Published in final edited form as:
Methods Mol Biol. 2012 ; 867: 307–323. doi:10.1007/978-1-61779-767-5_20.

Antisense-mediated exon inclusion

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Abstract

Exon skipping induced by gene mutations is a common mechanism responsible for many genetic diseases. A practical approach to correct the aberrant splicing of defective genes is to use antisense oligonucleotides (ASOs). The recognition of splice sites and the regulation of splicing involve multiple positive or negative cis-acting elements and trans-acting factors. Base-pairing of ASOs to a negative element in a targeted pre-mRNA blocks the binding of splicing repressors to this cis-element and/or disrupts an unfavorable secondary structure; as a result, the ASO restores exon inclusion. For example, we have recently shown that appropriate 2'-*O*-(2-methoxyethyl) (MOE) phosphorothioate-modified ASOs can efficiently correct *survival motor neuron 2* (*SMN2*) exon 7 splicing in a cell-free splicing assay, in cultured human cells—including patient fibroblasts—and in both peripheral tissues and the CNS of SMA mouse models. These ASOs are promising drug leads for SMA therapy.

Keywords

Exon skipping; antisense oligonucleotide; MOE; splicing; *SMN2*; SMA; ESS; ISS; cis-acting element; in vitro splicing assay; minigene; exon 7 inclusion; RT-PCR; ICV; ICV infusion; mouse tissue; spinal cord; CNS

1. Introduction

It has been estimated that over 60% of point mutations cause hereditary diseases by altering splicing (1). There are several ways in which mutations can lead to exon skipping: first, mutations at either splice site can inactivate or weaken the splice site; second, mutations near one of the splice sites, or even some distance away from it, can disrupt a critical exonic or intronic splicing enhancer (ESE or ISE) element; third, mutations away from the splice sites can fortuitously create an exonic or intronic splicing silencer (ESS or ISS); and fourth, mutations can alter RNA secondary structure in such a way as to disrupt the accessibility of signals important for exon recognition. As one example of the first type of splicing mutation, a T to C transition at position 6 of *IKBKAP* intron 20 results in skipping of exon 20, giving rise to familial dysautonomia (2). In principle, any approach that can restore exon inclusion in the context of disease-associated exon skipping should have therapeutic relevance.

A recent study showed that exon skipping is the most prevalent form of normal alternative splicing in human cells (3). Many exon skipping events are developmentally regulated or tissue-specific. Thus, similar methods could be used to restore exon inclusion for splicing-defective alleles, or to promote inclusion of alternative exons in normal genes. Redirecting the splicing of such normal alternatively spliced exons could be an important tool to dissect

the functions of splicing isoforms at different developmental stages or cell types, and to understand the complexity of gene-expression regulation by alternative splicing.

A powerful way to restore or promote exon inclusion is to use appropriately targeted antisense oligonucleotides (ASOs). Hybridization of the ASO to the target RNA to block an ESS or ISS should block the binding of the corresponding repressor—and/or disrupt an inhibitory secondary structure—resulting in more efficient recognition of the exon by the spliceosome. Antisense technology employs modified oligonucleotides to specifically bind target RNA sequences through Watson-Crick base pairing. Chemical modifications of the ASO are critical for its efficacy in splicing correction. They not only impart higher affinity for the targeted RNA, and resistance to both exo- and endonucleases, but also confer resistance to cleavage of the hybridized mRNA by RNase H (in contrast to DNA ASOs). In one study, ASOs with 2'-*O*-(2-methoxyethyl) (MOE)-phosphorothioate backbone outperformed other modifications, such as 2'-*O*-methyl (2'-OMe) and morpholino (4). MOE ASOs have been extensively characterized pharmacologically in animal models, and are a good option for redirecting RNA splicing.

Traditionally, deletion and point-mutagenesis analyses are used to find potential splicing silencers, and then an ASO is designed empirically to mask the silencer activity, i.e., to occlude repressor binding. However, this method is tedious, and error-prone. We have established a two-step ASO-tiling method involving ASO walks to systematically screen hundreds of MOE ASOs targeting a gene of interest; we have successfully applied this method to target *SMN2* exon 7 and its flanking introns in spinal muscular atrophy (SMA) (5, 6). The first, coarse walk involves a series of overlapping ASOs—e.g., 15mers with 10-nt overlap. The second, high-resolution walk focuses on regions of interest identified in the first walk, and uses a series of ASOs differing in length and/or position to map the exact silencer region and identify optimal ASOs; for example, we used 12mers, 15mers, and 18mers positioned along the target sequence in 1-nt steps. The efficacy of ASOs can be tested in several splicing assays: cell-free splicing with *in vitro* transcribed pre-mRNA; splicing of minigenes transfected into cultured cells; and splicing of RNA expressed from the endogenous genes in cultured cells, patient fibroblasts, or animal models.

Although MOE ASOs with a phosphorothioate backbone show superior antisense activities, MOE ASOs with a phosphodiester backbone are sufficiently effective in cell-free splicing assays and cultured cells. Less expensive modifications, such as 2'-OMe, can also be used in initial *in vitro* splicing assays and splicing assays with minigenes/endogenous-genes in cultured cells; however, 2'-OMe ASOs are not as effective when targeting the CNS, for example, and may induce CNS inflammation (7). Another important consideration is that for a given ASO sequence, different modifications may give different effects; one type of modification may be ineffective, or could even give opposite results as another, i.e., exon skipping instead of exon inclusion (unpublished data).

SMA is caused by loss-of-function mutations/deletions in the *SMN1* gene; an important feature of this disease is the existence of a paralog gene, *SMN2*, whose copy number is inversely proportional to disease severity (8). Both genes express an identical SMN protein, albeit at different levels. Exon 7 is efficiently included in spliced mRNA from *SMN1*; however, a silent C to T transition at position 6 of *SMN2* exon 7 results in marked skipping of this exon during pre-mRNA splicing (9, 10). The C to T transition abrogates an SF2/ASF-dependent exonic splicing enhancer (CAGACAA) and also appears to strengthen an hnRNP A1-dependent exonic splicing silencer (CAG to UAG) (11, 12). Because all SMA patients carry one or more copies of *SMN2*, increasing the extent of *SMN2* exon 7 inclusion holds great promise to treat or possibly cure SMA. Using *SMN2* exon 7 as an example, we

describe here how to identify and test MOE ASOs that promote exon inclusion, using various splicing assays.

2. Materials

2.1. *In vitro* splicing assay

1. *SMN1* and *SMN2* minigene plasmids pCI-SMN1 and pCI-SMN2 comprising the 111-nt exon 6, a shortened intron 6, the 54-nt exon 7, the 444-nt intron 7, the first 75 nt of exon 8, and a consensus 5' splice site (CAGGTAAGTACTT). The plasmids are linearized with restriction endonuclease *Sal* I or *Not* I. The original empty plasmid was pCI-neo.
2. Milli-Q water, 0.1% (v/v) diethylpyrocarbonate (DEPC)-treated.
3. RNA dye mixture: 90% (v/v) formamide, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF.
4. *In vitro* transcription reagents, including 10 mM ^{7Me}GpppG cap analog, T7 RNA polymerase, 5 × T7 RNA polymerase buffer, NTP mixture (5 mM ATP, 5 mM CTP 0.5 mM GTP and 0.5 mM UTP), α-^{32}P-UTP (250 μCi/25 μl, 800 Ci/mmol), 0.1 M dithiothreitol (DTT) and RNasin (RNase inhibitor, 40 units/μl) (13).
5. A 20 × 20 × 0.038 cm gel-casting cassette and an electrophoresis system.
6. 5.5% and 8% denatured acrylamide/8.3 M urea gels with dimensions of 18 × 18 × 0.038 cm (see Note 1).
7. 20× TBE electrophoresis buffer: add 216 g Tris Base, 110 g boric acid and 80 ml of 0.5 M EDTA in water to a total volume of 1 liter. Dilute to 1× TBE to run polyacrylamide gels.
8. Luminescent peel-off stickers
9. RNA elution buffer: 0.1% SDS, 0.5 M HN_4Ac.
10. 100% and 80% (v/v) ethanol.
11. 10 μg/μl glycogen.
12. Geiger counter, Beckman LS 6000 Series Liquid Scintillation System, scintillation fluid and scintillation vials.
13. 0.4 M Hepes-KOH, pH 7.3. Sterilize by filtration and store at 4°C or −20°C.
14. 80 mM $MgCl_2$, store at 4°C or −20°C.
15. 25× ATP/CP mixture: 12.5 mM ATP and 0.5 M creatine phosphate. Aliquot in 50 μl and store at −20°C.
16. 13% (w/v) low-molecular-weight polyvinyl alcohol (PVA). Store at −20°C.
17. Buffer D: 20 mM Hepes-KOH (pH 8.0), 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM PMSF (freshly added), 1 mM DTT (freshly added).
18. Nuclear extract, prepared from HeLa cells, store at −80°C. Refer to Mayeda A and Krainer AR (2000) (14).

[1]To make a denatured gel, prepare a 20 ml solution. For 5.5%, use 4.4 ml SequaGel concentrate, 2 ml SequaGel buffer and 13.6 ml SequaGel diluents; for 8%, use 6.4 ml SequaGel concentrate, 2 ml SequaGel buffer and 11.6 ml SequaGel diluent. Add 200 μl of 10% (w/v) ammonium persulfate (Aps) and 20 μl *N,N,N′, N′*-tetramethylethylenediamine (TEMED); mix the solution by swirling gently and pour into a gel-casting cassette. Wait for at least 30 min to let the gel polymerize.

19. 2 μM MOE ASOs with a phosphodiester backbone. Store at −20°C.
20. Splicing stop solution: 0.3 M NaAc (pH 5.2), 0.1% (w/v) SDS, 62.5 μg/ml tRNA. Store at room temperature.
21. Phenol solution saturated with Tris-HCl (pH 8.0).
22. Radiolabeled single-strand DNA size marker.
23. Gel Dryer, Model 583.
24. Image Reader FLA 5100.

2.2. Splicing assay with minigenes and endogenous genes in cultured cells

1. *SMN1* and *SMN2* minigene plasmids pCI-SMN1 and pCI-SMN2.
2. Dulbecco's Modified Eagle's Medium (DMEM), Opti-MEM I Reduced Serum Medium (Opti-MEM) and trypsin-EDTA solution.
3. Cell line HEK293, HeLa or C33A.
4. Hemocytometer.
5. 200 μM MOE ASOs with a phosphodiester backbone.
6. Optional materials: plasmids expressing GFP and pBabe-puro.
7. Gene Pulser II apparatus and 1-mm cuvettes.
8. Reagents to isolate RNA: Trizol, chloroform, isopropanol and 75% (v/v) ethanol (Note 2).
9. Milli-Q water, 0.1% (v/v) diethylpyrocarbonate (DEPC)-treated.
10. RQ1 RNase-free DNase, 10 × RQ1 RNase-free DNase buffer, RQ1 DNase Stop Solution.
11. RT-PCR reagents: SuperScript II reverse transcriptase (RT), 5 × SuperScript II RT buffer, 0.5 μg/μl custom 18mer oligo dT, desalted (see Note 3), dNTP mixture (10 mM each), 0.1 M DTT, AmpliTaq DNA polymerase (5 U/μl), 10 × AmpliTaq buffer containing 1.5 mM $MgCl_2$, and α-^{32}P-dCTP (3000 Ci/mmol, 10 mCi/ml).
12. Two sets of PCR primers. Primers SMN-T7F2 (forward, 5'-TACTTAATACGACTCACTATAGGCTAGCCTCG-3') and SMN8-75+5'R (reverse, 5'-AAGTACTTACCTGTAACGCTTCACATTCCAGATCTGTC-3') are used for amplifying *SMN1/2* minigene transcripts; the product size is 284 bp when exon 7 is included, or 230 bp when exon 7 is skipped. Primers Ex6-F (forward, 5'-ATAATTCCCCCACCACCTCCC-3') and Ex8-467-R (reverse, 5'-TTGCCACATACGCCTCACATAC-3') are used for amplifying *SMN1/2* endogenous gene transcripts; the size is 632 bp when exon 7 is included, or 578 bp when exon 7 is skipped.
13. Restriction endonuclease Dde I.
14. 6 × DNA loading buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol in Milli-Q water.

[2]RNeasy Mini Kit together with RNase-free DNase Set is another option to generate high-quality total RNA from both cells and tissues.
[3]Custom 18mer oligo-dT, desalted works well.

15. 6% native polyacrylamide gels with dimensions of 18 × 18 × 0.038 cm (see Note 4).
16. For the gel-casting cassette, electrophoresis apparatus, gel dryer, and phosphor-imaging instrument, refer to **Material 2.1** items 5, 23 and 24.

2.3. Analyzing ASO activity in peripheral tissues of SMA mouse model

1. 0.9% (w/v) saline.
2. MOE ASOs with a phosphorothioate backbone and all 5-methyl cytosines dissolved in 0.9% (w/v) saline.
3. 1- and 5-ml disposable syringes, 20–30 gauge needles, surgical scissors and forceps.
4. Mouse restraining tubes and a warming lamp.
5. Liquid nitrogen, mortar and pestle.
6. Human *SMN1/2* specific primers. Forward primer E4-33to55: 5'-AAGTGAGAACTCCAGGTCTCCTG-3', reverse primer Ex8-29to52-R: 5'-TCTGATCGTTTCTTTAGTGGTGTC-3'. PCR product size is 432 bp when exon 7 is included or 378 bp when exon 7 is skipped.
7. For reagents to isolate RNA and perform RT-PCR, see **Material 2.2** items 8–11.
8. For materials to analyze RT-PCR products, refer to **Material 2.2** items 14–16.
9. 1× SDS protein sample buffer: 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 6.8), and 0.1 M DTT.
10. TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20).
11. Western blot blocking buffer: 5% (w/v) non-fat milk in TBST.
12. Rabbit polyclonal anti-β-tubulin antibody, mouse monoclonal human-specific anti-SMN antibody (7, 15), secondary IRDye 800CW-conjugated goat anti-mouse or anti-rabbit antibodies.
13. Odyssey Infrared Imaging instrument to quantitate western blot signals.

2.4. Embryonic ICV delivery of ASOs and splicing assay in CNS tissues

1. MOE ASOs with a phosphorothioate backbone and all 5-methyl cytosines dissolved at 2.5 – 10 μg/μl in 0.9% (w/v) saline.
2. Fast Green FCF dissolved at 0.1% (w/v) stock solution in 0.9% (w/v) saline.
3. Anesthesia machine, Aerrane (isofluorane) and oxygen tanks.
4. Surgical materials, including 0.9% (w/v) saline, 37 °C water bath, fur clipper, scissors, forceps, ring forceps, gauze, and surgical sutures.
5. Capillary glass tubing with flame-polished end, OD=1.50 mm, ID=0.86 mm, length=10 cm; Needle pipette puller, model 730 and MF-900 Microforge. These are used to prepare glass micropipettes.

[4]To make a native gel, mix 4 ml of 30% acrylamide/bis solution (29:1), 1 ml of 20 × TBE (**Material 2.1** item 7), 15 ml Milli-Q water, 200 μl of 10% (w/v) Aps and 20 μl TEMED. Pour the mixture into a gel-casting cassette, and wait for at least 30 min to let the gel polymerize before use.

6. Materials used for preparing and analyzing tissue RNA/protein samples, see **Material 2.3** items 5–13.

2.5. Neonatal ICV delivery of ASOs and splicing assay in CNS tissues

1. MOE ASOs with a phosphorothioate backbone and all 5-methyl cytosines dissolved at 2.5 – 10 μg/μl in 0.9% (w/v) saline.
2. Fast Green FCF dissolved at 0.1% (w/v) stock solution in 0.9% (w/v) saline.
3. Fiber-optic illuminator with dual goose neck.
4. 5-μl RN (removable needle) syringe and 33-gauge custom needle (1-cm long, point style, 30° beveled).
5. Surgical tools: 0.9% (w/v) saline, surgical scissors and forceps.
6. Materials used for preparing and analyzing tissue RNA/protein samples, see **Material 2.3** items 5–13.

2.6. ICV infusion of ASOs and splicing assay in CNS tissues

1. MOE ASOs with a phosphorothioate backbone and all 5-methyl cytosines dissolved at 0.83, 2.08, 4.17, 8.34 and 12.51 μg/μl, respectively, in 0.9% (w/v) saline.
2. Surgical materials, including 0.9% (w/v) saline, 70% (v/v) alcohol, cotton tips, gauze, scissors, flat curved scissors, forceps, hemostats, knives, nail clipper, fur clipper, vetropolycin, betadine solution, and surgical sutures (or MikRon 9-mm Autoclip Applier).
3. Alzet micro-osmotic pumps, Model 1007D, 0.5 μl/hr.
4. Custom osmotic pump single cannula (3280 pm/spc 2.2 mm cut).
5. Vinyl catheter tubing (I.D. 0.027″, O.D. 0.045″).
6. Cyanoacrylate gel.
7. Dual digital small-animal stereotaxic instrument with digital display readout. Model 942, and mouse adaptor, Model 926.
8. Stereotaxic drill with a three-jaw chuck style handpiece and #75 drill bit (0.021”).
9. Anesthesia machine, Aerrane (isofluorane) and oxygen tanks.
10. Materials used for preparing and analyzing tissue RNA/protein samples, see **Material 2.3** items 5–13.

3. Methods

MOE ASOs with a phosphodiester backbone designed in the systematic ASO walks are first tested by a cell-free (*in vitro*) splicing assay, and/or in splicing assays with minigenes and endogenous genes in cultured cells. The in vitro splicing assay is a good system to study the splicing mechanism, free of any influence from coupled transcription or epigenetic effects exerted through genomic DNA or chromatin; effects on cell-free splicing provide good evidence that the ASO activity is through direct binding to the pre-mRNA target to redirect splicing. The splicing assays with cultured cells help to determine if the ASOs are sufficiently effective in human cells, in the more natural context of co-transcriptional splicing. In this chapter, we describe an efficient and inexpensive electroporation method to deliver ASOs into several types of human cells, such as HEK293. In some cases, it may be

desirable to use cultured motor neurons, or differentiated embryonic stem cells or induced pluripotent cells. Appropriate transfection protocols usually need to be selected and optimized for each cell type.

Individual ASOs can also be tested in patient fibroblasts. These are useful because they provide the correct genotype, such as a mutant allele of particular interest. ASOs can be efficiently transfected into primary fibroblasts with a lipid reagent, such as lipofectamine. The analysis of ASO activity in patient fibroblasts is similar to that in other human cells, so we will not reiterate it here.

The most promising ASOs, with a MOE-phosphorothioate backbone, are finally tested in SMA mouse models carrying a human *SMN2* transgene. ASOs cannot cross the blood brain barrier; thus both systemic and ICV delivery methods are employed to examine ASO activity in peripheral organs/tissues and the CNS, in either adult or neonatal SMA mice. Testing ASOs in various animal models is an essential goal in pre-clinical studies. The ASO distribution, its efficacy including pharmacokinetic (PK) and pharmacodynamic (PD) data in the affected tissues, and its toxicity in appropriate animal models, are essential to determine if an ASO can then be used for clinical trials. The mouse embryonic ICV injection procedure described here was modified from an earlier report (16); and the mouse ICV infusion procedure is based on a protocol previously used with rats (17).

3.1. ASOs in a cell-free splicing assay

1. To obtain pre-mRNA substrates, set up an *in vitro* transcription reaction including 1–1.5 μg template DNA (linearized plasmids pCI-SMN1 or pCI-SMN2), 5 μl of 5 × T7 RNA polymerase buffer, 2.5 μl NTP mixture, 5 μl of 10 mM ^{7me}GpppG cap analog, 2.5 μl of 0.1 M DTT, 1 μl RNasin, 2.5 μl α-^{32}P-UTP and 1.5 μl T7 RNA polymerase; add Milli-Q water to a total of 25 μl. (5, 13). Mix well by brief votexing and spinning, and incubate at 37°C for 2 hrs. Then add 25 μl RNA dye mixture to the reaction, mix and incubate at 68°C for 10 min to denature the transcript product. To purify the pre-RNA substrate, denatured reaction is loaded to a 5.5% acrylamide/8.3 M urea gel. Run at ~ 40 V/cm with 1× TBE buffer for 1 hr; carefully separate the glass plates using a thin spatula and cover the gel with Saran wrap. Expose a film on the gel for 1 min in a dark room with a luminescent peel-off sticker to locate the radio-labeled substrate. Cut off the band, put into a 1.5-ml eppendorf tube containing 400 μl RNA elution buffer and rotate at room temperature for 4 hrs or at 4°C overnight. Transfer eluted RNA to a fresh tube; add 1 ml ethanol and 1 μl of 10 μg/μl glycogen, and keep at −80°C for > 10 min. Spin at 13,000 rpm (~15,700 × g) at 4°C for 30 min to precipitate RNA. Rinse pellet with 1 ml of 80% ethanol and spin at 4°C for 30 min. Remove the ethanol to another tube and check with Geiger counter to make sure that the pellet is not disturbed. Re-suspend the pellet in 10–20 μl Milli-Q water and incubate at room temperature for 20 min. Take 1 μl substrate and add to a scintillation vial (containing 400 μl scintillation liquid) together with the pipette tip. Count by Beckman LS 6000 Series Liquid Scintillation System. Calculate the amount of transcription product by the count number (X cpm). For example, the *SMN1* substrate concentration = X / ($4.293 \times 10^4 \times 307 \times$ decay percentage) pmol/μl. In the formula, 4.731×10^4 is the specific activity (cpm / pmol) and 307 is the number of Us in the transcript. Dilute the substrate in Milli-Q water to 20 fmol/μl, add in 1 μl RNasin, and aliquot into several tubes. Store at –20°C. 8 fmol pre-mRNA substrate is used in a 10-μl splicing reaction.
2. To set up a 10-μl splicing reaction in a 1.5-ml eppendorf tube, first prepare 4 μl mixture A (buffer mixture): 0.8 μl of 0.4 mM Hepes-KOH, pH 7.3, 0.2 μl of 80

mM $MgCl_2$, 0.4 μl 25× ATP/CP mixture, 0.4 μl substrate (20 fmol/μl), 0.8 μl 13% (w/v) PVA (see Note 5), 1 μl of 2 μM ASO and 0.4 μl Milli-Q water. Mix Mixture A evenly by brief votexing and spinning.

3. Prepare 6 μl mixture B (N.E. mixture) on ice: 3 μl nuclear extract and 3 μl Buffer D. Nuclear extract is thawed at room temperature and kept on ice while setting up reaction; it is stored at −70°C and can be reused multiple times.
4. Add mixture B to mixture A, vortex gently, and spin briefly. Incubate at 30°C for 3–4 hrs.
5. Add 200 μl splicing stop solution and 200 μl Tris-saturated phenol to the splicing reaction. Then vortex for 2 min immediately, and spin at 13,000 rpm (~15,700 × g) for 8 min at 4°C.
6. Transfer the supernatant to a fresh tube and add 2.5× volumes of ethanol, vortex, and then keep at −20°C or −80°C for over 20 min.
7. Spin at 13,000 rpm (~15,700 × g) for 15 min and carefully remove the ethanol. Use a 1-ml tip first, and then a flat microtip to remove residual liquid. Dry by spinning in a speed-vac centrifuge for 5 min, without vacuum. Add 3 μl of RNA dye mixture, vortex for 2–3 min, and heat/denature at 85°C for 5–10 min.
8. Load the samples onto a denaturing 8% polyacrylamide gel containing 8.3 M urea. A radiolabeled single-strand DNA size marker is necessary.
9. Run the gel at ~40 V/cm using 1× TBE running buffer, until the dyes migrate out of gel. Carefully separate the glass plates using a thin spatula, and apply a piece of filter paper on top of the gel.
10. Carefully separate the gel stuck to the filter paper from the bottom glass piece. Cover the other side of the gel with Saran wrap.
11. Dry the gel under vacuum at 90°C for 20 min, and expose it onto a phosphor-imaging screen for 1 hr to overnight, depending on the signal intensity.
12. Scan the phosphor-imaging screen with an Image Reader FLA 5100 or equivalent. Calculate the exon 7 inclusion percentage, i.e., included mRNA × 100 / (included mRNA + skipped mRNA). The signal intensity of each mRNA isoform band is normalized according to its U content.

3.2. Splicing assays with minigenes and endogenous genes in cultured cells

1. On day 1, seed HEK293 cells in 10- or 15-cm dishes at 30~40% confluence. HEK293 cells are easily transfected. HeLa and C33A cells can be used instead.
2. On day 2, when the cells have grown to about 70% confluence, split the cells. For one 10-cm dish, use 1 ml trypsin-EDTA solution, and for a 15-cm dish, use 2 ml trypsin-EDTA solution; incubate at 37°C for 1–2 min. Add DMEM and disperse the cells by pipetting up and down several times; make sure the cells are evenly dispersed.
3. Pool the cells into one 15-ml tube and mix well. Take a 10 μl aliquot of the cell suspension for counting in a hemocytometer.

[5]In a standard *in vitro* splicing reaction, 2.6% (v/v) PVA is used. By testing different concentrations of PVA, it was observed that 1% (v/v) is optimal for *SMN1/2* minigene substrates.

4. Spin the cells in a 15-ml tube at 1,000 rpm for 10 min at room temperature or 4°C. Discard the medium by vacuum aspiration, and resuspend the cells in Opti-MEM at a concentration of 1×10^7 cells/ml.

5. Gently mix the cells with a P1000 pipetman, and aliquot 60 or 90 μL into each 1.5-ml eppendoff tube. The standard volume is 90 μl, but 60 μl is good enough, and can save reagents.

6. For 90 μl of cells, add 10 μl of DNA/ASO mixture that contains 3 μg minigene plasmid (pCI-SMN1 or pCI-SMN2) DNA and 5 μl of 200 μM MOE ASO. For 60 μl of cells, add 6.6 μl of DNA/ASO mixture, and scale down the DNA/ASO amount proportionately. To determine transfection efficiency, 0.5 μg GFP plasmid can be added into the cell/DNA/ASO mixture. pBabe-puro (2 μg) can be added into the cell/DNA/ASO mixture to allow removal of untransfected cells by treatment with 1~10 μg/ml of puromycin for 24 hrs. The DNA/ASO mixture can be prepared before splitting the cells. The final concentration of MOE ASO for electroporation is 10 μM. Mix the cell/DNA/ASO mixture well, using a P200 pipetman.

7. Place the cell/DNA/ASO mixture on ice, while preparing another set of 1.5-ml tubes, each containing 800 μl of complete DMEM and a set of 60-mm dishes containing 3 ml complete DMEM. Prepare an appropriate number of 1-mm cuvettes, and P200 and P1000 tips.

8. Use the P200 to transfer the cells into 1-mm cuvettes; set the voltage at 80 V and the capacitance at 500 μF; apply current by pushing the two pulse buttons at the same time. Pulse is delivered to the cuvette as tone sounds. Release the buttons after tone sounds; the pulse length (usually 6~10 mS) then appears on the screen. When the pulse length is more than 10 mS, fewer cells tend to survive the treatment. Pulse length is dependent on voltage, capacitance, cell purity, and DNA/ASO purity. A longer pulse does not imply a low transfection efficiency.

9. After electroporation, immediately add 800 μL DMEM with the P1000, mix with the sample in the cuvette at least 3 times, and then transfer the cells into a 60-mm dish containing 3 ml complete DMEM. Incubate the cells under normal growth conditions.

10. This electroporation protocol can yield >95% transfection efficiency with HEK293 cells; it is not necessary to use puromycin to treat this type of cells. For other types of cells, puromycin may be added to kill untransfected cells, one day before collecting the cells.

11. Two to three days after electroporation, collect the cells from each dish with 1 ml Trizol to extract total RNA (see Note 2). Add 200 μl chloroform to the Trizol homogenate and shake gently for 10 sec to mix well. Spin at 4°C at 12,000 rpm (~13,400 × g) for 10 min; transfer the 500 μl upper aqueous phase to a fresh tube; add 500 μl isopropanol and keep the tube at room temperature for 10 min. Spin at 4°C at >12,000 rpm (~13,400 × g) for 12 min to pellet RNA. After washing with 75% ethanol, dry the pellet and dissolve it in 20 μl Milli-Q water.

12. Remove contaminating genomic/plasmid DNA by treatment with RQ1 RNase-free DNase. A typical 10-μl reaction includes 1 μg RNA, 1 μl RQ1 DNase buffer, RQ1 RNase-free DNase at 1 U/μg RNA; add Milli-Q water to 10 μl. Incubate at 37°C for 30 min. Add 1μl of RQ1 DNase Stop Solution to terminate the reaction. Incubate at 65°C for 10 minutes to inactivate the DNase.

13. Set up a 20-μl reverse-transcription reaction to generate first-strand cDNA. First make oligo dT mixture: 1 μg total RNA, 1 μl custom 18mer oligo dT and 1 μl

dNTP mixture; add Milli-Q water to a total of 13 μl. Mix well and heat at 65–70°C for 5–10 min; quick chill on ice for > 2 min. Then make RT mixture: 4 μl of 5 × SuperScript II RT buffer, 2 μl of 0.1 M DTT and 1 μl SuperScript RT and mix gently. Add RT mixture to oligo dT mixture and mix by gentle votexing and brief spinning. Incubate the reaction at room temperature for 5 min and then at 42°C for 1 hr. Inactivate the reaction by heating at 70°C for 15 min. Store at –20°C.

14. Set up a 25-μl radiolabeled PCR reaction: 0.25 μl AmpliTaq DNA polymerase, 2.5 μl AmpliTaq buffer containing 1.5 mM $MgCl_2$, 0.75 μl dNTP mixture, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 2–4 μl cDNA (template), 0.1 – 0.2 μl α-^{32}P-dCTP. PCR program: 3 min at 94 °C, followed by 22–28 cycels of 35 sec at 94 °C, then 55°C for 50 sec and 72°C for 38 sec and a final extension step for 5 min at 72°C.

15. Add 5 μl of 6 × DNA loading buffer to the minigene PCR samples, and mix well by brief vortexing and spinning. Analyze the samples on a 6% native polyacrylamide gel.

16. Endogenous-gene PCR samples require separation of *SMN2* from *SMN1* PCR products by *Dde* I restriction digestion. Add 2.5 μl Buffer 3 (provided with the enzyme) and 1.2 μl restriction endonuclease *Dde* I (10 U/ μl) to a 25-μl PCR reaction, mix well, and incubate at 37°C for 2 hrs. PCR purification is not necessary for *Dde* I digestion. After digestion, add 6 μl of 6 × DNA loading buffer and mix well. Analyze the samples on a 6% native polyacrylamide gel.

17. Load the samples and run the gel at ~20 V/cm with 1 × TBE running buffer for 2–3 hrs.

18. Dismantle the gel, dry it, and expose to a phosphorimager screen as described in **Method 3.1**.

19. Calculate the exon 7 inclusion percentage, i.e., included mRNA × 100 / (included mRNA + skipped mRNA). The signal intensity of each mRNA isoform band is normalized according to its nucleotide composition.

3.3. Splicing assay in peripheral tissues of SMA mouse model

1. MOE ASOs can be directly injected intravenously (IV, such as from the tail vein), intraperitoneally (IP), or subcutaneously (SC) into adult SMA mice, e.g., twice a week for three weeks. For tail-vein injection, a dose of 25 mg/kg for each injection is used; for IP injection, 35–50 mg/kg is used. The injection volume is between 100 and 200 μl.

2. Three days after the last injection, the mice are sacrificed. Mouse tissues and organs, including liver, skeletal muscles (such as thigh muscles), kidneys, brain and spinal cord are first rinsed with 0.9% saline to remove blood contamination, and then snap-frozen in liquid N_2. Tissues and organs are kept at −70°C. Mouse tissues and organs are easy to collect, except for the spinal cord.

3. To collect the spinal cord, cut the whole spine into 3 parts: cervical, thoracic and lumbar. Use a 5-ml syringe and a needle of suitable size to inject 0.9% saline from one end, pushing the cord to come out at the other end.

4. To extract total RNA, 10–100 mg tissue is pulverized in liquid N_2 with mortar and pestle, and homogenized with 1 ml Trizol. Total RNA is isolated according to the manufacturer's directions.

5. As described in **Method 3.2**, total RNA is treated with DNase RQ1, and cDNA is synthesized with a suitable reverse transcriptase (see 3.2 step 13).

6. Set up 25-μl PCR reactions same as described in **Method 3.2**, but with a set of primers that are human *SMN1/2*-specific (i.e., do not cross-react with the mouse *Smn* gene, if it is present).

7. See **Method 3.2** steps 15 and 17–19 for the remaining steps in analyzing the PCR reactions.

8. To analyze the ASO effects at the protein level, 20 mg of each tissue is pulverized in liquid N_2, and homogenized in 0.4 ml of 1 × SDS protein sample buffer. Protein samples can be safely stored at −20°C for 1–2 weeks, or at −70°C for longer times.

9. Protein samples are separated by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes.

10. After blocking the membrane with blocking buffer for 1 h, add into the blocking buffer an antibody that specifically detects the human SMN protein, such as mAb SMN-KH, and gently shake the blot at room temperature for 3 hrs, or at 4°C overnight. Collect the blocking buffer containing antibody, which can be reused later, and rinse the membrane with TBST. Then incubate the membrane again with anti-β-tubulin pAb (dilution 1:5000) in fresh blocking buffer for 45 min. Wash the membrane thoroughly with TBST (> 3 times) and incubate it with secondary IRDye 800CW-conjugated goat anti-mouse and anti-rabbit antibodies in 5% fat-free milk in TBST for 2 h. Wash the blot thoroughly, and detect the protein signals with an Odyssey Infrared Imaging instrument. Alternatively, the membrane can be probed with two primary antibodies and their respective secondary antibodies in a sequential manner.

3.4. Embryonic ICV delivery of ASOs

1. Anesthetize a pregnant mouse with approximately E15 embryos with 2.5% isofluorane and place on its back on an operating board, with the head partially inside an anesthetizing tube that is blowing oxygen and isofluorane. Fasten the limbs to the board with sticking tape. Shave the fur in the abdomen area in advance. Spray 70% (v/v) ethanol on the abdomen and dry with a few pieces of gauze. Place on the abdomen a piece of gauze with a 3-cm long slit in the center.

2. Cut a 3-cm-long midline incision in the abdomen, through the skin and the abdominal wall, using scissors. Gently pull out the uterus from the abdominal cavity using ring forceps, and rinse the uterus with warm saline (37 °C) frequently, to make sure it remains wet.

3. A glass micropipette with a 12–20 μm tip is used to deliver the ASO solution into a lateral cerebral ventricle. The coordinates of the injection spot are approximately 1 mm lateral from the sagittal suture, 2–3 cm anterior from the lambdoidal suture, and 2 mm ventral (for FVB mice). ASO is dissolved in 0.9% (w/v) saline with 0.01% (w/v) Fast Green FCF, so that the shape of both lateral ventricles can be visualized if the ICV injection is successful (see Note 6). Inject a total of 2 μl of ASO solution with doses of 5, 10 and 20 μg ASO, respectively, or saline vehicle, into each embryo.

[6]A 2-μl mark is made on the glass micropipette. Generally, two people are required to perform the embryonic ICV injection. One person positions the tip of the glass micropipette in the lateral ventricle spot and holds it firmly; the other injects the ASO solution; practice (with dye) is needed to control the rate.

4. After injection, place the uterus back into the abdominal cavity; close the abdominal wall and the skin with surgical sutures.
5. Sacrifice ASO-treated pups that are 7-days old. Collect tissues and organs, such as brain, spinal cord, and liver. Collect spinal cord by squeezing the spine with the fingers from one end to the other end. Wash tissues and organs with 0.9% (w/v) saline, snap freeze them, and store at −70°C.
6. To examine the ASO activity on *SMN2* splicing at both the RNA and protein levels, see **Methods 3.2. and 3.3.**

3.5. Neonatal ICV delivery of ASOs

1. The ICV injection procedure with neonatal mouse pups is much easier than that with embryos. Pups that are 1-day or 2-days old (P1 or P2) are preferred to perform ICV injection. The ASO solution contains 0.01% (w/v) Fast Green FCF. Keep one flexible arm of a fiber-optic illuminator in a vertical position. With one hand, hold a pup directly on the top of the light; with the other hand, hold a 5-μl micro-syringe with a 33-gauge removable needle, and inject 2 μl of ASO solution into either of the two cerebral lateral ventricles. The coordinates of the injection spot are approximately 1 mm lateral from the sagittal suture, 2.5 mm anterior from the lambdoidal suture, and 2 mm ventral. The distal part of the needle can be marked with tape to indicate 2 mm from the tip, so that the injection is always 2-mm deep.
2. Sacrifice pups at P7 to collect tissues and organs. Process and analyze samples for ASO activity as described in **Methods 3.2.** and **3.3.**

3.6. ICV infusion of ASOs into adult mice

1. The day before surgery, or early in the morning before the surgery, connect a vinyl catheter tubing to an osmotic pump, and fill it with ASO solution using the accompanying needle, inserted through the tubing (carefully avoid introducing bubbles). Keep the pump/tubing in saline in a 37°C incubator for >4 hrs, with the tubing opening remaining above the saline-solution surface.
2. Place sterile tools and materials on a clean sterile mat: cotton tips, pumps, tubing, cannulas, nail clipper, vetropolycin, and betadine solution. Flat curved scissors, forceps, hemostats, and surgical knives are placed inside one or two 50-mL tubes filled with 70% (v/v) EtOH.
3. Anesthetize the mice with 2–2.5% (v/v) isofluorane blown with O_2.
4. Shave off the fur on the back, from the neck to the head.
5. Fasten the mouse on a KOPF Stereotaxic instrument. Place the front teeth in the tooth-hole of a mouse adaptor, so it cannot move; then secure the adaptor with two breathing tubes (one in and one out) blowing anesthetic gas. Place two iron earbars into the ears (0 matches 0 for both) and tighten the screws. Now the head is fastened.
6. Apply betadine solution on the head skin to sterilize the surgery area.
7. Cut the skin with a disposable surgical knife.
8. Use a cotton tip to remove soft tissues, and make sure you can clearly see the bregma (see Note 7).
9. Spray alcohol on the bit of the drill to sterilize it.

10. Move the drill bit just above the bregma, and tighten the screw at 90. There are 3 other screws you can use to adjust the position of the drill.
11. Use the digital display readout to adjust the drill bit to position 1.8 mm (lateral), -0.3 mm (sagittal), 2.2 mm (deep) from the bregma for FVB mice. For C57 mice, use coordinates of 1.6 mm/−0.7 mm/2.5 mm.
12. Drill a hole by pushing the foot pedal and turn the adjusting screw up and down quickly a few times. Stop when the bit cuts through the bone (and you see a red spot).
13. Make a subcutaneous pocket on the back using curved scissors from the surgery area, and cut off tissues that connect the skin with the body on the back.
14. Use a hemostat to hold the osmotic pump. Place the pump into the subcutaneous pocket. Cut the catheter tubing to a suitable length.
15. Sterilize your hands with alcohol and use a hemostat or forceps to hold a cannula at the neck. Insert the tiny metal tube on the side of the cannula (slowly and carefully) to the catheter that is connected to the pump.
16. Hold the hemostat with your left hand. Apply cyanoacrylate glue to the bottom surface of the cannula (use enough glue but avoid touching the stylet of the cannula), and insert the stylet into the hole drilled on the skull. Cut off the upper part of the cannula with a nail-clipper at the neck, and with a cotton tip press/hold the cannula gently for >30 seconds, to allow the glue to harden. At the same time, make sure the tubing points towards the back. Also, make sure skin is not caught under the cannula.
17. Close the skin using a curved needle and thread. Alternatively, use a MikRon 9-mm Autoclip Applier.
18. Warm the mouse for a few minutes, and place it back in its cage.
19. Collect tissues and analyze samples as described **Methods 3.2. and 3.3.**

Acknowledgments

The authors thank Drs C. Frank Bennett, Timothy A. Vickers, Brenda F. Baker, Gene Hung, and Frank Rigo at Isis Pharmaceuticals for providing MOE ASOs, for many helpful discussions, and sharing protocols, as well as Dr. Kentaro Sahashi at Cold Spring Harbor Laboratory for helping to implement the embryonic ICV injection procedure. The relevant research in the authors' laboratory was generously supported by the Muscular Dystrophy Association, the National Institute of Neurological Disorders and Stroke, and the SMA Foundation.

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[7]After cleaning the soft tissues on the skull, wait for about 2 min to let the bone dry a little bit, so that all the cranial sutures, including sagittal, coronal, and lambdoid can be clearly seen. The bregma is the spot where the coronal and sagittal sutures intersect. A microscope is not necessary for this ICV-infusion surgery.

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