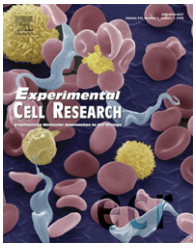
EXPERIMENTAL CELL RESEARCH 314 (2008) 878–886

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

Genetic conversion of an *SMN2* gene to *SMN1*: A novel approach to the treatment of spinal muscular atrophy

*Darlise DiMatteo, Stephanie Callahan, Eric B. Kmiec**

Department of Biological Sciences, University of Delaware, Newark, Delaware 19716, USA

ARTICLE INFORMATION

Article Chronology:
Received 3 August 2007
Revised version received
22 September 2007
Accepted 9 October 2007
Available online 24 October 2007

Keywords:
Single-stranded oligonucleotides
Gene alteration
SMA

ABSTRACT

Spinal muscular atrophy (SMA), a recessive, neuromuscular disease, is caused by a mutation or deletion in the *SMN1* gene. The *SMN2* gene is present in the same region of chromosome 5 and is similar in DNA sequence to *SMN1* except for a T at position +6 of exon 7 that is likely the predominant functional difference between the two genes. This change alters RNA splicing which results in the removal of exon 7 from the mature mRNA; only 10% full-length transcripts are produced from the *SMN2* gene. Our lab has shown that single-stranded oligonucleotides (ODN) can be used to repair genes with single base mutations within the context of the native chromosome. Here, we used SMN2-sequence-specific ODNs to direct the exchange of a T to a C in an SMA skin fibroblast cell line from a type 1 patient. The cells were transfected with ODNs of either 47 or 75 bases in length and designed to hybridize to either the transcribed or non-transcribed DNA strand of the *SMN2* gene. We analyzed the genotype of these cells using a well-established Taqman® probe-based PCR assay, restriction enzyme digestion, and cycle sequencing. Conversion of the *SMN2* genotype to *SMN1* was detected when the specific ODN was added. As a result, we observed an increase in production of full-length SMN mRNA, measured by qRT-PCR, and SMN protein, measured by western blotting. Finally, properly localized SMN protein was detected by the accretion of gemini of coiled bodies (gems) only in targeted cells. This is the first report of the use of ODNs to direct genetic conversion of *SMN2* to *SMN1* in human cells from SMA patients.

Introduction

Spinal muscular atrophy (SMA) is a recessive, neuromuscular disease characterized by symmetrical muscle wasting due to the death of motor neurons. The causal gene for this condition is known as survival motor neuron (*SMN*) which is located on chromosome 5q13 in two nearly identical copies — *SMN1* and *SMN2* [1]. The *SMN1* gene produces a full-length, functional protein and it is this gene that is deleted or mutated in SMA patients. In contrast, the *SMN2* gene contains a base alteration at the sixth nucleotide in exon 7 where a thymine in *SMN2* either disrupts an exonic splice enhancer or creates an exonic splice silencer [2,3]. As a result, exon 7 is skipped during RNA processing [3–5] and the predominant protein produced from this *SMN2* transcript is truncated and biologically inactive [4,6]. Disease severity correlates to *SMN2* gene copy number ([7] and other references therein), an observation that has guided the goals of clinical trials that aim to increase the overall amount of full-length mRNA using specific drugs [8,9]. While these drugs increase the amount of SMN *in vitro* and *in*

* *Corresponding author*. University of Delaware, DBI, 15 Innovation Way, Rm 270. Fax: +1 302 831 3427.
E-mail address: ekmiec@udel.edu (E.B. Kmiec).

0014-4827/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2007.10.012

vivo, they concomittedly require long-term administration to realize therapeutic benefits.

An alternate treatment for SMA has arisen from the field of molecular medicine. Since the genetics behind SMA are well established, various forms of gene therapy are a possibility as a therapeutic approach. Unlike most inheritable diseases, the SMN loci include two genes *SMN1* and *SMN2*, as described above [1]. Early studies focused on the potential use of gene therapy for SMA that involved introducing normal, cloned *SMN1* transgenes into cells in order to elevate levels of *SMN1* [10,11]. Although modest improvements *in vitro* and *in vivo* have been reported, episomal or extrachromosomal DNA templates have short half-lives and, in most cases, are not replicated. As a result, the transgene is diluted or its expression is suppressed as the cell population expands.

As the field of gene therapy evolved, methods to introduce inheritable, irreversible changes directly into the mammalian genome have been developed. Our laboratory has been using a method for directing site-specific, single base changes into dysfunctional and/or mutant genes [12]. With this method, short, single-stranded DNA oligonucleotides (ODNs) are introduced into a cell where they engage the processes of DNA repair and replication to catalyze specific base exchange [12]. The product of this activity is a corrected gene that encodes a normal protein. This approach avoids the problems of long-term drug treatment and transient expression systems which plague viral gene therapy, since a corrected base in the chromosome is permanent and inheritable. In addition, ODNs are known to exhibit low toxicity in humans and have been used extensively in clinical trials that encompass antisense and RNAi approaches. We used the framework of the studies from which potential drug therapies have been investigated to test our technology [11,13–15]. The outcomes measured in these studies were mRNA levels, total protein, and properly localized protein, but we took this one step further and measured DNA levels after base change as well. We chose to study SMA using the following strategy: a single base pair change in an existing gene (*SMN2*) would create a functional copy of the deleted gene and this gene would express normal mRNA and protein, leading to an inheritable and functional *SMN1* gene. In the present study, we delivered ODNs to cultured SMA patient cells lacking *SMN1* gene but containing wild-type *SMN2* gene. We were able to alter the genotype of *SMN2* and enhance the levels of full-length transcripts. As predicted, the genetic change resulted in the increased production of intact and properly localized SMN protein.

Materials and methods

Cell culture

Primary skin fibroblast cell line SP33 was obtained from an SMA type 1 patient and established at A.I. duPont Hospital for Children (Wilmington, DE). Cells were cultured in DMEM supplemented with 20% fetal bovine serum and 1% penicillin–streptomycin and incubated at 37 °C with 5% CO_2 at 95% humidity. No cells with a trypsinized culture number greater than 15 were used.

Transfections

Two hundred thousand cells were seeded into each well of a 6-well dish and incubated overnight. ODNs, 47-T, 47-NT, 75-T, 75-NT, targeted to either the transcribed (T) or non-transcribed (NT) strand of DNA were designed such that the center nucleotide directed the base change (Table 1). Three micrograms of ODN were mixed with jetPEI N/P=3 (Polyplus-transfection, Inc., San Marcos, CA) and the complex was added directly to the conditioned medium. The ODNs were allowed to incorporate for 6 h after which time the medium was replaced. The cells were harvested at 24 h for DNA and 24, 48, 72 h for RNA isolation.

Nucleic acid purification

Both DNA and RNA were isolated using kits from Qiagen (Valencia, CA) according to the manufacturer's instructions. In addition, total RNA was DNAse-treated during the isolation process as described by Qiagen. Concentrations were measured using Nanodrop spectrophotometer (Wilmington, DE) and only samples with ratios between 1.8 and 2.0 were used.

Reverse transcription with Sybr green PCR

One microgram of total RNA was reverse-transcribed at 42 °C for 50 min followed by 70 °C for 15 min using Superscript™

Table 1 – Targeted *SMN2* sequence and correcting oligonucleotides

Wild-type *SMN2*	*SMN1*-like
5′-GGGTTTTAGACAAAAT-3′	5′-GGGTTTCAGACAAAAT-3′
3′-CCCAAAATCTGTTTTA-5′	3′-CCCAAAGTCTGTTTTA-5′
Oligonucleotides used for correction experiments	
47-NT	5′cttCCTTCTTTTTGATTTTGTCTGAAACCCTGTAAGGAAAATAaagG3
47-T	5′cctTTATTTTCCTTACAGGGTTTCAGACAAAATCAAAAAGAAGgaaG3′
75-NT	5′aagAATGTGAGCACCTTCCTTCTTTTTGATTTTGTCTGAAACCCTGTAAGGAAAATAAAGGAAGTTAAAAAaaaT3′
75-T	5′attTTTTTTAACTTCCTTTATTTTCCTTACAGGGTTTCAGACAAAATCAAAAAGAAGGAAGGTGCTCACATtccT3′

The wild-type *SMN2* gene was targeted with ODNs that were either 47 or 75 bases in length and were specific for either the transcribed (T) or non-transcribed (NT) strand. The lower case bases in the ODN sequences designate phosphorothioate linkages that protect the ODN from nuclease degradation. The italicized base in the ODN is the base directing the targeting to the underlined base in the *SMN2* sequence which will be converted to the *SMN1*-like sequence shown above.

First Strand Synthesis System (Invitrogen, Carlsbad, CA). Two microliters of the cDNA were used as a template for separate PCR reactions with primers specific for full-length *SMN* and *β2M*, a housekeeping gene used as control for variations in the amount of template loaded to each reaction. The SMN primers, forward 5′-CCATATGTCCAGATTCTCTTGATGA-3′ and reverse 5′-ATGCCAGCATTTCTCCTTAATTTA-3′, were specific for full-length mRNA only because the reverse primer spanned the junction of exons 7/8. *β2M* primers — forward 5′-TTGTCTTTCAGCAAGGACTGGTC-3′ and reverse 5′-CATCTTCAAACCTCCATGATGC-3′, were designed using Primer Express software (Applied Biosystems, Foster City, CA). Real time PCR was performed using Applied Biosystem's 7300 Real Time PCR System under the following cycling conditions –95 °C denature followed by 55 °C annealing and extension. The cycle at which the amount of fluorescence was above the threshold (Ct) was detected and the relative amount of mRNA calculated using the RQ Software (Applied Biosystems).

Taqman® probe real time PCR

All primers were obtained from Sigma-Genosys (The Woodlands, TX) and all Taqman® probes were obtained from Applied Biosystems (Foster City, CA). Probes specific for either *SMN1* (6FAMCAGGGTTTCAGACAAAMGBNFQ) or *SMN2* (6FAMTGATTTTGTCTAAAACCCMGBNFQ) were used with the primer pair — forward 5′AATGCTTTTTAACATCCATATAAAGCT3′ and reverse 5′CCTTAATTTAAGGAATGTGAGCACC3′ which was the same for both reactions [16]. *E1α* (alpha-keto-acid dehydrogenase, housekeeping gene) gene-specific probe (6FAMCAGGAGATGCCCGCCCAGCTCTAMRA) was used with the primers — forward 5′CAACCTGCTCTTCTCAGACGTGTA3′and reverse 5′TCGAAGTGATCCAGTGGGTAGTG3′ and was assayed in separate reactions with 25 ng of the same DNA samples [17]. Real time PCR was performed using 25 ng DNA, 250 nM probe, and 900 nM both forward and reverse primers in each 25 μl reaction. All reactions were loaded into an optical plate in triplicate and run in the 7300 Real Time PCR System as recommended by Applied Biosystems. The data were collected and analyzed using the RQ software (Applied Biosystems).

Restriction enzyme digest

Cells were transfected as described above with the 47-NT ODN. Twenty-four hours later, the cells were trypsinized, counted using trypan blue exclusion, then diluted serially to a concentration of 1 cell per microliter. One cell (1 μl) was added to each well of a 96-well thermocycler plate. The cells were lysed and the DNA amplified in two rounds of PCR as described by Burlet et al. [18]. After the second round of amplification, a 10 μl PCR product was digested with *Dra*1 for 3 h, after which time the entire reaction was loaded onto a 4% agarose gel containing ethidium bromide and run at 100 V for 2 h. DNA bands were visualized under UV light using the AlphaImager™ (Alpha Innotech Corp., San Leandro, CA). The pattern in which the restriction enzyme cuts DNA is determined by the gene sequence present. If *SMN1* is present, the DNA is cut to produce bands of 203 bp and 33 bp in length. *SMN2* sequence DNA is cut into three bands — 178, 33, and 25 bp.

DNA sequencing

Genomic DNA was amplified using a two-round PCR assay as described above. After removing 10 μl for the restriction digest, the remaining product was purified using Qiagen's PCR Purification kit (Valencia, CA) and eluted in 30 μl water. Two microliters of the purified PCR product were run on a 4% agarose gel to confirm purity. DNA concentration was quantified using PicoGreen dsDNA Quantitation kit (Molecular Probes, Eugene, OR) and 30 μg of purified DNA combined with 2 μM primer (separate reactions for forward and reverse primers) were sent for cycle sequencing at the Molecular Biology Core Facility at the A.I. duPont Hospital for Children (Wilmington, DE).

Western blot assay

Cells, 4×10^5, were seeded onto a 60 mm^2 tissue culture dish and transfected the next day with 6 μg oligo complexed with jetPEI as described above. After transfection, plates were collected at 72 h, then again at 120 h post-transfection. To collect the cells, the plates were washed with cold phosphate-buffered saline (PBS) and collected by scrapping. Cell pellets were lysed on ice for 10 min in buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 100 mM NaF, 2.5 mM sodium pyrophosphate supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Pierce, Rockford, IL). The lysates were cleared by centrifugation at 13,500 rpm for 20 min at 4 °C. Protein concentration was measured by the BCA assay using BSA standards (Pierce, Rockford, IL). Ten micrograms of protein from the lysate was resolved on 12% SDS-PAGE and then transferred to nitrocellulose membrane. Membranes were probed with the human monoclonal antibodies anti-SMN (1:15,000, amino acids 14–174, BD Transduction Laboratories; BD Biosciences, San Jose, CA) and anti-α-tubulin (1:250, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with secondary HRP-conjugated antibody (Molecular Probes/Invitrogen) and proteins were detected using enhanced chemiluminescence reagents (Amersham, Piscataway, NJ).

Immunofluorescence and gem quantification

Cells were plated in 8-well chamber slides, allowed to grow overnight, and transfected with the ODN. Thirty-six hours post-transfection, the cells were washed with PBS and fixed for 20 min with 4% paraformaldehyde in PBS. After washing, the cells were permeabilized in 0.1% Triton X-100 in PBS for 5 min and blocked for 30 min in 10% normal goat serum (Zymed, South San Francisco, CA). Cells were then incubated with anti-SMN monoclonal antibody raised against amino acids 14–174 in 3% BSA for 1 h (1:500, BD Transduction Labroratories; BD Biosciences, San Jose, CA). Cells were washed four times with PBS–0.1% Triton X-100 and then incubated for 1 h with secondary antibody Alexa Fluor 555 (Molecular Probes/Invitrogen). After four more washes with PBS–Triton X-100, the slides were coverslipped with Prolong Gold mounting solution containing DAPI (Molecular Probes/Invitrogen). Slides were viewed and images captured using the

LSM 510 confocal microscope (Carl Zeiss Microimaging, Inc. Thornwood, NY). The number of gems was quantified using the exact protocol adopted by Andreassi et al. [19]. At least 100 cells, with fields of view selected randomly from each slide, were examined by two independent observers and the number of gems per hundred nuclei and percent of cells with gem-positive nuclei calculated as a function of treatment with ODN 75-T. Among the gem-positive nuclei, the number of gems in each nuclei was also scored for the treated and non-treated cells. As a positive control, the cell line 3814, heterozygous for *SMN1*, was used to provide a quantitative comparator which served to validate our protocol.

Statistical analysis

Differences in mRNA levels for each treatment group over time were determined by repeated measures ANOVA with Tukey's post hoc analysis using SPSS version 14 software (SPSS Inc., Chicago, IL). For the gem quantification, differences between the untreated and 75-T-treated cells were determined using Student's *t* test; Excel version 10.0 was used to perform all the Student's *t* tests. Significance was assigned when the *p* value was less than or equal to 0.05.

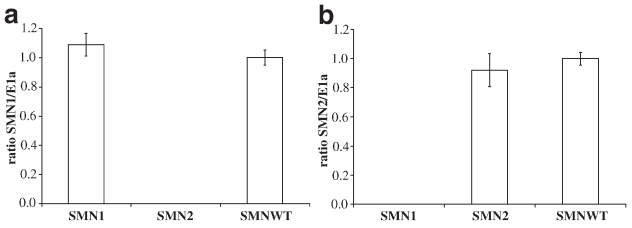


Fig. 1 – *Validation of Taqman® probe real time PCR assay*. SMN1 DNA contains no copies of *SMN2* gene, SMN2 DNA contains no copies of *SMN1* gene, and SMNWT DNA contains both *SMN1* and *SMN2* genes. Real time PCR was performed using 25 ng genomic DNA with Taqman® MGB probes specific for *SMN1* (a) and *SMN2* (b) and *E1α*, a housekeeping gene used to control for the amount of template added to each reaction. The amount of each product was determined from their respective standard curves and the bars in each graph represent the ratio of *SMN1* or *SMN2* to *E1α*. There was no fluorescence measured in those reactions where the *SMN* gene was missing, while all the reactions contained similar amounts of *E1α*. This implies that the lack of signal was due to the absence of the *SMN* gene measured with that specific probe.

Results

Validation of Taqman® probe real time PCR assays

An important primary task in assessing the efficiency of gene repair reactions at the DNA level is to validate the assay used to measure nucleotide exchange. We chose a well-established probe-based, quantitative real time PCR (qPCR) assay to identify the presence of genetically altered alleles. This type of assay is more sensitive and specific than sybr green PCR or semi-quantitative PCR because it allows for detection of minute levels of DNA. It can also distinguish between the unaltered and corrected genes with distinct clarity. The design of the experiment is based on using the ODNs depicted in Table 1 to direct gene editing in the SMA patient-derived cell line. In these cells, the *SMN1* gene is deleted, leaving an intact *SMN2* gene(s) which produces low levels of full-length mRNA and little functional protein (Fig. 1) [4,6]. Oligonucleotides of either 47 or 75 bases in length, which have been used previously in other systems [12], were designed to create a T/G mismatch with the *SMN2* at nucleotide position +6 of exon 7. The mismatched base pair will act as a signal for genetic repair enabling the exchange of the T residue with a C. The second repair event at the A/C mismatch converts the A to a G resulting in the establishment of a converted C–G pair at the target site. Since the protocol involves detection of a single nucleotide exchange, we sought to validate the assay by first measuring the specificity of our protocol which leads to an assurance that SMN2 or SMN1 DNA can be appropriately detected.

Test for specificity

Genomic DNA had been genotyped previously and donated by the Molecular Diagnostics Laboratory at A.I. duPont Hospital for Children (Wilmington, DE). In our control samples, "SMN1 DNA" contains no copies of the *SMN2* gene; "SMN2 DNA" contains no copies of *SMN1* gene; and "SMNWT DNA" contains DNA from both *SMN1* and *SMN2* genes. qPCR was performed using equal amounts of genomic DNA and primers/probes specific for *SMN1*, *SMN2*, and *E1α* in three separate reactions. In our experiments, *E1α*, alpha-keto-acid dehydrogenase gene, was used as a control to ensure that equal amounts of starting DNA were loaded into each reaction. As a result, *SMN1* was detected in only those reactions containing DNA known to have the *SMN1* gene (SMN1 DNA and SMAWT, Fig. 1a). Likewise, the assay produced a signal with SMN2 DNA and SMAWT (Fig. 1b). Notably, we do not detect any *SMN2* in the reaction where SMN1 DNA was the template, a sample previously determined to not have any copies of the *SMN2* gene. Similar levels of *E1α* were detected in the samples assayed above; suggesting that the lack of signal is due to the absence of this template, confirming that this probe-based PCR assay is able to efficiently distinguish between the two *SMN* genes. Beyond specificity, these results eliminate concerns of artifactual results emanating from contaminated samples.

Characterization of SMA skin fibroblast cell line

After validating the qPCR conditions, we used the Taqman® probe assay to confirm that intact *SMN1* was not present in SP33 and to quantitate the number of *SMN2* gene copies in this cell line. These cells were obtained from an SMA Type 1 patient, implying that the cells should have 1 to 2 copies of *SMN2* because *SMN2* copy number correlates to disease severity [7]. The amounts of each *SMN* gene were determined from their respective standard curves and related to the amount of *E1α*, also calculated from a separate standard curve (data not shown). Because *E1α* is a two-copy gene, a ratio of 1

Table 2 – Fold change in mRNA levels after correction with various length ODNs

Condition	24 h	48 h	72 h
No ODN	1.0±0.14	1.0±0.14	1.0±0.11
47-NT*	0.8±0.13	2.3±0.05	3.2±0.09
75-NT*	1.2±0.14	2.1±0.07	2.8±0.03
47-T	1.4±0.08	0.4±0.06	0.8±0.1
75-T**	5.8±0.12	1.7±0.17	3.4±0.13

SP33 cells were transfected with various length ODNs and allowed to express for 24, 48, 72 h. The total RNA was isolated every 24 h, then reverse-transcribed using the Superscript First Strand Synthesis kit from Invitrogen. cDNA was amplified using primers specific for full-length SMN mRNA and amount of PCR product quantified with sybr green. The amount of full-length SMN mRNA in the treated groups was calculated using the $2^{-\Delta\Delta Ct}$ method and the no ODN sample as the calibrator (RQ Software, Applied Biosystems). Each sample was assayed in triplicate and the average relative quantity plus/minus standard deviation was reported for each time point. Significance determined for each treatment over time using repeated measures ANOVA with Tukey's post hoc analysis (*$p<0.05$ as compared to no ODN; **$p<0.05$ as compared to all samples).

means that there were two copies of *SMN* present, where as a ratio of 0.5 would indicate that there is only one copy of *SMN* present. We confirmed that this cell line had no detectable levels of intact *SMN1* (up to 100 ng genomic DNA analyzed) and that only one copy of the *SMN2* gene is present.

Gene repair at the SMN2 locus in fibroblasts obtained from SMA patients

After validating the assay system, we proceeded to design experiments aimed at evaluating the gene repair activity of the 47- and 75-mers. As described above, these molecules align with perfect complementarity to the target site except for a single mismatch that is created between the target base in the gene and the center nucleotide of the ODN. Transfection efficiencies were optimized by evaluating a number of reagents complexed with an ODN bearing a fluorescent tag as a tracer. jetPEI generated a transfection efficiency of approximately 30% in fibroblasts; this combination of a polycation and the ODN was found to produce the highest levels of transfection with the lowest coincident toxicity (data not shown). To begin the gene repair reaction, SP33 cells were transfected with the jetPEI–ODN complex for 24 h with two reaction parameters — length and strand bias, evaluated simultaneously. Previous studies suggested that the length of the targeting ODN is an important parameter in maximizing correction in certain cell types [20]. In a similar fashion, strand bias is also a factor worth considering since targeting the transcribed or non-transcribed strand can also influence the level of correction [21,22]. The cells were harvested, DNA was isolated and 25 ng of genomic DNA was tested for the presence of *SMN1* using Taqman® probes in qPCR designed to distinguish between two alleles (allelic discrimination assay). Detectable SMN1 DNA would indicate that some percentage of *SMN2* genes had been converted to *SMN1*. In short, qPCR reactions were performed with the *SMN1* Taqman probe validated above and shown to be sequence-specific. Any reaction in which fluorescence above the background threshold is detected (Ct value above zero) would be scored as positive for a C at exon 7 nucleotide position +6. A reaction which does not produce a fluorescent signal would be scored as negative for the presence of the C base. Importantly, each ODN transfection was performed three times and in triplicate. To ensure that a lack of signal was not due to the absence of template, *E1α* was always run as a loading control. All DNA samples assayed produced signals with *E1α*-specific primers and Taqman® probes, even though the mock transfected SP33 cells had no detectable signal with *SMN1* primers and probes (data not shown). On the other hand, the cells treated with the appropriate targeting ODN contained *SMN1*-positive DNA. The

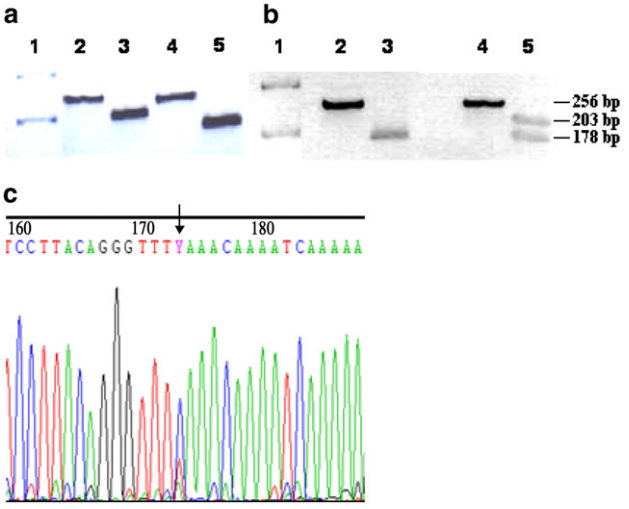


Fig. 2 – *Restriction digest and cycle sequencing confirm base conversion in SMN2*. DNA was amplified using a nested PCR strategy described by Burlet et al. [18]. For (a) and (b), digested and undigested PCR products were run on a 4% agarose gel. The predicted band sizes were: 256 bp for uncut DNA, 203 bp for cut *SMN1*, and 178 bp for cut *SMN2*. (a) SMN1 DNA and SMN2 DNA were used as positive controls for the digests. Lane 1 — 100 bp marker; Lane 2 — undigested SMN1 DNA; Lane 3 — digested SMN1 DNA; Lane 4 — undigested SMN2 DNA; Lane 5 — digested SMN2 DNA. All of the control reactions produced the correct band sizes before and after digestion. Also, one band is present in the digested DNA lanes, indicating that only one *SMN* gene is present in these samples, as determined by qPCR (see Fig. 1a and b). (b) DNA from SP33 cells before and after correction with the 47-NT ODN. Lane 1 — 100 bp marker; Lane 2 — mock transfected SP33; Lane 3 — digested, mock transfected SP33; Lane 4 — SP33+47-NT; Lane 5 — digested SP33+47-NT. As shown in Lane 5b, both the *SMN1* and *SMN2* genes were present in this sample after correction, but only one band of 178 bp, *SMN2*, was present before transfection. (c) The remaining PCR product from the SP33 cells transfected with the 47-NT ODN was purified and the DNA was sequenced. The results of the sequencing reaction using the forward primer from the second round of amplification are shown in the chromatogram. The arrow points to a double peak under the "Y" which means that both a T and C have been detected at that spot. This implies that this sample is heterozygous for *SMN1* and *SMN2*.

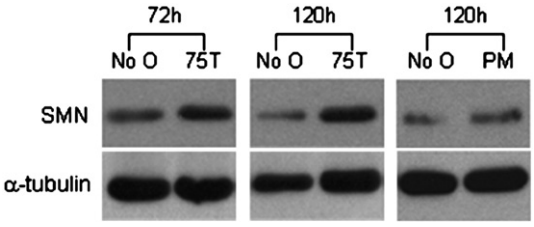


Fig. 3 – *SMN protein levels are increased after correction.* A representative western blot shows that SMN protein levels were increased in the 75-T treated cells as compared to the untreated (no ODN) or cells treated with a perfectly matched (PM) ODN. Ten micrograms of total protein were resolved on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, and stained with human monoclonal antibodies for SMN amino acids 14–174 (38 kD, BD Transduction) and α-tubulin (55 kD, Santa Cruz). SMN protein levels were quantified by densitometric analysis and normalized to α-tubulin. At 120 h, the amount of SMN protein detected in the 75-T treated cells was 2-fold higher than the untreated, while there was no difference in amounts detected between no ODN and PM (0.98 fold).

NT variants — both 47 and 75, produced modest, significant increases in full-length SMN mRNA over the 72 h studied ($p<0.05$), while the 47-T leads to a slight decrease. The 75-T oligo produced the highest significant increases in full-length mRNA over time as determined using repeated measures ANOVA with Tukey's post hoc analysis ($p<0.5$) as compared to all other ODNs. This increase in mRNA levels ranged from 3.4-fold to 5.8-fold in a reproducible and robust fashion (Table 2). When the latter experiment was extended to 144 h (over 7 doublings in this cell line), the elevation in full-length mRNA levels persisted in the 75-T-treated samples −2.4 ± 0.08 fold ($p<0.01$, Student's *t* test). Thus, targeting either the non-transcribed or transcribed strands to direct gene repair was successful in the SP33 cells.

The genomic DNA from single-cell dilutions was analyzed by restriction enzyme digestion and DNA cycle sequencing. In short, the genomic DNA from each cell was amplified using a nested PCR strategy designed by Burlet et al. [18] with both sets of primers surrounding the targeted region for correction. Two microliters of the PCR product were run on a 4% agarose gel and visualized with ethidium bromide to ensure the correct size product and product purity. Ten microliters of the PCR-amplified DNA were added to the restriction enzyme digestion while the remainder was cleaned using Qiagen's PCR Purification kit (Valencia, CA). SMN1 DNA and SMN2 DNA genomic DNA samples were used as controls in the restriction enzyme digestion because their genotype is known and the resulting band pattern after digestion would be predictable. As shown in Fig. 2a, the control samples produced the predicted band sizes. For instance, SMN1 DNA and SMN2 DNA produced single

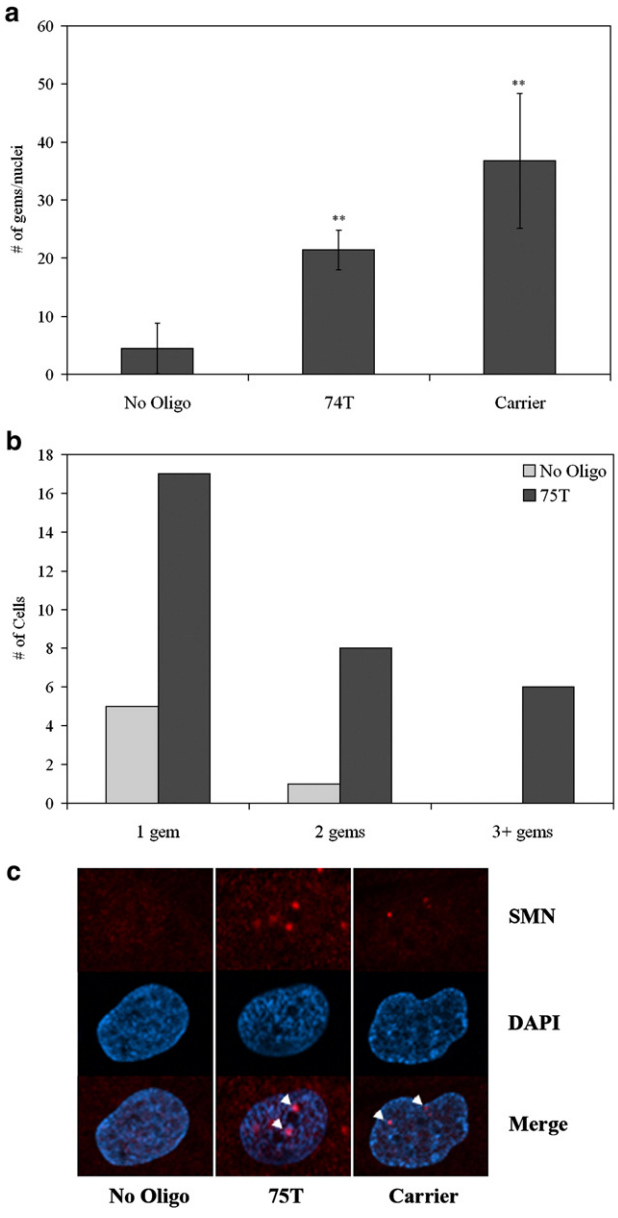


Fig. 4 – *Gem quantitation.* Cells were stained with a human SMN monoclonal antibody raised against amino acids 14–174 (BD Transduction) and DAPI nuclear stain to identify nuclear gems. Slides were viewed and images captured with a LSM 510 confocal microscope (Carl Zeiss, Inc.). Aminimum of 100 cells were analyzed for both the untreated and treated SP33 cells. (a) The number of nuclear gems and total number of nuclei were counted in randomly chosen fields on each slide by two independent observers. The number of gems per 100 nuclei was calculated and determined to be significantly higher in the 75-T treated and carrier cells (3814) when compared to no ODN ($p\leq0.01$). (b) Among the gem-positive nuclei, the number of gems in each cell was also scored. The number of oligo-treated cells containing 2 gems was significantly higher than the untreated cells (** $p\leq0.01$) and none of the "no ODN" cells had more than two gems. (c) Representative images of the gem staining in the SP33 cells treated with no ODN and 75-T treated and carrier cells. Upper panels show SMN in red (Alexa fluor 555) and the middle panels show nuclei stained in blue (DAPI). Arrows are pointing to gems which are purple in the merged images (bottom panels). Gems in the non-treated cells were rare, where as they occurred more frequently in the oligo-treated cells.**

bands of 203 bp and 178 bp, respectively, indicating the presence of either *SMN1* or *SMN2*. On the other hand, DNA from SP33 cells corrected with the ODN produced two distinct bands after digestion with *Dra*1, verifying that both "genes" are present, while the DNA from the mock transfected SP33 cells generated only the *SMN2*-specific band (Fig. 2b). We demonstrated that this cell line had no *SMN1* prior to exposure to the ODN, implying that this new gene is a result of base exchange. DNA samples from corrected SP33 cells were sequenced to confirm the presence of the C at nucleotide +6 in exon 7. Thirty nanograms of purified DNA combined with 2 μM primer (both forward and reverse used in separate reactions) were sent to the Molecular Biology Core Facility at the A.I. duPont Hospital for Children (Wilmington, DE) for cycle sequencing. As shown in Fig. 2c, the base conversion occurred at the targeted site and is designated with a "Y" in the chromatogram, denoting the presence of both a T and C at the same position indicating that a heterozygous state has been created. This was true for all samples that showed the base change (3 out of 96).

The DNA and mRNA findings predicted that increased SMN protein levels should be present in treated cells. We monitored SMN protein production by western blotting and proper protein localization by the increased presence of gems. It is important to note that the targeted cells do contain SMN protein and thus we are looking for an increase only. After transfection with the 75-T, the ODN which produced significantly higher levels of full-length mRNA, cells were harvested at 72 and 120 h for protein isolation. As shown in Fig. 3, SMN protein levels were increased 2-fold in the 75-T treated cells as compared to the untreated samples, while protein levels were unchanged with a perfectly matched oligo (0.98 fold). Coincidently, the number of gems per 100 nuclei was significantly increased in the cells transfected with 75-T compared to the no ODN group, 20.9 versus 5.2 ($p<0.01$, Fig. 4a). In addition, the number of cells containing gems was significantly increased from 4.5% to 12.7% after the correction experiment ($p<0.02$), as was the number of cells with 2 gems and 3 or more gems (Fig. 4b). A carrier cell line, 3814, had 36.5 gems/100 nuclei under our staining conditions, which was significantly higher than the SP33 cells alone ($p<0.01$). This was expected since gem number correlates to disease phenotype [23], the SP33 cells had an 86% reduction in the number of gems when compared to the carrier line 3814. Together, these data show that the amount of properly localized SMN protein was increased after treatment with a sequence-specific oligonucleotide and that the protein from the converted *SMN2* gene was functional in the assembly of gems.

Discussion

This is the first study to show that single-stranded ODNs can be used to convert an *SMN2* gene to an *SMN1*-like gene by changing the T to a C at nucleotide position +6 in exon 7. We confirmed this change by several methods, including direct sequence analyses, and showed that this change leads to higher levels of full-length SMN mRNA. It was however imperative to insure that this genetic change was manifest at the level of functional protein. Hence, we analyzed a cell population that contained corrected genes (SMN1 sequence) for the presence of functional SMN protein. Improvements in both protein production and functionality were observed. The oligonucleotide-based approach led to gene conversion at both the genotypic and phenotypic levels, an important consideration in the development of gene repair for SMA.

Other workers employed a similar technique: the use of DNA fragments of the *SMN1* gene to induce short fragment homologous recombination (SFHR) in *SMN1*-deleted chorionic villi cells [13]. In that study, Sangiuolo et al. were able to increase the level of full-length mRNA and showed that this change was inheritable over several passages. We also investigated this aspect of our approach by maintaining cells "corrected" with the 75-T in culture over many doublings. The significantly higher levels in full-length SMN mRNA reported at 72 h (Table 2) persisted over 7 days. We had to look at doublings as opposed to passages due to the limited capacity for primary cells to be expanded in culture without selection, in contrast to the fetal cell system described by Sangiuolo et al. This group used fetal cells which may be more amenable than adult cells to genetic manipulations in general. For SMA, however, fetal cells, specifically chorionic villi, are not applicable as a target for therapy since the target cells must be somatic in nature under the current regulations. Alternatively, transient episomal expression strategies that aim to introduce expression constructs retaining *SMN1* cDNA in SMA cells and animal models have also been utilized [10,11]. While these studies produced increased amounts of full-length mRNA *in vitro* and *in vivo*, the half-life of this effect is predictably short lived. In contrast to the latter treatments, however, our approach has the potential to provide long-lasting effects by altering the DNA and creating an inheritable functional gene [24–26]. Based on these studies, we do not anticipate that the change we have created in the *SMN2* gene would be lost over time because the change remains in the context of the gene's native environment. To be sure, studies are underway to test the persistence of the base exchange in our model system.

The work described in this manuscript suggests that gene repair for SMA could be a viable treatment option because the improvement in functional protein, as seen in our study, is in line with other reports describing drugs that are currently being evaluated as potential treatments for SMA [15,17,19,27,28]. Novel therapeutic approaches for SMA include drugs that lead to an increase in SMN full-length mRNA and functional protein [8–10,17,19,28,29]. While these drugs have been shown to produce modest improvements in SMN protein levels *in vitro*, such therapies would have to be administered indefinitely. It is likely that certain side effects of some of these drugs will hamper development and limit compliance.

Based on the results reported here, further studies are underway to test gene repair in SMA type 2 and type 3 cell lines, as well as, in an animal model. Preliminary data confirm that *SMN2* to *SMN1* conversion takes place in another patient cell line, suggesting a more universal application of this technique (S. Callahan, personal communication). Also to be evaluated are delivery methodology and timing of ODN administration. It is not expected that the ODN treatment we described would recover motor neurons or muscle cells that are already lost due to atrophy, but increased SMN protein

levels are expected to delay disease progression and improve muscle function as seen in other studies [30–32]. Whether direct muscle injection of the ODN, similar to what is being used in muscular dystrophy research [26,33], would be beneficial or whether central administration is required remains to be tested in animal models of SMA. These model systems will also allow us to evaluate the timing of the delivery. It is believed that earlier intervention would lead to better the outcomes [34], although Avila et al. [30] showed that treatment after disease onset lead to improvements in disease severity. The therapy that we describe herein, however, would enable long-lasting genomic changes, a distinct advantage for genomic reversal. As such, it will have the potential to improve SMN protein status which may delay the muscle atrophy associated with the SMA phenotype.

Acknowledgments

We thank Dr. Vicky Funanage and Dr. Wenlan Wang from the A.I. duPont Hospital for Children (Wilmington, Delaware) for the SP33 cell line, the SMN1 DNA, SMN2 DNA, SMAWT DNA genomic DNA samples. We are grateful to Hetal Parekh-Olmedo, Julia Engstrom, Katie Maguire and other members of the Kmiec laboratory for their critical evaluation of the work which was supported by a grant from the Delaware Department of Health and Human Resources (Tobacco Settlement Fund).

REFERENCES

[1] S. Lefebvre, L. Burglen, S. Reboullet, O. Clermont, P. Burlet, L. Viollet, B. Benichou, C. Cruaud, P. Millasseau, M. Zeviani, Identification and characterization of a spinal muscular atrophy-determining gene, Cell 80 (1995) 155–165.
[2] C.L. Lorson, E. Hahnen, E.J. Androphy, B. Wirth, A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 6307–6311.
[3] T. Kashima, J.L. Manley, A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy, Nat. Genet. 34 (2003) 460–463.
[4] U.R. Monani, D.D. Coovert, A.H. Burghes, Animal models of spinal muscular atrophy, Hum. Mol. Genet. 9 (2000) 2451–2457.
[5] L. Cartegni, A.R. Krainer, Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1, Nat. Genet. 30 (2002) 377–384.
[6] C.L. Lorson, E.J. Androphy, An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN, Hum. Mol. Genet. 9 (2000) 259–265.
[7] M. Feldkotter, V. Schwarzer, R. Wirth, T.F. Wienker, B. Wirth, Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy, Am. J. Hum. Genet. 70 (2002) 358–368.
[8] C. Brahe, T. Vitali, F.D. Tiziano, C. Angelozzi, A.M. Pinto, F. Borgo, U. Moscato, E. Bertini, E. Mercuri, G. Neri, Phenylbutyrate increases SMN gene expression in spinal muscular atrophy patients, Eur. J. Hum. Genet. 13 (2005) 256–259.
[9] L. Brichta, I. Holker, K. Haug, T. Klockgether, B. Wirth, In vivo activation of SMN in spinal muscular atrophy carriers and patients treated with valproate, Ann. Neurol. 59 (2006) 970–975.
[10] C.J. DiDonato, R.J. Parks, R. Kothary, Development of a gene therapy strategy for the restoration of survival motor neuron protein expression: implications for spinal muscular atrophy therapy, Hum. Gene Ther. 14 (2003) 179–188.
[11] M. Azzouz, T. Le, G.S. Ralph, L. Walmsley, U.R. Monani, D.C. Lee, F. Wilkes, K.A. Mitrophanous, S.M. Kingsman, A.H. Burghes, N.D. Mazarakis, Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy, J. Clin. Invest. 114 (2004) 1726–1731.
[12] H. Parekh-Olmedo, L. Ferrara, E. Brachman, E.B. Kmiec, Gene therapy progress and prospects: targeted gene repair, Gene Ther. 12 (2005) 639–646.
[13] F. Sangiuolo, A. Filareto, P. Spitalieri, M.L. Scaldaferri, R. Mango, E. Bruscia, G. Citro, E. Brunetti, M. De Felici, G. Novelli, In vitro restoration of functional SMN protein in human trophoblast cells affected by spinal muscular atrophy by small fragment homologous replacement, Hum. Gene Ther. 16 (2005) 869–880.
[14] T. Baughan, M. Shababi, T.H. Coady, A.M. Dickson, G.E. Tullis, C.L. Lorson, Stimulating full-length SMN2 expression by delivering bifunctional RNAs via a viral vector, Mol. Ther. 14 (2006) 54–62.
[15] Y. Hua, T.A. Vickers, B.F. Baker, C.F. Bennett, A.R. Krainer, Enhancement of SMN2 exon 7 inclusion by antisense oligonucleotides targeting the exon, PLoS Biol. 5 (2007) e73.
[16] D. Anhuf, T. Eggermann, S. Rudnik-Schoneborn, K. Zerres, Determination of SMN1 and SMN2 copy number using TaqMan technology, Human Mutat. 22 (2003) 74–78.
[17] S.M. Grzeschik, M. Ganta, T.W. Prior, W.D. Heavlin, C.H. Wang, Hydroxyurea enhances SMN2 gene expression in spinal muscular atrophy cells, Ann. Neurol. 58 (2005) 194–202.
[18] P. Burlet, N. Frydman, N. Gigarel, J.P. Bonnefont, V. Kerbrat, G. Tachdjian, R. Frydman, A. Munnich, J. Steffann, P.F. Ray, Improved single-cell protocol for preimplantation genetic diagnosis of spinal muscular atrophy, Fertil. Steril. 84 (2005) 734–739.
[19] C. Andreassi, C. Angelozzi, F.D. Tiziano, T. Vitali, E. De Vincenzi, A. Boninsegna, M. Villanova, E. Bertini, A. Pini, G. Neri, C. Brahe, Phenylbutyrate increases SMN expression in vitro: relevance for treatment of spinal muscular atrophy, Eur. J. Hum. Genet. 12 (2004) 59–65.
[20] L. Liu, M.C. Rice, E.B. Kmiec, In vivo gene repair of point and frameshift mutations directed by chimeric RNA/DNA oligonucleotides and modified single-stranded oligonucleotides, Nucleic Acids Res. 29 (2001) 4238–4250.
[21] L. Liu, M.C. Rice, M. Drury, S. Cheng, H. Gamper, E.B. Kmiec, Strand bias in targeted gene repair is influenced by transcriptional activity, Mol. Cell. Biol. 22 (2002) 3852–3863.
[22] E.E. Brachman, E.B. Kmiec, DNA replication and transcription direct a DNA strand bias in the process of targeted gene repair in mammalian cells, J. Cell Sci. 117 (2004) 3867–3874.
[23] D.D. Coovert, T.T. Le, P.E. McAndrew, J. Strasswimmer, T.O. Crawford, J.R. Mendell, S.E. Coulson, E.J. Androphy, T.W. Prior, A.H. Burghes, The survival motor neuron protein in spinal muscular atrophy, Hum. Mol. Genet. 6 (1997) 1205–1214.
[24] Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen, E.B. Kmiec, Reaction parameters of targeted gene repair in Mammalian cells, Mol. Biotechnol. 29 (2005) 197–210.
[25] M.D. Drury, M.J. Skogen, E.B. Kmiec, A tolerance of DNA heterology in the mammalian targeted gene repair reaction, Oligonucleotides 15 (2005) 155–171.
[26] C. Bertoni, G.E. Morris, T.A. Rando, Strand bias in oligonucleotide-mediated dystrophin gene editing, Hum. Mol. Genet. 14 (2005) 221–233.
[27] J. Jarecki, X. Chen, A. Bernardino, D.D. Coovert, M. Whitney, A. Burghes, J. Stack, B.A. Pollok, Diverse small-molecule

modulators of SMN expression found by high-throughput compound screening: early leads towards a therapeutic for spinal muscular atrophy, Hum. Mol. Genet. 14 (2005) 2003–2018.
[28] C.J. Sumner, T.N. Huynh, J.A. Markowitz, J.S. Perhac, B. Hill, D.D. Coovert, K. Schussler, X. Chen, J. Jarecki, A.H. Burghes, J.P. Taylor, K.H. Fischbeck, Valproic acid increases SMN levels in spinal muscular atrophy patient cells Ann. Neurol. 54 (2003) 647–654.
[29] V.B. Mattis, R. Rai, J. Wang, C.W. Chang, T. Coady, C.L. Lorson, Novel aminoglycosides increase SMN levels in spinal muscular atrophy fibroblasts, Hum. Genet. 120 (2006) 589–601.
[30] A.M. Avila, B.G. Burnett, A.A. Taye, F. Gabanella, M.A. Knight, P. Hartenstein, Z. Cizman, N.A. Di Prospero, L. Pellizzoni, K.H. Fischbeck, C.J. Sumner, Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy, J. Clin. Invest. 117 (2007) 659–671.
[31] J.G. Chang, H.M. Hsieh-Li, Y.J. Jong, N.M. Wang, C.H. Tsai, H. Li, Treatment of spinal muscular atrophy by sodium butyrate, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 9808–9813.
[32] L.K. Tsai, M.S. Tsai, T.B. Lin, W.L. Hwu, H. Li, Establishing a standardized therapeutic testing protocol for spinal muscular atrophy, Neurobiol. Dis. 24 (2006) 286–295.
[33] R.J. Bartlett, S. Stockinger, M.M. Denis, W.T. Bartlett, L. Inverardi, T.T. Le, M.N. thi, G.E. Morris, D.J. Bogan, J. Metcalf-Bogan, J.N. Kornegay, In vivo targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide, Nat. Biotechnol. 18 (2000) 615–622.
[34] T. Federici, N.M. Boulis, Gene-based treatment of motor neuron diseases, Muscle Nerve 33 (2006) 302–323.