Aging of Hutchinson-Gilford progeria syndrome fibroblasts is characterised by hyperproliferation and increased apoptosis.

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Abstract.

Hutchinson-Gilford Progeria Syndrome is a rare genetic disorder that mimics certain aspects of aging prematurely. Recent work has revealed that mutations in the lamin A gene are a cause of the disease. We show here that cellular aging of Hutchinson-Gilford progeria syndrome fibroblasts is characterised by a period of hyperproliferation and terminates with a large increase in the rate of apoptosis. The occurrence of cells with abnormal nuclear morphology reported by others is shown to be a result of cell division since the fraction of these abnormalities increases with cellular age. Similarly, the proportion of cells with an abnormal or absent A-type lamina increases with age. These data provide clues as to the cellular basis for premature aging in HGPS and support the view that cellular senescence and tissue homeostasis are important factors in the normal aging process.

1. Introduction.

Hutchinson-Gilford progeria syndrome (HGPS) is a rare genetic disorder that is characterized by aspects of accelerated and premature aging (Brown, 1992). Individuals affected with HGPS appear normal at birth but typically show growth defects within a year. Growth continues at a slow rate and by the second year there is usually a loss of scalp hair, eyelashes and eyebrows and lipodystrophy (redistribution of body fat, in particular, a marked reduction in subcutaneous fat). As the disorder develops, individuals suffer from stiffened joints and experience mild to severe limitation to motion. Death occurs at a mean age of 13.4 years usually from heart attacks or congestive heart failure (DeBusk, 1972). Widespread atherosclerosis is usually seen upon autopsy (Baker et al., 1981). Cytogenetic studies have led to chromosome 1 as the most likely candidate chromosome to carry the HGPS gene (Delgado Luengo et al., 2002) and recently it has been shown that HGPS is caused by mutations in the lamin A gene (Cao and Hegele, 2003; De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003), which is present on HSA1. This places HGPS within a class of syndromes termed the “laminopathies” (Gruenbaum et al., 2003) and includes Autosomal Emery-Dreifuss Muscular Dystrophy (Bonne et al., 1999), Dunnigan-type familial partial lipodystrophy (Cao and Hegele, 2000), autosomal dominant limb-girdle muscular dystrophy 1B (Muchir et al., 2000) and autosomal recessive axonal neuropathy (Charcot-Marie-Tooth type 2 disease 2B1) (De Sandre-Giovannoli et al., 2002).

Lamin A belongs to a family of nuclear intermediate-type filament proteins that form an insoluble network of filaments beneath the inner nuclear membrane. However, lamins have also been localised to the nuclear interior in speckles (Bridger

et al., 1993) and as part of the interior nuclear matrix (Barboro et al., 2002; Hozak et al., 1995). A-type lamins have also been found in association with transcription factors (Dreuillet et al., 2002; Lloyd et al., 2002). Furthermore, disruption of normal lamin organization by microinjection of a dominant-negative lamin A mutant results in inhibition of both DNA replication (Spann et al., 1997) and RNA polymerase II-dependent transcription (Spann et al., 2002) suggesting a role for lamin A in both these processes. Finally, lamins have the ablilty to bind dirctly to DNA (Stierle et al., 2003) and to chromatin (Glass et al., 1993; Taniura et al., 1995) and indirectly via associations with proteins containing a LEM box (Lee et al., 2001; Martins et al., 2003). Thus, the list of possible functions of the A-type lamins includes maintenance of nuclear structural integrity, organisation of higher-order chromatin structure and control of gene expression (Hutchison, 2002). Nevertheless, it is an important finding that mutations in the lamin A gene can give rise to a range of diseases and in particular to premature aging. Therefore it is essential to understand how lamin A mutations affect cell function and in the case of HGPS, how the mutations result in premature aging. In this study we have compared the rates of cellular aging and apoptosis in normal and HGPS fibroblast cultures. In addition, we have compared nuclear morphology and nuclear lamina organisation in young and senescent HGPS fibroblasts.

2. Materials and Methods.

2.1 Cell culture

Normal dermal fibroblast cultures 1BR (Arlett et al., 1975), 2DD (Bridger et al., 1993) and NB1 (Bolland, 2003) have been described previously. HGPS fibroblasts were obtained from the Coriell Cell Repository (see Table 1.). Cells were grown in DMEM containing 15% fetal bovine serum at 37^{o}C in 5% CO_2/95% air. Cultures

were passaged twice weekly and seeded at $2x10^5$ per 90mm diameter dish. Cell growth was measured by calculation of accumulated population doublings using the formula (logH – logS)/log2.0 where logH is the logarithm of the number of cells harvested after 3 or 4 days growth and logS is the logarithm of the number of cells seeded on the first day of each passage.

2.2 Indirect immunofluorescence

Cells grown on coverslips were washed in PBS then fixed with 4% parafomaldehyde in PBS for 10 minutes at room temperature. Fixed cells were washed with PBS and treated with ice-cold methanol/acetone (1:1) for 4 minutes followed by a wash in PBS. Cells on coverslips were incubated with primary antibodies (a polyclonal rabbit anti-pKi-67 antibody diluted 1:150, DAKO, Denmark); a polyclonal goat anti-lamin A antibody, diluted 1:100, Santa Cruz, USA) for 1 hour at room temperature, washed in PBS then incubated for 1 hour at room temperature with appropriate flurochrome-conjugated secondary antibody. Cells were then washed three times with PBS and once with water then mounted onto glass slides in 30% (v/v) glycerol containing 12% (w/v) Mowiol (Aldrich, UK), 1 mg ml^{-1} DAPI and 2.5% (w/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma, UK).

2.3 Apoptosis assay

Adherent cells were harvested by trypsinization, collected by centrifugation, washed and finally resuspended in binding buffer (10mM HEPES/NaOH, pH 7.5, containing 140mM NaCl and 2.5mM $CaCl_2$) at a concentration of 10^6 cells ml^{-1}. The cell suspension was incubated with 0.5μg ml^{-1} Annexin V FITC conjugate (Sigma, UK) and 2μg ml^{-1} propidium iodide solution (Sigma, UK) for 10 minutes in the absence of

light. Fluorescence was determined by flow cytometry using a Beckman Coulter Epics XL.MCL, with the counter set at 10,000 events.

3. Results.

3.1 Replicative lifespans of HGPS fibroblasts.

Since cellular aging is thought to contribute to organismal aging (Campisi, 1998; Faragher and Kipling, 1998) we compared the growth of 3 normal and 7 HGPS dermal fibroblasts throughout their reproductive lifespans (Table 1. A). In agreement with previous studies (Martin et al., 1970) we observed a wide range of growth potentials of HGPS fibroblasts (mean population doublings = 27.1 ± 18.5 s.d., n=7) compared with normal fibroblasts (mean population doublings = 58 ± 6.2 s.d., n=3). Thus, whilst some cultures of HGPS fibroblasts failed to thrive, others performed almost as well as normal cultures. The limited reproductive lifespan of normal fibroblasts is characterised by a steady decline in the fraction of proliferating cells throughout their reproductive lifespan (Kill et al., 1994). Accordingly, as normal cultures age, the fraction of reproductively-sterile senescent cells increases. Interestingly, the rate of accumulation of senescent fibroblasts in cultures derived from another progeroid syndrome, Werner Syndrome (WS) is accelerated by up to 6-fold (Kill et al., 1994). Therefore we wished to know how the rate of loss of proliferating cells within HGPS cultures compared with that of normal fibroblasts. At each passage fibroblasts grown on coverslips were fixed and stained for the presence of the Ki67 antigen (pKi67). Ki67 antibodies react with a nucleolar antigen that is present in proliferating cells only and thus provides a marker for the estimation of growth fraction (Kill, 1996). This fraction was plotted against the number of accumulated population doublings (APD) achieved by each culture. Thus, APD is a measure of growth whilst the percentage of Ki-67 positive cells represents the fraction

of cells contributing to that growth. In each HGPS case tested we found that the fraction of proliferating cells was maintained at relatively high levels compared with normal cultures at the same APD level. However, the duration of this period of sustained growth varied according to the maximum reproductive lifespan (Fig. 1, A-D). A striking difference between the growth of normal and HGPS fibroblasts is the rapid decline in proliferating cells observed towards the end of the HGPS culture's lifespan. This is in marked contrast to the growth of normal fibroblasts that shows no such rapid loss towards the end of the reproductive lifespan but rather a smooth decline of proliferative cells throughout. This can be seen clearly in Fig 1.E where the rates of cellular aging for two long-lived strains of HGPS fibroblasts and two normal fibroblast strains are plotted together. In this graph it is clear that young to late middle-aged HGPS cultures contain a relatively high fraction of proliferating cells compared with normal cultures. Subsequently, late passage HGPS experience a sharp decline in the fraction of proliferating cells and the values fall below those of normal cultures at an equivalent number of accumulated population doublings. Thus cellular aging in cultures of HGPS fibroblasts is characterised by an initial period of hyperproliferation followed by later rapid loss of proliferating cells. Hyperproliferation in HGPS fibroblasts is not likely to be caused by autocrine stimulation since these cells are serum-responsive and enter quiescence upon serum starvation (data not shown).

3.2 Apoptosis in HGPS fibroblasts

To investigate the possible cause of the rapid loss of proliferating cells from HGPS cultures we compared levels of apoptosis in young and old HGPS and normal fibroblast cultures (Table 1, B). There is only a small increase in the fraction of apoptotic cells during aging of normal fibroblasts, which is in agreement with

previous studies on adherent fibroblasts (DeJesus et al., 2002). However, in early passage cultures of HGPS fibroblasts the fractions of apoptotic cells are as high (2.2%) or higher (4.1%) than senescent, unaffected fibroblasts (2.2%). Within late passage HGPS cultures the fraction of apoptotic cells has risen dramatically to 16-17%. Thus there is a clear 4-8-fold increase in the rate of apoptosis during cellular aging of HGPS fibroblasts that is not observed with normal fibroblasts. This finding is in broad agreement with a report of increased rates of apoptosis in cultures of cells derived from a HGPS model mouse (Mounkes et al., 2003).

3.3 Changes in nuclear morphology during cellular aging of HGPS fibroblasts

Previous reports have noted the presence of abnormal nuclear morphology within HGPS cultures (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). However, we have noted that cellular aging within normal fibroblast cultures is characterised by an increase in the fraction of morphologically abnormal nuclei (Kill, I.R., unpublished data). Therefore, we wished to know whether the occurrence of abnormal nuclei in HGPS is linked to cellular aging or persists at a constant fraction throughout the reproductive lifespan. We scored the fractions of abnormal nuclei present in young and aged cultures of normal and HGPS fibroblasts (Fig. 2, Table 1. C). Abnormal nuclei are characterised by their deviation from a typical ellipsoid shape, displaying herniations, blebs and micronuclei. Within normal cultures the fraction of these nuclei rose from 5 to 32% from early to late passage. The occurrence of abnormal nuclei in cells of HGPS cultures was highly variable. However in each HGPS case tested we found there to be an increase in the fraction of abnormal nuclei with increasing cellular age. Thus within aged HGPS cultures the mean percentage of abnormal nuclei is 57.5 ±19.6 (SD, n=6) with a minimum value of 29.5% and a

maximum value of 87.1%. These data suggest that the generation of aberrant nuclear morphology seen in HGPS cells is as a result of cell division.

3.4 *Changes in nuclear lamina organisation and composition during cellular aging of HGPS fibroblasts*

Finally, we wanted to assess the consequences of having mutations in the lamin A gene upon lamina organisation. Early and late passage HGPS fibroblasts were grown on coverslips then fixed and stained for the presence of lamin A using a lamin A–specific antibody. After staining, cells were classified according to their presentation of lamin A-containing structures (Fig. 3). Essentially all interphase cells produced a positive stain using an antibody that cannot distinguish lamin A and C regardless of age, indicating the presence of lamin C throughout HGPS cells' lifespans (data not shown). In young HGPS cultures between 80 – 90% of nuclei showed typical nuclear rim staining (Fig 3, A). Another 7-14% showed rim staining but additionally showed speckles within the nuclear interior (Fig 3, B). Between 3-4% of young HGPS cells were negative for lamin A staining. In contrast, typical nuclear rim staining in aged HGPS cultures had decreased to 33% and the proportion of nuclei showing rim and speckles staining had risen to about 26%. Furthermore, between 12-22% of nuclei now displayed speckled lamin A staining in the absence of nuclear rim staining (fig 3, C) and between 17-29% showed no lamin A staining at all (Fig 3, D). Thus as HGPS fibroblasts age there is a loss of typical nuclear rim staining of lamin A structures and a concomitant increase in both intranuclear lamin A speckles and lamin A negative nuclei.

4. Discussion.

From these studies we are beginning to gain a picture of the possible processes leading to premature aging of HGPS individuals. Our data support the idea that a

HGPS cellular phenotype develops progressively from what appear to be essentially normal cells to cells that possess an abnormal lamina, abnormal nuclear morphology and are prone to apoptosis. A link between lamin A and apoptosis is clearly established (Rao et al., 1996), although lamin A cleavage seems to be a consequence rather than a cause of apoptosis. However, cleavage of only a small fraction of A-type lamins is sufficient to cause disintegration of the lamina (Broers et al., 2002). This finding may be highly relevant to our understanding of the cause of apoptosis in HGPS cells in which lamin A mutations may generate dominant-negative forms of lamin A.

Our finding that young HGPS cultures contain higher fractions of proliferating cells compared with young normal cultures suggests that proliferative control is deregulated. This process could be mediated by growth regulators known to interact with lamin-containing structures such as pRb (Markiewicz et al., 2002; Ozaki et al., 1994). Alternatively, since there is a strong link between genome organisation and structures at the nuclear envelope (Bridger and Bickmore, 1998; Holaska et al., 2002) then a compromised lamina organisation may result in aberrant gene expression leading to deregulated growth. In either case it is likely that a disorganised lamina is less able to protect the genome from physical trauma particularly encountered during the upheavals of mitosis and nuclear reassembly. Thus with increasing rounds of cell division there is an increasing likelihood that the resultant daughter nuclei will develop abnormally. In some cases the abnormality will lead to apoptosis. Superimposed on these processes is that of cellular senescence. Therefore with each successive round of cell division HGPS fibroblasts have an increasing probability of either entering senescence or proceeding to apoptosis. If apoptosis in HGPS cultures is driven by cell division then senescent HGPS fibroblasts will be refractory to

apoptosis since they are unable to divide. This idea is further supported by the finding that telomere lengths are reduced in HGPS fibroblasts (Allsopp et al., 1992). Thus, proliferating HGPS fibroblasts, presumably with long telomeres, are prone to apoptosis whilst senescent HGPS fibroblasts, presumably with short telomeres are resistant and accumulate within the cultures.

It is highly likely that accumulation of senescent cells within tissues with increasing age contributes to age-dependent decline in tissue function (Campisi, 1998; Faragher and Kipling, 1998). Thus, we propose that in HGPS individuals, mutations in the lamin A gene results in deregulation of growth control, hyperproliferation and increased rates of apoptosis. As a consequence there is premature loss of functional "young" cells and a concomitant premature accumulation of senescent cells, both of which promote the age-related decline in tissue function leading to premature senescence of the individual. Indeed, post-mortem studies have shown that progeric arteries are depleted of smooth muscle cells, which is consistent with atherosclerotic disease (Stehbens et al., 2001).

Interestingly, the rates of cellular aging in cultures of fibroblasts derived from another progeroid syndrome, Werner Syndrome are accelerated by up to 6-fold (Faragher et al., 1993; Kill et al., 1994). Thus, cellular aging of WS fibroblasts is characterised by premature accumulation of senescent cells whilst HGPS is characterised by premature loss of proliferating cells through apoptosis generating a culture also rich in senescent cells. Although mutations in the WRN helicase gene accounts for 80% of WS cases, it has recently been shown that 3% (4 out of 129 cases) are due to mutations in LMNA (Chen et al., 2003). Clinically, there is little overlap between WS and HGPS (Martin and Oshima, 2000). However, at the cellular level both syndromes seem to originate from genomic instability and both lead to

accelerated aging of certain tissues. There is no strong evidence to suggest that either HGPS or WS are characterised by premature aging of the brain or central nervous system. Since most of this tissue is post-mitotic then this is entirely consistent with the view that cell turnover and tissue homeostasis are important factors in the aging of certain tissues only. Indeed, in both HGPS and WS it is only those tissues undergoing turnover that are affected. Therefore, this provides powerful evidence that loss of normal cellular function, either through apoptosis or senescence, or a combination of both, leads to the decline in tissue-specific function characteristic of normal aging and which occurs prematurely in the human progeroid syndromes.

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Table 1. Comparison of normal and HGPS fibroblasts.

A. Maximum growth potential of normal and HGPS fibroblast cultures.

Cell Strain	Mutation	Max APD	Slope	Y-intercept	R^2	Standard error
AG10677	E145K*	6.3	nd	nd	nd	nd
AG03199	?	11.7	nd	nd	nd	nd
AG10750	?	13.8	nd	nd	nd	nd
AG10578	Not G608G+	18.9	nd	nd	nd	nd
AG06297	G608G*	44.3	nd	nd	nd	nd
AG08466	Not G608G+	45.5	-2.1	114	0.8350	12.41
AG11572	?	49.5	-1.5	95	0.6903	16.01
1Br	WT	65.0	-0.9	60	0.8007	5.86
2DD	WT	53	-0.9	56	0.7294	8.33
NB1	WT	56	-1.2	68	0.7892	7.22

B. Apoptosis.

Cell Strain	Early passage *(% replicative lifespan completed)*	Late passage *(% replicative lifespan completed)*
2DD	0.02 *(14.4)*	2.2 *(95.0)*
AG08466	2.2 *(18.9)*	16.2 *(99.6)*
AG11572	4.1 *(17.2)*	17.1 *(97.8)*

C. Percentages of abnormal nuclei.

Cell Strain	Early passage *(% replicative lifespan completed)*	Late passage *(% replicative lifespan completed)*
2DD	5.5 ± 0.1 *(14.4)*	32.1 ± 4.5 *(95)*
NB1	2.3 ± 0.8 *(7.6)*	17.9 ± 2.0 *(96.2)*
AG08466	8.5 ± 1.8 *(18.9)*	48.9 ± 1.5 *(99.6)*
AG11572	4.8 ± 0.5 *(17.2)*	52.6 ± 1.3 *(97.8)*
AG10578	14.1 ± 1.4 *(38.6)*	29.5 ± 0.3 *(93.0)*
AG06297	29.2 ± 2.1 *(78.8)*	70.3 ± 4.2 *(96.9)*
AG03199	n.d.	56.6 ± 2.2 *(99.5)*
AG10677	n.d.	87.1 ± 2.2 *(99.5)*

Growth potential determined by calculating the maximum number of population doublings (Max APD) and includes information supplied by the Coriell Cell Repository for HGPS fibroblast strains AG10677, AG03199, AG10750, AG10578, AG06297, AG08466 and AG11572. 1BR, 2DD and NB1 are all normal fibroblast strains. Mutation data is included where known. * indicates previously reported mutation analyses (Eriksson et al., 2003). + indicates data from Dr. N. Levy, personal communication. WT = wild type. Regression lines were fitted where appropriate. Slope values are %Ki-67 positive nuclei APD^{-1} , R^2 = correlation coefficient, nd = not done. Standard error of the regression line provides a measure of "goodness of fit".

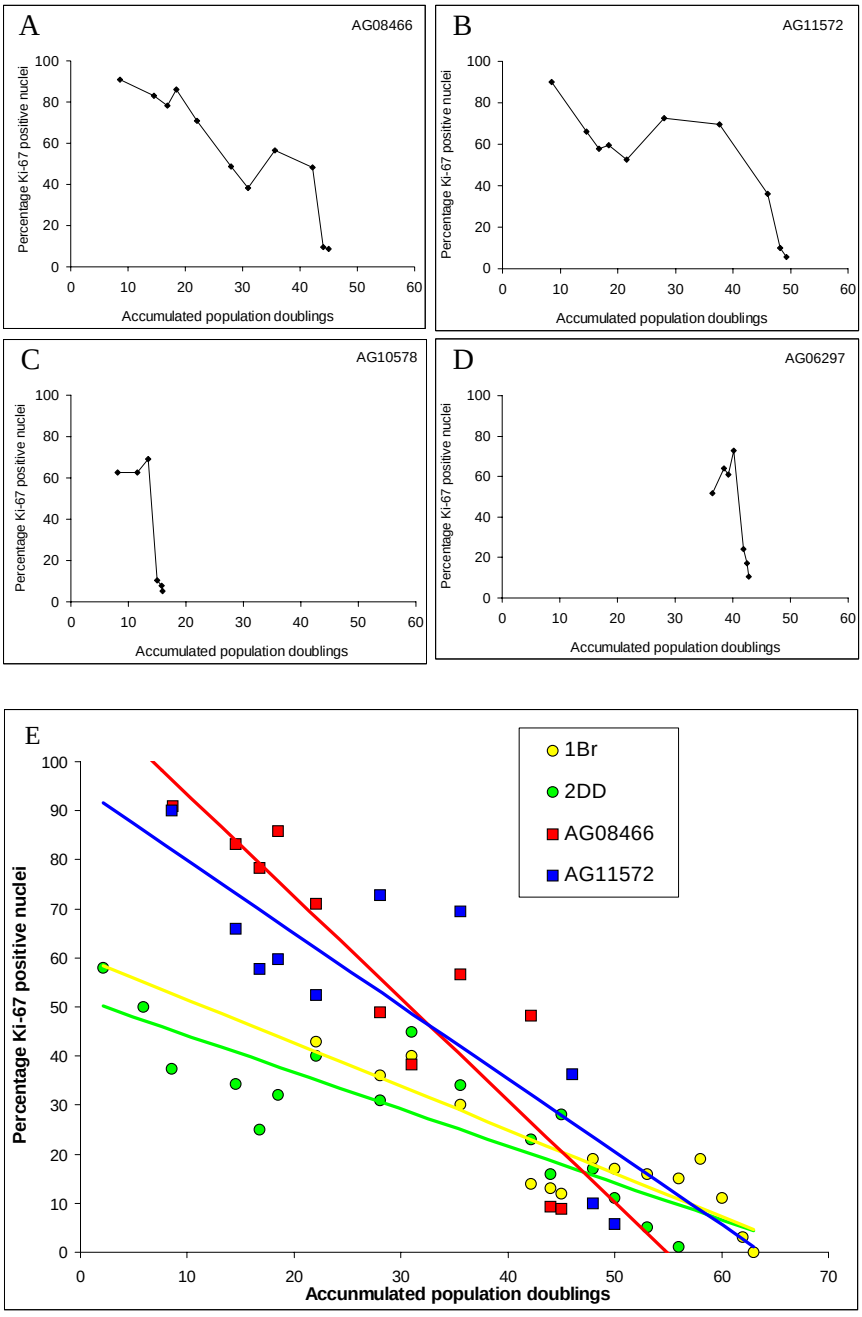


Figure 1. **Cellular aging of normal and Hutchinson-Gilford progeria fibroblasts.** Rates of cellular aging were assessed by estimating the percentage of proliferating cells remaining within fibroblasts cultures at increasing population doubling levels. Proliferating cells were identified by positive staining with Ki-67 antibodies. A) HGPS strain AG08466, B) HGPS strain AG11572, C) HGPS strain AG10578, D) HGPS strain AG06297. Early passage cells were not available for AG06297. E) shows a comparison between two normal fibroblasts cultures (1BR and 2DD) and two HGPS fibroblast cultures (AG08466 and AG11572). Regression lines were fitted using the method of least squares. Details of the graphs are shown in Table 1.

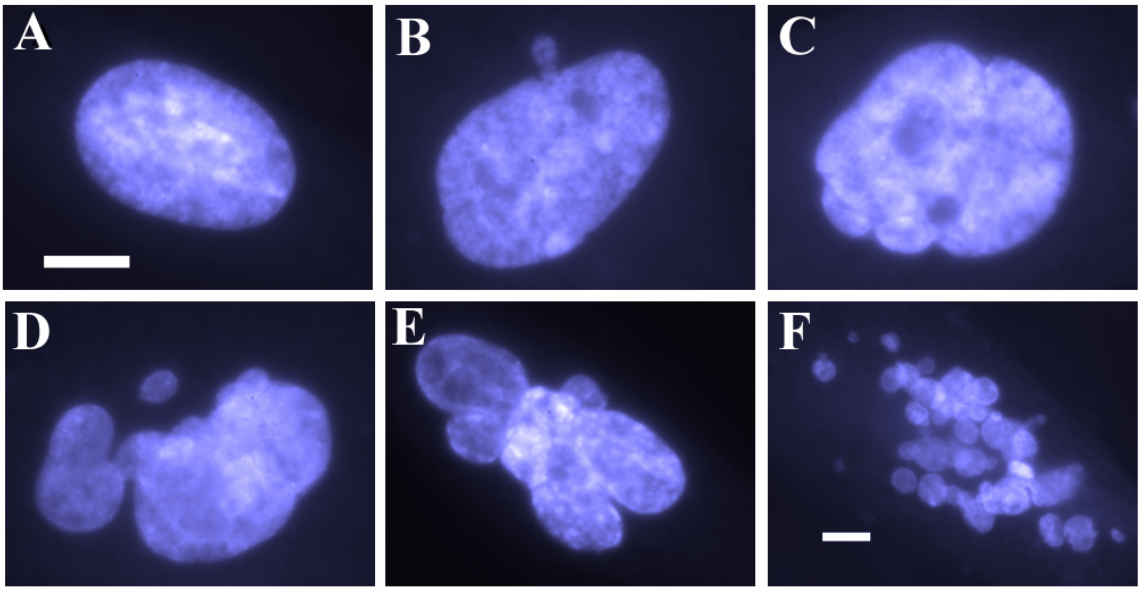
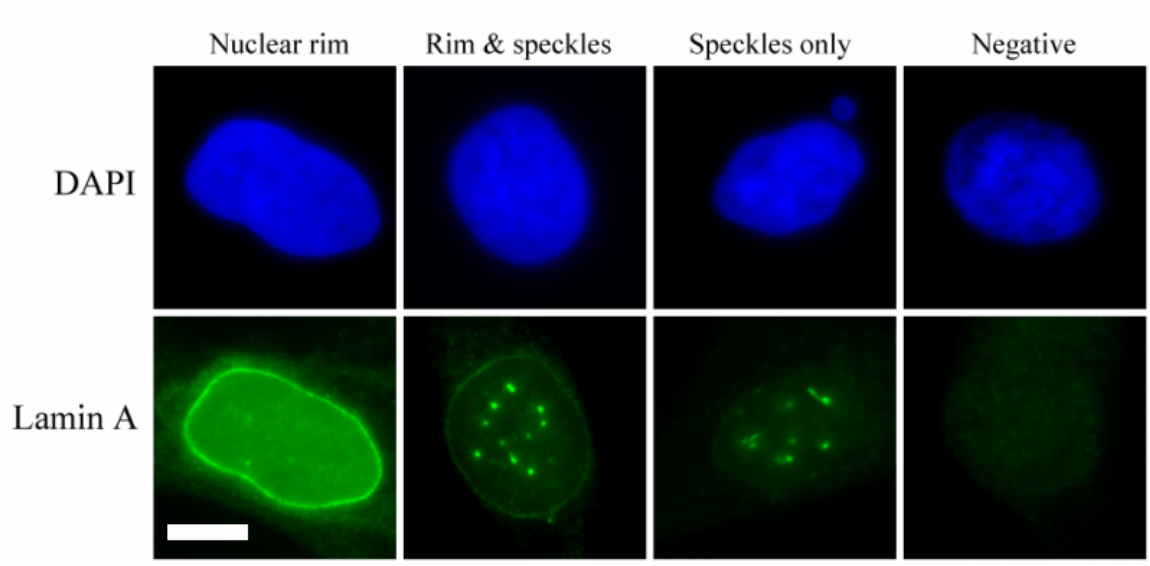


Figure 2. **Abnormal nuclear morpholgy in cultures of HGPS fibroblasts.** HGPS fibroblasts were grown on glass coverslips, fixed and stained with DAPI to reveal DNA. Representative images show a normal control nucleus (A) and the range of abnormal nuclear morphologies observed in HGPS cultures (B-F). Abnormalities could be minor such as herniations in the nuclear periphery (B) or folds and crevices (C). Abnormalites can be more severe such as fragments (D) and lobules (E). Panel F shows a striking fragmentation of the nucleus to generate micronuclei (note the change in scale). Scale bars = 10 μm.

A



B

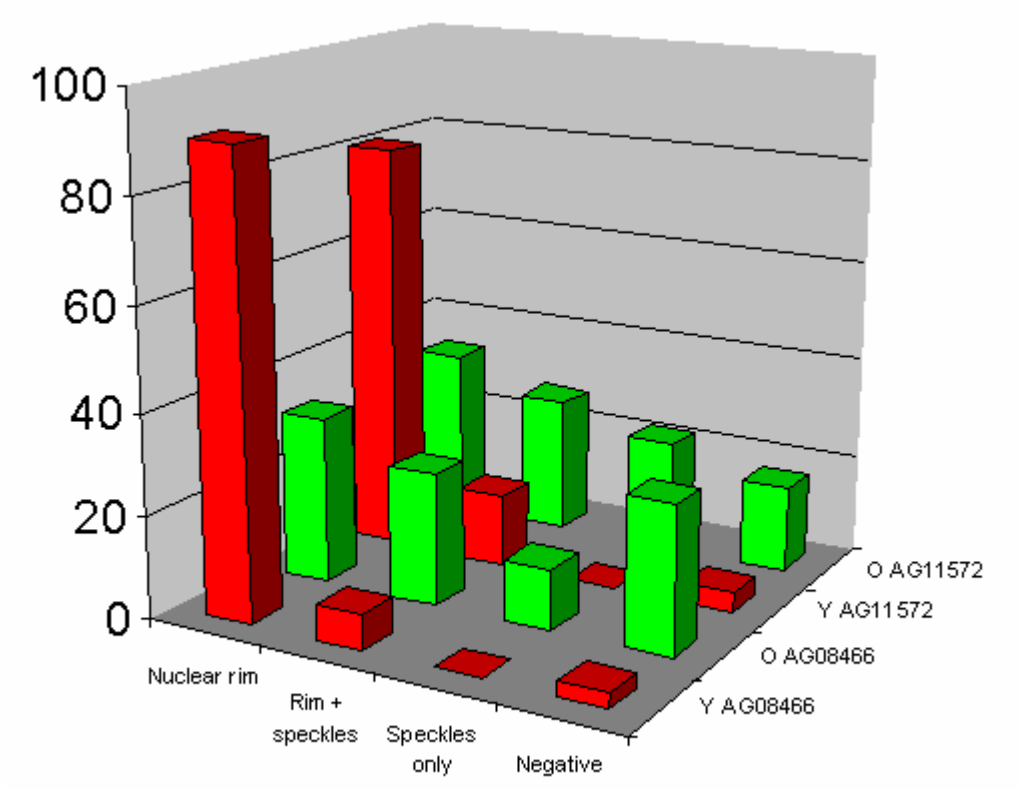


Figure 3. **Analysis of lamin A staining in Hutchison-Gilford Progeria Syndrome Fibroblasts**. (A) Cultures of HGPS fibroblasts were grown on coverslips, fixed and stained for the presence of lamin A and DAPI to reveal DNA. The panels show representative images of lamin A staining classified according to pattern. Scale bars = 10μm. (B) Percentages of HGPS fibroblasts presenting each staining pattern in young (Y) and senescent (O) HGPS strains AG08466 and AG11572.