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Special Focus Research Paper

A short antisense oligonucleotide masking a unique intronic motif prevents skipping of a critical exon in spinal muscular atrophy

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Abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neuron; ISS-N1, intronic splicing silencer N1; C6U, a C-to-T mutation at the 6th position of *SMN2* exon 7; ASO, antisense oligonucleotide; Anti-N1, a 20-mer stimulatory ASO; 3UP8, an 8-mer stimulatory ASO

Key words: survival motor neuron (SMN), SMN1, SMN2, alternative splicing, intron 7, exon 7, ISS-N1, GC-rich sequence, antisense oligonucleotide (ASO), 8-mer ASO, SMA

Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality. Most SMA cases are associated with the low levels of SMN owing to deletion of *Survival Motor Neuron 1* (*SMN1*). *SMN2*, a nearly identical copy of *SMN1*, fails to compensate for the loss of *SMN1* due to predominant skipping of exon 7. Hence, correction of aberrant splicing of *SMN2* exon 7 holds the potential for cure of SMA. Here we report an 8-mer antisense oligonucleotide (ASO) to have a profound stimulatory response on correction of aberrant splicing of *SMN2* exon 7 by binding to a unique GC-rich sequence located within intron 7 of *SMN2*. We confirm that the splicing-switching ability of this short ASO comes with a high degree of specificity and reduced off-target effect compared to larger ASOs targeting the same sequence. We further demonstrate that a single low nanomolar dose of this 8-mer ASO substantially increases the levels of SMN and a host of factors including Gemin 2, Gemin 8, ZPR1, hnRNP Q and Tra2-β1 known to be down-regulated in SMA. Our findings underscore the advantages and unmatched potential of very short ASOs in splicing modulation in vivo.

Introduction

Alternative splicing is an essential process in the generation of protein diversity and has been a major contributory force to genome evolution.[1] Splicing is catalyzed by a spliceosome, a complex macromolecular machine.[2,3] However, control of alternative splicing rests on non-spliceosomal factors that bind to pre-mRNA sequences called exonic or intronic splicing enhancers (ESEs or ISEs) and silencers (ESSs or ISSs).[4-6] Enhancer and silencer motifs promote or suppress splice-site (ss) selection, respectively. Methods to decipher critical splicing motifs are continuing to evolve.[7-10] An additional regulatory role is provided by RNA structures that enforce accessibility to splicing elements, as well as bring two distantly located cis-elements into close proximity.[11-14] Alternative splicing has been implicated in a growing number of human diseases.[15] In this regard antisense oligonucleotides (ASOs) targeting regulatory elements have emerged as the powerful tools to modulate alternative splicing in pathological conditions.[15-19]

Humans have two nearly identical copies of the *Survival Motor Neuron* (*SMN*) gene: *SMN1* and *SMN2*.[20] The two *SMN* genes code for identical proteins, however, *SMN2* predominantly generates a short transcript due to skipping of exon 7, producing a truncated SMN that is highly unstable.[21] Other non-productive *SMN2* mRNAs lacking exons 5 and 3 have also been reported.[22] The inability of *SMN2* to compensate for the loss of *SMN1* results in spinal muscular atrophy (SMA), a debilitating disease of children and infants.[15,16,23] Further, low levels of SMN are also associated with certain forms of amyotrophic lateral sclerosis (ALS), a progressive neurological disease that attacks the nerve cells responsible for controlling voluntary muscles.[24,25] SMN is a housekeeping protein, with its most important function being the assembly of U snRNPs, the essential components of the spliceosomal machinery.[26] Consistently, depletion of SMN causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing.[27] Other important functions associated with SMN include transcriptional regulation, telomerase regeneration and cellular trafficking.[28] Consistently, higher organisms lacking SMN are not viable.[28]

SMN1 and *SMN2* differ by a critical C to T substitution at position 6 (C6U transition) of exon 7 in *SMN2*.[29] The significance of an additional difference within intron 7 of *SMN1* and

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SMN2 has been reported.[30] Both *SMN1* and *SMN2* share the same branch site for the lariat formation during the removal of intron 6.[31] Yet C6U weakens the 3'-splice site (3' ss) of exon 7 in *SMN2*.[32] Published reports suggest C6U abrogates an enhancer and/or creates a silencer.[33-36] In vivo selection of the entire exon revealed three major cis-elements within exon 7.[37] Two of them, "Exinct" and "3'-Cluster," are negative regulators of splicing. They flank "Conserved Tract", the positive element located in the middle of exon 7.[29] The identification of "Conserved Tract" is in agreement with the role of the previously reported Tra2-β1-associated enhancer.[38] Recently, an antisense micro-walk within exon 7 independently supported the nature of these three cis-elements.[39] Several intronic cis-elements have been implicated in regulation of *SMN2* exon 7 splicing as well.[30,40-43]

Since *SMN2* is almost universally present in SMA patients, correction of *SMN2* exon 7 splicing using target-specific ASOs holds the promise for cure. A number of studies have focused on use of large ASOs with bifunctional properties to restore *SMN2* exon 7 inclusion.[44-47] Other studies have focused on targeting large regulatory motifs or a combination of regulatory motifs to induce *SMN2* exon 7 inclusion.[39,42,43,48,49] However, it is not known if sequestering of a short splicing motif by a short ASO could prevent exon skipping and restore *SMN2* exon 7 inclusion. Here we report the splicing-switching ability of an 8-mer ASO that binds to a unique regulatory sequence and fully restores *SMN2* exon 7 inclusion. We also demonstrate that this 8-mer ASO offers a high degree of specificity and reduced off-target effect compared to larger ASOs targeting the same region. Considering short ASOs offer several advantages including low cost of synthesis, ease of chemical modifications, reduced chances of immune response, and higher probability of crossing biological barriers, our findings serve as a major step forward for splicing modulation in a genetic disease.

Results

An ultra-refined antisense microwalk revealed shortest motif for splicing correction. Recent reports have confirmed the presence of a negative context located downstream of the 5' splice site (5' ss) of *SMN2* exon 7.[42,43] This negative context is defined by a 15-nucleiotide cis-element, ISS-N1 that harbors two putative hnRNP A1/A2 motifs (Fig. 1A).[42,43] ISS-N1 partially overlaps with an octamer sequence CUGCCAGC, which is the only GC-rich sequence in the first half of the intron 7 of human *SMN*. This sequence is predicted to reside in a single-stranded region sandwiched between two stem-loop structures (Fig. 1A).[28] Combined with an easy accessibility and the high GC-rich content (75%), this octamer sequence has a potential to provide an ideal ASO target. However, it is not known if a short RNA:RNA duplex formed between CUGCCAGC and an ASO could displace an interacting protein and/or drastically change the negative context to reverse the splicing pattern. To explore such possibility, we performed an ultra-refined antisense microwalk downstream of the 5' ss of *SMN2* exon 7. All ASOs used in our study incorporated 2'-O-methyl modification and phosphorothioate backbone (abbreviated as "2OMePS"), a widely used RNA modification with proven stability in vivo.[16,18,19] We performed our experiments in commercially available SMA type I patient cells (GM03813), which serves as an ideal system for testing of splicing-correcting compounds in the context of the disease caused by the lack of *SMN1*.[39,42,43]

Our ultra-refined antisense microwalk used four groups of ASOs of varying sizes. The ASOs from each group sequestered 0, 1, 2 or 3 residues upstream of ISS-N1. Accordingly, ASOs were named as F, 1UP, 2UP and 3UP, followed by a number representing the size of the ASO (Table 1). To discriminate between the most and the least efficient ASOs, the antisense microwalk was performed at four concentrations: 1 nM, 10 nM, 50 nM and 100 nM (Table 1). Figure 1 shows the splicing pattern of representative ASOs performed at 20 nM. We observed a decrease in the antisense effect on exon 7 inclusion with a decrease in the size of F, 1UP and 2UP ASOs (Table 1, Fig. 1B). However, the results were drastically different with 3UP ASOs: shortening of ASOs from 14 nucleotides to 8 nucleotides produced no significant changes in their stimulatory effects on exon 7 splicing (Fig. 1B). However, the stimulatory effect drastically decreased when ASO size was further reduced from 8 nucleotides to 7 nucleotides. Hence, we conclude that the shortest ASO to effectively restore *SMN2* exon 7 inclusion was 3UP8, an 8-mer ASO that sequestered the entire octamer sequence, CUGCCAGC, discussed above. Remarkably, 3UP8 was able to fully restore *SMN2* exon 7 inclusion at a relatively low concentration of 50 nM (Table 1).

The finding that 3UP8 restores *SMN2* exon 7 inclusion marks the discovery of the shortest ASO among ~200 ASOs tested thus far in SMA patient cells (Table 1).[39,42-49] In addition to revealing the shortest stimulatory ASO, the ultra-refined microwalk was able to accurately define the first five residues (CCAGC) of ISS-N1 as the core sequence of the antisense target. Our finding of core sequence brings a parallel with seed sequence required for miRNA and siRNA response.[50] Similar to seed sequence, sequestering of the core sequence was essential but not sufficient for the antisense response. In addition to sequestration of core sequence, sequestration of additional three residues (CUG) upstream of ISS-N1 was found to be essential to obtain the shortest stimulatory ASO. In the absence of the sequestration of CUG residues, an 11-nucleotide or longer target was required for realizing the stimulatory response (Table 1). Underscoring the overlapping nature of splicing cis-elements and their hard-to-predict accessibility during the dynamic process of splicing, we found no direct correlation between the size of ASOs and their stimulatory response. Similarly, we found no direct correlation between sequestration of any of the individual hnRNP A1 motifs and the level of stimulatory response. For instance, F10 and L13 fully sequestered the 1st and the 2nd hnRNP A1 motifs, respectively, and yet did not produce any significant stimulatory response even at higher concentration of 100 nM (Table 1). On the other hand, 3UP8 restored *SMN2* exon 7 by sequestering an 8-mer motif that only partially overlaps the first hnRNP A1 motif.

Antisense effect is specific to base paring with the target. Having discovered that a short intronic motif could be targeted for splicing modulation of endogenous pre-mRNA, we next examined the efficacy and specificity of short ASO in *SMN2*

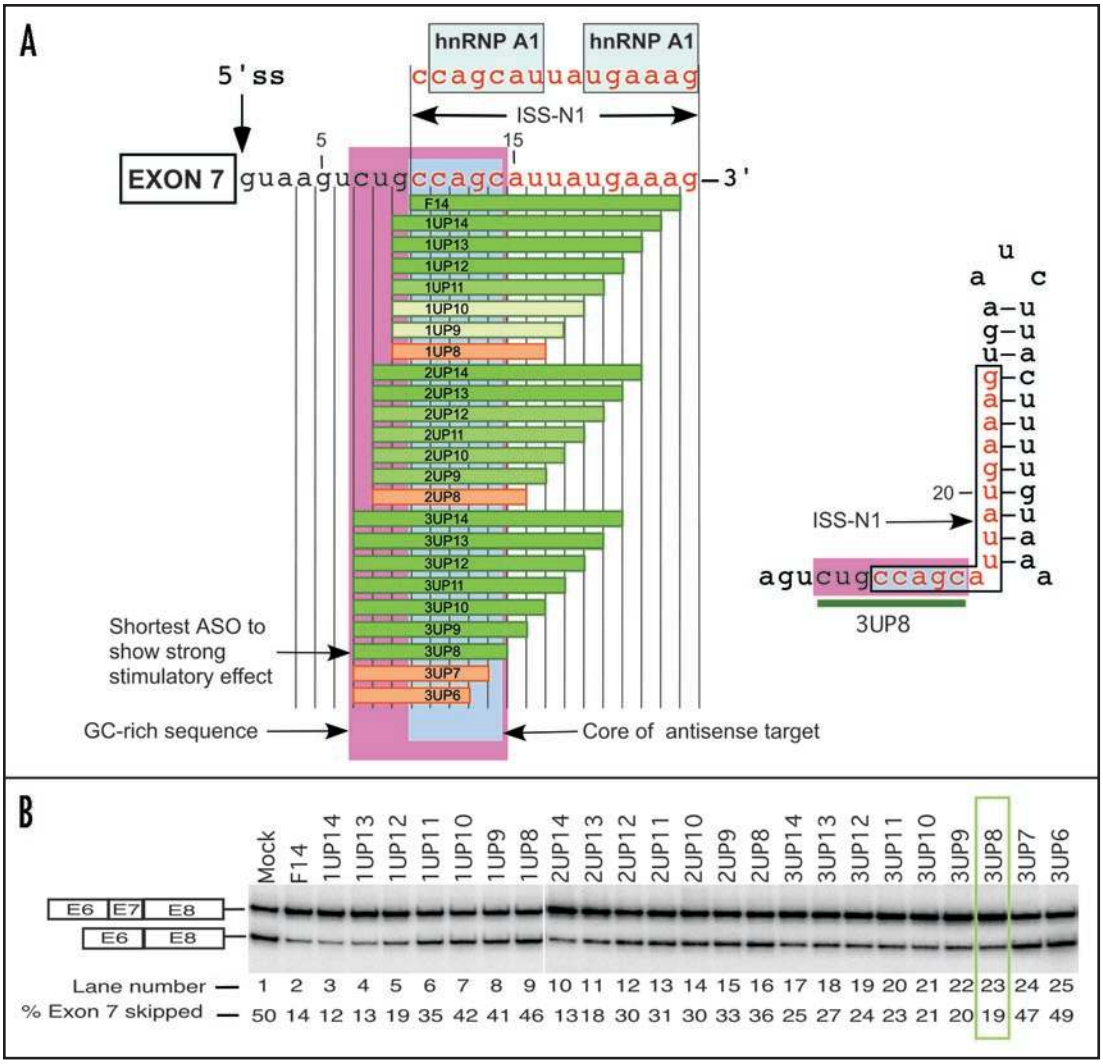

Figure 1. Untra-refined antisense microwalk to identify the shortest stimulatory ASO. (A) Diagrammatic representation of ASOs targeting sequences upstream of ISS-N1. Exon 7 is boxed and the first 24 residues of human *SMN* intron 7 are shown. Numbering starts from the first position of intron 7. The 5' ss of exon 7 is indicated by a vertical arrow. ASOs blocking different regions are shown as horizontal bars. Sequences of these ASOs are given in Table 1. Boundary of ISS-N1 is demarcated.[42] hnRNP A1 motifs are indicated.[43] Green bars represent ASOs that promote *SMN2* exon 7 inclusion. Intensity of green color reflects the strength of stimulatory effect. Tan bars represent ASOs that have no effect on *SMN2* exon 7 inclusion. Area highlighted in pink represents the only GC-rich sequence in the first half of human intron 7. Area highlighted in light blue represents the core sequence of the antisense target. Right panel shows the relative positioning of ISS-N1, GC-rich sequence in the context of predicted RNA structure. Green bar represents 3UP8, the shortest ASO to stimulate *SMN2* exon 7 inclusion. (B) Splicing pattern of endogenous *SMN2* in SMA type I patient fibroblasts (GM03813) treated with different ASOs. Cells were transfected with 20 nM of 2OMePS ASOs and the total RNA for splicing assay was isolated 24 h post transfection. Results were analyzed as described earlier.[35] 3UP8 was the shortest ASO to show stimulatory response (highlighted by green box).

minigene system. Here HeLa cells were co-transfected with the minigene (0.1 μg) and an ASO of interest (50 nM) and the effect on splicing was accessed by RT-PCR. As shown in Figure 2B, the ASO effect on splicing of minigene-derived exon 7 was consistent with the results for the endogenous *SMN2* with 3UP8 being the shortest ASO to fully restore exon 7 inclusion. To compare the target specificity between long and short ASOs, we generated a mutant minigene, *SMN2*/64A. This minigene has a single C to A substitution at the first position of ISS-N1, hence has capability to weaken the RNA:RNA duplex between the antisense and the target (Fig. 2A). Indeed, our shortest ASO (3UP8) lost all its stimulatory response in *SMN2*/64A. As expected, a mutant 8-mer ASO (3UP8/64A) that reinstated the base pairing with the mutated target fully restored exon 7 inclusion in *SMN2*/64A minigene; at the same time 3UP8/64A had no stimulatory effect on splicing of *SMN2* minigene (Fig. 2B). Note that the stimulatory impact of 3UP8/64A in *SMN2*/64A minigene was realized despite the fact that the C-to-A mutation reduced the GC content of the target from 75% to 62.5%. Unlike 8-mer ASOs, all four 15-mer ASOs we used effectively restored exon 7 inclusion in *SMN2*/64A. These results clearly suggest that an increase in ASO size could have drastic (negative) consequences on

Table 1 **Effect of ASOs on skipping of *SMN2* exon 7 in SMA type I fibroblasts (GM03183)**

No.	ASO Name	ASO Sequence	ASO Conc. (nM) 1	10	50	100
			% Exon Skipping			
1	Anti-N1	GGUCGUAAUACUUUCACUUA	24	19	6	4
2	F19	GGUCGUAAUACUUUCACUU	33	19	7	6
3	F18	GGUCGUAAUACUUUCAC	34	14	8	5
4	F17	GGUCGUAAUACUUUCAC	35	14	8	6
5	F16	GGUCGUAAUACUUUCA	35	15	8	5
6	F15	GGUCGUAAUACUUUC	39	23	18	6
7	F14	GGUCGUAAUACUUU	38	15	11	5
8	F13	GGUCGUAAUACUU	37	26	10	6
9	F12	GGUCGUAAUACU	49	35	14	7
10	F11	GGUCGUAAUAC	43	33	13	11
11	F10	GGUCGUAAUA	50	42	36	19
12	F9	GGUCGUAAU	50	48	44	36
13	F8	GGUCGUAA	46	46	41	42
14	F7	GGUCGUA	50	49	49	48
15	1UP15	CGGUCGUAAUACUUU	32	18	8	5
16	2UP15	ACGGUCGUAAUACUU	37	19	12	7
17	3UP15	GACGGUCGUAAUACU	40	25	15	5
18	L15Ex	UAAUACUUUCACUUA	52	51	50	45
19	L13	UCGUAAUACUUUC	50	51	50	46
20	L12	CGUAAUACUUUC	50	41	47	55
21	L10Ex	CUUUUCACUUA	49	49	48	45
22	L9Ex	ACUUUCACU	50	49	49	48
23	L8MEx	UACUUUCA	50	49	49	48
24	L7	AUACUUU	50	49	49	48
25	1UP14	CGGUCGUAAUACUU	27	14	6	4
26	1UP13	CGGUCGUAAUACU	34	15	7	5
27	1UP12	CGGUCGUAAUAC	44	20	8	7
28	1UP11	CGGUCGUAAUA	51	37	24	20
29	1UP10	CGGUCGUAAU	50	43	30	26
30	1UP9	CGGUCGUAA	50	48	31	29
31	1UP8	CGGUCGUA	50	48	42	37
32	2UP14	ACGGUCGUAAUACU	35	16	6	8
33	2UP13	ACGGUCGUAAUAC	39	20	7	8
34	2UP12	ACGGUCGUAAUA	46	35	13	10
35	2UP11	ACGGUCGUAAU	46	34	14	10
36	2UP10	ACGGUCGUAA	54	32	17	11
37	2UP9	ACGGUCGUA	49	37	21	17
38	2UP8	ACGGUCGU	48	48	22	17
39	3UP14	GACGGUCGUAAUAC	38	24	7	5
40	3UP13	GACGGUCGUAAUA	43	28	11	6
41	3UP12	GACGGUCGUAAU	41	27	9	6
42	3UP11	GACGGUCGUAA	40	25	12	7
43	3UP10	GACGGUCGUA	39	24	16	12
44	3UP9	GACGGUCGU	40	23	10	8
45	3UP8	GACGGUCG	40	21	9	7
46	3UP7	GACGGUC	49	46	42	38
47	3UP6	GACGGU	49	49	49	47

Cells were transfected with 1, 10, 50 and 100 nM of ASOs and the total RNA for splicing assay was isolated 24 h post transfection. First 29 residues of intron 7 of human *SMN* are shown in small-case letters. Numbering starts from position 1 of intron 7. GC-rich sequence is highlighted in pink. Positions of ISS-N1 residues are boxed. Two hnRNP A1 motifs within ISS-N1 are indicated.[43] First five residues of ISS-N1 constitute the core of the antisense target and are marked as "CORE" and highlighted in blue. Sequences of ASOs are shown in large case letters in 3' to 5' direction and are arranged against the target sequence of intron 7. Percentage of *SMN2* exon 7 skipping is shown on the right. Values highlighted in blue, green and light green colors represent 10% or less, 25% or less and 40% or less exon 7 skipping, respectively. Values highlighted in tan color represent no appreciable effect on *SMN2* exon 7 splicing. Mock transfection (without any ASO) produced 50% of *SMN2* exon 7 skipping.

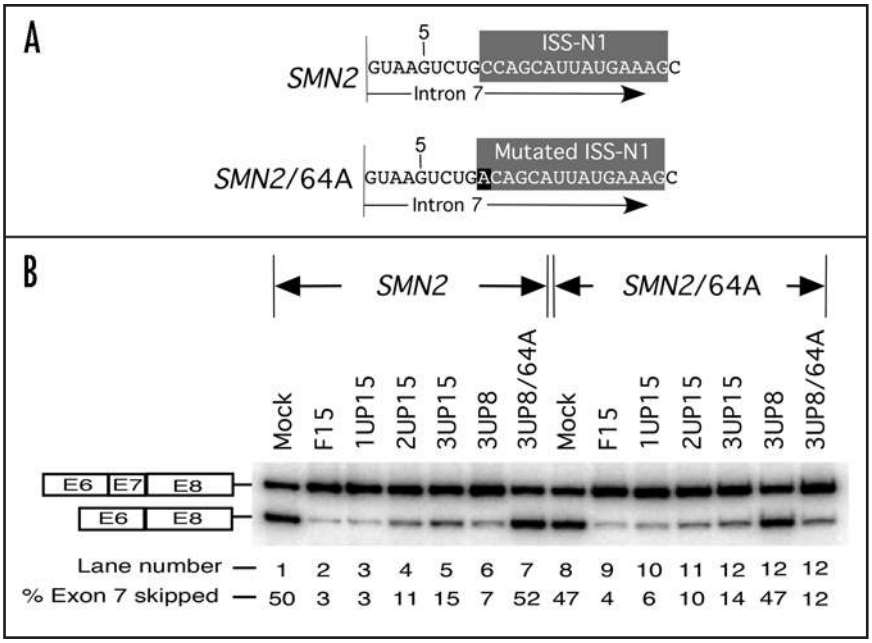

Figure 2. Antisense effect is specific to its target sequence. (A) Diagrammatic representation of intron 7 of *SMN2* minigene and its mutant, *SMN2*/64A. Numbering starts from the first position of human *SMN* intron 7. ISS-N1 sequence is highlighted in gray. Mutated residue is highlighted in black. (B) Effect of ASOs on splicing of *SMN2* minigene and its mutant, *SMN2*/64A. HeLa cells were transfected with 50 nM of a given ASO and 0.1 μg of minigene in a 24-well plate. Splicing was determined 24 h after transfection. Results were analyzed as described earlier.[35]

the specificity of the antisense response since longer ASO appear to be more "tolerant" to single-nucleotide mismatches.

The shortest stimulatory ASO has no off-target effect on other *SMN2* exons. To examine the possible off-target effect of ASOs that promote *SMN2* exon 7 inclusion in GM03813 cells, we focused on splicing of *SMN2* exons 3 and 5. These exons are known to undergo alternative splicing;[22] and therefore, have a potential to regulate the levels of full-length SMN. We have earlier reported that a 5 nM concentration of Anti-N1 had no detectable effect on splicing pattern of *SMN2* exons 3 and 5.[42] Here we increased the ASO concentration to 20 and 100 nM. We chose to use Anti-N1, F14 and 3UP8 to represent the longest, the intermediate and the shortest stimulatory ASO, respectively. F8 served as a negative control.

We started with the amplification of endogenous *SMN2*-spliced products using a pair of primers located within exons 4 and 8. This primer combination provided an added advantage of simultaneous detection of skipping of exons 5 and 7. To compare the amount of the spliced products in broad size range, we used an end-labeled primer. As shown in Figure 3A, all three functional ASOs: Anti-N1, F14 and 3UP8, were highly efficient in promoting exon 7 inclusion at 20 nM concentration, while F8 produced no effect. None of the ASOs appeared to have a pronounced effect on splicing of exon 5 at both ASO concentrations. At the same time, Anti-N1, F14 and 3UP8 caused a decrease in the amount of the co-exclusion product (mRNAs lacking both exons 5 and 7), especially at 100 nM (Fig. 3A). Skipping of exon 5 was separately measured with another primer pair that annealed to exons 4 and 6. We found no significant difference on effect on exon 5 splicing in cells treated with any of the tested ASOs (Fig. 3B). To monitor skipping of *SMN2* exon 3, we used a forward primer that annealed to exon 1 and the reverse primer that annealed to exon 4. As shown in Figure 3C, F14 and 3UP8 produced no detectable change in the level of exon 3 skipping as compared to the mock-transfected sample. Contrary to this, Anti-N1 produced a substantial increase in *SMN2* exon 3 skipping at 100 nM (Fig. 3C). Mechanism by which Anti-N1 elicits this off-target effect is not understood, although it clearly underscores the disadvantage of long ASOs as the therapeutic molecules.

Restoration of SMN levels by shortest ASO in SMA patient cells. The next goal of our study was to determine whether the correction of *SMN2* exon 7 splicing by 3UP8 resulted in SMN protein increase in patient cells. In particular, we wanted to compare the stimulatory effect of 3UP8 with a longer ASO, Anti-N1. F8 served as the negative control. The experiments were performed with 40 nM of a given ASO and protein levels were determined 48 hours after transfection. Simultaneously, we monitored the levels of *SMN2* exon 7 inclusion. As shown in Figure 4A, mock-treatment (mock) or treatment with F8 did not produce any change in SMN levels (left) as well as in levels of *SMN2* exon 7 inclusion (right). In contrast, treatment with 3UP8 resulted in a substantial upregulation of SMN levels (Fig. 4A, left) and *SMN2* exon 7 inclusion (Fig. 4A, right). Significantly, the effect of 3UP8 on SMN levels was comparable to the effect produced by Anti-N1 treatment. To determine whether increase in SMN levels in ASO-treated patient cells was accompanied by a change in cellular metabolism, we performed western blot for a number of proteins that are generally downregulated in SMA. As shown in Figure 4A (left), treatment of patient cells with 3UP8 was accompanied by a marked increase in the levels of Gemin 2 and Gemin 8. These factors are associated with SMN complex, a macromolecule essential for the housekeeping role of snRNP biogenesis.[26,27] We also observed an increase in the levels of ZPR1 (Fig. 4A), another SMN-interacting protein, reduced expression of which is associated with the progressive loss of motor neurons.[51,52] Interestingly, the correction of splicing by 3UP8 resulted in increase of levels of splicing factors Tra2-β1 and hnRNP Q. Tra2-β1 and hnRNP Q1 isoform have been shown to promote *SMN2* exon 7 inclusion and are generally downregulated in SMA.[38,53-55] Thus, our findings suggest that SMN may be a part of a positive feedback loop that provides signals to increase the levels of different splicing factors.

Cell division and degradation of ASOs are bound to attenuate the stimulatory effect of ASOs with respect to time. To determine the sustainability of a single 3UP8 treatment, we performed a time course analysis in which levels of SMN and other factors were

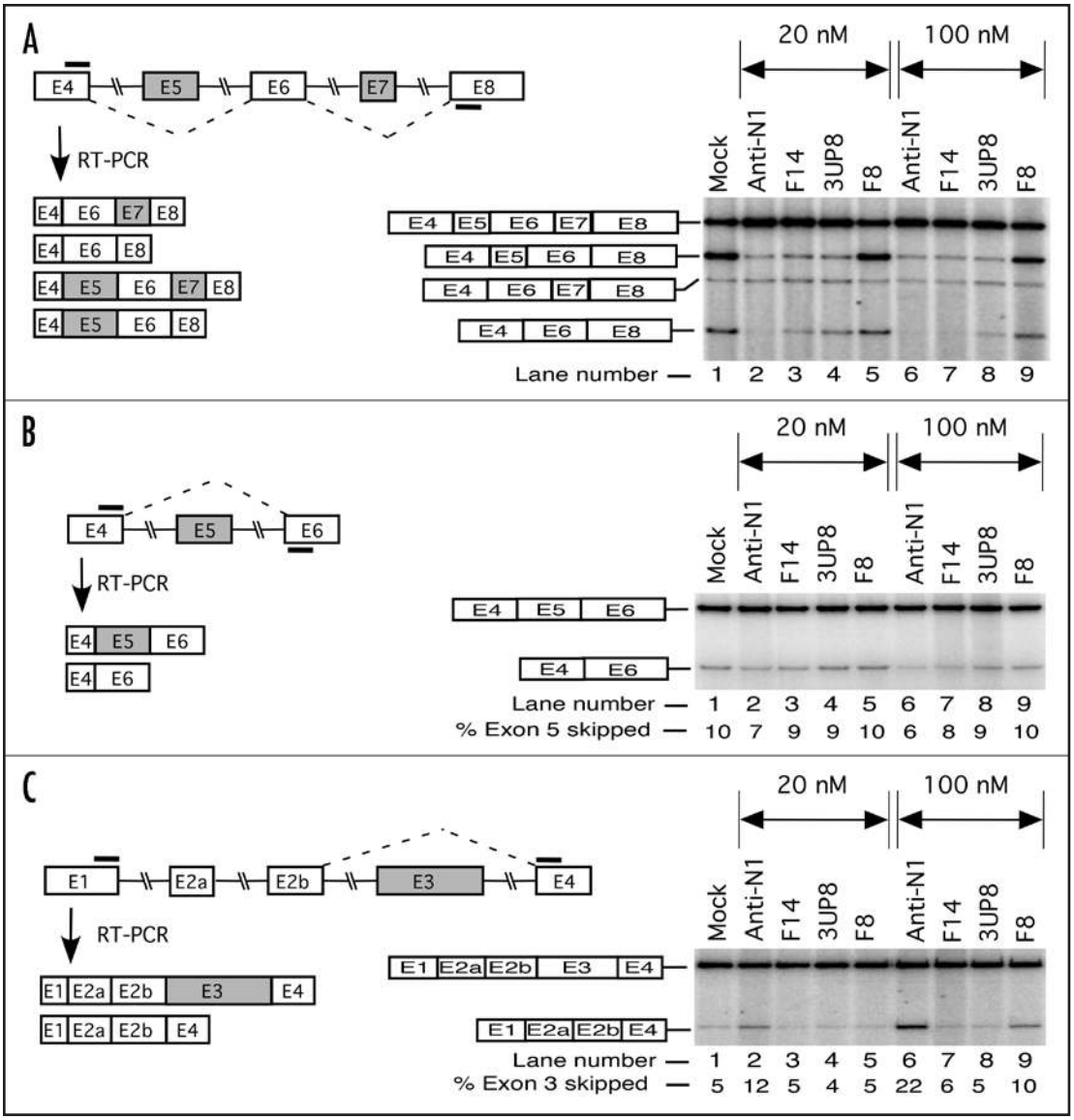

Figure 3. Effect of ASOs on alternative splicing of different exons of endogenous *SMN2*. SMA type I patient fibroblasts (GM03813) were transfected with 20 or 100 nM of selected ASOs in 6-well plates. The total RNA for splicing assay was isolated 24 h post transfection. Spliced products were amplified by RT-PCR with one of the primers being end-labeled. Annealing positions of primers are shown by bars. (A) Left panel depicts the diagrammatic representation of expected spliced products. Right panel shows the results of RT-PCR. Exon 7 skipped, exon 5 skipped and co-excluded products are marked. (B) Left panel depicts the diagrammatic representation of expected spliced products due to skipping of exon 5. Right panel shows the results of RT-PCR. Exon 5 included and exon 5 skipped products are marked. (C) Left panel depicts the diagrammatic representation of expected spliced products due to skipping of exon 3. Right panel shows the results of RT-PCR. Exon 3 included and exon 3 skipped products are marked.

examined at 24 hr intervals for six days. Simultaneously, we also monitored ASO effect on *SMN2* exon 7 splicing. A single dose of 40 nM of 3UP8 was sufficient to sustain the increased levels of SMN for five days (Fig. 4B, left). Effect on other proteins varied with respect to time. For example, levels of Gemin 2, Gemin 8, ZPR1 and hnRNP Q peaked at day three but started decreasing after that, whereas the levels of Tra2-β1 reached maximum on day three and remained high till day five (Fig. 4B, left). As for the effect on exon 7 splicing, levels of exon inclusion remained high for two days followed by a graduate decrease (Fig. 4B, right). It is possible that increase in Tra2-β1 and hnRNP Q levels contributed to exon 7 inclusion.

SMA patient cells are usually deficient in SMN-containing subnuclear bodies or gems. To test whether increase in SMN levels can induce its nuclear accumulation in gems, we performed immunofluorescence analysis of 3UP8 treated GM03813 cells. Here F8 was used as a negative control. As shown in Figure 5, transfection of cells with 3UP8 was accompanied by a profound increase in the number of gems containing SMN. We also observed that 3UP8 but not F8 resulted in increase and redistribution to gems of

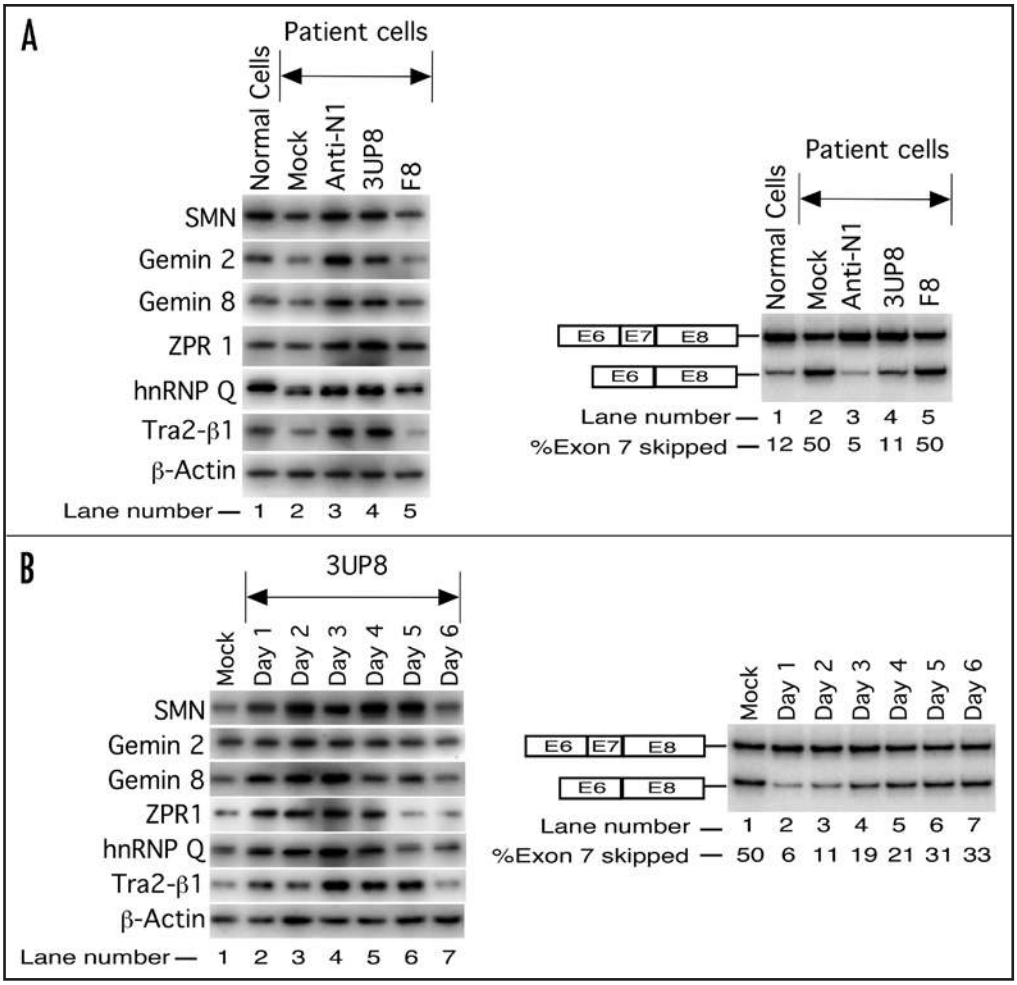

Figure 4. Effect of the shortest stimulatory ASO (3UP8) on levels of cellular proteins in SMA patient cells. (A) Western blot showing the effect of different ASOs. SMA type I patient cells (GM03813) were transfected with 40 nM of selected ASOs and cells were harvested 48 h after transfection. Left panel represents the results of western blot of different proteins, whereas the right panel represents the results of RT-PCR. (B) Time course of 3UP8 effect on the levels of SMN and other factors. SMA type I patient cells (GM03813) were transfected with a single dose of 40 nM of 3UP8 and harvested after every 24 h for six days. Left panel represents the results of western blot of different proteins, whereas the right panel represents the results of RT-PCR.

SMN-interacting protein, ZPR1 (Fig. 5). It is known that ZPR1 is required for accumulation of SMN in these sub-nuclear structures.[56,57] Our finding that 3UP8 is able to increase the number of gems confirms a proper assembly of SMN in the nucleus. This also marks the first evidence of a stimulatory response by a very short ASO leading to the massive macromolecular reorganization in the nucleus of a patient cell.

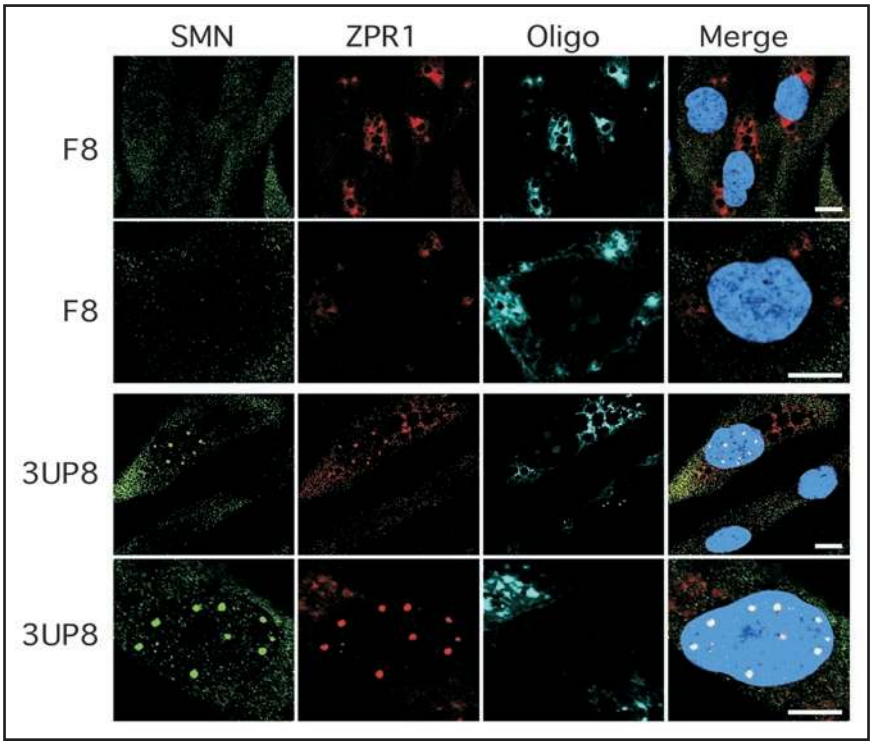

Figure 5. Confocal images confirming that treatment with short ASO (3UP8) promotes nuclear accumulation of SMN in SMA patient cells. The fibroblasts from SMA type I patient (GM03813) were cultured on coverslips and transfected with 40 nM of F8 (control) or 3UP8 CY3-labeled ASOs. Cells were fixed 48 h after transfection and stained with anti-SMN (Green) and anti-ZPR1 (Red) antibodies. Cells transfected with ASOs were detected by Cy3 fluorescence and presented in pseudo-color (Cyan). DNA was stained with DAPI (blue). The scale bar is 10 mm.

Discussion

SMA is the second most common genetic disorder of children and infants caused by insufficient levels of SMN protein due to the loss of the *SMN1* gene. Presence of a defective gene, *SMN2*, makes SMA a unique genetic disease that could be avoided and possibly cured by redirecting *SMN2* exon 7 splicing. Among several approaches to correct aberrant splicing, an ASO-based approach provides a superior

alternative due to the anticipated target specificity. Size of an ASO is an important determinant in success of an ASO-based strategy. Despite the expected advantages, it is not known if very short ASOs could anneal to the target and bring desired changes in a sequence-specific manner, particularly at the low nanomolar concentrations.

Here we report an 8-mer ASO (3UP8) as the shortest ASO to correct the aberrant splicing of *SMN2* exon 7 in SMA patient cells. To the best of our knowledge, this is the first report in which an 8-mer ASO is able to effectively correct aberrant splicing in a patient cell line. Identification of this ASO was achieved through a systematic approach of ultra-refined antisense microwalk in an intronic region adjacent to the 5' ss of exon 7. The 8-mer ASO exerts its stimulatory effect through binding to a GC-rich sequence (CUGCCAGC) spanning from the 7th to 14th position of intron 7 (Fig. 1). Underscoring an evolutionary significance, this intronic region is not conserved between human and mice.[42] CUGCCAGC target sequence seems to be highly accessible since low nanomolar concentrations of 3UP8 fully restores *SMN2* exon 7 inclusion (Table 1). Consistently, the predicted secondary structure puts this target sequence in an internal loop flanked by terminal stem-loop structures (Fig. 1A).[28]

Our ultra-refined antisense microwalk with about 50 ASOs captured relative strength of multiple antisense targets that differed by a single nucleotide. As a consequence, it also revealed positions of high significance, wherein sequestering of the last five residues (CCAGC) of the GC-rich target was found to be absolutely required for the stimulatory response on *SMN2* exon 7 inclusion (Table 1). Hence CCAGC residues could be considered as the core motif, analogous to the seed sequence of the micro-RNA target.[50] However, unlike microRNAs that require assembly of a RNA-induced silencing complex (RISCs) on an 18-mer or longer sequence, our antisense response is solely based on the short RNA:RNA duplex. Based on the published reports, it is highly unlikely that protein factors could form a stable complex with a short RNA:RNA duplex. However, we cannot rule out the possibility of secondary contacts that might have been affected.

The GC-rich target described here does not resemble any known binding motif of a splicing factor, although, it overlaps with the first five residues of ISS-N1, an intronic element that harbors two putative hnRNP A1 binding sites.[42,43] The C residue at the first position (^{1}C) of ISS-N1 is not the part of hnRNP A1 motif, yet sequestering of this position was found to be absolutely necessary for the antisense response. Further, several ASOs that did not sequester ^{1}C produced an inhibitory effect even though they fully sequestered both hnRNP A1 motifs (data not shown). These results suggest that the stimulatory response of ASOs is a combination of effects not necessarily caused by blocking of hnRNP A1 motifs.

Various mechanisms may account for the stimulatory response exerted by 3UP8. The most obvious among them is the strong target affinity of 3UP8 compared to an inhibitory factor that may transiently interact with the same target during the dynamic process of splicing. It is also possible that the RNA:RNA duplex formed between 3UP8 and the GC-rich target helps bring a subtle change in the RNA structure in the vicinity of the 5' ss. Such a structural change may help improve U1 snRNP recruitment and/or the 5' ss recognition. We have previously shown that recruitment of U1 snRNP at the 5' ss of exon 7 is a limiting step for *SMN2* exon 7 inclusion.[13,29] Our results also suggest that the catalytic core of splicing is not affected by a RNA:RNA duplex formed between an ASO and its target immediately downstream of the U1 snRNA binding site. However, dissociation of ASO from the target sequence through a helicase reaction during the catalytic core formation could not be ruled out. In this scenario, the same antisense will be recycled several times on different *SMN2* pre-mRNAs. This is an obvious advantage of short ASOs in an ASO-based therapy because frequency of drug (ASO) administration could be minimized.

Our work underscores the high target specificity of very short ASOs during RNA:RNA interactions. For instance, a single mismatch in the middle of the target caused a drastic decrease in the stimulatory response by 3UP8. On the contrary, longer ASOs tolerated this mismatch mutation due to a large duplex formed between an ASO and the target. Tolerance of mismatched mutations provides an inherent drawback and therapeutic risk associated with longer ASOs. Consistently, high concentrations of a 20-mer ASO (Anti-N1) targeting intron 7 produced an off-target effect on *SMN2* exon 3 splicing, whereas identical concentrations of 3UP8 had no effect (Fig. 3C).

Owing to the high target specificity and an efficient antisense response by a short ASO, 3UP8 increased levels of SMN in SMA patient cells. It also restored levels of several key proteins that are generally downregulated in SMA (Fig. 4B). These include factors involved in snRNP biogenesis (Gemin 2 and Gemin 8) and RNA splicing (Tra2-β1 and hnRNP Q).[38,55] hnRNP Q proteins have been also implicated in other aspect of RNA metabolism, such as RNA transcription, translation, stability and trafficking.[53-55] Increase in ZPR1 in 3UP8-treated cells suggests that a short ASO is capable of restoring SMN-interacting factors, reduced expressions of which are associated with the progressive loss of motor neurons.[56,57] Despite a gradual decrease in the levels of *SMN2* exon 7 inclusion after two days, high SMN levels were maintained for five days after single treatment with 40 nM 3UP8. These findings suggest a substantially longer half-life of SMN owing to the stabilization of SMN through association with itself and/or with other factors. Consistent with the restoration of the SMN-interacting partners, 3UP8-treated cells showed increased numbers of sub-nuclear bodies (gems) in the nucleus (Fig. 5).

Currently SMA has no cure, although several small compounds capable of increasing levels of SMN in SMA have been identified.[23] Mechanisms of actions and side effects of these compounds remain unknown. Earlier ASO-based strategies promised high target specificity and focused on large ASOs in the anticipation that small motifs could not be targeted by small ASOs.[42-49] In general, literature is replete with studies using 15-mer or longer ASOs for modulation of alternative splicing. Our work provides the first evidence of high target specificity for a very short ASO and sets a unique precedence of pathogenic splicing modulation by RNA molecules less than half the size of the most reported

ASOs. Compared to large ASOs that carry the inherent risk of partial sequestration of different kinds of small motifs and tolerate mismatch mutations, we show that the stimulatory activity of a small ASO is exclusively dependent upon the perfect match with a single motif that is uniquely located within an accessible region of a negative context. Short ASOs offer additional advantages including low cost of synthesis, ease of chemical modifications, reduced chances of immune response, and higher probability of crossing biological barriers.[58] When promotion of exon inclusion is the goal, a short intronic target brings the desired benefits of non-interference with nuclear export and translation. Hence, our findings represent further advancement towards an ASO-based therapy of SMA and bring a unique perspective to our understanding of splicing regulation of a defective gene associated with a major genetic disease of children and infants.

Materials and Methods

Plasmids, cells and ASOs. Construction of SMN2 minigene is described earlier.[35] Construct SMN2/64A contains a C-to-A mutation in SMN2 minigene and was generated by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs). HeLa cells were obtained from the American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary fibroblast cell line from SMA type I patient (Repository number GM03813) and a healthy control (Repository number AG06814) were obtained from Coriell Cell Repositories. These cells were maintained in MEM supplemented with 2 mM GlutaMAX-I and 15% FBS. All tissue culture media and supplements were purchased from Invitrogen. RNA ASOs used in our study were synthesized by Dharmacon Inc., These ASOs incorporated 2'-O-methyl modification and phosphorothioate backbone (2OMePS) as described earlier.[42]

Transfections and in vivo splicing assays. Transient transfections of cells with plasmid DNA and/or with ASOs were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. Briefly, cells were plated 24 h prior to transfection so that their density on the day of transfection was ~80%. Oligonucleotide concentration ranged from 1 to 100 nM. In a given experiment, the total amount of oligonucleotide was maintained constant by adding the control oligonucleotide (5'TGA CAT CCA CTT TGC CTT TCT CTC3'). Total RNA was isolated at the indicated time points using Trizol reagent (Invitrogen). To generate cDNA reverse-transcription was carried out using SuperScript III Reverse Transcriptase (Invitrogen) and Oligo (dT) primer (Invitrogen). 1 μg and 3 μg of total RNA were used per 20 μl of reaction for amplification of minigene-specific and endogenous spliced products, respectively. Minigene-specific spliced products were identified using Taq polymerase (Invitrogen) and the pair of primers P1 and P2 for *SMN2* minigenes.[35] For PCR amplification of endogenous exons the following primer combinations were used: N-24 and P2 for *SMN* exon 7;[42] 5'Ex4 hSMN-RP (5'GGC CAA GAC TGG GAC CAG G3') and 3'SPL8 (5'TGG TGT CAT TTA GTG CTG CT3') or 5'Ex4last1192 (5'AGG GCC AAG ACT GGG ACC AGG AAA GG3') and 3'Exon6SMN (5'CAT ATA ATA GCC AGT ATG ATA GCC3') for *SMN* exon 5; 5'Exon1SMN (5'CTG TTC CGG CGC GGC ACA GGC CAG3') and 3'Exon4SMN (5'TCA CTT TCA TCT GTT GAA ACT TGG3') for *SMN* exon 3. PCR reactions were performed either in the presence of a trace amount of [α-^{32}P] dATP (3,000 Ci/mmole) or with one of the primers being 5'end-labeled. Primers were end-labeled using [γ-^{32}P]ATP (3,000 Ci/mmole) and T4 polynucleotide kinase (New England Biolabs), followed by phenol:chloroform extraction and spinning through a Micro Bio-spin 30 Chromatography Column (Bio-Rad) to get rid of unincorporated [γ-^{32}P] ATP. Analysis and quantifications of spliced products were performed using a FPL-5000 Image Reader and Multi Gauge software (Fuji Photo Film Inc.,). Results were confirmed by at least three independent experiments.

Western blot analysis. Whole-cell extracts were prepared using ice-cold RIPA buffer (Boston BioProducts) supplemented with protease inhibitor cocktail (Roche Applied Science). Protein concentrations were determined using BSA Protein Assay Kit (Thermo Scientific). Cell extracts were resolved on a 10% (w/v) SDS-PAGE gel and transferred onto polyvinylidene fluoride (BioTrace PVDF) membrane (Pall Life Sciences). The following primary and secondary antobodies were used: mouse monoclonal anti-SMN (BD Transduction Laboratories), mouse monoclonal anti-hnRNP Q (Sigma), rabbit polyclonal anti-Tra2 (Abcam), rabbit polyclonal anti-β-actin (Sigma), horseradish-peroxidase-conjugated secondary antibodies against mouse (Sigma) and rabbit (Jackson Immuno Research). Mouse monoclonal anti-Gemin 2 and anti-Gemin 8 antibodies were kindly provided by Dr. Gideon Dreyfuss. Mouse monoclonal anti-ZPR was the same as described earlier.[51,57] In most cases, the membranes were stripped (15 min at room temperature) using Restore western Blot Stripping Buffer (Thermo Scientific) and re-probed. The membranes were scanned using UVP BioSpectrum AC Imaging System (UVP). Signal intensities were quantified using Vision works LS Image Acquisition and Analysis software (UVP). Results were confirmed by at least three independent experiments.

Immunofluorescence analysis. Patient fibroblasts (GM03813) were cultured on coverslips and transfected with 40 nM of F8 (control) and 3UP8 CY3-labeled ASOs using Lipofectamine 2000 as described above. Cell were harvested 48 hr post-transfection, washed, fixed and processed for immunofluorescence.[51,57] Double labeling (ZPR1/SMN) was carried out by sequential incubations with anti-SMN (clone 8, BD Transduction laboratories), Alexa 633-conjugated anti-mouse IgG secondary antibody (Molecular Probes) and then with FITC-conjugated LG1 (anti-ZPR1).[51,57] The cover slips were mounted on slides using Vectashield with DAPI (Vector Laboratories) and examined by indirect immunoflourescence using LSM510 confocal microscope (Carl Ziess) equipped with 405 nm diode laser.

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