MS. #10592 revised

Comparison of Human Mammary Epithelial Cells Immortalized by Simian Virus 40 T-Antigen or by the Telomerase Catalytic Subunit

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Keywords: hTERT, SV40-TAg, human mammary epithelial cells, immortalization, differentiation

Running title: Immortalized mammary epithelial cells

Abstract

We directly compared two methods of immortalizing human mammary epithelial cells (HMECs). Cells were transfected with an expression plasmid either for hTERT, the catalytic subunit of telomerase, or for the simian virus 40 (SV40) early region genes. Under standard culture conditions, HMECs were not immortalized by hTERT unless they had spontaneously ceased expression of the $p16^{INK4a}$ tumor suppressor gene. Untransfected HMECs had low levels of telomerase expression, and immortalization by both methods was associated with an increase in telomerase activity and prevention of telomere shortening. SV40-induced immortalization was accompanied by aberrant differentiation, loss of DNA damage response, karyotypic instability and, in some cases, tumorigenicity. hTERT-immortalized cells had fewer karyotypic changes, but had intact DNA damage responses, and features of normal differentiation. Although SV40-immortalized cells are useful for studies of carcinogenesis, hTERT-immortalized cells retain more properties of normal cells.

Introduction

Cultured human mammary epithelial cells (HMECs) are an important *in vitro* model system for normal mammary epithelium. However, the finite proliferative capacity of normal cells (Hayflick and Moorhead, 1961) limits the number of cells that can be grown from normal tissues. To overcome this limitation, HMECs have been immortalized by a variety of methods. These include exposure to chemical carcinogens such as benzo(a)pyrene (Stampfer and Bartley, 1985), transduction with DNA tumor viral oncogenes (Band *et al.*, 1990; Band *et al.*, 1991; Bartek *et al.*, 1991), mutant p53 (Cao *et al.*, 1997; Gao *et al.*, 1996; Gollahon and Shay, 1996), or the *ZNF217* gene (Nonet *et al.*, 2001), gamma irradiation (Wazer *et al.*, 1994), and the induction of telomerase activity (Elenbaas *et al.*, 2001; Farwell *et al.*, 2000; Kiyono *et al.*, 1998; Ramirez *et al.*, 2001; Stampfer *et al.*, 2001).

Under standard culture conditions, HMECs have an unusual pattern of growth that has not been observed for any other cell type. Primary HMECs proliferate for a small number of population doublings (PD) before entering a growth arrest phase, termed "selection" where the cells exhibit the large, flattened morphology characteristic of senescence (Foster and Galloway, 1996; Stampfer, 1985; Stampfer and Bartley, 1985). These arrested cell populations frequently give rise to outgrowths of small, rapidly dividing cells, termed post-selection HMECs. Post-selection HMECs have low or undetectable levels of $p16^{INK4a}$ expression, associated with hypermethylation of CpG islands in the $p16^{INK4a}$ gene (Brenner *et al.*, 1998; Foster *et al.*, 1998; Huschtscha *et al.*, 1998). These cells proliferate for up to 40 or 50 PDs before eventually undergoing a second growth arrest that has some features of senescence, and was recently called "agonescence" (Romanov *et al.*, 2001).

The telomere shortening occurring with each division of normal somatic cells may eventually trigger the onset of senescence (Harley, 1991; Olovnikov, 1973). Telomeres consist of repetitive DNA ([TTAGGG]n in vertebrates) at the ends of chromosomes which,

together with specific binding proteins, act as protective cap structures (Blackburn, 1991). In cells of the germ line, telomere shortening is prevented by telomerase (Greider and Blackburn, 1985), a holoenzyme complex that contains a number of subunits including an RNA template molecule and a catalytic subunit (TElomerase Reverse Transcriptase; TERT). Telomerase adds TTAGGG repeats to telomeres by reverse transcribing the RNA template and thus compensates for the loss of telomeric DNA associated with normal cell division (Nugent and Lundblad, 1998). Most normal human cells are telomerase-negative, but telomerase activity can be induced by expression of exogenous hTERT (Weinrich *et al.*, 1997). For some types of cells, expression of exogenous hTERT is sufficient for immortalization (Jiang *et al.*, 1999; Morales *et al.*, 1999; Vaziri *et al.*, 1999).

Immortalization of cells transformed with DNA tumor virus oncogenes is also associated with activation of a telomere maintenance mechanism, usually telomerase (Counter *et al.*, 1992). Transformation by such oncogenes depends on inactivation of the p53 and Rb tumor suppressor proteins (reviewed in (Bryan and Reddel, 1994)). Immortalization also requires additional, as yet unidentified, changes within the host cell genome. These additional changes occur in a minority of cells expressing the viral oncogenes (ranging from 1 in 10^5 to 1 in 10^9 cells) but, in every case, these changes result in the activation of a telomere maintenance mechanism (Colgin and Reddel, 1999).

Expression of viral oncogenes and treatment with DNA damaging agents has been shown to cause genotypic and phenotypic changes (reviewed in (Bryan and Reddel, 1994)). Analysis of cells immortalized by exogenous telomerase suggests that they are much closer to normal (Jiang *et al.*, 1999; Morales *et al.*, 1999; Vaziri *et al.*, 1999; Yang *et al.*, 1999). However, it has previously been shown that HMECs grown under standard culture conditions require loss of $p16^{INK4a}$ expression in addition to expression of hTERT for immortalization, and this was associated with various abnormalities (Kiyono *et al.*, 1998). We therefore

addressed the question of whether hTERT-induced immortalization of HMECs results in cell lines that are less abnormal than SV40-TAg immortalized cells. To do this we immortalized the same HMEC cell strain with either the SV40 oncogenes or expression of exogenous hTERT. This is the first direct comparison of these immortalization techniques, and showed that hTERT expression resulted in fewer karyotypic and phenotypic alterations than did expression of the SV40 oncogenes. The hTERT-transduced cells exhibited normal DNA damage responses, including expression of p53 and $p21^{CIP1/WAF1}$, and retained many differentiated features of normal HMECs, but we confirmed that immortalization by hTERT under these culture conditions was dependent on prior loss of $p16^{INK4a}$ expression. Furthermore, these cells were not karyotypically normal, although they were less abnormal than the SV40-immortalized lines. The latter had lost DNA damage response and displayed aberrant differentiation, and some of these lines were tumorigenic in athymic nude mice.

Results

Cell Transfection and Lifespan Extension

Pre- and post-selection HMECs (Bre-80 cells) were transfected with the expression plasmid, pCI-hTERT, encoding the telomerase catalytic subunit, or the pCI-Neo control plasmid; drug selection for the neomycin resistance marker was not used, however. hTERT transfection led to immortalization of post-selection HMECs without any noticeable period of growth arrest (Figure 1). In all cases hTERT expressing cells grew out of a background of senescent cells (Figure 1 Inset). Transfection with pCI-hTERT did not affect the lifespan of pre-selection HMECs (data not shown). Four independent hTERT-immortalized mass cultures obtained from three experiments were designated B80-TERT1, B80-TERT2, B80-TERT3a, and B80-TERT3b. B80-TERT3a and B80-TERT3b were derived from the same initial population of Bre-80 post-selection cells. The pCI-Neo transfected post-selection control HMECs acquired

a senescent phenotype 3-11 PD after transfection (Figure 1).

In contrast, when post-selection Bre-80 cells were transfected with an expression plasmid containing the SV40 early region, pRSV-T, T Antigen (TAg)-expressing cells were progressively diluted out of the cultures as previously reported (Huschtscha *et al.*, 2001). Briefly, expression of TAg was observed to lead to a changed cellular morphology, growth arrest and apoptosis. Similarly, post-selection HMECs expressing exogenous $p16^{INK4a}$ or endogenous $p16^{INK4a}$ induced by the demethylating agent 5-azacytidine, also arrested with TAg expression. As discussed elsewhere (Huschtscha *et al.*, 2001), this result suggests that there are genetic or epigenetic changes that occur in post-selection HMECs in addition to loss of $p16^{INK4a}$ expression that inhibit lifespan extension induced by TAg. Consequently, immortalization of post-selection HMECs by TAg was a very rare event, occurring only once in ten separate experiments (Huschtscha *et al.*, 2001). TAg expression, however, did transform pre-selection cells at a high frequency. Seven of nine such cultures became immortalized, one without crisis, and six of them after a period of culture crisis lasting 65 to 215 days (Table 1). These cells were compared with the hTERT-immortalized HMECs.

The growth rate of the hTERT HMECs was quite slow directly after transfection. This was primarily due to inhibitory effects of the transfection reagents. Like the control cells, the majority of cells within the hTERT-transfected populations became senescent within 3 to 11 PD of transfection, before the stably transfected, hTERT-expressing minority subpopulation overgrew the cultures. Differences in growth rates (or other characteristics) among the hTERT lines could possibly be explained by different plasmid integration sites. The growth rates of all of the hTERT- and most of the TAg-immortalized lines increased with increasing PDs (Table 1); this is suggestive of clonal evolution within each line. B80-T6 immortalized cells retained an essentially constant growth rate. Although the growth rate of half of the post-crisis immortal cell lines was observed to be increased when compared to pre-crisis

cells, B80-T5, B80-T9 and B80-T18 showed slower initial post-crisis growth rates: thus escape from crisis and immortalization is not necessarily associated with increased growth rate.

Within 1-2 PDs after escape from crisis, B80-T5, B80-T6, B80-T8, B80-T9 and B80-T18 TAg-immortalized cultures ceased dividing in the standard serum-free MCDB 170 medium and required a change in medium composition to allow continued growth. Various alternative media were tested to identify one that was able to permit continued growth. B80-T5 grew best in RPMI 1640 plus 10% fetal bovine serum (FBS), while the other cell lines grew in a mixture of MCDB 170 and RPMI 1640 medium plus 10% FBS. Interestingly, B80-TERT1 cells also showed a change in medium requirements at approximately PD 93.

Telomerase Activity and Telomere Length

Analysis of telomerase activity by the TRAP assay (Figure 2a) showed that the untransfected pre- and post-selection HMECs contained very low, but detectable levels of telomerase activity. The hTERT-transfected cells and the post-crisis TAg-transfected cells all had levels of telomerase activity that were substantially higher than their untransfected counterparts. RT-PCR analysis, using primers designed to detect transcripts from the pCI-hTERT plasmid, showed that the hTERT-transfected cultures were all expressing exogenous hTERT (data not shown).

TRF analysis (Figure 2b) indicated that most of the SV40-TAg- and hTERT-immortalized cultures had TRF lengths similar to those of telomerase-positive cancer cell lines (e.g., HeLa, Figure 2b, c). The telomere lengths of the immortal cell lines were generally shorter than those of the untransfected HMECs. Additional TRF analyses (Figure 2c, and data not shown) showed that the telomere lengths of all of the cell lines were stabilized, with some fluctuation around an apparent set-point telomere length that differed

for each cell line, as has been described previously (Sprung *et al.*, 1999). In contrast to the other cell lines, B80-TERT3b had a major TRF band with a mean length of approximately 12 kilobases (kb), but also had a TRF smear up to and beyond the 49 kb marker on the pulsed-field gel. Telomeres of this length have previously been seen in ALT cells, which are characterized by the absence of telomerase activity, heterogeneous telomere lengths ranging from very short to extremely long, and the presence of ALT-associated PML bodies (APBs) (Yeager *et al.*, 1999). APBs have been found only in ALT cell lines and tumors and are PML nuclear domains containing large aggregations of telomeric DNA and associated proteins (Yeager *et al.*, 1999). The B80-TERT3b cells were therefore indirectly immunostained with antibodies against PML and the TRF1 and TRF2 telomere-specific proteins, to test for APBs, but none were detected. We considered the possibility that the B80-TERT3b cell line may contain a mixture of telomerase-positive and ALT (telomerase-negative) sub-populations that account for it being both telomerase-positive and also having a small subset of telomeres that are long and heterogeneous. To test this, B80-TERT3b was subcloned by limiting dilution, and seven individual subclones were cultured separately. Telomerase activity was detected in each of these subclones by TRAP assay (data not shown). Thus, no ALT B80-TERT3b subclones were found, although each subclone had a telomere length phenotype that was similar to the parental cells (Figure 2c).

Expression of Tumor Suppressor Proteins

HMEC cultures transfected with pCI-hTERT or pRSV-T were analyzed for expression of tumor suppressor gene products that are commonly affected in immortalized cells. Western blotting (Figure 3) was used to detect expression of $p110^{Rb}$, $p16^{INK4a}$, p53 and $p21^{CIP1/WAF1}$ in the four hTERT cultures and four SV40-TAg cultures. All cell lines expressed $p110^{Rb}$ (Figure 3a). As expected, increased levels of hyper-phosphorylated $p110^{Rb}$ were observed in those

cells lines devoid of $p16^{INK4a}$ expression. $p16^{INK4a}$ was detected in the SV40-TAg cultures, but not in the hTERT cells (Figure 3b), consistent with their origin from HMECs that were transfected pre-selection and post-selection, respectively. Western analysis showed an increase in p53 levels in untransfected post-selection HMECs compared to their pre-selection counterparts (Figure 3c), as has previously been observed (Romanov *et al.*, 2001). p53 levels in B80-TERT2 and B80-TERT3a were similar to those in untransfected post-selection cells, however the levels in B80-TERT1 and B80-TERT3b were reduced. p53 levels were increased in all of the SV40-TAg cell lines, consistent with previous observations of HMECs (Garbe *et al.*, 1999). $p21^{WAF1/CIP1}$ protein levels in B80-TERT2 were similar to those observed in untransfected post-selection HMEC cells (Figure 3d), although there was a decrease in $p21^{WAF1/CIP1}$ expression observed in the other three hTERT cell lines (Figure 3d). All of the SV40-TAg cells showed lower levels of $p21^{WAF1/CIP1}$ expression (Figure 3d).

DNA Damage Response

To assess response to DNA damage, each cell line was exposed to the DNA intercalating agent, actinomycin D. Western analysis showed that after exposure to 7.5 nM actinomycin D for 24 h, p53 levels increased in untransfected post-selection and hTERT-immortalized HMECs (Figure 4a). Following exposure to actinomycin D, $p21^{WAF1/CIP1}$ expression levels also increased in each of the hTERT cell lines (Figure 4b). p53 expression did not discernibly change in either of the SV40-TAg cell lines tested, although there was a slight increase in $p21^{WAF1/CIP1}$ expression. Flow cytometry of propidium iodide-stained cells was performed to assess whether the actinomycin D treatment led to G_1 cell cycle arrest. Cell cycle analysis showed that the treated hTERT cells accumulated in the G_1 phase of the cell cycle with a concomitant decrease in the percentage of S phase cells (Figure 4c). There was no significant change in the cell cycle parameters of SV40-TAg cells treated with actinomycin D.

Differentiation status

HMEC hTERT and SV40-TAg cells were assessed by morphology and immunocytochemical analysis of keratin proteins and polymorphic epithelial mucins in comparison to untransfected HMECs (Figure 5a, b). At low cell density (Figure 5c) the hTERT immortalized HMECs resemble untransfected pre- and post-selection HMECs, although they tend to aggregate and grow as discrete colonies. At confluence, however, the hTERT HMECs generally have a smaller cytoplasmic volume, and become tightly packed. B80-TERT1 cells (Figure 5d) often contain small vacuoles in the cytoplasm that are not observed in the other cell lines. B80-TERT2 (Figure 5e) and B80-TERT3a (Figure 5f) maintain a phenotype that most closely resembles post-selection HMECs. B80-TERT3b cells (Figure 5g) resemble the other hTERT cell lines at low cell density, but at confluence the cells often form radial configurations which may have a central dome (Figure 5h). All of the hTERT HMEC lines are generally more uniform in shape at confluence than untransfected normal pre- and post-selection Bre-80 cells. Cellular morphologies of the SV40-TAg HMEC lines are much more varied (Figure 5i - l). B80-T8 cultures (Figure 5j) contain many floating cells and cellular debris in the medium while maintaining a high mitotic index, whereas B80-T17 cells (Figure 5k) maintain a morphology more similar to that observed in post-selection and hTERT-immortalized HMECs. The hTERT and SV40-TAg immortalized cells are able to grow to a high level of confluence without any diminution in their growth rate in subsequent passages. Confluence of untransfected HMECs, however, leads to slower growth in subsequent passages.

The untransfected post-selection, hTERT- and SV40-TAg-immortalized cells showed similar levels of expression for seven of the ten keratins assessed (keratins 1, 5, 6, 7, 10, 16 and 18; data not shown). Keratins 14 and 19 showed minimal changes in expression in the

immortal cell lines, however keratin 8 expression was upregulated in the SV40-TAg cells (Table 2). Polymorphic epithelial mucin markers were also observed to be upregulated in the SV40-TAg cells (Table 2), although they were expressed at normal levels in the hTERT cells. The upregulation of keratin 8 and the polymorphic epithelial mucin markers, generally highly expressed in luminal epithelial cells, indicate that SV40-TAg induced immortalization may induce trans-differentiation of HMECs to a somewhat more luminal phenotype.

Karyotypic Analysis

hTERT and SV40-TAg HMECs were analyzed by QFQ and GTG banding (Tables 3 and 4). Cytogenetic analysis revealed a large number of chromosomal aberrations in the SV40-TAg cells, with a moderate number in the hTERT cells (Table 3). SV40-TAg cells were observed to contain an abnormal number of chromosomes, leading to either a hypodiploid karyotype (B80-T8 and -T17) or an aneuploid karyotype (>60 chromosomes; B80-T5 and -T18). Observed chromosomal abnormalities include minutes and double minutes, acentric fragments, ring and dicentric chromosomes, and multiple marker chromosomes. GTG banding analysis of the hTERT HMEC lines showed an approximately diploid complement of chromosomes in the majority of metaphases analyzed (Table 3) with no consistent changes that could be associated with hTERT immortalization *per se*. However, changes leading to amplification and rearrangement at chromosome 20q13 and chromosome 7q21 were observed in each hTERT cell line, and amplification of chromosome 16p12 was observed in B80-TERT2 and B80-TERT3b (Table 4). Spectral karyotypic analysis (not shown) of B80-TERT2 and B80-TERT3a identified the chromosome 7 and 20 markers (Table 4) in these cells as der(7) t(4;7) (7)(7qter>p21/22::p15-p21:: 4p/q?) and t(1/7/15/20) respectively. The chromosome 6, 7, and 20 changes are likely to have occurred prior to hTERT transfection as they are common to more than one hTERT immortalized culture. These chromosomal

changes were not observed in the SV40-TAg HMEC lines examined.

Tumorigenicity

The tumorigenicity of some of the HMEC hTERT and SV40-TAg cell lines was assessed by subcutaneous injection of $5x10^6$ cells into ten BALB/c nude mice per cell line (Table 5). After an average of 125 and 107 days respectively, mice injected with B80-T8 and B80-T18 cell lines formed subcutaneous tumors of approximately 10mm diameter. At necropsy there was no evidence of metastasis. Tumors were resected and the cells were cultured *in vitro*. Fluorescence *in situ* hybridization of the tumor cells using biotinylated human and mouse specific COT-1 DNAs confirmed that the tumors contained human cells. Injection of the hTERT-immortalized cell lines into mice did not lead to tumor formation after nine months.

Discussion

This study provides the first direct comparison of immortalization of the same cells by expression of exogenous hTERT or SV40-TAg. Both procedures resulted in an increased level of telomerase activity, indicating that the pre-existing low levels of activity in HMECs (Figure 2a) are insufficient for telomere maintenance. Possible explanations for the inability of TAg to transform post-selection HMECs are discussed elsewhere (Huschtscha *et al.*, 2001). Immortalization of HMECs following expression of the TAg genes mostly involved a period of crisis, and was associated with karyotypic abnormalities, loss of some cell cycle checkpoint functions, and phenotypic alterations, including tumorigenicity in some cases. In contrast, the hTERT-immortalized cells were much more similar to the normal post-selection HMECs. Nevertheless, under the culture conditions used in this study, hTERT-immortalized HMECs were clearly abnormal.

In agreement with the observations by Kiyono and colleagues (1998), we found that

hTERT was only able to immortalize post-selection HMECs; it had previously been shown that such cells lack $p16^{INK4a}$ expression (Brenner *et al.*, 1998; Foster *et al.*, 1998; Huschtscha *et al.*, 1998). Similarly, immortalization of keratinocytes by hTERT occurred only in cells that had spontaneously downregulated expression of $p16^{INK4a}$ (Dickson *et al.*, 2000), or in conjunction with expression of the human papilloma virus E7 gene which inactivates $p110^{Rb}$ (Farwell *et al.*, 2000; Kiyono *et al.*, 1998). hTERT expression was also shown to immortalize primary pre-selection HMECs and human foreskin keratinocytes grown on fibroblast feeder layers without loss of $p16^{INK4a}$ expression (Ramirez *et al.*, 2001). The growth conditions included serum, which favors the growth of the luminal HMECs, whereas the growth conditions used in our study and other previous studies of hTERT-transduced HMECs select for basal HMECs, so that it is not clear to what extent the results can be compared. Further studies are required to determine whether other changes have occurred in the immortal cells grown on feeder layers. Nevertheless, the results clearly highlight the importance of cell culture conditions.

The $p16^{INK4a}$-related senescence-like growth arrest exhibited by normal HMECs grown without serum or feeder layers, and by other types of cells, is an important subject of study. *Ex vivo* growth and manipulation of cells for transplantation and other purposes will be facilitated by an understanding of how this arrest is triggered and whether it can be avoided. Also, the frequency with which $p110^{Rb}$ or $p16^{INK4a}$ function is inactivated in tumors, including breast carcinomas, raises the possibility that adverse growth conditions encountered during tumorigenesis may also activate a $p16^{INK4a}$-mediated growth arrest that must be overcome by inactivation of this tumor suppressor mechanism.

We recently described indirect evidence that post-selection HMECs have other changes in gene expression (Huschtscha *et al.*, 2001), and it is also possible that there are genetic alterations other than disruption of the Rb/$p16^{INK4a}$ pathway that cooperate with

hTERT for immortalization. The earliest immortalization studies utilizing hTERT showed that its expression is sufficient for the immortalization of fibroblasts and retinal pigment epithelial cells (Bodnar *et al.*, 1998; Jiang *et al.*, 1999; Morales *et al.*, 1999), and more recently it has also been shown to immortalize endothelial (Yang *et al.*, 1999) and mesothelial (Dickson *et al.*, 2000) cells. MRC-5 human fetal lung fibroblast cells infected with an hTERT retroviral construct, however, had an extended proliferative capacity but were not necessarily immortalized (MacKenzie *et al.*, 2000). Also, for CD8+ T lymphocytes hTERT transduction was found in one study to have no effect on the replicative lifespan (Migliaccio *et al.*, 2000), and in another study was found to result in immortalization of later passage cells (Hooijberg *et al.*, 2000). These data suggest that for some cell types and culture conditions altered gene expression was required to cooperate with hTERT expression for immortalization.

Karyotypic analysis of the SV40-TAg HMECs showed frequent aneuploidy and chromosomal abnormalities as expected (Sack, 1981). In comparison the hTERT HMECs remained approximately diploid in chromosome number, yet various marker chromosomes and other karyotypic aberrations were observed in metaphase spreads of each cell population. The presence of common changes in the hTERT cell lines suggested that at least some of the karyotypic changes occurred prior to transfection; in support of this, extensive karyotypic changes have previously been documented in post-selection HMECs (Romanov *et al.*, 2001). Cytogenetic alterations were also identified in hTERT immortalized human foreskin keratinocytes and adenoid epithelial cells (Farwell *et al.*, 2000). These observations contrast with those on hTERT immortalized human foreskin fibroblasts which were reported to exhibit minimal karyotypic changes, although further analysis did identify some aneuploid and tetraploid cells at around 200 PDs (Jiang *et al.*, 1999).

One of the hTERT lines (B80-TERT3b) had longer telomeres than the others, with a

minority of the telomeres being very long, although there was no obvious difference in levels of telomerase activity or of the negative telomere length regulators, TRF1 and TRF2 (van Steensel and de Lange, 1997). There is no evidence yet that this is deleterious to the cells, but dysregulation of telomere lengthening may need to be considered as a potential aberration associated with forced expression of exogenous hTERT from a heterologous promoter.

Immortal HMECs expressing TAg, but not those expressing hTERT, lacked normal G_1 checkpoint control. The TAg-immortalized cells upregulated $p21^{CIP1/WAF1}$ in reponse to actinomycin D exposure, possibly via p53-independent mechanisms or due to incomplete inactivation of p53 by TAg, but it was insufficient to activate the G_1 checkpoint.

Although the accelerated growth rate observed during the continuous culture of hTERT-immortalized HMECs, and the altered growth factor requirements of the B80-TERT1 line, may indicate accumulation of genetic or epigenetic changes, overall these cells show greater consistency in morphology, expression of keratin and polymorphic epithelial mucin markers, and growth dynamics than the SV40-TAg cells. In the SV40-TAg HMEC lines, although there was no distinguishable change in keratin 19 expression levels, the upregulation of the luminal cell markers keratin 8 and the polymorphic epithelial mucins HMGF-1 and -2, suggests a trend to a more luminal phenotype in these cells (Taylor-Papadimitriou *et al.*, 1989), as does the requirement for FBS in five of the TAg lines. The pre-selection HMECs contained a mixture of basal (inhibited by FBS) and luminal (stimulated by FBS) cell types (Kao *et al.*, 1995). These changes therefore suggest either that there was selective immortalization of luminal-like cells (Sun *et al.*, 1999), or a TAg-induced change in differentiation status.

The tumorigenicity of two of the SV40-TAg cell lines highlights the genetic changes that may occur in these cells. Previous analysis of SV40-TAg immortalization of HMECs has shown tumorigenicity in some, but not all studies (Elenbaas *et al.*, 2001; Lebeau *et al.*, 1995;

Yilmaz *et al.*, 1993). In one study, a combination of hTERT, SV40-TAg and oncogenic ras was required to induce tumorigenicity in HMECs (Elenbaas *et al.*, 2001). It is likely that the transformed phenotype results, at least in part, from the genetic instability caused by the loss of functional p53 and of G_1 cell cycle checkpoint control (Almasan *et al.*, 1995) and the shortening of telomeres to a critical length at crisis (Counter *et al.*, 1992), which may result in telomere-telomere fusion and fusion-breakage-fusion cycles.

This study provides the first direct comparison of cells immortalized by hTERT or by SV40-TAg. It demonstrates that hTERT-induced immortalization of HMECs is associated with fewer phenotypic and karyotypic changes than SV40 TAg-induced immortalization, providing a useful source of relatively normal cells for *in vitro* studies. These cells, together with the SV40-immortalized cells that underwent additional changes, in some cases including full malignant transformation, form a valuable set of related lines for studying the genetic requirements for tumorigenicity.

Materials and Methods

Cell Transfection and Culture

HMECs were grown from explanted mammary tissue obtained from reduction mammoplasty as described previously (Huschtscha *et al.*, 1998). Normal HMECs cultured from one individual were designated Bre-80, and pre-selection and post-selection cells were transfected with the plasmids pCI-hTERT (containing hTERT cDNA sequence from position 51-3456 bp, AF018167 (Colgin *et al.*, 2000)), pCI-Neo (empty vector control), pRSV-T (containing the SV40 early region genes, encoding the large and small TAg proteins (Sakamoto *et al.*, 1993)) or pRSV2 (empty vector control). pCI-hTERT transfections were performed utilizing FuGENE 6 transfection reagent (Roche, Basel, Switzerland), and pRSV-T transfections were performed utilizing either lipofectamine or DMRIE-C transfection reagents (Life

Technologies, Rockville, MD) respectively. Bre-80 HMECs transfected with pCI-hTERT were grown as mass cultures. Bre-80 HMECs transfected with pRSV-T were grown either as mass cultures (B80-T8, B80-T9, B80-T11) or selected as focal isolates of four individual colonies that grew out after transfection (B80-T5, B80-T6, B80-T17, and B80-T18) (Huschtscha *et al.*, 2001). Cells were cultured in a 5% CO_2 incubator using standard tissue culture procedures. All cultures were initially grown in MCDB 170 medium (Life Technologies). B80-TERT1, B80-T6, B80-T8, B80-T9 and B80-T18 were changed to a medium containing a 1:1 ratio of MCDB 170 and RPMI 1640 (Life Technologies) plus 10% FBS (Trace Biosciences, Castle Hill, NSW, Australia), and B80-T5 was changed to RPMI 1640 plus 10% FBS. MDA-MB-468 breast adenocarcinoma cells (American Type Culture Collection (ATCC; Manassas, VA)), HeLa epithelioid cervical carcinoma cells (ATCC), SaOS-2 osteosarcoma cells (ATCC), GM847 SV40-TAg immortalized Lesch-Nyhan Syndrome skin fibroblast cells (from O. Pereira-Smith, Baylor College of Medicine, TX), WMM1175 melanoma cells (from G. Mann, Millennium Institute, Westmead Hospital, Sydney, NSW, Australia), HFF5 human foreskin fibroblasts (provided by Ralph Böhmer, Ludwig Institute of Cancer Research, Melbourne, Victoria, Australia), and T24 bladder carcinoma cells (ATCC) were grown in 4.5g/l glucose Dulbecco's modified medium (DME-M; Life Technologies, Rockville, MD) plus 10% FBS. All media contained 50µg/ml gentamicin (Sigma, St. Louis, MO).

Cell cycle analysis

Single cell suspensions were stained for DNA analysis using a previously described method (Smyth *et al.*, 1993). Briefly, $1.0x10^6$ cells were added to 0.5 to 0.15ml 5% Triton X-100 (BDH (EM Sciences), Merck KGaA, Darmstadt, Germany), 0.5 mg ribonuclease A (Sigma), 25µg propidium iodide (Sigma) and phosphate buffered saline to a final volume of 0.5ml.

Cells were incubated for 1 h on ice before examining on a FACScan (Becton Dickinson, Franklin Lakes, NJ).

Cytogenetic Analysis

For chromosome studies exponentially growing cultures were treated as previously reported (Peterson *et al.*, 1979). The karyotypes were described according to standard nomenclature (Mitelman, 1995). Fluorescence *in situ* hybridization (FISH) with biotinylated COT-1 DNA (Gibco BRL, Rockville, MD) was used to identify mouse tumors as derived from human cells using standard techniques.

DNA Damage Response

Cell lines were exposed to 7.5 nM actinomycin D (Sigma) for 24 h. After exposure cells were counted and either analyzed for p53/p21$^{WAF1/CIP1}$ upregulation by Western blotting, or for cell cycle parameters by DNA flow cytometry.

Immunocytochemistry

Indirect immunostaining for TRF1, TRF2, and PML proteins was performed as described previously (Yeager *et al.*, 1999). Primary antibodies were 1:200 hTRF1 rabbit polyclonal, 1.66μg/ml hTRF2 mouse monoclonal (Upstate Biotechnology, Lake Placid, NY) and 2μg/ml PML mouse monoclonal (PG-M3; Santa Cruz Biotechnology). The secondary antibody used was either 1:200 goat anti-rabbit FITC labeled polyclonal IgG (Sigma, St. Louis, MO) or 1:128 goat anti-mouse FITC labeled polyclonal IgG (Sigma).

The keratin and polymorphic epithelial mucin differentiation marker profile of transfected cells was analyzed as described previously (Huschtscha *et al.*, 1998). Fluorescence intensity was assessed as either low, medium, or high, for individual slides in three separate

experiments. The consensus result for each cell type was then compared to that for post-selection Bre-80 cells. Primary monoclonal antibodies were against pan-keratin, keratin 19, HMFG-1, HMFG-2 and SM3 (gifts from Dr. Joyce Taylor-Papadimitriou, Imperial Cancer Research Fund Laboratories, London, UK), keratins 1, 6, 10 and 16 (gifts from Dr. Patricia Purkis, Imperial Cancer Research Fund Laboratories), and keratins 5, 7, 8, 14, and 18, and vimentin (Sigma). Secondary antibodies used were 1:128 FITC-conjugated goat anti-mouse IgG (Sigma) and 1:200 FITC-conjugated goat anti-rabbit IgG (Sigma) and 5ug/ml Texas Red-conjugated horse anti-mouse IgG (Vector, Burlingame, CA). Cell nuclei were counterstained with 0.2μg/ml DAPI (4',6-diamidino-2-phenylindol; Sigma).

Limiting Dilution Subcloning

Cells were trypsinized and then diluted and seeded at a concentration of approximately 1 cell per well into 96-well plates (IWAKI, Asahi Techno Glass Corporation, Tokyo, Japan), in MCDB 170 medium (Life Technologies) that had been conditioned by HMECs and then filtered through 0.2μm filters (Sartorius, Göttingen, Germany). Colonies were expanded separately and analyzed as separate subclones of the original parental cell line.

Telomerase Activity and Telomere Length Assays

Telomerase activity was assayed using the Telomere Repeat Amplification Protocol (TRAP) (Kim *et al.*, 1994) with modifications previously described (Perrem *et al.*, 1999). The PCR products were detected using SYBR Green I (Molecular Probes, Eugene, OR) staining and a STORM 860 utilizing ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Telomere length was determined as previously described (Bryan *et al.*, 1995) by pulsed-field gel electrophoresis (CHEF-DR II pulsed-field gel electrophoresis apparatus, Biorad, Hercules, CA) of terminal restriction fragments (TRF) generated by digesting extracted

genomic DNA with *Hin*f1 and *Rsa*1 restriction enzymes (Boehringer Mannheim).

Tumorigenicity Studies

For each cell line tested, ten 9-10 week old Balb/c nu/nu mice (Animal Resources Centre, Murdoch, WA, Australia) were injected subcutaneously in the interscapular region with $5x10^6$ cells. When the diameter of a tumor was >10 mm, the mouse was culled and necropsied, and the tumor was resected for analysis.

Western Analysis

Harvested cell pellets were lysed by addition of 0.5ml lysis buffer (10ml ECB buffer solution (50mM Tris-HCl at pH 8.0; 120nM NaCl; 100mM NaF; 0.2mM Na_2VO_4; 0.5% NP-40) containing leupeptin (10mg/ml; Boehringer Mannheim Corp., Indianapolis, IN) and 1 tablet of complete protease inhibitor cocktail (Boehringer Mannheim)) per 10^7 cells, followed by four freeze/thaw cycles. Protein concentration was quantitated with the BCA reagent (Pierce, Rockford, IL) and cellular protein (50µg) was separated on 7.5% ($p110^{Rb}$), 13% (p53) or 15% ($p16^{INK4a}$, $p21^{CIP1/WAF1}$) SDS-PAGE gels, then transferred to Immobilon-P® PVDF transfer membrane (Millipore) or Trans-Blot® transfer medium PVDF membrane (Bio-Rad, Hercules, CA). Primary mouse monoclonal antibodies used to detect proteins were 0.5µg/ml WAF ($p21^{CIP1/WAF1}$; Oncogene Research Products, CN Biosciences Inc., San Diego, CA) 0.5µg/ml p53, 0.5µg/ml $p16^{INK4a}$ and 0.5µg/ml $p110^{Rb}$ (Neomarkers, Lab Vision Corp., Fremont, CA) and 0.5µg/ml actin (Sigma). The secondary antibody used was horseradish peroxidase conjugated goat anti-mouse IgG (DAKO Corp., Santa Barbara, CA). Supersignal® chemiluminescence substrate for Western blots (Pierce, Rockford, IL) was used to detect the secondary antibody.

Acknowledgements

The research was supported by the National Breast Cancer Foundation, the National Health and Medical Research Council of Australia, the Carcinogenesis Fellowship of the New South Wales Cancer Council, and a Yass Memorial scholarship. The authors thank Christine Smyth for FACscan analysis, and CMRI Bioservices for nude mouse studies.

References

Almasan A, Linke SP, Paulson TG, Huang L and Wahl GM. (1995). Genetic instability as a consequence of inappropriate entry into and progression through S-phase. *Cancer Metastasis Rev.*, **14**: 59-73.

Band V, De Caprio JA, Delmolino L, Kulesa V and Sager R. (1991). Loss of p53 protein in human papillomavirus type 16 E6-immortalized human mammary epithelial cells. *J. Virol.*, **65** : 6671-6676.

Band V, Zajchowski D, Kulesa V and Sager R. (1990). Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc. Natl. Acad. Sci. U. S. A.*, **87**: 463-467.

Bartek J, Bartkova J, Kyprianou N, Lalani E-N, Staskova Z, Shearer M, Chang S and Taylor-Papadimitriou J. (1991). Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus. *Proc. Natl. Acad. Sci. U. S. A.*, **88**: 3520-3524.

Blackburn EH. (1991). Structure and function of telomeres. *Nature*, **350**: 569-573.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, Harley CB, Shay JW, Lichtsteiner S and Wright WE. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science*, **279**: 349-352.

Brenner AJ, Stampfer MR and Aldaz CM. (1998). Increased *p16* expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with *p16* inactivation. *Oncogene,* **17**: 199-205.

Bryan TM, Englezou A, Gupta J, Bacchetti S and Reddel RR. (1995). Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.,* **14**: 4240-4248.

Bryan TM and Reddel RR. (1994). SV40-induced immortalization of human cells. *Crit. Rev. Oncogenesis,* **5**: 331-357.

Cao Y-A, Gao Q, Water DE and Band V. (1997). Abrogation of wild-type p53-mediated transactivation is insufficient for mutant p53-induced immortalization of normal human mammary epithelial cells. *Cancer Res.,* **57**: 5584-5589.

Colgin LM and Reddel RR. (1999). Telomere maintenance mechanisms and cellular immortalization [Published erratum appears in Curr Opin Genet Dev 1999 Apr;9(2):247]. *Curr. Opin. Genet. Dev.,* **9**: 97-103.

Colgin LM, Wilkinson C, Englezou A, Kilian A, Robinson MO and Reddel RR. (2000). The hTERTα splice variant is a dominant negative inhibitor of telomerase activity. *Neoplasia,* **2**: 426-432.

Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB and Bacchetti S. (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.,* **11**: 1921-1929.

Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, Louis DN, Li FP and Rheinwald JG. (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.

Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Popescu NC, Hahn WC and Weinberg RA. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev.*, **15**: 50-65.

Farwell DG, Shera KA, Koop JI, Bonnet GA, Matthews CP, Reuther GW, Coltrera MD, McDougall JK and Klingelhutz AJ. (2000). Genetic and epigenetic changes in human epithelial cells immortalized by telomerase. *Am. J. Pathol.*, **156**: 1537-1547.

Foster SA and Galloway DA. (1996). Human papillomavirus type 16 E7 alleviates a proliferation block in early passage human mammary epithelial cells. *Oncogene*, **12**: 1773-1779.

Foster SA, Wong DJ, Barrett MT and Galloway DA. (1998). Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol. Cell. Biol.*, **18**: 1793-1801.

Gao Q, Hauser SH, Liu X-L, Wazer DE, Madoc-Jones H and Band V. (1996). Mutant *p53*-induced immortalization of primary human mammary epithelial cells. *Cancer Res.*, **56**: 3129-3133.

Garbe J, Wong M, Wigington D, Yaswen P and Stampfer MR. (1999). Viral oncogenes accelerate conversion to immortality of cultured conditionally immortal human mammary epithelial cells. *Oncogene,* **18**: 2169-2180.

Gollahon LS and Shay JW. (1996). Immortalization of human mammary epithelial cells transfected with mutant p53 (273^{his}). *Oncogene,* **12**: 715-725.

Greider CW and Blackburn EH. (1985). Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell,* **43**: 405-413.

Harley CB. (1991). Telomere loss: Mitotic clock or genetic time bomb? *Mutat. Res.,* **256**: 271-282.

Hayflick L and Moorhead PS. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell Res.,* **25**: 585-621.

Hooijberg E, Ruizendaal JJ, Snijders PJF, Kueter EWM, Walboomers JMM and Spits H. (2000). Immortalization of human $CD8^+$ T cell clones by ectopic expression of telomerase reverse transcriptase. *J. Immunol.,* **165**: 4239-4245.

Huschtscha LI, Neumann AA, Noble JR and Reddel RR. (2001). Effects of simian virus 40 T-antigens on normal human mammary epithelial cells reveal evidence for spontaneous alterations in addition to loss of $p16^{INK4a}$ expression. *Exp. Cell Res.,* **265**: 125-134.

Huschtscha LI, Noble JR, Neumann AA, Moy EL, Barry P, Melki JR, Clark SJ and Reddel RR. (1998). Loss of $p16^{INK4}$ expression by methylation is associated with lifespan extension of human mammary epithelial cells. *Cancer Res.,* **58**: 3508-3512.

Jiang X-R, Jimenez G, Chang E, Frolkis M, Kusler B, Sage M, Beeche M, Bodnar AG, Wahl GM, Tlsty TD and Chiu C-P. (1999). Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat. Genet.,* **21**: 111-114.

Kao C-Y, Nomata K, Oakley CS, Welsch CW and Chang C-C. (1995). Two types of normal human breast epithelial cells derived from reduction mammoplasty: Phenotypic characterization and response to SV40 transfection. *Carcinogenesis,* **16**: 531-538.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC, Coviello GM, Wright WE, Weinrich SL and Shay JW. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science,* **266**: 2011-2015.

Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA and Klingelhutz AJ. (1998). Both Rb/$p16^{INK4a}$ inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature,* **396**: 84-88.

Lebeau J, Gerbault-Seureau M, Lemieux N, Apiou F, Calvo F, Berthon P, Goubin G and Dutrillaux B. (1995). Loss of chromosome 3p arm differentiating tumorigenic from non-tumorigenic cells derived from the same SV40-transformed human mammary epithelial cells. *Int. J. Cancer,* **60**: 244-248.

MacKenzie KL, Franco S, May C, Sadelain M and Moore MAS. (2000). Mass cultured human fibroblasts overexpressing hTERT encounter a growth crisis following an extended period of proliferation. *Exp. Cell Res.,* **259**: 336-350.

Migliaccio M, Amacker M, Just T, Reichenbach P, Valmori D, Cerottini J-C, Romero P and Nabholz M. (2000). Ectopic human telomerase catalytic subunit expression maintains telomere length but is not sufficient for $CD8^+$ T lymphocyte immortalization. *J. Immunol.,* **165**: 4978-4984.

Morales CP, Holt SE, Ouellette M, Kaur KJ, Yan Y, Wilson KS, White MA, Wright WE and Shay JW. (1999). Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat. Genet.,* **21**: 115-118.

Nonet GH, Stampfer MR, Chin K, Gray JW, Collins CC and Yaswen P. (2001). The *ZNF217* gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. *Cancer Res.,* **61**: 1250-1254.

Nugent CI and Lundblad V. (1998). The telomerase reverse transcriptase: components and regulation. *Genes Dev.,* **12**: 1073-1085.

Olovnikov AM. (1973). A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J. Theor. Biol.,* **41**: 181-190.

Perrem K, Bryan TM, Englezou A, Hackl T, Moy EL and Reddel RR. (1999). Repression of an alternative mechanism for lengthening of telomeres in somatic cell hybrids. *Oncogene,* **18**: 3383-3390.

Peterson WD, Simpson WF and Hukku B. (1979). Cell culture characterization: monitoring for cell identification. *Methods Enzymol.,* **58**: 164-178.

Ramirez RD, Morales CP, Herbert B-S, Rohde JM, Passons C, Shay JW and Wright WE. (2001). Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev.*, **15**: 398-403.

Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM and Tlsty TD. (2001). Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. *Nature,* **409**: 633-637.

Sack GHJr. (1981). Human cell transformation by simian virus 40 - a review. *In Vitro,* **17**: 1-19.

Sakamoto K, Howard T, Ogryzko V, Xu N-Z, Corsico CC, Jones DH and Howard B. (1993). Relative mitogenic activities of wild-type and retinoblastoma binding-defective SV40 T antigens in serum-deprived and senescent human diploid fibroblasts. *Oncogene,* **8**: 1887-1893.

Smyth CM, Helmer MA, Dalla PL and Rowe PB. (1993). Flow cytometric DNA analyses of frozen samples from children's solid tumors. *Pathology,* **25**: 388-393.

Sprung CN, Sabatier L and Murnane JP. (1999). Telomere dynamics in a human cancer cell line. *Exp. Cell Res.,* **247**: 29-37.

Stampfer MR. (1985). Isolation and growth of human mammary epithelial cells. *J. Tissue Cult. Methods,* **9**: 107-115.

Stampfer MR and Bartley JC. (1985). Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. *Proc. Natl. Acad. Sci. U. S. A.,* **82**: 2394-2398.

Stampfer MR, Garbe J, Levine G, Lichtsteiner S, Vasserot AP 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.

Sun W, Kang K-S, Morita I, Trosko JE and Chang C-C. (1999). High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization. *Cancer Res.,* **59**: 6118-6123.

Taylor-Papadimitriou J, Stampfer M, Bartek J, Lewis A, Boshell M, Lane EB and Leigh IM. (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to *in vivo* phenotypes and influence of medium. *J. Cell Sci.,* **94**: 403-413.

van Steensel B and de Lange T. (1997). Control of telomere length by the human telomeric protein TRF1. *Nature,* **385**: 740-743.

Vaziri H, Squire JA, Pandita TK, Bradley G, Kuba RM, Zhang H, Gulyas S, Hill RP, Nolan GP and Benchimol S. (1999). Analysis of genomic integrity and p53-dependent G_1 checkpoint in telomerase-induced extended-life-span human fibroblasts. *Mol. Cell. Biol.,* **19**: 2373-2379.

Wazer DE, Chu Q, Liu X-L, Gao Q, Safaii H and Band V. (1994). Loss of p53 protein during radiation transformation of primary human mammary epithelial cells. *Mol. Cell. Biol.,* **14**: 2468-2478.

Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, Bodnar AG, Lichtsteiner S, Kim NW, Trager JB, Taylor RD, Carlos R, Andrews WH, Wright WE, Shay JW, Harley CB and Morin GB. (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat. Genet.,* **17**: 498-502.

Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A, Bronstein A, Chiu C-P and Herron GS. (1999). Human endothelial cell life extension by telomerase expression. *J. Biol. Chem.,* **274**: 26141-26148.

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR and Reddel RR. (1999). Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Res.,* **59**: 4175-4179.

Yilmaz A, Gaide A-C, Sordat B, Borbenyi Z, Lahm H, Imam A, Schreyer M and Odartchenko N. (1993). Malignant progression of SV40-immortalised human milk epithelial cells. *Br. J. Cancer,* **68**: 868-873.

Table 1 Growth of hTERT and SV40-TAg transfected HMECs

Cell line	Crisis period (days)[a]	Growth Rate (PD/day)		
		Pre-crisis[b]	Immortalized	
			Early[c]	Late[d]
B80-TERT1	-	n/a	0.18	0.24
B80-TERT2	-	n/a	0.19	0.33
B80-TERT3a	-	n/a	0.25	0.38
B80-TERT3b	-	n/a	0.27	0.37
B80-T17	-	n/a	0.53	n/a[e]
B80-T5	168	0.44	0.13	0.50
B80-T6[f]	77	0.29	0.35	0.37
B80-T8	215	0.21	0.29	n/a[e]
B80-T9[f]	65	0.37	0.26	0.55
B80-T11	212	0.27	0.55	n/a[e]
B80-T18	170	0.40	0.36	0.64

Growth characteristics of Bre-80 HMECs transfected with pCI-hTERT or pRSV-T. Four cultures (B80-TERT…) immortalized by expression of hTERT were compared with seven cultures (designated B80-T…) that became immortalized following transfection with the SV40 early region plasmid, pRSV-T. [a]Length of culture crisis prior to immortalization. Population doublings (PD)/day were calculated for each cell line for the following intervals. [b]Pre-crisis: growth rate was calculated for the 50 days prior to entering crisis. [c]Early: growth rate between days 100 and 200 for the hTERT cells and B80-T17, or the first 50 post-crisis days for the Tag cells. [d]Late: growth rate between days 300 and 400 for hTERT cells, and for the second 50 days of post-crisis culture for the TAg cells. [e]Immortal B80-T8 and B80-T11 HMECs were cultured for <100 days and B80-T17 was cultured for <400 days. [f]The data for B80-T6 and B80-T9 represent a minor correction to the data in Fig. 3a of (Huschtscha *et al.*, 2001). n/a = not applicable.

Table 2 Changes in differentiation marker expression in hTERT and SV40-TAg immortalized HMECs as compared to untransfected post-selection HMECs

Cell Line	PD[a]	Luminal/Basal Keratin Differentiation Markers			Polymorphic Epithelial Mucin Markers		
		Luminal Keratin 8	Basal Keratin 14	Luminal Keratin 19	Luminal SM3	Luminal HMFG-1	Luminal HMFG-2
B80-TERT1	81	+[b]	nc	nc	nc	+	nc
B80-TERT2	93	nc	+	nc	nc	nc	nc
B80-TERT3a	59	+	nc	nc	nc	nc	nc
B80-TERT3b	62	-	+	nc	nc	nc	nc
B80-T5	85	++	-	-	+	+	+
B80-T8	56	+	+	nc	+	+++	+
B80-T17	112	++	nc	nc	nc	++	++
B80-T18	76	++	-	-	nc	+	nc

[a]PD, population doubling level of the analyzed cells. [b]**+/-,** small up/down regulation of expression; **++,** moderate up regulation of expression; **+++,** large upregulation of expression; nc, no change in expression levels.

Table 3 Karyotypic analysis of hTERT and SV40-TAg HMEC lines

Cell Type	Modal Chromosome Number (range)[a]	4N metaphases (%)[b]	Aberrant Chromosomes / Karyotypes Examined[c]
B80-TERT1	46 (45 – 50)	0	37 /11
B80-TERT2	46 (45 – 49)	1	29 /11
B80-TERT3a	46 (46 – 47)	2	20 /10
B80-TERT3b	46 (46 – 47)	4	31 /10
B80-T5	63 (60 – 69)	4	165 /11
B80-T8	43 (40 – 45)	6	269 /11
B80-T17	43 (41 – 45)	10	73 /11
B80-T18	67 (65 – 71)	5	168 /10

[a]Modal chromosome number and range calculated from 30 metaphases. [b]The percentage of 4N karyotypes observed in 100 metaphases examined. [c]GTG banded metaphase spreads were examined for aberrant chromosomes. All Bre-80 metaphases contain a strain-specific strong GTG and QFQ band on chromosome 14p in addition to those counted. PD level at time of karyotypic analyses was approximately 80 for the hTERT cell lines, and approximately 70 for the SV40-TAg cell lines.

Table 4 Marker and aberrant chromosomes detected in the hTERT HMEC lines

Marker Name	Cell Line[a]			
	B80-TERT1	B80-TERT2	B80-TERT3a	B80-TERT3b
M1	dup(7)(q21-qter) [1][b]	dup(7)(q21-qter) [11]	dup (7)qter p21/ 22:: ?q21-qter) [10]	
M1a				der (7) t (7;?) (p21;?) [10]
M2	14p+	14p+	14p+	14p+
M3		add (20)(q13) ++ [6]	add (20) (p13/q13) +++ [10]	
M3A		add (20)(q13) +++[5]		
M3B	add (20)(q13) 20q+[11]			
M3C				20q+ [10]
M4		der (18) del/t (18;?)(q12;?) [3]		
M4A	der (18) 18q++ [11]			
M5		add (16)(p12) [1]		add (16) (p12?) [3]
M6		del (2)(p13) [1]		
M7		der (22?) t (1;22) (q21;q11) [2]		
M7A				dup (1) (q21-qter) [3]
M8	add (13) (p10) [10]			
M9	t (5q;10q) [2]			
M9A				10q+ [4]
M9B				del (10) (q10)
M10	del (6) (q21) [1]			
M11	t (4p;8q?) [1]			

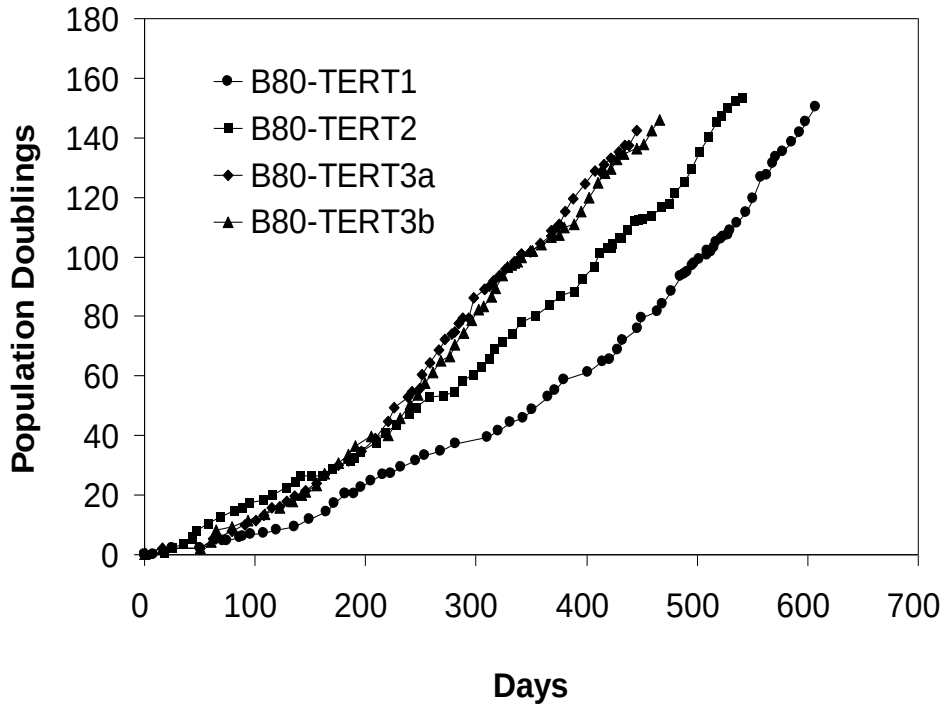
[a]GTG banding marker chromosome analysis was performed in eleven B80-TERT1 and B80-TERT2 metaphases, and ten B80-TERT3a and B80-TERT3b metaphases. [b]Number of karyotypes in which this marker was found.

Table 5 Tumorigenicity of hTERT and SV40-TAg HMEC lines

Cell Line	PD	No. of Mice Forming a Tumor[a]	Tumor Latency (Days)[b]	Time to form $1cm^2$ Tumor (Days)[c]
B80-TERT1	108	-	-	-
B80-TERT2	124	-	-	-
B80-TERT3a	75	-	-	-
B80-TERT3b	83	-	-	-
B80-T5	127	-	-	-
B80-T8	81	3	21, 21, 21	77, 95, 182
B80-T17	134	-	-	-
B80-T18	124	2	68, 68	98, 115
MDA-MB-468[d]	344[e]	2	9, 9	74, 161

[a]$5x10^6$ cells were injected subcutaneously into ten 9-10 week old Balb/c nu/nu mice. [b]Number of days until a tumor was first observed. [c]Number of days until the tumor reached approximately 10mm in diameter. [d]MDA-MB-468 human breast epithelial tumor cells are a positive control for tumor formation. [e]Passage number (not population doubling).

A



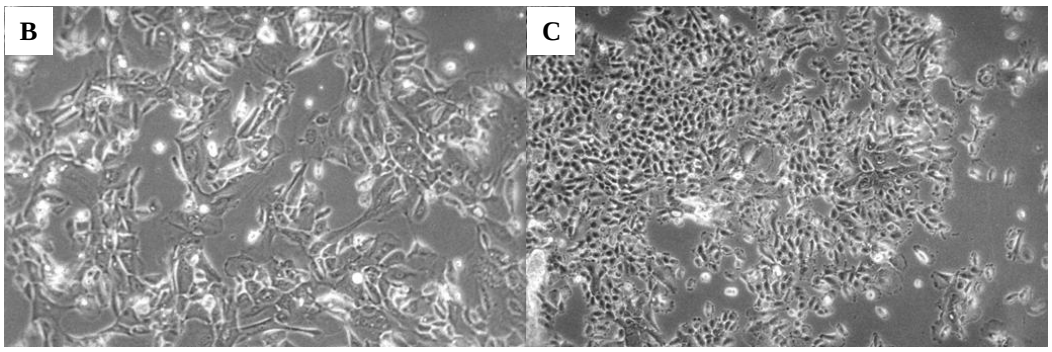
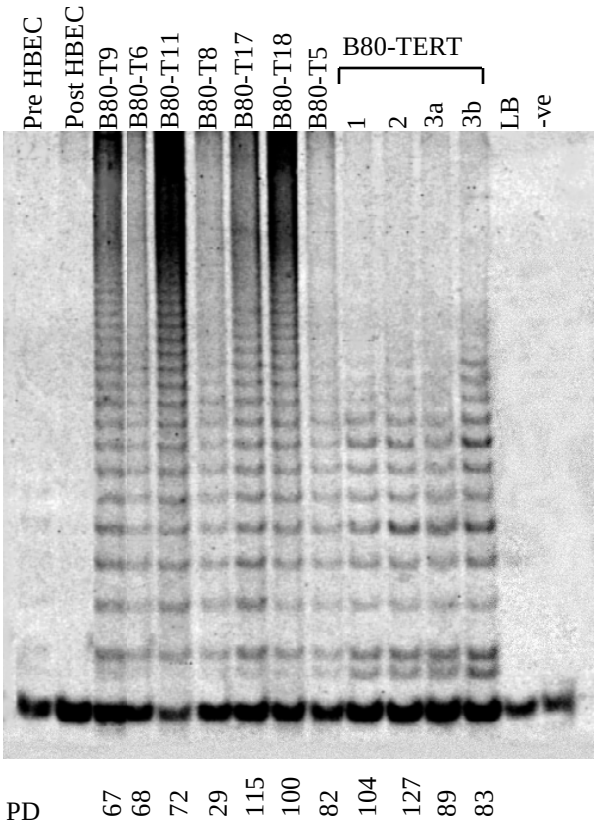
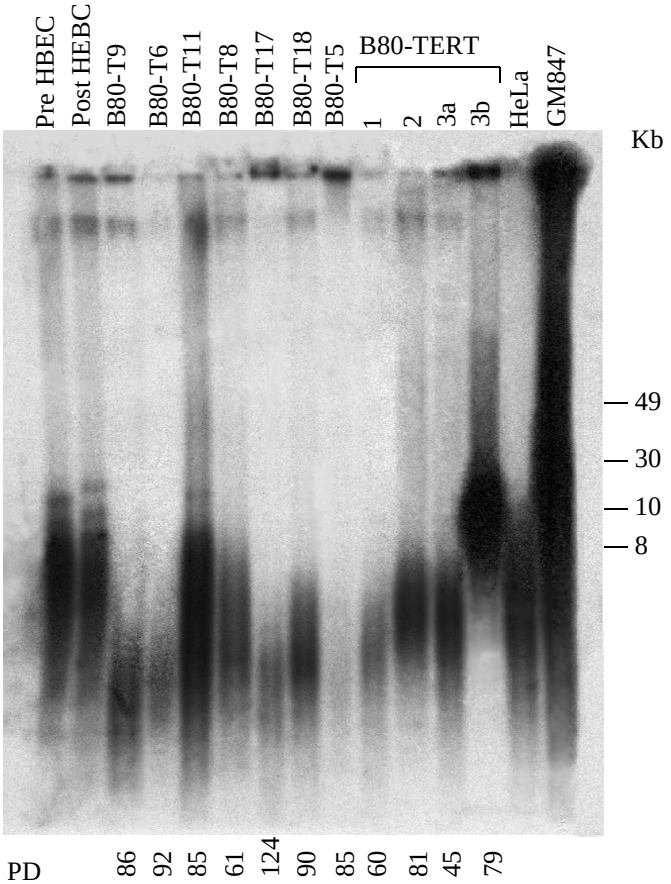


Figure 1 Growth of hTERT-transfected HMECs. Post-selection cells were transfected at day 0. Cumulative PDs were calculated at each passage. The pCI-Neo transfected control cells corresponding to the B80-TERT1, 2, 3a and 3b cultures grew for 5, 11, 3, and 3 PD, respectively, before ceasing cell division and acquiring a senescent morphology. Inset: Phase contrast photomicrograph of B80-TERT3a culture at PD 2 showing outgrowth of dividing cells on a background of senescent cells. Arrows indicate some of the senescent cells.

A


Pre HBEC
Post HBEC
B80-T9
B80-T6
B80-T11
B80-T8
B80-T17
B80-T18
B80-T5
B80-TERT
1
2
3a
3b
LB
-ve
PD
67
68
72
29
115
100
82
104
127
89
83

B


Pre HBEC
Post HEBC
B80-T9
B80-T6
B80-T11
B80-T8
B80-T17
B80-T18
B80-T5
B80-TERT
1
2
3a
3b
HeLa
GM847
Kb
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30
10
8
PD
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92
85
61
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45
79

C

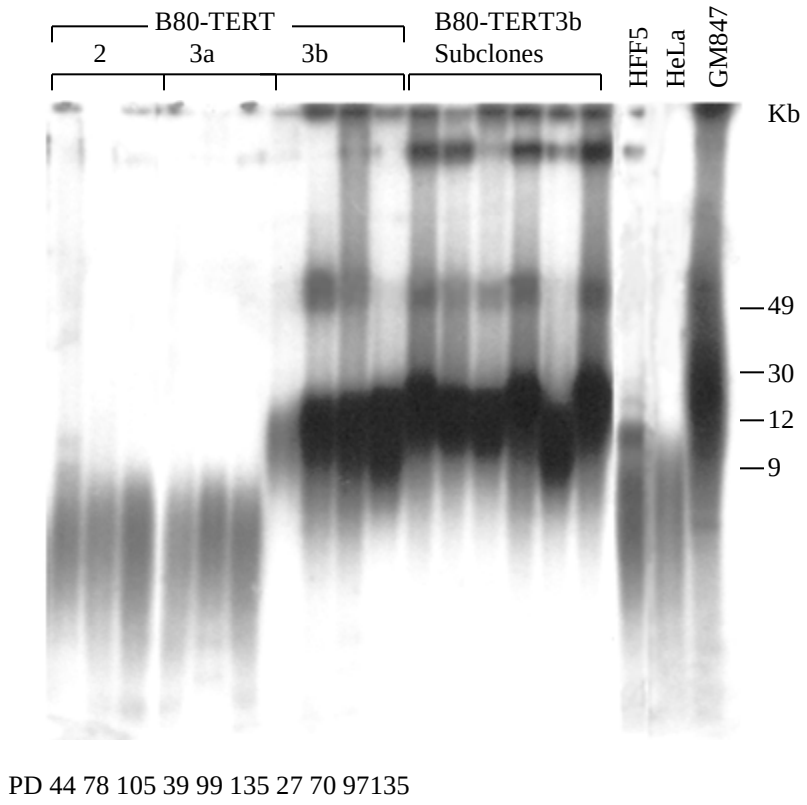

Figure 2 Telomerase activity and telomere maintenance in Bre-80 HMECs. (**a**) TRAP analysis of telomerase activity in hTERT and SV40-TAg HMECs. LB, TRAP lysis buffer; -ve, water instead of cell protein lysate; pre, pre-selection; post, post-selection. (**b**) Pulsed field terminal restriction fragment (TRF) analysis of telomere length in pre- and post-selection, hTERT, and SV40-TAg HMECs. (**c**) Pulsed field TRF analysis of telomere length with increasing PDs in B80-TERT2, 3a and 3b cells, and in 3b subclones; the subclones were obtained by limiting dilution at PD 107 and grown for 18-28 PD before TRF analysis. HeLa, GM847, and HFF5 cells are examples of telomerase-positive, ALT-positive and normal cells, respectively.

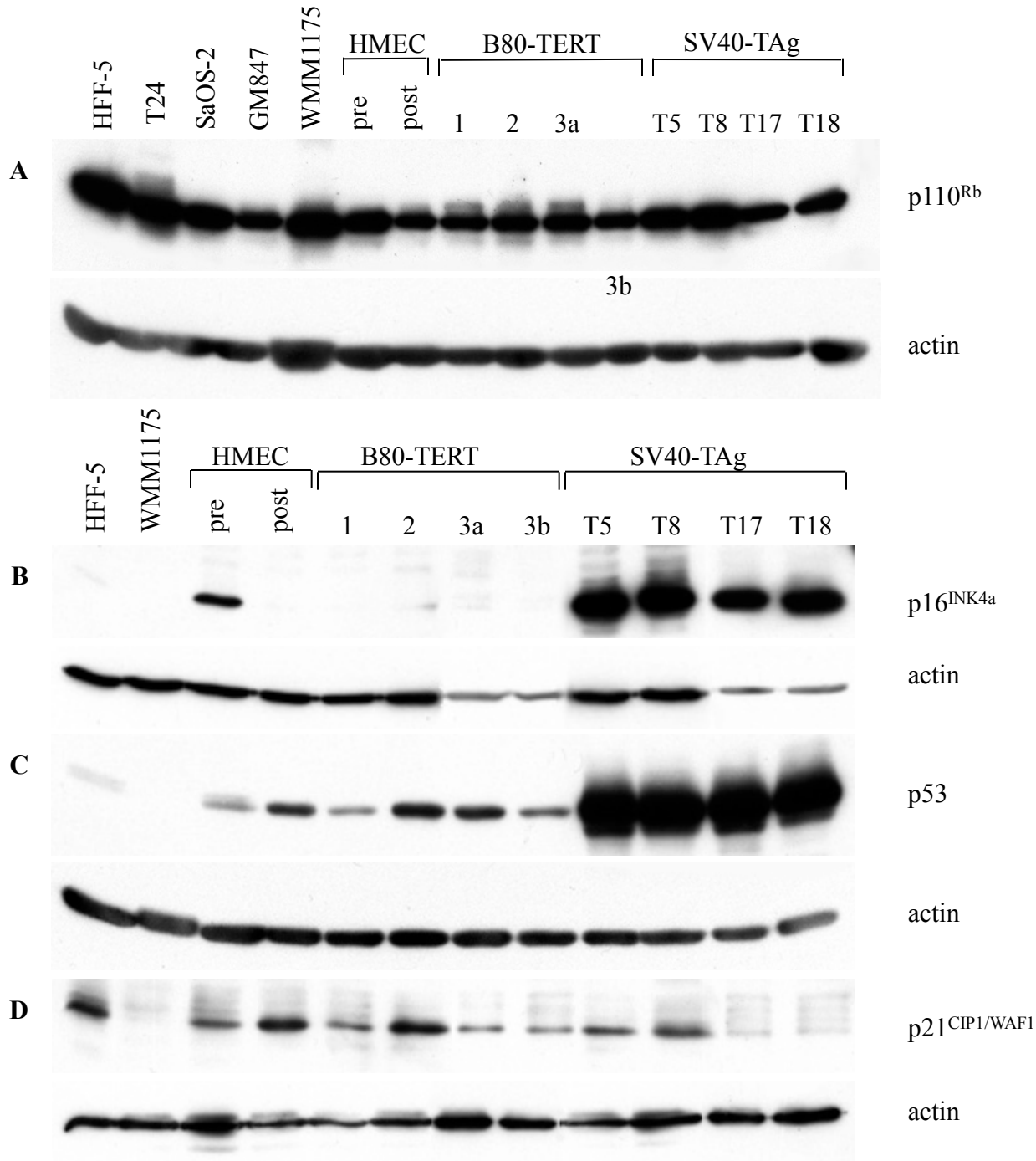

Figure 3 Western analysis of tumor suppressor protein expression in HMECs. (**a**) $p110^{Rb}$;(**b**) $p16^{INK4a}$; (**c**) p53; and (**d**) $p21^{CIP1/WAF1}$. PD levels at the time of analysis were: B80-TERT1, PD 136; -TERT2, 155; -TERT3a, 133; -TERT3b, 136; -T5, 131; -T8, 63; -T17, 128; and -T18, 151. Pre, pre-selection; post, post-selection. HFF5, T24, GM847 and WMM1175 cells act as positive controls for $p110^{Rb}$ expression. T24 and WMM1175 cells act as positive controls for increased expression of hyper-phosphorylated $p110^{Rb}$, associated with loss of expression of $p16^{INK4a}$. HFF5 and WM1175, positive and negative controls, respectively, for expression of $p16^{INK4a}$, p53 and $p21^{CIP1/WAF1}$. Actin expression is used as a loading control for each western blot.

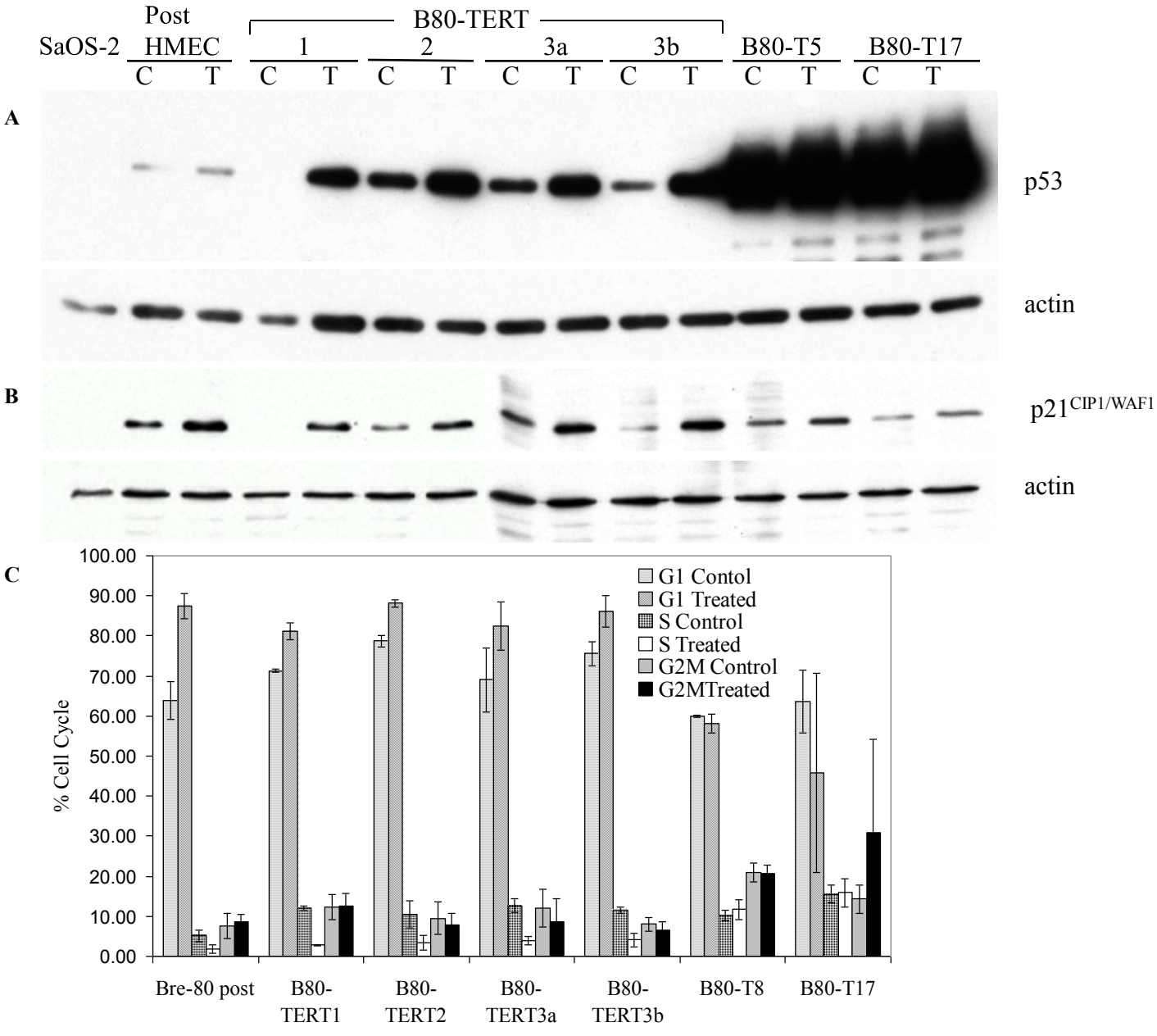

Figure 4 Response of HMECs to DNA damage. Cells were treated with 7.5 nM actinomycin D for 24 h. Western analysis was performed for (**a**) p53; and (**b**) $p21^{CIP1/WAF1}$. SaOS-2 cells, negative control for p53 expression. Post HMEC, post-selection Bre-80 cells; C, untreated control; T, treated. PD levels at the time of analysis: B80-TERT1, 136; B80-TERT2, 155; B80-TERT3a, 133; B80-TERT3b, 136; B80-T5, 131; and B80-T17, 128. Actin expression is shown as control for protein loading. (**c**) Cell cycle analysis. Propidium iodide-stained cells were analyzed by FACS. Percentage of cells in each phase of the cell cycle is shown; mean ± SEM, n=3 separate experiments.

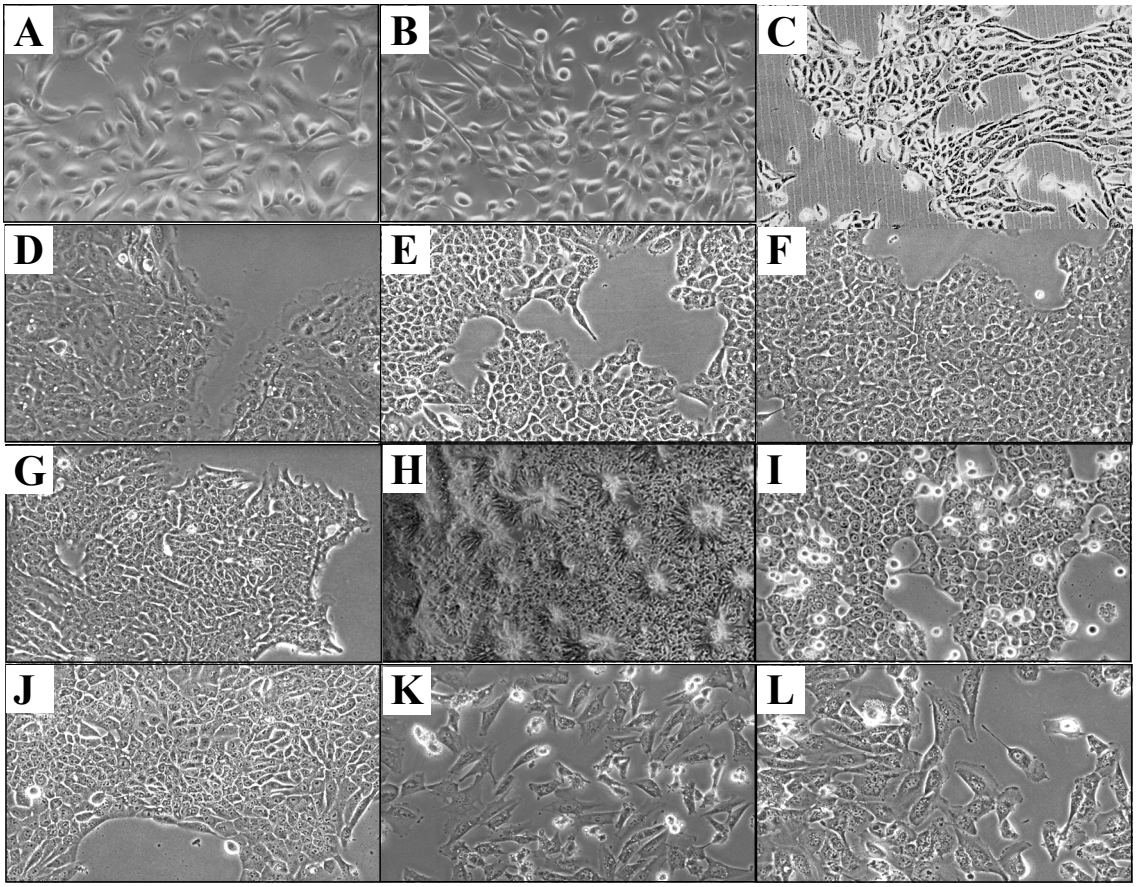

Figure 5 Morphologic changes observed in hTERT- and TAg-transfected HMECs (phase contrast microscopy using 20X objective, or 4X objective for (**h**)). (**a**) Pre-selection HMECs; (**b**) Post-selection HMECs; (**c**) B80-TERT2 at 50% confluence, PD 40; (**d**) B80-TERT1, PD 106; (**e**) B80-TERT2, PD 141; (**f**) B80-TERT3a, PD 89; (**g**) B80-TERT3b, PD 90; (**h**) B80-TERT3b cells at confluence, PD 40, showing dome-like structures; (**i**) B80-T5, PD 123; (**j**) B80-T8, PD 79; (**k**) B80-T17, PD 143; (**l**) B80-T18, PD 145.