

Original Article

Investigation of splicing changes and post-translational processing of *LMNA* in sporadic inclusion body myositis

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Abstract: Some features of sporadic inclusion body myositis (s-IBM) suggest that there is acceleration of the normal ageing process in muscle tissue. *LMNA* encodes the nuclear lamina proteins lamin A/C through alternative splicing, and aberrant splicing of exon 11 leads to the premature ageing disease, Hutchinson-Gilford progeria syndrome. Progerin, the pathogenic isoform expressed in HGPS tissues, has also been detected at low levels in tissues of normal individuals with aging. We therefore investigated the alternative splicing of *LMNA* gene transcripts, and the post-translational processing of prelamin A, in s-IBM and control muscle samples. Age-related low level expression of the progerin transcript was detected in both s-IBM and control muscles, but was not increased in s-IBM and there was no increase in progerin protein or demonstrable accumulation of intermediate prelamin isoforms in the s-IBM muscles. However, an age-related shift in the balance of splicing towards lamin A-related transcripts, which was present in normal muscles, was not found in s-IBM. Our findings indicate that while there are changes in the patterns of *LMNA* splicing in s-IBM muscle which are probably secondary to the underlying pathological process, it is unlikely that aberrant splicing of exon 11 or defective post-translational processing of prelamin A are involved in the pathogenesis of the disease.

Keywords: Inclusion body myositis, muscle, *LMNA*, progerin, prelamin A, lamin A/C

Introduction

Sporadic inclusion body myositis (s-IBM) is the most common myopathy of later life, with a prevalence in Caucasians of 51 per million over the age of 50 years [1]. There is still debate as to whether s-IBM is a T cell mediated autoimmune myopathy or a myodegenerative disorder of undetermined type with a secondary inflammatory component [2]. One of the pathological hallmarks of s-IBM is the presence of increased numbers of cytochrome C oxidase deficient muscle fibres harbouring clonally expanded mtDNA deletions and point mutations. Such fibres also occur in normal human muscle with ageing, but are much more frequent in s-IBM [3-6]. These findings suggest that there may be an acceleration of the normal ageing process-

es in skeletal muscle in s-IBM. One of the other pathological features of s-IBM is the presence of myonuclear abnormalities [7], leading to the hypothesis that a primary myonuclear defect may underlie the degeneration of muscle fibres and formation of rimmed vacuoles in muscle fibres [7, 8].

Mutations in *LMNA*, the gene that encodes the nuclear envelope proteins lamin A and C, result in a wide range of skeletal and cardiac myopathies and systemic disorders [9]. The premature ageing disease Hutchinson-Gilford progeria syndrome (HGPS) is caused by point mutations that partially activate a cryptic donor splice site in exon 11 [10]. The mutations compromise normal splicing and result in the loss of 150 bases from exon 11 of *LMNA* mRNA (*LMNA*

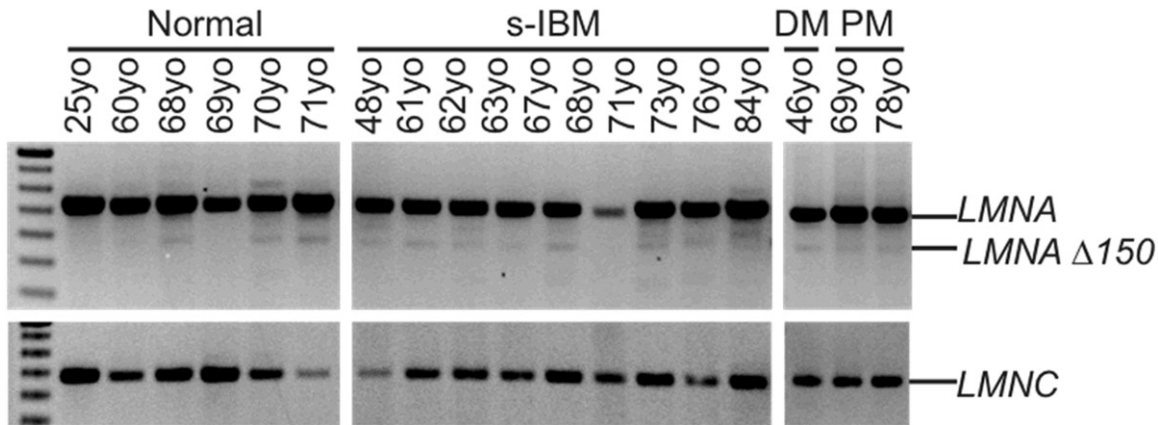


Figure 1. RT-PCR shows the presence of *LMNA* and *LMNC* in all samples. A much lower level of *LMNA* $\Delta 150$ transcript is shown in 8/11 s-IBM samples, 3/6 normal samples and 3/3 myositis controls. The lower intensity for *LMNA* in one sample (71 yr s-IBM) and for *LMNC* in two samples (71 yr normal and 61 yo s-IBM) is probably technical in origin as these samples did not show any obvious deviation of Ct values from the rest of the group in the subsequent real time qPCR analysis.

Table 1. Real time qPCR analysis of *LMNA* isoforms

| | s-IBM | Normal | p value |
|--------------------------|-------------------|-------------------|---------|
| <i>LMNA</i> $\Delta 150$ | 4.716 \pm 1.386 | 3.886 \pm 0.898 | 0.604 |
| <i>LMNA</i> | 2.685 \pm 0.506 | 2.030 \pm 0.515 | 0.395 |
| <i>LMNC</i> | 3.378 \pm 0.536 | 2.212 \pm 0.511 | 0.136 |
| <i>LMNA/LMNC</i> | 1.729 \pm 0.204 | 1.463 \pm 0.240 | 0.408 |

$\Delta 150$). The truncated protein isoform, named progerin, leads to premature ageing of multiple organs and tissues of mesodermal origin in affected individuals. It has been reported that progerin, or a progerin-like protein, is present in muscle biopsies from some older s-IBM patients [11], suggesting that altered splicing of exon 11 may be occurring in s-IBM. However, this finding requires confirmation and its significance is uncertain as we have recently shown that the *LMNA* $\Delta 150$ isoform is also expressed in normal human muscle with ageing [12].

In the present study, we investigated splicing of *LMNA* by comparing the expression of lamin A and C and the $\Delta 150$ progerin isoform in s-IBM and control muscle samples, as well as the post-translational processing of prelamin A.

Methods

Muscle samples

Muscle biopsy samples were available from 14 patients (9 males, 5 females; 48-82 years of age, mean 66.5 yr) with clinically and pathologically confirmed s-IBM who fulfilled the criteria

for definite s-IBM [2]. Control specimens were obtained from 13 biopsies from individuals investigated for suspected malignant hyperthermia (MH), all of whom were classified as being MH-negative after undergoing *in vitro* contracture testing (7 males, 6 females, 25-71 yr, mean 53.8 yr) and had normal muscle histology, and from 5 patients with other inflammatory myopathies (3 polymyositis, 2 dermatomyositis; 1 male, 4 females, aged 46-78 yrs, mean 63.0 yr). Ethical approval for the study was obtained from the Sir Charles Gairdner Hospital and Royal Perth Hospital Human Research Ethics Committees (reference number: 2006-073).

Muscle biopsies were snap frozen in isopentane chilled in liquid nitrogen and stored at -80°C . Muscle sections for the studies below were cut in a Leica CM1900 cryostat (Leica Microsystems, North Ryde, Australia).

Cell cultures

Primary HGPS fibroblasts were obtained from Coriell cell repositories (Coriell Institute for Medical Research, Camden, NJ, Cat # AG03513). Cells were proliferated in Dulbecco's Modified Eagle Medium (Invitrogen, Mulgrave, Australia) supplemented with 15% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin, and 250 ng/ml amphotericin B (Sigma Aldrich, Sydney, Australia) in a 37°C incubator with 5% CO_2 . Primary human myoblasts were proliferated as described previously [13],

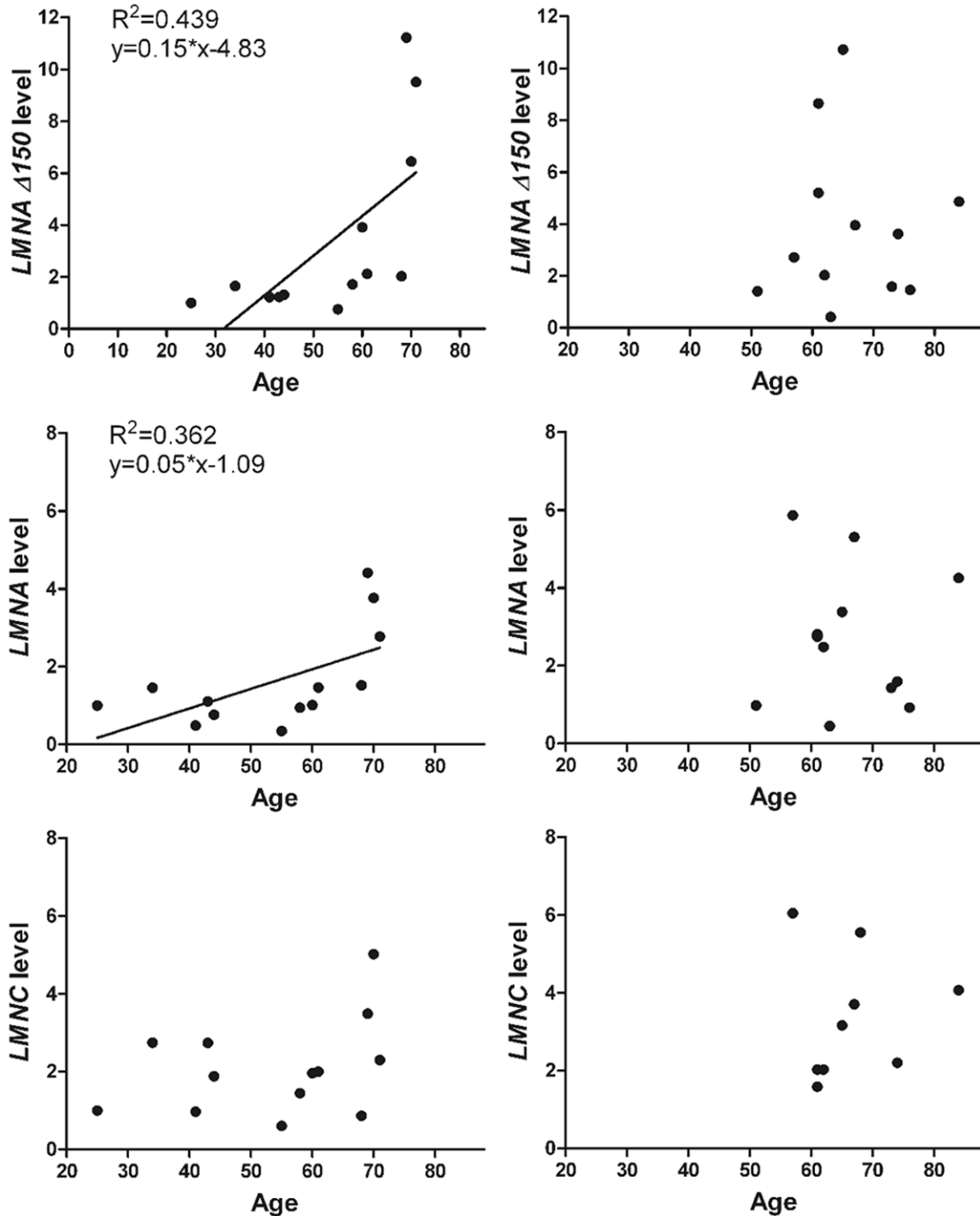


Figure 2. Real time qPCR analysis showing the comparison in the relative LMNA $\Delta 150$, LMNA and LMNC levels vs age between normal (left panel) and s-IBM groups (right panel).

360,000 myoblasts were seeded and differentiated in MatTek glass bottom petri dish (MatTek, Ashland, MA) for 48 hr, and were then treated with 20 mM farnesyltransferase inhibitor-277 (FTI-277, Sigma Aldrich), which induces

accumulation of non-farnesylated full-length prelamins A, or 40 mM N-acetyl-S L-cysteine-methyl ester (AFCMe, Enzo, Farmingdale, NY), which leads to accumulation of farnesylated prelamins A, every 24 hr for 72 hr.

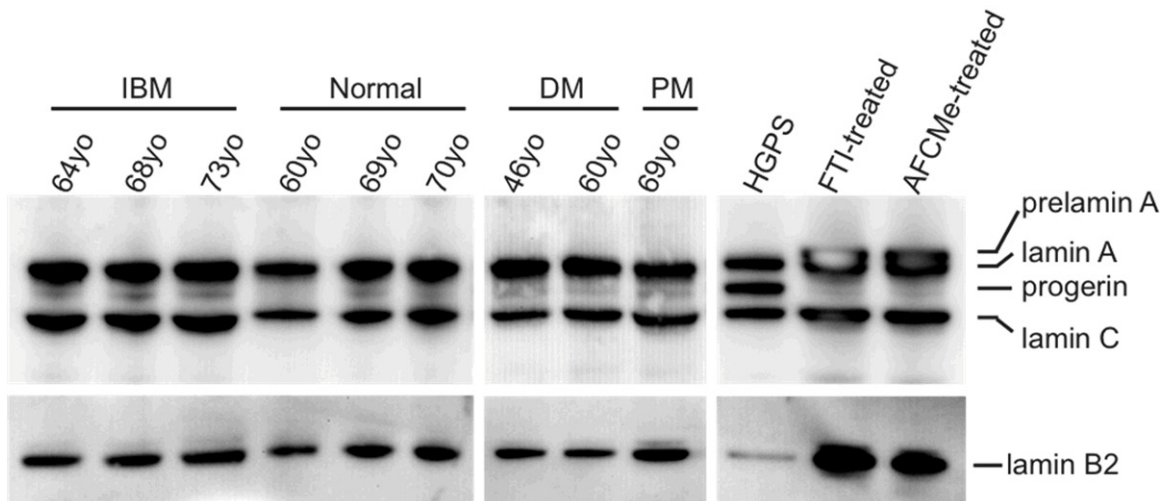


Figure 3. Western blots demonstrating lamin A and C bands in all s-IBM and normal control samples, but no prelamina A bands in these samples. A progerin band is also present in all s-IBM samples, but not in all normal individuals. HGPS fibroblasts, FTI-treated and AFCMe-treated myoblasts are used as positive control for progerin and prelamina A bands.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from HGPS fibroblast cultures and muscle sections were extracted using Trizol following the manufacturer's instructions (Invitrogen). One step RT-PCR using 100 ng of RNA and Superscript III (Invitrogen) was performed. Primers: *LMNA* and *LMNA* $\Delta 150$, (exon 7) AACTGGAGTCCCTGAGA, (exon 12): AGATTACATGATGCTGCAGTT; *LMNC*, (exon 6) GAGCGGGAGATGCCGAGAT, (exon 10) TCAGCGGCGGCTACACTCA. Reverse transcriptase-amplification reactions used the following conditions: 55°C for 30 min, 95°C for 10 min, 30 (for the amplification of *LMNC*) or 35 (for the amplification of *LMNA* and *LMNA* $\Delta 150$) cycles of 94°C for 30 sec, 60°C for 1 min, 68°C for 2 min. Amplicons were electrophoretically separated on 2% agarose gels and gel images were captured using the Chemi-Smart 3000 gel documentation system (Vilber Lourmat, Marne-la-Vallée, France).

Relative quantitative real time RT-PCR analysis

TaqMan qRT-PCR assays (Invitrogen) were used to detect *LMNA* $\Delta 150$, *LMNA* and *LMNC* transcripts [14]. A commercially available assay (Invitrogen) targeting human *RPLP0*, was included as an endogenous control. RNA was reverse-transcribed using the Superscript II cDNA synthesis kit (Invitrogen) with 50 ng random hexamers. Ten μ l real-time reactions with

5 μ l of TaqMan Universal Master Mix were performed in MicroAmp 384-well plate in the 7900HT Real-Time PCR system (Applied Biosystems). To increase the transcript detection capability, 4.8 μ l cDNA was used for *LMNA* $\Delta 150$ reactions and 2.5 μ l for *LMNA*, *LMNC* and *RPLP0* reactions. For *LMNA* $\Delta 150$ assays: 200 nM TaqMan MGB probe, 50 nM forward primer and 150 nM reverse primer were used, whereas for *LMNA* and *LMNC* assays: 200 nM MGB probe, 250 nM forward primer and 500 nM reverse primer were. Reactions were carried out in triplicate and end-point products were fractionated on 2% agarose gels and sequenced to confirm amplicon identities.

Standard curves for all three transcripts were obtained by diluting cDNA, prepared from RNA extracted from cultured HGPS fibroblasts. The detectable range was over a 5-log dilution, and the efficiencies were calculated to be 109.0%, 105.1%, 104.6% and 97.2% for *LMNA* $\Delta 150$, *LMNA*, *LMNC* and *RPLP0*, respectively.

Immunoblotting

Muscle tissue (10 mg) from 14 s-IBM, 3 disease control and 7 normal biopsies was cryosectioned and suspended in 200 μ l treatment buffer containing 125 mM Tris/HCl (pH 6.8), 15% SDS, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 9 μ l protease inhibitor cocktail (Sigma Aldrich) and 50 mM dithiothreitol.

Table 2. Densitometry analysis of western blots

| | s-IBM | Disease control | Normal | <i>p</i> value |
|------------------|-------------|-----------------|---------------|----------------|
| Lamin A | 2.35 ± 0.24 | 2.94 ± 0.74 | 2.20 ± 0.74 | 0.606 |
| Progerin | 0.09 ± 0.03 | 0.08 ± 0.01 | 0.07 ± 0.02 | 0.952 |
| Lamin C | 2.20 ± 0.15 | 1.80 ± 0.40 | 1.69 ± 0.43 | 0.317 |
| Lamin A/progerin | 24.84 ± 8.3 | 33.86 ± 7.10 | 33.89 ± 12.24 | 0.781 |
| Lamin A/C | 1.32 ± 0.21 | 1.64 ± 0.36 | 1.32 ± 0.37 | 0.412 |

Lamin B2 was used as loading control.

Protein extracts (15 µl) were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and electro-transferred to polyvinylidene fluoride membranes (Pall, Melbourne, Australia). Membranes were incubated with primary antibodies (polyclonal anti-lamin A/C, Santa Cruz, TX, 1:100; anti-progerin, Abcam, Sapphire Bioscience, 1:50; anti-lamin B2, Santa Cruz, 1:100) overnight at 4°C. The membranes probed with monoclonal antibodies were then incubated with Alkaline phosphatase labeled anti-mouse immunoglobulins (Novex Western-Breeze Immunodetection kit, Invitrogen), while membranes probed with polyclonal antibody were incubated with HRP labeled goat anti rabbit immunoglobulins (Dako, 1:2,000) for 1 hr. The membranes were then incubated with chemiluminescent substrate for 5 min. Images were captured by a Chemi-Smart 3000 gel documentation system (Vilber Lourmat) using Chemi-capt software and image densitometry analysis was performed using Bio-1D software. Lamin B2 was employed as the nuclear loading control because of its ubiquitous expression in muscle [15].

Immunocytochemistry

Sections from muscle biopsies from three cases of definite s-IBM were probed with progerin-specific antibody (using HGPS fibroblast cultures as a positive control), and with antibodies to full length or farnesylated prelamin A for the detection of non-farnesylated and farnesylated prelamin A. Human myoblast cultures treated with FTI-277 and AFCMe served as the positive controls [16].

After fixation in ice-cold methanol for 7 min, cultures and muscle sections were incubated with primary antibodies (anti-full length prelamin A, Diatheva, Fano, Italy, 1:200; anti-farnesylated prelamin A, Diatheva, 1:100; anti-progerin, 1:50, Abcam). Samples were then incubated with with Alexor Fluor 488 labelled

anti rabbit immunoglobulins (Invitrogen, 1:400) for 1 hr, and counterstained with Hoechst 33342 (Invitrogen, 1:4,000) for 5min. Petri dishes and slides were mounted with anti-fade glycerol reagent and viewed under a Nikon A1Si laser scanning confo-

cal microscope (Coherent Scientific, Hilton, Australia).

Data analysis

Real-time qPCR data were analyzed by the Sequence Detector System (SDS) software 1.3.1 (Applied Biosystems) with a standard curve analysis method to calculate the fold difference between samples and the calibrator sample (normal 25 years old). Linear regression was used to test the correlation between each transcript level (*LMNA*, *LMNC*, *LMNA* Δ150), the ratio of *LMNA*/*LMNA* Δ150 as well as *LMNA*/*LMNC* and age. For western blotting study, 2-way ANOVA was used to test the difference in lamin A/C and progerin levels among groups and if age is a confounding factor. Linear regression was used to test the relationship between lamin A/C, progerin, the lamin A/C and lamin A/progerin ratios, and age in each group. *P* values < 0.05 were considered statistically significant.

Results

RT-PCR of LMNA isoforms

As shown in **Figure 1**, RT-PCR showed clearly defined *LMNA* (740 bp) and *LMNC* (696 bp) bands in all s-IBM and control muscle samples. In addition, in the reactions employing *LMNA* primers, another smaller amplicon, which was shown by direct DNA sequencing to be the *LMNA* Δ150 progerin transcript, was present in some samples. A progerin band was identified in a higher proportion of s-IBM (8/11) and myositis control samples (4/4) than in normal controls (3/6), but the differences were not statistically significant after correcting for age.

Real-time qPCR of LMNA isoforms

Twelve s-IBM and 13 normal muscle samples were included for analysis of *LMNA* and *LMNA* Δ150, and 8 s-IBM and 13 normal samples for

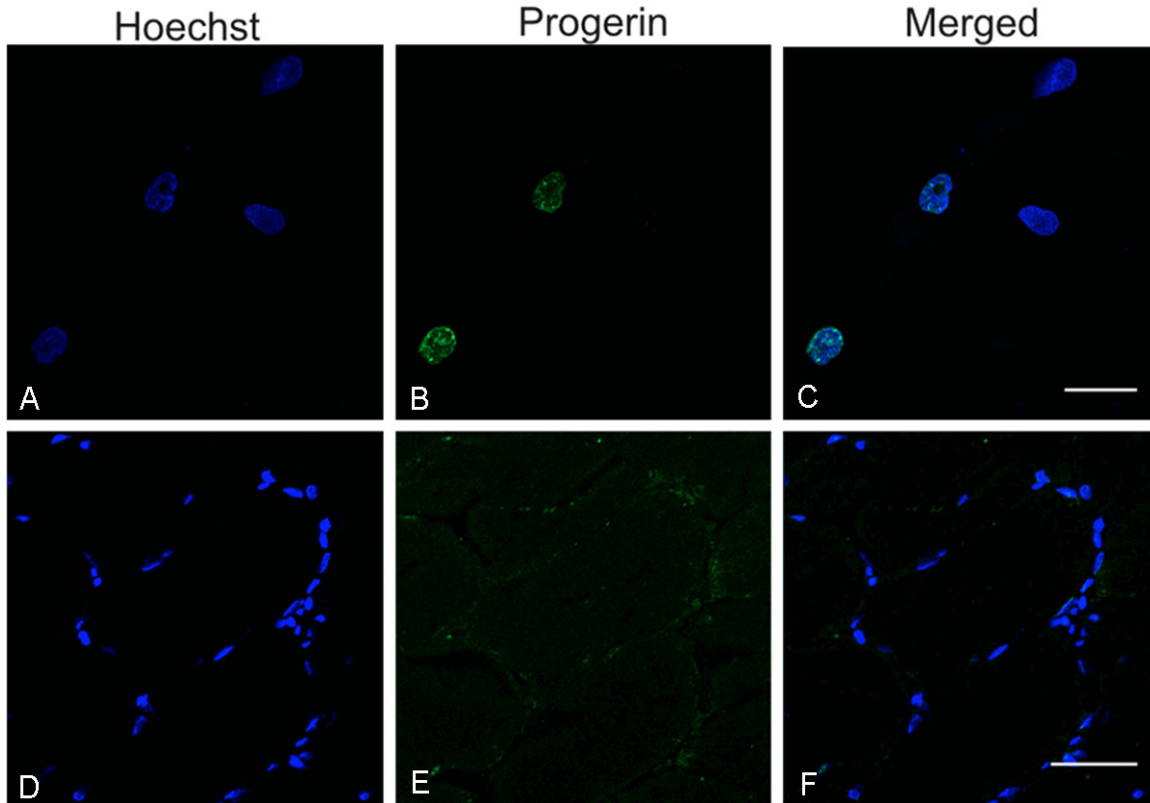


Figure 4. Immunostaining for progerin in HGPS fibroblast cultures (A-C) and s-IBM biopsy sections (D-F) showing strong nuclear staining in HGPS cells (B), but only non-specific interstitial staining without nuclear co-localisation in s-IBM muscle fibres (E). Primary antibody: anti-progerin. Magnification: $\times 600$. Scale bar: 50 μm .

LMNC. There was no difference in *LMNA* $\Delta 150$ levels between s-IBM and controls after correcting for age ($p = 0.604$). Although the levels of *LMNA* and *LMNC* were higher in the s-IBM than in the normal control group (**Table 1**), the differences were not statistically significant ($p = 0.395$ and 0.136) and the *LMNA*/*LMNC* ratio was not significantly different between s-IBM and normal individuals after correcting for age ($p = 0.408$). As shown in **Figure 2**, the *LMNA* and *LMNA* $\Delta 150$ transcripts increased with age in normal controls ($p = 0.014$ and 0.03 ; $R^2 = 0.439$ and 0.362 respectively), whereas this age-correlation was not found in the s-IBM samples ($p = 0.751$ and 0.858 ; $R^2 = 0.015$ and 0.005 respectively). The *LMNA*/*LMNA* $\Delta 150$ ratio decreased with age in the normal control group ($p = 0.028$, $R^2 = 0.368$), indicating that *LMNA* $\Delta 150$ increases at a faster rate than *LMNA*, but not in the s-IBM group ($p = 0.368$, $R^2 = 0.03$). There was no significant change in *LMNC* levels with age in either the normal control or s-IBM group ($p = 0.244$ and 0.905 ; $R^2 = 0.041$ and 0.002 respectively).

Western blotting

As shown in **Figure 3**, Lamin A and C bands were clearly identified in all samples. A faint band of an approximately 68 kDa molecular weight was present in most samples (s-IBM, 14/14; disease control, 3/3; age-matched normal, 6/7). This band was in alignment with the progerin band from HGPS fibroblast cultures, and is therefore deemed to be progerin. As shown in **Table 2**, there was no significant difference in lamin A, progerin or lamin C levels, or the lamin A/progerin, or lamin A/lamin C ratios, in the s-IBM, disease control and normal groups. There was no significant correlation with age of the levels of lamin A, progerin, lamin C, or the ratios of lamin A to progerin or lamin C in s-IBM or normal control muscle samples. There was no identifiable prelamin A band in either s-IBM or normal samples.

Immunocytochemistry

Progerin, farnesylated prelamin A and full length non-farnesylated prelamin A were readi-

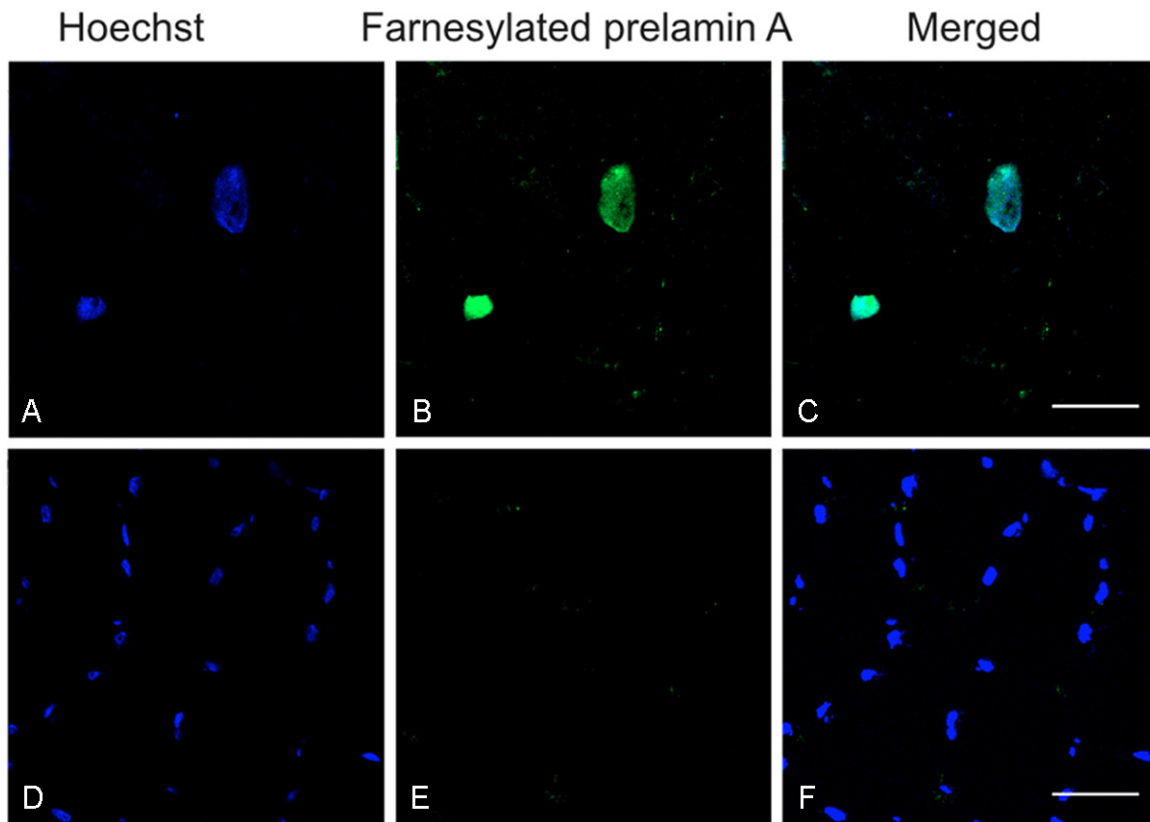


Figure 5. Immunofluorescence for farnesylated prelamin A on human myoblast culture treated with AFCMe (A-C), and s-IBM biopsy sections (D-F). Note nuclear staining in the myoblast culture (B), but only nonspecific interstitial staining in the s-IBM section, with no immunostaining of myonuclei (E). Primary antibody: antifarnesylated prelamin A. Magnification: $\times 600$. Scale bar: 50 μm .

ly demonstrated in HGPS fibroblast nuclei (20.1% of nuclei, **Figure 4**), in nuclei of human myogenic cells treated with AFCMe (10.2% of nuclei, **Figure 5**) and FTI-277 (12.7% of nuclei, **Figure 6**), but not in any myonuclei in the s-IBM muscle sections. No abnormalities of nuclear morphology of the type found in HGPS cells [17] were present in s-IBM muscle sections.

Discussion

Alternative processing of the *LMNA* transcript results in one of two isoforms through alternative 3' exon usage. The *LMNA* isoform consists of 12 exons, whereas the *LMNC* isoform is encoded by the first 10 exons and the first 123 bases of intron 10 which contributes to the 3' UTR of this transcript. The HGPS mutations activate a cryptic splice site that results in aberrant splicing with the loss of 150 bases from the end of exon 11. This cryptic splice site is occasionally used during processing of the normal transcript and low levels of the progerin

transcript have been detected in normal tissues [18, 19].

The present study explores the possible involvement of altered splicing during *LMNA* expression, and post-translational processing of prelamin A, in the pathogenesis of s-IBM. To our knowledge, this is the first study focusing on the role of normal and aberrant *LMNA* expression in s-IBM. We found that, as previously reported in normal muscle [12], there are low levels of both the progerin transcript and protein in s-IBM, however the levels of progerin mRNA and protein were not significantly different from those in normal muscle or other disease control samples. Moreover, we did not find any accumulation of unprocessed prelamin A in s-IBM muscle fibres, suggesting that a defect in the post-translational processing of prelamin A is unlikely to be present in s-IBM.

LMNA mRNA is first translated into a precursor protein prelamin A. It then goes through four post-translational processing steps at the C ter-

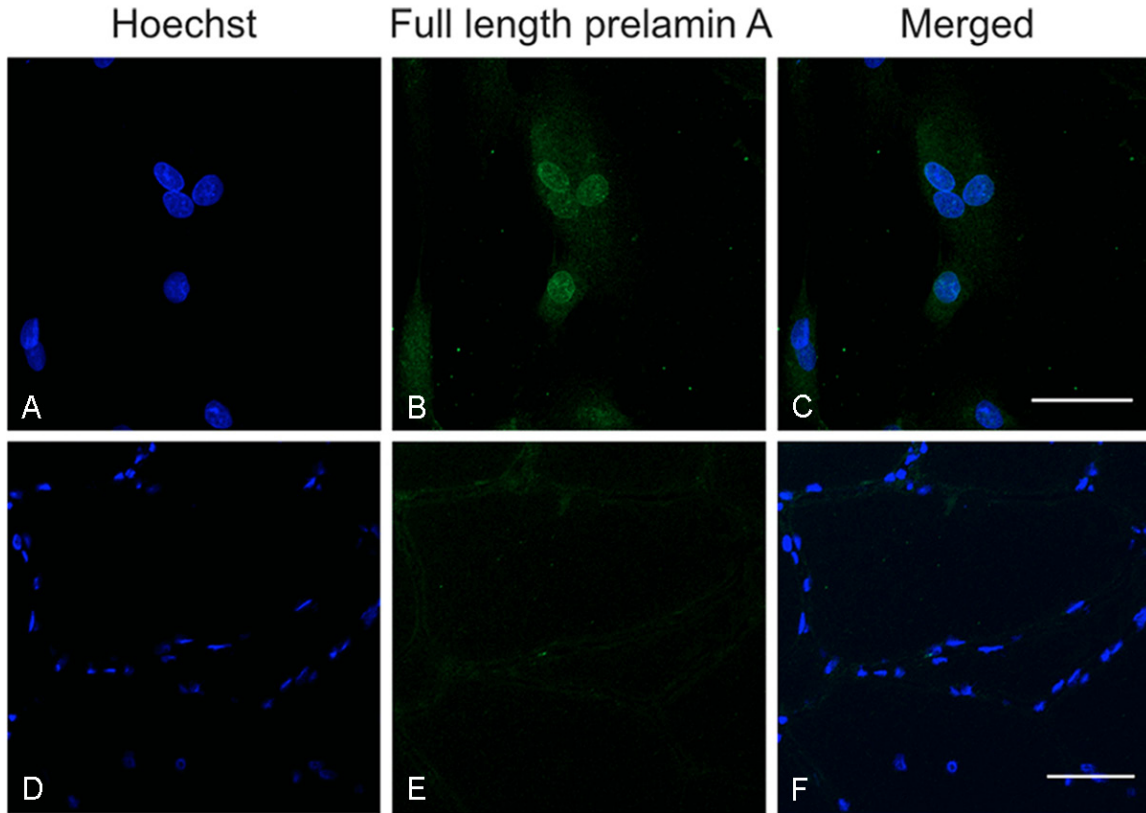


Figure 6. Immunofluorescence for full-length prelamin A on human myoblast culture treated with FTI-277 (A-C) and s-IBM muscle biopsy section (D-F), using anti-full-length prelamin antibody. Note positive staining for prelamin in myogenic cells (B), but no staining of myonuclei in s-IBM muscle fibres (E). Magnification: $\times 600$. Scale bar: 50 μm .

minal, namely farnesylation, cleaving of the last three amino acids, methylation, and finally cleaving of the last 15 amino acids that contains the previously farnesylated cysteine [20]. The half-life of prelamin A is approximately 100 minutes, and its intermediate forms are too low to be detected under normal conditions [21, 22]. The physiological functions of the processing of prelamin A include relocating prelamin A to the nuclear membrane and organization of chromatin [23, 24] and these functions are involved in muscle differentiation and maturation [25, 26]. While efficient production and processing of prelamin A is necessary for its proper positioning and function, pathogenic accumulation of the intermediate prelamin A isoforms can cause progeroid diseases, as well as cardiomyopathy [27-29]. The 50 amino acid deletion of progerin includes the cleavage site for the second endoproteolytic cleavage and retains the farnesyl group on the C terminal [17]. Insertion of the truncated progerin into the nuclear lamina causes defective cell mitotic behaviour and chromosome segregation,

genomic instability and widespread transcriptional changes, resulting in cellular senescence and premature ageing [30-32]. It is believed that the farnesyl group plays a major role in the negative dominant effect of progerin [33-35]. In this sense, progerin may be considered as a form of unprocessed prelamin A, and the accumulation of the farnesylated intermediate prelamin A isoforms are all potentially toxic to muscle cells. This point is exemplified by the lethal progeroid disease restrictive dermopathy, in which mutations in *LMNA* or *ZMPSTE* cause exon 11 skipping of *LMNA* resulting in a truncated isoform ($\Delta 11$), or an absence of Zmpste protein [36].

The age-dependent increase of *LMNA* and *LMNA* $\Delta 150$ in normal muscle is in accordance with our previous findings [12], and suggest a mechanism to compensate for deteriorating nuclear function in muscle cells with ageing [37]. In contrast, this age-related shift in *LMNA* expression was not found in s-IBM muscles. The significance of this is uncertain, but it is

likely to be secondary to the underlying pathological processes in the muscle, rather than a primary abnormality. It is known that there are active regenerative changes in s-IBM muscle [38], although there is some evidence that the regenerative capacity may be defective [39-41], and this may be accompanied by a change in the normal age-related shift in splicing, with upregulation of LMNC transcripts.

In conclusion, we found that there is a disruption of the normal age-related shift in the splicing pattern of LMNA and LMNC isoforms in s-IBM, which may be secondary to the ongoing processes of muscle damage and repair. However, we did not find any evidence of accumulation of prelamin A isoforms or increased progerin levels, or that these isoforms contribute to the pathogenesis of the disease or to accelerated ageing changes in s-IBM.

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Disclosure of conflict of interest

None.

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