The Relationship between the Aging- and Photo-Dependent T414G Mitochondrial DNA Mutation with Cellular Senescence and Reactive Oxygen Species Production in Cultured Skin Fibroblasts

Matthew J. Birket[1,2], João F. Passos[3], Thomas von Zglinicki[3] and Mark A. Birch-Machin[1]

Mutations in the mitochondrial genome (mtDNA) are thought to be one of the causes of age-dependent cellular decline through their detrimental effects on respiration or reactive oxygen species (ROS) production. However, for many mutations, this link has not been clearly established. This study aimed to further investigate the phenotypic importance of a T414G mutation within the control region of mtDNA, previously shown to accumulate in both chronologically and photoaged human skin. We demonstrate that during dermal skin fibroblast replication *in vitro* in five separate cultures obtained from elderly individuals, the T414G mutant load can either increase or decrease during progressive cell division, implying the absence of consistent selection against the mutation in this context. In support of this, by utilizing a cell-sorting approach, we demonstrate that the level of the T414G mutation does not directly correlate with increased or decreased mtDNA copy number, or markers of cellular ageing including lipofuscin accumulation or ROS production. By consequence, the mutation can be distributed with a bias towards either the proliferating or senescent cell populations depending on the cell line. In conclusion, we propose that this particular mutation may have little effect on ROS production and the onset of cellular senescence in cultured fibroblasts.

INTRODUCTION

Since its initial conception over 50 years ago, support has continually grown for the free-radical theory of ageing (Harman, 1956), and oxidative damage is now widely accepted to be an important component of age-dependent cellular decline in tissues such as skin. The principal source of reactive oxygen species (ROS) in the cell is the mitochondria, where superoxide radicals are generated at low levels during normal mitochondrial respiration when molecular oxygen is combined with free electrons along the respiratory chain (Barja, 1999). The close proximity of the

mitochondrial genome to this site of ROS production makes it highly vulnerable to oxidative damage, and the accumulation of mutations is considered a possible contributor to ageing (Chomyn and Attardi, 2003; Birch-Machin 2006; Krishnan et al., 2007). In addition, the ''vicious cycle'' theory proposes that as we age, the accumulation of mtDNA mutations may lead to even more ROS production and further escalating damage, but the fact remains that at present little *in vivo* evidence exists to support such an idea.

One interesting area of human ageing biology is the large age-dependent accumulation of mtDNA point mutations in the noncoding control region observed in skin, muscle, and brain (Michikawa et al., 1999; Del Bo et al., 2003; Coskun et al., 2004). The control region harbors genetic elements important for transcription and replication of mtDNA, giving potential functional relevance to these mutations and making them good candidates as contributors to ageing. In fibroblasts cultured from ageing skin, a T414G mutation has been shown to be one of the most common control region mutations (Michikawa et al., 1999) and we have recently shown that it accumulates in photodamaged skin (Birket and Birch-Machin, 2007). At present, however, the contribution of this mutation to skin fibroblast ageing is unknown. Fibroblasts derived from skin are able to maintain the T414G mutation in early

culture (Birket and Birch-Machin, 2007), which thereby presents an opportunity to address the functional relevance of this base change in terms of its effect on cell proliferation and mitochondrial ROS production, and thus its impact on the ageing phenotype. However, in addressing functional relationships with seemingly subtle mutations of this kind, it is difficult to differentiate cause from correlation, as cell cultures carrying mutations may show markers of dysfunctional for reasons independent of the mutation in question. Therefore, in an attempt to overcome this problem, as well as assessing the behavior of the T414G mutation during culture in different fibroblast lines, we have also examined correlations with mtDNA copy number and ROS production on a subpopulation basis through the use of a flow cytometry cell-sorting technique.

RESULTS

Differential behavior of the T414G mutation during long-term culture

Five separate fibroblast cultures obtained from skin of separate elderly individuals were selected based on the identification of the T414G mtDNA mutation in both the skin and fibroblasts. The T414G mutation is more common in photoaged skin, and four of these cultures were obtained from such sites and one mutant culture was identified from a sun-protected site. To assess whether long-term growth of T414G mutant fibroblasts would lead to the outgrowth of mutant cells by wild-type cells, as would be expected if the mutation was having severely detrimental effects on the cell, these five T414G mutant skin fibroblast cultures were grown for a period of over 100 days under standard conditions. This is the length of time over which a previous study showed consistent outgrowth of the same mutation (Michikawa et al., 2002). Results in Figure 1 show that in three of the five cultures, the level of the mutation gradually increased over time, whereas in the remaining two cultures, the level decreased. In cultures A, D, and E, the level of the mutation rose above or was maintained above heteroplasmy levels of

60% (that is, 60% of the mtDNA in the population carrying the mutation), a value that has been cited before as the threshold necessary for phenotypic expression for pathogenic mutations (Hayashi et al., 1991; Porteous et al., 1998). We note that even at these high mutant levels, the cells continued to proliferate and there was still no replicative selection against the mutation. In culture A, the T414G mutation even reached homoplasmy, that is, became fixed as 100% mutant.

Flow cytometry cell sorting by autofluorescence achieves good separation of cells based on their degree of senescence

There is some evidence to suggest that the T414G mutation, rather than being distributed evenly across all the cells in the skin, may instead be concentrated within a subset of the population (Michikawa et al., 2002; Birket and Birch-Machin, 2007). If this remains the case *in vitro* and if the T414G mutation is a contributor to ageing, then one would expect cells carrying high levels of this mutation to show markers of cellular senescence and/or else a reduced growth rate due to respiratory deficiency. However, our data showing an increase or decrease in the level of the mutation during culture suggest that this may not be the case and it may be that the mutation can associate with either the proliferative or slow growing cells; alternatively, it may be distributed evenly across the population and the fluctuations mediated simply through genetic drift. To investigate this, FACS was used to physically separate young and senescent cells within each of the five cultures. Fibroblasts accumulate lipofuscin as they approach replicative senescence and yellow-green autofluorescence has been shown to be a reliable marker of cellular lipofuscin content (Collins and Thaw, 1983; von Zglinicki et al., 2000). Cell sorting based on lipofuscin accumulation has been validated by others (Martin-Ruiz et al., 2004). In this study, we sorted the upper and lower 15% of the population based on autofluorescence measured in the FL1 channel (515–545 nm). β-Galactosidase activity is a reliable marker of senescence in fibroblasts (Dimri et al., 1995; Itahana et al.,
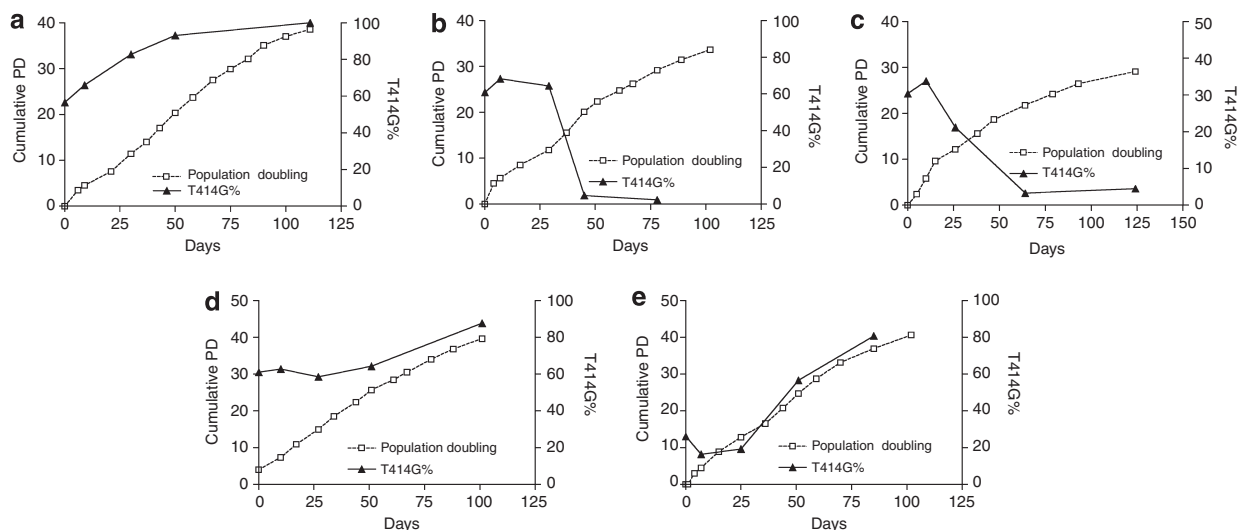

Figure 1. **Fibroblast growth curves and corresponding T414G levels in five separate cultures from different individuals.**

2007), and in each sorting experiment, we observed a much greater frequency of β-galactosidase-positive cells in the highly autofluorescent (High FL) population (Figure 2a).

Greater redox probe oxidation, higher mtDNA copy number, and mtDNA oxidative damage in prematurely senescent cells within a proliferating population

ROS production was measured with two probes with different species specificities: MitoSOX, a positively charged derivative of hydroethidine detects mitochondrial superoxide, and dihydrorhodamine 123 (DHR123) which detects hydrogen peroxide in the presence of peroxidase, cytochrome c, or Fe^{2+}. DHR123 also detects peroxynitrite.

Measurements in the sorted populations from the five different cell cultures each repeated two or three times showed a strikingly consistent differential level of ROS production in the sorted populations across all the five cultures. MitoSOX fluorescence was increased with a mean of twofold and DHR123 fluorescence increased 7.7-fold in the cells sorted for High FL cells compared with the Low FL cells once the differential autofluorescence had been subtracted (Figure 2b).

It has been demonstrated quite conclusively that relative mtDNA copy number increases in senescence along with a general increase in mitochondrial biogenesis (Lee *et al.*, 2000, 2002). In this study, relative mtDNA content was

assessed in the sorted populations and showed a mean of twofold increase in the High FL cells compared with the Low FL cells (Figure 2c).

Given the differential production of ROS in these sorted populations and the fact that mtDNA is highly susceptible to oxidative damage, we assessed the general integrity of the mitochondrial genome using a lesion-sensitive PCR-based assay. Using this technique, we have previously shown that there is an increase of this type of damage in replicative senescence and which is elevated when cells are grown under conditions of elevated oxidative stress (Passos *et al.*, 2007b; Ahmed *et al.*, 2008). The assay works by quantifying the amplification efficiency of a long (11 kb) versus a short (83 bp) amplicon and a decrease in this relative amplification efficiency indicates an increase in the frequency of polymerase blocking lesions. Assessment of the Low FL versus High FL populations showed a statistically significant increase in damage in the High FL populations, with a relative amplification efficiency of 0.84 ± 0.16 relating to an increase of approximately 0.164 lesions per 10 kb, or 1 lesion per 3.7 mtDNA molecules (Figure 2c), assuming a Poisson distribution.

The T414G mutation is not preferentially associated with cellular senescence *in vitro*

The level of the T414G mutation was quantified in each of the sorted populations. The results show that the T414G
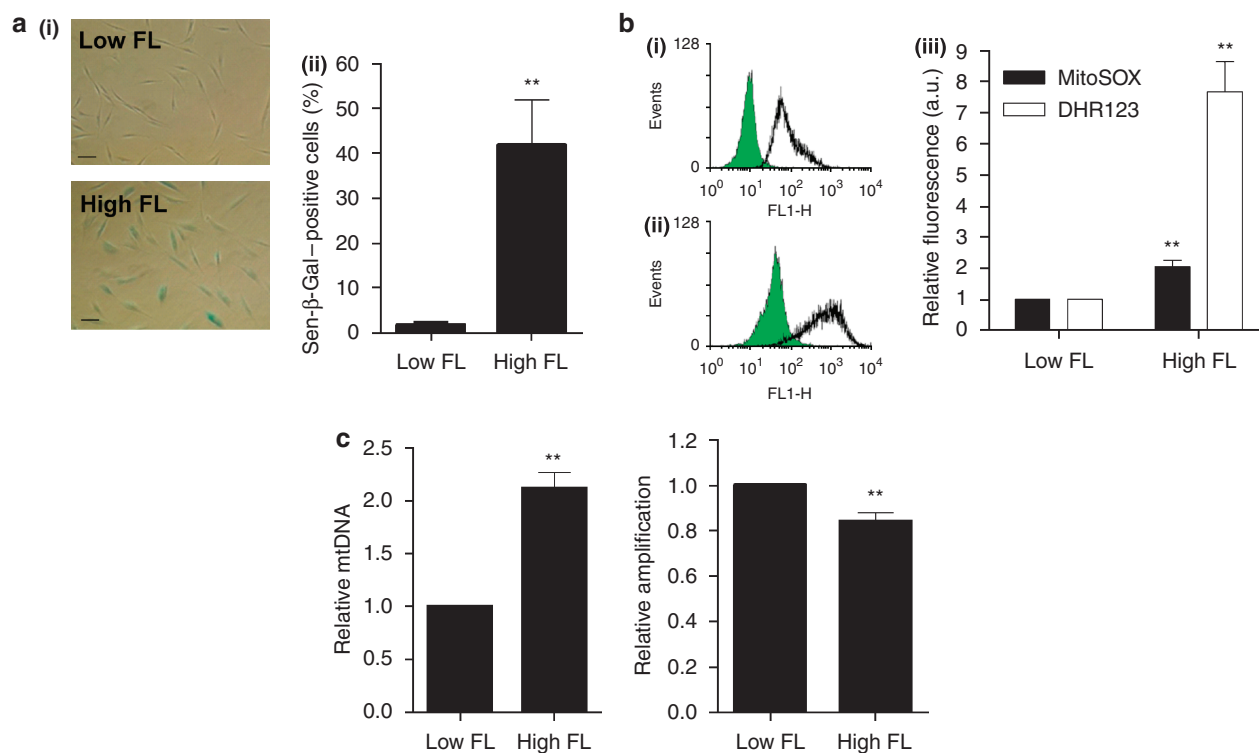
Figure 2. Markers of senescence and mitochondrial function in post-sorted populations. (**a**) Example staining for β-galactosidase activity in sorted cells (i) and the mean proportion of β-galactosidase positive cells following sorting, scale bar = 20 µm (ii). (**b**) Reactive oxygen species production in sorted cells. Typical fluorescence traces before (filled) and after (unfilled) DHR123 staining in Low FL (i) and High FL (ii) sorted cells. Quantification of relative MitoSOX and DHR 123 fluorescence is shown, after deduction of differential autofluorescence (iii). (**c**) Relative mtDNA copy number and long PCR relative amplification efficiency, indicating the integrity of the mtDNA population. Significance was assessed in all cases with a paired *t*-test **P < 0.01 and the quantitative data represent combined results from five different fibroblast lines.
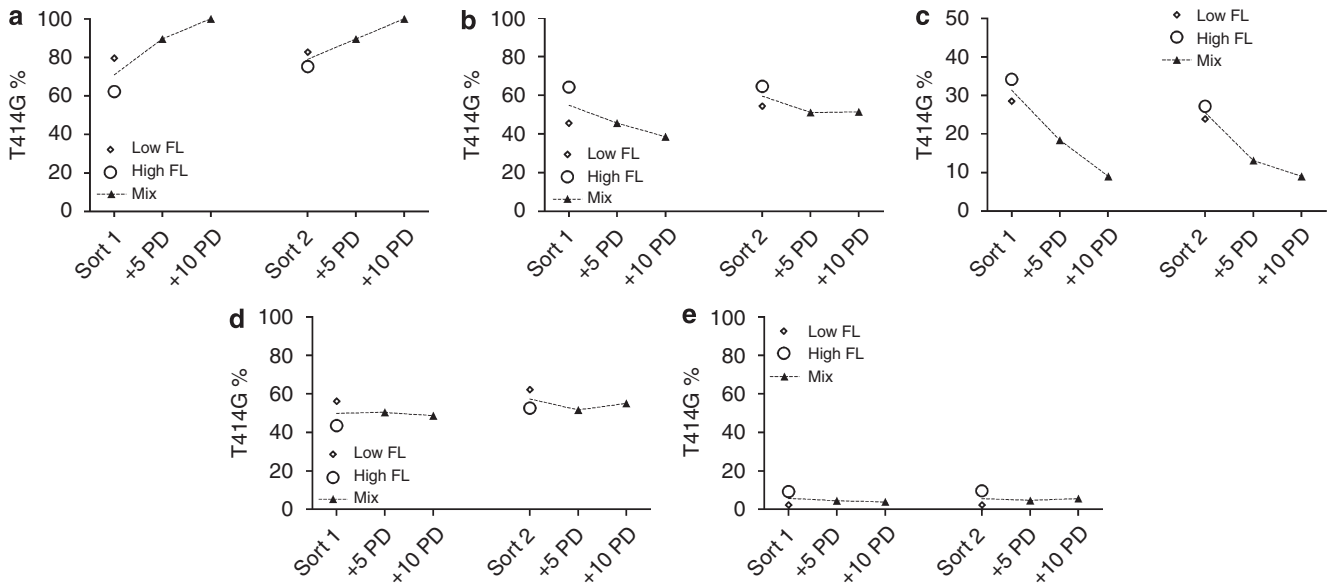
Figure 3. **T414G mutation level in sorted cells and mutation change during post-sorting growth.** T414G mutation level was quantified in sorted cells immediately after attachment and levels are indicated: small open diamonds = Low FL cells, large open circles = High FL cells, black triangles = mixed population without sorting. Unsorted ''mixed'' cells were grown as normal and DNA was extracted after 5 and 10 population doublings (PDs) and the T414G mutation was quantified. Sorting was carried out at the following PDs: (**a**) sort 1 = PD 11.7, sort 2 = PD 16; (**b**) sort 1 = PD 15.4, sort 2 = PD 16.6; (**c**) sort 1 = PD 9, sort 2 = PD 9.8; (**d**) sort 1 = PD 7.5, sort 2 = PD 9.9; (**e**) sort 1 = PD 7.5, sort 2 = PD 9.9.

mutation could segregate with a bias toward either the Low FL or High FL populations depending on the cell line (Figure 3). In lines B, C, and E, the mutation was at a consistently higher level in the High FL populations, whereas in lines A and D, the mutation showed a bias towards the Low FL cells.

In each culture, unsorted cells were grown and harvested at ~5 and ~10 population doublings (PDs) from the point of sorting to assess directly how the level of the mutation would change in these populations (Figure 3). The fluctuations in these ''post-sorting'' cultures were qualitatively similar to the changes observed in the initial cultures (Figure 1) in the cases of A–D, with A showing an increase over this period, B and C showing a decrease, and D showing little change. E was the exception; during the original culture of this cell line, the mutation was beginning to increase over this period (that is, between PD 10 and 20; Figure 1), but during the post-sort growth, the level of the mutation remained very low. The discrepancy between the behavior of culture E in the sorting experiments and its behavior in the original culture may possibly be explained by the fact that the initial level of the T414G mutation is very low compared with cultures A–D (that is, <20%), and thus would be more sensitive to a ''bottleneck'' effect the population may go through when restarting the culture from stocks. This could result in a different progression for the T414G mutation during long-term culture.

Comparing the distribution of T414G in these sorted populations (Figure 3) to the dynamics over the following 10 PDs in the mixed culture (Figure 1), it appears that if the mutation is more common in the High FL/more senescent cells, that is, in cultures B, C, and E, then there will be a tendency for the mutation to be selected out during cell division. Whereas if it is more common in the Low FL/less senescent cells, that is, in cultures A and D, then the mutation will either be maintained or gradually increase during cell division. Quantitative discrepancies between the original cultures (Figure 1) and the post-sort cultures (Figure 3) may be largely explained by the random nature of genetic drift that will play a factor when expanding the culture.

DISCUSSION

The T414G mutation has previously been shown to accumulate in skin as a function of age and UV exposure (Michikawa *et al.*, 1999; Birket and Birch-Machin, 2007). Indeed, we discovered frequent genetic linkage between a common photoageing-associated mtDNA deletion (that is, 3,895 bp) and the T414G mutation. It has previously been demonstrated that pathogenic mtDNA mutations such as the 4,977 bp ''common deletion'' become selected out during the culture of skin keratinocytes, fibroblasts, or 143B cell cybrids, even in nonselective media, whereas less detrimental defects such as tandem duplications have been shown to be maintained in fibroblasts (Bourgeron *et al.*, 1993; Koch *et al.*, 2001; Diaz *et al.*, 2002; Krishnan and Birch-Machin, 2006). Our observation that the T414G mutant load can either increase or decrease during fibroblast culture suggests the lack of a common selective pressure against the mutation under these conditions.

If the mutation is a contributor of ageing then one would expect that it might associate with markers of senescence on a cell by cell basis *in vivo* and for this distribution to be at least partially preserved *in vitro* considering that the mutation is well maintained in these cells (Birket and Birch-Machin, 2007). To test this, separate T414G mutation carrying fibroblast cultures from five elderly individuals were each

sorted into young and senescent populations by exploiting a known marker of cellular ageing, lipofuscin accumulation. The results obtained show that the cells with higher autofluorescence have consistently higher levels of ROS production, as measured by MitoSOX and DHR123 fluorescence. These values did not vary significantly among the different cultures and thus appear to be mostly a function of the level of autofluorescence/lipofuscin accumulation. The slight increase in mtDNA oxidative damage reported here in the more senescent population is in accordance with our previous findings that an increase in mtDNA copy number and damage tend to correlate and support the premise that overall oxidative stress is increased in these cells (Passos *et al.*, 2007a; Ahmed *et al.*, 2008).

Counter to the consistent segregation of the above markers, the T414G mutation showed an independent distribution between the two populations depending on the cell line. This indicates that the T414G mutation has little, if any, effect on ROS production or mtDNA copy number regulation and demonstrates that this particular point mutation is not preferentially associated with cellular senescence in this *in vitro* context.

The variable behavior in the change in T414G mutation load in the cultured fibroblasts examined here could be explained simply by genetic drift, but the fact that in each culture the mutation seems to take on a direction during the exponential growth phase, either to increase or decrease, suggests that selective forces may be exerting an influence. The growth of cells in primary cultures is heterogeneous and any population is made up of cells that are cycling and others which have exited the cell cycle (Sozou and Kirkwood, 2001). This dynamic of growing and senescent or quiescent cells may have a significant influence on the behavior of the T414G mutation during culture. Our data show that the distribution of the T414G mutation can favor either the young/highly proliferative cell population or the more senescent/slow growing cell population and that this information can be at least indicative of how the mutation will behave during culture. Therefore, it seems that this selective factor may at least partially govern the fluctuations observed *in vitro*. The data presented here have been limited to a full analysis of five fibroblast cultures; however, a similar trend in terms of an increasing or decreasing mutation load during culture was also observed in other fibroblast cultures (results not shown), strongly supporting these conclusions.

In summary, we have found that the level of the T414G mutation is independent of mtDNA copy number and general mtDNA oxidative damage, as well as lipofuscin accumulation, β-galactosidase activity, and overall ROS production on a sub-population basis, overall strongly pointing to a neutral phenotype for this mutation in these skin fibroblasts. The current data are therefore also in support of a neutral role for this particular control region mutation in senescence of cultured fibroblasts. In a wider context and in addition to point mutations, aged skin is characterized by the presence of large-scale deletions of mtDNA (Birch-Machin 2006; Krishnan *et al.*, 2007) which may have completely different functional consequences for fibroblast senescence. To our knowledge, this kind of study is previously unreported, that is, where primary cells derived from the skin of elderly donors have been sorted into subpopulations based on markers of ageing and then assessed for mutations in the mitochondrial genome.

MATERIALS AND METHODS

Cell culture

Dermal skin fibroblasts were isolated from skin biopsies taken from five different elderly individuals as previously described and with written informed consent (Birket and Birch-Machin, 2007). The medical ethical committee of the University of Newcastle has approved all of the described studies. In addition, the study was conducted according to the Declaration of Helsinki Principles. The donor age and sun-exposure status of each biopsy site are as follows: A = 65 years old, sun exposed (nose); B = 80 years old, sun protected (foreskin); C = 87 years old, sun exposed (back of neck); D = 88 years old, sun exposed (face although specific site unknown); and E = 84 years old, sun exposed (face although specific site unknown). Once the cells became confluent within the first flask, samples were taken for DNA extraction and T414G analysis. Cells were then repeatedly passaged and cumulative PDs were calculated. At early PDs, aliquots were taken and frozen in liquid nitrogen and these stocks were revived for the sorting experiments. Cells were grown in phenol red free DMEM (Invitrogen, Paisley, UK) containing glucose, supplemented with 10% fetal calf serum and penicillin/streptomycin. No pyruvate or uridine was included in the media.

Cell sorting using flow cytometry

Cell sorting was undertaken at relatively early PDs (between 7 and 17) in each culture. Sorting was carried out on a Becton Dickinson FACSVantage, using a 100 μm nozzle with the cells maintained at 4 °C during sorting. After initial acquisition, gates were created to exclude debris and cell doublets and then collection gates were set at 15% of upper and lower FL1 (515–545 nm) autofluorescence. Collected cells were seeded into 35 mm dishes and into duplicate wells of a 24-well plate for β-galactosidase staining. The following day, the cells in the dishes were trypsined and either frozen at −80 °C for DNA extraction and T414G quantification or used for flow cytometry analysis. Culturing was continued with the unsorted cells and DNA was extracted and the T414G mutation quantified at 5 and 10 PDs from the point of sorting. The T414G level in the mixed population at the time of sorting was inferred to be exactly in between that of the sorted populations to reduce the number of sequencing reactions performed.

Reactive oxygen species quantification

For the measurement of mitochondrial superoxide, cells were stained with 5 μM MitoSOX red (Molecular Probes; http://www.invitrogen.com) for 10 minutes at 37 °C in phosphate-buffered saline. Cellular peroxide levels were assessed by staining with 2.5 μM DHR123 (Molecular Probes) for 20 minutes at 37 °C in phosphate-buffered saline. Following staining with each probe, cells were washed once, resuspended in serum free DMEM, and placed on ice before analysis. Flow cytometry was performed on a Becton Dickinson FACScan. For analysis, gates were created to exclude debris and median fluorescence was recorded as an increase above autofluorescence in each sample. MitoSOX was measured in the FL3 channel and DHR123 in the FL1 channel.

Senescence-associated β-galactosidase staining

β-Galactosidase staining was performed using a commercially available kit (Sigma-Aldrich, Dorset, UK), following the manufacturer's instructions. Cells were visualized under a light microscope and the proportion of blue positively stained cells were counted from a total of 1,000 cells for each population.

T414G quantification

T414G mutant heteroplasmy was quantified using denaturing high-performance liquid chromatography WAVE heteroduplex analysis (Transgenomic, Cramlington, UK) and direct sequencing, as previously described (Birket and Birch-Machin, 2007).

mtDNA copy number and oxidative damage

As many types of DNA lesion block replication of DNA by Taq polymerase, mtDNA damage was measured as amplification efficiency for a large (11,095 bp) amplicon normalized to a short (83 bp) amplicon, performed using real-time PCR as previously described (Passos *et al.*, 2007a). Lesion frequency was calculated as previously described (Santos *et al.*, 2006). Relative mtDNA copy number was assessed by comparing amplification of mitochondrial versus nuclear DNA, as previously described (Passos *et al.*, 2007a; Ahmed *et al.*, 2008).

Statistical analyses

Differences between groups were determined by analysis of variance or *t*-test with correction for multiple groups using commercially available software (GraphPad Prism 5).

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