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# Reconstitution of active telomerase in primary human foreskin fibroblasts: effects on proliferative characteristics and response to ionizing radiation

H. H. KAMPINGA<sup>†\*</sup>, M. A. W. H. VAN WAARDE-VERHAGEN<sup>†</sup>,  
A. J. VAN ASSEN-BOLT<sup>†</sup>, B. NIEUWENHUIS<sup>†</sup>, H. P. RODEMANN<sup>‡</sup>,  
K. R. PROWSE<sup>§</sup> and M. H. K. LINSKENS<sup>§</sup>

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## Abstract.

**Purpose:** Telomere shortening has been proposed to trigger senescence, and since most primary cells do not express active telomerase, reactivation of telomerase activity was proposed as a safe and non-transforming way of immortalizing cells. However, to study radiation responses, it is as yet unclear whether cells immortalized by telomerase reactivation behave in a similar manner as their parental primary cells.

**Materials and methods:** Primary human foreskin fibroblasts were transfected with the human catalytic subunit of telomerase, the reverse transcriptase (hTERT), and their growth characteristics and response to DNA damage were characterized.

**Results:** The sole expression of the human hTERT was sufficient to immortalize the human foreskin fibroblasts. With time in culture, the immortalized cells almost doubled their average telomeric length and the clonal population contained almost no post-mitotic fibroblasts anymore. Up to 300 population doublings, no alterations compared with the parental primary cells were seen in terms of clonogenic radiosensitivity, DNA double-strand break repair, radiation-induced increases in p53 and p21<sup>WAF-1,CIP-1</sup> expression, and the G1/S and G2/M cell cycle checkpoints. Moreover, mitogen-induced mitotic arrest of fibroblasts was still possible in the hTERT-immortalized clones.

**Conclusions:** Immortalizing fibroblasts by reconstitution of active telomerase seems a good, reliable manner to generate a large source of cells with a radiation damage response similar to the primary cells.

## 1. Introduction

Shortening of telomeres due to the end-replication problem is likely the cause for ageing of primary cells in culture, finally resulting in a permanent arrest of cell growth (Hayflick 1997, Shay and Wright 2000). The inability to generate relatively normal immortal cells has limited research endeavours that require long-term culturing such as, for example, the generation of stable cell lines over-expressing a desired gene. Classical ways to immortalize normal cells have used viral infections, and although of some use, these lead to alterations in the growth characteristics and to impairment of cell cycle checkpoints. This induces a transformed phenotype that interferes with the stress response of cells, which requires the activation of such checkpoints.

In stem cells and reproductive cells, telomere length can be maintained by the activity of telomerase, consisting of a catalytic subunit (telomerase reverse transcriptase, TERT) and an integral RNA component (hTR). Whereas hTR remains expressed in most cells, TERT activity is lost in somatic cells and this has been linked to replicative senescence of cells in culture. Most convincingly, this was demonstrated by the introduction of active telomerase in normal human retinal pigment epithelial (RPE) cells and foreskin fibroblasts, which greatly extended their life span *in vitro* (Bodnar *et al.* 1998) and without being associated with a transformed phenotype (Jiang *et al.* 1999, Morales *et al.* 1999). The data suggest that introduction of human TERT (hTERT) into normal cells might be a safe procedure for immortalizing cells without affecting their normal phenotype.

However, the situation is not completely clear with regard to the response to DNA-damaging agents. Given the emerging direct link between telomerase and telomeres with DNA repair, telomerase-immortalized cells might behave differently than primary cells lacking telomerase activity (Gorbunova *et al.* 2003). In some cell types, telomerase activity is induced by ionizing radiation (Leteurtre *et al.* 1997, Hande *et al.* 1998), which suggests a role for

\*Author for correspondence; e-mail: h.h.kampinga@med.rug.nl

<sup>†</sup>Department of Radiation and Stress Cell Biology, DCB, Faculty of Medical Sciences, University of Groningen, Groningen 9713 AV, the Netherlands.

<sup>‡</sup>Section of Radiobiology and Molecular Environmental Research, Department of Radiation Oncology, Eberhard-Karls-University, Tübingen, Germany.

<sup>§</sup>Department of Biochemistry, Faculty of Mathematics and Natural Sciences, University of Groningen, Groningen, the Netherlands.

telomerase in DNA repair and chromosome healing (Leteurtre *et al.* 1997). Cells from patients with ataxia telangiectasia (A-T) known to be hypersensitive to ionizing radiation display increased rates of telomeric loss (Pandita *et al.* 1995) and telomeric proteins like Pin2/TRF1 are targets for the A-T-mutated (ATM) protein in response to DNA double-strand breaks (DSB) (Kishi *et al.* 2001). Furthermore, telomere maintenance requires several proteins also involved in non-homologous end-joining (NHEJ), the main DSB repair pathway in mammalian cells (Weaver 1998, Haber 1999, Martin *et al.* 1999, Mills *et al.* 1999, Gasser 2000). In fact, ectopic hTERT expression increased the transcription of several genes, including those encoding DNA repair proteins (Sharma *et al.* 2003). In addition, telomeres might act as a reservoir or depot for proteins that can be recruited immediately when DSB arise. Moreover, telomerase might not only protect against initiating chromosomal instability and cellular senescence by preventing telomere dysfunction, but also might change the spectrum and perhaps frequency of chromosome repair (e.g. Hackett *et al.* 2001 for a review) and thus alter the response of the immortalized cells to DNA damage. Only a few recent papers have tried to address these issues directly. Data from Gorbunova *et al.* (2002) showed that expression of hTERT in human lung and foreskin fibroblasts did not affect the induction of p21<sup>WAF-1,CIP-1</sup> or p53 after DNA damage and did not alter stress-induced senescence. Similarly, stress-induced senescence of human foreskin fibroblast by ceramide, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), LY294002 or trichostatin A was not altered by hTERT transfection (Matuoka and Chen 2002). Finally, it was found that p21<sup>WAF-1,CIP-1</sup> induction, cell growth delay and induction of senescence after ultraviolet B (UV-B) or H<sub>2</sub>O<sub>2</sub> was similar in primary and hTERT-immortalized human foreskin fibroblast (De Magalhaes *et al.* 2002). To address these issues further and to ask whether telomerase-immortalized human fibroblasts have a normal response to ionizing radiation, active hTERT was expressed ectopically in primary human foreskin fibroblasts. These fibroblasts were successfully rescued from telomere shortening during cell culturing without the need of additional gene inactivations. hTERT-expressing clones could undergo at least 300 population doublings (PD). With the exception of one clonal variant, the immortalized cells showed similar responses to radiation as the parental primary fibroblasts. The present data support the idea that fibroblast immortalization by the sole activation of hTERT does not induce changes associated with a transformed phenotype and does not alter the cellular radiation response. Hence, hTERT-immortalized fibroblasts are a

reliable model for studies in primary cells that would normally be impossible due to the finite supply of such cells.

## 2. Materials and methods

### 2.1. Cell culture and transfection

Normal human foreskin fibroblasts VH25 (kindly provided by Dr. L. H. F. Mullenders, Leiden, The Netherlands) were grown as monolayers at 5% carbon dioxide in a humidified 37°C incubator in Ham's F10 with 15% foetal calf serum (FCS) (all Gibco, Paisley, UK). The medium of the hTERT-positive clones was supplemented with 20 µg ml<sup>-1</sup> hygromycin B (Boehringer Mannheim, Mannheim, Germany). GLC4 cells derived from a human small cell lung carcinoma and MCF-7 cells (both kindly provided by Dr. E. G. E. de Vries, Groningen, The Netherlands) were cultured in respectively RPMI 1640 or DMEM medium each supplemented with 10% FCS.

For transfection, VH25 cells (PD23) were transfected with either 40 µg control vector without the hTERT insert or pGRN145 plus the hTERT insert following the standard calcium phosphate method (Kriegler 1990). The pGRN145 construct (Geron Corp., Menlo Park, USA) consists of a mammalian expression vector containing the hTERT open reading frame minus the 5' and the 3' UTR under the control of the myeloproliferative sarcoma virus promoter (MPSV LTR). After selection with hygromycin B, stable clones were frozen at PD50 at -140°C. Clones expressing the empty control vector senesced at PD45. For the present study, clones K15 and K20 were characterized over time in culture until PD105 and PD300, respectively. As a control in all experiments, the primary parental VH25 fibroblasts were used between PD15 and PD35.

### 2.2. Telomeric repeat amplification protocol (TRAP) assay for telomerase

Cells were lysed in 3-[(3-chloroamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) lysis buffer (10 mM Tris-Cl, pH 7.5, 1 mM MgCl<sub>2</sub>, 1 mM *O,O'*-bis(2-aminoethyl)ethylene-glycol-*N,N,N,N'*-tetra acetic acid (EGTA), 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol), placed on ice for 30 min, and microcentrifuged at 12 000g for 30 min at 4°C. TRAP assays were performed with a telomerase substrate (TS) oligo (5'-AATCCGTCGAGCAGAGTT-3') buffer, 50 µM dNTPs, 1 U Taq polymerase and 0.4 µl [α-<sup>32</sup>P]dCTP (10 µCi µl<sup>-1</sup>, 3000 Ci mmol<sup>-1</sup>) for 15 min at room

temperature. After 10 min heating at 94°C, reverse primer (CX) (5'-CCCTTACCCTTACCCTTACCC-TAA-3') was added followed by 27 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. Half the reaction was analysed by electrophoresis in 0.6 × Tris-boric acid-EDTA (TBE) in 10% polyacrylamide non-denaturing gels and the gels were exposed to a PhosphorImager plate (Molecular Dynamics, Boston, USA). The GLC4 human lung cancer cell line was used as an internal standard.

### 2.3. Terminal restriction fragment assay (TRF)

Genomic DNA was prepared from cell pellets and digested with *HinF1/RsaI* (2 units each per  $\mu\text{g}$  DNA) and DNase-free RNase at 37°C for 16 h. After separation on 0.5% agarose gels, the gels were incubated for 16 h at 37°C with a single-stranded telomeric oligonucleotide, (TTAGGG)<sub>3</sub>, end-labelled with 50  $\mu\text{Ci}$   $\gamma$ -<sup>32</sup>P adenosine triphosphate and the TRF length was determined using a PhosphorImager.

### 2.4. Phenotypic analysis of fibroblasts

The phenotype of replicative senescence and stress-induced premature senescence has been likened by several groups to a form of post-mitotic differentiation (Rodemann *et al.* 1991, Maas-Szabowski *et al.* 1999, Alaluf *et al.* 2000, Evans *et al.* 2003). To evaluate the phenotype of the fibroblasts, samples were decoded and sent for blind analysis to one of the authors (H. P. Rodemann). The amount of potentially mitotic active fibroblasts (MF) and of post-mitotic fibroblasts (PMF) of the cell lines analysed was determined using the senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) staining procedure (Dimri *et al.* 1995, Hakenjos *et al.* 2000). The amount of SA- $\beta$ -Gal-positive mitotic fibroblasts (senescent PMF) and SA- $\beta$ -Gal-negative mitotic fibroblast cells were determined by scoring  $5 \times 10^3$  cells of each cell line. In parallel, to quantify the amount of the three subtypes of progenitor fibroblasts (MF I-III) for each cell line, colony formation assays were performed and the differentiation state of individual colonies was characterized by applying published criteria (Bayreuther *et al.* 1988, Rodemann 1989).

Using mitomycin C ( $10^{-7}$  and  $5 \times 10^{-7}$  M), cell lines were stimulated into a post-mitotic state as described by Rodemann (1989). Therefore, 2 days after seeding the different cell lines at  $1.5 \times 10^3 \text{ cm}^{-2}$ , cells were treated twice for 48 h with mitomycin C with a 48-h interval between the two mitomycin C treatments. Three days after the last treatment, cultures

were scored for the amount of MFs and senescent PMFs using SA- $\beta$ -Gal staining as described above.

### 2.5. Irradiation protocol

Cells were either irradiated with a <sup>137</sup>Cs  $\gamma$ -ray machine (IBL 637, CIS Bio International Gif/Yvette, France) at 0.9 Gy min<sup>-1</sup> or with a X-ray machine (MG 226, Philips, Eindhoven, The Netherlands) at 200 kV, 10 mA shielded with 0.4 mm Cu. Dosimetry was performed with an ionization chamber (Philips 37489/19) calibrated with a 90Sr source (Philips 2011/00).

### 2.6. Pulsed-field gel electrophoresis (PFGE)

PFGE was essentially done as described (Rosemann *et al.* 1993, Woudstra *et al.* 1996). In short, fibroblasts were irradiated in flasks with graded doses of X-rays on ice and the cells were subsequently allowed to repair DNA damage at 37°C for 4 and 24 h. After repair, the cells were trypsinized, resuspended in culture medium and mixed with an equal volume of low melting point agarose to a final concentration of  $5 \times 10^6$  cells ml<sup>-1</sup> (about  $1.75 \times 10^5$  cells/plug) and 0.8% agarose. For induction of DSB (no repair) cells were poured into agarose and irradiated in the plugs on ice. After lysis (proteinase K) and RNase treatment, the samples were run on 0.8% agarose (chromosomal grade) for 30 h at 40 V and 5400 s switch time in a BioRad CHEF DR11. The gel was stained with ethidium bromide (0.5  $\mu\text{g ml}^{-1}$  final concentration) and analysed with an image analysis system as described by Rosemann *et al.* (1993). The percentage of DNA migrating from the plugs into the lane (per cent DNA extracted) was used as a measure of radiation-induced DSBs. The repair curves were expressed as Gy-equivalent damage calculated using the induction curve as a calibration curve.

### 2.7. Cell survival

For cell survival, cells were trypsinized and irradiated on ice with doses from 0 to 6 Gy. Cells were plated in triplicate in plastic Petri dishes (94 mm) (Greiner, Frickenhausen, Germany) containing 10 ml complete growth medium. After 14 days of incubation, colonies were fixed and stained. Colonies containing more than 50 cells were counted.

### 2.8. Western blot analysis

Cells were trypsinized, resuspended in phosphate-buffered saline (PBS), lysed by addition of sodium

dodecylsulphate-polyacrylamide gel electrophoresis sample buffer and sonicated before sodium dodecylsulphate-polyacrylamide gel electrophoresis/Western blot analysis. p21<sup>WAF1,CIP-1</sup> and p53 were detected by monoclonal antibodies to p21 WAF1 (AB-1, Oncogene, Amsterdam, The Netherlands) and p53 (DO-1, Santa Cruz Biotechnology, Santa Cruz, USA), respectively. To study the effect of irradiation, cells were irradiated with 6 Gy and harvested after a 6-h incubation at 37°C. Unirradiated cells were taken as a reference. As a loading control, antibodies to  $\alpha$ -tubulin (B-5-1-2, Sigma Lwijndrecht, The Netherlands) were used.

### 2.9. Flow cytometry

For cell cycle distribution assays,  $10^6$  cells were prepared from exponentially growing cultures. After irradiation with 6 Gy, cells were trypsinized at time points between 0 and 48 h. As a control for normal diploid cells, thymocytes from a C3H mouse were used. Cells were washed with PBS/5 mM MgCl<sub>2</sub>, fixed overnight in 80% ethanol/acetone 1:1 and incubated with propidium iodide ( $10 \mu\text{g ml}^{-1}$ ) and RNase A ( $0.5 \text{ mg ml}^{-1}$ ) at 37°C before flow cytometry. Flow cytometry was carried out with a fluorescence activated cell sorter (FACS) machine (Calibur, Becton Dickinson, Franklin Lakes, USA) using the histogram analysis software ModFit (Verity Software House, Topsham, USA).

## 3. Results

### 3.1. Immortalization of fibroblasts with hTERT can occur, albeit at low success rates

Human foreskin fibroblasts were transfected after 23 population doublings (PD23) with hTERT. Hygromycin-resistant colonies (containing  $>50$  cells: PD =  $\pm 30$ ) were selected and grown on T25 flasks to reach confluency ( $10^6$  cells: PD =  $\pm 45$ ). Non-transfected VH25 fibroblasts senesced after about 40–45 PD and none of the fibroblasts transfected with the hygromycin resistance gene without hTERT reached confluency and all senesced after the first subculturing of the flasks. Of the 32 hTERT-transfected, hygromycin-resistant clones, 12 clones continued to grow until confluency after which they were passaged. After passaging the cells, nine clones either did not grow at all or did so but only very slowly. Only three clones continued to grow and did not senesce over the period they were kept in culture (3–25 months). Of these three clones, clone 19 also started to show a decrease in growth rates and was not used for further analysis. Clones K15, and especially clone K20, were used for testing whether or not fibroblast immortalization by the sole activation of hTERT induces changes associated with a transformed phenotype and alters the cellular radiation response.

First, it was confirmed that these cells indeed expressed telomerase at all PD investigated (figure 1A,

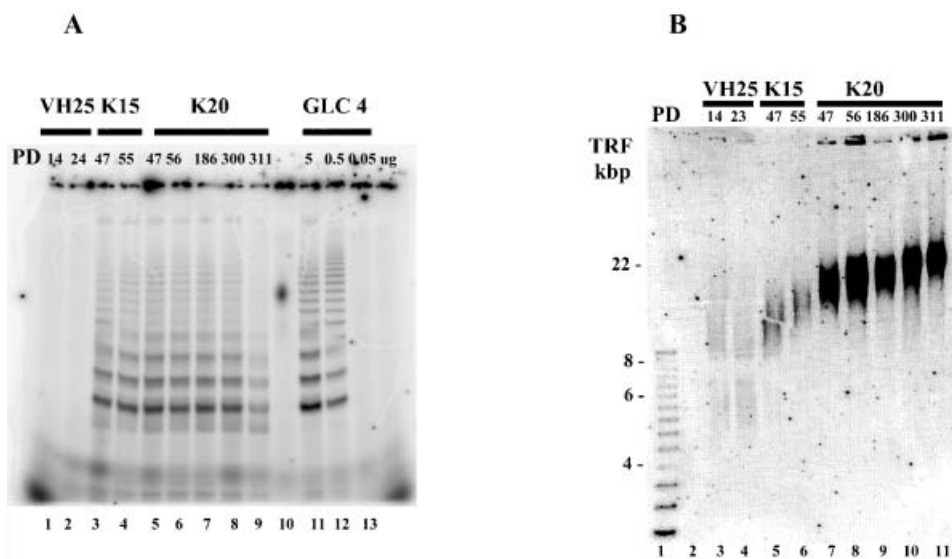


Figure 1. Telomerase activity and telomere lengths in hTERT-transfected human foreskin fibroblasts. VH25 cells at PD23 were stably transfected with hTERT. (A) Telomerase activity as determined by the TRAP assay is expressed in two transfected clones K15 (lanes 3 and 4) and K20 (lanes 5–9) at different population doublings (PD). The non-transfected, parental VH25 fibroblasts do not show any telomerase activity at any PD (lanes 1 and 2). The tumour-derived cell line GLC 4, assayed at different cell concentrations (0.05, 0.5 and 5  $\mu\text{g}$  protein), is shown as a positive control (lanes 11–13). (B) Terminal restriction fragment (TRF) length of DNA in the K15 (lanes 5 and 6) and K20 clones (lanes 7–11) is increased when compared with the parental VH25 cell strain at PD 14 and 23 (lanes 3 and 4). Lane 1 shows markers and molecular weight sizes are indicated; lane 2 is empty.

lanes 3–9) to comparable levels as in the small cell lung cancer cell line GLC4 (figure 1A, lanes 11–13) and that the parental VH25 fibroblasts lacks this activity (figure 1A, lanes 1 and 2). The expression of hTERT rescued the cells from telomere shortening during cell culturing (figure 1B). In fact, in clone K20, the TRF length initially increased rapidly from about 12 kbp in the parental, primary cells to about 17 kbp

within 40 PD. Hereafter, a gradual further increase in TRF length was seen to about 21 kbp after 300 PD (figures 1B and 2A).

### 3.2. Proliferation rates and phenotype of hTERT-immortalized fibroblasts

To estimate growth rates, cells were subcultured once a week. A T25-flask with cells was grown to near confluency ( $10^6$  cells), trypsinized and subcultured at several dilutions. After 1 week, the flask that had grown almost to confluency was chosen to continue the culture. The average number of PD was calculated for 5 consecutive weeks. As shown in figure 2B, the growth rate slightly decreased in clone 15 from PD50 to PD105. However, in the K20 clone, the number of population doublings gradually increased from about three per week to almost six per week at the later PD.

Normally, fibroblast cultures are a mixture of both mitotically active fibroblasts (MF) and non-dividing post-mitotic fibroblasts (PMF) (Bayreuter *et al.* 1988). The percentage of MF in the clones was scored at various time points in culture. Whereas the fraction of MF in the primary VH25 population declined with time in culture, an increase in the fraction of MF was seen in the K20 clone with age, with near to no PMF at the latest PD (figure 2C). When combining the data for all fibroblasts (both immortalized clones and VH25 primary cells) significant ( $p < 0.05$ ) correlations were found between the percentage of MF and the growth rate (figure 3C) between the percentage of MF and telomere length (figure 3B), and between growth rate and telomere length (figure 3A). The link between these three parameters indicates that upon immortalization, most cells no longer become post-mitotic, leading to a higher fraction of proliferating cells and hence shorter population doubling times. In parallel with the increases in growth fraction, the efficiency of single cells to form colonies (plating

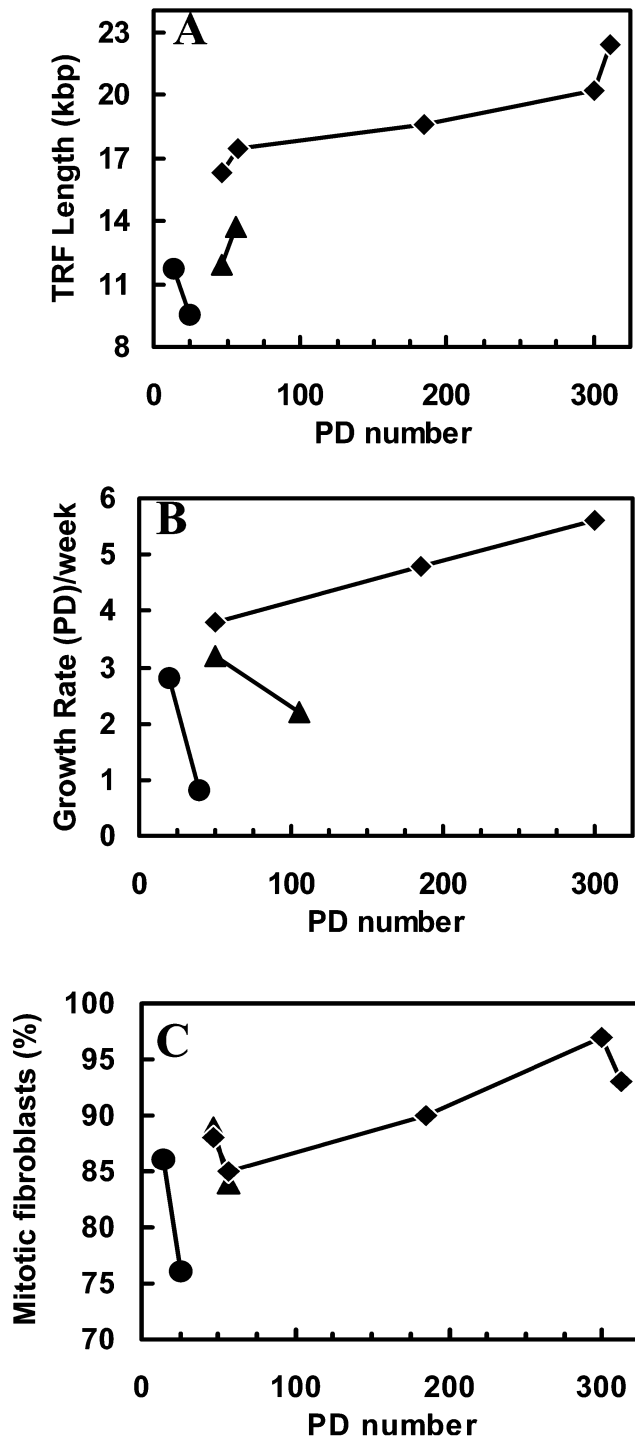


Figure 2. Effect of hTERT immortalization of fibroblasts on telomere lengths, growth rate and the fraction of proliferating cells. VH25 cells at PD23 were stably transfected with hTERT. Telomere lengths and growth characteristics of hTERT-positive clones K15 (▲) and K20 (◆) were compared with the parental VH25 fibroblasts (●) at the indicated population doublings (PD number). Non-transfected VH25 cells senesced after about 45 passages. (A) Mean telomere length, expressed as telomere restriction fragment size (TRF, kbp) plotted as a function of PD; (B) growth rates, expressed as the number of population doublings per week (PD/week), plotted as a function of PD; (C) percentage of proliferating fibroblasts in each cell population (representing the fraction of mitotic fibroblasts plotted as a function of their PD).

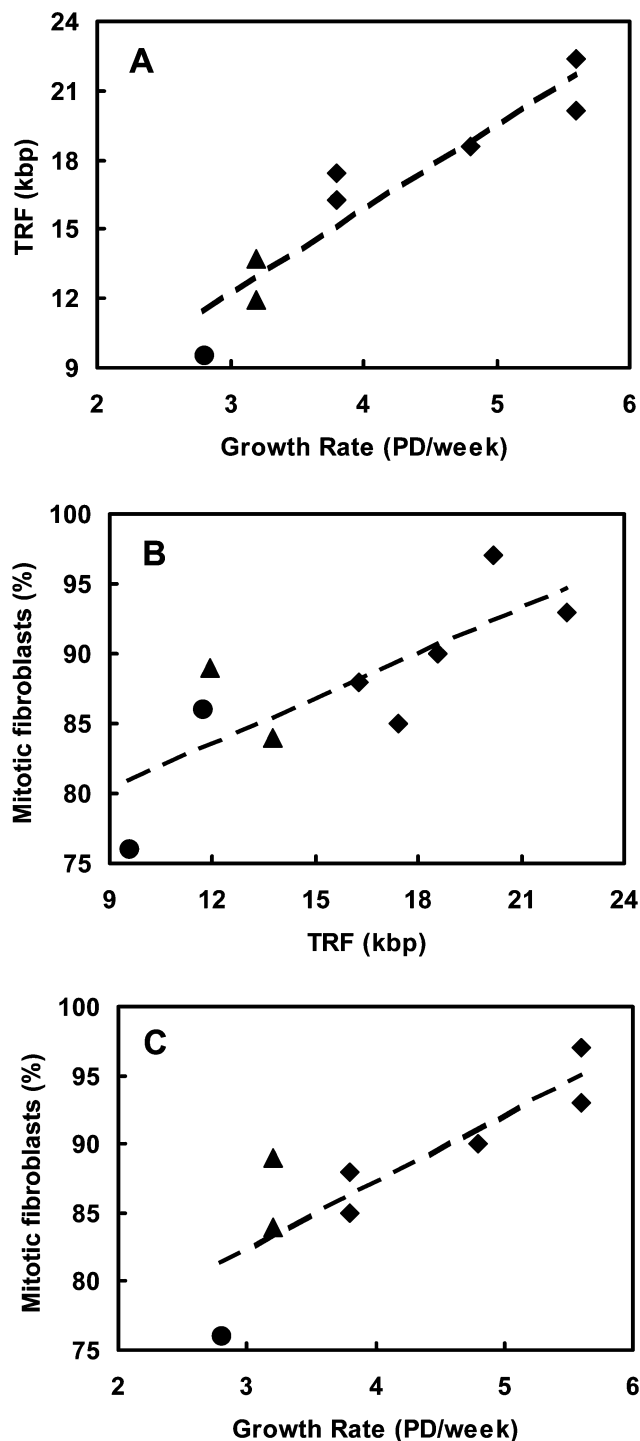


Figure 3. hTERT immortalization of fibroblasts: intercorrelations between telomere length, growth rate and the fraction of proliferating cells. Data from figure 2 for non-transfected VH25 fibroblasts (●), hTERT-positive fibroblasts clone K15 (▲) and clone K20 (◆) were pooled, and correlation plots showed significant correlations between the telomere length (TRF) and the growth rate (A:  $r^2=0.8859$ ,  $p<0.01$ ), the percentage of proliferating fibroblasts and TRF (B:  $r^2=0.6150$ ,  $p<0.05$ ), and the percentage of proliferating fibroblasts and the growth rate (C:  $r^2=0.7301$ ,  $p<0.01$ ). Data are derived from figure 2.

efficiency) increased by almost a factor of 3 (data not shown).

### 3.3. Radiation response in hTERT expressing fibroblasts

Considering the intimate relationship between telomere maintenance, DNA repair proteins and radioresponsiveness (see the Introduction), it was next tested whether hTERT-immortalized fibroblasts had an altered radiation response. All clones, irrespective of their PD number, showed the same radiation response in terms of clonogenic cell death as the parental, primary VH25 fibroblasts (figure 4) with the exception of one subclone of the K20 cells (clone PD310A) that was significantly more radioresistant (figure 4, open squares). Flow cytometric analysis showed that this clone had become tetraploid, which might have increased the tolerance of the cells to DNA damage and chromosome loss. However, yet another subclone of the K20 cells at the same PD (clone PD310C) was diploid and had an unaltered clonogenic response to radiation (figure 4, open diamonds).

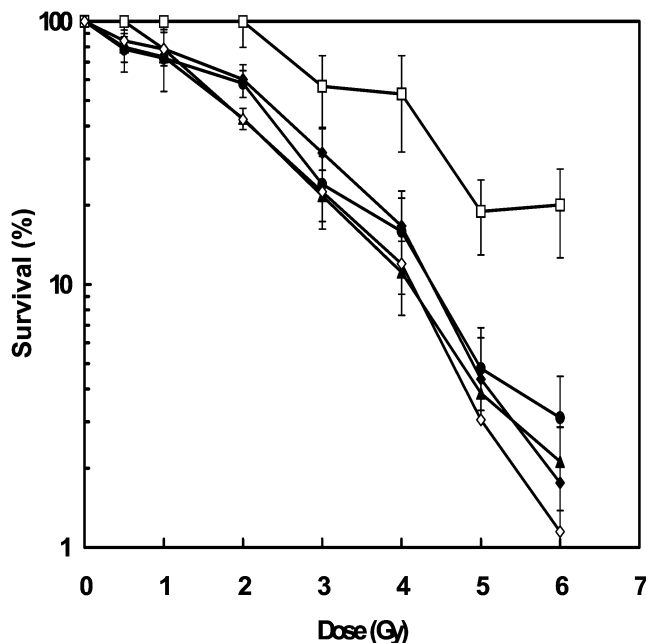


Figure 4. Radiation-induced cell death in immortalized fibroblasts. Non-transfected VH25 cells at PD20 (●), the hTERT-positive clone K15 at PD60 (▲) and the hTERT-positive clone K20 at PD60 (◆) and in two K20 subclones at PD310 (PD310A (□) and PD310C (◇)) were irradiated with graded doses of  $\gamma$ -rays. Cell survival was measured by colony formation. The radiation resistant subclone PD310A has characteristics of a transformed phenotype, like p21<sup>WAF-1,CIP-1</sup>/p53 abnormalities and tetraploidy (see also figure 6 and the text). Data points are the mean of three independent experiments; error bars indicate the standard deviation.

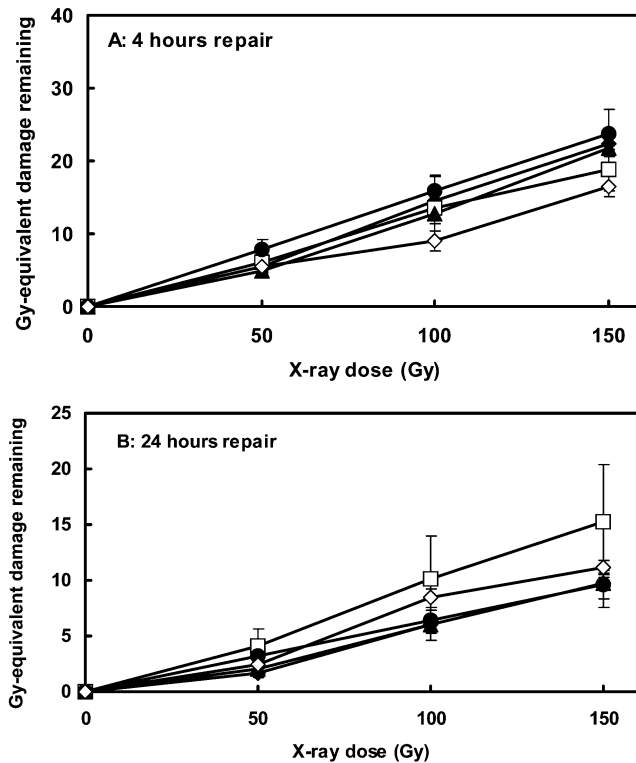


Figure 5. Rejoining of DNA double-strand breaks in immortalized fibroblasts. Non-transfected VH25 cells at PD20 (●), the hTERT-positive clones K15 at PD60 (▲) and the hTERT-positive clone K20 at PD60 (◆) and in two K20 subclones at PD310 (PD310A (□) and PD310C (◇)) were irradiated with graded doses of X-rays. The amount of DNA double-strand breaks remaining at either 4 (A) or 24 h (B) after radiation was measured. Data are the Gy-equivalent damage using the curves for initial DNA damage (no repair) as calibration curves. Data are the mean of three independent experiments; error bars indicate the standard deviation.

In parallel, the extent of DSB repair in the various clones was measured, which in mammalian cells is predominantly dependent on NHEJ (Jeggo 1997). Using pulsed-field gel electrophoresis, exclusively DSB can be measured (Blocher *et al.* 1989), although relatively high doses are required to measure residual DSB as an indicator for repair efficiency. It was found that induction of DSB was the same for all cells tested (data not shown). The level of residual DSB at 4 and 24 h after graded doses of radiation was also the same for all cells, independent of immortalization and PD (figure 5A, B). The unique tetraploid cell clone (PD310A) showed slightly elevated residual damage 24 h after radiation (figure 5B, open squares), suggesting impaired repair, despite its more radioresistant phenotype, consistent with an increased tolerance to DNA damage as a cause for its higher radioresistance. Similar to clonogenic survival, the DSB repair was unaltered in subclone PD310C, indicating that the aberrant response in PD310A is unrelated to the number of PD as such.

Next, it was tested whether or not the hTERT-immortalized cells showed normal induction of cell cycle checkpoints and arrest at the G1/S and G2/M border. Western analysis (figure 6) revealed normal p21<sup>WAF-1,CIP-1</sup> and p53 expression levels compared with VH25 fibroblasts except for the tetraploid PD310A cells in which p53 expression was elevated and p21<sup>WAF-1,CIP-1</sup> expression appeared to be down regulated. All cells showed similar elevations in p53 and p21<sup>WAF-1,CIP-1</sup> expression 6 h after irradiation with 6 Gy (figure 6).

Consistent with the faster growth rates, flow cytometric analysis showed that all immortalized cells had a higher proportion of cells in S phase

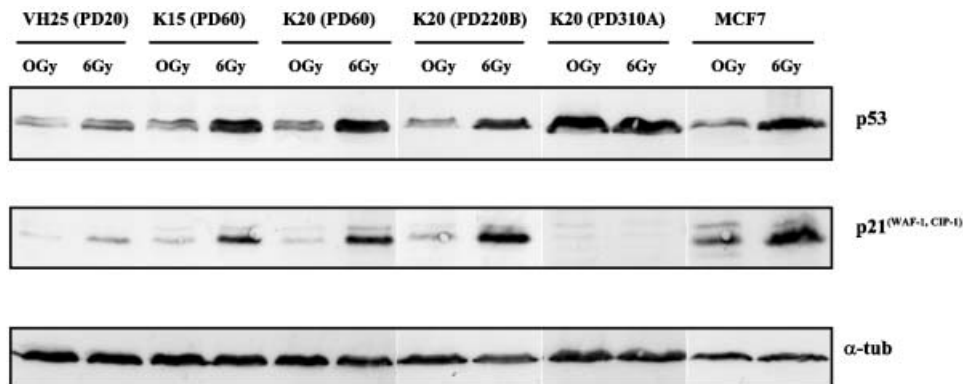


Figure 6. Expression of p53 and p21<sup>WAF-1,CIP-1</sup> in non-irradiated and irradiated hTERT-immortalized fibroblasts. The protein levels of p53 (top row) and p21<sup>WAF-1,CIP-1</sup> (middle row) were detected by Western blotting before (0 Gy) and 6 h after 6 Gy  $\gamma$ -irradiation in the non-transfected VH25 cells at PD20, the hTERT-positive clone K15 at PD60, the hTERT-positive clone K20 at PD60, and in two K20 subclones at PD220 (PD220B) and PD310 (PD310A).  $\alpha$ -Tubulin was used as a loading control (lower row). As a reference, the tumour-derived cell line MCF7 is shown.



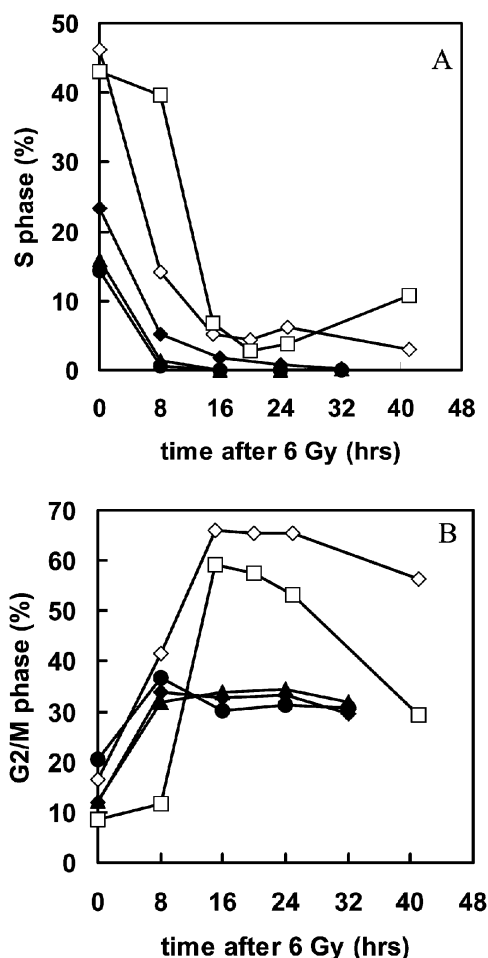


Figure 7. Radiation-induced cell cycle arrest in hTERT-immortalized fibroblasts. Exponentially growing VH25 fibroblasts at PD20 (●), the hTERT clone K15 at PD60 (▲), the hTERT-positive clone k20 at PD60 (◆), PD220 (PD220B: ◇), and PD310 (PD310A: □) were exposed to 6 Gy  $\gamma$ -irradiation. Cells were harvested and fixed at the indicated times, stained with propidium iodide, and cell cycle distribution was analysed by flow cytometry to calculate the fraction of S-phase cells (A) and G2/M cells (B). Figures show data from one typical experiment.

before irradiation (figure 7A, zero time point). In all cells, the fraction of S-phase cells declined 8 h after 6 Gy indicating proper G1/S arrest irrespective of hTERT activation (figure 7A). In addition, in all cells, the fraction of G2/M cells increased after irradiation indicating a proper G2/M arrest (figure 7B). Again, only the K20 subclone PD310A showed an aberrant response: consistent with its abnormal expression and lack of radiation-induced elevations in p53 and p21<sup>WAF-1,CIP-1</sup> levels, these cells had an 8-h delay in both the radiation-induced G1/S and G2/M cell cycle arrest.

In summary the combined data show that the activation of telomerase and increase in telomere

length have no impact on the radiation response of fibroblasts.

#### 3.4. Mitogen-induced phenotypic changes are unaltered in hTERT expressing fibroblasts

Since the hTERT-expressing cells showed a dramatic reduction in the percentage of PMF (figure 2C), it was finally asked whether or not the immortalized cells would still undergo mitotic arrest upon mitogenic stimuli (acute stress). The various fibroblasts were treated with mitomycin C, a treatment known to increase the fraction of PMF at the expense of MF in cultures of primary fibroblasts (Rodemann 1989). Consistently, a mitomycin C concentration-dependent increase in the PMF:MF ratio was induced in VH25 primary fibroblasts (figure 8A). Interestingly, the same extent of mitotic arrest could be induced by mitomycin C in the hTERT-expressing clones at PD60 and at PD190 (figure 8B, C) despite their immortalized phenotype. In the tetraploid subclone of K20 (PD300A; figure 8D), however, this response was lost and these cells did not show significant mitotic arrest after the mitogenic stimulus.

## 4. Discussion

Although it was proposed that reconstitution of telomerase activity alone would be sufficient for immortalization of human somatic cells (Bodnar *et al.* 1998, Vaziri and Benchimol 1998, Jiang *et al.* 1999, Morales *et al.* 1999), other studies have suggested that a second event is required (Counter *et al.* 1998, Kiyono *et al.* 1998, O'Hare *et al.* 2001). Part of this controversy may be due to cell type-specific features since most studies that did report immortalization by hTERT alone used primary fibroblasts (Bodnar *et al.* 1998, Vaziri and Benchimol 1998, Morales *et al.* 1999, Jiang *et al.* 1999, present study), whereas those that were not successful used either keratinocytes or mammary epithelial cells (Counter *et al.* 1998, Kiyono *et al.* 1998, Dickson *et al.* 2000, Rheinwald *et al.* 2002). However, even the situation in human fibroblasts remains not fully clear as O'Hare *et al.* (2001) failed to immortalize freshly isolated human mammary fibroblasts with hTERT alone and reported that immortalization also requires a second event. The present paper has shown that ectopic expression of hTERT in primary foreskin fibroblasts (having normal p53 and p21<sup>WAF-1,CIP-1</sup> status) can generate clones that can undergo at least 300 PDs in culture, confirming that the sole activation of telomerase activity is sufficient to immortalize primary foreskin fibroblasts. Intriguingly, all fibroblasts studies showing

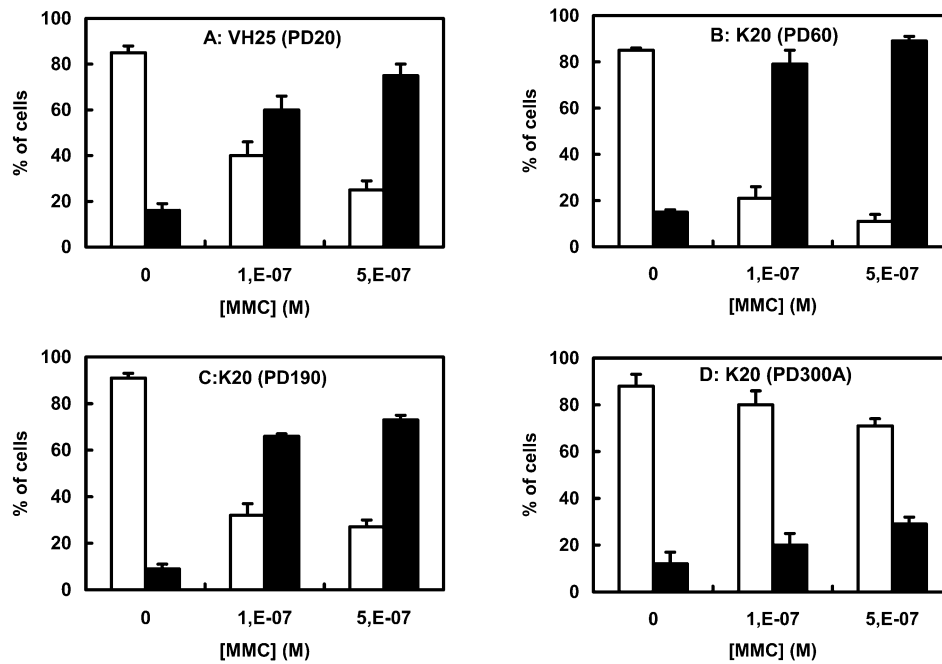


Figure 8. Mitomycin C-induced mitotic arrest in hTERT-immortalized cells. Parental VH25 fibroblasts (A) and hTERT clone K20 at PD60 (B), PD190 (C) or PD300A (D) were exposed to two concentrations of mitomycin C ( $10^{-7}$  and  $5 \times 10^{-7}$  M). Hereafter, the proportion of early mitotic fibroblasts (MFI/MFII) (white bars) and late or post-mitotic fibroblasts (MFIII/PMF) (black bars) was determined as described in the materials and methods. Figures show data from one typical experiment.

immortalization by hTERT alone used either foreskin fibroblasts (Bodnar *et al.* 1998, Morale *et al.* 1999, Jiang *et al.* 1999, present study) or neonatal fibroblasts (Vaziri and Benchimol 1998). In contrast, we could not immortalize human skin fibroblasts by the sole reconstitution of telomerase activity (data not shown). Together, these data suggest that the feasibility of immortalizing human primary cells by the sole reconstitution of telomerase activity might be highly dependent on the type of cells and even their origin, but that it is feasible without any second event in primary human foreskin fibroblasts.

A variety of data (see the Introduction) have indicated a relationship between telomeres and DNA repair. As such, one might expect that DSB repair and cellular radiosensitivity depend on telomerase activity or on telomere length. Indeed, Sharma *et al.* (2003) showed that ectopic expression of telomerase resulted in transcriptional up-regulation of several genes including those encoding the Ku80 and XRCC-3 proteins involved in NHEJ of radiation-induced DSB. In fact, the cells' expression of ectopic hTERT showed enhanced DSB repair kinetics, although end-joining *in vitro* was unaffected by hTERT suggesting that the effect must be indirect. The present study, however, found no evidence for an altered response of the hTERT-immortalized fibroblasts to ionizing irradiation. They show not only unaltered DSB repair, but also unaltered cellular

radiosensitivity in terms of clonogenicity. The present data are consistent with the findings of Vaziri *et al.* (1999) showing that telomerase activation does not affect clonogenic radiosensitivity and DNA-break rejoining measured by the comet assay. Note that the fibroblasts used by Vaziri and colleagues (until PD150) showed no increase in telomere length compared with the primary fibroblasts. In the present study, however, telomere length was almost doubled in the immortalized clone K20 grown until PD300. Despite this, these cells retained an unaltered response to ionizing radiation also showing that increasing telomere length does not confer radioresistance. Also consistent with the present findings Wood *et al.* (2001) published data on hTERT-immortalized A-T cells and found that the immortalized clones had retained their radiosensitive phenotypes, including increased sensitivity to chromosome damage after radiation and clonogenic hyper-radiosensitivity. Therefore, although shortening of telomeres below a critical length might render cells more radiosensitive (Goytisolo *et al.* 2000, Wong *et al.* 2000), increasing the length to above 'normal' does not result in radioresistance. Rubio *et al.* (2002) supported this idea even further: reversible manipulation of telomerase expression and telomere length by a retroviral Cre recombinase-hTERT system showed that telomerase activity or longer telomeres do not confer resistance to ionizing radiation, but that cells

with short telomeres can become particularly sensitive to DNA damaging agents.

Up to PD300, the hTERT-immortalized fibroblasts also showed normal radiation-induced G1/S and G2/M arrest and normal radiation-induced activation of p53 and p21<sup>WAF-1,CIP-1</sup>. This is consistent with data reported previously, albeit on cells with fewer PD (Jiang *et al.* 1999, Vaziri *et al.* 1999, Gorbunova *et al.* 2002). Furthermore, cell cycle checkpoint activation after other forms of DNA damage were unaffected by hTERT-mediated immortalization (Gorbunova *et al.* 2002, De Magalhaes *et al.* 2002). These combined data suggest that fibroblast immortalization by telomerase activation alone does not affect the response of cells to DNA damage in terms of cellular sensitivity and cell cycle response.

With increasing PD, the growth rates of the hTERT-immortalized cells increased in parallel with a shift of the population of cells within the clones towards a higher percentage of MF with almost no PMF present at the higher PD. Intriguingly, however, even after 300 PD, treatment of the hTERT expressing clones with mitomycin C still lead to a shift from MF to PMF. So, although hTERT lengthened the mean telomeric length in the fibroblasts and prevented replicative senescence during normal cell culturing (chronic stress), it did not prevent DNA damage (acute stress)-induced entry into senescence. This suggests that senescence due to end-replication problems might—at least in part—occur via separate pathways than senescence induced by DNA damage, which is in agreement with earlier observations using both ionizing radiation and/or other forms of DNA damage-inducing stresses like H<sub>2</sub>O<sub>2</sub> or UV-B (Chen *et al.* 2001, Gorbunova *et al.* 2002, De Magalhaes *et al.* 2002, Matuoka and Chen 2002). It is also consistent with recent findings by te Poele *et al.* (2002), who showed that DNA damage can induce senescence in tumour cells provided they express wild-type p53 and p21<sup>WAF-1,CIP-1</sup>. In analogy, the inability to induce mitomycin C-induced mitotic arrest in our tetraploid subclone might relate to its abnormal p53 and p21<sup>WAF-1,CIP-1</sup> expression.

In some studies (Farwell *et al.* 2000, Wang *et al.* 2000), the question was raised whether hTERT-immortalized cells could be considered as normal, non-transformed cells. Ectopic expression of hTERT enhanced the tumorigenic action of the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras (Hahn *et al.* 1999). Here the present authors found one subclonal variant that arose during prolonged cell culturing and that developed into a tetraploid clone with aberrant radiosensitivity and altered p53/p21<sup>WAF-1,CIP-1</sup> expression. Whether this single clone

represents a rare event in which the original population is overgrown by a single tetraploid cell clone or whether this is a systematic result of hTERT immortalization requires a separate study. However, other subclones from the same original K20 clone did not show abnormal phenotypes at the same late PD and none of the immortalized cells showed indications of a transformed phenotype and, for example, did not grow on soft agar (data not shown). As such, the present data confirm most published reports that human fibroblasts immortalized by telomerase activation alone show no transformed phenotypes (Bodnar *et al.* 1998, Jiang *et al.* 1999, Morales *et al.* 1999, Vaziri *et al.* 1999, Wood *et al.* 2002) and extend those observations to PD as high as 300.

In conclusion, immortalizing human (foreskin) fibroblasts by reconstitution of active telomerase seems a good, reliable manner to generate normal immortal cells with a normal radiation response (survival, DSB repair, cell cycle checkpoints, mitogen-induced differentiation). These cells provide a good source for otherwise limited research endeavours that require long-term culturing, especially such as the generation of stable cell lines over-expressing a desired gene.

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