Events in the immortalizing process of primary human mammary epithelial cells by the catalytic subunit of human telomerase

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The *in vitro* immortalization of primary human mammary epithelial (HME) cells solely by the exogenous introduction of the catalytic subunit of human telomerase (hTERT) has been achieved. Early passage hTERT-transfected HME (T-HME) cells continuously decreased the length and density of telomeres even in the presence of telomerase activity, with a significant number of cells staining positive for senescence-associated β -galactosidase (SA- β -gal). Subsequently, with the increase in cell passages, the copy number of the exogenously transfected hTERT gene and the percentage of SA- β -gal positive cells were found to decrease. Eventually, a single copy of the exogenous hTERT gene was observed in the relatively later passage T-HME cells in which telomere length was elongated and stabilized without obvious activation of endogenous hTERT and c-Myc expression. In T-HME cells, the expression of two p53 regulated genes p21^{WAF} and HDM2 increased (as in primary senescent HME

cells), and was found to be further elevated as the function of p53 was activated by treatment with DNA-damaging agents. p16^{INK4a} was shown to be significantly higher in the primary senescent HME and the early passage T-HME cells when compared with the primary presenescent HME cells, with a dramatic repression of p16^{INK4a} observed in the later passage T-HME cells. In addition, the expression of E2F1 and its transcription factor activity were found to be significantly higher in the later passage T-HME cells when compared with the earlier passage T-HME cells. Together, our results indicate that *in vitro* immortalization in HME cells may require the activation of the function of telomerase and other genetic alterations such as the spontaneous loss of p16^{INK4a} expression.

Key words: immortalization, myc, p16^{INK4a}, p53.

INTRODUCTION

After finite *in vitro* cell divisions, most of the human somatic cells face the loss of their replicative proliferating capacity and are termed as cellular senescent [1]. Among a variety of triggers for cellular senescence, the shortening of telomere that occurs in each cell division as a result of the end-replication problem of DNA polymerase is recognized as the 'mitotic clock' to control the cellular life span, at least in human somatic cells [2,3]. Critically shortened telomeres result in the loss of chromosomal integrity as manifested by chromosomal fusions, recombination and degradation, and contribute to the DNA damage responses that eventually lead to the irreversible cell-cycle arrest or cell death through the activation of p53- and Rb-dependent functions (reviewed in [4,5]). Furthermore, certain genetic alterations leading to the loss of p53 and Rb functions or the activation of telomerase have been shown to block the cellular senescence and cause cellular immortalization [5,6]. The recent molecular cloning of the catalytic subunit of human telomerase (hTERT) has provided an opportunity to investigate directly the functional role of telomerase in cellular senescence and immortalization [7,8].

Telomerase is a ribonucleoprotein complex that has a catalytic domain of reverse transcriptase and plays a key role in *de novo* synthesis and maintenance of telomeres by adding telomeric repeats on to the termini of chromosomes [9,10]. Telomerase activity is repressed in normal human somatic cells, but activated in most of the germline, immortal and tumour cells [7,11,12],

suggesting a strong correlation between the telomerase activity and the immortal phenotype. In fact, the enforced expression of hTERT was shown to be sufficient to reactivate the telomerase functions, to bypass senescent barriers and to lead *in vitro* life-span extensions in a variety of normal human premitotic cells including fibroblast, keratinocyte, retinal epithelial, mammary epithelial, mesothelial, and endothelial cells [13–25]. However, it remains unclear whether cellular immortalization can be caused by the exogenous introduction of hTERT alone, or by the combination of hTERT along with other regulatory factors, since the loss of other cellular factors such as p53, Rb and p16^{INK4a} have been found necessary for cellular immortalization in certain cell types [15,16,19,20,24,25]. In the present study, we have re-examined the functional roles of hTERT and other regulatory factors in the cellular immortalization of primary human mammary epithelial (HME) cells by reconstituting telomerase activity through the ectopic expression of exogenous hTERT.

MATERIALS AND METHODS

Cells, culture conditions and transfection of hTERT expression plasmid

Primary HME cells (Clonetics) were originally derived from a normal 51-year-old woman. HME cells were obtained at passage 9 and propagated for two additional passages for use in our

Abbreviations used: 5-aza-dC, 5-aza-2'-deoxycytidine; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HME, human mammary epithelial; hTERT, catalytic subunit of human telomerase; MEM, mammary epithelium basal medium; Mit C, mitomycin C; ODC, ornithine decarboxylase; RT, reverse transcription; SA- β -gal, senescence-associated β -galactosidase; T-HME, telomerized HME; TR, telomerase RNA component.

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present study. Primary presenescent HME-passage 11 cells were transfected with pCI-neo-hTERT expression plasmid (a gift from Dr Robert A. Weinberg, Whitehead Institute for Biomedical Research, MIT) using the electroporation method, and stable telomerized HME (T-HME) cells were obtained by antibiotic selection (G418; Calbiochem). Primary HME and T-HME cells were cultured in the mammary epithelium basal medium (MEBM; Clonetics) or MEBM plus high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen) at 37 °C with 5% CO₂ as described previously [26].

To determine the cell population doubling levels, T-HME cells were plated at a density of 5×10^5 cells/10 cm dish either in 100% MEBM or the MEBM/DMEM (1:1) mixture and transferred every 3 days. Primary and T-HME cells were stained with X-gal at pH 6.0 to detect the presence of senescence-associated β -galactosidase (SA- β -gal), a histochemical marker of replicative senescence [27].

Telomerase activity and telomere-length analysis

Telomerase activity was analysed using the TRAPeze kit (Intergen Company) following the manufacturer's instructions. Length of the telomere was determined by Southern-blot analysis using the genomic DNA isolated from the various passages of primary and T-HME cells using the Qiagen cell culture DNA kit (Qiagen). Genomic DNA was digested with *Hinf*I and *Rsa*I, resolved on a 0.8% agarose gel, blotted on to a positively charged nylon membrane and hybridized to a [γ -³²P]-end-labelled (CCCTAA)₆ telomeric repeat sequence probe [28].

RNA analysis

To determine the steady-state mRNA levels of the regulatory genes for p53 (p53, p21^{WAF}, HDM2), p16^{INK4a} (p16^{INK4a}, E2F1), telomere length [TERT, telomerase RNA component (TR), TRF1, TRF2, Tankyrase] and c-Myc [c-myc, ornithine decarboxylase (ODC)] as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in all primary and T-HME cells were tested and semi-quantitative reverse transcription (RT)-PCR was performed using reverse-transcribed cDNAs and sequence-specific primer pairs. (Information regarding primers and PCR parameters used in the RT-PCR will be provided upon request.) All RT-PCR amplifications were verified to be in linear ranges and detected by standard Southern-blot hybridization using their corresponding [α -³²P]-labelled cDNA probes prepared by RT-PCR. All image data were processed using the Eagle Eye II still video system (Stratagene) and analysed with the NIH Image software program.

Protein analysis

Total cell extracts were isolated from primary and T-HME cells using a cell lysis buffer containing 20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol and 1 × Protease Inhibitor Cocktail (Calbiochem). Cells were resuspended in cell lysis buffer and gently rocked at 4 °C for 1 h and then fractionated by centrifugation at 10000 *g* for 15 min. Supernatants were stored at -80 °C until used. The protein concentration of the cell extracts was determined by the Bio-Rad Protein Assay following the manufacturer's instructions (Bio-Rad). Cell extracts (100 μ g) were resolved by SDS/PAGE (12% gel) and

transferred on to Immobilon-P PVDF membranes (Millipore). Immunoblotting of the membranes was performed using an anti-human p53 antibody (Ab-3, 1:1000 dilution; Oncogene), an anti-mouse p21^{WAF} antibody (SX118, 1:2000 dilution; Pharmingen), an anti-human murine double minute clone 2 oncoprotein antibody (MDM2, sc-956, 1:1000 dilution; Santa Cruz Biotechnology), an anti-mouse p16^{INK4a} antibody (sc-1661, 1:250 dilution; Santa Cruz Biotechnology), an anti-human E2F1 antibody (sc-251, 1:1000 dilution; Santa Cruz Biotechnology), an anti-human c-Myc antibody (Ab-1, 1:1000 dilution; Oncogene) or an anti-chicken actin antibody (Ab-1, 1:2000 dilution; Oncogene), followed by treatment with horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham Pharmacia Biotech). All the proteins that were tested were visualized using the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Genomic DNA analysis

Genomic DNA was obtained from both primary and T-HME cells using the Genomic DNA Isolation Kit (Promega) according to the manufacturer's instructions. For genomic Southern-blot analysis, 15 μ g of DNA was digested with *Eco*RI, separated by electrophoresis, blotted and probed with an [α -³²P]-labelled full-length hTERT cDNA or a c-Myc cDNA fragment amplified by PCR. To determine changes in the copy number of the endogenous c-Myc gene in primary and various passages of T-HME cells, semi-quantitative PCR was performed using 50 ng of genomic DNA and a c-Myc-specific primer pair. The genomic c-Myc fragment was amplified using TaKaRa Ex Taq (Intergen) at 95 °C for 30 s, 65 °C for 1 min and 72 °C for 1 min for 23 cycles, which was verified to be in linear range.

Reporter-gene assay

The E2F1-Luc reporter plasmids were constructed in the pGL3 Luciferase reporter vector (Promega) using double-stranded oligonucleotides corresponding to tetramers of the E2F1 consensus-binding sequence as described previously [28]. All primary and T-HME cells were seeded at 5×10^6 cells/10 cm dish, 36 h before transfection. Transient transfection was performed using calcium phosphate precipitation and 10 μ g of promoter reporter plasmid (E2F1-Luc), which was co-transfected with 2 μ g of pcDNA 3.1 *LacZ* plasmid to normalize for efficient transfection. Reporter-gene activity was measured as described previously [28]. The relative expression levels of the luciferase reporter gene were normalized using the expression levels of GAPDH and *LacZ* genes.

DNA-damaging agent and DNA-methylation inhibitor treatments

To determine gene expression in response to DNA damage or demethylation, primary and T-HME cells were treated with a DNA-damaging agent mitomycin C (Mit C, 2 μ g/ml; Sigma) for 22 h or a DNA-methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC, 10 μ M; Sigma) for 3 days.

RESULTS

Telomere regulation in T-HME cells

We compared the phenotypic changes in T-HME cells (passage 35–103) with the presenescent passage 11 and the senescent passage 16 HME cells. As shown in Figure 1(A), primary

Figure 1 Telomere-length regulation and senescence rates in T-HME cells

(A) Representative photographs (magnification $\times 100$) show the cell phenotype in primary presenescent passage 11 and senescent passage 16 HME cells as well as passage 35 T-HME cells. (B) Genomic Southern-blot analysis was performed to determine the telomeric lengths of primary presenescent passage 11 and senescent passage 16 HME cells as well as passage 33, 53 and 63 T-HME cells. The DNA marker (in kb) appears on the left-hand side of the Figure. (C) The percentage of SA- β -gal-positive cells was determined in presenescent passage 11 HME and senescent passage 16 HME as well as passage 33, 53 and 103 T-HME cells. SA- β -gal-positive primary senescent HME cells and different passages of T-HME cells were compared with primary presenescent HME cells. Significant differences were determined by the two-tailed Student's *t* test. Bars with an * indicate a significant difference ($P < 0.01$).

presenescent HME (initially used for hTERT transfection) and T-HME cells displayed a cuboidal phenotype typical of the mammary epithelial cells. Conversely, the senescent HME cells showed an increased number of vacuoles, a flattened shape and an enlarged size, all phenotypical of replicative senescent HME cells [29]. The immortal T-HME cells were growth-arrested when grown to confluence but without evident foci formation, suggesting that these cells might not have properties common to transformed cells (results not shown).

We determined the reconstitution of telomerase activity and telomere-length regulation in T-HME cells transfected with the exogenous hTERT gene. Telomerase activity was detected in T-HME cells (passages 33 and 63) as determined by the telomeric repeat amplification protocol assay, whereas neither presenescent nor senescent primary HME cells displayed telomerase activity (results not shown). The length and density of telomere were shown to decrease in primary senescent passage 16 compared with primary presenescent passage 11 HME cells as determined by genomic Southern-blot analysis. Surprisingly, the telomere length was decreased in the early passage 33 T-HME cells, whereas it was found to be increased in the passage 53 cells and further stabilized in passage 63 cells as judged by the homogeneous sizes (Figure 1B).

As shown in Figure 1(C), approx. 90% of the senescent passage 16 HME cells were shown to be positive for SA- β -gal,

one of the histochemical markers for replicative senescence [27]. Interestingly, passage 33, 53 and 103 T-HME cells were shown to be positive for SA- β -gal (35–40, 20 and $< 2\%$ respectively) compared with the 5% positive for SA- β -gal in presenescent passage 11 HME cells. These results indicate that a cellular selection process may be occurring in the early passage T-HME cells.

Exogenous hTERT copy number and the expression of telomere-regulating factors in T-HME cells

We determined the hTERT copy number by Southern-blot analysis using the cellular T-HME genomic DNA. As shown in Figure 2(A), early passage 33 T-HME cells exhibited multiple copies of the exogenously transfected hTERT gene (in addition to endogenous hTERT) due to the pooled populations of the stable T-HME cells. Passage 53 T-HME cells showed that only three bands hybridized to the hTERT probe, whereas passage 63 and 103 cells showed only two bands (one corresponding to endogenous hTERT, and another corresponding to the exogenous hTERT gene). These results suggest that most of the early T-HME cell populations might be life-span-extended and simply selected against over time with increased cell passage, whereas only one homogeneous population of later passage T-HME cells has become immortalized.

Figure 2 Copy number of exogenous hTERT gene and expression of telomere regulating genes in T-HME cells

(A) Genomic Southern-blot analysis was used to determine the copy number of exogenously transfected hTERT in passage 33, 53, 63 and 103 T-HME cells as well as non-transfected primary presenescent passage 11 HME cells using [α - 32 P]-labelled full-length hTERT cDNA as probe. To distinguish between the two hTERT genes (endogenous and exogenous) that hybridized to the hTERT probe in passage 63 and 103 T-HME cells, DNA fragments from sites that corresponded to the two hybridization bands were gel-purified and subjected to PCR using two different primer pairs (one which amplifies the endogenous hTERT gene and another which amplifies the exogenously transfected hTERT gene). An arrow indicates a single exogenous hTERT gene fragment detected only in late passage 63 and 103 T-HME cells. *, endogenous hTERT gene fragment. (B) The mRNA expression patterns of the catalytic subunit of telomerase (TERT), TR and TRF1, TRF2 and tankyrase genes from primary presenescent passage 11 HME as well as passage 33, 53 and 63 T-HME cells were determined by semi-quantitative RT-PCR using their sequence-specific primer pairs and visualized by hybridizing the PCR products to their corresponding [α - 32 P]-labelled cDNA probes. GAPDH was used to confirm equal RNA levels for RT-PCR.

Several studies have demonstrated that telomere-regulating factors such as TR, TRF1, TRF2 and tankyrase are involved in maintaining the nature of the telomere [30–32]. To address the possible reasons for the telomere-length deregulation in early passage T-HME cells, we determined the steady-state expression levels of telomerase and telomere-regulating factors in primary HME and various passages of T-HME cells using RT-PCR. hTERT was not expressed in the primary presenescent passage 11 HME cells, whereas it was expressed in the various passages (33, 53 and 63) of T-HME cells, although its mRNA expression was found to decrease significantly in passages 53 and 63 of T-HME cells compared with their early passage 33 cell counterparts (Figure 2B). Interestingly, the dramatic down-regulation of TR expression was observed in early passage 33 T-HME cells (Figure 2B). Similar to previous results for TR-knockout mice [30], this significant loss of TR expression in the dominant T-HME cells may account for the telomere shortening in early passage 33 T-HME cells even in the presence of hTERT mRNA expression. In addition, it is plausible that the relatively low levels of telomerase activity in the early passage T-HME cells

compared with the later passage cells may be derived from heterogeneous cell populations that express functionally relevant levels of hTR (results not shown). At present, the regulation of TR expression in primary and immortal cells remains largely unclear, although previous studies [33,34] have demonstrated that the expression of the TR gene is promiscuously regulated in various passages of human and mouse cells, and is relatively abundant in immortal and transformed cell lines when compared with primary cells. Other telomere-regulating factors including TRF1, TRF2 and tankyrase were expressed in T-HME cells at levels similar to those observed in primary presenescent cells (Figure 2B). The steady-state expression levels of these genes were not changed in all different passages of T-HME cells tested, whereas the significant down-regulation of these genes was observed in the primary senescent cells (results not shown).

Expression of endogenous hTERT and c-Myc in T-HME cells

Previous studies [36] have demonstrated that the expression of endogenous hTERT and c-Myc (the later being one of the

Figure 3 Expression of endogenous hTERT mRNA and regulation of the c-Myc gene in primary HME and T-HME cells

(A) Expression of endogenous hTERT mRNA in primary presenescent passage 11 and senescent HME passage 16 as well as passage 33 and 63 T-HME cells was determined by semi-quantitative RT-PCR using a specific primer pair that amplifies the endogenous hTERT mRNA (but not exogenous hTERT). PCR products were visualized by hybridization to an [α - 32 P]-labelled TERT cDNA fragment. Human HeLa and HepG2 cell lines were used as telomerase positive controls for endogenous hTERT gene expression. GAPDH was used to confirm equal RNA levels for RT-PCR. (B) Upper panel: genomic Southern-blot analysis was used to determine the possible translocation of c-Myc in the genomic DNA of passage 33, 53 and 63 T-HME cells as well as non-transfected primary presenescent passage 11 and senescent passage 16 HME cells using an [α - 32 P]-labelled c-Myc cDNA fragment prepared by PCR as probe. Lower panel: semi-quantitative genomic DNA PCR using a sequence-specific c-Myc primer pair was used to determine the possible changes in the copy number of c-Myc in the genomic DNA of the same cells described above. Equal amounts of genomic DNA used for PCR was confirmed by amplifying GAPDH (results not shown). (C) RT-PCR: expression levels of c-Myc and ODC (one of the transcriptional target genes of c-Myc) mRNA in primary presenescent passage 11 and senescent passage 16 as well as passage 33 and 53 T-HME cells were determined by semi-quantitative RT-PCR using their sequence-specific primer pairs. PCR products were visualized by hybridization to their corresponding [α - 32 P]-labelled cDNA probes. GAPDH was used to confirm equal RNA levels for RT-PCR. Protein: expression levels of the c-Myc protein in passage 33, 63, 103 and 123 T-HME cells as well as primary presenescent passage 11 HME cells were determined by Western-blot analysis. Actin protein was used to confirm equal protein loading.

transcriptional activators for hTERT [35]) was shown to increase in later passage T-HME cells. We determined the endogenous expression levels of these genes in the early and later passages of our T-HME cells. The expression of endogenous hTERT in primary presenescent and the various passages of T-HME cells was determined by RT-PCR using a primer pair that specifically amplified the endogenous hTERT mRNA. All primary and T-HME cells that were tested did not express physiologically significant levels of endogenous hTERT mRNA when compared with HeLa and HepG2 cell lines, both known to be positive for telomerase activity (Figure 3A).

We also examined the possible alteration of the endogenous c-Myc gene in the various passages of T-HME cells. The translocation of the c-Myc gene was not detected either in primary HME or in the various passages of T-HME cells as determined by genomic Southern-blot analysis (Figure 3B, top panel). Using semi-quantitative genomic PCR, no evident alteration in the c-Myc copy number was observed in all of the primary HME and T-HME cells tested (Figure 3B, bottom panel). Furthermore, neither c-Myc nor ODC (one of the c-Myc downstream-regulated genes [37]) mRNA increased either in early or in later passage T-HME cells when compared with their presenescent cell counterparts (Figure 3C; RT-PCR analysis). Consistent with mRNA levels, c-Myc protein expression did not change from the early to the later passages of T-HME cells (Figure 3C, protein-blot analysis). Our results suggest that the activation of endogenous c-Myc and hTERT genes in later passage T-HME cells that has

been reported by Wang et al. [36] may not be a common event in T-HME cells which are long-term passaged.

Expression and functional activity of p53- and p16^{INK4a}-dependent genes in T-HME cells

To address the possibility that certain cell-cycle regulatory factors may be functionally altered in T-HME cells, we compared the steady-state expression levels of p53- and p16^{INK4a}-regulated genes, including p53, p21^{WAF}, HDM2, p16^{INK4a} and E2F1, in primary presenescent with senescent HME as well as T-HME cells.

In senescent HME and T-HME cells, p53, p21^{WAF} and HDM2 protein levels were found to be higher than in their presenescent cell counterparts (results not shown). The functional integrity of p53 and its downstream regulation were further determined by treating the cells with Mit C, a DNA-damaging agent. The expression of p21^{WAF} and HDM2 mRNA was shown to increase by 4–5-fold in both early passage 33 and relatively later passage T-HME cells (Figure 4A). The expression of p53, p21^{WAF} and HDM2 proteins was also elevated in these T-HME cells in the presence of Mit C (Figure 4B), which suggests that T-HME cells have a functionally normal p53 and downstream-regulatory pathway.

Further, we determined the expression of p16^{INK4a} in T-HME cells, since spontaneous loss of p16^{INK4a} expression has been detected frequently in life-span-extended or immortalized HME

Figure 4 p53-dependent regulation in T-HME cells

(A) Expression levels of p21^{WAF} and HDM2 mRNA in passage 33 and 123 T-HME cells grown in the presence (5 μ g/ml) or absence of Mit C, were determined by semi-quantitative RT-PCR using their sequence-specific primer pairs. PCR products were visualized by hybridization to their corresponding [α -³²P]-labelled cDNA probes and normalized to expression levels of GAPDH. The data (means $\pm$ S.D.) were plotted with relative expression levels of p21^{WAF} and HDM2 mRNA from three independent experiments. (B) Expression levels of p53, p21^{WAF} and HDM2 protein in passage 123 T-HME cells, grown in the presence (5 μ g/ml) or absence of Mit C, were determined by Western-blot analysis. Actin protein was used to confirm equal protein loading.

Figure 5 Expression of p16^{INK4a} and transcriptional activity of E2F1 in T-HME cells

(A) Expression levels of p16^{INK4a} mRNA in primary presenescent passage 11 and senescent passage 16 HME as well as passage 33 and 123 T-HME cells grown in the presence (10 μ M) or absence of the DNA methylation inhibitor 5-aza-dC were determined by semi-quantitative RT-PCR using their sequence-specific primer pairs. PCR products were visualized by hybridization to their corresponding [α -³²P]-labelled cDNA probes and normalized to expression levels of GAPDH. The data (means $\pm$ S.D.) were plotted with relative expression levels of p16^{INK4a} mRNA from three independent experiments. (B) Expression levels of p16^{INK4a} and E2F1 protein in passage 123 T-HME cells grown in the presence (10 μ M) or absence of 5-aza-dC and non-treated passage 33 T-HME cells were determined by Western-blot analysis. Actin protein was used to confirm equal protein loading. (C) Primary presenescent passage 11 and senescent passage 16 HME as well as passage 33 and 123 T-HME cells were transiently co-transfected with an E2F1 reporter plasmid (E2F1-Luc) along with pcDNA3.1 LacZ plasmid. The β -lactamase (*LacZ*) was amplified by PCR to normalize transfection efficiency. Luciferase mRNA levels determined by semi-quantitative RT-PCR indicates the transcription-factor activity of endogenous E2F1. The luciferase mRNA level in primary presenescent passage 16 HME cells was set to 1.0. Normalized values indicate the relative expression levels with standard deviation from three independent experiments. Bars with an * indicate a significant difference ($P < 0.01$).

cells [29,38]. Expression levels of $p16^{INK4a}$ mRNA were elevated in senescent HME and early passage 33 T-HME cells as compared with their presenescent cell counterparts (Figure 5A). Interestingly, $p16^{INK4a}$ mRNA was dramatically down-regulated in passage 123 T-HME cells and was detected at even lower levels when compared with presenescent HME cells (Figure 5A). Numerous reports have documented that the decrease of $p16^{INK4a}$ mRNA is the result of hypermethylation of CpG islands in its promoter [39–41]. We compared the expression levels of $p16^{INK4a}$ in presenescent and senescent HME as well as early and relatively later passage T-HME cells grown in the presence or absence of the DNA methylation inhibitor 5-aza-dC, which is known to activate $p16^{INK4a}$ mRNA expression by demethylation of the CpG islands in its promoter. The expression of $p16^{INK4a}$ was found to be elevated by 15-fold in presenescent HME cells and only slightly increased in senescent HME and early passage 33 T-HME cells grown in the presence of 5-aza-dC (Figure 5A). These results indicate that demethylation of CpG islands in the $p16^{INK4a}$ promoter might be associated with increased $p16^{INK4a}$ mRNA expression in senescent HME and the early passage T-HME cells. However, $p16^{INK4a}$ mRNA expression was not restored in the later passage 123 T-HME cells, which were treated with 5-aza-dC (Figure 5A). This indicates that the decrease in mRNA levels is not due to the methylation of CpG islands in its promoter. Similarly, Figure 5(B) shows that $p16^{INK4a}$ protein was neither detected in immortalized HME cells nor restored by 5-aza-dC treatment.

The down-regulation of $p16^{INK4a}$ is known to be associated with the increased phosphorylation of Rb protein, thereby losing its inhibitory effects on the cyclin-dependent kinases 4 and 6 (reviewed in [42,43]). The E2F1 transcription factor (normally inactivated if bound to dephosphorylated Rb) released from the phosphorylated Rb, activates its transcription factor activity, and eventually leads to the up-regulation of its downstream genes, such as E2F1 itself, and cyclins A and E. Therefore we determined the transcription-factor activity of E2F1 and its expression levels to address the functional consequences of decreased $p16^{INK4a}$ in the later passage T-HME cells. As determined by promoter-reporter gene assays, the transcription-factor activity of E2F1 was found to be elevated by 6-fold in passage 123 T-HME cells when compared with the primary senescent passage 16 HME and passage 33 T-HME cells (Figure 5C). In addition, expression of E2F1 protein was significantly up-regulated in the passage 123 T-HME cells that expressed dramatically lower levels of $p16^{INK4a}$ (Figure 5B), suggesting that the increased E2F1 transcription-factor activity in the later passage T-HME cells may result from the loss of $p16^{INK4a}$ expression.

DISCUSSION

HME, as well as several other cell types, have been shown to require the telomerase activity in conjunction with loss of $p16^{INK4a}$ - or Rb-dependent function and/or loss of p53 function for cellular immortalization [15,16,19,25,44]. Consistent with these reports, our immortalized T-HME cells exhibited telomere-length maintenance through telomerase activity (by expression of hTERT and hTR) and spontaneous loss of $p16^{INK4a}$ expression at the relatively later cell passages. Since $p16^{INK4a}$ -positive HME cells generally senesce after 10–20 population doublings [45], it is possible that the population of the primary HME cells used in the present study may have included residual $p16^{INK4a}$ -negative cells. These $p16^{INK4a}$ -negative HME cells could have been immortalized in the initial step of hTERT transfection, and eventually and advantageously overgrown and dominated in the

later passages due to the loss of a cell-cycle checkpoint ($p16^{INK4a}$ /Rb function). However, we observed no obvious increase in the proliferating capacity of the later passage immortalized T-HME cells when compared with the early passages, as judged by growth rates over the course of increased T-HME cell passage (results not shown). Furthermore, approx. 40 % of the T-HME cells exhibited positive SA- β -gal staining in early cell passages, suggesting that at least these cell populations might be continuously selected through senescence-associated cell arrest, but not through the overgrowth of a population of T-HME cells. In addition, the primary HME cells used in the present study may be $p16^{INK4a}$ positive, because both semi-quantitative RT-PCR and Western-blot analyses showed that significant levels of $p16^{INK4a}$ expression were detectable in the primary HME cells that were used. $p16^{INK4a}$ expression increased further over the course of primary and early telomerized cell passages, suggesting that the subsequent loss of $p16^{INK4a}$ expression may have occurred in later passages of T-HME cells than in the initial population of primary cells. Therefore it is more plausible that the reconstitution of telomerase activity by exogenous transfection of hTERT may allow the primary HME cells to be life-span-extended. Very rarely, these T-HME cells undergo another genetic alteration (loss of $p16^{INK4a}$), which is a critical event for cellular immortalization.

The tumour suppressor p53 plays an important functional role in maintaining the chromosomal integrity in response to the DNA damage, and the loss of p53 function increases the genomic instability that is a common event in a variety of transformed cells and tumours. The intact p53 function observed in the T-HME cells might be allied with the senescence-associated selection observed in the early passage T-HME cell populations that may have lost the telomere-length maintenance (Figures 1B and 1C). In contrast, it is possible that the p53 function in T-HME cells immortalized at later passages may be involved in the maintenance of an immortal phenotype by preventing immortal cells from undergoing tumourigenic conversion via the inhibition of other genetic alterations.

Interestingly, the T-HME cells grown in a 1:1 mixture of MEBM with high-glucose DMEM exhibited significantly higher cell division rates (an average of 0.93 population doublings) compared with an average of 0.78 population doublings for the same cells grown in 100 % MEBM (results not shown). Conversely, the growth rates for primary HME cells decreased when grown in a 1:1 mixture of MEBM and DMEM when compared with the cells grown in 100 % MEBM (results not shown). This observation suggests that T-HME cells may have different growth factor requirements for *in vitro* proliferation. It is possible that either the increased mitogenic effects of growth factors or the increased desensitization of receptor-mediated signal transduction (e.g. increased growth-factor receptor internalization for degradation) or the possible additional genetic alteration (e.g. activation of AKT) could relieve the observed exogenous growth-factor dependence [46].

In conclusion, we report that when telomerase activity was reconstituted in presenescent HME cells by the ectopic expression of exogenous hTERT, the *in vitro* life span was extended in the presence of functional p53- and $p16^{INK4a}$ /Rb-dependent regulation. However, despite hTERT expression, T-HME cells were found to reach the senescent barriers eventually, possibly due to either deregulated telomere-length maintenance by loss of TR expression or functional p53- and $p16^{INK4a}$ /Rb-dependent regulation. Nevertheless, the homogeneous population of immortal cells observed in the later passages of T-HME cells was quite possibly the result of clonal selection over the course of cell passage.

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REFERENCES

- Hayflick, L. and Moorehead, P. S. (1961) The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **35**, 585–621
- Sedivy, J. M. (1998) Can ends justify the means?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9078–9081
- Aviv, A. and Harley, C. B. (2001) How long should telomeres be? *Curr. Hypertens. Rep.* **3**, 145–151
- Greider, C. W. (1996) Telomere length regulation. *Annu. Rev. Biochem.* **65**, 337–365
- Sherr, C. J. and DePinho, R. A. (2000) Cellular senescence: mitotic clock or culture shock? *Cell (Cambridge Mass.)* **102**, 407–410
- Campisi, J. (2001) From cells to organisms: can we learn about aging from cells in culture? *Exp. Gerontol.* **36**, 607–618
- Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q. et al. (1997) hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell (Cambridge Mass.)* **90**, 785–795
- Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Linger, J., Harley, C. B. and Cech, T. R. (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science* **227**, 955–959
- Greider, C. W. and Blackburn, E. H. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature (London)* **337**, 331–337
- Yu, G.-L., Bradley, J. D., Attardi, L. D. and Blackburn, E. H. (1990) *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature (London)* **344**, 126–130
- Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L. and Shay, J. W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015
- Venkatesan, R. N. and Price, C. (1998) Telomerase expression in chickens: constitutive activity in somatic tissues and down-regulation in culture. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14763–14768
- Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Temer, V. M., Holt, S. E., Bodnar, A., Lichtsteiner, S., Kim, N., Trager, J. B. et al. (1997) Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. *Nat. Genet.* **17**, 498–502
- Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S. and Wright, W. E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352
- Counter, C. M., Hahn, W. C., Wei, W., Caddle, S., Beijersbergen, R. L., Landsdorp, P. M., Sedivy, J. M. and Weinberg, R. A. (1998) Dissociation among *in vitro* telomerase activity, telomere maintenance, and cellular immortalization. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14723–14728
- Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A. and Klingelutz, A. J. (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature (London)* **396**, 84–88
- Nakayama, J.-I., Tahara, H., Tahara, E., Saito, M., Ito, K., Nakamura, H., Nakanishi, T., Tahara, N., Ide, T. and Ishikawa, F. (1998) Telomerase activation by hTERT in human normal fibroblasts and hepatocellular carcinomas. *Nat. Genet.* **18**, 65–68
- Vaziri, H. and Benchimol, S. (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol.* **8**, 279–282
- Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W. and Weinberg, R. A. (1999) Creation of human tumour cells with defined genetic elements. *Nature (London)* **400**, 464–468
- Halvorsen, T. L., Leibowitz, G. and Levine, F. (1999) Telomerase activity is sufficient to allow transformed cells to escape from crisis. *Mol. Cell. Biol.* **19**, 1864–1870
- Jiang, X.-R., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A. G., Wahl, G. M., Tlsty, T. D. et al. (1999) Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat. Genet.* **21**, 111–114
- Morales, C. P., Holt, S. E., Ouellette, M., Kaur, J. K., Yan, Y., Wilson, K. S., White, M. A., Wright, W. E. and Shay, J. W. (1999) Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat. Genet.* **21**, 115–118
- Yang, J., Chang, E., Cherry, A. M., Bangs, C. D., Oei, Y., Bodnar, A., Bronstein, A., Chiu, C.-P. and Herron, G. S. (1999) Human endothelial cell life extension by telomerase expression. *J. Biol. Chem.* **274**, 26141–26148
- Zhu, J., Wang, H., Bishop, J. M. and Blackburn, E. H. (1999) Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3723–3728
- Dickinson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., Louis, D. N., Li, F. P. and Rheinwald, J. G. (2000) Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol. Cell. Biol.* **20**, 1436–1447
- Stampfer, M. R. (1985) Isolation and growth of human mammary epithelial cells. *J. Tissue Culture Methods* **9**, 107–115
- Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, D., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O. et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9363–9367
- Kim, H., You, S., Kim, I.-J., Foster, L. K., Farris, J., Ambady, S., Ponce de León, F. A. and Foster, D. N. (2001) Alterations in p53 and E2F-1 function common to immortalized chicken embryo fibroblasts. *Oncogene* **20**, 2671–2682
- Romanov, S. R., Kozakiewicz, B. K., Holst, C. R., Stampfer, M. R., Haupt, L. M. and Tlsty, T. D. (2001) Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. *Nature (London)* **409**, 633–637
- Blasco, M. A., Lee, H. W., Hande, M. P., Samper, E., Lansdorp, P. M., DePinho, R. A. and Greider, C. W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell (Cambridge Mass.)* **91**, 25–34
- Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M. R., Schnapp, G. and de Lange, T. (2000) Control of human telomere length by TRF1 and TRF2. *Mol. Cell. Biol.* **20**, 1659–1668
- Smith, S., Gariat, I., Schmitt, A. and de Lange, T. (1998) Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* **282**, 1484–1487
- Blasco, M. A., Rizen, M., Greider, C. W. and Hanahan, D. (1996) Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nat. Genet.* **12**, 200–204
- Kim, H., You, S., Farris, J., Kong, B.-W., Christman, S. A., Foster, L. K. and Foster, D. N. (2002) Expression profiles of p53, p16^{INK4a} and telomere regulating genes in the replicative senescent human, mouse and chicken fibroblast cells. *Exp. Cell Res.* **272**, 199–208
- Wang, J., Xie, L. Y., Allan, S., Beach, D. and Hannon, G. J. (1998) Myc activates telomerase. *Genes Dev.* **12**, 1769–1774
- Wang, J., Hannon, G. J. and Beach, D. H. (2000) Risky immortalization by telomerase. *Nature (London)* **405**, 755–756
- Bello-Fernandez, C., Packman, G. and Cleveland, J. L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7804–7808
- Stampfer, M. R., Bodnar, A., Garbe, J., Wong, M., Pan, A., Villeponteau, B. and Yaswen, P. (1997) Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. *Mol. Biol. Cell* **8**, 2391–2405
- Bender, C. M., Pao, M. M. and Jones, P. A. (1998) Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res.* **58**, 95–101
- Foster, S. A., Wong, D. J., Barrett, M. T. and Galloway, D. A. (1998) Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol. Cell. Biol.* **18**, 1793–1801
- Robertson, K. D. and Jones, P. A. (1998) The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol. Cell. Biol.* **18**, 6457–6473
- Sherr, C. J. (1996) Cancer cell cycle. *Science* **274**, 1672–1677
- Dyson, N. (1998) The regulation of E2F by pRB-family proteins. *Genes Dev.* **12**, 2245–2262
- Elenbaas, B., Spirio, L., Koerner, F., Fleming, M. D., Zimonjic, D. B., Donaher, J. L., Popescu, N. C., Hahn, W. C. and Weinberg, R. A. (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev.* **15**, 50–65
- Stampfer, M. R., Garbe, J., Levine, G., Lichtsteiner, S., Vassero, A. and Yaswen, P. (2001) Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor β growth inhibition in p16^{INK4a}(-) human mammary epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4498–4503
- DiRenzo, J., Signoretti, S., Nakamura, N., Rivera-Gonzalez, R., Sellers, W., Loda, M. and Brown, M. (2002) Growth factor requirements and basal phenotype of an immortalized mammary epithelial cell line. *Cancer Res.* **62**, 89–98

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