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Increased expression of the Hutchinson–Gilford progeria syndrome truncated lamin A transcript during cell aging

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Most cases of the segmental progeroid syndrome, Hutchinson–Gilford progeria syndrome (HGPS), are caused by a *de novo* dominant mutation within a single codon of the *LMNA* gene. This mutation leads to the increased usage of an internal splice site that generates an alternative lamin A transcript with an internal deletion of 150 nucleotides, called lamin A Δ 150. The *LMNA* gene encodes two major proteins of the inner nuclear lamina, lamins A and C, but not much is known about their expression levels. Determination of the overall expression levels of the *LMNA* gene transcripts is an important step to further the understanding of the HGPS. In this study, we have performed absolute quantification of the lamins A, C and A Δ 150 transcripts in primary dermal fibroblasts from HGPS patients and unaffected age-matched and parent controls. We show that the lamin A Δ 150 transcript is present in unaffected controls but its expression is >160-fold lower than that in samples from HGPS patients. Analysis of transcript expression during *in vitro* aging shows that although the levels of lamin A and lamin C transcripts remain unchanged, the lamin A Δ 150 transcript increases in late passage cells from HGPS patients and parental controls. This study provides a new method for *LMNA* transcript analysis and insights into the expression of the *LMNA* gene in HGPS and normal cells.

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Introduction

Hutchinson–Gilford progeria syndrome (HGPS) or progeria is a severe genetic disorder affecting about 1 per 4–8 million live births. HGPS is classified as a segmental progeroid syndrome as multiple organs and tissues replicate phenotypes associated with normal aging.¹ Children with HGPS appear healthy at birth but develop distinctive clinical features during the first years of their

life, including severe growth retardation, and loss of subcutaneous fat and skin appendages. Most children suffer from osteoporosis and die in their early teens from heart attacks and strokes caused by progressive atherosclerotic disease.² Approximately 90% of classical HGPS is caused by a *de novo* mutation in exon 11 of the *LMNA* gene (1824C>T).^{3,4} The *LMNA* gene encodes the A-type lamins, which are intermediate filament proteins of the inner nuclear lamina. The inner nuclear lamina is functionally involved in the maintenance of nuclear shape and structure, DNA replication and gene expression.⁵

At least four different proteins are generated from the *LMNA* gene through alternative exon splicing and termination codon usage. Lamin A, encoded by exons 1–12, and lamin C, encoded by exon 1–10, are the major proteins

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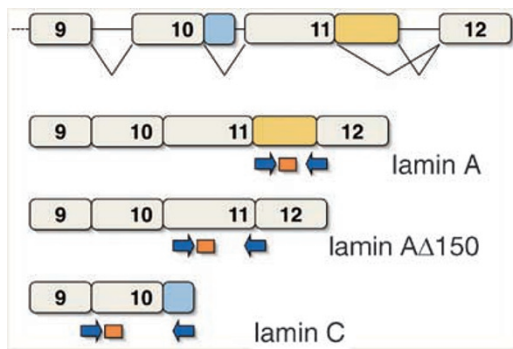


Figure 1 LMNA gene transcript quantification. Amplicons for lamins A, AΔ150 and C transcript quantification located in the 3' end of the LMNA gene (exons 9–12). Blue arrows and orange bars show the location of primers and TaqMan probes, respectively. The reverse primers for the lamins A and AΔ150 assays, and the forward primer for the lamin C assay covered exon–exon borders. The part of exon 10 specific for lamin C is marked in blue. The 150 nucleotide region of exon 11, which is deleted because of alternative splicing in the lamin AΔ150 transcript is marked in yellow. Not to scale.

expressed from the LMNA locus in differentiated cells (see Figure 1).^{6,7} The lamin AΔ10 protein is identical to lamin A except that it lacks exon 10. Lamin AΔ10 has been detected in the normal colon and a variety of carcinoma cell lines.⁸ Lamin C2 has an alternative exon 1 compared with lamin C, and is present in germ cells.⁹ In contrast to lamins C and C2, the lamins A and AΔ10 proteins contain CAAX boxes at their C-terminal ends, which undergo farnesylation and other post-translational modifications to become mature proteins.¹⁰

In addition to HGPS, there are 10 other different genetic disorders caused by mutations in the LMNA gene, and these disorders are collectively named laminopathies.¹¹ The majority of the laminopathies are caused by substitutions located in exons shared by all A-type lamins (LMNA exons 2–9; for a collection of LMNA variants, see the Leiden Muscular Dystrophy pages: <http://www.dmd.nl>). The most common HGPS mutation (1824C>T) is located in exon 11, and therefore only affects the lamin A protein isoforms. This mutation leads to the activation of a cryptic splice donor site that removes 150 nucleotides from exon 11. The resulting lamin AΔ150 mRNA gives rise to a lamin A isoform containing an internal deletion of 50 amino acids, referred to as lamin AΔ50 or progerin.^{3,4} This truncated lamin A protein is improperly processed because of the lack of a proteolytic cleavage site for the prelamins A processing enzyme, Zmpste24. In wild-type cells, Zmpste24 removes 15 amino acids from the C terminus, including a farnesylated and carboxymethylated cysteine.¹² In the absence of this cleavage site, this abnormal processing leads to the generation of a stably farnesylated and carboxymethylated lamin A protein isoform, referred to as progerin.¹³ In HGPS, the expression of progerin leads to irregular shaped nuclei, DNA repair defects, loss

of heterochromatin, changes in histone methylation, downregulation of nuclear proteins and interferes with the onset and progression of cytokinesis.^{13–18}

It is not clear how HGPS relates to normal physiological aging, but the identification of mutations in the LMNA gene has increased interest in the search for the role of the lamin proteins in the aging process. Studying HGPS cells, Goldman *et al*¹⁴ showed that nuclear defects accumulate as the cells become older in culture, which is accompanied by an increase in the amount of progerin. Progressive alterations in nuclear architecture also accompany aging in the nematode, *Caenorhabditis elegans*.¹⁹ Scaffidi and Misteli²⁰ showed that cells from normal individuals of old age have similar defects compared with those reported in cells from HGPS patients. This study also showed that the cryptic splice site, which is active in HGPS cells, is also used in cells and tissues from both young and old healthy individuals, and inhibiting the splice site in cells from old individuals reversed their nuclear abnormalities.²⁰ Additional evidence implicating progerin in the normal aging process comes from recent reports that show that the number of progerin protein positive cells increase with age, both *in vitro* and *in vivo*, in samples from apparently healthy individuals.^{18,21}

Cellular research in aging has been stimulated by the observation that human diploid cells have a limited number of cell divisions in culture, and therefore an analysis of cell cultures may provide valuable tools for studying the molecular mechanism of physiological senescence.²² In addition, isolated fibroblasts obtained from patients affected with premature aging syndromes, such as HGPS, show a markedly reduced life span when grown in culture.²³ Determination of the overall expression levels of the LMNA gene is an important step to further understanding of the progeria disease. In this study, we have developed a method for absolute quantification of LMNA locus transcripts (lamins A, AΔ150, and C). We utilized this method on RNA extracted from different passaged cells of HGPS affected individuals and controls to study changes during *in vitro* cell aging. Our data indicate that although the abundance of lamins A and C transcripts was unchanged, lamin AΔ150 transcripts increased during *in vitro* cell aging in both HGPS and unaffected parental control cells.

Materials and methods

Cell cultures and growth conditions

Primary dermal fibroblasts were obtained from the Coriell Cell Repositories (Camden, NJ, USA). Selection of HGPS cultures was according to confirmed G608G mutation and stock availability.⁴ The HGPS cultures were AG06917, AG06297, AG03198, AG03199, AG03513, AG11513, AG11498, AG01972, and GM01972. The age-matched control cultures were GM00499, GM00316, and GM01651. HGPS parent control cultures were AG06299, AG03257, AG03258, and AG03512. In some cases, multiple

Table 1 Primary dermal fibroblasts for the quantification of LMNA locus transcripts

Sample ID	Lot ^a	Passage ^b	Classification	Age (years) ^c	Gender
AG06917	A	15	HGPS (G608G)	3	Male
	B	13–15			
AG06297	C	21	HGPS (G608G)	8	Male
AG03198	A	10–12	HGPS (G608G)	10	Female
AG03199	A	10	HGPS (G608G)	10	Female
	B	8–11			
AG03513	E	12–15	HGPS (G608G)	13	Male
AG11513		6–12	HGPS (G608G)	13	Female
	A	9–15			
AG11498		6–12	HGPS (G608G)	14	Male
	A	8–12			
AG01972	B	13–21	HGPS (G608G)	14	Female
GM01972	B	15–21	HGPS (G608G)	14	Female
	B	13–18			
GM00499	B	13–18	Unaffected	8	Male
GM00316	D	14–24	Unaffected	12	Male
GM01651	B	12–24	Unaffected	13	Female
AG06299	B	27–42	Unaffected (HGPS parent)	34	Female
AG03257		1–12 ^d	Unaffected (HGPS parent)	35	Female
AG03258		3–9 ^d	Unaffected (HGPS parent)	36	Male
		1–12 ^d			
AG03512		4–15	Unaffected (HGPS parent)	41	Female

^aLot specified by Coriell cell repositories (CCR). Two lots from the same sample ID represent cultures ordered in different years and organized with the first ordered culture first and the most recent ordered culture second.

^bPassage or span of passages that was used for transcript quantification.

^cAge of patient when skin biopsy was obtained.

^dThe number of times the cell culture was subcultured at CCR was not determined.

cultures and/or lots from the same donor were included (see Table 1). Cells were grown in a monolayer in T25 flasks (Sarstedt) in a humidified 37°C 5% CO₂ incubator. Growth medium was minimal essential medium + earle's salt (Gibco) supplemented with 15% fetal bovine serum (Biowest), and 2 mM L-glutamine and 1 × penicillin–streptomycin (Gibco). Cells were split 1:4 on reaching confluency.

RNA isolation and cDNA synthesis

Total RNA was extracted from confluent T25 flasks of primary dermal fibroblasts using 2.5 ml of Trizol[™], according to standard procedure (Invitrogen). RNA pellets were dissolved in 70 µl of nuclease-free water (Ultrapure, Gibco), quantified using a spectrophotometer (BioPhotometer, Eppendorf), and treated with DNase I, according to the manufacturer's recommendations (RQ1 RNase-Free DNase, Promega). DNase was removed using RNeasy mini columns (Qiagen). 0.5 µg RNA was reverse transcribed with random hexamers using the SuperScript[™] cDNA Synthesis Kit (Invitrogen). cDNA samples were diluted in nuclease-free water to a final volume of 150 µl and stored at –80°C.

Taqman[®] assays

Primer and Taqman MGB probe sets were designed using Primer Express[®] Software version 2.0. (Applied Biosystems) and Primer 3.²⁴ One primer per amplicon was designed to cover an exon–exon boundary (Figure 1). Primer

sequences were lamin A (exons 11/12), 5'-TCTTCTGCCTC CAGTGTACAG-3' and 5'-AGTTCTGGGGGCTCTGGGT-3'; lamin C (exons 9/10), 5'-CAACTCCACTGGGGAAGAA GTG-3' and 5'-CGGCGGCTACCACTCAC-3'; lamin AΔ150 (exons 11/12), 5'-ACTGCAGCAGCTCGGGG-3' and 5'-TC TGGGGGCTCTGGGC-3'. Taqman MGB probe sequences were lamin A (exon 11), 5'-ACTCGCAGCTACCG-3'; lamin C (exon10), 5'-ATGCGCAAGCTGGTG-3'; lamin AΔ150 (exon 11), 5'-CGCTGAGTACAACCT-3'. Reporter and quencher dyes for the LMNA locus assays were 5'- 6FAM[™] and 3'- nonfluorescent quencher dye (NFQ; Applied Biosystems). Amplicon sizes were lamin A, 139 base pairs (bp); lamin C, 123 bp; lamin AΔ150, 123 bp. Real-time PCR reactions were performed in a 20 µl reaction volume, including 5 µl of template, in MicroAmp 96-well plates sealed with optical adhesive covers (Applied Biosystems). Each plate contained triplicates of cDNA samples, no template controls, and a minimum of six different dilutions of cloned standards. All assays were performed in a 1 × Taqman Universal master mix (Applied Biosystems). The concentration of oligos was initially optimized for each amplicon and the same batch of primers (Medprobe), and probes were used throughout this study. For lamin A, 300 nM forward primer, 900 nM reverse primer, and 200 nM MGB probe were used. For lamin C; 300 nM forward primer, 900 nM of reverse primer, and 200 nM MGB probe were used. For lamin AΔ150, 50 nM forward primer, 300 nM reverse primer, and 200 nM MGB probe were used.

For endogenous controls, we utilized validated assays for β -glucuronidase (GUSB) and ribosomal protein large PO (RPLPO) with VIC[®] and NFQ as reporter and quencher dyes, respectively (Applied Biosystems). Immediately after preparation, the plates were loaded on an ABI7500 fast system sequence detection instrument and the cycling conditions were 2 min at 50°C, 10 min at 95°C, then 50 cycles each of 15 s at 95°C and 40 s at 60°C.

Standard curves

The amplicons (lamins A, C, and A Δ 150, β -glucuronidase, and RPLPO) were amplified using standard PCR conditions from human cDNA prepared from EBV-transformed lymphoblastoid cell lines, AG03506 or AG03259 (Coriell Cell Repositories, Camden, NJ, USA). The PCR products were cloned into a pCR 2.1 vector (TOPO TA-cloning Kit, Invitrogen). The clones were purified using the Qiagen plasmid purification kit followed by a phenol–chloroform extraction and an ethanol precipitation. Plasmids were resuspended in nuclease-free water and sequenced to check that the insert had the correct sequence, and was only present in one copy. In addition, PCR using vector primers and restriction digest with EcoR1 confirmed single copy integration. Plasmid concentrations were determined using spectrophotometry (BioPhotometer, Eppendorf) and purity was confirmed by agarose gel electrophoresis. Purified plasmids were diluted in nuclease-free water and stored in single-use aliquots at –80°C. The same diluted plasmid preparations were used throughout this study. The standard dilutions ranged between 11 and 1.1×10^7 estimated copies of plasmids.

Data analysis

The fluorescence data were analyzed using the Sequence Detector System (SDS) Software 1.3.1 (Applied Biosystems). Amplification plots were analyzed by the automatic baseline option and fixed threshold values for each amplicon. Data were only considered acceptable if the correlation coefficient of the standard curve, with a minimum of five standards, exceeded 0.99, and the C_t values did not differ by >0.3 units among triplicates for C_t values <30, and not by >0.5 units for C_t values >30 units.²⁵ All three LMNA locus transcripts were normalized to the endogenous control β -glucuronidase in 1 μ g of total RNA. The transcript copy numbers for each of the LMNA locus transcripts were normalized to the transcript copy number for β -glucuronidase by dividing the mean of the triplicates for each LMNA locus transcript by the mean of the triplicates of β -glucuronidase for each individual sample.

Statistical analysis

All samples were assumed to be from normally distributed populations. One-way ANOVA or repeated measurement ANOVA was used to compare three independent or dependent groups, respectively. Significantly different means were post-tested using Tukey's multiple comparison test. The paired student's *t*-test, two-tailed, was used for statistical analysis of difference between early and late passages. First, normalized mean transcript levels for individual cell cultures and batches were calculated. Second, mean transcripts for each individual were calculated and used to represent normalized mean transcript values per individual (illustrated in Figure 2). Mean values

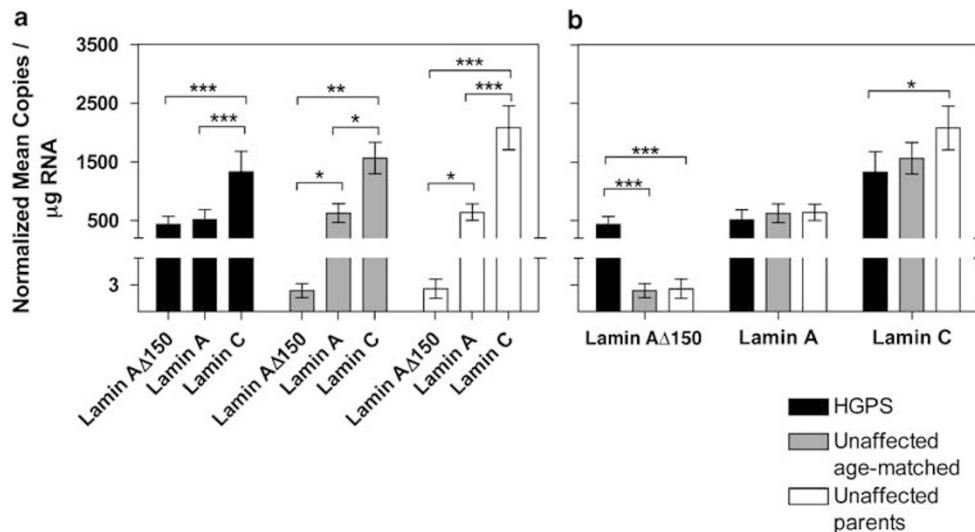


Figure 2 Absolute quantification of lamin A Δ 150, A and C transcripts in primary fibroblast from HGPS patients and unaffected controls. Same data were plotted in (a) and (b), and results represent means \pm SD. RNA was extracted from different passages of primary dermal fibroblasts from seven Hutchinson–Gilford progeria syndrome patients (HGPS, $n = 34$), three unaffected age-matched ($n = 12$), and four unaffected parent controls ($n = 24$). Asterisk represent significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

of early and late passages were calculated from all cell cultures where data from more than one passage were available (first per culture, second per individual; $n = 13$). For the HGPS sample group ($n = 6$), the early and late passages for RNA extraction ranged between passages 6–15 and 11–21, respectively. For the age-matched control group ($n = 3$), the early passage group included RNA from passage 12–13, and the late passage group included RNA from passage 18–24. For the adult control group ($n = 4$), the early passages included RNA from passages 4–27, and late passages included RNA from passage 15–42. However, as the record of earlier passaging was not available from the Coriell cell repositories for some of the cultures in the adult control group, the actual range of passages might be wider (Table 1).

RT-PCR analysis of LMNA splice forms

cDNA samples from HGPS samples and unaffected controls were tested for alternative LMNA splice forms. Expression of alternative lamins A and C transcript isoforms were analyzed using forward primers in exon 1, 5'-ACCTGCAGGAGCTCAATGAT-3', exon 2, 5'-CAAGAAGGAGGGTGACCTGA-3', and exon 3, 5'-ACCATGAAGGAGGAAGTGA-3', combined with a reverse primer in exon 12 (for lamin A), 5'-GTCCCAGATTACATGATGC-3' or exon 10 (for lamin C), 5'-CGGCGGCTACCACTCAC-3'. Additional forward primers for amplification of lamin A and/or C were exon 7/8, 5'-GCAACAAGTCCAATGAGGACCA-3'; exon 8/9, 5'-AGGTGGTGACGATCTGGG-3'; and exon 9/11, 5'-ACTCCACTGGGAAGGCTC-3'.

Protein analysis

Cells were collected from confluent T25 flasks, washed in PBS and lysed in RIPA buffer (including a cocktail of proteinase inhibitors (Roche)). Samples were stored at -80°C . Before loading, samples were thawed on ice and diluted in 16M of urea to a final concentration of 1.3M in a 5 × SDS loading buffer (including freshly added β -mercaptoethanol). The samples were denatured at 100°C for 5 min. Enhanced protein separation was accomplished using Protean II xi cell (Biorad) according to the previously described procedure.²⁶ To test for changes in protein levels during *in vitro* aging, protein extracts from multiple passages from the same culture were loaded on the same gel. For the HGPS sample group ($n = 6$), the early and late passages for protein extraction included passages 6–12 and 9–18, respectively. For the age-matched and adult unaffected control group ($n = 6$), the early and late passages for protein extraction included passages 1–27 and 6–42, respectively. As noted above, the actual range of passages may be wider. Primary antibodies used for western blot were mouse monoclonal antihuman lamin A/C (mab3211, Chemicon), goat polyclonal antihuman lamin A/C (sc-6215, Santa Cruz Biotechnology), mouse monoclonal antihuman lamin A (ab8980, Abcam Ltd) and rabbit

antihuman lamin C (BP4505, Acris antibodies). All blots were reincubated with antiactin (mouse monoclonal anti- β -actin, A5441, Sigma), which was used for normalization. Protein quantification was performed on western filters by densitometry using the Versa Doc Imaging system (Biorad) and analyzed with the Quantity One software (Biorad). The ratio of the normalized protein value from late passage of a culture to the normalized protein value from early passage obtained from the same culture was used to analyze changes in protein quantities with increased passages between the HGPS sample group and the unaffected control group. Statistical analysis was performed using the two-tailed unpaired student's *t*-test. To test for differences with aging, a one-sample *t*-test comparing the mean ratio for each protein and sample group to 1 was performed.

RESULTS

Validation of TaqMan assays

RNA was extracted at different passages from primary dermal fibroblasts from HGPS patients and controls (Table 1). Taqman assays for absolute quantification of lamins A, AΔ150, and C transcripts were designed (Figure 1), and conditions were optimized using different amounts of primers and probe. The combination with the highest ΔRn and the lowest C_t was considered to be the optimal assay. Agarose gel electrophoresis of PCR products from the cDNA template confirmed the specificity of the assays with a single band of expected length. There was no PCR product generated when genomic DNA, no RT, and no template controls were tested. Plasmids containing a single copy of the amplicon of correct identity were used for the construction of standard curves.

Endogenous controls were initially selected based on low inter-sample variability on RNA microarrays with human fibroblast cultures (data not shown), and earlier usage in quantitative RT-PCR experiments on HGPS cell lines.^{17,27} The selected endogenous controls, β -glucuronidase, and RPLPO were evaluated on our sample material ($n = 67$), and the absolute values for estimated copies were compared. Similar expression profiles were obtained with both endogenous controls (data not shown); but as the expression level of β -glucuronidase was closer to the levels of the LMNA locus transcripts, it was used for normalization.

LMNA locus transcript expression in HGPS and control dermal fibroblasts

Real-time RT-PCR of LMNA locus transcripts revealed expression of all three splice forms in all cDNA samples tested. As our approach used absolute quantification based on individual standard curves, we were able to compare different transcripts' expression levels within and between different samples (Figure 2). Comparison of the normalized mean copies of lamins A, C, and AΔ150

showed that lamin C was the most highly expressed transcript in all the sample groups (Figure 2a). In contrast to the HGPS sample group, where the lamin A and AΔ150 transcripts were observed to be at a similar level (normalized mean transcript of 516 and 439 copies/μg RNA, respectively), the levels of the lamin A transcripts were >240-fold higher than the lamin AΔ150 transcripts in the unaffected age-matched and parent control groups (normalized mean lamin A transcript copies were 627 and 644 copies/μg RNA, compared with the normalized mean lamin AΔ150 transcript copies of 2.4 and 2.6/μg RNA for the age-matched and parent control groups, respectively; Figure 2a).

Comparison of the normalized mean transcript levels of each transcript between the different sample groups (Figure 2b) showed that although there was no significant difference in the levels of lamin A transcripts, the levels of lamin AΔ150 differed significantly between the different sample groups (Figure 2b). As expected, the lamin AΔ150 transcript levels were higher, >160-fold, in the HGPS group compared with the control groups (HGPS (mean 439 copies/μg RNA) vs unaffected age-matched group (mean 2.4 copies/μg RNA), $P < 0.001$, and HGPS vs unaffected parent controls (mean 2.6 copies/μg RNA), $P < 0.001$). However, there was no significant difference in lamin AΔ150 transcript levels between the unaffected age-matched and parent control groups ($P > 0.05$).

Comparison of the lamin C transcript levels between the different sample groups revealed a significant difference in the mean normalized levels between the HGPS and the unaffected parental control group ($P < 0.05$; Figure 2b). However, there was no difference between the HGPS sample and the age-matched control groups in the level of lamin C transcripts ($P > 0.05$). This might indicate that lamin C expression is upregulated with age. However, comparison of lamin C levels between the differently aged control groups did not show any significant difference ($P > 0.05$; Figure 2b).

Lamin AΔ150 transcript level increases with *in vitro* cell aging

To analyze the changes in expression associated with *in vitro* cell aging, we calculated the mean expression of each transcript in each sample from early and late passages (Figure 3). Only cultures where multiple passages were collected for RNA extraction were included. A comparison of lamin AΔ150 transcripts in early passage HGPS cells with late passage HGPS cells revealed a significantly higher expression in later passages ($P = 0.047$; Figure 3a). A significantly higher expression of lamin AΔ150 transcripts was also seen in late passage cells from unaffected adult controls, when compared with early passage cells of the same control group ($P = 0.007$; Figure 3a). No other significant difference was seen when

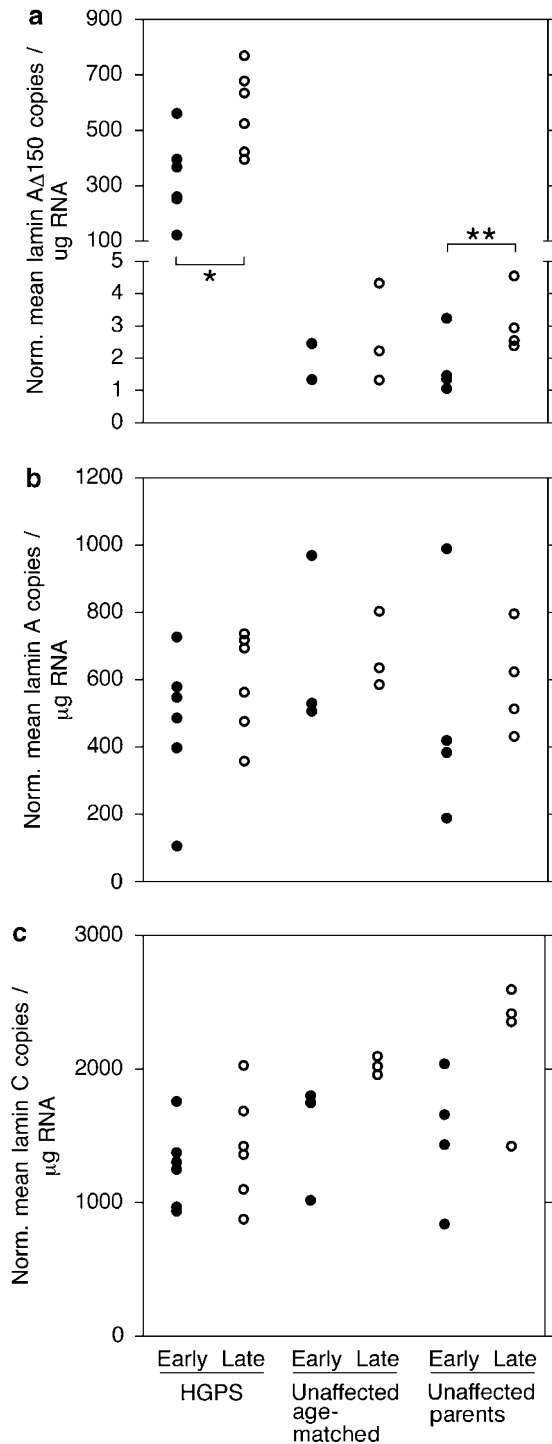


Figure 3 LMNA locus transcripts in early and late passage cells. Results represent means and \pm SD. Asterisk represent significant differences: * $P < 0.05$, ** $P < 0.01$.

analyzing differences in the expression of LMNA locus transcripts between early and late passages for the different sample groups (Figure 3b–c).

Lamin A/C protein isoforms

To test whether the changes in the RNA level were also evident at the protein level, protein was extracted from HGPS and unaffected control cells of different passages. Western blots with antibodies to human lamin A/C were used to study lamin A and C protein isoform expression during passaging (Figure 4). Enhanced protein separation revealed proteins corresponding in size to prelamin A, lamin A, progerin, and lamin C, when hybridized with antibodies to human lamin A/C (Figure 4a right top panel, and 4c). In addition, one extra fragment migrating between progerin and lamin C was seen in protein extracts from all individuals (HGPS and controls) (Figure 4c). The extra fragment was also seen when blots were hybridized with a lamin C-specific antibody (Figure 4a middle top panel).

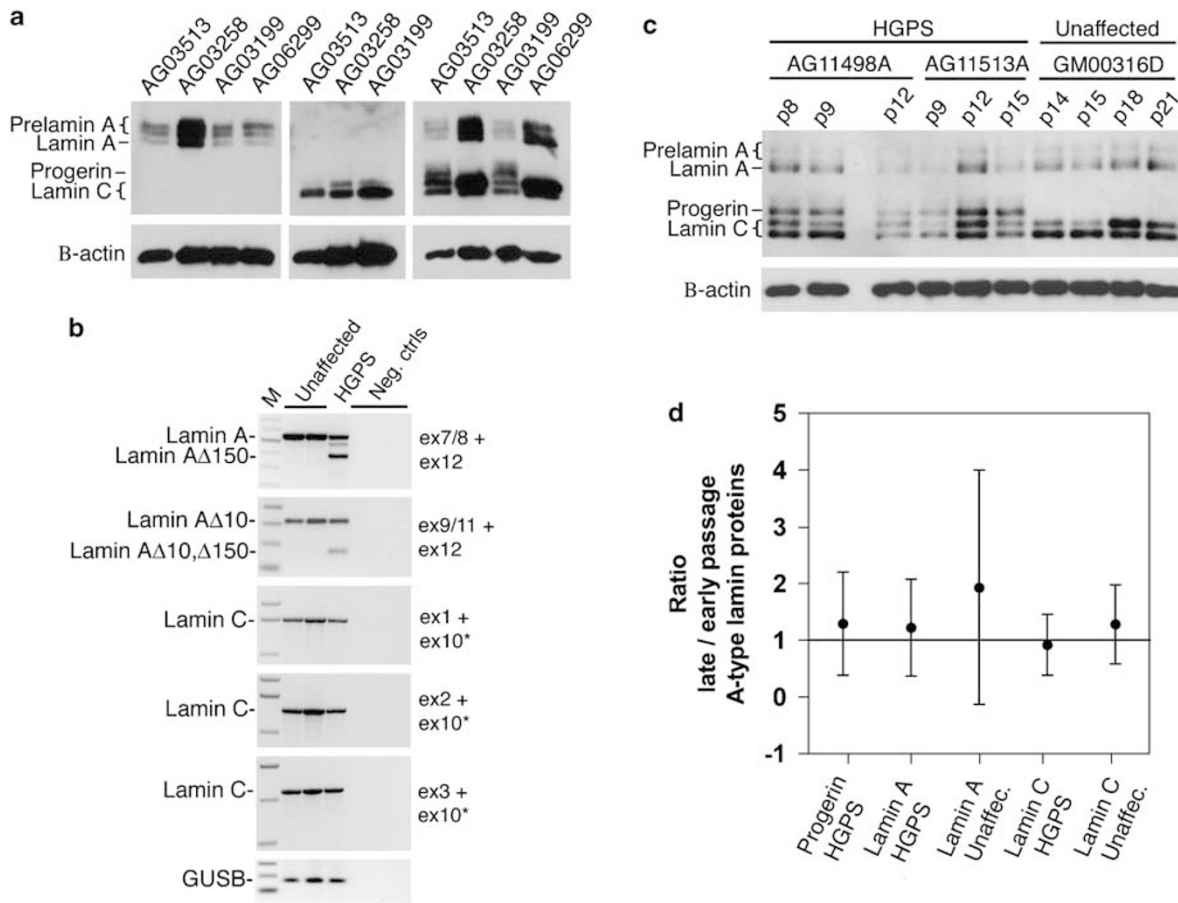


Figure 4 A-type lamins in primary fibroblasts from HGPS and unaffected controls. (a) Western blot experiments with antibodies to lamins A (left panel), C (middle panel), and A/C (right panel). (b) RT-PCR screening with exon (ex) 7/8 or ex9/11, and ex12 primer showed fragments corresponding to lamins A (640 base pairs, bp) and A Δ 150 (490 bp), or lamins A Δ 10 (319 bp) and A Δ 10, Δ 150 (169 bp), respectively. RT-PCR with primers in ex1, 2, or 3, and ex10, resulted in fragments corresponding to lamin C (1612, 1350, and 1118 bp, respectively). Asterisk (*) indicates that the primer was located in the region of exon 10 specific for lamin C. Negative control lanes contained PCR samples with genomic DNA, no RT, and no template controls (M, 100 bp DNA ladder (Invitrogen)). (c) Representative western blot with protein extracts from multiple passages of fibroblast cell cultures. (d) Changes in protein levels during passaging illustrated as the ratio of normalized band intensities for late and early passage protein extracts of the same culture. Results represent means \pm SD.

In accordance with the unknown fragment being an isoform of lamin C, the extra fragment was not seen when filters were hybridized with the lamin A-specific antibody (Figure 4a left top panel). To elucidate whether the extra fragment seen on the western blots corresponded to novel LMNA splicing event, we performed an RT-PCR analysis of the LMNA gene. RT-PCR analysis using forward primers located in exons 1, 2, 3, spanning the junction of exons 7 and 8 (7/8), and 8/9, and reverse primers in the region of exon 10 specific to lamin C, or exon 12 (specific for lamin A) did not reveal any novel LMNA splice form that could explain the extra fragment seen on western blot (Figure 4b, data not shown). RT-PCR products corresponding in size to the expected fragments for lamins A or C were present in all tested cDNA samples. In cDNA samples from HGPS patients,

there was an additional band corresponding to lamin A Δ 150 whenever the reverse primer located in exon 12 was used (Figure 4b, top panel).

Earlier reports have identified a splice variant of lamin A that lacked exon 10, lamin A Δ 10.⁸ This splice form was detected in our cDNA samples when using a forward primer spanning the junction of exons 9 and 11, and a reverse primer in exon 12 (Figure 4b, second panel). This same primer pair also amplified a shorter fragment in HGPS samples, which showed that both lamin A and A Δ 150 transcripts have alternative splice forms that lack exon 10.

Quantification of LMNA protein isoforms

Protein extracts from different passages of primary fibroblast cultures from HGPS patients and unaffected controls were analyzed using western blot, with antibodies to human lamin A/C (Figure 4c). All protein quantifications were performed on western blots hybridized to the human lamin A/C antibody, mab3211 (Figure 4c). Similar results were also seen with an N-terminal lamin A/C antibody, N-18 (data not shown). Samples were normalized to β -actin. Quantification of lamin A included the band densities for proteins corresponding in size to prelamin A and lamin A (Figure 4c). Quantification of lamin C included the band densities for the two fragments recognized by the lamin C-specific antibody and that corresponded to lamin C in size. A fragment corresponding in size to progerin was only seen in samples from HGPS patients (Figure 4a and c, and data not shown). To analyze changes in lamins A, C, and progerin proteins during *in vitro* cell aging, the normalized band densities from late passage cells were divided by the normalized band densities from early passage cells of the same cell culture. If the obtained ratio was higher than 1, the protein expression had increased with passaging, but if the ratio was lower than 1, the protein expression had decreased with passaging.

Analysis of changes in lamins A, C, and progerin protein levels with aging in HGPS cells, did not show a statistical significant difference to 1 for any of the proteins (mean values were 1.2 ($P=0.586$), 0.9 ($P=0.741$), and 1.3 ($P=0.458$) for lamins A, C, and progerin, respectively; Figure 4c and d). Similarly, there was no significant increase in lamins A and C protein levels with aging in unaffected control cells (sample group included samples from both age-matched and parents; mean values were 1.9 ($P=0.318$) and 1.3 ($P=0.363$), respectively). In addition, large standard deviations showed that all sample groups contained samples in which protein levels had both increased and decreased with passage number (Figure 4c and d). There was no significant difference between the mean ratios of lamin A or C when comparing the HGPS and unaffected control group ($P=0.494$ and $P=0.339$, respectively; Figure 4d).

Discussion

Determination of the overall expression levels of the LMNA locus transcripts is an important step in further understanding the molecular mechanisms of the HGPS. Existing data on the expression of the LMNA gene in HGPS are based on semiquantitative RT-PCR techniques, or there is a lack of data on the expression of lamin C.^{20,21,28} In this study, we have developed a method for absolute quantification of the lamins A, C, and A Δ 150 transcripts. Absolute quantification of these transcripts during *in vitro* aging of HGPS patient cell cultures and unaffected controls showed different expression levels and regulation. To analyze normal changes in the LMNA gene expression during aging, samples from two differently aged normal control groups were included. Our results showed that lamin C is the most highly expressed LMNA locus transcript, in both HGPS and control groups. Considering that lamins A and C are regulated by the same promoter, our results would suggest that the splicing efficiency for the lamin C transcript was favored and/or the lamin C transcripts were more stable. Lamin C transcripts were significantly higher in the unaffected parent control samples when compared with those in samples from HGPS. However, as there was no significant difference in lamin C expression between HGPS and age-matched controls, or between the two control groups, these results are hard to interpret.

In accordance with earlier studies,^{20,21} we have shown that the lamin A Δ 150 transcript is present in cells from normal individuals, in addition to cells from HGPS patients. Our results showed that the lamin A Δ 150 transcript level was >160-fold higher in samples from HGPS compared with that in the unaffected controls. An earlier study has reported a 50-fold lower expression of the lamin A Δ 150 transcript in normal cells compared with that in HGPS cells.²⁰ The difference in the results could be a result of differences in methodology or by variations in progerin mRNA levels between HGPS patients.²⁸

In this study, we have shown that lamin A Δ 150 transcript levels in HGPS cells are present at the same levels as lamin A transcripts in unaffected controls. This is in agreement with findings from an earlier study,²⁹ which showed that both the wild-type and mutant alleles in HGPS patients are capable of comparable levels of transcription. Perhaps a surprising finding in this study was that the presence of lamin A Δ 150 transcripts in HGPS cells did not affect the normal transcript levels of lamin A. Absolute quantification of the LMNA locus transcripts in HGPS cells showed that the combined amounts of lamins A and A Δ 150 are about twice the amount compared with what are found in normal controls (Figure 2). This might be explained by differences in mRNA stability and accumulation of the lamin A Δ 150 transcript, or increased LMNA gene expression in HGPS cells. Given that both the lamins C and A transcript copy numbers in HGPS cells were similar to what was seen in unaffected age-matched control

samples, an upregulation of LMNA gene expression would then have to be combined with a decreased splicing to form lamin C, and/or increased splicing to form lamins A and AΔ150.

Our quantification of lamins A, C and AΔ150 transcripts in RNA from different passages of cells showed that although the lamin A and C transcripts were unchanged, the lamin AΔ150 transcript levels increased in both late passage HGPS and unaffected parent control cells compared with that in early passage cells. These results might indicate a similar mechanism in progeria and normal aging cells with a reduced stringency of the splicing machinery with aging. Cells from normal younger individuals are somehow protected, and therefore less prone to abnormal splicing during *in vitro* aging. However, in this study and in agreement with earlier reports, using a different experimental approach,^{20,21} there was no difference in the lamin AΔ150 transcripts levels between differently aged normal control samples (Figure 2b), which argues against age as a significant modifier of lamin AΔ150 expression. Future studies with larger control sample sets and perhaps with samples from older individuals might relate our results to *in vivo* cellular aging.³⁰

Extensive RT-PCR analysis of novel LMNA splicing did not reveal any alternative splice forms, which suggests that the two lamin C fragments, seen in our samples using enhanced protein separation, migrate differently because of differences in post-translational modification. Our results emphasize the importance of using larger gels or high separation when analyzing proteins of similar molecular weights.

In contrast with earlier studies on HGPS cells,^{14,31} we have, in this study, shown that the amount of progerin protein does not increase with passaging. This is in agreement with a recent study from Verstraeten *et al*³² where progerin levels remained constant during passaging of HGPS cells. It is unclear whether the differences between the different studies reflect actual inter-sample variations of progerin or is a consequence of differences in experimental techniques. In our relative quantification of lamins A, C, and progerin proteins in early and late passages, we have seen varied results within the sample groups, which are reflected by extensive standard deviations. This would argue in favor of inter-sample variation of protein levels.

In summary, we have developed a method for absolute quantification of LMNA locus transcripts, which should be useful for future studies on HGPS, laminopathies, and lamins involvement in physiological aging.

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Conflict of interest

None.

References

- Martin GM: Genetic modulation of senescent phenotypes in *Homo sapiens*. *Cell* 2005; **120**: 523–532.
- Pollex RL, Hegele RA: Hutchinson–Gilford progeria syndrome. *Clin Genet* 2004; **66**: 375–381.
- De Sandre-Giovannoli A, Bernard R, Cau P *et al*: Lamin a truncation in Hutchinson–Gilford progeria. *Science* 2003; **300**: 2055.
- Eriksson M, Brown WT, Gordon LB *et al*: Recurrent *de novo* point mutations in lamin A cause Hutchinson–Gilford progeria syndrome. *Nature* 2003; **423**: 293–298.
- Goldman RD, Gruenbaum Y, Moir RD, Shumaker DK, Spann TP: Nuclear lamins: building blocks of nuclear architecture. *Genes Dev* 2002; **16**: 533–547.
- Fisher DZ, Chaudhary N, Blobel G: cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proc Natl Acad Sci USA* 1986; **83**: 6450–6454.
- Rober RA, Weber K, Osborn M: Differential timing of nuclear lamin A/C expression in the various organs of the mouse embryo and the young animal: a developmental study. *Development* 1989; **105**: 365–378.
- Machiels BM, Zorenc AH, Ender JM: An alternative splicing product of the lamin A/C gene lacks exon 10. *J Biol Chem* 1996; **271**: 9249–9253.
- Furukawa K, Inagaki H, Hotta Y: Identification and cloning of an mRNA coding for a germ cell-specific A-type lamin in mice. *Exp Cell Res* 1994; **212**: 426–430.
- Beck LA, Hosick TJ, Sinensky M: Isoprenylation is required for the processing of the lamin A precursor. *J Cell Biol* 1990; **110**: 1489–1499.
- Capell BC, Collins FS: Human laminopathies: nuclei gone genetically awry. *Nat Rev Genet* 2006; **7**: 940–952.
- Sinensky M, Fantle K, Trujillo M, McLain T, Kupfer A, Dalton M: The processing pathway of prelamin A. *J Cell Sci* 1994; **107**: 61–67.
- Dechat T, Shimi T, Adam SA *et al*: Alterations in mitosis and cell cycle progression caused by a mutant lamin A known to accelerate human aging. *Proc Natl Acad Sci USA* 2006; **104**: 4955–4960.
- Goldman RD, Shumaker DK, Erdos MR *et al*: Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson–Gilford progeria syndrome. *Proc Natl Acad Sci USA* 2004; **101**: 8963–8968.
- Shumaker DK, Dechat T, Kohlmaier A *et al*: Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging. *Proc Natl Acad Sci USA* 2006; **103**: 8703–8708.
- Ly DH, Lockhart DJ, Lerner RA, Schultz PG: Mitotic misregulation and human aging. *Science* 2000; **287**: 2486–2492.
- Csoka AB, English SB, Simkevich CP *et al*: Genome-scale expression profiling of Hutchinson–Gilford progeria syndrome reveals widespread transcriptional misregulation leading to mesodermal/mesenchymal defects and accelerated atherosclerosis. *Aging Cell* 2004; **3**: 235–243.
- Cao K, Capell BC, Erdos MR, Djabali K, Collins FS: A lamin A protein isoform overexpressed in Hutchinson–Gilford progeria syndrome interferes with mitosis in progeria and normal cells. *Proc Natl Acad Sci USA* 2007; **104**: 4949–4954.

- 19 Haithcock E, Dayani Y, Neufeld E *et al*: Age-related changes of nuclear architecture in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 2005; **102**: 16690–16695.
- 20 Scaffidi P, Misteli T: Lamin A-dependent nuclear defects in human aging. *Science* 2006; **312**: 1059–1063.
- 21 McClintock D, Ratner D, Lokuge M *et al*: The mutant form of lamin A that causes Hutchinson–Gilford progeria is a biomarker of cellular aging in human skin. *PLoS ONE* 2007; **2**: e1269.
- 22 Hayflick L: The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 1965; **37**: 614–636.
- 23 Brown WT: Genetic diseases of premature aging as models of senescence. *Annu Rev Gerontol Geriatr* 1990; **10**: 23–42.
- 24 Rozen S, Skaletsky H: Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000; **132**: 365–386.
- 25 Eriksson M: Real-time RT-PCR for CTG repeat-containing genes. *Methods Mol Biol* 2004; **277**: 77–84.
- 26 Sagelius H, Rosengardten Y, Hanif M *et al*: Targeted transgenic expression of the mutation causing Hutchinson–Gilford progeria syndrome leads to proliferative and degenerative epidermal disease. *J Cell Sci* 2008; **121**: 969–978.
- 27 Nagasaka Y, Dillner K, Ebise H *et al*: A unique gene expression signature discriminates familial Alzheimer's disease mutation carriers from their wild-type siblings. *Proc Natl Acad Sci USA* 2005; **102**: 14854–14859.
- 28 Moulson CL, Fong LG, Gardner JM *et al*: Increased progerin expression associated with unusual LMNA mutations causes severe progeroid syndromes. *Hum Mutat* 2007; **28**: 882–889.
- 29 Reddel CJ, Weiss AS: Lamin A expression levels are unperturbed at the normal and mutant alleles but display partial splice site selection in Hutchinson–Gilford progeria syndrome. *J Med Genet* 2004; **41**: 715–717.
- 30 Reff M, Schneider EL: Cell culture aging. *Mol Cell Biochem* 1981; **36**: 169–176.
- 31 McClintock D, Gordon LB, Djabali K: Hutchinson–Gilford progeria mutant lamin A primarily targets human vascular cells as detected by an anti-lamin A G608G antibody. *Proc Natl Acad Sci USA* 2006; **103**: 2154–2159.
- 32 Verstraeten VL, Ji JY, Cummings KS, Lee RT, Lammerding J: Increased mechanosensitivity and nuclear stiffness in Hutchinson–Gilford progeria cells: effects of farnesyltransferase inhibitors. *Aging Cell* 2008; **7**: 383–393.