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Xiaoming Zhang, … , Barry London, Ryan L. Boudreau


$SCN5A$ encodes the voltage-gated Na\(^+\) channel Na\(_v\)1.5 that is responsible for depolarization of the cardiac action potential and rapid intercellular conduction. Mutations disrupting the $SCN5A$ coding sequence cause inherited arrhythmias and cardiomyopathy, and single-nucleotide polymorphisms (SNPs) linked to $SCN5A$ splicing, localization, and function associate with heart failure--related sudden cardiac death. However, the clinical relevance of SNPs that modulate $SCN5A$ expression levels remains understudied. We recently generated a transcriptome-wide map of microRNA (miR) binding sites in human heart, evaluated their overlap with common SNPs, and identified a synonymous SNP (rs1805126) adjacent to a miR-24 site within the $SCN5A$ coding sequence. This SNP was previously shown to reproducibly associate with cardiac electrophysiological parameters, but was not considered to be causal. Here, we show that miR-24 potently suppresses $SCN5A$ expression and that rs1805126 modulates this regulation. We found that the rs1805126 minor allele associates with decreased cardiac $SCN5A$ expression and that heart failure subjects homozygous for the minor allele have decreased ejection fraction and increased mortality, but not increased ventricular tachyarrhythmias. In mice, we identified a potential basis for this in discovering that decreased $Scn5a$ expression leads to accumulation of myocardial reactive oxygen species. Together, these data reiterate the importance of considering the mechanistic significance of synonymous SNPs as they relate to miRs and disease, and highlight a surprising link between $SCN5A$ expression […]
A common variant alters SCN5A–miR-24 interaction and associates with heart failure mortality

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SCN5A encodes the voltage-gated Na+ channel \( \text{Na}_v \)1.5 that is responsible for depolarization of the cardiac action potential and rapid intercellular conduction. Mutations disrupting the SCN5A coding sequence cause inherited arrhythmias and cardiomyopathy, and single-nucleotide polymorphisms (SNPs) linked to SCN5A splicing, localization, and function associate with heart failure–related sudden cardiac death. However, the clinical relevance of SNPs that modulate SCN5A expression levels remains understudied. We recently generated a transcriptome-wide map of microRNA (miR) binding sites in human heart, evaluated their overlap with common SNPs, and identified a synonymous SNP (rs1805126) adjacent to a miR-24 site within the SCN5A coding sequence. This SNP was previously shown to reproducibly associate with cardiac electrophysiological parameters, but was not considered to be causal. Here, we show that miR-24 potently suppresses SCN5A expression and that rs1805126 modulates this regulation. We found that the rs1805126 minor allele associates with decreased cardiac SCN5A expression and that heart failure subjects homozygous for the minor allele have decreased ejection fraction and increased mortality, but not increased ventricular tachyarrhythmias. In mice, we identified a potential basis for this in discovering that decreased Scn5a expression leads to accumulation of myocardial reactive oxygen species. Together, these data reiterate the importance of considering the mechanistic significance of synonymous SNPs as they relate to miRs and disease, and highlight a surprising link between SCN5A expression and nonarrhythmic death in heart failure.

Introduction

The onset and clinical course of heart failure is shaped by a complex interplay of environmental and genetic factors that influence a host of biological processes, including gene regulatory mechanisms. Optimal cardiac function relies on finely tuned expression of genes related to cardiac structure, energetics, conduction, and contraction. Tight regulation of cardiac ion channels governing heart rate and rhythm is vital, since slight changes in ion conductance can trigger arrhythmia, elevating one’s risk for sudden cardiac death. While prior research has established that heart failure invokes cardiac ion channel remodeling with arrhythmias and that some mutations in ion channels cause cardiomyopathy with heart failure (1, 2), it remains unclear how alterations in channels related to cardiac conduction may influence nonarrhythmic deaths in heart failure patients.

The cardiac action potential is initiated by sodium current through the heart’s primary voltage-gated sodium channel, \( \text{Na}_v \)1.5, encoded by SCN5A. \( \text{Na}_v \)1.5 is critical for normal cardiac function, and mutations in \( \text{Na}_v \)1.5 and \( \text{Na}_v \)1.5-interacting genes that modify its function and trafficking cause arrhythmic syndromes (Brugada, long QT, inherited conduction) (3–8) and cardiomyopathies with contractile dysfunction (9, 10). Moreover, genome-wide association studies (GWAS) have identified several common SNPs within the SCN5A locus linked to electrocardiographic measures (PR, QT, and QRS intervals) (11–16) and Brugada syndrome (17), an inherited arrhythmic disease with sudden cardiac death. The clear relevance of SCN5A to arrhythmias has fueled vigorous research of cellular mechanisms controlling \( \text{Na}_v \)1.5 biosynthesis, posttranslational processing, localization, and function (18–20). Among these efforts, researchers have identified alternatively spliced SCN5A transcript isoforms that associate with fatal arrhythmias in heart failure subjects (21) and have begun characterizing miR-mediated regulation via the SCN5A 3′-untranslated region (3′-UTR) (22–24). However,
given the complex nature of this sizable gene (nearly 30 exons spanning > 80 kb), continued efforts are needed to further define SCN5A transcript regulatory mechanisms; for example, no studies have yet assessed miR functions within the expansive SCN5A coding region.

MiRs have been established as key effectors in cardiovascular biology and disease (25–27). These short RNAs are incorporated into Argonaute (Ago) proteins, producing effector complexes capable of base-pairing with and repressing target transcripts via translational inhibition and mRNA destabilization (28–30). Canonic ally, miRs engage 3′-UTRs containing sequences complementary to their seed (miR nucleotide positions 2–8; ref. 31). This minimal degree of sequence recognition complicates research efforts to assess miR functions within the expansive SCN5A coding region.

Translational inhibition and mRNA destabilization (28–30). Canonical, miRs engage 3′-UTRs containing sequences complementary to their seed (miR nucleotide positions 2–8; ref. 31). This minimal degree of sequence recognition complicates research efforts to identify biologically relevant miR-target sites. To overcome this, we recently performed high-throughput sequencing of cross-linked immunoprecipitates (HITS-CLIP) to identify Ago2-associated miRs and their bound sequences in human myocardial tissues, yielding approximately 4,000 miR-target sites across more than 2,000 mRNAs (32), with approximately 50% of the sites overlapping coding regions. In exploring the interface of these sites with human genetic variations, we identified an intriguing interaction identified by Ago2 HITS-CLIP (read coverage shown above). The site resides within the coding region of the SCN5A terminal exon and is adjacent to the synonymous SNP rs1805126 (*) that has previously been linked to heart rhythm abnormalities by GWAS. (B-D) The effect of miR-24 on SCN5A expression, and the potential impact of rs1805126 on this interaction, was tested in cell culture experiments. Mouse N2a cells were cotransfected in triplicate with synthetic pre-miRs (4 nM) and human full-length SCN5A expression plasmids harboring either the C or T allele for rs1805126, or synonymous mutations that disrupt the miR-24 seed site (mut*, base mutations are indicated in bold in panel A). At 48 hours after transfection, Western blot and QPCR analyses were used to measure protein and mRNA expression. (B) Representative Western blots show that miR-24 strongly suppresses NaV1.5 expression, having a more robust effect on the C allele, relative to T. Densitometry analysis of Western blot data (n = 9 biological replicates from 3 separate studies for C versus T, n = 4 for mutated) supports a significant allele-specific difference in miR-24 suppression of NaV1.5 levels (C), as does QPCR analyses measuring SCN5A mRNA levels (D; n = 6 biological replicates from 2 studies). Data are represented as the mean ± SEM, and P values were obtained using 2-tailed unpaired t test comparing the indicated groups.

Results

Regulation of human SCN5A by miR-24 is modulated by the common SNP rs1805126. The SCN5A coding SNP rs1805126 was previously found to associate with electrocardiographic measures across several independent GWAS cohorts (12, 13, 15, 16, 33), but is synonymous (i.e., does not alter the amino acid sequence), and thus has been overlooked as being a causal variant. However, our new cardiac Ago2 HITS-CLIP data point to the possibility that this polymorphism may modulate the function of an adjacent miR-24 site (Figure 1A); notably, this was the most robustly engaged region by Ago2 across the entire SCN5A mRNA (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI95710DS1). As this SNP does not interfere with miR-target base-pairing (Figure 1A), we speculated that a change in mRNA structure (supported by computational RNA-fold analysis; Supplemental Figure 2) might confer an allele-specific response of SCN5A mRNAs to miR-24 activity. Indeed, PITA miR target prediction analysis, which incorporates target-site accessibility parameters (34), indicated that the C allele represents the more thermodynamically favorable miR-24 target, compared with T (32). This was supported by our previous experiments testing traditional luciferase-based 3′-UTR reporters for each allele, revealing that miR-24 more strongly suppresses the C allele compared with the T allele (32). To move beyond this artificial system (i.e., coding region placed into reporter gene 3′-UTR), we assessed the impact of miR-24 on SCN5A gene expression derived from full-length cDNA expression plasmids engineered to harbor either the rs1805126 T or C allele. Cotransfection studies in both mouse N2a and human HEK293 cells demonstrated that miR-24 mimics strongly suppress NaV1.5 expression (Figure 1, B and C and Supplemental Figure 3),
**Table 1. Demographic and clinical data for GRADE subjects by rs1805126 genotype**

<table>
<thead>
<tr>
<th></th>
<th>TT</th>
<th>TC</th>
<th>CC</th>
<th>P value (TT vs. CC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age: mean ± SEM</td>
<td>62.7 ± 11.7</td>
<td>63.0 ± 12.1</td>
<td>62.0 ± 12.7</td>
<td>0.46</td>
</tr>
<tr>
<td>Ethnicity: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>26 (5)</td>
<td>112 (18)</td>
<td>118 (34)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Caucasian</td>
<td>262 (55)</td>
<td>308 (51)</td>
<td>175 (28)</td>
<td>0.09</td>
</tr>
<tr>
<td>Ethnicity: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>103 (19)</td>
<td>123 (20)</td>
<td>78 (22)</td>
<td>0.62</td>
</tr>
<tr>
<td>Gender: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tobacco use history</td>
<td>267 (52)</td>
<td>305 (51)</td>
<td>175 (28)</td>
<td>0.06</td>
</tr>
<tr>
<td>NYHA: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With Class III-IV</td>
<td>139 (27)</td>
<td>175 (28)</td>
<td>120 (34)</td>
<td>0.06</td>
</tr>
<tr>
<td>Etiology: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic</td>
<td>148 (28)</td>
<td>179 (29)</td>
<td>120 (34)</td>
<td>0.14</td>
</tr>
<tr>
<td>EF: mean ± SEM</td>
<td>21.3 ± 5.9</td>
<td>20.8 ± 6.0</td>
<td>19.8 ± 6.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diabetes: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With history</td>
<td>171 (33)</td>
<td>209 (34)</td>
<td>128 (38)</td>
<td>0.39</td>
</tr>
<tr>
<td>Hypertension: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With history</td>
<td>322 (62)</td>
<td>384 (63)</td>
<td>220 (65)</td>
<td>0.7</td>
</tr>
<tr>
<td>β-Blocker: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prescribed</td>
<td>451 (85)</td>
<td>532 (85)</td>
<td>304 (86)</td>
<td>0.98</td>
</tr>
<tr>
<td>Ace Inhibitor: N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prescribed</td>
<td>418 (81)</td>
<td>489 (80)</td>
<td>267 (79)</td>
<td>0.69</td>
</tr>
<tr>
<td>QRS (ms; mean ± SEM)</td>
<td>136.9 ± 35.8</td>
<td>135.6 ± 37.5</td>
<td>137.2 ± 36.3</td>
<td>0.78</td>
</tr>
<tr>
<td>PR (ms; mean ± SEM)</td>
<td>173.4 ± 38.8</td>
<td>171.8 ± 49.0</td>
<td>170.8 ± 39.7</td>
<td>0.77</td>
</tr>
</tbody>
</table>

P values for categorical clinical factors were tested by Fisher’s exact test, and for continuous clinical factors by ANOVA. Cox proportional hazard ratio (HR) (CC versus TT), after adjusting for the above variables: appropriate shocks: HR = 1.1, 95% CI (0.8, 1.6), P = 0.47; death: HR = 1.5, 95% CI (1.1, 1.9), P = 0.006.

and consistent with the luciferase reporter data, the rs1805126 C allele was repressed to a greater degree than the T allele (relative to control miR mimics, P < 0.01). The enhanced response to miR-24 treatment was further supported by corresponding quantitative PCR (QPCR) data in N2a cell studies, showing a nearly 40% decrease in SCN5A C allele transcripts and virtually no change in T allele transcripts (Figure 1D, P < 0.01). To address the possibility that additional miR-24 sites and/or other mechanisms may contribute to the robust miR-24–mediated SCN5A silencing, we introduced synonymous mutations into the experimental miR-24 seed sequence within the full-length SCN5A expression construct, and this completely abolished the miR-24 inhibitory effect on SCN5A expression (Figure 1, B and D).

**miR-24 overexpression inhibits Na\(_{\text{v}}\) 1.5 expression and sodium current density.** To determine if miR-24 functionally inhibits the activity of the SCN5A-encoded Na\(_{\text{v}}\)1.5 channel in heart cells, we performed single-cell patch-clamp analyses in cultured neonatal rat cardiomyocytes (NRCMs); of note, the miR-24 SCN5A target seed sequence is conserved in rodents (Figure 1A), and NRCMs have been shown to express high levels of endogenous miR-24 (35). Consistent with this, we found that a luciferase-based miR-24 sensor transgene was suppressed in NRCMs and that this was blocked by treatment with miR-24 inhibitors (i.e., anti-miRs, Supplemental Figure 4A). We next tested whether miR-24 overexpression could provoke a functional interaction with rat Scn5a mRNA in NRCMs. NRCMs treated with miR-24 mimics displayed a marked decrease in sodium current density (>4-fold, P < 0.001), as compared with cells treated with control miR (Figure 2). Notably, the degree of miR-24–induced sodium current suppression was similar to that observed in NRCMs treated with Scn5a-targeted RNAi, and in complementary studies, NRCMs treated with miR-24 mimics showed an approximately 50% decrease in Na\(_{\text{v}}\)1.5 protein expression (Figure 2, D and E, P < 0.01). However, anti-miR-24 treatment in NRCMs had no effect on Na\(_{\text{v}}\)1.5 sodium current density or expression (Supplemental Figure 4, B–E). It is possible that miR-24 does not naturally or strongly engage Scn5a mRNA in cultured NRCMs, since proximal nucleotide differences make the site considerably less favorable compared with human SCN5A (Supplemental Figure 5).

The rs1805126 C allele associates with adverse outcomes in heart failure patient cohorts. Next, we assessed the clinical significance of the miR-24–SCN5A–SNP interaction in heart failure patients. We determined rs1805126 genotypes in 1,658 cardiomyopathy patients (mean age = 62 years, mean ejection fraction [EF] = 21%; 79% male, 79% Caucasian and 20% African American, 70% ischemic) with implantable cardioverter defibrillators (ICDs) from the prospective, multicenter, NIH-funded Genetic Risk Assessment of Defibrillator Events (GRADE) study (36), identifying 565 TT (34%), 694 TC (42%), and 399 CC (24%) subjects. Kaplan-Meier analyses revealed that CC homozygotes had markedly higher mortality rates, as compared with T allele carriers (hazard ratio [HR] = 1.5, P = 0.002; Figure 3A). This risk appeared to be independent of other potential confounding variables, including heart failure etiology (ischemic or idiopathic) and ethnicity (Supplemental Figure 6), the latter being of notable significance given that the CC genotype is approximately 3 times more prevalent among African Americans. This was supported by multivariate analyses to estimate the effect of rs1805126 genotype on mortality, adjusting for other clinically relevant variables and comorbidities (listed in Table 1); this yielded a Cox proportional HR = 1.5, 95% CI (1.1, 1.9), P = 0.006 (CC versus TT). Interestingly, rs1805126 genotype did not significantly associate with appropriate ICD shocks for ventricular tachycardia or ventricular fibrillation, a surrogate for arrhythmic sudden cardiac death (Figure 3B), as might be expected given the well-established relationships among SCN5A, rs1805126, and heart rhythm. Moreover, whole-cohort analyses did not reveal significant genotype-based differences in PR or QRS intervals (Table 1); of note, the aforementioned GWAS links were established in very large cohorts without heart failure, whereas heart failure subjects in GRADE had significantly greater variability in PR and QRS intervals due to conduction disease. By contrast, the CC genotype in GRADE was associated with indications of worsening heart failure, with CC subjects having significantly reduced left ventricular ejection fractions (LVEFs) compared with TT subjects (P = 0.002) and trending towards poorer New York Heart Association (NYHA) heart failure classification scores (P = 0.06; Figure 3C and Table 1).

To determine if these observations extend beyond the GRADE cohort, we evaluated the association of rs1805126 genotypes with heart failure outcomes in 270 African American cardiomyopathy patients (mean age = 57 years, mean EF = 24%; 61% male, 26% ischemic) from the Genetic Risk of Heart Failure in African Americans (GRAHF) study, a subgroup of the African American Heart Failure Trial (A-HeFT) with available DNA samples (37). Geno-
typing of rs1805126 identified 32 TT (12%), 99 TC (37%), and 139 CC (51%) subjects, consistent with previous observations that the C allele is more common in African Americans. Association of these genotypes with accompanying clinical data revealed that CC patients showed a near-significant trend towards having worse A-HeFT composite scores (trial primary endpoint, which summarizes death, hospitalization, and change in quality of life; Figure 3D, \( P = 0.06 \) for CC versus TT). Of note, genotype-based subgroups showed no difference in other clinically relevant variables and comorbidities (Supplemental Table 1), and adjusting for sex, age, etiology and diabetes yielded a statistically significant association \( (P = 0.05) \), further reinforcing the link between the rs1805126-CC genotype and adverse outcomes in heart failure patients.

The rs1805126 C allele associates with decreased SCN5A expression in human hearts. To understand the molecular basis of the association between the rs1805126 SNP and heart failure mortality, we first evaluated genotype-related alterations in myocardial gene expression, given the effects of rs1805126 on miR-24-mediated SCN5A silencing and the absence of nonsynonymous SNPs in linkage disequilibrium with it \( (r^2 \geq 0.4); \) Supplemental Table 2. For this, we assessed human cardiac tissue samples from nonfailing hearts \((n = 6)\) total biological replicates per treatment; mean ± SEM) is plotted. \( \star \) Pre-miR-29a and -Neg1 serve as negative controls, and \( \beta \)-actin or GAPDH levels were used for normalization. \( *P \) value derived using 1-way ANOVA with Dunnett’s post hoc test (versus miR-Neg1). (D) NRCMs were transfected with 25 nM synthetic pre-miR mimics and endogenous Na\(_{\text{A,1.5}}\) protein levels were measured by Western blot 48 hours later; a representative Western blot image is shown. (E) Combined Western blot (WB) densitometry analysis from 2 separate experiments \((n = 3)\) mouse strain. These mice are known to show intermediate expression (Supplemental Figure 8). In follow-up work with the Margulies lab, we further interrogated this association by allel-specific expression analysis using myocardial RNA sequencing \( (RNA-seq) \) data derived from 29 individuals heterozygous for rs1805126 (Supplemental Table 5). In the majority of these heterozygous samples, C allele SCN5A transcripts were expressed at lower levels, as compared with those harboring the T allele \((-12\% \) mean difference, \( P < 0.001; \) Figure 4D), providing strong evidence to further support this reproducible association.

Decreased Scn5a expression leads to accumulation of cardiac reactive oxygen species in mice. As an initial step towards understanding how decreased SCN5A expression may compromise myocardial health and function, we focused on the established Scn5a heterozygous knockout \( (\text{Scn5a}^{+/-}) \) mouse strain. These mice are known to
express Scn5a mRNA and Na\textsubscript{1.5} protein at 50% levels, relative to wild-type littermates, and develop aging-related signs of adverse myocardial remodeling (e.g., increased fibrosis and conduction slowing), which manifest after 12 months of age. Considering that oxidative stress is often at the root of profibrotic signaling cascades slowing), which manifest after 12 months of age. Considering that oxidative stress is often at the root of profibrotic signaling cascades, we hypothesized that oxidative stress is often at the root of profibrotic signaling cascades, we hypothesized that oxidative stress is often at the root of profibrotic signaling cascades. Relative to wild-type littermate hearts, Scn5a\textsuperscript{−/−} hearts showed a striking and highly significant increase in DHE oxidation (2.5-fold, \( P < 1 \times 10^{-5} \); Figure 5), and Western blot analysis done in parallel confirmed a 50% decrease in cardiac Na\textsubscript{1.5} expression in Scn5a\textsuperscript{−/−} mice (Supplementary Figure 9). Together, these data support a potentially novel link between reduced expression of the cardiac sodium channel and downstream escalation in oxidative stress, which represents an established pathway towards adverse myocardial remodeling and dysfunction and could explain the worsened LV function and increased mortality in rs1805126-CC heart failure patients.

**Discussion**

Previous studies have demonstrated that SCN5A is under regulatory control by miRs targeting the 3′-UTR (22–24); however, our studies are the first to our knowledge to evaluate miR binding within the coding sequence, an often overlooked region in miR-related investigations. Indeed, our previous HTS-CLIP data revealed that the most prominent human cardiac Ago2 binding site within SCN5A lay within the terminal coding exon. Here, our follow-up studies indicate that this site is functionally engaged by miR-24, a miR that is commonly upregulated in failing human and rodent hearts (40, 41), revealing a potentially novel and potent disease-relevant suppressor of SCN5A expression. Furthermore, we demonstrate that the synonymous SNP rs1805126 modulates this regulation, providing a potential molecular mechanism to account for established associations between this variant and heart rhythm changes in human subjects.

Specifically, our data link lower myocardial SCN5A expression to the rs1805126-CC genotype, which was previously associated with highly significant increases in PR and QRS intervals (13); this is consistent with the prolonged PR that coincides with decreased Na\textsubscript{1.5} expression in patients with loss-of-function SCN5A mutations and in Scn5a heterozygous knockout mice (42, 43). In our current work, we did not find a significant association between rs1805126 genotypes and PR and QRS intervals in GRADE subjects; our study was underpowered for this, especially considering that heart failure causes these measures to become highly variable across patients with frequent bundle branch block and need for pacing and biventricular pacing. In addition, the prior related GWAS were done on very large populations of normal individuals and found only relatively small alterations in PR and QRS intervals (2.5 and 0.7 ms, respectively).

Beyond cardiac conduction, we uncovered an unanticipated and significant association between rs1805126 and all-cause mortality in the GRADE cohort, signifying the first observed link to our knowledge between a common SCN5A SNP and nonarrhythmic death in heart failure. We did not see evidence of an association between rs1805126 genotypes and PR and QRS intervals in GRADE subjects; our study was underpowered for this, especially considering that heart failure causes these measures to become highly variable across patients with frequent bundle branch block and need for pacing and biventricular pacing. In addition, the prior related GWAS were done on very large populations of normal individuals and found only relatively small alterations in PR and QRS intervals (2.5 and 0.7 ms, respectively).

As an initial step towards interrogating the mechanistic basis of the association between decreased SCN5A expression and worse heart failure outcomes, we considered previous observations that Scn5a\textsuperscript{−/−} mice develop aging-related signs of adverse myocardial remodeling (43), and we speculated that elevated ROS may be
at the root of this pathology. Interestingly, previous reports have shown that mitochondrial ROS can alter Na\textsubscript{v}1.5 channel activity (44); however, our new data showing elevated ROS in hearts from 

\textit{Scn5a}\textsuperscript{+/–} mice provide the first evidence to our knowledge that suggest a bidirectional relationship between Na\textsubscript{v}1.5 and ROS. Along these lines, one recent study demonstrated that increasing persistent late Na\textsuperscript{+} current in mouse hearts leads to structural derangements, mitochondrial injury, and fibrosis (45).

Overall, the results presented herein support future studies to look beyond 3′-UTRs for clinically significant miR-SNP interactions. To our knowledge, we have discovered only the second known example of a disease-associated miR-SNP interaction found within a coding region (46), likely due to related studies having a restricted focus on 3′-UTRs (47, 48). Beyond this, our data highlight the need for follow-up investigations to further define the extensive regulatory controls that influence \textit{SCN5A} expression, assessing if rs1805126-TCC genotype risk is compounded by the extensive regulatory controls that influence \textit{SCN5A} expression in heterozygous individuals was done using RNA-seq data obtained from nonfailing and failing human hearts (n = 29), offering further support that the C allele is expressed at lower levels, relative to T. **P < 0.001 by 1-sample t test.

Methods

\textit{Human cardiac tissue samples}. LV cardiac tissues were obtained from the Myocardial Applied Genomics Network (MAGNet; www.med.upenn.edu/magnet). All subjects donating tissue provided consent under an approved IRB protocol, and provided clinical information that is confidentially linked to the specimens by a study number. LV free-wall tissue was harvested at the time of cardiac surgery from subjects with heart failure undergoing transplantation and from unused donor hearts. The heart was perfused with cold cardioplegia prior to cardiectomy to arrest contraction and prevent ischemic damage. Tissue specimens were then obtained and frozen in liquid nitrogen and stored at ~80°C until used. For Western blot analysis, total protein of human left ventricles was harvested by homogenizing tissues in lysis buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS) containing protease inhibitors (Roche). Protein concentrations were determined using the Pierce BCA Assay (Thermo Fisher Scientific).

\textit{Plasmids}. The rs1805126 C allele expression plasmid was generated by site-directed mutagenesis performed on a plasmid containing full-length human \textit{SCN5A} cDNA (rs1805126 T allele) under control of the CMV promoter. For this, the Phusion site-directed mutagenesis protocol (New England BioLabs) was used with the following 5′-phosphorylated primers: 5′-/Phos/GCCCTGTCTGAGCCACTCCGGTATC-3′ and 5′-/Phos/GTCGGCAAAGTCAGACAGGACCGAATAC-3′ (Integrated DNA Technologies). For the miR-24 dual luciferase sensor plasmid, DNA oligos were used to insert the following sequence: 5′-GACCGAATAC-3′ (Integrated DNA Technologies). For the miR-24 8mer binding sites downstream of the Renilla luciferase expression cassette (using Xhol and NotI restriction enzyme sites) in the psiCheck2 plasmid (Promega).

\textit{Full-length Scn5a knockdown studies}. Mouse N2a and human HEK293 cells (which do not express endogenous \textit{SCN5A} at detectable levels by Western blot, data not shown; ATCC cell lines) were seeded in 24-well plates (80,000 and 200,000 cells/well for N2a and HEK293, respectively), and the next day, cells were cotransfected with synthetic pre-miRs (4 nM) along with human full-length \textit{SCN5A} expression plasmids (100 ng) using Lipofectamine 2000 (Life Technologies). After 48 hours, cells were harvested in 200 μl of RIPA buffer, and half of the lysate was immediately added to 900 μl of TRizol reagent (Invitrogen) for RNA isolation. Western blot and QPCR analyses were performed as described below.

\textit{Western blot}. Protein samples were resolved by standard SDS-PAGE before being transferred to a 0.45-μm PVDF membrane (Millipore). The membrane was blocked with 2%-5% milk in 1× PBST (0.05% Tween 20), after which rabbit anti-Na\textsubscript{v}1.5 antibody (1:200; Alomone...
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Figure 5. Evaluation of oxidative stress in Scn5a heterozygous knockout mouse hearts. Oxidation of dihydroethidium (DHE, a surrogate marker of steady-state levels of superoxide) was measured in fresh-frozen cardiac tissue sections collected from 7- to 8-month-old male Scn5a+/− mice and wild-type littermates (n = 3 each). Positive controls included sections treated with antimycin A (AntA, a mitochondrial electron transport chain blocker that is known increase ROS generation). Images were captured at ×40 magnification. Scale bars: 100 μm. (A) Representative photomicrographs of DHE-stained cardiac sections are shown. (B) Signal intensity quantified in 90 cells per group (i.e., 30 per mouse) is plotted (mean ± SEM). ****P < 0.0001 by 2-tailed t test.

Labs, ASC-005), anti-β-catenin (1:200; Abcam, ab2365), or anti-β-actin (1:2,000; Sigma-Aldrich, A5441) diluted in blocking buffer was added and incubated overnight at 4°C. Membranes were washed with 1× PBST, incubated for 1 hour with HRP-conjugated goat anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch, 115-035-146 and 111-035-144) diluted 1:10,000 in glass coverslips coated with laminin, and stored at 37°C in a 5% CO2 incubator. Culture reagents were purchased from Gibco-BRL. After 2 hours, cells were cotransfected with synthetic pre-miRs (25 nM) along with miR or siRNA expression plasmids coexpressing EGFP (100 ng), using Lipofectamine 2000 (Life Technologies). For anti-miR studies, LNA-based or 2′-O-methyl-based miR inhibitors (Exiqon and Integrated DNA Technologies) were transfected into NRCMs at 10–50 nM along with 100 ng of an EGFP expression plasmid. Media were then changed 3 hours later to cardiomocyte medium with gentamycin (50 μg/ml, Life Technologies), and 5% stripped horse serum.

Whole-cell patch clamp. After 2–4 days of transfection, a conventional whole-cell patch-clamp technique was used to record Na+ currents from EGFP-positive NRCMs. Patch pipettes with a resistance of 1.5 ± 0.2 MΩ when filled with pipette solution contained (in mmol/l) 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, 10 HEPES (titrated to pH 7.35 with CsOH) and the bath solution contained (in mmol/l) 145 NaCl, 4.5 KCl, 1 MgCl2, 1.5 CaCl2, 10 HEPES (pH 7.35 with CsOH). In all recordings, the potentials were corrected for the liquid junction and 80% of the series resistance was compensated, yielding a maximum voltage error of ±1 mV. Low-pass filtered signals (5 kHz) from a patch clamp amplifier (Axopatch 200B, Molecular Devices) were digitized in an AD/DA converter (Digidata 1200, Molecular Devices) at 20 kHz and stored in a PC for later analysis. Na+ channel data were analyzed with Origin software (Microcal). Cell capacitance was recorded directly from the patch amplifier after nullifying the transients following patch rupture. Data were accepted only when access resistance in voltage-clamp mode was below 7 MΩ. To minimize time-dependent drift in gating parameters, all protocols were initiated 5 minutes after whole-cell configuration was obtained. A 200-ms prepulse to −120 mV was used to eliminate inactivation, and 200-ms test pulses between −80 and +30 mV were used to activate the channel. All electrophysiological experiments were performed at room temperature (23°C–25°C).

GRADE and GRAHF samples and genotyping. The GRADE and GRAHF patient cohorts have been previously reported (36, 37). In brief, subjects included in this study are from the National Heart, Lung, and Blood Institute-sponsored (NHBLI-sponsored), prospective, observational, multicenter GRADE study, designed to identify genetic modifiers of arrhythmic risk and heart failure outcomes.

as follows: human SCN5A (forward: 5′-GCCATCTTCACAGGC- GAGTGTATTG-3′, reverse: 5′-GGGGAGAAGAAGTACTTCTG- GATGATG-3′), mouse β-actin (forward: 5′-CTGAACCTCAAGGC- CAACCGTG-3′, reverse: 5′-GTGTACGACCAAGGGCATACG-3′), and human GAPDH (forward: 5′-GAAGGTGAAGGTCGAGGTCT-3′, reverse: 5′-GCAAAATATGCACATTACGAGA-3′). Small RNA QPCR analyses were performed using commercially available stem-loop reverse transcription primers and accompanying TaqMan assays for miR-24, U6, and RNU48 (Life Technologies). Standard curve and melt curves were evaluated for quality control as needed, and relative mRNA expression was determined by the ddCt method.

GRADE and GRAHF patient cohorts have been previously reported (36, 37). In brief, subjects included in this study are from the National Heart, Lung, and Blood Institute-sponsored (NHBLI-sponsored), prospective, observational, multicenter GRADE study, designed to identify genetic modifiers of arrhythmic risk and heart failure outcomes.
Inclusion criteria were patients who were 18 years of age or older with a diagnosis of at least moderate-to-severe systolic LV dysfunction (EF ≤ 30%) and who had an ICD. Subjects were excluded if they had intractable class IV heart failure and conditions (other than heart failure) that were expected to limit survival to less than 6 months. The primary endpoint in this study was time to first appropriate ICD shock, and secondary endpoints included all-cause death. The GRAHF cohort derives from a subgroup of the A-HeFT study; inclusion criteria include self-designation as African Americans, heart failure due to systolic dysfunction, and standard background therapy for heart failure with neurohormonal blockade, including angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists, and β-blockers. Based on available DNA and successful genotyping, a total of 1,658 GRADE patients and 270 GRAHF patients were considered for the current analyses. Genotyping was performed using a commercially available TaqMan SNP assay (rs1805126) and TaqMan Genotyping Universal Master Mix (Life Technologies). Detection was done on a Viia 7 QPCR machine and genotyping calls were obtained using the analysis software’s default settings.

**LD analysis.** The rs1805126 SNP id was input into the HaploReg v4.1 web-based tool (Broad Institute), and the options were adjusted to set the linkage disequilibrium (LD) threshold to 0.4. The analysis was run using the 1000G Phase 1 population data for EUR (European) and AFR (African) populations, which are most relevant to the GRADE cohort. Data are provided in Supplemental Table 2.

**Microarray-based SCN5A eQTL analyses.** Previously published human cardiac transcriptomic data were obtained from the NCBI Gene Expression Omnibus (GEO) database (University of Pennsylvania, GSE57338 and The Netherlands, GSE55231), and accompanying sample genome-wide genotyping data were either downloaded (GSE55230) or made available through collaboration with Kenneth B. Margulies. For the latter, DNA samples were genotyped using Affymetrix Genome-Wide SNP Array 6.0, and quality control (QC) filters were applied to exclude unreliable samples, samples with cryptic relatedness, and samples that were not genetically inferred Caucasian; in addition, SNPs were eliminated if there was significant departure from Hardy-Weinberg equilibrium (P < 10^-8). Subject SNP genotypes for rs1805126 and normalized SCN5A mRNA probe intensities were retrieved from the data for subsequent genotype-based expression quantitative trait loci (eQTL) analyses.

**Allele-specific expression analyses.** Total RNA was extracted from human cardiac tissue samples (n = 60) using the miRNeasy Kit (Qiagen) including DNase treatment. For RNA-seq, library prep was conducted using Illumina trueSeq stranded mRNA kit followed by the Nugen Ovation amplification kit. Resultant fastq files were assessed for quality control using the FastQC program. Fastq files were aligned against the human reference genome (hg19/hgRGC37) using the GSNAP aligner in SNP tolerant mode and converted to BAM files using SAMtools (56). Duplicate reads in the BAM files were flagged using the MarkDuplicates program from Picard tools. The R package assEQ and custom R scripts were used to generate per-allele specific counts of SCN5A from RNA-seq and genotype data (57). Briefly, per-sample haplotype information was estimated from Affymetrix SNP genotyping data using the software package SHAPEIT with the 1000 Genomes reference panel (58). Then for each sample a list of heterozygous SNPs was used to create a new BAM file for each haplotype per sample. Reads mapping to the SCN5A exons and UTRs were counted from each haplotype-specific BAM file using the htseq-count application. Allele-specific expression was plotted as C allele counts/T allele counts (Figure 4D), and 1-sample 2-sided t test was performed on the allele-specific expression values for C allele counts/sum (T and C allele counts) against the hypothetical mean value of 0.5.

**DHE oxidation levels in wild-type and Scn5a+/- mouse heart tissue sections.** Scn5a+/- mice (backcrossed more than 10 times to the C56BL6/J strain) were a gift from Dan Roden (Vanderbilt University, Nashville, Tennessee, USA). Mice were housed in a controlled temperature environment on a 12-hour light/dark cycle, and food and water were provided ad libitum. Fresh frozen hearts were collected from 7- to 8-month-old male Scn5a+/- and wild-type littermate mice (n = 3 per group) and then cut into 10-μm sections for DHE analysis. Briefly, the tissue sections were labeled with 10 μM DHE in Dulbecco’s PBS containing 5 mM sodium pyruvate for approximately 20-25 minutes at 37°C, after which they were imaged by confocal microscopy. The red fluorescence intensity of each section was quantified using ImageJ software (NIH) (30 cells per mouse, i.e., 90 cells per group) and then converted to the Netherlands cohort (CC sample with probe intensity = 6.48; Figure 4C and Supplemental Figure 7), as detected by ROUT (definitive outlier stringency, Q = 0.1%). Power analyses considered event rates for death and shock and SNP allele frequencies; based on the minor-allele frequency of rs1805126, the study was powered to greater than 90% to detect an HR of 1.5.

**Study approval.** All animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Iowa. Deidentified human samples and accompanying clinical and genetic data were used for this study. Sample and data collection were previously approved by Institutional Review Boards at GRADE and A-HeFT (GRAHF) study sites and at the University of Pennsylvania. All human subjects provided written informed consent.

**Author contributions**

RLB and BL designed and conceived the project, supervised the research, and interpreted the data. XZ, JYY, JMM, and KAM carried out experimental work, analyzed data, and participated in data...
interpretation. RLB, MM, and PB carried out computational and statistical analyses. RG, HLB, SCD, PTE, AAS, RW, MS, HM, and BL played key roles in overseeing GRADE patient data acquisition, organization, and interpretation. ALT, CWY, AMF, and DMM played key roles in overseeing GRAHF patient data acquisition, organization, and interpretation. KRM, WHWT, and CSM provided critical human cardiac tissue samples and accompanying clinical and genotyping data. KI and DRS guided experimental design and helped with data interpretation. RLB, BL, and XZ wrote the manuscript.

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