Telomeres are specialized structures found at the end of chromosomes, consisting of proteins and tandem G-rich repeats that are conserved in all vertebrae as (TTAGGG)n (1). They are essential elements that protect chromosomal ends from nucle-clease degradation, interchromosomal fusion, and improper recombination, thereby, contributing to genome stability. Telomerase is a specialized cellular ribonucleoprotein reverse transcriptase that synthesizes telomeric repeats onto chromosomal ends (2, 3). It consists of two main components that are required for core enzyme activity, which is measured by the telomeric repeat amplification protocol (TRAP) assay. These are a telomerase RNA, used as template for the addition of new telomeric repeats, and a catalytic protein subunit (TERT). In humans, telomerase is preferentially expressed in germ-line cells and in early embryonic tissues but is not detectable in somatic cells (4). In the absence of telomerase activity, somatic cells undergo a progressive shortening of telomeres with each cell division, because DNA polymerase is unable to replicate the chromosomal ends completely. Eventually when the telomeres on one or more chromosomes become critically short, the cells stop dividing and enter replicative senescence (5, 6).

Telomerase catalytic protein subunit (TERT) has been indicated as a key factor limiting telomerase activity in human somatic cells, because the RNA subunit is constitutively expressed at a low basal level in cells (7). This was confirmed by experiments showing that forced expression of hTERT in human fibroblast, epithelial, endothelial, and osteoblast cells reconstitutes telomerase activity and extends their proliferative lifespan (8–13). However, telomere lengths are not always maintained in these cells, despite the fact that telomerase activity is restored. Similarly, the telomeres of many tumor cell lines are shorter than those in normal cells despite the fact that they have high levels of telomerase activity. For example, telomeres in colorectal and ovarian carcinoma tissues are shorter than in the corresponding normal tissue from the same individual (14, 15). It has been accepted generally that telomere length in human cells is controlled by a homeostatic mechanism that involves telomerase and other factors, such as the TTAGGG-repeat binding proteins, TRF1 and TRF2 (16), but it is not fully understood how these factors cooperate with each other or whether other factors are involved.

We have shown recently that sheep fibroblasts are similar to human fibroblasts in several aspects of telomere biology and replicative senescence. Thus, they have no detectable telomerase activity and undergo only a limited number of cell divisions before entering replicative senescence. Telomere lengths in these cells shorten with cell proliferation at a rate of 50–200 bp per cell division. Finally, forcing hTERT expression in transfected cells restores telomerase activity and extends their proliferative lifespan. In this report, we have followed the continuous growth of these cells for more than 15 months and investigated the telomere maintenance and karyotypic stability as a function of hTERT mRNA and protein levels, as well as telomerase catalytic activity in cell lysates. Our results show that the expression of hTERT reconstitutes telomerase catalytic activity, resulting in cell immortalization. At higher levels of hTERT expression full-length telomeres are maintained and karyotypic stability is preserved. At lower levels, telomere erosion and genomic stability occur to an extent that is directly determined by the level of hTERT expression.
Telomere Length and Telomerase Gene Expression

sodium pyruvate, 1% non-essential amino acids, and 10% fetal calf serum (Globe Farm, Surrey, UK) in a humidified incubator at 37 °C and 5% CO₂.

Plasmid GRN145 was kindly provided by the Geron Corp., which contains hTERT cDNA driven by myeloproliferative sarcoma virus promoter, a human polyadenylation signal, and a human CMV immediate-early enhancer. An empty vector control was constructed by digesting GRN145 plasmid with EcoRI followed by re-ligation to remove the hTERT cDNA fragment. Linearized plasmids were electroporated at 250 microfarads/400 V to B6W6F2 cells at passage 6. Cells were then plated into 96-well plates at 5000 cells per well. Purumycin (750 ng to 1 µg/ml) was added into the medium at least 48 h after transfection to select purumycin-resistant colonies.

Each colony was expanded to confluence in a T₂ flask, from which 3 x 10⁶ cells were plated into a T₁ flask, and the growth curve was started at this point. The growth curve of the parental mass culture, B6W6F2, was started at passage 6, which is the stage the transfection was carried out. Cells were sub-cultured twice weekly and maintained at log phase growth.

Telomerase Activity Assay—Telomerase activity of cell extracts was analyzed by telomeric repeat amplification protocol (TRAP) assay as described previously (18, 19) with a TRAPEze Telomerase Detection kit (Intergen) using either radioactive or non-radioactive labeling of TS primer (19). Each reaction product was resolved in 10% polyacrylamide gel and visualized in a PhosphorImager screen (Amersham Biosciences) and visualized by scanning. To quantify the activities, cell extracts from 0.01 µg to 1 µg were used for TRAP assay, and telomerase activity was calculated as the ratio of the intensity of telomere ladder over the intensity of 36 bp of internal control band. The TRAP assay was calibrated by quantifying activity in serial dilutions of cell extracts. Although the activities decreased with dilution in each cell line within a narrow range, the differences in activity between the various cell lines were consistent at each dilution.

Telomere Length Assay—Genomic DNA from cultured cells was isolated by phenol/chloroform extraction using tubes containing phase lock gel (Eppendorf, Hamburg, Germany). Telomere length was determined by telomere restriction fragment (TRF) Southern blot analysis with either [γ⁻³²P]ATP or digoxigenin labeled (TTAGGG)₃ probe as described previously (20). Mean TRF was calculated as described previously (12).

Fluorescence in Situ Hybridization on Metaphase Spreads and Cyto- genetics—Sheep fibroblasts were harvested after sub-culturing in growth medium for 26 h and incubated in 0.075 M KCl at room temperature for about 10 min. Subsequently, cells were fixed in methanol-acetic acid (3:1, v/v) and were dropped onto clean microscopic slides. FISH was as described previously (21) with a fluorescein isothiocyanate-conjugated telomere peptide nucleic acid probe (Dako, Denmark). The slides were mounted in Vectashield containing 0.1 mg/ml 4',6-diamidino-2-phenylindole. For karyotype analysis, slides with metaphase spreads were stained in 5% Gurro R66 Giemsa at room temperature for 8 min and mounted with DPX mountant. Thirty cells were analyzed from each culture.

RNA Extraction, RT-PCR, and qRT-PCR—Cells were trypsinized and counted and then washed with phosphate-buffered saline. Total RNA was extracted with RNeasy B and digested with DNase I. First-strand cDNA was synthesized from 5 ng of total RNA by reverse transcriptase in a 20-µl volume with oligo(Pd(T)₁₅). Each PCR was carried out with 5 µl of the reverse transcription product and amplified for 26 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The primer sequences used are as follows: hTERT-W1, 5'-AGCGACTACTCCAGCTATG-3', hTERT-W2, 5'-GTTCCTTGGCTTTAGGTTG-3', sheep GAPDH-F, 5'-TGTCCGTGTGAGTACGCCC-3', sheep GAPDH-R, 5'-CGTACGCAAGAATGAGCTTGCAG-3'. Real-time qRT-PCR primers and TaqMan probes were selected for hTERT and sheep GAPDH using Primer Express software (Applied Biosystems). Forward primer for hTERT, 5'-TCACGCCGCTTCAA-3'; reverse primer, 5'-TCCAGAACACGTGGTGACACT-3'. The hTERT TaqMan probe was 5'-Fam-TTGGCGAGCCATGGTCCCTCCAG-Tamara-3'. Forward primer for sheep GAPDH, 5'-TTGCTCTCTGAGCTTTAAC-3'; reverse primer, 5'-AACCAGAAATGAGCTTGCAGAAG-3'. The TaqMan probe for sheep GAPDH was 5'-Vic-GAAGAATGAGCTTGCAGAAG-3'. Protein extracts were pre-treated with Trizol (Invitrogen) and used for reverse transcription in a final volume of 5 µl of reverse transcription master mix (Invitrogen). Total RNA and 1 µl of reverse transcription product were 1:30–60. Assays were performed in quadruplicate using an Applied Biosystems Model 7700 sequence detection system. All data were normalized to sheep GAPDH internal mRNA control (∆∆CT analysis). The same real-time PCR was also performed with hTERT cDNA plasmid at concentration equivalent to 0.01, 0.1, 1, 10, 100, and 1000 copies per cell, and a standard curve was generated. The hTERT mRNA copy number was calculated with the standard curve.

Protein Extract and Immunoblotting—Protein extracts were prepared from logarithmically growing cells by lysis in buffer (25 mM Tris·HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA, 2 mM EGTA). Protein concentration was determined using bicinchoninic acid (BCA) assay (Pierce) according to the manufacturer’s instruction. Protein (40 µg) was separated on a SDS 7.5% polyacrylamide gel and transferred onto nitrocellulose membrane. After blocking for 1 h in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 1% bovine serum albumin, blot were incubated with mouse monoclonal anti-hTERT antibody 1A4 (from Geron Corp., Menlo Park, CA) at 1:10,000 dilution in the blocking solution for 1 h at room temperature. The blots were incubated with secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse IgG, at 1:5000 dilution for 1 h. The signals were detected by incubation with Streptavidin Texas red (Vector laboratories) at 1:200 dilutions for 20 min, and the slides were mounted with Vectashield containing 4',6-diamidino-2-phenylindole.

RESULTS

Immortalization of Sheep Fibroblasts with hTERT—Primary cultures of sheep fetal fibroblasts show similar characteristics to human fibroblasts in that there is no detectable telomerase activity by TRAP assay and the cells undergo only a limited number of cell divisions. Stable transfection and expression of human telomerase catalytic subunit hTERT in these cells constituted telomerase activity (Fig. 1A) and extended their proliferative lifespan. The proliferative capacities of these colonies have been followed for more than 400 days of continuous culture. They maintained a stable growth rate, similar to young fibroblasts and have accumulated 400–500 population doublings (PDs) to date. This is between 8- and 25-fold more than the population doublings achieved by empty vector-transfected clones or clones without hTERT expression, which only grew for a maximum of 61 PDs and then senesced (Fig. 1B). This is also more than 3-fold that of the parental mass culture, B6W6F2, which senesced after 110 PDs (Fig. 1B). hTERT-expressing clones exhibited a similar morphology to young fibroblasts even at high PDs and were negative with senescence associated β-galactosidase staining. Therefore, we consider these cells to be functionally immortal.

Immortalized Lines Exhibit Maintenance or Shortening of Telomeres—The most obvious function of active telomerase is to add telomeric repeats to telomere ends to prevent telomeres from shortening. From a number of recent reports, however, it is unclear whether reconstituted telomerase catalytic activity as measured by TRAP assay is always sufficient to maintain telomere lengths in somatic cells (8–10, 12). In some experiments, overexpression of hTERT reconstituted telomerase catalytic activity, indefinitely extended proliferative lifespan, and maintained the telomere length (8, 9, 22). By contrast, in other experiments, forced expression of hTERT, although extending proliferative lifespan, did not maintain telomere length (10, 12). To assess the effects of reconstituted telomerase catalytic activity on telomere length in the hTERT-expressing sheep fibroblasts, the telomere lengths were measured by Southern blot with a (TTAGGG)₃ probe, and the mean TRF was calculated. We measured telomere lengths at the beginning of growth curve and after about 260–280 days in culture (Fig. 2A). The results showed that all clones have similar telomere length at early passage (PDs = 2). However, after more than
200 cell divisions, some clones (2-1, 2-5, and 2-7) maintained the telomere length (Fig. 2A, lane 3–8), whereas others (1-1, 2-8, 2-12, and 2-13) exhibited telomere shortening (Fig. 2A, lanes 1–2 and 9–14). These results were confirmed by telomere FISH analysis (Fig. 2B). Clones 1-1, 2-8, 2-12, and 2-13 had much lower telomere signals in compare with clones 2-1, 2-5, and 2-7.

Telomere lengths in these cell lines were re-analyzed after further population doublings. In each line, the telomeres became stabilized at a characteristic length, although there was considerable variation between individual cell lines (Fig. 2C and Table I). Clones 2-1, 2-5, and 2-7 maintained their telomere lengths at ~21.0 kb throughout culture. Telomere lengths were the same as they were at the earlier passages (Fig. 2C, lanes 1–6) and, moreover, were the same as those in early passage primary parental cells (Fig. 2C, lane 15). Clones 1-1, 2-8, and 2-12, in which the telomeres had already shortened, did not exhibit further shortening of telomeres and showed no significant difference in telomere length at the two later passages (Fig. 2C, lanes 7–12). By contrast, clone 2-13 continued to exhibit further shortening (Fig. 2C, lane 13 versus 14), but this subsequently stabilized after approximately four hundred population doublings (data not shown). In the cell lines that exhibited telomere shortening, the new, stabilized telomere lengths varied considerably, with mean TRF at about 8.4, 10.8, 14.6, and 6.0 kb in clones 1-1, 2-8, 2-12, and 2-13, respectively (Table I). The stabilized mean TRF in clones 1-1 and 2-13 were substantially shorter than that in parental mass culture, BW6F2, at the time of senescence (mean TRF ~11.3 kb) (Fig. 2C, lane 16) even though these cell lines still grew vigorously.

Telomere Length and Telomerase Gene Expression

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All Immortalized Cell Lines Exhibit Consistent Levels of Telomerase Activity throughout Culture—We next addressed whether the telomere length maintenance was correlated with the levels of telomerase catalytic activity in extracts from these various cell lines. Telomerase catalytic activity was measured by TRAP assay in these clones at early and later passages, and their relative levels were calculated to that in human tumor cell line 293 as described under “Experimental Procedures.” All the clones showed relatively high levels of telomerase catalytic activity, between 30 and 60% of that measured in 293 cells.
FIG. 2. Telomere length in the hTERT-expressing sheep cells. A, telomere length was measured by Southern blot analysis with (TTAGGG)₃ probe at the early passage (PDs = 2) and the later passages (over 200 PDs). There is no telomere shortening in clones 2-1, 2-5, and 2-7, whereas in clones 1-1, 2-8, 2-12, and 2-13 telomere length shortening is clear. B, fluorescent in situ hybridization with telomere peptide nucleic acid probe in representative clones. Clone 2-5 showed much stronger signals than clone 1-1. Population doublings are indicated. C, telomere lengths were measured again after further population doublings and show the stabilization at new levels. PDs are indicated above each lane. Telomere lengths are also shown in the young parental cells BW6F2 (lane 15) at the stage the transfection was carried out, and in the senescent BW6F2 (lane 16).
The mean TRFs at this stage (shorter length, showed a moderately strong correlation with telomere length had been maintained or was stabilized at a decrease of telomerase activity was observed after cell passage and lowest expressing cell lines. No consistent increase or (Table I). The range was narrow, and there was no more than a 2-fold difference in telomerase activity between the highest and lowest expressing cell lines. No consistent increase or decrease of telomerase activity was observed after cell passages. The TRAP activity measured in later passage cells, when telomere length had been maintained or was stabilized at a shorter length, showed a moderately strong correlation with the mean TRFs at this stage (r = 0.696).

**Stabilized Telomere Length Correlates with hTERT mRNA Levels**—Expression of hTERT mRNA in the later passages (PDs > 250) was first analyzed by semi-quantitative reverse transcription-PCR (RT-PCR) in each of the cell lines. In contrast to the TRAP assays the RT-PCR showed there was a broad range of steady-state hTERT mRNA levels (Fig. 3A). Thus in lines 2-1, 2-5, and 2-7 hTERT transcripts were readily detected after 26 PCR cycles, whereas they were only just detectable in lines 2-8 and 2-12. In lines 1-1 and 2-13 as well as in human 293 cells (not shown) the steady-state mRNA level of hTERT were so low that the transcript could not be detected under these conditions. However, when the RT-PCR was repeated using 35 cycles of amplification all samples, including 1-1 and 2-13 and human 293 cells showed detectable hTERT transcripts. To confirm these results and to quantitate the steady-state mRNA levels accurately, we carried out real-time quantitative RT-PCR (qRT-PCR) on these samples. The results are shown in Fig. 3B and tabulated in Table I. They show that the steady-state levels of hTERT mRNA vary over an enormous, 6000-fold range in these lines. Assigning a relative value of 1 to the lowest expressor (2-13) we grouped the lines into three classes according to the level of expression: very low (1-8), low (8-10, lines 2-8 and 2-12), and high (1000–6000, in lines 2-1, 2-5, and 2-7) and corresponded to the result obtained by semi-quantitative RT-PCR (Fig. 3A). We also independently measured the copy number of hTERT mRNA per cell in these clones using real-time quantitative PCR with diluted hTERT cDNA plasmid as standard curve. The results are shown in Table I. The low and very low hTERT expression clones had less than one copy of hTERT mRNA transcript per cell, whereas the highly expressing clones were estimated to contain 80–400 copies per cell.

hTERT protein levels were measured in the cell extracts of each of the lines at both early (4 PDs), middle (105–165 PDs), and later passages (270–360 PDs) by Western blotting using an antibody against hTERT. In all Western blotting experiments clones 2-1, 2-5, and 2-7 showed detectable levels of the correctly sized 127-kDa hTERT protein, whereas, no detectable level of hTERT protein was found in the other clones (Fig. 4A). Human tumor cell line 293, which has high telomerase catalytic activity, did not exhibit detectable levels of hTERT protein (Fig. 4A, lane 9). Although initially surprising to us, this observation is consistent with recent reports using a different hTERT antibody in Western blotting experiments, which, again, failed to detect hTERT protein in 293 cells, despite high levels of telomerase catalytic activity (23, 24). Protein expression was also investigated by immunocytochemistry. Cell lines 2-1, 2-5, and 2-7, in which hTERT was detectable Western blotting showed strong nuclear staining, whereas cells from the other clones, including human tumor 293 were negative (Fig. 4B).

Our measurements on hTERT mRNA and protein levels are fully consistent, and the three cell lines expressing high steady-state levels of hTERT mRNA were the only ones with detectable level of hTERT protein. Indeed, the lowest expressor of these three lines (2-5, which had about 1/5 the steady-state hTERT mRNA level as compared with the other two, Table I) also had the lowest level of detectable hTERT protein (Fig. 4A). We conclude that there is a large range of hTERT expression in these cell lines that is reflected in both the steady-state mRNA and protein levels but not in the telomerase catalytic activity. Shortening of telomeres during extended culture correlated with the hTERT mRNA levels. Thus lines 2-8 and 2-12, which had low levels of hTERT mRNA and undetectable levels of hTERT protein, exhibited telomere shortening, and the telomeres were stabilized with a mean TRF greater than 10 kb. Lines 1-1 and 2-13, having even lower levels of hTERT mRNA, also exhibited telomere shortening. Their telomeres shortened to an even greater extent than 2-8 or 2-12 and only stabilized when the mean TRF was below 10 kb. In these four lowly expressing lines there was a very strong correlation (r = 0.940) between the steady-state mRNA levels and the stabilized telomere length. By contrast, the cell lines exhibiting high steady-state hTERT mRNA levels and detectable hTERT protein (2-1, 2-5, and 2-7) did not undergo telomere shortening and maintained full-length telomere throughout the extended culture period.

**Telomere Shortening Causes Genomic Instability**—Recent reports in yeast and plants suggest that the telomeres play an important role in genome stability (25, 26). To assess the effects of hTERT expression on the genome stability of the stably transfected sheep fibroblast, we examined the chromosomes in these cell lines by cytogenetic analysis (Table II). At beginning of their proliferative lifespan, all hTERT-transfected clones showed normal karyotype (Fig. 5A). However, after about 220 population doublings, clones 1-1 and 2-13, started to exhibit a high frequency of abnormal karyotype (20% or more cells).
These abnormalities included abnormal sub-metrocentric, dicentric, and ring chromosomes (Fig. 5, B–D), which likely resulted from chromosomal end-to-end fusions. The frequency of these abnormalities increased with cell aging; for example, after 266 PDs, line 2-13 exhibited a 100% abnormal karyotype. Lines 2-8 and 2-12 also started to exhibit chromosomal abnormalities after extended culture, although this occurred at later PDs than was the case for lines 1-1 or 2-13. By contrast, cells 2-1, 2-5, and 2-7 essentially maintained a normal karyotype even at high population doublings, although very occasionally an abnormal karyotype was observed (Table II). The genomic stability of these cell lines showed a clear correlation with the level of hTERT gene expression. For example, all the lines were karyotyped after about 280 days in culture. The highly expressing lines, 2-1, 2-5, and 2-7, showed no abnormal karyotype, the lowly expressing lines 2-12 and 2-8, showed a low frequency of abnormality (3 and 20%, respectively), whereas the very lowly expressing lines 1-1 and 2-13 showed high levels of karyotypic abnormalities (63 and 83%, respectively). We conclude that higher levels of expression of hTERT are required to maintain genomic stability and that this is reflected in the maintenance of full-length telomeres. When lower levels of hTERT are present the telomeres shorten and stabilize at a length that is determined directly by the level of hTERT expression. Further-
more, the degree of genomic instability, in terms of the timing and degree of karyotypic abnormalities, is inversely correlated with the level of hTERT expression.

**DISCUSSION**

In this report we show that the forced expression of hTERT in sheep fibroblasts functionally immortalizes these cells in culture. This extends our previous observations showing that hTERT expression can extend the proliferative lifespan of sheep fibroblasts and demonstrates that the hTERT is functionally compatible with the other components of the sheep telomerase complex, including sheep telomerase RNA component. Although hTERT expression leads to immortalization, this does not necessarily lead to the maintenance of full-length telomeres in these cells. Only higher levels of hTERT expression are able to prevent telomere erosion. At lower levels of expression the telomeres shortened and stabilized at a final length that is a direct function of hTERT gene expression level. To our knowledge this is the first demonstration of a direct and quantifiable relationship between telomere length and the level of hTERT expression in mammalian cells. Cell lines with shortened telomere lengths show no evidence of senescence, and they continue to proliferate at the same rate as early passage cells. In some lines the stabilized telomere length is considerably shorter than the telomere length in the senescent parental line. Thus telomerase length per se is not the factor determining whether or not these cells escape senescence and become immortalized. The increased tumor incidence in telomerase null mice suggests that telomere dysfunction induces genetic instability (27). This has been tested directly in a telomerase null strain of yeast (25). The absence of telomerase resulted in an increased frequency of terminal deletions and chromosomal rearrangements. We show here that there is a direct relationship between hTERT expression and genetic instability. Thus highly expressing lines showed a virtually normal karyotype, even after extended culture (Table II). By contrast, lower expressing lines exhibited chromosomal abnormalities. Furthermore, the timing and degree of genomic instability was critically dependent on the level of expression of hTERT, and the lowest expressing line exhibited the earliest and most frequent karyotypic abnormalities. This relationship was clearly reflected in telomere lengths. The highly expressing, karyotypically stable lines exhibited full-length telomeres that were the same size as the telomeres in early passage parental cells (Fig. 2). Although these lines were expressing very high levels of hTERT mRNA, as judged by qRT-PCR and detectable hTERT protein in Western blots, their telomeres were no longer than those in early passage parental cells. This shows that there is a mechanism limiting maximal telomere length in these cells. This is similar to the situation reported for primary human cells where hTERT expression can elongate shortened telomeres present in later passage cells but generally only up to the length characteristic of the early passage cells (8, 22).

In contrast to the high expressers, the four lowly expressing hTERT lines all showed telomere shortening and eventual stabilization at a reduced length. This was exclusively sensitive to hTERT mRNA expression levels with an ∼9-fold reduction in hTERT mRNA level resulting in a reduction of ∼9 kb of the stabilized mean TRF. By extrapolation of the correlation between hTERT mRNA levels and mean TRF lengths, we estimate that it may only require one to two copies of hTERT mRNA per cell to maintain full-length telomeres.

Using 293 cells as a positive control, all the hTERT-immortalized sheep cell lines showed consistent TRAP activities at both early and late passages. This shows that the forced expression of low steady-state hTERT mRNA levels can restore relatively high levels of telomerase catalytic activity in these cells. The catalytic activity varied over only a 2-fold range, in contrast to the ∼6000-fold range of steady-state mRNA levels in the cell lines. Within this limited range there was a moderately strong correlation between mRNA and TRAP activity (r = 0.747) for the four lines expressing less than one copy of hTERT mRNA. The three lines expressing high levels of hTERT mRNA had TRAP activities at the upper end of the range. We conclude that in these lines the level of hTERT mRNA/protein is far in excess of that required to restore maximal telomerase activity in these cells. This is consistent with our estimate of requiring only one to two copies of hTERT mRNA to maintain full-length telomeres. Presumably, at levels of hTERT gene expression above this other factors operate to limit telomere length.

RT-PCR data indicate that most cancer cells contain only a very few (<2 copies per cell) molecules of hTERT full-length mRNA (28). This is in the range of hTERT gene expression we report for the four lowly expressing sheep fibroblast lines in this study. Like tumor cells, these cell lines are immortal. They are also remarkably like tumor cells in that they exhibit shortened telomeres and genomic instability while having relatively high levels of telomerase activity. We would suggest then that low levels of forced TERT mRNA expression in these cells mimic the biological situation in most tumors. The levels of TERT catalytic activity are sufficient to bypass senescence but do not protect against telomere erosion and, ultimately, genomic instability. Although shortening does occur in the presence of these low levels of telomerase, the telomeres are eventually stabilized in both tumors and sheep fibroblasts. The results suggest that the length that the telomeres stabilize is a...
direct function of hTERT expression level and, although this has not been tested, we presume the same will be the case for tumor cells.

In conclusion we have shown that the reconstitution of telomerase activity in sheep primary fibroblasts is necessary but not sufficient to maintain the genomic stability of these cells in culture. To maintain genomic stability over an extended period, higher levels of hTERT gene expression are required. In our cells we estimate that this corresponds to at least one to two copies of hTERT mRNA/cell. This reconstitutes maximal catalytic activity enabling the telomeres to avoid erosion, although it is conceivable that a high level of hTERT expression is itself protective, independent of catalytic activity.

These findings may have important practical consequences

### TABLE II

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*a* DIC, dicentric; LM, large metacentric; Mar, marker chromosome; Ring, ring chromosome; SM, small metacentric; SubM, submetacentric.

**FIG. 5.** Cytogenetic analysis of chromosomal abnormalities. A, normal karyotype in BW6F2 sheep fibroblasts, 54XY, including six metacentric chromosomes. B–D, abnormal karyotype in the hTERT-transfected clones after extended culture. Seven metacentric plus a dicentric (B), abnormal submetacentric (C), and ring chromosome (D) chromosomes. *Arrowheads* indicate metacentric chromosomes, and *arrows* indicate some abnormal chromosomes. *Dic*, dicentric; *Ab SubM*, abnormal submetacentric.
for human cells. Inhibition of hTERT gene expression has been proposed as a potential therapeutic approach to treat cancer (29, 30). Given that the levels of TERT catalytic activity, which are sufficient to bypass senescence, are achieved with very low levels of mRNA, it will be necessary to have extremely effective methods that are capable of reducing steady-state levels to below 0.05 copy per cell (this work and Ref. 28). Second, it has been reported that stem cells from many tissues used in transplantation have active telomerase, but, consistent with our data, this does not prevent telomere shortening with aging of the donors (4, 31–33). We suggest that this is due to the fact that these cells express only insufficient TERT mRNA to restore maximal telomerase activity. Therefore, even after a limited number of cell divisions, the telomeres shorten leading to karyotypic abnormalities and potentially tumorigenic cells in the graft. Introduction of the hTERT gene has been proposed as a means of extending the lifespan of grafted cells for a number of tissues (13, 34). Clearly, from our data, the absolute expression level of hTERT in the grafted cells must be considered as a crucial parameter. This is because grafted cells expressing sub-maximal levels of TERT would allow them to bypassing senescence, but they would still be prone to telomere shortening, genomic instability, and tumorigenic transformation.

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Stabilization of Telomere Length and Karyotypic Stability Are Directly Correlated with the Level of hTERT Gene Expression in Primary Fibroblasts
Wei Cui, Samena Aslam, Judy Fletcher, Diana Wylie, Michael Clinton and A. John Clark

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