

# Developmental differences in the immortalization of lung fibroblasts by telomerase

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## Summary

**The role of ambient (21%) and physiological oxygen (2–5%) in the immortalization of fetal vs. adult human lung fibroblasts was examined. Growth in low oxygen and antioxidants extended the lifespan of both fetal and adult strains. As the ectopic expression of telomerase could immortalize adult lung fibroblasts cultured in ambient oxygen, the lifespan-shortening effects of 21% oxygen must have been largely limited to telomeres. By contrast, fetal lung fibroblasts could not be immortalized in ambient oxygen in spite of telomere elongation by telomerase, suggesting more widespread oxidative damage. The long-term culture requirements for the immortalization of WI-38 fetal lung fibroblasts included supplementation with N-(tert) butyl hydroxylamine, dexamethasone, zinc and vitamin B12, in addition to growth in physiological oxygen. The mechanisms regulating telomere shortening remain controversial. The present results suggest that both end-replication and oxidative damage events contribute to telomere shortening in lung fibroblasts *in vitro*. These observations emphasize the need for better analytical techniques to distinguish whether the correlation of short telomeres with disease and mortality in humans reflects the consequences of increased proliferation, telomere shortening as a result of oxidative damage or some combination of these processes.**

**Key words:** oxidative damage; replicative aging; senescence; telomeres.

## Introduction

The original concept of the mechanism regulating telomere shortening was that the end-replication problem (the inability of lagging-strand synthesis to copy to the end of a linear DNA molecule) was the primary factor driving telomere shortening

(Olovnikov, 1973; Harley *et al.*, 1990). It is now becoming clear that the blunt-ended product of leading strand synthesis also develops a single-stranded 3'G-rich overhang (Makarov *et al.*, 1997; Wellinger *et al.*, 1996; Wright *et al.*, 1997, 1999), so that processing events at leading strands (and perhaps lagging strands as well) may contribute to telomere shortening. An alternative view is that oxidative damage drives telomere shortening (von Zglinicki *et al.*, 1995; von Zglinicki, 2002). Free radicals preferentially induce lesions at triplet G's (Oikawa & Kawanishi, 1999) making telomeres, with many kilobases of the triplet G containing repetitive sequence TTAGGG, an ideal target for oxidative damage.

One problem in evaluating the effects of oxidative damage on shortening is that most reported experiments utilize hyperoxia (40% O<sub>2</sub>) or other oxidative insults (e.g. H<sub>2</sub>O<sub>2</sub>) to induce accelerated telomere shortening. This is further complicated, because normal tissue oxygen concentrations are not the 21% oxygen concentration of the culture room, but much lower (estimated to be roughly 1–6%; Guyton & Hall, 1966). Furthermore, earlier experiments have shown that although growing human IMR-90 fibroblasts in low oxygen extended their lifespan by approximately 10 doublings, this extension was independent of whether they were grown in low oxygen for the entire culture period (65 doublings) or only the final 30 divisions (Saito *et al.*, 1995). This lack of proportional effect suggested that oxidative damage was not influencing telomere shortening during the bulk of the culture period. IMR-90 cells have now been shown to undergo an oxidative crisis during their last 10 or so doublings (Atamna *et al.*, 2000), providing an explanation for why low oxygen might have a protective effect only at the end of their lifespan.

Much of our work emphasizing the importance of end-processing events for regulating the rates of shortening used the neonatal foreskin strain BJ (Wright *et al.*, 1997; Huffman *et al.*, 2000), whereas the investigations of oxidative damage in telomere shortening have primarily used fetal lung fibroblast strains such as WI-38, IMR-90 and MRC-5 (von Zglinicki, 2002; Saretzki *et al.*, 2003). Although we and others have successfully immortalized a large number of different cell types with telomerase, we have been unable to immortalize IMR-90 cells successfully even following transfection with h-TERT. Although we observed a significant extension of lifespan in IMR-90, the cells slowed down and growth arrested shortly after the point we reported (Ouellette *et al.*, 1999). BJ fibroblasts have been shown to be much more resistant to hyperoxia-induced telomere shortening than IMR-90 (Lorenz *et al.*, 2001). We thus decided to re-examine the effects of oxygen and other tissue culture conditions on IMR-90, WI-38 and two adult lung cell strains. Low oxygen extended replicative lifespan of both the fetal and the adult lines. In the adult strains, oxidative damage appeared to be primarily restricted to

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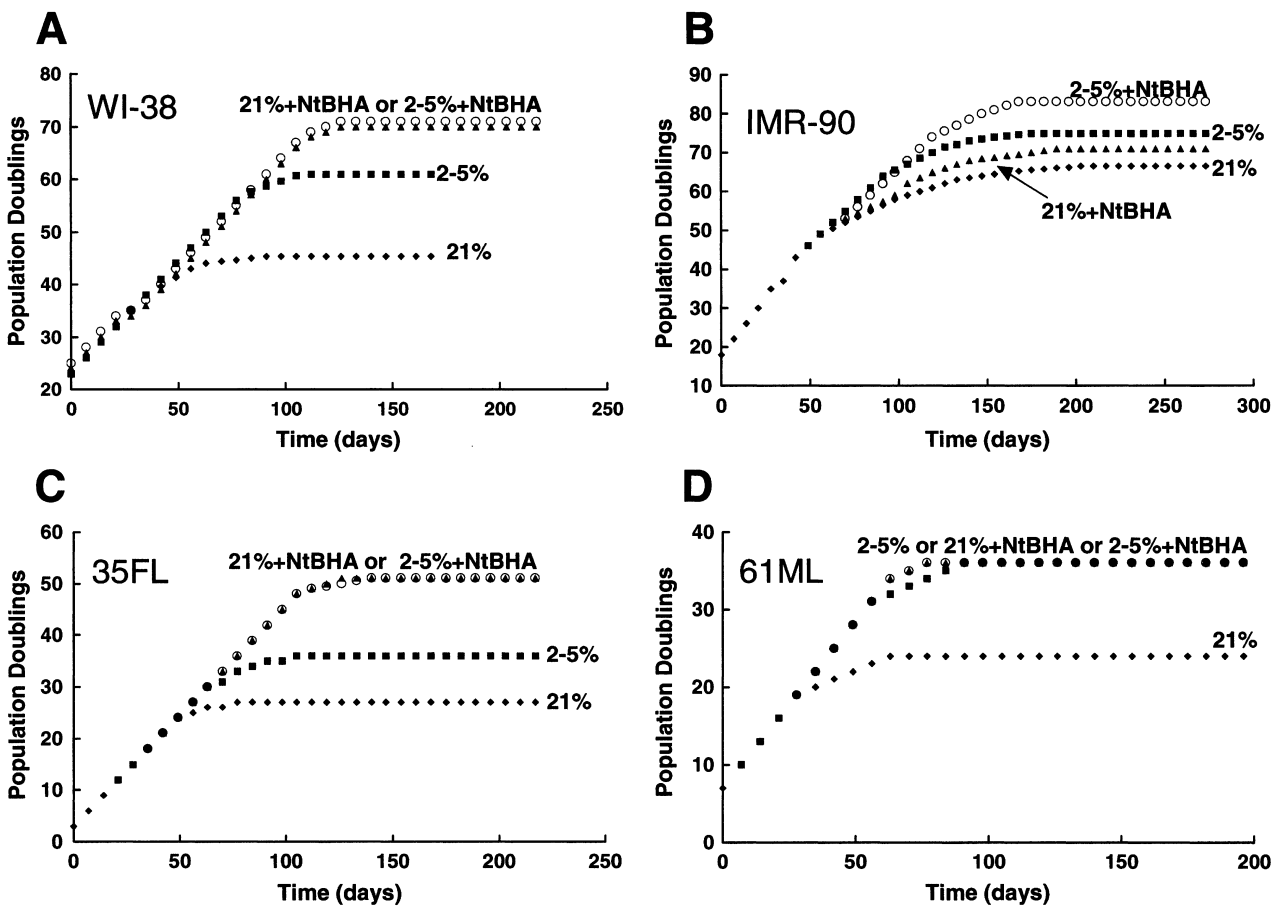
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telomeres, because telomerase immortalized the cells even when grown under ambient oxygen. Ongoing oxidative damage to telomeres by ambient oxygen in these immortal cells was suggested by the much lower rates of telomere elongation by telomerase in 21% vs. low (2–5%) oxygen. By contrast, in the fetal strains the oxidative damage produced by ambient oxygen was apparently not limited to telomeres, because neither WI-38 nor IMR-90 cells were immortalized by telomerase in 21% oxygen, in spite of considerable telomere elongation. Reducing oxygen concentrations in combination with antioxidants, corticosteroids and micronutrients (vitamin B12 and zinc) permitted the direct immortalization of telomerase-expressing WI-38 cells, but not IMR-90. Collectively, these results demonstrate that the effectiveness of mechanisms which protect against oxidative damage may be developmentally regulated in cells cultured from fetal vs. adult tissues. In the present report we demonstrate that damage induced by ambient oxygen concentrations can contribute significantly to rates of telomere shortening under conditions that do not induce growth arrest, and that cell strain differences in levels of oxidative protection or growth requirements are sufficient to explain much of the variation in the ability of telomerase to immortalize different strains of lung fibroblasts.

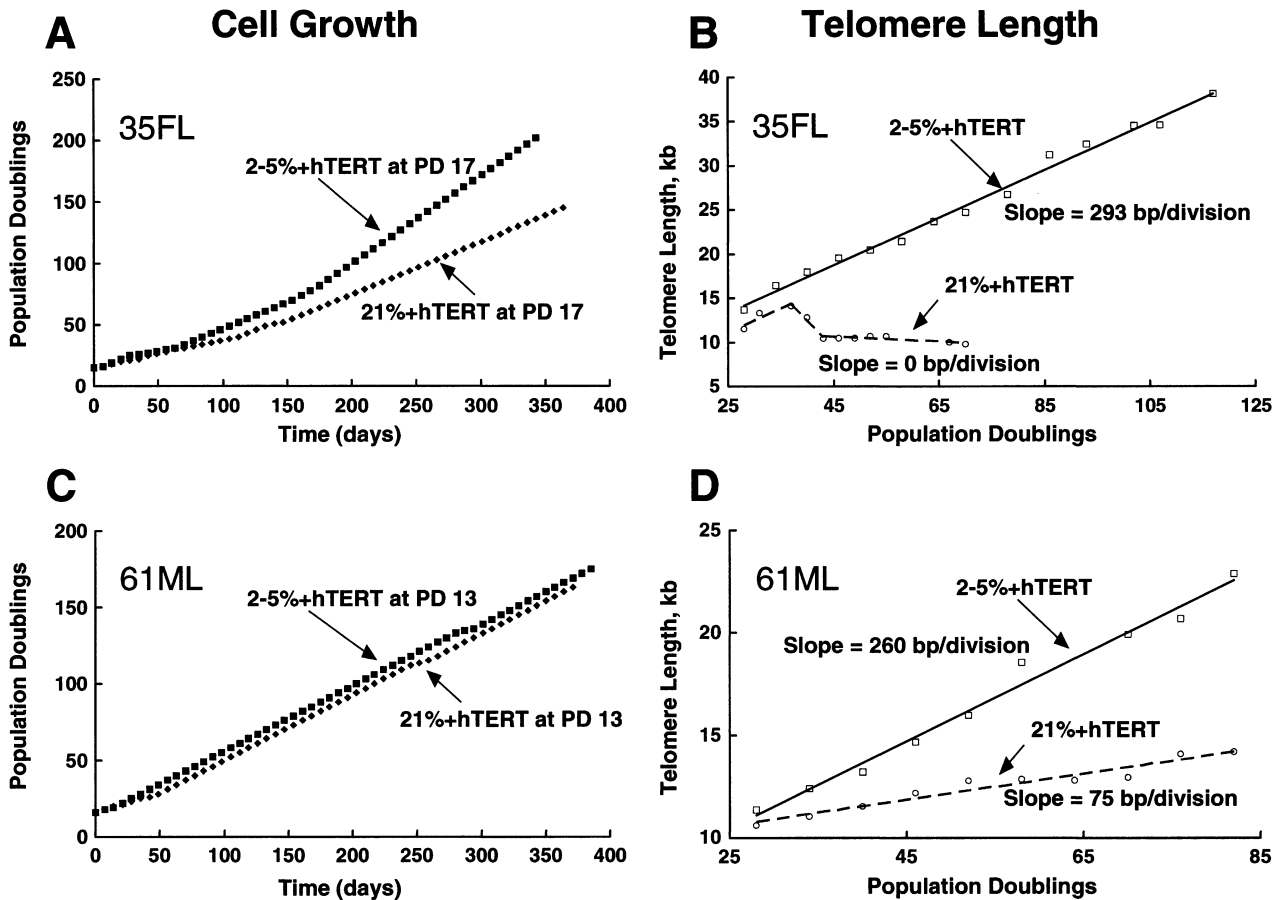
## Results

### Ambient oxygen shortens the lifespan of fetal and adult lung fibroblasts

Physiological oxygen in most tissues is much lower than the ambient sea-level oxygen concentration of 21%. Growing fetal lung fibroblasts in low oxygen atmospheres (Packer & Fuehr, 1977; Saito *et al.*, 1995), or in the presence of oxidation spin-trap compounds such as N-(*tert*) butyl hydroxylamine, extends their lifespan (Atamna *et al.*, 2000). We thus compared the response of cultured fetal and adult lung fibroblasts to ambient and low oxygen environments in the presence or absence of the oxygen spin-trap molecule N-(*tert*) butyl hydroxylamine. Figure 1 shows the proliferative history of the fetal lung strains, WI-38 and IMR-90, and adult strains from a 35-year-old female lung (35FL) and a 61-year-old male lung (61ML). All strains showed an approximately 10–20 population doubling (PD) extension of lifespan under optimal oxidation-protection conditions. Western analysis of WI-38 and IMR-90 time courses displayed up-regulated p16<sup>INK4A</sup> and p21<sup>WAF1</sup> as cells approached growth arrest, regardless of oxidative conditions, in the manner described previously for diploid fibroblasts (Atadja *et al.*, 1995; Alcorta *et al.*, 1996;



**Fig. 1** Effects of oxygen concentration and N-(*tert*)-butyl hydroxylamine on the growth of fetal and adult lung fibroblasts. The proliferation of cells in normoxia (◆), low oxygen (■), normoxia with NtBHA (▲), and low oxygen with NtBHA (○) are shown for the fetal strains WI-38 (A) and IMR-90 (B), and the adult strains 35FL (C) and 61ML (D).



**Fig. 2** Adult lung fibroblast hTERT immortalization and telomere dynamics. The growth curves and changes in telomere lengths are shown for 35FL (A and B) and 61ML (C and D) cells following the introduction of telomerase, and grown under both ambient and low oxygen conditions.

Hara *et al.*, 1996). The expression status of p16<sup>INK4A</sup> and p21<sup>WAF1</sup> was not analysed in 35FL or 61ML because they readily immortalized with telomerase regardless of oxygen conditions (Fig. 2).

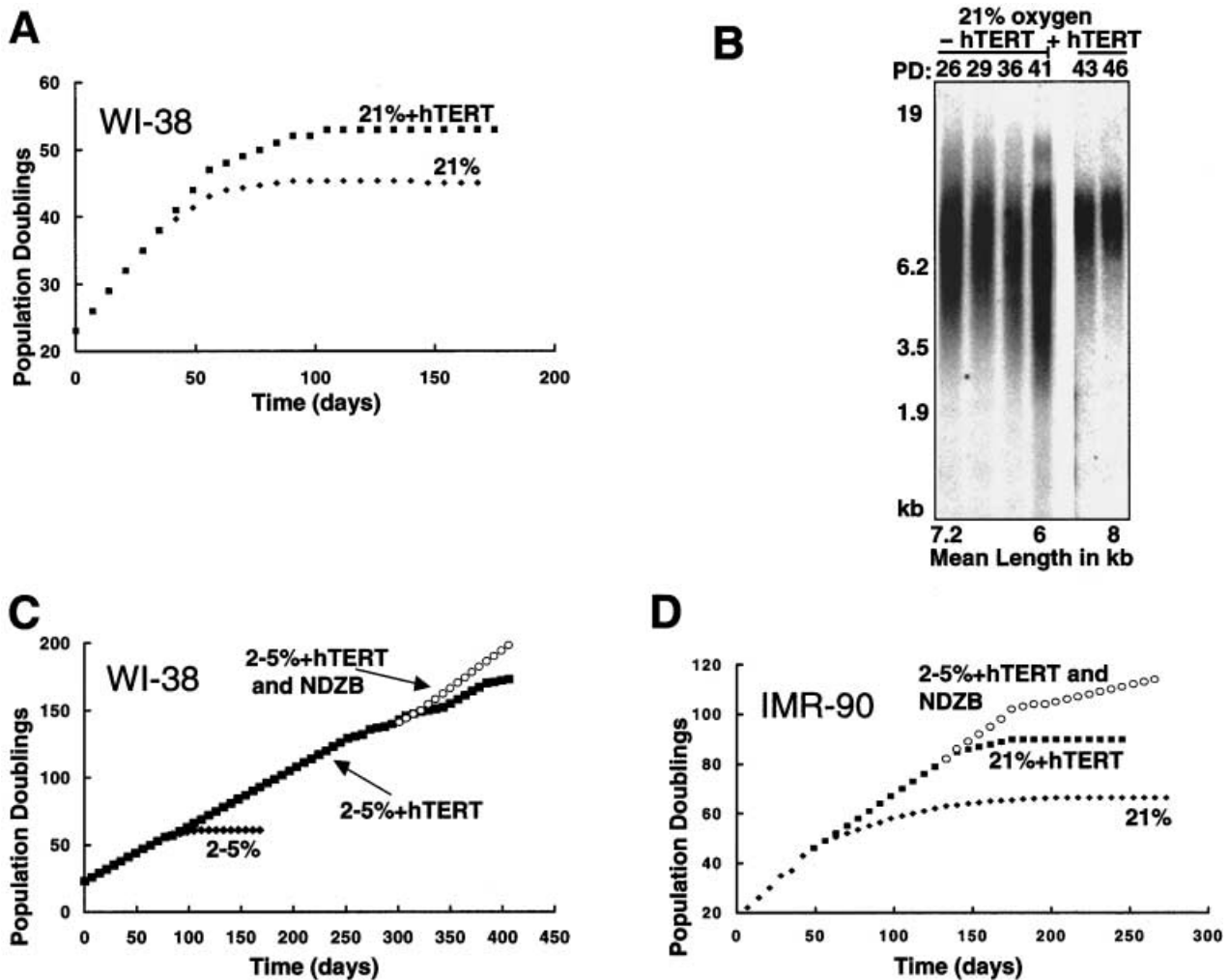
### Telomerase immortalizes adult lung fibroblasts under ambient oxygen conditions

Although their lifespan in culture was shortened by 21% oxygen, the adult lung strains became immortal following the introduction of the catalytic subunit of telomerase, hTERT, regardless of whether they were grown in 21% or low oxygen (Fig. 2A,C). However, the rate of telomere elongation by telomerase differed dramatically under the two conditions (Fig. 2B,D). In low oxygen, telomeres exhibited rates of elongation of approximately 250–300 bp per division in both strains. In the 35FL strain in 21% oxygen, telomeres initially elongated, but at population doublings 30–35 there was a stabilization of telomere length. Telomeres in the 61-year-old male lung strain elongated at a much lower rate in 21% oxygen (75 bp per division) than in low oxygen (260 bp per division). One explanation for this behaviour is that oxidative damage-induced telomere shortening continued to occur under ambient oxygen conditions, but that

telomerase elongated telomeres at a rate equal to or greater than the rate of shortening. Importantly, the ability of cells to immortalize under both oxygen concentrations implies that the oxidative insults occurring in 21% oxygen produced insufficient general genomic damage to induce growth arrest, and thus senescence would have resulted specifically from oxidative damage to telomeres.

### Telomerase does not immortalize fetal lung fibroblasts under ambient oxygen conditions

In contrast to adult lung fibroblasts, neither WI-38 nor IMR-90 fetal lung fibroblasts expressing telomerase were able to grow indefinitely in 21% oxygen (Fig. 3). Figure 3(A) shows that the introduction of the catalytic subunit of telomerase only modestly extended the lifespan of WI-38 cells in 21% oxygen, in spite of successfully elongating telomeres to a greater length than the parental line (Fig. 3B). WI-38 expressing hTERT and grown in low oxygen extended their proliferative capacity from 60 to about 130 doublings, but then growth slowed dramatically (Fig. 3C). The cells continued to divide but at a greatly reduced rate and were removed from culture. Telomere lengths at this time were approximately 25 kb (data not shown).



**Fig. 3** Immortalization of WI-38 fetal lung fibroblasts requires low oxygen and micronutrients. WI-38 cells infected with retroviral hTERT do not immortalize in ambient oxygen (A) in spite of elongating their telomeres (B). The slowed growth of telomerase expressing WI-38 cells grown in low oxygen that occurred after 130 doublings (C) was prevented by supplementing the media with NDZB (N-(*tert*)-butyl hydroxylamine, dexamethasone, zinc and vitamin B12) from 140 doublings onwards. (◆) Uninfected control, (■) expressing telomerase and (○) further supplemented with NDZB. IMR-90 cells do not immortalize with supplementation and low oxygen (D). IMR-90 cells were grown in normoxia (◆), or infected with an hTERT retrovirus and cultured in ambient oxygen (■) or low oxygen with NDZB supplementation (○).

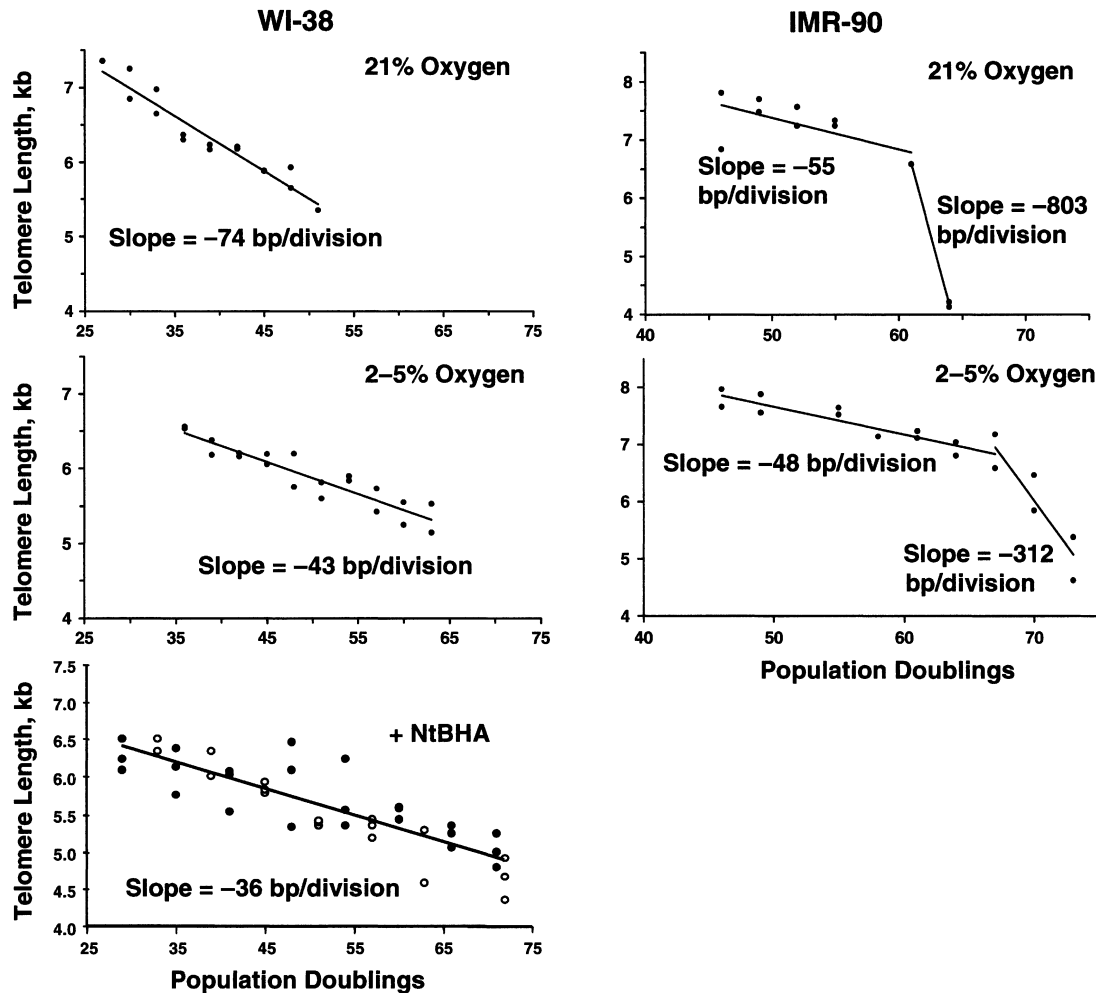
Dexamethasone is a growth factor for many cell types, and extends the lifespan of fetal lung fibroblasts by 5–10 doublings (Li *et al.*, 1998; Macieira-Coelho, 1966), and the spin-trap N-(*tert*) butyl hydroxylamine extends the lifespan of WI-38 beyond that obtained with low oxygen alone (Fig. 1A). Deficiencies in any one of six different micronutrients can compromise oxidation-protection mechanisms (Ames, 1999). Our medium lacked two of these: vitamin B12 and zinc. When WI-38 hTERT cells were grown in low oxygen in medium supplemented with all four components (N-(*tert*) butyl hydroxylamine, dexamethasone, zinc and B12: 'NDZB') from PD 140 onwards, cells became immortal (Fig. 3C). Telomere length in these cells was approximately 30 kb when the culture was discontinued after PD 200. Normoxic WI-38 hTERT cells did not immortalize when supplemented with NDZB. In addition to low oxygen, WI-38 fetal lung fibroblasts thus require a variety of medium supplements in

order to provide an adequate culture environment for their long-term proliferation and immortalization.

Telomerase-expressing IMR-90 fetal lung fibroblasts failed to immortalize in 21% oxygen, low oxygen or low oxygen supplemented with NDZB (Fig. 3D). To our knowledge there are no successful reports of IMR-90 hTERT immortalization. Their proliferative capacity was progressively extended under each of these conditions, but the cells eventually exhibited a dramatically slowed growth rate (at approximately PD 110 in low oxygen with NDZB). Telomeres elongated to approximately 20 kb by PD 110, so it is unlikely that telomere length was limiting for their growth.

### Responses to oxidative stress

IMR-90 fibroblasts undergo an oxidative crisis during the last 10–15 doublings of their lifespan when grown in 21% oxygen

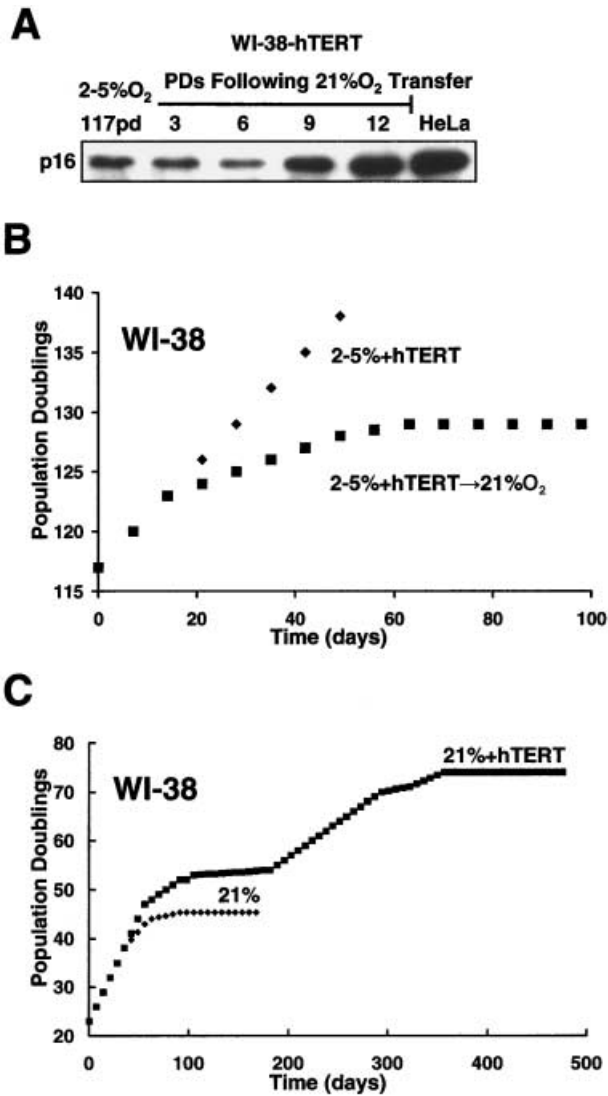


**Fig. 4** Oxygen and telomere shortening in fetal lung fibroblast strains. Telomeric DNA from cells was analysed every 3 PD throughout the proliferative lifespan, visualized and quantified as described in Materials and methods. Individual points of two separate experiments are indicated. Best-fit linear shortening trends are illustrated by the solid line, with slope values indicated. Telomere length in cells grown under normoxia vs. physiological oxygen is compared for the two fetal strains WI-38 and IMR-90. Data for WI-38 grown in the presence of NtBHA under normoxic (●) or physiological (○) oxygen are also shown.

(Atamna *et al.*, 2000). We compared the rates of telomere shortening in the different fetal lung fibroblast strains in order to determine the generality of this response. The rate of telomere shortening increases dramatically, from about 55 bp per division to 200–800 bp per division, in IMR-90 during the last few doublings (Fig. 4). Low oxygen at best only slightly reduced the rate of telomere shortening throughout the bulk of the lifespan (from 55 to 48 doublings per division, which is within experimental error), but slightly delayed the onset and significantly reduced the rate of telomere shortening once the 'oxidative crisis' had begun. WI-38 responded to low oxygen by reducing the rate of telomere shortening by 60% (from 74 to 44 bp per division), and did not show an accelerated rate of shortening at the end of their lifespan. Antioxidants may have slightly further reduced the rate of telomere shortening to 36 bp per division, irrespective of oxygen concentration (Fig. 4).

#### Checkpoint controls and the emergence of variants

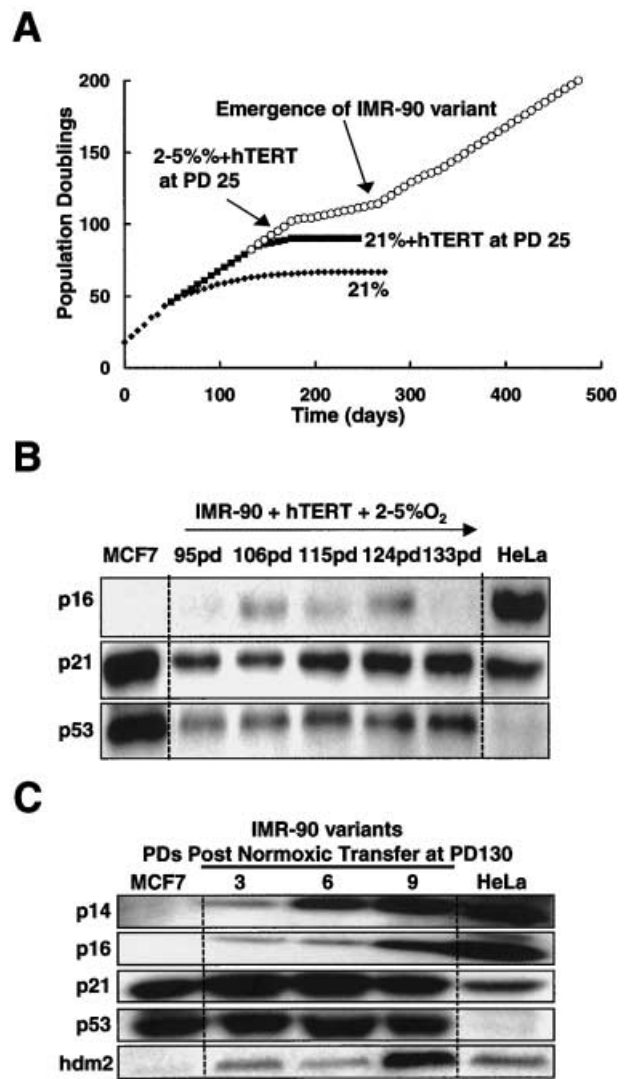
The p16/pRB pathway has been implicated in the growth arrest (stasis) that occurs when cells are grown under inadequate conditions (Ramirez *et al.*, 2001; Drayton & Peters, 2002; Herbert *et al.*, 2002). Consistent with these observations, telomerase expressing WI-38 cells grown in low oxygen exhibited a growth arrest accompanied by gradual elevation of p16<sup>INK4A</sup>, p21<sup>WAF1</sup> and p53 following their transfer to a 21% oxygen environment at PD 117 (Fig. 5A,B, only p16<sup>INK4A</sup> data are shown). The very slow kinetics of induction of p16 following transfer to ambient oxygen is consistent with previous reports of its role in response to DNA damage. Although its levels did not increase acutely following irreparable doses of irradiation or bleomycin treatment in normal human fibroblasts, p16 levels subsequently rose after 2–3 weeks as part of what appeared to be a chronic response (Robles & Adami, 1998). The data in Fig. 5 were obtained prior



**Fig. 5** Characterization of WI-38hTERT variants. (A) Cell lysates from telomerase immortalized WI-38 cells grown in low oxygen were analysed using an antibody directed against p16<sup>INK4A</sup> at the time of and following transfer to 21% oxygen. HeLa cell lysate is included as a positive control. Ponceau S staining was used to verify equal loading. (B) Growth kinetics of WI-38 cells following normoxic transfer (■) and low oxygen controls (◆). (C) Normoxic WI-38 cultured cells with (■) and without (◆) hTERT infection. A slow growing variant emerged at PD 55.

to the establishment of NDZB media as a requirement for continuous growth of WI-38-hTERT cells.

We have observed several instances in which cell variants appeared, where a more rapid proliferation began after a prolonged period of slow growth. For example, after 100 days without significant growth, cell division resumed in WI-38 cells expressing telomerase cultured in 21% oxygen (Fig. 5C). Cell death in this population was highly elevated, and eventually growth arrest occurred after an additional 20 doublings. The growth rate of telomerase-expressing IMR-90 cells grown in low oxygen increased after approximately 25 doublings of very slow growth between PD 105 and PD 125 (Fig. 6A). Figure 6(B)



**Fig. 6** Characterization of IMR-90hTERT variants. (A) IMR-90 cells cultured in normoxia with (◆) and without (■) hTERT expression and low oxygen with hTERT expression (○). A variant overgrew the low oxygen with hTERT culture at PD 125. (B) Immunoblot analysis of low oxygen cultured cells expressing telomerase during the period characterized by slow growth from 90 to 130 PD, using antibodies against p16<sup>INK4A</sup>, p21<sup>WAF1</sup> and p53. HeLa was included as a p16<sup>INK4A</sup>-positive control, MCF7 as a positive control for p21<sup>WAF1</sup> and p53. p16<sup>INK4A</sup> expression declined in the variant that appeared after PD 125. (C) Immunoblot of the variant cells cultured continuously in low oxygen and then transferred to a normoxic incubator, using antibodies against p14<sup>ARF</sup>, p16<sup>INK4A</sup>, p21<sup>WAF1</sup>, p53 and hdm2. An additional variant overgrowing this culture nine doublings after transfer had up-regulated hdm2. Controls were used to verify loading and are described in Materials and methods.

shows that p53, p21 and p16 all increased during this period of slow growth, but that p16 expression disappeared following the outgrowth of the cell variant after PD 125. At PD 130, these variant cells were transferred to a 21% oxygen environment. Although they initially behaved similarly to the WI-38 cells and growth arrested after about six doublings, more rapidly growing additional variants in turn emerged. Figure 6(C) shows an elevation of p16 in these cells, indicating that the earlier decrease of p16 possibly represented a partial methylation event that did

not completely silence both alleles. The other gene in the INK4A locus, p14<sup>ARF</sup>, became significantly elevated during this growth arrest, whereas the negative regulator of p14<sup>ARF</sup>, hdm2, became elevated in the variants that resumed growth by nine population doublings after ambient oxygen exposure. These observations demonstrate that fibroblasts, as well as epithelial cells, can undergo changes in gene expression that allow abnormal variants to overgrow cultures grown under inadequate conditions.

## Discussion

A variety of diseases involving reduced telomerase [dyskeratosis congenita (Mitchell *et al.*, 1999)] or increased cell turnover [ulcerative colitis (O'Sullivan *et al.*, 2002), aplastic anaemia (Ball *et al.*, 1998), Duchenne muscular dystrophy (Decary *et al.*, 2000)] are associated with shortened telomere length *in vivo*. Short telomeres may also contribute more broadly to human aging, as evidence accumulates for their association with hypertension (Aviv & Aviv, 1999), heart attacks (Brouillette *et al.*, 2003) or overall rates of human mortality after age 60 years (Cawthon *et al.*, 2003). It is thus important to understand mechanisms regulating rates of telomere shortening and to develop appropriate model systems for their studies. Although the effects of oxidative stress *in vivo* on telomere length have not been examined, previous studies using cultured cells have employed hyperoxia (40% oxygen), hydrogen peroxide or other mechanisms to exceed the endogenous protective mechanisms and produce telomeric damage (von Zglinicki, 2002). In the present study we examined whether ambient oxygen levels might have been responsible for our failure to immortalize IMR-90 fetal lung fibroblasts following the introduction of the catalytic protein subunit of telomerase, hTERT.

The behaviour of WI-38, IMR-90 and two adult lung fibroblast strains suggests a developmental difference in their ability to restrict oxidative damage to telomeres. Only the adult strains could be immortalized by telomerase under normoxic conditions. Although fetal WI-38 cells showed a rapid elongation of telomeres by telomerase in 21% oxygen, there was only a minimal prolongation of lifespan. This strongly suggests that the damage induced by normoxia was not limited to telomeres but also affected other cellular structures.

The level of oxidative protection mechanisms within a particular cell strain is not constant, but can decline after prolonged culture in fetal IMR-90 fibroblasts (Atamna *et al.*, 2000). This might reflect changes in gene expression during long-term adaptation to the culture environment, clonal overgrowth of cells with different requirements, consequences of cumulative damage due to the lack of particular micronutrients such as vitamin B12 (Ames, 1999), or positive-feedback loops involving changes to mitochondria or other mechanisms. Irrespective of this, it complicates the detailed interpretation of the causes of growth arrest. Because WI-38 cells do not immortalize in 21% oxygen following the introduction of telomerase, it is tempting to speculate that they do not stop growing as a result of replicative aging in culture but rather stop as a result of stasis

induced by genomic oxidative damage. This would have important experimental consequences because most of the data on the phenotype of senescent cells have come from studies of IMR-90 and WI-38 cells. However, Fig. 5 here shows that rather than just delaying an oxidative crisis, low oxygen slowed the rate of telomere shortening, and that the telomere length at senescence in 21% and low oxygen were roughly equivalent. This suggests that oxidative damage is primarily having a telomeric effect in WI-38 up to PD 65, and that increasing oxidative damage that induces non-telomeric effects in 21% oxygen only occurs after the lifespan has been extended by telomerase. If this is the case, WI-38 would be growth-arresting as a result of short-telomere-induced replicative senescence rather than stasis in both oxygen concentrations, which would only affect the rate of telomere shortening rather than the actual cause of cell cycle checkpoint activation. In contrast to WI-38, IMR-90 cells are arresting as a result of stasis rather than replicative aging. There is an oxidative crisis that occurs at the end of lifespan in IMR-90, and telomerase in 21% oxygen does not extend the lifespan much beyond that which can be achieved by low oxygen alone. Thus, general cellular damage rather than replicative aging is probably producing the growth arrest observed in IMR-90 cells grown under normoxic conditions, and the phenotype studied in 'senescent' IMR-90 probably reflects stasis.

Telomerase also fails to immortalize the fetal lung fibroblast strains MRC-5 (MacKenzie *et al.*, 2000) and TIG-3 (H. Tahara, personal communication) when grown under normoxic conditions. Telomerase thus fails to immortalize four strains of fetal lung fibroblasts under ambient oxygen conditions, whereas it can immortalize both tested strains of adult lung fibroblasts. These results are consistent with our interpretation that a developmental change occurs so that fetal, but not adult, lung fibroblasts develop non-telomeric oxidative damage when grown in 21% oxygen, preventing immortalization by telomerase.

It has long been known that oxidation-protective mechanisms vary both with tissue type and development (e.g. Vlessis & Mela-Riker, 1989; Sohal *et al.*, 1990). One study compared two fetal lung fibroblasts (WI-38 and MRC-5) with five different neonatal-adult skin fibroblasts and found both fetal lung strains to have lower levels of oxidant defences and altered levels of a variety of factors (Serra *et al.*, 2003). However, the changes between fetal and adult lung fibroblasts were not examined.

The present results demonstrate that there is considerable cell-strain variability in the response to oxygen, and all tested lung fibroblasts, from different donors or at different developmental stages, exhibit altered telomere biology in ambient vs. low oxygen conditions. A recent report analysing the rate of shortening of individual telomeres using allele-specific markers found that most of the shortening could be explained by progressive events consistent with the end-replication problem, but that occasional stochastic events caused a rapid decrease in the size of an individual telomere, even within a subcloned population (Baird *et al.*, 2003). This is consistent with both progressive telomere shortening and oxidative damage operating in

tandem. The present results support the hypothesis that both the size of the G-rich single-stranded telomeric overhangs and the level of oxidative damage might contribute to telomere size changes *in vivo*. Additional experiments will be needed to determine whether the growing evidence for the association of short telomeres with human aging and disease reflects the consequences of replicative aging, or serves as a marker for other processes such as oxidative damage to a wide variety of cellular structures, including telomeres.

## Experimental procedures

### Cell culture

WI-38 (ATCC (American Type Culture Collection), CCL-75), IMR-90 (ATCC, CCL-186), 35FL (CIMR (Coriell Institute for Medical Research), AGO2603), and 61ML (CIMR, AG02262A) cells were maintained in four parts Dulbecco's modified Eagle's medium to one part Medium 199 supplemented with 10% Cosmic calf serum (HyClone) and 50 µg mL<sup>-1</sup> gentamicin sulphate. Cells were passaged 1 : 8 approximately once per week, with each passage taken to represent three population doublings. Cells were considered growth-arrested after 100 days in culture with no obvious proliferation. Normoxic cells were maintained in 37 °C humidified incubators containing 5% CO<sub>2</sub> and ambient oxygen (21%). To create a low oxygen environment, plexiglass containers with sealed lids were flushed with a mixture of 2% O<sub>2</sub>, 7% CO<sub>2</sub> and 91% N<sub>2</sub>. Measurements with a Fyrite O<sub>2</sub> meter indicated that oxygen concentrations gradually rose from 2% to 5% over a 5-day period. Containers were thus gassed twice per week. Further supplementation was provided by adding to the media the antioxidant N-(*tert*-butyl hydroxylamine (100 µM), dexamethasone (1 µM), ZnSO<sub>4</sub> (1.4 mg L<sup>-1</sup>) and vitamin B12 (0.03 mg L<sup>-1</sup>).

### Vectors and retroviruses

Retroviral infections were performed using supernatant from PA317 cells infected with pBabePuro (Morgenstern & Land, 1990) containing hTERT or control vectors. Independent infections were performed in both low oxygen and normoxia. The efficiency of retroviral infections was estimated to be approximately 30–60% based on cell density 5 days post selection.

### Telomere length analysis

Telomere length was measured after DNA extraction at multiple population doubling levels by digestion with the restriction enzymes: *AluI*, *CfoI*, *HaeIII*, *HinfI*, *MspI* and *RsaI*, and resolution by agarose gel electrophoresis as detailed elsewhere (Ouellette *et al.*, 2000). High-molecular-weight telomeric DNA was resolved using programmed pulse field gel electrophoresis (Mapper system, Bio-Rad). After hybridizing to radiolabelled (T<sub>2</sub>AG<sub>3</sub>)<sub>3</sub> probes, signals were analysed with the program Telorun ([http://www.swmed.edu/home\\_pages/cellbio/shay-wright/](http://www.swmed.edu/home_pages/cellbio/shay-wright/)

research) using weighted mean calculations designed to normalize signal intensity relative to the digestion product size.

### Western analysis

Chemiluminescence was used to detect signals from secondary antibody (ECL, Amersham Pharmacia). Primary antibodies recognizing p21<sup>WAF1</sup> (Ab-1) and p53 (DO-1) were obtained from Oncogene Research Products, p14<sup>ARF</sup> from Novus Biologicals, p16<sup>INK4A</sup> from BD Pharmingen, and hdm2 (SMP14) from Santa Cruz Biotechnology. Ponceau staining was used to verify equal protein loading in all instances.

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