Heart Failure

Role of RBM25/LUC7L3 in Abnormal Cardiac Sodium Channel Splicing Regulation in Human Heart Failure

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Background—Human heart failure is associated with decreased cardiac voltage-gated Na+ channel current (encoded by SCN5A), and the changes have been implicated in the increased risk of sudden death in heart failure. Nevertheless, the mechanism of SCN5A downregulation is unclear. A number of human diseases are associated with alternative mRNA splicing, which has received comparatively little attention in the study of cardiac disease. Splicing factor expression profiles during human heart failure and a specific splicing pathway for SCN5A regulation were explored in this study.

Methods and Results—Gene array comparisons between normal human and heart failure tissues demonstrated that 17 splicing factors, associated with all major spliceosome components, were upregulated. Two of these splicing factors, RBM25 and LUC7L3, were elevated in human heart failure tissue and mediated truncation of SCN5A mRNA in both Jurkat cells and human embryonic stem cell–derived cardiomyocytes. RBM25/LUC7L3-mediated abnormal SCN5A mRNA splicing reduced Na+ channel current 91.1±9.3% to a range known to cause sudden death. Overexpression of either splicing factor resulted in an increase in truncated mRNA and a concomitant decrease in the full-length SCN5A transcript.

Conclusions—Of the 17 mRNA splicing factors upregulated in heart failure, RBM25 and LUC7L3 were sufficient to explain the increase in truncated forms and the reduction in full-length Na+ channel transcript. Because the reduction in channels was in the range known to be associated with sudden death, interruption of this abnormal mRNA processing may reduce arrhythmic risk in heart failure. (Circulation. 2011;124:1124-1131.)

Key Words: angiotensin II ■ arrhythmia ■ cardiomyopathy ■ gene expression ■ sodium

Up to 95% of human genes have multiexon alternative spliced forms, suggesting that alternative splicing is one of the most significant components of the functional complexity of the human genome.1,2 Although our understanding of the role of alternative mRNA splicing is elemental, a growing number of human diseases such as cancer,3 neurodegenerative disorders,4 and autoimmune diseases5 are associated with alternative splicing. Alternative splicing events have been identified in cancer allowing for splicing signatures associated with different tumor subgroups.6 Important goals of future studies of alternative splicing regulation include understanding how it occurs, its implications for disease, and its stimuli.7

Clinical Perspective on p 1131

Alternative splicing regulation has received comparatively little attention in the study of cardiac diseases. Splicing variants associated with the cardiac sodium,8 calcium,9 and HERG potassium channels10 have been reported. SCN5A is the gene encoding the cardiac sodium channel (Na,1,5), a protein responsible for generating the main current for excitation propagation in cardiomyocytes.11 Previously, we have shown that cardiac Na+ channel mRNA is alternatively spliced in human heart failure (HF) and that this splicing contributes to a reduction in current of a magnitude likely to contribute to the arrhythmic risk in this condition.9 Here, we identified the upstream stimuli, hypoxia and angiotensin II (Ang II), and the mediators, RBM25 and LUC7L3, of this abnormal splicing event.

RBM25 belongs to a family of RNA-binding proteins whose members share an arginine-glutamic acid/arginine-aspartic acid–rich central region and a C-terminal proline-tryptophan-isoleucine motif.12 RBM25 localizes to the nu-
clear speckles and associates with multiple splicing components such as splicing cofactors SRm160/300, U1 small nuclear (sn) RNAs, assembled splicing complexes, and spliced mRNAs. Characterization of RBM25 strongly suggests that it functions in premRNA processing and that this regulation is gene specific.\textsuperscript{12,13} LUC7L3, a human homolog of yeast U1 snRNP-associated factor, is also a nuclear protein with a role in premRNA spliceosome. LUC7L3 has 2 zinc finger motifs.\textsuperscript{12,13} The first crosslinks the premRNA and is required for LUC7L3 splicing activity. LUC7L3 acts as a bridge between the premRNA and the U1 snRNP through its second zinc finger. According to a recent report, RBM25 associates selectively with the LUC7L3 and activates proapoptotic Bcl-x-S selective with the LUC7L3 and activates proapoptotic Bcl-x-S and activates proapoptotic Bcl-x-S.

\textbf{Methods}

\textbf{Microarray Assay}  
See the only online Data Supplement for details.

\textbf{Cell Culture}  
Jurkat T-cell clones E6.1 (ATCC, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 4 mmol/L glutamine, 75 U/mL streptomycin, and 100 U/mL penicillin.

Human embryonic stem cells (hESCs) were maintained on mouse embryonic fibroblasts as previously described.\textsuperscript{14} Cardiomyocytes were differentiated from WA09 (H9) embryonic stem cells with a directed differentiation approach in defined media for efficient cardiogenesis. After 30 days of differentiation, the hESC-derived cardiomyocytes (hESC-CMs) were used in this study.

\textbf{Real-Time Polymerase Chain Reaction Quantification}  
Total RNA was isolated from cultured cells and human ventricular tissue with the RNAeasy Mini Kit and RNAeasy Lipid Tissue Mini Kit, respectively (Qiagen, Valencia, CA). Human heart tissue was obtained from a tissue bank maintained at Advocate Christ Cardiac Surgery Clinical Research Center. For primers for the target genes, see the only online Data Supplement.

\textbf{Transfection and Infection Assays}  
Fugene 6 reagents from Roche (Madison, WI) were used for transfection assays by following the manufacturer’s instructions. Small inhibitory RNAs (siRNAs) for LUC7L3 and RBM25 were purchased from ORIGENE (Rockville, MD). The Mini-PROTEAN Tetra Electrophoresis System from BioRad (Hercules, CA) was used for Western blot analysis. Anti-RBM25 antibodies were purchased from chimeric reagents (Pierce, Appleton, WI). In brief, biotinylated wild-type (CAGCAGGGCAGGGCCU) and mutant (CAGCAGGUUAGAGGCG) chemiluminescent RNA electrophoretic mobility shift assay kit (Carl Zeiss GmbH, Oberkochen, Germany) and quantitative polymerase chain reaction (qPCR), respectively, on days 2 and 3. Green fluorescent protein–tagged open reading frame clones of Homo sapiens LUC73 and RBM25 were purchased from ORIGENE (Rockville, MD). The transfection assays followed the manufacturer’s instructions.\textsuperscript{12}

\textbf{Electrophysiology}  
The hESC-CMs were trypsinized (0.25%; Invitrogen) for 10 minutes and plated on 35-mm glass-bottomed culture dish (MatTek, Ashland, MA) at a cell density of 40 000 cells per dish on the day before the experiments. Na\textsuperscript{+} channel currents were measured with the whole-cell patch-clamp technique in the voltage-clamp configuration at room temperature. To measure Na\textsuperscript{+} channel currents, pipettes (3 to 4 mol/L) were filled with a pipette solution containing (in mmol/L) CsCl 80, cesium aspartate 80, EGTA 11, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 1, HEPES 10, and Na\textsubscript{2}ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L) NaCl 130, CsCl 5, CaCl\textsubscript{2} 2, MgCl\textsubscript{2} 1.2, HEPES 10, and glucose 5 (adjusted to pH 7.4 with CsOH).\textsuperscript{15} The holding potential was –100 mV. A voltage step protocol ranging from –80 to 70 mV with steps of 10 mV was applied to establish the presence of Na\textsuperscript{+} channel currents. The peak current density was used to plot current-voltage curves. Nifedipine (10 μmol/L; Sigma) was added in the bath solution to block L-type Ca\textsuperscript{2+} channel currents.

\textbf{Gel Mobility Shift Assays}  
DNA gel mobility shift assays were performed with the LightShift chemiluminescent RNA electrophoretic mobility shift assay kit (Pierce, Appleton, WI). In brief, biotinylated wild-type (CAGCAGGGCAGGGCCU) and mutant (CAGCAGGUAGGGGCCU) RNA substrates were synthesized by Invitrogen. Binding of biotinylated RNA to RBM25 was achieved by incubating 0.2 mmol/L RNA and variable amounts of protein for 30 minutes at 4°C in 20 μL binding buffer. For the competition assays, a molar excess of unlabeled competitor RNAs at various fold levels was added to the preincubated reaction mixture. Samples were fractionated in a native 5% polyacrylamide gel and transferred to Hybond-N+ nylon membranes (Pierce). The biotin-labeled RNA was detected with the streptavidin horseradish peroxidase conjugate and a chemiluminescent substrate.\textsuperscript{12}

\textbf{Western Blots Assays}  
The Mini-PROTEAN Tetra Electrophoresis System from BioRad (Hercules, CA) was used for Western blot analysis. Anti-RBM25 antibodies were provided by Dr Shu-Ching Huang (Dana-Farber Cancer Institute). Anti-LUC7L3 antibodies were purchased from Millipore (Billerica, MA). Anti–green fluorescent protein was purchased from ORIGENE (Rockville, MD).

\textbf{Statistics}  
Data are presented as mean±SEM. Means were compared by use of the unpaired Student t test or 1-way ANOVA. Values of P<0.05 were considered statistically significant.

\textbf{Results}  
Altered mRNA Profiles of Splicing Factors in Human Heart Failure Tissue  
A mRNA microarray analysis was used to identify and to compare splicing factors in both normal and human HF tissues. Of the 181 known human splicing factors analyzed, 17 were upregulated in HF. These splicing factors were grouped according to known pathogenic regulators such as hypoxia,\textsuperscript{16} inflammation,\textsuperscript{17} wall tension,\textsuperscript{18} or hormonal factors\textsuperscript{19} involved in HF (see the Table and the only online Data Supplement).

\begin{table}[h]
\centering
\small
\begin{tabular}{|l|l|}
\hline
\textbf{Pathogenic Regulation Involved} & \textbf{Gene Symbol} \\
\hline
Hypoxia & RBM25, LUC7L3, TARDBP, HNRNP3, PP1G \\
\hline
Inflammation & BAT1, RBM39, IRS1, ABP \\
\hline
Hormone level changing & BAT1 \\
\hline
Overloading pressure & RBM39 \\
\hline
Others & YTHDC1, SFPO, OKI, HNRNPA1, TRA2A \\
\hline
\end{tabular}
\caption{Comprehensive List of Human Heart Failure–Related Splicing Factors}
\end{table}
Upregulation of RBM25 and LUC7L3 in Human Heart Failure Tissue

The cis element CGGGCA of splicing factor RBM25 was found to be near the splicing sites of SCN5A variants E28C and E28D. RBM25 requires LUC7L3 to be active in splicing regulation, so both RBM25 and LUC7L3 were evaluated further for a role in SCN5A mRNA splicing regulation. Of the other 15 splicing factors upregulated, on the basis of the known cis element sequence, none is known to bind to or has canonical binding sequences that are present in SCN5A.

The upregulation of splicing factors RBM25 and LUC7L3 was confirmed in human HF tissue by qPCR. Compared with the normal human heart tissue, results indicated that the relative abundances of RBM25 and LUC7L3 were increased by 1.1- and 0.6-fold, respectively, in HF tissue (P<0.05; Figure 1A). Full-length SCN5A mRNA was reduced by 0.6-fold in HF tissue (P<0.05; Figure 1A), a result similar to our previous report. mRNA findings were correlated with protein expression by Western blots. The representative Western blots are shown in Figure 1B. Compared with the control group (mixture of 4 normal human heart tissue samples), Western blot quantification showed that RBM25 was increased by 0.5- to 0.6-fold in HF tissue samples 1 to 4 (P<0.05; 3 replications for each sample). The gel density of LUC7L3 was increased by 0.6- to 0.7-fold in the same HF tissue samples (P<0.05; 3 replications for each sample). The clinical information on these HF tissue samples is shown in Table III in the online-only Data Supplement.

RBM25 Associates With SCN5A and Interacts With CGGGCA

Gel mobility shift assays showed that RMB25 was bound to the canonical sequence CGGGCA in SCN5A exon 28 (Figure 2). Scanning the entire SCN5A RNA sequence revealed only a single binding site for RBM25 at the place where SCN5A splicing variants were detected (Figure 2A). Binding of biotinylated wild-type (CAGCAGGCGGGCAGCGGCCU) RNA to RBM25 is shown in Figure 2B through 2D. The results illustrated that RBM25 binding was specific to the sequence CGGGCA. RBM25 was bound to the wild-type SCN5A sequence in a concentration-dependent manner (Figure 2B). For the competition assays, a molar excess of unlabeled competitor RNAs at various fold levels was added to the preincubated reaction mixture (Figure 2D). Specificity was confirmed by showing a lack of this binding to a mutated canonical binding sequence (Figure 2C) and the inability of unlabeled probe to compete with labeled probe for RBM25 binding (Figure 2D).

Angiotensin II and Hypoxia-Regulated RBM25, LUC7L3 Expression, and SCN5A mRNA Splicing

Hypoxia and Ang II, common pathogenic factors in HF, were identified in the microarray analysis as possible upstream stimuli responsible for the changes in mRNA splicing factors (the Table). SCN5A mRNA is known to be transcribed in skeletal muscle and leukocytes. We have reported that leukocytes have an mRNA splicing pattern similar to that in the heart. Moreover, circulating leukocytes from HF patients showed a 4-fold increase in the Ang II type 1 receptor (data not shown). Therefore, Jurkat cells, an immortalized line of T lymphocyte cells that prominently express SCN5A, were chosen to be as an initial model to study the SCN5A regulation mechanism.

Jurkat cells were divided into 3 experiment groups: an untreated control group, a hypoxia-treated (1% O2) group, and an Ang II–treated (200 nmol/L) group. The cells were harvested from each experiment group at 4 time points (30 minutes, 24 hours, 48 hours, and 72 hours), and total mRNA was extracted. The expression of RBM25 and LUC7L3 was examined by qPCR, and the results at 48 hours are shown in Figure 3A. Hypoxia-inducible factor-1α was used to indicate cellular hypoxic stress. Under the hypoxia-treated condition, the expression of RBM25 and LUC7L3 in Jurkat cells was...
increased by 2.4- and 4.9-fold, respectively \( (P<0.05) \). Under the Ang II–treated condition, the expression of RBM25 and LUC7L3 in Jurkat cells was increased by 2.1- and 1.9-fold, respectively \( (P<0.05) \). The expression of RBM25 and LUC7L3 was analyzed by Western blots at 3 time points (12, 24, and 48 hours) for the hypoxia-treated group and at 4 time points (24, 48, 72, and 96 hours) for the Ang II–treated group. Western blot quantification showed that RBM25 was increased by 1.9-, 2.0-, and 1.5-fold in the hypoxia-treated group at 12, 24, and 48 hours, respectively, and was increased by 2.1-, 2.1-, 1.9-, and 2.0-fold in the Ang II–treated group at 24, 48, 72, and 96 hours, respectively \( (P<0.05) \). The gel density of LUC7L3 was increased by 2.4-, 2.4- and 2.7-fold in the hypoxia-treated group at 12, 24, and 48 hours, respectively, and was increased by 2.6-, 2.5-, 2.8-, and 2.8-fold in the Ang II–treated group at 24, 48, 72, and 96 hours, respectively \( (P<0.05) \). The representative Western blots and quantification (based on 3 replications for each group) are shown in Figure 3B.

The effect of hypoxia and Ang II on SCN5A variants E28C and E28D in Jurkat cells was also studied to correlate SCN5A variants with RBM25 and LUC7L3 abundances. The expression of the full-length SCN5A transcript and SCN5A variants E28C and E28D at 48 hours is shown in Figure 3C. With hypoxia, the expression of SCN5A variants E28C and E28D was increased by 3.7- and 6.4-fold, respectively \( (P<0.05) \), whereas the expression of the full-length SCN5A transcript was decreased by 0.7-fold \( (P<0.05) \). With Ang II, the expression of SCN5A variants E28C and E28D was increased by 2.9- and 4.3-fold, respectively \( (P<0.05) \), whereas the expression of the full-length SCN5A transcript was decreased by 0.8-fold \( (P<0.05) \).

siRNAs for these 2 splicing factors were found to partially block the increases in the hypoxia- or Ang II–induced SCN5A variants E28C and E28D at 48 hours (Figure 3D and 3E). The partial effect on reducing abnormal splicing may be due to the siRNA knockdown efficiencies were 60±5% and 70±5% estimated by qPCR and 63±7% and 69±6% by Western blots for RBM25 and LUC7L3, respectively.

Although downregulation of the 2 splicing factors in Jurkat cells reduced SCN5A variants E28C and E28D, overexpression of RBM25 and LUC7L3 increased E28C and E28D and decreased the full-length SCN5A mRNA abundances. Expression of the full-length SCN5A transcript and SCN5A variants E28C and E28D at 48 hours is shown in Figure 3F. With overexpression of RBM25, the expression of SCN5A variants E28C and E28D was increased by 1.6- and 2.5-fold, respectively \( (P<0.05) \), whereas the expression of the full-length SCN5A transcript was decreased by 0.6-fold \( (P<0.05) \). With overexpression of LUC7L3, the expression of SCN5A variants E28C and E28D was increased by 1.1- and 1.9-fold, respectively \( (P<0.05) \), whereas the expression of the full-length SCN5A transcript was decreased by 0.7-fold \( (P<0.05) \).

Effect of Angiotensin II on Na\(^+\) Channels in Human Embryonic Stem Cell–Derived Cardiomyocytes

The effect of Ang II on the cardiac Na\(^+\) channel was investigated in hESC-CMs. The hESC-CMs were plated on a 24-well culture plate on day 30 of differentiation. The cells were divided into 3 experiment groups: an Ang II–treated (200 nmol/L) group, an Ang II–treated (200 nmol/L) group preinfected by pGIPZ lentiviral RBM25 shRNAmir, and an Ang II–treated (200 nmol/L) group preinfected by scrambled shRNA. All the experiment groups were given Ang II (200 nmol/L) treatment on infection day 3. When cells were preinfected by RBM25 shRNA, the expression of the full-length SCN5A transcript was decreased by 0.4-fold, whereas the expression of SCN5A variants E28C and E28D was decreased by 0.4- and 0.5-fold, respectively \( (P<0.05) \). The qPCR measurements were performed in each experiment group 24 hours after Ang II treatment and normalized by β-actin. No changes were observed when cells were preinfected by scrambled shRNA, however. The results indicated that Ang II–mediated SCN5A downregulation was dependent on the splicing factor RBM25 (Figure 4A). The infection rate was 90±6%, evaluated by the ratio of green fluorescent protein–positive cells (pGIPZ lentiviral infected cells) to total 70±5% estimated by qPCR and 63±7% and 69±6% by Western blots for RBM25 and LUC7L3, respectively.

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Figure 2. Illustration of the C-terminal structure of SCN5A and the variants E28C and E28D. A, Gel mobility shift assays are performed using the biotinylated wild-type (WT) probe (B) or the mutant (Mu) probe (C) and purified RBM25 protein. For loading samples from left to right, the amount of RBM25 in each binding reaction is increased by 1-fold. For the competition assay (D), 0 or 1-, 5-, or 20-fold molar excesses of unlabeled WT or Mu probes are added in each binding reaction. *The RBM25 binding site CGGGCA in exon 28 (982 to 987 bp).
Figure 3. RBM25 and LUC7L3 are involved in SCN5A regulation in Jurkat cells. A, Quantitative polymerase chain reaction (PCR) demonstrates the upregulation of RBM25 and LUC7L3. The expression changes of RBM25 and LUC7L3 in hypoxia-treated (shaded bars) and angiotensin II (Ang II)–treated (black bars) vs normal control (white bars) Jurkat cells are shown at 48 hours. Hypoxia-inducible factor-1α is an indicator of hypoxia. mRNA abundances are normalized by β-actin. *P < 0.05 vs control (n = 6). B, Western blot quantification confirms the upregulation of RBM25 and LUC7L3 in Jurkat cells. Expressions of RBM25 and LUC7L3 are analyzed by time course. *P < 0.05 vs control (n = 6). C, The expression changes of SCN5A and the variants E28C and E28D in hypoxia-treated (shaded bars) and Ang II–treated (black bars) vs untreated (white bars) Jurkat cells are shown at 48 hours. Western blots indicate the downregulation of SCN5A in Jurkat cells with Ang II or hypoxia. *P < 0.05 vs control (n = 6). D, Quantitative PCR demonstrates that RBM25 and LUC7L3 siRNAs could block the induction of hypoxia on variants E28C and E28D. The representative results at 24 hours are shown. *P < 0.05 vs control (n = 6). E, Quantitative PCR demonstrates that RBM25 and LUC7L3 siRNAs could block the induction of variants E28C and E28D by Ang II. Representative results at 48 hours are shown. Scrambled siRNA had no effect on the induction by Ang II (data not shown). The knockdown efficiency of RBM25 and LUC7L3 siRNAs are evaluated by Western blots and compared with control and scrambled RNA. *P < 0.05 vs control (n = 6). F, Quantitative PCR demonstrates that RBM25 and LUC7L3 overexpression decreases the full-length SCN5A transcript and increases the variants E28C and E28D at 48 hours. Exogenously expressed RBM25–green fluorescent protein (GFP) and LUC7L3–GFP are detected by Western blot analysis with anti-GFP at 48 hours after transfection in Jurkat cells. The representative Western blots show the downregulation of the full-length SCN5A transcript in Jurkat cells with exogenously expressed RBM25 and LUC7L3 vs the control group. Full-length SCN5A RNA is unchanged with GFP expression alone. *P < 0.05 vs control (n = 6).
Altered Na+ were tested by measuring Na+ channel mRNA processing changes from domain IV, S3, or S4 to the C terminus. A mouse model truncation variant showed a 80% reduction in peak current and a significant reduction in electric current-voltage relationship compared with Ang II alone (data not shown). The results indicated that Ang II could downregulate Na+ channel currents in hESC-CMs and that this downregulation was dependent on the splicing factor RBM25.

**Discussion**

HF remains a substantial clinical problem affecting millions of Americans, and HF-associated arrhythmia remains a cause of the high morbidity and mortality.19 The role of abnormal mRNA splicing in this condition is unknown. Gene array comparisons of splicing factor transcription between normal and HF tissues demonstrated that 17 splicing factors were upregulated during HF. The spliceosome assembles onto the premRNA in a stepwise manner. U1 snRNP binds to the 5′ splice site, SF1 binds to the branch site, and the U2 auxiliary factor binds to the 3′ splice site to form the E complex. Next, U2 snRNP replaces SF1 at the branch site to form the A complex. Subsequently, the B complex is formed when the U4, U5, and U6 trisnRNP enters the spliceosome. Finally, a rearrangement occurs to form the catalytically active C complex.23 Splicing factors in all major spliceosome components were altered in human HF. Upregulated splicing factors were located in U1-related components (RBM25, LUC7L3), EJC/TREX (BAT1), and common components (SRSF11, NCBP 2, HNRNPC). This suggested that alternative mRNA splicing may be a major pathological feature of and contribute to the arrhythmic risk in HF.

We evaluated the effects of abnormal mRNA splicing in HF on the cardiac Na+ channel (encoded by SCN5A), the main ion channel generating current for conduction in the heart. Previously, we have demonstrated that HF results in an increase in 2 SCN5A variants, designated E28C (39 bp) and E28D (114 bp).8 SCN5A mRNA variants result from splicing at cryptic splice sequences in the terminal exon of SCN5A (exon 28). Compared with the full-length Na+ channel, SCN5A variants are shorter and encode prematurely truncated, nonfunctional Na+ channel proteins missing the segments from domain IV, S3, or S4 to the C terminus.8 A mouse model in which 1 allele of the SCN5A gene is substituted by a model truncation variant showed a >80% reduction in cardiac Na+ current and a significant reduction in electric conduction velocity.8,24 On the basis of the presence of a cis-element binding site in exon 28 of SCN5A, the role of the upregulation of U1-related components RBM25 and LUC7L3 in abnormal splicing by recruiting LUC7L3,12 loss of function of RBM25 with lentiviral shRNA was used exclusively to suppress abnormal channel splicing. The macroscopic Na+ channel currents in each experiment group were measured. The results from the first 3 experiment groups at 24 hours after Ang II treatment are shown in Figure 5. There was a significant difference in peak currents between control cells and Ang II–treated cells at membrane potentials ranging from −40 to 30 mV (P<0.05). The effect of Ang II on peak current was not observed when the cells were preinfected by RBM25 shRNA before Ang II treatment. Nonspecific effects of the lentivirus were excluded by comparison with the cells preinfected by scrambled shRNA, which had no effect on the current-voltage relationship compared with Ang II alone (data not shown). The results indicated that Ang II could downregulate Na+ channel currents in hESC-CMs and that this downregulation was dependent on the splicing factor RBM25.
SCN5A mRNA splicing was investigated. RBM25 was bound to the canonical CGGGCA sequence in exon 28 near the splicing sites of SCN5A variants E28C and E28D. The binding specificity of RBM25 in exon 28 was confirmed by gel mobility shift assays. The upstream signal pathway of RBM25/LUC7L3-mediated splicing is not clear. Nevertheless, we showed that 2 common features present in HF, Ang II and hypoxia,25 were able to induce these splicing factors. These results are consistent with clinical data suggesting that renin-angiotensin system inhibition and revascularization have antiarrhythmic effects.26 Both hypoxia and Ang II increase oxidative stress and hypoxia-inducible factor-1α.27 LUC7L3 is known to be regulated by hypoxia, acidosis, and hypoxia-inducible factor-1α.28 It is possible that hypoxia-inducible factor-1α is an intermediate mediator of RBM25/LUC7L3-dependent splicing regulation of SCN5A. These signals are unlikely to be the only upstream regulators of abnormal Na⁺ channel splicing. Other factors such as inflammation29 and stretch30 may be important in mediated abnormal Na⁺ channel splicing in HF. Finally, although experimental manipulations of RBM25 and LUC7L3 were sufficient to cause or to prevent SCN5A mRNA dysregulation, it remains possible that some of the other splicing factors increased in HF contribute to abnormal SCN5A splicing.

Abnormal SCN5A splicing variants appear to be confined to humans for unclear reasons.8 The mechanism of splicing does not appear to be tissue restricted, however, because a similar pattern of SCN5A splicing variants has been found in both lymphoblasts and skeletal muscle.8 Moreover, Jurkat cells showed similar responses to cardiomyocytes.

Because there are no animal models of human abnormal Na⁺ channel splicing, it was impossible to determine directly the physiological significance of the downregulation of Na⁺ channel current mediated by abnormal splicing. Nevertheless, Ang II treatment of hESC-CMs showed current changes in the range known to contribute to arrhythmic risk.31

Conclusions

We have shown that human HF is associated with dysregulation of mRNA splicing factors and that 2 splicing factors contribute to abnormal SCN5A regulation. SCN5A regulation was similar in Jurkat cells and hESC-CMs. LUC7L3 and RBM25 were elevated in human HF tissue and mediated abnormal SCN5A splicing regulation in the 2 cell types tested. The resultant current reductions were of an order large enough to contribute to arrhythmic risk, especially in patients challenged by Na⁺ channel blockers. Although Ang II and hypoxia were sufficient to signal SCN5A abnormal splicing, other HF-associated factors could contribute to SCN5A downregulation in vivo. Therapies directed at reducing the activation of RBM25 and LUC7L3 or their upstream inducing stimuli may prevent the reduction in cardiac Na⁺ channels seen in HF and reduce arrhythmic risk in this condition.

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Disclosures

Dr Dudley holds patents related to this work, Human Sodium Channel Isoforms (11, 707882) and Potential Drug Targets to Prevent Arrhythmia in Heart Disease. The other authors report no conflicts.

References

Finally, this work defines potential therapeutic targets to address arrhythmic risk in human heart failure.

**Clinical Perspective**

We have previously shown that human heart failure is associated with abnormal mRNA splicing of the cardiac sodium channel. This abnormal mRNA splicing results in truncated sodium channels that are nonfunctional and a reduction in sodium channel current to levels known to cause sudden death. Here, we explored the mechanisms by which this abnormal splicing occurs. Using microarray comparisons of diseased and normal human hearts, we identified 2 splicing factors, RBM25 and LUC7L3, that were necessary and sufficient to cause the abnormal sodium channel splicing. These factors were upregulated by hypoxia and elevated angiotensin II, conditions known to be present in heart failure. Moreover, we showed that the responses of white heart cells and to these 2 inciting stimuli were equivalent. The potential clinical implications of these findings include a possible mechanism whereby hypoxia is arrhythmogenic and blockade of the renin-angiotensin system is antiarrhythmic. Moreover, if white cell sodium channel splicing in vivo correlates with that in the myocardium, it may be possible to develop a blood test to assess sodium channel availability and arrhythmic proclivity. Finally, this work defines potential therapeutic targets to address arrhythmic risk in human heart failure.
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Supplemental Material

The Role of RBM25/LUC7L3 in Abnormal Cardiac Sodium Channel Splicing Regulation in Human Heart Failure

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Comparison of Microarray Gene Expression in Human Heart Failure Tissue

Clinical Specimens

The microarray study samples were composed of end-stage cardiomyopathy hearts (n=10) and nonfailing control hearts (n=6). End-stage cardiomyopathy heart samples were obtained at the time of left ventricular assist device (LVAD) placement or cardiac transplantation. Subjects with end-stage cardiomyopathy exhibited severely reduced ejection fraction, left ventricular dilation, elevated pulmonary arterial and wedge pressures, and a reduced cardiac index. The control subjects were younger (median age 42 years with an interquartile range of 24–50 years) and predominantly male.

Data Analysis Methods

The GeneSifter gene expression microarray data analysis system was used to identify and compare significant differentially expressed genes from human (GEO accession: GSE1869) heart failure tissue-derived gene expression data. The data were uploaded to GeneSifter by Batch Upload with the option to use Affymetrix probe IDs. No data points were missing from any
of the files. The z score was used as a measure of the significance of observed genes compared to a normal distribution. A z score was considered significant if it was > 2 or < -2, implying the genes were significantly over-represented or under-represented. Statistically significant gene changes were identified using an unpaired Student's t test, $p$ value < 0.05 and a 5% Benjamini and Hochberg false discovery rate (FDR) correction. These settings are similar to those used by Kittleson et al.\textsuperscript{1} A range of fold change cutoffs was used. For functional analysis, gene ontology (GO) reports were generated according to the method of Doniger et al.\textsuperscript{2} Genes associated with RNA splicing were found under the biological process GO term “GO:0008380 RNA splicing”. The upregulated splicing factors are listed in Table 1. No significant downregulation of splicing factors was observed. Hierarchical clustering of these genes across samples was done using the average correlation approach through Bioconductor (Fred Hutchinson Cancer Research Center). The heatmap (Figure 1) represents the relationship of genes and samples to one another with green representing downregulated and red representing upregulated genes relative to the mean expression value of each gene.

References


Results

Table 1. RNA splicing genes differentially regulated in human heart failure

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene ID</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)</td>
<td>SFPQ</td>
<td>2.05</td>
</tr>
<tr>
<td>Ubiquitin specific peptidase 39</td>
<td>Usp39</td>
<td>2.02</td>
</tr>
<tr>
<td>Quaking homolog, KH domain RNA binding (mouse)</td>
<td>QKI</td>
<td>1.91</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein H3 (2H9)</td>
<td>HNRPH3</td>
<td>1.86</td>
</tr>
<tr>
<td>Cisplatin resistance-associated overexpressed protein</td>
<td>LUC7L3</td>
<td>1.77</td>
</tr>
<tr>
<td>YTH domain containing 1</td>
<td>YTHDC1</td>
<td>1.77</td>
</tr>
<tr>
<td>Splicing factor, arginine/serine-rich 7, 35kDa</td>
<td>SFRS7</td>
<td>1.66</td>
</tr>
<tr>
<td>HLA-B associated transcript 1</td>
<td>BAT1</td>
<td>1.65</td>
</tr>
<tr>
<td>TAR DNA binding protein</td>
<td>TARDBP</td>
<td>1.61</td>
</tr>
<tr>
<td>Influenza virus NS1A binding protein</td>
<td>IVNS1BP</td>
<td>1.61</td>
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<tr>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 1</td>
<td>Ddx1</td>
<td>1.60</td>
</tr>
<tr>
<td>Transformer-2 alpha</td>
<td>TRA2A</td>
<td>1.57</td>
</tr>
<tr>
<td>Peptidylprolyl isomerase G (cyclophilin G)</td>
<td>PPIG</td>
<td>1.55</td>
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<td>RNA binding motif protein 39</td>
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<td>1.49</td>
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<td>Splicing factor, arginine/serine-rich 11</td>
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<td>RNA binding motif protein 25</td>
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<td>Heterogeneous nuclear ribonucleoprotein A1</td>
<td>HNRNPA1</td>
<td>1.49</td>
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Table 2. Primers for target genes

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<tr>
<th>Gene</th>
<th>Primers</th>
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<tr>
<td>SCN5A</td>
<td>5'-TTACGCACCTTCCGAGTCTCC-3'</td>
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<tr>
<td></td>
<td>5'-GATGAGGCAAGAGCCTGAGG-3'</td>
</tr>
<tr>
<td>HSCN5AE28C/R</td>
<td>5'-TCTTCTTCCCTCCTGCTGTA-3'</td>
</tr>
<tr>
<td>HSCN5AE28D/R</td>
<td>5'-GGAAGACGTCGGGAGAGAAGTA-3'</td>
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<tr>
<td>RMB25</td>
<td>5'-TGTCTTTTCCACCTTATTGAATTC-3'</td>
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<td></td>
<td>5'-ATTGTACAGGAATTGCGGTTAAT-3'</td>
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<tr>
<td>LUC7L3</td>
<td>5'-GGACCAAGATCAAGCTGTTTGGTATTTG-3'</td>
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<tr>
<td>β-actin</td>
<td>5'-GATCCAGCCGCTCCAT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CATACTCCTGCTCCTTATCCA-3'</td>
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Table 3. Clinical information on heart failure tissue samples
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>M/F*</th>
<th>RACE</th>
<th>CAD*</th>
<th>Beta Blocker</th>
<th>ACEI*</th>
<th>Antiarrhythmic</th>
<th>LVEF* (%)</th>
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<tr>
<td>HF-1</td>
<td>59</td>
<td>M</td>
<td>Black</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
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<td>HF-2</td>
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<td>HF-7</td>
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<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

* M/F: male/female; CAD: coronary artery disease; ACEI: Angiotensin I-converting enzyme inhibitor; LVEF: left ventricular ejection fraction
Figure 1. Heatmap representing a hierarchical clustering of genes differentially regulated in human heart failure.