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ORIGINAL INVESTIGATION

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# Correction of cellular phenotypes of Hutchinson-Gilford Progeria cells by RNA interference

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**Abstract** The great majority of cases of the Hutchinson-Gilford progeroid syndrome (HGPS) ("Progeria of Childhood") are caused by a single nucleotide mutation (1824 C->T) in the *LMNA* gene which encodes lamin A and C, nuclear intermediate filaments that are important components of the nuclear lamina. The resultant mutant protein (Δ50 lamin A) is thought to act in a dominant fashion. We exploited RNA interference technology to suppress Δ50 lamin A expression, with the long range goal of intervening in the pathogenesis of the coronary artery atherosclerosis that typically leads to the death of HGPS patients. Short hairpin RNA (shRNA) constructs were designed to target the mutated pre-spliced or mature *LMNA* mRNAs, and were expressed in HGPS fibroblasts carrying the 1824 C->T mutations using lentiviruses. One of the shRNAs targeted to the mutated mRNA reduced the expression levels of Δ50 lamin A to 26% or lower. The reduced expression was associated with amelioration of abnormal nuclear morphology, improvement of proliferative potential, and reduction in the numbers of senescent cells. These findings provide a rationale for potential gene therapy.

**Keywords** Progeroid syndrome · LMNA · Aging · Atherosclerosis

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## Introduction

Hutchinson-Gilford progeria syndrome (HGPS) is a rare genetic disorder that is characterized by childhood onset of several symptoms associated with aging (Brown 1992; DeBusk 1972). At birth, HGPS patients appear normal. However, usually within one year, they begin developing various features consistent with premature aging. These features include hair loss, lipodystrophy (especially loss of subcutaneous fat), reduced bone density, stiffened joints, and progressive arteriosclerosis. Owing to severe growth retardation, HGPS patients generally attain an average height of ~1 m and an average weight of less than 15 kg, even as teenagers. Age at death ranges from 7 to 28 years, with a median age of 13.4 years, predominately due to cardiovascular or cerebrovascular diseases. Autopsy examinations reveal severe and widespread atherosclerosis in HGPS patients upon death (Baker et al. 1981). However, many other aspects of aging, such as cancer, cataracts, and cognitive degeneration—features often associated with normal aging—are absent in HGPS patients. HGPS is therefore considered a segmental progeroid syndrome, as it only manifests a portion of the normal aging process (Martin and Oshima 2000). A better understanding of HGPS, as well as other progeroid syndromes with features of accelerated aging, may lead to a better understanding of normal aging and age-related diseases in humans (Martin 1978).

Hutchinson-Gilford progeria syndrome is caused by mutations in *LMNA*, the gene coding for the A-type lamins (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003). The lamins are type-V intermediate filament (IF) proteins, possessing a central α-helical coiled-coil rod domain, flanked by globular N-terminal head and C-terminal tail domains (Stuurman et al. 1998). Polymerization and higher-order assembly of the lamin proteins produce a network of lamin IFs, which comprise the 20–50 nm thick fibers of the nuclear lamina that lies between the inner membrane of the nuclear

envelope and chromatin. The nuclear lamina structurally supports the nuclear envelope (NE) and plays a key role in determining the overall shape of the interphase nucleus (Sullivan et al. 1999). In addition, the lamins connect to the chromatin in the nucleoplasm and are probably involved in numerous functions, including DNA replication, transcription, chromatin organization, and nuclear positioning, as well as assembly and disassembly of the nucleus during cell division (Goldman et al. 2002).

The great majority of HGPS patients are heterozygous for a single nucleotide substitution at position 1824, C->T, within exon 11 of *LMNA*. This mutation does not cause an amino acid change (GGC->GGT, G608G), but partially activates a cryptic splicing site leading to an in-frame deletion of 50 amino acids (Δ50 mutation) in lamin A (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003). The Δ50 lamin A has been shown to cause the aberrant nuclear morphology typical of HGPS cells, even in the presence of wild type lamin A, suggesting it acts in a dominant negative manner (Goldman et al. 2004). Currently, there is no effective treatment for HGPS. Of greatest concern is the fulminating coronary artery atherosclerosis, the usual cause of premature death. Motivated by progress in the use of gene therapy as an approach to prevent coronary artery re-stenosis (Karthikeyan and Bhargava 2004; Luo et al. 2004), we explored the feasibility of gene therapy, ultimately directed to the coronary arteries of HGPS patients, using RNA interference (RNAi) to correct cellular phenotypes associated with HGPS.

## Materials and methods

### Cell lines

Primary human fibroblast cell strains AG3513D and 75-8 (AG01972) were obtained from the Coriell Institute (Camden, NJ, USA) and International Registry of Werner Syndrome (Seattle, WA, USA), respectively. We confirmed that both cell lines were heterozygous for the 1824 C->T (G608G) mutation in the *LMNA* gene, as reported previously (Eriksson et al. 2003). The cells were immortalized by expression of the catalytic subunit of human telomerase (hTERT), using a reversible retroviral expression vector, previously described (Rubio et al. 2002). Cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen/GIBCO) containing 15% fetal bovine serum (FBS, Atlanta Biologicals), 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in a 5% $CO_2$ atmosphere.

### shRNA vectors and generation of shRNA-expressing cell lines

Nine shRNAs targeted to either the unspliced G608G mutant pre-mRNA or the mature, spliced Δ50 LMNA mutant mRNA were designed and cloned into a previously reported lentiviral system (Bridge et al. 2003). Three of these vectors (shRNA1, shRNA2, and shRNA3) were targeted to the spliced mature mRNA (i.e., Δ50 LMNA mRNA), whereas the other six (shRNA 4-9) were targeted to the unspliced pre-mRNA (G608G mRNA). The sequences of the shRNAs were as follows (sequences in italics are loop sequences):

- shRNA1, CUCAGGAGCCCAGAGCCCCCA*UUCAAGAGA*UGGGGGCUCUGGGCUCCUGAG;
- shRNA2, CAGGAGCCCAGAGCCCCCAGA*UUCAAGAGA*UCUGGGGGCUCUGGGCUCCUG;
- shRNA3, GGCUCAGGAGCCCAGAGCCCC*UUCAAGAGA*GGGGCUCUGGGCUCCUGAGCC;
- shRNA4, GUGGACCCAUCUCCUCUGG*UCUCUUGAA*CCAGAGGAGAUGGGUCCAC;
- shRNA5, UGGACCCAUCUCCUCUGGC*UCUCUUGAA*GCCAGAGGAGAUGGGUCCA;
- shRNA6, CAGGUGGGUGGACCCAUCU*UCUCUUGAA*AGAUGGGUCCACCCACCTG;
- shRNA7, CCCAGGUGGGUGGACCCAUCU*UCUCUUGAA*AGAUGGGUCCACCCACCTGGG;
- shRNA8, AGCCCAGGUGGGUGGACCCAU*UCUCUUGAA*AUGGGUCCACCCACCTGGGCT;
- shRNA9, AGGUGGGUGGACCCAUCUCCU*UCUCUUGAA*AGGAGAUGGGUCCACCCACCT.

A control shRNA was designed using a sequence with no significant homology to the *LMNA* gene or other genes in the human genome, as determined by a BLAST search and analysis. The control shRNA (ctrRNA) sequence was AGGUGGGUGGACCCAUCUCCU*UCUCUUGAA*AGGAGATGGGTCCAGCCACCT. Lentiviruses expressing these shRNAs were prepared as described (Bridge et al. 2003) and used to infect telomerase-immortalized AG3513D and 75-8 cells, followed by drug selection with 50 ug/ml hygromycin for 1 week. These shRNA-expressing cells were then converted to mortal cells by removing hTERT expression following Cre recombinase expression and puromycin selection (Rubio et al. 2002).

### Western analysis

Western analysis was performed, as described (Chen et al. 2003). Briefly, total proteins were extracted using a denaturing buffer containing 10 mM Tris–HCl, pH 7.0, 1 mM EDTA, and 5% SDS. After boiling for 5 min, the protein concentrations of the samples were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) as per the supplier's instructions. Proteins were separated by a NuPAGE 10% Bis–Tris gel and blotted onto a PVDF membrane as per the supplier's instructions (Invitrogen, Carlsbad, CA). Nonspecific epitopes were blocked with 5% nonfat dry milk. The lamin A/C proteins were detected by a rabbit polyclonal anti-lamin A/C antibody (Cell Signaling,

Beverly, MA, USA) and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA), following the suppliers' instructions. The relative lamin A/C protein levels were determined by densitometry of the autoradiography.

### Life span, clone size distribution and nuclear morphology determination

Following removal of hTERT expression and conversion to mortal cells (Rubio et al. 2002), cell cultures were passaged by trypsinizing at confluence and subculturing. Cumulative population doublings were determined immediately after hTERT removal using the formula $(\log H - \log S)/\log 2.0$, where $\log H$ is the logarithm of the number of cells harvested when the cells were confluent, and $\log S$ is the logarithm of the number of cells seeded on the first day of each passage. The fractions of proliferating cells were determined by plating cells at $10^3/\text{cm}^2$ and labeling with $^3H$-thymidine for 48 and 72 h. The percentage of labeled nuclei was determined by autoradiography. The senescence status of the cells was determined by staining for the senescence-associated $\beta$-galactosidase, as described (Dimri et al. 1995).

Clonal size distribution was determined by culturing the cells at clonal densities in 100 mm culture dishes for 1 week, followed by staining with crystal violet and counting. The experiment was performed in duplicate with four different serial dilutions of the cells, as described (Pendergrass et al. 1995; Smith et al. 1978).

To determine nuclear morphology, cells were plated on glass coverslips, fixed, permeabilized, and stained with DAPI as described (Chen et al. 2003). Nuclear images were obtained with a Nikon Upright Microscope at the Keck Center for Imaging, University of Washington, Seattle, WA. To analyze changes in nuclear morphology between cells with or without shRNA expression, the perimeter and area of the nuclei were measured. The extent of nuclear lobulation was determined by measuring the roundness or nuclear contour ratios ($4\pi \times \text{area}/\text{perimeter}^2$) from randomly chosen nuclei. Measurements were taken using the MetaMorph program. The contour ratio for a circle is 1 and approaches 0 as the nucleus becomes more and more lobulated. Blebbed nuclei were determined as those with one or more lobulations. Three samples of 200 nuclei each were analyzed for each cell line. Statistical significance for the cellular phenotype measurements was determined by a two-tailed student $t$ test.

## Results

We established a series of cell lines carrying the 1824 C->T (G608G) HGPS *LMNA* mutations and short hairpin RNAs (shRNAs) targeted to either the unspliced G608G mutant pre-mRNA or the mature, spliced Δ50 *LMNA* mutant mRNA. Due to the extremely short replicative life spans of HGPS cells, we first enhanced the replicative life spans of HGPS fibroblast strains AG03513D and 75-8, using ectopic expression of an excisable hTERT cDNA (encoding the catalytic subunit of human telomerase) (Rubio et al. 2002). Nine different shRNAs targeted to either G608G or Δ50 mutant mRNAs were introduced into the cells using a recombinant lentiviral system in which shRNAs are expressed from the H1 promoter (Bridge et al. 2003). Western analysis showed that one shRNA (shRNA3) reduced Δ50 mutant lamin A to 26% of control cells without shRNA in AG3513D/hTERT cultures (on the basis of normalization to lamin C levels) (* in Fig. 1a). This shRNA was chosen for further experiments, using AG3513D and 75-8 cell lines.

After expansion of the shRNA-expressing cultures, hTERT was removed by a retrovirally expressed Cre

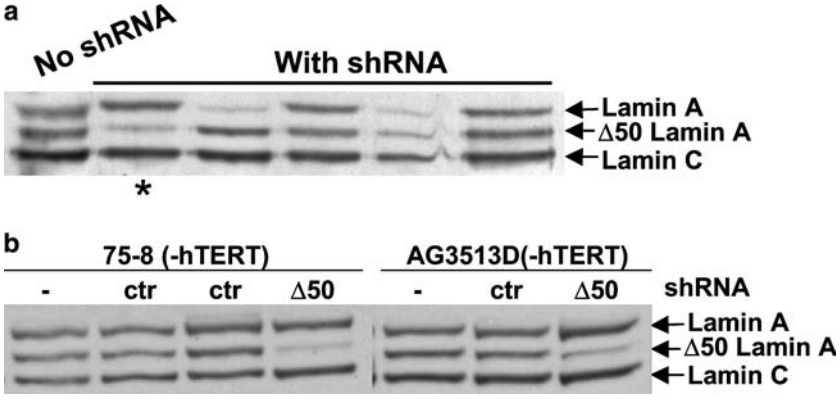


**Fig. 1** Effect of shRNA "knockdown" on Δ50 lamin A expression in HGPS fibroblasts. Proteins were extracted from the indicated cell lines, separated by 10% SDS-PAGE, transferred to a PVDF membrane, and probed with a rabbit anti-lamin A polyclonal antibody. **a** Western analysis of lamin A and C in AG3513D/hTERT cells to screen for effective shRNAs. *Lane 1* AG3513D/hTERT without shRNA. *Lane 2–6* AG3513D/hTERT expressing shRNA3, 4, 7, 8, and 9, respectively. shRNA3, targeted to Δ50 lamin A mature mRNA, suppressed Δ50 lamin A level to 26% the level in cells lacking shRNA expression. **b** Western analysis of lamin A and C in HGPS cells after hTERT removal. - Cells without shRNA; *ctr* cells infected with the control shRNA; Δ50 cells infected with shRNA3

recombinase, followed by 7 days of selection for hTERT excision cultures lacking telomerase(exhTERT), as described (Rubio et al. 2002). Western analysis showed that AG3513D/exhTERT cells continued to express reduced Δ50 mutant lamin A levels (and slightly increased wild-type lamin A levels) after hTERT removal (Fig. 1b). The changes in wild-type lamin A levels after shRNA3 expression varied from a 21% increase to a 19% decrease, and were not statistically significant (data not shown). 75-8/exhTERT cells also showed reduced Δ50 lamin A expression (15% of control cells) after hTERT removal (Fig. 1b). These results indicated that shRNA3 was able to suppress the expression of Δ50 mutant lamin A, while having minimal effects on wild-type lamin A expression.

The initial thymidine labeling indices of 75-8/exhTERT cultures lacking shRNA immediately following removal of hTERT and selection were 31% (48 h labeling) and 29% (72 h labeling) and the fraction of cells expressing the senescence-associated $\beta$-galactosidase (SA-$\beta$gal) was 8%. These data are consistent with the very long doubling times of HGPS fibroblasts. Expression of shRNA3 increased the labeling indices to 39% (48 h labeling) and 49% (72 h labeling) and decreased the fraction of cells expressing SA-$\beta$gal to 4.5% (Table 1). Cells infected with a control shRNA had labeling and SA-$\beta$gal indices similar to cells without shRNA (Table 1). Likewise, AG03513D/exhTERT cultures without shRNA had an initial labeling index of 31% (48 h labeling) and 29% SA-$\beta$gal positive cells, while the culture expressing shRNA3 had a 40% labeling index (48 h labeling) and 13% SA-$\beta$gal positive cells. AG3513D/exhTERT cells infected with control shRNA had indices similar to those of cells without shRNA (Table 1). Although the differences in labeling and SA–$\beta$gal indices between control and shRNA3-expressing cells were small, they were statistically significant (Table 1). These data suggest that soon after the introduction of shRNA3 the HGPS fibroblast strains show a partial amelioration of their poor growth phenotypes.

To assess the replicative potential of the cultures, we determined their clone size distributions (Pendergrass et al. 1995; Smith et al. 1978). After 7 days in culture, approximately 75% of control 75-8 (exhTERT) cells did not divide at all, while approximately 50% of 75-8 + shRNA3 cells (exhTERT) divided at least once (Fig. 2a). These numbers are consistent with the labeling indices of the cultures. Moreover, overall clone sizes of 75-8 + shRNA3 (exhTERT) were larger than those of 75-8 (exhTERT). For example, clones containing at least eight cells (undergoing three or more divisions) accounted for 6% of 75-8 (exhTERT) colonies, but 13.3% of 75-8 + shRNA3 (exhTERT) colonies ($P = 0.01$). Control shRNA had no significant effect on 75-8 (exhTERT) colony sizes. Consistent with enhanced clonal growth mediated by shRNA3, suppression of Δ50 mutant lamin A expression by shRNA3 also significantly extended the replicative life span of 75-8 (exhTERT) mass clutures (Fig. 2b). 75-8/exhTERT cells expressing shRNA3 achieved eight population doublings, at which point their thymidine labeling indices were still 20%. In contrast, the cells without shRNA reached thymidine labeling index of 8% after only about five population doublings, similar to the behavior of cells expressing a control shRNA.

One hallmark of HGPS cells is their markedly abnormal nuclear morphologies. Unlike the typical ellipsoid shape seen in normal cells, the nuclei of HGPS cells display a variety of herniations, pronounced blebbing, and micronuclei (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003). Introduction of shRNA3 restored the roundness of HGPS nuclei as measured by nuclear contour ratios (from 0.85 to 0.91 for 75-8 cells, and 0.78 to 0.88 for AG3513D cells). Expression of shRNA3 markedly reduced the fraction of cells with visibly misshapen nuclei, defined by the presence of one or more lobules (Table 1 and Fig. 3). Cells with misshapen nuclei comprised 60.5% of AG03513D (exhTERT) cultures, while they comprised only16.8% of AG03513D (exhTERT) +shRNA3 cultures. For 75-8 cells, the percentage of misshapen nuclei was 49.9% for control cultures and 13.2% for cultures expressing shRNA3. In control cells, misshapen nuclei were characterized by multiple large lobulations, whereas in shRNA3-expressing cells, the misshapen nuclei consisted primarily of relatively shallow and single lobulations.

**Table 1** Cellular characteristics of HGPS fibroblasts with and without shRNA

| Cell Lines | Labeling Index (%) | | $\beta$-gal (%) | Nuclear contour ratio | Nuclear blebbing (%) |
|---|---|---|---|---|---|
| | 48 h | 72 h | | | |
| 75-8/exhTERT | 31 | 29 | 8.0 ± 0.4 | 0.85 ± 0.093 | 49.9 ± 5.80 |
| 75-8/exhTERT + ctrRNA | 28 | 33 | 7.1 ± 1.0 | 0.84 ± 0.071 | 49.8 ± 3.53 |
| 75-8/exhTERT + shRNA3 | 39 | 49 | 4.5 ± 0.3 | 0.91 ± 0.041 | 13.2 ± 2.67 |
| *P* value | 0.014 | 0.014 | 0.002 | < 0.001 | < 0.001 |
| AG3513D/exhTERT | 31 | NA | 29 ± 0.4 | 0.78 ± 0.130 | 60.5 ± 2.34 |
| AG3513D/exhTERT + ctrRNA3 | 32 | NA | 24 ± 0.5 | 0.77 ± 0.072 | 59.3 ± 2.80 |
| AG3513D/exhTERT + shRNA3 | 40 | NA | 13 ± 0.6 | 0.88 ± 0.047 | 16.8 ± 4.28 |
| *P* value | 0.037 | NA | 0.001 | < 0.001 | < 0.001 |

Data were obtained as described in "Methods". *ctrRNA* control shRNA. *NA* not available

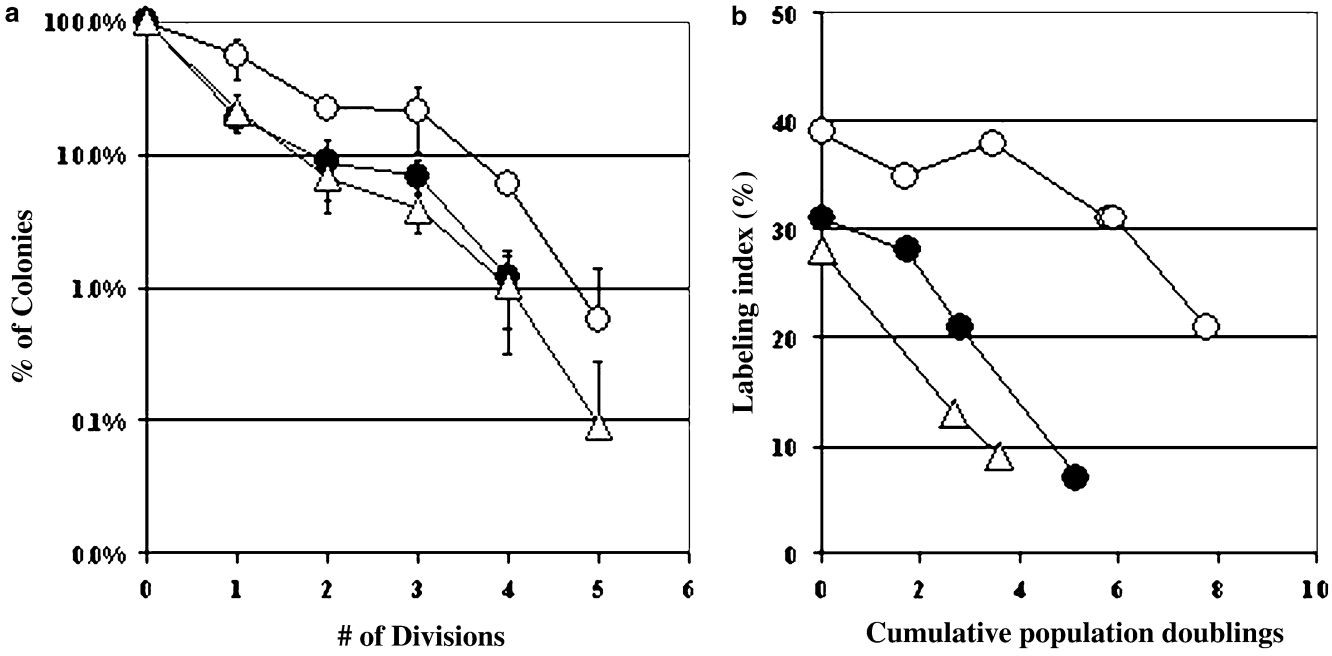


**Fig. 2** Replicative capacities of HGPS cells with and without shRNA3 expression. **a** Clone size distributions of 75-8 (exhTERT) (*filled circle*), 75-8 + ctrRNA (exhTERT)(Δ) and 75-8 + shRNA3 (exhTERT) (*open circle*). Cells were plated and cultured in 100 mm dishes for 1 week, followed by staining with crystal blue and counting as described in "Methods". Data are presented as cumulative percentages, starting from the highest number of divisions. **b** Improvement of replicative potentials by shRNA3 suppression of Δ50 lamin A expression. Cumulative population doublings were determined as described in "Methods," with a 0 value assigned to cells immediately after hTERT removal. *Symbols* designating the cultures are the same as in **a**

## Discussion

The identification of mutations in *LMNA* as the cause of HGPS laid the groundwork for molecular analysis of HGPS. It is still not clear how mutations in *LMNA* lead to the severe clinical manifestations seen in HGPS patients. Cells cultured from HGPS subjects generally have a much shorter replicative life span than normal cells, although there is substantial variation in growth potentials of different HGPS fibroblast strains (Bridger and Kill 2004; Martin 1978). Telomere lengths are relatively reduced in HGPS fibroblasts (Allsopp et al. 1992), which may at least in part explain their limited replicative life span. HGPS cells also show striking nuclear abnormalities, which may also play a key role in the short replicative life span and/or an independent role in the pathogenesis of HGPS. Cellular senescence and apoptosis are critical for maintaining tissue homeostasis and are thought to play important roles in the normal aging process of humans (Campisi 2003). Because of their inability to divide and altered differentiated functions, senescent cells that accumulate within tissues could compromise tissue functions and contribute to aging (Campisi 2005; Faragher and Kipling 1998). In addition, loss of functional "young" cells due to apoptosis, senescence, or other forms of proliferative failure may also contribute to the premature aging manifested in HGPS patients (Bridger and Kill 2004). For example, smooth muscle cells have been found to be severely depleted in the arteries of HGPS patients, which may well play an important role in the pathogenesis of their accelerated atherosclerotic diseases (Stehbens et al. 2001). The coronary lesions of HGPS probably have, as their primary events, mechanical injuries to highly susceptible vascular smooth muscle and endothelial cells (Worman and Courvalin 2004). A major medical lesson from HGPS and from the Werner syndrome ("Progeria of the Adult") (Martin et al. 1970) is that these syndromes support the proposition that the pathogenesis of common forms of atherosclerosis may involve primary events within cells of the vascular wall, including premature replicative senescence (Martin and Sprague 1972).

The nuclear lamina plays an important role in providing structural support to the nuclear envelope and largely determines the overall shape of the interphase nucleus (Sullivan et al. 1999). In addition, the lamins are distributed throughout the nucleoplasm, connecting directly or indirectly to the chromatin. The Δ50 lamin A mutation disrupts the normal processing, turnover, and assembly state of lamin A, ultimately leading to nuclear shape abnormalities and loss of peripheral heterochromatin in HGPS nuclei (Goldman et al. 2004). These changes, in turn, may disturb DNA synthesis and alter the regulation of gene expression (Goldman et al. 2002). The mutant Δ50 lamin A protein was predicted to function as a dominant negative based on the fact that the majority of HGPS patients are heterozygous for the mutation (Eriksson et al. 2003). This idea was

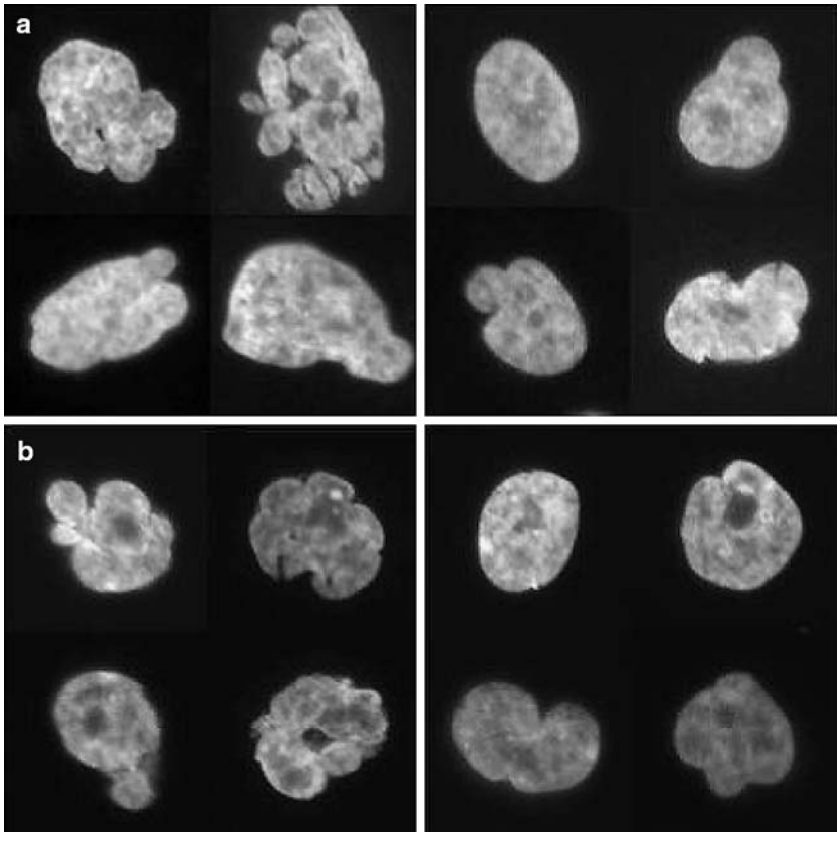


**Fig. 3** Representative nuclear morphologies of HGPS cells with and without shRNA. AG3513D (exhTERT) (*left panel*, **a**); AG3513D + shRNA3 (exhTERT) (*right panel*, **a**); 75-8 (exhTERT) (*left panel*, **b**); and 75-8 + shRNA3 (exhTERT) (*right panel*, **b**). Cells were cultured on glass coverslips, fixed, permeabilized, and stained with DAPI as described (Chen et al. 2003)

strengthened by a recent report showing that ecotopic expression of Δ50 lamin A altered nuclear morphology in the presence of wild-type lamin A (Goldman et al. 2004) . Our data are also consistent with the hypothesis that the mutant Δ50 lamin A protein exert dominant negative effects.

Reduced Δ50 lamin A expression by shRNA significantly improved the replicative potentials and nuclear morphology of HGPS cells. We were unable to assess the extent to which the abnormalities were corrected because variations among normal fibroblasts were larger than differences between HGPS fibroblasts with or without shRNA. Complete restoration of HGPS cells to normal phenotypes may not have been achieved by this shRNA because suppression of mutant lamin A was not complete (26 and 15% unsuppressed for AG3513D and 75-8, respectively). Another reason why abnormal nuclear morphology was sustained may be that the shRNA was not expressed long enough in the cells—that is, not enough time elapsed for the cells to recover from the toxicity caused by mutant Δ50 lamin A. A third possibility is that mutant Δ50 lamin A may have caused uncorrectable damage to HGPS cells prior to introduction of shRNA.

Stable, partial suppressions of a specific mutated allele by shRNA have been demonstrated in vivo (Caplen 2004; Xia et al. 2002). Current research on the delivery of genetic constructs to cells of the arterial wall is still in its infancy and is motivated primarily by the clinical problem of restenosis. Because we demonstrated shRNA-mediated suppression of the mutant Δ50 lamin A and improvement of cellular properties (replicative life span and nuclear morphologies) of HGPS fibroblast cells, it may be possible to treat arterial wall cells in HGPS patients as methods for gene therapy to treat the arterial wall improve. More research will need to be done to further evaluate the feasibility of exploiting shRNA as a path to gene therapy for HGPS.

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