failure might also be relevant for arrhythmia generation (73).

In hearts isolated from canines with chronic heart failure, qRT-PCR revealed that the levels of miR-1 and miR-133 were significantly increased in cardiomyocytes compared with controls. Western blot analyses demonstrated that expression levels of the protein phosphatase 2A (PP2A) catalytic and regulatory subunits, putative targets of miR-133 and miR-1, were decreased in cardiomyocytes with chronic heart failure. PP2A catalytic subunit mRNAs were validated as targets of miR-133 by luciferase reporter assays. Pharmacological inhibition of phosphatase activity increased the frequency of diastolic Ca^{2+} waves and afterdepolarizations in control myocytes. The decreased PP2A activity observed in heart failure was accompanied by enhanced Ca^{2+} /calmodulin-dependent protein kinase (CaMKII)-mediated phosphorylation of the ryanodine receptor (RyR2) at sites Serine-2814 and Serine-2030 and increased frequency of diastolic Ca^{2+} waves and afterdepolarizations in heart failure myocytes compared with controls. These findings suggest that altered levels of major muscle-specific miRNAs contribute to abnormal RyR2 function in chronic heart failure by depressing phosphatase activity localized to the channel, which in turn, leads to the excessive phosphorylation of RyR2s, abnormal Ca^{2+} cycling, and increased propensity to arrhythmogenesis (74).

Terentyev *et al* investigated the effects of increased expression of miR-1 on excitation-contraction coupling and Ca^{2+} cycling in rat ventricular myocytes. Adenoviral-mediated overexpression of miR-1 in myocytes resulted in a marked increase in the amplitude of the inward Ca^{2+} current, flattening of Ca^{2+} transients voltage dependence, and enhanced frequency of spontaneous Ca^{2+} sparks while reducing the sarcoplasmic reticulum Ca^{2+} content as compared with control. In the presence of isoproterenol, miR-1-overexpressing cardiomyocytes exhibited spontaneous arrhythmogenic oscillations of intracellular Ca^{2+} events. The effects of miR-1 were completely reversed by the Ca^{2+} /calmodulin-dependent protein kinase (CaMKII) inhibitor KN93. Although phosphorylation of phospholamban was not altered, miR-1 overexpression increased phosphorylation of the ryanodine receptor (RyR2) at Serine2814 (a target of CaMKII) but not at Serine2808 (a target for protein kinase A). Overexpression of miR-1 was accompanied by a selective decrease in expression of the protein phosphatase PP2A regulatory subunit B56 α involved in PP2A targeting to specialized subcellular domains. These data demonstrated that miR-1 enhances cardiac excitation-contraction coupling by selectively increasing phosphorylation of the L-type and RyR2 channels via disrupting localization of PP2A activity to these channels (60).

MicroRNA misregulation in models of atrial fibrillation

Atrial fibrillation is the most common sustained arrhythmia associated with substantial cardiovascular morbidity and mortality. As already discussed, target genes of miRNAs have the potential to influence AF through the effects on conduction properties. Structural remodeling, either through atrial dilatation or fibrosis also impacts the development of atrial arrhythmias. AF maintenance is importantly related to changes in atrial electrical and structural properties. Electrical remodeling occurs early during the course of AF and leads to characteristic changes in action potential shape and duration, whereas structural alterations develop more slowly. Atrial fibrillation, as a complex electrical phenotype involving complex factors beyond electrophysiology, can occur in a variety of pathological settings (63). Atrial fibrillation of different types has distinct underlying mechanisms, and different miRNAs may be involved in different types of atrial fibrillation (Table 2). Cooley *et al* examined miRNA expression profiles in right and left atrial appendage tissue from valvular heart disease patients. There was no detectable effect of chronic AF on miRNA expression in left atrial tissue, but miRNA expression in the right atrium was strongly influenced by AF, with forty-seven miRNAs showing differential expression between the AF and control sinus rhythm groups. Valvular heart disease induced different changes in miRNA expression in the left atrium compared with the right atrium. Fifty-three miRNAs were altered by the presence of valve disease in left atrium, compared with five in right atrial tissue.

In a study of patients with mitral stenosis and sinus rhythm or AF, miRNA arrays revealed that 136 miRNAs were differentially expressed in mitral stenosis patients with AF and 96 miRNAs were dysregulated in mitral stenosis patients without AF, compared with healthy controls. More importantly, 28 miRNAs were expressed differently in the MS patients with AF compared with the MS patients without AF (58). Taking these two studies together, the presence of valvular heart disease and atrial fibrillation influences miRNA expression patterns in left atrium and right atrium (75). In addition, the differential miRNA expression between left and right atria in valve disease not only highlights the differences from one cardiac chamber to the next, but also the conditions in which the arrhythmia occurs.

Structural remodeling of the atrium is important for the pathogenesis of atrial fibrillation but the underlying signal transduction is incompletely understood. Adam *et al* studied the role of microRNA-21 (miR-21) and its downstream target Sprouty 1 (Spry1) during atrial fibrillation and development of atrial fibrosis. Left atria from patients with AF showed a 2.5-fold increased expression of miR-21 compared to matched left atrium of patients in sinus rhythm. Increased miR-21 expression correlated positively with atrial collagen content and was associated with a reduced protein expression of Spry1 and increased expression of connective tissue growth factor (CTGF), lysyl oxidase and Rac1-GTPase. Transgenic mice with cardiac overexpression of Rac1, which develop spontaneous AF and atrial fibrosis with increasing age, showed upregulation of miR-21 expression associated with reduced Spry1 expression. Inhibition of miR-21 by antagomir-21 prevented fibrosis of the atrial myocardium post-myocardial infarction (76).

As discussed previously, the development of interstitial fibrosis affects chamber geometry and the mechanical performance of the heart and thereby increases the likelihood of cardiac arrhythmias. Several studies have shown that nicotine abuse is associated with the occurrence of cardiac arrhythmias and that nicotine accelerates atrial collagen accumulation (77-79). In a study by Shan *et al*, the authors utilized a canine model of AF by nicotine administration and rapid pacing. Administration of nicotine for 30 days increased AF vulnerability by approximately 8- to 15-fold in dogs. The role of microRNAs (miRNAs) on the expression and regulation of transforming growth factor-beta1 (TGF- β 1), TGF-beta receptor type II (TGF- β RII), and collagen production was evaluated *in vivo* and *in vitro*. For

in vitro studies, atrial fibroblasts isolated from healthy dogs were treated with nicotine. Nicotine stimulated remarkable collagen production and atrial fibrosis both *in vitro* in cultured canine atrial fibroblasts and *in vivo* in atrial tissues. Nicotine produced significant upregulation of expression of TGF- β 1 and TGF- β RII at the protein level, and a 60-70% decrease in the levels of miRNAs miR-133 and miR-590. Luciferase reporter assays in HEK293 cells confirmed the ability of miR-133 to repress TGF- β 1 expression and of miR-590 to repress TGF- β RII expression. To test the notion that miR-133 or miR-590 could cause suppression of collagen production in atrial fibroblasts, the authors transfected synthetic miR-133 or miR-590 into cultured canine atrial fibroblasts. They found that transfection of miR-133 or miR-590 into these fibroblasts decreased TGF- β 1 and TGF- β RII levels and collagen content. These effects were abolished by the antisense oligonucleotides against miR-133 or miR-590. The authors concluded that the profibrotic response to nicotine in canine atrium was critically dependent upon downregulation of miR-133 and miR-590 (80). However, how nicotine results in this downregulation of these miRNAs remains unclear.

Conclusions

The available data from experimental studies demonstrate that miRNAs regulate numerous properties of cardiac excitability including conduction, repolarization, automaticity, Ca²⁺ handling, spatial heterogeneity, and apoptosis and fibrosis. MicroRNAs can also impose their regulatory actions on cardiac excitability indirectly through targeting non-ion channel genes, such as transcription factors, that in turn regulate expression of ion channel genes (Table 1). This mode of action reinforces the complex nature and fine-tuning regulation of miRNA actions. It must be noted that we have just begun to appreciate the role of miRNAs in controlling cardiac excitability and our understanding is still rather preliminary.

As the study of microRNAs begins to move into the arena of therapeutics, a thorough understanding of the broad functions of any particular miRNA is necessary to avoid unappreciated or even harmful “off target” effects. More importantly, although the role of miRNAs has started to be elucidated in the basis of individual entity as a part of the miRNA network, how these miRNAs integrate and interplay to regulate cardiac excitability is unknown. It is likely that multiple miRNAs contribute to controlling arrhythmogenicity of the heart and that different miRNAs are involved in different types of arrhythmias under different pathological conditions of the heart. Thus, it will be crucial to identify sets of miRNAs acting cooperatively within the cardiovascular system. This information will be particularly relevant to the development of miRNA-based therapeutics, as cocktails of miRNA inhibitors may prove more efficacious than targeting a single miRNA.

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Non-standard Abbreviations and Acronyms

3'UTR	3' untranslated region
AF	atrial fibrillation
AP	action potential
AV	atrioventricular
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase

MI	myocardial infarction
miRNA	microRNA
RISC	RNA-induced silencing complex
RyR2	ryanodine receptor
SA	sinoatrial

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Figure 1.

Schematic diagram of the ventricular action potential with ion currents activated during the various phases of the action potential and possible microRNA influence of ion current. I_{Na} , fast sodium current; I_{CaL} , calcium current through L-type calcium channels; I_{Kr} , rapid delayed rectifier potassium current; I_{Ks} , slow delayed rectifier potassium current; I_{to} , transient outward current; I_{K1} , inward rectifier potassium current.

Figure 2.

Schematic diagram of ventricular cardiomyocyte ion channels regulated by microRNAs. NCX, sodium-calcium exchanger; SCN5A, sodium channel, voltage-gated, type V, alpha subunit; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase 2a. Ca²⁺-induced Ca²⁺ release involves the ryanodine receptor (RyR2) channels on the sarcoplasmic reticulum and is essential for activation of contractile filaments during myocardial contraction.

Table 1

MicroRNAs and identified targets in arrhythmia

MicroRNA	Targets	Function
miR-1	KCNJ2	Decrease I_{K1}
	GJA1	Conduction slowing
	CACNA1C	Decrease I_{CaL}
	Irx5 $\rightarrow$ KCND2	Decrease I_{to}
	B56a $\rightarrow$ RyR2	Altered Ca^{2+} cycling
miR-133	PP2A $\rightarrow$ RyR2	Altered Ca^{2+} cycling
	?	Decreased $I_{to,f}$
	ERG	Decreased I_{Kr}
miR-328	CACNA1C, CACNB1	Decrease I_{CaL}
miR-212	KCNJ2	Decrease I_{K1}
miR-208a	GJA5	Conduction slowing

Table 2

Arrhythmia/Conduction	Model	microRNA
Atrial Fibrillation	Tachy-paced canine	↑ miR-328
	Tachy + nicotine	↓ miR-133, miR-590
	Human atrial tissue	↓ miR-1, ↑ miR-21
	Human atria+VD	↑ miR-212
	Transgenic mouse (Rac1)	↑ miR-21
	Mouse knockout	miR-208a KO
QRS prolongation	Mouse knockout	miR-1-2 KO
	As ₂ O ₃ Guinea Pig	↑ miR-1, ↑ miR-133
QT prolongation	Diabetic rabbit heart	↑ miR-133
	αMHC transgenic (miR-133)	miR-133 TG
	As ₂ O ₃ Guinea Pig	↑ miR-1, ↑ miR-133
Ventricular Arrhythmia	Rat myocardial infarction	↑ miR-1
	Human Myotonic Dystrophy	↓ miR-1
Conduction Block	αMHC transgenic (miR-208a)	miR-208a TG
	Human Myotonic Dystrophy	↓ miR-1