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# Regulation of cardiac CACNB2 by microRNA-499: Potential role in atrial fibrillation

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ABSTRACT

The L-type calcium channel (LTCC) is one of the major ion channels that are known to be associated with the electrical remodeling of atrial fibrillation (AF). In AF, there is significant downregulation of the LTCC, but the underlying mechanism for such downregulation is not clear. We have previously reported that microRNA-499 (miR-499) is significantly upregulated in patients with permanent AF and that *KCNN3*, the gene that encodes the small-conductance calcium-activated potassium channel 3 (SK3), is a target of miR-499. We found that CACNB2, an important subunit of the LTCC, is also a target of miR-499. We hypothesize that miR-499 plays an important role in AF electrical remodeling by regulating the expression of *CACNB2* and the LTCC. In atrial tissue from patients with permanent AF, CACNB2 was significantly downregulated by 67% ($n = 4$, $p < 0.05$) compared to those from patients with no history of AF. Transfection of miR-499 mimic into HL-1 cells, a mouse hyperplastic atrial cardiac myocyte cell-line, resulted in the downregulation of CACNB2 protein expression, while that of miR-499 inhibitor upregulated CACNB2 protein expression. Binding of miR-499 to the 3′ untranslated region of *CACNB2* was confirmed by luciferase reporter assay and by the increased presence of *CACNB2* mRNA in Argonaute pulled-down microRNA-induced silencing complexes after transfection with the miR-499 mimic. In addition, downregulation of CACNB2 resulted in the downregulation of protein levels of the pore-forming α-subunit (CACNA1C). In conclusion, upregulation of atrial miR-499 induces the downregulation of CACNB2 expression and may contribute to the electrical remodeling in AF.



## 1. Introduction

Atrial fibrillation (AF) results in substantial changes in the electrical and structural properties of the fibrillating atria and is termed "remodeling" [1–3]. AF electrical remodeling is associated with significant shortening of the atrial action potential and refractoriness as well as loss of rate adaptation [4–6]. Central to these changes is the profound downregulation of the LTCC, which accounts for the signature changes in AF electrical remodeling [1,7,8]. The functional cardiac voltage-gated LTCC channel is a multimeric protein consisting of a α1 pore-forming subunit and several accessory subunits denoted β, $\alpha_2$-δ, and γ [9]. The CACNB2 subunit is encoded by the *CACNB2* gene in humans and is known to increase the current amplitude, accelerate activation and slow inactivation kinetics of the L-type calcium current ($I_{Ca,L}$) when co-expressed with *CACNA1C* [10–13]. The clinical significance of *CACNB2* is underscored by the association of its mutations with sudden death syndromes. Patients with a mutation in the C-terminus of *CACNB2* (S481 L) demonstrate short QT intervals, elevated ST segments, and sudden death, [14] while those with a mutation in the N-terminus (T11I) develop Brugada syndrome with accelerated inactivation of cardiac L-type calcium current [15]. In addition, *CACNB2* is implicated in a wide range of psychiatric and neurologic disorders including migraine, autism, bipolar disorder, major depressive disorder, and schizophrenia [16–18].

MicroRNAs (miRs) have emerged as important regulators of gene expression, and are predicted to regulate at least a third of all human

*Abbreviations:* AF, atrial fibrillation; Ago, Argonaute; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LTCC, L-type calcium channel; miR-499, microRNA-499; miRISC, microRNA-inducing silencing complex; CACNA1C, voltage-dependent calcium channel α-1C subunit; CACNB2, voltage-dependent calcium channel β-2 subunit; SR, sinus rhythm; UTR, untranslated region.

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genes [19]. Recent reports have shown that microRNAs are involved in the regulation of cardiac electrophysiology [20–22]. We have previously reported that miR-499 was significantly elevated in patients with permanent AF, and that *KCNN3*, which encodes the small-conductance calcium-activated potassium channel 3 (SK3), was a target of miR-499 [23]. A key observation gained from miR research is that miRs target multiple functionally related mRNAs, modulating specific pathological processes at multiple levels and sites [24]. Since the LTCC is known to be important players in the electrical remodeling of AF, we examined the 3′-untranslated region (3'UTR) of the LTCC subunits and noted that the binding motif for miR-499 is present in *CACNB2*, which could be a target of miR-499 regulation. In this study, we found that expression of CACNB2 is downregulated in patients with AF. Hence, we sought to determine whether the downregulation of *CACNB2* expression in AF is mediated through the action of miR-499.

## 2. Materials and methods

### 2.1. Patients

This study was conducted according to the principles of the Declaration of Helsinki and informed consent was obtained from all patients. This study was approved by the Mayo Clinic Institutional Review Board. Right atrial appendages were obtained during cardiac surgery and were immediately snap-frozen and stored at −80 °C. Patients with dilated cardiomyopathy, congenital heart disease, uncontrolled hypertension (>160/90 mm Hg), type 1 diabetes mellitus, or untreated obstructive sleep apnea were excluded. The clinical characteristics of the patients from whom samples were obtained are summarized in Table 1 of Supplemental Materials.

### 2.2. MicroRNA target prediction

Potential targets of miR regulation were predicted by using the miRwalk database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) [25] with the following algorithms: Targetscan, miRanda, DIANA-microT, PITA, and miRwalk. The miR best predicted by these algorithms to regulate the *CACNB2* gene was selected for further analysis.

### 2.3. HL-1 cell culture

HL-1, a mouse atrial hyperplastic myocyte cell line, was kindly provided by Dr. William C. Claycomb and cultured with Claycomb Medium [26]. Transfection of 100 nM mouse miR-499-5p mimic (Qiagen, Germantown, MD, Cat. No. MSY0003482, 5′UUAAGACUUGCAGUGAUGUUU3′), miR-499 mimic negative control (Qiagen, AllStars Negative Control siRNA, Cat. No. 1027280), miR-499 inhibitor (Qiagen, Cat. No. MIN0003482, 5′ AAACAUCACUGCAAGUCUUAA3′), or miR-499 inhibitor negative control (Qiagen, Cat. No. 10272715) into HL-1 cells was performed with Lipofectamine 2000 (Invitrogen, Grand Island, NY) as reported previously [23]. Cells were used for experiments 48 h after transfection unless otherwise specified. The miR-499 mimic consisted of chemically synthesized double-stranded RNAs that simulate native miR-499. The miR-499 inhibitor consisted of single-stranded 2′-O-methylated (for stability against degradation) oligonucleotides complementary to miR-499 and inhibited endogenous miR-499 from regulating its targets. For the miR-499 mimic negative control, we used the AllStars Negative Control siRNA, which consisted of a scrambled RNA sequence, that has been thoroughly tested and validated and has no homology to any known mammalian gene. The miScript Inhibitor Negative Control, without homology to any known mammalian gene, was used as the miR-499 inhibitor negative control.

### 2.4. Western blot analysis

Western blotting was performed as previous described [23,27,28]. Polyclonal anti-CACNB1 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-CACNB2 (1:100 dilution; Abcam Inc., Cambridge, MA), monoclonal anti-CACNB3 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-CACNA1C (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal anti-actin (1:2500 dilution; Oncogene Research Products, Boston, MA) antibodies, were used in this study. Results were normalized against the expression of loading controls of GAPDH or actin.

### 2.5. Real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was performed as previously described [23]. The primer pairs for *CACNB2* were as follows:

Forward: 5′-TGC CTC TTG CTC CTG TTG TT-3′.

Reverse: 5′-GGG CTC TTG CGG CTA ATA CA-3′.

The primer pairs for *GAPDH* were as follows:

Forward: 5′-CCA CCT TCG ATG CCG GGG CT-3′.

Reverse: 5′-GGG GCC GAG TTG GGA TAG GG-3′.

The reaction had an initial denaturation step at 95 °C for 3 min, followed by 50 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s, and extension at 72 °C for 45 s. Fluorescence was measured at the end of each extension step, and data were normalized to *GAPDH* from the same preparation.

### 2.6. Luciferase gene expression reporter assay

The luciferase reporter assay was performed as previously described [23]. A fragment of the 3′-UTR of *CACNB2* containing the predicted binding site for miR-499 was subcloned into the luciferase reporter vector (Switchgear Genomics, Menlo Park, CA). As recommended by the manufacturer, the vector was cotransfected with miR-499 mimic or mimic negative control into HL-1 cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY) for 24 h, the time period required for the mimic to produce optimal results. The cells were then lysed and assayed using the LightSwitch Luciferase Assay Reagent LS010 (SwitchGear Genomics, Menlo Park, CA) and the luciferase reporter signal was measured by a plate luminometer (Synergy 2; BioTek, Winooski, VT).

### 2.7. Co-immunoprecipitation and Argonaute pull-down assay

Immunoprecipitation was performed as we have previously described [23]. Briefly, HL-1 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors. After centrifugation, the supernatant (500 μg in 300 μL) from each sample was incubated with Argonaute (Ago) 2 antibodies (1:50 dilution; Cell Signaling Technology, Danvers, MA) with rotation overnight at 4 °C. The immune complexes were pulled down by incubation with 40 μL of Protein A/G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 4 h with rotation. Immune complexes were collected by centrifugation at 3000 rpm for 3 min and washed three times with 1 mL lysis buffer. One milliliter of TRIzol reagent (Invitrogen, Grand Island, NY) was added to each sample to isolate RNA. Purified RNA was solubilized in RNAse-free water, kept on ice, and immediately reverse-transcribed to cDNA. RNA and miRNA were always dissolved in RNAse-free water and stored in −80 °C. CACNB2 mRNA levels were measured by using quantitative real-time PCR.

### 2.8. Statistical analysis

Data were presented as mean ± SEM. The unpaired *t*-test was used to compare data between groups. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Human atrial CACNB2 protein expression was downregulated in permanent AF

Using atrial myocardial biopsies obtained from patients who underwent open heart surgeries, Western blot analysis showed that CACNB2 protein expression was significantly downregulated by 67% in patients with permanent AF (0.33 ± 0.06 vs. 1.0 ± 0.22) compared to those in sinus rhythm without history of AF ( $n = 3$ for both; $P < 0.05$; Fig. 1). Specificity of the anti-CACNB2 antibody was validated by siRNA knockdown of CACNB2 expression in HL-1 cells (Supplementary Materials, Suppl. Fig. 1).

We have previously reported that miR-499 is significantly increased in the atrial tissues from patients with permanent AF [23]. Using the miR target prediction algorithm described in Materials and methods, we have found that *CACNB2* is a potential target of miR-499. Hence, this study was focused on determining the role of miR-499 in the regulation of cardiac CACNB2 expression.

### 3.2. The effects of miR-499 on CACNB2 expression in HL-1 cells

MiRs induce gene silencing by binding to specific sites in the 3'UTR of the targeted mRNA. This interaction inhibits protein production by suppressing protein synthesis and/or by promoting mRNA degradation. To determine the effects of miR-499 on CACNB2 protein expression, we transfected the miR-499 mimic into HL-1 cells, and then measured CACNB2 protein expression by Western blot analysis. The results showed a significant down-regulation of CACNB2 protein by 75% in miR-499 mimic transfected cells (0.25 ± 0.02 relative units with miR-499 mimic vs. 1.0 ± 0.22 relative units with control; $n = 3$; $P < 0.05$; Fig. 2A). In contrast, transfection with the miR-499 inhibitor upregulated CACNB2 expression by more than two-fold (2.27 ± 0.40 relative units with miR-499 mimic vs. 1.0 ± 0.21 relative units with control; $n = 3$; $P < 0.05$; Fig. 2B). These results indicate that the levels of CACNB2 protein expression are regulated by miR-499 in HL-1 cells. In contrast, CACNB1 and CACNB3 protein expression were not affected by miR-499 (Supplementary Materials, Suppl. Fig. 2).

We further determined the effect of miR-499 mimic on the transcript levels of CACNB2 by quantitative RT-PCR. The results showed that CACNB2 mRNA expression was not significantly altered in HL-1 cells 48 h after transfection with miR-499 mimic (0.90 ± 0.13 vs. 1.0 ± 0.04 relative units, $n = 3$, $P = 0.48$) (Fig. 2C). These results suggest that the downregulation of CACNB2 expression by miR-499 in cardiac cells may be due to the inhibition of protein synthesis, rather than through accelerated degradation of *CACNB2* mRNA.

### 3.3. MiR-499 targets the 3′ UTR of CACNB2

Examination of the *CACNB2* gene sequence showed the presence of a conserved sequence in its 3′ UTR that is complementary to the seed site of miR-499 (Fig. 3A). We subcloned this sequence into the luciferase reporter vector as described in Materials and methods. Using the luciferase reporter assay in HL-1 cells, we confirmed the direct binding of miR-499 to the 3′ UTR of *CACNB2*. Co-transfection of the miR-499 mimic (100 nM) with the *CACNB2* luciferase reporter vector in HL-1 cells resulted in a significantly decreased luciferase activity compared with cells transfected with the miR-499 negative control (0.42 ± 0.03 relative units with miR-499 mimic vs. 1.0 ± 0.05 relative units with control; $n = 4$; $P < 0.001$; Fig. 3B). These results provided supporting functional evidence that miR-499 binds to the 3′ UTR of *CACNB2*, resulting in reduced expression of CACNB2.

MiRs silence mRNAs by the association of miR/mRNA with Ago proteins to form miR-induced silencing complexes (miRISCs), which leads to translational repression and/or mRNA destabilization [29,30]. To further confirm the interactions of miR-499 with *CACNB2* mRNA, we determined whether miR-499 promote the formation of miRISCs with *CACNB2* mRNA and Ago proteins. Using a pull-down assay with Ago 2, a partner of miRISCs in HL-1 cells, the miRISC pulled down by anti-Ago 2 contained *CACNB2* mRNA that was 4.24-fold higher in cells transfected with miR-499 mimic compared with those transfected with the mimic negative control (4.24 ± 0.72 relative units with miR-

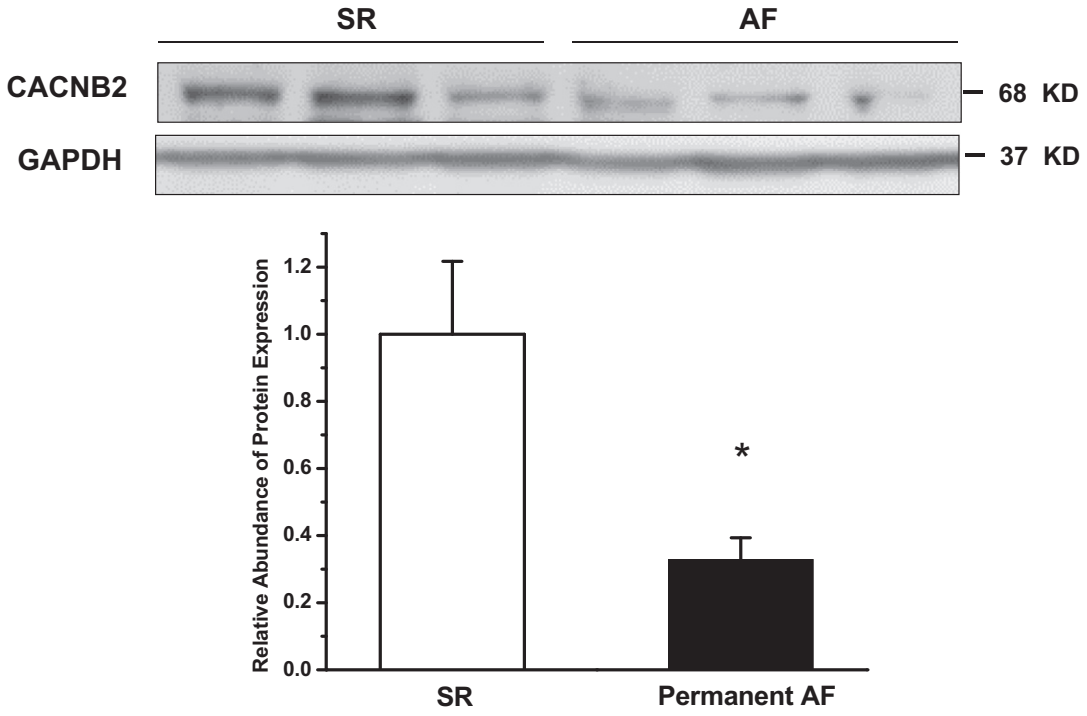


**Fig. 1.** CACNB2 protein expression is downregulated in AF. Down-regulation of CACNB2 protein expression in the atrial tissues from patients with permanent AF compared to those with SR. *$P < 0.05$ vs. SR, $n = 3$ for each group.

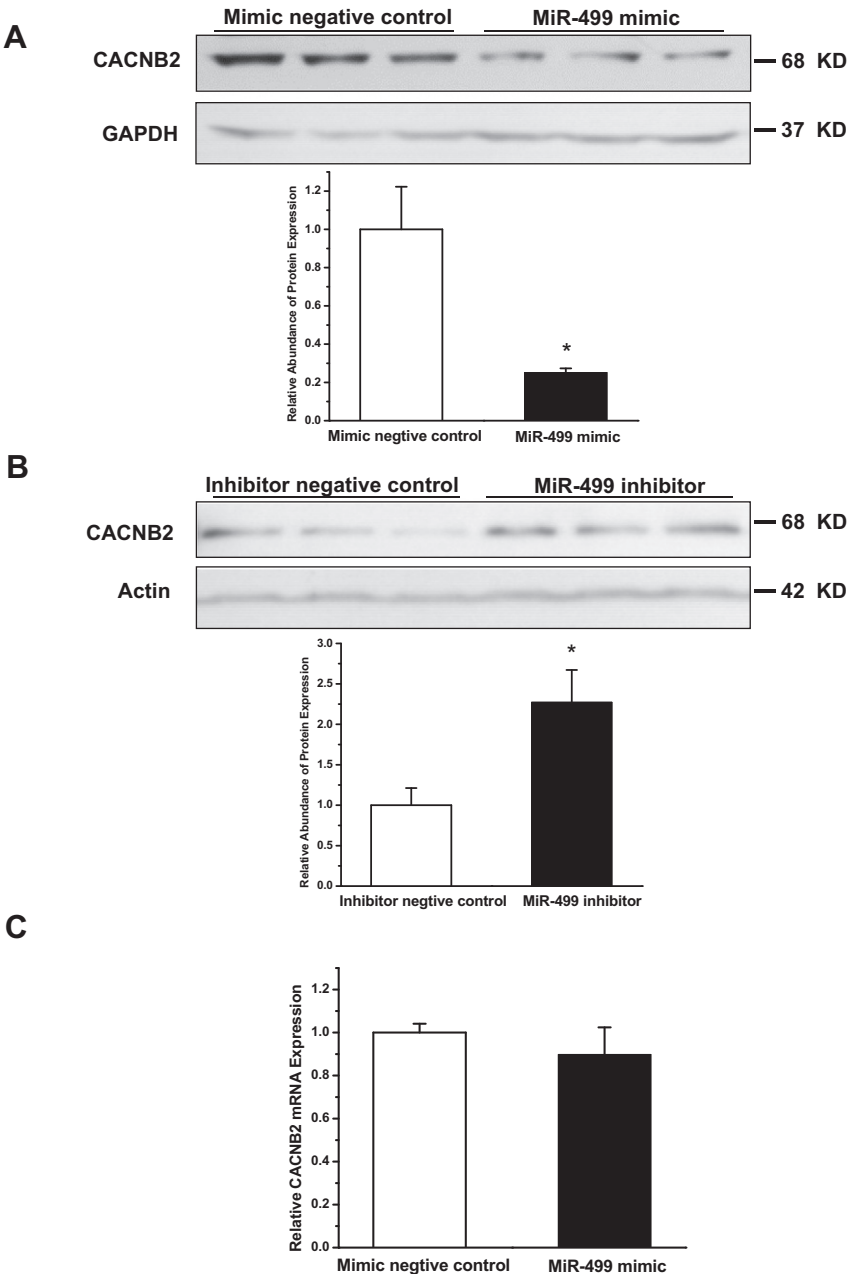


**Fig. 2.** Regulation of CACNB2 protein expression by miR-499. (A) Protein expression of CACNB2 was significantly reduced in HL-1 cells transfected with the miR-499 mimic, compared to those with mimic negative control (* $P < 0.05$ vs. control, $n = 3$ for each group). (B) Protein expression of CACNB2 expression was increased in HL-1 cells transfected with the miR-499 inhibitor compared to those with inhibitor negative control (*$P < 0.05$ vs. control, $n = 3$ for each group). (C) Real-time PCR analysis showing *CACNB2* mRNA expression was not changed by miR-499 in HL-1 cells. $P = 0.48$ vs. control, $n = 3$ for each group.

499 mimic vs. 1.0 ± 0.41 relative units with control; $n = 4$; $P = 0.007$; Fig. 3C). These findings indicate that miR-499 targets *CACNB2* mRNA and facilitates its recruitment into miRISCs, which may result in the translational repression of CACNB2 expression.

### *3.4. Knockdown of CACNB2 by miR-499 affects L-type calcium channel α subunit*

To determine the effects of reduced level of CACNB2 expression on the pore-forming CACNA1C subunit, we transfected HL-1 cells with miR-499 every 72 h, consecutively, for 3 times over a 12-day period. These experiments were performed to mimic the effects of long-term CACNB2 downregulation on the cardiac LTCC. After 48 h of miR-499 transfection, the expression of CACNA1C was not significantly altered in HL-1 cells (Fig. 4A). However, after 12 days of miR-499 transfection, there was significant down-regulation in the protein expression of CACNB2 by 72% and CACNA1C by 58% (0.28 ± 0.40 relative units with miR-499 mimic vs. 1.0 ± 0.17 relative units with control; $n = 3$; $P < 0.05$; and 0.38 ± 0.24 relative units with miR-499 mimic vs. 1.0 ± 0.18 relative units with control; $n = 3$; $P < 0.05$, respectively) (Fig. 4B). These findings suggest that long-term suppression of CACNB2 expression by miR-499 is associated with the downregulation of the pore-forming unit of the LTCC.

## 4. Discussion

In this study, we have made several important observations regarding the regulation of CACNB2 by miR-499. First, we found that the protein expression of CACNB2 was downregulated in human atria with long-standing persistent AF. Second, the miR-499 mimic significantly

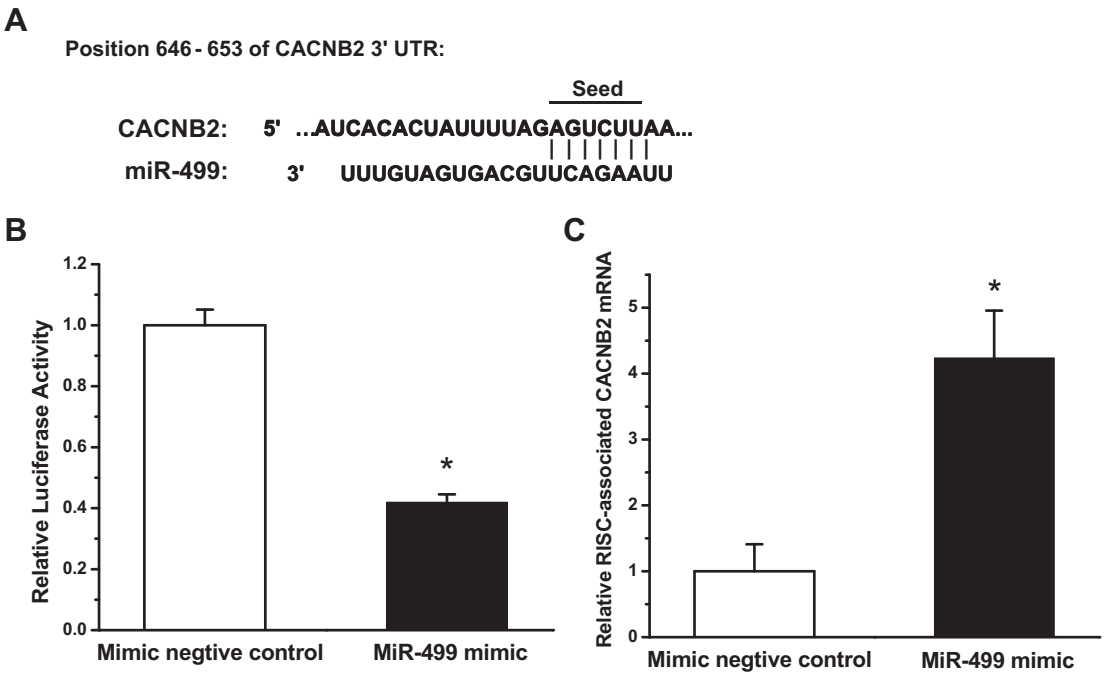


**Fig. 3.** MiR-499 targets *CACNB2*. (A) Alignment of the sequences of miR-499 with its target sites in the 3′ UTR of *CACNB2*. The miR-499 sequence is identical between human and mouse, and the binding site of *CACNB2* is conserved among mammals. The nucleotides marked represent the miR-499 "seed site" and its paired sequence. (B) Luciferase reporter assay in HL-1 cells showing co-transfection of miR-499 mimic significantly reduced luciferase activity in the luciferase reporter vector containing the 3′ UTR of *CACNB2* (*$P < 0.05$ vs. control, $n = 4$ for each group). (C) Argonaute 2 pull-down assays in HL-1 cells. The levels of *CACNB2* transcripts were determined by quantitative real-time RT-PCR. Transfection with the miR-499 mimic increased the RISC-associated *CACNB2* mRNA.

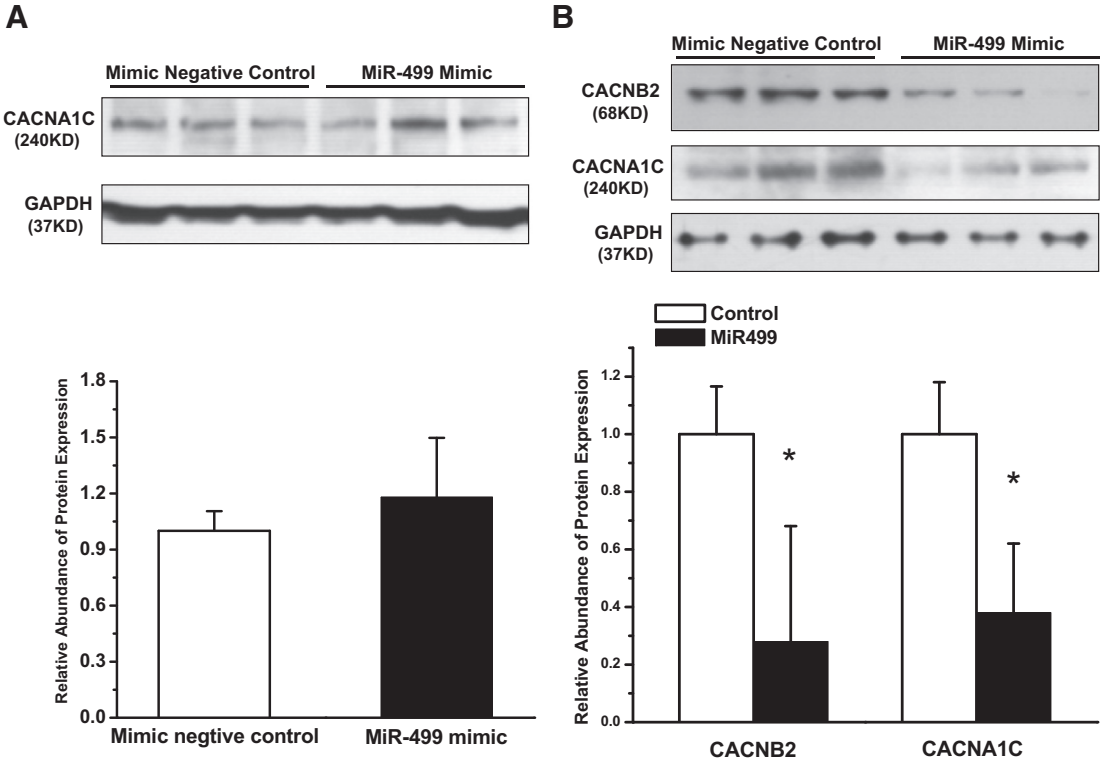


**Fig. 4.** Prolonged inhibition of CACNB2 by miR-499 downregulates the expression of CACNA1C. (A) Protein expression of the CACNA1C subunit was not significantly altered in HL-1 cells 48 h after transfection with miR-499 mimic, compared with mimic negative control ($P > 0.05$ vs. control, $n = 3$ for each group). (B) After 12-day of miR-499 transfection in cultured HL-1 cells, protein expression of CACNB2 and CACNA1C were significantly reduced in HL-1 cells transfected with miR-499 mimic, compared to those with mimic negative control (*$P < 0.05$ vs. control, $n = 3$ for each group).

reduced CACNB2 protein expression, whereas the miR-499 inhibitor significantly enhanced the level of CACNB2 expression in cultured atrial HL-1 cell. Third, using a *CACNB2* 3′UTR-luciferase reporter construct, we confirmed that miR-499 binds to the 3′UTR of *CACNB2* mRNA and inhibits expression of the reporter gene. In addition, results from the Ago protein pull-down assay showed that co-expression with miR-499 enriched *CACNB2* mRNA in the miRISCs. Fourth, downregulation of CACNB2 expression was associated with the downregulation of the pore-forming subunits of the LTCC. These observations provide compelling evidence that miR-499 regulates the expression of atrial CACNB2 channel subunit and may contribute to the electrical remodeling in AF.

Electrical remodeling is a maladaptation of the atria in response to the development of AF resulting in increased cardiac vulnerability to the initiation and maintenance of AF [1,31]. Hence, AF begets AF [6]. Two fundamental characteristics of electrical remodeling in AF are the shortening of the atrial action potential duration and the loss of rate adaptation [4–6]. These signature changes are due to the profound downregulation of the L-type calcium currents in AF [1,7,8]. Our findings in this study suggest that the upregulation of miR-499 in the fibrillating atrial myocardium may contribute to the electrical remodeling of L-type calcium currents through regulation of CACNB2.

The LTCC are highly expressed in cardiac muscle and play a central role in cardiac excitation-contraction coupling [32]. The major L-type voltage-gated calcium channel pore-forming subunit in the heart is CACNA1C, which is present in all cardiac myocytes [9]. CACNB2 is the predominant β subunit of the voltage-dependent LTCC in the heart [33] and is an intracellular protein that binds with high affinity to the α interaction domain (AID) in the loop connecting domains I and II of CACNA1C [34]. This interaction is critical for the surface expression of calcium channels, [35] potentially through the masking or interruption of an endoplasmic reticulum retention motif on CACNA1C by CACNB2, thereby prevents degradation by the ubiquitin-proteasomal system [36].

The biophysical properties of the LTCC are significantly modulated by CACNB2, which shifts the voltage of activation to more negative voltages but that of inactivation to more positive voltages. These changes make the channel easier to open with an increase in the rate of channel opening, but inactivate the channel at more depolarized potentials with slow kinetics of channel inactivation [10,12]. Knockout of *CACNB2* is embryo-lethal with failure of proper cardiac development [37]. The clinical importance of CACNB2 in cardiac electrophysiology is beyond question as mutations of the *CACNB2* are known to be associated with short QT sudden death syndrome [14] and with Brugada syndrome [15]. The findings of our study provide further support that CACNB2 plays a critical role in the regulation of cardiac electrophysiology.

MiRs have been reported to participate in the electrical remodeling in AF. MiR-1, -21, -26, -208a, -328, and -499 are all implicated in the electrical remodeling of AF, targeting a wide range of genes including *CACNA1C, CACNB1, CACNB2, KCNB2, KCNE1, KCNJ2,* and *KCNN3* [20–22, 38]. MiR-328 was found to be upregulated more than two-fold in patients with AF and rheumatic heart disease and in dogs with AF. Molecular manipulation of miR-328 resulted in a reciprocal relationship between the protein expression of CACNA1C/CACNB2 and miR-328 [39]. A recent study reported that miR-21 is upregulated in atrial myocytes from patients with chronic AF and there is an inverse relationship between miR-21 and the mRNA levels of *CACNA1C* and *CACNB2* [38]. These findings together with the results of our study suggest that there are multiple miR-mediated mechanisms that result in the downregulation of the LTCC in AF. The aggregate effects of these mechanisms may contribute to the profound downregulation of the LTCC in AF with current reduction of 60–70% [1,7,40]. This is vital for preserving myocardial viability by preventing excessive intra-calcium overload, but at the expense of electrical maladaptation with increased vulnerability for AF [41,42]. Another target of dual miR regulation in electrical remodeling is *KCNJ2* which has been shown to be upregulated in AF due to the downregulation of miR-1 and miR-26 [21,43,44].

We have previously reported that miR-499 contributes to the development of AF through the downregulation of KCNN3 expression [23]. It is known that one single miR can regulate multiple different transcripts, whereas a single transcript may also have multiple binding sites and be regulated by several miRs of the same or different sequence [45]. The results of this study affirm this: miR-499 contributes to the electrical remodeling of AF through its effects on both KCNN3 as well as CACNB2 [23]. Similar examples are noted in the upregulation of miR-328 in AF, which has been shown to target both *CACNA1C* and *CACNB2*, causing the downregulation of both [39]. All these findings indicate that miRs play an important role in the regulation of cardiac electrophysiology, especially in the electrical remodeling of AF.

In this study, we found that *CACNB2* is a primary target of miR-499 modulation. The level of CACNB2 protein expression showed an inverse relationship with miR-499 but the mRNA levels of *CACNB2* was not affected by miR-499 (Fig. 2C). Hence, miR-499 suppresses the expression of *CACNB2* through inhibition of translation. Although *CACNA1C* is not a direct target of miR-499, downregulation of CACNB2 in cultured atrial HL-1 cells was associated with downregulation of the pore-forming subunit, CACNA1C (Fig. 4B). The mechanisms through which miR-499 produced the downregulation of CACNA1C after 12 days of transfection are not clear, but based on previous studies it might be due to the dependence of CACNA1C on CACNB2 for surface targeting and/or due to the destabilization of the multimeric ion channel complex with the absence of CACNB2, resulting in accelerated degradation of the channel by the ubiquitin-proteasomal system [35,36]. However, the process of CACNA1C protein downregulation in association with miR-499-mediated CACNB2 suppression takes days. It is known that microRNAs could have multiple targets. Therefore, we cannot rule out that long-term elevation of miR-499 in AF may affect other factors leading to the downregulation of CACNA1C. Nevertheless, our results confirm the important function of CACNB2 in the LTCC and the role of miR-499 in mediating the cardiac electrical remodeling in AF.

## Transparency document

The Transparency document associated with this article can be found, in online version.

## Acknowledgment

All authors have read the journal's policy on conflicts of interest and report no potential conflicts of interest. All authors have read the journal's authorship agreement and have reviewed and approved the manuscript. This manuscript has not been published and is not under consideration by another journal.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbacli.2017.02.002.

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