Human Heart Failure Is Associated With Abnormal C-Terminal Splicing Variants in the Cardiac Sodium Channel


Abstract—Heart failure (HF) is associated with reduced cardiac Na⁺ channel (SCN5A) current. We hypothesized that abnormal transcriptional regulation of this ion channel during HF could help explain the reduced current. Using human hearts explanted at the transplantation, we have identified 3 human C-terminal SCN5A mRNA splicing variants predicted to result in truncated, nonfunctional channels. As compared with normal hearts, the explanted ventricles showed an upregulation of 2 of the variants and a downregulation of the full-length mRNA transcript such that the E28A transcript represented only 48.5% (P<0.01) of the total SCN5A mRNA. This correlated with a 62.8% (P<0.01) reduction in Na⁺ channel protein. Lymphoblasts and skeletal muscle expressing SCN5A also showed identical C-terminal splicing variants. Variants showed reduced membrane protein and no functional current. Transfection of truncation variants into a cell line stably transfected with the full-length Na⁺ channel resulted in dose-dependent reductions in channel mRNA and current. Introduction of a premature truncation in the C-terminal region in a single allele of the mouse SCN5A resulted in embryonic lethality. Embryonic stem cell–derived cardiomyocytes expressing the construct showed reductions in Na⁺ channel–dependent electrophysiological parameters, suggesting that the presence of truncated Na⁺ channel mRNA at levels seen in HF is likely to be physiologically significant. In summary, chronic HF was associated with an increase in 2 truncated SCN5A variants and a decrease in the native mRNA. These splice variations may help explain a loss of Na⁺ channel protein and may contribute to the increased arrhythmic risk in clinical HF. (Circ Res. 2007;101:1146-1154.)

Key Words: sodium channels | transcriptional regulation | mRNA splice variations | heart failure | arrhythmia

Human heart failure (HF) is associated with decreased cardiac voltage-gated sodium channel current.1,2 Genetically mediated decreases in Na⁺ current have been implicated in the risk for sudden death,3–5 and Na⁺ channel changes may contribute to the increased risk of sudden death in HF.5,7 Because transcriptional alterations in other ion channels have been noted to contribute to current changes in HF,8,9 we investigated Na⁺ channel protein and mRNA abundance in hearts explanted during cardiac transplantation to determine whether there were changes that might explain the reduced Na⁺ current previously reported in this tissue.

Materials and Methods

Detection of Human SCN5A 3′ UTR Variants by Rapid Amplification of cDNA Ends PCR

Total human RNA from normal fetal and adult whole hearts was purchased from Clontech (Mountain View, Calif). The RNA ligase-mediated rapid amplification of cDNA ends (RACE) method was used to characterize the 3′ ends of the human SCN5A mRNA using the GeneRacer kit (Invitrogen, Carlsbad, Calif). Primary and nested PCR reactions were performed with primers HE26F (on exon 26) and HE27F (on exon 27) specific to the human SCN5A gene and the GeneRacer 3′ primer for amplifying the 3′-end fragment. The nested PCR products were cloned into pCR4-TOPO vector (Invitrogen) and sequenced. Sequences were compared with that of SCN5A using Vector NTI 7 software (Invitrogen). All primers are presented in Table I in the online data supplement at http://circres.ahajournals.org.

Isolation and Culture of Lymphoblasts

Human lymphoblast cell lines were developed from peripheral blood mononuclear cells of volunteers with normal cardiac function according to a previously published protocol.10 RNA isolated was from ∼5.0×10⁸ lymphoblasts. Procedures and consent forms were approved by the Institutional Review Board.

Real-Time SYBR Green PCR Quantification of SCN5A Transcript Variants

Ventricular tissue from hearts removed at the time of cardiac transplantation at Emory University Hospital under a protocol approved by the Emory Institutional Review Board was homoge-
nized, and total RNA was isolated using the TRIzol reagent (Invitrogen). The total RNA from ventricles and skeletal muscle of the normal adults was bought from Ambion (Austin, Tex) and Clontech, respectively. The quantitative SYBR real time RT-PCR was performed as previously described.11 The reversed primers for exon 28 variants were HSCN5AE28A/R (E28A and E28D), HSCN5AE28B/R (E28B), HSCN5AE28C/R (E28C), and HSCN5AE28D/R (E28A only), respectively. The forward primer, HE27F, was constant in each case. The reactions gave rise to 124-, 170-, 143-, and 211-bp PCR products, respectively. Amplification with primers HE27F and HSCN5AE28D/R produced the full-length variant, E28A. Amplification with HE27F and HSCN5AE28A/R produced a product comprised of both variants E28A and E28D. The amount of E28D was calculated by subtraction of the products of these 2 reactions.

/H9252-A-Actin was used as an internal reference when making quantitative comparison.

In Vitro Studies of Native and Truncated SCN5A cDNA

The open reading frame of truncated SCN5A E28C and E28D variants was cloned into the expression vectors, pLP-IRES-GFP and pLP-AcGFP1-N, using the BD Creator DNA cloning kit (Clontech, Mountain View, Calif) as described previously.12 The truncated SCN5A cDNAs labeled either with internal ribosome entry site (IRES)-mediated green fluorescent protein (GFP) or GFP fused to the channel C terminus were transfected into human embryonic kidney (HEK) cells or a HEK cell line stably expressing the full-length human SCN5A cDNA (HEK-SCN5A). HEK cell lines stably expressing the truncation variants were created by selection for 3 weeks with geneticin after transfection.

Western Blot Analysis

Normal human ventricular tissue was kindly offered by Dr J. Andrew Wasserstrom (Northwestern University, Chicago, Ill). For Western blot analysis, lysates from cells transfected with SCN5A cDNA expression constructs or the protein extracts from human ventricles were separated by SDS-PAGE and probed with an anti-Nav1.5 antibody that should recognize all channel variants, a gift from Dr Mohler (University of Iowa, Iowa City) and with anti-β-tubulin (Rockland Immunochemicals Inc, Gilbertsville, Pa). The signal was visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

Laser Confocal Microscopy

HEK cells transfected with SCN5A fused to GFP were plated for 4 hours at room temperature and then fixed in 4% paraformaldehyde in PBS for 10 minutes. Fluorescent images were taken using a Zeiss LSM 510 confocal microscope (Thornwood, NY).
In Vitro Differentiation of Embryonic Stem Cell Into Cardiomyocytes

Experimental studies were performed on an embryonic stem (ES) cell line in which 1 allele of the Na⁺ channel was successfully targeted as described in the online data supplement. R1 mouse ES cells with or without the mutation were maintained in the undifferentiated state as described previously. For patch clamp experiments, areas of beating cardiomyocytes (CMs) were mechanically dissected from 19-day-old embryoid bodies, and single CMs were obtained by enzymatic digestion. For the multielectrode array (MEA) experiments, areas of beating CMs were mechanically dissected from 17-day-old embryoid bodies, placed on top of a MEA, and cultured for another 2 days before recording.

Functional Assessment of a Truncation Mutant by MEA Recording

Extracellular recording from wild-type (WT) and truncation syncytial CMs derived from ES cells was performed and analyzed using a MEA data acquisition system (Multi Channel System, Reutlingen, Germany) at 37°C as described previously. Conduction velocity was calculated using the activation time at each point and a threshold-crossing algorithm to form an isochrone map. Three noncollinear points from an area with uniform, parallel isochrones were chosen to calculate conduction velocity in 2 orthogonal directions (eg, vx and vy). The final conduction velocity was calculated as \( v = (\frac{1}{v_x^2} + \frac{1}{v_y^2})^{\frac{1}{2}} \).

Recording of Na⁺ Current

In ES-derived CMs, patch clamp experiments were performed 1 to 5 days after cell isolation. CMs with uniform contractions and beating rates were used in the study. Current and voltage clamp experiments were performed as previously described at 37°C. Current and voltage clamp experiments were performed and analyzed using a MEA data acquisition system (Multi Channel System, Reutlingen, Germany) at 37°C as described previously. Conduction velocity was calculated using the activation time at each point and a threshold-crossing algorithm to form an isochrone map. Three noncollinear points from an area with uniform, parallel isochrones were chosen to calculate conduction velocity in 2 orthogonal directions (eg, vx and vy). The final conduction velocity was calculated as \( v = (\frac{1}{v_x^2} + \frac{1}{v_y^2})^{\frac{1}{2}} \).

Statistical Evaluations

All data are presented as means±SEM. Means were compared using unpaired t tests or 1-way ANOVAs. A probability value of <0.05 was considered statistically significant.

Results

Detection of Three Novel Human SCN5A C-Terminal mRNA Splicing Variants

Two SCN5A mRNA variants that do not alter the coding sequence have been reported previously from mouse heart that differed in the length of the poly-A tail. Using RACE-PCR, we found analogous Na⁺ channel mRNA variants in human heart (Figure 1A). In addition to these bands, nested RT-PCR revealed shorter bands in fetal and adult human heart. Sequence analysis revealed 3 new mRNA splice variants in exon 28 designated as E28B (27 bp), E28C (39 bp), and E28D (114 bp) (GenBank accession nos. EF092292, EF092293, and EF092294, respectively; Figure 1B). In comparison with the full-length E28A variant, all 3 new variants were shorter and were predicted to result in prematurely truncated Na⁺ channel proteins missing the segments from domain IV, S3, or S4 to the C terminus (Figure 1C). Sequence alignments are shown in the supplemental material (Figure IV in the online data supplement). Preliminary data suggest that these variants were specific to humans because they were not seen in rats or mice.

The Relative Abundances of the SCN5A Variants Are Developmentally Regulated

Splice variants of Na,1.5 in the C terminus are known to vary during development. Quantitative real-time RT-PCR indicated that the relative abundances of each of the variants increased by 41.6% \( (P<0.001) \), 5.1-fold \( (P<0.01) \), 1.1-fold \( (P<0.01) \), and 4.8-fold \( (P<0.001) \) for E28A, E28B, E28C, and E28D from fetal to adult heart, respectively (Figure 2A). Figure 2B shows that as a percentage of the total SCN5A transcripts splice variants, E28B and E28D increased significantly, the full-length E28A decreased, and the E28C abundance was unchanged during development.

HF Increased Two of the Na⁺ Channel C-Terminal Splice Variants

The presence of splice variants was compared between explanted ventricles and 3 control patients with no known cardiac disease (supplemental Table II). RT-PCR results indicated that the relative mRNA abundance of E28A full-length variant was decreased by 24.7% in HF patients compared with controls \( (P<0.001) \). E28C and E28D mRNA abundances were increased 14.2-fold \( (P<0.001) \) and 3.8-fold \( (P<0.001) \), respectively, when comparing controls with HF patients (Figure 3A). The least abundant variant, E28B, decreased 73.8% \( (P<0.01) \) in HF patients. As a percentage of the total SCN5A transcript, E28A and E28B decreased significantly from 87.5% \( (\pm 0.4) \) and E28C and E28D from fetal to adult heart, respectively (Figure 2A). The total percentage of short variants went from 12.5% \( (\pm 0.4) \) of the total SCN5A mRNA in control subjects to 54.9% \( (\pm 4.5) \) in HF patients. Similar
amounts of truncated channel variants are known to cause Brugada syndrome.\textsuperscript{5,20–23}

The relative RNA abundance of the SCN5A variants were compared in the left (Figure 3B) and right (Figure 3C) ventricles of controls and HF patients. Normalized to the total SCN5A mRNA, E28A abundances were decreased in both the left and right ventricles. The pattern of changes for the truncation variants was similar in both ventricles with increases in E28C and E28D. The percentage of truncated mRNAs was increased more in the left ventricle when compared with the right ventricle ($P<0.0003$). Corresponding to the RNA effects, Western blot analysis of human control and HF tissue revealed a 62.8\% ($P=0.01$) protein reduction in HF compared with normal heart (Figure 3D and 3E). No bands that might correspond to truncation variants were observed in normal and failing heart.

**Tissue-Specific SCN5A Splicing**

In addition to heart, cardiac Na\textsuperscript{+} channel mRNA is known to be transcribed in skeletal muscle\textsuperscript{8,9,17,24} and leukocytes.\textsuperscript{25} RT-PCR result showed that only 2 of the four splice variants were transcribed in skeletal muscle, but all four variants were detected in the human lymphoblast (Figure 4). The distribution of those 4 variants in lymphoblasts was different from that in heart and was 22.3\% ($\pm 4.2$), 12.6\% ($\pm 1.7$), 50.8\% ($\pm 8.2$), and 14.4\% ($\pm 3.1$) for E28A, E28B, E28C, and E28D, respectively. In lymphoblasts from patients (n=4) with no cardiac dysfunction, the presence of identical variants confirm the specificity of findings in explanted heart.

**Truncation Variants Reduce Na\textsuperscript{+} Channel Protein and Current**

Variant cDNA was expressed in the HEK-SCN5A cell line stably expressing the full-length E28A channel. E28D transcript was found in HEK cells transfected with the E28D

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\caption{C-terminal variant mRNA abundances and protein vary between control and diseased hearts. Real-time PCR results show that the variant abundances with respect to the total mRNA abundance in each sample. The changes were determined for the 4 exon 28 variants in control (black bars) and HF (open bars) patients for both ventricles (A), left ventricle (B), and right ventricle (C). Comparison of the left and right ventricles in B and C, respectively, shows that the variant abundance changes are more prominent in the left ventricle. $\beta$-Actin was used as a reference in all cases. All mRNA abundances were normalized to 1 of the normal human ventricular exon 28D mRNA abundances. *$P<0.05$ compared with controls with HF. D, A representative Western blot for the cardiac Na\textsuperscript{+} channel in normal human hearts and failing hearts normalized to tubulin expression. HEK cells expressing the E28A, E28D, or neither (-) variant were used as controls. E, Failing human hearts showed reduced Na\textsuperscript{+} channel protein.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Tissue-specific expression of human SCN5A exon 28 variants. RT-PCR results show that variants E28A and E28D were expressed in skeletal muscle (SKM), as well as in human heart. All 4 variants were found in lymphoblasts. $\beta$-Actin was used as internal reference.}
\end{figure}
variant linked to GFP expression (IRES-GFP; D1) or fused to GFP (D2). E28D variant expression reduced the E28A variant mRNA abundance (Figure 5A). The use of increasing ratios of E28D vector resulted in progressive reductions in full-length transcript mRNA abundance (Figure 5B). Neither the E28C nor E28D variants generated current when transfected into HEK cells alone, and when transfected into the HEK-SCN5A cell line stably expressing the full-length Na⁺ channel, both variants reduced Na⁺ current (Figure 5C). The presence of the E28C or E28D variants resulted in 54.6% (±8.5, P<0.01, n=14) and 56.0% (±8.9, P<0.01, n=10) reductions in peak current, respectively, when compared with the native channel alone (Figure 5D and 5E). The reduction of current was dependent on the ratio of variant to full-length vector used (Figure 5F and 5G). Fluorescent microscopy of HEK cells transfected with Na⁺ channel C-terminal–labeled variants demonstrated markedly reduced amounts of E28C or E28D variant Na⁺ channel protein when compared with cells transfected with an equal amount of the full-length E28A variant (Figure 6).

**Figure 5.** Truncation variants suppress function of the full-length cardiac Na⁺ channel. A, RT-PCR to detect the full-length SCN5A (A) and truncation variant E28D in HEK cells transfected with the E28D variant linked to GFP expression (IRES-GFP; D1) or fused to GFP (D2). HEK cells stably transfected with the full-length variant served as a positive control (+). The presence of the E28D variant decreases E28A mRNA abundance. B, Quantitative real-time PCR shows a dose-dependent reduction in reduced full-length mRNA with increasing ratios of the E28D variant vector. C, Current trace families in HEK cells expressing the E28A, E28C, or E28D variants alone or combinations of the full-length channel with the E28C or E28D variants. D, Peak current–voltage relationship of the E28A with or without the E28C or E28D variants in a 1:1 ratio normalized to cell capacitance. F, Peak current was reduced with E28C or E28D variants present. G and H, Current–voltage curves and peak currents showing a dose-dependent reduction in peak Na⁺ current as a function of the ratio of E28A to E28D variant. *P<0.05.
A Mouse Model of the Effect of Truncation Variants

The physiological significance of truncations in exon 28 was tested by making a gene-targeted mouse model with a nonsense mutation in exon 28 between the truncations caused by the E28C and E28D variants. This mutation was lethal to embryos. Undifferentiated mouse ES cells heterozygous for the SCN5A1652stop had normal growth characteristics and could be differentiated into spontaneously beating CMs. The Na⁺ channel current–voltage relationships from contracting CMs isolated from WT and truncation embryonic bodies derived from the respective ES cell lines are shown in Figure 7A. The peak \( I_{Na} \) was decreased by 86.1% (±5.2, \( n=8 \), \( P=0.0002 \)) in differentiated CMs containing the truncation when compared with that of WT (Figure 7B). Action potentials recorded in the current clamp mode from spontaneously beating CMs showed significant slowing of the beating frequency (\( P=0.02 \), \( n=11 \)) in the truncation mutant. Action potentials also showed a significant reduction in the maximum rate of rise of the AP in the truncation mutation (\( P<0.01 \), \( n=11 \)) and a reduced amplitude (\( P<0.01 \), \( n=11 \)) in comparison with WT (Figure 5C and 5D). These changes were consistent with reduced Na⁺ channel function.26–28

Syncytial properties of these CMs were studied using MEAs.29,30 Consistent with a physiologically significant reduction in Na⁺ current as a result of the truncated mRNA, MEA recordings of CMs with the truncation mutation showed the minimum FP decreased by 70.5% (\( P<0.05 \)), the FP rise slowed by 45.5% (\( P<0.05 \)), and the conduction velocity was decreased by 64.2% (\( P<0.03 \)) as compared with the WT (Figure 8).31

Discussion

HF is known to be associated with reduced Na⁺ current.1,2 In this study, we report 3 new cardiac Na⁺ channel C-terminal splicing variants. Each of these variants is expected to result in prematurely truncated, nonfunctional Na⁺ channels. These variants are differentially regulated during development. As compared with controls, patients with HF showed an increase in the total truncated forms of up to \( \approx 50\% \) and a reduction in the number of Na⁺ channels by 63%. These effects were in line with the \( \approx 50\% \) reduction in current seen when HEK cells were transfected with equal amounts of full-length and E28C or E28D variants. Introducing a premature stop codon in exon 28 of a single allele of the SCN5A gene to mimic the \( \approx 50\% \)
Alternative C-terminal splicing variants were seen in all tissues known to transcribe SCN5A, implying a specific mechanism. Nevertheless, the number of variants and their relative abundances changed between cell types. Because lymphoblast and skeletal muscle mRNA was obtained from subjects without HF, it remains unclear whether the variant abundances would be related among tissues under similar clinical circumstances.

Identical C-terminal splicing variants were seen in all hearts. During HF, these alternatively spliced mRNA may contribute to reductions in Na⁺ current in HF.

The E28C and E28D variant mRNAs reduced Na⁺ channel full-length mRNA and current in the absence of encoding for significant protein or current themselves. The mechanism for this effect at the mRNA level was unclear, but dominant-negative effects have been seen with the cardiac Na⁺ channel and other ion channels. The physiological significance of the HF variants was supported by a premature stop codon in exon 28 of 1 of the 2 SCN5A alleles, resulting in an 86% reduction in Na⁺ current and presumably explaining the embryonic lethality. Another Na⁺ channel disruption mouse model, in the heterozygous state, showed reduced Na⁺ current, impaired conduction, and a tendency toward ventricular tachycardia. The lack of a dominant-negative effect in this model may have been because this mouse had a disruption of the transcription start site, preventing the production of abnormal channel mRNA. In humans, frameshift errors, splice-site defects, and premature stop codons in a single allele of the SCN5A gene result in the Brugada sudden death syndrome. The predilection for changes to be most prominent in the left ventricle may explain why HF patients did not show the classic right heart electrocardiographic changes of Brugada syndrome.

There are several limitations to our study. First, although the mouse gene targeting was designed to yield truncated mRNA at a similar proportion to that seen in HF, the mutation introduced was not identical to any of the 3 novel splice variations. Nevertheless, the E28C variant showed similar electrophysiological effects on the full-length channel to that of E28D, suggesting that electrophysiological results were somewhat insensitive to the location of the truncation. It cannot be ruled out, however, that the site of truncation may have contributed to the quantitative differences between the in vitro and in vivo results. Second, we were unable to confirm the presence of truncated protein in human heart by Western blotting, most likely because the level of protein produced by the variants was below the detection limit of this technique. Third, the mechanism for abnormal splicing remains to be elucidated, and the variant splicing sites are not near recognized splice sequences. Fourth, in this study, most RNA samples were from males with dilated ischemic cardiomyopathy. Similar splice variations were seen in the single female and 2 ischemic cardiomyopathy samples, but sex- and pathology-specific differences may exist that were not uncovered here. Finally, although the control subjects were younger than the HF patients, we could find no evidence of splice variation changes with age (supplemental Figure I).

In conclusion, we demonstrate that there are several alternatively spliced forms of SCN5A mRNA in human hearts. During HF, these alternatively spliced mRNA variants are likely to reduce Na⁺ current to levels that might contribute to arrhythmic risk alone or in combination with other inciting causes. In the future, it would be conceivable to incorporate the relative abundances of these variants into an arrhythmic risk prediction algorithm. Ultimately, treatment strategies could be developed to reduce arrhythmic risk based on the mechanisms underlying the increase in abnormal splicing during the natural history of chronic HF.
Sources of Funding
This study was supported by NIH grants HL64828 and HL073753, the Emory University General Clinical Research Center (M01-RR00039), a Department of Veterans Affairs Merit grant (to S.C.D.), an American Heart Association Established Investigator Award (to S.C.D.), and a research fellowship from the American Heart Association (to A.E.P.).

Disclosures
S.C.D. and L.L.S. have filed a patent (11/707,882) based on this work.

References


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Heart failure is associated with abnormal C-terminus splicing variants in the cardiac sodium channel

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Generation of a truncated Scn5a mouse model

A 4.0-kb fragment of 129Sv/J mouse genomic DNA was cloned from a mouse ES cell genomic library by PCR amplification using primers RHI28F/R¹ corresponding to the known mouse SCN5A exon 28 sequences.² One of the PCR positive clones was used to construct the scn5a truncation targeting vector pBSK.SCN5A¹⁶⁵²stop by HindIII-site subcloning. The floxed neomycin cassette was inserted into AatII site (Figure S2). A deletion of an adenosine (A) in codon resulted in a frame shift turning the 1652 codon that formerly encoded for a methionine into a stop codon (TGA; NCBI Accession NP_932173). After electroporating the target construct into the mouse embryonic stem cells (R1), targeted clones were screened and identified by PCR using primers P1/neoR and neoF/P2 (Figures S2B and S3A), and using primers P3/P4 surrounding the mutation site (Figures S2 and S3E) and Southern blotting (Figures S2, S3B, and S3C). Cre recombinase was expressed in correctly
targeted clones confirmed by PCR using neorF/R (Figure S2D). Heterozygosity was confirmed by P3-P4 PCR product with or without BspHI digestion (Figure S2E).

References


Table S1. PCR primers

Table S2. Patient characteristics

Figure S1. A comparison of the abundances of SCN5A splice variants as a function of age. Comparing the relative mRNA abundances of the splice variants after dividing subjects into three age groups of 40-49 (40's), 50-59 (50's) and 60-69 (60's) shows that splice variant abundances did not appear to be a function of age.

Figure S2. The targeting strategy to create a mouse SCN5A truncation model. Panel A: Targeting vector pBSK.SCN5A\textsuperscript{1652stop} mapped to the native SCN5A exon 28 region (WT SCN5A allele). Panel B: Map showing incorporation of the targeting vector into the WT allele. Panel C: Map of the truncation mutation introduced into WT SCN5A allele after Cre-mediated excision of the neomycin resistance cassette to create SCN5A\textsuperscript{1652stop}. Restriction digests, PCR primers, and hybridization probes A (3.1-kb PvuII fragment) and B (3.71-kb PvuII fragment) for genotyping are indicated.

Figure S3. Genotyping for homologous recombination of the SCN5A\textsuperscript{1652stop}. Panel A: PCR analysis using upper and lower PCR amplicons (see methods and figure S2) demonstrates proper recombination in heterozygous (+/-) and not in wild-type (WT) mice (+/+). Panel B and C: Southern blot analysis with external probes A and B showing proper incorporation of the truncation vector in a single allele of targeted ES cells (+/-). Panel D shows PCR result of a properly targeted ES cell clone before (neo+) and after (neo-) successful excision of the neomycin resistance cassette. The targeting vector was used as a control. Panel E shows BspHI restriction digests to demonstrate
incorporation of the targeting vector. Introduction of the coding mutation resulted in elimination of a BspHI restriction site. Therefore, the properly targeting allele displayed an additional 545 bp fragment representing the targeted allele (heterozygous, +/-) as compared to the 395bp and 150 bp fragments resulting from the native sequences (wild-type, WT) when performing a BspHI digest of a PCR amplicon spanning this region.

Figure S4. Alignment of the nucleotide and amino acid sequences (single letter) of the four SCN5A transcriptional variants. The variant name and nucleotide base pairs numbering starting at the initial AUG codon are indicated at the left. The sequences start from exon 27 (shaded) and continue to the poly-A tail. Introns are shown as dashed lines. Splicing of exons B, C, and D result in frame shifts and premature stop codons. Methionine at amino acid 1652 is bolded to indicate the site of introduction of a stop codon in the gene-targeted mouse.
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DCM: dilated cardiomyopathy  
ICM: ischemic cardiomyopathy  
CM: cardiomyopathy  
M: male  
F: female
FIGURE S1

![Bar chart showing relative RNA abundance (log10) for different groups labeled E28A, E28B, E28C, and E28D. The chart compares 40's n=4, 50's n=3, and 60's n=5 groups.]
FIGURE S2

A. pBSK.SCN5A<sup>wt/samp-<i>+-</i></sup>

WT SCN5A allele

B. SCN5A<sup>samp-<i>+</i>/samp-<i>-</i></sup> allele

C. SCN5A<sup>samp-<i>-</i>/samp-<i>-</i></sup> allele

Cre-excision

BapHI digest on PCR product

395 & 1500bp

PvuII 5.2kb

XhoI 2.5kb

PvuII 3.1kb

XhoI 4.1kb

PvuII 3.7kb

545bp

BamHI digest on PCR product

PvuII 5.2kb

XhoI 2.5kb

545bp

BamHI digest on PCR product
FIGURE S3

A.

B. Probe A

C. Probe B

D. Neo-cassette excision

E. Mutant site confirmation

Undigested PCR

BspHI digested PCR