Review Article • DOI: 10.2478/s13380-013-0109-2 • Translational Neuroscience • 4(1) • 2013 • 1-7

VERSITA

ANTISENSE OLIGONUCLEOTIDE MEDIATED THERAPY OF SPINAL MUSCULAR ATROPHY

Senthilkumar Sivanesan[1],
Matthew D. Howell[1],
Christine J. DiDonato[2,3],
Ravindra N. Singh[1,*]

[1]*Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50011*

[2]*Department of Pediatrics, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611*

[3]*Human Molecular Genetics Program, Ann & Robert H. Lurie Children's Hospital of Chicago, Research Center, Chicago, IL 60614*

Received 10 February 2013
accepted 13 February 2013

Abstract

Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality. SMA results from deletions or mutations of *survival motor neuron 1* (*SMN1*), an essential gene. *SMN2*, a nearly identical copy, can compensate for *SMN1* loss if *SMN2* exon 7 skipping is prevented. Among the many cis-elements involved in the splicing regulation of *SMN* exon 7, intronic splicing silencer N1 (ISS-N1) has emerged as the most effective target for an antisense oligonucleotide (ASO)-mediated splicing correction of *SMN2* exon 7. Blocking of ISS-N1 by an ASO has been shown to fully restore *SMN2* exon 7 inclusion in SMA patient cells as well as *in vivo*. Here we review how ISS-N1 targeting ASOs that use different chemistries respond differently in the various SMA mouse models. We also compare other ASO-based strategies for therapeutic splicing correction in SMA. Given that substantial progress on ASO-based strategies to promote *SMN2* exon 7 inclusion in SMA has been made, and that similar approaches in a growing number of genetic diseases are possible, this report has wide implications.

Keywords

• Antisense oligonucleotide • Splicing • ISS-N1 • ASO • SMA • SMN • PMO • MOE

Introduction

Spinal muscular atrophy (SMA) is a leading neurodegenerative disease of children and infants [1-7]. SMA is characterized by the progressive loss of α-motor neurons in the spinal cord, which leads to paralysis of the trunk and limbs and respiratory insufficiency [2-7]. SMA is caused by the homozygous functional loss of the *survival motor neuron 1* (*SMN1*) gene due to deletion, subtle mutation, or gene conversion [2-5]. *SMN2*, a nearly identical copy of *SMN1*, fails to compensate for *SMN1* loss due to a critical C to T transition at the 6th position (C6U in transcript) in exon 7 [8]. C6U leads to exon 7 skipping during pre-mRNA splicing of *SMN2* [9]; as a consequence, a truncated, dysfunctional and rapidly degraded protein (SMNΔ7) is produced [10,11]. The defects caused by *SMN1* deficiency can be compensated by increased copies of *SMN2*, which produces low levels of full-length SMN [12]. Considering all SMA patients retain at least one functional copy of *SMN2*, it is reasonable to assume that strategies aimed at correcting *SMN2* exon 7 splicing hold the promise for a treatment. Indeed, the postnatal increase of SMN levels through *SMN2* exon 7 splicing correction—by both small compounds and antisense oligonucleotides (ASOs)—provides substantial therapeutic benefits in animal models of SMA [13-16]. Several recent reviews summarized the general progress in the rapidly evolving field of oligonucleotide research [17-21]. This review is inspired by several independent reports in which different ASO chemistries against a single intronic target have shown unprecedented therapeutic benefits in various mouse models of SMA. Here we focus on challenges and lessons drawn from the recent ASO-based therapeutic approaches in SMA, and their implications for nucleic-acid-based therapy of a growing number of genetic diseases.

Developing ASO-based splicing correction therapeutics is not a straight-forward task. Splicing modulation using an ASO-mediated approach requires sequestration of a splicing cis-element [18]. Since the majority of the human genome is transcribed [22], there is a substantial risk that an ASO could anneal to analogous sequences through mismatch base pairing and have off-target effects. Furthermore, transcribed RNAs form secondary structures within microseconds of emerging from polymerase II [23-25]. Thus, low annealing efficiency of ASOs with targets located in the structured region of transcripts results in reduced antisense response [26]. Most RNA-interacting proteins recognize small sequence motifs. Therefore, unwanted interactions of proteins with ASOs could also contribute to reduced antisense efficacy. Hence, designing an ASO that does not interact with protein factors and anneals to a desired cis-element located within a structurally accessible region of RNA remains the most challenging aspect of developing an ASO-based therapeutic strategy.

The degree of antisense response is dependent upon the strength of the targeted splicing cis-element as well as the nature of the context created by the duplex formed between the ASO and its target. Splicing is a dynamic process that involves numerous RNA-protein and RNA-RNA interactions [27-30], so it is safe

* *E-mail: singhr@iastate.edu*

to assume that permanent annealing of an ASO to its target affects several interactions and enforces remodeling of the RNA structure of the target and associated sequences. Therefore, the location of the target, ASO length and ASO chemistry play critical roles in determining the extent of antisense response. *In vivo* efficacy of an ASO is dependent upon additional factors including but not limited to dose, frequency, route and method of administration. The specific animal model and immune tolerance for the administered ASO also critically influence the phenotype of the ASO-treated animals.

ISS-N1 as a promising therapeutic target

To restore *SMN2* exon 7 inclusion in SMA, it is essential that splice-switching ASOs target an inhibitory cis-element. It is also desirable that the inhibitory cis-element is located within an intronic sequence in order to avoid interfering with the translation and transport of mRNA. Discovery of ISS-N1 (intronic splicing silencer N1) in 2006 provided a major breakthrough for an ASO-mediated splicing correction approach in SMA [31]. ISS-N1 is a 15-nucleotide sequence spanning the 10th to 24th positions of intron 7 (Figure 1A). ISS-N1 creates a strong negative context at the 5′ splice site (5′ ss) of exon 7, and ISS-N1 deletion fully restores *SMN2* exon 7 inclusion [31]. Remarkably, the deletion of ISS-N1 confers such a stimulatory effect on *SMN2* exon 7 splicing that all known positive cis-elements within exon 7 become dispensable. Therefore, ISS-N1 is described as the master checkpoint of *SMN2* exon 7 splicing regulation [32]. The therapeutic efficacy of ISS-N1 was first assessed by blocking ISS-N1 using a 20-mer ASO (Anti-N1) carrying phosphorothioate backbone and 2′-O-methyl modification (2′-OMe). Even at a very low concentration (5 nM), Anti-N1 substantially elevated SMN levels in type I SMA patient cells [31]. These unprecedented results established that ASO-mediated sequestration of an intronic sequence could fully correct a splicing defect linked to an exonic mutation.

Two years after the discovery of ISS-N1, the Krainer group in collaboration with ISIS Pharmaceuticals conducted a systematic antisense microwalk employing ASOs carrying phosphorothioate backbone and 2′-O-methoxyethyl modification (MOE) chemistry [33]. MOE chemistry, a proprietary product of ISIS Pharmaceuticals, is considered to confer increased nuclease resistance and decrease non-specific protein interactions [34]. The authors concluded that the stimulatory effect of ISS-N1 targeting MOE ASO was higher than other MOE ASOs targeting different regions of *SMN2* [33]. They independently validated the strong inhibitory nature of ISS-N1 and generated interest in an ASO-mediated therapy for SMA.

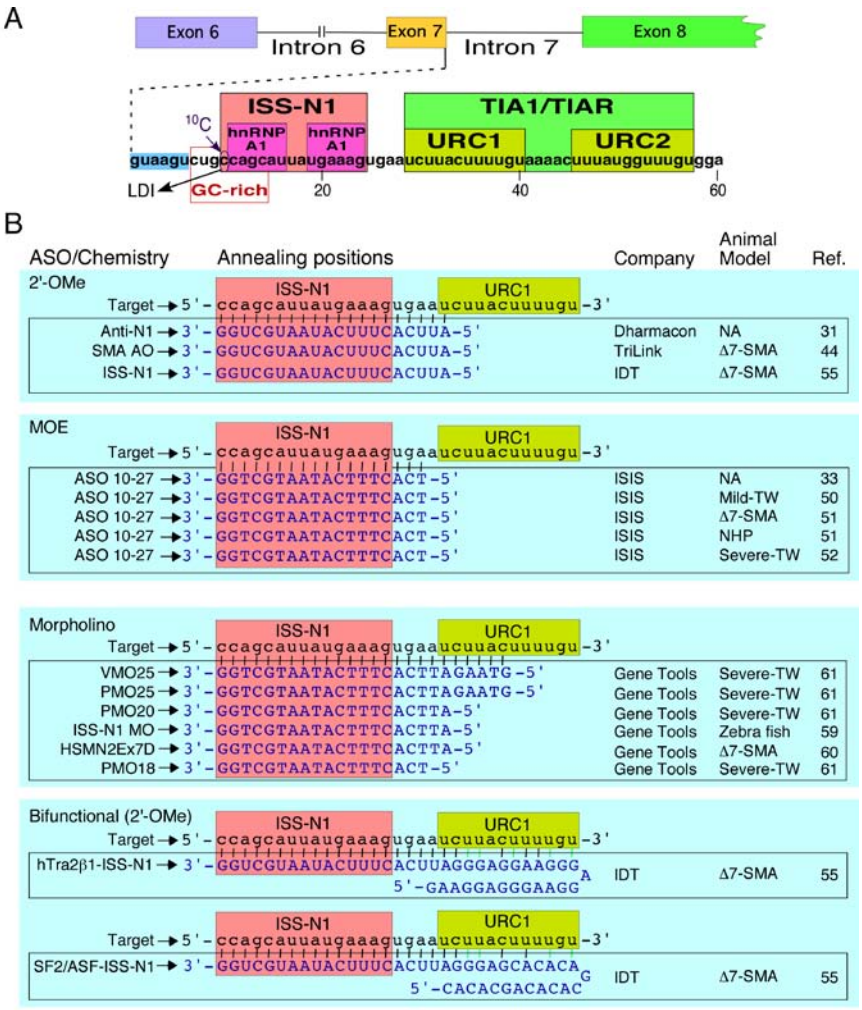

Figure 1. ASO-mediated splicing correction in SMA. (A) Diagrammatic representation of splicing cis elements. Splicing cis-elements are indicated by boxes. Numbering starts from the first position of *SMN2* intron 7. Cis-elements shown here are described in detail in a recent review by Singh and Singh [9]. ISS-N1 is negative cis-element that has emerged as the leading therapeutic target for an ASO-mediated splicing correction in SMA [31-33,50-52,55,60,61]. Binding sites of inhibitory factor hnRNP A1 [33] and stimulatory factors TIA1 and TIAR [39] have been indicated. GC-rich sequence constitutes the target for splicing correction by the shortest ASO [35]. A cytosine residue at the 10th intronic position (^{10}C) associates with a long-distance interaction (LDI) with the downstream intronic sequences [36]. Sequestration of ISS-N1 or GC-rich sequence appears to abrogate the inhibitory LDI associated with ^{10}C. (B) Diagrammatic representation of annealing positions of ISS-N1 targeting ASOs. All ISS-N1 targeting ASOs sequestered the first residue of ISS-N1. Reference numbers of studies using different ASOs targeting ISS-N1 are shown. Abbreviations: ISS-N1, Intronic splicing silencer N1; LDI, long-distance interaction; URC, U-rich cluster; ASO, antisense oligonucleotide; 2′-OMe, an ASO with phosphorothioate backbone and 2′-O-methyl modification; MOE, an ASO with phosphorothioate backbone and 2′-O-methoxyethyl modification; Δ7-SMA, severe SMA model [45]; NHP, non-human primate; Mild-TW, mild Taiwanese model of SMA [53]; Severe-TW, severe Taiwanese model of SMA [56,57].

ISS-N1 encompasses two hnRNP A1/A2 motifs and overlaps with a unique GC-rich motif (Figure 1A; 33,35). A cytosine residue at the 10th position (^{10}C) of intron 7 occupies the first position of ISS-N1. Sequestration of the first five residues of ISS-N1, including ^{10}C, was necessary for the stimulatory effect of the ISS-N1-targeting ASOs [36]. Research data supports that ISS-N1-targeting ASOs stimulate *SMN2* exon 7 inclusion by reconstructing a positive context in addition to displacing inhibitory factors (i.e. hnRNP A1/A2) at the 5′ ss of *SMN2* exon 7 [9]. The 5′ ss of exon 7 is weakened by the presence of a number of inhibitory cis-elements [31,35-38]. The only intronic positive cis-element in the vicinity of the 5′ ss of *SMN2* exon 7 is the U-rich clusters (URCs) immediately downstream of ISS-N1 (Figure 1A) [39]. In particular, URC1 and URC2 have been shown to interact with T-cell-restricted intracellular antigen 1 (TIA1) and TIA1-related (TIAR) proteins that are splicing stimulatory factors [39]. Recently, a point mutation within the glutamine-rich domain of TIA1 has been shown to affect *SMN2* exon 7 splicing in the patients of Welander distal myopathy (WDM), an adult onset autosomal-dominant disorder characterized by distal limb weakness [40]. These findings are significant as they reveal for the first time that a single point mutation within a splicing factor could have an adverse effect on *SMN2* exon 7 splicing.

It has been suggested that the ASO-mediated sequestration of ISS-N1 increases the interaction efficiency of TIA1/TIAR and/or other members of the glutamine-rich-RNA-binding proteins that stimulate *SMN2* exon 7 inclusion by binding to U-rich sequences immediately downstream of ISS-N1 [39]. Such interaction is necessary to recruit U1 snRNP at the weak 5′ ss of exon 7. Recruitment of U1 snRNP at the 5′ ss is the first and most critical step of spliceosomal assembly, which is a multistep dynamic process of intron removal [41]. Recent reports suggest U1 snRNP recruitment plays a role in regulating isoform expression and polyadenylation [42,43]. Considering intron 7 is the last intron of *SMN2*, it is possible that the enhanced recruitment of U1 snRNP upon sequestration of ISS-N1 provides additional benefits unrelated to splicing.

In vivo studies with ISS-N1 targeting ASOs

The first *in vivo* study of an ISS-N1 targeting ASO was conducted by Williams et al. with a 20-mer 2′-OMe ASO (SMA AO) employing the Δ7-SMA mouse model [44]. The median survival age of Δ7-SMA mice is ~13 days, and this model has been widely used for understanding the pathogenesis of SMA as well as pre-clinical testing of potential SMA drugs [45-49]. This particular study administered ASOs on P1, 3, 5, 7, and 10 by bilateral intracerebroventricular (ICV) injections (1 μg per lateral ventricle), and tissues were harvested for analysis at P12. SMA AO increased SMN levels in brain and spinal cord, whereas control AO had no appreciable effect. In addition, SMA AO increased body weight and improved righting reflex of the Δ7-SMA mice, whereas control AO provided no phenotypic benefit. Despite the limited nature of this study, the findings were encouraging for subsequent studies that employed different oligonucleotide chemistries, routes of oligonucleotide delivery and mouse models.

The Krainer group at Cold Spring Harbor Laboratory in collaboration with ISIS Pharmaceuticals conducted a series of *in vivo* studies with ISS-N1 targeting MOE ASOs [50-52]. In particular, they used an 18-mer MOE ASO (ASO-10-27) that sequesters the entire ISS-N1 and three residues downstream of ISS-N1 (Figure 1B). The initial study employed a mild SMA model (Taiwanese SMA model; four copies of *SMN2*) that displays tail and ear necroses [53]. As expected, ICV injections of ASO-10-27 in adult mice stimulated *SMN2* exon 7 inclusion in brain and spinal cord [50], and immunostaining confirmed the ASO-induced upregulation of SMN in brain and spinal cord. Single embryonic or neonatal ICV injection of ASO-10-27 successfully rescued the phenotype. For instance, tail and ear necroses were either completely prevented or substantially delayed by treatment with ASO-10-27. Embryonic injections were more effective than neonatal injections. Overall, the study demonstrated the efficacy of MOE ASO in treating mild SMA mice when given very early.

The authors also compared the MOE ASO (ASO-10-27) with the 2′-OMe ASO used by Williams et al. [44,50]. In contrast to the results obtained by Williams et al. in Δ7-SMA mice [44], ICV injection of 2′-OMe ASO in the Taiwanese SMA model did not stimulate *SMN2* exon 7 inclusion [50]. In addition, the 2′-OMe ASO, but not the MOE ASO, produced proinflammatory effects. The discrepancy between these two studies is not entirely unexpected since they used different sources of 2′-OMe oligonucleotide synthesis and different mouse models (Figure 1B). A recent study that involved repeated long-term subcutaneous treatment of Duchenne muscular dystrophy (DMD) mice with a 2′-OMe ASO produced tangible therapeutic benefits without any safety concerns [54]. Therefore, it is possible that the proinflammatory effect of 2′-OMe ASO is a characteristic of the Taiwanese SMA model, and/or this model is more tolerant to MOE chemistry than to 2′-OMe chemistry. Recently, the Lorson group used a third source of oligonucleotide synthesis and independently confirmed upregulation of SMN levels in brain and spinal cord upon ICV administration of an ISS-N1-targeting 2′-OMe ASO in Δ7-SMA mice [55].

The Passini group at Genzyme Corporation, in collaboration with the Krainer group and ISIS Pharmaceuticals, tested the efficacy of ASO-10-27 in Δ7-SMA mice [51]. A single ICV injection of ASO-10-27 (4 μg) at P0 increased median life expectancy from 16 to 26 days. Treated mice had increased numbers of motor neurons within the spinal cord and improved motor function. Interestingly, higher and lower doses of ASO delivered by ICV were less effective. The authors also performed preliminary experiments in nonhuman primates (NHP) and demonstrated that 1) intrathecal infusion over a 24 hr period of a fixed dose of ASO 10-27 (3 μg) was well tolerated and could achieve therapeutically relevant levels of ASO 10-27 and 2) intrathecal infusion is an effective delivery method for global distribution of ASOs to the spinal cord of NHPs. The latter finding will be important for the future therapeutic development of other ASOs for SMA, and more broadly for other therapies that will require delivery to the spinal cord [51].

Encouraged by the results of ICV injections of MOE ASOs in two SMA mouse models

[50,51], the Krainer group in collaboration with ISIS Pharmaceuticals performed an elaborate study using the Taiwanese type I SMA mice (two copies of *SMN2*), a more severe SMA mouse model [52]. This model was generated from mild Taiwanese SMA mice and has a median lifespan of ~10 days [56,57]. The major finding of this study was an unexpectedly high efficacy of ASO-10-27 when delivered peripherally [52]. The animals tolerated a very high dose of subcutaneously (SC) administered ASO-10-27. The best survival outcome was observed when animals received two SC administrations (one at P0-P1 and the other at P2-P3) each at 160 µg/g body weight [52]. The median life expectancy of treated mice increased ~25 fold (from ~10 days to ~273 days). Interestingly, ICV delivery was substantially less efficacious than SC delivery. For instance, ICV delivery of ASO-10-27 (20 µg) at P1 only increased median life expectancy of Taiwanese type 1 SMA mice from 10 days to 16 days [52]. Although somewhat controversial, these results underscore the peripheral requirement of SMN protein and demonstrate the long-term benefits of early ASO administration in severe SMA mice.

In vivo studies with ISS-N1 targeting morpholinos

Phosphorodiamidate morpholino oligomers (PMOs) are a class of compounds in which the ribose sugar moiety is replaced with a morpholino ring and the phosphorothioate group is replaced with an uncharged phosphorodiamidate group. PMOs and their derivatives have been widely used for therapeutic splicing correction in animal models [18,58,59]. Porensky and coworkers from the Burghes laboratory employed Δ7-SMA mice to test the efficacy of an ISS-N1 targeting PMO (HSMN2Ex7D) that sequestered the entire ISS-N1 and 5 residues downstream of ISS-N1 (60; Figure 1B). ICV administration of HSMN2Ex7D corrected *SMN2* exon 7 splicing and increased levels of SMN in brain and spinal cord. A single ICV administration of 54 µg HSMN2Ex7D at P0 prolonged median life expectancy of Δ7-SMA mice from ~15 days to more than 100 days. Interestingly, peripheral delivery of HSMN2Ex7D produced no therapeutic benefit in Δ7-SMA mice. These results are consistent with the prevailing view that brain and spinal cord are the primary tissues in which high levels of SMN are required for an effective SMA therapy. The results also demonstrate PMO as superior to MOE chemistry when delivered through ICV injections.

A recent study by the Muntoni group and coworkers utilizing Taiwanese type I SMA mice independently confirmed the high therapeutic efficacy of ISS-N1 targeting PMOs [61]. The authors used PMOs of three different sizes and showed 25-mer PMO (PMO25) that sequestered the entire ISS-N1 and 10 residues downstream of ISS-N1 conferred better therapeutic efficacy than the 18-mer or 20-mer PMO (Figure 1B). A single ICV administration of PMO25 (40 µg/g body weight) at P0 increased median life expectancy of Taiwanese type I SMA mice from ~10 days to more than 200 days. Contrary to findings in the Δ7-SMA mouse model [60], the authors found no significant difference in therapeutic efficacy of PMOs when delivered peripherally or ICV [61]. These results again underscore the difference between the Δ7-SMA and Taiwanese type I SMA models with regards to the efficacy of an identical ASO. The observation that peripheral delivery of MOE and PMO25 has therapeutic benefit in the Taiwanese type I SMA model but not in the Δ7-SMA model supports that timings of Blood-brain barrier formation between these two mouse models could be distinct.

In vivo studies with ISS-N1 targeting bifunctional ASOs

Bifunctional ASOs anneal to specific RNA sequences and recruit additional factors through hanging tails [62]. The Lorson group used bifunctional 2′-OMe ASOs to block ISS-N1 and recruit either Tra2-β1 or SF2/ASF in the vicinity of ISS-N1 [55]. Upon ICV administration (6 µg/day) at P1, P3 and P5, these ASOs increased weight and gross motor functions of Δ7-SMA mice. Treatment also increased the median life expectancy of Δ7-SMA mice from ~16 days to 18-20 days. The efficacy of the ASO carrying a Tra2-β1 motif was higher than the ASO carrying a SF2/ASF motif; however, the therapeutic benefit of these bifunctional ASOs was substantially lower than ISS-N1-targeting MOEs and PMOs [52,60,61].

The low efficacy of ISS-N1 targeting bifunctional ASOs could result from a limited ability of Tra2-β1 and/or SF2/ASF to activate the 5′ ss of *SMN2* exon 7. Since these bifunctional ASOs are larger, they may fold into secondary and/or higher order structures that could sequester critical residues responsible for annealing and/or protein recruitment. In addition, large oligonucleotides are notorious for non-specific trapping of protein factors. Some of these problems could be overcome through trial and error by testing different tailed sequences in the context of different chemistries. However, since a bifunctional ASO is designed to trap an arbitrary chosen splicing factor, there is a risk of unwanted secondary effect on splicing of other genes.

Other ASO-based approaches

Several other ASO-based studies independent of ISS-N1 have been used for splice correction in SMA. A bifunctional ASO targeting element 1 located within intron 6 exhibited efficacy in SMA mice [63,64]. Other bifunctional ASOs that anneal to *SMN2* exon 7 and recruit splicing factors either at the 3′ or 5′ ss of exon 7 have shown encouraging results *in vitro*, *ex vivo* and in SMA mice [65-68]. Blocking the 3′ ss of exon 8 by an ASO embedded in U7 snRNAs was shown to prevent exon 7 skipping and increase SMN levels in SMA patient-derived cells [69]. These results underscore that the transcripts retaining *SMN2* intron 7 are capable of being polyadenylated, exported out of the nucleus, and translated in the cytoplasm. Recently, an 8-mer ASO (3UP8) that sequesters a GC-rich sequence within intron 7 was shown to have a strong stimulatory effect on *SMN2* exon 7 inclusion in SMA patient fibroblast cell lines [35,70]. In addition to being cost effective, small ASOs offer the advantage of being less tolerant towards non-canonical and mismatch base pairing, a major cause for the nonspecific effect associated with large ASOs. Consistently, 3UP8 showed zero tolerance for a single mismatch base pair mutation at the target site [35]. However, it remains to be seen whether the

expected benefit of a small ASO is realized in a mouse model of SMA.

Conclusions and future directions

SMA is a rare disease in which a correctable copy of the gene (*SMN2*) is universally present in all SMA patients. In addition, SMA is unique in that all patients have the opportunity for an ASO-based therapy to increase SMN levels by correcting the pre-mRNA splicing of one exon. In the absence of structure-specific targets, an ASO-based approach remains the best option for gene-specific splicing correction. In general, the success of an ASO-based approach for splicing correction depends on the potency of the target. Characterizing an efficient intronic target for inducing exon inclusion remains a challenging endeavor.

The discovery of ISS-N1 provided a major breakthrough as it demonstrated for the first time that a large intronic motif could serve as a master regulator of *SMN2* exon 7 splicing. The finding that an ASO-mediated sequestration of ISS-N1 fully restores *SMN2* exon 7 inclusion led to several *in vivo* studies in mouse models of SMA. Consistently, ISS-N1 stands as the most scrutinized antisense target for the potential treatment of a genetic disease. The last few years have witnessed remarkable progress towards the development of ISS-N1-based therapy. For instance, ISIS Pharmaceuticals successfully concluded a phase 1 clinical trial of ISIS-SMN_{Rx}, an ISS-N1 targeting ASO with MOE chemistry. This is the first clinical trial for an ASO-mediated restoration of exon inclusion in a human disease. Consistent with the results in NHP [51], intrathecal infusion of ISIS-SMN_{Rx} was well tolerated in SMA patients during phase 1 clinical trial. If successful, this would be the first mechanism-based therapy for SMA.

The great promise of an ASO based therapy is derived from the limitless number of compounds that could be tested against the same target. Two recent pre-clinical studies conducted independently support ISS-N1 targeting PMOs as an entirely different class of very effectives ASOs [60,61]. When delivered peripherally, ISS-N1 targeting PMOs appear to offer an advantage of being active at a substantially lower dose than a MOE ASO [52,61]. In a recent Duchenne muscular dystrophy clinical trial, the PMO backbone was well tolerated when delivered systemically [71]. However, it remains to be seen whether intrathecal administration of PMOs are tolerated in NHPs. It will be also tempting to determine if systemic delivery of PMOs is a viable alternative in SMA. Although in preliminary stages, bifunctional and small ASOs hold potential to offer additional antisense compounds. With the ongoing progress in clinical trials and advancement in oligonucleotide chemistry and delivery schemes, it is only a matter of time until an array of effective ASO-based drugs becomes available for the treatment of SMA, a devastating disease of children and infants. The lessons learned from ASO-based therapy development in SMA will be extremely informative for other diseases that could utilize similar approaches.

Acknowledgements

This work was supported by grants from the National Institutes of Health (R01 NS055925, R21 NS072259 and R21 NS080294) and Salsbury Endowment (Iowa State University, Ames, IA, USA) to RNS. CJD is supported by Families of SMA grant DID1214 and the Muscular Dystrophy Association grant 255785. The authors acknowledge Dr. Natalia N. Singh and Joonbae Seo for providing critical comments on the manuscript.

Disclosures and competing interests

ISS-N1 target (US patent # 7,838,657) was discovered in the Singh lab at UMASS Medical School (Worcester, MA, USA). Inventors, including RNS and UMASS Medical School, are currently benefiting from licensing of ISS-N1 target (US patent # 7,838,657) to ISIS Pharmaceuticals. A GC-rich target for a small ASO (Patent# US 20110269820) was discovered in the Singh lab at Iowa State University (Ames, IA, USA). Therefore, inventors including RNS and Iowa State University could potentially benefit from any future commercial exploitation of the above-mentioned target.

References

[1] Pearn J., Classification of spinal muscular atrophies, Lancet, 1980, 8174, 919-922

[2] Lefebvre S., Burglen L., Reboullet S., Clermont O., Burlet P., Viollet L., et al., Identification and characterization of a spinal muscular atrophy-determining gene, Cell, 1995, 80, 1-5

[3] McAndrew P. E., Parsons D. W., Simard L. R., Rochette C., Ray P. N., Mendell J. R., et al., Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number, Am. J. Hum. Genet., 1997, 60, 1411-1422

[4] Prior T. W., Spinal muscular atrophy diagnostics, J. Child. Neurol., 2007, 22, 952-956

[5] Wirth B., An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA), Hum. Mutat., 2000, 15, 228-237

[6] Lannaccone S. T., Modern management of spinal muscular atrophy, J. Child. Neurol., 2007, 22, 974-980

[7] Araujo A. P., Araujo M., Swoboda K. J., Vascular perfusion abnormalities in infants with spinal muscular atrophy, J. Pediatr., 2009, 155, 292-294

[8] Lorson C. L., Hahnen E., Androphy E. J., Wirth B. A., Single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy, Proc. Natl. Acad. Sci. USA, 1999, 96, 6307-6311

[9] Singh N. N., Singh R. N., Alternative splicing in spinal muscular atrophy underscores the role of an intron definition model, RNA Biol., 2011, 8, 600-606

[10] Vitte J., Fassier C., Tiziano F. D., Dalard C., Soave S., Roblot N., et al., Refined characterization of the expression and stability of the SMN gene products, Am. J. Pathol., 2007, 171, 1269-1280

[11] Cho S., Dreyfuss G., A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity, Genes Dev., 2010, 24, 438-442

[12] Monani U. R., Sendtner M., Coovert D. D., Parsons D. W., Andreassi C., Le T. T., Jablonka S., et al., The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy, Hum. Mol. Genet., 2000, 9, 333-339

[13] Lorson C. L., Rindt H., Shababi M., Spinal muscular atrophy: mechanisms and therapeutic strategies, Hum. Mol. Genet., 2010,19, R111-118

[14] Van Meerbeke J. P., Sumner C. J., Progress and promise: the current status of spinal muscular atrophy therapeutics, Discov. Med., 2011, 12, 291-305

[15] Kolb S. J., Kissel J. T., Spinal muscular atrophy: a timely review, Arch. Neurol., 2011, 68, 979-984

[16] Markowitz J. A., Singh P., Darras B. T., Spinal muscular atrophy: a clinical and research update, Pediatr. Neurol., 2012, 46, 1-12

[17] Hammond S. M., Wood M. J., Genetic therapies for RNA mis-splicing diseases, Trends Genet., 2011, 27, 196-205

[18] Kole R., Krainer A. R., Altman S., RNA therapeutics: beyond RNA interference and antisense oligonucleotides, Nat. Rev. Drug Discov., 2012, 11, 125-140

[19] Southwell A. L., Skotte N. H., Bennett C. F., Hayden M. R., Antisense oligonucleotide therapeutics for inherited neurodegenerative diseases,Trends Mol. Med., 2012,18, 634-643

[20] Deleavey G. F., Damha M J., Designing chemically modified oligonucleotides for targeted gene silencing, Chem. Biol., 2012, 19, 937-954

[21] Aartsma-Rus A., Overview on AON design, Methods Mol. Biol., 2012, 867,117-129

[22] ENCODE Project Consortium, Birney E., Stamatoyannopoulos J. A., Dutta A., Guigó R., Gingeras T. R., et al., Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project, Nature, 2007, 447, 799–816

[23] Gralla J., Crothers D. M., Free energy of imperfect nucleic acid helices. II. Small hairpin loops, J. Mol. Biol., 1973, 73, 497–502

[24] Pörschke D., Thermodynamic and kinetic parameters of an oligonucleotide hairpin helix, Biophys. Chem., 1974, 1, 381-386

[25] Xu Z., Almudevar A., Mathews D. H., Statistical evaluation of improvement in RNA secondary structure prediction, Nucleic Acids Res., 2012, 40, e26

[26] Freier S. M., Watt A. T., Basic principles of antisense drug discovery, In: Antisense drug technologies, 2nd Ed., Taylor & Francis, UK, 2007, 118–138

[27] Xing Y., Lee C., Relating alternative splicing to proteome complexity and genome evolution, Adv. Exp. Med. Biol., 2007, 623, 36-49

[28] Nilsen T. W., Graveley B. R., Expansion of the eukaryotic proteome by alternative splicing, Nature, 2007, 463, 457-463

[29] Matlin A. J., Moore M. J., Spliceosome assembly and composition, Adv. Exp. Med. Biol., 2007, 623, 14-35

[30] Wahl M. C., Will C. L., Lührmann R., The spliceosome: design principles of a dynamic RNP machine, Cell, 2009, 136, 701-718

[31] Singh N. K., Singh N. N., Androphy E. J., Singh R. N., Splicing of a critical exon of human Survival Motor Neuron is regulated by a unique silencer element located in the last intron, Mol. Cell. Biol., 2006, 26, 1333-1346

[32] Buratti E., Baralle M., Baralle F. E., Defective splicing, disease and therapy: searching for master checkpoints in exon definition, Nucleic Acids Res., 2006, 34, 3494-3510

[33] Hua Y., Vickers T. A., Okunola H. L., Bennett C. F., Krainer A. R., Antisense masking of an hnRNP A1/A2 intronic splicing silencer corrects SMN2 splicing in transgenic mice, Am. J. Hum. Genet., 2008, 82, 834-848

[34] Teplova M., Minasov G., Tereshko V., Inamati G. B., Cook P. D., Manoharan M., et al., Crystal structure and improved antisense properties of 2'-O-(2-methoxyethyl)-RNA, Nat. Struct. Biol., 1999, 6, 535-539

[35] Singh N. N., Shishimorova M., Cao L. C., Gangwani L., Singh R. N., A short antisense oligonucleotide masking a unique intronic motif prevents skipping of a critical exon in spinal muscular atrophy, RNA Biol., 2009, 6, 341-350

[36] Singh N. N., Hollinger K., Bhattacharya D., Singh R. N., An antisense microwalk reveals critical role of an intronic position linked to a unique long-distance interaction in pre-mRNA splicing, RNA, 2010, 16, 1167-1181

[37] Singh N. N., Androphy E. J., Singh R. N., In vivo selection reveals combinatorial controls that define a critical exon in the spinal muscular atrophy genes, RNA, 2004, 10, 1291-1305

[38] Singh N. N., Singh R. N., Androphy E. J., Modulating role of RNA structure in alternative splicing of a critical exon in the spinal muscular atrophy genes, Nucleic Acids Res., 2007, 35, 371-389

[39] Singh N. N., Seo J., Ottesen E. W., Shishimorova M., Bhattacharya D., Singh R. N., TIA1 prevents skipping of a critical exon associated with spinal muscular atrophy, Mol. Cell. Biol., 2011, 31, 935-954

[40] Klar J., Sobol M., Melberg A., Mäbert K., Ameur A., Johansson A. C., et al., Welander distal myopathy caused by an ancient founder mutation in TIA1 associated with perturbed splicing, Hum. Mutat., 2013, doi: 10.1002/humu.22282 [Epub ahead of print]

[41] Hicks M. J., Mueller W. F., Shepard P. J., Hertel K. J., Competing upstream 5'-splice sites enhance the rate of proximal splicing, Mol. Cell. Biol., 2010, 30,1878-1886

[42] Berg M. G., Singh L. N., Younis I., Liu Q., Pinto A. M., Kaida D., et al., U1 snRNP determines mRNA length and regulates isoform expression, Cell, 2012,150, 53-64

[43] Kaida D., Berg M. G., Younis I., Kasim M., Singh L. N., Wan L., et al., U1 snRNP protects pre-mRNAs from premature cleavage and polyadenylation, Nature, 2010, 468, 664-668

[44] Williams J. H., Schray R. C., Patterson C. A., Ayitey S. O., Tallent M. K., Lutz G.J., Oligonucleotide-mediated survival of motor neuron protein expression in CNS improves phenotype in a mouse model of spinal muscular atrophy, J. Neurosci., 2009, 29, 7633-7638

[45] Le T. T., Pham L. T., Butchbach M. E., Zhang H. L., Monani U. R., Coovert D. D., et al., SMNDelta7, the major product of the centromeric survival

motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN, Hum. Mol. Genet., 2005, 4, 845-857

[46] Avila A. M., Burnett B. G., Taye A. A., Gabanella F., Knight M. A., Hartenstein P., et al., Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy, J. Clin. Invest., 2007, 117, 659-671

[47] Butchbach M. E., Singh J., Thorsteinsdóttir M., Saieva L., Slominski E., Thurmond J., et al., Effects of 2,4-diaminoquinazoline derivatives on SMN expression and phenotype in a mouse model for spinal muscular atrophy, Hum. Mol. Genet., 2010, 19, 454-467

[48] Mentis G. Z., Blivis D., Liu W., Drobac E., Crowder M. E., Kong L., et al., Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy, Neuron, 2011, 69, 453-467

[49] Bebee T. W., Dominguez C. E., Chandler D. S., Mouse models of SMA: tools for disease characterization and therapeutic development, Hum. Genet., 2012, 131, 1277-1293

[50] Hua Y., Sahashi K., Hung D., Rigo F., Passini M. A., Bennett C. F., et al., Antisense correction of SMN splicing in the CNS rescues necrosis in a type III SMA mouse model, Genes Dev., 2010, 24,1634-1644

[51] Passini M. A., Bu J., Richards A. M., Kinnecom C., Sardi S., Stanek L. M., et al., Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy, Sci. Transl. Med., 2011, 3, 72ra18

[52] Hua Y., Sahashi K., Rigo F., Hung G., Horev G., Bennett C. F., et al., Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model, Nature, 2011, 478, 123-126

[53] Hsieh-Li H. M., Chang J. G., Jong Y. J., Wu M. H., Wang N. M., Tsai C. H., et al., A mouse model for spinal muscular atrophy, Nat. Genet., 2000, 24, 66-70

[54] Tanganyika-de Winter C. L., Heemskerk H., Karnaoukh T. G., van Putten M., de Kimpe S. J., van Deutekom J., et al., Long-term exon skipping studies with 2'-O-methyl phosphorothioate antisense oligonucleotides in dystrophic mouse models, Mol. Ther. Nucleic Acids, 2012, 1, e44

[55] Osman E. Y., Yen P. F., Lorson C. L., Bifunctional RNAs targeting the intronic splicing silencer N1 increase SMN levels and reduce disease severity in an animal model of spinal muscular atrophy, Mol. Ther., 2012, 20, 119-126

[56] Gogliotti R. G., Hammond S. M., Lutz C., Didonato C. J., Molecular and phenotypic reassessment of an infrequently used mouse model for spinal muscular atrophy, Biochem. Biophys. Res. Commun., 2010, 391, 517–522

[57] Riessland M., Ackermann B., Förster A., Jakubik M., Hauke J., Garbes L., et al., SAHA ameliorates the SMA phenotype in two mouse models for spinal muscular atrophy, Hum. Mol. Genet., 2010, 19, 1492–1506

[58] Ellett F., Lieschke G. J., Zebrafish as a model for vertebrate hematopoiesis, Curr. Opin. Pharmacol., 2010, 10, 563–570

[59] Hao le T., Burghes A. H., Beattie C. E., Generation and characterization of a genetic zebrafish model of SMA carrying the human SMN2 gene, Mol. Neurodegener., 2011, 6, 24

[60] Porensky P. N., Mitrpant C., McGovern V. L., Bevan A. K., Foust K. D., Kaspar B. K., et al., A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse, Hum. Mol. Genet., 2012, 21, 1625-1638

[61] Zhou H., Janghra N., Mitrpant C., Dickinson R. L., Anthony K., Price L., et al., A novel morpholino oligomer targeting ISS-N1 improves resuce of severe SMA transgenic mice, Hum. Gene Ther., 2013, doi: 10.1089/hum.2012.211 [Epub ahead of print]

[62] Gendron D., Carriero S., Garneau D., Villemaire J., Klinck R., Elela S. A., et al., Modulation of 5' splice site selection using tailed oligonucleotides carrying splicing signals, BMC Biotechnol., 2006, 6, 5

[63] Miyajima H., Miyaso H., Okumura M., Kurisu J., Imaizumi K., Identification of a cis-acting element for the regulation of SMN exon 7 splicing, J. Biol. Chem., 2002, 277, 23271-23277

[64] Baughan T. D., Dickson A., Osman E. Y., Lorson C. L., Delivery of bifunctional RNAs that target an intronic repressor and incease SMN levels in an animal model of spinal muscular atrophy, Hum. Mol. Genet., 2009, 18, 1600-1611

[65] Cartegni L., Krainer A. R., Correction of disease-associated exon skipping by synthetic exon-specific activators, Nat. Struct. Biol., 2003, 10, 120-125

[66] Skordis L. A., Dunckley M. G., Yue B., Eperon I. C., Muntoni F., Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates SMN2 gene expression in patient fibroblasts, Proc. Natl. Acad. Sci. USA, 2003, 100, 4114-4119

[67] Owen N., Zhou H., Malygin A. A., Sangha J., Smith L. D., Muntoni F., et al., Design principles for bifunctional targeted oligonucleotide enhancers of splicing, Nucleic Acids Res., 2011, 39, 7194-7208

[68] Meyer K., Marquis J., Trüb J., Nlend Nlend R., Verp S., Ruepp M. D., et al., Rescue of a severe mouse model for spinal muscular atrophy by U7 snRNA-mediated splicing modulation, Hum. Mol. Genet., 2009, 18, 546-555

[69] Geib T., Hertel K. J., Restoration of full-length SMN promoted by adenoviral vectors expressing RNA antisense oligonucleotides embedded in U7 snRNAs, PLoS One, 2009, 4, e8204

[70] Singh N. N., Seo J., Rahn S. J., Singh R. N., A multi-exon-skipping detection assay reveals surprising diversity of splice isoforms of spinal muscular atrophy genes, PLoS One, 2012, 7, e49595

[71] Cirak S., Arechavala-Gomeza V., Guglieri M., Feng L., Torelli S., Anthony K., et al., Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study, Lancet, 2011, 378, 595-605