Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress

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Summary

Telomerase is a ribonucleoprotein that counteracts telomere shortening and can immortalise human cells. There is also evidence for a telomere-independent survival function of telomerase. However, its mechanism is not understood. We show here that TERT, the catalytic subunit of human telomerase, protects human fibroblasts against oxidative stress. While TERT maintains telomere length under standard conditions, telomeres under increased stress shorten as fast as in cells without active telomerase. This is because TERT is reversibly excluded from the nucleus under stress in a dose- and time-dependent manner. Extranuclear telomerase colocalises with mitochondria. In TERT-overexpressing cells, mtDNA is protected, mitochondrial membrane potential is increased and mitochondrial superoxide production and cell peroxide levels are decreased, all indicating improved mitochondrial function and diminished retrograde response. We propose protection of mitochondria under mild stress as a novel function of TERT.

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Key words: Telomerase, TERT, Mitochondria, Oxidative stress, Reactive oxygen

Introduction

Normal human somatic cells shorten their telomeres during their lifespan leading eventually to dysfunctional telomeres, growth arrest and replicative senescence. Overexpression of *TERT*, the catalytic subunit of telomerase, counteracts telomere shortening, extends the replicative potential and prevents replicative senescence (Bodnar et al., 1998). In addition, there is evidence for telomere length-independent functions of telomerase, which appear to promote cell survival and stress resistance (del Bufalo et al., 2005; Kang et al., 2004; Ludwig et al., 2001; Oh et al., 2001; Zhang et al., 2003). For example, telomerase expression conferred increased resistance to specific DNA damaging agents (Kondo et al., 1998; Ludwig et al., 2001; Sharma et al., 2003) and decreased apoptosis (Fu et al., 2000; Massard et al., 2006; Zhang et al., 2003; Zhu et al., 2000). Telomerase transgenic and knockout mice have been used to discriminate between telomere-dependent and -independent functions of telomerase. Increased metabolic turnover and augmented antioxidant responses in germ line cells (Franco et al., 2005) and increased sensitivity to chemotherapeutic (Lee et al., 2001) or alkylating (Gonzalez-Suarez et al., 2003) agents have been related to late generation $Terc^{-/-}$ mice specifically, suggesting a predominant dependency of these phenotypes on the telomere capping state in this model. By contrast, overexpression of mouse TERT confers increased stress resistance, improved antioxidant defence and improved differentiation capacity to mouse embryonic stem cells (Armstrong et al., 2005), and promotes proliferation of resting stem cells in the skin epithelium in a telomere-independent manner (Sarin et al., 2005). $Tert^{-/-}$ mice also show reduced glutamate+malate (complexI-dependent) respiration of heart mitochondria (J. Haendeler et al., unpublished).

The cell protective function of telomerase has been related to improved DNA damage repair (Sharma et al., 2003) or to increased apoptosis resistance (Fu et al., 2000; Massard et al., 2006; Zhang et al., 2003; Zhu et al., 2000). A better understanding of the mechanisms involved is of obvious importance, as telomerase is active in the vast majority of human cancers (Shay and Bacchetti, 1997) and might significantly contribute to tumorigenic potential (Bagheri et al., 2006; Stewart et al., 2002) and to the low sensitivity of many cancer cells to chemo- and radiotherapeutic treatments (Bakalova et al., 2003).

It had been shown recently that telomerase is excluded from the nucleus upon oxidative stress (Haendeler et al., 2003; Haendeler et al., 2004; Santos et al., 2004; Santos et al., 2006b). Santos et al. have found a mitochondrial localisation signal in the TERT sequence that directs the protein to mitochondria and have demonstrated a mitochondrial localisation for TERT (Santos et al., 2004). Stress-dependent nuclear exclusion has been demonstrated not only for ectopically overexpressed TERT, but also for wild-type TERT in endothelial cells (Haendeler et al., 2004), indicating that this shuttling is not an overexpression artefact. Possible biological function(s) of telomerase in an extra-nuclear localisation remained unclear, however. Liu et al. have described phosphorylation-dependent nuclear import as a method for regulation of telomerase activity in human lymphocytes (Liu et al., 2001). Santos et al. found

an aggravation of mitochondrial DNA (mtDNA) damage in cells expressing TERT (Santos et al., 2004; Santos et al., 2006b).

By contrast, we describe here improved mitochondrial function, including better protection of mtDNA together with export of TERT towards mitochondria. We used chronic mild oxidative stress to explore the roles of an *TERT* transgene in MRC-5 lung fibroblasts for both telomere maintenance and stress resistance. Telomerase did not prevent telomere shortening under hyperoxia as it translocated gradually from the nucleus to mitochondria. However, *TERT* overexpression reduced mtDNA damage levels under oxidative stress and improved mitochondrial function, e.g. resulted in lower mitochondrial production of reactive oxygen species (ROS) together with an enhanced mitochondrial membrane potential (MMP). This was accompanied by indicators for a reduced retrograde response in terms of gene expression pattern and mitochondrial biogenesis. We suggest that protection of mitochondria against oxidative stress is an important telomere length-independent function for telomerase in cell survival.

Results

TERT overexpression does not maintain telomere length under chronic oxidative stress

Transfection of human MRC5 fibroblasts with *TERT* provides high levels of telomerase activity, stabilises telomere length (Fig. 1A) and allows greatly extended replicative lifespan under normoxic (21% oxygen) culture conditions (Bodnar et al., 1998). Increasing the ambient oxygen concentration to 40% (hyperoxia) generates increased peroxide levels and shortens the replicative lifespan of primary human fibroblasts because of stress-induced accelerated telomere shortening (Passos et al., 2007; von Zglinicki, 2002). Net growth of MRC5-TERT cells under hyperoxia decreased as in control MRC5 cells under the same conditions, albeit somewhat slower (data not shown). Growth arrest was confirmed by loss of expression of Ki67, a marker for cell proliferation, as well as increased frequencies of cells positive for senescence-associated β-galactosidase (SA-β-Gal) or phosphorylated histone H2A variant X (γ-H2A.X), both markers for cellular senescence (d'Adda di Fagagna et al., 2003; Dimri et al., 1995; von Zglinicki et al., 2005). Although expression of *TERT* delayed the occurrence of these senescence markers under hyperoxia, eventually MRC5-TERT cells showed maximal marker expression similar to senescent parental cells (supplementary material Fig. S1).

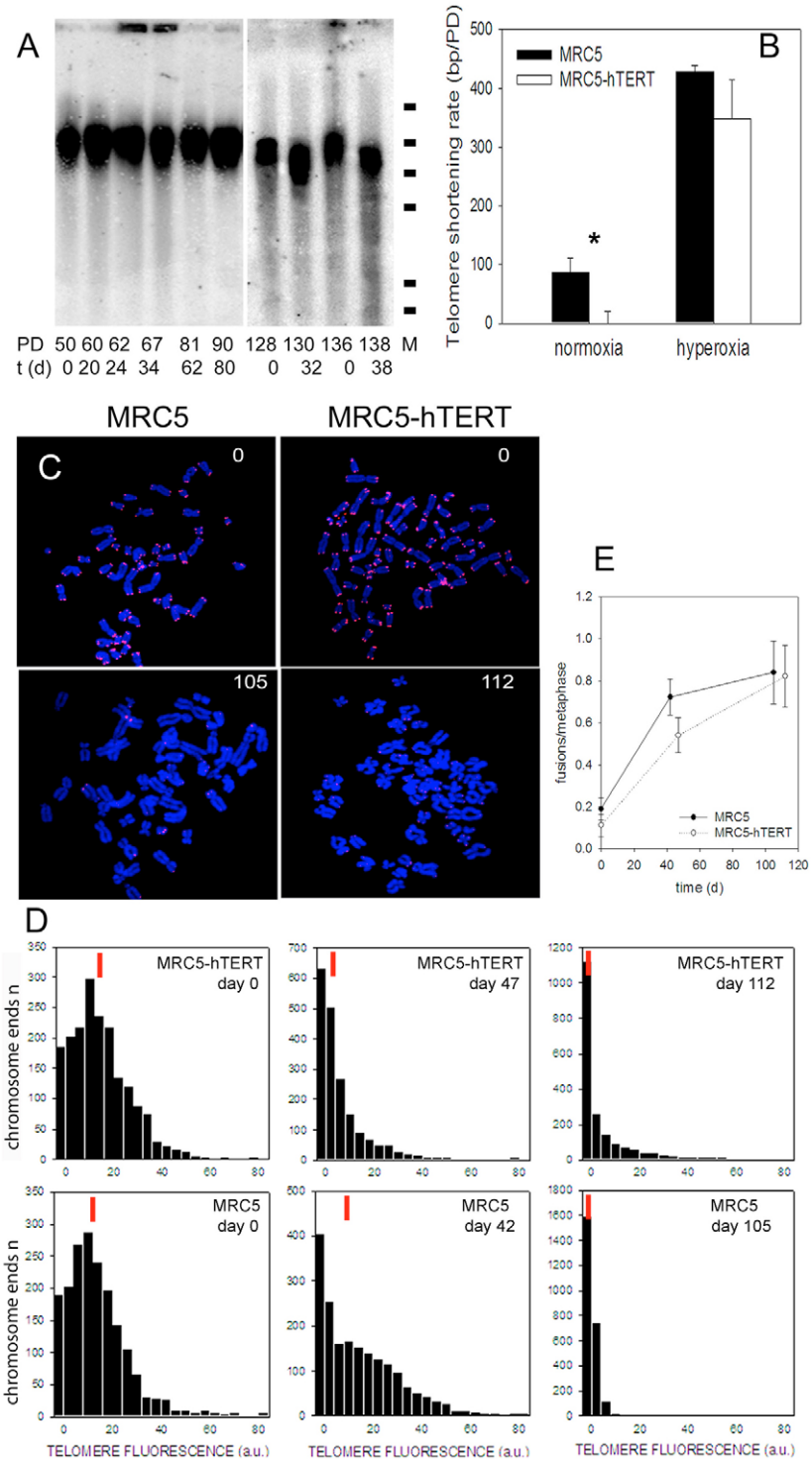

Fig. 1. *TERT* overexpression does not maintain telomere length under hyperoxia. (A) Telomere restriction fragment length in MRC5-TERT cells grown for the indicated times (in days) under normoxia (left) or under 40% hyperoxia (right). Population doublings are indicated (PD). M indicates the positions of a λHINDIII size marker. (B) Telomere shortening rates per PD in MRC5 (black bars) and MRC5-TERT (white bars) under normoxia (left) and hyperoxia (right). Data are mean±s.e.m. from four experiments measured in quadruplicate. The asterisk indicates a significant difference between parental and TERT-overexpressing cells with $P<0.05$ (ANOVA). (C) Metaphase spreads from MRC5 (left) and MRC5-TERT (right) cells grown under hyperoxia for the indicated times (in days) hybridised with a telomeric PNA probe (red). Chromosomes are stained with DAPI (blue). (D) Frequency distributions of telomere fluorescence signal intensities in MRC5-TERT (top) and MRC5 (bottom) cells grown under hyperoxia for the indicated times. Red bars indicate the median telomere length. (E) Frequencies of chromosomal end-to-end fusions per metaphase in MRC5 (black) and MRC5-TERT (white, broken lines) under hyperoxia. Data are mean±s.e.m. from at least 25 metaphases per condition.

Under hyperoxia, telomere restriction fragment length in MRC5-TERT cells decreased (Fig. 1A) with the same rate as in parental MRC5 cells (Fig. 1B). This was confirmed by fluorescence in situ hybridization (FISH; Fig. 1C): metaphases from TERT-overexpressing fibroblasts under hyperoxia showed the same telomere loss as those from parental MRC5 cells (Fig. 1D). Moreover, frequencies of chromosomal end-to-end fusions were very similar in MRC5 and MRC5-TERT cells after prolonged hyperoxia (Fig. 1E). Thus, overexpression of *TERT* is sufficient to maintain telomere length in MRC5 fibroblasts growing under 21% oxygen, but not under 40% oxygen.

Growth arrest of *TERT*-overexpressing cells under oxidative stress is telomere-dependent but reversible

Colocalisation of telomeres and γ-H2A.X-containing DNA damage is a hallmark of telomere-dependent senescence (d'Adda di Fagagna et al., 2003). Telomere-induced DNA damage foci were found at similar frequencies in both MRC5 and MRC5-TERT cells following long-term culture under hyperoxia (Fig. 2A,B). Quantitatively, the correlation between telomeres and DNA damage foci was as good in MRC5-TERT cells as in senescent parental MRC5 (Fig. 2C).

The growth arrest of parental fibroblasts under chronic mild hyperoxia is telomere length dependent (Martin-Ruiz et al., 2004) and remains irreversible even if the cells are set back into normoxia, because short telomeres cannot be re-elongated. However, if MRC5-TERT cells were transferred back to normoxic conditions after a period of hyperoxia, they resumed growth, the frequency of Ki67-positive cells increased, cells lost γH2A.X staining and telomeres became re-elongated (supplementary material Fig. S2), indicating that telomerase regains the ability to re-elongate telomeres even after long periods of increased oxidative stress.

TERT is excluded from nuclei under oxidative stress and accumulates in mitochondria

Total activity of telomerase in MRC5-TERT cells as measured by TRAP assay was not decreased under hyperoxia (Martin-Ruiz et al., 2004) (and data not shown). However, telomerase can be excluded from the nucleus in parallel with ROS generation (Haendeler et al., 2003; Haendeler et al., 2004). Telomerase activity as measured by Telomere Repeat Amplification Protocol (TRAP) in the extranuclear fraction (including mitochondria) increased from about 25-30% of the total cell activity in controls to 80–90% after both H_2O_2 treatment (Fig. 3A) and under hyperoxia (Fig. 3B). This was mostly due to an increase of TRAP activity in mitochondria and a corresponding decrease of nuclear TRAP activity (Fig. 3C).

Using an antibody that allows specific detection of TERT (Wu et al., 2006), we confirmed concentration-dependent H_2O_2-mediated nuclear exclusion of ectopically expressed TERT (Fig. 3D) and gradual nuclear exclusion of TERT under chronic hyperoxia (Fig. 3E). Western blotting of mitochondrial and nuclear fractions confirmed an increase of TERT protein specifically in mitochondria following treatment with hydrogen peroxide (supplementary

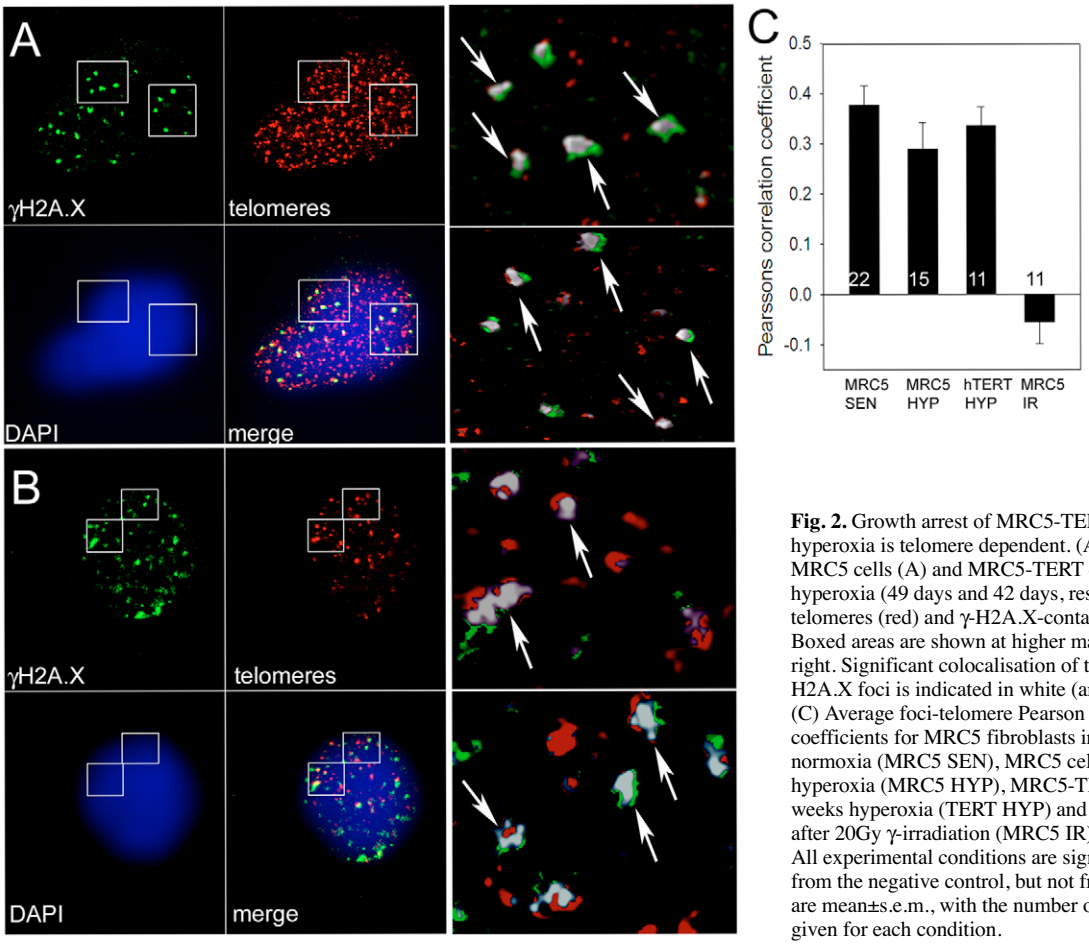

Fig. 2. Growth arrest of MRC5-TERT cells under hyperoxia is telomere dependent. (A,B) ImmunoFISH of MRC5 cells (A) and MRC5-TERT cells (B) under hyperoxia (49 days and 42 days, respectively) showing telomeres (red) and γ-H2A.X-containing foci (green). Boxed areas are shown at higher magnification on the right. Significant colocalisation of telomeres and γ-H2A.X foci is indicated in white (arrowheads). (C) Average foci-telomere Pearson correlation coefficients for MRC5 fibroblasts in senescence under normoxia (MRC5 SEN), MRC5 cells after 7 weeks hyperoxia (MRC5 HYP), MRC5-TERT cells after 6 weeks hyperoxia (TERT HYP) and MRC5 cells 48 hours after 20Gy γ-irradiation (MRC5 IR) as negative control. All experimental conditions are significantly different from the negative control, but not from each other. Data are mean±s.e.m., with the number of cells evaluated given for each condition.

material Fig. S3). Exclusion of TERT from the nuclei was reversible: when cells were shifted back from hyperoxia to normoxia, control conditions were restored after about 4 weeks (Fig. 3F). This time course corresponded well to the re-elongation of telomeres and resumption of cell growth.

TERT immunofluorescence in the cytoplasm significantly (Pearson's correlation coefficient P=0.98) colocalised with Mitotracker Green staining (Fig. 3G), thus confirming mitochondrial localisation of TERT in accordance with earlier reports (Santos et al., 2004; Santos et al., 2006b).

TERT protects mitochondria

One group recently reported higher susceptibility of mtDNA to damage in TERT-overexpressing fibroblasts (Santos et al., 2004; Santos et al., 2006b). We could not confirm this. On the contrary, we found that mtDNA in MRC5-TERT was protected against acute (Fig. 4A) or chronic (Fig. 4B) oxidative damage. We noted that mtDNA damage levels actually decreased under long-term hyperoxia, i.e. when most of the TERT protein (Fig. 3E) and telomerase activity (Fig. 3C) was translocated to mitochondria. This result was confirmed using an independent technique that measured damage via its effect on the supercoiled state of mtDNA (supplementary material Fig. S4).

To analyse the impact of *TERT* expression on mitochondrial function, we measured mitochondrial superoxide production by MitoSOX fluorescence. Fluorescence increased when either MRC5 or MRC5-TERT cells were grown under hyperoxia. However, fluorescence levels were always lower in MRC5-TERT cells (Fig. 4C). This result was confirmed by measuring cellular peroxide levels by dihydrorhodamine 123 (DHR) fluorescence in MRC5/MRC5-TERT fibroblasts (Fig. 4D). It was further confirmed in an independent human fibroblast strain, comparing parental BJ fibroblasts versus an *TERT*-immortalised clone derived from them (Bodnar et al., 1998) (supplementary material Fig. S5). No significant differences were found between parental and empty vector (pBabe)-transfected cells (data not shown). Thus, TERT expression diminishes mitochondrial superoxide production and cellular ROS levels.

Mitochondrial membrane potential (MMP) is a direct indicator of the efficiency of coupling between respiration and ATP production. However, high MMP increases the probability of ROS generation (Balaban et al., 2005), and MMP in human fibroblasts responds to cellular ROS levels in an adaptative fashion, so that high ROS levels transcriptionally upregulate the uncoupling protein UCP2, resulting in MMP downregulation (Passos et al., 2007). Measuring MMP in intact cells by JC1 fluorescence we found higher membrane potential in MRC5-TERT cells (Fig. 4E). *UCP2* expression was elevated by hyperoxia, but less so in MRC5-TERT cells (Fig. 4F). These data are in accordance with the idea that overexpression of *TERT* improves mitochondrial function, allowing low ROS levels despite tight coupling of mitochondria.

To test whether endogeneous TERT might also protect mitochondria, we knocked down endogeneous TERT in primary human umbilical vein endothelial cells (HUVECs) using two separate siRNAs (Fig. 4G). Mitochondrial superoxide production as measured by MitoSOX fluorescence and cellular peroxides measured by DHR fluorescence were significantly increased 2 days after transfection with both siRNAs in combination (Fig. 4H).

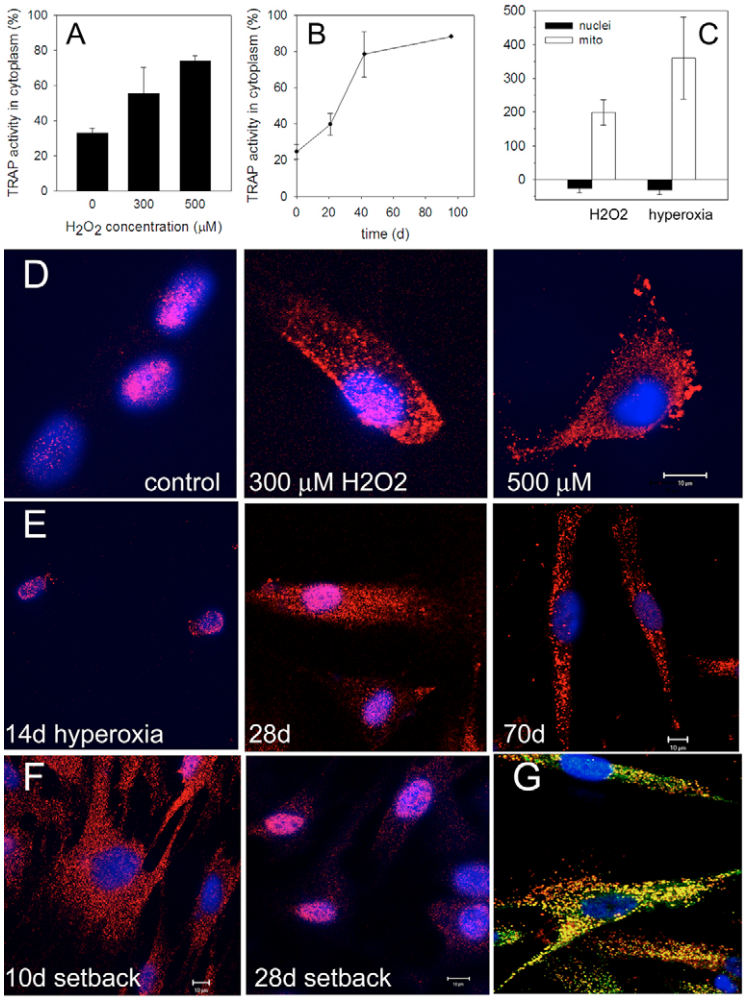

Fig. 3. Telomerase is excluded from the nucleus under oxidative stress and colocalises with mitochondria. (A) Percentage of total TRAP activity in the extranuclear fraction of MRC5-TERT after H_2O_2. (B) Percentage of total TRAP activity in the extranuclear fraction following hyperoxic treatment for the indicated times. Data are mean±s.e.m. from three independent experiments. (C) Change in mitochondrial (white) and nuclear (black) TRAP activity after 3 hours of 500 mM H_2O_2 exposure or 52 days of hyperoxia. Data are mean±s.e.m. from six replicate measurements. All values are significantly different from controls (100%). (D-G) TERT immunofluorescence (red) in MRC5-TERT under normoxia (D, left), following treatment with H_2O_2 at the indicated concentrations (D, middle and right), under hyperoxia for the indicated times (E), in cells grown under hyperoxia for 50 days followed by shift back to normoxia for the indicated times (F), and co-stained after 70 days hyperoxia with Mitotracker green (G). Colocalisation of mitochondria and TERT-containing foci appears in yellow.

TERT overexpression counteracts retrograde response

Mitochondrial dysfunction induces major adaptative changes in global gene expression patterns often termed retrograde response, both in yeast (Jazwinski, 2005) and mammalian cells (Biswas et al., 2005), and this is also part of replicative senescence of human fibroblasts (Passos et al., 2007). In retrograde signalling, lower MMP results in the activation of cytoplasmic Ca^{2+} signalling, leading to metabolic readjustment, activation of mitochondrial biogenesis (Biswas et al., 2005; Passos et al., 2007) and downregulation of apoptotic signals. Having seen that *TERT* overexpression was able

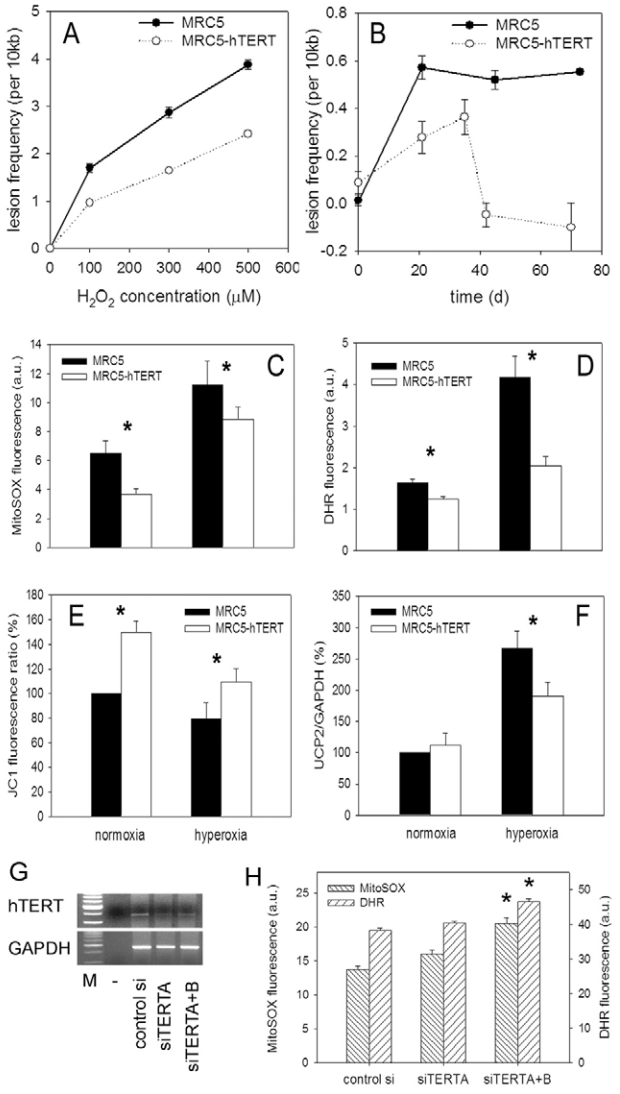

Fig. 4. *TERT* overexpression protects mitochondrial DNA integrity and mitochondrial function under oxidative stress. Frequency of lesions in mtDNA as measured by the relative amplification efficiency of an 11 kb mtDNA fragment in MRC5 (black circles) and MRC5-TERT (white circles) following treatment with hydrogen peroxide in the indicated concentrations (A) and under long-term hyperoxia (B). Data are mean±s.e.m. from quadruplicate measurements. (C) Mitochondrial superoxide generation as measured by MitoSOX fluorescence intensity per cell. (D) Cellular peroxide levels as measured by DHR123 fluorescence. (E) MMP as measured by JC1 fluorescence ratio. (F) *UCP2* expression as measured by duplex RT-PCR with *Gapdh* as control. All data in C to F were measured in MRC5 (black bars) and MRC5-TERT (white bars) under normoxia (left) and after 1 week of hyperoxia (right), and are mean±s.e.m. from three experiments. Differences between parental and TERT-overexpressing cells marked by an asterisk are significant with $P<0.05$ (ANOVA). (G) Expression of endogeneous TERT was measured by semi-quantitative TERT RT-PCR 2 days after transfection with the indicated siRNAs. GAPDH was measured as control. (H) MitoSOX and DHR fluorescence intensity in HUVECs at 2 days after transfection with the indicated siRNAs. Data are mean±s.e.m. from quadruplicate measurements. Asterisks indicate significant differences to cells treated with control siRNA with $P<0.05$ (ANOVA).

to improve mitochondrial function, we wanted to find out whether *TERT* would also downregulate retrograde response. *TERT*-overexpressing fibroblasts showed lower mitochondrial mass per cell under both normoxia and hyperoxia (Fig. 5A), mtDNA copy number was lower in MRC5-TERT cells under normoxia, and it did not increase under hyperoxia (Fig. 5B), altogether indicating diminished mitochondrial biogenesis.

We recently identified 92 genes (120 Affymetrix probesets) with functions in cellular signalling, glycolysis and Krebs cycle metabolism, mitochondrial function and stress response, which were differentially expressed in senescent when compared with young MRC5 fibroblasts and, thus, might be candidate marker genes for retrograde response in human fibroblasts (Passos et al., 2007). Many of these genes changed expression in the opposite direction in MRC5-TERT when compared with senescent MRC5, while the majority of the remaining genes were not differentially expressed between young MRC5 and MRC5-TERT cells (Fig. 5C-F). Expression levels for nine selected genes were also evaluated by RT-PCR, and differential regulation was confirmed for all of them (supplementary material Fig. S6). In particular, we noted that many genes coding for enzymes with major functions in metabolism, including *GLS*, *AK3*, *PDK4*, *RODH*, *GCLM* and *MAOA*, were upregulated in senescence, but downregulated in MRC5-TERT cells (Fig. 5D,E). The same was true for genes involved in cellular signalling, including *IGFBP3*, *SULF1* and *BAMBI*, while some signalling genes (e.g. *ADCY3*, *AURKB*, *GPSM2*) that were downregulated in senescence became upregulated following *TERT* overexpression (Fig. 5C). Interestingly, relatively few apoptosis-related genes were regulated in opposite fashion by senescence and *TERT* overexpression (*ASC*, *BCOR* and *PHLDA1*; Fig. 5F). Accordingly, we found that MRC5-TERT cells were significantly more resistant against apoptosis induced by either hydrogen peroxide (Fig. 5G) or etoposide (Fig. 5H).

Together, these data suggest that *TERT* overexpression lowers cellular ROS production, improves mitochondrial coupling, counteracts retrograde response and thus 'rejuvenates' fibroblasts in terms of mitochondrial function. This is associated with improved resistance to apoptosis.

Discussion

Chronic mild oxidative stress accelerated telomere shortening. Unexpectedly, ectopically overexpressed telomerase in human fibroblasts did not counteract it to any significant degree. It has been shown before that both acute oxidative stress induced by treatment of 293 cells with H_2O_2 (Haendeler et al., 2003) and endogenous chronic oxidative stress in senescing endothelial cells (Haendeler et al., 2004) induced the export of TERT from the nucleus. So far, the effect of nuclear exclusion of TERT on telomere maintenance has not been examined intensively, although it had been noted that increased oxidative stress accelerated telomere shortening not only in human fibroblasts (von Zglinicki, 2002) but also in telomerase-positive endothelial cells (Furumoto et al., 1998; Kurz et al., 2004). Our data now show that chronic oxidative stress interferes with telomere maintenance at two levels: it increases the basal rate of telomere shortening by induction of telomeric DNA damage, as shown before in telomerase-negative fibroblasts (Petersen et al., 1998), and it prevents telomerase from counteracting telomere shortening by inducing its export from the nucleus and to mitochondria.

In terms of the effect of TERT on mtDNA integrity, our results are contrary to those published recently by one other group. Santos et al. (Santos et al., 2004; Santos et al., 2006b) have reported that ectopically expressed *TERT* in human fibroblasts under acute oxidative stress resulted in increased mtDNA damage. At present, we cannot resolve this discrepancy to our data. However, we note

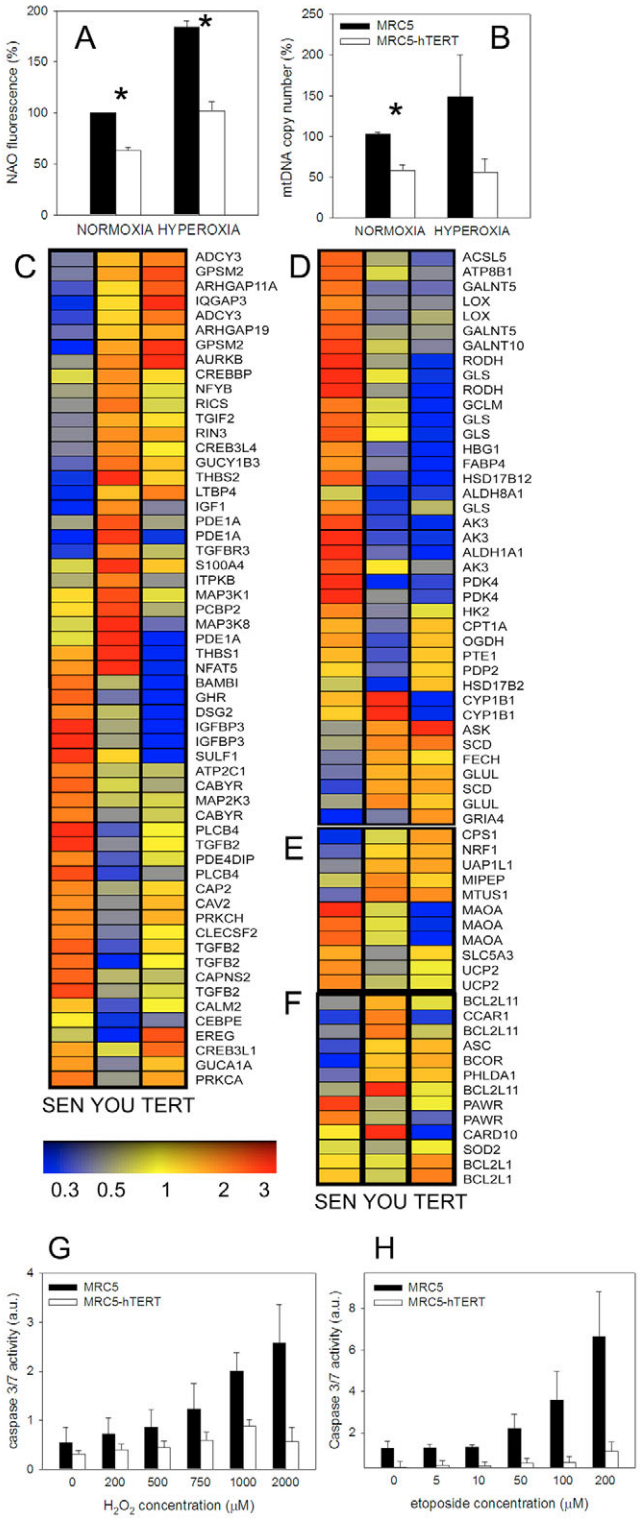

Fig. 5. *TERT* overexpression decreases mitochondrial mass and mtDNA content, reverses expression of candidate genes for retrograde response and improves resistance against apoptosis. (A) Relative mitochondrial mass per cell (relative to that in MRC5 cells under normoxia) as measured by NAO fluorescence in flow cytometry. (B) Relative MtDNA copy number as measured by real-time PCR. All data are mean±s.e.m. from at least three experiments. Asterisks indicate significant differences between parental and TERT-overexpressing cells with $P<0.05$. (C-F) Relative mRNA expression levels of candidate marker genes for retrograde response (Passos et al., 2007) in senescent MRC-5 (SEN), young MRC-5 (YOU) and MRC5-TERT cells (TERT). Expression colour code is indicated at the bottom. (C) Ca^{2+}-related signalling, (D) metabolism, (E) mitochondrial function and (F) stress response. (G) Caspase 3/7 activity (in arbitrary units) in MRC5 and MRC5-TERT cells treated for 2 hours with H_2O_2 at the indicated concentrations and assayed after 24 hours. Data are mean±s.e.m. from three experiments. (H) Caspase 3/7 activity (in arbitrary units) in MRC5 and MRC5-TERT cells treated for 48 hours with etoposide at the indicated concentrations and assayed after 3 days. Data are mean±s.d. from two experiments.

that our data not only demonstrate a protective effect of *TERT* on mtDNA integrity, but that we also find decreased mitochondrial ROS generation and increased MMP under both acute and chronic oxidative stress conditions, as well as indications for diminished retrograde response and improved apoptosis resistance, all demonstrating improved mitochondrial function in TERT-overexpressing cells. Our results are in accordance with recent data showing protection of mtDNA from UV- and ethidium bromide-mediated damage, as well as protection of mitochondrial function due to *TERT* expression (J. Haendeler et al., unpublished). Importantly, the fact that knock-down of TERT in HUVECs (this paper) as well as knock out of mouse TERT in mouse heart (J. Haendeler et al., unpublished) resulted in increased mitochondrial production of superoxide indicates that the mitochondrial effects of TERT are not simply overexpression artefacts, but describe a physiological function of telomerase. Kang et al. also found increased MMP and enhanced Ca^{2+} storage capacity in mouse neurons overexpressing mouse TERT (Kang et al., 2004). Moreover, in a model of apoptosis induction by BCL2 inhibition, *TERT* overexpression protected mitochondrial membrane potential and function independently of its catalytic activity with respect to telomere maintenance (del Bufalo et al., 2005).

The protective effect of telomerase against a variety of apoptosis- or senescence-inducing stressors has been related to TERT-mediated regulation of expression of genes implicated in cell proliferation and differentiation (Geserick et al., 2006), to improved DNA damage repair (Sharma et al., 2003; Smith et al., 2003), increased apoptosis resistance (del Bufalo et al., 2005; Zhang et al., 2003) or decreased apoptosis signalling (Dudognon et al., 2004). We show here evidence for the existence of a candidate protective mechanism that might be able to integrate some of these observations into a common concept: cells that overexpress *TERT* show evidence for improved mitochondrial function, specifically less mitochondrial superoxide production and lower levels of cellular ROS, improved mitochondrial coupling and suppressed retrograde response. An effect of telomerase on mitochondrial gene expression and function has been noted before in knock-down experiments in yeast (Nautiyal et al., 2002) and in mouse melanoma cells (Bagheri et al., 2006), although the direction and magnitude of the effect might be modified in tumour cells by their dependency on glycolysis as well as on telomerase (Li et al., 2005). Retrograde response has been described as a major reprogramming of nuclear gene expression patterns, including genes involved in metabolism, stress response and growth signalling, as result of mitochondrial dysfunction and resultant Ca^{2+}-dependent signalling (Biswas et al., 2005; Butow and Avadhani, 2004). It is important to note in this respect that mitochondrial dysfunction and retrograde response is a characteristic feature of replicative senescence in both yeast (Jazwinski, 2005) and human fibroblasts (Passos et al., 2007). Thus, the effect of ectopic *TERT* expression can be described as a 'rejuvenation' of mitochondria that impacts on global gene expression pattern via

suppression of mitochondrial dysfunction and, possibly, retrograde signalling.

Our results suggest that mitochondrial location of TERT might be necessary for its protection of mitochondrial function. It has recently been shown that TERT binds to mtDNA (J. Haendeler et al., unpublished). However, we do not know yet whether TERT actually protects mtDNA against oxidative damage, whether it activates repair of mtDNA or whether it accelerates degradation of mitochondria with damaged DNA. It is also not clear yet whether the improved maintenance of mtDNA is a cause or a consequence of the lower levels of ROS production in *TERT*-expressing fibroblasts. However, it is important to note that a relatively mild mitochondrial dysfunction as induced here by an increase in ambient oxygen concentration might occur under a wide variety of physiological or mildly pathological situations. Thus, we propose a telomere-independent function for telomerase, namely a protective role against mtDNA damage by mitochondrially derived ROS. The fact that essentially all of TERT is transported out of the nucleus under long-term low level oxidative stress raises the issue of whether protection of mitochondrial DNA might be of more immediate consequence than protection of telomeres under stress.

Materials and Methods

Cell culture and measurements

Human embryonic lung MRC5 fibroblasts were obtained from ECACC (Salisbury, UK) and grown in DMEM supplemented with 10% foetal calf serum, Pen/Strep and 2 mM glutamine under control conditions (air plus 5% CO_2) or hyperoxia (40% O_2 and 5% CO_2) in a three-gas incubator. Human umbilical vein endothelial cells (HUVEC) were grown in M199 with 10% FCS, Pen/Strep and 2 mM Glutamine, supplemented with endothelial cell growth factor (Sigma). Cells were treated with H_2O_2 at the indicated concentrations in serum-free medium for 3 hours. MRC5 cells were transfected retro-virally at population doubling (PD) 30 with the human catalytic subunit (*TERT*) of the enzyme telomerase. Transfected cells were cytogenetically tested at PD100. No cytogenetic abnormalities were observed. Immunofluorescence with anti-phospho-Histone H2AX (Upstate Biotechnology), anti Ki-67 Mab (BD Transduction Labs) and anti-TERT (Rockland), colocalisation analysis of telomeres and γ-H2A.X foci, and flow cytometric analysis of cells stained with MitoSOX, dihydrorhodamine 123, JC-1 or nonyl Acridine Orange (NAO, all Molecular Probes) were performed as described previously (Passos et al., 2007). To measure apoptosis, the activity of the 'executioner caspases' 3 and 7 was assessed using Apo-one homogeneous caspase-3/7 assay (Promega).

siRNAs A and B against TERT as described (Shammas et al., 2005) and control siRNA (AM4611, Ambion) were transfected using siPORT Amine (Ambion) at a concentration of 30 nM. TERT expression was measured by RT-PCR using primers 5′-GCCTGAGCTGTACTTTGTCAA-3′ (forward) and 5′-GCAAACAGCTTGTTTC-TCCATGTC-3′ (reverse).

Gene expression

Total RNA was extracted from the following independent cultures using RNAeasy (Qiagen): four mid-passage parental MRC5, three last passage MRC5 (senescent) and three MRC5-TERT, all grown under normoxia. All cultures were confluency-arrested in G0. Biotin-labelled cRNA was prepared and processed according to the manufacturer's instructions and analysed using U133 2.0 plus gene chips (Affymetrix, Buckingham, UK). Data were analysed using GeneSpring v7.0 using the following criteria: at least three out of 10 calls marginal or present, average fold change between conditions at least twofold.

Telomere length and telomerase activity

Telomere length was measured by in gel hybridisation and Telomere Q-FISH on metaphases as described (Passos et al., 2007; von Zglinicki et al., 2000). Telomerase activity was analysed with a TeloTAGGG Telomerase PCR ELISA kit (Roche). Signal linearity was established over an input range of 0.1 to 20 ng of protein, and measurements were performed with 2 and 10 ng protein per sample. A DNA/Cytosol fractionation kit (Bio Vision) was used to separate nuclei. A crude mitochondrial fraction was isolated as described (Stuart et al., 2004). Purity of fractions was tested by western blotting using antibodies against HDAC2 (Abcam) and COX2 (SantaCruz).

mtDNA damage and copy number

Relative mtDNA copy number was determined as amplification efficiency of an 83 bp mtDNA amplicon using real-time PCR as described (Passos et al., 2007); however, the heat denaturation step preceding the PCR reaction was prolonged to 6 minutes to allow full denaturation of supercoiled mtDNA.

mtDNA damage was measured by two independent methods. First, we used long-range PCR (Santos et al., 2006a) to measure the amplification efficiency for a large (11,095bp) mtDNA fragment in real-time PCR as described (Passos et al., 2007). Second, we assessed damage by its effect on the supercoiled state of mtDNA. After 2 minutes of heat denaturation at 94°C, intact mtDNA remains largely in a supercoiled state (being resistant to amplification by PCR), while damaged mtDNA is relaxed and can be amplified. After 6 minutes at 94°C, all mtDNA is relaxed and can be amplified (Chen et al., 2007). Thus, relative mtDNA damage was measured as the ratio of amplification efficiencies for the 83 bp amplicon [primer nucleotide sequences 5′-GATTTGGGTACCACCCAAGTATTG-3′ (16042-16064) and 5′-AATATTCAT-GGTGGCTGGCATGTA-3′ (16125-16102)] following 2 and 6 minutes of heat denaturation. Both amplifications were performed using SYBR Green JumpStart Taq ReadyMix (Sigma) in an MJ Chromo4. Cycle conditions following heat denaturation were: 30 seconds at 94°C, 45 seconds at 60°C, 45 seconds at 72°C.

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