Delineating RBM20 Regulation of Alternative Splicing in Dilated Cardiomyopathy

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Study Hypothesis

Dilated cardiomyopathy (DCM) is a progressive disease of heart muscle characterized by chamber enlargement and wall dilatation, resulting in reduced systolic function. DCM is the most common form of nonischemic cardiomyopathy, and up to 30% of cases are estimated to be familial in nature.1 The majority of identified causative genes encode proteins responsible for muscle contraction or cellular architecture, yet DCM genes may be quite functionally distinct. An intriguing example is RBM20, which encodes RNA-binding motif protein 20 (RBM20), a pre-mRNA splicing factor that is highly expressed in striated muscle, particularly the heart.2 Genetic association between DCM and this post-transcriptional RNA processing protein was established in 2009 when missense mutations from 2 large families with autosomal-dominant DCM were mapped to RBM20. Subsequent profiling of a large cohort of idiopathic DCM patients identified additional missense mutations, all of which cluster within an arginine/serine–rich protein domain between amino acids 634 and 638.3 RBM20-dependent DCM (CMD1DD, OMIM #613172) occurs in ≈3% of DCM patients,4 and disease pathology recapitulated in RBM20 loss-of-function and deletion models involves altered splicing of numerous genes, including several linked to cardiomyopathy, ion transport, and contractile function.5,6 Presumably, these splice variants are directly regulated by RBM20 activity; however, TTN remains the only validated direct mRNA target.2 In a recent study in the Journal of Clinical Investigation,7 Maatz and colleagues reasoned that identification and mapping of the RBM20 RNA-recognition element (RRE) and protein interaction partners would facilitate greater understanding of how it regulates alternative splicing in the heart, thereby providing key mechanistic insights into how RBM20 mutations contribute to DCM.

How Was the Hypothesis Tested?

To identify and map general and cardiomyocyte-specific RREs for RBM20, 2 different RNA–protein interaction techniques were applied. Photocleavable ribonucleoside cross-linking immunoprecipitation (CLIP)8 was performed on human embryonic kidney 293 cells expressing N-terminally tagged human RBM20, whereas heart-specific transcriptome-wide analysis was conducted using high throughput sequencing of RNA isolated by CLIP, known as HITS-CLIP or CLIP-Seq.9 CLIP enables identification of the RNA-binding protein RRE sequence motif and, by comparing CLIP data to standard RNA-Seq data, determines whether binding occurs preferentially at exonic versus intronic and coding versus untranslated transcript regions.7 RBM20 target genes were identified by the presence of the RRE, with subsequent cardiomyocyte-specific analysis of differential exon expression used to determine if RBM20 RREs were localized primarily at or around activated or repressed exons and whether there was enrichment in particular exon categories, that is, mutually exclusive, cassette, or constitutive.3 A reporter assay was used to determine whether RRE mutation affected splicing activity, and a mobility shift assay was applied to confirm that reporter assay constructs were indeed interacting with RBM20. To complement RNA binding experiments, broad spectrum RBM20 protein–protein interactions were investigated by differential quantitative affinity mass spectrometry of stable isotope-labeled human embryonic kidney 293 cells. Background control during immunoprecipitation was
compared with coimmunoprecipitation with either RBM20 or an RBM20 missense mutant (S635A) at the known mutational hotspot in the arginine–serine domain, an essential region for protein–protein interaction. Finally, cardiac-specific interaction partners were examined via label-free proteomic quantification of cardiomyocyte-derived proteins in both the presence and absence of endogenous RBM20.

**Principal Findings**

In human embryonic kidney 293 cells, photoactivatable ribonucleoside CLIP profiling defined the consensus RRE, consisting of a UCUU oligonucleotide core with photoactivatable ribonucleoside CLIP/RNA-Seq comparison revealing core localization primarily to intronic sequences. This pattern was consistent with HITS-CLIP data in cardiomyocytes, which showed the RRE located almost exclusively at introns and exhibiting a strong UCUU oligonucleotide core signature. Alternative splicing effects between normal and mutant rat hearts were confirmed for several target proteins, with RBM20 found to act as a splicing repressor of cassette exons and regulating splicing of mutually exclusive exons. A total of 97 RBM20 regulated exons were identified, over 80% of which mapped to only 18 genes. TTN, the only previously confirmed target of RBM20 splicing effects, contained a large number of intronic RREs, aligning with splicing events detected in its elastic region.

A large proportion of proteins found to immunoprecipitate with RBM20 are frequently detected in spliceosome complexes and predominantly belong to U1 and U2 snRNPs as part of the complex A-specific spliceosome present in early-stage spliceosome assembly. Pull-downs with an RBM20 arginine/serine domain mutant indicated that mutation did not influence interaction with core spliceosome subunits, but significantly reduced interactions with 38 alternative spliceosomal proteins, 13 of which were not dependent on RNA for RBM20 connectivity. Ten of these 13 interacting partners detected in human embryonic kidney cells also immunoprecipitated with endogenous RBM20 in cardiomyocytes.

Comparison of splicing patterns in rat cardiomyocytes and human cardiac transcripts revealed conserved splicing of orthologous targets at the exon level for 7 genes, including TTN, CAMK2D, LDB3, LMO7, PDLIM3, RTN4, and RYR2. In all instances, RBM20 RREs were clustered in surrounding introns. Of note, RBM20 was previously implicated in regulating TTN, LDB3, and CAMK2D, but this article provided the first mechanistic evidence of how their pre-mRNA is directly regulated by this splicing factor. Besides the effect of missense mutations, the consequence of RBM20 differential expression was also investigated in heart tissue samples from nearly 150 end-stage heart failure patients. RBM20 was found to be highly variable in expression, and comparisons between the splicing patterns of RBM20-regulated exons in individuals with the highest and lowest RBM20 expression revealed significant differences in splicing of CAMK2D, LDB3, RYR2, and TTN, consistent with their previous findings.

**Implications**

Combined application of high throughput genetic and proteomic profiling has revealed the mechanism of RBM20-regulated splicing repression, including the splicing factor RRE, its direct gene targets, and differential splicing outcomes, as well as clinical consequences arising from both RBM20 dysfunction and altered expression. The network of functional influence for this splicing repressor on its direct targets may also extend to indirect effects on other genes and proteins, including those previously implicated in RBM20-dependent DCM. As a result of its wide ranging impact, RBM20 is a promising target for therapeutic intervention. Differential expression of RBM20 in heart failure patients may arise, however, from a response to disease rather than serving a causative role. In this regard, characterization of upstream regulators of RBM20 expression may offer other candidate genes and proteins responsible for familial DCM. In conclusion, this study has moved our knowledge of RBM20-dependent DCM from the realm of genetic association to that of mechanistic comprehension.

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**Disclosures**

None.

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