

Antisense Oligonucleotides for the Treatment of Spinal Muscular Atrophy

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive disease affecting ~1 in 10,000 live births. The most striking component is the loss of α -motor neurons in the ventral horn of the spinal cord, resulting in progressive paralysis and eventually premature death. There is no current treatment paradigm other than supportive care, though the past 15 years has seen a striking advancement in understanding of both SMA genetics and molecular mechanisms. A variety of disease-modifying interventions are rapidly bridging the translational gap from the laboratory to clinical trials, including the application of antisense oligonucleotide (ASO) therapy for the correction of aberrant RNA splicing characteristic of SMA. Survival motor neuron (SMN) is a ubiquitously expressed 38-kD protein. Humans have two genes that produce SMN, *SMN1* and *SMN2*, the former of which is deleted or nonfunctional in the majority of patients with SMA. These two genes are nearly identical with one exception, a C to T transition (C6T) within exon 7 of *SMN2*. C6T disrupts a modulator of splicing, leading to the exclusion of exon 7 from ~90% of the mRNA transcript. The resultant truncated $\Delta 7$ SMN protein does not oligomerize efficiently and is rapidly degraded. SMA can therefore be considered a disease of too little SMN protein. A number of *cis*-acting splice modifiers have been identified in the region of exon 7, the steric block of which enhances the retention of the exon and a resultant full-length mRNA sequence. ASOs targeted to these splice motifs have shown impressive phenotype rescue in multiple SMA mouse models.

Introduction

SPINAL MUSCULAR ATROPHY (SMA) IS A neuromuscular disease that is characterized by dysfunction and eventually death of α -motor neurons in the spinal cord ventral horn (Crawford and Pardo, 1996). SMA is considered common among National Institutes of Health–designated “orphan” diseases (<http://rarediseases.info.nih.gov/>) with a prevalence of 1 case per 10,000 live births (Pearn, 1978; Prior *et al.*, 2010). Rapid disease progression and the severity of paralysis-related morbidity leaves SMA as one of the leading causes of genetic infant mortality (Roberts *et al.*, 1970). There is no current treatment except for supportive care, although some promising therapies are preparing for phase 1/2a clinical trials.

SMA is classified into a spectrum of disease severity (Type I through Type IV; Zerres and Rudnik-Schoneborn, 1995). Type I patients are never able to sit upright and typically show rapid loss of motor tone before 6 months of age. Death from respiratory insufficiency or other associated comorbidities is typical before 1 year without aggressive in-

tervention including positive-pressure ventilation and enteric tube feeds. Alternatively, type II patients can sit upright but not ambulate without assistance, and those with type III can ambulate but lose this function in passing years. Type IV patients may only have evidence of neuromuscular decline in later decades (Piepers *et al.*, 2008).

Advances in SMA genetics support a molecular classification scheme that directly correlates with the range of clinical phenotypes. These disease insights have generated multiple proposed treatment paradigms. All species require the critical survival motor neuron (SMN) protein, and all cells require at least a basal production of SMN. The deletion of *Smn* results in embryonic lethality in mice and death of that cell if removed (Cifuentes-Diaz *et al.*, 2001; Frugier *et al.*, 2000; Schrank *et al.*, 1997; Vitte *et al.*, 2004). Humans have two similar genes that produce SMN, *SMN1* and *SMN2*, the latter of which is unique to our species (Rochette *et al.*, 2001). *SMN2* is therefore postulated to be a relatively recent mutation since no other species has two SMN-producing genes with the exception of chimpanzees (with two copies of *SMN1*).

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SMN2 essentially differs from *SMN1* by a single nucleotide (C6T) within exon 7. While the coded amino-acid sequence is unchanged, the mutation does disrupt a modulator of RNA splicing (Lorson *et al.*, 1999; Monani *et al.*, 1999). Efficiency of exon 7 retention is radically altered with ~90% of the *SMN2* RNA transcript undergoing alternative splicing-out of the exon (Fig. 1A; Gennarelli *et al.*, 1995). The $\Delta 7$ SMN protein does not oligomerize efficiently and is rapidly degraded by cellular enzymatic activity (Burnett *et al.*, 2009; Lorson *et al.*, 1998). *SMN1* is deleted or nonfunctional in the majority of patients with SMA. Affected individuals survive by virtue of the low levels of FL-SMN produced by *SMN2*, but it is clear that these levels are insufficient for normal cell maintenance (Coovert *et al.*, 1997; Lefebvre *et al.*, 1997; Parsons *et al.*, 1996). Different cell and tissue populations likely require unique basal and peak levels of SMN (and at different time points) in order to meet metabolic demands. This could be one reason why the motor neuron unit is so exquisitely susceptible to dysfunction (Burghes and Beattie, 2009). Regardless of the vagaries of timing and location of SMN depletion, SMA is at its core a disease of insufficient SMN.

A direct modifier of disease severity is therefore the relative quantity of FL-SMN produced inefficiently by *SMN2*. An inverse correlation is seen between *SMN2* copy number and clinical disease classification from type I (single *SMN2* copy) through type IV (more than three copies) (McAndrew *et al.*, 1997; Parsons *et al.*, 1998). This copy-number scheme is supported by the multiple murine models of disease (Mon-

ani *et al.*, 2000a; Schmid and DiDonato, 2007). Mice have only one gene that produces SMN; animals without native *Smn* and with two inserted copies of *SMN2* (i.e., "severe mouse" *Smn*^{-/-}; *SMN2*^{+/+}) display a striking motor decline and die by postnatal day 5 (P5) if on a complete null background, and survive to P10 if the deletion allele is capable of making the mouse *Smn* transcript lacking exon 7 (Hsieh-Li *et al.*, 2000; Monani *et al.*, 2000b). To effect a longer survival and thus facilitate therapeutic pharmacological trials, the *SMN Δ 7* mouse incorporates an additional transgene expressing *SMN2* cDNA (*Smn*^{-/-}; *SMN2*^{+/+}; *SMN Δ 7*^{+/+}) which prolongs survival to P15 (Le *et al.*, 2005). $\Delta 7$ SMN is capable of interacting with FL-SMN and providing some minimal function. The amount of FL-SMN produced by an *SMN2* gene is critical in determining the severity of SMA phenotype (Prior *et al.*, 2009; Vezain *et al.*, 2010). More intermediate mouse models have increasing copies of *SMN2*; these animals develop ear and tail necrosis but not necessarily weakness (Hsieh-Li *et al.*, 2000). Necrosis does not occur in patients with mild SMA, and clear electrophysiological abnormalities indicating reduced motor neuron number and sprouting are not available.

Five percent of *SMN1* mutations are small mutations. Some of these are missense mutations that can produce both severe and mild phenotypes in a low *SMN2* copy background, depending on whether the mutation can complement the FL-SMN produced by *SMN2* (Burghes and Beattie 2009; Workman *et al.*, 2009). The major function of SMN in complex with other proteins is the assembly of Sm proteins

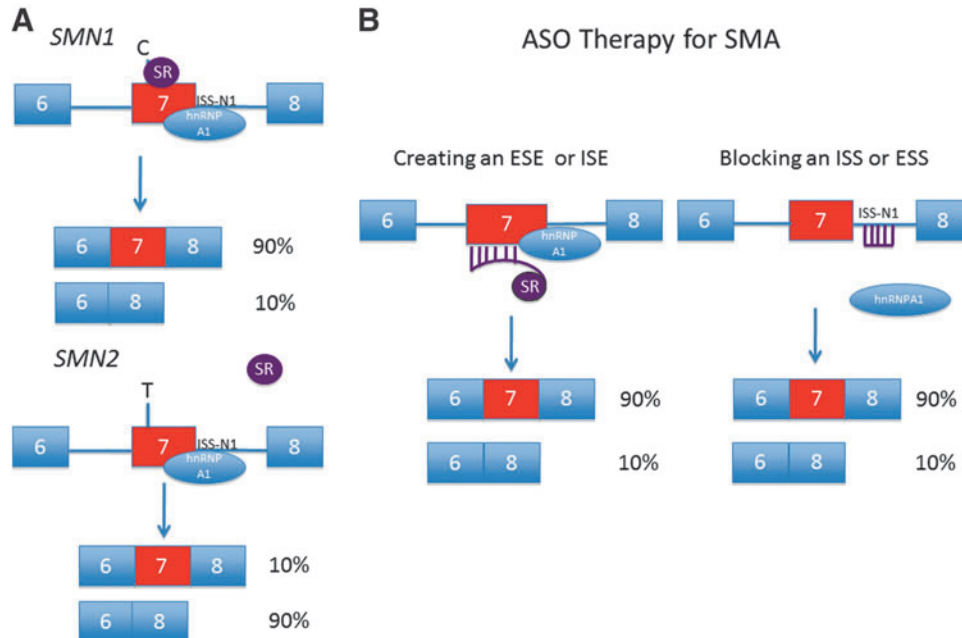


FIG. 1. (A) *SMN1* and *SMN2* splice dynamics. The C to T change within *SMN2* exon 7 position 6 either disrupts a splice enhancer or creates a splice silencer. The *SMN1* transcript binds both the splice activator ASF/SF2 (SR protein) and the splice repressor hrRNPA1 (at intron 7 ISS-N1). The resulting mRNA transcript includes exon 7. *SMN2* does not bind the splice activator, and the majority of mRNA transcript lacks exon 7. *SMN1* is missing or nonfunctional in SMA, and the patient survives on the small amount of full-length SMN transcript produced by *SMN2*. (B) *SMN2* gene splicing can be modified by either bifunctional ASOs that promote binding of SR proteins, or through steric block of hrRNPA1 binding at splice suppressor sites (e.g., ISS-N1). Figure adapted from Burghes and McGovern (2010). SMA, spinal muscular atrophy; SMN, survival motor neuron; ASO, antisense oligonucleotide; ISS, intron splice suppressor; ESS, exon splice suppressor; ESE, exon splice enhancer; ISE, intron splice enhancer.

onto snRNA, which is then transported into the nucleus and plays a critical function in gene splicing (Pellizzoni *et al.*, 1998). SMN reduction drastically alters snRNP assembly and results in gene splicing changes in spinal cord tissue of $\Delta 7$ SMA mice (Baumer *et al.*, 2009; Gabanella *et al.*, 2007). It is unclear if alternative splicing is a primary driver of disease or rather a secondary effect of generalized organism decline. Further characterization of the splicing changes unique to snRNP dysfunction in SMA, specifically within the motor neuron, should shed light on the molecular and cellular pathways affected. Future investigation will define the critical splicing changes that contribute to all or part of the SMA phenotype.

The facilitation of RNA axonal transport by SMN is debated and under investigation. Shorter axons are observed with knockdown of SMN in zebrafish as well as in motor neuron cultures from SMA mice (Jablonka *et al.*, 2006; McWhorter *et al.*, 2003; Rossoll *et al.*, 2003). However, SMA mice do not show axonal growth defects (McGovern *et al.*, 2008). There is disrupted synaptic morphology and function in these animals, which is corrected by central nervous system (CNS) SMN expression (Gavrilina *et al.*, 2008; Gogliotti *et al.*, 2012; Martinez *et al.*, 2012; Park *et al.*, 2010). It is therefore indeterminate what direct function SMN has with respect to axonal and synaptic SMA pathology (Burghes and Beattie, 2009).

The above insights into the genesis of SMA due to the depletion of $_{FL}$ SMN have yielded two main concepts of therapy: first is the wholesale replacement of SMN above that afforded by *SMN2* through the introduction of a construct expressing $_{FL}$ SMN, facilitated by viral vectors carrying the full-length construct. This method has yielded impressive results in severe mouse models after neonatal delivery (Dominguez *et al.*, 2011; Foust *et al.*, 2010; Passini *et al.*, 2010; Valori *et al.*, 2010). Investigation has continued in larger and more mature animals to determine rates of motor neuron transfection as well as to analyze biodistribution (Bevan *et al.*, 2011; Marco Passini, personal communication, Families of SMA International SMA Research Group Meeting, June 23, 2012). While viral delivery of an intact SMN construct has progressed well and is approaching phase I/IIa clinical trials, impediments remain, including the ability to produce sufficient virus for a large clinical study.

An alternative method to increase $_{FL}$ SMN levels is to manipulate *cis*-acting RNA sequences to increase exon 7 incorporation during *SMN2* RNA processing. As with other pre-mRNA transcripts, *SMN2* contains multiple intronic and

exonic conserved coding regions that recruit components of the splicing machinery (Beebe *et al.*, 2010). Many of these sequences, both those that inhibit splicing (exon splice suppressor [ESS], intron splice suppressor [ISS]) and those that activate splicing (exon splice enhancer [ESE], intron splice enhancer [ISE]), have been identified adjacent to or within exon 7, and antisense oligonucleotides (ASOs) targeting these regions can alter RNA processing dynamics and increase $_{FL}$ SMN (Fig. 1B). Optimization of the target sequence and chemistry of ASO are ongoing; nevertheless, *in vitro* and recent *in vivo* investigations have shown this line of therapeutic attack can vastly increase the complement of $_{FL}$ SMN to levels seen with *SMN1*. Fortuitously, ASOs have shown impressive disease amelioration in murine models. As this model of treatment edges closer from the laboratory towards the clinic, important questions remain regarding dosing, toxicity, timing, and location of ASO delivery.

Splice Regulation of *SMN2* Exon-7

Post-transcriptional mRNA processing is a complex process involving both local splicing elements as well as a *trans* acting modulators of splicing. A series of defined features are necessary but not altogether sufficient for splicing, including weakly conserved 5' and 3' splice-sites (ss), a polypyrimidine tract, and the branch point (Cartegni *et al.*, 2002). Additional splice effectors (ESE, ESS, ISE, ISS) flanking the intron–exon junctions are especially important in the setting of alternative splicing or intrinsically weak splice-sites. These specialized regions can conscript proteins that can either positively or negatively influence spliceosome recruitment, aid in exon definition, and promote favorable or unfavorable tertiary structures.

The translationally silent mutation at exon 7 C6T drastically alters exon inclusion from >90% in *SMN1* to 10% in *SMN2*, leading to protein instability and rapid cellular degradation (Burnett *et al.*, 2009; Lorson and Androphy, 2000; Lorson *et al.*, 1998). The 3' ss of both *SMN1* and *SMN2* are inherently weak due to a suboptimal polypyrimidine tract and intron 6 branch point (Lim and Hertel, 2001; Scholl *et al.*, 2007), and therefore, the alternative splice inclusion of exon 7 is heavily dependent on the combinatorial effects of local enhancers and repressors. A systemic examination of the exon–intron region adjacent to and within exon 7 during the past decade has defined these alternative splicing mechanisms (Fig. 2).

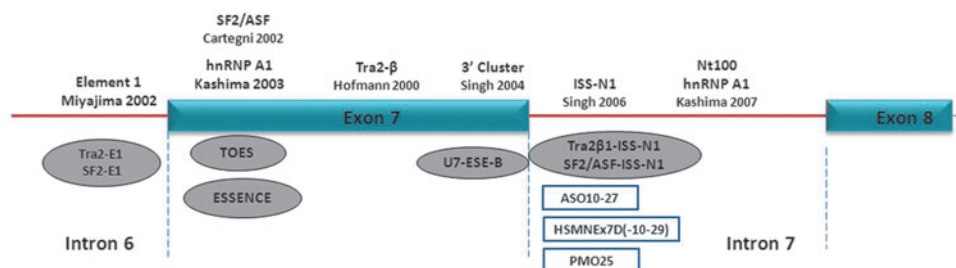


FIG. 2. Schematic representation of *SMN2* exon 7 splicing regulatory elements. The functional elements regulating exon 7 splicing in *SMN2* are depicted along the top. ASOs targeting these regulatory elements are listed at the bottom, including bifunctional ASOs (gray ovals) and conventional ASOs (open rectangles). Figure and legend reprinted by permission from Zhou *et al.* (2013).

A handful of ESEs, including both binding sites for the positive regulators SF2/ASF and TRA2 β 1, heavily influence exon inclusion. One of the most powerful, *Conserved*, rests within the middle of the 54-nucleotide exon 7. *Conserved* is an AG-rich TRA2 β 1 binding site that is both necessary and sufficient for basal levels of exon retention (Hofmann *et al.*, 2000; Lorson and Androphy, 2000; Singh *et al.*, 2004a, 2004b; Singh 2007). Blocking protein recognition of *Conserved* inhibits U2 snRNP recruitment, while elevated TRA2 β 1 expression increases exon retention (Hofmann *et al.*, 2000; Martins de Araujo *et al.*, 2009).

The splice modulatory effect of C6T suggests either ESE or ESS characteristics within this sequence adjacent to the 3' ss. SF2/ASF protein binds to a hexamer motif overlapping position 6 in *SMN1*, defining the exon and recruiting spliceosomal machinery. The C6T substitution of *SMN2* obliterates SF2/ASF binding, suggesting the abrogation of a strong ESE (Cartegni *et al.*, 2002; Lorson and Androphy, 2000). Alternatively, C6T may conversely create an ESS by defining an efficient binding site for hnRNP A1, a protein that blocks spliceosome binding (Kashima and Manley, 2003). Refinement of the 5' region splice mechanisms suggests that C6T may both decrease enhancer and increase suppressor protein binding, though impedance of the former mechanism likely plays a greater role in alternative splicing (Cartegni *et al.*, 2006). An additional consequence of C6T is the creation of an extended inhibitory context (Exinct) that further represses exon inclusion (Singh *et al.*, 2004a, 2004b).

Regional mRNA processing dynamics strongly affect exon 7 3' ss efficiency. The exon 7 5' ss is intrinsically weak and is flanked by dual suppressors including the exon 7 3' cluster and intron 7 *ISS-N1*. Additional splice-suppressing elements have been identified within intron 6 and intron 7 that negatively influence *SMN2* exon 7 inclusion (Kashima *et al.*, 2007; Miyajima *et al.*, 2002; Miyaso *et al.*, 2003). *ISS-N1*, first identified by Singh *et al.* (2006) and further characterized by Hua *et al.* (2008), has been at the forefront of therapeutic ASO applications for exon 7 inclusion. The splice suppressor is located immediately beyond the conserved junction binding region for U1snRNA (intron-7 positions 10–24, CCAGCAUUAUGAAAG), and its adjacent hnRNP A1 and A2 motifs are instrumental in decreasing accessibility and thus binding of this positive splicing factor. *ISS-N1* deletion in the *SMN2* minigene increases $_{FL}SMN$ to *SMN1* levels, an effect that is continued even after abolishment of multiple local splice enhancer elements. Patient-derived SMA cells (*SMN*^{-/-}; *SMN2*^{+/+}) show striking increases in $_{FL}SMN$ and SMN protein after application of a 2'-O-methyl phosphorothioate (2OMePS) ASO targeting *ISS-N1*. The splice modulation was effective in multiple types of cells in culture and did not show off-target splicing effects (Singh *et al.*, 2006).

ASO Therapy

Deletion or dysfunction of *SMN1* is a common denominator in all SMA cases, but it is the alternatively spliced *SMN2* gene that both rescues from embryonic lethality and permits the SMA phenotype. The presence of the deficient *SMN2* rescue gene, together with the duality of both weak 3' and 5' exon 7 splice sites and a multitude of local and regional splice silencers and enhancers, makes SMA uniquely suited for therapeutic ASO intervention. The goal of splice

modulation for *SMN2* is to increase exon 7 incorporation and thus $_{FL}SMN$. Targeting ASOs should increase exon retention with high efficiency, have durable resistance to cellular degradation and robust target specificity, and have low toxicity and high penetrance to target cells including neurons within the CNS. A variety of sequences have been targeted by three different chemistries of ASO: 2OMePS, 2'-O-methoxyethyl (MOE), and morpholino.

Bifunctional ASOs

Sequence targeting within or adjacent to exon 7 can take advantage of the multitude of splice suppressors and activators by combining a sequence-specific ASO with a tethered oligomer that functions as a binding platform for positive splice activators. Two groups using different ASO chemistries (2OMePS and MOE) showed this proof-of-concept by targeting the putative ESE abolished by C6T transition, combined with a tailed splice-factor recruiting ASO (Cartegni and Krainer, 2003; Skordis *et al.*, 2003). Splicing was restored to *SMN1* levels, though the groups did not extend their investigations towards *in vivo* animal analysis. A similar 3' ss exon 7 targeting scheme utilizing lentiviral germline transgenesis also drastically increased exon 7 retention and SMN protein levels (Marquis *et al.*, 2007). The vector expression product, a bifunctional U7 snRNA with a positive splice-factor recruiting tail, increased median survival to 123 days in a severe SMA model (*Smn*^{-/-}; *SMN2*^{+/+}; Meyer *et al.*, 2009).

Two studies have combined bifunctional ASOs targeting splice suppressor sequences with tethered splice activator recruiters of SR-like proteins (SF2/ASF, hTra2 β 1). Osman *et al.* (2012) selected steric block of the *ISS-N1* sequence with a 2OMePS chemistry ASO. Delivery was by intracerebroventricular (ICV) injection at three time points before P5. SMN protein was increased up to 3.5-fold within the CNS, yet doses up to 60 μ g ASO per injection extended median survival to only ~20 days in the *SMN Δ 7* mouse. Similarly, Baughan *et al.* (2009), blocked the E1 splice suppressor upstream of exon 7 with a 2OMePS chemistry. Despite early 2-fold increases in SMN protein throughout the neuraxis after ICV injection, delayed analysis at 5 days showed little or no effect. Severe SMA mice (*Smn*^{-/-}; *SMN2*^{+/+}) had very modest increased survival and weight gain. Low ASO doses, multiple cerebral cannulations, and delayed initial treatment at P2 may have reduced the potential for disease amelioration (Baughan *et al.*, 2009).

Other strategies have directed ASOs to the intron 7/exon-8 junction with the goal of reducing recognition of the exon 8 3' ss, thereby shifting favor to the 3' ss of exon 7 and thus increasing exon 7 retention (Lim and Hertel, 2001; Madocsai *et al.*, 2005). A bifunctional ASO with a tail designed to recruit negative splice effectors (hnRNP A1/A2) can further facilitate splice suppression (Gendron *et al.*, 2006). One such 2OMePS ASO design targeted *SMN2* exon-8; ICV injection at low dose into the *SMN Δ 7* mouse increased exon 7 retention, though survival in SMA animals was not assessed (Dickson *et al.*, 2008).

Suppressor Sequence Steric Block

Chemically modified ASOs that resist cellular endonuclease activity and operate independent of RNase-H degradation can

be used as powerful steric blocks of splice suppressors, thereby promoting robust exon retention. Perhaps the most important splice modulator yet targeted by ASOs for SMA has been the *ISS-N1* sequence adjacent to the exon-7 5' ss. ASO microwalks have revealed strong splice modification when targeting intron positions 10–27, 10–29, 10–34, and 07–14 (Hua *et al.*, 2008; Mitrpant *et al.*, 2013; Porensky *et al.*, 2012; Singh *et al.*, 2009; Zhou *et al.*, 2013).

Recent studies of ASO *ISS-N1* therapy have continued the refinement of delivery methods, ASO chemistries, and dosing strategies, resulting in concurrent incremental disease amelioration in SMA mouse models. A 20-mer 2OMePS ICV injection into SMNΔ7 mice increased $_{FL}SMN$ throughout the CNS and improved righting and weight gain; survival was not assessed. The study was hindered by a total of 10 ICV cannulations delivering a relatively low dose (10 μ g total) of ASO (Williams *et al.*, 2009).

Dose elevation analysis of an 18-mer MOE into a mild mouse model (*Smn*^{-/-}, four copies *SMN2*; ear and tail necrosis at 6 weeks of age) showed that continuous CNS infusion into adult animals of up to 100 μ g/d were well tolerated and produced effective and long-lasting splice modulation (Hua *et al.*, 2010). Embryonic and neonatal ICV bolus therapy (20 μ g maximum dose) delayed necrosis onset up to 12 weeks. In a direct comparison of 2OMePS and MOE chemistries, the former showed increased spinal cord inflammatory markers as well as reduced capacity for exon 7 inclusion after CNS infusion. Further investigation of the MOE chemistry after ICV bolus delivery in the neonatal SMNΔ7 mouse showed strong exon 7 retention throughout the entire neuraxis (Passini *et al.*, 2011). Despite improved weight gain and motor function, as well as increased α -motor neuron counts and concurrent neuromuscular junction architecture, median survival was extended only modestly (26 days). Interestingly, a >8- μ g bolus dose led to rapid animal decline, suggesting acute CNS toxicity of the MOE chemistry in neonates.

Recent studies report long-lasting survival of >100 days after ASO treatment. Hua *et al.* (2011) found marginal increased survival (severe mouse, *Smn*^{-/-}; *SMN2*^{+/+}) after a 20- μ g ICV bolus MOE treatment. Strikingly, multiple subcutaneous or intraperitoneal injections of large dose MOE (320 μ g ASO/g) resulted in 248-day median survival, with some animals living >500 days. Peripheral tissue, particularly liver, showed large increases in SMN protein; brain and spinal cord also showed a 4-fold increase in exon 7 retention, suggesting ASO translocation across the immature blood-brain barrier (BBB) of the neonatal mouse. Recent research has found that even a modest elevation of SMN within the CNS can affect survival (Bowerman *et al.*, 2012; Gavrilina *et al.*, 2008; Marco Passini, personal communication, Families of SMA International SMA Research Group Meeting, June 23, 2012). One factor to consider with different routes of ASO administration is whether the same neurons are transduced and at what times. Our ASO investigation, using a 20-mer morpholino chemistry delivered by single-bolus ICV injection, also showed dose-dependent animal survival (SMNΔ7 mouse) to a median of 112 days without evidence of toxicity. CNS splice modulation was elevated through 60 days after P0 injection, suggesting strong endonuclease resistance (Porensky *et al.*, 2012). Subsequent investigations (morpholino chemistry, 25-mer, ICV injection) have confirmed the

efficacy for *ISS-N1* targeting (Mitrpant *et al.*, 2013; Zhou *et al.*, 2013). These results are confirmation that ASO therapy can drastically increase survival in animal SMA models and should proceed forward as potential strong disease modifiers. Indeed, ISIS Pharmaceuticals is embarking on early clinical trials to assess safety and pharmacokinetics of single dose MOE ASO delivered by lumbar intrathecal injection for children with Type II and III SMA (Chirboga *et al.*, 2013). It remains to be seen whether these patients, rather than newly diagnosed infants with SMA, will show molecular or clinical benefit with such delayed treatment.

When Is Treatment Effective?

SMA disease severity is largely dependent on the amount of full-length SMN protein, which, in the absence of *SMN1*, varies directly with the number of gene copies of *SMN2*. As previously described, disease classification (groups I–IV, with decreasing severity) correlates with *SMN2* copy number (McAndrew *et al.*, 1997; Monani *et al.*, 2000b; Parsons *et al.*, 1998; Schmid and DiDonato, 2007). One of the key differences among patient disease severity scoring is the age at which symptoms manifest. Electrodiagnostic studies in pre-symptomatic patients show early preservation of the motor unit, followed by a precipitous drop, then a much more gradual decline (Finkel, 2013; Swoboda *et al.*, 2005). Surviving motor neurons likely sprout and compensate for the acute loss. These results have been mirrored in the SMNΔ7 mouse (W. Arnold, personal communication, November 2012). Motor neuron and synapse dysfunction have been identified as key early events in SMA mice (Baumer *et al.*, 2009). Two groups have recently developed mouse models with an inducible SMN transgene; embryonic and early neonatal induction yielded much improved survival when compared to a delayed start (Le *et al.*, 2011; Lutz *et al.*, 2011). These studies suggest a critical temporal window for motor neuron loss.

Therapeutic trials in murine SMA models advocate for early intervention yielding the greatest survival benefit. Foust *et al.* (2010) used a self-complementary adeno-associated virus (scAAV9) to replace SMN in SMA pups, vastly improving motor function and survival. When the P1 treatment was delayed to P5 a much more modest effect was seen, and P10 dosed animals were indistinguishable from controls. ASO trials have shown similar results with delayed treatment. Embryonic ICV injection delayed onset of peripheral necrosis in mild SMA mice (four copies of *SMN2*) to a much greater extent than P1 dosing, despite similar rates of splice modulation (Hua *et al.*, 2010). Both ICV and peripheral delayed injection of *ISS-N1* blocking ASO showed decreased survival compared to P0 administration (Hua *et al.*, 2011; Porensky *et al.*, 2012). These and others results have shown that complete restoration of $_{FL}SMN$ yields only moderate phenotypic advantage when achieved at a delayed time point.

It is becoming increasingly clear that therapeutic interventions, including ASO therapy, are most advantageous when delivered before motor neuron collapse. What is uncertain is the effect of increasing SMN in human patients who manifest disease over a time course that is an order of magnitude greater than murine models. Will increasing SMN stabilize and perhaps promote recovery in dysfunctional motor units,

or will SMN therapy only protect the remaining healthy neurons? SMA is not currently part of any standard prenatal or neonatal screening, though the capacity for such exists. Until recently the argument against screening was predicated on the lack of effective therapy, a condition that should change as ASOs and gene therapy proceed toward human clinical trials.

Where Is Treatment Necessary?

SMA has been shown to be a disease first and foremost of the spinal cord motor neuron unit. The most glaring symptoms in human patients relate to a progressive voluntary motor paresis, leading to paralysis-related secondary morbidity. While all cell populations require a basal production of SMN and complete elimination of the protein from any organ leads to cellular and organism death, it is likely that the decreased levels within spinal cord neurons lead to the unique neuromuscular pathology of SMA (Burghees and Beattie, 2009). Furthermore, analysis of laser microdissected motor neurons from SMA mice reveals a lower production of FL_{SMN} from *SMN2* when compared to production levels in other neural populations, suggesting that motor neurons have a further reduction in SMN levels than other cell types (Ruggiu *et al.*, 2012). A logical corollary is whether elevation of SMN within the motor neuron alone will resolve the spectrum of morbidity and mortality seen with SMA.

Recent studies have investigated both increased and decreased expression of SMN in mouse SMA models using tissue-specific promoters targeting skeletal muscle or CNS cells. Increasing SMN from basal rates produced by *SMN2* within spinal cord ventral horn cells, including neurons and supporting glia, yields striking improvement in motor neuron retention, synaptic integrity, muscle physiology, and animal survival (Gavrilina *et al.*, 2008; Martinez *et al.*, 2012; Vicki McGovern, personal communication, Families of SMA International SMA Research Group Meeting, June 22, 2012). Focal SMN reduction in motor neurons and some glial populations results in an early SMA neuromuscular phenotype, though without the spectrum of morbidity and infant mortality seen in SMA mice with ubiquitously low levels of SMN (Park *et al.*, 2010). Inversely, elevation of SMN solely in motor neurons will correct motor neuron counts and restore sensory-motor spinal circuitry, but does not have a major impact on SMA survival (Gogliotti *et al.*, 2012; Mentis *et al.*, 2011). These results suggest that dysfunction of the motor neuron is the major contributor to the neuromuscular symptoms of SMA, but that other neuronal cells likely have a strong effect on disease-related mortality. Increasing SMN in motor neurons is necessary but probably not sufficient to treat the full disease spectrum.

Skeletal muscle is also postulated to contribute to SMA pathology independent of motor neuron dysfunction. Inhibition of myostatin, itself a potent inhibitor of cell growth and regeneration, does not alter SMA survival in murine models (Rindt *et al.*, 2012; Rose *et al.*, 2009; Sumner *et al.*, 2009). Skeletal muscle-specific SMN restoration did not affect mortality in one study and increased survival and myofiber size in another, suggesting that SMN may regulate muscle growth autonomously from CNS innervation (Gavrilina *et al.*, 2008; Martinez *et al.*, 2012). Future studies should clarify muscle-SMA dynamics by developing conditions that

correct or create SMA, rather than showing only a lack of correction. Therefore, a future goal should be to demonstrate a physiologic impairment in force generation after decreasing muscle SMN to levels seen in SMA (with two copies of *SMN2*).

Similarly, multiple groups have documented dysfunction of the autonomic nervous system (ANS) in SMA models, largely manifest by cardiac dysfunction (Bevan *et al.*, 2010; Gogliotti *et al.*, 2012; Heier *et al.*, 2010; Shababi *et al.*, 2010). The autonomic instability and resulting bradyarrhythmias are at least partially corrected after systemic restoration of SMN (Bevan *et al.*, 2010; Shababi *et al.*, 2012). There is growing clinical evidence of autonomic dysfunction in SMA, again largely manifest by cardiac arrhythmia, though these reports are limited to retrospective or small cohort reports (Arai *et al.*, 2005; Araujo Ade *et al.*, 2009; Bach 2007; Finsterer and Stollberger, 1999; Hachiya *et al.*, 2005). Intrinsic basal heart-rate differences between mice and humans may be a reason for the purported paucity of clinical bradyarrhythmia. The extent and frequency of autonomic dysfunction in human SMA are unknown, and a more comprehensive prospective clinical study would be of great benefit.

Therapeutic trials with both viral-SMN vectors and ASOs have offered conflicting evidence of the importance of extra-CNS SMN elevation. Vector delivery of SMN to the CNS but not peripheral tissue results in extended SMA survival, but peripheral injection with diffuse CNS, skeletal and cardiac muscle, and ANS transduction showed even more impressive survival (Foust *et al.*, 2010; Passini *et al.*, 2010). Likewise, ASO therapy delivered directly into the CNS, without evidence of subsequent peripheral splice correction, strongly modulated SMA phenotype in various mouse models (Hua *et al.*, 2010; Mitrpant *et al.*, 2013; Porensky *et al.*, 2012; Zhou *et al.*, 2013). In contrast, peripheral ASO injection, albeit at a very high dose and at multiple neonatal time points, also produced extended SMA mouse survival (Hua *et al.*, 2011). The authors found concurrent elevated levels of liver-specific IGF-1 in treated mice, and postulate that strong splice modulation in the liver may be an important condition for prolonged survival.

Future therapeutic development of ASOs for SMA must consider whether mice can be more completely rescued (*i.e.*, longer survival) with repeat CNS dosing and whether peripheral delivery is required. CNS delivery is essential, whether by bare ASOs injected directly by intrathecal or intraventricular cannulation or by tagged oligomers that can cross the BBB and subsequently escape into cerebrospinal fluid pathways for full neuraxis distribution. The importance of extra-CNS targeting, such as autonomic nervous system ganglia, requires further investigation. Optimal treatment strategies may require a hybridized delivery mechanism into multiple compartments.

Future Considerations/Outlook

ASO therapy for SMA has made great strides during the past decade, including optimization of target mRNA sequence and impressive effects in severe models of disease. ASO splice modulation, together with SMN gene therapy and small molecules, has altered the conversation of SMA intervention from symptom palliation toward disease prevention and stabilization. Many questions remain before

efficacy in human patients can be assessed. As already detailed, questions yet to be answered include selection of the optimal oligomer chemistry, as well as extensive questions regarding frequency and location of dosing. ASOs have illuminated disease insights that were not previously appreciated; the most interesting and exciting is that additional neuron groups other than the motor neuron could be involved. The role of peripheral tissues is harder to ascertain.

A logical next step towards human clinical trials would be translation into a large animal with anatomy and physiology similar to humans. Timing, volume, and location of dosing could be optimized, and potential toxicity better observed. Moreover, recapitulation of the unique human morphology, including a fully intact BBB and large-volume cerebrospinal fluid space, would better simulate human applications. No large SMA models currently exist, though both transgenic and vector-induced porcine models are in development (Lorson *et al.*, 2008; personal communication, Monique Lorson, Families of SMA International SMA Research Group Meeting, June 24, 2011; personal communication, Sandra Duque, Families of SMA International SMA Research Group Meeting, June 21, 2012). Whatever the path taken, it is clear that ASO therapy for SMA is rapidly approaching human applications.

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Author Disclosure Statement

The authors declare that they have no competing interests.

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