Antisense Oligonucleotide Induction of Progerin in Human Myogenic Cells

Yue-Bei Luo1,2, Chalermchai Mitrpant1,3, Abbie M. Adams1,4, Russell D. Johnsen1,4, Sue Fletcher1,4, Frank L. Mastaglia1,5, Steve D. Wilton1,4,*

1 Centre for Neuromuscular and Neurological Disorders, Australian Neuro-Muscular Research Institute, University of Western Australia, Perth, Australia, 2 Department of Neurology, Xiangya Hospital, Central South University, Changsha, China, 3 Department of Biochemistry, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, 4 Centre for Comparative Genomics, Murdoch University, Perth, Australia, 5 Institute for Immunology & Infectious Diseases, Murdoch University, Perth, Australia

Abstract

We sought to use splice-switching antisense oligonucleotides to produce a model of accelerated ageing by enhancing expression of progerin, translated from a mis-spliced lamin A gene (LMNA) transcript in human myogenic cells. The progerin transcript (LMNA Δ150) lacks the last 150 bases of exon 11, and is translated into a truncated protein associated with the severe premature ageing disease, Hutchinson-Gilford progeria syndrome (HGPS). HGPS arises from de novo mutations that activate a cryptic splice site in exon 11 of LMNA and result in progerin accumulation in tissues of mesodermal origin. Progerin has also been proposed to play a role in the ‘natural’ ageing process in tissues. We sought to test this hypothesis by producing a model of accelerated muscle ageing in human myogenic cells. A panel of splice-switching antisense oligonucleotides were designed to anneal across exon 11 of the LMNA pre-mRNA, and these compounds were transfected into primary human myogenic cells. RT-PCR showed that the majority of oligonucleotides were able to modify transcript processing. Oligonucleotides that annealed within the 150 base region of exon 11 that is missing in the progerin transcript, as well as those that targeted the normal exon 11 donor site induced the LMNA Δ150 transcript processing. Oligonucleotides that annealed within the 150 base region of exon 11 that is missing in the progerin transcript, as well as those that targeted the normal exon 11 donor site induced the LMNA Δ150 transcript processing, but most oligonucleotides also generated variable levels of LMNA transcript missing the entire exon 11. Upon evaluation of different oligomer chemistries, the morpholino phosphorodiamidate oligonucleotides were found to be more efficient than the equivalent sequences prepared as oligonucleotides with 2′-O-methyl modified bases on a phosphorothioate backbone. The morpholino oligonucleotides induced nuclear localised progerin, demonstrated by immunostaining, and morphological nuclear changes typical of HGPS cells. We show that it is possible to induce progerin expression in myogenic cells using splice-switching oligonucleotides to redirect splicing of LMNA. This may offer a model to investigate the role of progerin in premature muscle ageing.

Introduction

Hutchinson-Gilford progeria syndrome (HGPS) is a rare premature ageing disease caused by mutations in LMNA that activate a cryptic splice site in exon 11 [1]. Induction of this inappropriate alternative splicing leads to the loss of 150 bases from the end of exon 11, and results in the translation of a truncated protein isoform, progerin. Compared with the normal translation product prelamin A, progerin lacks an endoproteolytic site and retains a farnesylation site on its carboxyl terminal. How progerin overexpression causes premature ageing is still uncertain. Accumulation of the permanently farnesylated progerin in the nuclear membrane results in abnormalities of nuclear shape, genome instability, and downstream activation of Notch and p53 pathways [2,3]. Trace amounts of progerin have also been observed in several normal human tissues, although its biological significance and role in normal ageing remain to be determined [3–5].

Antisense oligonucleotides (AOs) can be designed to anneal to RNA by Watson-Crick hybridisation, and depending upon the base modifications and backbone chemistry, may exert their effects on gene expression through different mechanisms. An early application of AOs was to suppress expression of target gene and this was commonly achieved by recruitment of RNase H to degrade mRNA of a RNA:DNA oligonucleotide hybrid [6,7]. AOs can also be used to redirect pre-mRNA processing [8,9]. Since at least 74% of gene transcripts are alternatively spliced, splice-switching strategies could be broadly applicable to many different conditions [10]. Furthermore, it is estimated that 10-15% of pathogenic mutations affect gene splicing, although this number is now considered to be an underestimate [11,12].

AO induced exon skipping, exon retention and abrogation of the usage of alternative splice sites have been reported to by-pass or suppress pathogenic mutations in Duchenne muscular dystrophy, spinal muscular atrophy and thalassemia, respectively [13–15]. Splice-switching AOs were able to mask abnormal splice sites in β-globin introns and force the aberrant splicing to default back...
AO-Mediated Progerin Induction in Myogenic Cells

Antisense oligonucleotides

2'-O-methyl modified bases on a phosphorothioate backbone (2OMe AOs) were synthesised in-house on an Expedite 8909 Nucleic Acid Synthesiser (Applied Biosystems, Framingham, MA) using the 1 μmol thioate synthesis protocol. Phosphorodiamidate morpholino oligonucleotides (PMOs) were obtained from Gene-Logic (Philmorn, OR).

Nomenclature of AOs adopted the method described by Mann et al. [27]: species ('h' for homo sapiens), exon number, acceptor (A)/donor (D) site, coordinate (+ for exon, − for intron).

Tissue samples

Surplus material from de-identified vastus lateralis muscle biopsies, obtained from individuals undergoing screening for malignant hyperthermia (MH) was provided by the Department of Pathology, Royal Perth Hospital, with informed consent. These individuals were found to be MH-negative based upon in vitro contracture testing, and had normal muscle histology. Additional muscle tissues and skin tissues from healthy individuals were obtained after informed consent and stored at −80°C. All procedures were approved by the Royal Perth Human Ethics Committee (reference number: 2006-073).

Cell culture and AO transfection

Primary human myogenic cells were prepared and differentiated as described previously [28]. Human cells were transfected with 2OMe AOs complexed with Lipofectamine 2000 (Invitrogen, Melbourne, Australia) and 2OMe at 1:1 (w:w) ratio.

Human myogenic cells were transfected with PMOs using the Amaxa Nucleofector electroporation system (Lonza, Basel, Switzerland) with P3 primary cell 4D-Nucleofector X kit and pulsed with the programme CM-138 according to the manufacturer’s instructions.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from cells 48 hr (2OMe AOs) or 72 hr (PMOs) after transfection using Trizol (Invitrogen) according to manufacturer’s instructions. One-step RT-PCR was undertaken essentially as described previously [26]. Briefly, samples were incubated at 75°C for 30 minutes to reverse transcription step, followed by 3 minutes incubation at 94°C to denature the templates, followed by 30 cycles of PCR (denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes). Amplification primers were: LAf (exon 9/10 junction), 5'-ATCACTCCACTGGGGAAGAAGT-3', LAr (exon 12) 5'-ATGTGGAGTTTCCTGGAAGCAG-3', LCl (exon 6), 5'-GAGCCTGAGATGCGAGAT-3', LCr (exon 10) 5'-TCAGCCGGCGCCACCCTCA-3'. Amplification products were separated on 2% agarose gel and images captured using a Chemi-smart 3000 system (Vilber Lourmat, Marne-la-Vallée, France). The identity of the PCR amplicons were confirmed by direct DNA sequencing.

Western blotting

Three hundred and sixty thousand human myogenic cells were seeded into T25 flasks and incubated for 48 hr before transfection with AOs as described. Forty-eight hr after transfection, cells were harvested from the wells and centrifuged at 14,000 rcf for 3 minutes to collect the cell pellets. Approximately 4.5 mg of cells were lysed with 100 μl of 125 mM Tris-HCl (pH 6.8), 15% SDS (w:v), 10% glycerol (v:v), 0.5 mM phenylmethylsulfonyl fluoride and 9 μl protease inhibitor cocktail (Sigma Aldrich, Sydney, Australia). Western blots were carried out essentially as described by Cooper [29]. Briefly, 4 μl aliquots of protein extract were separated on NuPAGE 4–12% Bis-Tris gels (Life Technologies, Mulgrave, Australia) and stained with 0.2% Coomassie blue and destained with 0.7% acetic acid. Gel densitometry was used to estimate relative myosin expression to ensure equal protein loading on subsequent gels for western blotting. Protein extracts were fractionated on NuPAGE 4–12% Bis-Tris gels (Life Technologies) and electro-transferred to polyvinylidene fluoride membrane (Pall, Melbourne, Australia). The membranes were incubated with primary antibodies (anti-lamin A/C, Millipore, Kilsyth, Australia, 1:100; anti-dysferlin, Leica Microsystems, North Ryde, Australia, 1:1,500) overnight and then labeled with anti-mouse secondary antibody (Novex Western Breeze Immunodetection kit, Life Technologies) for 1 hr. After incubation with Chemiluminescent substrate for 5 min, images were captured by a Chemi-smart 3000 gel documentation system (Vilber Lourmat) using Chemi-capt software with image analysis performed using Bio-1D software.

Confocal microscopy

After PMO or 2OMe transfection, 180,000 myogenic cells were placed in a glass bottom petri dish (MatTek, Ashland, MA) and cultured in 5% horse serum in Dulbecco’s modified Eagle medium for 72 hr before immunostaining. Cultures were incubated with anti-progerin (Abcam, Sapphire Bioscience, Waterloo, Australia) or lamin A/C (Millipore) antibody for 2 hr and followed by incubation with Alexa Fluor 488 goat anti mouse immunoglobulin (Invitrogen, 1:400) for 1 hr at room temperature, and then counterstained with Hoechst 33342 (Sigma Aldrich, 1:4,000) for 5 min. After rinsing with PBS, slides were viewed under a Nikon A1Si laser scanning confocal microscope (Coherent Scientific, Hilson, Australia).
Results

AO induction of LMNA Δ150 and LMNA ΔE11 transcripts

Forty-two 2OMeAOs, 18–30 bases in length, were designed to target the LMNA pre-mRNA sequence of the end of intron 10 and the beginning of intron 11 (Figure 1, Table 1). AOs targeting the pre-mRNA from 30 bases downstream of the cryptic splice site to the donor site, were able to induce some LMNA Δ150 transcript production, as assessed by RT-PCR (Figure 2). In addition to the LMNA Δ150 transcripts, there were also variable levels of exon 11 skipping (LMNA ΔE11), particularly with AOs annealing close to the donor site. Cryptic splice site activation and exon 11 skipping was generally stronger when AOs were targeted to the area near the donor site than the domain 30 bases downstream to the cryptic splice site. AOs 11A(+152+181), 11A(+157+186) and 11A(+162+186) were the most efficient LMNA Δ150-inducing AOs targeting the domain 30–70 bases downstream of the HGPS splice site (Figure 2B,C). The AOs 11A(+221+245) and 11A(+231+255) annealed upstream of the wild-type donor site, and induced the highest level of LMNA Δ150 induction of all the 2OMe AOs tested (Figure 2B,C). Transfection of AOs that anneal to the acceptor site or the first 120 bases upstream of the cryptic splice site did not have any obvious effect on the splicing of LMNA exon 11 (Figure 2B). The identities of the LMNA Δ150 and LMNA ΔE11 transcripts were confirmed by direct DNA sequencing.

Further refinement of AOs that induced the most pronounced induction of the LMNA Δ150 transcript was undertaken. Lengthening the AO, 11A(+157+181), by five bases at the 3’ end (11A(+157+186)) increased cryptic splicing, whereas removing bases from each end (11A(+159+176)) or moving the annealing coordinates 5 bases downstream, as well as extending the 3’ end again (11A(+162+191)) resulted in less splice switching activity (Figure 2C). Moving 11A(+211+235) 10 bases further toward the donor site (11A(+221+245)) or 20 bases further (11A(+231+255)) dramatically increased cryptic splicing (Figure 2C).

Two 2OMe AOs, shown to effectively modify LMNA splicing were selected for further evaluation after being synthesised as PMOs: 11A(+221+245) was selected since LMNA Δ150 induction was greater than exon 11 skipping whereas 11D(+2–23) induced robust exon 11 skipping with reduced LMNA Δ150 generation (Figure 3). Compared with its 2OMe equivalent, the PMO 11A(+221+245) appeared more specific in terms of cryptic splicing site activation. Both PMOs induced higher levels of LMNA Δ150 than their 2OMe counterparts (Figure 2, 11A(+221+245) PMO 80.2% vs 2OMe 44.7%, 11D(+2–23) PMO 33.7% vs 2OMe 18.4%). The level of LMNA Δ150 was even higher in myogenic cells treated with 11A(+221+245) PMO than in HGPS fibroblast cultures (Figure 3).

Progerin induction in PMO transfected myogenic cells

Despite inducing robust expression of the LMNA Δ150 transcript, the western blots of extracts from 2OMe AO transfected cells demonstrated only wild-type lamin A and C bands, with no detectable progerin (Figure 4A). In contrast, both PMOs induced sufficient splice-switching to generate detectable levels of progerin (Figure 4B). Theoretically, the lamin A AE11 protein should go through the first three steps of post-translational processing, and since it is only one amino acid smaller than lamin C, it is not distinguishable from lamin C using our current protein detection system.

Accumulation of progerin induces abnormalities in nuclear shape

PMO-treated myogenic cells and HGPS fibroblasts were stained with a progerin-specific antibody to assess its distribution. In HGPS fibroblast cultures, 25.2% (115/456) of nuclei were immuno-reactive for progerin. In human myoblast cultures, consistent with the RT-PCR results, cells transfected with the PMO 11D(+2–23) at 0.5 and 1 μM concentration induced 11.2% (71/632) and 15.2% (247/1625) progerin positive nuclei, whereas 11A(+221+245) induced marginally more positive nuclei (13.2% (93/705) and 17.1% (114/667) respectively). Nuclei from the PMO-treated cells that stained positive for progerin generally demonstrated abnormal shapes (e.g. lobulation and pouching) similar to those of HGPS nuclei, and some contained progerin aggregates (Figure 5A–I). Progerin-positive nuclei were not found in untreated human myogenic cells (0/541, Figure 5J–L) or cells transfected with 2OMe AOs (data not shown).

Cells were labelled with anti-lamin A/C antibody to evaluate nuclear shape abnormalities. There were 8.97% (14/156) and 11.80% (42/356) abnormally shaped nuclei in cells nucleofected with PMO11D(+2–23) at 0.5 and 1 μM concentration respectively, while 5.37% (18/335) and 5.99% (10/167) in cells transfected with 2OMe11D(+2–23) at 0.5 and 1 μM concentration. In comparison, the percentage of aberrant nuclei in cells nucleofected with 0.5 and 1 μM PMO11A(+2–23) at 0.5 and 1 μM concentration respectively, while 5.37% (18/335) and 5.99% (10/167) in cells transfected with 2OMe11D(+2–23) at 0.5 and 1 μM concentration. In comparison, the percentage of aberrant nuclei in cells nucleofected with 0.5 and 1 μM PMO11A(+2–23) at 0.5 and 1 μM concentration respectively, whereas that in cells transfected with 0.5 and 1 μM 2OMe11A(+221+245) was 5.74% (7/122) and 8.12% (19/234) respectively, whereas that in cells transfected with 0.5 and 1 μM 2OMe11A(+221+245) was 4.67% (7/150) and 6.29% (21/334). Cells transfected with 0.5 and 1 μM scrambled 2OMe AO 8.9–11.7 also demonstrated 5.13% (4/78) and 5.32% (5/94) aberrant nuclei, and untreated cells 2.13% (2/93).

Figure 1. Schematic of LMNA exon 11 and annealing AOs. The grey bar represents the 150 bases omitted from the LMNA Δ150 transcript. The AOs assessed in this study are shown according to their coordinates on exon 11. AOs that have minimal splicing modulatory effect are shown in black, AOs inducing predominantly cryptic splicing activation in red, AOs inducing mainly exon 11 skipping in green. Splicing strength scores are calculated by Human Splice Finder (http://www.umd.be/HSF/).

Figure 2. AOs targeting the cryptic splice site downstream of the HGPS splice site. The identities of the AOs are shown according to their coordinates on exon 11. AOs that have minimal splicing modulatory effect are shown in black, AOs inducing predominantly cryptic splicing activation in red, AOs inducing mainly exon 11 skipping in green. Splicing strength scores are calculated by Human Splice Finder (http://www.umd.be/HSF/).
Figure 2. RT-PCR showing changes in LMNA splicing after transfecting with 2OMe AOs. (A) AO annealing location within the 150 base region of exon 11 excluded in HGPS (in grey). The arrowhead denotes the site of the classic HGPS C>T mutation. AOs that induce the greatest degree of cryptic splicing activation are shown in red. (B) Representative gel images of RT-PCR LMNA-related products from cells transfected over a range of concentrations. A smaller fourth LMNA transcript product induced in cells transfected with 11A(+211-235) to 11A(+231-25S) was identified as missing exons 10-11. (C) Semi quantitative analysis by densitometry of gel band intensity, indicating levels of different LMNA transcripts. Bars denote mean ± SE.
doi:10.1371/journal.pone.0098306.g002

Table 1. Antisense oligonucleotides tested in the present study.

| Number | Nomenclature and Coordinates | Sequence (5'-3') | GC content |
|--------|------------------------------|------------------|------------|
| 1      | HLmnA11A (−5−23)             | aag gga gac aag acu cag g | 52.63%     |
| 2      | HLmnA11A (−15+10)            | agu ggg agc ccu ggg aag gga gac a | 60.00%     |
| 3      | HLmnA11A (−5+20)             | gag cug cug cag ugg gag ccc ugg g | 72.60%     |
| 4      | HLmnA11A (+2+26)             | ucc ccc gag cug cug cag ugg gac c | 72.60%     |
| 5      | HLmnA11A (+11+35)            | uca ggc ggg ucc ccc gag cug cuc g | 76.00%     |
| 6      | HLmnA11A (+21+45)            | cag guu gua cuc agc ggc ggg ggc ccc c | 72.00%     |
| 7      | HLmnA11A (+31+55)            | ugc ggc agc gca ggu ugu acu cag c | 64.00%     |
| 8      | HLmnA11A (+41+65)            | cac agc acg gug cgc gag cgc agg u | 72.00%     |
| 9      | HLmnA11A (+51+75)            | gca ggu ccc gca gag cag ggu gcg c | 76.00%     |
| 10     | HLmnA11A (+61+85)            | cag gcu ggc gcg agg ucc gcg cca c | 79.17%     |
| 11     | HLmnA11A (+71+95)            | gcc ugg ugc gca ggc ugc cgc cag g | 76.00%     |
| 12     | HLmnA11A (+81+105)           | gcu ggc aga ugc cuu ggc ggc agg c | 68.00%     |
| 13     | HLmnA11A (+91+115)           | cuc gcu agc cgc ugg cag aug ccc u | 64.00%     |
| 14     | HLmnA11A (+101+125)          | ccc acc ugg gcu ccc gag ccc cu g | 76.00%     |
| 15     | HLmnA11A (+111+135)          | gau ggg ucc gcc ccc cag cug ugc u | 72.00%     |
| 16     | HLmnA11A (+121+145)          | agg cag agg aga ugg ggc gcg cca c | 68.00%     |
| 17     | HLmnA11A (+131+155)          | gag gca gaa gag ccc gag cag aug g | 60.00%     |
| 18     | HLmnA11A (+141+165)          | cgu gac acu gga ggc aga aga gcc a | 60.00%     |
| 19     | HLmnA11A (+147+176)          | cug cga gug acc gug acu cag gca gaa | 60.00%     |
| 20     | HLmnA11A (+152+176)          | cug cga gug acc acc acu cag gac | 64.00%     |
| 21     | HLmnA11A (+152+181)          | ggu agc ugc gag ugc cca gaa cug agg | 63.33%     |
| 22     | HLmnA11A (+157+181)          | ggu agc ugc gag ugc cca gaa cag u | 60.00%     |
| 23     | HLmnA11A (+157+186)          | acu ggc gau gcu gcg aga gcg cgu gac acu | 60.00%     |
| 24     | HLmnA11A (+159+176)          | cgu cga gug acc gug acu | 61.11%     |
| 25     | HLmnA11A (+162+186)          | acu ggc gua ggc ggc agu cgu | 64.00%     |
| 26     | HLmnA11A (+162+191)          | ccc cca cgg cgg uag cug cga gug acc gug | 66.67%     |
| 27     | HLmnA11A (+167+191)          | ccc cca cgg uag uag cug cga gua | 64.00%     |
| 28     | HLmnA11A (+171+195)          | gcc ccc cac acu gcg una gcg cga a | 72.00%     |
| 29     | HLmnA11A (+181+205)          | cac ccc cac ucg cca cca cac ugc g | 76.00%     |
| 30     | HLmnA11A (+191+215)          | cgc aag cug ccc cca cca cug ccc c | 76.00%     |
| 31     | HLmnA11A (+196+220)          | ugg cca cga agc ugc ccc ccc cug u | 68.00%     |
| 32     | HLmnA11A (+201+225)          | cag auu guc ccc gaa gcu gcc acc c | 64.00%     |
| 33     | HLmnA11A (+211+235)          | agc ggg uga cca gau ugu cca cga a | 60.00%     |
| 34     | HLmnA11A (+221+245)          | agg agg uag gac gcg ggc acc uga u | 60.00%     |
| 35     | HLmnA11A (+231+255)          | gga guu gcc cag gag gua ggc gcg g | 68.00%     |
| 36     | HLmnA11A (+241+265)          | ugc ggg ggc ugg agu ugc cca gcg a | 68.00%     |
| 37     | HLmnA11D (+11−18)            | aaa gca gac aca acu cag ggu ugc gg | 55.17%     |
| 38     | HLmnA11D (+7−18)             | aaa gca gac aca acu cac cug ggu u | 48.00%     |
| 39     | HLmnA11D (+7−23)             | gag aca aag cag aca cca cac acc ugg guu | 50.00%     |
| 40     | HLmnA11D (+2−23)             | gag aca aag cga aca cac acc u | 48.00%     |
| 41     | HLmnA11D (−2−26)             | ugg gag aca aag cag aca cca ca c | 44.00%     |
| 42     | HLmnA11D (−5−29)             | gau ugg gag aca aag cag aca ca c | 44.00%     |

doi:10.1371/journal.pone.0098306.t001
Discussion

Under normal conditions, alternative splicing of \textit{LMNA} gives rise to at least three different isoforms, lamin A, C and lamin A D10 [30,31]. The predominant isoforms, lamin A and C, are involved in a myriad of physiological processes, including maintaining nuclear shape, DNA replication and transcription, and enabling interaction between nucleoplasm and cytoplasm by connecting the nucleo- with the cyto-skeleton of the cell [32–35]. It is therefore not surprising that in HGPS, aberrant splicing arising from activation of a cryptic splice site and production of the progerin isoform lead to a wide range of downstream events culminating in premature cellular senescence [2,36,37]. 

\textit{LMNA} mutations have been associated with several clinically distinct neuromuscular disorders including Emery-Dreifuss muscular dystrophy, limb girdle muscular dystrophy type 1B and Charcot-Marie-Tooth diseases type 2B1 [38–40]. Lamin A/C expression is also important in muscle differentiation and maintenance of muscle function [41–43]. We have demonstrated the presence of progerin in normal skeletal muscles [26] and other researchers have reported detecting progerin in other normal tissues including blood vessels, skin, liver and heart [3,4,44]. By using splice-switching AOs, we show here that progerin-overexpressing myonuclei exhibit aberrant shapes similar to those in HGPS cells, and to nuclei in normal ageing cells [3], that may be a relevant \textit{in vitro} model of accelerated muscle ageing.

The AOs annealing to motifs across exon 11 of \textit{LMNA} pre-mRNA could be divided into 3 classes according to their effects on \textit{LMNA} splicing: 1- those that exerted no or minimal effects on \textit{LMNA} pre-mRNA processing, 2- those that induced primarily exon 11 skipping, and progerin production to a lesser extent, and 3- those that promoted selection and usage of the cryptic splice site leading to the production of the truncated lamin A isoform, progerin with some exon 11 skipping.

Our experience with the design of splice-switching AOs to induce exon skipping in the dystrophin gene transcript is that the donor sites are generally unresponsive splice switching targets for the majority of constitutively expressed exons. On the other hand, the dystrophin acceptor sites and the first half of exons have proved to be more amenable targets for exon skipping [45]. Directing AOs to mask either donor or acceptor splice sites, both crucial motifs in the splicing process, is not guaranteed to identify a compound capable of modifying processing of the target transcript. In direct contrast to our previous studies on dystrophin, the acceptor site and first half of \textit{LMNA} exon 11 were unresponsive to AO splice modulation, while AOs targeting the latter half of \textit{LMNA} exon 11 and the donor splice site did modify processing of the transcript. There may be restricted access to the \textit{LMNA} exon 11 acceptor site because of secondary RNA conformation or an enrichment of proteins binding in this domain that prevent oligonucleotide binding. By masking the latter half of \textit{LMNA} exon 11 and the constitutive donor splice site, the splicing machinery...
cells transfected with PMOs also demonstrated abnormally shaped nuclei are mostly lobulated or trabeculated (A–C). Human myogenic spliceosome [46]. The HGPS structure of the pre-mRNA, which limits its accessibility by the splicing machinery. This study also proposed that the exon 11 cryptic splice site is engaged in a stem-loop like loop structure and facilitates recognition of the cryptic splice site by the splicing machinery. This study also proposed that the exon 11 cryptic splice site of exon 11 activated in HGPS. Another difference between this study and that by Fong et al., is that the 11A(324) described by Fong et al., and caused both cryptic splice site activation and exon 11 skipping in our study. Several factors may contribute to the discrepancies between the two studies, including the use of different cell strains (i.e. fibroblasts vs myogenic cells) and different AO chemistries (2'-MOE vs 2OMe). However, we also transfected normal human skin fibroblasts with our AOs and different AO chemistries (2'-MOE vs 2OMe). Untreated cells did not contain any detectable progerin positive nuclei (J–L). Magnification: 60×. Scale bar: 50 μm.

doi:10.1371/journal.pone.0098306.g005

Figure 5. Confocal fluorescence microscopy with false colour showing the localization of progerin (green) in nuclei (blue) in human myogenic cells. In HGPS fibroblast cultures, progerin positive nuclei are mostly lobulated or trabeculated (A–C). Human myogenic cells transfected with PMOs also demonstrated abnormally shaped progerin reactive nuclei (D–F: transfected with 1 μM PMO 421; G–I: 0.5 μM PMO 422). Untreated cells did not contain any detectable progerin positive nuclei (J–L). Magnification: 60×. Scale bar: 50 μm.

either fails to recognise the entire exon or is forced to use the cryptic splice site of exon 11 activated in HGPS. Most of the AOs found to influence LMNA splicing induced a mixture of transcripts, some missing exon 11 and others missing the 150 bases downstream of the cryptic splice site. This implies two mechanisms, either enhancing recognition of the cryptic splice site, or blocking selection of the entire exon and inducing its loss from the mature mRNA. Some AOs may influence exonic splicing enhancer (ESE) and/or an exonic splicing silencer (ESS) and direct the splicing machinery to use the cryptic splice site or mask the entire exon. The GC content of AOs targeting this area are similar (Table 1), therefore it is unlikely that the annealing capacity of these AOs plays a significant role in the different levels of alternative splicing. Instead, the results suggest that the motifs targeted by 11A(+177+186) (57 bases downstream to the cryptic splice site) and 11A(+236+255) (116 bases downstream to the cryptic splice site, 15 bases upstream to the donor site) may act as ESEs for the consensus donor site or ESSs for the cryptic splice site. A previous study by Lopez-Mejia and colleagues demonstrated that the exon 11 cryptic splice site is engaged in a stem-loop like structure of the pre-mRNA, which limits its accessibility by the spliceosome [46]. The HGCS C>T mutation potentially opens up the loop structure and facilitates recognition of the cryptic splice site by the splicing machinery. This study also proposed that the region 50 to 66 bases downstream of the cryptic splice site is in a single-stranded region and is likely to be highly accessible to splicing factors, as well as to the AOs. Oligonucleotides targeting this area may have higher affinity for the pre-mRNA and cause more dramatic effects on LMNA splicing.
redirect in the different studies [46,52]. The variable efficiencies with which progerin was induced by our 2OMe AOs and PMOs also support the possibility that the oligonucleotide chemistry has a major impact on transfection outcomes. But other factors may also contribute: different AO length (25–50 mer vs. 16–20 mer), transfection (ion concentrations [100–400 nM vs 2.5–100 nM] and PCR amplification conditions.

We could induce the accumulation of progerin as well as lamin A ΔE11 in human myogenic cells using splicing switching AOs. Both progerin and lamin A ΔE11 lack a proteolytic site for post-translational modification of the precursor protein prelamin A. Consequently, both aberrant proteins retain a farnesyl group at the C terminal, which is normally cleaved from the wild-type mature lamin A. It is proposed that the farnesyl group plays a key role in the pathogenesis of farnesylated prelamin A-accumulating diseases [53,54]. The retention of the farnesyl group prevents the progerin from disassociating from the nuclear lamina during the cell cycle and disrupts mitosis [56].

Accumulation of lamin A ΔE11 causes another fatal progeroid disease, restrictive dermopathy [55]. To date there are few studies regarding the pathophysiology of lamin A ΔE11, hence the splice-switching method here may offer an inducible model to further study this disease. Given that the lamin A ΔE11 product, like progerin, is presumably permanently farnesylated and that restrictive dermopathy demonstrates similar nuclear abnormalities to HGPS, it is possible that lamin A ΔE11 will have similar downstream effects to those caused by progerin. Lamin A ΔE11 is probably as deleterious as, if not more so, progerin in HGPS, considering the extreme phenotype of restrictive dermopathy. Indeed, the fact that accumulation of progerin and lamin A ΔE11 can both cause restrictive dermopathy suggests that HGPS and restrictive dermopathy belong to the same clinical spectrum of diseases caused by farnesylated prelamin A [56]. Therefore, although there is a mixture of cryptic splicing activation and exon 11 skipping in the AO treated myogenic cells in the present study, it is our belief that the induced products, progerin and lamin A ΔE11, exert similar effects in cells to cause accelerated ageing. Consistent with this hypothesis, similarly mis-shaped myonuclei were found in myogenic cells treated with the PMOs that induced progerin alone and both progerin and lamin A ΔE11.

Premature ageing can be induced in fibroblasts and human midfield dopamine neurons derived from induced pluripotent stem (iPS) cells by transfection with a synthetic RNA that encodes progerin tagged with GFP [57]. Enhanced expression of progerin was only achieved after 3 and 5 repeats of daily transfection in iPS-fibroblasts and iPS-neurons respectively. In contrast, the splice switching PMOs in this study induced more readily detectable amounts of progerin 36 hours after transfection. It will be interesting to evaluate the consequences of progerin expression arising from PMO induced splice switching in iPS-fibroblasts and iPS-neurons.

In conclusion, we have shown that AOs targeting the putative ESEs/ESSs within exon 11 of LMNA or the donor site, can be used to redirect splicing in human myogenic cells, and lead to the production of two distinctive, yet functionally similar, farnesylated prelamin A isoforms (progerin and lamin A ΔE11). The PMO chemistry was found to be more effective than the 2OMe chemistry in terms of specificity and progerin production. The PMOs increased production of progerin and induced the nuclear changes associated with premature ageing, similar to those that occur in HGPS. AOs therefore have the potential to manipulate splicing and induce pathogenic splicing, and changes of premature ageing in cells in vitro. PMO 11D(+2)–23 leads to predominant exon 11 skipping and may serve as a suitable model to study the pathophysiology of lamin A ΔE11.

Acknowledgments

The authors thank Professor Paul Rigby from Centre for Microscopy, Characterisation and Analysis for assistance in confocal microscopy.

Author Contributions

Conceived and designed the experiments: YBL CM FLM SDW. Performed the experiments: YBL CM AMA RJ. Analyzed the data: YBL CM AMA RJ SF FLM SDW. Contributed reagents/materials/analysis tools: SF FLM SDW. Wrote the paper: YBL SF FLM SDW.

References

1. Eriksson M, Brown WT, Gordon LB, Glynn MW, Singer J, et al. (2003) Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. Nature 423: 293–298.
2. Scaffidi P, Misteli T (2000) Lamin A-dependent misregulation of adult stem cells associated with accelerated ageing. Nat Cell Biol 10: 452–459.
3. Scaffidi P, Misteli T (2006) Lamin A-dependent nuclear defects in human aging. Science 312: 1059–1063.
4. McClintock D, Ratner D, Lokaye M, Owens DM, Gordon L, et al. (2007) The mutant form of lamin A that causes Hutchinson- Gilford progeria is a biomarker of cellular aging in human skin. PLoS One 2: e1269.
5. Luo YB, Fabian V, Johnsen R, Fletcher S, Wilton S, et al. (2011) Alternative splicing of lamin A leads to age-dependent accumulation of progerin transcript in normal human muscle and sporadic IBM [abstract]. Neuro muscular Disorder 21: 734.
6. Crooke RM, Graham MJ, Lemonidis KM, Whipple CP, Koo S, et al. (2005) An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. J Lipid Res 46: 872–884.
7. Gleave M, Chi KN (2005) Knock-down of the cytoprotective gene, clustatin, to enhance hormone and chemosensitivity in prostate and other cancers. Ann N Y Acad Sci 1058: 1–15.
8. Sazani P, Kole R (2003) Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. J Clin Invest 112: 481–486.
9. Wilton SD, Fletcher S (2005) RNA splicing manipulation- strategies to modify gene expression for a variety of therapeutic outcomes.Curr Gene Ther 5: 467–483.
10. Johnson JM, Castle J, Garrett-Engle P, Kan Z, Loehr PM, et al. (2003) Genome-wide study of human alternative pre-mRNA splicing with exon microarrays. Science 302: 2141–2144.
11. López-Bigas N, Audit B, Ouzounis C, Parra G, Guigo R (2005) Are splicing regulatory elements involved in the pathogenesis of lamin A? In: Mastaglia FL, Hilton-Jones D, editors. Handbook of clinical neurology Vol62 Myopathies. Edinburgh: Elsevier. pp. 357–388.
12. Stenson PD, Mort M, Ball EV, Howells K, Phillips AD, et al. (2009) The Human Gene Mutation Database: 2008 update. Genome Med 1: 13.
13. Cirak S, Arechavala-Gomez V, Gaglieri M, Feng L, Torelli S, et al. (2011) Eon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. Lancet 378: 593–603.
14. Porrenys PN, Mitrpant C, McGovern VL, Bevan AK, Foust KD, et al. (2012) A single administration of morpholino antisense oligonucleotides rescues spinal muscular atrophy in mouse. Hum Mol Genet 21: 1625–1636.
15. Guo S, Casu G, Gardenghi S, Bottoni S, Aghajani M, et al. (2013) Reducing TMRP58 ameliorates hemochromatosis and beta-thalassemia in mice. J Clin Invest 123: 1531–1541.
16. Dominski Z, Kole R (1995) Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. Proc Nad Acad Sci U S A 90: 3673–3677.
17. Scaffidi P, Misteli T (2005) Reversal of the cellular phenotype in the premature aging disease Hutchinson-Gilford progeria syndrome. Nat Med 11: 440–445.
18. Fong LG, Vickers TA, Farber EA, Choi C, Yun UJ, et al. (2009) Activating the synthesis of progerin, the mutant prelamin A in Hutchinson-Gilford progeria syndrome, with antisense oligonucleotides. Hum Mol Genet 18: 2462–2471.
19. Serdaroglu P (2007) Muscle diseases and ageing. In: Mastaglia FL, Hilton-Jones D, editors. Handbook of clinical neurology Vol62 Myopathies. Edinburgh: Elsevier. pp. 357–388.
20. Carmeli E, Coleman R, Reznick AZ (2002) The biochemistry of aging muscle. Exp Gerontol 37: 477–489.
21. Clark DJ, Fielding RA (2012) Neuromuscular contributions to age-related sarcopenia. J Gerontol A Biol Sci Med Sci 67: 41–47.
22. Lee HC, Wei YH (2001) Mitochondrial alterations, cellular response to oxidative stress and defective degradation of proteins in aging. Biogerontology 2: 231–244.
23. Schap LA, Pluim SM, Dreg DJ, Visser M (2006) Inflammation markers and loss of muscle mass (sarcopenia) and strength. Am J Med 119: 526 e529–517.
24. Lahousse C, Sotiropoulos A, Favier M, Guillet-Denai I, Charvet C, et al. (2008) Premature aging in skeletal muscle lacking serum response factor. PLoS One 3: e3910.

25. Muller FL, Song W, Liu Y, Chaudhuri A, Pieke-Dahl S, et al. (2006) Absence of Cu/Zn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. Free Radic Biol Med 40: 1993–2004.

26. Luo YB, Mitriapnt C, Johnsen R, Fabian V, Needham M, et al. (2013) Investigation of splicing changes and post-translational processing of LMNA in sporadic inclusion body myositis. Int J Clin Exp Pathol 6: 1725–1729.

27. Mann CJ, Honeyman K, McClorey G, Fletcher S, Wilson SD (2002) Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. J Gene Med 4: 649–654.

28. Harding PL, Fall AM, Hensyman K, Fletcher S, Wilson SD (2007) The influence of antisense oligonucleotide length on dystrophin exon skipping. Mol Ther 15: 157–166.

29. Cooper ST, Lo HP, North KN (2003) Single section Western blot: improving the molecular diagnosis of the muscular dystrophies. Neurology 61: 93–97.

30. Machics BM, Zorenne AH, Endert JM, Knijgers HJ, van Eys GJ, et al. (1996) An alternative splicing product of the lamin A/C gene lacks exon 10. J Biol Chem 271: 9249–9253.

31. Fisher DZ, Chauhdhary N, Blobel G (1986) cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. Proc Natl Acad Sci U S A 83: 6450–6454.

32. Kumaran RI, Murakikshma B, Parmaik VK (2002) Lamin A/C speckles mediate spatial organization of splicing factor compartments and RNA polymerase II transcription. J Cell Biol 159: 783–793.

33. Olins AL, Olins DE (2004) Cytoskeletal influences on nuclear shape in granulocytic HL-60 cells. BMC Cell Biol 5: 30.

34. Kennedy BK, Barbie DA, Classon M, Dyson N, Lu Y, et al. (2000) Nuclear organization of DNA replication in primary mammalian cells. Genes Dev 14: 2553–2568.

35. Shimizu T, Ben-Israel V, Goldman RD (2012) The functions of the nuclear envelope in mediating the molecular crosstalk between the nucleus and the cytoplasm. Curr Opin Cell Biol 24: 71–78.

36. Dechat T, Shiomi T, Adam SA, Rusinol AE, Andres DA, et al. (2007) Alterations in mitosis and cell cycle progression caused by a mutant lamin A known to accelerate human aging. Proc Natl Acad Sci USA 104: 4955–4960.

37. Liu Y, Rusinol A, Sinensky M, Wang Y, Zou Y (2006) DNA damage responses in progeroid syndromes arise from defective maturation of prelamin A. J Cell Sci 119: 4644–4649.

38. Bonne G, Di Barletta MR, Varnous S, Becane HM, Hammouda EH, et al. (1999) Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. Nat Genet 21: 285–288.

39. Genschel J, Schmidt HH (2000) Mutations in the LMNA gene encoding lamin A/C. Hum Mutat 16: 451–459.

40. De Sandre-Giovannoli A, Chauhch M, Kozlov S, Vallat JM, Tazir M, et al. (2002) Homozygous defects in LMNA, encoding lamin A/C nuclear-envelope proteins, cause autosomal recessive axonal neuropathy in human (Charcot-Marie-Tooth disorder type 2) and mouse. Am J Hum Genet 70: 726–736.

41. Frock RL, Kudlow BA, Evans AM, Jameson SA, Hauschka SD, et al. (2000) Lamin A/C and emerin are critical for skeletal muscle satellite cell differentiation. Genes Dev 20: 498–500.

42. Mattioli E, Columbaro M, Capanna C, Maraldi NM, Cenni V, et al. (2011) Prelamin A-mediated recruitment of SUN1 to the nuclear envelope directs nuclear positioning in human muscle. Cell Death Differ 18: 1303–1315.

43. Zhang Q, Ragnauth CD, Skerpet JM, Worth NF, Warren DT, et al. (2005) Nesprin-2 is a multi-isomeric protein that binds lamin and emerin at the nuclear envelope and forms a subcellular network in skeletal muscle. J Cell Sci 118: 673–687.

44. Ragnauth CD, Warren DT, Liu Y, McNair R, Tajic T, et al. (2010) Prelamin A acts to accelerate smooth muscle cell senescence and is a novel biomarker of human vascular aging. Circulation 121: 2200–2210.

45. Wilton SD, Fall AM, Harding PL, McClorey G, Coleman C, et al. (2007) Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript. Mol Ther 15: 1289–1296.

46. Lopez-Mejia IC, Vautrot V, De Toledo M, Behm-Ansamt I, Bourgeois CF, et al. (2011) A conserved splicing mechanism of the LMNA gene controls premature aging. Hum Mol Genet 20: 4540–4555.

47. Fletcher S, Honeyman K, Fall AM, Harding PL, Johnson RD, et al. (2006) Dystrophin expression in the mdx mouse after localised and systemic administration of a morpholino antisense oligonucleotide. J Gene Med 8: 207–216.

48. Fletcher S, Ashin CF, Meloni P, Wong B, Muntoni F, et al. (2012) Targeted exon skipping to address ‘leaky’ mutations in the dystrophin gene. Mol Ther Nucleic Acids 1: e48.

49. McClorey G, Moulton HM, Iverson PL, Fletcher S, Wilson SD (2006) Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. Gene Ther 13: 1373–1381.

50. Heemskerk HA, de Winter CL, de Kimpe SJ, van Kuik-Rompeijn P, Henehlmans N, et al. (2009) In vivo comparison of 2’-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. J Gene Med 11: 257–266.

51. Mendell JR, Rodino-Klapac LR, Sahenk Z, Roush K, Bird L, et al. (2013) Eteplirsen for the treatment of Duchenne muscular dystrophy. Ann Neurol 74: 637–647.

52. Black DL (2005) Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem 72: 291–336.

53. Navarro CL, Cadamanso J, De Sandre-Giovannoli A, Bernard R, Currier S, et al. (2005) Loss of ZMPSTE24 (FACE-1) causes autosomal recessive restrictive dermopathy and accumulation of Lamin A precursors. Hum Mol Genet 14: 1503–1513.

54. Toth JI, Yang SH, Qiao X, Beigneux AP, Gelb MH, et al. (2005) Blocking protein farnesylation improves nuclear shape in fibroblasts from humans with progeroid syndromes. Proc Natl Acad Sci U S A 102: 12873–12878.

55. Navarro CL, De Sandre-Giovannoli A, Bernard R, Boccaccio I, Boyer A, et al. (2004) Lamin A and ZMPSTE24 (FACE-1) defects cause nuclear disorganization and identify restrictive dermopathy as a lethal neonatal laminopathy. Hum Mol Genet 13: 2071–2078.

56. Pereira S, Bourgeois P, Navarro C, Esteves-Vieira V, Cau P, et al. (2008) HGPS and related premature aging disorders: from genomic identification to the first therapeutic approaches. Mech Ageing Dev 129: 449–459.

57. Miller JD, Ganat YM, Kishinevsky S, Bowman RL, Liu B, et al. (2013) Human iPSC-based modelling of late-onset disease via progerin-induced aging. Cell Stem Cell 13: 691–705.