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ORIGINAL RESEARCH

Nonproductive Splicing Prevents Expression of MYH7b Protein in the Mammalian Heart

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BACKGROUND: Although the roles of alpha-myosin heavy chain (α-MyHC) and beta-myosin heavy chain (β-MyHC) proteins in cardiac contractility have long been appreciated, the biological contribution of another closely related sarcomeric myosin family member, MYH7b (myosin heavy chain 7b), has become a matter of debate. In mammals, MYH7b mRNA is transcribed but undergoes non-productive alternative splicing that prevents protein expression in a tissue-specific manner, including in the heart. However, several studies have recently linked MYH7b variants to different cardiomyopathies or have reported MYH7b protein expression in mammalian hearts.

METHODS AND RESULTS: By analyzing mammalian cardiac transcriptome and proteome data, we show that the vast majority of MYH7b RNA is subject to exon skipping and cannot be translated into a functional myosin molecule. Notably, we discovered a lag in the removal of introns flanking the alternatively spliced exon, which could retain the non-coding RNA in the nucleus. This process could play a significant role in controlling MYH7b expression as well as the activity of other cardiac genes. Consistent with the negligible level of full-length protein coding mRNA, no MYH7b protein expression was detected in adult mouse, rat, and human hearts by Western blot analysis. Furthermore, proteome surveys including quantitative mass spectrometry analyses revealed only traces of cardiac MYH7b protein and even then, only in a subset of individual samples.

CONCLUSIONS: The comprehensive analysis presented here suggests that previous studies showing cardiac MYH7b protein expression were likely attributable to antibody cross-reactivity. More importantly, our data predict that the MYH7b disease-associated variants may operate through the alternately spliced RNA itself.

Key Words: exon skipping ■ heart ■ mass spectrometry ■ MYH7b ■ RNA-seq

The mammalian heart maintains a controlled balance of 2 functionally distinct myosin motors, alpha-myosin heavy chain (α-MyHC) and beta-myosin heavy chain (β-MyHC), to regulate cardiac contractility. More recently, MYH7b (myosin heavy chain 7b), a third myosin isoform similar in sequence to both α-MyHC and β-MyHC, has been implicated in mammalian cardiac function and disease.[1–3] MYH7b is considered an ancient myosin as its genomic locus predates the emergence of both canonical cardiac myosins.[4] However, unlike the other sarcomeric myosin isoforms, MYH7b is post-transcriptionally regulated and has a unique expression pattern in mammals.[5,6] Although MYH7b RNA is present in mammalian heart and skeletal muscles, the vast majority of the transcripts are subject to an alternative splicing event that induces exon skipping. As a result, a premature termination codon is introduced by a frame-shift in the transcript's open reading frame, which blocks protein production and activates the nonsense-mediated mRNA decay pathway.[6] This process does not affect the maturation or expression of the intronic microRNA miR-499 that is part of the MYH7b primary transcript.[6] Our recent finding that forced expression of MYH7b protein in the mouse heart causes severe dilated cardiomyopathy corroborates the hypothesis that MYH7b synthesis has

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CLINICAL PERSPECTIVE

What Is New?

- Analysis of mammalian cardiac transcriptome and proteome data reveal that the vast majority of MYH7b (myosin heavy chain 7b) RNA is subjected to exon skipping and cannot encode MYH7b protein.
- Identification of retained introns flanking the skipped exon suggests that MYH7b noncoding RNA could operate as a novel regulator of cardiac function.

What Are the Clinical Implications?

- MYH7b disease-associated variants likely exert their effects through the noncoding RNA rather than affecting protein activity.

Nonstandard Abbreviations and Acronyms

HCM	hypertrophic cardiomyopathy
MyHC	myosin heavy chain

been silenced during evolutionary adaptation of the heart.[7] Nevertheless, MYH7b protein encoded by full-length mRNA is co-expressed with several other myosin isoforms in a subset of specialized muscles such as extraocular muscles, muscle spindles, and muscle fibers within the esophagus.[5,8] Despite these published data, a substantive misunderstanding surrounding the presence of MYH7b protein in mammalian hearts still persists. One report that erroneously concluded that MYH7b protein is present in the mouse heart has since been corrected after antibody cross-reactivity was demonstrated.[1] However, this incorrect observation continues to be cited, generating misinformation about the role/presence of MYH7b protein in the mammalian heart. More recently, a second study concluded that a cardiac phenotype observed in MYH7b-null rats was attributable to the loss of MYH7b protein.[3] Strikingly, neither of these papers assessed the protein coding ability of MYH7b RNA, but only relied on Western blot analysis. To clarify and further broaden our knowledge surrounding the cardiac biology of MYH7b, we have analyzed numerous mammalian RNA-sequencing (RNA-seq) and both qualitative and quantitative mass spectrometry databases. We report here that our deeper analysis substantiates efficient repression of MYH7b protein in the heart by exon skipping and suggests a potential biological role for the non-coding RNA. Moreover, based on the extremely low levels of MYH7b protein detected in mammalian hearts by high-sensitivity mass spectrometry, our study confirms the hypothesis that MYH7b protein cannot significantly influence mammalian heart structure and function.

METHODS

The data that support the findings of this study are available in public databases or are available from the corresponding author upon reasonable request. Institutional Review Board approval was therefore exempted for this study.

RNA-seq

The raw RNA-seq data from healthy left ventricular heart samples in GEO data set GSE141910 were retrieved via SRA and processed through the quality control and read mapping pipeline nf-core/rna-seq v1.4.2. Reads were mapped against hg38 and gene quantification used Gencode v34. We appended the exon-skipped version of MYH7b to Gencode v34 before quantifying gene counts. The same analysis was run for 6-week-old wild-type mouse left ventricles (n=6), adult rat ventricular myocytes (GEO accession number GSE95231), and mouse extraocular muscle (GEO accession number GSE100505) RNA-seq data, using Gencode v23 (mouse) and Rn6 (rat). We investigated intron retention by appending a transcript with both introns, intron 7 alone, or intron 8 alone. As the transcript file with either individual intron would also identify transcripts that contain both, we subtracted the percentage of transcripts containing both introns (29%) from those that contained each individual intron (40% intron 7 and 29% intron 8). Details are available at Github repository: https://github.com/libr8211/MYH7b_exon_skipping.

Western Blotting

Protein lysates were prepared by homogenizing in ice cold sample buffer containing 8 M urea, 2 M thiourea, 0.05 M Tris pH 6.8, 75 mmol/L DTT, 3% SDS, 0.05% bromophenol blue; 15 µg total protein was resolved by 8% PAGE and transferred to nitrocellulose membrane. All antibodies were diluted in TBST+5% non-fat dry milk. The antibodies used were against MYH7b (1:50; double affinity purified in-house), F59 (1:50; DSHB), and GAPDH (1:5000; CST 2118S).

RESULTS

MYH7b RNA Exon-Skipping Analysis

Recent advances in sequencing technology and public databases allowed us to expand our original reverse transcription-polymerase chain reaction analysis of MYH7b alternative splicing to large cardiac RNA-seq

data sets. To quantify the exon 8 skipping event (referred to as exon 7 in an earlier report[6]) that introduces a premature termination codon into the RNA and blocks protein production, we first analyzed an RNA-seq data set derived from 19 healthy human heart ventricles (GSE14 1910). Our analysis of these deep sequencing data showed minimal read coverage of the skipped exon 8 as well as a substantially higher signal at the beginning and end of the 2 introns flanking exon 8, introns 7 and 8, respectively (Figure 1A). The latter finding, which suggests that a proportion of MYH7b transcripts are subjected to intron retention, prompted us to expand our analysis of the MYH7b genomic region spanning exons 6 to 10. As shown in Figure 1B, the initial reads observed across the unspliced exon–intron junctions are maintained along the entire lengths of both introns 7 and 8, while no read accumulation is detected in the introns separating exons 6 and 7 and 9 and 10. Moreover, we found that ≈29% and 11% of transcripts retain either introns 7 and 8, or only intron 7, respectively. Interestingly, no transcripts retaining intron 8 alone were detected. Thus, transcripts containing the retained introns, which are subjected to slower removal kinetics than the constitutive introns, could be actively retained in the nucleus by associated spliceosomal components.

We next analyzed the human heart data set and data sets collected from adult rat ventricular myocytes and mouse left ventricles for the presence of the MYH7b RNA containing exon 8, whose inclusion restores the transcript's open reading frame. Full-length transcripts were either undetectable or observed at extremely low levels in all 3 data sets (Figure 1C). This analysis demonstrates that all but a few cardiac MYH7b RNAs lack full length protein-coding capacity. In contrast, RNA profiling of extraocular muscles, which express MYH7b protein in a small population of slow-tonic fibers[5] revealed a significant shift in the exon 8 skipping/inclusion ratio with ≈60% of the transcripts encoding the MYH7b protein (Figure 1C). It is worth noting that the small amount of the protein-coding mRNA detected in extraocular muscles is concentrated in a small number of muscle fibers. In contrast, the amount detected in the heart is shared by all of the cardiomyocytes[9,10] and becomes physiologically irrelevant at the single-cell level. Taken together, these results illustrate the principal role of alternative splicing in controlling MYH7b tissue-specific expression and suggest that MYH7b mutations associated with cardiomyopathy do so via the skipped transcripts, and not the MYH7b protein.

Probing Mass Spectrometry Data for the Presence of MYH7b Protein in the Mammalian Heart

We did not detect MYH7b protein in mammalian hearts by Western blot analysis using a highly specific antibody raised against an MYH7b N-terminal peptide sequence and further affinity purified (Figure 2A and 2B). We next surveyed published rodent and human heart mass spectrometry data sets. Since the sarcomeric myosin gene family is so highly conserved, only peptides that are unique to MYH7b were scored. In accordance with our RNA-seq analysis, only half of the 16 studies we queried detected any cardiac MYH7b-specific peptides; in the other half, only extremely low levels were identified in a subset of individual heart samples. For example, a study performed on the developing postnatal mouse heart detected MYH7b peptides in only 2 of 91 left ventricular samples examined. Furthermore, the intensity score for MYH7b in those 2 samples was ≈4000-fold lower than α-MyHC (MYH6), which is the major ventricular myosin in the postnatal mouse heart.[11] Correspondingly, a quantitative proteomics analysis performed in adult rat ventricular myocytes identified MYH7b levels that were >37 000-fold and 10 billion-fold less than α-MyHC and β-MyHC (MYH7), respectively.[12] Importantly, MYH7b was the lowest quantified sarcomeric myosin, even when compared with the skeletal muscle-"specific" MyHC-IIx (MYH1), MyHC-IIa (MYH2), and MyHC-IIb (MYH4) and the developmental myosins, MyHC-emb (MYH3) and MyHC-peri (MYH8), which are not thought to be expressed in the adult heart.[12] The result of quantitative mass spectrometry analysis obtained from 3 independent human studies again shows the extremely low level of cardiac MYH7b compared with the other sarcomeric myosins (Figure 3). In a multi-tissue proteomic analysis, peptides unique to MYH7b were found in 3 healthy human heart samples (dark purple columns); however, MYH7b peptides were also identified in the kidney and liver.[13] A second cardiac study quantified region and cell-type specific proteomes and detected low levels of MYH7b in the left ventricle estimated to be <1 nmol/L compared with >100 000 nmol/L for β-MyHC (blue columns).[14] Lastly, in a separate study of 7 adult human male hearts, MYH7b peptides were identified in the left ventricle; however, similar to the Kim et al 2014 study[13], they were 3000-fold less abundant than α-MyHC and β-MyHC, the 2 major cardiac myosins (teal green columns).[15] It is worth emphasizing that all 3 studies analyzed above identified MYH7b at lower levels than other sarcomeric myosins that are normally absent in adult heart, but are expressed only during development or in skeletal muscle (Figure 3). Moreover, the observed low sequence coverage for MYH7b (11.5%–21% versus 84%–94% and 81%–90%, for β-MyHC and α-MyHC, respectively) is reflective of a low confidence protein identification in the samples analyzed.[13–15] Additionally, mass spectrometry did not identify MYH7b in human fetal ventricular and atrial samples[16] or in hypertrophic cardiomyopathy, dilated cardiomyopathy, or ischemic cardiomyopathy patient

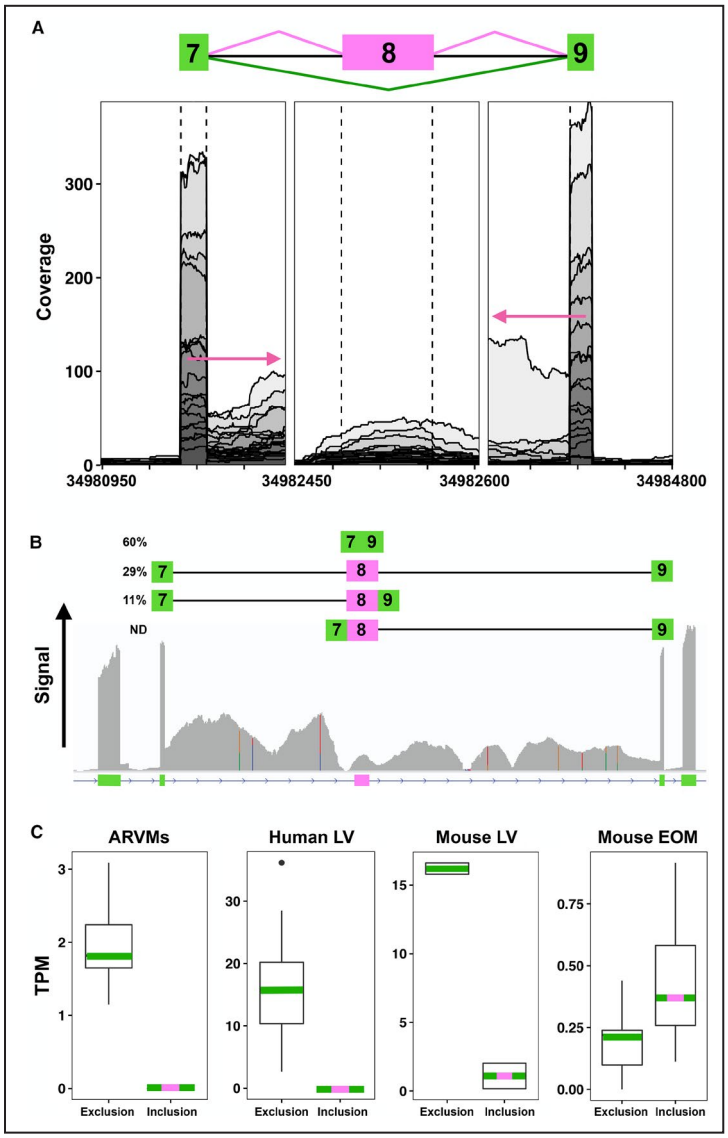

Figure 1. The majority of MYH7b RNA undergoes exon 8 skipping, which blocks MYH7b protein synthesis in the mammalian heart by introducing a premature termination codon.

A, Top: schematic representation of MYH7b (myosin heavy chain 7b) exon 8 cassette splicing (skipping/inclusion). The regulated exon originally reported as exon 7,[6] has been annotated as exon 8 in the databases analyzed in this study. Bottom: mapping RNA-seq reads to MYH7b exons 7, 8, and 9. Data sets of 19 healthy human left ventricles (each shade of gray corresponding to individual samples) were surveyed. Genomic coordinates are reported on the x-axis. The 2 magenta arrows indicate unspliced exon-intron junction reads. **B**, Sashimi plot that converts genomic reads into read densities showing the MYH7b gene region spanning exons 6 to10. Percentage of transcripts that skip exon 8 without retaining introns 7 and 8 (≈60%), retain either intron 7 and 8 (≈29%), or intron 7 only (≈11%). No transcripts retaining intron 8 alone were detected. Vertical lines in introns 8 and 9 correspond to mismatches/short reads. **C**, Quantification of the MYH7b transcripts, which either skipped or included exon 8, in adult rat ventricular myocytes, human and mouse left ventricle, and mouse extraocular muscles. Left green and right green-magenta-green medians inside each box correspond to transcripts that respectively skip or include exon 8. ARVM indicates adult rat ventricular myocytes; EOM, extraocular muscles; LV, left ventricle; ND, not detected; and TPM, transcripts per million.

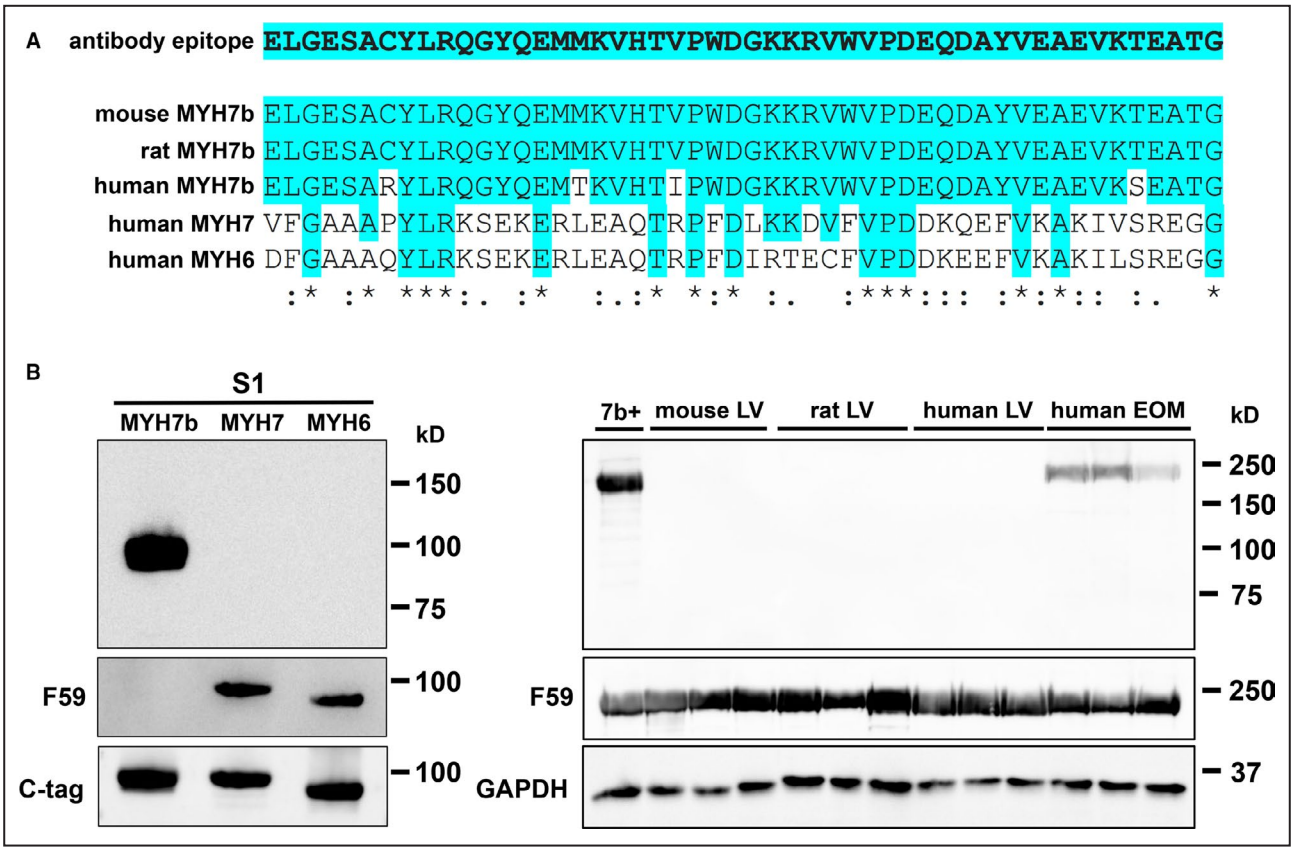

Figure 2. MYH7b is not detected in mammalian hearts by Western blot.
A, Amino acid sequence and comparative sequence analysis of the peptide used to generate the polyclonal antibodies against MYH7b (myosin heavy chain 7b) used in this study (mouse residues 6–56). **B**, Left panel: Western blot of purified human MYH7b, β-MyHC (beta-myosin heavy chain; MYH7), and α-MyHC (alpha-myosin heavy chain; MYH6) motor domains (myosin subfragment-1; S1). Right panel: Western blot analysis of homogenates collected from mouse, rat, and human left ventricle and human extraocular muscle; n=3 for each tissue type. Left ventricle extract prepared from a transgenic mouse forcibly expressing MYH7b in the heart was used as a positive control (7b+)[7]. The double-affinity purified antibodies detect MYH7b only in the positive control lanes containing the purified MYH7b S1, the MYH7b cardiac transgene, and the human extraocular muscles samples (top blots). The F59 antibody that does not recognize MYH7b, but it is known to react with both β- and α-MyHC, was used as a control for determining the presence of the myosin isoforms in the samples analyzed. GAPDH and ePDZ-His antibodies, the latter detecting the purified C-tagged myosin motors, were used as loading controls. EOM indicates extraocular muscles; and LV, left ventricle.

samples.[17] Finally, our analysis of mass spectrometry data agrees with a large human cardiac ribosomal profiling study reporting low MYH7b translation levels.[18]

DISCUSSION

Here we comprehensively profiled MYH7b post-transcriptional regulation and protein expression in the mammalian heart. Our RNA-seq analysis indicates that the vast majority of rodent and human cardiac MYH7b transcripts undergo exon 8 skipping. This alternative splicing event disrupts the mRNA open reading frame and triggers an early translational arrest. Conversely, we found that exon 8 inclusion was predominant in extraocular muscles that are known to express MYH7b protein.[5] Consistent with our RNA observations, we did not detect MYH7b in human, rat, and mouse hearts by Western blot. Finally, our analysis of several quantitative proteomic studies indicates that MYH7b is either not detected or detected at negligible levels in mammalian hearts, which are even lower than levels detected for non-cardiac myosin isoforms. Consistent with a lack of cardiac function for MYH7b is the finding that the tolerance of the MYH7b gene to non-synonymous amino acid changes is significantly higher (5- to 10-fold) than the cardiac myosin isoforms α-MyHC and β-MyHC (z=−0.34; z=0.86, z=3.93, respectively; gnomAD; https://gnomad.broadinstitute.org). This is a clear indication that MYH7b function is subjected to different evolutionary constraints. The absence of MYH7b protein in the heart is apparently at odds with several GWAS that have linked MYH7b variants to

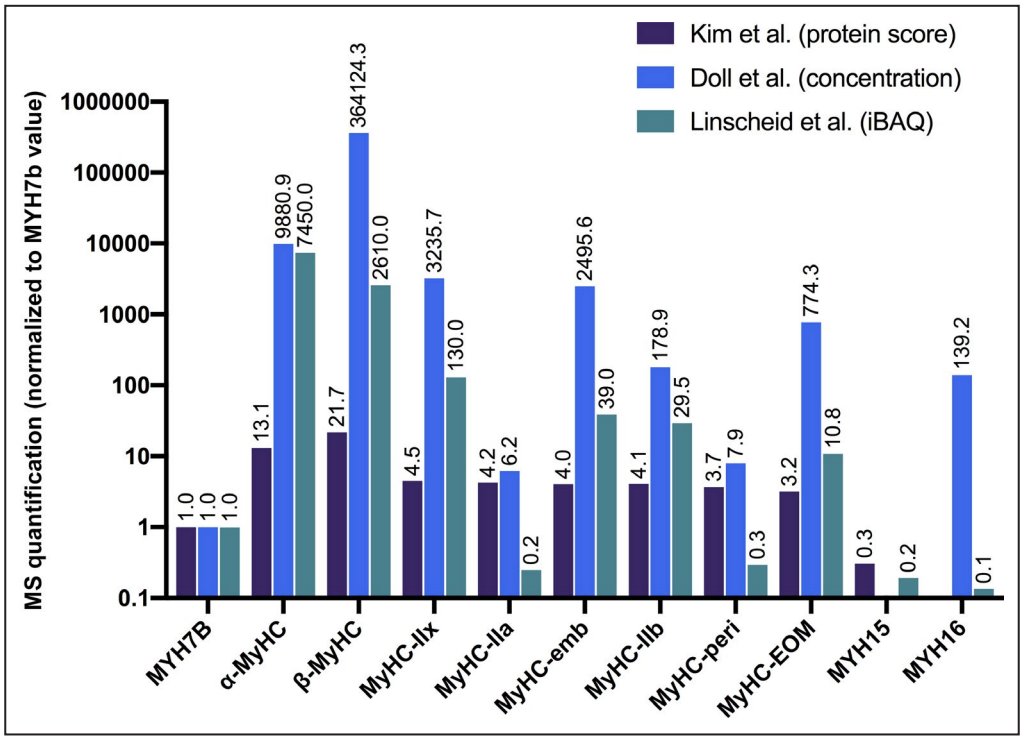

Figure 3. Mass spectrometry quantification of sarcomeric myosins detected in 3 human cardiac studies.

Protein score (adult heart[13]), concentration (adult left ventricle[14]), and intensity-based absolute quantification (adult heart[15]) were normalized to values for MYH7b (myosin heavy chain 7b) and then graphed on a log-scale y-axis. iBAQ indicates intensity-based absolute quantification. MS, mass spectrometry; MYH7b, myosin heavy chain 7b; α-MyHC, alpha-myosin heavy chain; β-MyHC, beta-myosin heavy chain; MyHC-IIx, myosin heavy chain-IIx; MyHC-IIa, myosin heavy chain-IIa; MyHC-emb, myosin heavy chain-embryonic; MyHC-IIb, myosin heavy chain-IIb; MyHC-peri, myosin heavy chain-perinatal; MyHC-EOM, myosin heavy chain-extraocular muscle; MYH15, myosin heavy chain 15, MYH16, myosin heavy chain 16.

cardiac diseases such as congenital left-sided heart lesions and QRS duration.[19] A search for MYH7b on Clinvar returns 4 likely pathogenic variants associated with familial hypertrophic cardiomyopathy,[20] and 8 patients with hypertrophic cardiomyopathy (1.46% of the cohort studied) carrying pathogenic or likely pathogenic variants in MYH7b were recently identified.[3] Finally, a digenic mutation of ITGA7 and MYH7b has been linked to left-ventricular non-compaction.[2] However, none of these exome-sequencing analyses assessed MYH7b protein expression and/or a survey of exon 8 non-productive alternative splicing. It is worth noting that the precise quantification of individual myosin isoforms, which share high sequence homology, presents some degree of uncertainty because of the presence of identical peptides obtained after digestion. Despite this limitation, it is evident that the amount of MYH7b detected by mass spectrometry is so small that it cannot influence heart function; a bipolar thick filament contains ≈300 myosin molecules, and the highest ratio of β-MyHC and α-MyHC over MYH7b found in human heart samples is ≈3000:1[15]; thus, only 1 MYH7b myosin molecule would be expected to be incorporated in 10 thick filaments. Hence, the trace quantity of cardiac MYH7b detected corresponds to the limited number of MYH7b transcripts that escape exon 8 skipping.

Pre-mRNA splicing requires accurate selection of sequences located at the 5' and 3' ends of introns (donor and acceptor sites, respectively) by the spliceosome, a multi-megadalton macromolecular ribonucleoprotein complex. Alternative splicing patterns are controlled by mRNA architecture and exon recognition, the latter driven by both splice sites intrinsic strength, which is measured as their homology to 5' and 3' splice site consensus sequences. To substantiate our findings and gain new insight into the cis-elements and mechanisms inducing exon 8 skipping, we inspected and compared the regions spanning human MYH7 (β-MyHC) and MYH7b exons 7 to 9. This analysis revealed a significant reduction of MYH7b exon 8 length from 93 to 24 nucleotides (the majority of human exons average ≈170 nucleotides, with optimal length between 50 and 250 nucleotides for efficient splicing[21]) as well as an increase in intron 7 length from 83 to 2136 nucleotides. While inclusion of small exons (<50 nt) often

requires the presence of specialized intronic splicing enhancers that bind dedicated trans-acting factors, the probability for an exon to be alternatively spliced is higher when the upstream intron is long.[22] Moreover, cumulative scoring of the 5' and 3' splice site information content of the human MYH7b versus MYH7 exons 7 to 9, used as best predictor for spliceosome exon recognition[23] and calculated by MaxEntScan,[24] reveals that the MYH7b exon 8 splice sites are much weaker than the homologous MYH7 sites (MaxEnt, 8.76 versus 17.61 respectively). Importantly, the same decrease in splice site strength is also present in those species that were previously shown to undergo exon 8 skipping.[6] In contrast, both MYH7b and MYH7 exons 7 and 9 splice site scoring/comparison shows a strong correlation (MaxEnt exon 7, 21.78 versus 22.37; MaxEnt exon 9, 17.83 versus 17.66). Collectively, these results indicate that the weak 5' and 3' splice sites of exon 8 impair efficient exon recognition with subsequent activation of the stronger and competing exon 7 and 9 5' and 3' splice sites, respectively. As a result, exon 8 is spliced out with its flanking introns. When MYH7b protein expression is required, for example in extraocular muscles, exon 8 inclusion could be triggered by different mechanisms that include activation of the weak splice sites by tissue-specific spicing factors or modulation of RNA polymerase II elongation rate.[25] Another alternative splicing event that controls MYH7b protein expression has been identified in chicken heart. In this context, CUG-BP, Elav-like family member 1 (CELF1) binding to the first intron of the MYH7b pre-mRNA promotes inclusion of an out-of-frame exon that induces an early arrest of protein translation.[26] It will be noteworthy in future studies to determine if CELF1 can also control exon 8 alternative splicing in mammals.

By further analyzing MYH7b RNA-seq data we also discovered a high read coverage of both introns bordering exon 8. This finding reveals that MYH7b transcripts can skip/include exon 8 but can also undergo non-productive intron 7 and/or intron 8 retention. Since the removal rate of the 2 introns is probably slow because of their 3' and 5' splice site intrinsic weakness, their retention could reflect sporadic failure of the spliceosome to join exons 7 and 9. Detained introns appear to have an important role in fine-tuning post-transcriptional regulation of many mRNAs by increasing the transcript half-life, promoting nuclear retention, and inducing nonsense-mediated decay resistance.[27] Investigating how this intron retention affects RNA trafficking, which could affect the mRNA's function in the cell should be considered in future studies of this locus. In light of the data presented in this study, which show that non-productive splicing silences cardiac MYH7b protein expression, we propose that the reported pathogenic MYH7b missense mutations do not alter protein function, but rather impact an unforeseen activity of the MYH7b RNA generated by non-productive splicing. The finding that MYH7b RNA is subjected to intron retention bolsters this hypothesis: nuclear localization of the MYH7b unspliced RNA isoform could, for instance, convert the non-coding RNA into a trans-acting modulator of cardiac gene expression.[28] Overall, by redirecting future studies on the proposed functional link between MYH7b non-coding RNA and cardiac gene regulation, our findings provide a basis for better understanding the functional role of MYH7b in health and disease.

ARTICLE INFORMATION

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Disclosures

None.

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