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The expression of proliferation-dependent antigens during the lifespan of normal and progeroid human fibroblasts in culture

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SUMMARY

Normal human fibroblasts display a limited lifespan in culture, which is due to a steadily decreasing fraction of cells that are able to proliferate. Using antibodies that react with antigens present in proliferating cells only, in an indirect immunofluorescence assay, we have estimated the fraction of proliferating cells in cultures of normal human fibroblasts. Furthermore, we have estimated the rate of decline in the fraction of proliferating cells during the process of cellular ageing by application of the assay to normal human fibroblasts throughout their lifespan in culture. Werner's Syndrome is an autosomal recessive disease in which individuals display symptoms of ageing prematurely. Werner's Syndrome fibroblasts display a reduced lifespan in culture compared with normal human fibroblasts. Like normal human fibroblasts, the growth of Werner's Syndrome fibroblasts is characterised by a decreasing fraction of cells reacting with the proliferation-associated antibodies throughout their lifespan in culture. However, the rate of loss of proliferating cells in Werner's Syndrome fibroblasts during the process of cellular ageing is accelerated 5- to 6-fold compared with the rate determined for normal human fibroblasts.

Key words: cellular ageing, proliferation assay, Werner's syndrome, human fibroblast, cell mortalization

INTRODUCTION

The growth of normal human fibroblasts is characterised by a finite and predictable lifespan (Hayflick and Moorhead, 1961; Shall, 1987). The limited lifespan is due to a steadily decreasing fraction of cells that are able to proliferate (Cristofalo and Sharf, 1973; Smith and Hayflick, 1974; Westermark, 1978; Stein et al., 1978; Shall and Stein, 1979; Ponten et al., 1983). Cultures of normal human fibroblasts have been widely used both as a model for ageing in vitro and as an example of cell growth regulation.

Werner's Syndrome (WS) is a rare autosomal recessive disease in which the affected patients display symptoms of ageing prematurely. Individuals suffering from WS may exhibit a number of a wide range of symptoms including short stature, hypogonadism, juvenile cataracts, premature greying of hair with alopecia, diabetes, atherosclerosis, skin changes and osteoporosis. However, individuals do not usually suffer the behavioral changes and Alzheimer-type neuropathologies commonly associated with old age. Diagnosis of WS therefore relies upon assessment of a number of criteria. All WS individuals appear to display two additional diagnostic features: firstly, affected individuals display increased levels of urinary hyaluronic acid; secondly, fibroblasts derived from affected individuals display a markedly reduced in vitro lifespan compared with normal fibroblasts (Epstein et al., 1966; Tollefsbol and Cohen, 1984). Since only a single Mendelian genetic locus is involved, it is likely that the mechanisms responsible for the shorter lifespan of WS fibroblasts in culture are related to the mechanisms causing the onset of premature ageing in individuals. Therefore, a comparison of the behaviour of normal and WS fibroblasts in vitro may provide insight into the processes involved in ageing in vivo. The relative rates of ageing (loss of proliferative potential) of normal and WS fibroblasts in culture are unknown. In order to compare the proliferative behaviour of normal and WS fibroblasts it is necessary to determine the population 'age' of cultures and to determine how this 'age' varies with passage.

Two parameters are needed to identify a meaningful biological age of cells in culture; the number of cell generations the culture has survived and the fraction of cells in the culture that are still capable of proliferation. Passage number and accumulated population doublings achieved, provide only a very rough estimate of the progress of cell cultures. The most commonly used method to determine the population 'age' of cultures has been to estimate by autoradiography the fraction of cells incorporating radioactive nucleosides after a long exposure (Cristofalo and Sharf, 1973). The estimates obtained could then be compared with previous estimates made at each passage level, and from this an estimate of the population age could be deduced. This method has been cogently criticised by Macieira-Coelho (1974), because it is acutely dependent upon unknown factors like the duration of the cell cycle phases and the variances of their distributions. This method is very sensitive to the period used for labelling compared to the cell cycle phases; too short a label fails to reveal all cycling cells,

while too long a label overestimates cycling cells because some of the labelled cells divide and are counted twice.

Immunohistochemical markers of cellular proliferation have been widely used to analyze the proportion of proliferating cells in histological sections of tumours (Hall and Woods, 1990). We present here a simple, reliable method for the determination of the population age of cultures of human cells. The method is based upon the estimation of the fraction of human fibroblasts expressing a number of antigens commonly associated with proliferation, in an indirect immunofluorescence assay performed at each passage level. In addition, we have estimated the fractions of cells expressing a number of antigens that are expressed independently of the cell cycle in a similar assay performed at each passage level, and these serve as a control for the total metabolising population of cells.

The results we have obtained indicate that there is a smooth and progressive decline in the fraction of cells expressing each of the cell cycle-dependent antigens, whilst the fraction of cells expressing each of the cell cycle-independent antigens remains constant and high throughout the in vitro ageing process.

Thus, estimation of the fraction of cells expressing the cell cycle-dependent antigens throughout the lifespan of a culture provides a useful means for the estimation of the population age of mortal human cultures.

When a similar indirect immunofluorescence assay was applied to cultures of WS fibroblasts, we found that as with normal fibroblasts there was a smooth decline in the fraction of cells displaying the proliferation-dependent antigens. However, in the case of WS fibroblasts the rate of decline in the fraction of proliferating cells was accelerated by between 5- and 6-fold compared with normal fibroblasts.

MATERIALS AND METHODS

Cells and cultures

Human dermal fibroblasts (Strain 1BR.3) were obtained by punch biopsy from a 23-year-old male and have been described previously (Arlett et al., 1975). Mass cultures were grown in 90 mm dishes (Gibco/Nunc) in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum (NCS) and antibiotics (10 units ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin) at 37°C in a 5% CO_2 atmosphere. Cells were released from the dish every 3 or 4 days by incubation with 0.01% (w/v) trypsin and 0.02% (w/v) EDTA, counted in an haemacytometer and replated into fresh dishes at a density of 3×10^3 cells cm^{-2}. This procedure was carried out until fewer cells were obtained upon subculture than were seeded. Accumulated population doublings (APD) were calculated from the increase in cell numbers in successive passages.

WS fibroblasts (strain BJ846) were obtained from a skin punch biopsy from a 24-year-old male patient initially diagnosed as a mute, hypogonadial dwarf. The patient was subsequently diagnosed as suffering from Werners Syndrome. Cultures were prepared, passaged and maintained as described for normal fibroblasts above.

Fibroblast cultures were made quiescent by washing twice in serum-free medium followed by incubation in medium supplemented with 0.5% (v/v) NCS for two days.

Antibodies

All antibody solutions were diluted in PBS containing 1% (v/v) NCS. Anti-Ki-67 (Dako PC, Denmark) was used at 1:25; anti-PCNA (anti-proliferating cell nuclear antigen, a kind gift from Dr R. Bravo, Heidelberg) was used at 1:1000; anti-topo II (anti-topoisomerase II, a kind gift from Dr L. Lui, Baltimore) was used at 1:250; SCL 70 (anti-topoisomerase I) and AAC 14 (anti-nucleolar antigens), were obtained from PRU Antiserum Purchasing Unit, Protein Reference Unit, Royal Hallasham Hospital, Sheffield S10 2JF) and both used at 1:500 and L68A7 (anti-lamin A/C, a kind gift from Dr R. Stick, Tubingen) was used at 1:500. Fluorochrome-conjugated second antibodies (Dako, Denmark) were used as follows: fluorescein-conjugated rabbit anti-mouse, 1:20 (anti-Ki-67, L68A7); rhodamine-conjugated goat anti-human, 1:25 (anti-PCNA, SCL 70, AAC 14) and fluorescein-conjugated goat anti-rabbit, 1:40 (anti-topo II).

Description of the antibodies

Antibodies reacting with proliferation-dependent antigens: anti-Ki-67, anti-PCNA and anti-topo II. Antibodies reacting with proliferation-independent antigens: AAC 14 (anti-nucleolar antigens), L68A7 (anti-A/C-type lamins), SCL 70 (anti-DNA topoisomerase I)

Anti-Ki-67 is a monoclonal antibody that reacts with nuclear antigens expressed in proliferating cells only (Gerdes et al., 1983, 1984, 1991).

Proliferating cell nuclear antigen (PCNA) was initially identified as a nuclear autoantigen from patients with systemic lupus erythematosus (Miyachi et al., 1978), and has subsequently been shown to be an auxiliary protein of DNA polymerase delta (Bravo et al., 1987; Prelich et al., 1987).

Topoisomerase II (topo II) is a well characterised nuclear enzyme involved in both DNA replication (Yang et al., 1987), and the segre-

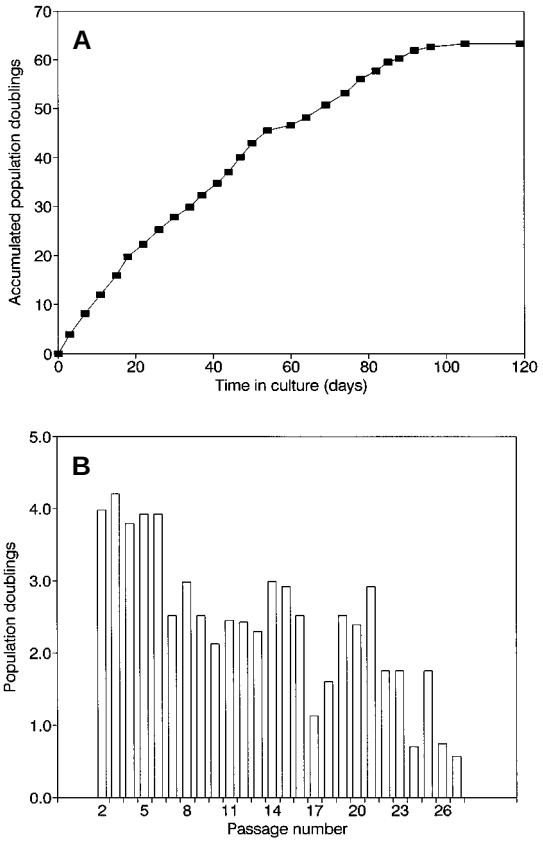

Fig. 1. Growth characteristics of the normal human fibroblast cell strain 1Br.3. (A) Growth curve for the human diploid cell strain 1Br.3. (B) Number of population doublings achieved at the indicated passage levels for the human dermal fibroblast cell strain 1Br.3.

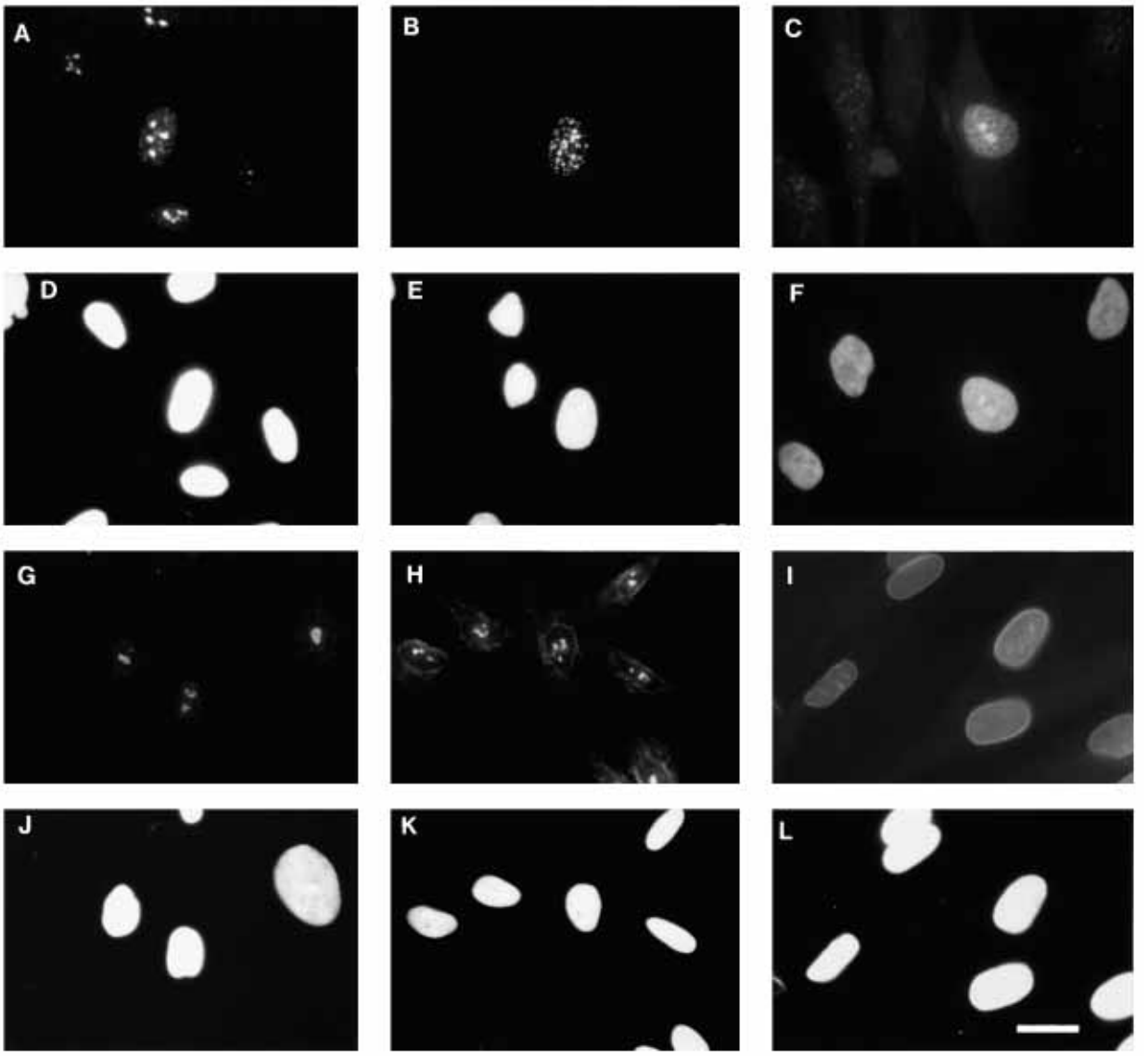

Fig. 2. Indirect immunofluorescence staining patterns obtained using the antibodies (A) anti-Ki-67, (B) anti-PCNA, (C) anti-topoisomerase II, (G) AAC 14, anti-nucleolar, (H) SCL 70, anti-topoisomerase I and (I) L6 8A7, anti lamin A/C. The identical fields are stained with DAPI to reveal DNA (D-F, and J-K) All photomicrographs were taken at the same magnification. Bar, 20 μm.

gation of replicated DNA molecules (DiNardo et al., 1984; Uemura and Yanagida, 1984; Earnshaw and Heck, 1985).

AAC14 is an antibody present in the serum of a human patient suffering from an autoimmune disease. The antibody recognises nucleolar antigens.

The nuclear lamins are a group of polypeptides that form a meshwork of intermediate filaments contiguous with the inner surface of the nuclear membrane (reviewed by Cox and Hutchison, 1993). The lamins are present in the nucleus throughout interphase although immunolocalisation studies have demonstrated cell-cycle-dependent patterns of distribution (Bridger et al., 1993).

Topo I has been shown to regulate the superhelical density of DNA by transiently nicking one strand on the DNA helix (Wang, 1985). The relative level of expression of topo I compared with total cellular protein has been demonstrated to remain essentially constant as cells progress through the cell cycle, and is thus regulated independently of the cell cycle (Heck et al., 1988).

Indirect immunofluorescence

Cells were plated onto glass coverslips at the same density as used for the mass cultures. On the day of subcultivation of the mass cultures, the cells on coverslips were washed extensively with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) and fixed with methanol/acetone (1:1 v/v) at 4°C for 10 minutes, rehydrated in PBS and incubated with 10 μl of antibody solutions at the dilutions given above, for 1 hour at room temperature in an humidified atmosphere. After washing with PBS the cells were covered with 10 μl of second antibody solutions for 1 hour at room temperature in an humidified atmosphere then washed with PBS and mounted on glass slides in 50% (v/v) glycerol in 50 mM Tris-HCl (pH 8.0) containing 1 μg/ml 4,6-diamidino-2-phenylindole and 1 mg/ml *p*-phenylenediamine and sealed with nail polish. Stained cells were viewed with a Zeiss Photomicroscope III fitted with a ×60 water-immersion objective using epifluorescence. For assessment of the proportion of stained cells, 1000 total or 200 positive nuclei, whichever came first, were counted in randomly selected fields.

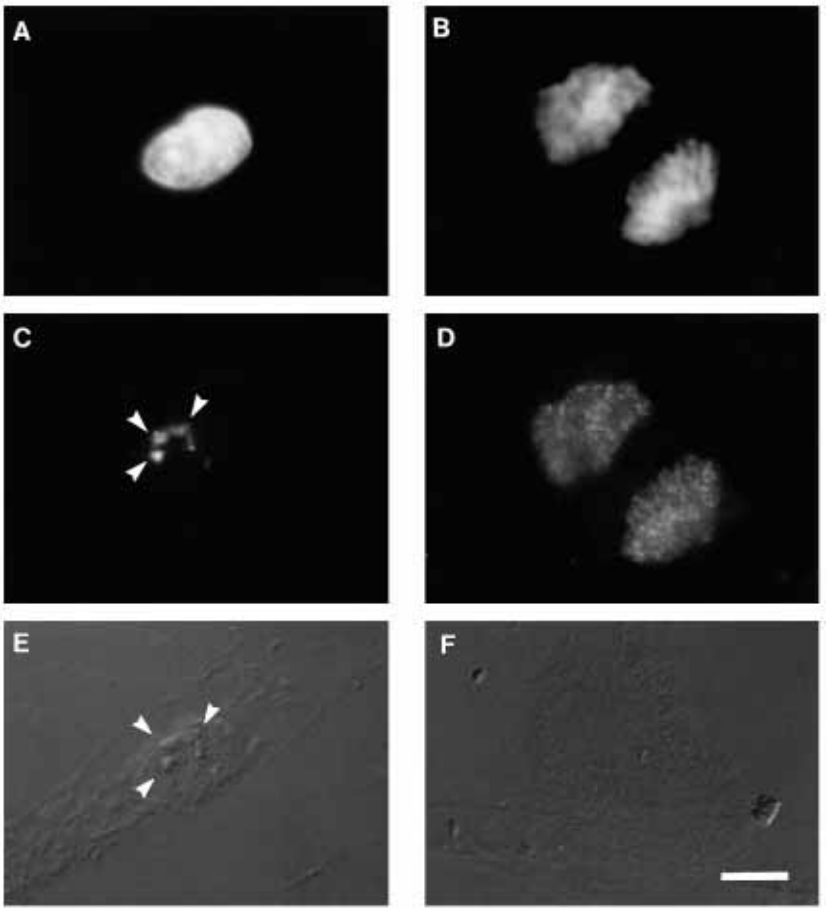

Fig. 3. The distribution of Ki-67 during interphase and mitosis. Immunolocalisation of Ki-67 in normal human fibroblasts during interphase: (A) shows the distribution of DNA, revealed by DAPI staining, (C) shows the distribution of Ki-67 and (E) shows the same field viewed using Nomarski optics. Arrowheads indicate the association of Ki-67 with components of the nucleoli. Immunolocalisation of Ki-67 during mitosis: (B) shows the distribution of DNA, (D) shows the distribution of Ki-67 and (F) shows the same field viewed using Nomarski optics indicating that nuclear envelope breakdown has occurred. Bar, 10 μm.

RESULTS

Growth of normal human dermal fibroblasts

The complete lifespan growth curve for the cell strain 1BR.3 is shown in Fig. 1A. Regular twice-weekly passaging was used to maintain the cells in log-phase growth, thereby minimising proliferative decline due to topoinhibition. The culture was passaged 27 times over 120 days, having undergone some 65 APD, before no increase in cell number was observed after 7 days. This result is consistent with those obtained with other human fibroblast cell strains (Hayflick, 1965; Martin et al., 1970; Cristofalo and Sharf, 1973; Ponten et al., 1983). Early passage cultures (passages 1-6) were unavailable, the growth curve for this period being supplied by Dr C. Arlett, University of Sussex. Even this presentation of the data shows a gradually declining growth rate from the outset of the culture. The decline in growth rate is clearly seen when the growth achieved at each passage is shown graphically (Fig. 1B).

Indirect immunofluorescence of the antigens

Representative photomicrographs of the staining patterns obtained with each antibody are shown in Fig. 2A-C and G-I. Cells were also stained with DAPI to reveal the distribution of DNA and thus to visualise all the cells in each field (Fig. 2D-F and J-L).

Staining for Ki-67 under the conditions reported here gave reasonable discrimination between positive and negative nuclei, although variation in intensity and staining pattern was observed between positive nuclei (Fig. 2A,D). Since the majority of studies involving immunolocalisation of the Ki-67 antigen have been performed in transformed cell lines, it was necessary to determine whether the Ki-67 antigen behaved in a similar manner in normal fibroblasts (Fig. 3). During interphase the Ki-67 antigen seems to be associated with several discrete nuclear structures (Fig. 3A,C and E, arrows) and is not uniformly dispersed through the nucleus. The nuclear structures with which Ki-67 associates have been identified as nucleoli (Verheijen et al., 1989a,b). In contrast, during mitosis the Ki-67 antigen is clearly associated with condensing chromosomes and not with the mitotic spindle (Fig. 3B,D and F). Thus, we conclude that the Ki-67 antigen does behave in a similar manner in normal fibroblasts and in transformed cells.

Staining for PCNA following fixation by methanol/acetone gave a bright punctate pattern, easily discernable from negatively stained nuclei (Fig. 2B,E). This method of fixation and staining with antibodies to PCNA has been shown to reveal sites of active DNA synthesis (Bravo and Macdonald-Bravo, 1987) and thus represents S phase nuclei.

All cells showed a positive reaction with the antibody to topo II, however, a fraction of nuclei displaying a diffuse bright stain were apparent, and it is these nuclei that are used as the data presented here as topo II-positive (Fig. 2C,F). During cell cycle progression, nucleoli undergo characteristic re-organisations (Ghosh, 1987). Immediately following mitosis nucleoli are present as a number of small foci that coalesce during G_1 to form larger aggregates. Co-staining of fibroblasts with topo II

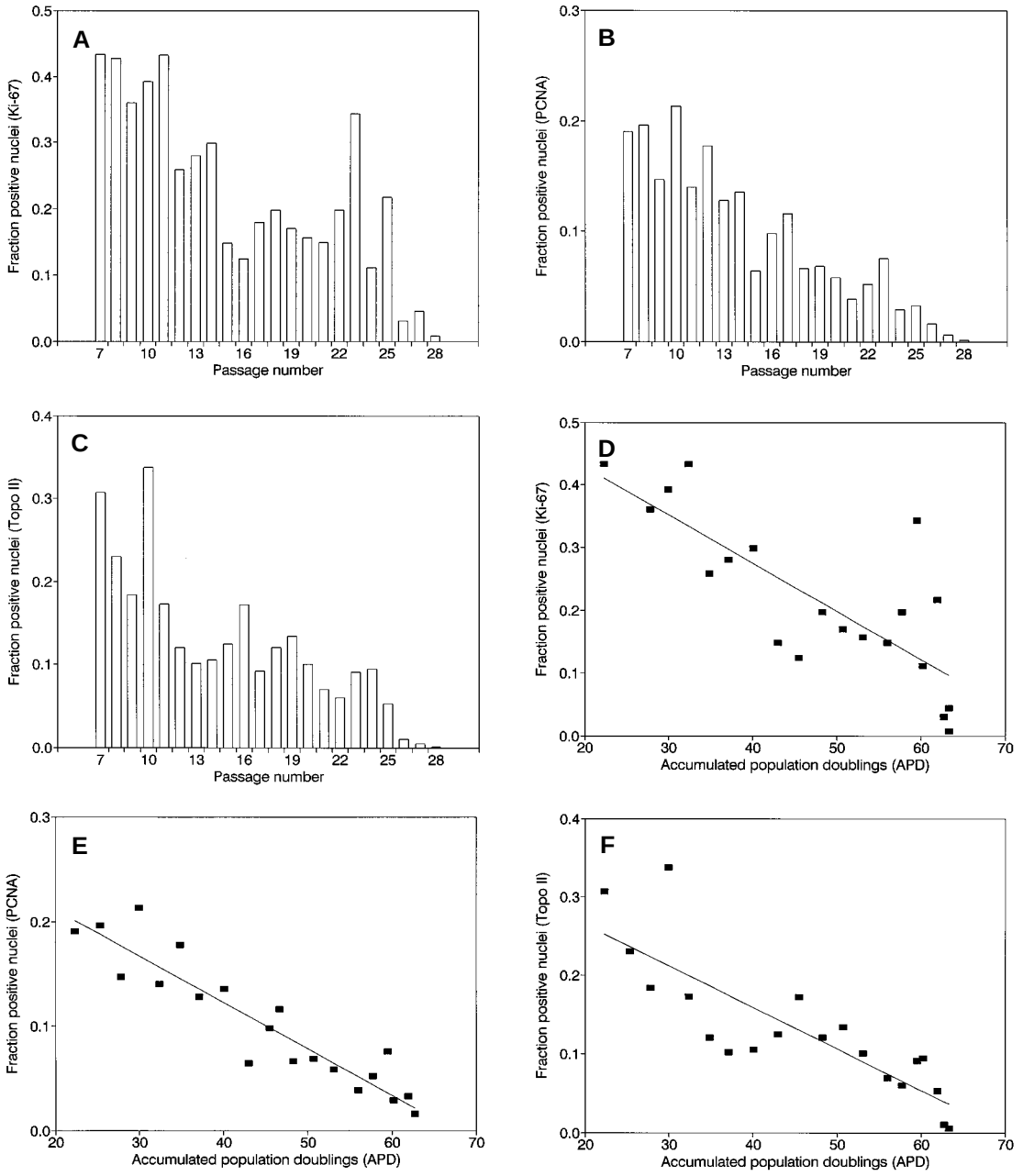

Fig. 4. The fraction of nuclei reacting with the antibodies anti-Ki-67, anti-PCNA and anti-topo II during cellular ageing of the normal fibroblast cell strain 1Br.3. The fractions of nuclei reacting with the indicated antibodies were plotted as a function of passage (A,B and C), and accumulated population doublings (APD; D,E and F). Regression lines were fitted using the method of least squares, and the details of the fitted lines are given in Table 1.

and anti-nucleolar antibodies have revealed that the diffuse bright topo II stain correlates with cells that have passed through mitosis and are in the process of nuclear reformation (I. R. Kill, J. M. Bridger and C. T. Hutchison, unpublished data).

The staining patterns obtained using both AAC14 (anti-nucleolar antigens, Fig. 2G,J) and SCL 70 (anti-topo I, Fig. 2H,K) were similar, being largely restricted to large, discrete regions present in the nucleus. Staining for L68A7 (anti-nuclear lamins, Fig. 2I,L) revealed intense staining of the nuclear rim.

The fraction of cells displaying the antigens during ageing of normal human dermal fibroblasts in culture

The fraction of cells that display each of the antigens Ki-67, PCNA and topo II as the cultures age show a smooth decline (Fig. 4A,B and C). The anti-Ki-67 antibody is thought to detect all proliferating cells whilst anti-PCNA detects only S-phase cells; thus, we observe that at any given point during the lifespan, the fraction of cells reacting with anti-Ki-67 antibodies is greater than the fraction of cells reacting with anti-PCNA.

Table 1. Decline in proliferation of the normal human fibroblast culture 1BR.3 during in vitro cellular ageing

Antibody	Assay	*y*-intercept	slope	*x*-intercept	r^2	*P*
Anti-Ki-67	days	47.5±7.8*	–0.39±0.06*	122±20	0.664	<0.001
	APD	59.2±7.8*	–0.79±0.13*	75±10	0.662	<0.001
Anti-PCNA	days	23.3±2.5*	–0.22±0.02*	106±11	0.857	<0.001
	APD	30.3±2.3*	–0.45±0.04*	67±6	0.886	<0.001
Anti-topo II	days	29.2±4.8*	–0.27±0.04*	108±18	0.709	<0.001
	APD	37.5±4.7*	–0.54±0.08*	69±8	0.723	<0.001

Details of the regression lines fitted to the data shown in Fig. 4 (normal fibroblasts). Regression lines were fitted using the least squares method. Days, number of days in culture. APD, accumulated population doublings. *y*-intercept, the fraction of nuclei staining for the indicated antigen at the initiation of the culture, determined by back-extrapolation of the regression line to $x = 0$. Slope, the slope of the regression line generated by the least squares method. Dimensions of the slopes are: days, fraction positive nuclei day^{-1}; APD, fraction positive nuclei APD^{-1}. *x*-intercept, the predicted maximum number of APD achieved by the culture, determined by extrapolation of the regression line to the *x* axis when *y* (the fraction of nuclei positive for the indicated antigen) = 0. r^2, regression coefficient. *P*, significance of the slopes. Values are shown ± standard error of the coefficient. Values marked with an asterisk are multiplied by 100.

The fraction of cells reacting with the antibody to topo II was intermediate between Ki-67 and PCNA, although we note that the assay for topo II-reactive cells is not as sensitive as the assays for Ki-67 and PCNA.

Since passage number is only a poor indicator of cell ageing, we have expressed the fraction of cells reacting with each of the antibodies as a function of the number of accumulated population doublings (Fig. 4D,E and F). By extrapolation of the regression lines generated for each curve to the horizontal axis, we obtained the maximum number of APD achieved by the culture for each antibody used in the assay. Using anti-Ki-67, the maximum predicted lifespan was 75.0 APD compared with 68.9 APD using anti-topo II and 67.0 APD using anti-PCNA (Table 1, APD). The small differences between these estimates arise from the relative sensitivity of the assay with each antibody. We have included in Table 1 details of the regression lines generated using the fraction of positive nuclei for each antibody plotted against time in culture (days).

Less than 1% of cells in cultures made quiescent by serum starvation for 48 hours at passage levels 9, 20 and 27 were positive for each of the antigens Ki-67, PCNA and topo II, confirming that expression of these antigens is dependent upon progression through the cell cycle.

Since expression of these antigens is dependent upon progression through the cell cycle, then clearly there is a smooth decline in the fraction of cells that are proliferating as the culture develops from early to late passage. The rate of decline in the fraction of positive cells is essentially the same with all three antibodies. This implies that we are measuring the same underlying event each time. This is presumably the frequency of irreversible exit from the cell cycle and here we estimate the fraction of cells remaining that are cycling.

Essentially all cells were positive throughout the lifespan of these cultures for the antigens recognised by the antibodies AAC14, L68A7 and SCL 70. In cultures that were made quiescent by growth in medium containing low (0.5% v/v) levels of serum these antigens were still expressed in all cells (data not shown). Since these antigens are expressed independently of the cell cycle, this indicates that the decline in proliferative potential of the culture is not accompanied by a substantial loss of viability during in vitro ageing.

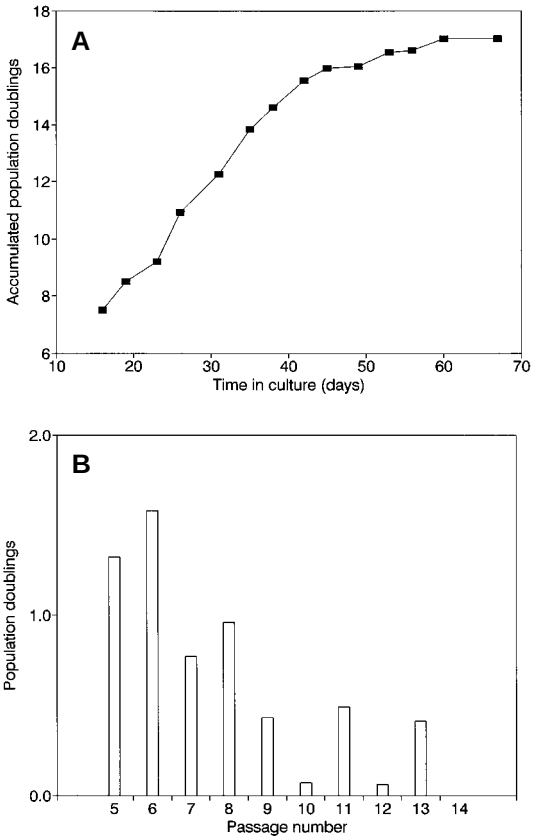

Fig. 5. Growth characteristics of the Werner's Syndrome fibroblast cell strain BJ846. (A) Growth curve for the WS cell strain BJ846. (B) Number of population doublings achieved by the WS cell strain BJ846 at the indicated passage levels.

The growth of WS fibroblasts

The growth curve for the WS cell strain (BJ846) is shown in Fig. 5A. In our hands the culture could achieve only 17 APD before no further increases in cell number was observed. This result is consistent with other WS cell strains (Tollefsbol and Cohen, 1984). The growth rate of the culture measured as the number of population doublings achieved at each passage by this culture reveals a decline in the growth rate with each passage, although the data show considerable scatter (Fig. 5B).

The fraction of cells displaying Ki-67 and PCNA during the ageing of WS fibroblasts in culture

The data described above using normal fibroblasts indicated that anti-Ki-67 and anti-PCNA antibodies provided the most consistent results in the indirect immunofluorescence assay. With this in mind, and because both anti-Ki-67 and anti-PCNA are obtainable commercially, we have concentrated on these two proliferation-dependent antibodies for our assays of WS fibroblasts. Both antibodies displayed staining patterns indistinguishable from those obtained with normal fibroblasts (data not shown). The fraction of WS fibroblasts that reacts with anti-Ki-67 and anti-PCNA at each passage level shows a gradual decline as with normal fibroblasts (Fig. 6A,B). Similarly, when plotted against the number of accumulated

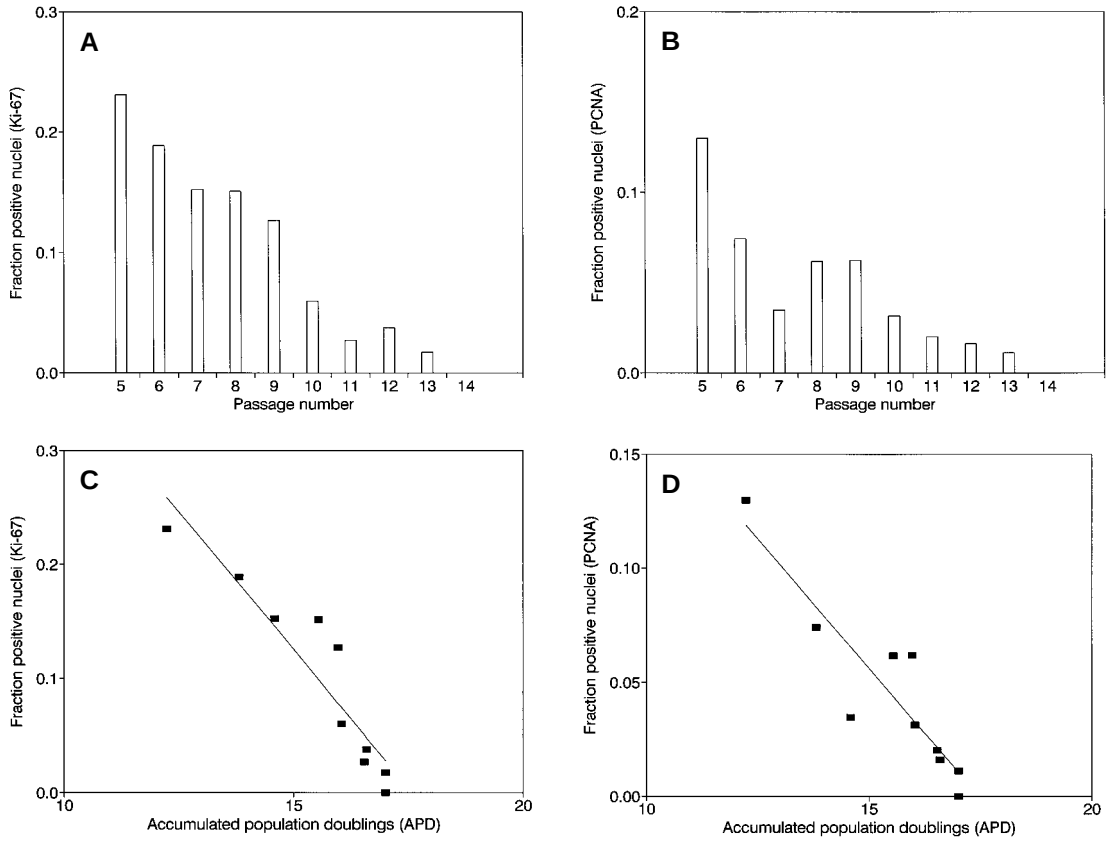

Fig. 6. The fraction of nuclei reacting with anti-Ki-67 and anti-PCNA during the cellular ageing of the WS cell strain BJ846. The fraction of nuclei reacting with the indicated antibodies were plotted as a function of passage (A,B) and accumulated population doublings (APD; C,D). Regression lines were fitted using the method of least squares and the details of the fitted lines are given in Table 2.

population doublings we observed a gradual decline in the fraction of cells reacting with each antibody (Fig. 6C,D). Regression lines were fitted to the data using the method of least squares (Table 2). The predicted maximum lifespan using each antibody was determined by extrapolation of the regression lines. The parameters of these regression lines are shown in Table 2. Using anti-Ki-67 the maximum predicted lifespan of WS fibroblasts is estimated at 17.6 APD and using anti-PCNA, 17.5 APD. We have included in Table 2 details of the regression lines generated using the fraction of positive nuclei for each antibody plotted against time in culture (days).

Essentially all WS fibroblasts were positive for the antigens recognised by the two proliferation-independent antibodies L6 8A7 and SCL 70 throughout the lifespan of these cultures (data not shown). Therefore, ageing of WS fibroblasts in culture is not characterised by a detectable increase in non-metabolising, moribund cells.

The data show that WS fibroblast cultures undergo a gradual decline in the fraction of proliferating cells during serial passage in the same manner as do normal cells. However, the rate of decline in the fraction of proliferating cells is accelerated when compared with normal fibroblasts (compare Table 1 with Table 2). It is interesting to note that extrapolation of the regression lines to the vertical axis (time 0, or the point of explantation of the culture) for the WS cell strain predicts a high fraction of cells reacting with either antibody at the initiation of the culture. For anti-Ki-67 a value of 0.59 was obtained for normal fibroblasts compared with 0.85 obtained for WS fibroblasts and for anti-PCNA a value of 0.30 was obtained for normal fibroblasts compared with 0.40 with WS fibroblasts.

Clearly, the rate of decline in the fraction of proliferating cells in WS cultures is accelerated when compared with the rate of decline in the fraction of proliferating cells in normal

Table 2. Decline in proliferation of WS fibroblast cultures during in vitro cellular ageing

Antibody	Assay	*y*-intercept	slope	*x*-intercept	r^2	*P*
Anti-Ki-67	days	30.8±3.5*	−0.54±0.08*	57±6	0.838	<0.001
	APD	85.3±3.1*	−4.85±0.67*	18±2	0.868	<0.001
Anti-PCNA	days	13.5±2.3*	−0.24±0.06*	57±10	0.687	<0.001
	APD	39.6±1.7*	−2.27±0.37*	17±2	0.824	<0.001

Details of the regression lines fitted to the data shown in Fig. 6 (Werners Syndrome fibroblasts). Regression lines were fitted using the least squares method. Days, number of days in culture. APD, accumulated population doublings. *y*-intercept, the fraction of nuclei staining for the indicated antigen at the initiation of the culture, determined by back-extrapolation of the regression line to $x = 0$. Slope, the slope of the regression line generated by the least squares method. Dimensions of the slopes are: days, fraction positive nuclei day^{-1}; APD, fraction positive nuclei APD^{-1}. *x*-intercept, the predicted maximum number of APD achieved by the culture, determined by extrapolation of the regression line to the *x* axis when *y* (the fraction of nuclei positive for the indicated antigen) = 0. r^2, regression coefficient. *P*, significance of the slopes. Values are shown ± standard error of the coefficient. Values marked with an asterisk are multiplied by 100.

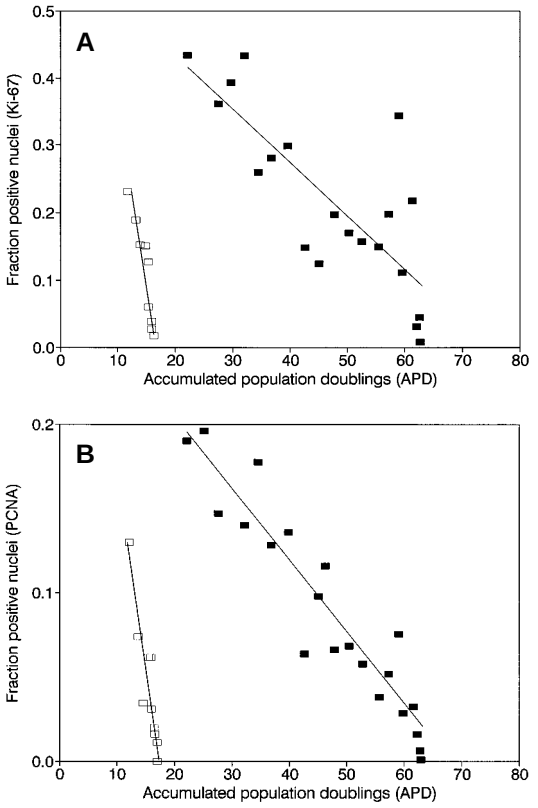

Fig. 7. Comparison of the rate of cellular ageing of normal human fibroblasts and WS fibroblasts. The fraction of nuclei reacting with (A) anti-Ki-67 and (B) anti-PCNA, in normal fibroblasts (closed squares) and WS fibroblasts (open squares) plotted against the number of accumulated population doublings (APD). Regression lines were fitted using the method of least squares.

fibroblast cultures using either antibody for the assay. To obtain estimates of the increased rate of decline in the fraction of proliferating cells between WS and normal fibroblasts during ageing in vitro, we calculated the ratio of the values of the slopes derived in the assays plotted against APD for the antibodies anti-Ki-67 and anti-PCNA. Using anti-Ki-67 a ratio of 6.14 is obtained (F_S=6.35, $F_{\alpha[1,27]}$, P=0.02) and using anti-PCNA a ratio of 5.04 is obtained (F_S=12.44, $F_{\alpha[1,27]}$, P=0.01). This is shown graphically in Fig. 7, where we have plotted the data obtained using each antibody with both normal and WS fibroblasts on the same axes (Fig. 7A,B).

DISCUSSION

We have demonstrated that the human dermal fibroblast strain 1BR.3 has a finite lifespan. The growth of these cultures is characterised by a gradual decrease in the fraction of cells that express each of three cell cycle-regulated antigens. Furthermore, the growth of these cultures is not characterised by a gradual accumulation of dead cells, since the fraction of cells expressing each of three cell cycle-independent antigens remains high and constant during in vitro ageing. The fraction of cells expressing these antigens remains high and constant in cultures made quiescent by culture in low concentrations of serum, confirming that these antigens are expressed independently of the cell cycle, and in the non-growing cells. Senescent cultures consist of non-proliferating, mortalized (Stein et al., 1978; Shall and Stein, 1979) cells that are quite competent in metabolic activities; the process of cell senescence or mortalization is one of reproductive sterility not cell death.

We have also confirmed that WS fibroblasts have a finite lifespan that is markedly reduced when compared with the lifespan of normal fibroblasts. WS fibroblasts also display a gradual decrease in the fraction of cells expressing two cell cycle-regulated antigens. The data clearly show an increase in the rate of decline in the fraction of cells reacting with either antibody in the WS cultures compared with the normal fibroblast cultures. The ratio of the slopes obtained through assays of normal and WS cultures using either antibody provide an estimate of the increase in the frequency of mortalization in WS cultures compared with normal fibroblasts. Using Ki-67, a ratio of 6.14 is obtained, and using anti-PCNA, a ratio of 5.04 is obtained. Thus, an indirect immunofluorescence assay of normal and WS fibroblasts, shows that the foreshortened lifespan of WS fibroblasts compared with normal fibroblasts is due to a 5- to 6-fold increase in the rate of loss of proliferating cells in these cultures during the process of cellular ageing. One possible explanation for the foreshortened lifespan of WS fibroblasts compared with normal fibroblasts would be that WS fibroblasts at an early passage contain a decreased fraction of proliferating cells, and that these cells exited the proliferative pool at a rate comparable with that of normal fibroblasts. This explanation is inconsistent with our data. In addition, it has been shown that the plating efficiencies of both normal fibroblasts and WS fibroblasts remain stable at levels of above 80-90% (Salk et al., 1981; Ponten et al., 1983).

The fraction of cells expressing any one of the three cell cycle-regulated antigens at any given point in the lifespan of the culture provides an estimate of the in vitro age of the culture. Using average estimates obtained using all three antibodies at any given point in the assay increases the reliability of the estimate of the age of the culture. In addition to allowing data obtained from different laboratories using different cell strains to be compared directly, this method may prove useful in the evaluation of the effects of any treatments intended to alter the proliferative potential of these cultures, such as treatment of cultures with 5-azacytosine (Holliday, 1986; Fairweather et al., 1987).

Although a growing body of evidence confirms that the growth of normal mammalian cultures is characterised by a smooth and gradual decline in the fraction of proliferating cells, this has not been adequately incorporated into the literature. It is not uncommon to find reference to the age of cultures in terms of phases I, II and III as defined by Hayflick (1965). We suggest that this classification is inappropriate, since it is inferred that there are intrinsic differences between young, middle aged and aged cultures. It is now clear that the difference between cultures at different in vitro ages is the relative proportions of proliferating and non-proliferating, post-mitotic cells.

Comparison of WS fibroblasts with normal fibroblasts revealed that the rate of decline of antibody-reactive cells in WS cultures was accelerated by 5- to 6-fold. This observation suggests that the genetic lesion present in WS fibroblasts may affect the regulation of a biological ‘counter’ gene that in turn

affects the rate of entry into a post-mitotic, non-proliferating pool of cells. The Werner's Syndrome phenotype is known to be due to a recessive mutation in a single autosomal locus giving rise to a rare disease in the homozygous state (Tollefsbol and Cohen, 1984). Presumably, the large foreshortening of the in vitro lifespan of WS fibroblasts is due to mutation of the same gene that is responsible for the other features of Werner's Syndrome. What then is the relationship between the characteristics of Werner's Syndrome observed in vivo and the increase in the rate of irreversible exit from the cell cycle of WS fibroblasts in vitro? We suggest that those tissues that show turnover in the adult are dependent upon the proliferative capacity of their cells to maintain tissue homeostasis. When the reproductive ability of the cycling cells in the tissue approaches exhaustion, then that tissue will show significant deficiency in its characteristic function. Perhaps then, the in vitro lifespan of cultured cells is a measure of the ability of the tissue to maintain itself in the intact body. In Werner's Syndrome patients, the ability of tissues to maintain themselves may be compromised because the cells are losing their reproductive ability at an increased rate.

The molecular mechanisms responsible for the loss of proliferative ability of cells as they age in culture remain obscure. Elucidation of these mechanisms and of the lesion involved in Werner's syndrome will greatly enhance our understanding of cellular ageing and ageing in vivo, as well as providing insight into cellular transformation and tumorigenesis.

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