A Negatively Acting Bifunctional RNA Increases *Survival Motor Neuron* Both *In Vitro* and *In Vivo*

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder and is the leading genetic cause of infant mortality. SMA is caused by the loss of *survival motor neuron-1* (*SMN1*). In humans, a nearly identical copy gene is present called *SMN2*, but this gene cannot compensate for the loss of *SMN1* because of a single silent nucleotide difference in *SMN2* exon 7. This single-nucleotide difference attenuates an exonic splice enhancer, resulting in the production of an alternatively spliced isoform lacking exon 7, which is essential for protein function. *SMN2*, however, is a critical disease modifier and is an outstanding target for therapeutic intervention because all SMA patients retain *SMN2* and *SMN2* maintains the same coding sequence as *SMN1*. Therefore, compounds or molecules that increase *SMN2* exon 7 inclusion hold great promise for SMA therapeutics. Bifunctional RNAs have been previously used to increase SMN protein levels and derive their name from the presence of two domains: an antisense RNA sequence specific to the target RNA and an untethered RNA segment that serves as a binding platform for splicing factors. This study was designed to develop negatively acting bifunctional RNAs that recruit hnRNPA1 to exon 8 and block the general splicing machinery from the exon 8. By blocking the downstream splice site, this could competitively favor the inclusion of *SMN* exon 7 and therefore increase full-length SMN production. Here we identify a bifunctional RNA that stimulated full-length SMN expression in a variety of cell-based assays including SMA patient fibroblasts. Importantly, this molecule was also able to induce SMN expression in a previously described mouse model of SMA and demonstrates a novel therapeutic approach for SMA as well as a variety of diseases caused by a defect in splicing.

Introduction

SPINAL MUSCULAR ATROPHY (SMA) is an autosomal recessive neuromuscular disorder and is the leading genetic cause of infant mortality. SMA has a carrier frequency of nearly 1 in 35 and an incidence of 1 in 6000 live births (Pearn, 1980; Melki, 1997). SMA is caused by the loss of *survival motor neuron-1* (*SMN1*). In humans, a nearly identical copy gene, called *SMN2*, is present, but this gene cannot compensate for the loss of *SMN1* because of a single silent nucleotide difference in *SMN2* exon 7 (Lefebvre *et al.*, 1997; Lorson *et al.*, 1999; Monani *et al.*, 1999). This single-nucleotide difference attenuates an exonic splice enhancer and at the same time creates an exonic splice silencer, resulting in the production of an alternatively spliced isoform lacking exon 7 (Cartegni and Krainer, 2002; Kashima and Manley, 2003; Kashima *et al.*, 2007). The majority of *SMN2*-derived transcripts lack exon 7, and these transcripts produce an unstable protein called SMN Δ 7, which cannot provide protection from SMA

development when *SMN1* is not present (Lorson and Androphy, 2000).

SMN exon 7 is highly regulated by a complex cohort of positive and negative regulatory *cis* and *trans* factors. SF2/ASF, a serine-arginine (SR)-rich protein, has been shown to interact with an enhancer that overlaps the critical C/T transition in *SMN* exon 7, and the protein is unable to bind the region in the *SMN2* context (Cartegni and Krainer, 2002). The C/T transition not only disrupts the SF2/ASF enhancer, but it creates a novel hnRNPA1-dependent splicing silencer (Kashima *et al.*, 2007). Other SR or SR-like proteins such as hTra2 β 1, SRp30c, RBMY, and hnRNP-G have all been shown to associate either directly or indirectly with *SMN* exon 7 (Hofmann *et al.*, 2000; Hofmann and Wirth, 2002; Young *et al.*, 2002). There are also several *cis*-acting negative regulatory regions that have been identified that surround *SMN* exon 7, including a negative element upstream and several negative elements including the extended inhibitory context (Miyajima *et al.*, 2002; Singh *et al.*, 2004a–c, 2007).

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Increasing expression from *SMN2*, which is retained in essentially all SMA patients, may be a viable treatment option because none of the nonpolymorphic nucleotide differences between *SMN1* and *SMN2* alters the coding sequence when properly spliced. Several strategies have been employed toward this end. Stimulating the *SMN2* locus to increase total transcripts would increase *SMNΔ7* and full-length transcripts. Increasing both transcripts would be beneficial from a therapeutic perspective because the *SMNΔ7* protein product lessens the disease phenotype (Le *et al.*, 2005). Pharmacologically increasing full-length *SMN* expression from *SMN2* or altering *SMN2* splicing to increase exon 7 inclusion has also been reported for a variety of compounds, including valproic acid, phenylbutyrate, polyphenol-based botanical compounds, hydroxyurea, suberoylanilide hydroxamic acid (SAHA), and quinazoline-based compounds (Andreassi *et al.*, 2001, 2004; Chang *et al.*, 2001; Lunn *et al.*, 2004; Brahe *et al.*, 2005; Grzeschik *et al.*, 2005; Jarecki *et al.*, 2005; Kernochan *et al.*, 2005; Brichta *et al.*, 2006; Avila *et al.*, 2007; Sakla and Lorton, 2008; Thurmond *et al.*, 2008). In addition, aminoglycosides, which can promote translational read-through of cellular stop codons, may prove beneficial for therapy by stabilization of the *SMN2*-derived *SMN* protein (Wolstencroft *et al.*, 2005; Mattis *et al.*, 2006).

Several nucleic-acid based SMA therapeutics have been developed that are designed to increase production of functional *SMN* protein by promoting inclusion of *SMN2* exon 7 (Hua *et al.*, 2007, 2008). Oligonucleotide therapies that have been designed to block the exon 8 splice site increase splicing to exon 7 (Lim and Hertel, 2001; Madocsai *et al.*, 2005). Novel peptide-nucleic acids that comprise an exon 7 antisense domain and a synthetic splicing-activation domain peptide were shown to increase *SMN2* exon 7 inclusion (Cartegni and Krainer, 2003). Similar methods using bifunctional RNAs have also been previously shown to increase full-length *SMN* transcripts as well as *SMN* protein levels and derive their name from the presence of two domains: an antisense RNA sequence specific to the target RNA and an untethered RNA segment that serves as a binding platform for splicing factors (Skordis *et al.*, 2003; Baughan *et al.*, 2006). In other genetic models, negatively acting bifunctional RNAs that recruit negatively acting proteins to an intron/exon boundary successfully redirected pre-mRNA splice site decisions (Villemaire *et al.*, 2003; Gendron *et al.*, 2006).

This study aimed to discover whether a negatively acting bifunctional RNA, designed to recruit hnRNP1 to the exon 8 splice site, could block the general splicing machinery from the exon 8 boundary, thereby competitively favoring the slightly weaker *SMN* exon 7 splice site. A bifunctional RNA was identified that specifically bound the *SMN* exon 8 splice boundary and stimulated full-length *SMN* protein in a variety of cell-based assays, including SMA patient fibroblasts. Furthermore, *SMN* protein levels were elevated *in vivo* after administration of the bifunctional RNA in a mouse model of SMA.

Materials and Methods

Construction of plasmids

Bifunctional clones were generated with annealed complementary pairs of DNA oligonucleotides (Integrated DNA Technologies, Coralville, IA) that were cloned into the previously published pMU2 vector between the *Bam*HI and *Spe*I

sites (Baughan *et al.*, 2006). A *Kpn*I site was engineered as the spacer region between the antisense and tandem repeats of the splicing factor, targeting for ease of transition between different antisense and targeting domains. Sequences used for the exon8-hnRNP1 bifunctional RNA are as follows: top, 5'-GAT CCA GCA TTT CCT GCA AAT GAG GGT ACC TAG GGA TAG GGA TAG GGA TTT TTT A-3'; bottom, 5'-CTA GTA AAA AAT CCC TAT CCC TAT CCC TAG GTA CCC TCA TTT GCA GGA AAT GCT G-3'. Plasmids were sequenced for verification. The *SMN* intron 7/exon 8 sequence targeted by the antisense RNA within the bifunctional RNA and the antisense sequence used previously (Madocsai *et al.*, 2005) is as follows: 5'-tttctcattgcaggaattctgcatagag-3'.

Luciferase assay

Subconfluent HeLa cells were plated in 12-well dishes and cotransfected with a previously described *SMN2*-luciferase plasmid (Zhang *et al.*, 2001) and the exon8-hnRNP1 bifunctional plasmid, using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). pCI-SMN1-Luc and pCI-SMN2-Luc were transfected at 1 μ g and, to equalize DNA content, were also transfected with 3 μ g of sheared salmon sperm DNA. The experimental cells were transfected with 1 μ g of pCI-SMN2-Luc and 3 μ g of pMU2-exon8-hnRNP1. Transfected cells were collected with a luciferase assay system (Promega, Madison, WI) and luminescence was measured with a luminometer.

2'-O-Methyl RNA transfection

Sequences of the 2'-O-methyl RNAs (Integrated DNA Technologies) are as follows: Exon8-hnRNP1, 5'-CCA GCA UUU CCU GCA AAU GAG GGU ACC UAG GGA UAG GGA UAG GGA-3'; G2, 5'-CCU UUA CGA CCG UAU CUC-3'; D2-2, 5'-AAG AUU AAA GAG UAA ACG UCC-3'; D2-2 hnRNP1, 5'-AAG AUU AAA GAG UAA ACG UCC GGU ACC UAG GGA UAG GGA UAG GGA-3'; and hnRNP1-act, 5'-UAG GGA UAG GGA UAG GGA-3'. The D2-2 sequence was based on a previously published sequence that was unable to increase splicing to exon 7 (Madocsai *et al.*, 2005). Subconfluent type I patient fibroblast cells (GM03813 cells; Coriell Cell Repositories, Camden, NJ) were transfected with 100 ng of each indicated RNA, using Lipofectamine 2000 reagent (Invitrogen).

Immunofluorescence imaging

For all immunofluorescence staining, subconfluent GM03813 cells were transfected for 48 hr with either exon8-hnRNP1-pMU2 or 2'-O-methyl RNAs, using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were fixed with an acetone-methanol (1:1) solution and washed with phosphate-buffered saline (PBS). Cells were blocked with 5% bovine serum albumin (BSA) in PBS and subsequently washed with PBS. A previously described pooled collection of mouse anti-SMN monoclonal antibodies (Wolstencroft *et al.*, 2005) was added, diluted 1:10 in 1.5% BSA in PBS. After PBS washing, the secondary antibody, anti-mouse antibody conjugated to either Texas red (Jackson ImmunoResearch Laboratories, West Grove, PA) or fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO), was added diluted 1:200 in 1.5% BSA in PBS. After another wash, 4',6-diamidino-2-phenylindole (DAPI) was added to each sample for 5 min and the samples were washed again. Coverslips

were then mounted to samples, using mounting medium (1,4-diazabicyclo[2.2.2]octane [DABCO, 2.3% (w/v)], 10% PBS, 87.7% glycerol), and sealed with nail polish. Microscope images were captured with a Nikon Eclipse E1000 using Meta-Morph software. Gem counts were performed in triplicate, counting 500 cells for each treatment.

Western blot analysis

Patient fibroblast cells transfected with 2'-O-methyl RNAs were harvested 48 hr posttransfection. Cells were added to 10 μ l of sodium dodecyl sulfate (SDS) loading dye, boiled for 5 min, and resolved on an SDS-10% polyacrylamide gel. The gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) and an SMN immunoblot was performed with 4F11 monoclonal SMN antibody (Wolstencroft *et al.*, 2005). Horseradish peroxidase-conjugated goat anti-mouse antibody was used as the secondary antibody and was detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Blots were H_2O_2 inactivated (H_2O_2 /PBS, 1:1) and reprobed with anti-actin antibodies and developed with anti-rabbit secondary antibody by similar methods. All Western blots were performed in triplicate or quadruplicate and representative images are shown.

Injected mouse tissues were harvested 24 hr postinjection for brain samples and 48 hr postinjection for kidney and liver samples and snap frozen in liquid nitrogen. Samples were then thawed on ice and prepared for Western blot as previously described. All Western blot images were captured with a Fuji Imager and Multi-Gage V2.3 system. Each Western

blot was performed in triplicate or quadruplicate and representative results are shown.

Animal injection

All animals were housed and treated in accordance with animal care and use committee guidelines. *SMN2*^{+/+}, *SMNΔ7*^{+/+}, and *Smn*^{-/-} mice (Le *et al.*, 2005) were genotyped on the day of their birth, designated day 0, and injected on day 1. Intravenous injection was performed as previously described (Sands and Barker, 1999), using 2'-O-methyl RNA at 12.5 mg/kg. Intraventricular injection was performed as previously described (Passini and Wolfe, 2001), using 2 μ g of 2'-O-methyl RNA per ventricle. Neonates were then returned to their mothers for 24–48 hr, and then anesthetized with isoflurane and killed by decapitation.

Results

Development of a negatively acting bifunctional RNA

Antisense RNAs that recruit negatively acting proteins have been successfully developed to modulate alternative pre-mRNA splicing in other systems (Villemaire *et al.*, 2003; Gendron *et al.*, 2006). To determine whether negatively acting bifunctional RNAs could be developed that stimulated full-length SMN expression, we used a previously published antisense region (Madocsai *et al.*, 2005) and three tandem repeats of the high-affinity hnRNP A1 sense region (Burd and Dreyfuss, 1994) (Fig. 1A). The SELEX-determined hnRNP A1-binding site was used because this binding motif would have a high probability of re-

FIG. 2. Cotransfection of *SMN2*-luciferase reporter with exon8-hnRNPA1 bifunctional RNA increases luminescence. HeLa cells were transfected with a cassette expressing *SMN2*-luciferase and exon8-hnRNPA1 bifunctional-expressing plasmid. These cells showed a significant increase in luminescence, showing an increase in splicing to exon 7 ($p < 0.08 \times 10^{-8}$).

cruiting hnRNPA1. To initially screen these bifunctional RNAs, cassettes encoding the bifunctional RNAs were cloned into a previously published delivery vector, pMU2 (Baughan *et al.*, 2006). The bifunctional RNA is driven by a polymerase III-dependent U6 promoter and a separate polymerase II-dependent cytomegalovirus (CMV) promoter drives expression of green fluorescent protein (GFP), thus allowing detection of transfected cells.

Exon8-hnRNPA1 RNA increases SMN2 full-length expression via luciferase

To determine whether the exon8-hnRNPA1 bifunctional RNA could alter *SMN2* exon 7 splicing, a previously described *SMN2* luciferase reporter plasmid was used to analyze the exon8-hnRNPA1 bifunctional RNA (Zhang *et al.*, 2001). The *SMN2*-luciferase vector is designed to produce luciferase only when exon 7 is in frame; therefore, compounds or RNAs that induce exon 7 inclusion should result in an increase in luciferase activity. When the *SMN1* construct was transiently expressed in HeLa cells, expected high levels of luminescence were detected, whereas the *SMN2* construct expressed lower levels of luminescence (Fig. 2). Cotransfection of the plasmid expressing the exon8-hnRNPA1 bifunctional RNA, exon8-hnRNPA1-pMU2, resulted in a significant increase in luciferase activity compared with *SMN2* alone (Fig. 2), whereas cotransfection of the *SMN2* luciferase plasmid and the parental pMU2 plasmid that expresses an RNA of similar size did not increase luminescence (Fig. 2).

Exon8-hnRNPA1 RNA increases gem numbers in fibroblasts from patients with severe disease

SMA type I patient fibroblasts (GM03813 cells) are an excellent cell-based system with which to study potential SMA therapies because these cells lack *SMN1* and retain two copies of *SMN2* (Coovert *et al.*, 1997). These cells contain low levels of SMN protein and few SMN-positive nuclear foci, termed “gems,” which are a biomarker for disease severity as carriers and unaffected cell lines have higher gem numbers than do SMA cells (Coovert *et al.*, 1997). Because the luciferase assay demonstrated an increase in splicing to *SMN2* exon 7 in exon8-hnRNPA1-transfected cells, we next sought to determine whether exon8-hnRNPA1-pMU2 induced increased SMN levels in a more complex and disease-relevant cellular context. To this end, GM03813 cells were transfected with the plasmid expressing the exon8-hnRNPA1 bifunctional RNA. Plasmid DNA transfection levels of GM03813 cells, which are primary patient fibroblasts, are low, as expected. However, transfected cells are easily identified because of the plasmid-derived GFP moiety encoded by a separate promoter within the pMU2 derivatives. After transient transfection of the exon8-hnRNPA1-expressing plasmid for 48 hr, SMN-positive gem numbers were increased severalfold above those of untransfected or parental pMU2-transfected cells, as determined by indirect immunofluorescence (Fig. 3). The parental vector, pMU2, expresses a similarly sized scrambled RNA that has no homology to the SMN sequences.

FIG. 3. Transfection of exon8-hnRNPA1 bifunctional RNA increases gem numbers in fibroblasts from patients with severe disease. Exon8-hnRNPA1 bifunctional RNA-expressing plasmid was transfected into GM03813 cells (type I patient fibroblast cells) and harvested after 48 hr. Transfected cells were identified by GFP expression and SMN protein was analyzed with a previously published SMN antibody.

2'-O-Methyl exon8-hnRNPA1 RNA increases gem numbers in fibroblasts from patients with severe disease

As it is not currently practical to deliver plasmid-derived bifunctional RNAs as an SMA therapeutic, the previous qualitative analysis of bifunctional RNAs served as a screen to determine whether negatively acting bifunctional RNAs could increase SMN levels. 2'-O-Methyl RNAs are a more tractable therapeutic candidate and are more easily transfected into primary patient fibroblasts, and were therefore selected as the chemistry to analyze in the second generation of bifunctional RNAs. The addition of the 2'-O-methyl moiety stabilizes the RNA and allows for sustained expression *in vivo*. To evaluate the ability of the exon8-hnRNPA1 bifunctional 2'-O-methyl RNA to induce SMN levels, several additional RNAs were designed: "ex8 antisense" corresponds exclusively to the antisense segment within the exon8-hnRNPA1 bifunctional RNA and is a previously published antisense that increased splicing to exon 7 (Madocsai *et al.*, 2005); "hnRNPA1-act" is an RNA that lacks any antisense component and is composed solely of the hnRNPA1-binding motifs present in the bifunctional RNAs, and can be thought of as the activation (act) alone; "int7/ex8" is a previously published antisense RNA that targets the intron 7/exon 8 boundary and was only minimally able to increase splicing to exon 7 (Madocsai *et al.*, 2005); "int7/ex8-hnRNPA1" is a bifunctional RNA with the same antisense domain as "int7/ex8" and the same hnRNPA1-binding motifs as "exon8-hnRNPA1" (Fig. 1B).

To determine whether these RNAs increase total SMN levels, the RNAs were transfected into GM03813 cells. RNA transfection levels in these cells are significantly higher than plasmid transfection levels, and can range from 10 to 30% (data not shown). After 48 hr, cells were analyzed by indirect immunofluorescence to identify SMN-positive gems (Fig. 4A). In cells transfected with the exon8-hnRNPA1 bifunctional RNA, gem numbers were significantly elevated when 500 randomly selected cells were analyzed (Fig. 4B). In this system, it was not possible to identify the transfected cells because the RNA molecules were not tagged with a fluorescent moiety. The additional 2'-O-methyl RNAs were analyzed similarly, and were shown to have a modest impact on gem numbers, albeit greater than untransfected GM03813 levels. However, gem numbers from the additional RNAs were significantly lower than those associated with the exon8-hnRNPA1 bifunctional RNA (Fig. 4B).

2'-O-Methyl exon8-hnRNPA1 RNA increases total SMN protein level in fibroblasts from patients with severe disease

As the previous results demonstrated an increase in SMN-containing gems, we next sought to determine whether bifunctional RNAs altered steady state levels of total SMN protein in GM03813 cells. GM03813 cells were transfected with the panel of 2'-O-methyl RNAs, extracts were generated from transfected cells, and SMN levels were measured by Western blotting with an anti-SMN monoclonal antibody. In extracts from cells transfected with the exon8-hnRNPA1 bifunctional RNA, total SMN levels were consistently increased, resulting in levels of SMN similar to those detected in extracts from unaffected 3814 cells. Additional RNAs that

resulted in lower levels of gems were less robust inducers of SMN (Fig. 5).

Intravenous delivery of 2'-O-methyl exon 8-hnRNPA1 RNA increases SMN protein levels in a mouse model of SMA

To determine whether the exon8-hnRNPA1 bifunctional RNA could elevate SMN levels in an SMA-specific context, the facial vein of 1-day-old SMA mice was injected with either the exon8-hnRNPA1 bifunctional RNA or int7/ex8 antisense RNA. Facial vein delivery in neonates is an effective method to achieve systemic delivery (Sands and Barker, 1999). The SMA mouse model used in these experiments is well characterized, lacks endogenous murine Smn protein, and has the following genotype: *SMN2*^{+/+}; *SMNΔ7*; *Smn*^{-/-}. Importantly, the genomic *SMN2* is present, which allows alternative splicing and the examination of potential therapeutics; therefore, increases in SMN protein are due to an induction of the human *SMN2* transgene. Forty-eight hours postinjection, SMN levels were measured in kidney and liver, as these are the tissues most likely to be exposed to the RNAs by this type of delivery protocol. Consistent with the cellular analysis of the exon8-hnRNPA1 bifunctional RNA, Western blot analysis demonstrated that SMN levels were increased in the kidney and liver of exon8-hnRNPA1 bifunctional RNA-injected animals (Fig. 6A). SMN levels were poorly elevated in one mouse, potentially because of an injection error or because of a lack of responsiveness to the exon8-hnRNPA1 bifunctional RNA either at the uptake level or intracellularly. In addition, one int7/ex8-injected mouse showed a small SMN increase in liver. Although this latter result is incongruous with the previous cell-based analysis, it is not completely unexpected as the antisense sequence within the int7/ex8 bifunctional RNA corresponds to a previously published antisense sequence that was capable of modestly altering *SMN2* pre-mRNA splicing (Madocsai *et al.*, 2005). In this previously published study, however, SMN protein levels were not examined in cells or *in vivo*.

Intraventricular delivery of 2'-O-methyl exon 8-hnRNPA1 RNA increases SMN protein levels in SMA mice

Because SMA is a neurodegenerative disease, SMA mice were used to determine whether the exon8-hnRNPA1 RNA could increase SMN levels in central nervous system tissue. To deliver the RNAs to the central nervous system, intraventricular injections of exon8-hnRNPA1 and int7/ex8-hnRNPA1 bifunctional RNAs were performed on 1-day-old SMA mice. Twenty-four hours postinjection, brain tissue was harvested and Western blotting of total protein extracts was performed to determine total SMN protein levels in the brain. Brain extract from five exon8-hnRNPA1-injected SMA mice was examined. Consistent with the analysis of the intravenous injections, mice that received the intraventricular exon8-hnRNPA1 RNA injection exhibited a consistent increase in total SMN protein levels compared with the PBS-injected animal (Fig. 6B). D2-2 and hnRNPA1-act exhibited only modest levels of activation compared with PBS-treated samples. Taken together, these results identify negatively acting bifunctional RNAs as an efficient means to increase SMN protein levels in primary patient fibroblasts, as well as in the more complex *in vivo* context of the SMA mouse model.

A

FIG. 4. (A and B) Transfection of 2'-O-methyl exon8-hnRNPA1 bifunctional RNA increases gem numbers in fibroblasts from patients with severe disease. 2'-O-Methyl bifunctional RNAs were transfected into GM03813 cells (type I patient fibroblast cells) and SMN localization was observed with an SMN-specific antibody 48 hr posttransfection. Gem distribution showed that the majority of transfected cells had one or two gems per nucleus. Exon8-hnRNPA1 transfection significantly increased gem numbers ($p < 0.006$).

B

FIG. 5. Transfection of 2'-O-methyl exon8-hnRNA1 bifunctional RNA increases total SMN protein in fibroblasts from a patient with severe disease. 2'-O-Methyl bifunctional RNAs were transfected into GM03813 cells (type I patient fibroblast cells) and total SMN protein was analyzed by Western blotting 48 hr posttransfection.

Discussion

SMA represents a unique genetic context for therapeutic development for several reasons. Approximately 97% of SMA cases are caused by the loss of *SMN1*, but retain at least one copy of *SMN2* (Wirth *et al.*, 1999). As *SMN2* is a nearly identical copy of *SMN1* and has the ability to encode the same protein as *SMN1* when exon 7 is included in the fully spliced transcript, this makes the disease extremely amenable to therapies designed to promote full-length *SMN* mRNA. In addition, transcripts derived from *SMN2* appear relatively stable (Heier *et al.*, 2007), and similarly abundant compared with *SMN1* transcripts.

The results presented in this study include the first description of a 2'-O-methyl bifunctional RNA functioning *in vitro*, and more importantly, *in vivo* in the mouse model of SMA. These RNAs supply *in trans* a binding platform for regulatory splicing factors, in this case hnRNA1. The binding site for this study used an *in vitro*-determined hnRNA1-binding sequence; however, it is possible that an empirically determined binding site may stimulate greater levels of full-length *SMN* production. The antisense region may contribute to the production of full-length *SMN* because the same targeting region was previously shown to increase exon 7 inclusion in cells transfected with synthetic antisense RNAs with U7 moieties (Madocsai *et al.*, 2005). However, in these experiments, the antisense alone was less effective in producing endogenous *SMN*, demonstrating that the combined activity of the antisense and *trans*-factor-binding sites contribute to *SMN* production.

The results described herein show that a negatively acting bifunctional RNA designed to recruit hnRNA1 to the *SMN* intron7/exon8 boundary is able to increase splicing to exon 7. As the exon8-hnRNA1-based RNA blocks the exon 8 splice site decision, it is possible that although the splicing to exon 7 is increased via the *SMN2*-luciferase assay, this may not accurately represent the extent to which the bifunctional RNA is working, because if intron 7 is not removed, the luciferase cassette will be out of frame. It is possible, therefore, that the increase in *SMN* protein we observed does not di-

rectly relate to the increase in luminescence observed. In addition, it is important to examine *SMN2* expression in a variety of experimental contexts because it appears that some of the oligonucleotides induced *SMN* levels to various degrees, depending on the cellular context. Although these are important considerations, the results presented here demonstrate that the leading candidate, exon8-hnRNA1, was robust in all of the assays examined, ranging from reporter assays to a complex *in vivo* disease context within the central nervous system.

The ideal time during development that an SMA therapy must be administered in order to be effective is still unclear. This time line would need to be evaluated clearly in order to have an effective therapy. Another challenge facing SMA therapy is the necessity that the therapy cross the blood-brain barrier. As direct delivery to the ventricles may not be ideal for a human treatment, it is possible to express these small RNAs using recombinant adeno-associated virus (rAAV). This virus is an ideal candidate for gene therapy as it is replication deficient and certain AAV serotypes have been shown to have high tropism for both muscles and neurons and can use retrograde transport to travel from muscle to neurons *in vivo*. Previous studies have successfully used the retrograde transport ability of a pseudotyped lentivirus to deliver *SMN* cDNA (Azzouz *et al.*, 2004). Although these data have direct implications for SMA therapy, they could also be transitioned to be applicable to many disease states in which it would be desirable to skip an exon, such as in muscular dystrophy, where skipping an exon or set of exons may return the proper reading frame.

FIG. 6. Injection of 2'-O-methyl exon8-hnRNA1 bifunctional RNA increases total SMN protein in the SMA mouse model. (A) Intrafacial vein injection of 2'-O-methyl exon8-hnRNA1 bifunctional RNA increases SMN protein in liver and kidney after 48 hr. (B) Intraventricular injection of 2'-O-methyl exon8-hnRNA1 bifunctional RNA increases SMN protein in brain after 24 hr. Equivalent levels of protein were quantitated and confirmed by staining with actin (data not shown).

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Author Disclosure Statement

There are no competing or financial interests to disclose.

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