A Multi-Exon-Skipping Detection Assay Reveals Surprising Diversity of Splice Isoforms of Spinal Muscular Atrophy Genes

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Abstract

Humans have two near identical copies of Survival Motor Neuron gene: SMN1 and SMN2. Loss of SMN1 coupled with the predominant skipping of SMN2 exon 7 causes spinal muscular atrophy (SMA), a neurodegenerative disease. SMA patient cells devoid of SMN1 provide a powerful system to examine splicing pattern of various SMN2 exons. Until now, similar system to examine splicing of SMN1 exons was unavailable. We have recently screened several patient cell lines derived from various diseases, including SMA, Alzheimer’s disease, Parkinson’s disease and Batten disease. Here we report a Batten disease cell line that lacks functional SMN2, as an ideal system to examine pre-mRNA splicing of SMN1. We employ a multi-exon-skipping detection assay (MESDA) to capture simultaneously skipping of multiple exons. Our results show surprising diversity of splice isoforms and reveal novel splicing events that include skipping of exon 4 and co-skipping of three adjacent exons of SMN. Contrary to the general belief, MESDA captured oxidative-stress induced skipping of SMN1 exon 5 in several cell types, including non-neuronal cells. We further demonstrate that the predominant SMN2 exon 7 skipping induced by oxidative stress is modulated by a combinatorial control that includes promoter sequence, endogenous context, and the weak splice sites. We also show that an 8-mer antisense oligonucleotide blocking a recently described GC-rich sequence prevents SMN2 exon 7 skipping under the conditions of oxidative stress. Our findings bring new insight into splicing regulation of an essential housekeeping gene linked to neurodegeneration and infant mortality.

Introduction

Alternative pre-mRNA splicing increases the coding potential of eukaryotic genome by producing multiple proteins from a single gene [1]. As per current estimate, more than 95% of human genes with two or more exons are alternatively spliced [2]. Splicing is catalyzed by spliceosome, a macromolecular machine, which is assembled de novo for the removal of each intron [3,4]. Splicing is also coupled with transcription as several splicing factors are recruited to spliceosome and/or pre-mRNA sequence through RNA polymerase [5]. Regulation 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) [6–8]. Enhancer and silencer motifs promote or suppress splice-site (ss) selection, respectively. Due to the difference in arrangement of cis-elements within exonic and flanking intronic sequences, regulation of alternative splicing of each exon is unique [9,10]. Mutations within regulatory sequences and/or aberrant expression of splicing factors due to genotoxic and/or oxidative stress (OS) result in defective splicing [11–17]. However, there are very limited studies capturing OS-triggered aberrant splicing of multiple exons in a single transcript of an essential human gene. Also it is not known if deleterious effect of OS on splicing of a specific exon could be prevented by strengthening of a ss.

Humans have two near identical copies of Survival Motor Neuron gene: SMN1 and SMN2 [18]. Hereafter, SMN (in italics) refers to a gene or a transcript, whereas SMN (in normal case capital letters) refers to a protein. Both SMN genes code for identical proteins; however, SMN2 predominantly generates a short transcript due to skipping of exon 7, producing a truncated protein (SMN7) that is highly unstable [19–21]. Therefore, SMN1 serves as the primary gene for production of full-length SMN, a multifunctional protein containing nucleic acid binding, tudor, Sm binding, Calpain cleavage, ZPR1 binding and Gemini2 binding domains (Figure 1) [22–28]. Interaction of SMN with Gemini2 is essential for the formation of a large heteromeric complex (SMN complex) that participates in snRNP biogenesis, an important housekeeping function [29,30]. SMN is also implicated in transcription, DNA recombination, signal transduction, stress granule formation, vesicular transport and motor neuron trafficking [31–37]. The
inability of SMN2 to compensate for the loss of SMN1 results in spinal muscular atrophy (SMA), a leading genetic cause of infant mortality [30]. The exact function of SMN2 remains unknown, although, several lines of evidence support its role in cellular metabolism. For instance, a SMA mouse model expressing very high levels of SMNΔ7 showed prolonged lifespan [39]. Further, deletion of SMN2 has been associated with higher incidence of amyotrophic lateral sclerosis (ALS) and lower motor neuron disease [40,41]. In addition, SMN2 serves as a spare gene with a potential to be corrected in SMA. Indeed, recent reports of correction of SMN2 exon 7 splicing in animal models have shown promise for SMA therapy [42–47]. Most lead compounds to show therapeutic potential in animal models have been initially found to correct SMN2 exon 7 splicing in cultured SMA patient cells. In particular, publically available GM03813 cell line that lacks SMN1 has emerged as a cell-based model system for the preliminary screening of potential SMA drugs [48–54]. GM03813 cell line has also been useful in validating regulatory cis-elements and transacting factors that modulate SMN2 exon 7 splicing [48,51–55]. On the other hand, there is no systematic study on SMN7 splicing regulation in a publically available SMN2-lacking cell line.

Both SMN1 and SMN2 have similar gene organization i.e. nine exons and eight introns (Figure 2A). A critical cytosine (C) to thymidine (T) mutation at the 6th position (C6U transition in transcript) of exon 7 and an adenosine (A) to guanosine (G) transition at the 100th position (A100G) of intron 7 cause SMN2 exon 7 skipping [19,56]. Both, C6U and A100G mutations create binding sites for an inhibitory protein hnRNP A1 that weakens the 3' ss of SMN2 exon 7 [56]. An additional G to A mutation at the 236th position (G236A) of non-coding exon 8 creates a SMN2-specific signature motif (CTNAG) that could be cleaved by DdeI restriction endonuclease (Figure 2A). Therefore, DdeI digestion has been useful in distinguishing SMN2 transcripts from SMN1 transcripts [19]. Based on studies in SMA patient cells as well as in mouse models carrying SMN2, skipping of SMN2 exons 3, 5 and 7 have been confirmed [18,48,53,57]. There is also evidence to suggest very small but detectable skipping of SMN1 exon 5 and exon 7 in certain cell types [18,19,58]. However, it is not known if splicing of two or more exons of SMN1 is co-regulated. Also, there is no report of skipping of SMN1 exon 3. In general, there is a lack of a reliable assay to capture the relative abundance of the major splice variants of SMN1 and SMN2.

Paraquat (PQ), an herbicide and oxidative stress (OS)-causing agent, has been linked to the increased risks of neurological disorders, including Parkinson’s disease [59]. Incidentally, PQ treatment of neuronal cells have been shown to cause enhanced skipping of exons 5 and 7 of SMN2 but not SMN1 [60]. Although not independently validated, these findings put SMA patients into the category of high-risk candidates who are likely to be affected the most under the conditions of OS. In addition, OS-induced enhanced skipping of SMN2 exon 7 raises the fundamental question whether any of the strategies aimed at the correction of SMN2 exon 7 splicing in SMA will retain its efficacy under the conditions of OS. Given a high degree of sequence conservation between SMN1 and SMN2, it is likely that some of the yet uncharacterized splicing events in OS are common to both, SMN1 and SMN2. A definite answer to this question awaits further experimentation in specific cell types that express either SMN1 or SMN2.

Here we report a systematic analysis of splice isoforms generated by SMN1 and SMN2 under normal and OS conditions. Our study also addresses an important question of neuronal versus non-neuronal regulation of alternative splicing of various SMN exons. One of the defining aspects of this study is the application of a multiple-exon-skipping detection assay (MESDA) that captures the relative abundance of all major splice variants of SMN. We also take advantage of a unique cell type (GM20384) that we serendipitously discovered to lack SMN2 transcripts probably due to a partial or complete deletion of SMN2 alleles. Our findings reveal novel splice variants, including those generated by an unexpected skipping of exon 4 and/or several adjacent exons of SMN. We show that OS affects alternative splicing of several exons of SMN1 and SMN2 in both, neuronal and non-neuronal cells. Our results suggest an OS-induced collaborative skipping of SMN2 exons 5 and 7. Further, we demonstrate that skipping of SMN2 exon 7 under OS is dependent upon several factors including promoter sequence, endogenous context and the strength of ss. We also demonstrate that an antisense oligonucleotide (ASO)-based strategy to correct SMN2 exon 7 splicing retains its efficacy under the conditions of OS.

Results

Identification of a Cell Line that Lacks SMN2

To explore the possibility that different pathological conditions can affect splicing of SMN exon 7, we screened a number of publically available patient cell lines, including batten disease (BD), Parkinson’s disease and Alzheimer’s disease cell lines (Table 1, Figure 2B). As a control, we also used undifferentiated neuronal SH-SY5Y cells (lane 1, Figure 2B). SMN1/SMN2 transcripts were analyzed by a sensitive radioactive RT-PCR using forward and reverse primers annealing to exon 6 and exon 8, respectively. Importantly, PCR products contained the SMN2-specific DdeI restriction site within exon 8 (Figure 2A). Therefore, after DdeI digestion of PCR products, samples from cells containing both, SMN1 and SMN2, produced four bands (Figure 2B). The slowest migrating band (top band) in a polyacrylamide gel represented the

Figure 1. Diagrammatic representation of SMN mRNA and SMN protein. Number of amino acids encoded by each exon is indicated. Exons as well as domains they encode are shown as boxes. Domain functions are indicated.

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exon 7 included transcripts from SMN1, whereas, three fast migrating bands originated from SMN2.

Despite similar amount of starting material (RNA) used for RT-PCR, we observed varied intensity of expected four bands in different cell types. This could be due to a combination of factors, including but not limited to variations in SMN1/SMN2 copy numbers, transcription rate and splicing regulation. In order to compare side-by-side the relative proportions of SMN1 versus SMN2 transcripts in various cell types, loading of PCR products in polyacrylamide gel was adjusted (Figure 2B). Our assay reliably detected the presence and/or absence of major transcripts specific to SMN1 and SMN2, including Ddel restriction site, as well as location of primers used for amplification of endogenous SMN transcripts are indicated.

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Figure 2. Splicing of endogenous SMN exon 7. A, Diagrammatic representation of exon/intron organization of SMN1 and SMN2 genes. Exonic sequences are shown as colored boxes and intronic sequences are shown as lines/broken lines. Sizes of exons and introns are given. Size of the 3' UTR is based on the prevalently reported SMN1 transcript (NCBI accession number NM_000344). B, Splicing patterns of endogenous SMN1 and SMN2 exon 7 in different cell lines. Name of cell lines used are given on the top of the gel. Spliced products were analyzed by RT-PCR. We used 1 μg of total RNA per 5 μl of reverse transcription reaction and generated cDNA employing random primers. SMN transcripts were amplified using primers P25 and P31. To distinguish transcripts originated from SMN2, PCR products were digested with Ddel [55]. SMN1 and SMN2 transcripts are indicated on the right of the gel. Exon 7-included (+) and exon 7-skipped (−) products are indicated on the left of the gel. Based on RT-PCR results, the presence and absence of SMN1/SMN2 gene is marked by (+) and (−) respectively. The percentage of SMN2 exon 7 skipping was calculated based on the total value of SMN2 exon 7-included and exon 7-skipped products [55]. The last 81 nucleotides of exon 1 are translated. Several mutations that distinguish SMN1 and SMN2, including Ddel restriction site, as well as location of primers used for amplification of endogenous SMN transcripts are indicated.

To determine the relative abundance of major splice variants of SMN1/SMN2 in a one-step reaction, we developed a PCR-based assay that we named “MESDA”. The 5’ and 3’ primers used in MESDA annealed to SMN exons 2b and 8, respectively. Our rationale to use 5’ primer in exon 2b was based on the fact that
this exon is constitutively spliced. Also, use of 5′ primer in exon 2b versus constitutively spliced exon 1 or exon 2a resulted in shorter PCR products that can be better resolved on a polyacrylamide gel. To maintain the sensitivity and an accurate estimate of the relative molecular abundance of amplified products of different sizes, we performed a limited-cycle radioactive PCR in which only one of the primers was 5′-radiolabeled. Considering that the 5′ and 3′ primers employed in MESDA annealed to exons 2B and 8 of \( \text{SMN} \), respectively, we were able to capture multiple splice variants, including the transcripts generated by synonymous skipping of three exons of \( \text{SMN} \). MESDA also identified novel splice variants due to an entirely unexpected splice site pairings.

Table 1. Cell lines used in this study.

| No. | Cell type | Source | Disease Description | Cell Type |
|-----|------------|--------|---------------------|-----------|
| 1   | SH-SY5Y (CRL-2266) | ATCC | NB Unique marker on Chromosome 1 | Neuroblastoma |
| 2   | GM 20383  | CCR | BD/CL Juvenile CL, CLN3 mutations | B-Lymphocyte |
| 3   | GM 20384  | CCR | BD/CL Juvenile CL, CLN3 mutations | B-Lymphocyte |
| 4   | GM 20381  | CCR | BD/CL Juvenile CL, CLN3 mutations | B-Lymphocyte |
| 5   | GM 20382  | CCR | BD/CL Juvenile CL, CLN3 mutations | B-Lymphocyte |
| 6   | GM 08820  | CCR | BD/CL Juvenile CL, CLN3 mutations | B-Lymphocyte |
| 7   | GM 08794  | CCR | BD/CL Juvenile CL, CLN3 mutations | B-Lymphocyte |
| 8   | ND 00013  | CCR | PD Late onset DNA panel | B-Lymphocyte |
| 9   | ND 00002  | CCR | PD Young onset DNA panel | B-Lymphocyte |
| 10  | ND 00092  | CCR | Ctrl PD Convenience control | B-Lymphocyte |
| 11  | AG 04159  | CCR | AD Familial, type 3 | Fibroblast |
| 12  | GM 03814  | CCR | SMA Carr Mother (\( \text{SMN}^+/- \)) | Fibroblast |
| 13  | GM 03815  | CCR | SMA Carr Father (\( \text{SMN}^+/- \)) | Fibroblast |
| 14  | GM 03813  | CCR | SMA Child (\( \text{SMN}^+/- \)), SMA Type I | Fibroblast |

Abbreviations: ATCC, American type cell collection; CCR, Coriell cell repositories; NB, Neuroblastoma; BD, Batten disease; CL, Ceroid lipofuscinosis; PD, Parkinson’s disease; Ctrl, Control; AD, Alzheimer’s disease; SMA, Spinal muscular atrophy; Carr, Carrier.

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We employed MESDA to first profile major splice variants of \( \text{SMN}^1 \) and \( \text{SMN}^2 \) in GM20384 and GM03813 cells, respectively. \( \text{SMN}^1 \) in GM20384 cells produced one predominant band corresponding to the full-length transcript (Figure 3B, lane 2). In addition, \( \text{SMN}^1 \) showed very small but detectable skipping of exon 5 and exon 3. These results reveal for the first time the possibility of alternative splicing of \( \text{SMN}^1 \) exon 3 that codes for a critical tudor domain. Previous studies have shown that tudor domain plays an important role in nucleocytoplasmic trafficking of SMN [25]. GM03813 cells showed two prominent bands corresponding to the full-length and exon 7-skipped transcripts of \( \text{SMN}^2 \). Similar to \( \text{SMN}^1 \) splice variants in GM20384 cells, we also detected skipping of \( \text{SMN}^2 \) exon 5 and exon 3 in GM03813 cells. However, unlike \( \text{SMN}^1 \), \( \text{SMN}^2 \) showed co-skipping of exons 5 and 7 as well as exons 3 and 7 (Figure 3B, lanes 1 and 2). We also observed much weaker bands representing \( \text{D}_5 \) and \( \text{D}_3,7 \) transcripts in GM03813 and GM20384 cells. As controls, we used GM20383 lymphocytes and undifferentiated neuronal SH-SY5Y cells. Since these cells contain both, \( \text{SMN}^1 \) and \( \text{SMN}^2 \), they produced a mixture of transcripts representing all \( \text{SMN} \) splice isoforms.

Table 2. Description and GenBank accession numbers of splice variants reported in this study.

| Exon | Gene | Cell line | Treatment* | GenBank accession number |
|------|------|-----------|------------|-------------------------|
| \( \text{D}_5 \) | \( \text{SMN}^1 \) | GM20384 | PQ-treated | JQ657798 |
| \( \text{D}_3 \) | \( \text{SMN}^1 \) | GM20384 | PQ-treated | JQ657800 |
| \( \text{D}_5,7 \) | \( \text{SMN}^1 \) | GM20384 | PQ-treated | JQ657799 |
| \( \text{D}_5,6,7 \) | \( \text{SMN}^1 \) | GM20384 | PQ-treated | JQ657801 |
| \( \text{D}_5,6 \) | \( \text{SMN}^1 \) | GM20383 | Untreated | JQ732166 |
| \( \text{D}_5,6 \) | \( \text{SMN}^1 \) | GM20383 | Untreated | JQ732167 |
| \( \text{D}_5 \) | \( \text{SMN}^1 \) | SH-SY5Y | PQ-treated | JQ657802 |
| \( \text{D}_3 \) | \( \text{SMN}^2 \) | SH-SY5Y | PQ-treated | JQ690861 |
| \( \text{D}_3,4 \) | \( \text{SMN}^2 \) | SH-SY5Y | PQ-treated | JQ745297 |
| \( \text{D}_4,7 \) | \( \text{SMN}^2 \) | SH-SY5Y | PQ-treated | JQ690864 |
| \( \text{D}_3,7 \) | \( \text{SMN}^2 \) | SH-SY5Y | PQ-treated | JQ690862 |
| \( \text{D}_3,5 \) | \( \text{SMN}^1 \) | SH-SY5Y | PQ-treated | JQ657803 |
| \( \text{D}_3,5,7 \) | \( \text{SMN}^2 \) | SH-SY5Y | PQ-treated | JQ690863 |
| \( \text{D}_5 \) | \( \text{SMN}^2 \) | SH-SY5Y | Untreated | JQ690865 |
| \( \text{D}_3 \) | \( \text{SMN}^1 \) | SH-SY5Y | Untreated | JQ690867 |
| \( \text{D}_3 \) | \( \text{SMN}^2 \) | SH-SY5Y | Untreated | JQ690866 |
| \( \text{D}_3,5,7 \) | \( \text{SMN}^1 \) | SH-SY5Y | Untreated | JQ690868 |

*Represents whether splice isoforms were detected in untreated and/or PQ-treated cells. PQ-treated refers to treatment of cells with 1 mM PQ for 24 h.
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amplified by MESDA. DdeI digestion combined with sequence analysis confirmed that isoforms lacking exon 7 came mostly from SMN1 (Figure 4C and Figure S1). Based on sequence analysis, transcripts containing exon 7 but lacking exon 5 or exon 3 were generated mostly from SMN1 (Figure S1). It remains to be seen if these results were affected by potentially different copy numbers of SMN1 and SMN2 genes in SH-SY5Y cells. Our detection of SMN1 transcript lacking exons 5 and 6 (Δ5,6) in GM20383 lymphocytes was a novel and an unexpected finding. This transcript is generated by an unusual pairing between the 5’ ss exon 4 and the 3’ ss exon 7. Considering that the 3’ ss SMN1 exon 7 is much stronger than the 3’ ss SMN2 exon 7, we expected a higher occurrence of SMN1Δ5,6 compared to SMN2Δ5,6. Indeed, sequence analysis revealed that an overwhelming majority of Δ5,6 transcripts generated were from SMN1 (Figure S1A).

Effect of OS on Splicing of SMN Exons

Having established that various exons of SMN1/SMN2 are alternatively spliced, we set out to distinguish between general versus neuronal cell-specific effect of OS on SMN pre-mRNA splicing. To induce OS, neuronal and non-neuronal cells were treated with 1 mM PQ and transcripts were analyzed by MESDA 24 h post treatment. Of note, a previous study has shown that 1 mM of PQ is sufficient to produce a significant oxidative stress in neuronal SH-SY5Y cells [60]. We observed OS-induced increase in skipping of SMN1 exon 7 in all cell types treated with PQ (Figure 4 and Figure S2). Based on results of DdeI digestion (Figure 4C), the strongest effect of OS on splicing of SMN2 exon 7 was observed in GM20383 lymphocytes followed by neuronal SH-SY5Y cells. Substantial skipping of SMN2 exon 7 was also observed in GM03813 fibroblasts treated with PQ. Based on these findings, we conclude that response to PQ-induced OS on splicing of SMN2 exon 7 is universal. In addition, DdeI digestion analysis underscores that OS-induced skipping of exon 7 is associated mostly with SMN2 (Figure 4C). Supporting the reduced vulnerability of SMN1 exon 7 to skipping under OS, we detected very low levels of SMN1 exon 7-skipped products in PQ-treated SH-SY5Y cells (Figure 4C, lane 8).

We observed an increase in PQ-induced skipping of SMN1 exon 5 in GM20384 lymphocytes, suggesting that pre-mRNA splicing of human SMN1 is sensitive to OS (Figure 4B). To validate that the effect of OS on SMN1 exon 5 skipping is not specific to lymphocytes and/or related to BD-causing mutations in GLN3 gene, we isolated and cloned RT-PCR products lacking exon 5 from PQ-treated SH-SY5Y cells. Based on sequence analysis, conditions of OS led to an appreciable increase in SMN1Δ5 transcripts compared to SMN1Δ5 (Figure S1). These results confirm that skipping of exon 5 is one of the major SMN1-associated events in cells undergoing OS. Unlike SMN1, most SMN2 transcripts that went through exon 5 skipping also lacked exon 7. Consistently, we observed an enhanced co-skipping of SMN2 exons 5 and 7 in PQ-treated GM03813 fibroblasts, GM20383 lymphocytes and neuronal SH-SY5Y cells (Figure 4B).

Interestingly, PQ-induced OS caused a noticeable reduction in intensity of several fast migrating exon 3-skipped splice variants (Δ3, Δ3,5 and Δ3,7) in SH-SY5Y cells. Consistent with these findings, results of quantitative real-time PCR (qPCR) using various junction primers supported a decrease in skipping of exon 3 in PQ treated SH-SY5Y cells (Figure S3). However, due to a near background level of expression of Δ3 transcripts, suppression of exon 3 skipping under OS was not accompanied by an appreciable increase in the exon 3-included transcripts.

Conditions of OS produced three additional novel SMN splice variants that we captured in neuronal SH-SY5Y cells (Figures 4A and 4B). The first such variant lacked SMN1 exons 4 and 7 (ΔSMN1Δ4,7) and co-migrated with the SMNΔ3 transcript in a polyacrylamide gel (Figure 4B). Considering skipping of constitutive exon 7 has not been previously reported and skipping of SMN1 exon 7 is an infrequent occurrence, presence of SMNΔ1A4,7 was very surprising. The second novel variant lacked SMN1 exons 3 and 4 (SMN1Δ3,4) and co-migrated with Δ3,5,7 transcripts on a polyacrylamide gel (Figures 4A and 4B). The third novel SMN1 splice variant that we captured lacked exons 5, 6 and 7 (SMN1Δ5,6,7). Occurrence of SMN1Δ5,6,7 reveals for the first time the feasibility of a rare phenomenon where three adjacent exons of SMN1 are skipped.

All internal exons of SMN are divisible by three. Thus, skipping of one or more internal exons of SMN does not create a premature termination codon. Consequently, none of the alternatively spliced variants of SMN are natural substrates of nonsense-mediated decay (NMD). To analyze the translated products of various alternatively spliced transcripts of SMN1 and SMN2, we performed western blot analysis of PQ-treated GM20384 and GM03813 cells that carry SMN1 and SMN2, respectively. In both cell types, we noticed a decrease in SMN levels at 24 h post PQ treatment (Figure 4D). However, we were unable to detect translated products corresponding to any of the short transcripts generated from either SMN1 or SMN2. This could be attributed to several factors, including low levels of exon-skipped transcripts, slow rate of translation under the conditions of OS and unstable nature of Δ7-translated products. Of note, skipping of SMN exon 7 is known to create a degradation signal [21].

Translation Efficiency of SMN Transcripts Lacking Internal Exons

In order to determine whether proteins encoded by transcripts that lack one or more internal SMN exons could be produced, we employed an alternative approach. We induced SMN2 exon skipping in HeLa cells using two ASOs: E3/I3Jxn and E5/I5Jxn (Figure 5A). E3/I3Jxn and E5/I5Jxn blocked the 5’ ss of exon 3 and exon 5, causing massive skipping of exon 3 and exon 5, respectively (Figure 5B). Targeting ASOs did not discriminate between SMN1 and SMN2 transcripts that code for identical proteins. Also, ASOs were designed not to interfere with the translational machinery because they annealed to sequences that are removed during pre-mRNA splicing. Our approach provided high levels of SMN exon-skipped transcripts that served as the needed templates for protein synthesis. As a result, we were able to detect proteins generated from transcripts lacking either exon 3 or 5 (Figure 5C, lanes 2 and 5). SMNΔ5 appeared to be stable and migrated very close to the full-length SMN. On the other hand, SMNΔ3 seems to be less stable. Poor stability of SMNΔ3 could be attributed to its misfolding due to the loss of the tudor-domain coded by exon 3. Our results validate that SMNΔ3 and SMNΔ5 transcripts could be translated. Whether SMNΔ3 and SMNΔ5 proteins play any physiological role is a matter of future investigation.

Effect of Promoter on OS-induced Skipping of SMN Exon 7

Promoter structure (sequence) has been shown to affect alternative splicing of specific exons in several genes including fibronectin (FN), calcitonin-gene-related product (CGRP) and CD44 [62,63,64]. SMN promoter has been localized in a 2 kb region upstream of the coding sequence that starts within exon 1 [65]. Using reporter assays, two earlier studies support similarity of promoter activity between SMN1 and SMN2 [65,66]. Based on
effect of small compounds that act as inhibitors of histone deacetylase 1, recent reports suggest the role of promoter sequences in splicing regulation of SMN exon 7 [50,67]. However, there is no study to implicate a direct role of SMN promoter sequence on usage of exon 7. To address the promoter-specific splicing regulation of SMN2 exon 7 under normal and OS conditions, we employed SMN minigenes with three different promoters: cytomegalovirus (CMV), thymidine kinase (TK) and wild type SMN1/SMN2 promoters (Figure 6A). Several CMV promoter-containing minigenes encompassing SMY genomic sequences from exon 6 through exon 8 cloned in pCI vector (Promega) have been reported [19,68,69]. We took advantage of CMV promoter containing short minigenes that maintained an earlier reported deletion within intron 6 [68]. Due to decreased size, these minigenes provide desired benefit of high transfection efficiency without any apparent change in splicing pattern of SMN exon 7. To generate minigenes under the control of wild type SMN1 and SMN2 promoters, we replaced CMV promoter with

Figure 3. Detection of multiple exon skipping events in endogenous SMN. A, Diagrammatic representation of the SMN gene. Exonic and intronic sequences are depicted as in (Figure 2A). Dotted lines indicate skipping of previously reported exons and/or novel splice variants. Annealing positions of primers used for MESDA are shown. B, Multiple splice isoforms of endogenous SMN pre-mRNA in different cell lines. Name of cell lines used are given on the top of the gel. Splice products were analyzed by RT-PCR. We used 1.4 μg of total RNA per 10 μl of reverse transcription reaction and generated cDNA employing oligo(dT)12-18 primer. SMN transcripts were amplified using primers 5’hSMN-E2b (anneals within Exon 2b) and P2-2 (anneals within Exon 8). Diagrammatic representation of splice variants is given on the left of the gel; their sizes are indicated on the right of the gel with names of skipped exons given in brackets. For intra-lane comparison of bands, pictograms (shown on right) were generated for each lane by MultiGauge software version 3.0 (FUJIFILM). The identity of splice variants was established by sequencing. Novel splice variant is marked by a star. Note that 567-nucleotide-long Δ5,6 variant moves slower than 573-nucleotide-long Δ3 variant. FL, full length; NS, non-specific. The number of SMN1 and SMN2 transcripts for each of splice variants, except for the full-length and Δ7, was determined by sequencing of at least 8 randomly selected clones per variant (Figure S1). doi:10.1371/journal.pone.0049595.g003
Figure 4. Effect of PQ treatment on splicing of \textit{SMN}. \textbf{A}, Diagrammatic representation of the \textit{SMN} gene. Exonic and intronic sequences are depicted as in (Figure 2A). Dotted lines indicate exon skipping that generated novel splice variants. Annealing positions of primers used for MESDA and RT-PCR are shown. \textbf{B}, Detection of multiple exon skipping variants in endogenous \textit{SMN} in the presence (+) and absence (−) of PQ. Names of cell lines used are given on the top of the gel. Total RNA was prepared from cells treated with 1 mM of PQ for 24 hours. Splice products were analyzed by RT-PCR as described in Figure 3B. Diagrammatic representation of splice variants is given on the left of the gel; their sizes are indicated on the right of the gel with names of skipped exons given in brackets. The identity of splice variants was confirmed by sequencing. Note that 513-nucleotide-long \textit{A5,6,7} variant moves slower than 519-nucleotide-long \textit{A3,7} variant. Novel splice variants are marked by stars. FL, full length; NS, non-specific. For intra-lane comparison of bands, pictograms were generated for each lane (Figure S2). \textbf{C}, Splicing patterns of endogenous \textit{SMN} exon 7 in the presence (+) or absence (−) of PQ. Names of cell lines used are given on the top of the gel. Total RNA was prepared from cells treated with 1 mM of PQ for 24 hours. Spliced products were analyzed by RT-PCR as described in panel B except primers P25 and P31 were used for PCR amplification step. To distinguish transcripts originated from \textit{SMN2}, PCR products were digested with DdeI [55]. \textit{SMN1} and \textit{SMN2} transcripts are indicated on the right of the gel. Exon 7-included (+) and exon 7-skipped (−) products are indicated on the left of the gel. The percentage of \textit{SMN2} exon 7 skipping was calculated as described in Figure 2B. \textbf{D}, Western blot showing translated products generated from \textit{SMN} in GM03813 and GM03813 cells treated with PQ for 24 h. We loaded 50 and 80 μg of protein from GM03813 and GM03813 cell lysates, respectively. Primary antibodies used for probing are indicated on the left of the gel. Although the predicted molecular mass of \textit{SMN} is 32 kDa, it migrates as 40 kDa band due to posttranslational modifications.

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∼3.5 kb genomic sequences harboring promoter region of \textit{SMN1} and \textit{SMN2}, respectively. Wild type \textit{SMN1} and \textit{SMN2} promoters used in this study were the same as reported in an earlier study that confirmed similarity of transcriptional regulation between two \textit{SMN} genes [66]. To generate TK promoter-containing minigenes, we subcloned \textit{SMN} genomic sequences from pCI-based \textit{SMN} minigenes into commercially available pTK-Gluc vector (New England Biolabs). Other than variations in promoter structures, vector-specific sequences downstream of promoters brought additional differences in the contexts of the three minigenes we used (Figure 6A and Figure S4). Therefore, the design of our minigene constructs allowed us to simultaneously examine the effect of promoters as well as sequences upstream of the \textit{SMN} splicing cassette. We employed neuronal SH-SY5Y cells to examine the splicing pattern of \textit{SMN} minigenes expressed under the control of different promoters. We simultaneously monitored the splicing pattern of exon 7 derived from endogenous \textit{SMN1} and \textit{SMN2}.

Among three promoters used in this study, we observed substantially higher levels of \textit{SMN} expression with CMV promoter. For the purposes of comparison of splice variants, we adjusted loading of PCR products for different promoter samples. All minigenes expressing \textit{SMN} under the control of various promoters recapitulated the splicing pattern of endogenous gene, with predominant exon 7 skipping in \textit{SMN2}, and predominant exon 7 inclusion in \textit{SMN1} (Figure 6B). However, unlike minigenes under the control of TK and CMV promoters, wild type promoter produced noticeably less exon 7 skipping (Figure 6B, lane 4). These results provide the first direct evidence of the role of promoter structure in modulation of \textit{SMN} exon 7 splicing. However, compared to a reported 10-fold decrease in usage of the extra domain 1 (ED1) exon of \textit{FN} when expressed under control of a CMV promoter [64], impact of \textit{SMN} promoter on percentage of exon 7 skipping could be considered as less prominent (Figure 6B, compare lanes 1 and 2 with lanes 9 and 10). Effect of promoter structure on \textit{SMN} exon 7 splicing was further supported by an appreciable change in the levels of exon 7 inclusion when CMV promoter was exchanged with TK promoter in \textit{SMN} minigenes (Figure 6B, compare lanes 5 and 6 with lanes 9 and 10). Interestingly, \textit{SMN2} transcripts derived from endogenous gene showed ∼13% less exon 7 skipping as compared to \textit{SMN2} minigene expressed under wild type promoter (compare Figure 6B with Figure 6C). This difference could be attributed to the context of the endogenous gene, which is subjected to chromatin remodeling during transcription elongation. Considering rate of transcription elongation and transcriptional pausing affects the outcome of alternative splicing [5,70,71], a moderate difference in splicing of \textit{SMN2} exon 7 between full endogenous gene and minigene is expected.

Treatment of SH-SY5Y cells with PQ caused noticeable increase in skipping of \textit{SMN} exon 7 from all minigenes expressed under different promoters. However, distinctions between \textit{SMN1} and \textit{SMN2} exon 7 splicing under the conditions of OS were more pronounced in the context of endogenous promoter followed by the expression under wild type promoter. For instance, under the conditions of OS, levels of exon 7-containing \textit{SMN2} transcripts decreased ∼2-fold and more than 2.5-fold and in the context of wild type and endogenous promoters, respectively (Figure 6B, lanes 1 and 2; Figure 6C). At the same time, levels of exon 7-containing \textit{SMN1} transcripts in these contexts decreased only marginally. \textit{SMN1} expressed under the control of TK promoter produced appreciable exon 7 skipping. Also, \textit{SMN2} expressed under the control of TK promoter caused the highest levels exon 7 skipping under the conditions of OS (Figure 6B, lanes 7 and 8). Overall, our results support the role of promoter sequence in regulation of \textit{SMN} exon 7 splicing under the conditions of OS. However, promoter sequences were not the sole regulatory elements to affect OS-induced \textit{SMN1} exon 7 splicing. Considering \textit{SMN1} expressed under all promoters maintained the high levels (>60%) of exon 7-included transcripts even under the conditions of OS, cis-elements within \textit{SMN1} exon 7 and/or within flanking intronic sequences also contribute towards OS-induced exon 7 skipping (Figure 6B, lanes 4, 8 and 12).

A Short ASO Targeting an Intrinsic GC-rich Silencer Fully Prevents OS-induced Skipping of \textit{SMN2} Exon 7

One of the fundamental questions in stress-related studies is to establish whether exon-specific aberrant splicing under OS is preventable. Considering the well-characterized nature of various negative cis-elements, \textit{SMN2} exon 7 splicing offers an ideal system to test this hypothesis. We have earlier reported that a 15-nucleotide-long intrinsic splicing silencer N1 (I5S-N1) and an overlapping 8-nucleotide-long GC-rich sequence play critical role in \textit{SMN2} exon 7 skipping (Figure 7A) [48,53,72,73]. An 8-mer ASO (3UP8) targeting GC-rich sequence prevents \textit{SMN2} exon 7 skipping with high target specificity without any off-target effect on splicing of other \textit{SMN} exons [53]. Therefore, we used 3UP8 to examine whether it will alleviate the negative effect of PQ-induced OS on splicing of \textit{SMN2} exon 7. We first treated GM03813 cells with 50 nM of 3UP8 for 24 h and then induced OS by exposing the cells to 1 mM PQ. Cells were harvested 24 h post PQ treatment and transcripts were isolated for analysis by MESDA. As shown in Figure 7B, 3UP8 was able to fully prevent \textit{SMN2} exon 7 skipping even under PQ-induced OS. As expected, the effect of 3UP8 was exon 7 specific since this ASO did not change the splicing pattern of other \textit{SMN2} exons. We also used a control ASO with a single mismatch mutation. The control ASO had no effect.
on splicing of SMN2 (Figure 7B). To validate that the effect of 3UP8 is not due to a general stimulation of splicing machinery, we examined the splicing pattern of endogenous SMN in HeLa cells treated with various ASOs. Names of ASO used are given on the top of the gel. HeLa cells were transfected with 100 nM of an individual ASO. Cells were collected for total RNA preparation 38 hours post transfection. Spliced products were analyzed by RT-PCR as described in Figure 3B. Diagrammatic representation of spliced variants is given on the left of the gel; their sizes are indicated on the right of the gel with names of skipped exons given in brackets. All spliced products represent mixture of transcripts generated from both, SMN1 and SMN2. Western blot showing SMN protein isoforms generated from transcripts in panel B. Cells were collected for whole-cell lysate preparation 38 hours post transfection. In order to capture the low abundance of SMNΔ3 isoform, we loaded 50 μg of protein from sample treated with E3/I3 Jxn ASO. Protein loading was confirmed by re-probing the blot with anti-β-actin antibodies. Primary antibodies used for probing are indicated on the left of the gel; whereas, SMN isoforms are shown on the right of the gel. SMN is present in all lanes. SMNΔ3 is detectable in lane 2, whereas SMNΔ5 is detectable in lane 3.

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Diversity of Splice Isoforms of SMA Genes
critical SMN-interacting partner responsible for the formation of SMN complex that participates in snRNP biogenesis [24–26]. PQ-induced decrease in SMN levels was accompanied by a similar reduction in Gemin2, suggesting a potential adverse effect of PQ on snRNP biogenesis. Remarkably, ASO treatment that restored SMN2 exon 7 inclusion also produced increased levels of SMN and

Figure 6. Effect of PQ on splicing of different SMN minigenes. A, Diagrammatic representation of promoter and exon/intron organization in different minigenes as compared to endogenous SMN. Promoters and exons are shown as boxes, introns are indicated as lines. Alternatively spliced exons 3, 5 and 7 are shown as colored boxes. Dotted lines indicate deleted sequences. Wild type refers to the minigenes under the control of human SMN1/2 promoters; TK, minigenes under the control of thymidine kinase promoter; CMV, minigenes under the control of CMV promoter. In CMV and TK minigenes, solid bar before exon 6 refers to a vector-specific sequences (Figure S4). Locations of primer pairs for the amplification of spliced products have been marked by arrows.

B, In vivo splicing pattern of different SMN1 and SMN2 minigenes. Data were generated using total RNA from SH-SY5Y cells transfected with 1 μg of an indicated minigene and treated with PQ for 21 h. Minigene splice products were analyzed by RT-PCR. We used 0.5 μg of total RNA for 5 μl of reverse transcription reaction and generated cDNA employing oligo(dT)12–18 primer. Loading was adjusted so that the intensity of bands between samples was comparable. Exon 7-included and skipped products are indicated on the left of the gel. Due to the change in location of the primer annealing sites, SMN spliced products generated from different vectors have variable lengths. The results were analyzed as previously described [55]. Abbreviations are same as described in panel A. C, Effect of PQ on splicing of endogenous SMN exon 7. Splice products were analyzed by RT-PCR as described in panel B, except we used N-24 and P26 primers for PCR to specifically amplify endogenous SMN. Of note, P26 anneals to exon 8 region, which is absent in minigenes [55]. To distinguish spliced variants originated from SMN2, PCR products were digested with DdeI [55]. The percentage of SMN2 exon 7 skipping was calculated as described in Figure 2B. Labeling was the same as described in panel A and Figure 2B.

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Diversity of Splice Isoforms of SMA Genes

A

5' ss

EXON 7

ISS-N1

GC-rich sequence

hnRNP A1

hnRNP A1

5

3'GUAGUGCGCAGCAUAAUGAAAG

3' GACGUGCG

3UP8

CONT

ASOs

B

(i) ASO (50 nM) →
PQ (1 mM) →

(ii) FULL

Δ7

Δ5

Δ5,7

Relative abundance of transcripts (% of total)

(iii) FULL

Δ7

Δ5

Δ5,7

3 vs 4

Δ7

Δ5

Δ5,7

5 vs 6

FULL

Δ7

Δ5

Δ5,7

Δ7,1

Δ7

Δ5

Δ5,7

C

ASO (50 nM) →
PQ (1 mM) →

SMN

Gemin2

β-Actin

Lane number — 1 2 3 4 5 6

Mock CONT 3UP8
Gemin2 (Figure 7C). Considering several ASO-based strategies to correct SMN2 splicing in SMA have been proposed [42–47], our findings are significant as they suggest that these strategies will retain their efficacy even under OS conditions.

Discussion

Occurrence of aberrant splicing under the conditions of OS is an area of growing interest due to its correlation with major human diseases including cancer, cardiovascular and neurodegenerative disorders. The fundamental issue of specificity with which OS affects splicing of certain exons of particular genes in specific tissues remains poorly understood. Here, we use human spinal muscular atrophy genes (SMV1 and SMV2) as a representative system to understand the impact of OS on alternative splicing of various exons of two nearly identical genes. The full-length transcripts from both genes code for SMN, an essential protein that plays a central role in gene regulation through snRNP biogenesis [24]. Skipping of any of the seven internal exons of SMV results in the loss of a fully functional protein that contains several overlapping domains with defined roles. Our study addresses an important question of prioritization of splicing events by which each copy of a duplicate gene responds differently to the conditions of OS.

Publically available SMA patient fibroblast cell line (GM03813) that lacks SMV1 has been widely used for drug screening as well as for understanding transcriptional and posttranscriptional regulation of SMV2. However, analogous cell line to examine SMV1-specific transcriptional and posttranscriptional regulation has not been found. Consequently, a side-by-side comparison of the major splice variants of SMV1 and SMV2 has not been reported. We serendipitously discovered a BD patient cell line (GM20384) that lacked major transcripts specific to SMV2. Such occurrence could be due to complete or partial deletion of SMV2 genes. The splicing pattern of SMV1 exon 7 in GM20384 cells appeared to be identical to those observed in other cell types including BD, Parkinson’s disease, Alzheimer’s disease and neuronal SH-SY5Y cell lines, all of which carried SMV2. Here, we took advantage of GM20384 cell line as a model system to examine SMV1-specific splicing regulation.

In order to reliably capture the relative abundance of major transcripts of SMV, we resorted to develop MESDA. The defining feature of MESDA was the simultaneous evaluation of splicing of five internal exons (exons 3, 4, 5, 6 and 7), among which exons 3, 5 and 7 are known to be alternatively spliced. On the expected lines, GM03813 cells produced two abundantly expressed splice variants corresponding to the full-length and SMV2 exon 7-skipped (SMV2Δ7) transcripts (Figure 3B, lane 1). In addition, GM03813 cells generated SMV2Δ5,7 and SMV2Δ5 as the third and fourth most abundant transcripts, respectively. Low levels of SMV2Δ5 as compared to SMV2Δ5,7 was somewhat surprising as it suggested a cooperative mode of action in which spliced intermediates lacking SMV2 exon 7 served as a preferred substrate for exon 5 skipping. GM03813 cells produced very low levels of SMV2Δ3, SMV2Δ3,7, SMV2Δ3,5 and SMV2Δ3,5,7 transcripts, demonstrating the feasibility of all combinations of co-skipping events of three alternate exons of SMV2. Among low abundant novel isoforms, we identified Δ3,6 transcript in GM20383 lymphocytes (Figure 3B, lane 3). The infrequent occurrence of this splice variant could be ascribed to a rare paring of the 5’ ss of exon 4 with the 3’ ss of exon 7, which itself is an alternatively spliced exon. Considering C6U mutation in SMV2 creates a weak 3’ ss of exon 7, we observed substantially less SMV2Δ3,6 transcripts compared to SMV1Δ3,6 transcripts in GM20383 lymphocytes.

Beyond a handful studies reported more than a decade ago on exon 7- and exon 5-skipped transcripts [18,19,58], our understanding of transcript diversity generated by endogenous SMV1 remains very limited. Therefore, several of our findings reported here on SMV1 splicing constitute a significant advancement towards a better understanding of an overall posttranscriptional regulation of SMV1, which serves as the primary source for maintaining healthy SMN levels in general population. Our results established that the skipping of SMV1 exons 3 and 5 is a general phenomenon that occurs in neuronal and non-neuronal cells alike. Based on the conserved nature of a sequence spanning from exon 2a through exon 6 of SMV1 genes, one could speculate that the mechanism of splicing of exons 3 and 5 is the same for SMV1 and SMV2. However, lack of SMV1Δ5,7 transcripts owing to the absence of SMV1 exon 7 skipping was not accompanied by a proportionate gain in SMV1Δ5 transcripts, suggesting that inclusion of SMV1 exon 7 has a favorable effect on inclusion of exon 5. This could be due to the supporting role of a new sequence and/or structural context created by the inclusion of exon 7. Interestingly, we observed about 13-fold more SMV1 exon 3 skipping as compared to SMV2 exon 3 skipping in neuronal SH-SY5Y cells. Also, as compared to SMV2Δ3 transcripts, less proportion of SMV1Δ3 transcripts underwent through co-skipping with exon 5 (Figure S1). These results suggest an inverse correlation between skipping events of exon 3 and exon 5 of SMV1. Our subsequent finding that PQ-induced enhanced skipping of SMV1 exon 5 suppresses generation of Δ3 transcripts supports such mechanism (Figure 4B). It remains to be seen if such correlation is due to the predominant inclusion of exon 7 in SMV1.

Effect of PQ-induced OS on splicing in different cell types (GM03813, GM20384, GM20383 and SH-SY5Y) revealed remarkable similarities as well as differences between two SMV1 genes. Supporting an earlier report [60], PQ-induced OS produced a significant skipping of SMV1 exon 2, whereas SMV1 exon 7 splicing remained mostly unaffected (Figure 4C). However, the sensitivity of our assay combined with the cell types used demonstrated that high susceptibility of SMV2 exon 7 to skipping...
under the conditions of OS is more general than previously thought. Consistently, all SMN2-containing cells in our study showed substantial SMN2 exon 7 skipping under conditions of OS. We show that OS-induced skipping of SMN2 exon 5 happens primarily (if not exclusively) as co-skipping of SMN2 exons 5 and 7 (Figure 4B, lane 2). By contrast, skipping of SMN1 exons 5 under conditions of OS takes place almost always without skipping of SMN1 exon 7 (Figure 4B, lane 4). Our results also demonstrate that OS-induced skipping of SMN exon 5 occurs in both, neuronal and non-neuronal cells. Exon 5 of SMN codes for a recently described proline-rich calpain cleavage domain [27]. Owing to the low levels of SMN/Δ5 transcripts, we could not detect SMNΔ5 protein under the conditions of OS (Figure 4D). However, our finding that SMNΔ5 is stably translated is significant (Figure 5C). Future studies will address if the calpain cleavage domain lacking protein (SMNΔ5) generated by SMN/Δ5 has any physiological significance.

Skipping of any of the internal exons of SMN maintains the reading frame. Therefore, NMD pathway that degrades mRNAs carrying a premature termination codon is not applicable for the reduced levels of any of the short SMN transcripts. We detected three novel SMN isotypes (SMN/Δ1,7; SMN/Δ3,4 and SMN/Δ5,6,7) generated under the conditions of OS. It is not known if low abundance of these splice variants are in part due to their poor stability caused by a non-NMD mechanism. Presence of SMN/Δ1,7 underscores the occurrence of a rare splicing event of exon 4 skipping in which the 5′ ss of exon 3 and the 3′ ss of exon 5 are required to pair. Considering exons 3 and 5 are also alternatively spliced, skipping of exon 4 represents a unique event that guarantees promotion of inclusion of both, exons 3 and 5. Therefore, our finding of SMN/Δ1,7 reveals the first mutually exclusive event with a significance to the prevention of skipping of two alternatively spliced exons of SMN. Also, generation of SMN/Δ1,7 comes at the expense of competing events that lead to production of two novel isoforms: SMN/Δ3,4 and SMN/Δ5,6,7. Presence of SMN/Δ5,6,7 underscores a unique splicing event requiring a rare long-distance pairing between the 5′ ss of exon 4 and the 3′ ss of exon 8. Another significant observation of our study was the stimulatory effect of OS on splicing of exon 3 (Figures S2 and S3). It remains to be seen if decrease in exon 3 skipping under the conditions of OS contributes at least in part towards generation of some of the novel splice variants described above.

Increasing evidence support transcription-coupled splicing regulation. Effect of transcription on alternative splicing could be exerted through transcription initiation at specific promoters as well as through transcriptional pausing [5]. Well-known factors that affect alternative splicing in a promoter-specific manner include steroid hormone nuclear receptor coactivators, human papilloma virus (HPV) transcriptional activator E2 and peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) [64]. Our finding that large wild type promoter sequence in our reporter system suppresses skipping of SMN exon 7 provided the first direct evidence of the role of promoter in regulation of SMN exon 7 splicing. Effect of promoter sequence on regulation of SMN exon 7 splicing was also observed under the conditions of OS. In particular, OS-induced differential splicing regulation between SMN and SMN2 was much more apparent in the context of the wild type promoter as compared to CMV and TK promoters. Decreased ATP level in OS is likely to slow down or even pause transcription elongation with a significant consequence to ss selection and exon usage [70,71]. Generation of a long endogenous transcript requires an extensive transcription elongation step. Therefore, it is likely that the effect of OS is exerted mostly at the level of transcription elongation. Our finding that endogenous SMN2 produced the highest degree of exon 7 skipping under the conditions of OS also supports the role of transcription elongation in OS-induced splicing regulation of SMN2 exon 7. Recently, transcriptional elongation regulator 1 (TCERG1) has been found to regulate alternative splicing of the short isoform of B-cell lymphoma-extra (BCL-xL) [74]. It remains to be seen if analogous mechanism accounts for the regulation of SMN2 exon 7 skipping under the conditions of OS. However, our results do not preclude the role of additional factors that act through splicing initiation albeit variably at different promoters expressing SMN minigene under the conditions of OS.

Translation of specific transcripts is selectively affected under the conditions of stress [75]. Our finding that ASO-mediated prevention of SMN2 exon 7 skipping under the conditions of OS is able to restore the levels of SMN and Gemini2 suggests that OS does not affect selective repression of SMN translation. Given the prominent role of SMN in cellular metabolism, it is imperative that cells maintain a minimum SMN level even under the conditions of OS. Selective skipping of exon 7 from one gene (SMN2) but not from the other (SMN) supports this argument. Although Δ7 and Δ5,7 were the most abundant SMN2 transcripts generated under OS, we could not detect their corresponding translated products. This could be due to a protein degradation signal coded by the exon 7-skipped transcripts [21]. Our ASO-based approach ruled out an analogous degradation mechanisms for the translated product generated from Δ3 and Δ5 splice variants. Cells prioritize mRNA translation and storage under stress-associated conditions [75]. Hence, it is possible that shorter splice variants of SMN served as decoy molecules to capture microRNAs (miRNAs) and relieve full-length transcripts of miRNA repression. This mechanism will allow a better synthesis of SMN even from the low levels of full-length transcripts. A recent report provides a strong proof of principle for such mechanism in an analogous system [76], miRNA-associated translational repression is generally associated with the 3′ untranslated regions (UTRs) [77]. However, it (repression) could also occur through targets within isoform-specific coding sequences [77,78]. Given the fact that P/Q treatment generates an altered 3′ UTRs due to overwhelming skipping of the last coding exon (exon 7) and also produces additional splice variants, there is a plausible possibility of miRNA-associated control of SMN levels in stress-associated conditions. Now that we have confirmed the vulnerability of various SMN exons to skipping under the conditions of OS, future experiments would address the mechanism of OS-induced aberrant splicing regulation of SMN and the physiological role of various SMN transcripts generated under stress-associated conditions.

In summary, our findings uncover the surprising diversity of SMN transcripts expressed under normal and OS conditions. We validate our findings employing several complementary approaches including MESDA and a unique cell type devoid of major SMN2 transcripts. Our findings underscore an added vulnerability of SMA patients to the conditions of OS and demonstrate the efficacy of an ASO-based strategy in splicing correction under OS. Our results provide the first direct evidence of role of SMN promoter sequence in regulation of SMN exon 7 splicing under normal and OS conditions. In addition to a better understanding of SMA pathogenesis, our findings bring new perspective to splicing regulation of a model housekeeping gene associated with one of the leading genetic causes of infant mortality.
Materials and Methods

Plasmid Constructs

Minigene splicing cassettes pSMN1Δ16 and pSMN2Δ16 have been described previously [68]. Designs of newly constructed minigenes and primer sequences for cloning are given in supporting data (Table S1, Figure S4). Briefly, pTK-SMN1Δ16 and pTK-SMN2Δ16 constructs, in which SMN minigene sequences were placed under the control of Herpes Simplex Virus (HSV) thymidine kinase (TK) promoter, were generated as follows. SMN1 and SMN2 sequences were amplified by polymerase chain reaction (PCR) using pSMN1Δ16 and pSMN2Δ16 as templates, respectively. The amplification was performed with Phusion DNA Polymerase (New England Biolabs) and a pair of primers, P12 and P14. PCR products were then digested with HindIII and BamHI and inserted into pTK-GLuc vector (New England Biolabs) treated with the same restriction enzymes. To generate splicing cassettes pWTP-SMN1 and pWTP-SMN2 in which SMN1 and SMN2 minigene sequences were placed under the control of human SMN1 and SMN2 promoters, respectively, we started with PCR amplification of the SMN1 and SMN2 promoter sequences (~3.5 kb) using T1 and C1 clones, respectively [66]. The promoter sequences were amplified using Phusion DNA Polymerase and primers 5’-SMN-pro-BglII and 3’-SMN-pro-XhoI. The amplified SMN1 and SMN2 promoter sequences were subsequently digested with BglII and XhoI and ligated into pSMN1Δ16 and pSMN2Δ16 minigenes subjected to partial digestion with the same restriction enzymes. Partial digestion was used since pSMN1Δ16 and pSMN2Δ16 minigenes contain two BglII restriction site. For partial digestion several μg of pSMN1Δ16/pSMN2Δ16 were treated with XhoI (6.4 U) at 37°C overnight; next morning BglII (4 U) was added to the reaction mixture and digestion continued for another 15 min followed by phenol/chloroform extraction and ethanol precipitation. The precipitated products were separated on a 1% agarose gel, the band of interest corresponding to 4.2 kb was excised from the gel and the DNA product was recovered from the gel using QIAquick Gel Extraction Kit (Qiagen) as per manufacturer’s instructions. The recovered DNA corresponded to either BglII- and XhoI-digested pSMN1Δ16 or pSMN2Δ16, which, as mentioned above, were used in ligation reaction with SMN1 and SMN2 promoters and Quick Ligation Kit (New England Biolabs). The identity of novel minigene clones was verified by sequencing. All primers were obtained from Integrated DNA Technologies (Table S1). All restriction enzymes used were from New England Biolabs.

Cell Culture and ASOs

All tissue culture media and supplies were purchased from Life Technologies. Human neuroblastoma SH-SY5Y cells were cultured in 1:1 mixture of Minimum Essential Medium (MEM, catalog # 11095) and F12 medium supplemented with 10% fetal bovine serum (FBS). Primary patient fibroblasts, immortalized B-lymphocytes as well as cells from healthy individuals were obtained from Coriell Cell Repositories (CCR). Description of cells from CCR used in this study is given in Table 1. These cells were grown according to the provided instructions. In brief, all primary fibroblasts were grown in minimal essential medium (MEM, catalog # 10370) supplemented with 2 mM GlutaMAX-I and 15% FBS. Only in case of primary fibroblasts from Alzheimer patient (Repository number AG04159), the amount of FBS in growth medium was 10%. B-lymphocytes were grown in RPMI1640 (catalog # 11875) medium supplemented with 15% FBS. The following ASOs were used in this study: 3UP8, 5’-mG*mC*mU*mG*mG*mU*mG*mA*mG-3’; 3UP8/64A (CONT), 5’-mG*mC*mU*mG*mU*mC*mG*mA*mA*mG-3’; E3/I3Jxn, 5’-mU*mA*mU*mC*mU*mC*mU*mC*mU*mU*mG*mA*mG*mU*mA-3’; E5/I5Jxn, 5’-mC*mG*mU*mC*mC*mU*mC*mC*mC*mC*mC-3’. All ASOs were obtained from Dharmacon Inc. They incorporated 2’-O-methyl modification (indicated by “m”) and phosphorothioate backbone (indicated by “*”) as described earlier [48].

PQ Treatment

PQ (Paraquat, methyl viologen dichloride hydrate, catalog # 856177) was obtained from Sigma. PQ treatment of pre-plated adherent cells was done as follows. Sixteen to eighteen hours before the treatment GM03813 cells were plated at a density of ~1.1 x 10^5 cells per well of 6-well plate or ~6.2 x 10^4 per 100 mm tissue culture dish. For SH-SY5Y, ~1.9 x 10^5 and 0.5 x 10^6 SH-SY5Y cells were seeded in 100 mm dishes. In case of SH-SY5Y, cells plated at a higher density were subjected to PQ treatment, while cells seeded at a lower density served as an untreated control. Lower cell density insured that untreated control SH-SY5Y cells do not become confluent at the end of PQ treatment. PQ was added with fresh growth medium at a final concentration of 1 mM; fresh medium without PQ was also added to control untreated cells. 200 mM stock solution of PQ in phosphate buffered saline (PBS) was prepared immediately before usage. In case of B-lymphocytes that grow in suspension as aggregates, right before PQ addition cell clumps were disaggregated by pipetting and cells were seeded at a density of ~1.4 x 10^6 cells per ml in a desired volume. PQ was added to a final concentration of 1 mM. In case of untreated control lymphocytes, PQ was omitted from growth medium. Unless noted otherwise, PQ treatment continued for 24 h, after which cells were collected for whole cell lysates/total RNA preparation.

PQ treatment combined with transfection of minigenes was done as follows. SH-SY5Y cells were plated at a density of ~2.8 x 10^5 cells per one well of a 6-well plate. Next day the cells were transfected with 1 μg of different minigenes using Lipofectamine 2000 (Life Technologies) following the manufacturer’s recommendations. Six hours after transfection cells were washed once and conditioned medium harvested from growing SH-SY5Y cells was added to the wells. Twelve hours post transfection, cells in each well were trypsinized and collected in a total volume of 4 ml; 2.5 ml of this cell suspension were transferred to a new well of 6-well plate and freshly prepared PQ was immediately added to a final concentration of 1 mM (PQ treated cell), while 1.5 ml of this suspension were plated in another well of 6-well plate and used as an untreated control. Following 21 hours of PQ treatment, cells were collected for total RNA preparation.

PQ treatment combined with transfection of ASOs was done as follows. GM03813 primary fibroblasts were plated at a density of ~8 x 10^5 cells per 100 mm tissue culture dish. Total number of six dishes was seeded. Twenty-four hours later cells were transfected with 50 nM of a given ASO (two plates were transfected with each ASO) using Lipofectamine 2000 following the manufacturer’s recommendations. Following another 24 hours the medium was changed, and freshly prepared PQ was added to one of each pair of transfected plates; the other plate served as a PQ untreated control. PQ treatment continued for 24 hours, after which cell were washed several times with ice-cold PBS and collected by scraping. For total RNA preparation ~1/4 of cells from each dish was used, while 3/4 were used for making cell lysates.
ASO Transfections

Delivery of ASOs into HeLa cells was done by reverse transfection using Lipofectamine 2000. Complexes of Lipofectamine 2000 and ASOs were prepared following the manufacturer’s recommendations. The complexes were allowed to form for 20 minutes at room temperature, after what each complex (1 ml, in Opti-MEM I Reduced Serum Medium) was mixed with 4 ml of HeLa cell suspension in a 60 mm dish. Total number of HeLa cells used for transfection varied from ~2.0×10^6 to ~2.5×10^6 cells. The final ASO concentration was 100 nM. Next day culture medium was changed for the fresh one. About 30 hour post transfection the cells were washed several times with ice-cold PBS and collected by scraping. About 4/5th of cells were used for making cell lysates, while 1/5th of cells were used for preparing total RNA.

RT-PCR

For reverse transcription and PCR (RT-PCR), total RNA was isolated using Trizol reagent (Life Technologies) following the manufacturer’s recommendations. To generate cDNA, reverse transcription was carried out using a SuperScript III reaction kit (Life Technologies). For cDNA synthesis random primers (Promega), an oligo(dT)_{12-18} primer (Life Technologies) or gene-specific primer 3’E6-Dde were employed. Generally, 0.5 to 1 µg of total RNA was used per 5 µl of Reverse transcriptase (RTase) reaction. Minigene-specific spliced products were resolved on a 6% native polyacrylamide gel. Analysis and quantifications of splice products were performed using a FPL-5000 Image Reader and Multi Gauge software version 3.0 (FUJIFILM). Abbreviation: FL, the presence of a trace amount of [α-32P] dATP (3,000 Ci/mmole; Perkin-Elmer Life Sciences) or with the following pairs of primers: P1 and P2 for SMN (RTase) reaction. Minigene-specific spliced products were resolved on a 6% native polyacrylamide gel. Analysis and quantifications of splice products were performed using a FPL-5000 Image Reader and Multi Gauge software version 3.0 (FUJIFILM).

Supporting Information

Figure S1 Distribution of splice forms revealed by cloning and sequencing. A, Relative abundance of SMN splice isoforms. All splice variants except Δ5,6 were cloned from SH-SY5Y cells. Splice variant Δ5,6 was cloned from GM20383 cells. B, Evaluation of SMN splice isoforms generated under OS caused by PQ treatment of SH-SY5Y cells. Cells were treated with 1 mM PQ for 24 hours and analyzed by RT-PCR, cloning and sequencing. (DOCX)

Figure S2 Pictogram showing relative abundance of transcripts generated in various cell types undergoing through OS. Peak values are derived from the band intensities in lanes shown in Figure 4B. Pictograms were generated by MultiGauge software version 3.0 (FUJIFILM). Abbreviation: FL, Full-length transcript. (DOCX)

Figure S3 Comparison of different splice variants in PQ treated and untreated SH-SY5Y cells as determined by qRT-PCR. A, Relative expression of splice isoforms from control untreated and 1 mM PQ treated cells. qRT-PCR was performed as described in Materials and Methods S1. Total SMN from untreated SH-SY5Y was used for normalization. The values are mean ± SD. Abbreviations for transcripts: Total, total SMN; 7+, exon 7-included; Δ7, exon 7 skipped; 5+, exon 5-included; Δ5, exon 5 skipped; 3+, exon 3-included; Δ3, exon 3 skipped. *, P<0.05; **, P<0.01. B, Log2 transformed fold changes in SMN splice isoform levels between control untreated and 1 mM PQ treated calculated based on the results shown in Panel (A). The values are mean ± SD. Abbreviations for transcripts: Total, total SMN; 7+, exon 7-included; Δ7, exon 7 skipped; 5+, exon 5-included; Δ5, exon 5 skipped; 3+, exon 3-included; Δ3, exon 3 skipped. *, P<0.05; **, P<0.01. (DOCX)

Figure S4 Diagrammatic representation of minigenes placed under the control of different promoters. A, SMN1 and SMN2 minigenes (pSMN1ΔI6 and pSMN2ΔI6) under the control of CMV promoter. Cloning strategy is described earlier [61]. B, SMN1 and SMN2 minigenes (pTK-SMN1ΔI6 and pTK-SMN2ΔI6) under the control of TK promoter were generated by subcloning into pTK-GLuc vector (New England Biolabs).
Cloning strategy is described in Materials and methods. C, SMN1 and SMN2 minigenes (pWTP-SMN1A68 and pWTP-SMN2A68) under the control of wild type SMN promoters were generated by replacing CMV promoter with human SMN1 and SMN2 promoters, respectively. Cloning strategy is described in Materials and Methods.

(DOCX)

Figure S5 Results showing specificity of 8-mer ASO targeting GC-rich sequence. A, Diagrammatic representation of the ASO target area as described in Figure 7A. B, Splicing pattern of endogenous PLOD2 in SMA patient fibroblasts transfected with different ASOs in the presence (+) or absence (−) of PQ. Transfection, PQ treatment and RNA isolation procedures were the same as described in Figure 7. PLOD2 spliced products were analyzed by RT-PCR. RNA was converted to cDNA using an oligo(dT)12–18 primer. PLOD2 transcripts were amplified using E13-PLOD2 and E15-PLOD2 primers that anneal to exons 13 and 15, respectively. Primer sequences are given in Table S1.

(DOCX)

Table S1 List of primers used in this study.

(DOCX)

Materials and Methods S1 Detailed information on methods used in this study.

(DOCX)

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Author Contributions

Conceived and designed the experiments: RNS NNS JS SJR. Performed the experiments: NNS JS SJR. Analyzed the data: RNS NNS JS SJR. Contributed reagents/materials/analysis tools: RNS. Wrote the paper: RNS NNS.

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