

MicroRNA-34a mediates atrial fibrillation through regulation of Ankyrin-B expression

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Abstract. Atrial fibrillation (AF) has a high prevalence and recurrence rate, and is associated with substantial mortality. However, its underlying mechanisms are not thoroughly understood. Increasing attention has been paid to the roles of microRNAs (miRs) in the pathogenesis of cardiovascular disease, including miR-1 and miR-133 (in the electrophysiological response), and miR-34a (in cardiac fibrosis). Recently, Ankyrin-B (Ank-B), an adaptor protein, has been demonstrated to be associated with AF. As a predicted target gene of miR-34a, the present study aimed to investigate if miR-34a has a role in AF via regulation of Ank-B expression. Western blot analysis revealed that the expression levels of Ank-B was lower in the atrial tissue of AF patients than in individuals with sinus rate (SR); however, reverse transcription-quantitative polymerase chain reaction data demonstrated that miR-34a expression exhibited the opposite pattern. Dual-luciferase assays following the specific overexpression or inhibition of miR-34a indicated that the 3' untranslated region of *Ankyrin 2* (the gene encoding Ank-B) contained binding sites for miR-34a. Furthermore, the expression levels of Ank-B and sodium-calcium exchanger 1 (an Ank-B binding partner important in Ca²⁺ homeostasis), as well as intracellular Ca²⁺ signaling detected by Fluoro-3 AM, were altered following the modulation of miR-34a expression. Thus, miR-34a may serve an important role in early electrophysiological remodeling and the development of AF via the regulation of Ank-B expression. These results offer valuable insight into the underlying

mechanism of AF, and provide a promising target for developing clinical diagnostic tools and potential therapies for patients with AF.

Introduction

Atrial fibrillation (AF) is the most common symptomatic cardiac arrhythmia in clinical practice, which exhibits an increasing prevalence with age. As the currently available therapeutic approaches for treating AF have a number of limitations, including the adverse side effects that may occur with antiarrhythmic drugs and arrhythmia recurrence following AF ablation (1), AF contributes substantially to worldwide morbidity and mortality (1-3). To date, there are 4 principal pathophysiological mechanisms known to contribute to AF including electrical and structural remodeling, alterations in autonomic nervous system function, and abnormalities in intracellular Ca²⁺ handling (4). Each of these may arise from underlying cardiac disease and contribute to the subsequent development of AF, which in turn may cause additional abnormalities in the above aspects, further enhancing a patient's susceptibility to AF induction and maintenance (4). Therefore, innovative studies of the pathophysiology and underlying molecular mechanisms of AF are required to develop novel therapeutic approaches for this disease.

Ankyrins (Anks), a ubiquitously expressed family of intracellular scaffolding proteins, are associated with a diverse set of membrane, cytoskeletal and cytoplasmic proteins; they are known to tether these proteins to specialized membrane signaling domains. The Ank family is comprised of Ank-B, Ank-G and Ank-R, which are encoded by the genes, *Ankyrin-2* (*ANK2*), *ANK3* and *ANK1*, respectively (5). In particular, the targeting and stability of sodium-calcium exchanger 1 (NCX1), inositol triphosphate receptor and Na⁺K⁺-adenosine triphosphatase (NKA) at the membrane of the transverse-tubule (T-tubule)/sarcoplasmic reticulum microdomain in cardiomyocytes are heavily reliant on Ank-B (6). Ank-B also regulates the protein expression and membrane targeting of the K_{adenosine 5'-triphosphate (ATP)} channel subunit Kir6.2 to T-tubules, in addition to modulating K_{ATP} channel ATP sensitivity (7). NCX1, in turn, is an integral membrane protein that is expressed in various tissues and is involved in Ca²⁺ homeostasis (8). As aforementioned, a previous study revealed that Ank-B binds to

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and targets NCX1, and participates in the regulation of Ca^{2+} homeostasis (9).

MicroRNAs (miR/miRNA) inhibit the expression of specific mRNA targets through a base-pairing reaction between the miRNA 'seed region' and sequences commonly located in the 3' untranslated region (3'UTR) of the target mRNA (10). Recently, miR-1, together with miR-133, were observed to regulate protein levels by repressing the translation of genes involved in cardiac contractility, hypertrophy and electrical conductance (11). MiR-34a was reported to serve an important role in cardiac fibrosis in patients and in mice, and it has been suggested that therapeutic inhibition of members of the miR-34 family may attenuate pathological cardiac remodeling and improve cardiac function in patients, as this approach has been proven to be effective in mouse models of cardiac disease (12,13). Electrophysiological alterations and fibrosis are closely associated with AF (5); however, the potential functions of miR-1, miR-133 and miR-34a in AF have been studied to a lesser extent.

In the present study, right atrial tissue samples were collected from rheumatic heart disease patients with AF or sinus rate (SR). As the aforementioned previous studies have reported that miRNAs are associated with AF or with fundamental pathophysiological processes of AF, the present study investigated the expression differences between patient groups. By manipulating the levels of miRNAs in cell culture through overexpression and inhibition strategies, the role of miRNAs in the molecular mechanisms underlying the early electrophysiological changes observed in AF were further examined.

Materials and methods

Human tissue and animal samples. The present study, including all sample collection procedures for human and animal studies, was approved by the Ethics Committee of Xinqiao Hospital (Chongqing, China). Patients participating in the present study were all recruited from the Department of Cardiovascular Surgery of Xinqiao Hospital between the July and October 2013. All patients provided written informed consent. Right atrial tissue (100 mg/patient) was removed from patients with rheumatic heart disease whilst undergoing valve replacement (n=40; Table I); these tissues were then either immediately snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde at 4°C for 48 h. Right atrial tissue was obtained from 20 patients with permanent AF (AF group; n=20) and 20 patients with normal SR (SR group; n=20).

The male neonatal Sprague Dawley (SD) rat pups (age, <5 days; n=20) used in the present study were supplied by the Laboratory Animal Center of the Third Military Medical University (Chongqing, China). Neonatal rats were sacrificed as soon as they were received.

Reagents. The reagents used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The agomir, antagomir and the corresponding negative controls (NC) for miR-34a, as well as the recombinant plasmid vector containing the predicted ANK-2-miR-34a sites and mutations of those sites were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The pmirGLO Dual-Luciferase

miRNA Target Expression Vector and Dual luciferase assay system were purchased from Promega Corporation (Madison, WI, USA). The recombinant plasmid containing the ANK2 wild type (WT) and mutant (MUT) 3'UTR sequences were purchased from Shanghai GenePharma Co., Ltd.

Cell culture. Atrial primary cardiomyocytes were isolated from the auricular appendages and atrial tissues of male neonatal SD rats. Briefly, the auricular appendages and atrial tissues were cut into 1 mm³ granules and digested with pancreatin and collagenase II, filtered through a 200-mesh screen filter to remove undigested cellular aggregates, non-cellular tissue components and debris, and centrifuged at 700 x g for 5 min at 4°C. The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) Cells (1.5x10⁵ cm²) were subsequently placed in a 25 cm culture flask and incubated in 95% air and 5% CO₂ at 37°C for 2 h to allow for the differential attachment of non-myocardial cells. The non-adhesive cells (cardiomyocytes) were transferred into a sterile tube, centrifuged at 700 x g for 5 min at 4°C and subsequently plated into a novel culture flask.

293T cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in 96-well plates in DMEM with 10% FBS in 5% CO₂ at 37°C at 24 h, at which point transfection was performed. All cell culture reagents were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Bioinformatics analysis. Potential miR-34a targets were predicted using the following online databases: TargetScan (version 7.1; www.targetscan.org/vert_71/), PicTar (2006 release; pictar.mdc-berlin.de), miRanda (2010 release; 34.236.212.39/microrna/home.do), RNA22-HAS (2006 release; <https://cm.jefferson.edu/rna22>), miRTarbase (version 6.0; <http://mirtarbase.mbc.nctu.edu.tw/php/index.php>) and miRBD (2015 release; www.mirdb.org).

Transfection. Atrial cardiomyocytes were cultured to 70% confluence in 6-well plates and transfected with a specific miR-34a agomir (0.5 µM), antagomir (1 µM) or the respective matched miRNA NC (Ago-N, 0.5 µM; Ant-N, 1 µM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the transfection reagent. Briefly, transfection experiments were carried out in 500 µl Opti-minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) with the desired concentration of the specific miR-34a interference reagent (agomir, Ago-N, antagomir and Ant-N). Cells were removed following a 6 h incubation period with the transfection reagent in serum free Opti-MEM medium at 37°C; the medium was subsequently replaced with DMEM containing 10% FBS for 48 h at 37°C for the following experiments. The cells of untransfected (Blank) group underwent the same procedure but the interference reagent was replaced with serum free Opti-MEM.

293T cells were cultured to 50% confluence in 96-well plates and transfected with the WT or MUT vector, containing the 3'UTR of ANK2 or the mutant 3'UTR sequence of ANK2 (Shanghai GenePharma Co., Ltd.), respectively, along with the

Table I. Clinical data of patients with atrial fibrillation and sinus rate.

Clinical data	AF group	SR group	<i>t</i> / <i>x</i> ²	P-value
Age (years)	52.9±5.8	52.4±6.2	0.237	0.814
Sex (M/F)	8/12	9/11	0.102	0.749
Body weight (kg)	51.9±8.7	53.5±8.4	0.593	0.557
LVEF (%)	51.7±6.3	53.7±7.9	0.881	0.384
LVFS (%)	33.6±3.3	33.4±3.2	0.163	0.872
Preoperative LA (mm)	49.6±5.0	48.5±6.6	0.617	0.541
DVR/MVR (n)	12/8	10/10	0.404	0.525
Postoperative hospital stay (days)	10.7±2.0	11.1±2.0	0.720	0.476

Values are presented as the mean ± standard error mean. AF, atrial fibrillation; SR, sinus rate; DVR, double valve replacement; MVR, mitral valve replacement; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LA, left atrial diameter.

miR-34a agomir, antagomir, Ago-N or Ant-N. Transfection was carried out in 200 μ l Opti-MEM with 2 μ M of WT or MUT vector and the specific miR-34a reagent. For all experiments, transfection took place 24 h following the starvation of cells in serum-free medium. Transfection experiments for each construct and/or miRNA were performed in triplicate in each assay and three assays were performed in total.

RT-qPCR. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) from cells transfected with each specific miR-34a reagent or from untransfected controls. RT was carried out using the RTPrimeScript™ RT reagent kit (Perfect Real Time) as per the manufacturer's instructions (Takara Biotechnology Co., Ltd.). The temperature protocol was as follows: 42°C for 30 min and 85°C for 5 sec. Amplification of cDNA products was performed with the SYBR® Premix Ex Taq™ II (Perfect Real Time) amplification kit (Takara Biotechnology Co., Ltd.) using U6 snRNA as the reference gene, and the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Initial denaturation occurred at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Bulge-Loop™ Primers for miR-34a (hsa-miR-34a, cat. no. miRQ0000255-1-21; mmu-miR-34a, cat. no. miRQ0000542-1-2; sequences unavailable) and U6 (cat. no. MQP-0202; sequence unavailable) detection were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). PCR results were quantified using the 2^{- $\Delta\Delta C_q$} method (14).

Isoprenaline (ISO) mediated Ca²⁺ signaling detection. Primary SD neonatal atrial cardiomyocytes were treated with 10M ISO for different periods at 37°C (30 min, and 1, 1.5, 3, 6, 12, 24, 48 or 72 h) to mimic the early electrophysiological change of atrial cardiomyocytes in AF (15). Transfection of miR-34a interference reagents was performed and miR-34a expression levels from the various cultures were determined as aforementioned. The calcium fluorescent dye Fluro-3 AM (10 μ M; Thermo Fisher Scientific, Inc.) was added into the serum-free DMEM and subsequently added to atrial cardiomyocytes at 37°C for 30 min. Cellular Ca²⁺ signaling of each group was detected at 37°C using a laser scanning confocal microscope in the line scanning mode. Quantification of fluorescence intensity was

performed with the Leica Las AF lite software (version 2.6; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Luciferase activity assay. miRNA function following transfection of miRNA modifier reagents was measured using a luciferase activity assay. 293T cells were transfected with pmirGLO™ luciferase miRNA expression reporter vectors (Promega Corporation) carrying the 3'UTR of the potential miR-34a target gene ANK-2 or its mutant variant, along with miR-34a modifier reagents as described above using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection for 48 h, luciferase activities were measured using a dual luciferase reporter assay kit (Promega Corporation) on a luminometer (Lumat LB9507; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) according to the manufacturer's instructions. *Renilla* luciferase activities were measured for normalization.

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded explanted human atrial tissue fixed in 4% paraformaldehyde at 4°C. Paraffin sections (6 μ m) were immersed in 1,2-dimethylbenzene I for 10 min, 1,2-dimethylbenzene II for 10 min, 1,2-dimethylbenzene with ethyl alcohol (1:1), absolute ethyl alcohol for 5 min, 95% ethyl alcohol for 5 min, 75% ethyl alcohol for 5 min, 50% ethyl alcohol for 5 min in turn. Sections were subsequently washed in PBS for 3 min three times. Following microwave antigen retrieval at 120°C for 5 min and 85°C for 15 min with the addition of citric acid buffer every 4 min to prevent drying, 3% H₂O₂ was added for 15 min at room temperature and sections were incubated overnight with the primary antibody anti-Ank-B (1:50; cat. no. sc-12718; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C. After washing the sections with PBS-0.1% Triton X-100 for 5 min five times, sections were incubated with horseradish peroxidase-labeled goat anti-mouse secondary antibody (1:1,000, cat. no. A0216; Beyotime Institute of Biotechnology, Shanghai, China) at 37°C for 50 min and subsequently washed with PBS-0.1% Triton X-100 for 5 min five times. Immunoreaction was revealed with 3,3'-diaminobenzidine and observed using an inverted microscope. Results were analyzed with Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Protein extraction and western blot analysis. The expression of Ank-B in the atrial tissue of patients and in the primary atrial cardiomyocytes of neonatal SD rat pups transfected with miR-34a specific interference sequences or in untransfected controls was determined using western blotting. Total protein from tissue and cell cultures was extracted with SDS Lysis Buffer (Beyotime Institute of Biotechnology) and protein concentration was determined with the bicinchoninic acid protein assay and proteins (20 μ g/lane) were separated using 10% SDS-PAGE. Gels were transferred using a wet transfer method to a polyvinylidene fluoride membrane (Roche Diagnostics, Basel, Switzerland) and blocked with 5% non-fat dried milk at room temperature. Membranes were subsequently incubated overnight at 4°C with the primary antibodies Ank-B (1:200; cat. no. sc-12718; Santa Cruz Biotechnology, Inc.), NCX1 (1:100; cat. no. bs-1550R; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) and β -actin (1:1,000; cat. no. bs-0061R; Beijing Biosynthesis Biotechnology Co., Ltd.). The membranes were washed in PBS with 0.1% Tween for 5 min five times, prior to incubation with horseradish peroxidase-labeled goat anti-rabbit (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) and anti-mouse (1:1,000; cat. no. A0216; Beyotime Institute of Biotechnology) for 1 h at 37°C. Following washing the membrane in PBS with 0.1% Tween for 5 min five times, immunoreactivity was revealed by chemiluminescence using an Alpha Innotech FluorChem imaging system (Canberra Packard Central Europe GmbH, Schwadorf, Austria) with Fluorchem SP software (version 2.2; ProteinSimple, San Jose, CA, USA). The relative band densities were analyzed with ImageJ 1.5.0 (National Institutes of Health, Bethesda, MD, USA). β -actin was used as the internal reference. The SR group was used as the control for protein level analysis in atrial tissue, and the untransfected or untransfected without ISO groups served as the controls for *in vitro* analyses. All samples were run at least 3 times.

Statistical analysis. Student's t-test, chi-square test or one-way analysis of variance followed by Tukey's post-hoc test was used to analyze the statistical significance of data with SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean \pm standard error mean of at least 3 independent experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ank-B expression was inversely proportional to miR-34a expression in the AF and SR patient groups. The present study examined the protein expression levels of Ank-B, an intracellular scaffolding protein that is important for ion homeostasis and is associated with AF (16), in cardiac tissue from patients with AF or SR. As presented in Fig. 1A, Ank-B expression in the atrial tissue from the AF group (Fig. 1A-a and c) was lower than that observed in the SR group (Fig. 1A-b and d), as determined by the decreased amount of positive brown signaling in the AF images. The results of western blot analysis of atrial tissue protein were consistent with those observed from immunohistochemistry staining, as measured by chemiluminescent quantitation (Fig. 1B and C). Notably, as shown in Fig. 1D, the relative expression of miR-34a, as measured by RT-qPCR,

was higher in the atrial tissue of the AF group when compared with the SR group; however, the relative expression of miR-1 and miR-133 was lower in the AF group than in the SR group. MiR-1 and miR-133 serve important roles in cardiac electrophysiological remodeling, and are known to be closely associated with congenital and acquired heart disease (11,17). Recently, miR-34a was noted for its significant effect on the development of fibrosis (18) and, in particular, of cardiac fibrosis (12,13). Therefore, the present study performed bioinformatics analysis using online databases, which revealed that 4 of the 6 databases predicted that the 3'UTR of *ANK2* (Ank-B) contained potential miR-34a target binding sites; while one website predicted that the 3'UTR of *ANK2* (Ank-B) also contained potential miR-133 target sites. These results, combined with the accepted mechanism of miRNA function, indicated that miR-34a may have some co-regulatory connection with Ank-B.

miR-34a overexpression enhances Ca^{2+} signaling in atrial cardiomyocytes treated with ISO. Atrial electrical remodeling is an important component of the primary pathophysiological process in the development of AF, and intracellular calcium signaling is the principle constituent of early electrical remodeling (4). To imitate the early electrophysiological change of atrial cardiomyocytes in AF, the present study treated primary SD neonatal atrial cardiomyocytes with 10M ISO (15). ISO, a non-selective β -adrenergic agonist, has been shown to increase the intracellular Ca^{2+} transient and contraction amplitudes in cardiomyocytes via the β -adrenergic receptor-adenylyl cyclase-cyclic adenosine 5'-phosphate-protein kinase A signaling pathway (19). qPCR analysis demonstrated that the relative expression of endogenous miR-34a increased with ISO treatment duration, prior to peaking at 12 h and then declining (Fig. 2A). In order to observe and analyze the effects of miR-34a on intracellular Ca^{2+} signaling, the present study induced the overexpression or silencing of miR-34a in primary SD neonatal atrial cardiomyocytes via transfection with an miR-34a specific agomir or antagomir (chemically modified double stranded miRNA mimic, or single stranded complementary, interfering, oligonucleotides), respectively (Fig. 2B). Following 12 h of ISO treatment, the intracellular Ca^{2+} signal of treated cells was stronger than that of the untreated group. Simultaneous overexpression of miR-34a further enhanced intracellular Ca^{2+} signaling. The strength and frequency of the signal were increased in treated cells (Agomir+ISO; Fig. 2C-d), compared with the blank (Fig. 2C-a), Ago (Fig. 2C-b) and ISO groups (Fig. 2C-c). By contrast, inhibition of miR-34a did not lead to a significant difference between antagomir transfected and untransfected control groups. Thus, inhibition of miR-34a did not appear to be sufficient to counterbalance the change in intracellular Ca^{2+} signaling caused by ISO; however, miR-34a overexpression did enhance it. It was therefore hypothesized that the increased expression of miR-34a may elicit, or cause the further deterioration of, early electrophysiological changes in the atrial cardiomyocytes.

***ANK2* is a target of miR-34a.** *ANK2*, the gene encoding Ank-B, was the predicted potential target gene of miR-34a, as predicted by 4 of the 6 bioinformatics databases (Fig. 3A). Ank-B participates in cellular Ca^{2+} signaling via direct physical connections with a number of ion channels and exchangers (6);

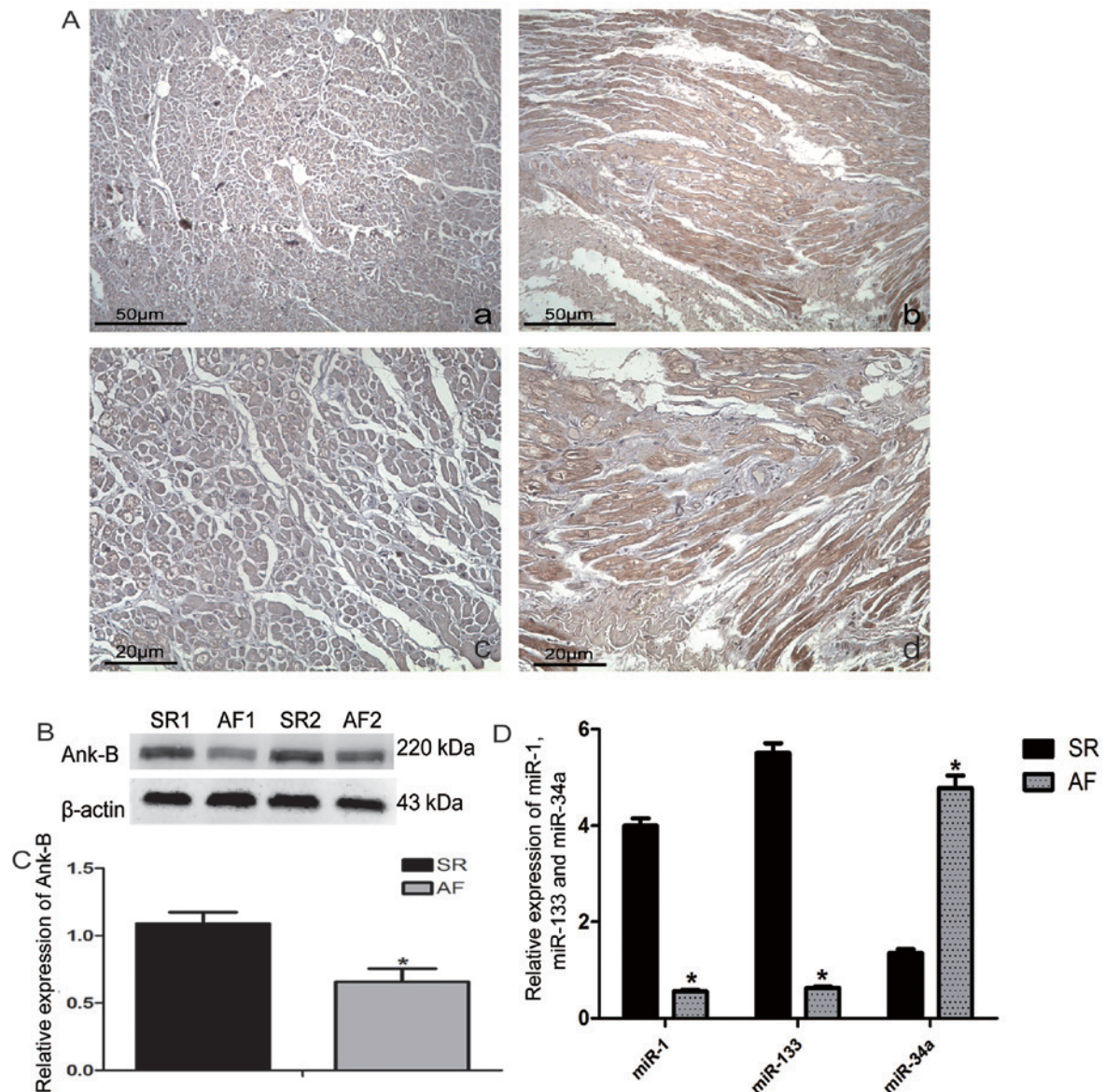


Figure 1. Expression of miR-1, miR-133 and Ank-B were lower while the expression of miR-34a was higher in the AF group when compared with the SR group. (A) The expression of Ank-B expression was evaluated using immunohistochemistry where images (a and c) are the SR group and images (b and d) are the AF group. (B) Ank-B protein expression was also determined by western blotting, which revealed that (C) Ank-B expression was significantly decreased in the AF group. (D) The expression of miR-1, miR-133 and miR-34a was investigated in the SR and AF groups reverse transcription-quantitative polymerase chain reaction. Values are presented as the mean \pm standard error mean. * $P < 0.05$ vs. SR group. miR, microRNA; Ank-B, Ankyrin-B; AF, atrial fibrillation; SR, sinus rate.

it is unknown whether miR-34a affects intracellular Ca^{2+} signaling via a direct physical connection with *ANK2* as well, albeit through a completely different mechanism. To further elucidate the underlying mechanism of the apparent miR-34a Ank-B co-regulation, and to confirm our previous hypothesis, the present study cloned the WT 3'UTR of *ANK2* into the pmirGLO-vector, a reporter construct for the measurement of miRNA activity, and co-transfected the miR-34a agomir or antagomir with the vector into 293T cells (Fig. 3B). The results revealed that the normalized luciferase activity of the agomir-transfected group was significantly reduced, while the normalized luciferase activity of the antagomir-transfected group increased, when compared with their respective negative (scrambled) and untransfected controls (Fig. 3C). In addition, a vector construct containing a version of the *ANK2* 3'UTR sequence was generated, in which the predicted miR-34a

binding site had been mutated (MUT type) and co-transfected along with the miR-34a agomir and antagomir into 293T cells. In these experiments, no significant difference in the normalized luciferase activities between the groups was observed ($P > 0.05$; Fig. 3C). Furthermore, transfection of primary SD neonatal atrial cardiomyocytes with the miR-34a agomir (mimicking overexpression) significantly reduced the protein levels of Ank-B; however, transfection with the miR-34a antagomir increased the levels of Ank-B ($P < 0.05$; Fig. 3D). Together, these results suggested that miR-34a may inhibit the expression of Ank-B by directly binding to the 3'UTR of *ANK2*, and that the effect of miR-34a expression on intracellular Ca^{2+} signaling may also be explained by this mechanism.

Effects of miR-34a modulation on Ank-B and NCX1 expression in primary rat neonatal atrial cardiomyocytes treated

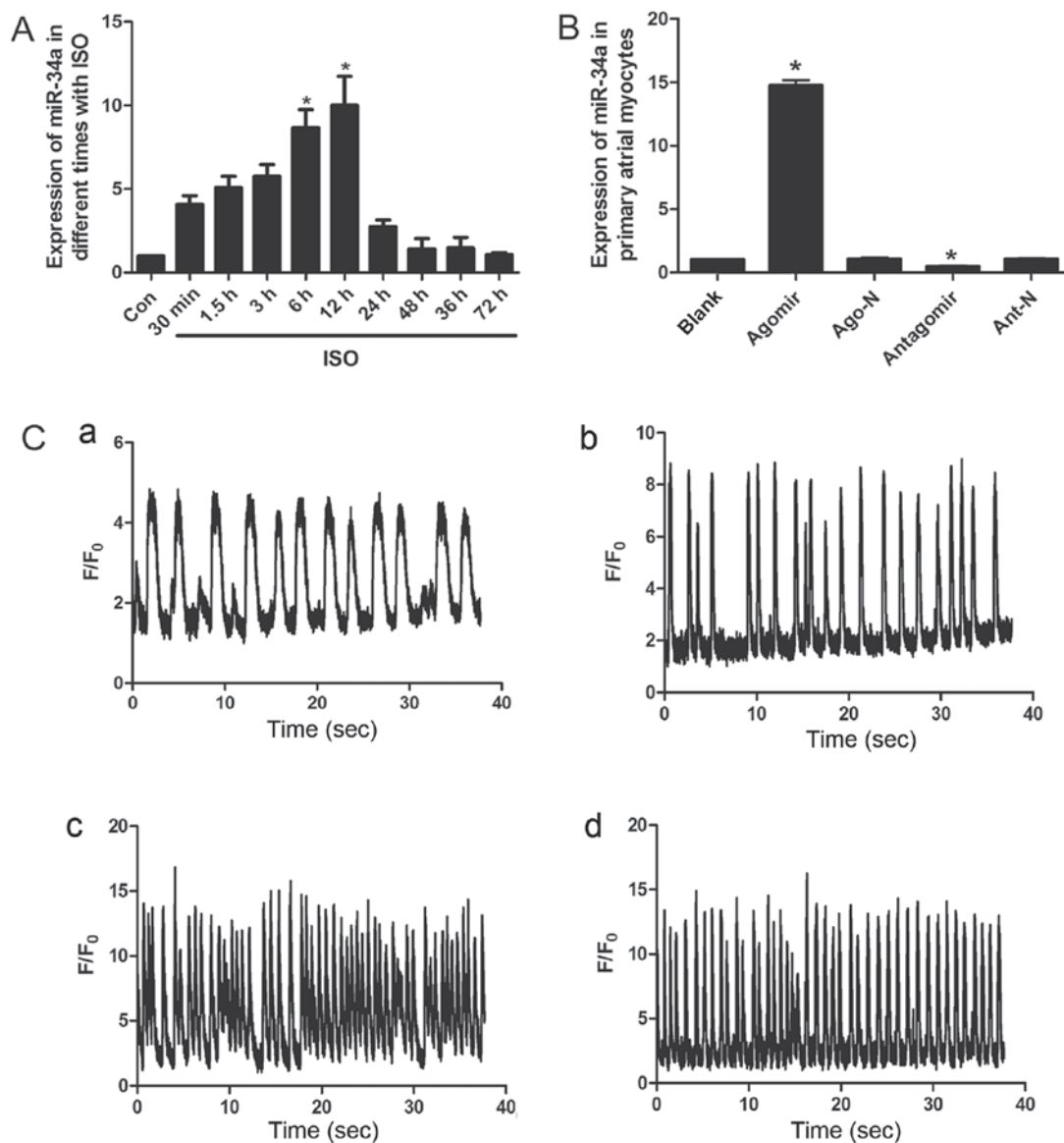


Figure 2. (A) Expression of miR-34a in neonatal SD primary atrial myocytes increased with different ISO treated durations, prior to peaking at 12 h of ISO treatment and then decreasing. The 6 and 12 h groups were significantly different. (B) The expression of miR-34a in neonatal SD primary atrial myocytes in the agomir and antagomir groups was significantly different. (C) The Ca^{2+} signaling of neonatal SD primary atrial myocytes in the different groups: a) blank, b) treated with agomir, c) treated with ISO and d) treated with agomir + ISO. Values are presented as the mean \pm standard error mean. * $P < 0.05$ vs. the respective controls. Con, control; miR, microRNA; SD, Sprague Dawley; ISO, Isoprenaline; Ago-N, agomir-negative control; Ant-N, antagomir-negative control.

with ISO. One of the membrane proteins that serves as a binding partner with Ank-B is NCX1, a Na^+/Ca^{2+} exchanger involved in the regulation of Ca^{2+} homeostasis (9). The present study demonstrated that overexpression of miR-34a in primary rat neonatal atrial cardiomyocytes led to lower levels of Ank-B and NCX1, while inhibition of miR-34a led to higher levels (Fig. 3D-F), suggesting that miR-34a may impact cellular Ca^{2+} signaling directly by regulating the expression of Ank-B and indirectly by affecting NCX1.

Following exposure to ISO, the expression of Ank-B and NCX1 was lower than that in cells without ISO treatment; however, the trends in the expression of these proteins following the overexpression and inhibition of miR-34a in cells exposed to ISO were consistent with those exhibited by cells without ISO treatment (Fig. 3E and F). Therefore, ISO exposure had no influence on the regulation of miR-34a to the expression of

Ank-B and NCX-1, while miR-34a with ISO exposure had a synergistic effect on calcium signaling.

Discussion

Atrial electrical remodeling, which can cause impairment in atrial conduction, is characterized by a reduction of the effective refractory period and the action potential duration (APD), the dispersion in refractoriness and a reduction in the conduction velocity of impulse propagation (20). These alterations may often have been elicited during the early process of remodeling, and are maintained in the development of AF (20). The atrial enlargement and fibrosis observed in AF is characterized by structural remodeling, which causes further conduction disturbances. These electrophysiological changes have, until recently, provided the basis

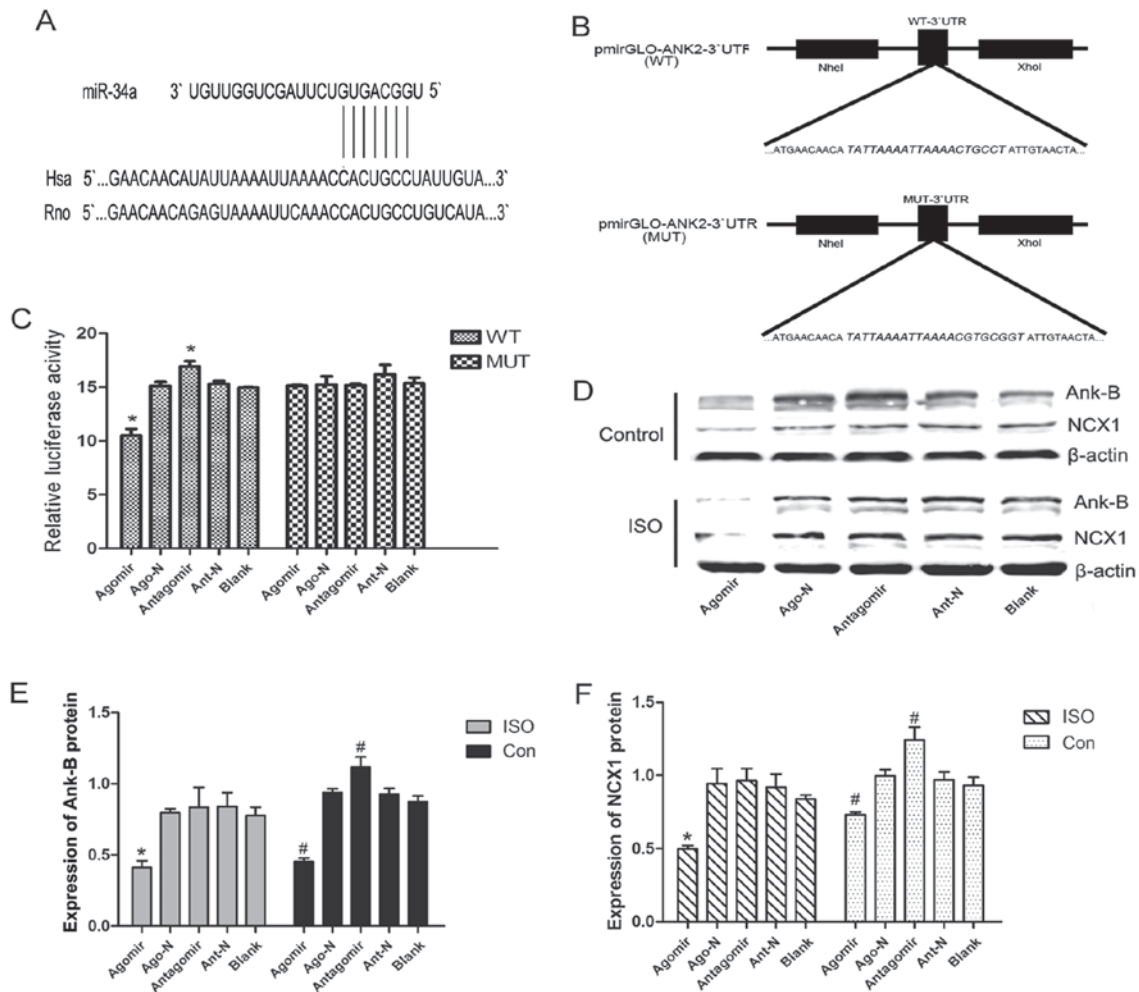


Figure 3. *ANK2* is a target of miR-34a. (A) Sequence alignment of miR-34a and 3'UTR of *ANK2* using the TargetScan algorithm. (B) Plasmid construction: 3'UTR of *ANK2* or a mutated sequence was cloned into pmirGLO-vector. (C) Luciferase reporter assay with co-transfection of WT or MUT 3'UTR, and agomir, Ago-N, antagomir, Ant-N or blank control in 293T cells. or MUT group, WT, wild-type; MUT, mutant (D) Western blotting results revealed the relative expression of (E) Ank-B and (F) NCX1. Values are presented as the mean \pm standard error. * $P < 0.05$ vs. the corresponding blank group. Con, control; *ANK2*, Ankyrin 2; Ank-B, Ankyrin B; miR, microRNA; UTR, untranslated region; NCX1, sodium-calcium exchanger 1; ISO, Isoprenaline; Ago-N, agomir-negative control; Ant-N, antagomir-negative control.

for the majority of the therapies for AF; however, AF itself promotes the remodeling process, which in turn contributes to the therapeutic resistance observed in patients with long-standing arrhythmia (21).

The fundamental molecular basis of electrical remodeling is an alteration in the function and/or expression of ion channels and exchangers, including the NCX1, NKA, and L-type Ca^{2+} -channels and ryanodine receptors (4). As aforementioned, Ank-B binds to a number of different types of ion channels and exchangers in cardiomyocytes, and serves an important role in maintaining cellular homeostasis for various ions, contributing to proper cell functioning (6). *ANK2* gene variants are associated with sinus node dysfunction in humans, which are often accompanied by AF (22). A previous study revealed that mice heterozygous for an *ANK2* deletion (Ank-B^{+/-}) exhibited atrial electrophysiological dysfunction and increased susceptibility to AF, and that Ank-B^{+/-} atrial myocytes displayed shortened action potentials (23). In the present study, the expression of Ank-B was decreased in cardiac dysfunction patients with AF when compared with those with SR, and that the level of Ank-B

was decreased in atrial myocytes treated with ISO, which mimics the early electrophysiological changes seen in AF. Furthermore, it was observed that the cells with decreased levels of Ank-B expression displayed abnormal Ca^{2+} signaling as well. It was therefore hypothesized that a hereditary *ANK2* gene mutation may cause AF, and an acquired aberrant function and/or expression of Ank-B may also be associated with AF. However, the underlying mechanism that may be accountable for the acquired aberrant expression of Ank-B in these experiments was not immediately evident.

The role of miRNAs in the development of the heart and of heart diseases has also become more apparent. In the present study, some of the miRNAs associated with electrophysiological processes, such as miR-1 and miR-133, and with the development of fibrosis, such as miR-34a, were investigated. The results demonstrated that the levels of miR-1 and miR-133 were decreased in the atrial tissue from patients with AF when compared with those in tissues from patients with SR, while miR-34a exhibited the opposite trend. Bioinformatics analysis predicted that the 3'UTR of *ANK2* may contain homology to the 'seed region' of miR-34a and miR-133, with

a higher likelihood of being assigned to miR-34a as it was flagged by several different analysis programs. However, the changes in miR-1 and miR-133 expression presented a similar trend to that of Ank-B in the atrial tissue of AF patients. Although miR-1 and miR-133 are associated with various ion channels (10,24), of which, some directly bind to Ank-B (7,25), as the majority of miRNAs tend to downregulate the expression of their target genes, it was suggested that miR-1 and miR-133 may participate in the development of AF via different signaling pathways, as opposed to through the direct regulation of Ank-B expression.

MiR-34a was revealed to be induced in the aging heart and was associated with heart fibrosis; the inhibition of members of the miR-34 family could also attenuate pathological cardiac remodeling and improve cardiac function (12). The present study identified direct potential binding sites for miR-34a in the 3'UTR of *ANK2* and demonstrated that the expression of Ank-B could be regulated by the modulation of miR-34a. It was also demonstrated that the levels of NCX1, an ion exchanger, were indirectly affected by miR-34a modulation, potentially through Ank-B as an intermediary, which may account for the smaller degree of alterations observed in the expression of NCX1 than of Ank-B. In addition, it was further deduced that the changes in intracellular Ca^{2+} signaling associated with modulation of miR-34a expression may be as a result of Ank-B functioning. Lower levels of Ank-B and NCX1 influenced the outward currents in the plateau phase of atrial myocytes, leading to the reduction in APD and Ca^{2+} signal enhancement. Upon treatment with ISO, the overexpression of miR-34a enhanced the Ca^{2+} signal in terms of strength and frequency, while inhibition of miR-34a was unable to counteract the effect of ISO, which was attributed to the complexities of the response to ISO treatment. The aberrant cellular Ca^{2+} signaling observed in the present study following miR-34a modulation was similar to the early electrophysiological changes seen in AF. Therefore, it was hypothesized that miR-34a may be involved in the development of AF by regulating the expression of Ank-B, and in turn, disrupting normal cell signaling.

In conclusion, the results of the present study demonstrated that miR-34a is upregulated in AF, and that it may serve an important role in the early electrophysiological changes and development of AF via the regulation of the expression of Ank-B. Notably, miR-34a was also a miRNA identified as having a role in fibrosis, and the present study revealed that it is also associated with electrophysiological changes. Taken together, these results provide a significant and valuable basis for the further study of the underlying mechanism of AF, and also a promising target for the development of clinical diagnosis strategies and therapies to treat patients with AF.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ performed the experiments and was a major contributor in writing the manuscript. YZ and ZF analyzed the data. WC and YX conceptualized the study design and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study, including all sample collection procedures for human and animal studies, were approved by the Ethics Committee of Xinqiao Hospital (Chongqing, China). All participants provided written informed consent.

Consent for publication

All participants provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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