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Accelerated Telomere Shortening and Replicative Senescence in Human Fibroblasts Overexpressing Mutant and Wild Type Lamin A

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Abstract

LMNA mutations are responsible for a variety of genetic disorders, including muscular dystrophy, lipodystrophy, and certain progeroid syndromes, notably Hutchinson-Gilford Progeria. Although a number of clinical features of these disorders are suggestive of accelerated aging, it is not known whether cells derived from these patients exhibit cellular phenotypes associated with accelerated aging. We examined a series of isogenic skin fibroblast lines transfected with *LMNA* constructs bearing known pathogenic point mutations or deletion mutations found in progeroid syndromes. Fibroblasts overexpressing mutant lamin A exhibited accelerated rates of loss of telomeres and shortened replicative lifespans, in addition to abnormal nuclear morphology. To our surprise, these abnormalities were also observed in lines overexpressing wild-type lamin A. Copy number variants are common in human populations; those involving *LMNA*, whether arising meiotically or mitotically, might lead to progeroid phenotypes. In an initial pilot study of 23 progeroid cases without detectable *WRN* or *LMNA* mutations, however, no cases of altered *LMNA* copy number were detected. Nevertheless, our findings raise a hypothesis that changes in lamina organization may cause accelerated telomere attrition, with different kinetics for overexpression of wild-type and mutant lamin A, which leads to rapid replicative senescence and progeroid phenotypes.

Keywords

Lamin; laminopathies; progeroid syndromes; Hutchinson-Gilford Progeroid Syndrome; Aging

Introduction

The *LMNA* gene encodes two nuclear intermediate filament proteins, lamin A and lamin C, via alternative splicing. *LMNA* mutations are responsible for at least nine classes of disorders; Emery-Dreifuss muscular dystrophy [1], dilated cardiomyopathy type 1A with or without atrio-ventricular conduction disturbance [2], limb-girdle muscular dystrophy type 1B [3], Charcot-Marie-Tooth disease type 2 [4], Dunnigan-type familial partial lipodystrophy [5], mandibuloacral dysplasia [6], Hutchinson-Gilford Progeria Syndrome (HGPS) [7,8], restrictive dermopathy (RD) [9] and atypical Werner syndrome (WS) [10]. Overlapping syndromes of various combinations have been widely reported as well [11].

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A majority of the disease mutations of *LMNA* are heterozygous amino acid substitutions that are located across the *LMNA* gene [12]. Cases with haploinsufficiency or homozygous mutations have also been reported [4,6]. A hierarchical cluster analysis revealed interesting genotype/phenotype correlations [13]. There was a trend for mutations in the alpha-helical regions to cause muscular dystrophy, while mutations in globular regions were more likely to have been responsible for lipodystrophies. Progeroid syndromes, reported as either atypical HGPS or atypical WS, tend to be associated with amino acid substitutions in the heptad region of lamin A/C [8,10].

Classical HGPS and RD are due to point mutations that generate new splicing sites, resulting in in-frame deletions of the C-terminal end of prelamin A [7–9]. The deleted regions include the endoproteolytic sites for Zmpste24, sites that are required for the conversion of prelamin A to mature lamin A [14]. The diseases caused by splicing mutations are more severe than those resulting from missense mutations. Therefore, it has been postulated that the accumulation of prelamin A is the proximal cytotoxic abnormality in HGPS [15]. Correlation has also been observed between the lengths of the in-frame deletions and the severity of the diseases. The common HGPS mutation results in a 50 amino acid deletion; these patients have a median lifespan of 13 years. An atypical HGPS patient, who lived until his 5th decade, had a 35 amino acid deletion [16].

Abnormal nuclear morphology is a hallmark of *LMNA* mutant cells. Cells isolated from *Lmna*^{−/−} mice exhibit nuclear deformations, defective mechanotransduction, and impaired viability following mechanical strain [17]. In HGPS cells, nuclear lamina had a significantly reduced ability to rearrange in response to mechanical stress and the nuclei had a reduced deformability [18]. This may partly explain the increase in apoptosis of primary HGPS fibroblasts as they age in culture [19].

Global gene expression has been examined both at the protein [20] and mRNA levels [21]. The latter showed an increase in the expression of a transcription factor implicated as a negative regulator of mesodermal tissue proliferation, consistent with clinical phenotypes that reflect developmental abnormalities of mesenchymal cell lineages.

Most of our knowledge of the impact of *LMNA* mutations upon the replicative potential of somatic cells comes from studies of fibroblasts from patients with HGPS. Early studies gave mixed results [22,23] and, while showing impaired proliferation compared to most age-matched controls, cultures from a single HGPS patient did not exhibit the dramatic replicative senescence observed with cultures from patients with the Werner syndrome [24]. More recent reports have documented significant limitations of the replicative lifespans of primary fibroblasts from HGPS subjects when cultured under the usual hyperoxic conditions of ambient oxygen [25]. Cumulative population doublings (CPDs) of HGPS fibroblasts ranged from 20–30, while CPDs of age-matched controls achieved approximately 60 CPDs [19]. Telomeres of skin fibroblasts derived from the HGPS patients were also shown to be shorter than those of age-matched controls [26]. These measurements were only made at a single point, however. The replicative life spans and the kinetics of telomere loss associated with other *LMNA* mutations, including those found in atypical Werner syndrome, have not been well studied. We have therefore investigated how these major cellular phenotypes are impacted by a range of pathogenic *LMNA* mutations.

Materials and Methods

Cell lines and cell culture

A line of human diploid fibroblasts, 82-6, had been previously “immortalized” with a construct encoding an excisable *hTERT* [27] and an L-histidinol resistance gene. The resulting line was

named 82-6pBlox. Several human full length *LMNA* cDNAs, wild-type, R133L, L140R and $\Delta 50$ mutants, were obtained by RT-PCR from cells derived from the patients carrying these mutations [10,28]. The $\Delta 35$ mutant *LMNA* cDNA was generated by mutagenesis of wild-type *LMNA* cDNA. The *LMNA* cDNAs were subcloned into a pLXSN vector with a neomycin resistant gene and introduced into 82-6pBlox to generate lamin A overexpression lines. After cultures were expanded, the hTERT was removed by superinfection with pLXSH-Cre to express a Cre recombinase. This results in the excision of hTERT and L-histidinol and activates puromycin expression from the pBlox vector. After hTERT was removed, the cultures were maintained in 0.4 μ g/ml puromycin to continue to select against *hTERT* (+) clones [27].

Western blot analysis

20 μ g of total proteins were separated on a 4–12% gradient Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and transferred to nylon filters. Filters were incubated with anti-lamin A/C (clone JoL2, Chemicon International, Temecula, CA), or anti- β -actin (clone AC-15, Sigma, St Louis, MO), then with secondary antibodies against mouse IgG (BA-9200, Vector Laboratories, Burlingame, CA), and visualized by chemiluminescence [10,28].

Immunostaining and Nuclear morphology

Exponentially growing cultures were plated on glass coverslips, fixed with 3% paraformaldehyde in PBS, pH 7.4, permeabilized with 1% Triton-X in PBS, and incubated with anti-lamin A/C (clone JoL2, Chemicon International, Temecula, CA) followed by rhodamin-conjugated anti-mouse antibody, as described previously [10,28]. Cells were mounted with DAPI (diaminophenylindole), and nuclei were visualized with a Nikon Eclipse E600 microscope at the Keck Center for Imaging, University of Washington, Seattle, WA. For the quantitation of nuclear irregularity, nuclear contour ratios were determined in a minimum of two hundred randomly chosen nuclei using the MetaMorph program [10,28,29]. Statistical significance was determined by the Student t-test and f-test [10,28,29].

Replicative lifespan and the labeling index

Replicative lifespans of the fibroblasts were determined by cell counting at each passage. At each passage, the labeling index was measured as previously described [30]. Briefly, cells were incubated in media containing 5 μ Ci/ml (67 Ci/mmol) of [3 H]methyl-thymidine for 48hrs, fixed with methanol and exposed to Kodak NTB2 nuclear emulsion for 2 days. The plates were then developed and counterstained with Giemsa, and the average percentages of cells with radiolabeled nuclei were determined by counting 300 cells per data point.

Measurement of relative mean telomere length

Genomic DNA was isolated via the salting out method [31]. Relative telomere length was determined as previously described [32], with several modifications. Quantitative PCR was performed with a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) in a final volume of 20 μ l. Each reaction included 1X PCR buffer (Invitrogen, Carlsbad, CA), 0.2mM dNTPs, 0.4X SybrGreen (Molecular Probes, Eugene, OR), 2.5mM DTT, 1% DMSO, and 5ng of DNA. The telomere PCR used 0.8 units of Platinum Taq (Invitrogen, Carlsbad, CA), 1.5mM MgCl₂, 300nM of each primer (tel1b:

CGGTTTGTGGTTTGGGTTTGGGTTTGGGTTTGGGTT; tel2b:

GGCTTGCCCTTACCCTTACCCTTACCCTTACCCTTACCCT) and 30 cycles of

amplification at 95°C for 15 seconds and at 56°C for 60 seconds. The control gene PCR used 0.5 units of Platinum Taq, 3.5mM MgCl₂, 300nM of forward primer (36B4u:

CAGCAAGTGGAAGGTGTAATCC) and 500nM (36B4d:

CCCATTCTATCATCAACGGGTACAA) of reverse primer and 35 cycles of amplification at 95°C for 15 seconds, at 56°C for 20 seconds, and 72°C for 20 seconds. A four-point standard

curve (2-fold serial dilutions from 10 to 1.25 ng DNA) was used to allow transformation of cycle threshold into nanograms of DNA. Baseline background subtraction was performed in Excel by aligning amplification plots to a baseline height of 2% in the first 5 cycles. The cycle threshold was set at 20% of maximum product. All samples were run in triplicate and the median was used for subsequent calculations. The amount of telomeric DNA (T) was divided by the amount of single-copy control gene DNA (S), producing a relative measurement of the telomere length (T/S ratio). Two control DNA samples were run in each experiment to allow for normalization between experiments, and periodic reproducibility experiments were performed to guarantee correct measurements. The intra- and inter-assay variability (coefficient of variation) for Q-PCR was 6 and 7%, respectively. Linear regressions of telomere shortening versus CPD and labeling index were calculated, and the differences between two regression slopes were compared using the t-test [33].

Results

Generation of cell lines overexpressing mutant and wild-type lamin A

We chose two disease mutations found in atypical WS and two mutations found in HGPS patients for this study because of our interests in such progeroid syndromes. We identified R133L and L140R as heterozygous mutations in “atypical” WS patients referred to our International Registry of Werner Syndrome (Department of Pathology, University of Washington: www.wernersyndrome.org.) and found to have wild type *WRN* genes. [10]. The HGPS patients had the anticipated 50 amino acid deletion ($\Delta 50$) in lamin A resulting from G608G and G608S mutations previously documented in HGPS children [7,8]. A T623S mutation had been previously documented in a 45-year old HGPS patient, leading to a 35 amino acid deletion ($\Delta 35$) in lamin A and a less severe phenotype [16].

In order to compare the consequences of *LMNA* mutations in isogenic cell lines, we introduced a full length human *LMNA* cDNA carrying either wild-type, R133L, L140R, $\Delta 50$, or $\Delta 35$ mutations (Fig. 1A) into human control fibroblasts that had been immortalized with excisable hTERT (82-6pBlox). Western analysis prior to the removal of hTERT showed that the transfected cultures were expressing introduced *LMNA* of the expected sizes (Fig. 1B). The levels of lamin A were increased by approximately 90% in wild-type, R133L, and L140R mutant lines. In $\Delta 50$ and $\Delta 35$ mutant lines, the mutant lamin A were expressed at 91 and 92%, respectively, of the endogenous lamin A and there was no compensatory decrease of endogenous lamin A. There was also no compensatory decrease of lamin C after normalization with β actin in these lines. Therefore, the established lines expressed lamin A, either wild-type or mutant, at levels equivalent to 4 copies of the *LMNA* gene.

A hallmark of *LMNA* mutant cells is a characteristic abnormal nuclear morphology, often described as “blebbing” and “herniation” or “denting”. When we examined the nuclear morphologies of these cell lines prior to the removal of hTERT, we observed little or no difference compared to the control pBlox line, as noted previously [28]. However, following hTERT removal via superinfection with Cre and subsequent selection for 1 week, we found increased fractions of misshapen nuclei among the *LMNA* transfected cultures (Fig. 2). The control culture, 82-6, contained uniformly oval nuclei, and lamin A/C was mostly visualized as a thin, smooth perinuclear lining, with occasional small nuclear foci. Approximately 6% of fibroblasts overexpressing wild-type lamin A showed misshapen nuclei (those containing two or more blebs or herniations), while 2% of control fibroblasts had similar variations. Lamin A/C staining revealed irregular thickening of the peripheral lamin layers in wild-type lamin A overexpressing lines. Fibroblasts overexpressing R133L and L140R mutant lamin A showed various degrees of irregular nuclei. Misshapen nuclei were seen in 22% and 6% in R133L and L140R lines, respectively, with occasional nucleoplasmic lamin aggregates and lamina layer thickening. DAPI staining revealed misshapen nuclei in 23% and 22% of the $\Delta 50$ and $\Delta 35$

lines, respectively. In addition, lamin A/C staining revealed irregular nuclear edges, irregular thickening of the peripheral lamin layer, and nucleoplasmic lamin aggregates.

The degree of nuclear morphological abnormality was quantitated via nuclear contour ratios (NCR) (Tab. 1). NCR were calculated as $(2\pi/\text{perimeter})^2$ [29]. As noted previously, the average NCR for the several mutant lamin A lines were not significantly different, except for the $\Delta 50$ lamin A line (t-test $P < 0.001$). However, there was a trend for cells containing more misshapen nuclei to exhibit lower NCR. The variance of NCR, however, showed significant differences among cell lines expressing lamin A mutations (f-test $P < 0.0001$ in R133L, $\Delta 50$, and $\Delta 35$; f-test $P = 0.047$ in L140R).

There is a suggestion that, within comparable mutation types (missense or in-frame deletion), higher variances for NCR were observed for the case of mutations associated with more severe clinical phenotypes. Cases with R133L mutations were diagnosed in their late teens, while the diagnosis for the L140R subject was made in his late twenties [10]. The mean age of death for the classical $\Delta 50$ mutation cases was reported to be 13 years, while the single case of a $\Delta 35$ mutation survived to his forties [16]. Given the small number of cases, however, no firm conclusions can be made.

Accelerated replicative senescence of lamin A overexpressing lines

We then compared rates of replicative senescence and telomere shortening. Baselines of zero population doublings were established following Cre infection and 1-week of selection. In the subsequent serial passages, cell numbers and [^3H]thymidine incorporation were determined until the labeling index (dividing fraction) of the culture declined to 10%. Figure 3 illustrates culture lifespans as functions of 48hr labeling indices. Fibroblast cultures expressing lamin A with the HGPS deletion mutation and cultures with missense mutations found in atypical WS had reduced replicative lifespans. Total CPD were between 22 and 24 for R133L and L140R, 25 for $\Delta 35$ and 20 for $\Delta 50$ mutant lines. These are in contrast to the results for the parental fibroblast cultures, 82-6, which continued to divide until they reached CPDs beyond 45, as previously reported [27]. The persistence of introduced *LMNA* constructs were confirmed by nucleotide sequencing at the conclusion of the culture studies.

Interestingly, a fibroblast line overexpressing wild-type lamin A (82-6 +wt) also exhibited a diminished replicative lifespan, comparable to those expressing mutant forms of lamin A (Fig. 3). The kinetics of declines in replicative potentials appeared to differ somewhat, however. Cultures overexpressing wild-type lamin A began to decline early and steadily, beginning at CPD3.

Accelerated telomere shortening of lamin A overexpressing lines

To assess the rate of telomere loss during replicative senescence, mean telomere lengths were determined by quantitative PCR. Telomeres of fibroblasts overexpressing lamin A, either wild-type or mutant, exhibited accelerated rates of shortening as compared to the parental control, 82-6, (Fig. 4A). Linear regression analyses were statistically significant for R133L, $\Delta 50$ and $\Delta 35$ mutants ($P < 0.01$) and for overexpressing wt line ($P < 0.05$). For L140R, this test was not significant ($P = 0.18$) probably due to the insufficient data points near ends of replicative lifespan. There were no notable differences in the rates of telomere shortening among the various mutant fibroblast lines in this analysis.

When the rates of the telomere loss were plotted against the labeling indices, however, fibroblasts overexpressing wt lamin A showed the fastest rate of telomere loss (Fig 4B). This is consistent with the data of Fig. 3 indicating that the decline of labeling index started very early in the 82-6+wt line. The slope of the linear regression for the line overexpressing wt

lamin A was statistically different from those for the L140R, $\Delta 35$ and $\Delta 50$ mutant lines ($P < 0.01$ for R140L and $\Delta 50$; $P < 0.05$ for $\Delta 35$ mutant). Differences between the slope of overexpressing wt line and those of R133L and parental control lines did not reach statistical significance ($P = 0.13$ for R133L and $P = 0.09$ for control).

The relative telomere length at which replicative senescence occurs appears to differ depending on the strain. Cells that overexpress wt lamin A senesce with an average telomere length similar to control cells. Cells that express mutant lamin A, however, seem to senesce with telomeres that are substantially shorter than control cells. As these observations reflect the mean telomere length of cells in the culture, there is a further need to examine telomere lengths of individual chromosomes at the cellular level. It remains possible that the shortest subset of telomeres are of the same length in both cells, as the most critically short telomeres have been proposed to be limiting in senescence [35,36].

Our results with overexpressed wild type *LMNA* raise the possibility that gene duplication of that locus may be among the causes of progeroid syndromes. In an initial study to test that hypothesis, we assessed the copy number of *LMNA* in a small subset ($N = 23$) of atypical Werner syndrome cases from our International Registry of Werner Syndrome. Mutations of *LMNA* and *WRN* had previously been ruled out in these cases. Gene dosage of *LMNA* was determined by quantitative PCR with RnaseP as an internal standard [34]. We did not find evidence of an altered *LMNA* copy number among these 23 cases (data not shown).

Discussion

In our previous study, we examined well known parameters of replicative senescence in HGPS cells to demonstrate that senescence is delayed when the expression of mutant alleles is suppressed by RNAi [28]. We were unable to make comparisons with control lines, however, because of differences in the genetic backgrounds, which could independently impact differential rates of replicative senescence. In the present study, we have established a series of isogenic fibroblast lines to obviate that problem. *LMNA* cDNAs were introduced into a standard control parental fibroblast line that had been “immortalized” with an excisable hTERT construct. Replicative lifespans, rates of telomere shortening, and nuclear morphologies were assessed after the hTERT was removed. We were thus able to observe these phenotypes in cultures that were representative of normal diploid somatic cells, which lack telomerase and invariably undergo replicative senescence.

Control strain showed substantial fluctuations in labeling index shortly after CPD 30. A plausible explanation is that this reflects a phenomenon termed clonal succession, described in Salk et al [37]. In this report, mass cultures of skin fibroblasts derived from Werner syndrome patients were examined cytogenetically throughout their entire replicative lifespans. Primary fibroblasts from such subjects carry multiple, stable, structural chromosome rearrangements (“variegated translocation mosaicism”) which were used to identify cytogenetically marked clones of cells within a mass culture. They observed clear evidence of expansions and attenuations of individually marked clones during sequential passaging of the mass cultures. This is consistent with other published lines of evidence demonstrating heterogeneity of the replicative potentials of clones from such cultures [38,39]. The spikes of increased labeling index in our study may therefore reflect phases of clonal attenuation and expansion.

The kinetics of telomere length shortening in fibroblasts overexpressing lamin A and those of control parent fibroblasts differ in two regards. First, lamin A overexpressing cells lose more telomeres per population doubling compared to the control. Second, cells overexpressing mutant lamin A, but not wt lamin A, seem to senesce with telomeres that are considerably shorter than control cells.

What might be the mechanism of accelerated telomere loss in our lamin A overexpressing fibroblasts? In mammalian cells, telomeres are packaged within telomere-specific chromatin domains attached to the nuclear matrix [40,41]. Lamina-associated peptide (LAP) 2 α interacts with nucleoplasmic lamin A and exhibits dynamic associations with telomeric regions of chromosomes during the cell cycle [42]. LAP2 α gr; was also shown to partially co-localize with a telomere binding protein, TRF2 [43], a protein that plays a key role in the protection of telomeric ends [42]. A possible explanation for the accelerated rates of telomere loss in our *LMNA* mutant fibroblasts might be an altered assembly and distribution of the nuclear lamina and associated chromatin structures. Such altered structures might also arise from overexpression of wild type forms of lamin A via perturbations of multimeric proteins that can be presumed to act, in part, to protect telomeric DNA. One can speculate that such diminished “shielding” could lead to accelerated rates of loss of telomeres and consequent premature replicative senescence.

Why do cells overexpressing mutant lamin A, but not wt lamin A, appear to senesce with telomeres that are considerably shorter than control cells? It is conceivable that abnormal lamins could suppress signals from shortened telomere, thereby allowing mutant cells to proliferate with telomere lengths that are shorter than those that would signal normal cells to senesce. Mechanisms whereby telomere shortening triggers replicative senescence have been reviewed by Campisi [44]. Telomeres of fibroblasts in serial passages eventually reach a critical length [45] at which they elicit DNA damage responses similar to the cellular DNA response involving p53 tumor suppressor proteins. Activation of the retinoblastoma tumor suppressor protein (pRB) also provides a barrier to cell proliferation, one that cannot be overcome by the loss of p53 [44]. It has been reported that telomere shortening triggers senescence through p53, but not p16^{INK4a}, which positively regulates pRB in some fibroblast strains [46]. Those authors cautioned, however, that the extent to which cells rely on signaling from the p53 versus p16^{INK4a}-pRB pathways may vary from strain to strain, or even among clones within the same strain.

Johnson et al [47] reported that lamin A co-localizes pRB and that the abolishment of lamin A causes a marked reduction pRB and the mislocalization of the remaining pRB. Mutant lamin A introduced into HeLa cells was shown to form intranuclear aggregates containing pRB [48]. How mutant lamin A affects the function of pRB to inhibit initiation of DNA synthesis is not entirely clear. It is conceivable that abnormal lamins might fail to maintain the inhibitory effect of pRB, thus allowing cells to continue to proliferate, resulting in even shorter telomeres.

Telomere shortening results in either permanent growth arrest (replicative senescence) or cell death [44,49]. Critically shortened telomeres can lead to end fusion [45]. Covalent fusion of the chromosomal ends may induce genomic instability due to the random DNA break during anaphase [50]. It has recently been proposed that replication-associated telomere loss and resultant chromosome fusions may be the cause of genomic instability seen in the classical Werner syndrome caused by WRN helicase gene mutations [51]. It is conceivable that the increased apoptosis [19] and DNA accumulation [52] seen in HGPS may be, at least in part, secondary to accelerated telomere loss. DNA damage accumulation and aberrant nuclear morphology, however, may be independent phenotypes [52].

Abnormal nuclear morphology has been widely used to assess the toxicity of mutant lamins. They are often correlated with the severity of the complications within patients carrying the same *LMNA* mutations [53,54]. For instance, we reported two female patients, one African-American and one Caucasian, who shared the same R133L mutation but had vastly different body fat distribution. The extent of the lipodystrophy appeared to be correlated with the severity of the metabolic complications and also the degree of abnormal nuclear morphology [53]. A study examining three homozygotes for the R527H mutation and the same clinical phenotype

demonstrated that the extent of nuclear and heterochromatin irregularities were correlated with pre-lamin A accumulation and, interestingly, patient age [54]. Our results are also consistent with these findings. Within the same class of mutations, in-frame deletions at the C-terminal end or amino acid substitution in the heptad repeat region, the degree of nuclear morphological abnormality correlates with the severity of the disease. The number of mutations examined in our study was quite modest, however; additional disease mutations need to be examined in order to make more definitive conclusions.

An intriguing finding emerging from our study is that overexpression of wild-type lamin A also causes accelerated telomere loss and shorter replicative lifespan of fibroblasts, although with kinetics somewhat different from that observed in lines with mutant lamin A. Altering the relative amount of any lamina component could potentially affect the assembly and integrity of nuclear lamina [55]. Excessive amounts of lamin A may lead to altered lamina and chromatin structures and destabilization in the tethering of telomere ends. Overexpressed wild-type lamin A is synthesized as prelamin A, which needs to be sequentially processed and converted to mature lamin A [14]. The adverse effects of introduced wild-type lamin A on telomeres can potentially be attributed to increased amounts of prelamin A and/or mature lamin A. To address this question, one must generate cell lines in which endogenous lamin A is reduced while introduced lamin A is expressed at comparable levels.

Our observations of the pathophysiological impacts of overexpressing lamin A have potential clinical relevance. Reports of genetic disorders that result from genomic rearrangements leading to gene copy number variants are increasing in number [56–59]. These now include a recent report of a gene duplication of *LMNB1* in the patient with autosomal dominant leukodystrophy [34]. Tandem genomic duplication of *LMNB1* resulted in 3 copies of *LMNB1* per cell and a marked elevation of the lamin B1 protein level. Previous studies have had also shown that overexpression of wild-lamin A lead to abnormal nuclear morphologies [55]. The maintenance of appropriate stoichiometric amounts of laminar protein components may therefore be essential for normal lamina assembly. Although there are no known segmental duplications of the chromosome region within 1q21.1–21.3, the region that spans the *LMNA* locus [59] (<http://humanparalogy.gs.washington.edu>), the presence of flanking repeats could lead to unequal crossing over and copy number variants. Thus, increases in *LMNA* copy number could be among the many causes of segmental progeroid syndromes [61]. It has already been established that haploinsufficiency can lead to such syndromes [62]. For example, a heterozygous stop codon mutation at amino acid codon 6, leading to functional haploinsufficiency, was found in a patient with Emery-Dreifuss muscular dystrophy [1]. Given the results reported in this paper, however, we shall continue to screen additional such cases for gene dosage variations in *LMNA*.

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Figure 1.

Diagram of the *LMNA* mutants and Western analysis of 82-6pBlox lines overexpressing lamin A. (A) Sites of the *LMNA* mutations used for this study are shown within the several functional domains of lamin A. The arrow indicates the endoproteolytic site (absent in $\Delta 50$ and $\Delta 50$ mutants). (B) Western analysis of lamin A/C shows protein levels of introduced lamin A for the corresponding mutations as well as endogenous lamin A/C on a 82-6 pBloxTSH background. β -actin is shown as the loading control.

Figure 2.

Nuclear morphology and lamin A/C subnuclear localization within fibroblasts overexpressing lamin A. After hTERT removal, cells were stained for lamin A/C and nuclei were counter-stained with DAPI. Typical confocal images from the parent line, 82-6 (left top), and lines overexpressing lamin A are shown. The labels correspond to the mutations in Fig 1.

Figure 3. Replicative lifespan of the fibroblasts overexpressing lamin A. Fibroblast cultures correspond to those of Fig. 1 and 2. X-axis indicates the cumulative population doublings and Y-axis indicates the labeling index (dividing fraction of the cells in culture).

Figure 4. Mean telomere length during replicative senescence in fibroblasts overexpressing lamin A. The mean telomere lengths were determined in the fibroblast cultures used in Fig. 3. Left 3 panels show the declines of mean telomere lengths as a function of CPD. Regressions of 82-6 were shown in all 3 panels. Right panels show them as a function of labeling index. Regressions of 82-6+wt were shown in all 3 panels.

Table 1**Nuclear morphological abnormalities of LMNA mutant fibroblasts**

Nuclear contour ratios (NCR) were obtained for a minimum of 200 cells in each fibroblast line. Fibroblast cultures correspond to those given in Fig. 2. When a nuclear image is that of a complete circle, the NCR was scored as 1. The NCR averages were compared to that of the parental line, 82-6, using the student t-test (1 tailed) [29, 33]. Variances of NCR were assessed with the f-test [29,33].

Cell line	Nuclear Contour Ratio	t-test	f-test
82-6	0.778 ± 0.102	na	na
+wt	0.769 ± 0.116	0.516	0.120
+R133L	0.755 ± 0.1511	0.45	<0.0001
+L140R	0.788 ± 0.125	0.345	0.047
+Δ50	0.718 ± 0.165	<0.001	<0.0001
+Δ35	0.769 ± 0.152	0.554	<0.0001