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Medina, Marisa W
Krauss, Ronald M

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Alternative splicing in regulation of cholesterol homeostasis

Marisa W. Medina and **Ronald M. Krauss**
Children's Hospital Oakland Research Institute

Abstract

Purpose of review—With the advent of whole-transcriptome sequencing, or RNA-seq, we now know that alternative splicing is a generalized phenomenon, with nearly all multi-exonic genes subject to alternative splicing. In this review we highlight recent studies examining alternative splicing as a modulator of cellular cholesterol homeostasis, and as an underlying mechanism of dyslipidemia.

Recent findings—A number of key genes involved in cholesterol metabolism are known to undergo functionally relevant alternative splicing. Recently, we have identified coordinated changes in alternative splicing in multiple genes in response to alteration in cellular sterol content. We and others have implicated several splicing factors as regulators of lipid metabolism. Furthermore, a number of cis-acting human gene variants that modulate alternative splicing have been implicated in a variety of human metabolic diseases.

Summary—Alternative splicing is of importance in various types of genetically influenced dyslipidemias, and in the regulation of cellular cholesterol metabolism.

Keywords

PTBP1; HMGCR; LDLR; statin; SFRS10

Introduction

Alternative splicing is the process by which a single gene produces more than one transcript, and is considered to be the principal mechanism of expanding diversity within the eukaryotic proteome. The most common forms of alternative splicing include exon skipping, alternative 5′ or 3′ splice-site selection, intron retention and mutually exclusive exon utilization [1]. Molecular analyses during the past two decades have demonstrated that alternative splicing influences binding properties, intracellular localization of both transcripts as well as the proteins they encode, enzymatic activity, protein stability and post-translational modifications [2].

As recently as 20 years ago, alternative splicing was thought to be a relatively rare phenomenon, with only 5% of human genes subject to alternative splicing [3]. However, with the development of new detection technologies, this value has been repeatedly revised upwards. Analyses based on expressed sequence tags (ESTs) increased estimates of alternative splicing to 40–60% [4, 5], with a later jump to 74% using exon-junction microarrays [6]. With the advent of whole transcriptome sequencing, or RNA-seq, we now know that nearly every multi-exonic gene undergoes some form of alternative splicing,

Author of correspondence: Marisa W. Medina, 5700 Martin Luther King Jr. Way, Oakland, CA 94609, 510-450-7977, mwmedina@chori.org.

demonstrating that this is a universal phenomenon [7, 8]. Furthermore, the large number of RNA-seq datasets on a wide variety of primary human tissues currently being generated through The Common Fund's Genotype-Tissue Expression (GTEx) program will likely reveal a plethora of yet unidentified tissue-specific and rare splice variants. With further improvements in sequencing technology coupled with decreasing costs, transcript quantification platforms that include information on both transcript levels as well as structure will replace the gene expression array as the standard technology.

While it has been known for several years that many key regulators of cholesterol metabolism undergo functionally relevant alternative splicing [9–12], the wider extent of this process has recently become evident. The objective of this paper is to provide an overview of the role of alternative splicing in cellular cholesterol homeostasis and plasma lipid metabolism.

The functional effects of alternative splicing

Alternative splicing events are often divided into one of two classes, 1) "functional" or "productive alternative splicing", by which transcripts with biological functions are created, and 2) splice variants with no known functional effect, called "unproductive" [13], "erroneous" [14], or "aberrant alternative splicing" [15]". Often these unproductive splicing events either encode defective proteins, or stimulate one of the many mechanisms that prevent mRNA translation, such as nonsense-mediated decay [16], non-stop decay [17] or no-go decay [18]. With 60% of the alternatively spliced transcripts characterized in the ENCODE pilot project lacking an annotated protein coding sequence, it is now clear that alternative splicing is not simply a mechanism for increasing protein diversity [19, 20].

There has been debate about whether these unproductive splicing events are derived from errors in the splicing machinery [15, 21], or represent targeted changes that influence cellular processes [22]. Proposed functions for these variants include roles in gene expression regulation or gene evolution [14]. Indeed, it has been shown through evolutionary conservation studies of the DNA polymerase β transcript that, regardless of whether the specific RNA transcripts are functional, alternative splicing yielding unproductive splice variants may have adaptive significance [13]. Furthermore, given evidence of widespread coupling of alternative spicing and nonsense-mediated decay in humans, regulated unproductive splicing may be an under appreciated means of regulating protein expression [23].

Alternative splicing as a regulatory mechanism

Although it has been suggested by some that unproductive alternative splicing is not regulated [15], recently we reported evidence that alternative splicing may be a generalized mechanism for regulating genes in the cholesterol biosynthesis and uptake pathways [10]. 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGCR*), the gene that encodes the rate limiting enzyme of the cholesterol biosynthesis pathway, undergoes alternative splicing of exon 13 to generate a second transcript called designated *HMGCR13(−)*. Although the open reading frame is maintained past the exon 12–14 splice junction, the resulting HMGCR protein cannot catalyze the conversion of HMG-CoA into mevalonate, and thus exon 13 skipping could be considered a form of "unproductive" alternative splicing. Using a variety of cellular models, we found that while sterol depletion up-regulated overall expression levels of both the canonical full-length *HMGCR* transcript, *HMGCR13(+)*, as well as *HMGCR13(−)*, the relative degree on induction of the *13(−)* variant was smaller than that of the *13(+)* variant, causing an overall reduction in the proportion of alternatively spliced transcripts. This effect was reversed by add-back of either LDL-C or 25-hydroxycholesterol. Similar sterol induced changes in the relative number of alternatively spliced transcripts

were observed in other genes encoding enzymes in the cholesterol biosynthetic pathway, including HMG-CoA synthase 1 (*HMGCS1*) and mevalonate kinase (*MVK*) as well as key regulators of cholesterol uptake, namely the low density lipoprotein receptor (*LDLR*) and proprotein convertase subtilisin/kexin type 9 (*PCSK9*). Similarly, cholesterol feeding of African Green monkeys increased the relative levels of alternatively spliced variants of *MVK, LDLR* and *PCSK9.* Furthermore, inter-individual variation in sterol-induced increases in expression of an *LDLR* splice variant lacking exon 12, *LDLR12(–),* was directly correlated with degree of change in hepatic cholesterol ester content. Since the splice variants studied were all either known or predicted to attenuate or abolish protein activity, reductions in alternative splicing under conditions of sterol depletion would be expected to increase activity of these two pathways, while increased alternative splicing observed under conditions of sterol excess would decrease both cholesterol synthesis and uptake. Thus, these changes were consistent with the expected directionality necessary to achieve cholesterol homeostasis, and suggested that cellular sterol levels may directly regulate alternative splicing.

These results strongly suggest that alternative splicing is a novel and generalized mechanism of regulating the cholesterol biosynthesis and uptake pathways, and that it functions in conjunction with SREBP-induced increases in transcription (Figure 1). Although it may be argued that sterol-induced changes in alternative splicing are quite modest in comparison to SREBP2-mediated regulation of transcription, since we observed changes in alternative splicing within 15 minutes of sterol depletion, whereas SREBP-mediated transcriptional regulation did not appear until 4–6 hours after sterol depletion, we have proposed that this may be a mechanism to rapidly modulate or fine tune the effects of SREBP-mediated transcriptional regulation in response to changes in intracellular sterol content.

Splicing factors implicated in cholesterol metabolism

The process of alternative splicing is regulated in part by interactions between RNA-binding proteins (also called splicing factors) and specific sequences contained within the pre-mRNA. There are two major classes of splicing factors, serine-arginine (SR) proteins and heteronuclear riboprotein (hnRNP) proteins. These two classes tend to work in opposition to one another with SR proteins generally promoting splicing by recruiting components of the spliceosome to the exon (leading to exon inclusion, or canonical splicing), while hnRNP proteins generally repress splicing by interfering with spliceosome binding (leading to complete or partial exon skipping, or alternative splicing) [1]. To date, only a few splicing factors have been shown to modulate splicing of genes involved in lipid metabolism: polypyrimidine tract binding protein 1 (PTBP1), Transformer-2alpha (TRA2A) and Transformer-2beta (TRA2B).

Polypyrimidine tract binding protein 1 (PTBP1 also known as hnRNP I) is a member of the hnRNP family. PTBP1 is found mainly in the nucleus, where it is thought to mediate repression of splicing of a number of pre-mRNAs; however, it is also found in the cytoplasm where it has been shown to bind both the 3′ and 5′ UTRs of mRNA to regulate mRNA localization, stability, and translational initiation [24]. While intracellular localization of PTBP1 has been shown to be influenced by glucose [25], recently we have also found that *PTBP1* transcript levels are regulated by sterols, with sterol depletion decreasing *PTBP1* transcript levels by almost 50%, an effect that is reversed with either LDLC or 25-hydroxycholesterol add-back [10]. We have shown that PTBP1 modulates alternative splicing of several genes involved in cholesterol biosynthesis and uptake including *LDLR, MVK, HMGCS1* and *PSCK9* [10]. PTBP1 knock-down in HepG2 cells reduced the relative levels of alternatively spliced variants of *LDLR, MVK, HMGCS1* and *PSCK9* but did not appear to regulate *HMGCR* exon 13 alternative splicing. Notably, PTBP1 knockdown either

attenuated or ameliorated sterol-induced changes in alternative splicing of *HMGCS1*, *MVK*, *LDLR* and *PCSK9*, consistent with the likelihood that these changes are mediated at least in part by sterol regulation of PTBP1.

PTBP1 has also been implicated in the production on both omega-3 and omega-6 polyunsaturated fatty acids. Notably, Reardon *et al* reported that PTBP1 regulates alternative splicing of both fatty acid desaturase 2 (*FADS2*), the gene that encodes the first and rate limiting enzyme in the biosynthesis of long chain polyunsaturated fatty acids, as well as fatty acid desaturase (*FADS3*). In addition, they found that PTBP1 knock-down reduced both omega-3 and omega-6 fatty acids in HepG2 cells, but did not affect either monounsaturated or saturated fatty acids. Since the most dramatic effects (50% reduction) were observed with reduction in eicosapentaenoic acid, PTBP1 knock-down modulated the ratio of omega-3 to omega-6 fatty acids [26].

Transformer-2 alpha and beta (TRA2A and TRA2B) belong to the SR-like protein family of splicing factors, and are the human homologs of the Drosophila transformer -2 (TRA-2) protein, one of the most well studied splicing regulators in Drosophila [27]. Both TRA2A and TRA2B have been implicated in human cholesterol metabolism. TRA2A has been shown to target the scavenger receptor class B, member 1 (*SCARB1*) gene, which, through alternative splicing of exon 12, encodes both the scavenger receptor class B type I and II (SR-BI and SR-BII) proteins [12, 28]. Although both SR-BI and SR-II bind high density lipoprotein (HDL) particles and serve as the major receptor mediating reverse cholesterol transport from HDL to the liver, the two isoforms differ in their intracellular localization and efficiency in promoting cholesterol efflux. *SCARB1* alternative splicing is regulated by estrogen, and similar to what we observed with sterol regulation of PTBP1, estrogen-induced changes in *SCARB1* alternative splicing appear to be mediated by estrogen regulation of TRA2A [12].

Pihlajamaki *et al* reported that genes involved in RNA splicing were down-regulated in liver and muscle of insulin-resistant obese humans, as well as a mouse model of diet-induced obesity [29]. Knock-down of one of these genes, *TRA2B* (also known as *SFRS10*) in HepG2 cells was shown to increase expression levels of lipogenic genes such as *SREBP1c, FASN, ACC1* and *DGAT2*, leading to a 60% increase in lipogenesis. These effects were confirmed *in vivo*, as heterozygous *Sfrs10*$^{+/-}$ mice had increased expression of lipogenic and triacylglycerol (TAG) synthesis genes, leading to elevated hepatic VLDL secretion as well as a 3-fold increase in plasma VLDL. This effect appeared to be attributed in part to altered expression of lipin 1 (*LPIN1*), a key regulator of lipoprotein metabolism. Using mini-gene constructs as well as knockdown in HepG2 cells, *SFRS10* was shown to modulate the relative expression levels of the *LPIN1* α versus β splice variants, with no effect on overall *LPIN1* levels. Notably, SFRS10-induced changes in expression of lipogenic genes, as well as measures of cellular lipogenesis, TAG and lysophosphatidic acid, were reversed with *LPIN1*β knock-down. Very recently, the physiological relevance of this finding has been called into question as Brosch *et al* were unable to replicate the reduced *SFRS10* expression levels in an independent cohort of lean versus obese human subjects [30]. However, given the complexities of accurately quantifying *SFRS10* due to the expression of numerous splice variants, and the fact that different quantitation methods were employed by the two groups [31], further studies are required to determine the true relationship between *SFRS10* and human metabolic disease.

Alternative splicing and human genetic variation in cholesterol metabolism and statin response

Originally, the relationships between alternative splicing and cholesterol metabolism were identified through human genetics. For example, a number of studies demonstrated that a SNP within *HMGCR* intron 13, rs3846662, or other SNPs in tight linkage disequilibrium, are associated with variation in endogenous levels of plasma cholesterol [32, 33]. Associations between rs3846662 and statin response have also been reported in populations treated with a number of statins including simvastatin, pravastatin, rosuvastatin and atorvastatin, indicating a relationship with statin efficacy as a class effect versus a specific statin drug [34–37]. These relationships have been attributed to alternative splicing of *HMGCR* since rs3846662 was shown to regulate exon 13 skipping [33,38], and inter-individual variation in the magnitude of statin-induced change in exon 13 skipping was directly correlated with variation in *in vivo* plasma LDL-C reduction with statin treatment [38]. Furthermore, loss of exon 13 abolishes the catalytic activity of the resulting HMGCR isoform [33, 39], while enrichment of the *HMGCR13(−)* splice variant reduces statin sensitivity of the HMGCR enzyme [38, 40].

There are numerous examples in the literature of other variants (both common and rare) in *cis*-elements causing aberrant splicing in a number of cholesterol related diseases. The most well-known example is familial hypercholesterolemia, in which many of the mutations identified within the low density lipoprotein receptor (*LDLR*) have been shown to cause skipping of various exons in the transcript, thus attenuating or abolishing LDLR activity [9, 11, 41]. Very recently, mutation analysis found that the molecular basis for a form of primary hereditary hypertriglyceridemia was a single base mutation in the glycerol-3-phosphate dehydrogenase 1 (*GPD1*) gene causing irregular splicing and generation of a truncated pathogenic GPD1 isoform [42]. Similarly, cases of hyperalphalipoproteinemia have been attributed to SNPs causing aberrant splicing of cholesterol ester transfer protein (*CETP*) in multiple independent populations [43, 44]. Splicing mutations in microsomal triglyceride transfer protein (*MTTP*) have been identified in cases of abetalipoproteinemia [45, 46], while splicing mutations in 7-dehydrocholesterol reductase (*DHCR7*) were found in individuals with Smith-Lemli-Optiz syndrome [47].

Remaining questions and challenges

Although we and others have started to define the relationship between alternative splicing and its effects on cellular cholesterol metabolism as well as its role in human lipid disorders, there are still a number of questions remaining. For example, while sterol-induced changes in alternative splicing are mediated by sterol regulated splicing factors; the molecular mechanism for how sterols regulate expression and activity of these splicing factors remains unknown. In addition, since functional studies are usually limited to testing for effects of alternative splicing on a specific catalytic activity or known function of the canonical protein isoform, it is possible that expression of alternatively spliced variants may affect cellular processes beyond cholesterol metabolism. For example, mevalonate kinase, the enzyme immediately following HMGCR in the cholesterol biosynthesis pathway, has also been shown to act as a mRNA binding protein whereby it specifically binds the luteinizing hormone receptor (*LHR*) mRNA to suppresses its translation [48]. While splice variants that are not translated are often assumed to be non-functional, both the prevalence of expression of non-coding transcripts as well as recent findings for specific regulatory roles of non-coding transcripts challenge this assumption [49].

Conclusion

With recent advances in sequencing technology, we now know that alternative splicing is a universal phenomenon that impacts the whole transcriptome, and thus potentially every cellular process. Numerous key regulatory genes involved in cholesterol metabolism are known to undergo functionally relevant changes in alternative splicing, and these changes appear to be part of an orchestrated mechanism of increasing or decreasing activity of the cholesterol biosynthesis and uptake pathways in conjunction with SREBP-mediated transcriptional regulation in response to changes in intracellular sterol content. A number of splicing factors have been shown to not only modulate this regulation, but also to directly affect plasma lipid metabolism suggesting the possibility that there may be both cis-acting and trans-acting gene variants underlying various dyslipidemias. Overall these studies validate the physiological relevance of alternative splicing as a mechanism that influences cholesterol homeostasis, and highlight the likelihood that our current understanding represents only a glimpse of the extent of the role of alternative splicing in lipid metabolism.

Acknowledgments

Abbreviations

PTBP1	polypyrimidine tract binding protein 1
TRA2A	Transformer-2alpha
TRA2B	Transformer-2beta
SFRS10	arginine/serine rich 10
ESTs	expressed sequence tags
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HMGCR13(−)	HMGCR transcript lacking exon 13
HMGCR13(+)	HMGCR transcript with exon 13
LDL	low density lipoprotein
HMGCS1	3-hydroxy-3-methylglutaryl coenzyme A synthase 1
MVK	mevalonate kinase
LDLR	low density lipoprotein receptor
PCSK9	proprotein convertase subtilisin/kexin type 9
LDLR12(−)	LDLR splice variant lacking exon 12
SREBP	sterol response element binding protein
hnRNP	heterogenous nuclear ribonuclear protein
SR	serine-arginine
FADS2	fatty acid desaturase 2
FADS3	fatty acid desaturase 3
UTR	untranslated region
FASN	fatty acid synthase

ACC1	acetyl-CoA carboxylase 1
DGAT2	diacylglycerol O-acyltransferase 2
LPIN1	lipin 1
SNP	single nucleotide polymorphism
GPD1	glycerol-3-phosphate dehydrogenase 1
CETP	cholesterol ester transfer protein
MTTP	microsomal triglyceride transfer protein
DHCR7	7-dehydrocholesterol reductase

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Key points

- Alternative splicing is a generalized phenomenon.

- Expression of splice variants, whether they are translated into active proteins or not, can impact cholesterol metabolism.

- Alternative splicing is a coordinated and generalized mechanism of regulating genes in the cholesterol biosynthesis and uptake pathways.

- Splicing factors can modulate cholesterol metabolism by altering expression of splice variants.

- One of the major challenges to the field is functionalizing the impact alternative spliced transcripts.
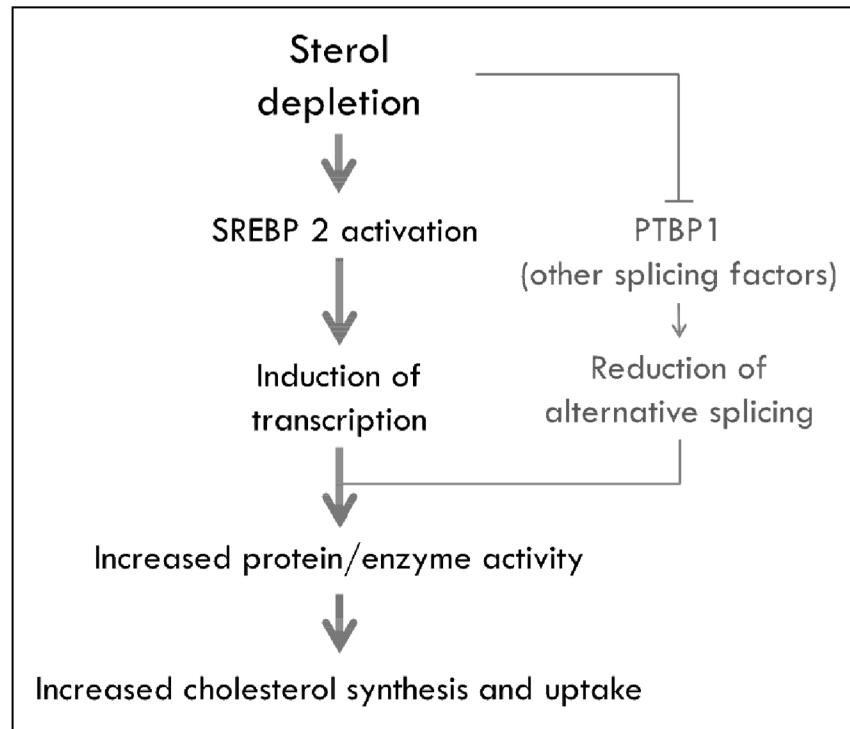
Figure 1.
Proposed model of alternative splicing as a regulatory mechanism of cellular cholesterol homeostasis.