MicroRNAs and atrial fibrillation: new fundamentals

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Abstract

Atrial fibrillation (AF) is the most commonly encountered clinical arrhythmia associated with pronounced morbidity, mortality, and socio-economic burden. This pathological entity is associated with an altered expression profile of genes that are important for atrial function. MicroRNAs (miRNAs), a new class of non-coding mRNAs of around 22 nucleotides in length, have rapidly emerged as one of the key players in the gene expression regulatory network. The potential roles of miRNAs in controlling AF have recently been investigated. The studies have provided some promising results for our better understanding of the molecular mechanisms of AF. In this review article, we provide a synopsis of the studies linking miRNAs to cardiac excitability and other processes pertinent to AF. To introduce the main topic, we discuss basic knowledge about miRNA biology and our current understanding of mechanisms for AF. The most up-to-date research data on the possible roles of miRNAs in AF initiation and maintenance are presented, and the available experimental results on miRNA and AF are discussed. Some speculations pertinent to the subject are made. Finally, perspectives on future directions of research on miRNAs in AF are provided.

Keywords

MicroRNA • Atrial fibrillation • Electrical remodelling • Ion channel • Gene expression

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1. Introduction

Atrial fibrillation (AF) is the most commonly encountered clinical arrhythmia, with an estimated prevalence in the general population up to 1%1 and 7.1% in those >85 years.2 It is associated with pronounced morbidity, mortality, and socio-economic burden, accounting for more hospitalization days than any other type of arrhythmia, because it can cause lethal ventricular arrhythmias, exacerbate heart failure, and constitute a risk factor for ischaemic stroke due to thrombo-embolism.3–8 AF, appearing as irregular atrial activation in the electrocardiogram and electrical disorders at the cellular level, is a manifestation of diverse abnormalities (electrical, structural, metabolic, neurohormonal, or molecular alterations) in multiple pathological conditions (heart failure, hypertension, diabetes mellitus, hyperthyroidism, ageing, etc.). Genetic mutation, functional impairment, and expression deregulation of ion channels, transporters, intracellular Ca2+-handling proteins, and other relevant proteins that can create structural and metabolic substrates all have the potential to render electrical disturbances predisposing to AF. Recently, deregulation of gene expression as a molecular mechanism for arrhythmogenesis has attracted tremendous attention of researchers. In this regard, the role of transcription factors in regulating transcription of genes has been a major focus of research. However, frequent mismatch between mRNA and protein levels of gene expression has turned many studies to the regulatory mechanisms at the post-transcriptional level. Particularly with the recent rapid evolution of microRNA (miRNA) research, researchers have begun to appreciate the roles of these small non-protein-coding mRNAs in the cardiovascular system. Unlike transcription factors, which regulate gene expression at the transcriptional level by acting on the 5’-flanking region of genes to define the transcriptome of cells, miRNAs primarily silence gene expression at the post-transcriptional level by acting on the 3’-untranslated region of genes to help define the proteome of cells. Several original studies have been reported that point to the direct involvement of miRNAs in controlling cardiac excitability and arrhythmogenesis in particular pathological conditions as well.9–13 A group of miRNAs that are able to regulate the genes encoding cardiac ion channels/transporters/Ca2+-handling proteins and other relevant genes have been identified. Some of these miRNAs have been shown to be involved in AF, and some are considered to have
the potential to regulate AF based on their target genes (Table 1). Although published studies focusing on miRNAs and AF are still sparse, available results have demonstrated the potential of miRNAs being a new mechanism for AF. Systematic and insightful investigations on miRNA and AF will no doubt advance our understanding of the fundamentals of the disorder and may offer new strategies for the treatment of AF.

This review article aims to provide a synopsis of the studies linking miRNAs to cardiac excitability and other processes pertinent to AF. Basic knowledge of miRNA biology is introduced and our current understanding of mechanisms for AF discussed, to pave the path leading to the main topic. Possible roles of miRNAs in AF are commented and speculated upon, based on the published studies on the related subjects. In addition, perspectives on future directions for research on miRNAs and AF are also provided.

2. miRNA biology

2.1 miRNA biogenesis

Genes for miRNAs, like the protein-coding genes, are located in the chromosomes as an integral part of the complex genome; however, the miRNA genes appear more genomically diverse and dynamic, as follows: (i) some miRNAs are transcribed from multiple copies of their genes; (ii) many miRNAs are identified in clusters that can be transcribed as polycistronic primary transcripts; and (iii) some miRNAs are encoded by their own genes and others are encoded by the sequences within introns (mirtrons) as a part of the host protein-coding genes. Accordingly, miRNAs are generated by two pathways: the canonical pathway and the mirtronic pathway.

The first step of the canonical pathway of miRNA biogenesis involves generation of primary miRNAs (pri-miRNAs) with a stem–loop structure through transcription of intergenic miRNA genes operated mainly by RNA polymerase II (Figure 1). The pri-miRNA is subsequently processed to become precursor miRNAs (pre-miRNAs) by the nuclear RNase endonuclease III, Drosha, and its partner proteins Drosha and refold into the typical stem–loop structure. The subsequent steps converge into the canonical miRNA-processing pathway, being processed by another RNase III, Dicer, to become mature miRNAs. The pre-miRNAs are then exported to the cytoplasm from the nucleus through the Ran–GTP-dependent nuclear pores exportin-5. In the cytoplasm, pre-miRNAs are further processed by another RNase III, Dicer, to become mature miRNAs. The intronic miRNAs are processed by sharing the same promoter and other regulatory elements of the host genes. They are first transcribed along with their host genes by RNA polymerase II (the canonical pathway) and spliced by spliceosome out of their host genes to form looped intermediates, bypassing the Drosha pathway. These intermediates are then debranched and refolded into the typical stem–loop structure. The subsequent steps converge into the canonical miRNA-processing pathway, being processed by Dicer.

2.2 miRNA action

Incorporation of mature miRNA into the protein complex called RNA-induced silencing complex (RISC) to form miRISC is the first step for its action in gene regulation (Figure 1). One of the double strands, the passenger strand, is eliminated, while the remaining strand, the guide strand, serves to guide miRISC to find its complementary motif(s), mainly in the 3′-untranslated region of the target mRNA through a Watson–Crick base-pairing mechanism with its 5′ end two to eight nucleotides exactly complementary to the recognition motif within the target. This 5′ end two-to-eight nucleotide region is termed the ‘seed site’ because it is critical for miRNA actions. Partial complementarity with rest of the nucleotide region is termed the ‘seed site’ because it is critical for miRNA actions.30,31 Partial complementarity with rest of the nucleotide region is termed the ‘seed site’ because it is critical for miRNA actions.30,31
transcriptional regulation of gene expression, presumably by stabilizing the miRNA:mRNA interaction. The binding primarily inhibits the translation of the protein that the target mRNA encodes, although a number of miRNAs have been reported to promote gene silencing through mRNA degradation.32,33

2.3 miRNA function

miRNAs have been demonstrated to play important roles in diverse fundamental biological processes, including cell growth, cell death, cell differentiation, development, and ageing.34–36 They have also been implicated in a variety of pathological conditions, such as cancer, heart disease (myocardial infarction, cardiac hypertrophy/heart failure, arrhythmogenesis, and atrial fibrillation), and Alzheimer’s disease.10,11,16,37–40 These pathophysiological functions are conferred by their gene-silencing properties and cell type-specific and disease-dependent expression profiles.

2.4 miRNA expression profiles in heart

Expression of miRNAs in mammalian species in normal conditions is genetically programmed with certain spatial (depending on cell, tissue, or organ type) and temporal patterns (depending on developmental stage). For example, the miRNA expression profile in arteries is
different from that in the heart. The most abundant miRNAs in cardiac muscles are miR-1, let-7, miR-133, miR-126–3p, miR-30c, and miR-26a. However, in artery smooth muscles the most abundant miRNAs are miR-145, let-7, miR-125b, miR-125a, miR-23, and miR-143.

2.4.1 Cardiac-specific miRNAs

In adult heart, the MYH6 gene, encoding a fast myosin, the α-myosin heavy chain (αMHC), co-expresses the intronic miR-208a, which regulates the expression of genes encoding two slow myosins (βMHC and Myh7b) and their intronic miRNAs, MYH7/miR-208b and MYH7b/miR-499, respectively.43,44

2.4.2 Muscle-specific miRNAs

miR-1 and miR-133 are muscle-specific miRNAs preferentially expressed in cardiac and skeletal muscle (Figure 2), and have been shown to regulate differentiation and proliferation of these cells. They represent the most abundant miRNAs expressed in the heart.45,46

2.4.3 Cardiac-enriched miRNAs

According to the results reported by Liang et al.,45 for human heart, the top 20 abundant miRNAs in human heart are miR-1, miR-133a/b, miR-16, miR-100, miR-125a/b, miR-126, miR-145, miR-195, miR-199a, miR-20a/b, miR-21, miR-26a/b, miR-24, miR-23, miR-29a/b, miR-27a/b, miR-30a/b/c, miR-92a/b, miR-99, and let-7a/c/f/g. We verified the expression abundance of several selected miRNAs (miR-1, miR-133a/b, miR-125a/b, miR-30a/b/c, miR-26a/b, miR-24, miR-27a/b, miR-23, miR-29a/b, miR-101, miR-21, miR-150, and miR-328) using RNA samples isolated from left ventricular tissues of healthy human subjects46 (Figure 2). A recent study by Rao et al.47 reported a similar array of abundant miRNAs in mouse heart.

2.4.4 Other cardiac-expressed miRNAs

Some miRNAs are expressed at relatively low levels in myocardium but have been shown to have an important involvement in regulating cardiovascular function in particular situations. These include miR-19544 and miR-948 in cardiac hypertrophy, miR-143 in smooth muscle differentiation,49 miR-320 in myocardial infarction,50 miR-138 in cardiac development,51 miR-223 in glucose metabolism,52 miR-155 in neurohormonal activation,53 and miR-590 in cardiac fibrosis.54

In addition, the expression profile of miRNAs is also disease status dependent. A particular pathological process is associated with the expression of a particular group of miRNAs, the signature expression pattern of miRNAs. These signature patterns could aid in the diagnosis and prognosis of human disease.

3. Mechanisms of atrial fibrillation

AF can be induced by a variety of detrimental alterations as substrates, such as electrical disturbance, structural abnormality, intracellular Ca2+ overload, automaticity enhancement, and oxidative stress. All these factors act by altering, directly or indirectly, atrial electrical activities through the modulation of ion channel activity and/or expression.58–59

3.1 Ionic remodelling

Tachycardia-induced AF is characterized by a process termed atrial electrical remodelling (A-ER) that promotes the recurrence and maintenance of AF. A prominent alteration in A-ER is shortening of the atrial effective refractory period (ERP), which favours re-entrant arrhythmias, primarily because of the shortening of atrial action potential duration (APD) as a result of two critical changes. The first change is the reduction of L-type Ca2+ current (\(I_{\text{CaL}}\)) that primarily serves to shorten the plateau duration.3–6,60–64 The second change is the increase in inward rectifier K+ current (\(I_{\text{K1}}\)), a hallmark of A-ER in AF, which underlies the shortening of the terminal phase.64–73 Reduction of \(I_{\text{CaL}}\) and augmentation of \(I_{\text{K1}}\) are critically involved in the adverse A-ER to shorten atrial APD and favour the generation and perpetuation of multiple re-entrant wavelets in a limited mass of atrial tissue in both animal models and patients with AF (Figure 3).74

Decreased expression of the Cav1.2-channel subunit in tachycardia-induced AF or in patients with paroxysmal and persistent atrial fibrillation has been documented to account partly for the reduced \(I_{\text{CaL}}\) in several studies,75–77 although in chronic AF53,78,79 or in AF patients with valvular heart disease,68 the expression was unchanged. In addition, levels of accessory β1, β2a, β2b, β3, and α2δ2 were also found to be reduced during AF, potentially contributing to the reduction of \(I_{\text{CaL}}\).68,77 Gaborit et al.68 evaluated the ion-channel gene expression profiles in valvular heart disease patients with persistent AF and with sinus rhythm, and they found that Kir2.1 (the main K+ channel subunit for \(I_{\text{K1}}\))80–82 was up-regulated in the AF patients, and the up-regulation of Kir2.1 protein (two-fold) was remarkably more pronounced than that of KCNJ2 mRNA (only 20%), which encodes Kir2.1. The authors proposed that Kir2.1 is regulated post-transcriptionally. In addition, expression of other ion channels, such as KCND3 (encoding Kv4.3 for the transient outward current, \(I_{\text{o}}\))83,84 and KCNAN5 (encoding Kv1.5 for ultrarapid delayed rectifier K+ current, \(I_{\text{Ks}}\)),85 has consistently been found to be down-regulated in AF,3,4,86 although the role of the changes in AF is yet to be elucidated.

![Figure 2](image-url)
Figure 3  Schematic illustration of various factors involved in induction and perpetuation of AF by generating substrates and triggers for AF. AF, atrial fibrillation; APD, action potential duration; CHF, congestive heart failure; ERP, effective refractory period; HCN, hyperpolarization-activated cAMP-gated non-selective cation channel; \( I_{\text{CaL}} \), L-type \( \text{Ca}^{2+} \) current; \( I_f \), funny current; \( I_{\text{Kr}} \), inward rectifier \( \text{K}^+ \) current; \( I_{\text{NCX}} \), \( \text{Na}^+/\text{Ca}^{2+} \) exchanger current.

Figure 4  Schematic summary of regulation of various genes pertinent to AF by miRNAs based on published studies. This diagram shows how some pathological conditions alter expression of \( \text{miR-1, miR-26, miR-328, and miR-328} \) and how these miRNAs regulate their target genes to affect the properties of atrial cells leading to AF. AMI, acute myocardial infarction; CH/HF, cardiac hypertrophy/heart failure; AF, atrial fibrillation; OS, oxidative stress. Solid line-arrows indicate positive regulation and dashed line-arrows negative regulation.
3.2 Structural remodelling

The A-ER seen with the atrial tachypacing model of AF has little in the way of atrial structural remodelling (A-SR) in AF associated with congestive heart failure (CHF) and in other heart conditions. The role of alterations in the architecture of the myocardium in the initiation, development, and maintenance of arrhythmias has been recently appreciated. Apoptosis resulting from pathological triggers may create gaps leading to destruction of tissues and discontinuous conduction. In contrast, in tissues composed of post-mitotic cells, such as the heart, new cells cannot be regenerated after apoptotic cell death; instead, fibroblasts proliferate to fill the gaps. Scar formation at the site of the interstitial fibrosis of adjacent myocardium prevents myocardial repair and predisposes individuals to arrhythmias. It appears that the atrium is more susceptible to fibrosis than the ventricle, and that three interconnected pathways are involved: the renin–angiotensin system, transforming growth factor-β1 (TGF-β1), and the oxidative stress pathways. The increase in atrial fibrosis was shown to correspond to an increase in conduction heterogeneity and AF vulnerability in animal models of CHF and in transgenic mouse model for selective atrial fibrosis. Increased amounts of fibrosis have also been seen in the atria of AF patients as opposed to those in sinus rhythm.

Slowing of conduction, consequent to malfunction and/or down-regulation of the gap junction proteins (connexins), can result in unidirectional block of cardiac excitation conduction, leading to re-entrant arrhythmias. In mammalian heart, connexins 37, 40, 43, 45, 46 and 50 are present, and in atrium, connexin 43 (Cx43) and Cx40 co-localize to a considerable extent. In the mouse heart, the absence of Cx40 significantly increased its susceptibility to atrial arrhythmias. In humans, complex chronic AF was reportedly associated with about 50% less Cx40 compared with simple chronic AF. In contrast, Cx40 was expressed at significantly higher levels in samples from patients who had developed post-operative AF. Likewise, atrial tachypacing in the dog revealed an increased expression of Cx43.

3.3 Abnormalities of intracellular Ca2+ homeostasis

Intracellular Ca2+ homeostasis is crucial to cardiac rhythmic activities, and emerging evidence suggests Ca2+ overload as a causal factor for arrhythmogenesis, promoting focal firing, substrate evolution, and A-ER during AF. Ectopic activity contributes to AF by acting as a trigger to initiate re-entry in a vulnerable substrate and is governed by factors controlling after-depolarizations and triggered activity: early after-depolarizations with prolonged APD and delayed after-depolarizations due to spontaneous sarcoplasmic reticulum (SR) Ca2+ release. AF frequently occurs in the context of underlying cardiac disease, such as CHF. Stambler et al. suggested that AF in the setting of CHF in dogs was focal in origin, caused by triggered activity. Drugs that reduce intracellular Ca2+ levels are able to terminate AF. Fenelon et al. expanded on this study by performing biatrial mapping in dogs with CHF and showed that a majority of AF episodes had a focal mechanism. The Na+-Ca2+ exchanger (I(Ca XC)) is believed to be critically involved in triggered activity. Indeed, increase in I(Ca XC) has been noted in CHF and in AF. In addition, other Ca2+-handling proteins, protein kinase A (PKA), calmodulin-dependent protein kinase II, type 1 phosphatase (PP1) and type 2 phosphatase (PP2A), have also been shown to regulate AF vulnerability, probably through phosphorylation of the L-type Ca2+ channel, ryanodine receptor 2 (RyR2), phospholamban (PLB), and inhibitory troponin subunit (Tn-I).

3.4 Enhanced spontaneous activity

Excessive supraventricular ectopic activity has been clinically linked to increased risk of AF. In addition to abnormal intracellular Ca2+- handling, leading to triggered activity, other factors may also be involved in the generation of ectopic activity. Pacemaker channels, which carry the non-selective cation current (hyperpolarization-activated cation current or funny current, I(f)) and/or T-type Ca2+ channel current, are critical for generating sinus rhythm and arrhythmias in abnormal conditions by enhancing spontaneous membrane depolarization. The focal excitation is often caused by dysfunction of these channels. A higher left atrial filling pressure, an indicator of CHF, was associated with a higher mRNA level of HCN2 (the gene encoding fchannels), and the mRNA amount was significantly higher in patients with AF than in patients without AF.4

4. Involvement of miRNAs in atrial fibrillation

In principle, any miRNA that is involved in modulating A-ER, structural remodelling, Ca2+- handling and automaticity described above could contribute to AF induction and/or perpetuation. Numerous published studies have documented the ability of miRNAs to target the genes associated with AF substrates and triggers. In this regard, certain miRNAs can be viewed as pro-arrhythmic and others as anti-arrhythmic. With the same logic, some miRNAs produce pro-fibrotic and others anti-fibrotic actions; some are pro-apoptotic and others anti-apoptotic. These miRNAs might be involved directly or indirectly in the control of AF.

5. Regulation of ion channels/transporters/Ca2+-handling proteins by miRNAs

5.1 Role of miR-1

The first evidence for the role of miRNAs in controlling cardiac excitability came from our study on acute myocardial ischaemia. In this study, we demonstrated that miR-1 is overexpressed (~2.8-fold increase) in the myocardium of individuals with coronary artery disease relative to healthy hearts. Overexpression of miR-1 slows cardiac conduction, as indicated by the widening of the QRS complex and increase in conduction time, and depolarizes the cytoplasmic membrane, which are likely to be the cellular mechanisms for the arrhythmogenic potential of miR-1. Knockdown of endogenous miR-1 by the specific antisense in rats with infarcted hearts suppresses arrhythmogenesis. miR-1 represses GJA1 (encoding connexin43) and KCNJ2 (encoding Kir2.1 protein for I(K1)), leading to slowing of cardiac conduction (Figure 4), which was assessed by the widening of the QRS complex and increase in conduction time, and depolarizes the cytoplasmic membrane, which are likely to be the cellular mechanisms for the arrhythmogenic potential of miR-1. Knockdown of endogenous miR-1 by the specific antisense in rats with infarcted hearts suppresses arrhythmogenesis.
infarction. In contrast, knockdown of miR-1 can also slow cardiac conduction, as demonstrated by Srivastava’s group with targeted deletion of miR-1-2 in mice.\(^{156}\) Evidently, miR-1 in myocardium must be kept within a proper range to maintain normal cardiac conduction; an excessive decrease or increase in the level of miR-1 can promote arrhythmogenesis, which supports a central role for miR-1 for fine-tuning the regulation of cardiac electrical activities.

The arrhythmogenic potential of miR-1 initially identified by us was reproduced in a subsequent study in rats,\(^{151}\) but a different mechanism was identified. Terentyev et al. investigated the effects of increased expression of miR-1 on excitation–contraction coupling and Ca\(^{2+}\) cycling in rat ventricular myocytes.\(^{151}\) Overexpression of miR-1 resulted in a marked increase in the amplitude of the inward Ca\(^{2+}\) current, flattening of the voltage dependence of Ca\(^{2+}\) transients, and increased frequency of spontaneous Ca\(^{2+}\) sparks, while reducing the sarcoplasmic reticulum Ca\(^{2+}\) content compared with control myocytes. Overexpression of miR-1 increased phosphorylation of RyR at S2814 for calmodulin-dependent protein kinase II but not at S2808 for PKA, which was accompanied by a selective decrease in expression of B56\(x\) involved in PP2A targeting to specialized subcellular domains. The authors suggested that miR-1 enhanced the functional activity of RyR2 channels by disrupting localization of PP2A activity to these channels and thus resulted in elevated diastolic SR Ca\(^{2+}\) leak, reduced SR Ca\(^{2+}\) content and promoted arrhythmogenic disturbances in myocyte Ca\(^{2+}\) cycling. In light of the importance of GJA1/Cx43/L and KCNJ2/Kir2.1/I\(\text{K}_{\text{1}}\) and Ca\(^{2+}\) handling in atrial electrophysiology, it can be speculated that miR-1 might contribute to AF if deregulated in its expression. However, in contrast to the overexpression of miR-1 in these studies, the miR-1 level was found to be reduced or unaltered in the setting of AF, as described below. Moreover, phosphorylation of RyR2 at Ser2814 is reportedly increased in AF patients and animal AF models\(^{134}\) despite increased PP2A activity in AF,\(^{63,79}\) making the mechanism described by Terentyev et al.\(^{151}\) unlikely in AF.

In a later study by Girmatsion et al.,\(^{152}\) the authors evaluated changes in miR-1 and Kir2 subunit expression in relation to I\(\text{K}_{\text{1}}\) alterations in the left atrium (LA) of patients with persistent AF. Atrial tissue was obtained from 62 patients (including 31 with AF) undergoing mitral valve repair or bypass grafting. The I\(\text{K}_{\text{1}}\) density was found to be significantly increased in LA cells from patients with AF. There was a corresponding increase (by \(\sim 1.5\) fold) in Kir2.1 protein expression, but no change in other Kir subunits (Kir2.3, Kir3.1, and Kir3.4). Kir2.1 mRNA was more dramatically increased (three-fold). An approximately 86% reduction of miR-1 level was observed in atrial samples from the AF patients. \textit{Ex vivo} tachystimulation of human atrial slices simultaneously up-regulated Kir2.1 (\(\sim 86\%\)) and down-regulated miR-1 (\(\sim 77\%\)). These results established a clear inverse relationship between Kir2.1 and miR-1 expression levels and provided a suggestive evidence for a role of miR-1 down-regulation in I\(\text{K}_{\text{1}}\) up-regulation in AF patients. This link was proposed based on the previous study showing the ability of miR-1 to target Kir2.1.\(^{148}\) However, this study leaves a few unresolved issues. First, no confirmative evidence for the role of miR-1 in AF was presented, which requires studies with animal models of AF to examine whether artificial manipulation of miR-1 expression is able to alter the propensity of AF. Second, Kir2.1 was found to be up-regulated by only 1.5-fold at the protein level but by three-fold at the mRNA level in this study. These results are inconsistent with the expected effect of miR-1, because miR-1 has been documented not to affect the level of KCNJ2 (the mRNA encoding Kir2.1).\(^{148}\) Third, the authors showed that connexin43, another validated target of miR-1,\(^{148}\) was unaltered in its expression, though it is expected to have an inverse relationship to miR-1. Furthermore, based on the work by Terentyev et al.,\(^{151}\) down-regulation of miR-1 may improve Ca\(^{2+}\) handling to elicit a benefit effect against AF. Moreover, in the study by Voigt et al.\(^{153}\) the increase of basal inward rectifier current in patients with paroxysmal AF occurred without accompanying changes in Kir2.1 protein expression, suggesting a more complex regulation of Kir2.1 in AF. (For detailed discussion about the expression data of I\(\text{K}_{\text{1}}\) subunits in relation to current amplitude, please see reference no. 154.) It appears quite conceivable that in addition to miR-1, other miRNAs may also play roles via targeting genes relevant to AF.

### 5.2 Role of miR-26

Our recent study demonstrated that miR-26 plays a significant role in up-regulating I\(\text{K}_{\text{1}}\) in AF.\(^{155}\) We found that miR-26 is significantly down-regulated in AF. This down-regulation results in an increase in I\(\text{K}_{\text{1}}\) density owing to the ability of miR-26 to repress KCNJ2 (Figure 4). Transfection of miR-26 reduced Kir2.1 protein level, whereas application of the miR-26 antisense (AMO-26) to knockdown the endogenous miR-26 caused a robust increase in Kir2.1 level, indicating a relief of tonic repression of Kir2.1 by endogenous miR-26. Correspondingly, miR-26 depresses whilst AMO-26 enhances I\(\text{K}_{\text{1}}\) current density, as revealed by whole-cell patch-clamp recordings. We characterized the core promoter region of the miR-26 genes and identified nuclear factor of activated T-cells (NFAT), a transcription factor which has been shown to be enhanced in its activity in AF, as a transcriptional repressor of this miRNA gene. We further confirmed the nuclear accumulation of NFAT in the atrial samples from AF patients with rheumatic heart disease and from tachypaced dogs. Furthermore, application of miR-26 antagoniR (anti-miR-26 antisense conjugated with a cholesterol moiety) to mice enhanced the AF vulnerability as indicated by the reduced number of animals with successful induction of AF and shortened duration of AF once induced. Based on these results, it is likely that enhanced NFAT activity in AF down-regulates miR-26 transcription, which results in relief of repression of KCNJ2/ Kir2.1, and thereby an increase in I\(\text{K}_{\text{1}}\), leading to shortening of atrial action potential and creating the substrate for AF and AF remodelling.\(^{155}\)

### 5.3 Role of miR-328

Our most recent study revealed repression of I\(_{\text{cal}}\) by miR-328 as an independent mechanism for AF.\(^{156}\) miRNA transcriptome analysis by microarray and real-time reverse transcriptase–polymerase chain reaction showed around 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. As a control, the miR-1 level was found to be unaltered in these human atrial samples. Forced expression of miR-328 through adenovirus infection in canine atrium and a transgenic approach in mice recapitulated the phenotypes of AF, exemplified by enhanced AF vulnerability, diminished I\(_{\text{cal}}\), and shortened atrial APD. Normalization of miR-328 level by its antagoniR reversed the conditions, and genetic knockdown of endogenous miR-328 dampened AF vulnerability. CACNA1C and CACNB1, which encode cardiac I\(_{\text{cal}}\) channel a1c- and b1-subunits, respectively, 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\(_{\text{cal}}\) channel protein subunits (Figure 4).
The results indicate that miR-328 contributes to the adverse A-ER in AF through targeting HCN2 channel genes. Intriguingly, one study from Nattel’s group showed that Ca<sup>2+</sup>-dependent activation of the calmodulin–calcineurin–NFAT system causes transcriptional down-regulation of HCN subunits in dogs with atrial tachycardia remodeling. This suggests that enhanced NFAT activity in AF may simultaneously affect HCN<sub>1</sub> and HCN<sub>2</sub> through regulation of the transcription of HCN-encoding genes and miR-26 as described in the previous section, respectively.

### 5.4 Potential role of miR-133

In addition to miR-1, another muscle-specific miRNA, miR-133, has also been documented to be a miRNA regulator of cardiac ion channels. We exploited the possible miRNA mechanism for the abnormal QT interval prolongation in a rabbit model of insulin-dependent diabetes mellitus. We found a remarkable up-regulation of miR-133 in diabetic hearts. Delivery of exogenous miR-133 into the rabbit myocytes and H9c2 cell line produced post-transcriptional repression of the KCNH2 gene encoding ether-a-go-go-related gene protein (ERG), responsible for the rapid delayed rectifier K<sup>+</sup> current (I<sub>Ks</sub>), the major repolarizing current in cardiac cells of various species, including humans. miR-133 down-regulated the ERG protein level without altering its transcript level. Consistently, forced expression of miR-133 caused a substantial depression of I<sub>Ks</sub>. The data indicate that miR-133 may contribute to the abnormal QT prolongation and the associated arrhythmogenesis in diabetic hearts. The QT-shortening effect of miR-133 was further demonstrated in the study by Matkovich et al. The authors described that increased miR-133a levels prolonged QT intervals in surface electrocardiographic recordings in mice and APD in isolated ventricular myocytes, with a decrease in the fast component of the transient outward K<sup>+</sup> current, I<sub>to</sub>, at baseline. Another study from our group revealed that KCNQ1, encoding KvLQT1 K<sup>+</sup> channel α-subunit for the slow delayed rectifier K<sup>+</sup> current (I<sub>Kr</sub>), is also a target for miR-133. Notably, down-regulation of miR-133 along with corresponding up-regulation of KvLQT1/I<sub>Kr</sub>, was observed in cultured canine ventricular cells exposed to dofetilide (an I<sub>Kr</sub> blocker), which was proposed to be a feedback control mechanism of ion channel expression. Gain-of-function of I<sub>Kr</sub> leading to shortening of atrial APD has been shown to account for a type of familial AF. The possibility thus exists for miR-133 to play a role in AF; down-regulation of miR-133 is expected to shorten APD, favouring A-ER through derepression of KCNH2/ERG/I<sub>Kr</sub>, and KCNQ1/KvLQT1/I<sub>Kr</sub>. However, it remains unproved whether miR-1 and miR-133 indeed affect I<sub>Kr</sub>, I<sub>Ks</sub>, or I<sub>Kf</sub> in AF. In addition, as discussed in the next section, miR-133 may participate in AF via a different pathway.

In addition to the automatic cells (e.g. sinus nodal cells), I<sub>Kr</sub> also exists in non-automatic regions of the heart, such as atria and ventricles, where it contributes to abnormal automatic activities. In ventricular myocytes, I<sub>Kr</sub> is abundantly expressed during foetal and neonatal life but progressively decreases towards adulthood. This results in a loss of the capacity of adult ventricular cells to generate spontaneous activity. Strikingly, substantial up-regulation of I<sub>Kr</sub> expression has been observed in animal models of cardiac hypertrophy and heart failure, and in human failing hearts. We performed a study on post-transcriptional regulation of HCN2 and HCN4 by miRNAs, experimentally establishing HCN2 as a target for miR-1 and miR-133, and HCN4 as a target for miR-1 only. We unravelled robust increases in HCN2 and HCN4 transcripts and protein levels in a rat model of left ventricular hypertrophy and in angiotensin II-induced neonatal cardiomyocyte hypertrophy. The up-regulation was accompanied by reduction of miR-1 and miR-133. Our data indicate that miR-1 and miR-133 act to limit overexpression of HCN2 and HCN4, and down-regulation of miR-1/miR-133 partly underlies the abnormal re-expression of HCN2 and HCN4 in hypertrophic hearts, possibly contributing to the associated arrhythmias. It is speculated that possible re-expression of HCN2/I<sub>channel</I<sub>Kr</sub> consequent to the down-regulation of miR-1 in AF patients observed by Girmatsion et al contributes to AF pathology. However, it is unclear whether miR-1 and miR-133 affect HCN channels in the setting of AF.

### 6. Regulation of fibrosis and apoptosis by miRNAs

#### 6.1 Role of miR-133 and miR-590

A recent study from our group demonstrated that down-regulation of miR-133 is able to promote AF, through a mechanism favouring atrial structural remodelling (A-SR). This study was designed to elucidate molecular mechanisms underlying the AF-promoting effects of nicotine by inducing A-SR. The idea was originated from the fact that a large number of people smoke cigarettes and/or use over-the-counter nicotine products (patches and gums) to satisfy nicotine addiction; however, use of a nicotine product has been frequently associated with serious, sometimes fatal, cases of AF. We found that nicotine stimulated remarkable collagen production and atrial fibrosis, both in vitro in cultured canine atrial fibroblasts and in vivo in canine atrium subjected to rapid atrial pacing to induce AF. Nicotine significantly up-regulated expression of TGF-β1 and TGF-β1 receptor II (TGFBRII) at the protein level and meanwhile down-regulated the levels of miR-133 and miR-590. This down-regulation resulted in up-regulation of TGF-β1 and TGFBRII, which we established to be target genes for miR-133 and miR-590. Transfection of miR-133 or miR-590 into cultured atrial fibroblasts reduced the protein level of TGF-β1 and TGF-βRII and collagen content. The antisense oligonucleotides to miR-133 or miR-590 abolished these effects. The data prompted us to conclude that the pro-fibrotic response to nicotine in canine atrium critically depends upon down-regulation of the anti-fibrotic miRNAs miR-133 and miR-590. It is interesting to note that possible up-regulation of I<sub>Kr</I<sub>/I<sub>Ks</I<sub> due to down-regulation of miR-133 may also contribute to the AF-promoting effect of nicotine, which was unfortunately not tested in this study.

#### 6.2 Potential role of other miRNAs

In addition to miR-133 and miR-590, several other miRNAs have also been reported to regulate fibrogenesis in cardiac tissues. miR-208 and miR-21 were characterized as pro-fibrotic miRNAs implicated in cardiac hypertrophy/heart failure and myocardial infarction, respectively, and miR-29, miR-30, miR-133 and miR-590 are evidenced as anti-fibrotic miRNAs. While these miRNAs all have the potential to affect AF vulnerability, no experimental data have been documented thus far.

Another layer of regulation of anatomical/structural components by miRNAs is apoptotic cell death. The first evidence for the role of miRNAs in cardiomyocyte apoptosis was obtained in 2007 from our group, demonstrating the pro-apoptotic effect of miR-1 and the anti-apoptotic effect of miR-133 in response to oxidative stress. Subsequent studies revealed the involvement of other miRNAs, such as
miR-21, miR-29, miR-199a, and miR-320, in regulating cardiomyocyte apoptosis.\textsuperscript{177–179} Among these, miR-29 and miR-320 are linked to pro-apoptotic pathways,\textsuperscript{53,177} whereas miR-21 and miR-199a are linked to anti-apoptotic pathways,\textsuperscript{78,179} and outcomes. Whether these miRNAs are involved in AF remains unknown.

7. Concluding remarks

Although published data testify to the critical roles of many miRNAs in regulating the expression of a variety of genes that may constitute the substrates or triggers for AF when deregulated, interest in investigating the roles of miRNAs in AF has just begun to warm up. The studies revealing the regulation of \textit{CAGNA1C/Cav1.2/L} and \textit{KCNJ2/Kir2.1/L} by miR-328\textsuperscript{156} and miR-26,\textsuperscript{155} respectively, represent the first documentation of the roles of miRNAs in AF in animal models and the target gene mechanisms. The messages we have received from available data are as follows: (i) multiple miRNAs are involved in controlling AF, but our current knowledge in this regard is still rather preliminary; and (ii) AF, as a complex electrical phenotype involving complex factors beyond electrophysiology, can occur in a variety of pathological settings; AF of different sorts has distinct underlying mechanisms and different miRNAs may be involved in different types of AF. The very limited data currently available do not allow us to view the whole picture of AF regulation by miRNAs in different pathophysiological contexts. Future studies are absolutely encouraged to shed light on the matter. Several approaches outlined below may be taken to facilitate the pace of research on miRNAs and AF.

(i) A conventional and logical approach to step into the door for exploring the roles of miRNAs in AF will be to acquire miRNA expression signatures in animal models and patients having different sorts of AF; such profiling data are still presently missing. These data will allow us to gain the information on the miRNAs that could most probably contribute to AF.

(ii) Alternatively, a short-cut leading to rapid identification of AF-regulating miRNAs is to take the advantage of published results or theoretical predictions regarding the regulation of pertinent genes by miRNAs. For example, ACh-gated inward rectifier K$^+$ current (\textit{I}_{\text{K(ACh)}}) has been shown to develop agonist-independent constitutive activity, contributing to the higher basal inward rectifier currents in AF, in addition to \textit{I}_{\text{K1}}.\textsuperscript{180,181} Computational analysis predicts \textit{KCNJ3/Kir3.1/I}_{\text{K(ACh)}} and \textit{KCNJ5/Kir3.4/I}_{\text{K(ACh)}} as targets for miR-30 and miR-150, both of which are down-regulated in CHF.\textsuperscript{182–184} There is a possibility that miR-30 and miR-150 contribute to AF by enhancing \textit{KCNJ3/Kir3.1/I}_{\text{K(ACh)}} through derepression. Likewise, \textit{NCX1} (cardiac sodium-calcium exchanger) is predicted to be a target for miR-1, miR-27 and miR-214, the cardiac-enriched miRNAs.\textsuperscript{185} \textit{SERCA2} (calcium ATPase) may be regulated by miR-30 and all members of the \textit{let}-7 family. All these theoretical analyses require bench-top experiments for verification.

(iii) As already mentioned, AF of different types in different pathological situations may have different miRNAs involved. It would be highly desirable to identify the respective key miRNAs and their responsible target genes using different animal models of AF.

(iv) Finally, simulation studies making use of existing AF models, miRNA expression signatures and miRNA target gene prediction should give rise to an overall picture with integrated information on the roles that miRNAs play in AF initiation and maintenance.

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Role of miRNAs in atrial fibrillation


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