

Splicing therapeutics in SMN2 and APOB

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Splicing therapeutics are defined as the deliberate modification of RNA splicing to achieve therapeutic goals. Various techniques for splicing therapeutics have been described, and most of these involve the use of antisense oligonucleotide-based compounds that target key elements in the pre-mRNA to control splicing in the nucleus. In this review, recent developments in splicing therapeutics for the treatment of two specific diseases are described: correcting the alternative splicing of survival of motor neuron (SMN)2 pre-mRNA to compensate for the defective SMN1 gene in spinal muscular atrophy, and re-engineering the splicing of apolipoprotein B pre-mRNA to lower circulating cholesterol levels.

Keywords Antisense oligonucleotide, apolipoprotein B, cholesterol, RNA splicing, SMN2, spinal muscular atrophy

Introduction

During the process of pre-mRNA splicing the spliceosome, a dynamic complex of five small nuclear ribonucleoprotein (snRNP) particles and various proteins remove introns from the transcribed pre-mRNA and join the exons to produce a translatable mRNA [1]. Exon-intron junctions are defined by degenerate *cis*-elements: the 5' splice site, the 3' splice site and the branch-point sequence (BPS). The degenerate *cis*-elements are necessary but not sufficient for accurate splicing. Other auxiliary sequences and their cognate factors influence the selection of splice sites. 'Splicing enhancers' are sequences within the pre-mRNA that promote the use of particular splice sites. Splicing enhancers are present in exons or introns, and are recognized by activator proteins, such as serine/arginine (SR)-rich proteins [2,3], which promote spliceosomal assembly on adjacent splice sites [4]. Conversely, 'splicing silencers' are sequences that inhibit the use of particular splice sites. Elements of the splicing silencer class are recognized by various repressors, such as polypyrimidine tract binding protein (PTB) [5] and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) [6].

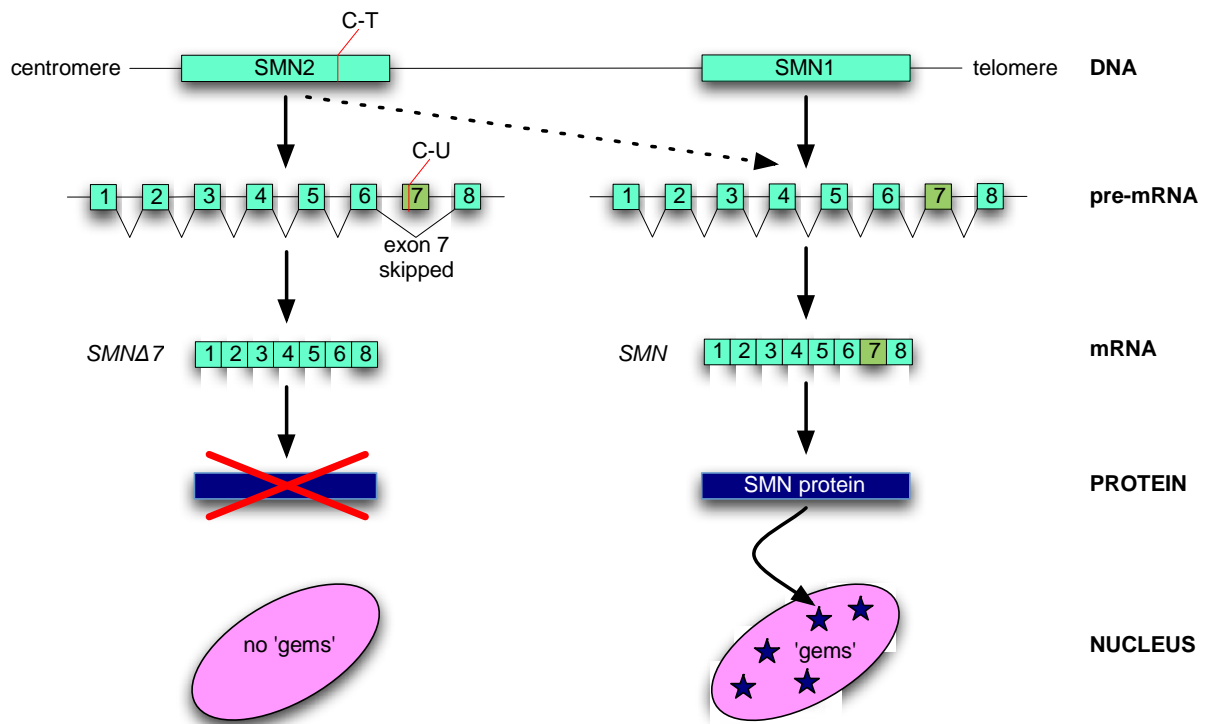
In the last 15 years, increasing interest has centered on the deliberate modification of RNA splicing for therapeutic purposes. The impetus for this interest arises from the observation that various mutations that cause human genetic diseases affect splicing, with approximately 10% of mutations affecting the RNA splice sites *per se* [7]. This review describes two therapeutic target pre-mRNAs – survival of motor neuron (SMN)2 and apolipoprotein B (APOB) – to highlight the developments in splicing therapeutics in 2007 and 2008.

SMN2: Stimulating inclusion of exon 7

Spinal muscular atrophy (SMA) is a degenerative disease of the motor neurons of the spinal cord anterior horn and lower brainstem [8]. SMA presents in childhood with muscle weakness and occasionally fatal respiratory failure, and is caused by mutations of the *SMN* locus. In humans, the *SMN* locus is part of an inverted chromosomal-segment duplication that comprises the *SMN1* gene and its paralog *SMN2*. The *SMN* protein is ubiquitously expressed, and the majority (60 to 90%) of the *SMN* protein is translated from *SMN1* mRNA. Point mutations or deletions in *SMN1* have been observed in patients with SMA [9]. The *SMN* protein is localized in subnuclear complexes known as Gemini or coiled bodies or 'gems', which are involved in snRNP assembly and recycling; these gems are absent or greatly reduced in the cells of patients with SMA [10].

There is only a partial deficiency of the *SMN* protein in SMA, as the mutated *SMN1* is partially complemented by the wild-type copy of *SMN2*. The copy number of *SMN2* is variable because of *SMN2* gene duplication or conversion. The higher the copy numbers of *SMN2*, the milder the SMA phenotype [11]. The *SMN2* gene differs from *SMN1* by five nucleotide changes, two of which occur in the exons and three of which occur in the introns [12]. One of these nucleotide changes, a translationally silent cytosine to thymine transition at position 6 in exon 7, causes skipping of exon 7 in the majority of transcripts from *SMN2* and give rise to the *SMN Δ 7* RNA isoform. The alternative splicing of *SMN2* exon 7 is caused by the disruption of an exonic splicing enhancer (ESE) sequence that is recognized by the SR protein SF2 (splicing factor 2)/ASF (alternative SF) [13] and/or by the creation of an exonic splicing silencer (ESS) recognized by hnRNP A1 and A2 [14]. Exon 7 is the last coding exon in *SMN1*, and the resulting *SMN Δ 7* protein isoform lacks the C-terminal 16 amino acid residues of the *SMN* protein, which are replaced by 4 amino acid residues derived from exon 8 [15]. The *SMN Δ 7* protein is unable to self-associate, is degraded more quickly, and is unable to effectively replace the function of the full-length *SMN* protein [16-18]. Approximately 20% of *SMN2* mRNA transcripts are spliced to include exon 7 and are translated to the full-length, functional *SMN* protein; the higher the level of *SMN* protein, the milder the SMA phenotype (Figure 1) [19,20].

Figure 1. The genomic structure of the *SMN* locus.



The survival of motor neuron (*SMN*)1 gene (located at the telomeric end of the chromosome) is transcribed and spliced normally to generate the SMN mRNA. The SMN mRNA is translated into the SMN protein, which is assembled into intranuclear Gemini of coiled bodies ('**gems**'). The effect of the cytosine (C) to thymine (T) transition (C to uridine [U] in the pre-mRNA) in the SMN2 gene (located at the centromeric end of the chromosome) on SMN2 exon 7 splicing and SMN protein expression is shown. A minority of the SMN2 pre-mRNA is spliced to include exon 7 and generate the wild-type SMN mRNA (indicated by the dashed arrow). In the majority of the SMN2 pre-mRNA exon 7 is spliced out generating the SMNΔ7 mRNA. The SMNΔ7 mRNA is translated to a shortened form of the SMN protein that is unstable and is unable to self-associate and assemble into intranuclear gems.

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The potential for therapy in SMA is based on correcting the aberrant splicing of the SMN2 gene, and relies on increasing the inclusion of exon 7 in the SMN2 pre-mRNA. The correction of the aberrant splicing of SMN2 is in contrast to the treatment of dystrophin-related diseases, in which exon skipping is desired [21]. Progress has been made in identifying small molecules that increase SMN2 exon 7 inclusion in the mRNA and expression of the SMN protein, including treatment with various histone deacetylase inhibitors. Examples of these and other molecules include sodium butyrate [22], phenylbutyrate [23], hydroxyurea [24], sodium valproate [25], salbutamol [26], plant-derived polyphenols (such as resveratrol and curcumin [27]), and a quinazoline compound series and an indole compound identified by HTS [28]. The anthracycline aclarubicin is also effective in increasing SMN2 exon 7 inclusion and SMN protein expression, although the mode of action of this agent is unclear [29]. Aminoglycoside compounds that promote read-through of premature termination codons also increase SMN protein expression levels in fibroblasts derived from patients with SMA [15]. The NSAID drug indoprofen increases levels of SMN protein expressed from the SMN2 gene; this agent does not have an effect on exon 7 splicing, but may instead act co-translationally to increase expression of the wild-type SMN protein [30]. Although these small molecules are promising as modulators of exon 7 splicing, they are likely to be pleiotropic in their effects. To minimize off-target effects, methods that correct the defective splicing of SMN2 exon 7 with improved specificity have been explored. The mechanics of SMN2 exon 7 alternative splicing and

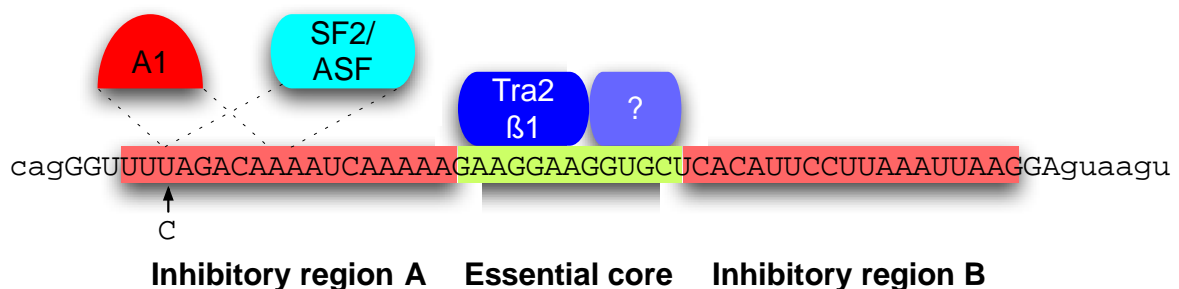
the potential splicing therapies under development in 2007 and 2008 that specifically correct SMN2 exon 7 splicing to compensate for the loss of SMN1 are described.

(sub) Enhancement of exon 7 inclusion by antisense oligonucleotides

Following on from the observation that the ESE in SMN1 exon 7 is disrupted in SMN2 [13], a synthetic SR protein analog to foster exon 27 inclusion was designed. This comprised a chimeric molecule with a peptide-nucleic acid (PNA; a peptide-like backbone of nucleobases capable of hybridizing with RNA [31]) covalently joined to a peptide. The constructed hybrid comprised a PNA antisense oligonucleotide (ASO) moiety, which targeted nucleotide positions 7 to 18 in SMN2 exon 7, coupled to 10 serine-arginine dipeptide repeats (the RS10 domain), to form an oligopeptide that can substitute for the natural serine-arginine rich domain of SF2/ASF. This compound, SMN-RS10, was effective at inducing SMN2 exon 7 inclusion in the mRNA transcript *in vitro* [32]. An important observation was that the PNA ASO moiety alone was partially effective in augmenting the inclusion of exon 7 in SMN2 mRNA, although the effect was strongly enhanced when the RS10 domain was attached to the ASO. The enhancement of exon 7 inclusion in SMN2 mRNA by the addition of the RS10 domain to the ASO suggests that either SMN-RS10 displaces a repressor protein from an ESS or that the formation of a secondary structure unfavorable to spliceosomal assembly is inhibited.

To investigate the mechanism of action of SMN-RS10 and optimize the placement of the ASO target (ie, to identify the SMN1 nucleotide sequence that when targeted by the ASO leads to the most improvement in exon 7 inclusion), a systematic ASO 'walk' along the entire length of SMN2 exon 7 using 2'-O-methoxyethyl (MOE) ASOs was undertaken [33]. The effects of the MOE ASOs on SMN2 splicing were evaluated with *in vitro* splicing assays and verified by transfection of HEK293 cells. MOE ASOs, which have a charged backbone, were transfected more efficiently than PNA ASOs. Two regions that were inhibitory to exon 7 inclusion in the SMN2 mRNA transcript were identified: one region extends from the nucleotide positions 4 to 22 (region A) and the other from 34 to 51 (region B), sandwiching a central core region from position 22 to 33 containing a 5' motif that binds the splicing activator protein transformer 2 β 1 (Tra2 β 1) [34], as well as another less well-characterized 3' ESE (Figure 2). Individual MOE ASOs targeting the nucleotide positions 7 to 21 and 34 to 48 were able to increase SMN2 exon 7 inclusion in the mRNA transcript to similar levels observed in the SMN1 mRNA. These MOE ASOs also increased the number of gems in fibroblasts derived from a patient with SMA suggesting a physiologically significant increase in the amount of functional SMN protein.

Figure 2. Regions in the SMN2 pre-mRNA that inhibit the inclusion of exon 7.



The exon 7 sequence of the survival of motor neuron (SMN)2 gene is shown in capital letters, with flanking intronic sequences shown in lower case letters. The position of the cytosine (C) to uracil (U) transition in exon 7 is indicated by the arrow. Inhibitory regions A and B that inhibit the inclusion of exon 7 in the SMN2 mRNA are shown in pink, and the central core region, which appears to be essential for exon 7 inclusion, is shown in green. The splicing factors **A1** (heterogeneous nuclear ribonucleoprotein A1) [14], **SF2/ASF** (splicing factor 2/alternative splicing factor) [13], **Tra2 β 1** (transformer 2 β 1) [34], and an unknown splicing activator (?), which are thought to bind to exon 7, are shown.

Androphy and coworkers characterized an inhibitory stem-loop structure that incorporates the 3' end of SMN2 exon 7, including two intronic residues of the 5' splice site [35]. This stem-loop structure was postulated to sequester the weak 5' splice site of exon 7 and prevent its recognition by the spliceosomal component U1 snRNP. In this regard, it is interesting that ASO 34 to 48 is complementary to the 5' strand of the stem-loop structure, and may foster exon 7 inclusion by inhibiting the formation of the stem-loop. Similarly, MOE ASO 7 to 21 and the PNA ASO 7 to 18 may encourage the inclusion of exon 7 by inhibiting the binding of splicing repressor proteins, such as hnRNP A/B proteins; these repressor proteins are thought to bind an ESS motif at positions 6 to 11 [14], or to spread from other regions when the SF2/ASF ESE motif is absent, such as in SMN2 [36].

Weakening the 3' splice site of exon 8 of the SMN2 gene (e.g., by targeting this site with an ASO) favors exon 7 inclusion, perhaps by a competitive mechanism [37]. Dickson *et al* increased full-length SMN protein expression as well as restoration of gems in transfected fibroblasts from patients with SMA with bifunctional 2'-O-methyl RNA oligonucleotides, which incorporated an antisense moiety that bound to the 3' splice site of exon 8 with an hnRNP A1-binding motif [38].

Intronic inhibitory elements within the SMN2 pre-mRNA repress the inclusion of exon 7 [39]. Targeting intronic inhibitory elements instead of exonic elements is an attractive strategy, because this is less likely to interfere with subsequent steps in mRNA processing, such as mRNA transport or translation. One such element, intronic splicing silencer element (ISS)-N1, is present within intron 7 of the SMN2 gene and downstream of the 5' splice site of exon 7. Singh and colleagues demonstrated that binding an ASO to the ISS-N1 element restored SMN2 exon 7 inclusion to the levels observed in SMN1 mRNA [39]. The mapping of the SMN2 ISS-N1 silencer motif has been refined by conducting a high-resolution ASO walk in intron 7 [40]. This silencer element binds strongly and specifically to hnRNP A1/A2, and binding of hnRNP A1/A2 appears to underlie the silencing activity of the ISS-N1 element [40]. The ASOs that were most effective at increasing SMN2 exon 7 inclusion in the mRNA transcript in HEK293 cells were infused into transgenic mice harboring the human SMN2 gene [40]. These ASOs increased SMN2 exon 7 inclusion in the SMN2 mRNA in transgenic mice, and the most striking effect was observed in the liver. The inclusion of exon 7 in SMN2 mRNA was increased from a baseline of 21 to 91% over a 4-week ASO-treatment period. Significant increases of 2 to 3-fold in exon 7 inclusion in the SMN2 mRNA were also observed in kidney and muscle. Because these ASOs do not cross the blood-brain barrier, no increase in exon 7 inclusion was observed in the spinal cord [40].

(sub) Enhancement of exon 7 inclusion by bifunctional tailed ASOs

Bifunctional antisense oligonucleotides were generated by Skordis *et al*, in which a 5' phosphorothioate RNA tail containing ESE motifs that bind endogenous SR proteins, or other splicing activator proteins, were combined at the 3' end of the oligonucleotide with a 2'-O-methyl phosphorothioate ASO moiety targeting positions 2 to 16 of SMN2 exon 7 [41]. This strategy increased exon 7 inclusion in the SMN2 mRNA from 57% to 84%, both *in vitro* and *in vivo* in fibroblasts isolated from patients with SMA, and partially restored the appearance of gems in the nuclei of these fibroblasts [41]. Subsequently, similar but unmodified bifunctional RNA ASOs expressed in recombinant adeno-associated virus (rAAV) vectors increased exon 7 inclusion in the SMN2 mRNA, partially restored the appearance of gems and increased SMN protein levels [42]. In 2004, Schümperli and Pillai utilized the same bifunctional oligonucleotide design plus an additional feature wherein the oligonucleotides were coupled to a modified U7 small nuclear RNA (U7 Sm OPT, reviewed in reference [43]) at the 3' end to target the bifunctional oligonucleotide to the spliceosome and increase its efficacy in modifying splicing. Interestingly, targeting the ASO moiety to position 2 to 19 of the SMN2 pre-mRNA (ASO 02-19-U7 Sm OPT, approximately corresponding to inhibitory region A) was only partially effective and induced exon 7 inclusion in SMN2 mRNA only to a maximum of 41% from a baseline value of 10%. Furthermore, by removing the ESE tail, ASO 02-19-

U7 Sm OPT had the reverse effect and induced exon 7 skipping in both SMN1 and SMN2 pre-mRNA. The increased exon skipping was attributed to steric hindrance of the 3' splice site in both SMN1 and SMN2 by the U7 Sm OPT stem-loop. Targeting positions 31 to 48 (ASO 31-48-U7 Sm OPT, approximately corresponding to inhibitory region B) was more successful, with the ESE-ASO 31-48-U7 Sm OPT construct able to boost exon 7 inclusion in SMN2 mRNA to 89% [44].

A series of experiments evaluated the ability of various ESE sequences within the 5' phosphorothioate RNA tail to increase exon 7 inclusion in the SMN2 mRNA. The first series of experiments by Skordis *et al* utilized a purine-rich ESE sequence (GGAGGAC) to recruit SF2/ASF [41]. A bifunctional ASO incorporating a tail with a different ESE sequence (TGCCTC) optimized for the binding of the SRp55 SR protein, achieved only partial efficacy, with an increase to 42% of exon 7 inclusion [45]. When a second ESE sequence optimized for SF2/ASF binding (CACACGA) was used, the construct had the reverse effect and caused exon 7 skipping. Similarly, ESE tails optimized for binding of the splicing enhancer proteins SRp40, SC35 and TIA-1 caused exon 7 skipping in this context [45]. This firstly demonstrates that not all splicing enhancer proteins are effective in causing exon 7 inclusion and some might even be counterproductive in causing skipping. Secondly, it is likely that the original purine-rich ESE sequence (GGAGGAC) did not recruit SF2/ASF, but actually recruited Tra2 β 1, a splicing activator protein, which naturally binds to a similar purine-rich ESE in the middle of exon 7 (Figure 2) [34].

Infection of immortalized fibroblasts from a patient with SMA with bifunctional U7 cassettes coupled to lentiviral vectors, increased SMN2 mRNA exon 7 inclusion from 54% at baseline to 75% using the ESE-ASO 02-19-U7 Sm OPT and to 97% using the ESE-ASO 31-48-U7 Sm OPT [44]. SMN protein levels were increased from the control levels of approximately 17% to approximately 50% for both constructs, and intranuclear gem numbers were also significantly increased from 17 gems/100 nuclei to 49 gems/100 nuclei [44].

(sub) *Trans*-splicing as a means of replacing exon 7

Lorson and colleagues developed a *trans*-splicing strategy to replace exon 7 in SMN2 mRNA. This approach relied on the expression of a therapeutic *trans*-splicing RNA (tsRNA – reviewed by Garcia-Blanco [46]), which was engineered to base-pair to intron 6 of SMN2 [47]. The tsRNA also contained an optimized BPS and a 3' splice site, the SMN1 exon 7 sequence and a hemagglutinin tag to allow for easy monitoring of *trans*-splicing. Splicing occurred *in trans* between the SMN2 pre-mRNA and the tsRNA, such that the SMN1 exon 7 sequence was spliced into the SMN2 mRNA. In their proof-of-principle study, *trans*-splicing was demonstrated between the tsRNA and the native SMN2 pre-mRNA in HeLa cells. Furthermore, by placing the tsRNA within an rAAV vector and infecting fibroblasts from patients with SMA, *trans*-splicing was observed between the native SMN2 pre-mRNA and the tsRNA, and intranuclear gems and snRNP assembly in nuclear extracts from these cells (measured using an *in vitro* assay) were restored [47].

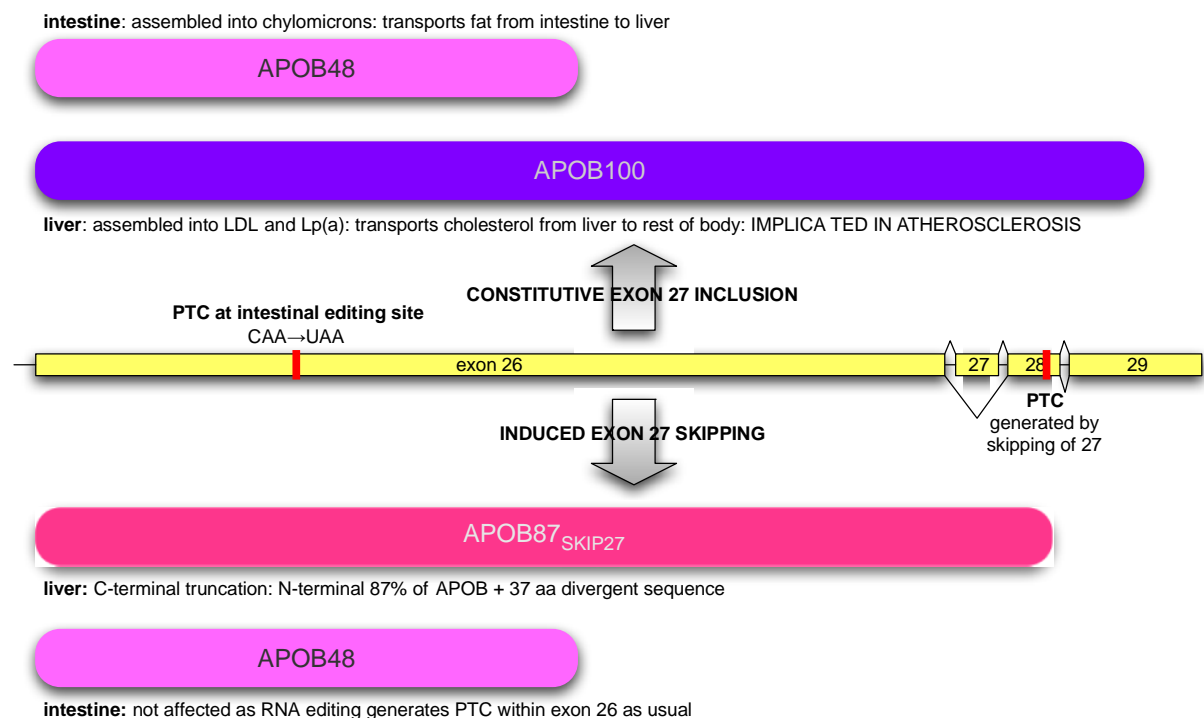
Re-engineering APOB mRNA to lower cholesterol

APOB is the principal structural apolipoprotein in LDL, very (V)LDL, intermediate-density lipoprotein (IDL), lipoprotein(a) (Lp(a)) and chylomicron particles [48]. The APOB pre-mRNA is composed of 29 exons, all of which are constitutively spliced into the mature APOB mRNA. There are two natural protein isoforms, the full-length APOB100 and the C-terminal truncated APOB48 forms. APOB100 is synthesized in the liver and is required for the assembly of VLDL, IDL, LDL and Lp(a) particles. Along with APOE, APOB100 is the ligand for the LDL receptor. Excess levels of the APOB100-containing particles LDL and Lp(a) have been implicated in atherogenesis [49]. The APOB48 isoform is synthesized in the intestine, is identical to the N-terminal 48% of the APOB100 protein, and is required for chylomicron assembly and intestinal fat transport. These chylomicrons are cleared from the circulation by interaction of APOE with the chylomicron remnant receptor; APOB48 cannot bind to the LDL receptor as it lacks the C-terminal domain necessary for binding. The APOB48 isoform is

generated by tissue-specific RNA editing of the CAA codon to a premature UAA termination codon in the intestine. The APOB48 mRNA is edited by a protein complex known as the editosome, which consists of the catalytic subunit APOBEC-1 (APOB mRNA-editing enzyme, catalytic polypeptide-1) and accessory factors. The RNA editing site and mooring sequence necessary for editosome binding and function are within exon 26 of the APOB mRNA [50]. Because of the central role of APOB100 in atherosclerosis, this isoform has become a major therapeutic target. Down-regulation of APOB100 expression is expected to decrease total cholesterol, LDL and Lp(a) levels, and therefore prevent the development of atherosclerosis.

Modifying APOB splicing was hypothesized to cause the expression of an alternative isoform of APOB. Exon 27 was selected as the target for three reasons. First, translation of the APOB mRNA lacking exon 27 (skip 27 mRNA) would generate a C-terminally truncated isoform of APOB100, APOB87_{SKIP27}, which is similar to the C-terminal truncations observed in some patients with mutations in APOB causing hypobetalipoproteinemia [51]. Heterozygotes for these mutant alleles of APOB have low cholesterol and LDL levels, have lowered risks of heart attacks, and live significantly longer than normal, although these individuals may be susceptible to fat accumulation in the liver [52]. Second, the 5' splice site of exon 27, when scored for its similarity to the splice-site consensus sequence using the Shapiro and Senapathy position weight matrix, was the weakest of all the 5' splice sites of the internal exons of APOB [53]. As constitutive 5' splice sites in general demonstrate better scores than the alternatively spliced 5' splice sites [54], APOB exon 27 would be the most amenable to alternative splicing. Third, as the sequences necessary for RNA editing are present in exon 26, the skip 27 mRNA should be edited as usual in the intestine and APOB48 expression should be unaffected (Figure 3).

Figure 3. Skipping APOB exon 27 causes the expression of APOB87_{SKIP27}, a C-terminally truncated isoform of APOB.



Constitutive exon 27 inclusion. Two isoforms of apolipoprotein B (APOB), APOB48 and APOB100, which both include exon 27 can be generated from the APOB mRNA. The APOB100 isoform is synthesized in the liver and is assembled into LDL particles, which transport cholesterol from the liver to the rest of the body and which have been implicated in atherosclerosis. APOB48 is identical to the N-terminal 48% of APOB100 and is synthesized in the intestine by tissue-specific RNA editing within exon 26, which generates a premature

termination codon (**PTC**; indicated by the red box – sequence changed from CAA to UAA, where **A** = adenine, **C** = cytosine and **U** = uracil) and the C-terminally truncated protein. The APOB48 isoform is assembled into chylomicrons that transport dietary fat from the intestine. **Induced exon 27 skipping.** Modifying APOB splicing by the skipping of exon 27 can lead to the expression of two alternative isoforms of APOB, APOB87_{SKIP27} and APOB48. Because skipping exon 27 induces a frameshift, a PTC is generated within exon 28 (indicated by the red box). The skip 27 mRNA is translated in the liver to the APOB87_{SKIP27} isoform, which contains the N-terminal 87% of APOB100 plus a 37 amino acid divergent sequence derived from the frameshifted sequence of exon 28. However, as RNA editing in the intestine generates the PTC within exon 26, intestinal APOB48 expression is unaffected.

Therefore 2'-O-methyl ASOs targeting the splice sites flanking APOB exon 27 and to the BPS of intron 26-27 were designed and transfected into HepG2 cells, which naturally express APOB100 [55]. Combination ASOs targeting two elements simultaneously in a single ASO induced exon 27 skipping, and this was most effective when both the 3' splice site and BPS were targeted. In contrast, targeting predicted ESE motifs within exon 27 to interfere with SR protein binding did not have a significant effect on exon skipping, suggesting that the constitutive incorporation of exon 27 is more dependent on the splice sites than on exonic elements, or that there is functional redundancy between the exonic elements [55].

Chabot and colleagues successfully utilized A1-tailed bifunctional oligonucleotides to induce alternative splicing in the Bcl-x pre-mRNA [56]. A bifunctional oligonucleotide consisting of a combination ASO targeting two splice sites plus an RNA tail was designed to bind the hnRNP A1 protein to encourage skipping of exon 27; however, this A1 tail did not augment exon-skipping in APOB pre-mRNA, but paradoxically partially reversed the skipping of exon 27 [55]. Therefore, the splice site and BPS ASOs may not function through a simple interference with splicing-factor binding. Instead, hybridization of the combination ASOs is likely to induce a secondary structure that is unfavorable to exon 27 inclusion. It is possible that recruitment of hnRNP A1 is counterproductive, as this may cause unwinding of the secondary structure [55].

Lastly, we demonstrated that combination ASO-transfected HepG2 cells were able to translate the skip 27 mRNA to a shortened isoform of APOB100, APOB87_{SKIP27}, which included the N-terminal 87% (3929 amino acids) of APOB100, along with a divergent 37 amino acid peptide at the C-terminus and ending in a premature termination codon within the exon 28 sequence [55]. Therefore, the induction of APOB87_{SKIP27} expression *in vivo* should lead to decreased LDL and cholesterol levels, such as occurs in patients with hypobetalipoproteinemia.

As mentioned previously, because intestinal APOB mRNA editing and APOB48 expression rely on sequences within exon 26, exon 27 skipping should not affect APOB48 expression. This is in contrast to methods that rely on a generalized downregulation of APOB mRNA levels, such as chimeric ASO-mediated RNase H degradation [57], and RNA i [58]. These latter methods have been demonstrated to reduce APOB100 levels and therefore circulating levels of cholesterol and LDL, but they also reduce the levels of APOB48 and circulating levels of chylomicrons, an unwanted side effect expected to interfere with the transport of fat from the intestine.

Conclusion

The potential treatment of SMA and high cholesterol by splicing therapeutics exemplify two key uses of this strategy: first, correcting defective splicing caused by gene mutations, such as the replacement of the defective SMN1 gene in SMA with SMN2; second, to re-engineer normal pre-mRNAs to achieve the desired physiological effects, such as the re-engineering of APOB pre-mRNA to reduce cholesterol levels.

In 2008, the development of various therapeutic strategies to correct SMN2 splicing has been particularly active. The challenge now is to identify reliable and safe methods of applying these therapeutic concepts to the bedside. ASOs are known to accumulate in the liver when administered *in vivo* [59], and have been shown to switch splicing in this organ when administered to mice intraperitoneally and orally [60]. In addition, ASOs targeted against the ISS-N1 element and infused into mice are able to increase exon 7 inclusion in SMN2 mRNA in the liver, kidney, and to a lesser extent, in muscle [40]. With regards to splicing therapeutics directed against APOB, as the liver is the principal target organ, ASOs are natural candidates for such an application. The next stage in the development of these APOB ASOs will be to demonstrate their effectiveness in lowering LDL cholesterol *in vivo*, and then to demonstrate that the APOB ASOs reduce the risk of clinically relevant endpoints.

With regards to SMN2, however, there are obstacles remaining in the delivery of ASO-based therapies, particularly because the blood-brain barrier protects the CNS from these agents [61]. SMN2 ASOs administered into the systemic circulation are not able to modify the splicing of SMN2 in the spinal cord because the ASOs cannot cross the blood-brain barrier [40]. The blood-brain barrier can be bypassed by administering ASOs to the CSF, either intrathecally or intracerebroventricularly; this strategy has been shown to be effective in downregulating superoxide dismutase in spinal motor neurons and slowing disease progression in a mouse model of amyotrophic lateral sclerosis [62]. Lentiviral- or rAAV-based vectors represent an alternative technology that could allow splicing-based therapies to be administered efficiently to the CNS. The rAAV-based vectors can be used to transduce spinal cord neurons by retrograde transport [63]. However, other concerns exist with regard to the use of viral vectors in the CNS, particularly related to the induction of an immune response in patients previously exposed to AAV [64]. Therefore, both delivery strategies for splicing therapy (viral and nonviral), will be worth pursuing.

Lastly, it is not yet known if the modification of SMN2 splicing and restoration of the expression of the SMN protein can reverse the pathophysiology of SMA and the death and degeneration of spinal motor neurons to achieve clinically relevant outcomes. This is analogous to the situation with Duchenne muscular dystrophy (DMD). The intramuscular injection of ASOs has been demonstrated to successfully restore local dystrophin expression in patients with DMD. However, in a short follow-up study of 28 days, restoration of local dystrophin expression was not accompanied by an increase in muscular strength [65]. Thus, the practical application of splicing therapeutics with regard to SMN2 is at least some distance away and awaits further development *in vivo*.

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- of outstanding interest
 - of special interest
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