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REGULATION OF SCN5A BY MICRORNAS: MIR-219 MODULATES *SCN5A* TRANSCRIPT EXPRESSION AND THE EFFECTS OF FLECAINIDE INTOXICATION IN MICE

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Short title: Regulation of Scn5a by microRNAs

The authors declare that there is no conflict of interest concerning any commercial or funding relationship with topic related enterprises. A patent is granted in Spain on the utility and usage of miR-219 modified microRNAs.

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Abbreviations

AP: action potential
E: embryonic day
ECG: electrocardiogram
GFP: Green fluorescent protein
LQTS; long QT syndrome
SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis

ABSTRACT

Background: The human cardiac action potential in atrial and ventricular cells is initiated by a fast-activating fast-inactivating Na^+ current generated by the Nav1.5/*SCN5A* channel, in association with its β1/*SCN1B* subunit. The role of Nav1.5 in the etiology of many cardiac diseases strongly suggests that proper regulation of cell biology and function of the channel is critical for normal cardiac function. Hence, numerous recent studies have focused on the regulatory mechanisms of Nav1.5 biosynthetic and degradation processes as well as its subcellular localization.

Objective: To investigate the role of microRNAs on the *Scn5a*/Nav1.5 post-trancriptional regulation.

Methods: qPCR, immunohistochemical and electrophysiological measurements of distinct microRNA gain-of-function experiments.

Results: Functional studies in HL-1 cardiomyocytes and luciferase assays in fibroblasts demonstrate that *Scn5a* is directly (miR-98, miR-106, miR-200, miR-219) and indirectly (miR-125 and miR-153) regulated by multiple microRNAs displaying distinct time-dependent profiles. Co-transfection experiments, demonstrated that miR-219 and miR-200 have independent opposite effects on *Scn5a* expression modulation. Among all microRNAs studied, only miR-219 increases Scn5a expression levels, leading to altered contraction rhythm of HL-1 cardiomyocytes. Electrophysiological analyses in HL-1 cells revealed that miR-219 increases sodium current (I_{Na}). *In vivo* administration of miR-219 does not alter normal cardiac rhythm but abolishes some of the effects of flecainide intoxication in mice, particularly QRS prolongation.

Conclusions: This study demonstrates the involvement of multiple microRNAs on the regulation of *Scn5a*. Particularly, miR-219 increases *Scn5a*/Nav1.5 transcript and protein expression. Our data suggest that microRNAs, such as miR-219, constitute a promising therapeutical tool to treat sodium cardiac arrhythmias.

INTRODUCTION

Voltage-gated sodium channels are large, multimeric complexes, composed of an α subunit and one or more smaller β subunits[1]. Voltage-gated sodium channels are crucial for the generation and propagation of the all-or-none action potentials (APs) in excitable cells, such as neurons and myocytes. In the heart, the main voltage-gated sodium channel is Nav1.5, encoded by *Scn5a*, providing large and rapid influx of sodium ions (I_{Na}) during the rapid upstroke (phase 0) of the cardiac action potential in atrial and ventricular cells. I_{Na} underlies the initiation and propagation of action potential, determining thus the cardiac excitability and conduction velocity of electrical stimuli through the heart. Dysfunction of cardiac sodium channel is associated with cardiac arrhythmias such as long QT syndrome (LQTS), idiopathic ventricular fibrillation, Brugada syndrome and cardiac conduction disease[2-4], which are commonly associated in many cases with sudden cardiac death. Thus it is becoming evident that cellular and subcellular processing of the cardiac sodium channel must be tightly controlled to provide normal cardiac function[5], including post-transcriptional mechanisms.

microRNAs are small non-coding RNAs (20-24 nt) that mediate translational repression and/or mRNA cleavage. microRNAs have extensively reported as post-transcriptional regulators of cardiac gene expression[6,7], i.e. in cardiac remodeling, growth, conductance and contractility by regulating ion channel expression[8,9]. However, there is little information concerning the role of microRNAs in the regulation of *Scn5a*/Nav1.5 despite its major role in several cardiac arrhythmogenic syndromes[10].

In the present study we demonstrate the functional role of several microRNAs in the post-transcriptional regulation of *Scn5a* expression. miR-219 increases while miR-200 decreases *Scn5a* expression. We also demonstrate that miR-219 increases *Scn5a*/Nav1.5 protein levels, thus leading to increasing cellular sodium current activity. Furthermore, miR-219 over-expression does not alter basal cardiac rhythm in mice, but ameliorates the effects of flecainide intoxication. In addition, we provide evidence that partial modification of the miR-219 seed sequence provides a mean to tightly modulate *Scn5a* expression, thus opening new avenues for the therapeutic application of these biomolecules in arrhythmogenic syndromes.

METHODS

Cell culture and microRNAs transfection assays

Mouse HL-1 immortalized atrial myocardial cells[11] were cultured ($6*10^5$ cells per dish) under appropriate cell culture condition[11] and primary cultures of fetal (17.5 mouse embryos) cardiomyocytes were isolated using standard procedures, cultured accordingly and transfected as detailed in Supplementary Methods. Pre-miRs (Ambion, USA) and anti-miR (Eugentec, Belgium) were transfected with lipofectamine 2000 (Invitrogen, USA) according to manufacturer's guidelines, respectively.

Tissue samples, RNA isolation and qPCR analsyes

C57BL6 mouse embryos were isolated at embryonic day (E) 12.5, E15.5 and E17.5 and the hearts corresponding to all embryos of a single litter were carefully dissected, pooled and snapped frozen in liquid nitrogen and stored at -80ºC until used. In addition, one day neonate and adult hearts were also collected, pooled (n=5) and processed accordingly. The investigation conforms to the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health and it was approved by the University of Jaen ethical committee. Total RNA was isolated and qPCR was performed following standard procedures as detailed in Supplementary Methods.

Luciferase assays

Generation of SNC5A 3´UTR constructs and the corresponding mutated versions is detailed in Supplementary Methods. LC5 human lung fibroblasts (Vircell, Granada, Spain) were co-transfected with 100 ng of the corresponding *Scn5a* 3’UTR-luciferase vector and 100 ng of pmiR- β-galactosidase vector (Ambion) for internal normalization as described in Supplementary Methods. Luciferase activity were normalized to □-galactosidase and compared to non-transfected controls as detailed in Supplementary Methods..

Nav1.5 immunofluorescencestaining and Western blot analyses

Nav1.5 immunofluorescence staining was performed in 100-mm dishes containing ~1-4× 10^6 HL-1 cells as detailed in Supplementary Methods. Control and miR-200 HL-1 transfected cells were homogenized in standard 2x Laemmli buffer supplemented with complete protease inhibitor cocktail (Roche, Madrid, Spain). Protein extracts were normalized and 20 mg were separated by SDS-PAGE electrophoresis and immunoassayed as detailed in Supplementary Methods.

Recording of I_{Na} in HL-1 myocytes

HL-1 cardiomyocytes were plated as described above. HL-1 cells were transiently co-transfected with miR-219- GFP expression vector using lipofectamine 2000 (Invitrogen, USA) following manufacturer instructions. Currents were recorded 48 h after transfection in cells identified by GFP fluorescence under fluorescent microscopy (Nikon Eclipse TE2000S, Nikon) as detailed in Supplementary Methods.

Electrocardiographic Measurements

Adult C57BL/6 mice (~ 3 months old) were tail vein injected with 100µL (10nM) of pre-miR-219 (n=12) or with 100µL of vehicle solution (n=12) and allowed to recover for one day. In a subset of mice (n=6 from each group), the sodium channel blocker flecainide acetate (Sigma Aldrich, Spain) was administered intraperitoneally (20 mg/kg body weight). Twenty minutes after injection, all mice (n=24) were intraperitoneally anesthetized with 2 mg/Kg Ketamine (Parke-Davis, Spain). Surface electrocardiograms (ECG) were recorded and analyzed using a digital acquisition and analysis system (Power Lab/4SP; www.adinstrument.com). QT intervals were corrected for heart rate using Bazett's formula (RR in ms): $QTc=QT/\sqrt{RR}$ as previously reported[12-13].

Statistical analysis

Statistical significance between control and experimental conditions was assessed by the unpaired Student’s t-test when appropriate. Individual measurements were averaged together for each animal/experiment followed by determination of group averages. Statistical analysis of microRNAs effects on *Scn5a* was based on two-way analysis of variance (ANOVA) with microRNAs and time as variables. Following a significant difference in the ANOVA at $p < 0.05$, groups differing from each other were analyzed by Bonferroni’s post-test. Group differences were declared significant at $p < 0.05$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

RESULTS

Bioinformatic algorithms such as TargetScan (http://www.targetscan.org/) and miRanda (http://*www.mirbase.org*) provides a mean to predict putative microRNAs binding sites. In this context, the mouse and human Scn5a genes, but no other *ScnXa* genes (**Supplementary Figures 1-3**), are predicted to be targeted by miR-125, miR-200, miR-219, miR-153, miR-106 and miR-98. qPCR analyses demonstrate that these microRNAs are expressed during mouse and adult cardiogenesis as depicted in **Supplementary Figure 1**. It is important to highlight in this context that miR-200 presents a complementary expression profile to *Scn5a*/Nav1.5[14] while miR-219 and miR-98 display a similar profile to *Scn5a*/Nav1.5, suggesting therefore a putative role for these microRNAs in the regulation of *Scn5a* expression.

Scn5a is regulated by multiple microRNAs that display distinct time-dependent profiles

Analyses of *Scn5a* expression at different time point after microRNA transfection revealed a different profile for each microRNA (**Figure 1A**). Two-way ANOVA analyses demonstrated a statistically significant difference on the regulation of microRNAs depending on time ($F_{(3,4)} = 255.8$, $p < 0.001$) and on the distinct microRNA assayed ($F_{(6,56)} = 380.8$, $p < 0.001$). miR-98 and miR-106 over-expression display no significant changes at 12h/24h after transfection but decreased *Scn5a* levels were observed at 48h. Over-expression of miR-125 and miR-153 leads to no significant changes on *Scn5a* expression at 12h, but consistently increased *Scn5a* expression at 24h/48h after transfection. Interestingly, miR-219 increases while miR-200 decreases *Scn5a* expression at all stages analyzed. Thus, these data demonstrate that microRNAs can differently modulate *Scn5a* transcript levels. Moreover, it is important to note that over-expression of these microRNAs does not modify the expression levels of other cardiac enriched genes such as *Nkx2.5* and *Mef2c* (**Supplementary Figure 4**) neither *Scn3a* nor *Scn10a* sodium channels (**Supplementary Figure 4**).

Functional analyses using luciferase-based *Scn5a* 3´UTR constructs showed that miR-98, miR-106,miR-200 and miR-219 directly target the human *Scn5a* 3´UTR(**Figure 1B**) but not miR-125 and miR-153, since similar luciferase levels were observed 48 hours after transfection. These finding were further validated using expression vectors containing miR-219 and miR-200 sequences in HL-1, respectively **(Supplementary Figure 5A,B)** as well as using plasmids that include mutations in the corresponding in miR-219 and miR-200 binding sites, respectively (**Figure 1C**). Importantly co-transfection both microRNAs (miR-219 and miR-200) resulted in similar *Scn5a* expression levels as compared to non-transfected controls (**Figure 1D**), supporting opposite independent but additive effects for miR-200 and miR-219 modulating *Scn5a* gene expression. Furthermore, transfection of primary cultures of fetal mouse cardiomyocytes with gain-of-function pre-miR-219 and loss-of-function anti-miR-

219, respectively, reveals that miR-219 increases *Scn5a*, while blockage of miR-219 diminishes *Scn5a* expression (**Figure 1E)**. Overall these data demonstrates that miR-219 and miR-200 directly regulates *Scn5a* expression.

miR-219 impairs Nav1.5 subcellular localization

Confocal microscopy analysis demonstrated that miR-219 over-expression leads to significant changes in Nav1.5 protein expression (**Figure 1F**), with a conspicuous accumulation of Nav1.5 around the nuclei (**Figure 1F**) as well as an increase at the plasma membrane. Co-localization with ER-specific markers was not detected (data not shown). Importantly, such a perturbed Nav1.5 distribution was observed exclusively for miR-219. Western blot analyses demonstrated that miR-200 significantly diminished Nav1.5 expression (**Figure 1G**), in line with qPCR analyses. Interestingly, *Mog1* expression is significantly decreased in miR-219 over-expressing HL-1 cardiomyocytes **(Supplementary Figure 6A)**, providing thus preliminary evidences on the molecular mechanisms underlying aberrant Nav1.5 subcellular localization. Furthermore, transcriptional inhibition experiments using α-amanitin suggest that miR-219 action is exerted at post-transcriptional level (**Figure 2A,B**), either by increasing *Snc5a* mRNA stability and/or by enhancing *Scn5a*/Nav1.5 protein translation.

miR-219 increases current amplitude and shifts voltage-dependent activation of I_{Na} in HL-1 cells

Contraction rhythm and frequency analyses demonstrate that miR-219 over-expression increases the contraction rate (85±3 beat/min) as compared to controls (77±3 beat/min) (**Supplementary Figure 6B and Supplementary Video control and miR-219)**, consistent with the observed increased *Scn5a* transcript and protein expression (**Figures 1A and 1F**).

Furthermore, to determine whether miR-219 over-expression directly alters the function of the cardiac sodium channel Nav1.5, I_{Na} was recorded in HL-1 cardiomyocytes transfected with miR-219 conjugated with GFP (+miR-219) and compared to controls. As detailed in **Figure 3A** and **3B**, density of I_{Na} recorded in miR-219 transfected cells was markedly increased (≈2-fold greater; $p<0.05$, n=21) compared with that recorded in control cells, although miR-219 did not modify cell capacitance (14.2±1.4 vs 16.1±1.5 pF). Transfection of miR-219 did not modify activation and inactivation kinetics or the E_{rev} ($p>0.05$, **Table 1**). Interestingly, transfection of miR-219 slightly but significantly shifted the midpoint of the conductance-voltage and availability curves to more negative potentials ($p<0.05$), without modifying the slope values (**Table 1**). In addition, the amplitude of the window current was slightly decreased (0.018%) and the peak was shifted to more negative potentials (-95 mV) (**Figure 3D**). Since the voltage-dependent effects cannot be attributed to the increase in the expression of Nav1.5, we surmised that miR-219 may be affecting the expression of some of the regulatory proteins that forms the Nav1.5 channelosome. In this context, our results demonstrated that miR-219 also increased *Scn1b* mRNA expression, which in turn can be translated into increase expression of Navβ1 and might explain, at least in part, the effects on the shift of voltage dependence of activation and inactivation[15] induced by miR-219 overexpression (**Supplementary Figure 7**). However, putative effects of miR-219 on other regulatory proteins cannot be ruled out.

miR-219 in vivo administration does not alter basal cardiac rhythm but it reverses flecainide-induced arrhythmogenesis

Flecainide is a class 1C anti-arrhythmic drug[16] and severe flecainide intoxication frequently leads to rapid hypotension onset and ventricular arrhythmias, mainly due to sodium channel blocking effects. Mice injected with flecainide show progressive prolongation of QRS complex of about 50% of the cases[16] (**Figures 4A and 4B**). qRT-PCR results show that flecainide poisoning does not alter *Scn5a* transcript levels (**Figure 4C**). Interestingly, miR-219 injected mice demonstrate no ECG parameters differences yet significant higher *Scn5a* expression levels were observed. Importantly, injection of both miR-219 and flecainide rescues the QRS and QT prolongations observed by flecainide administration alone (**Figure 4B**) restoring *Scn5a* transcript expression to control levels (**Figures 4C and 4D**). Overall, these data demonstrate that miR-219 administration did not affect the cardiac rhythm in normal mice, but it rescued QRS and QT prolongation of flecainide intoxication.

Modulator roles of modified miR-219 gain- and loss-of-function microRNAs

We tested the range of modulation obtained by discretely altering miR-219 sequence complementary to the *Scn5a* 3´ UTR. Two distinct chimerical biomolecules, mimicking pre-miR-219 endogenous sequence (miR-219) but with three extra nucleotides pairing (miR-219+) or impairing (miR-219-) to *Scn5a* 3 ´UTR were synthesized and functionally assessed. qRT-PCR data show that miR-219+ over-expression increases (~40%) *Scn5a* expression while miR-219- decreases (~25%) *Scn5a* transcript levels (**Figure 5A,B)**.

DISCUSSION

miRNAs have attracted much attention as regulators of cardiac remodeling, growth, conductance and contractility[17] and aberrant microRNA expression has recently been reported in arrhythmogenic syndromes[18]. Conditional deletion of microRNA function, has revealed a crucial role of microRNAs during cardiogenesis, resulting in aberrant cardiac morphogenesis and conductive defects[6]. To date, only a limited number of microRNAs have been reported to play essential roles in cardiac electrophysiology[19]; e.g. miR-1 has been reported to regulate *Gja1* (Cx43) and *Kcnj2* (Kir2.1) demonstrating a crucial role for the maintenance of normal cardiac conduction[8,20], However, despite the relevant role of the *Scn5a*/Nav1.5 in cardiac electrophysiology, scarce information is available about regulation of *Scn5a* by microRNAs.

Previous studies have demonstrated Nav1.5 is dynamically expressed during heart development[14]. In this study, we provide the first evidence that *Scn5a* dynamic expression is accompanied by a differential expression of microRNAs predicted to be its post-transcriptional regulators. In this context, it is important to note that miR-125, miR-200, miR-219, miR-153a, miR-106 and miR-98 are expressed at different levels during cardiac development. Some of these microRNAs i.e. miR-125 have been already implicated in heart diseases such as cardiac hypertrophy[15] while others, such as miR-219, have been reported to be involved in neurological disorders or miR-200, miR-98, miR-106 and miR-153 in cancer development[16-23]. In this context, miR-219 and miR-98 display an expression profile similar to *Scn5a*, while miR-200 presents a complementary expression profile, supporting a putative role of these microRNAs regulating *Scn5a* expression.

Functional analyses demonstrate that that each microRNA exerts its effect at different time points regulating *Scn5a* levels. All microRNAs assayed except miR-219, resulted in a progressive decrease of *Scn5a* levels with longer transfection times. Comparative analyses of gain-of-function experiments in HL-1 cardiomyocytes, primary culture of cardiomyocytes and *Scn5a* 3´UTR luciferase based experiments in fibroblasts, demonstrate that miR-98, miR-106, miR-200 and miR-219 directly target the *Scn5a* 3´UTR, while miR-125 and miR-153 seems to indirectly regulate *Scn5a*. Interestingly, none of the tested microRNAs except miR-219 leads to a subcellular changes in Nav1.5 protein expression by over-expression in HL-1 cardiomyocytes. Nav1.5 subcellular localization was preferentially perinuclear, probably within the Golgi apparatus, a notion supported by additional post-transcriptional targeting of *Mog1*[25] by miR-219 as demonstrated herein.

Although there is evidence that in specific settings, microRNAs can increase, rather than decrease the levels of target transcripts[24], the exact mechanisms by which microRNAs lead to increased gene transcript remains to be understood. A recent study by Place *et al.*[26] reported that miR-373 induced the expression of genes by targeting their promoter sequence rather than the 3’UTR. In the present study, we described for the first time the role of miR-219 as an enhancer of *Scn5a* expression in cardiomyocytes. Our data demonstrate that the increase of *Scn5a* transcript and protein expression in miR-219 over-expressing HL-1 cells is mediated at post-transcriptional level, either by increasing *Scn5a* mRNA stability or by enhancing Scn5a/Nav1.5 translation. Our patch-clamp analyses further support those findingas as the increase of Nav1.5 protein levels in HL-1 cardiomyocytes leads to an increased I_{Na} density. Thus our data reveal a novel means to modulate *Scn5a* expression in channelopathies characterized by Scn5a gain-of-function (LQT syndrome; LQT3) and/or loss-of-function (Brugada syndrome), opening new therapeutical strategies.

microRNAs have been recently shown to play crucial roles not only in modulating cardiac ion channel expression. Yang et al.[27] described that gain-of-function miR-1 transfection assays into healthy hearts significantly ($p<0.05$) widened the QRS complex and prolonged the QT interval, indicating a cardiac conduction arrest, while using loss-of-function miR-1 experimental settings narrowed the QRS complex. In the present study we have observed that miR-219 gain-of-function did not modify the heart rhythm of healthy mice. Interestingly, miR-219 gain-of-function in flecainide intoxicated mice lead to a recuperation of the normal ECG parameters caused by flecainide overdose. It is well known actually that flecainide administration in healthy hearts leads to a functional block of the cardiac sodium channel protein[16]. Here we have demonstrated that flecainide toxicity in mice basically does not alter *Scn5a* transcript levels while miR-219 administration significantly increases it, but importantly, co-administration of miR-219 and flecainide leads to a significant restoration of normal *Scn5a* transcript levels. Thus as a proof-of-concept, our data support the notion that miR-219 administration can reverse the effects of flecanide intoxication both at molecular and electrophysiological level.

Modification of a discrete number of nucleotides from the endogenous mature microRNA has a direct and subtle effect on the *Scn5a* post-transcriptional regulation. Although there are several reports that have indeed nicely identified six nucleotide seed sequence of the mature microRNA as a hallmark to drive post-transcriptional regulation of the targeted mRNA and a functional role of discrete flanking nucleotides to the seed sequence have been suggested[28], this is to our knowledge the first study reporting the modulatory properties of a modified native microRNA. These modified microRNAs can open new avenues to design biomolecules that can fine-tune modulate *Scn5a* bioavailability, especially in cardiac electrophysiological dysfunctional syndromes such as Brugada syndrome or long QT syndrome (LQT3)[29], which are characterized by *Scn5a*/Nav1.5 loss-of-function and gain-of-function, respectively.

CONCLUSIONS

In summary, our data reveal that the regulation of *Scn5a*are modulated by the action of multiple microRNAs,. Our data also provide evidences that a single microRNA might target several members of a discrete signaling pathway, in line with previous reports[28]. In addition, we demonstrate that miR-219 and miR-200 have opposite and complementary roles in modulating post-transcriptional regulation of *Scn5a*, with impact on channel function. Thus, these findings open the possibility of that microRNAs may be used to modulate *Scn5a* transcript bioavailability and thus to be used as putative therapeutic tools to regulate loss-of function sodium channelopathies such as Brugada and/or gain-of function sodium channelopathies such as long QT syndrome[29].

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CLINICAL PERSPECTIVES

The pore-forming subunit of the cardiac sodium channel (SCN5A/Nav1.5) plays a pivotal role controlling the upstroke phase of cardiac action potential and the deregulation of its function underlies distinct cardiac arrhythmogenic syndromes, such as Brugada and long QT syndrome. In this study we demonstrate that distinct microRNAs can post-transcriptional modulate the expression of the pore-forming subunit of SCN5A/Nav1.5, and thus influence its electrophysiological output. Furthermore, subtle molecular changes within the microRNAs sequences leads also to discrete SCN5A/Nav1.5 expression changes. Our findings open up the possibility of using microRNAs as therapeutical tools to control and restore cardiac sodium channel deficiencies. Our preliminary *in vivo* data support such claims. Exploring the biological effects of engineered microRNAs would be desirable to optimize the pharmacological-electrophysiological correlates. In addition larger animal cohorts are required to be analyzed before considering to enter into the clinical arena.

FIGURE LEGENDS

Figure 1: ***Scn5a is regulated by several microRNAs with a specific time of action.*** **Panel A.** qRT-PCR analyses of *Scn5a* expression in HL-1 cardiomyocytes transfected with distinct microRNAs at different time points after transfection, i.e. 12, 24, 48 and 72 hours, respectively. Two-way ANOVA revealed a significant microRNAs effect ($F_{(6,56)} = 380.8$, $p < 0.001$) and a significant time effect on *Scn5a* expression ($F_{(3,4)} = 255.8$, $p < 0.001$). $p<0.05$ were considered statistically significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Bonferroni’s post test. **Panel B.** Luciferase-based assays displaying the relative luciferase values after miR-98, miR-106, miR-125, miR-153, miR-200 and miR-219 overexpresion (white bars) as compared to non-transfected controls (black bars). **Panel C (Left).** Luciferase-based assays showing the relative luciferase values after miR-219 co-transfection with native (UTR1+miR-219) or mutated (UTR1 mut+miR-219) UTR1 3’UTR construction. The created mutations are localized in miR-219 binding site. **(Right)** Luciferase-based assays showing the relative luciferase values after miR-200 co-transfection with native (UTR1+miR-200) or mutated (UTR1 mut+miR-200) UTR1 3’UTR construction. Similarly, the created mutations are localized in miR-200 binding site. **Panel D.** Co-transfection assays of miR-200 and miR-219 in HL-1 cardiomyocytes at 24 and 48 hours after transfection (white bars) display similar Scn5a expression levels as compared to non-transfected controls (black bars), respectively as revealed by qRT-PCR. **Panel E**. qRT-PCR analyses of *Scn5a* expression in primary culture of mouse fetal cardiomyocytes transfected (24 hours) with pre-miR-219 or anti-miR-219 as compared to controls (non-transfected cells). **Panel F.** Confocal imaging of Nav1.5 immunostaining in HL-1 cardiomyocytes (Control) and HL-1 cells transfected with miR-98, miR-106, miR-125, miR-153, miR-200 and miR-219 respectively and fixed at 72 hours post-transfection and . Semi-quantitative analysis using Leica LCS software of Nav1.5 sodium channel α-subunits in HL-1 cardiomyocytes over-expressing *miR-219* compared to non transfected cells. Nuclei are labeled in red using DRAQ5 and Nav1.5 in green. All measured intensities were expressed as a percentage of the overage intensity value. Results are averaged from measures performed in three experiments and three fields per experiment (Total number of fields analyzed=9). **Panel G**. Western blot

analyses of Nav1.5 expression after miR-200 over-expression in HL-1 cells. Note that expression of Nav1.5 is significantly decreased, in line with qPCR analyses. Mb; cell membrane, N, nucleus, G, perinuclear cytoplasmatic organelles, including mainly the Golgi apparatus and endoplasmatic reticulum. White bars correspond to controls and black bars correspond to the corresponding experimental condition * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 2. miR-219 stabilizes Scn5a expression. **Panel A.** qRT-PCR analyses of *Scn5a*, *5S*, *β-actin* , *Gusb* and *Nkx2.5* expression in HL-1 cardiomyocytes treated with α-amanitin alone (α-amanitin) or followed with miR-219 over-expression (α-amanitin+miR-219) (white bars) compared to control condition (Black bars).**Panel B.** Confocal imaging of Nav1.5 immunostaining in HL-1 cardiomyocytes (Control), and HL-1 cells treated with α-amanitin alone (α-amanitin) or followed with miR-219 over-expression (α-amanitin+miR-219). * $p<0.05$.

Figure 3: miR-219 increases I_{Na} density. **Panel A:** I_{Na} traces recorded in HL-1 cells transfected with miR-219 conjugated with GFP (+miR-219) or with GFP alone (Control). **Panels B and C:** Current density- (**B**) and conductance-voltage and availability curves (**C**) for I_{Na} recorded in HL-1 cells in the absence or presence of miR-219. The inset in panel C shows current traces recorded in the presence of miR-219 by applying test pulses to -20 mV after prepulses to potentials ranging -130 and -30 mV to construct the availability curves. In **Pane**l **C**, the solid lines represent the fit of a Boltzmann function to the data. *$P<0.05$ vs control. **Panel D:** Effects of miR-219 overexpression on the window current. The plots show the probability of being within this window for currents recorded in cells expressing or not miR-219. The probability of Na^+ channel opening within the window was calculated from the product of mean activation and inactivation parameters.

Figure 4: ***miR-219 does not alter normal mouse cardiac rhythm but it reverts flecainide intoxication.*** **Panel A.** Electrocardiogram recordings of adult C57BL/6 mice without (Control) or with flecainide administration and/or miR-219. **Panel B.** Measurements of the electrocardiogram parameters of adult C57BL/6 mice after flecainide and/or miR-219 administration compared to controls. **Panel C.** qRT-PCR quantification of *Scn5a* and *miR-219* expression levels of adult C57BL/6 mice hearts in controls, flecainide, miR-219 and miR-219 and flecainide administration. ** $p<0.01$, *** $p<0.001$.

Figure 5. ***miR-219 modified biomolecules modulate Scn5a expression. Panel A.*** qRT-PCR quantification of *Scn5a* expression levels in HL-1 cardiomyocytes transfected with native miR-219 (miR-219), loss-of-function mutated miR-219 (miR-219-) and gain-of-function mutated miR-219 (miR-219+). **Panel B.** Luciferase activity quantification in human fibroblasts co-transfected with pmiR-Scn5a-3'UTR1 vector and native or modified miR-219 molecules, respectively. Native miR-219 is noted as (miR-219), loss-of-function mutated miR-219 is indicated as (miR-219-) and gain-of-function mutated miR-219 is illustrated as (miR-219+). * $p<0.05$, ** $p<0.01$.

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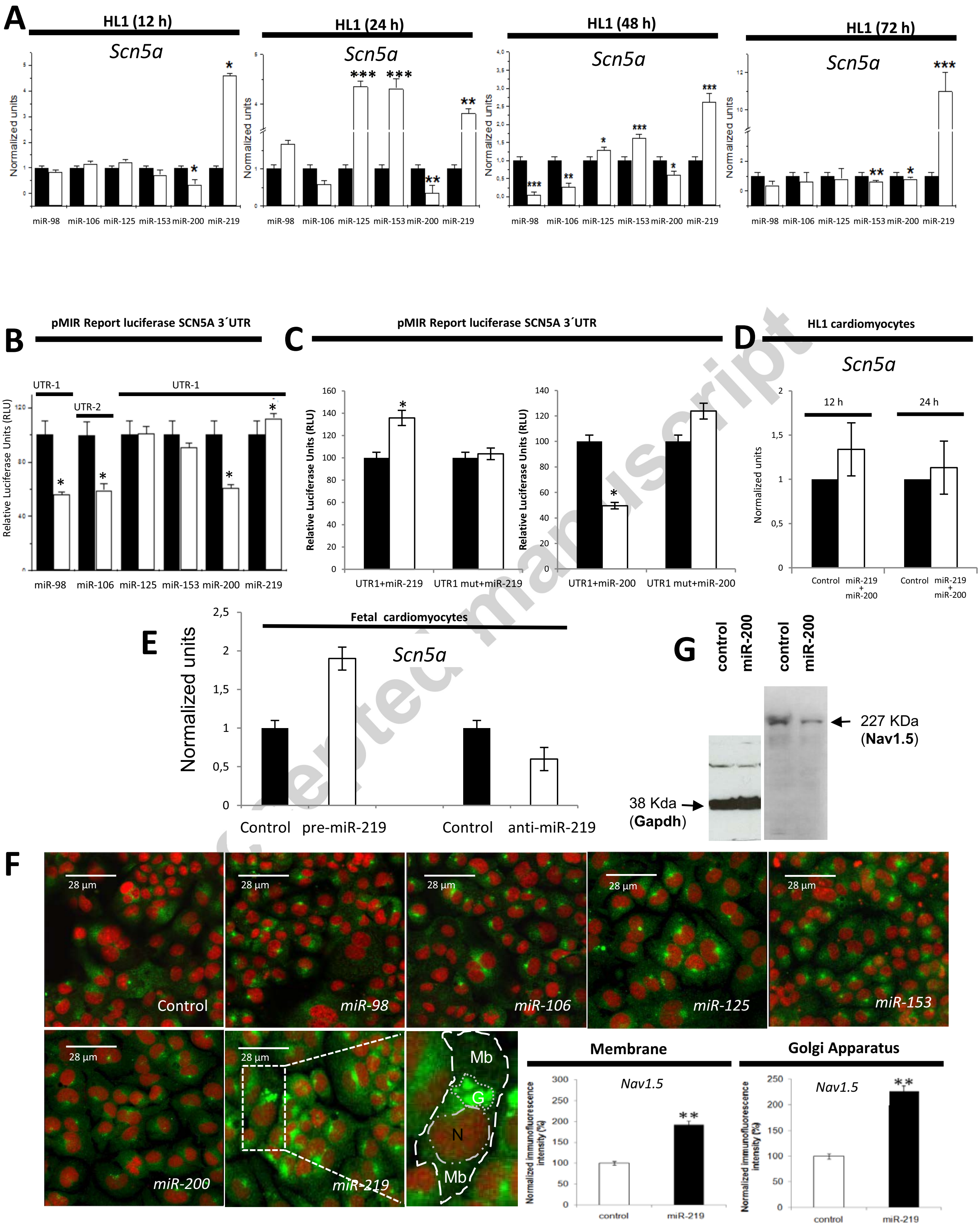
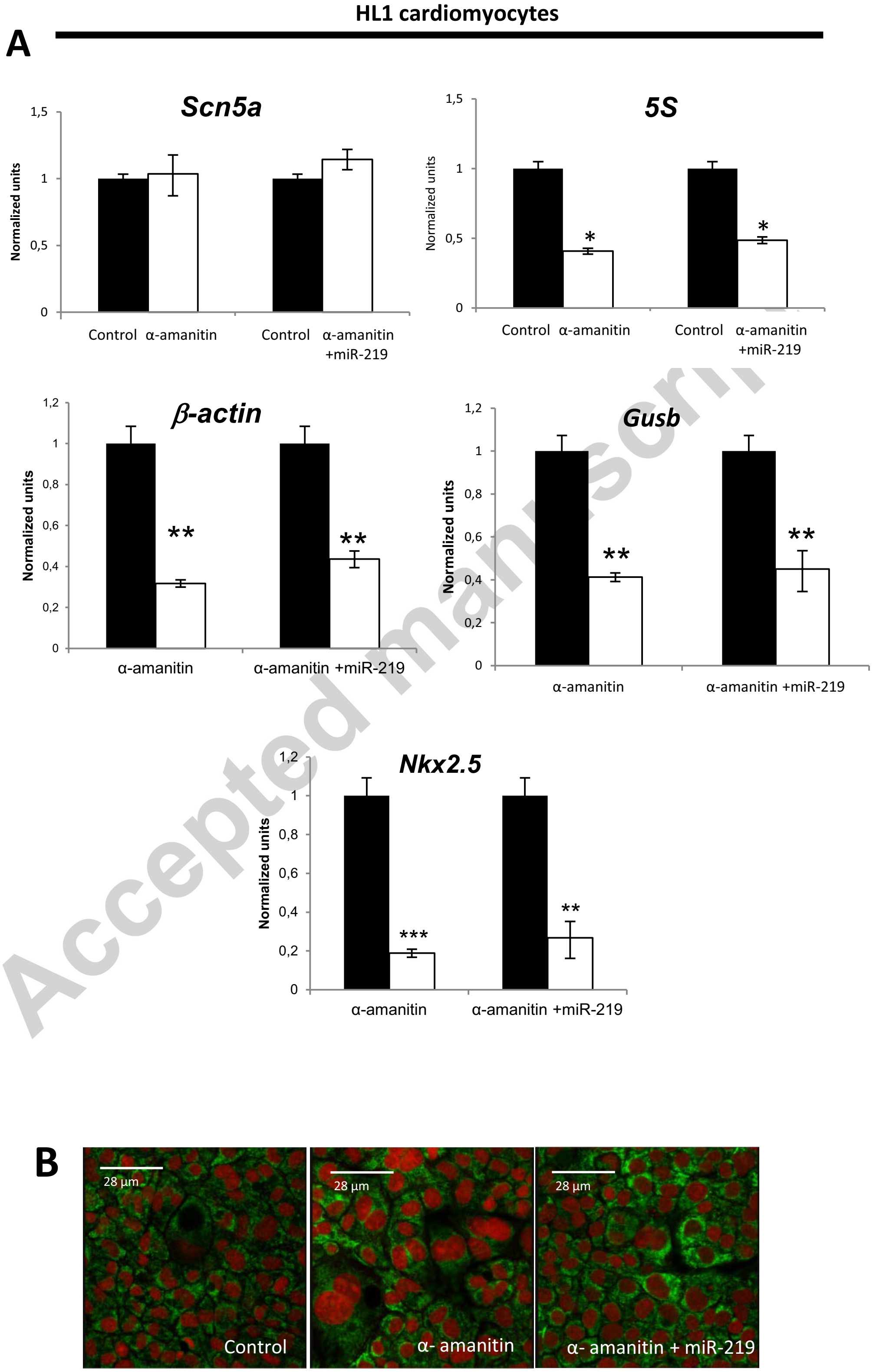
Table 1. Effects of miR-219 on time- and voltage-dependent properties of I_{Na} recorded in HL-1 cells.

			Time course of I_{Na} activation $^{\delta}$	**Time course of I_{Na} decay$^{\delta}$**		**Voltage dependence of I_{Na} activation**		**Voltage dependence of I_{Na} inactivation**	
	Maximum I_{Na} density (pA/pF)	**E_{rev} (mV)**	**τ_{act} (ms)**	**$\tau_{f\ inact}$ (ms)**	**$\tau_{s\ inact}$ (ms)**	**V_h (mV)**	***k***	**V_h (mV)**	***k***
Control (n=12)	-51.3±10.7	28.1±2.0	0.3±0.05	1.0±0.1	7.7±1.3	-35.9±3.0	7.3±1.0	-91.5±1.8	4.5±0.2
+miR219 (n=21)	-97.4±11.8*	27.2±2.7	0.3±0.03	1.1±0.1	7.0±1.2	-42.6±1.8*	6.9±0.4	-97.6±2.0*	4.6±0.2

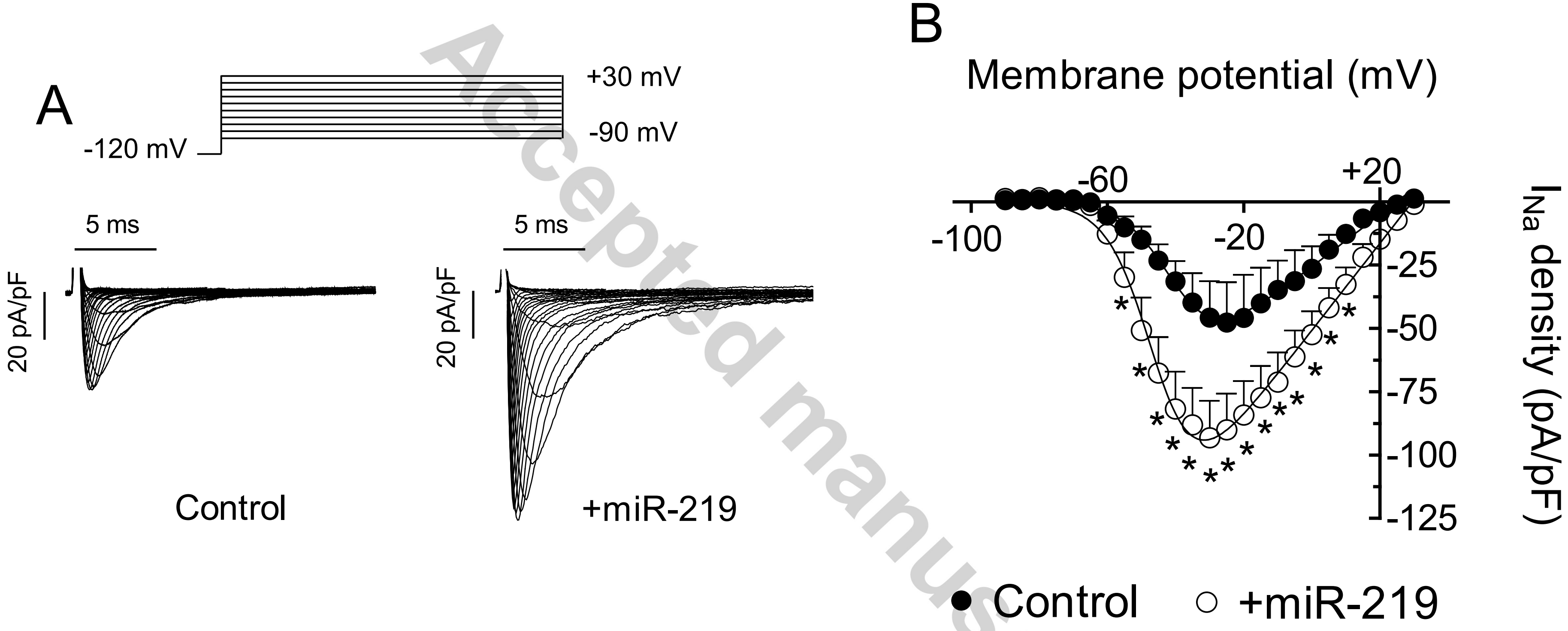
* $P<0.05$ vs control values. $^{\delta}$ Activation and inactivation kinetics were measured by fitting monoexponential and biexponential, respectively, functions to the traces corresponding to the maximal I_{Na} amplitude. E_{rev}= Reversal potential for Na^+; τ_{act}= time constant of activation. $\tau_{f\ inact}$ and $\tau_{s\ inact}$ = fast and slow time constants, respectively, of current decay; V_h and *k*= midpoint and slope, respectively, of the conductance-voltage and availability curves.



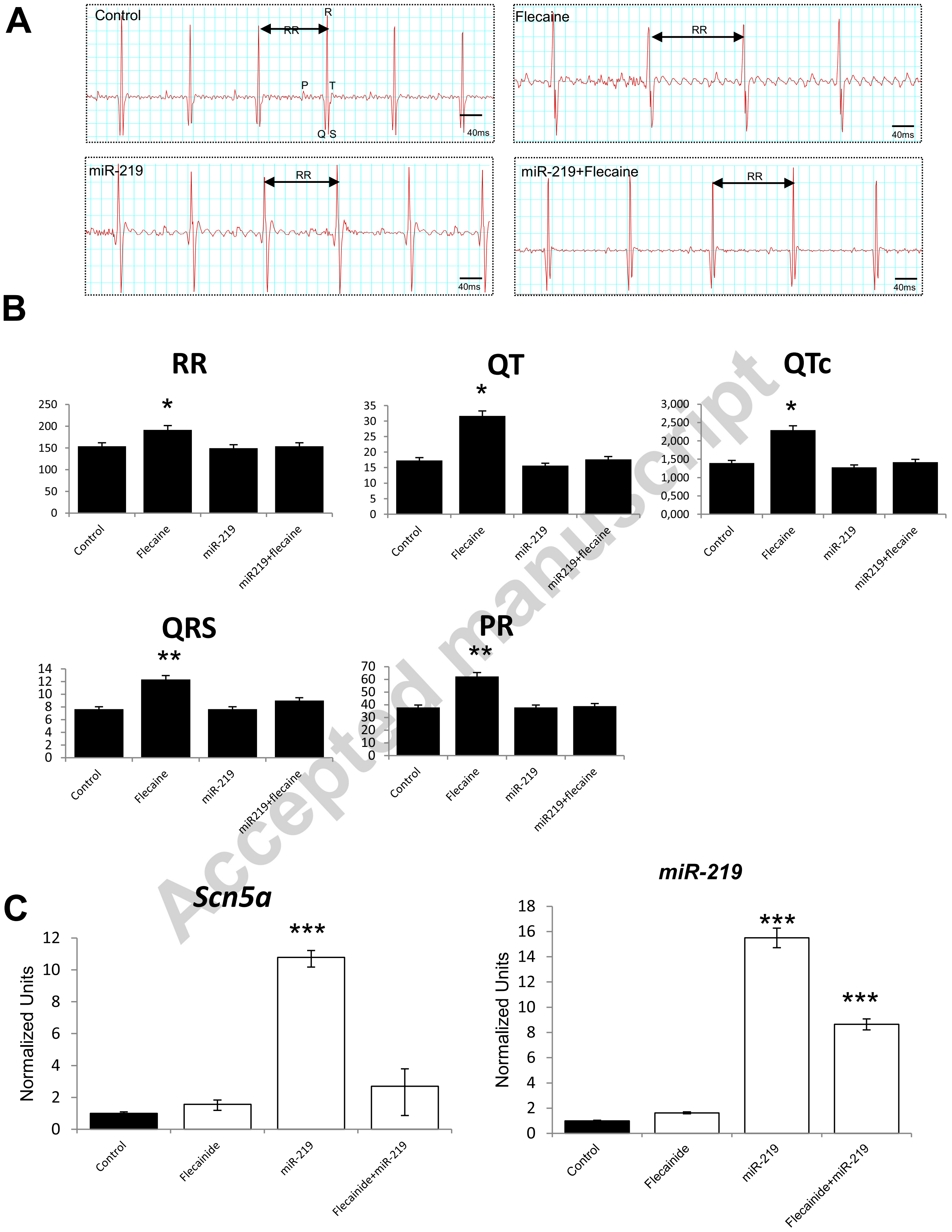
Daimi et al., Figure 1



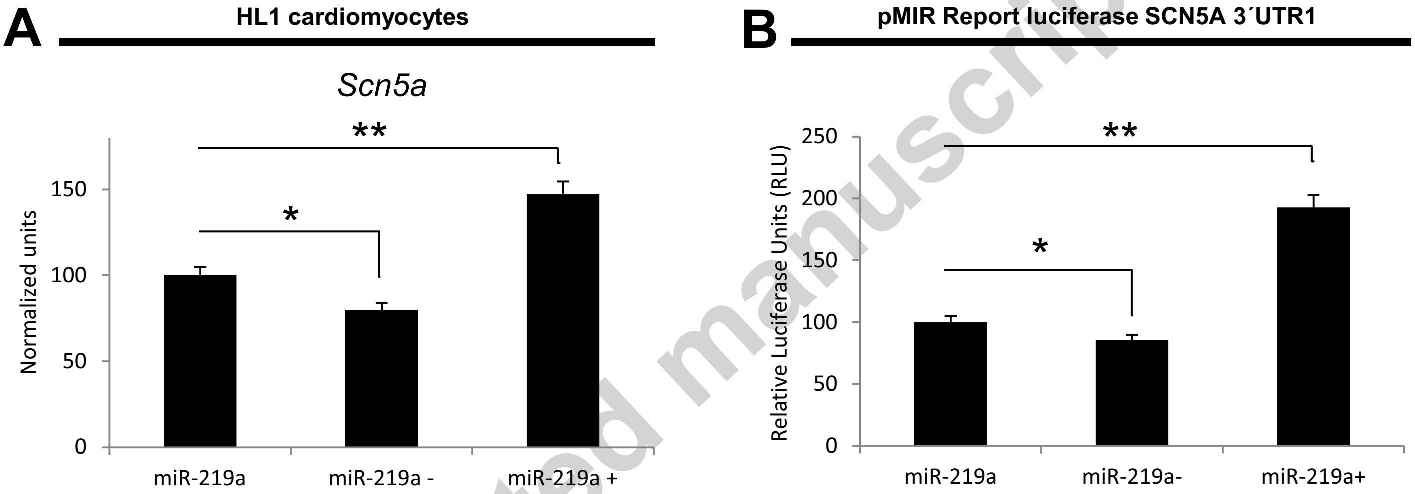
Daimi et al., Figure 2



Daimi et al., Figure 3



Daimi et al., Figure 4



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