MicroRNA-328 Contributes to Adverse Electrical Remodeling in Atrial Fibrillation

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Background—A characteristic of both clinical and experimental atrial fibrillation (AF) is atrial electric remodeling associated with profound reduction of L-type Ca\(^{2+}\) current and shortening of the action potential duration. The possibility that microRNAs (miRNAs) may be involved in this process has not been tested. Accordingly, we assessed the potential role of miRNAs in regulating experimental AF.

Methods and Results—The miRNA transcriptome was analyzed by microarray and verified by real-time reverse-transcription polymerase chain reaction with 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. miR-223, miR-328, and miR-664 were found to be upregulated by >2 fold, whereas miR-101, miR-320, and miR-499 were downregulated by at least 50%. In particular, miR-328 level was elevated by 3.9-fold in AF dogs and 3.5-fold in AF patients relative to non-AF subjects. Computational prediction identified CACNA1C and CACNB1, which encode cardiac L-type Ca\(^{2+}\) channel α1c- and β1 subunits, respectively, as potential targets for miR-328. Forced expression of miR-328 through adenovirus infection in canine atrium and transgenic 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 antagoniR reversed the conditions, and genetic knockdown of endogenous miR-328 dampened AF vulnerability. CACNA1C and CACNB1 as the cognate target genes for miR-328 were confirmed by Western blot and luciferase activity assay showing the reciprocal relationship between the levels of miR-328 and L-type Ca\(^{2+}\) channel protein subunits.

Conclusions—miR-328 contributes to the adverse atrial electric remodeling in AF through targeting L-type Ca\(^{2+}\) channel genes. The study therefore uncovered a novel molecular mechanism for AF and indicated miR-328 as a potential therapeutic target for AF. (Circulation. 2010;122:2378-2387.)

Key Words: arrhythmia ■ atrial fibrillation ■ gene expression ■ ion channels ■ pacing

Atrial fibrillation (AF) is a highly prevalent condition associated with pronounced morbidity, mortality, and socioeconomic burden, accounting for more hospitalization days than any other arrhythmias. It can cause or exacerbate heart failure and is a potent risk factor for ischemic stroke resulting from thromboembolism.1–5 A characteristic of both clinical and experimental AF is the alteration of electrophysiology that promotes persistent AF even in the absence of progressive underlying heart disease, so-called atrial remodeling or “AF begets AF.”1–3 This remodeling process is associated with a profound reduction of L-type Ca\(^{2+}\) current (I\(_{\text{CaL}}\)) density and shortening of atrial action potential duration (APD),6–14 an adaptation mechanism to avoid Ca\(^{2+}\) overload to minimize the threat to cell viability in response to increased firing rate during AF. However, this adaptation, while providing protective negative feedback on Ca\(^{2+}\) loading, is at the cost of generating positive feedback on AF vulnerability. Indeed, it has been shown that α\(_{1b}\) (an α subunit of atrial L-type Ca\(^{2+}\) channel) knockout mice had atrial ECG abnormalities and enhanced AF vulnerability.15 Although expression deregulation of Ca\(^{2+}\) channel-encoding genes in AF has been documented, the molecular determinants have remained incompletely understood.

Clinical Perspective on p 2387

With the recent surge of research into microRNAs (miRNAs), this category of endogenous noncoding small RNAs has...
emerged as one of the central players in gene expression regulation, participating in many essential biological processes.

The implications of miRNAs in the pathological process of the cardiovascular system have also been recently appreciated. In particular relevance to this study, miRNAs may also be involved in AF and the associated atrial remodeling process. In this regard, 2 recent studies suggested an indirect role of miR-133 and miR-590 in I K1 alterations in the left atrium (LA). These authors did not provide further insight into delineating the targeting mechanism and the potential role of miR-1 downregulation in AF initiation and maintenance, but on the basis of our previously established relationship between miR-1 and Kir2.1,24 they proposed a primary role of miR-1 downregulation in I K1 upregulation in AF. Another study from our laboratory suggested an indirect role of miR-133 and miR-590 in smoking/nicotine-induced AF via promoting atrial fibrosis on their expression downregulation.34

Although these studies provided promising evidence in support of the role of miRNAs in AF, mechanistic data on these small molecules in AF and the associated atrial remodeling in animal models with minimal confounding factors in conjunction with human samples are still missing. The present study was designed to shed light on this issue.

Methods

Induction of AF in Wild-Type Mice

Wild-type (WT) C57BL/6 mice were divided into 2 groups: a control (n=14) group and an antagomiR-328 (n=10) group. Intracardiac pacing was performed in these animals by inserting an 8-electrode catheter (1.1F, octapolar EP catheter, Scisense, London, Ontario, Canada) through the jugular vein and advancing it into the right atrium and ventricle.35,36 Inducibility of atrial arrhythmias was tested by applying 6-second bursts through the catheter electrodes using the automated stimulator that was part of the data acquisition system. By applying 6-second bursts through the catheter electrodes using the automated stimulator that was part of the data acquisition system. The procedures were similar to the study reported by van Rooij et al.21

Knockdown of endogenous miR-328 in F0 was verified, and the same procedures described above for miR-328 were followed.38 Knockdown of endogenous miR-328 in F0 was verified, and the 2-month-old Tg mice were used in the experimental studies.

Data Analysis

Group data are expressed as mean±SEM. Statistical comparisons among multiple groups were performed by ANOVA. If significant effects were indicated by ANOVA, a t test with Bonferroni correction for microarray data or a Dunnett test for patch-clamp data was used to evaluate the significance of differences between individual
means. Otherwise, baseline and drug data were compared by paired Student \(t\) test, and age-matched comparisons between control and treatment were done by unpaired Student \(t\) test. Group comparisons for AF incidence were performed with the /H9273 test. A 2-tailed \(P < 0.05\) was taken to indicate a statistically significant difference. Linear and nonlinear least squares curve fitting was performed with CLAMPFIT in pCLAMP 8.0 or GraphPad Prism 4.0. More information on materials and methods is provided in the online-only Data Supplement.

Results

miRNA Expression Profile in a Canine Model of AF Induced by Atrial Tachypacing

As an initial step to examine our hypothesis that miRNAs are involved in AF, we used a canine model of AF by atrial tachypacing (A-TP) for 8 weeks to create AF-induced remodeling.3,4,21,39 As illustrated in Figure 1 A, A-TP increased the vulnerability to AF as indicated by the facilitated induction of AF by electric stimuli and prolonged duration of electrically induced AF. As in previous studies, the cellular and ionic alterations were also consistent with the atrial remodeling process during AF: APD was markedly shortened and APD accommodation (use-dependent shortening of APD) was lost (Figure 1B). These electrophysiological abnormalities were presumably caused by reduction of \(I_{\text{Ca,L}}\) density (Figure 1C).

We then conducted expression profiling to identify deregulated miRNAs in the atrial tissues of this canine model of AF using miRNA microarray analysis comparing the differential expressions of miRNAs between control and AF dogs. Four miRNAs—miR-223, miR-328, miR-664, and miR-517—were found to be increased by \(>2\) fold, and 6—miR-101, miR-133, miR-145, miR-320, miR-373, and miR-499—were decreased by at least \(50\%\) (Figure 2A). Real-time quantitative reverse-transcription PCR (RT-PCR) analysis confirmed the significant upregulation of miR-223, miR-328, and miR-664 (miR-517 was undetectable) and the significant downregulation of miR-101, miR-320, and miR-499 (Figure 2B). In particular, miR-328 demonstrated the most pronounced change among all, being elevated by 4 times in AF over control. Furthermore, by computational prediction of target genes, we identified 2 genes, \(\text{CACNA1C}\) and \(\text{CACNB1}\), encoding the cardiac L-type \(\text{Ca}^{2+}\) channel \(\alpha_{1c}\)- and \(\beta_{1}\) subunits, respectively, as potential targets for miR-328, an intergenic monocistronic gene located in human chromosome 16. The expression of endogenous miR-328 in heart and the upregulation of miR-328 were verified with Northern blot analysis (Figure 2C). These findings prompted us to focus on exploring the role of miR-328 in AF and the associated atrial remodeling.

Figure 1. Characterization of the canine model of AF and associated electric remodeling. AF was induced by A-TP for 8 weeks. AF was induced by single premature extrastimuli at a basic pacing cycle length of 300 milliseconds. A, Verification of AF vulnerability. Left, Representative direct atrial activation recordings obtained in the LA wall of a control (Ctl) dog with sinus rhythm and an A-TP dog. Right, Averaged data of AF incidence and AF duration. AF incidence was assessed by the number of animals that demonstrated at least 1 run of induced AF and the duration of AF once induced. \(P < 0.05\) vs Ctl, unpaired \(t\) test; \(n = 7\) dogs for each group. B and C, Verification of atrial electric remodeling. Note the shortening of APD to 90% full repolarization \((\text{APD}_{90})\) and loss of rate-dependent shortening of \(\text{APD}_{90}\) (B) and reduction of L-type \(\text{Ca}^{2+}\) current density \((I_{\text{Ca,L}}, \text{pA/pF})\) (C) in A-TP animals relative to Ctl animals. Action potentials were recorded in freshly isolated single atrial myocytes under the current-clamp mode in the whole-cell patch-clamp configuration. \(I_{\text{Ca,L}}\) was recorded with the voltage-clamp mode with the whole-cell patch-clamp configuration, with the voltage protocols shown in the inset. \(\dagger P < 0.05\) vs Ctl, unpaired \(t\) test; \(n = 19\) cells for each group. †\(P < 0.05\) vs control, ANOVA \(F\) test for rate dependence; \(n = 17\) cells for each group.

Forced Expression of miR-328 Recapitulates the Phenotypes of AF and Atrial Electric Remodeling

On the basis of the above findings, we decided to examine whether forced expression of miR-328 is able to recapitulate
The phenotypes of experimental AF by taking 2 different approaches. In the first approach, we constructed adenovirus vector carrying the premiR-328 sequence (Figure 1 in the online-only Data Supplement) and then performed in vivo gene transfer procedures through direct intramuscular injection of the construct into multiple sites (10 sites within 1 cm in diameter) in the right atrial free wall of healthy dogs. This infection procedure resulted in significant overexpression of miR-328 in the tissue mass 12 hours after injection; the miR-328 level was 4.9-fold higher than in the tissues injected with premiR-328–free adenovirus vector (Adv-pDC316) (Figure II in the online-only Data Supplement). This forced expression of miR-328 clearly increased the vulnerability of canine to electric induction of AF with marked prolonged AF duration (Figure 3A). Notably, the AF maintenance by miR-328 was significantly attenuated when the antimiR-328 was significantly attenuated when the antimiR-328 lost-of-function approaches. In the first approach, we generated an miR-328 antisense Tg mouse line (AMO/Tg) (Figure III in the online-only Data Supplement). As expected, all 55 miR-328/Tg mice tested developed AF phenotype and miR-328 level. Moreover, this transgene model recapitulated the AF remodeling properties. As depicted in Figure 3C, atrial APD was markedly shortened, APD accommodation was abolished, and ionic remodeling was substantially decreased (Figure 3D), typical of atrial tachycardia remodeling seen in experimental and clinical AF. Noticeably, these changes were all reversed by the antisense oligo-miR-328 (AMO-328; Figure 3C and 3D), indicating the specificity of action of miR-328. In comparison, inward rectifier K⁺ current (Iₖ₁), transient outward K⁺ current (Iₖₒ), and ultrarapid delayed rectifier K⁺ current (Iₖ₃), the 3 major K⁺ currents in mouse atrial cells, were unaltered in the miR-328/Tg mice relative to WT littermates (Figure V in the online-only Data Supplement). Furthermore, administration of antagoniR-328, AMO-328 conjugated with a cholesterol moiety, normalized the miR-328 level in miR-328/Tg mice and abrogated or even stopped AF (Figure 3E and 3F). AntagoniR has been documented by numerous studies to be highly effective in knocking down target miRNA with long-lasting efficacy under in vivo conditions.

Loss of Function of miR-328 Reduces AF Vulnerability

We reasoned that if overexpression of miR-328 promotes AF, then downregulation of this miRNA of endogenous origin should prevent AF. To test this hypothesis, we used 2 miR-328 loss-of-function approaches. In the first approach, we generated an miR-328 antisense Tg mouse line (AMO/Tg) to genetically knock down endogenous miR-328. In the second approach, we injected antagoniR-328 into WT mice to knock down the miR-328 of endogenous origin. The animals were all pretreated with low-dose carbachol through the tail vein for 7 consecutive days before attempts were made to induce AF by intracardiac pacing. These treatments facilitated induction of AF (Figure 4A and 4B), upregulated miR-328 (Figure 4C), and decreased Iₖ₃ (Figure VI in the online-only Data Supplement). As expected, the AF induction was substantially dampened in both antagoniR-328–treated WT mice (Figure 4A) and AMO/Tg mice (Figure 4B), as indicated by the reduced number of animals with successful induction of AF by electric stimulation, and shortened AF duration once induced compared with that in the sham-operated, age-matched mice. Quantitative RT-PCR confirmed the downregulation of miR-328 expression; in AMO-Tg mice, the miR-328 level was only 40% of the control value of the WT littermates (Figure 4C), and antagoniR-328 reduced the endogenous miR-328 level by ≈80% in WT mice relative to the animals treated with mismatched antagoniR.

Correspondingly, the Cav1.2 and Cavβ1 protein levels were reciprocally increased by ≈2-fold in AMO/Tg mice.
These above changes were not observed in the WT mice treated with mismatched antagomiR-328 or in the mismatched AMO/Tg mice. We also performed additional experiments to exclude the possible contribution of structural components to the ability of miR-328 to induce AF (Figure VII in the online-only Data Supplement).

**CACNA1C and CACNB1 Genes as Targets for miR-328**

Having established that gain of function of miR-328 promotes while loss of function of miR-328 abrogates AF, we went on to delineate the target genes of miR-328 that play a key role in AF and the associated electric remodeling process. As mentioned, our computational analysis pointed to a possibility of miR-328 regulating *CACNA1C* and *CACNB1* genes because they contain in their 3’ untranslated regions and coding regions multiple sequence motifs complementary to the “seed site” of miR-328 (Figure VIII in the online-only Data Supplement). To experimentally establish these 2 genes as cognate targets of miR-328, we engineered the fragments containing the binding sites for miR-328 in these candidate target genes into the 3’ untranslated region of the luciferase gene and carried out luciferase reporter activity assays. As illustrated in Figure 5A, transfection of miR-328 (10 nmol/L) with the luciferase expression constructs carrying each of the target fragments caused a substantial reduction of luciferase activities. The decreases were efficiently reversed by AMO-328 (10 nmol/L). Furthermore, a negative control construct did not affect the luciferase activities.

The ability of miR-328 to repress the expression of these genes was further verified by Western blot analysis of the levels of the proteins encoded by their corresponding mRNAs.
in neonatal rat atrial myocytes transfected with miR-328 (Figure 5B). This downregulation was efficiently prevented by AMO-328. The efficacy of AMO-328 to knock down the mature miR-328 in all our experimental settings was confirmed (Figure II in the online-only Data Supplement).

In line with the above data, the protein levels of Cav1.2 and Cavβ1 were found to be significantly reduced in the atrial tissues of A-TP dogs (Figure 5C). As a negative control, an unrelated protein connexin43 was also analyzed, and no change was found between A-TP and control dogs. Similar downregulation of Cav1.2 and Cavβ1 protein levels was consistently observed in miR-328/Tg mice (Figure 5D).

Effects of miR-328 on the transcript level of CACNA1C and CACNB1 were also evaluated in miR-328/Tg mice and in cultured neonatal rat atrial myocytes. miR-328 induced \( \approx 30\% \) decreases in CACNA1C and CACNB1 transcript levels (Figure 5E). Significant downregulation of Cav1.2 and Cavβ1 protein levels was also observed in AF patients (Figure 5F).

The relative abundances of miR-328 and CACNA1C in the RNA samples from healthy human atrial tissues were quantified by real-time RT-PCR. The results revealed that the copy number of miR-328 is \( \approx 4000 \) in a cardiac cell, some 7-times higher than that of CACNA1C (copy number, \( \approx 600 \)), indicating that the endogenous concentration of miR-328 should be sufficiently high to produce posttranscriptional repression of CACNA1C in a cardiac cell (Figure IX in the online-only Data Supplement). We then further ruled out the possibility that miR-328 acts by targeting ion channels other than CACNA1C and CACNB1 (Figure X in the online-only Data Supplement).

**Discussion**

Sustained atrial tachycardia as a stress, like clinical AF, can modify atrial properties so that AF recurs and maintains itself more readily; the rapid atrial rate of AF is the primary stimulus of the remodeling process, and such a remodeling has also been called atrial tachycardia remodeling.\(^{1,2,42,43}\) Atrial tachycardia remodeling is suggested to contribute to a variety of clinically important phenomena, including the tendency of paroxysmal AF to become persistent, the tendency of AF to recur soon after cardioversion, and the tendency for longer-lasting AF to become refractory to pharmacological cardioversion. Experimentally, AF and atrial tachycardia remodeling can readily be induced by A-TP.\(^{3,4,37–39,42}\) Intensive studies have been done to understand ionic mechanisms for AF and the associated atrial electric remodeling. It has been widely accepted that for the A-TP–induced electric remodeling, diminished \( I_{\text{calc}} \) density resulting from expression downregulation and functional impairment of L-type Ca\(^{2+} \) channel protein is a hallmark and a critical causal factor. In other words, excessive reduction of
I_Ca,L predicts enhanced AF vulnerability. In the present study, we found that miR-328 is aberrantly upregulated in dogs with AF induced by A-TP and in patients with AF, and this overexpression is accompanied by reduction of I_Ca,L and shortening of atrial APD. Moreover, gain of function of miR-328 through forced expression facilitates AF and recapitulates the major characteristics of atrial electric remodeling; in sharp contrast, loss of function of miR-328 by genetic knockdown or miRNA interference approaches did the opposite. We further established CACNA1C and CACNB1, which encode I_Ca,L channel α1c- and β1 subunits, respectively, as cognate target genes for miR-328. The study therefore provides the first detailed characterization of the role of miRNAs in AF and the associated atrial electric remodeling.

A recent study reported by Girmatsion et al. showed that the miR-1 level is decreased in AF patients and, in parallel, the inward rectifier K^+ current I_K1 and I_K1 channel protein Kir2.1 were increased. It was speculated that downregulation of miR-1 may underlie the upregulation of I_K1, which is another critical factor for AF. Unfortunately, no detailed experimental investigation was conducted in this study to clarify whether downregulation of miR-1 plays a role in AF induction and maintenance and to elucidate whether the cause-effect relationship between the changes of miR-1 and I_K1 exists in this particular case. Moreover, Kir2.1 was found to be upregulated by only 1.5 times at the protein level but by 3 times at the mRNA level. These results are inconsistent with the expected effect of miR-1 because miR-1 has been predicted to target I_K1.
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Lu et al  Control of Atrial Fibrillation by miR-328  2385
documented not to affect the KCNJ2 ( Kir2.1 mRNA) level.24 Further evidence against the role of miR-1 in AF came from the same study showing that connexin 43, another validated target of miR-1,24 was found to be unaltered in expression.33 Furthermore, in the present study, we observed no significant changes of miR-1 level in either the canine model of AF by A-TP or the atrial samples from patients with clinically defined AF. The reason for the discrepancy in miR-1 expression in AF patients between the study by Girmatsion et al33 and this study remained unclear. Although the patient populations recruited for the 2 studies both had rheumatic heart disease, many confounding factors (eg, degree of heart damage, ethnic groups, age, and gender) may contribute. Consistently in our study, the increase in miR-328 level found in AF dogs was also observed in the AF patients. This fact points to a rationale for using animal models to minimize confounding factors for addressing some clinical questions and dissecting the molecular mechanisms.

Considering the previous studies that identified miR-1 as an arrhythmogenic miRNA,24,26,28,33 it appears that in addition to the muscle-specific miRNA, ubiquitously distributed miRNAs like miR-328 can have strong arrhythmogenic potential. In addition, 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. miR-1 has been shown to be critical in ischemic ventricular arrhythmias by slowing cardiac conduction via targeting connexin43 and inward rectifier K+ channel.24 miR-133, another muscle-specific miRNA, may play a role in the abnormal QT prolongation in diabetic cardiomyopathy through repressing KCNH2 encoding human ether-a-go-go–related gene K+ channel.29 The present study revealed, for the first time, the role of miRNA in AF and miR-328 as a new arrhythmogenic miRNA acting on L-type Ca2+ channel subunits. In addition to miR-328, we found that miR-223 and miR-664 were significantly upregulated in AF dogs and patients (Figure 2B and 2D). However, miR-223 was predicted not to target any cardiac-expressed ion channel transporter genes, and miR-664 is expressed at an extremely low level (Figure IX in the online-only Data Supplement). These 2 miRNAs could hardly play a significant role in AF and the associated atrial electric remodeling process. Further evidence in support of this notion is the fact that manipulating miR-328 alone, either gain of function or loss of function, is insufficient to alter the propensity of AF accordingly.

Our data also present the first demonstration that miR-328 targets the genes encoding L-type Ca2+ channel proteins to reduce ICaL density and to shorten APD in atrial myocytes, which is likely a mechanism underlying the atrial arrhythmogenic potential of miR-328. In agreement with this notion, Mancarella et al15 have shown that knockout of α1D (another α subunit of atrial L-type Ca2+ channel) alone is sufficient to cause atrial ECG abnormalities and to enhance AF vulnerability in the absence of changes of other currents such as INa, ICa,T, IKr, and IK1. Moreover, atrial tachyarrhythmias cause atrial mechanical dysfunction related to abnormal handling of cellular Ca2+, which contributes to thrombus formation. Our findings that miR-328 represses L-type Ca2+ channel proteins may also explain the impaired atrial contraction during AF because ICaL is known to be crucial for excitation-contraction coupling, and reduction of ICaL could well lead to weakening of cardiac contraction.1,2

Finally, the ability of the antagoniR-328 to rescue miR-328/Tg mice from the AF phenotype and the associated atrial remodeling properties suggests the potential of miR-328 as a target for molecular conversion of AF to sinus rhythm. Normalization of the miR-328 level to the normal range may well be a novel strategy for converting AF to sinus rhythm in the clinical setting. In this sense, antagoniR approach appears to represent a wise choice, for this technique has been reported to have superior cellular stability and miRNA specificity under in vivo conditions.40

It should be noted that although our study identified miR-328 as an important factor in controlling experimental AF, it does not exclude other mechanisms for determining AF, and our findings in experimental models may not be applied directly to human AF. Nonetheless, our study does provide novel insight into experimental AF and a new aspect of the role of miRNAs in cardiac disease. Moreover, we are well aware of other limitations in our study. First, although downregulation of ICaL has been commonly accepted as a key event causing AF and atrial electric remodeling, our study did not give provide evidence for the downregulation of ICaL to mediate AF occurring with miR-328 overexpression. The second limitation of our study is that atrial electric remodeling properties are different among different types of underlying heart disease. Our findings obtained from AF induced by A-TP should not be extrapolated to other types of AF. For example, the electric remodeling in AF occurring in failing heart has different properties and ionic mechanisms: Atrial APD is not shortened and ICaL may not be reduced. Although miR-328 is involved in tachyarrhythmic AF, whether it also plays a role in AF of other causes is unclear.

It is worth mentioning that miR-328 has been implicated in many other pathological conditions. For example, miR-328 is thought to play a role in Alzheimer disease by regulating the β-amyloid precursor protein-converting enzyme,44 in the malignant progression of gliomas,45 in primary biliary cirrhosis,46 in the expression of breast cancer resistance protein,47 and in chronic bladder pain syndrome.48 These findings indicate the widespread pathophysiological function of miR-328 in humans. Moreover, in all these cases, abnormal upregulation of miR-328 is a common finding; therefore, knockdown of miR-328 expression may be a useful strategy for many diseases associated with miR-328.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Atrial fibrillation (AF) is highly prevalent and associated with pronounced morbidity, mortality, and socioeconomic burden. AF accounts for more hospitalization days compared with other arrhythmias, can contribute to other arrhythmias, can exacerbate heart failure, and is a risk factor for ischemic stroke resulting from thromboembolism. A characteristic of both clinical and experimental AF is alterations in electrophysiology, promoting persistent AF even in the absence of progressive underlying heart disease, so-called atrial remodeling or “AF begets AF.” Despite extensive research, AF remains a difficult clinical problem. In the present study, we report that a single microRNA (miRNA), miR-328, can contribute to a key component of the adverse electric remodeling process leading to AF. miRNAs are a class of newly discovered endogenous 22-nt noncoding double-stranded RNAs that fine-tune expression of protein-coding genes, primarily repression of protein level. miR-328 is aberrantly upregulated in its expression level in patients with AF and in animal models of AF. This upregulation results in silencing of a gene critical to cardiac electric function; knockdown of miR-328 terminates AF. The study represents a detailed characterization of the role of a select miRNA in AF. Our findings suggest the potential of miR-328 as a target for AF management with normalization of miR-328 level for converting AF to sinus rhythm in the clinical setting.
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Correction

In the article by Lu et al, “MicroRNA-328 Contributes to Adverse Electrical Remodeling in Atrial Fibrillation,” which was published in the December 7, 2010 issue of the journal (Circulation. 2010;122:2378-2387), an institution was omitted from the list of author affiliations. The Montreal Heart Institute and the Department of Medicine, Université de Montréal should be listed as an affiliation for Xiaobin Luo and Zhiguo Wang.

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The authors apologize for the error.
SUPPLEMENTAL MATERIAL

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This PDF file includes:
Supplementary Methods
Supplementary Results
Figs. S1 to S10
Tables S1
Supplementary Methods

Computational Prediction of miRNA Target

We used five established miRNA target prediction algorithms to identify the candidate miRNAs that have the potential to target the ion channel genes; these algorithms include DIANA-microT3.1, miRanda, PITA, RNAhybrid, and TargetScan5.1. Only the gene predicted by at least three of the five algorithms to be a target for a given miRNA (miR-328, miR-223, or miR-664) was considered as a candidate for further analysis.

Canine Model of Atrial Fibrillation (AF)

Mongrel dogs (20 to 30 kg) of either sex were randomly divided into two groups: sham control (Ctl, n=10) and atrial tachypacing (A-TP, n=17) groups. For animals in the A-TP group, dogs were sedated and anesthetized with morphine (2 mg/kg SC) and α-chloralose (120 mg/kg IV load, 29.25 mg/kg/h infusion), for electrode implantation via the jugular veins. A programmable pacemaker was inserted in a subcutaneous pocket with sterile techniques, and a tined atrial pacing lead was positioned in the right atrial appendage under fluoroscopic guidance. The dogs were subjected to continuous right atrial pacing at 400 bpm for 56 days (8 weeks) before experimental studies. The control dogs were sham-operated in the same way as A-TP dogs but without tachypacing. On study days, dogs were anaesthetized with morphine and α-chloralose and ventilated to maintain physiological arterial blood gases. Body temperature was maintained at 37°C. A median sternotomy was performed, and bipolar, Teflon-coated, stainless steel electrodes were hooked into the right and left atrial appendages for recording and stimulation. A programmable stimulator was used to deliver 2-ms pulses at twice-threshold current. The surface ECG and direct atrial activation electrograms were recorded. All animal procedures were
previously approved by the Animal Care and Use Committee at the Harbin Medical University (same below).

AF vulnerability was tested at a basic cycle length (S1–S1 interval) of 300 ms, with single premature S2 extrastimuli delivered at each site by setting the coupling interval initially to 200 ms and decreasing by 10 ms decrements until AF was induced or failure to capture occurs. For the purpose of measuring AF duration, AF was induced by burst atrial pacing with 4× threshold 4-ms pulses at 20 Hz at a basic cycle length (BCL) of 300 ms. AF was considered sustained if it required electrical cardioversion for termination (cardioversion was performed after 30 min AF). To estimate the mean duration of AF, AF was induced 10 times if AF duration was <5 min, 5 times for AF between 5 and 20 min and 3 times for AF >20 min. Measurements were made before drug treatment and repeated 12 h after treatment (adenovirus infection or lipofectamine transfection of miR-328 and other constructs).

Atrial Samples from Patients

Human tissues (right atrium appendage) were provided by the Second Affiliated Hospital of the Harbin Medical University under the procedures approved by the Ethnic Committee for Use of Human Samples of Harbin Medical University. The tissues were obtained from 22 individuals undergoing heart surgery, ten of them with no atrial fibrillation (AF) and twelve with AF, who undergoing surgical procedures (see Supplementary Table 1 online). These preparations were used to isolate total RNA for real-time RT-PCR quantification of miRNAs.

Microarray Analysis

The hearts were removed from dogs and RNA samples were extracted for miRNA expression analysis. The RNA samples from 7 AF dogs and from 7 control dogs were pooled into three pairs, respectively for miRNA profiling. miRNA expression profile was analyzed using the
miRNA microarray technology miRCURY™ LNA Array (Exiqon Company, Denmark). miRCURY™ LNA Array, representing 540 mature human miRNAs plus 576 mature rodent miRNAs, incorporates Locked Nucleic Acid into an oligonucleotide probe, which greatly increases the affinity and specificity of that oligonucleotide for its complementary DNA or RNA target. Slides were scanned by the Genepix 4000B at 635 nm and the expression level was analyzed by Genepix Pro 6.0. The array output was received in Excel spreadsheets as lists of raw data and also as “simple detectable” data, which were the average of 4 signal values for each miRNA on the array. Differentially regulated miRNAs were defined as those with >2-fold increase and >50% decrease of miRNA levels in AF dogs compared with the baseline expression levels from sham-operated dogs.

**Quantitative Real-Time RT-PCR Analysis**

The mirVana™ qRT-PCR miRNA Detection Kit (Ambion) was used in conjunction with real-time PCR with TaqMan for quantification of miRNAs in our study, as previously described in detail.²⁴ The total RNA samples were isolated with Ambion’s mirVana miRNA Isolation Kit, from canine left atrial preparations, from cultured neonatal rat atrial myocytes, and from mouse left atrium. Reactions contained mirVana qRT-PCR Primer sets specific for human, canine, rat and mouse miR-328, and a scrambled miRNA as a negative control. qRT-PCR was performed on a thermocycler ABI Prism® 7500 fast (Applied Biosystems) for 40 cycles. Fold variations in expression of an mRNA between RNA samples were calculated. The threshold cycle (Cₜ) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. To estimate copy numbers of transcript in a cardiac cells, a standard curve was generated by using a series of concentrations of synthetic miR-328 and converting TaqMan Cₜ values into absolute copy numbers using the standard curve assuming 30 pg of total RNA in each cell.
**Northern Blot Analysis**

Total miRNAs were extracted from dog tissue with mirVana mRNA isolation kit (Ambion, Cat.No. AM1560). RNA samples were run on 12.5% acrylamide denaturing (urea) gels and then transferred to Nylon Positively Charged Membranes (Roche, Cat. No. 1 209 272) by semi-dry electrophoresis (OWL SEPARATION SYSTEMS, HEP-1Semidry Electroblotter). After transfer, they were crosslinked with 120 mjoules of UV and baked at 80°C for 1 hour. Oligonucleotide probes were labeled using DIG oligonucleotide tailing Kit, 2nd generation (Roche, Cat. No. 3353583) and hybridized to the membranes at 50°C overnight. Membranes were washed twice with 2XSSC, 0.1% SDS and twice with 0.5XSSC, 0.1% SDS. The blots were exposed on X-ray film (Clonex corporation, Bioflex MSI Film for maximum sensitivity imaging, Cat. CLMS810). The oligonucleotide probes used were all LNA-modified (synthesized by IDT) including miR-328 probe: ACGGAAGGGCAGAGAGGGCCAG; miR-1 probe: TACATACTTCTTTACATTCCA; and U6 snRNA probe: TAAAATATGGAACGCTTCACGAATTTGCGTGCATCCTTGCGCAGGGCCATGCTAAT.

**Western Blot Analysis**

The protein samples (membrane and cytosolic samples separately) were extracted from the left atrium of the dogs, cultured rat atrial myocytes, and atrial tissues of transgenic mice for immunoblotting analysis, with the procedures essentially the same as described in detail elsewhere. The protein content was determined by BCA Protein Assay Kit using bovine serum albumin as the standard. Protein sample (~50 µg) was fractionated by SDS-PAGE (12% polyacrylamide gels) and transferred to PVDF membrane (Millipore, Bedford, MA). The sample was incubated overnight at 4°C with the primary antibodies in 1:200. Affinity purified goat
polyclonal anti-CACNB1 (Santa Cruz Biotechnology Inc.) and goat polyclonal anti-Cav1.2 (Alomone Labs), and goat polyclonal antibodies to Kir2.1, Kv4.2, PLN and Cx43 (Santa Cruz Biotechnology Inc.) were used as the primary antibodies. Inhibitory peptide for each antibody was used to test the antibody specificity. Next day, the membrane was incubated with secondary antibodies (Molecular Probes) diluted in PBS for 2 h at room temperature. Finally, the membrane was rinsed with PBS before scanning using the Infrared Imaging System (LI-COR Biosciences). GAPDH was used as an internal control for equal input of protein samples, using anti-GAPDH antibody. Western blot bands were quantified using QuantityOne software by measuring the band intensity (Area x OD) for each group and normalizing to GAPDH. The final results are expressed as fold changes by normalizing the data to the control values.

**Synthesis of miRNAs and anti-miRNA Antisense Inhibitors**

miR-328 (5’-CUGGCCCUCCUCUGCCCUUCGU-3’) and its antisense oligonucleotides (AMOs: 5’-ACGGAAGGGCGAGAGGGCCAG-3’) were synthesized by Integrated DNA Technologies Inc (IDT), as described previously. Five nucleotides or deoxynucleotides at both ends of the antisense molecules were locked (the ribose ring is constrained by a methylene bridge between the 2’-O- and the 4’-C atoms). Additionally, a scrambled RNA was used as negative control; sense: 5’-UUCUCCGAACGUGUCACGUAA-3’ and antisense: 5’-ACGUGACACGUUCCGGAGAAUU-3’.

**Construction of Luciferase-miRNA-Target Site Fusion Plasmids**

To construct reporter vectors bearing miRNA-target sites, we synthesized fragments containing the exact target sites for miR-328 through Invitrogen, and the 3’UTR of miR-328 target genes (CACNA1C and CACNB1) by PCR amplification. The sense and antisense strands of the oligonucleotides were annealed by adding 2 µg of each oligonucleotides to 46 µl of annealing
solution (100 mM K-acetate, 30 mM HEPES-KOH, pH 7.4 and 2 mM Mg-acetate) and incubated at 90°C for 5 min and then at 37°C for 1 h. The annealed oligonucleotides were digested with HindIII and SpeI. These inserts were ligated into HindIII and SpeI sites in the pMIR-REPORT™ luciferase miRNA expression reporter vector (Ambion).  

Construction of Adenovirus and Infection

The procedures were similar to the study reported by van Rooij et al6 Rno-miR-328 precursor DNA (5'-GGATCCgACCCCGTCCCCGTCCTCCCGAGTCCCTCTTTCGTAGATGTCGGGGACCGGGAGAGACGGGAAGGCAGGGGACAAGGGTTTAttttttAAGCTT-3') was synthesized by GenScript (Nanjing, P.R. China). The fragment was first inserted into adenovirus shuttle plasmid pDC316-EGFP-U6 (Microbix Biosystems Inc, Canada). pDC316-EGFP-U6 was then cotransfected with the infectious adenovirus genomic plasmid pBHGloxΔE1,3Cre into 293 cells by liposome reagent. Following co-transfection of these two DNAs, homologous recombination occurred to generate a recombinant adenovirus in which the transgene (pre-miR-328) is incorporated into the viral genome, replacing the ΔE1 region7 (Supplementary Figure 1). Mismatched miR-328 was generated by substituting 6 nts within the seed motif as indicated by underlined and italic letters (5'-

GGATCCgACCCCGTCCCCGTCCTCCCGAGTCCCTCTTTCGTAGATGTCGGGGGTTGTT
GAAACGAGACGGAAAGGCAGGGGACAGGGGTATtttttAAGCTT-3').

In vivo Gene Transfer

Mongrel dogs (20 to 30 kg) of either sex were randomly divided into 4 groups: sham-operated control (control, n=7), adenovirus empty vector control (Adv-pDC316, n=7), adenovirus pre-miR-328 (n=7), and Adv pre-miR-328+AMO-328 (n=7). The dogs were initially anesthetized
using 30 mg/kg of sodium pentobarbital delivered intravenously. Additional anesthesia was administered as needed throughout the experimental study. A right-sided thoracotomy was performed at the first intercostal space. A pericardial cradle was created, and adenovirus (1x10⁹ pfu/ml, 300 µl) was injected through a 26-gauge needle into multiple sites (~10 sites within an area of 1 cm²) of the right atrium. After that, a stimulus electrode with five pairs of electrodes was hooked into the injected sites of the right atrium. Twelve hours after drug injection, atrial activation electrograms were recorded.

**Generation of miR-328 Transgenic Mice**

A fragment (320 bp) containing precursor miR-328 (pre-miR-328) sequence was PCR amplified from the mouse genomic DNA (accession no.: NT_078575). The fragment was then subcloned into the Sal I and Hind III sites of Bluescript vector (Promega) carrying the cardiac-specific α myosin heavy chain (αMHC) promoter and human growth hormone poly(A)⁺ signal (Supplementary Figure 3). The plasmid was digested at the Spe I site to release the pre-miR-328 sequence flanked by 5’end αMHC promoter and 3’end poly(A). The fragment was separated on an agarose gel and purified by QIAEX II gel extraction kit (Qiagen # C 04539). The DNA sample was prepared at a concentration of 3 ng/µl ready for injection. A Tg mouse line carrying a mismached pre-miR-328 sequence as indicated above was also generated for negative control experiments.

Sexually immature female mice (4-5 weeks of age) were superovulated by consecutive PMS and HCG hormone injections to obtain sufficient quantity of (>250) eggs for injection. These female mice were mated with stud males immediately following the HCG injection. Eggs were harvested the next day from the ampulla of the oviduct of the mated females, and treated with hyaluronidase to remove nurse cells. Fertilized eggs were then stored in M16 media (37°C,
5% CO₂) until injection. Each egg was individually micro-injected with the DNA fragment and the eggs which did not survive injection were removed. Pseudo-pregnant female mice were prepared by mating with the vasectomized males. On the day of micro-injection, the pseudo-pregnant females were anesthetized with 0.5% pentobarbital (20 ml/kg). The injected eggs were then implanted in a group of 10-15 bilaterally into the oviduct of these animals. The animals were allowed to recover from anesthesia on a warming plate, and then returned to the animal room. They were kept under sterile conditions throughout their pregnancy.

The genomic DNA was prepared from tail tissue of the transgenic mice and subjected to PCR verification for the presence of miR-328 transgene. The forward primer was designed to recognize αMHC (position: 5250-5268): 5'-CCTTACCCACATAGACCT-3', and the reverse primer was for miR-328 (position: 57-39): 5'-CTGTAGATACTTTCTCCCT-3'. The PCR profiling was composed of an initial denaturing step at 94°C for 2 min and 35 cycles of 94°C (20 s), 60°C (7 s) and 72°C (20 s), followed by a final extension step at 72°C for 5 min.

**Generation of miR-328 Sponge Transgenic Mice**

A fragment containing six anti-miR-328 antisense units 5’-gtcgacacggaaggggagagggccagtc-aggccagatacgaaggggagagggccagtc-aggccagatacgaaggggagagggccagtc-aggccagatacgaaggggagagggccagtc-aggccagatacgaaggggagagggccagtc-aggccagatacgaaggggagagggccagtc-aggccagatacgaaggggagagggccagtt-3’ was synthesized by Shanghai Biological Engineering Inc. The fragment was then subcloned into the Sal I and Hind III sites of Bluescript vector (Promega) carrying the cardiac-specific α myosin heavy chain (αMHC) promoter and human growth hormone poly(A)+ signal. The same procedures as described above for miR-328 were followed.8 Knockdown of endogenous miR-328 in F0 was verified and the transgenic mice of 2 months old were used experimental studies.

**Myocyte Isolation and Primary Cell Culture**
The enzymatic dispersion techniques used to isolate single atrial myocytes from dog, neonatal rat, and mouse have been previously described in detail.\textsuperscript{1,2} Canine myocardial specimens from left atria were cut into chunks and washed three times in oxygenated Ca\textsuperscript{2+}-free Tyrode solution at 37\textdegree C. The tissues were then incubated in 10 ml Ca\textsuperscript{2+}-free Tyrode’s solution containing collagenase (0.25 mg/ml, Type α, Sigma) and BSA (0.2 mg/ml) for 40 min at 37\textdegree C, with the solution constantly gassed with 100% oxygen. Afterwards, tissue were transferred to fresh Ca\textsuperscript{2+}-free Tyrode’s solution containing collagenase (0.13 mg/ml, Type α) until atrial myocytes were dispersed. Isolated myocytes were stored in KB solution (in mM: glutamic acid 70, taurine 15, KCl 30, KH\textsubscript{2}PO\textsubscript{4} 10, HEPES 10, MgCl\textsubscript{2}·6H\textsubscript{2}O 0.5, glucose 10, and EGTA 0.5; pH 7.4 with KOH) at 4\textdegree C until use.

Neonatal rat atrial cardiomyocytes were isolated and cultured with the procedures similar to previously described.\textsuperscript{2} Briefly, 1-3 days old rats were decapitated and their hearts were aseptically removed. The atria were dissected, minced and trypsinized at 37\textdegree C for 10 min. Dissociated cells were plated in 24-well plates in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) containing 10% FBS and 0.1 mM bromodeoxyuridine (Sigma) and the non-adherent cardiomyocytes were removed. The cells (1x10\textsuperscript{5}/well) were seeded in a 24-well plate for further experiments. This procedure yielded cultures with 90±5% myocytes, as assessed by microscopic observation of cell beating. The cardiomyocytes were also verified by positive staining with an anti-\textalpha-actin monoclonal antibody through immunocytochemistry.

For mice, wild-type and transgenic animals of 2–3 months of age were heparinized, anaesthetized with 1% pentobarbital (16 ml/kg). The hearts were rapidly removed, and retrogradely perfused through the aorta using a modified Langendorff apparatus. The preparation was perfused with standard Tyrode’s solution (in mM: NaCl 126, KCl 5.4, HEPES 10,
NaH$_2$PO$_4$·2H$_2$O 0.33, MgCl$_2$·6H$_2$O 1.0, CaCl$_2$ 1.8, and glucose 10; pH adjusted to 7.4 with NaOH) for 5 min, then switched to Ca$^{2+}$-free Tyrode’s solution until it stopped beating, followed by perfusion with the same solution containing collagenase II (7 mg/50 ml) and BSA. The freshly isolated myocytes from the atrial free wall were gently centrifuged and resuspended in the KB medium. All solutions were gassed with 100% oxygen and warmed to (37 ± 0.5°C). Only single rod-shaped, Ca$^{2+}$-tolerant, and quiescent cells with clear cross-striations were selected for electrophysiological recording.

**Cell Culture**

HEK293 (human embryonic kidney cell line) used in this study was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM).

**Transfection Procedures**

Neonatal rat atrial myocytes were transfected with 1 µg miRNA and/or AMOs, and negative control AMOs with lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were used for luciferase assay or were collected for total RNA or protein purification.

**Synthesis and Administration of miR-328 AntagomiR**

miR-328 antagomiR was synthesized by Ribobio Co. (Guangzhou, China). The antagomiR is a single-stranded RNA analogue complementary to the mature miR-328 (5’-GACCGGGAGAGACGGGAAGGCA-3’), which was chemically modified and cholesterol-conjugated from a hydroxyprolinol-linked cholesterol solid support and 2’-OMe phosphoramideites. For negative control experiments, a mismatched miR-328 antagomiR (5’-GGCAAGACGAAACGAGACGACA-3’) was also synthesized. miR-328 antagomiR was injected
into WT or TG mice through the tail-vein at a dosage of 80 mg/kg/d in 0.2 ml saline once a day for three consecutive days. The surface ECG (lead II) was recorded in anesthetized mice once a day (1 h) for 2 weeks.

**Luciferase Activity Assay**

For luciferase assay involving miRNA function, HEK293 cells were transfected with the pMIR-REPORT™ luciferase miRNA expression reporter vector carrying the 3’UTR of miR-328 target genes.2-4

For luciferase assay involving analysis of miR-328 promoter activities, neonatal rat atrial myocytes were similarly transfected with 1 µg PGL3–target DNA (firefly luciferase vector) and 0.1 µg PRL-TK (TK-driven Renilla luciferase expression vector) with lipofectamine 2000. Following transfection (48 h), luciferase activities were measured with a dual luciferase reporter assay kit (Promega) on a luminometer (Lumat LB9507). For all experiments, transfection took place 24 h after starvation of cells in serum-free medium.

**Whole-Cell Patch-Clamp Recording**

Patch-clamp techniques were applied to isolated atrial myocytes from A-TP dogs and transgenic mice. The procedures have been described in detail elsewhere.1-3 Briefly, the pipette of patch electrodes had the tip resistance of 2-3 MΩ when filled with pipette solution. The isolated single cells were placed in a 1-ml chamber mounted on an inverted microscope (IX-70, Olympus) and perfused with Tyrode solution. Whole-cell recording were performed using an amplifier (Axopatch 200B, Axon instrument, USA). Signals were filtered at 1 kHz and data were acquired by A/D conversion (Digidata 1320, Axon Instrument). Ion currents were recorded in the whole-cell voltage-clamp mode. For the recording of L-type Ca²⁺ current (I_{CaL}), the pipette solution contained (in mM) 20 CsCl, 110 Cs-aspartate, 1 MgCl₂, 5 MgATP, 0.1 GTP, 5 Na₂
phosphocreatine, 10 EGTA, and 10 HEPES (pH 7.2 with CsOH). The external Tyrode solution contained (in mM) 136 tetraethylammonium chloride, 5.4 CsCl, 2 CaCl₂, 0.8 MgCl₂, 10 HEPES, and 10 dextrose (pH 7.4 with CsOH). Niflumic acid (50 µM) was added to inhibit Ca²⁺-dependent Cl⁻ current. For recording inward rectifier K⁺ current ($I_{K1}$), transient outward K⁺ current ($I_{to}$), and ultrarapid delayed rectifier K⁺ current ($I_{Kur}$), The pipette solution contained (in mM) 20 KCl, 110 K-aspartate, 1 MgCl₂, 5 MgATP, 0.1 GTP, 5 Na₂ phosphocreatine, 10 EGTA, and 10 HEPES (pH 7.2 with KOH); the external Tyrode solution contained (in mM) 136 NaCl, 5.4 KCl, 2 CaCl₂, 0.8 MgCl₂, 10 HEPES, and 10 dextrose (pH 7.4 with NaOH). And BaCl₂ (2 mM) was included to inhibit $I_{Ca,L}$. Experiments were conducted at 36 ± 1°C. Junction potentials were zeroed before formation of the membrane-pipette seal and they were not corrected for our data analyses. Series resistance and capacitance were compensated and leak currents were subtracted. Cells with significant leak currents were rejected. For analysis, the data were collected to an IBM-compatible computer and analyzed with the use of pCLAMP software system 9.2.

$I_{Ca,L}$ was elicited by 300-ms depolarizing pulses delivered from a holding potential of -50 mV at a frequency of 0.1 Hz. $I_{K1}$ was recorded by 300-ms square pulses ranging from -120 mV to +10 mV at a holding potential of -20 mV at a frequency of 0.1 Hz. $I_{to}$ and $I_{Kur}$ were evoked by 1000-ms depolarizing pulses ranging from -40 mV to +50 mV from a holding potential of -50 mV at a frequency of 0.1 Hz. $I_{to}$ was measured as the difference between the peak current amplitude and the sustained current level, and $I_{Kur}$ was defined as the current amplitude at the end of the 1000-ms pulse. For all recordings, sodium current was inactivated by the holding potentials at or more positive than -50 mV. Since our study was designed for group comparisons of the experimental results, the currents were all recorded immediately after membrane rupture and series resistance compensation in order to minimize the possible time-dependent rundown of
currents. Individual currents were normalized to the membrane capacity to control for differences in cell size, being expressed as current density pA/pF.

Single cell action potentials were recorded under the current-clamp mode and a stimulatory current which is sufficient to induce action potential was used in this experiment. The action potential duration for both 50% and 90% repolarization (APD\textsubscript{50} and APD\textsubscript{90}) was analyzed. To verify rate-dependent APD changes, different stimulatory frequency (0.1 Hz, 1 Hz, and 3 Hz) was applied to record action potential.

**Masson Trichrome Staining of Atrial Tissue**

Atrial tissues collected from different ages of transgenic mice (28 days and 2 month) and the age-matched wild-type littermates were fixed in 4% paraformaldehyde solution and embedded in paraffin and sectioned into 4-μm slices. Slides were hydrated through a series of down-graded alcohols (100%, 95%, and 75%) for 15 min each. The slides were then stained with Masson trichrome for the presence of interstitial collagen fiber accumulation indicative of cardiac fibrosis. After gently rinsing with water, slides were dehydrated through up-graded alcohols for 15 min each, and finally cleared in xylene and coverslipped. Fibrotic areas were stained blue. Photomicrographs were obtained using Olympus microscopes (100×). The ratio of interstitial fibrosis to the total atrial area was calculated from 10 randomly selected microscopic fields (n=5 mice per condition).

**Supplementary Results**

To exclude the possible involvement of structural components such as cardiac fibrosis (in addition to the electrical alterations) in the observed AF in our miR-328/Tg model, we assessed
the anatomical properties of the transgenic heart. In our study, miR-328/Tg mice of 2-month age were used for data collection. We did not see significant differences in the morphology and size of the hearts and the thickness of ventricular walls between miR-328/Tg mice of 2-month age. We observed slightly higher cardiac fibrosis in miR-328/Tg mice than in WT littermates, which might contribute to the sustained AF in addition to the electrical remodeling process in our miR-328/Tg mouse model (Supplementary Figure 7 online). Additionally, we consistently observed sustained AF in younger miR-328/Tg mice (28 days or 4 weeks) after birth that had no cardiac fibrosis.

To exclude the possibility that miR-328 induces AF by targeting ion channel subunits other than CACNA1C and CACNB1, we assessed the effects of miR-328 and antagomiR-328 on the protein levels of several ion channel genes including KCNJ2 (encoding Kir2.1 for \(I_{K1}\)), KCND2 (encoding Kv4.2 for \(I_{to}\) in mice), GJA1 (encoding gap junction channel protein connexin43 or Cx43), and phospholamban (a regulator of the \(Ca^{2+}\) pump). As expected, the protein levels of these genes in miR-328/Tg mice were not different from those in WT littermates and administration of antagomiR-328 did not affect the expression levels either (Supplementary Figure 10 online).

**Supplementary References**


Supplemental Figure Legends

**Supplementary Fig. 1.** Schematic illustration of construction of adenovirus vector carrying pre-miR-328. Rat miR-328 precursor DNA (5’-GGATCCgACCCCGTCCCCCCGTCCTCCCTGAGTCCCTCTTTCGTAGATGTCGGGGACCGGGAGAGACGGGAAGGCAGGGGACAGGGGTTTAttttttAAGCTT -3’) was inserted into adenovirus shuttle plasmid pDC316-EGFP-U6. pDC316-EGFP-U6 was then cotransfected with the infectious adenovirus genomic plasmid pBHGlox∆E1,3Cre into 293 cells by lipofectamine. Following co-transfection of these two DNAs, homologous recombination occurred to generate a recombinant adenovirus in which pre-miR-328 is incorporated into the viral genome, replacing the ∆E1 region.

**Supplementary Fig. 2.** Verification of knockdown of miR-328 by its antisense oligo and antagomiR. Upper panels: data generated by real-time RT-PCR expressed as mean±SEM; lower panels: examples of Northern blot bands. (A) Co-application of AMO-328 with Adv-miR-328 effectively prevents the increase in miR-328 level in tissue mass from canine right atrial free wall subjected to intramuscular injection. *p<0.05 vs pDC316, +p<0.05 vs Adv-miR-328 alone; unpaired student t-test; n=5 tissue samples or cell batches for each group. pDC316: miR-328-free adenovirus vector; Adv-miR-328: miR-328-carrying adenovirus vector; +AMO-328: co-infection with the anti-miR-328 antisense oligo. (B) Co-transfection of AMO-328 with miR-328 prevents the increase in miR-328 level in neonatal rat atrial cells. *p<0.05 vs Lipo, +p<0.05 vs miR-328 alone; unpaired student t-test; n=5 tissue samples or cell batches for each group. Lipo: lipofectamine 2000; +AMO-328: co-transfection with the anti-miR-328 antisense oligo. (C) Tail vein injection of antagomiR-328 rescues overexpression of miR-328 in TG mice (Left panels) and knockdown endogenous miR-328 in WT mice (right panels). WT: wild-type mice; TG: pre-
miR-328 transgenic mice; A-328: antagomiR-328; d0, d7, and d14: day zero, day 7 and day 14 after injection of antagomiR-328. *p<0.05 vs. WT, unpaired t-test; +p<0.05 vs. TG alone, unpaired t-test; n=14 for control/WT group, n=10 for TG group, n=10 for antagomiR-328/TG group, and n=10 for antagomiR-328/WT group.

**Supplementary Fig. 3.** Schematic illustration of engineering the vector carrying pre-miR-328 for generating transgenic mice. A fragment (350 bp) containing pre-miR-328 sequence was PCR amplified from the mouse genomic DNA. The fragment was subcloned into the Sal I and Hind III sites of Bluescript vector (Promega) carrying the cardiac-specific α myosin heavy chain (αMHC) promoter and human growth hormone poly(A)’ signal. The plasmid was digested at the Spe I site to release the pre-miR-328 sequence flanked by 5’end αMHC promoter and 3’end poly(A). The DNA fragment was individually micro-injected into mouse to generate heterozygous miR-328 transgene mice.

**Supplementary Fig. 4.** “Dose-response” relationship between miR-328 level and AF duration in pre-miR-328 transgenic mice (Tg mice). miR-328 level, expressed in Log_{10} scale, represents the fold increase of miR-328 protein level in Tg mice over wild-type littermates. AF duration, expressed as %AF duration, represents the percentage of the AF duration over the total length of time for ECG recordings (120 min) in each mouse. Note that %AF duration increases with increasing miR-328 level in Tg mice, indicating a “dose-response” relationship between AF phenotype and miR-328 level. The circles are the original data and the line is the fit by the Boltzmann distribution using GrpahPad Prism software.
Supplementary Fig. 5. Lack of effects of miR-328 overexpression and antagomiR-328 on inward rectifier $K^+$ current $I_{K1}$ (A) and transient outward $K^+$ current and ultra-rapid delayed rectifier $K^+$ current $I_o$ and $I_{Kur}$ (B) in miR-328 transgenic mice. Unpaired student $t$-test was done between groups and n=7 cells for each group were studied. A-328: antagomiR-328;

Supplementary Fig. 6. Effects of intracardiac pacing (ICP) in the presence of carbachol on miR-328 expression (A) and L-type Ca$^{2+}$ current ($I_{CaL}$, B) in the atrial tissue of wild-type mice. *$p<0.05$ vs Ctl, unpaired $t$-test, n=5 for each group.

Supplementary Fig. 7. Examination of atrial fibrosis in miR-328 transgenic mice. Shown are examples of images of atrial tissue slices with masson trichrome staining. Fibrotic tissue is stained blue. Comparisons between age-matched WT and TG mice are presented for mice 28 days (28d) or two months (2m) after birth. Similar results were consistently observed in another 5 mice for each group.

Supplementary Fig. 8. Alignment of the sequences of miR-328 with its target sites in the 3’-UTRs of CACNCA1 and CACNB1. Shown is complementarity between human miR-328 and their target sites in the 3’-UTRs (3’-untranslated regions) of CACNCA1 (encoding L-type Ca$^{2+}$ channel α1c subunit, Cav1.2; Accession No.: NM_000719) and CACNB1 (L-type Ca$^{2+}$ channel β subunit; Accession No.: BC037311). For each pairs, upper sequence is hsa-miR-328 and the lower sequence represents the motif in the 3’UTR and in the CDR (coding region) of the target mRNA with the position indicated by the numbers. The hsa-miR-328 and rno-miR-328 are identical, and the binding motifs are conserved between human and rat. Boldface letters indicate
that the seed site is critical for miRNA-mRNA binding and interaction and the miRNA::mRNA base pairings.

**Supplementary Fig. 9.** Relative abundance of miR-328, miR-664 and *CACNA1C* transcripts in the RNA samples from healthy canine atrial tissues (A) and human atrial tissue (B), determined by real-time RT-PCR. *p* >0.05, unpaired *t*-test, n=5 batches of cells.

**Supplementary Fig. 10.** Effects of miR-328 overexpression and antagomiR-328 on the protein levels of Kv4.2, Kir2.1, connexin43 (Cx43) and phospholamban (PLN), as a comparison with the effects on Cav1.2 and Cavβ1. A-miR: antagomiR-328.
Supplementary Figure 1
Supplementary Figure 2

Real-Time RT-PCR

A

B

C

Northern Blot

- Ctl
- pDC316
- Adv-miR-328
- +AMO-328

- Ctl
- Lipo
- miR-328
- +AMO-328

- WT
- TG (d0)
- A-328/TG (d7)
- A-328/TG (d14)

- Ctl/WT
- A-328/WT
Supplementary Figure 3

α MHC □ Pre-miR-328 Vector

Spe I

α MHC promoter
β

α1 2 3

9 kb

Sal I

Pre-miR-328

Hind III

hGH

PolyA

Spe I
Supplementary Figure 4

![Graph showing the relationship between %AF Duration and \( \text{LOG}_{10}[\text{miR-328 Level}] \).]
Supplementary Figure 5

A

WT  TG  A-328/TG

$\mathbf{I_{K1}}$

$\mathbf{I_{to}}$

$\mathbf{I_{Kur}}$

B

$\mathbf{I_{to}}$

$\mathbf{I_{Kur}}$

$\mathbf{10 \ pA/pF}$

$\mathbf{100 \ ms}$

$\mathbf{500 \ ms}$
Supplementary Figure 6

A

$\text{I}_{\text{cal}}$ Density

B

Atrial APD$_{90}$

C

Protein Level

D

Surface ECG in Mice
Supplementary Figure 7

WT (28d)  TG (28d)

WT (2m)  TG (2m)
Supplementary Figure 8

**CACNA1C**

3' UGCCUGCUCCUCUCUCCGAC 5'  
1210-UGGCGGCGGACUACGCAC-1231  
3' UGCCUGCUCCUCUCUCCGAC 5'  
1719-AGGAGGCAAAACUCCGGGCGAG-1740  
3' UGCCUGCUCCUCUCUCCGAC 5'  
3270-UAGAGGCCGCAUCAACAGGCGCCA-3291  
3' UGCCUGCUCCUCUCUCCGAC 5'  
3282-UAGAGGCCGCAUCAACAGGCGCCA-3303  
3' UGCCUGCUCCUCUCUCCGAC 5'  
5251-GGCCUGGAAUCGCACGGGCCCA-5272  
3' UGCCUGCUCCUCUCUCCGAC 5'  
5628-GGUCAGCAUUGGAGGCGACA-5647  
3' UGCCUGCUCCUCUCUCCGAC 5'  
7260-UCCGGGGAGCGCCACGGGCCCA-7281  

**CACNB1**

3' UGCCUGCUCCUCUCUCCGAC 5'  
1-UGGCGGCA-22  
3' UGC-GUG---------CCGUCUC-UGCCCGC-3'UTR  
1702-ACGAGUACCCGGG-GAGCUGGCGACAG-1723
Supplementary Figure 9

(A) Human Atrial RNA

(B) Canine Atrial RNA

Copy Number per Cardiac Cell

miR-328  miR-223  miR-664  CACNA1C

miR-328  miR-223  miR-664  CACNA1C

*
Supplementary Table 1. Clinical characteristics of the patients used in our study

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<th>Patient No.</th>
<th>Gender</th>
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<th>Diagnosis</th>
<th>Treatment</th>
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</table>
AF, atrial fibrillation; AVR, aortic valve replacement; AVS, aortic valve stenosis; CAD, coronary artery disease; CHD, congenital heart disease; MVP, mitral valve prolapse; MVR, mitral valve replacement; MVD, mitral valve disease; MVS, mitral valve stenosis; RFA, radiofrequency ablation; RHD, rheumatic heart disease; SR, sinus rhythm; TVP, tricuspid valvuloplasty.