



Post-transcriptional regulation of cardiac sodium channel gene *SCN5A* expression and function by *miR-192-5p*



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ABSTRACT

The *SCN5A* gene encodes cardiac sodium channel $Na_v1.5$ and causes lethal ventricular arrhythmias/sudden death and atrial fibrillation (AF) when mutated. MicroRNAs (miRNAs) are important post-transcriptional regulators of gene expression, and involved in the pathogenesis of many diseases. However, little is known about the regulation of *SCN5A* by miRNAs. Here we reveal a novel post-transcriptional regulatory mechanism for expression and function of *SCN5A/Na_v1.5* via *miR-192-5p*. Bioinformatic analysis revealed that the 3'-UTR of human and rhesus *SCN5A*, but not elephant, pig, rabbit, mouse, and rat *SCN5A*, contained a target binding site for *miR-192-5p* and dual luciferase reporter assays showed that the site was critical for down-regulation of human *SCN5A*. With Western blot assays and electrophysiological studies, we demonstrated that *miR-192-5p* significantly reduced expression of *SCN5A* and $Na_v1.5$ as well as peak sodium current density I_{Na} generated by $Na_v1.5$. Notably, in situ hybridization, immunohistochemistry and real-time qPCR analyses showed that *miR-192-5p* was up-regulated in tissue samples from AF patients, which was associated with down-regulation of *SCN5A/Na_v1.5*. These results demonstrate an important post-transcriptional role of *miR-192-5p* in post-transcriptional regulation of $Na_v1.5$, reveal a novel role of *miR-192-5p* in cardiac physiology and disease, and provide a new target for novel miRNA-based antiarrhythmic therapy for diseases with reduced I_{Na} .

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1. Introduction

$Na_v1.5$ is the primary voltage-gated sodium channel in the heart and responsible for the fast depolarization and generation of the cardiac action potential (AP) and conduction of the cardiac AP [1]. It is encoded by the *SCN5A* gene on chromosome 3p21 [1]. *SCN5A* is one of the first two genes identified for long QT syndrome (LQTS), an inherited cardiac arrhythmia [2,3,4,5]. LQTS mutations in *SCN5A* act by a gain-of-function mechanism, mostly by generating late persistent sodium currents [6]. On the other hand, loss-of-function mutations were later identified by

us and others in *SCN5A* in patients with Brugada syndrome (BrS), cardiac conduction defects, sudden infant death, sick sinus syndrome, dilated cardiomyopathy and atrial fibrillation (AF) [7,8,9,10]. Moreover, I_{Na} density was reduced in AF patients [11] and a dog model for AF [12], but the underlying molecular mechanism is unknown. Reduced $Na_v1.5$ expression and I_{Na} were also detected in the atria in a mouse model for catecholamine-induced VT with the RyR2-P2328S gain-of-function mutation [13]. Similarly, reduced I_{Na} density has also been associated with more common cardiovascular diseases such as heart failure [14, 15,16] and myocardial ischemia [17], but the underlying molecular mechanism is unknown either.

Post-transcriptional regulation by microRNAs (miRNAs) may be an attractive, putative mechanism for the observed reduction of I_{Na} in AF, heart failure, and myocardial ischemia [18]. MiRNAs are small, non-coding RNAs (about 22 nucleotide long) that regulate gene expression through translational repression or mRNA degradation, typically by binding to their targeted sites located in the 3' untranslated regions (3'-UTRs) of mRNAs [19,20]. For example, *miR-1*, *miR-133*, *miR590*, *miR-208a* and *miR-328* have been found to be significantly associated

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² These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

with AF and heart failure [21,22]. Many miRNAs that regulate the expression of cardiac ion channels have been reported. For example, *miR-1* regulates *KCNJ2*, *CACNA1C*, and *GJA1*; *miR-133* regulates *ERG*; *miR-328* regulates *CACNA1C* and *CACNB1*; *miR-212* regulates *KCNJ2*; *miR-208a* regulates *GJA5* [23]. In this study, we found that cardiac sodium channel gene *SCN5A* was regulated by *miR-192-5p*. *MiR-192-5p* was first identified by Lagos Quintana et al. [24] and later by Lim et al. [25]. *MiR-192-5p* was shown to be highly expressed in kidney and hepatocellular carcinoma tissue samples [26,27], but its expression in cardiac tissues was not reported. In this study, we reveal that *miR-192-5p* binds to the 3'-UTR of human *SCN5A* to negatively regulate the expression of $\text{Na}_v1.5$ and reduce I_{Na} density. We further show that the expression of *miR-192-5p* is significantly reduced in human AF tissues compared with normal tissue samples and associated with down-regulation of *SCN5A/Na_v1.5*.

2. Methods

2.1. Study subjects

This study was approved by the appropriate local institutional review boards on human subject research at the First Affiliated Hospital of Xiamen University and Huazhong University of Science and Technology and conformed to the guidelines set forth by the Declaration of Helsinki. Written informed consent was obtained from the participants. We studied left atrial appendage tissue samples from AF patients with rheumatic valve disease and control subjects with rheumatic valve disease alone for the expression levels of *miR-192-5p* and *SCN5A/Na_v1.5*. The demographic and clinical characteristics of study subjects are shown in Table 1.

2.2. Mutational analysis by sequencing analysis

Extraction of human genomic DNA from blood samples was as described previously by us [28]. All exons, intron–exon boundaries and 5' and 3' UTRs of *SCN5A* were amplified by polymerase chain reactions (PCR) using primers published before [29]. The PCR products were purified by agarose gel electrophoresis, followed by direct sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies).

2.3. Bioinformatic analysis

The sequences at SNP rs41310757 and in its flanking regions were analyzed for potential binding sites for miRNAs by bioinformatic analyses. We used several online miRNA binding site prediction software programs with different algorithms, including TargetScan (<http://www.targetscan.org/>), RNA Hybrid (<http://bibiserv.tenchfak.uni-bielefeld.de/rnahybrid>), miRWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>), DIANA-microT v3.0 (<http://diana.cslab.ece.ntua.gr/microT/>), and microRNA.org (<http://www.microna.org/microna/getGeneForm.do>).

2.4. Cell lines, plasmids and miRNAs

HCT116 cells (human colorectal carcinoma cell), SW620 cells (human colorectal adenocarcinoma cell) and HEK293 cells (human embryonic kidney cell) were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Gaithersburg, MD, USA) in a humidified incubator with 5% CO_2 at 37 °C.

We amplified a region of the 3'-UTR of *SCN5A* that contains the predicted *miR-192-5p* target binding site using human genomic DNA. The PCR product included two restriction sites, *Sac I* at the left end and *Hind III* at the right end. The PCR product was cut with the two enzymes and sub-cloned into the multiple cloning site of the pMIR-REPORT luciferase vector (Applied Biosystems, Foster City, CA, USA) (Fig. 1A). Two reporter genes were created. Reporter gene pMIR-REPORT-SCN5A-3' UTR-T contains the wild type binding sequence for *miR-192-5p*, whereas pMIR-REPORT-SCN5A-3' UTR-C contains a variant, a T to C substitution (single nucleotide polymorphism or SNP rs41310757), at the *miR-192-5p* binding sequence. The sequences of primers are 5'ACTCTGGC CCCCTATTGTCT3' for the forward primer and 5'CACCCTGTGTCTTT GGTT3' for the reverse primer.

The putative target binding site of *miR-192-5p* at the 3'-UTR of human *SCN5A*, AGGTCAT, was deleted from pMIR-REPORT-SCN5A-3' UTR-T by overlapping PCR-based mutagenesis as previously described [30]. This generated a mutant reporter, pMIR-REPORT-SCN5A-3' UTR-T-Mut.

The pHL3 expression plasmid for *SCN5A* was described previously [31,32,33]. The whole 3'-UTR of *SCN5A* with the *miR-192-5p* binding

Table 1

Clinical and demographic characteristics of study subjects.

| ID | Gender | Age | Height (cm) | Weight (kg) | BMI (kg/m ²) | Systolic (mm Hg) | Diastolic (mm Hg) | AF | Other cardiovascular disease | Test |
|----|--------|-----|-------------|-------------|--------------------------|------------------|-------------------|-----|--|------------------|
| O2 | F | 49 | 158 | 47 | 0.30 | 110 | 72 | No | Mitral stenosis and incompetence | qPCR |
| 7 | F | 27 | 163 | 50 | 0.31 | 122 | 71 | No | Mitral stenosis and incompetence | qPCR |
| 5 | F | 40 | 160 | 56 | 0.35 | 130 | 60 | No | Aortic valve stenosis and incompetence | qPCR |
| Y2 | F | 60 | – | – | – | 105 | 70 | Yes | Heart valve disease | qPCR |
| 3 | F | 43 | 163 | 57 | 0.35 | 112 | 73 | Yes | Heart valve disease | qPCR |
| 8 | F | 61 | 161 | 53 | 0.33 | 130 | 82 | Yes | Heart valve disease | qPCR |
| A | F | 51 | 156 | 53 | 0.34 | 104 | 88 | Yes | Heart valve disease | ISH |
| B | F | 43 | – | – | – | – | – | Yes | Heart valve disease | Immunohis ISH |
| M | M | 62 | – | – | – | – | – | Yes | Congenital heart disease, ventricular septal defect | Immunohis ISH |
| L | F | 48 | 145 | 40 | 0.28 | 100 | 65 | No | Heart valve disease | Immunohis ISH |
| G | F | 35 | 160 | 54 | 0.34 | 102 | 74 | No | Heart valve disease | Immunohis ISH |
| F | M | 73 | – | – | – | 123 | 60 | No | Heart valve disease | Immunohis ISH |

qPCR, real-time quantitative RT-PCR analysis; ISH, in situ hybridization; Immunohis, immunohistochemical analysis.

site was then sub-cloned into pHL3 by GeneWiz (Suzhou, Jiangsu, China) and used for Western blot and electrophysiological studies.

MiR-192-5p mimics and negative control miRNA mimics were purchased from Guangzhou RioboBio (Guangzhou, Guangdong, China). The *miR-192-5p* mimics were chemically synthesized double-stranded RNA molecules with the sequence identical to the mature *miR-192-5p* sequence. When the mimics were transfected into cells, it would be recognized and processed by Argonaute (AGO) and RNA-induced silencing complex (RISC) into single-stranded, functional *miR-192-5p*. The negative control miRNA mimics were miR-mimics with sequences that do not bind any mRNA in humans.

2.5. Dual luciferase reporter assays

HCT116 cells were cultured in 24-well plates for 24 h and transfected with 200 ng of either pMIR-REPORT-SCN5A-3'UTR-T or pMIR-REPORT-SCN5A-3'UTR-C and 100 nM of *miR-192-5p* mimics or negative control NC-miRNA mimics, along with 20 ng of the pRL-TK vector containing the *Renilla* luciferase gene (Promega, Madison, WI, USA). The transfection was carried out using 2 μ l of lipofectamine 2000 and 500 μ l of Opti-MEM reduced serum medium according to the manufacturer's the protocol (Gibco Life Technologies, Gaithersburg, MD, USA). Cells were harvested 48 h after transfection and lysed using 1 \times passive lysis buffer (Promega, Madison, WI, USA). Luciferase assays were performed as described by us previously [34] and using the Dual-Glo luciferase assay kit (Gibco Life Technologies, Gaithersburg, MD, USA). The ratio of firefly over *Renilla* luciferase activities was calculated and considered as the final luciferase activity value. Each assay was performed in triplicate and repeated at least three times.

2.6. Quantitative real time RT-PCR analysis

To examine the effect of *miR-192-5p* on the expression of *SCN5A* mRNA, we searched literature for a cell line with expression of *SCN5A* and found that SW620 cells expressed a high level of endogenous $\text{Na}_v1.5$ protein [35]. Therefore, SW620 cells were cultured in 24-well plates for 24 h and transfected with 100 nM of *miR-192-5p* mimics or NC-miRNA mimics using lipofectamine 2000 and Opti-MEM reduced serum medium according to the manufacturer's the protocol (Gibco Life Technologies, Gaithersburg, MD, USA). Cells were collected 48 h after transfection and lysed using RNAiso plus (TaKaRa, Dalian, China). Total RNA was isolated from cells using the Trizol reagent (TaKaRa, Dalian, China) and converted into cDNA by reverse transcription using oligo(dT)₁₅ with the First-Strand cDNA Synthesis kit (Toyobo, Japan).

Total RNA samples were also isolated from left atrial appendage samples from three AF patients (Y2, 3, 8) and three non-AF control patients (O2, 7, 5) (Table 1) using the Trizol reagent (TaKaRa, Dalian, China). Total RNA was converted into cDNA as described above.

Real time qPCR analysis was carried out using the FastStart Universal SYBR Green Master kit (Roche Applied Science, Mannheim, Germany) in a 10 μ l reaction volume on an ABI 7900 Genome Analyzer System. The reaction system contained 2 μ l of cDNA template, 5 μ l of SYBR green mix (including ROX), 200 nM of forward primer and 200 nM of reverse primer (a total volume of 10 μ l). Human *GAPDH* was used as an internal standard. The primers for RT-PCR analyses were listed in Table 2. The PCR products were verified by melting curve analysis and

the results were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method as described [36]. Each assay was performed in triplicate and repeated at least three times.

Expression of *miR-192-5p* was carried out for left atrial appendage samples from three AF patients and three non-AF controls (Table 1) using the stem-loop real-time PCR as described previously [37]. Total RNA was isolated as described above. Bulge-loopTM miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for *miR-192-5p* were purchased from RiboBio (Guangzhou, Guangdong, China). MiRNA bulge-loop was reverse transcribed with the First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) using the specific *miR-192-5p* RT primer. Real time qPCR analysis was carried out as described for *SCN5A* above except that the reaction volume was 20 μ l and the internal control was U6 small RNA.

2.7. Electrophysiological studies

HEK293 cells are the most commonly used cells for electrophysiological studies of cardiac sodium channels. HEK293 cells were cultured at 37 °C under 5% CO₂ in high glucose DMEM media supplemented with 10% FBS (Invitrogen). When cell culture was 70–80% confluent in 3.5-cm plates, transfection was carried out with 2 μ g of pcDNA3-SCN5A-whole 3'UTR and 200 ng of pEGFP-N1 with *miR-192-5p* mimics or negative control NC-miRNA mimics using lipofectamine 2000 and Opti-MEM reduced serum medium. Cells were cultured for 48 h and EGFP-positive cells were selected for patch-clamp recordings. We recorded sodium current at room temperature using a Multiclamp 700B amplifier (Axon Instruments, Sunnyvale, CA). The patch pipette (tip resistance was 2–3 M Ω) was filled with following solutions as described previously [3,38,39]: 20 mM NaCl, 130 mM CsCl, 10 mM HEPES, and 10 mM EGTA, pH 7.2 (adjusted with CsOH). The components of bath solution was 70 mM NaCl, 80 mM CsCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.3 (adjusted with CsOH). All chemicals were purchased from Sigma (Madison, WI, USA). The junction potential, capacitance and series resistance were automatically compensated in the whole cell configuration. The holding potential was maintained at –120 mV, and the voltage clamp protocols were as described previously. The sodium currents were filtered at 5 kHz, sampled at 50 kHz, and stored on a desktop computer equipped with an AD converter (Digidata 1440A, Molecular Devices). All current measurements were normalized using the cell capacitance. The Clamp fit 10.2 (Axon Instruments), Excel (Microsoft), and Origin 8.5 (Microcal Software) were used for data acquisition and analysis.

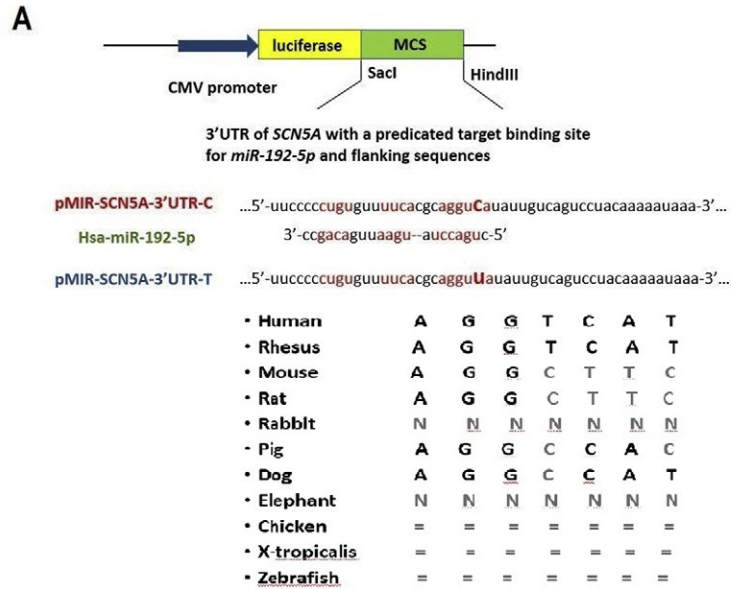
2.8. Western blot analysis

Cells were cultured and transfected as described above. Transfected cells were collected and incubated in ice-cold TNEN lysis buffer (in mmol/l: 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2.0 mM EDTA, 1.0% Nonidet P-40) with 1 mini tab of EDTA-free protease inhibitors (Roche) and 1 mmol/l PMSF (phenylmethylsulfonyl fluoride) for 30 min at 4 °C. The insoluble fraction was pelleted by centrifugation at 12,000 \times g for 15 min at 4 °C. 100 μ l of supernatant was mixed with 20 μ l of 6 \times Laemmli buffer (0.3 mol/l Tris-HCl, 6% SDS, 60% glycerol, 120 mmol/l dithiothreitol (DDT) and proprietary pink tracking dye), and heated at 37 °C for 10 min. Then, 20 μ l of samples was subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto a 0.45 μ m polyvinylidene fluoride (PVDF) membrane (Millipore). The

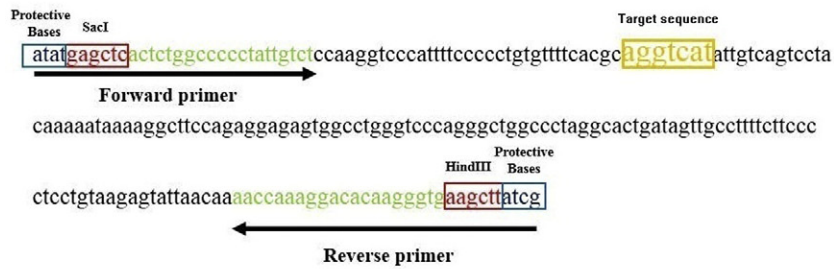
Fig. 1. *MiR-192-5p* down-regulates expression of the *SCN5A* gene by targeting the 3'-UTR. A. Bioinformatic analysis identified a potential binding site for *miR-192-5p* at the 3'-UTR of *SCN5A*. A diagram showing luciferase reporter constructs containing the *miR-192-5p* targeting sequences with the C allele or T allele of rs41310757 is shown. The *miR-192-5p* "seed region" and the predicted *miR-192-5p* target sequences are indicated. The evolutionary conservation of the *miR-192-5p* binding sequences at the 3'-UTR of *SCN5A* is shown for several different species at the bottom. B. The sequence of the 3'-UTR fragment of human *SCN5A*, which contains the target binding sequence of *miR-192-5p* and was subcloned into the pMIR-REPORT luciferase vector. Forward and reverse primers used for PCR-amplification of the 3'-UTR fragment of *SCN5A* are shown. C. Effects of *miR-192-5p* mimics on the pMIR-SCN5A-3'UTR luciferase reporters compared with negative control mimics (NC-control) transfected into HCT116 cells. Three luciferase reporters were analyzed, including the pMIR-SCN5A-3'UTR luciferase reporter containing the C allele of rs41310757, the reporter with the T allele, and the reporter with the *miR-192-5p* target sequence (aggctcat) deleted. Luciferase activities were calculated as the ratio of firefly/*Renilla* activities and normalized to the negative control mimic group. Results were obtained from three independent experiments. The data was normalized to NC-miR. Data is shown as means \pm SD.

membrane was probed with an anti-Na_v1.5 rabbit polyclonal antibody (Alomone, ASC-005), followed by incubation with a HRP-conjugated secondary goat anti-rabbit antibody (Millipore). The protein signal

was visualized by a Super Signal West Pico Chemiluminescent substrate according to the manufacturer's instructions (Pierce Chemical Co., Rockford, Illinois, USA). Human GAPDH was used as loading control.



B



C

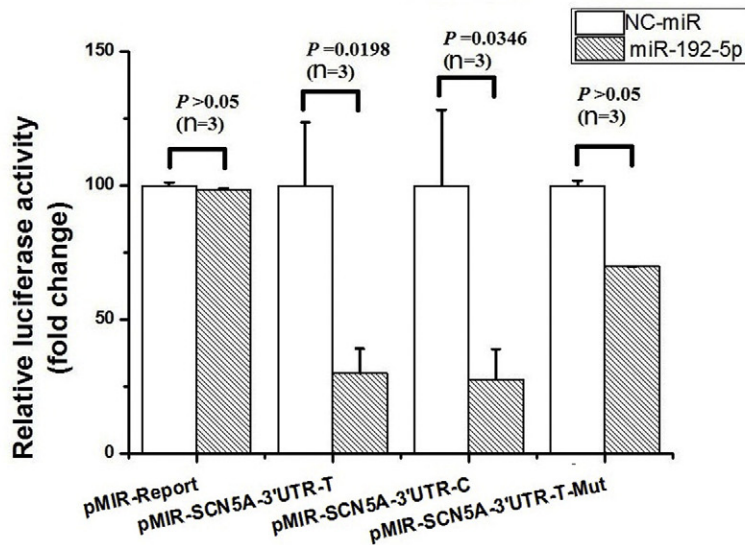


Table 2
The primer sequences for RT-PCR analysis of *SCN5A* and control *GAPDH*.

| Gene | Forward | Reverse |
|--------------|-----------------------|----------------------|
| <i>SCN5A</i> | CCAGATCTCTATGGCAATCCA | GAATCTTCACAGCCGCTCTC |
| <i>GAPDH</i> | AGTCAGCCGCATCTTCT | GCCCAATACGACCAAATCC |

Each assay was performed in triplicate and repeated at least three times.

2.9. In situ hybridization analysis (ISH)

ISH for *miR-192-5p* was carried out using a Dig-labeled *miR-192-5p* antisense probe. The sequence of the probe was 5'-GGCTGCAATTCATAGGTCAG-3'. Human left atrial appendage samples from three AF patients (A, B, M) and three non-AF controls (L, G, F) (Table 1) were fixed in 4% paraformaldehyde (PFA) and paraffin-embedded. Serial sections of 5 μ m were cut and hybridized in 100 ml hybridization buffer with 50 pM of denatured Dig-labeled probe at 42 °C overnight. The sections were then washed with 1 \times SSPE buffer (8.765 g NaCl, 1.38 g NaH₂PO₄ and 0.37 g EDTA-Na₂·2H₂O dissolved with 500 ml DEPC H₂O, pH 7.4) and incubated with an anti-DIG-AP antibody in 1 \times blocking buffer (2% sheep serum and 0.1% Triton X-100 dissolved with 100 mM Tris-HCl and 150 mM NaCl, pH 7.5) for 2 h. The sections were then stained with 45 μ g Nitroblue tetrazolium (NBT) in 10 ml TNM-50 (50 ml Tris, pH 7.5, 10 ml 5 M NaCl and 25 ml 1 M MgCl₂ dissolved with 500 ml DEPC H₂O, pH 9.0) for 12 h and imaged under a microscope (LEICA). The florescent optical density for *miR-192-5p* was quantified using a computer image analysis program (Image J) and analyzed for differences between the AF group and the control group by Student's *t*-test.

2.10. Immunohistochemical analysis

Immunohistochemistry was performed for Na_v1.5 with human left atrial appendage samples from three AF patients (A, B, M) and three non-AF controls (L, G, F) (Table 1) as described [37]. A polyclonal anti-Na_v1.5 antibody from Sigma-Aldrich (St. Louis, MO, USA) was used for immunohistochemistry staining.

2.11. Statistical analysis

Experimental data from at least three independent experiments was presented as means \pm standard deviation (S.D.). Statistical analysis was performed with Student's *t*-test using SPSS version 17.0 software (SPSS, Chicago, IL, USA). A *P* value of 0.05 or less was considered to be significant.

For analysis of the data from real-time RT-PCR analysis, we calculated the means for RQ values from the 3 wells and then compare the means from three independent experiments between two different groups with Student's *t*-test. For Western blot analysis, the images from three independent experiments were scanned with Quantity One 4.6.8 (Basic) (Bio-Rad, Hercules, California, USA) and quantified. The density of the Na_v1.5 protein band for the *miR-192-5p* treatment group was calibrated to that for the control sample (NC-miR). The means from three independent experiments were compared between two different groups with Student's *t*-test.

3. Results

3.1. Identification of *miR-192-5p* as a potential posttranscriptional regulator of cardiac sodium channel gene *SCN5A*

During mutational screening of the *SCN5A* gene in a panel of 45 BrS patients, we identified a T to C variant in the 3'-UTR of *SCN5A* (754 bps from the stop codon), which appeared to be a common polymorphism (SNP rs41310757). The minor allele frequency (MAF) of the

rs41310757 variant was 37.30% in the BrS group, which was higher than that in a group of 100 Chinese Han controls (31.2%) (*P* = 0.04). The MAF of the rs41310757 variant was similar to that in the NCBI database for the East Asian population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=41310757) (32.50%). Bioinformatic analysis of the sequences flanking SNP rs41310757 revealed a potential binding site for *miR-192-5p* (Fig. 1A). The *miR-192-5p* binding site on *SCN5A* shows Watson-Crick matches to the seed region of *miR-192-5p* (Fig. 1A). The mature sequence of *miR-192-5p* and potential binding regions are shown in Fig. 1A. Interestingly, the putative *miR-192-5p* target binding sequences at the 3'UTR of human *SCN5A* are not evolutionarily conserved. High conservation was observed only with rhesus and dogs, but not with the pig, elephant, rabbit, mouse, rat, chicken, zebrafish and *Xenopus tropicalis* (Fig. 1A). The data supports the hypothesis that human *SCN5A* may be regulated by *miR-192-5p*.

3.2. Analysis of regulation of *miR-192-5p* on the expression of *SCN5A* using pMIR-REPORT luciferase reporters

Based on the data from the bioinformatic analysis described above, we hypothesized that *miR-192-5p* could bind to the 3'-UTR of *SCN5A* and regulate the expression of *SCN5A*. To test the hypothesis, we cloned a 194 bp region that contains the predicted *miR-192-5p* binding site and flanking sequences at the 3'-UTR of *SCN5A* into the pMIR-REPORT luciferase vector, resulting in a reporter gene pMIR-SCN5A-3'UTR-T with the T allele of SNP rs41310757 and pMIR-SCN5A-3'UTR-C with the C allele (Fig. 1A, B).

Each reporter was co-transfected with *miR-192-5p* mimics and negative NC-control mimics into cells and luciferase assays were carried out. Compared to NC-control mimics, over-expression of *miR-192-5p* (i.e. *miR-192-5p* mimics) reduced luciferase activities by 3.3-fold (*P* = 0.0198) for the pMIR-SCN5A-3'UTR-T reporter gene (Fig. 1C). The *miR-192-5p* mimics reduced the activity from pMIR-SCN5A-3'UTR-C by 3.8-fold (*P* = 0.0346) (Fig. 1C). The data suggests that *miR-192-5p* negatively regulates the activity of pMIR-SCN5A-3'UTR luciferase reporters containing the *miR-192-5p* target binding site. We also analyzed the luciferase activity from a mutant pMIR-SCN5A-3'UTR-T reporter gene with the putative *miR-192-5p* target sequence (AGGTCAT) deleted (pMIR-SCN5A-3'UTR-T-Mut). No significance difference was found between *miR-192-5p* mimics and NC-control mimics. The data suggests that *miR-192-5p* negatively regulates the activity of pMIR-SCN5A-3'UTR luciferase reporters by targeting the *miR-192-5p* target binding sequence (AGGTCAT).

There was no statistical significant difference between pMIR-SCN5A-3'UTR-T and pMIR-SCN5A-3'UTR-C with or without *miR-192-5p* mimics (*P* = 0.444 and 0.351, respectively) (Fig. 1B). The data suggests that the T to C substitution (SNP rs41310757) at the *miR-192-5p* binding site does not affect the expression *SCN5A*, consistent with the finding that it is a benign polymorphism.

3.3. *miR-192-5p* negatively regulates expression of *SCN5A* at both mRNA and protein levels

To further validate the finding that *miR-192-5p* regulates the expression of *SCN5A*, we assessed the expression level of *SCN5A* mRNA using real-time qPCR analysis in SW620 cells with a relatively high expression level of endogenous *SCN5A*. SW620 cells were transfected with *miR-192-5p* mimics or NC-control miRNA mimics. Real-time qPCR analysis showed that compared to control mimics, *miR-192-5p* mimics significantly reduced the level of *SCN5A* mRNA, although the magnitude of reduction was only 17% (*P* = 0.0064) (Fig. 2A).

We then further examined the regulation of Na_v1.5 encoded by *SCN5A* at the protein level using Western blot analysis. Protein extracts were isolated from SW620 cells transfected with *miR-192-5p* mimics or NC-control miRNA mimics. Western blotting analysis showed that *miR-192-5p* mimics significantly reduced the expression level of

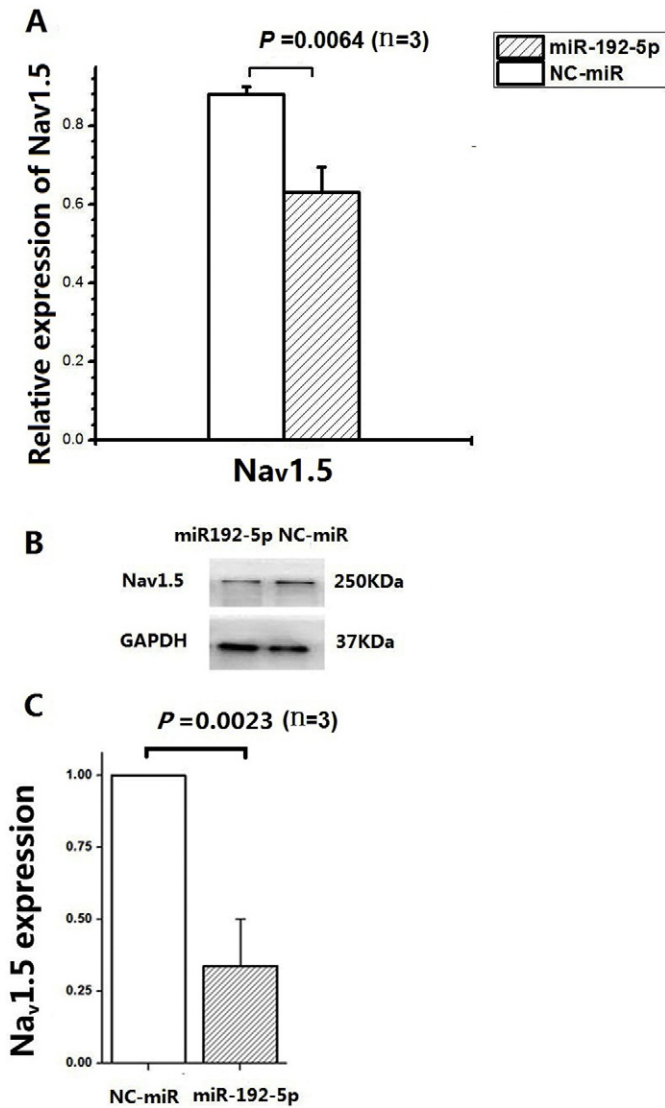


Fig. 2. *MiR-192-5p* negatively regulates expression of *SCN5A* mRNA or $Na_v1.5$ protein. A. *MiR-192-5p* overexpression with mimics significantly reduced the expression level of *SCN5A* mRNA in SW620 cells compared with negative control mimics (NC-control) by real time qPCR analysis. *GAPDH* was used as internal control and the fold change was calculated by RQ values ($2^{-\Delta\Delta Ct}$). B, C. *MiR-192-5p* overexpression with mimics significantly reduced the expression level of $Na_v1.5$ protein in SW620 cells compared with negative control mimics (NC-control) by Western blot analysis. *GAPDH* was used as loading control. The images of Western blot analysis shown in (B) were scanned, quantified and plotted in (C). The data was normalized to NC-miR. Data is shown as means \pm SD.

$Na_v1.5$ protein by 66% compared with the negative control mimics ($P = 0.0023$) (Fig. 2B and C).

Taken together, these results indicated that *miR-192-5p* significantly down-regulated the expression of cardiac sodium channel mRNA (*SCN5A*) and protein ($Na_v1.5$). The regulation is more on the protein level than on the mRNA level, 66% and 17%, respectively. These features are consistent with the general regulatory characteristics by miRNAs.

3.4. *MiR-192-5p* reduces the density of cardiac sodium current

$Na_v1.5$ is the sodium channel subunit that generates sodium current in the heart. The sodium current is commonly analyzed by patch-clamping of HEK293 cells transfected with the expression plasmid for *SCN5A*. We co-transfected HEK293 cells with 2 μ g of pcDNA3-*SCN5A*-whole 3'UTR and 200 ng of pEGFP-N1 together with *miR-192-5p* mimics or negative control miRNA mimics and selected cells with a green signal for recordings of sodium current. Fig. 3A shows representative whole-

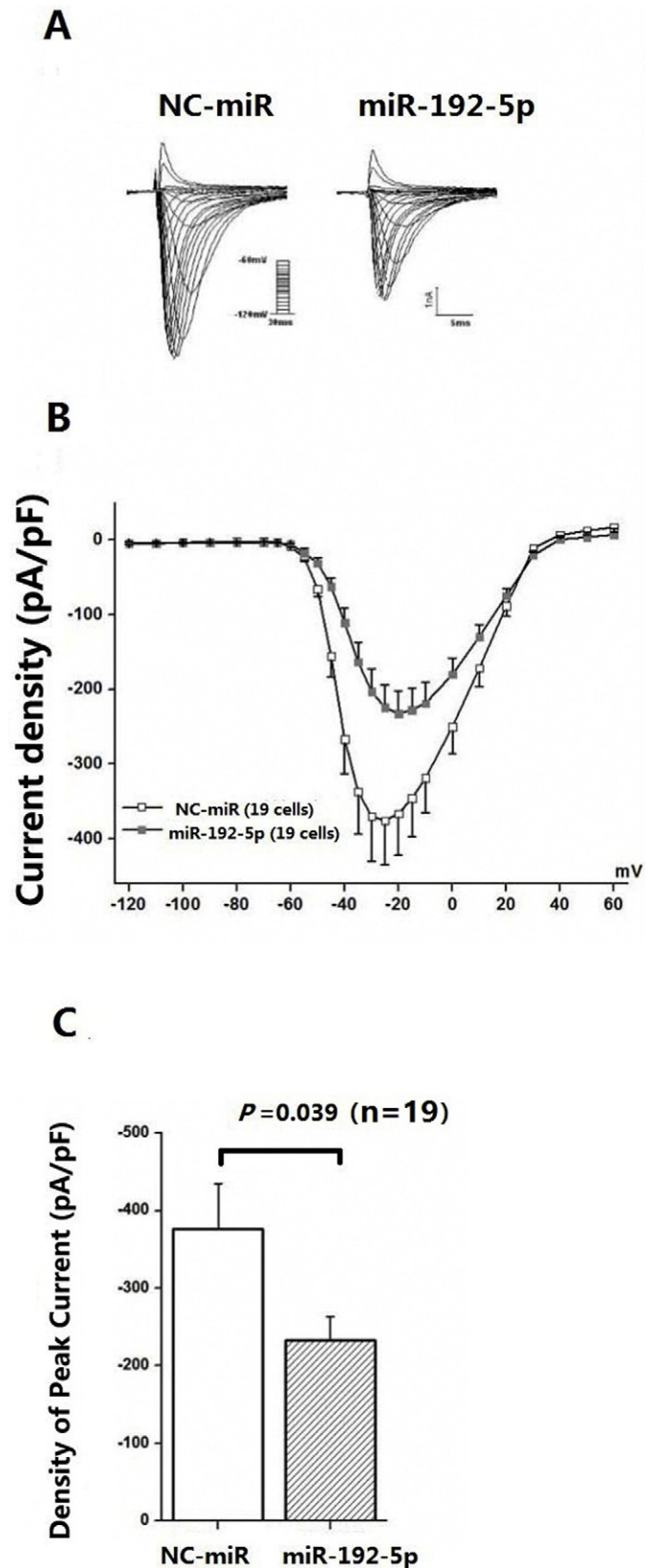


Fig. 3. *MiR-192-5p* significantly reduces the sodium current density. A. Representative traces for sodium currents from HEK293 cells co-transfected with pcDNA3-*SCN5A*-whole-3'UTR and negative control mimics (NC-control) (left) or *miR-192-5p* mimics (right). The current protocol is depicted in the inset. B. Current-voltage (I-V) relation for sodium currents. The average sodium current density is significantly smaller in cells transfected with *miR-192-5p* mimics than those transfected with NC-control mimics. C. Histogram of sodium current densities at -20 mV. Data is in means \pm SEM.

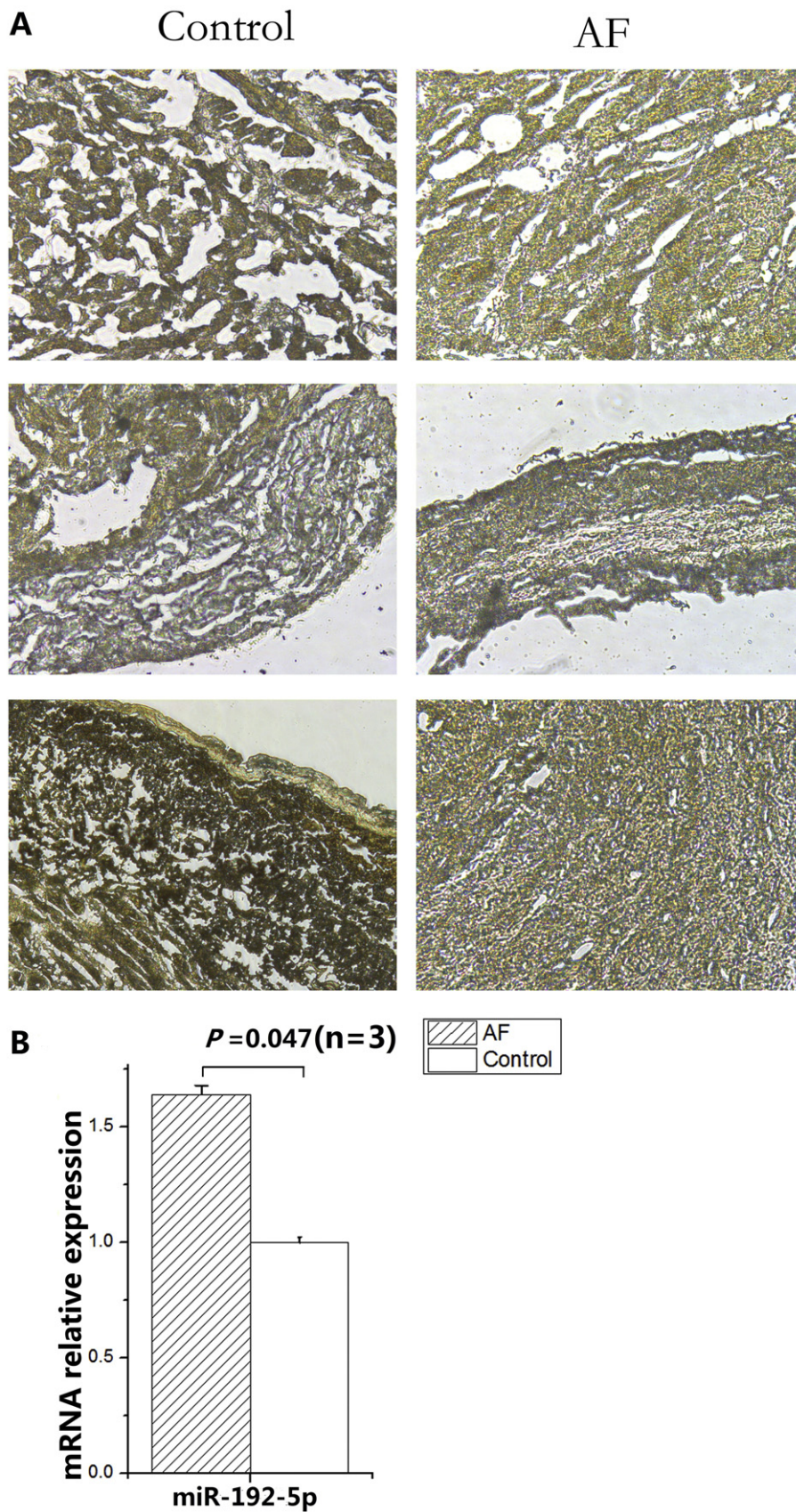


Fig. 4. Up-regulation of *miR-192-5p* expression in tissue samples from AF patients compared with non-AF controls by in situ hybridization. Representative images of in situ hybridization are shown on the left. The images were scanned, quantified and plotted on the right. The data was normalized to non-AF control. Data is shown as means \pm SD.

cell sodium currents recorded from HEK293 cells transfected with a plasmid expressing human *SCN5A* with its whole 3'UTR and PEGFP-N1 (marker for successful transfection) together with miRNA mimics. Compared with NC-control miRNA mimics, cells expressing the *miR-192-5p* mimics had a markedly decreased sodium current density (peak current normalized to cell capacitance, pA/pF, $n = 19$ cells/group) under test potentials. As shown in Fig. 3C, compared with NC-control miRNA mimics, *miR-192-5p* mimics significantly reduced the sodium current density recorded at -25 mV by 40% (-375.63 ± 59.14 pA/pF vs. -224.21 ± 30.83 pA/pF). We also carried out Western blot analysis to measure the expression level of $Na_v1.5$. Consistent with electrophysiological results, *miR-192-5p* mimics significantly reduced the expression level of $Na_v1.5$ in transfected HEK293 cells (data not shown).

3.5. Correlation of increased *miR-192-5p* expression and down-regulation of *SCN5A/Na_v1.5* in human AF tissue

A previous study with right atrial appendages from AF patients showed that the expression level of the $Na_v1.5$ protein and the peak sodium current density were reduced [11]. Therefore, we hypothesized that the expression level of *miR-192-5p*, the negative regulator of *SCN5A*, may be altered in AF. To test the hypothesis, we studied six left appendage tissue samples, three of which were from AF patients and three were from normal non-AF controls (Table 1). We analyzed the expression of *miR-192-5p* using in situ hybridization with a *miR-192-5p* anti-sense oligonucleotide probe. As shown in Fig. 4, *miR-192-5p* expression was significantly up-regulated in AF samples compared with non-AF control samples by 1.6-fold ($P = 0.047$). Consistent with up-regulation of *miR-192-5p*, the expression level of $Na_v1.5$ was significantly down-regulated ($P = 0.015$) (Fig. 5). Therefore, there was a correlation between up-regulation of *miR-192-5p* and down-regulation of $Na_v1.5$ in AF.

To confirm the correlation between up-regulation of *miR-192-5p* and down-regulation of $Na_v1.5$ in AF, we studied another independent set of AF samples and non-AF controls (left appendages) (Table 1) by real-time qPCR analysis. As shown in Fig. 6A, *miR-192-5p* expression was significantly up-regulated in AF samples compared with non-AF control samples by 3.9-fold ($P = 0.020$). The expression of *SCN5A* was significantly down-regulated in AF samples compared with control samples by 3.3-fold ($P = 0.028$) (Fig. 6B). Together, the data suggests that up-regulation of *miR-192-5p* is associated with down-regulation of $Na_v1.5$ in AF.

4. Discussion

Cardiac sodium channel gene *SCN5A* is critical to the generation of cardiac action potentials and, when mutated, causes many cardiac arrhythmias and sudden death. Therefore, the regulation of *SCN5A* expression and function must be in a delicate balance in cells and human bodies. In this study, we report a novel post-transcriptional regulatory mechanism for *SCN5A* expression and function. Our view of how target genes are regulated has been dramatically altered by the important role of miRNAs in the regulation of post-transcriptional protein expression. In this study, we report that *miR-192-5p* is involved in the negative regulation of expression of both *SCN5A* mRNA and $Na_v1.5$ protein and in reducing the density of peak sodium current generated by $Na_v1.5$. Several pieces of evidence strongly support the new finding. First, luciferase assays from Luc-*SCN5A*-3'-UTR reporter genes with the *miR-192-5p* binding site showed that *miR-192-5p* mimics significantly decreased the expression of *SCN5A* by 3.3-fold compared with negative control miRNA mimics (Fig. 1C). When the *miR-192-5p* binding site was deleted from the Luc-*SCN5A*-3'-UTR reporter, the effect of *miR-192-5p* mimics was lost (Fig. 1C). Second, real time qPCR analysis and Western blot analysis showed that *miR-192-5p* mimics significantly reduced the levels of both *SCN5A* mRNA and $Na_v1.5$ protein compared with negative control miRNA mimics (Fig. 2A). Third, *miR-192-5p* mimics significantly

reduced the sodium current density by about 40% compared with NC control mimics (Fig. 3). Together, our study demonstrates an important role of miRNAs in the posttranscriptional regulation of *SCN5A*. Moreover, because *SCN5A* is a target for anti-arrhythmic therapies, *miR-192-5p* becomes a target for anti-arrhythmic therapies.

Very little is known about the function of *miR-192-5p*. It was reported that *miR-192-5p* was usually down-regulated in tumors [27, 40] and in patients with wet age-related macular degeneration (plasma miRNA levels) [41]. In contrast, in this study we found that *miR-192-5p* was significantly up-regulated in tissue samples from AF patients by both in situ hybridization and real-time qRT-PCR analysis (Figs. 4 and 6). Therefore, *miR-192-5p* may play a role in several different human diseases.

AF is the most common arrhythmia encountered at the clinical setting and causes more than 15% of stroke cases. Reduced expression of *SCN5A/Na_v1.5* and reduction of I_{Na} density have been reported in AF [11]. In this study, we found that $Na_v1.5$ expression was significantly down-regulated in AF left atrial appendage samples compared with non-AF control samples by immunohistochemistry (Fig. 5). Similarly, real-time qPCR analysis showed that the expression of *SCN5A* was also significantly down-regulated (Fig. 6B). Interestingly, the expression level of *miR-192-5p* was significantly up-regulated in AF samples compared with non-AF controls (Figs. 4 and 6A). Considering our demonstration that *miR-192-5p* directly down-regulates the expression of *SCN5A/Na_v1.5*, our data suggests that up-regulation of *miR-192-5p* may be one of the causes for down-regulation of *SCN5A/Na_v1.5* in AF tissue samples.

Reduction of I_{Na} density was reported not only in AF, but also in heart failure and myocardial ischemia. The cardiac diseases with reduced I_{Na} density may be potentially treated by modalities that increase I_{Na} density or *SCN5A* expression. Our finding that *SCN5A* is the first downstream target of *miR-192-5p* in the heart makes it an attractive target to modulate the expression of *SCN5A* and I_{Na} density in diseased states. For example, inhibitors of *miR-192-5p* may be used to increase *SCN5A* expression or enhance density of I_{Na} , and treat patients with down-regulation of *SCN5A* or reduced I_{Na} density as in AF, heart failure, myocardial ischemia, BrS and other arrhythmias.

Houria et al. recently showed that *miR-219* also regulated the expression of *SCN5A*, however, it increased the expression of both *SCN5A* mRNA and $Na_v1.5$ protein as well as I_{Na} in HL1 cells derived from mouse atria [42]. MiRNAs normally down-regulates the expression of its downstream target genes either by inducing mRNA degradation or by inhibiting translation. Thus, it is surprising that *miR-219* increases the expression of *SCN5A/Na_v1.5*, thus, the underlying mechanism needs further clarification. On the contrary, here we show that *miR-192-5p* down-regulates the expression of *SCN5A/Na_v1.5*. Therefore, the finding of regulation of *SCN5A/Na_v1.5* by *miR-192-5p* in this study distinguishes the regulation by *miR-219* into two aspects. First, as with almost all miRNAs, *miR-192-5p* down-regulates the expression of *SCN5A/Na_v1.5* and I_{Na} . Second, the *miR-192-5p* binding sequences at the 3'-UTR of *SCN5A* are not evolutionarily conserved in the elephant, rabbit, pig, mouse, rat and other species (Fig. 1A). Therefore, regulation of *SCN5A/Na_v1.5/I_{Na}* by *miR-192-5p* may be specific for some species including humans.

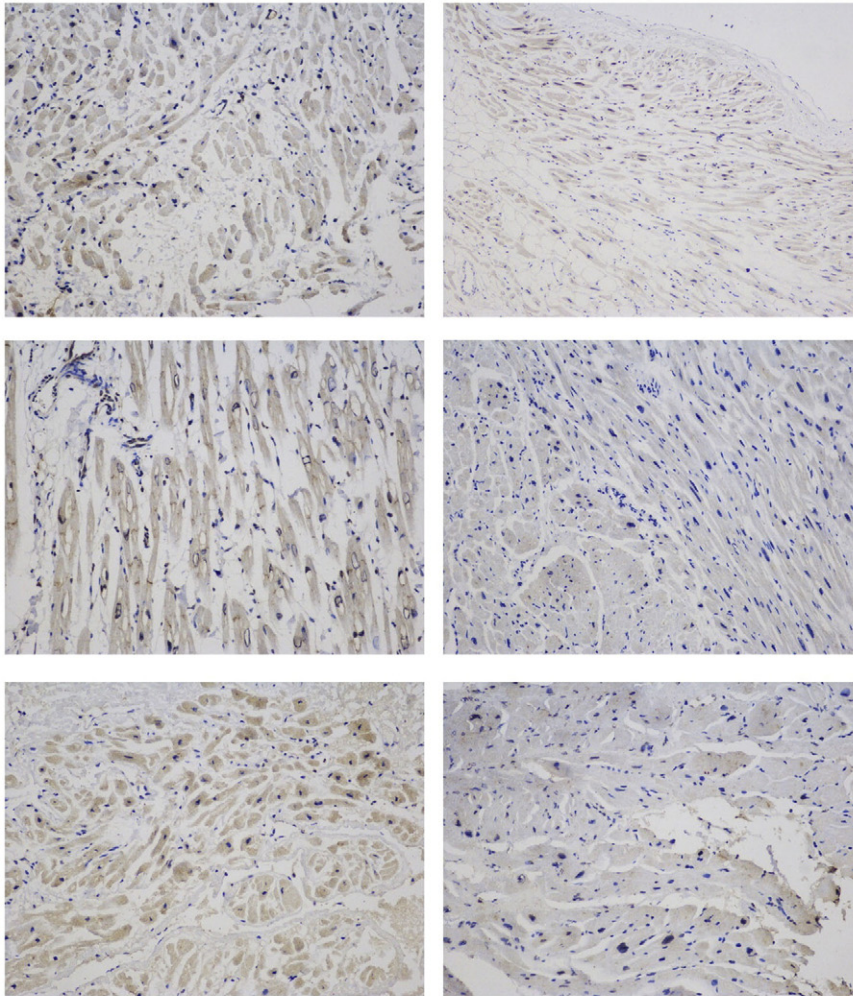
There is a limitation with the present study. The sample size for the human AF study was small due to difficulties in collecting human tissue samples. Future studies with larger sample sizes are needed to further validate the finding on the up-regulation of *miR-192-5p* in AF. Moreover, the up-regulation of *miR-192-5p* may be only one mechanism for the reduction of *SCN5A* expression and I_{Na} in AF. There may be other plausible mechanisms for the reduction of I_{Na} . For example, Eleonora and Herren showed that Ca^{2+} /calmodulin-dependent kinase II (CaMKII) can also regulate I_{Na} [43].

In conclusion, our results in this study demonstrate that the expression of cardiac sodium channel gene *SCN5A* and its function can be

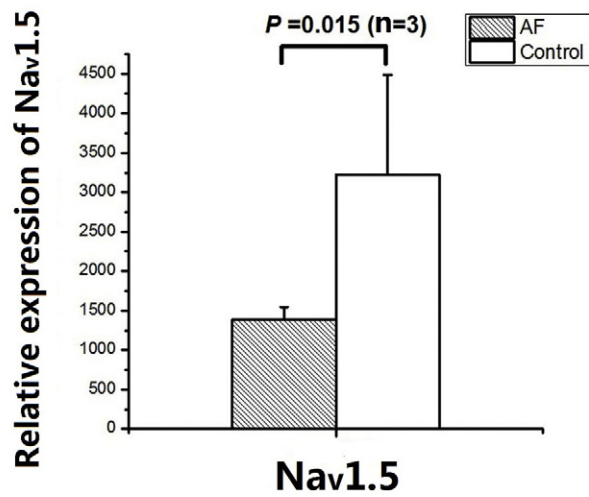
A

Control

AF



B



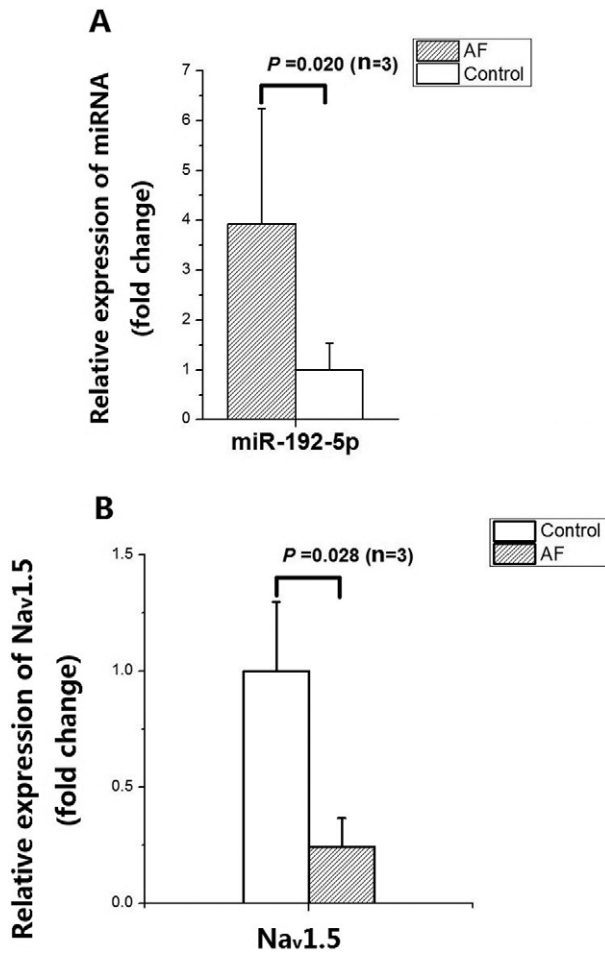


Fig. 6. Correlation between up-regulation of *miR-192-5p* expression and down-regulation of *SCN5A* mRNA expression in tissue samples from AF patients compared with non-AF controls by qRT-PCR analysis. A. Up-regulation of *miR-192-5p* expression in tissue samples from three AF patients compared with three non-AF controls. B. Down-regulation of *SCN5A* expression in tissue samples from three AF patients compared with three non-AF controls. The data was normalized to non-AF control. Data is shown as means \pm SD.

regulated at the posttranscriptional level by *miR-192-5p*. Importantly, we showed that the expression of *miR-192-5p* was up-regulated in AF patients, which may promote the arrhythmia by down-regulating the expression of *SCN5A*. This study provides important insights into the posttranscriptional regulation of cardiac ion channels by miRNAs and suggests that *miR-192-5p* may be an attractive target for novel antiarrhythmia therapies for treating cardiac diseases with reduced *SCN5A* expression and decreased I_{Na} density as in AF, heart failure, myocardial ischemia, BrS and other arrhythmias.

Author contributions

Y-Y. Z., Y. H., Z-J. W., M-C. Z., Y-F. H., Z-P. Z. and Y-X. H. carried out the experimental work. W-H. L. and Z-R. H. provided and assisted in the clinical sample analysis. Q. K. W., Z-R. H., K-T.L., Y-Y. Z., Y. H., Z-J. W., M-C. Z., Y-F. H., Z-P. Z., Y-X. H., Q.C., and X. T. analyzed data and provided advice. Y-Y. Z., Y. H., Q. K. W. and Z-R. H. designed experiments. Q. K. W., Y-Y. Z. and Y. H. wrote the manuscript. Z-R. H., Q.C. and Q. K. W. obtained funding for this project, and directed and supervised the research. Z-R. H. and Q. K. W. are co-corresponding authors.

Competing interests

None.

Acknowledgments

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Fig. 5. Down-regulation of $Na_v1.5$ expression in tissue samples from AF patients compared with non-AF controls by immunohistochemistry with an anti- $Na_v1.5$ antibody. A. The immunohistochemical images from three AF patients and three non-AF patients. B. The immunohistochemical images in (A) were scanned, quantified and graphed. Data is shown as means \pm SD.

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