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Validation of trans-acting elements that promote exon 7 skipping of SMN2 in SMN2-GFP stable cell line

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ABSTRACT

Spinal muscular atrophy is a genetic disease in which the SMN1 gene is deleted. The SMN2 gene exists in all of the patients. Alternative splicing of these two genes are different. More than 90% of exon 7 included form is produced from SMN1 pre-mRNA, whereas only ~20% of exon 7 included form is produced from SMN2 pre-mRNA. Only exon 7 inclusion form produces functional protein. Exon 7 skipped SMN isoform is unstable. Here we constructed a GFP reporter system that recapitulates the alternative splicing of SMN1 and SMN2 pre-mRNA. We designed a system in which GFP protein is expressed only when exon 7 of is included in alternative splicing. The stable cell that expresses SMN1-GFP produces ~4 times more GFP protein than the stable cell line that expresses SMN2-GFP; as demonstrated by microscopy, FACS analysis and immunoblotting. In addition the ratio of exon 7 inclusion and skipping of SMN1-GFP and SMN2-GFP pre-mRNA was similar to endogenous SMN1 and SMN2 pre-mRNA as shown in RT-PCR. Furthermore the knockdown with hnRNP A1 shRNA, a known protein which promotes exon 7 skipping of SMN2, induces exon 7 inclusion of exon 7 in SMN2-GFP pre-mRNA in SMN2-GFP cell line. We conclude that we have established the stable cell lines that recapitulate alternative splicing of the SMN1 and SMN2 genes. The stable cell line can be used to identify the trans-acting elements with siRNA.

1. Introduction

Spinal muscular atrophy is an autosomal recessive genetic disease and the leading cause of infant mortality [1,2]. The genetic cause of spinal muscular atrophy is the deletion or mutation of the SMN1 gene that encodes a spinal motor neuron (SMN) protein [3–5]. SMN protein functions in the assembly and disassembly of U snRNP particles which play an important role in pre-mRNA splicing [6]. In addition, SMN protein promotes β-actin mRNA transport in neuronal cells. The lucky fact is that the SMN2 gene, a duplicate of the SMN1 gene, exists in patient cells. However, because of a few nucleotide differences of genomic sequences between SMN1 and SMN2 gene [7], the alternative splicing of the two genes is varied, especially the ratio of exon 7 inclusion and skipping [8–11]. In the case of SMN1 gene, more than 90% of pre-mRNA undergoes splicing with exon 7 inclusion that produces functional SMN protein; whereas ~20% of SMN2 pre-mRNA undergoes splicing with exon 7 included and other ~80% SMN2 pre-mRNA undergoes exon 7 skipping [4,8,10]. The products of exon 7 skipping are degraded [12].

Pre-mRNA splicing is a key regulatory step required for gene expression in higher eukaryotes [13–15]. Alternative splicing produces different mRNA isoforms from a single pre-mRNA molecule, greatly contributing to protein diversity. Alternative splicing is regulated by cis-acting elements, the sequences on pre-mRNA, and trans-acting elements, the proteins that function on the alternative splicing [16–19]. The well-established regulatory systems include that the SR proteins promote splicing through binding to the enhancer sequences, and that hnRNP proteins inhibit splicing through binding to the inhibitor sequences [18,20–27].

siRNA is an efficient tool to study the function of a protein [28–31]. siRNA is produced through the consecutive cleavage by drosa and dicer. A single stranded shRNA (small hairpin RNA) is a modified version of siRNA that is produced from the transfected plasmid [32]. shRNA can be produced from a viral vector to knock down the genes through effective virus infection [33]. In order to identify the trans-regulatory elements that regulate SMN2 pre-mRNA splicing through gene knockdown with shRNA, a reporter system that recapitulates alternative splicing of SMN2 pre-mRNA is valuable [34]. Here we have established a stable cell line with a GFP reporter system that correctly recapitulates alternative

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splicing of SMN2 pre-mRNA. The treatment with a shRNA which targets hnRNP A1, a gene, that is known to repress exon 7 inclusion of SMN2 pre-mRNA [35], promotes exon 7 inclusion of SMN2 pre-mRNA in our stable cell lines. The results of our studies reveal that our SMN2-GFP system can be used as a new tool for identifying regulatory elements of SMN2 alternative splicing.

2. Materials and methods

2.1. Construction of SMN1-GFP and SMN2-GFP reporter system

In order to perform the siRNA screen, SMN1, SMN2-GFP stable cell lines were generated with plasmids that were constructed by Zhang et al. [36]. In brief, to inactivate the translation termination codon at the 3′ end of exon 7, a single nucleotide G was inserted into exon 7 after the 48th nucleotide. The reporter gene GFP was fused to 21 nucleotides downstream from the 5′ end of exon 8. The initiation codon at the 5′ end of the reporter gene was modified by removing the 'A' thereby preventing internal translation and background expression.

2.2. Transfection and stable cell lines

Plasmid transfections into C33A cells were carried out with polyethyleneimide (PEI) according to the manufacturer's instruction. 4 μg of PEI were mixed with 2 μg of SMN1-GFP or SMN2-GFP reporter plasmids in 100 μl of DMEM. The mixture was applied to cells in 900 μl of DMEM supplemented with FBS. Four hours later, media was changed. To generate the stable cell lines, transfected C33A cells were treated by 0.75 mg/ml G418 for 10 days.

2.3. RT-PCR

Total RNA was extracted from transfected C33A cells using RiboEx reagent (GeneAll) following the manufacturer's protocol. One microgram of total RNA was reverse transcribed using oligo (dT) primer using ImProm-II™ reverse transcriptase (Promega) following the manufacturer's protocol. One microlitre of the reverse transcription reaction was amplified by PCR using G-Taq polymerase (Cosmo Genetech). The primers and PCR condition for each gene were used as following: SMN1, SMN2 mini-genes: pCIFW (5′-GCT AAC GCA GTC AGT GCT TC-3′), GFP-220AS (5′-CTG AAG CAC TGC ACG CCG TAG-3′); GAPDH Fwd (5′-ACC ACA GTC CAT GCC ATC A-3′) and GAPDH Rev (5′-TCC ACC ACC CTG TTG CTG TA-3′). PCR products from endogenous SMN mRNAs were obtained with primers Ex5Fwd (5′-CTA TCA TGC TGG CTG CCT-3′) and Ex8-Rev (5′-CTA CAA CAC CCT TCT CAC AG-3′) and digested with *Dde*I to distinguish SMN and SMN2.

2.4. Western Blotting

Cells were lysed in ice-cold lysis buffer for 20 min at 4 °C and then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was mixed with SDS sample buffer, and heated for 5 min. The proteins were separated through 12% SDS–PAGE gels and were transferred into a nitrocellulose membrane. The membranes were blocked using 5% skim milk in TBS-T and then incubated with first antibody (GFP, α-tubulin) in TBS-T for 2 h. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse Igg-HRP, Santa Cruz biotechnology) for 1 h. After three washes with TBS-T buffer, the bands were visualized by ECL Western Blotting detection reagents after exposed to X-ray film.

2.5. Cell culture

C33A and SMN1, 2-GFP stable cells were maintained in Dulbecco's Modified Eagle's Medium(DMEM) supplemented with 10% of Fetal Bovine Serum(FBS) at 37 °C in a humidified 5% CO_2 condition.

2.6. Flow cytometry

2.0×10^6 SMN1 and SMN2-GFP stable cells were washed with PBS (Hyclone) 3 times. To detach the cells trypsin–EDTA (Hyclone) was added and incubated at 37 °C in a humidified 5% CO_2 condition for 3 min. Then cells were centrifuged at 14,000 rpm for 15 min at room temperature. After centrifuge cells were suspended using phenol red free DMEM (Hyclone). These cells analyzed by with FACSAriaII (BD Biosciences) FACS analyzer [37–39].

2.7. shRNA treatment

shRNA lentivirus were generated by the cotransfection of pLKO.1 plasmid encoding the hnRNP A1 mRNA matching sequence or non-silencing sequence and PSPAX2 and PMD2G helper plasmids into 293T cells using polyethyleneimide (PEI). After 24 h transfection, the supernatants containing lentiviruses were harvested with a 0.45 μm filter. SMN2-GFP stable cells were seeded in 6-well plate before infection. Lentivirus containing supernatants were added to the cells supplemented with 10 μg/ml polybrene. After 72 h infection, virus containing medium was removed then total RNA was extracted for RT-PCR.

3. Results

3.1. GFP reporter stable cell lines which recapitulate exon 7 inclusion of SMN2 pre-mRNA are established

In order to identify the trans-acting elements quickly with shRNA screening, we need a reporter system that recapitulates alternative splicing of SMN2 pre-mRNA. In addition, the cell lines that stably express the reporter system should be alive after shRNA treatment. Because luciferase assay has to be performed after cell lysis treatment, previously published luciferase reporter is not a recommendable system in this case [36]. We choose GFP as a reporter system, as GFP is easily observable with microscopy and FACS analysis while the cells are alive. As shown in Fig. 1, we inserted a minigene which spans from exon 6 through exon 8 in front of GFP in pCI-neo vector. Translation initiation codon is inserted in exon 6 to produce SMN2-GFP fusion protein. One nucleotide was inserted into exon 7 to avoid a stop codon. One nucleotide was deleted from exon 8 to fit the translation codon. GFP is expressed only when exon 7 is included. In the case of exon 7 skipping, GFP is not expressed because GFP reading frame is out of order.

3.2. Alternative splicing of SMN1-GFP and SMN2-GFP mini-gene is similar to endogenous pre-mRNA splicing of SMN1 and SMN2 gene

We asked if the pre-mRNA splicing patterns of SMN1-GFP or SMN2-GFP minigene are similar to the pattern of endogenous SMN1 and SMN2 pre-mRNA splicing. Fig. 2A shows SMN1 pre-mRNA undergoes splicing with ~95% exon 7 inclusion and ~5% of exon 7 exclusion in C33A cell line. SMN2 pre-mRNAs are spliced with ~10% of exon 7 inclusion and ~90% of exon 7 exclusion.

In order to discriminate the splicing of mini-genes with endogeneous ones, we used a primer which basepairs with pCI-neo plasmid; the other primer used basepairs with GFP coding region. The results in Fig. 2B shows that ~85% of exon 7 included mRNAs

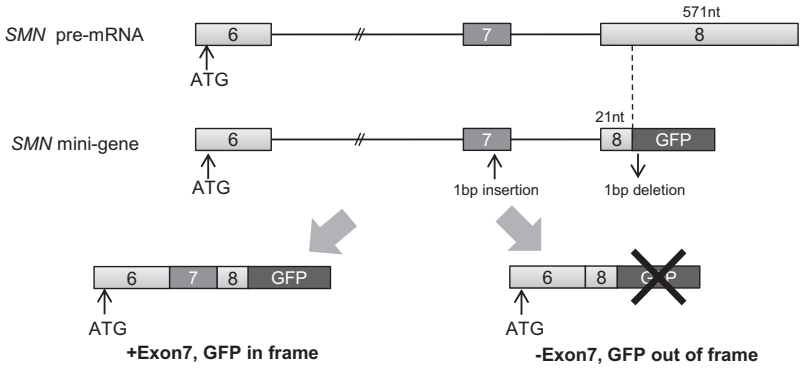

Fig. 1. The design of SMN1-GFP and SMN2-GFP minigene. Genomic sequences which covers exon 6, intron 6, exon 7, intron 7 and 21 nt of exon 8 of SMN1 and SMN2 gene are inserted at N-terminal of GFP coding region. Initiation codon is included in exon 6. One nucleotide is deleted from exon 7 to avoid stop codon. One nucleotide is inserted into exon 8 to supplement the 1 bp deletion in exon 7. GFP is expressed when exon 7 is included because GFP coding is in the frame. GFP is not expressed when exon 7 is skipped because GFP coding region is out of frame.

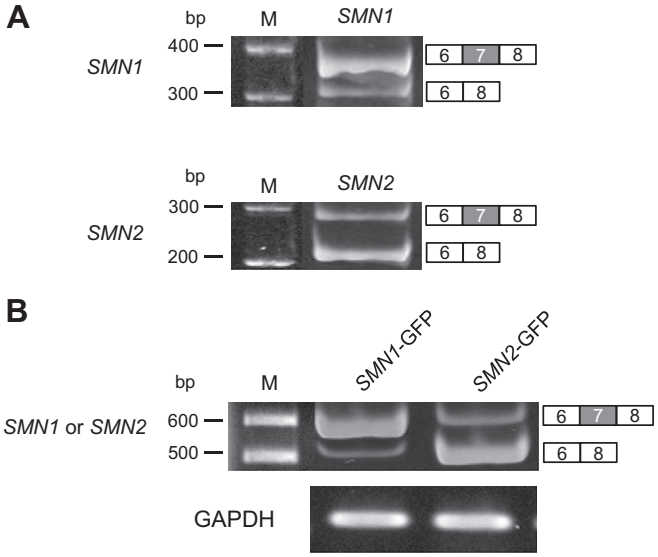

Fig. 2. Alternative splicing of SMN1-GFP and SMN2-GFP are analogous to splicing of endogenous SMN1 and SMN2 pre-mRNA. (A) RT-PCR products of endogenous SMN1 and SMN2 pre-mRNA are shown. (B)RT-PCR analysis of pre-mRNA splicing SMN1-GFP and SMN2-GFP minigenes are shown. RT-PCR of GAPDH gene is show as a control.

are produced from SMN1-GFP minigene construct, whereas only ~15% of exon 7 included mRNAs are produced from SMN2-GFP minigene. We conclude that pre-mRNAs of SMN1-GFP and SMN2-GFP minigene are spliced in the similar ways to that of endogenous ones.

3.3. SMN1-GFP stable cell line expresses ~fourfold GFP protein than SMN2-GFP stable cell line does

We next use the SMN1-GFP and SMN2-GFP plasmids to establish the stable cell lines. In order to make stable cell lines that express SMN1-GFP and SMN2-GFP, we transfected the plasmids into C33A cell line. Multi round selection through G418 treatment in single cell seeding and FACS sorting, the cell lines that stably express GFP were established. From the florescence microscopy pictures shown in Fig. 3A, SMN2-GFP stable cell line has much lower GFP intensity than SMN1-GFP stable cell line. FACS analysis confirmed above results that SMN1-GFP stable cell line has higher GFP intensity than SMN2-GFP stable cell line (Fig. 3B). Immunoblotting assay shows that SMN1-GFP stable cell line produces ~fourfold GFP expression of SMN2-GFP stable cell line (Fig. 3C). As SMN1 and SMN2 pre-mRNA produces ~90% and ~20% of exon 7 inclusion, respectively, and GFP expression is dependent only on exon 7 inclusion, the fourfold change of GFP expression exactly reveal the alternative splicing of SMN1 and SMN2 pre-mRNA. We conclude that GFP expression level of SMN1-GFP and SMN2-GFP stable cell line reveals exon 7 inclusion levels of endogenous SMN1 and SMN2 pre-mRNA splicing.

3.4. shRNA knockdown of hnRNP A1 in SMN2-GFP stable cell line promotes exon 7 inclusion of SMN2-GFP minigene

We have demonstrated that SMN2-GFP stable cell line recapitulates the exon 7 inclusion level of endogenous pre-mRNA splicing. We assume that the cell line can be applied to usage of shRNA to identify the trans-acting elements which repress exon 7 inclusion of SMN2-pre-mRNA. In order to test the possibility, we choose hnRNP A1, a well-known repressor for exon 7 inclusion, for shRNA knockdown. We infected SMN2-GFP cell lines with lenti-viruses which harbor hnRNP A1 shRNAs. RT-PCR results (Fig. 4B) show the efficient knockdown of hnRNP A1 level. Fig. 4B shows that knockdown of hnRNP A1 induced significant increase of exon 7 inclusion as expected. Furthermore hnRNP A1 knockdown induced the increase of GFP intensity as shown in Fig. 4A with microscopy, FACS analysis and Western Blotting. Our results reveal that SMN2-GFP stable cell line provides a system that can be used to identify the trans-acting elements using shRNA screening.

4. Discussion

In this study, we constructed a cell line which can be applied for investigation of siRNA effects on pre-mRNA splicing of the SMN2 gene, because it recapitulates the pre-mRNA splicing of the SMN2 gene. In SMA (Spinal Muscular Atrophy) patients, SMN1 gene is deleted or mutated but a duplicate of SMN1 gene, SMN2 gene exists. Largely due to one single nucleotide difference in exon 7 between SMN1 and SMN2, pre-mRNA splicing of SMN2 gene mostly produces exon 7 skipped mRNAs which encode non-functional proteins [40]. We designed a SMN2 and SMN1 minigene which contains exon 6 through exon 8 of SMN2 gene with a GFP reporter at its C-terminal [36]. Through genetic engineering, GFP is supposed to be expressed only in the case that exon 7 is included. In the contrary, GFP is not expected to be expressed when exon 7 is skipped. The SMN1-GFP and SMN2-GFP minigene constructs were transfected into cells to stably express the minigenes. The stable cell lines acted as we expected. GFP expression is

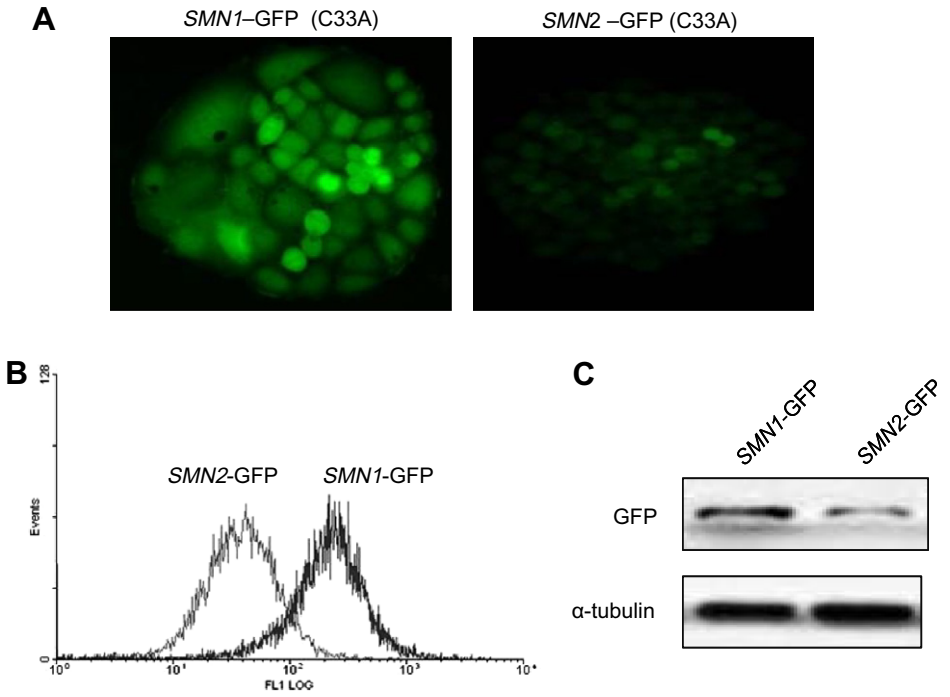

Fig. 3. The amounts of GFP protein expressed by SMN2-GFP and SMN1-GFP cells reflect the exon 7 inclusion level of SMN2 and SMN1 pre-mRNA splicing. (A) GFP intensities of SMN1-GFP and SMN2-GFP are shown by fluorescence microscopy. The intensity of SMN1-GFP cells is much higher than SMN2-GFP cells. (B) FACS analysis show the GFP expression level is much higher in SMN1-GFP cells than SMN2-GFP. (C) Immunoblotting analysis of SMN1-GFP and SMN2-GFP cells with anti-GFP antibody are shown (34 kDa). SMN1-GFP cell express ~fourfold GFP protein of that SMN2-GFP cell expresses. α-Tubulin is used as a control.

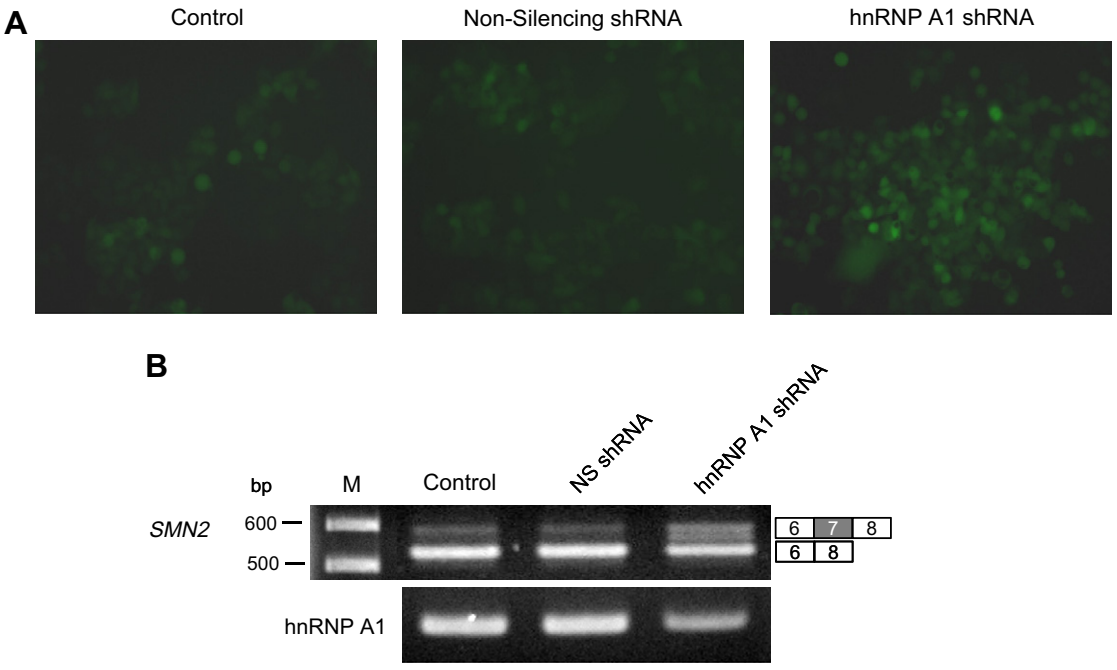

Fig. 4. shRNA treatment for hnRNP A1 gene induced significant increase of GFP expression of SMN2-GFP cell line. (A) GFP intensity is increased after shRNA treatment for hnRNP A1 gene as shown with florescent microscopy. The treatment with non-silencing shRNA did not induce any change in GFP intensity. (B) shRNA treatment for hnRNP A1 gene induces the increase of exon 7 inclusion of SMN2 minigene.

~fourfold more in SMN1-GFP cells than SMN2-GFP cells as shown with microscopy, FACS analysis and Western Blotting. In addition the alternative splicing events of these minigenes are analogous to the splicing of endogenous genes. In SMN2-GFP minigene, ~20% of exon 7 included isoform is produced whereas ~80% of exon included isoform is produced from SMN1-GFP minigene.

siRNA is an efficient tool to investigate the physiological effects of proteins by interfering its expression. shRNA we used here is a small hairpin RNA which produces siRNA from a viral vector. We tested if our SMN2-GFP cell line can be applied for identification of proteins that affect alternative splicing of SMN2 with shRNAs. We choose hnRNP A1, a well-known repressor of exon 7 inclusion

of SMN2 pre-mRNA [35], to test the effects of its shRNA on SMN2-GFP cell line. Our results showed that the treatment of hnRNP A1 shRNA induced significant increase of GFP expression as expected. In addition, the shRNA treatment induced a significant increase of exon 7 inclusion of the SMN2-GFP minigene pre-mRNA.

In conclusion, SMN2-GFP cell line we have established is an efficient system for the shRNA-applied detection of proteins which regulate alternative splicing of SMN2 pre-mRNA. These Proteins could be targeted for SMA treatment.

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