Brief Communications

Trans-Splicing-Mediated Improvement in a Severe Mouse Model of Spinal Muscular Atrophy

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Spinal muscular atrophy is a leading genetic cause of infantile death and occurs in ∼1/6000 live births. SMA is caused by the loss of *Survival Motor Neuron-1* (*SMN1*), however, all patients retain at least one copy of a nearly identical gene called *SMN2*. While *SMN2* and *SMN1* are comprised of identical coding sequences, the majority of *SMN2* transcripts are alternatively spliced, encoding a truncated protein that is unstable and nonfunctional. Considerable effort has focused upon modulating the *SMN2* alternative splicing event since this would produce more wild-type protein. Recently we reported the development of an optimized *trans*-splicing system that involved the coexpression of a *SMN2 trans*-splicing RNA and an antisense RNA that blocks a downstream splice site in *SMN2* pre-mRNA. Here, we demonstrate that *in vivo* delivery of the optimized *trans*-splicing vector increases an important SMN-dependent activity, snRNP assembly, in disease-relevant tissue in the SMA mouse model. A single injection of the vector into the intracerebral-ventricular space in SMA neonates also lessens the severity of the SMA phenotype in a severe SMA mouse model, extending survival ∼70%. Collectively, these results provide the first *in vivo* demonstration that *SMN2 trans*-splicing leads to a lessening of the severity of the SMA phenotype and provide evidence for the power of this strategy for reprogramming genetic diseases at the pre-mRNA level.

Introduction

Spinal muscular atrophy (SMA), a neurodegenerative disorder, is caused by homozygous loss of *survival motor neuron 1* (*SMN1*) and is a leading genetic cause of infantile death (Crawford and Pardo, 1996; Oskoui et al., 2007). The human genome contains *SMN1* and *SMN2* genes and while these genes are nearly identical, only *SMN1* serves as the SMA-determining gene (Lefebvre et al., 1995; Rochette et al., 2001). The critical distinction between the *SMN1* and *SMN2* occurs at the RNA processing level: *SMN1* produces primarily full-length transcripts, while *SMN2* produces an alternatively spliced isoform lacking the final coding exon (Lefebvre et al., 1995). A single silent C to T nonpolymorphic nucleotide difference is responsible for disrupting a critical splice enhancer element in *SMN2* exon 7 (Lorson et al., 1999; Cartegni and Krainer, 2002). *SMN2* is retained in all SMA patients and is an ideal target for SMA therapeutic development (Sumner, 2006). The presence of *SMN2* and the fact that SMA is monogenic have allowed progress in a number of therapeutic avenues. In addition to the identification and development of small molecules that stimulate promote exon 7 inclusion and/or full-length *SMN2* expression, RNA modalities such as antisense oligonucleotides (ASO), TOES/bifunctional RNAs, and *trans*-splicing RNAs have shown promise in cell-based and animal models of SMA (Lim and Hertel, 2001; Cartegni and Krainer, 2003; Skordis et al., 2003; Madocsai et al., 2005; Baughan et al., 2006, 2009; Singh et al., 2006, 2009; Coady et al., 2007, 2008; Hua et al., 2007, 2008; Marquis et al.,

2007, 2009; Dickson et al., 2008; DiMatteo et al., 2008; Meyer et al., 2009; Williams et al., 2009).

Trans-splicing is a natural, albeit relatively uncommon process in mammals (Wood et al., 2007). *Trans*-splicing has recently been envisioned as a potential therapeutic intervention for a variety of genetic diseases, including spinal muscular atrophy, cystic fibrosis, hyper-IgM X-linked immunodeficiency, hemophilia A, Alzheimer's disease, and epidermolysis bullosa simplex with muscular dystrophy (Garcia-Blanco, 2003, 2004; Rodriguez-Martin et al., 2005; Coady et al., 2007; Zayed et al., 2007; Coady et al., 2008; Hengge, 2008; Wally et al., 2008). Unlike normal pre-mRNA splicing that occurs within a single pre-mRNA substrate, *trans*-splicing occurs between two different molecules: (1) the mutant endogenous RNA and (2) the exogenous therapeutic RNA that provides the correct RNA sequence via a *trans*-splicing event. An important benefit to *trans*-splicing is that it maintains the native expression patterns since *trans*-splicing relies directly on endogenous mRNA expression.

Recently, we developed a strategy to increase *trans*-splicing efficiency by blocking the downstream splice site in *cis*-with an antisense RNA molecule (Coady et al., 2008). To examine the activity of *trans*-splicing in a severe model of SMA, plasmids expressing the SMN *trans*-splicing RNA and antisense RNA were transfected *in vivo* into the severe SMA mouse model. The severe SMA mouse ($mSMN^{-/-}$, $hSMN2^{+/+}$) phenotype is very severe, with early synaptic occupation defects and an average life span of 3–4 d (Monani et al., 2000; McGovern et al., 2008). Here, we demonstrate that *SMN2 trans*-splicing functionally restores in important SMN-dependent pathway, snRNP assembly, within the CNS and that the average life span of severe SMA mice is significantly extended following a single administration of the optimized *trans*-splicing vector.
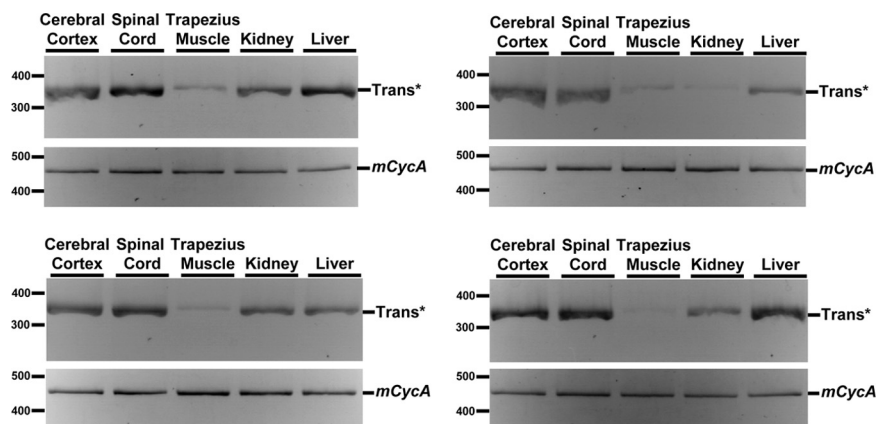
Figure 1. *Trans*-splicing detection in SMA tissues. Four representative severe SMA mice (Smn$^{-/-}$;SMN2$^{+/+}$) were assayed for *trans*-splicing using *trans*-splicing specific primers. The panel of gels shows RT-PCR results indicating the biodistribution of the *trans*-SMN RNA. Tissues were harvested 24 h posttransfection and mCycA was used as a positive control for loading and RT-PCR. Molecular weight markers are indicated on the left of each gel.

Materials and Methods

Plasmids. The ASO-tsRNA-expressing pMU3 plasmid and ptsRNA^{KO} have been previously described (Coady et al., 2008). Plasmid was purified using a QIAgen kit and dissolved into phosphate buffered saline for injections.

Genotyping and mouse protocol. Animals were handled according to the University of Missouri Animal Care and Use Committee approved protocols. The *mSmn* heterozygous mother would birth, counting as postnatal day 0 (PND0) and the neonates were genotyped within PND1. The genotyping, PCR conditions were 94°C denature for 2 min (94°C denature for 15 s, 68°C anneal) for 29 cycles followed by an elongation at 68°C for 10 min (Coady et al., 2008). Results indicating SMA mice (null for Smn) were repeated in triplicate. Severe SMA mice were raised with 4 heterozygous siblings. Additional heterozygous and wild-type animals were culled within the first hours of PND1 in experimental cages. Each mouse examination was equal in its duration throughout the cohort with markings occurring on each mouse up to the last day of life. The mice were individually weighed at the same time each day to maintain feeding patterns and intraday metabolic differences. Dissections were as follows: the vertebral column was separated from the torso then divided into the cervical, thoracic, and lumbar (C-T-L) regions. The spinal cord was excised by performing two incisions in a posterior to anterior manner with a ventral approach. The ventral vertebral strip was then removed exposing the spinal cord white tissue. The C-T-L sections were further disrupted by shearing with a 1000 µl pipette tip. The resulting homogenates were aliquoted and immediately frozen.

SMA trans-*splicing RT-PCR. In vivo* RT-PCR method is described previously (Coady et al., 2008). To remove possible genomic contaminants, DNase I-treated whole RNA stocks were seeded into RT reactions at 9.5 µg of total RNA. *In vivo trans*-splicing primer and reaction are previously described (Coady et al., 2008). The PCR amplification used a Roche High Fidelity kit. Negative controls were performed with no RT and no RNA to control for nonspecific amplification during the PCR.

Vector plasmid degradation and expression. Heterozygous mouse brain tissues were extracted and lysed in HiRT buffer. Total DNA was extracted with chloroform and precipitated with ethanol. The DNA reconstitution buffer was then treated with 1.5 units of RNase A to remove residual ribonucleic acids. DNA concentration was determined with the Bio-Rad slot blot vacuum. DNA (5 µg) was used in Invitrogen Platinum Taq reactions with 1.25 U of polymerase and 5 µl of PCR buffer and 2.25 µl of enhancer solution. The PCR cycle number was empirically determined to be 17 by the amplification of the positive control band and band appearance comparable to a 1:1 ratio with 1 µg of standard in the 1 kb molecular marker. The positive-control heterozygous mouse was intracerebroventricularly injected on PND 1 and was harvested 24 h later. The brain was dissected out and the cerebellum and olfactory lobes excised leaving the cerebral cortex. This brain section contains the majority of the ventricle space and therefore the highest exposure to the vector. The cortex was further divided through the sagittal and then the coronal plane creating four sections. These were titled the anterior (A) and the posterior (P) samples. The negative control heterozygous mouse was from a different litter injected with Mock solution described previously which contains no plasmid.

In vivo UsnRNP assembly. The procedure was adapted as previously described for tissue-derived snRNP assembly assays (Gabanella et al., 2007). The lumbar section of the spine was isolated and passed through the tip of a 1000 µl pipette tip to disperse the tissue in cold PBS. The fresh cells were counted on a hemocytometer then 4×10^6 cells aliquots placed into new tubes by spinning at 500 rpm for 6 min in a cold centrifuge. The spinal cord pellets were resuspended evenly in RB-buffer and then Triton X-100 was added to a final percentage of 0.1% to separate the cytoplasm from the nucleus. The cytoplasmic fractions were then quantitated using the Bradford/Lowry method to calculate 25 µg of total protein used in the assembly reactions.
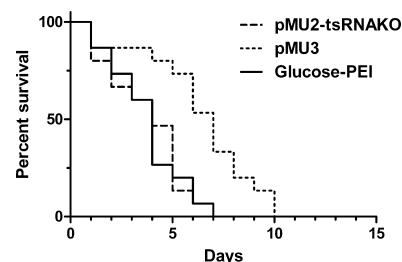
Results

The determine whether *in vivo* increases in SMN protein in a severe mouse model of SMA could lessen the severity of the phenotype, an *in vivo* assessment of *trans*-splicing in the severe SMA model was initiated. This model lacks endogenous murine *Smn* but contains two genomic copies of the human *SMN2* gene, and is therefore ideally suited for translational studies that target *SMN2* alternative splicing. To determine the biodistribution and efficiency of *trans*-splicing in disease-relevant tissues, SMA pups were received intracerebral-ventricular injections with vector expressing the tsRNA and antisense RNA, pMU3. Tissues from four separate SMA animals were examined. 24 h after receiving a single injection, SMN *trans*-splicing was readily detectable within the CNS, as well as distal sites, including kidney and liver. To a lesser degree, *trans*-splicing was also detected within skeletal muscle (Fig. 1). It is possible that in neonatal animals, the blood brain barrier is more permeable, allowing greater levels of transport into the circulatory system, helping to explain the detectable levels of *trans*-splicing in distal sites. SMN *trans*-splicing was not detected in any tissue from untreated SMA pups or from a pMU3 derivative that lacked the tsRNA promoter (tsRNA^{KO}) (data not shown and supplemental Fig. 1, available at www.jneurosci.org as supplemental material). GFP expression derived from the tsRNA vector was monitored and was detected throughout the spinal column, consistent with the *trans*-splicing results (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Plasmid-derived GFP tissue expression correlated with the observed *trans*-splicing (data not shown). Based upon these results and a recent report that demonstrates that high levels of neuronal SMN significantly rescues the SMA phenotype (Gavrilina et al., 2008), subsequent experiments were focused upon the CNS.

SMN is a multifunctional protein, however, it is not clear which specific function is connected to the development of SMA. The best described biochemical function of SMN centers around the assembly of small nuclear ribonuclear protein complexes, snRNPs, which are the building blocks for the spliceosome. To determine whether SMA mice treated with the *trans*-splicing vector exhibited increased levels of snRNP assembly activity, protein
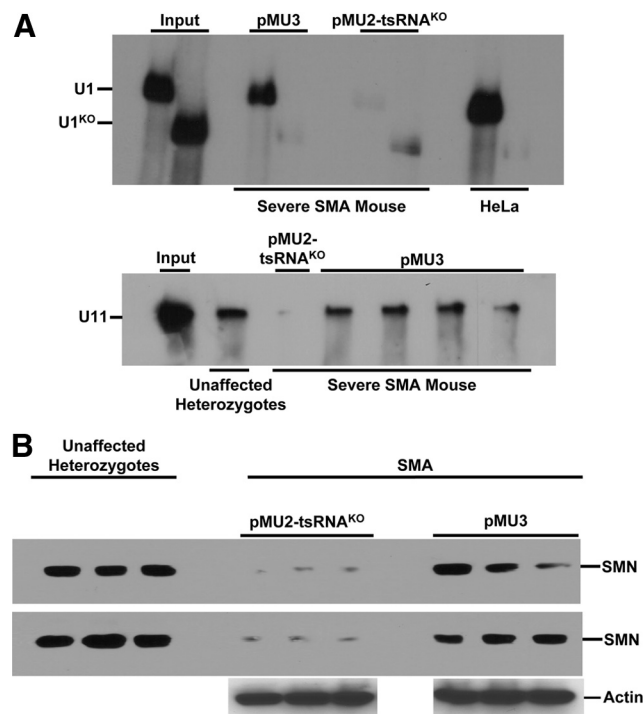
Figure 2. *Trans*-splicing mediated increases in functional SMN levels *in vivo*. ***A****, In vivo* restoration of severe SMA neonatal UsnRNP assembly capacity is mediated by *SMN trans*-splicing RNA plasmid injections. U1 and U11 snRNAs were radiolabeled *in vitro* and incubated with the respective extracts. Y12 antibody was used to immunoprecipitate snRNP complexes from tissue extracts derived from treated SMA mice or unaffected heterozygote mice. U1^{KO} is a negative control RNA that lacks the Sm nucleation site. Mouse lumbar spinal cord sections were removed from the vertebrae and passed through a 25 gauge needle before the assembly process. The negative control SMA mouse was injected with pMU2-tsRNA^{KO} plasmid. HeLa cell extract served as a positive control. Representative results from three independent experiments are shown. ***B****,* Western blots of spinal cords from ASO-tsRNA-treated mice demonstrated increases in SMN protein. 10% SDS-PAGE gels are shown developed with an anti-SMN monoclonal antibody. Six separate SMA mice were used to generate statistical significance between treatment and control transfections. Equal amounts of protein were loaded in each well as determined by Lowry assay.

Figure 3. Intracerebroventricular delivery of optimized *trans*-splicing vector extends average life span in a severe model of SMA. Neonatal pups were injected with a mixture of PEI/Glucose and plasmid vector via a single intracerebral-ventricular injection. The pMU3 intracerebroventricular injection produced an approximate ∼70% increase in SMA mouse survival. Kaplan–Meier curve depicts negative controls vehicle (Glucose/PEI) and tsRNA^{KO} dying at a maximum 7 d. $n = 15$ mice for each group. $p < 0.001$.

assembly is the critical function underlying SMA development, these results demonstrate that SMN levels can be significantly increased in disease-relevant tissue and that this increase correlates with elevated snRNP assembly levels.

To examine the potential for physiological changes in the SMA mice we used a more severe mouse model for two reasons: (1) The severe mouse model experimental duration is much shorter than the Δ7 mice and the ability to deliver via repeated intracerebroventricular injection is limited; (2) the severity of the model could potentially be a benefit because a small change would be measurable rather than in a less severe model. To determine whether the previous biochemical and molecular increases in SMN splicing and protein levels resulted in detectable changes in the SMA phenotype in SMA mice, weight and life span data were collected following a single intracerebral-ventricular injection at postnatal day 1. Three cohorts consisting of 15 SMA pups per group were examined: the *trans*-splicing vector (pMU3); an early generation *trans*-splicing vector that lacked the *trans*-splicing RNA promoter; and vehicle alone (Glucose-PEI). As previously published, this model of SMA is very severe, resulting in low birth weights and death for the majority of the vehicle-treated animals in <5 d (Monani et al., 2000). Similarly, the control vector lacking the promoter resulted in nearly identical data, with no animal living past 7 d, with an average life span of ∼4 d (Fig. 3). SMA pups transiently transfected with the *trans*-splicing vector, however, had an average life span of ∼7 d, significantly longer than the control cohorts (Mantel-Cox test, $P = 0.001$) (Fig. 3). Additionally, the longest lived animals reached 9 and 10 d (Fig. 3). Weight differences were not observed between untreated and treated SMA groups, suggesting that this is still an extremely severe model of neurodegeneration (data not shown). Even though this model is exceptionally severe, collectively, these results demonstrate that increases in functional SMN protein can be achieved *in vivo* and that these increases can significantly alter the disease phenotype.

Discussion

SMN2 has been envisioned as an outstanding target for therapeutic intervention since it is retained in all SMA patients and it has the potential to encode a fully functional SMN protein. Strategies that redirect the *SMN2* alternative splicing event have been particularly intriguing for RNA-based therapeutics, including a variety of antisense oligonucleotides as well as *trans*-splicing. Recent reports highlight the potential application of RNA-based therapeutics designed to increase full-length expression from *SMN2*, including the delivery of ASOs directly to the CNS of SMA pups

extracts were generated from pMU3-treated and control-treated animals. Extracts from the lumbar spinal cord region were generated and incubated with radiolabeled U1 snRNA. As anticipated, HeLa cell extracts supported very high levels of snRNP assembly, consistent with the high levels of endogenous SMN protein (Fig. 2*A*). In pMU3-treated SMA extracts, snRNP assembly levels were dramatically elevated compared to control vector-treated extracts (Fig. 2*A*). U1 is a component of the major splicing pathway, however, defects in the assembly of minor splicing snRNP components have also been identified in SMA animals (Gabanella et al., 2007). To examine SMN activity in this alternative pathway, snRNP assembly on minor pathway snRNA, U11, was examined in similar extracts (Fig. 2*A*, bottom panel). Consistent with the increased activity observed in the U1-dependent assays, snRNP assembly was elevated using the U11 snRNA template (Fig. 2*A*, bottom panel). SMN protein levels were examined by Western blot in lumbar spinal cord extracts to correlate the apparent increase in SMN-dependent snRNP assembly activity with increased SMN protein levels (Fig. 2*B*; supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Consistent with the increased snRNP assembly activity, steady-state SMN protein levels were increased in *trans*-splicing-treated tissues (Fig. 2*B*). While it is not clear that snRNP

through intracerebroventricular injection (Dickson et al., 2008; Baughan et al., 2009; Williams et al., 2009). In this report we demonstrate that following intracerebroventricular administration of an optimized *trans*-splicing vector, *in vivo trans*-splicing of the *SMN2* gene results in increased levels of functional SMN, consistent with a significant increase in average life span for *trans*-splicing treated SMA pups.

We chose to measure snRNP assembly as a means of monitoring SMN functionality. The role of SMN in snRNP assembly has been extremely well defined and analyzed at a very high-resolution level from a biochemical perspective (Pellizzoni, 2007). While the exact role of SMN in major versus minor splicing pathways remains unclear, simply using the snRNP assembly assay in spinal cord extracts provides a tractable biochemical assay to monitor functional SMN levels. It remains a possibility that an alternative function such as axonal transport of mRNAs is the primary cause of SMA development; however, a well defined assay system for this type of activity has not been described and we are making a logical assumption that full-length SMN restores or significantly increases all SMN-dependent deficiencies.

While these are clearly proof-of-concept experiments, the experiments raise in intriguing question in SMA: when can SMA therapeutics be delivered to elicit a response? Two SMA animal models have been the focus of therapeutic analysis: the severe model used in this report and the less severe "Δ7" SMA mouse model. Intraperitoneal injections of the HDAC inhibitor trichostatin A (TSA) in the Δ7 model significantly extended survival (Narver et al., 2008), suggesting that this model may not be intractable and can be fully rescued by more potent SMN activators or gene replacement strategies. To date, the extreme disease progression observed with the "severe" model suggests that prenatal deficiencies exist. While further studies will be required to draw accurate conclusions, the severe model still serves as an important proof-of-concept model that is exceptionally simple at the genetic level and allows that rapid analysis of *in vivo* applications such as *trans*-splicing.

Intracerebroventricular delivery provides a robust means to directly deliver SMA therapeutics to disease-relevant tissues within the CNS. As the benefits for oligonucleotide-mediated and vector-mediated strategies are weighed, sustained expression will likely be an important factor. It is likely that SMN will continue to be required throughout life; therefore, either a continuous pump or longer-term viral vector will likely move closer toward clinical reality. These results provide the first *in vivo* proof-of-concept evidence that SMN *trans*-splicing can lessen the severity of the SMA phenotype and provide a platform to move forward into a viral vector system that can provide sustained and robust expression of functional SMN.

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