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# The establishment of telomerase-immortalized cell lines representing human chromosome instability syndromes

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**The limited life span of normal human cells represents a substantial obstacle for biochemical analysis, genetic manipulation and genetic screens. To overcome this technical barrier, immortal human cell lines are often derived from tumors or produced by transformation with viral oncogenes such as SV40 large T antigen. Cell lines produced by these approaches are invariably transformed, genomically unstable and display cellular properties that differ from their normal counterpart. It was recently shown that the ectopic expression of hTERT, encoding the catalytic subunit of human telomerase, can extend the life span of normal human cells without causing cellular transformation and genomic instability. In the present study, we have used hTERT to extend the life span of normal human skin fibroblasts derived from patients afflicted with syndromes of genomic instability and/or premature aging. Our results show that hTERT efficiently extends the life span without altering the characteristic phenotypic properties of the cells. Thus, the ectopic expression of telomerase represents a major improvement over the use of viral oncogenes for the establishment of human cell lines.**

## INTRODUCTION

Normal human somatic cells have a limited life span and enter senescence after a limited number of cell divisions (1–3). At senescence, cells are viable but no longer divide. This limit on cell proliferation represents an obstacle to the study of normal human cells, especially since many rounds of cell division are used as cells are shared between laboratories or to produce large quantities of cells required for biochemical analysis, for genetic manipulations or for genetic screens. This limitation is of particular concern for the study of rare hereditary human diseases, since the volume of the biological samples collected (biopsies or blood) is usually small and contains a limited number of cells. The establishment of permanent cell lines is one way to circumvent this lack of critical material. Some tumor cells yield cultures with unlimited growth potential, and *in vitro* transformation with oncogenes or carcinogens have proven a successful means to establish permanent fibroblast and lymphoblast cell lines. Such cell lines have been valuable in the analysis of mammalian biochemistry and the identification of disease-related genes. However, such transformed cells typically exhibit significant alterations in physiological and biological properties. Most notably, these cells are associated with aneuploidy, spontaneous hypermutability, loss of contact inhibition and alterations in biochemical functions related to cell cycle checkpoints. These cellular properties that differ from their normal counterparts pose significant limitations to the analysis of many cellular functions, in particular those related to genomic integrity and the study of the human chromosome instability syndromes.

Recent advances have shown that the onset of replicative senescence is controlled by the shortening of the telomeres that occurs each time normal human cells divide (4–7). This loss of telomeric DNA is a consequence of the inability of DNA polymerase alpha to fully replicate the ends of linear DNA molecules (8,9). It has been proposed that senescence is induced when the shortest one or two telomeres can no longer be protected by telomere-binding proteins and thus is recognized as a double-stranded (ds) DNA break. In cells with functional checkpoints, the introduction of dsDNA breaks leads to the activation of p53 and of the p16/pRB checkpoint and to a growth arrest state that mimics senescence (10–12). Cell cycle progression in senescent cells is also blocked by the same two mechanisms (13–15). This block can be overcome by viral oncogenes such as SV40 large T antigen that can inactivate both p53 and pRB. Cells that express SV40 large T antigen escape senescence but continue to lose telomeric repeats during their extended life span. These cells are not yet immortal and terminal telomere shortening eventually causes the cells to reach a second non-proliferative stage termed 'crisis' (16,17). Escape from crisis is a very rare event (1 in $10^7$) usually accompanied by the reactivation of telomerase (18).

Telomerase is a specialized cellular reverse transcriptase that can compensate for the erosion of telomeres by synthesizing new

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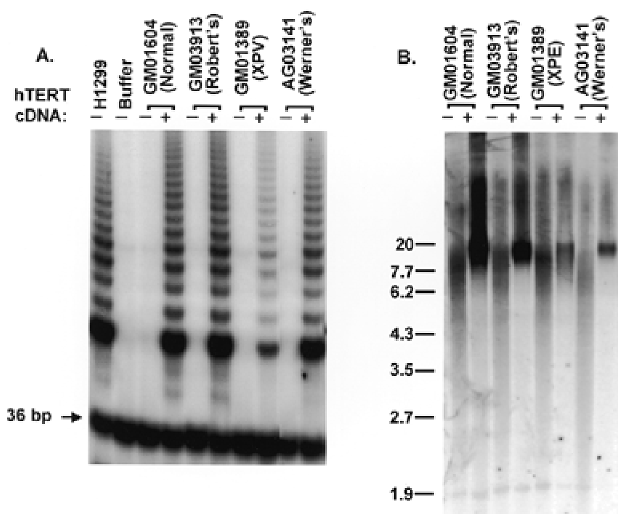


**Figure 1.** Telomerase activity and telomere length in the control and hTERT-infected cells. (**A**) Telomerase activities for a representative set of uninfected control cells and cells infected with the hTERT vector. Similar results were obtained for all other sets. The tumor-derived cell line, H1299, serves as a positive control and the lysis buffer lane as a negative control. (**B**) The telomere lengths for the same representative set of samples is shown. The results show the typical smear characteristic of human telomeres and also show that the telomeres are longer in cells that have been infected with the hTERT vector. Positions of molecular weight markers are indicated on the left side (in kDa).

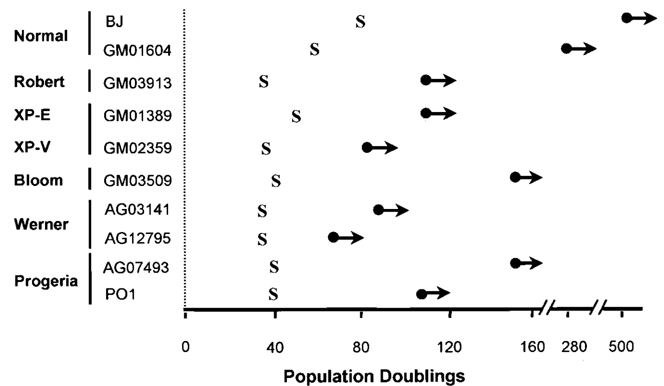


**Figure 2.** Life span of control and hTERT-infected cells with the results expressed in population doublings (PDs). All uninfected control cells eventually entered replicative senescence (S) after a cumulative number of PDs between 30 and 60. All cells infected with the hTERT vector grew well past the PD number at which senescence was induced in the corresponding control strain with the current PD of the hTERT-immortalized lines specified by a closed circle. All hTERT-expressing cells were still dividing at the time of publication (indicated by arrows).

telomeric DNA. The activity of telomerase is present in certain germline cells but is repressed during development in most somatic tissues with the exception of proliferative descendants of stem cells such as those in the skin, intestine and blood (19–23). The enzyme telomerase is a ribonuclear protein composed of at least two subunits; an integral RNA that serves as a template for the synthesis of telomeric repeats (hTR) and a protein (hTERT) that has reverse transcriptase activity (24–28). The RNA component (hTR) is ubiquitous in human cells, but the presence of the mRNA encoding hTERT is restricted to the cells with telomerase activity. We and others have shown that the forced expression of exogenous hTERT in normal human cells is sufficient to produce telomerase activity in these cells and prevent the erosion of telomeres and circumvent the induction of both senescence and crisis (6,7).

Recent studies have shown that telomerase can immortalize a variety of cell types. Cells immortalized with hTERT have normal cell cycle controls, functional p53 and pRB checkpoints, are contact inhibited, are anchorage dependent, require growth factors for proliferation and possess a normal karyotype (29,30). In the present study, we have used hTERT to immortalize skin fibroblasts from patients that suffer from a number of rare hereditary diseases with relevance to genomic integrity. Our results show that the expression of exogenous hTERT efficiently extends their life span without changing the characteristic phenotypic properties of any of the various cells strains examined.

## RESULTS

### Immortalization of control and mutant cell lines

The hTERT cDNA was transferred into cells derived from normal individuals or patients afflicted with rare hereditary disorders associated with chromosome instability. Subsequently, each cell line was followed for extension of cellular life span and compared with the parental cells to determine whether characteristic cellular and biochemical properties were retained.

Primary fibroblasts were infected with a defective retrovirus encoding hTERT (pBabepuro-hTERT). All fibroblasts were derived from skin except where noted. One control cell line, hTERT-BJ, has been described elsewhere (6,30) and a second was derived from the lung tissue of an apparently normal individual (GM01604) (referred to as NOR1 and NOR2, respectively). Genomic instability and/or premature aging syndromes examined included Roberts syndrome (RBS) (GM03913), xeroderma pigmentosum complementation group E (XP-E) (GM01389), xeroderma pigmentosum variant complementation group (XP-V) (GM02359), Bloom syndrome (BLM) (GM03509), Werner syndrome (WRN) (AG03141 and AG12795; WRN1 and WRN2, respectively), and Hutchinson–Gilford progeria syndrome (HGPS) (AG07493 and PO1; HGPS1 and HGPS2, respectively). Each sample was divided into two populations: a control population which was uninfected and a test population that was infected and subsequently selected for retroviral integration with puromycin. In some infections cells were also infected with a vector lacking the hTERT gene and selected in puromycin. In these cases vector alone had no effect on the replicative life span of the primary cells in culture as had previously been shown (6). All immortalized cell lines were compared with the uninfected cells to demonstrate: (i) the presence of telomerase activity; (ii) the capacity to maintain extended telomere size; and (iii) the ability to grow beyond the number of population doublings of uninfected control cells. Using the telomere repeat amplification protocol (TRAP) assay, telomerase activity was found to be absent from all uninfected samples but present in samples that had been infected with the hTERT vector (Fig. 1A). The level of activity detected in the infected cells was comparable with that detected in a human lung cancer cell line, H1299. Telomere size was larger in the immortalized cells than in their uninfected counterpart (Fig. 1B). These results imply that the exogenous telomerase

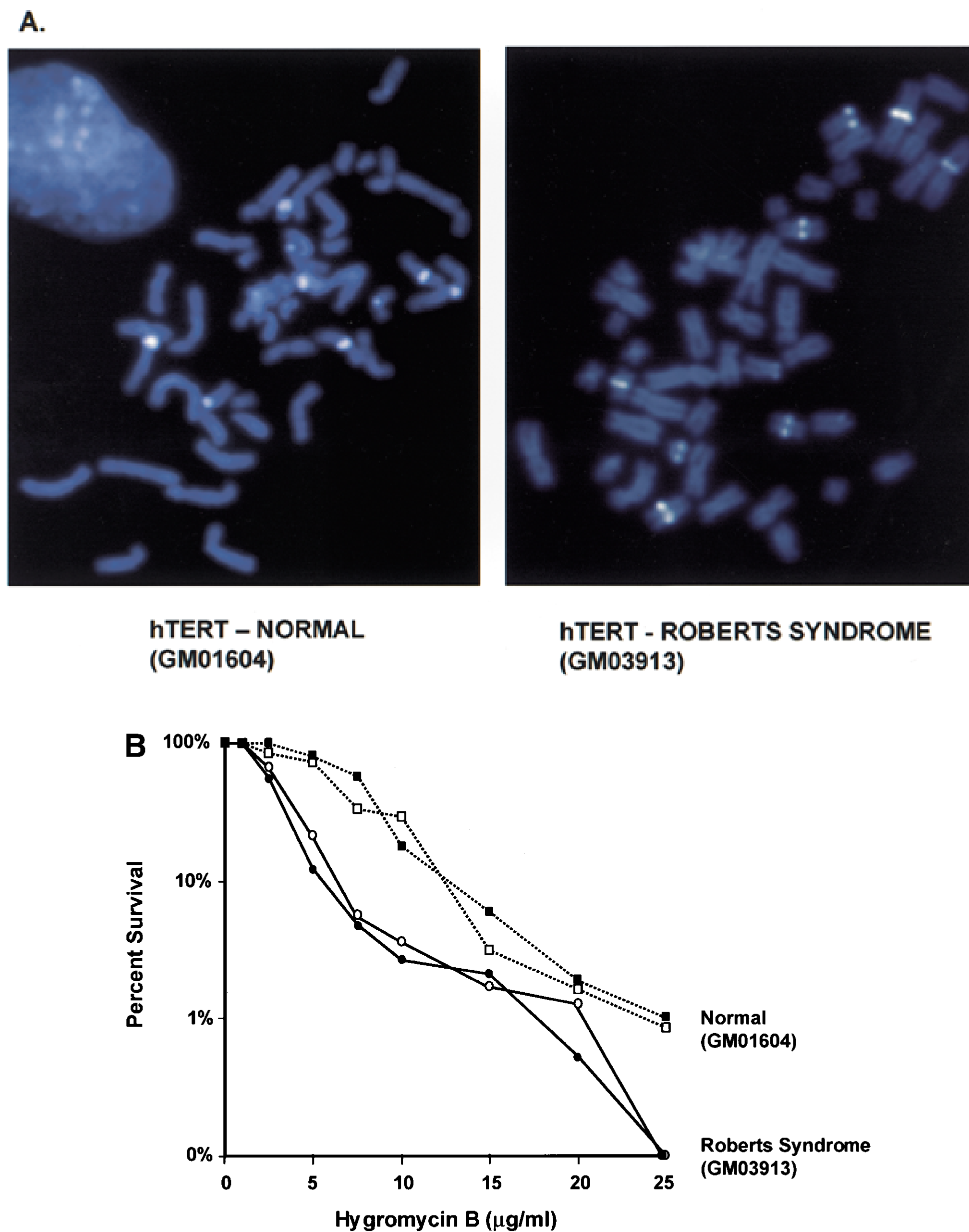


**Figure 3.** Retention of cellular phenotypes in hTERT-infected RBS cells. (**A**) HR phenotype of RBS cells. Metaphase chromosomes from hTERT-normal 2 (GM01604) (left) and hTERT-RBS (GM03913) (right) were stained with DA/DAPI, which causes bright centromeric heterochromatin staining on chromosomes 1, 9, 15, 16 and Y (appears white). The hTERT-RBS cells retain the HR phenotype. (**B**) Sensitivity of both primary (open symbols) and hTERT-immortalized cell lines (closed symbols) to hygromycin B at the indicated doses confirming that RBS cells are more sensitive than 'normal' cell lines.

activity is able to elongate telomeres. All samples were continuously passaged to determine cellular life span. Uninfected samples entered senescence after 30–60 population doublings, typical of the range normally seen for different donors (3) (Fig. 2). The presence of exogenous telomerase extended the life span of all samples irrespective of their origin. At the present time, all telomerase-expressing cultures have exceeded at least twice the life span at which the uninfected cultures senesced. Since these cells are still dividing, they fulfill the functional criteria for describing them as being immortal (29). Cells from each line were characterized for their respective phenotypes only after telomerase expression was confirmed by TRAP analysis, after telomere extension was confirmed by TRF and after they had divided beyond the life span of the primary cells in culture.

## Conservation of mutant cellular phenotypes in cell lines representing chromosome instability syndromes

*Roberts syndrome (RBS).* RBS is characterized clinically by tetraphocomelia, growth retardation and a variety of craniofacial malformations including cleft lip and palate (31). At the cellular level, the disease features genomic instability that is characterized by a mild sensitivity to DNA-damaging agents and protein

**Table 1.** Numerical chromosome instability in hTERT-expressing RBS clones

| Sample | Clone | *n* | HR | Average | Range |
|---|---|---|---|---|---|
| hTERT-normal (BJ) | 1 | 20 | – | 46 | 46 |
| | 2 | 20 | – | 46 | 46 |
| | 3 | 20 | – | 46 | 46 |
| | 4 | 20 | – | 46 | 46 |
| hTERT-RBS (GM03913) | 1 | 20 | + | 48.15 | 46–53 |
| | 2 | 20 | + | 48.05 | 46–50 |
| | 3 | 20 | + | 47.65 | 46–50 |
| | 4 | 20 | + | 47.40 | 46–51 |
| | 5 | 20 | + | 47.15 | 46–49 |
| | 6 | 20 | + | 47.00 | 46–50 |

synthesis inhibitors, and random chromosome gain and loss (32). Metaphase chromosome preparations from RBS cells reveal an unusual chromatin structure referred to as heterochromatic repulsion (HR) (33). In normal metaphase chromosomes constitutive heterochromatin stains as a single structure. In contrast, for RBS chromosomes each individual sister chromatid bears a separately staining heterochromatic block, yielding the appearance of premature separation or HR. Although the aforementioned RBS phenotypes can be complemented *in vitro* by whole cell fusion, the gene responsible for the syndrome has not yet been cloned.

Infection of hTERT was found to extend the life span of RBS cells almost 3-fold (Fig. 2). To verify that the presence of hTERT did not alter the phenotypes associated with RBS cells in culture, the cellular phenotypes of HR, random chromosome gain and loss, and hypersensitivity to hygromycin B were examined. The HR phenotype can be visualized by examining fixed metaphase chromosomes stained by conventional C-banding (34) or with distamycin A (DA) and 4′-6-diamidino-2-phenylindole (DAPI) (35). Examination of hTERT-RBS1 cells by DA/DAPI staining confirmed that the large pericentromeric heterochromatin blocks on chromosomes 1, 9, 15, 16 and Y retained the HR phenotype, whereas no such repulsion was detected in hTERT-NOR2 control cells (Fig. 3A).

Random chromosome gain and loss is a second important characteristic of RBS cells in culture. GM03913 primary RBS fibroblasts exhibit variable numbers (between 46 and 53) of chromosomes. To examine this phenotype in hTERT-expressing RBS cells, hTERT-RBS cells were plated at low density and individual clones were isolated, expanded and analyzed for chromosome content. If introducing hTERT eliminated the random chromosome gain and loss phenotype, each clone would be expected to show a uniform chromosome number. However, all hTERT-RBS clones isolated in this experiment displayed substantial variation in the number of chromosomes seen in individual metaphases (Table 1). These clonal isolates also exhibited the HR phenotype. Normal cells immortalized with hTERT were also examined and, as expected, revealed a stable chromosome content of 46 chromosomes per metaphase in all clones examined.

A third hallmark of RBS is mild *in vitro* hypersensitivity to agents such as hygromycin B. Cells plated at low density were exposed to various doses of hygromycin B. Cell viability was scored to evaluate survival (Fig. 3B). RBS cells exhibited an increased sensitivity compared with normal cells, and the presence or absence of exogenous hTERT did not affect the outcome. These results indicate that the phenotypic properties of RBS cells are unaltered by the presence of exogenous hTERT during their extended life span and also indicate that these apparently immortalized cells should prove useful for characterizing the defect that causes the syndrome.

*Xeroderma pigmentosum (XP).* XP is a rare autosomal recessive disorder which is characterized by a dramatic photosensitivity and early development of ocular and cutaneous neoplasia (36). At the cellular level, the disease results from a defect in the repair of ultraviolet (UV) light-induced DNA damage. XP is a heterogeneous disorder that includes eight genetic complementation groups, seven of which (XP-A, -B, -C, -D, -E, -F and -G) have been associated with defective nucleotide excision repair (NER). The eighth group, the variant (XP-V), represents a defect in DNA replication bypass. The least well understood XP gene products include XP-E and XP-V. Although XP-E cellular extracts are generally regarded as deficient in a damage-specific binding factor, only a few XP-E cell lines reveal mutations in the gene encoding this activity (37). The recently cloned XP-V gene has been shown to encode a DNA polymerase which functions in a poorly understood mechanism associated with the bypass of lesions during DNA replication (38,39). To provide reagents useful for the further characterization of these unique cellular pathways, XP-E and XP-V hTERT-expressing cell lines were generated. Introduction of hTERT was sufficient to extend the life span 2-fold (Fig. 2). Cells plated at low density were exposed to increasing doses of UV light and the number of surviving cells was quantified by scoring colony formation ability (Fig. 4A). Normal cells, as well as SV40-transformed XP-A cells, were included as controls. As expected, hTERT-infected XP-E and XP-V cells exhibited a sensitivity to UV light intermediate between that of XP-A cells and normal cells. Furthermore, the presence or absence of exogenous hTERT does not alter the UV sensitivity of the normal, XP-E or XP-V samples.

XP-E cells also show reduced unscheduled DNA synthesis (UDS), a measure of NER (36). Following exposure to UV light, control cells exhibit a delay in semi-conservative DNA synthesis and initiate NER, demonstrated by the incorporation of [$^3$H]Tdr into repair patches as a consequence of repair (unscheduled) DNA synthesis. All classic XP genetic complementation groups (excluding XP-V) are deficient in UDS. Whereas XP-A cells are most deficient, XP-E cells exhibit only a modest deficiency in NER as measured by UDS. Examination of UDS in hTERT-expressing XP-E cells confirmed that this phenotype was preserved. As expected, UDS was normal in XP-V cells (Fig. 4B). SV40-transformed XP-A cells were included as a control and demonstrated the expected profound defect in UDS.

Normal control cells recover from the UV light-induced inhibition of DNA synthesis more rapidly if the irradiation is delivered as a split dose (a low dose followed 1 h later by a high dose) rather than a single high dose. This split dose response is presumed to reflect the induction, by the initial dose, of the cellular mechanism(s) necessary to repair damage and recover normal semi-conservative DNA synthesis. XP-V cells fail to demonstrate this split-dose damage induction, suggesting that the DNA replication bypass mechanism is an important component of this response (40). Figure 4C shows that control samples re-enter S phase more rapidly under split-dose conditions whether or not exogenous hTERT was present. Although the rate of cell division,

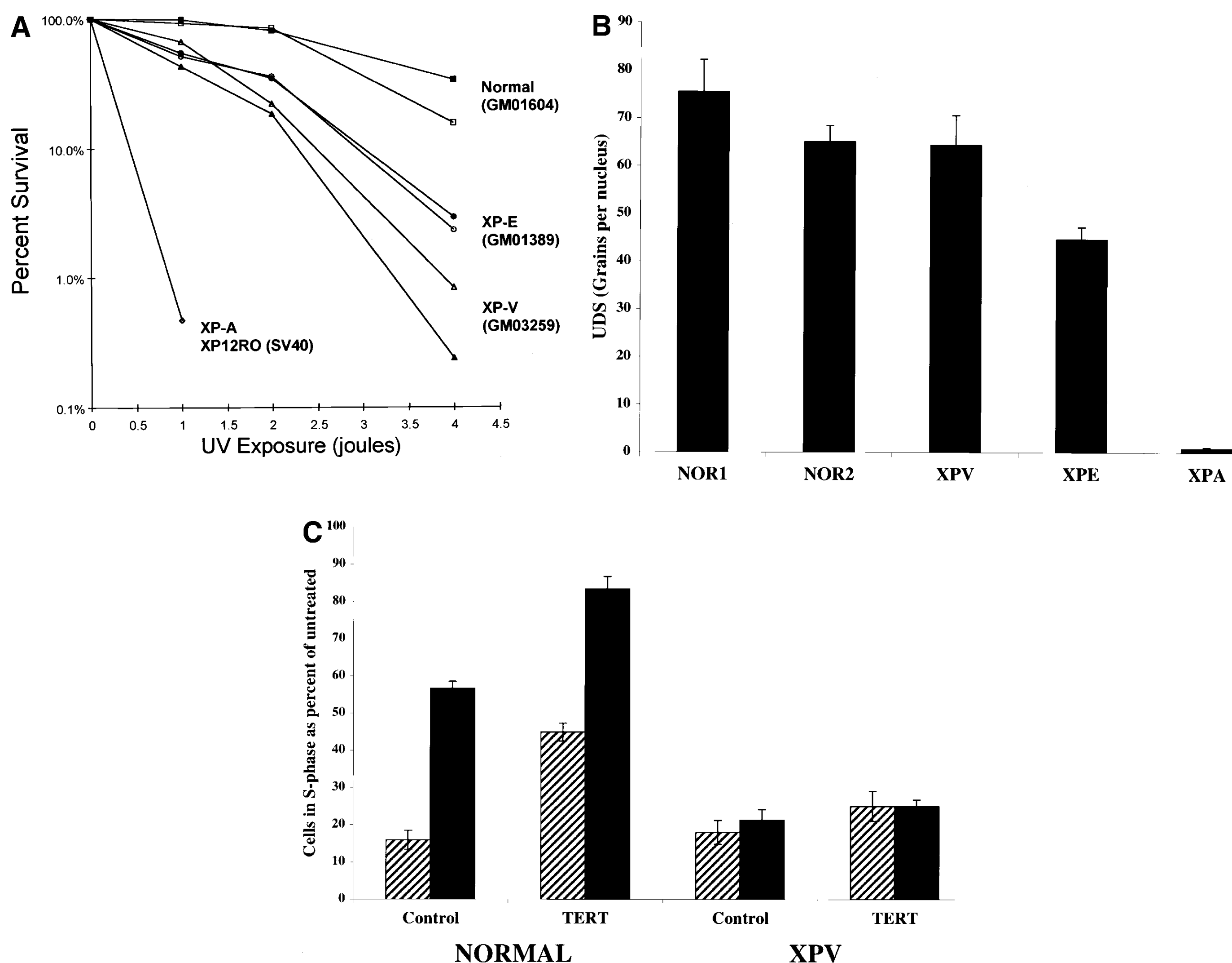


**Figure 4.** Retention of cellular phenotypes in hTERT-infected xeroderma pigmentosum cells. (**A**) UV survival curve demonstrating the intermediate sensitivity of both XP-E [GM01389 (open circles) and hTERT-XPE (closed circles)] and XP-V [GM02359 (open triangles) and hTERT-XPV (closed triangles)] relative to normal cells [GM01604 (open squares) and hTERT-GM01604 (closed squares)] and XP-A cells [XP12R0SV (open diamonds)] at the indicated doses of UV light. (**B**) A UDS assay was performed on hTERT-expressing cell lines representing normal [NOR1 (hTERT-BJ) and NOR2 (hTERT-GM01604)], XPV (hTERT-GM02359), XPE (hTERT-GM01389) and SV40-transformed XPA (XP12ROSV)] cells, illustrating the levels of UDS in XP-V and XP-E cells to be intermediate between normal and XP-A cells. Standard errors are shown. (**C**) Cells were exposed to either 10 J/m$^2$ UV light (hatched) or 1 J/m$^2$ followed 1 h later by an additional 9 J/m$^2$ (black). Results confirm the defective split-dose response in the XP-V (GM02359) cells relative to that seen in normal (GM01604) cells. This result was obtained for both uninfected (Control) and hTERT-infected (TERT) cultures. Over 1000 nuclei were examined for each point and error bars reflect standard error among the six to eight slides examined for each group.

and thus the fraction of cells in S phase, is less in the ‘middle aged’ control cells compared with their hTERT-immortalized derivative, both show the increased response to the split-dose protocol. In contrast, neither XP-V nor hTERT-XP-V samples demonstrate rapid recovery of DNA synthesis in response to the split-dose protocol. Taken together, these results suggest that the phenotypic properties of XP-E and XP-V cells are unaltered by the presence of exogenous hTERT or during their extended life span and also indicate that the immortalized cells still express the defects that are believed to cause the diseases.

*Bloom syndrome (BLM).* BLM is rare autosomal recessive disorder characterized clinically by growth retardation, immunodeficiency, a sun-sensitive facial erythema and significant predisposition to neoplasia. The gene responsible for BLM encodes a protein that is a member of the DNA-dependent DNA helicases with homology to the *Escherichia coli* RecQ protein (41). Mutations in the BLM gene yield increased chromosome breakage and an increased frequency of sister chromatid exchange (SCE). Introduction of hTERT into BLM cells produced at least a 3-fold extension of life span (Fig. 2). SCE frequencies were compared for normal control cells (NOR2), uninfected BLM cells (GM03509; BLM) and BLM cells immortalized by hTERT (hTERT-BLM) or SV40-transformation (GM08505; SV40-BLM) (Table 2). Both hTERT-immortalized and SV40-transformed BLM cells retain a frequency of SCEs >10 times that seen in controls. Interestingly, the frequency of SCE per chromosome was identical in the hTERT-BLM and the SV40-BLM cells despite the aneuploidy that developed in the latter.

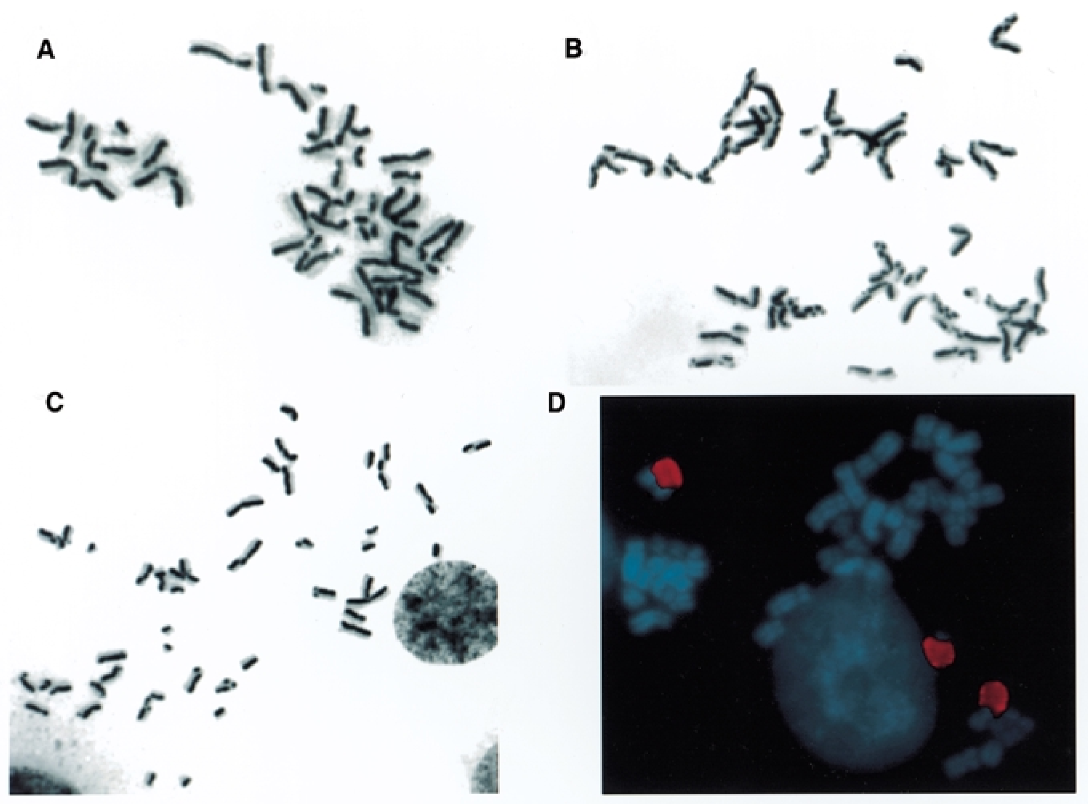


**Figure 5.** Retention of elevated SCE phenotype in hTERT-infected BLM cells. SCE-stained metaphase chromosomes are shown for hTERT-normal (GM01604) (**A**), h-TERT-BLM (GM03913) (**B**) and phenotypically complemented clone hTERT-BLM × A15 resulting from microcell-mediated chromosome transfer of a normal human chromosome 15 into hTERT-BLM cells (**C**). (**D**) FISH results using a chromosome 15-specific probe on a metaphase from the recipient hTERT-BLM × A15 clone detecting three chromosomes 15.

**Table 2.** Sister chromatid exchange frequency of hTERT-expressing BLM and normal cell lines

| | *n* | Chromosome no. | SCEs per cell | SCEs per chromosome |
|---|---|---|---|---|
| TERT-normal (GM01604) | 30 | 46 | 7.2 | 0.16 |
| TERT-BLM (GM03509) | 30 | 46 | 80.1 | 1.74 |
| SV40-BLM (GM08505) | 30 | 77 | 156.0 | 1.93 |
| TERT-BLM × A15 | 30 | 47 | 11.0 | 0.24 |

These results demonstrate retention of the genomic instability phenotype in hTERT-expressing BLM cells.

As a demonstration of the utility of hTERT-immortalized cells for molecular manipulations and genetic complementation, microcell-mediated chromosome transfer was used to introduce a normal chromosome 15 into hTERT-BLM cells. After selection in Geneticin (G418 sulfate), resistant clones were stained for SCEs. The SCE frequency was reduced to control levels in recipient clones demonstrating that complementation had occurred (Table 2, Fig. 5), a result consistent with previous mapping of the BLM gene to chromosome 15 (42). This demonstrates the potential utility of hTERT-immortalized cell lines in genetic screens. Moreover, the hTERT-BLM recipient clones could be shown to exhibit a modal chromosome number of 47 and fluorescence *in situ* hybridization (FISH) revealed the presence of three chromosomes 15 (Fig. 5). Propagation of hTERT-expressing cells in the absence of selection for a newly introduced chromosome revealed no appreciable loss of Geneticin resistance even after several weeks, suggesting that the newly introduced chromosomes are relatively stable. Thus, despite the karyotypic stability and normal checkpoint controls associated with hTERT immortalization, the results indicate that these cells can be molecularly manipulated and are capable of retaining extra chromosomal material.

### Extended life span in cells associated with premature aging syndromes

*Werner syndrome (WRN).* WRN is characterized by the early onset of major geriatric disorders including atherosclerosis, diabetes mellitus, osteoporosis, ocular cataracts and various neoplasms (43,44). Atherosclerosis is the usual cause of death at a median age of 47. Cultures of skin fibroblasts from WRN patients exhibit a mutator phenotype, chromosomal translocation and deletions, and have a shorter cultured life span. The gene responsible for WRN has been identified and encodes a protein

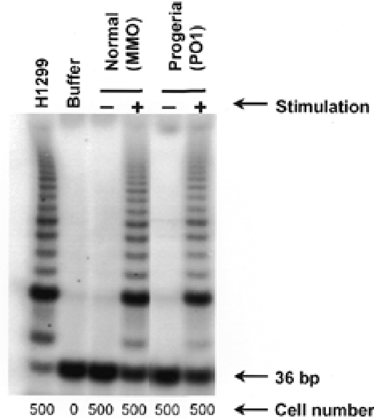


**Figure 6.** Telomerase activity detected in the white blood cells of a patient afflicted with HGPS. Fresh blood lymphocytes were either unstimulated or stimulated for 72 h using an immobilized antibody against CD3 (mouse monoclonal antibody 640-1). Telomerase activity was detected following antibody stimulation in both the normal and the HGPS samples, as well as the positive control (H1299).

with homology to the budding yeast SGS1 DNA-dependent DNA helicase (45). In yeast cells, mutations of the SGS1 helicase lead to the premature senescence of mother cells without any alteration in telomere biology (46). In yeast, the shortening of the telomeres does not control the onset of senescence. Instead, this process is induced by the progressive accumulation of extrachromosomal episomes carrying rDNA sequences. Abrogation of SGS1 function promotes this accumulation and leads to premature senescence (46). Therefore, if mutations in the WRN helicase produce similar defects in human cells, one would predict that exogenous hTERT would not suffice in extending the life span of WRN cells. However, our results show that exogenous hTERT introduced into skin fibroblasts obtained from two WRN patients is sufficient to produce at least a 3-fold extension in life span (Fig. 2).

*Hutchinson–Gilford progeria syndrome (HGPS).* HGPS is characterized by an early-onset premature aging that includes short stature, alopecia micrognathia, premature arteriosclerosis, coronary artery disease, angina pectoris, myocardial infarction, congestive heart failure and absence of subcutaneous fat (47,48). At the cellular level, very few abnormalities have been observed, but skin fibroblasts from some HGPS patients have been reported to have shorter telomeres and a shorter *in vitro* life span compared with skin fibroblasts derived from normal age-matched individuals (49). One possibility is that the symptoms of premature aging associated with progeria might be the consequence of a defect in telomerase or telomeres. To exclude this possibility, we tested the expression of telomerase in white blood cells of patients afflicted with HGPS to determine whether telomerase activity could be induced. The results indicate that a strong and inducible endogenous level of hTERT activity can be detected in these cells and that progeria is not due to a simple lack of telomerase function (Fig. 6). We then examined the consequences of expressing exogenous hTERT in HGPS fibroblasts. Similar to those results seen with all other hTERT-expressing cell lines reported in this study, the expression of exogenous telomerase was able to prevent telomere shortening (data not shown) and was able to extend cellular life span (Fig. 2). These results also imply that the HGPS defect does not limit the access of telomerase to native telomeres present in fibroblast cells. Thus, the 'telomerized' cell lines described here are likely to carry the primary mutation responsible for HGPS and could prove to be valuable reagents in identifying that defect, provided that the gene of interest is expressed in skin fibroblasts.

## DISCUSSION

The present study demonstrates that the expression of exogenous hTERT can extend the life span of skin fibroblasts from patients with genetic abnormalities without changing their phenotypic properties. The establishment of such cells was achieved with high efficiency and in the absence of the biological uncertainties associated with viral transformation. With the exception of the hTERT-RBS cell line, all of the cell lines maintain stable diploid karyotypes, a result consistent with previous studies of telomerized cultures. The instability seen in the RBS cells is consistant with the *in vitro* phenotypes associated with this disease. Based on previous studies, it is also reasonable to expect that these telomerized cells will be free of p53, p16 and pRB mutations (29,30). The genomic stability associated with hTERT infection should offer significant advantages for the study of cells representing chromosome instability syndromes. Despite the karyotypic stability associated with hTERT infection, data presented here demonstrate that these cells can be molecularly manipulated with relative ease including the stable introduction of an entire chromosome (Fig. 5C and D).

Skin fibroblasts from WRN patients have a shorter cultured life span but the mechanism responsible may be different from normal replicative senescence. WRN cells stop dividing with telomeres longer than normally seen at senescence (50), tend to become arrested in the S phase rather than $G_1$ as in telomere-controlled senescence (51), and stop dividing while still being able to express FOS (senescent fibroblasts repress the expression of FOS) (52). The expression of exogenous hTERT in WRN cells none the less extended their life span and these cells are likely immortalized (Fig. 2). It is known that telomere shortening induces replicative senescence by a mechanism involving p53 (10). Our interpretation of these results is that an ongoing low-level damage response produced by the defective WRN helicase only reaches a threshold sufficient to produce growth arrest when combined with the signal produced by shortening telomeres. These telomeres would have shortened to the point where they began to induce a DNA damage signal, but where the strength of the signal would be insufficient by itself to have produced a growth arrest in normal cells. In normal cells, additional telomere shortening would be required before the signal became sufficiently strong to induce senescence. The maintenance of telomeres by hTERT thus prevents this additive effect and WRN cells are able to continue to divide. The availability of hTERT-immortalized WRN cells should contribute to studies attempting to determine the biological function of the WRN helicase.

The symptoms of progeria might be the consequences of alteration in telomere biology based on the fact that some affected individuals have cell types with shorter telomeres. Our results clearly indicate that progeria is not due to a simple lack of telomerase since the enzymatic activity can be detected in the patient's white blood cells where it is properly induced following T cell stimulation. Our results also show that factors in patient cells do not prevent an exogenous telomerase from acting on

native telomeres. The possibility remains that in progeria the endogenous hTERT gene is misregulated or telomerase malfunctional, such that the telomeres are shorter at birth.

Cells from progeria patients have a limited proliferative life span in culture. Studies of their phenotypes have been difficult to interpret because of difficulties in distinguishing between effects due to the use of near-senescent cultures versus effects intrinsic to the genetic defect. The ability to immortalize progeria fibroblasts removes this complication and should greatly facilitate the analysis of this disease.

Established tumor and SV40-immortalized cell lines have been key reagents for the discovery of many fundamental biochemical pathways. The ability demonstrated in this report to use hTERT to immortalize fibroblasts obtained from patients with a wide variety of genetic diseases should now provide a new generation of cellular reagents for the discovery of pathogenic mechanisms.

## MATERIALS AND METHODS

### Introduction of telomerase into skin fibroblasts

Retroviral supernatants were obtained from PA317 packaging cells stably expressing hTERT cloned into the pBabepuro vector (53). Cells were infected and then selected for 2 weeks using puromycin at 750 ng/ml.

### Cell culture

Cells were grown at 37°C in 5% $CO_2$ in a 4:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Media 199 containing 10% iron-supplemented calf serum (Hyclone Laboratories, Logan, UT) and Gentamicin (25 µg/ml) or in DMEM supplemented with 15% fetal bovine serum, 2× non-essential amino acids, and antibiotic/antimycotic (Gibco BRL, Rockville, MD). Metaphase chromosomes were made by standard methods (34). DA/DAPI staining was according to Verma and Babu (35). For hygromycin B sensitivity, cells were grown at various doses for 7 days, stained for viability and counted. UV survival was scored by the methods of Schultz *et al.* (54). SCE staining utilized 5-bromodeoxyuridine (BrdU) incorporation and differential staining (55). Microcell-mediated chromosome transfer and FISH were used as previously described (42,54).

### UDS

UDS was performed as previously described (56). Cells (~$2 \times 10^4$) were seeded into petri dishes containing glass microscope slides. After attachment, cells were exposed to 20 J/M$^2$ UV radiation at a dose of 3 J/M$^2$/s. and subsequently incubated for 4 h in medium containing methyl-[$^3$H]thymidine (49 Ci/mmol) at 15 µCi/ml. Autoradiographic grains were scored on nuclei which were not in S phase and corrected for both background and nuclear size.

### Defective split-dose response in XP-V cells

The experiment examining the split-dose enhancement of the recovery of DNA synthesis following exposure to UV radiation was performed following a previously published protocol (40), with minor modifications. Cells were exposed to 1 J/M$^2$ UV at a dose of 3 J/M$^2$/s, followed 1 h later by an exposure to 9 J/M$^2$. Alternatively, cells were exposed to no initial dose and were subjected to a single dose of 10 J/M$^2$ at the later time point. Non-irradiated cells were included as controls. Following 8 h of post-UV recovery, normal growth medium was supplemented with 10 µg/ml BrdU for 30 min. Cells were washed briefly in phosphate-buffered saline and fixed in methanol:acetic acid (3:1) for 5 min. The percentage of cells that had incorporated BrdU was determined by immunofluorescent staining using a mouse anti-BrdU antibody (Sigma, St Louis, MO) and a fluorescently labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

### Telomerase activity

The TRAP assay was performed using the TRAPeze kit (Intergen, Purchase, NY) as previously described (53). PCR products were electrophoresed on 10% polyacrylamide gels and quantified using the Phosphorimaging system and IMAGEQUANT (Molecular Dynamics, Amersham Pharmacia Biotech, Piscataway, NJ). Quantitation of telomerase activity was done by determining the ratio of the 36 bp internal standard to the telomerase ladder.

### Telomere length

Total genomic DNA was isolated as described previously (53). The DNA was then digested with a battery of six enzymes (*Hin*fI, *Rsa*I, *Cfo*I, *Alu*I, *Hae*III, *Msp*I) and resolved on a 1% agarose gel. The gel was denatured and dried, neutralized and the signal detected *in situ* using a telomeric probe end-labeled with [$\gamma$-$^{32}$P]ATP.

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