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Minireview

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# Targeting RNA-Splicing for SMA Treatment

**Jianhua Zhou[1], Xuexiu Zheng, and Haihong Shen***

**The central dogma of DNA-RNA-protein was established more than 40 years ago. However, important biological processes have been identified since the central dogma was developed. For example, methylation is important in the regulation of transcription. In contrast, proteins, are more complex due to modifications such as phosphorylation, glycosylation, ubiquitination, or cleavage. RNA is the mediator between DNA and protein, but it can also be modulated at several levels. Among the most profound discoveries of RNA regulation is RNA splicing. It has been estimated that 80% of pre-mRNA undergo alternative splicing, which exponentially increases biological information flow in cellular processes. However, an increased number of regulated steps inevitably accompanies an increased number of errors. Abnormal splicing is often found in cells, resulting in protein dysfunction that causes disease. Splicing of the survival motor neuron (SMN) gene has been extensively studied during the last two decades. Accumulating knowledge on SMN splicing has led to speculation and search for spinal muscular atrophy (SMA) treatment by stimulating the inclusion of exon 7 into SMN mRNA. This mini-review summaries the latest progress on SMN splicing research as a potential treatment for SMA disease.**

## INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive, early childhood disease that affects 20,000-30,000 patients in the US. Although SMA is an orphan disease, it is the most common genetic cause of infant mortality in the US and western Europe (Pearn, 1980). The carrier frequency of SMA is estimated to be as high as 1 in 40, leading to the disease in 1 in 6000 newborns (Pearn, 1980). Patients with SMA are intellectually normal but have problems with movement, muscle tone, and muscle-related functions such as breathing and swallowing. These symptoms result from abnormally low levels of the survival motor neuron (SMN) protein (Pearn, 1980), leading to degeneration of alpha-motor neurons in the anterior horn of the spinal cord. More recent studies have indicated that other organs such as the heart and liver are also damaged in patients with SMA (Bevan et al., 2010; Hua et al., 2011). Respiratory failure due to diaphragm weakness is the main cause of death in patients with SMA. SMA is categorized into four types based on severity. The mildest form of SMA is type IV, which is an adult SMA, with symptoms beginning in the middle 30s. Patients with the milder type III SMA start to experience muscle weakness from as early at 2 years of age to their teen years. They are frequently wheelchair bound when they reach their 20s. Patients with type III and type IV SMA have a normal lifespan. Patients with type II SMA usually experience muscle weakness at around 2 years of age, are wheelchair bound at early ages, and die before they reach 20 years of age. The most severe type of SMA is type I with onset at a few months old and patients usually die before 2 years of age.

Based on linkage analysis and mapping, the survival motor neuron (*SMN1*) gene is responsible for SMA. Humans have two highly homologous copies of the *SMN* gene, *SMN1* and *SMN2,* which are located on chromosome 5q13 as inverted repeats (Lefebvre et al., 1995) (Fig. 1). In > 95% of patients with SMA, the telomeric *SMN1* copy is deleted or mutated (DiDonato et al., 1997; Lefebvre et al., 1995; Parsons et al., 1996; Wirth, 2000), whereas the *SMN2* centromeric copy remains in most patients with SMA but predominantly (> 85%) produces exon7-skipped transcripts, generating truncated, unstable proteins that fail to compensate for the loss of *SMN1* (Coovert et al., 1997; Lefebvre et al., 1995). The skipping of exon 7 in *SMN2* is caused by alternative splicing of exon 7 (Lorson et al., 1999) (Fig. 1).

*SMN* encodes a 294 amino acid protein with a molecular weight of 34 kD and is conserved across species. In mammalian cells, SMN forms a characteristic dot-like gem structure in the nucleus (Liu and Dreyfuss, 1996). SMN interacts with many cellular proteins (reviewed by Nicole et al., 2002) and is involved in the assembly of large macromolecular ribonucleoprotein particle (RNP) complexes (Buhler et al., 1999; Carvalho et al., 1999; Fischer et al., 1997; Massenet et al., 2002; Meister, 2001; Pellizzoni et al., 2002), playing an important role in RNA metabolism. We found that SMN is co-localized with the apoptosis-related proteins TIA-1/R and G3BP in stress granules (SGs) (Forch and Valcarcel, 2001; Hua and Zhou, 2004a; 2004b; Le Guiner et al., 2003), a cellular structure that stores translationally silenced snRNPs during the stress response. Additional studies have demonstrated that SMN initiates the stress response by facilitating the formation of SGs under stressful conditions (Hua and Zhou, 2004b). Therefore, SMN may function as an anti-apoptotic chaperone protein in SMN/SGs by stabilizing RNA/protein species against stress-induced apoptosis.

School of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea, [1]Department of Neuroregeneration, Nantong University, JiangSu, P. R. China
*Correspondence: haihongshen@gist.ac.kr

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Consistent with our results, SMN has been implicated in transporting specific mRNAs to neuron neurites (Pagliardini et al., 2000; Zhang et al., 2003), possibly in neuronal RNA granules (Bassell et al., 1998; Hu et al., 2003; Tiruchinapalli et al., 2003), a structure with a function reminiscent of SGs in other cell types (Wickens and Goldstrohm, 2003). SMN overexpression in cultured forebrain neurons induce granules that are actively transported to developing neurites and growth cones in a manner similar to that of RNA granules (Zhang et al., 2003). Based on these findings and our findings of SMN in SGs (Hua and Zhou, 2004b), we hypothesize that SMN plays a key role protecting and storing mRNAs/proteins in both neuronal cell bodies and processes (axons and dendrites). Low levels of SMN may fail to protect specific mRNA/protein species in long axons, particularly under stressful conditions, resulting in failure of synaptic plasticity.

**SMA animal models**

Unlike humans, mice contain one copy of the *SMN* gene, equivalent to the *SMN*1 gene in humans. Homozygous knockout of the mouse *SMN* gene is embryo lethal at a very early developmental stage (Schrank et al., 1997). Animals with an *SMN*$^{-/-}$ h*SMN2* genotype were generated by crossing mice transgenic for human *SMN2* into a *SMN*$^{+/-}$ heterozygous mouse (Monani et al., 2000). Because hSMN2 expresses a low level of the full length SMN protein, these mice do not die *in utero*, suggesting that human *SMN2* can rescue the lethality of homozygous knockout. A single copy of human *SMN*2 was sufficient to restore mouse viability from *SMN*$^{-/-}$ lethality, but severe weakness developed, and the mice died within a few days of birth. In contrast, mice with eight copies of h*SMN*2 appeared normal, consistent with studies in human patients with SMA, in which *SMN2* functions as a disease-modifying gene. and multiple copies of SMN2 gene can produce full-length SMN at a high enough level to compensate for the loss of SMN1; thus, explaining the correlation between disease severity and *SMN2* copy number (Monani et al., 2000).

*SMN* homologues have also been identified from *Caenorhabditis elegans* (Miguel-Aliaga et al., 1999), *Danio rerio* (zebrafish) (Bertrandy et al., 1999; McWhorter et al., 2003), *Schizosaccharomyces pombe* (Hannus et al., 2000; Owen et al., 2000; Paushkin et al., 2000), and *Drosophila* (Miguel-Aliaga et al., 2000). The SMN proteins from these species retain a number of biochemical properties identified in the human SMN, including RNA binding activity and self-association. SMN models of *C. elegans* (Miguel-Aliaga et al., 1999), *D. rerio* (Bertrandy et al., 1999; McWhorter et al., 2003), and *Drosophila* (Miguel-Aliaga et al., 2000) show numerous SMA disease phenotypes. Defects of SMN proteins in *C. elegans*, *D. rerio*, and *S. pombe* affect cell viability and growth (Bertrandy et al., 1999; Hannus et al., 2000; McWhorter et al., 2003; Miguel-Aliaga et al., 1999; Owen et al., 2000; Paushkin et al., 2000). *Drosophila SMN* mutants (*dSMN*) result in abnormal neuromuscular junctions phenotypes (Chan et al., 2003). Ectopic overexpression of human SMN protein domains, which may function as dominant negatives to disrupt the endogenous dSMN protein, result in abnormally positioned wings and legs or pupal lethality (Miguel-Aliaga et al., 2000). Additionally, suppressing SMN expression in zebrafish induces motor axon-specific pathfinding defects, indicating that SMN functions in motor axon development, and that these early developmental defects may lead to subsequent motor neuron loss (McWhorter et al., 2003).

**Mechanisms of RNA splicing and alternative splicing**

Pre-mRNA splicing is a process in which introns are removed and exons are ligated together. Pre-mRNA splicing is divided into two steps. In the first step, the phosphate group at the 5′ splice site attacks the hydroxyl group of the adenine at the branch point to form a 2′-5′ phosphodiester bond. In the second step, the phosphate group at the 3′ splice site attacks the hydroxyl group at the 5′ splice site to form a 3′-5′ phosphodiester bond. The cleaved exons are ligated to form a mature RNA. Sequences required for pre-mRNA splicing include the 5′ splice site, the 3′ splice site, the branch point, and the polypyrimidine tract. These sequences are also called splicing signals. Pre-mRNA splicing of some introns requires a pre-mRNA enhancer region. Pre-mRNA splicing of some introns is regulated by pre-mRNA inhibitors.

Pre-mRNA splicing occurs in a large RNA protein complex called a spliceosome. A spliceosome contains two component parts: the first part is U1, U2, U4, U5, and U6 small nuclear RNA protein complexes (snRNPs); the second part includes proteins such as U2AF65 and SR. The formation of a spliceosome is a stepwise process. The first complex formed is an early spliceosome (complex E). In complex E, U1 snRNP is recruited into the pre-mRNA, and U1 snRNA in the U2 snRNP forms a base-pair with the 5′ splice site. The next complex formed is a pre-spliceosome. In the prespliceosome, U2 snRNP is recruited into the pre-mRNA, and the U2 snRNA in U2 snRNP forms a base-pair with the branch-point on pre-mRNA. U4/U5/U6 snRNPs are recruited into the mature spliceosome to perform catalytic activities.

In alternative splicing, different exons are included in different mRNAs. In humans, at least 75% of genes proceed by alternative splicing. Alternative splicing regulates almost every biological process in metazoans including signal transduction and energy transfer. A variety of diseases are caused by defects in pre-mRNA splicing; SMA is one example.

**Is SMA caused by splicing?**

Unlike many other diseases, SMA is a unique genetic disorder, because there are two copies of SMN genes in humans (Fig. 1). Yet, only when *SMN1* is deleted or mutated, do patients suffer from the disease. *SMN2* remains but is not able to produce sufficient full-length SMN protein to compensate for the loss of the *SMN1* gene due to alternative splicing of exon 7. Strictly speaking, SMA is not a disease that is caused by splicing because it is the *SMN1* gene deletions or mutations that cause the disease. However, the difference in splicing between *SMN1* and *SMN2* plays a critical role in how SMA disease develops, progresses, and is ultimately defined. For this reason, exon 7 splicing in SMN genes has been extensively studied during the last 15 years.

The primary sequences of *SMN1* and *SMN2* are rather similar. By examining mini-genes constructed from exon 6, intron 6, exon 7, intron 7, and exon 8, one of the major variations, a change in a C in *SMN1* to a T in *SMN2* at position 6 in exon 7 (Fig. 1), weakens the effect of SF2/ASF for enhancing inclusion of exon 7. This C6T change also creates a silencer element for hnRNP A1 to exclude exon 7. As a result, the C6T nucleotide largely determines the splicing patterns of the *SMN1* and *SMN2* genes, leading to 80-90% of *SMN2* mRNA without exon 7 (see review by Singh, 2007). However, many studies have revealed that in addition to the C6T nucleotide in exon 7, several other elements regulate exon 7 alternative splicing in the *SMN2* gene. On the positive site, 5′ and 3′ splicing sites, SF2/ASF binding sites in exon 7 of the *SMN1* gene, and the "conserved tract", which is located in the middle of exon 7, apparently stimulate the inclusion of exon 7, resulting in normal splicing of the exon in the *SMN1* gene. In contrast, multiple

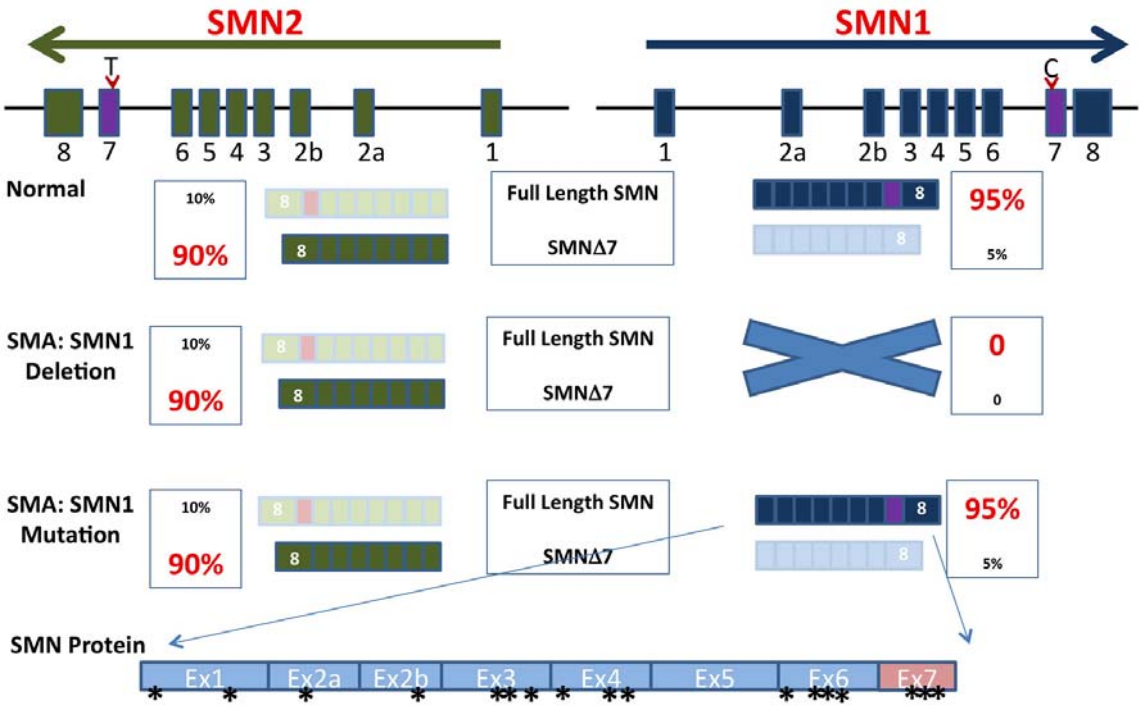


**Fig. 1.** Survival motor neuron (SMN) genes and spinal muscular atrophy (SMA) disease. Two inverted *SMN* genes, *SMN1* and *SMN2* are located on chromosome 5. *SMN1* and *SMN2* have nine exons. Exon 7 is alternatively spliced. Under normal conditions (Normal), > 95% of *SMN1* mRNA includes exon 7, whereas only 10% of *SMN2* mRNA has exon 7. Most patients with SMA have a deletion of the *SMN1* gene (SMA: *SMN1* deletion). In these patients, 10% of full-length mRNA produced from the *SMN2* gene is unable to compensate for the loss of the *SMN1* gene. SMA can also be caused by point mutations (SMA: *SMN1* mutation). Missense mutations have been identified across the gene (indicated by *).

negative elements are present in both the *SMN1* and *SMN2* genes, which overrun positive elements in the *SMN2* gene. For example, a diversified intronic hnRNA A1 motif between *SMN1* and *SMN2* plays a role in exon 7 inclusion/skipping. Other negative elements including extended inhibitory context (Exinct), a 3′ cluster, the negative elements towards 5′ and 3′ ends of exon 7, two RNA secondary structures (terminal stem loop: TSL1 and TSL2) and more significantly intronic splicing silencer sequences which was identified by *in vivo* anti-sense oligo walking seem to have a great impact on regulation of exon 7 skipping (reviewed by Singh, 2007). Slight changes in any of these elements could tip the balance towards exclusion of exon 7 from *SMN* mRNA. How these elements regulate exon 7 has been comprehensively reviewed (Singh, 2007). In addition to cis-elements, multiple trans splicing factors such as hTra2β1 (Hofmann et al., 2000), SF2/ASF (Singh et al., 2004), hnRNP A1 (Harahap et al., 2012), and SRp30C (Young et al., 2002) have been identified and implicated to affect exon 7 splicing by directly or indirectly modulating silencers or enhancers.

**Alternative splicing in SMN2 is targeted for SMA treatment**

Unlike many other neurodegenerative diseases, SMA is a single gene disorder. More than 95% of SMA cases are directly caused by deletion or mutation defects in the *SMN1* gene. Although there are only 20,000-30,000 patients in the US, this simple and unique genetic defect has lead to an extensive search for treating SMA disease. SMA is autosomal recessive; thus, exogenous expression of the SMN protein could be a viable approach for therapeutic treatment of patients with SMA. Not surprisingly, extensive efforts have been undertaken to examine constructs and vectors that can safely boost SMN expression in motor neurons of SMA models (Bevan et al., 2010; Dominguez et al., 2011; Foust et al., 2010; Glascock et al., 2011; Passini et al., 2010). This has been one of the mainstream objectives in the search for drugs to treat SMA. Significant progress has been made to deliver SMN products into SMA animal models during the last few years. Several groups have reported that *SMN1* gene transfer into SMA models using self-complementary adeno-associated virus type 9 (scAAV9) or adeno-associated virus type 9 (AAV) can extend the life of mice with SMA from 15 to > 200 days without apparent disease symptoms.

While gene therapy for SMA is promising, targeting exon 7 splicing of the *SMN2* gene has been gaining momentum during the last few years. Initial efforts to identify drugs aimed at exon 7 in *SMN2* were focused on small molecules. We developed a cell-based screen to identify small molecules capable of promoting inclusion of exon 7 in the mature *SMN2* message using a mini-gene with minimal exon 7 modifications and a luciferase reporter fused to exon 8. We and others have used this cell-based assay and identified several compounds including sodium vanadate, aclarubicin, and indoprofen that increases inclusion of exon 7 (Andreassi et al., 2001; Lunn et al., 2004; Zhang et al., 2001). Identification of other compounds such as hydroxyurea (Grzeschik et al., 2005), valproate (Weihl et al., 2006), and phenylbutyrate (Andreassi et al., 2004) have been also described. In fact, several of these compounds have been examined for clinical trials in patients with SMA. However, se-

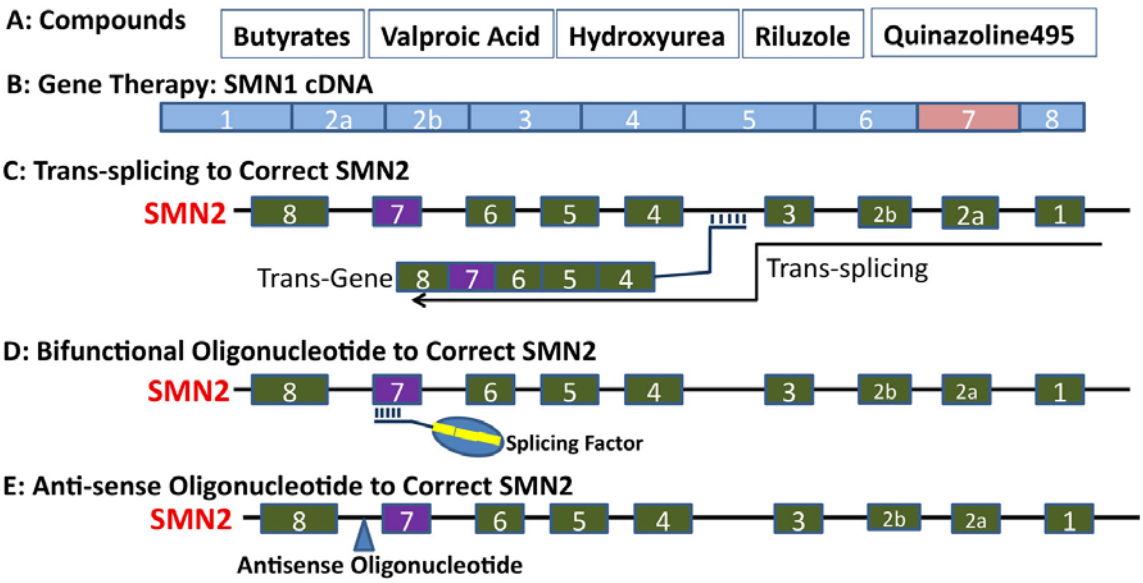


**Fig. 2.** Potential therapeutic approaches for spinal muscular atrophy (SMA). (A) Compounds that have been or will be tested in clinical studies. (B) Gene therapy can be achieved by delivering full length SMN1 cDNA into patients with SMA; (C) Trans-splicing can be used to correct exon 7 skipping in the *SMN2* gene; (D) A bifunctional oligonucleotide can recruit splicing factors to exon 7, leading to more exon 7 inclusion in SMN2 mRNA; (E) Anti-sense oligonucleotides would block negative elements in *SMN* splicing, stimulating exon 7 inclusion into SMN2 mRNA.

lectivity and side effects of these compounds is still a concern. To increase specificities, multiple technologies to target aberrant splicing (Gallo et al., 2007) have been examined for *SMN* genes. One strategy that is employed to stimulate inclusion or exclusion of exon 7 is to use bifunctional oligoribonucleotides, which are molecules made of an anti-sense sequence that bind to an exonic element and a sequence tethering a trans factor, or link to a peptide domain mimicking a trans splicing factor (Cartegni and Krainer, 2003; Skordis et al., 2003; Villemaire et al., 2003). Bifunctional oligoribonucleotides effectively increase insertion of exon 7 into *SMN2* mRNA. Relative to bifunctional oligoribonucleotides (Baughan et al., 2006; 2009; Dickson et al., 2008; Horne and Young 2009; Osman et al., 2012; Skordis et al., 2003; Voigt et al., 2010), the strategy of trans-splicing is new but has offered great promise to correct abnormal splicing of several other genes (Coady et al., 2007; Puttaraju et al., 1999; Rodriguez-Martin et al., 2005). Trans-splicing is based on studies showing that two pre-mRNAs can undergo trans-splicing if sufficient homologies between the two pre-mRNAs exist, leading to the first part of the mRNA from exons of pre-mRNA1 and the second part from exons of pre-mRNA2. This strategy has been comprehensively explored by Lorson's group during the last few years to correct *SMN2* splicing. They optimized trans-splicing strategies and demonstrated that trans-splicing increases exon 7 insertion into SMN2 mRNA and extend the life of animals with SMA (Coady and Lorson, 2010; Coady et al., 2007; 2008; Shababi and Lorson, 2011; Shababi et al., 2011).

While all of these strategies that target splicing hold promise, the most intriguing results come from anti-sense oligonucleotides which target negative elements of exon 7 splicing in the SMN gene (Hua et al., 2007; 2008; 2010; 2011; Passini et al., 2011; Porensky et al., 2011; Singh et al., 2009). More interestingly, it has been shown that systemic administration of an anti-sense oligonucleotide (ASO-10-27) to neonates with SMA robustly rescues mice with severe SMA, much more effectively than intracerebroventricular administration, and subcutaneous injections extended median lifespan of animals with SMA 25-fold. These authors also demonstrated that not only the central nervous system but also peripheral tissues, in particular the liver, are involved in the pathogenesis of SMA. Restoration of liver function and other tissues may be essential for SMA treatment. Nonetheless, studies of *SMN* with documented methods to target exon 7 splicing provide multiple drug candidates for SMA therapy.

## CONCLUSIONS

### Can SMA be a model for disease treatment by targeting splicing?

Similar to transcription, splicing is a ubiquitous cellular process. Targeted splicing machinery may induce global effects on splicing events in cells. Therefore developing drugs that target splicing is difficult, if not impossible. However, compounds that were identified to regulate SMN splicing have shown relative selectivity to the *SMN* genes, leading scientists to believe that it may be possible to find safer drugs that can reverse abnormal splicing in disease (Andreassi et al., 2001). This promising perspective seems to be greatly boosted by recent developments in SMN/SMA studies in which technologies such as trans-splicing, bifunctional oligonucleotides, and anti-sense oligonucleotides very specifically increase the inclusion of exon 7 into SMN2 mRNA (Fig. 2) (Baughan et al., 2006; 2009; Coady and Lorson, 2010; Coady et al., 2007; 2008; Dickson et al., 2008; Hua et al., 2010; 2011; Lorson et al., 2010; Osman et al., 2012; Shababi and Lorson, 2011; Shababi et al., 2011; Skordis et al., 2003). In particular, the effectiveness of anti-sense oligos in animals with SMA has excited the scientific community so that SMA may be treatable in the near future. While clinical trials in humans are being developed, obstacles such as effectiveness and safety of these technologies in humans need to be overcome. In conclusion, SMA studies have set an example that splicing can be targeted for treatment of many diseases involved with abnormal splicing.

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