Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells

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Human bone marrow stromal cells (hMSCs) were stably transduced by a retroviral vector containing the gene for the catalytic subunit of human telomerase (hTERT). Transduced cells (hMSC-TERTs) had telomerase activity, and the mean telomere length was increased as compared with that of control cells. The transduced cells have now undergone more than 260 population doublings (PD) and continue to proliferate, whereas control cells underwent senescence-associated proliferation arrest after 26 PD. The cells maintained production of osteoblastic markers and differentiation potential during continuous subculturing, did not form tumors, and had a normal karyotype. When implanted subcutaneously in immunodeficient mice, the transduced cells formed more bone than did normal cells. These results suggest that ectopic expression of telomerase in hMSCs prevents senescence-associated impairment of osteoblast functions.

Decreased bone formation and impaired osteoblast function are important mechanisms underlying age-related bone loss[1]. During *in vitro* subculturing, human osteoblasts show a replicative senescence phenotype culminating in growth arrest[2,3]. They also show impaired function evidenced by decreased expression of osteoblast-specific genes, such as core-binding factor 1/Runt-related transcription factor 2 (Cbfa1/Runx2), alkaline phosphatase (AP), collagen type I (Col I), and osteocalcin (OC)[2,4,5]. These alterations may be related to progressive shortening of telomeres[3] leading to defects in chromosome integrity and stability.

Telomere length is maintained by telomerase, a ribonuclear protein complex consisting of an integral RNA (hTR), which serves as the telomeric template, a catalytic subunit (hTERT), which has reverse transcriptase activity, and associated protein components. In the absence of hTERT[6,7], telomeres shorten during cell division because the DNA replication complex cannot completely copy telomeric DNA. Cellular senescence and growth arrest are proposed to occur when telomeres in one or more chromosomes reach a critical length[8]. Supporting this hypothesis is the presence of hTERT and stable telomere lengths in germ cells and most cancer cells[7,9]. Furthermore, ectopic expression of hTERT leads to telomere elongation and extended life-span in a number of cell types, including fibroblasts, retinal pigment cells, and endothelial cells[10,11].

We report here that ectopic expression of hTERT in hMSCs abolishes the senescence-associated phenotype and maintains cell functions including unlimited proliferative ability, capacity to differentiate into multiple cell lineages, and *in vivo* bone-forming ability.

Results

Expression of ectopic hTERT induces telomerase activity and lengthens telomeres. We monitored the transduction efficiency of hMSC-TERTs by examining parallel experiments using a retroviral vector containing green fluorescent protein (GFP) and analyzing the cells (hMSC-GFPs) for GFP expression. The efficiency of transduction was 98%. The presence of the ectopic hTERT gene was verified by Southern blot analysis (Fig. 1A), which showed two bands corresponding to endogenous and ectopic hTERT. Integration site and copy number were determined by fluorescent *in situ* hybridization (FISH). Twenty metaphases and 100 nuclei of hMSC-TERTs at population-doubling level (PDL) 186 were evaluated, and the ectopic hTERT was found to be integrated at chromosome 5q23–31 (Fig. 1B). All metaphases and nuclei showed only one copy of ectopic hTERT and two copies of endogenous hTERT. These data were supported by the results of a second Southern blot using the restriction enzyme *Xho*I, which digests at one site in the retroviral vector. DNA from hMSC-TERTs at PDL 75 gave rise to one extra band in addition to bands from the endogenous genomic hTERT gene, consistent with the presence of a single integration site (data not shown).

Using the telomeric repeat amplification protocol (TRAP) assay, we found telomerase activity in hMSC-TERTs but not in the parental hMSCs or hMSC-GFPs (Fig. 1C). In addition, telomerase activity was not detectable in several other normal strains of hMSCs, but was maintained during continuous subculturing of hMSC-TERTs. The ectopic expression of hTERT resulted in an increased mean telomere length of 18.6 kb at PDL 75 as compared with mean telomere lengths of 9.2 kb and 9.5 kb in normal hMSCs at PDL 15 (Fig. 1D).

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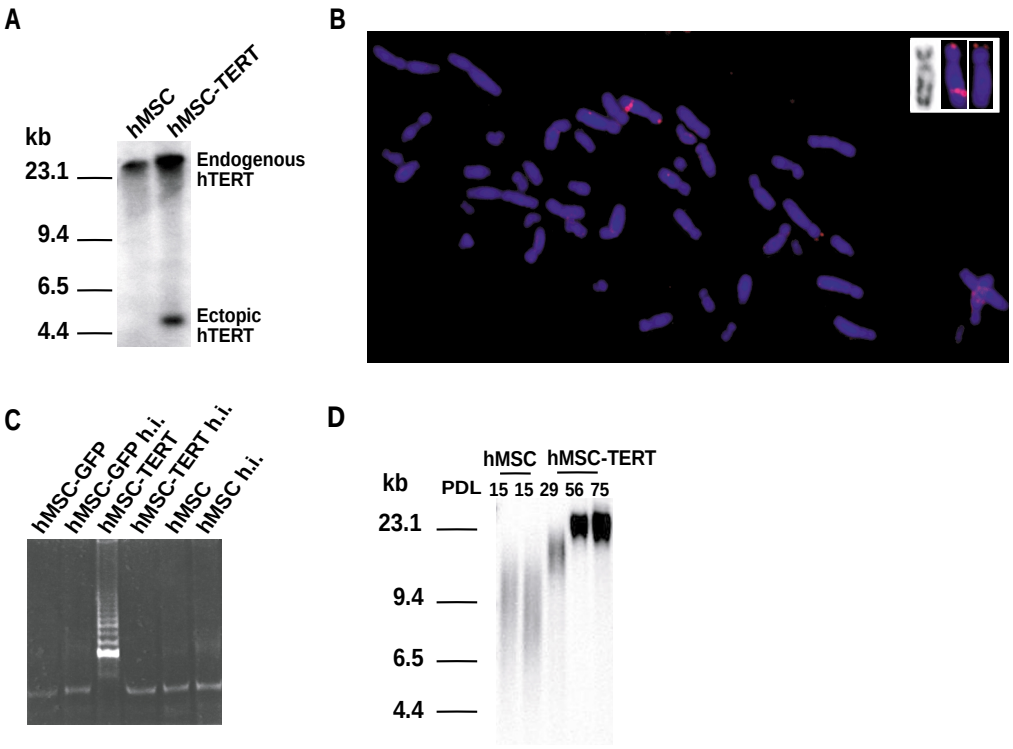

Figure 1. Presence of ectopic hTERT in hMSC-TERTs. (A) Ectopic hTERT was detected by Southern blot using restriction enzyme *Nhe*I to cleave sites flanking the ectopic hTERT cDNA. (B) FISH of hMSC-TERTs. A metaphase spread with GCsamTERT hybridization showed two copies of endogenous hTERT loci and one copy of the ectopic hTERT locus. Inset, homologs of chromosome 5 in comparison with G-banding, showing that ectopic hTERT is situated at chromosome 5q23–31. The inset shows G-banding and FISH in one metaphase chromosome 5. (C) Telomerase activity by hMSC-TERTs is shown, with parental hMSCs and control cell line hMSC-GFP as negative controls. Telomerase activity is revealed by the characteristic 6 bp ladder of bands that disappears upon heat inactivation (h.i.). (D) Mean telomere length assessed by telomeric restriction fragment analysis of hMSCs at PDL 15 and hMSC-TERTs at PDL 29, 56, and 75.

Extended life-span of hMSC-TERTs. The parental hMSC strain underwent senescence-associated proliferation arrest after 26 PD (Fig. 2). hMSC-TERTs were grown as a polyclonal population and have undergone more than 260 PD during the past 2.5 years (Fig. 2). We did not detect any morphological evidence of cellular senescence[2], and we saw positive staining for senescence-associated β-galactosidase (SA β-gal) in a stable proportion of <5% of hMSC-TERTs. More than 90% of senescent normal hMSCs stained positive for SA β-gal.

Expression of osteoblastic markers by hMSC-TERTs. We found that, like normal cells, hMSC-TERTs constitutively express Cbfa1/Runx2. As determined by real-time PCR analysis, Cbfa1/Runx2 expression was 112% (PDL 25) and 138% (PDL 109) of that in normal hMSCs. In the presence of calcitriol, OC mRNA levels in hMSC-TERTs increased 13–18-fold. Measurements of Col I and OC concentrations and AP enzymatic activity (Table 1), together with cellular staining for AP and Col I, demonstrated the production of these osteoblastic markers in hMSC-TERTs.

Enhanced *in vivo* bone formation by hMSC-TERTs. hMSC-TERTs implanted subcutaneously with hydroxyapatite/tricalcium phosphate (HA/TCP) powder in immunodeficient mice formed normal lamellar bone that enclosed marrow cells and adipocytes (Fig. 3A). The amount of bone formed by hMSC-TERTs at PDL 41 was larger than that formed by an equal number of normal hMSCs at PDL 15 (% bone volume/total volume: 17% ± 6% vs. 9% ± 6% (mean ± s.d.), respectively, $P < 0.001$; Fig. 3B). Immunocytochemical staining of implants using human-specific Col I antibody revealed that the bone formed was of donor origin (Fig. 3C). The specificity of the Col I antibody for human tissue was confirmed, as no staining was detected in mouse bone (Fig. 3D) and positive staining was seen in human bone (Fig. 3E).

hMSC-TERTs maintain differentiation potential. After incubation in an adipogenic medium, hMSC-TERT cells formed mature adipocytes with lipid-filled cytoplasm. Furthermore, hMSC-TERTs cultured in micropellets in chondrogenic medium formed morphologically distinct chondrocytes that stained positive for collagen type II (data not shown).

Karyotypic evaluation and *in vivo* tumorigenicity assay. Giemsa banding (G-banding) and comparative genomic hybridization (Fig. 4) revealed that hMSC-TERTs did not develop any numerical or structural chromosomal abnormalities and maintained a normal diploid male karyotype.

hMSC-TERT cells did not form tumors in the immunodeficient mice after subcutaneous implantation for up to six months. In contrast, the fibrosarcoma cell line HT1080 formed tumors after two months.

Discussion

hMSCs are present in the bone marrow stroma and include stem cells and progenitor cells that are able to differentiate into multiple cell lineages[12,13]. Although some stem cells have telomerase activity[9], no telomerase activity was detectable in several hMSC strains established from different donors. In agreement with this, hMSCs had a

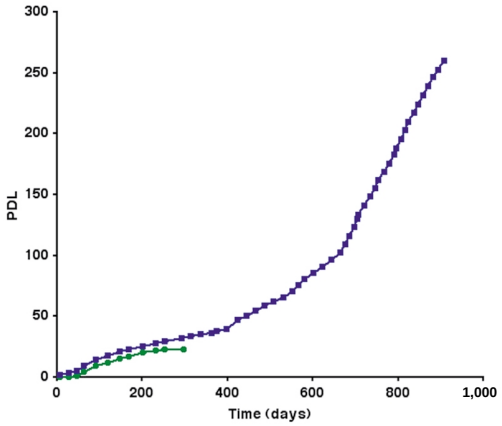

Figure 2. Effect of hTERT expression on life-span of hMSC-TERTs. Growth curves of hMSC-TERTs (blue squares) and parental hMSCs (green circles).

limited proliferative potential *in vitro* and showed characteristics of senescent phenotype with continuous subculture.

We found that ectopic expression of hTERT in hMSCs inhibited the replicative senescence phenotype, maintained normal differentiation potential, and enhanced the cells' bone-forming ability *in vivo*. Telomerase activity was detected in hMSC-TERTs and was maintained despite the long-term cell growth *in vitro*. Mean telomere length in hMSC-TERTs increased, suggesting that hTERT-reconstituted telomerase elongates the telomeres. In other cell lines with ectopic expression of hTERT, either telomere lengthening[11,14] or telomere-length stabilization[15] has been observed.

Whereas several cell strains of hMSCs showed senescence-associated proliferation arrest at 40–45 population doublings (K.S., J.J., and M.K., data not shown), the hMSC-TERTs have undergone more than 260 PD. The PD rate is 0.69/day, similar to that of early-passage young cells. These growth characteristics are typical of established immortalized cell lines[16] and are similar to those of other hTERT-immortalized cell lines[11,15]. However, some investigators have been unable to immortalize human cells through ectopic expression of hTERT alone[17,18]. Clonal analysis of mass cultures of hTERT-transduced MRC-5 fibroblasts showed that there are large interclonal variations in life-span extension and that cellular immortalization cannot be explained solely by telomerase activity or mean telomere length[19]. Thus the ability of ectopic hTERT to extend life-span may be related to the site of integration and the levels of telomere- or telomerase-associated proteins in a cell type–specific manner.

hTERT has been increasingly recognized to affect cellular functions other than proliferation. We found that hMSC-TERTs maintained both expression of osteoblastic markers and differentiation potential. hTERT expression also enhanced the bone-forming ability of hMSCs. In agreement with this, ectopic expression of hTERT in senescent fibroblasts restored their functional capacity in a dermal reconstitution model[20]. Similarly, hTERT expression in human endothelial cells did not affect their phenotype[15] and enhanced their ability to form microvascular structures[21]. These findings suggest that cellular dysfunction associated with replicative senescence is linked to telomere shortening.

Like other cell types that ectopically express hTERT[22,23], hMSC-TERTs did not form tumors when implanted in immunodeficient mice. Furthermore, chromosomal analysis showed a normal karyotype and no evidence of abnormalities associated with malignancy. The nonclonal and transient chromosomal abnormalities reported in some cell lines that ectopically express hTERT[19,24] therefore seem to be rare events. Assuming that hMSC-TERTs maintain a normal phenotype, hTERT reactivation may be useful for tissue regeneration and engineering[25]. A better understanding of the hTERT-activated pathways controlling bone formation may also lead to therapeutic approaches for preventing bone loss during aging and in osteoporosis.

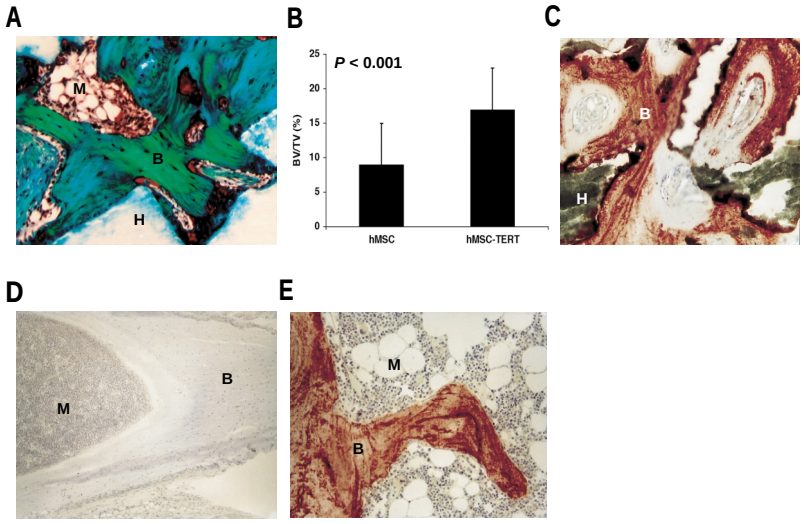

Figure 3. *In vivo* bone formation by hMSC-TERTs. (A) hMSC-TERTs formed normal lamellar bone and osteoid in HA/TCP implants in immunodeficient mice (Goldner's Trichrome staining; magnification 80×) (B) Percent bone volume/total volume (BV/TV) was quantified in implants of normal hMSCs obtained from young donors and in hMSC-TERTs. (C) Immunohistochemical stainings for Col I using a human-specific antibody in a section from a hMSC-TERT implant (magnification 80×). (D) Section from mouse bone (magnification 40×). (E) Section from human bone (magnification 80×). B, bone; M, marrow with adipocytes; H, HA/TCP.

Experimental protocol

Cell culture and retroviral transduction. hMSC cultures were established from bone marrow aspirates as described[26,27]. The hTERT cDNA (plasmid pGRN145, provided by Calvin Hurley, Geron Corporation, Menlo Park, CA) was inserted in the GCsam retroviral vector[28], in which the expression of the transgene was driven by a Moloney murine leukemia virus long terminal repeat. The retroviral construct GCsamTERT was packaged in PG13 (American Type Culture Collection (ATCC), Manassas, VA). hMSC cultures established from a healthy male donor (age 33) were transduced with the retroviral vector after nine days in culture (PDL 12).

Detection of hTERT gene in hMSC-TERT cells. Southern blotting was performed according to standard procedures after genomic DNA was digested with restriction enzyme *Nhe*I or *Xho*I. A 3.4 kb hTERT cDNA randomly labeled with ^{32}P was used as the probe.

The probe used for FISH of the chromosome 5p15.33 region (hTERT) was a bacterial artificial chromosome clone (provided by Nicole Keith, Glasgow, United Kingdom) and was labeled using a nick translation kit (Roche, Lilleroe, Denmark). Hybridizations were performed as described[29].

Assays for telomerase activity and telomere length. The telomerase activity was assessed by the TRAP protocol using the Telomerase PCR ELISA kit (Roche) according to the manufacturer's protocol. Mean telomere lengths were determined by telomeric restriction fragment length analysis using restriction enzymes *Rsa*I and *Hin*fI (Roche).

Assays for cell differentiation. Cells were incubated either in control medium (MEM with 10% FCS) or medium inducing the osteoblastic phenotype (control medium and 10^{-8} M calcitriol (1,25-$(OH)_2$vitamin D_3))

Table 1. Production of osteoblastic proteins by hMSC and hMCS-TERT at different population doubling levels

Cell type	PDL	Calcitriol (10^{-8} M)	PINP (ng/10^5 cells)	AP (nmol PNP/min/10^5 cells)	OC (ng/10^5 cells)
hMSC	12	–	10.0	15.6	ND
		+	20.2	48.3	4.7
hMSC-TERT	41	–	8.5	11.6	ND
		+	16.9	44.4	3.8
	135	–	16.2	24.7	ND
		+	29.4	59.5	14.2

PDL, population doubling levels; PINP, pro-collagen type I N-terminal pro-peptide; AP, alkaline phosphatase; PNP, *p*-nitrophenol; OC, osteocalcin; ND, not detectable.

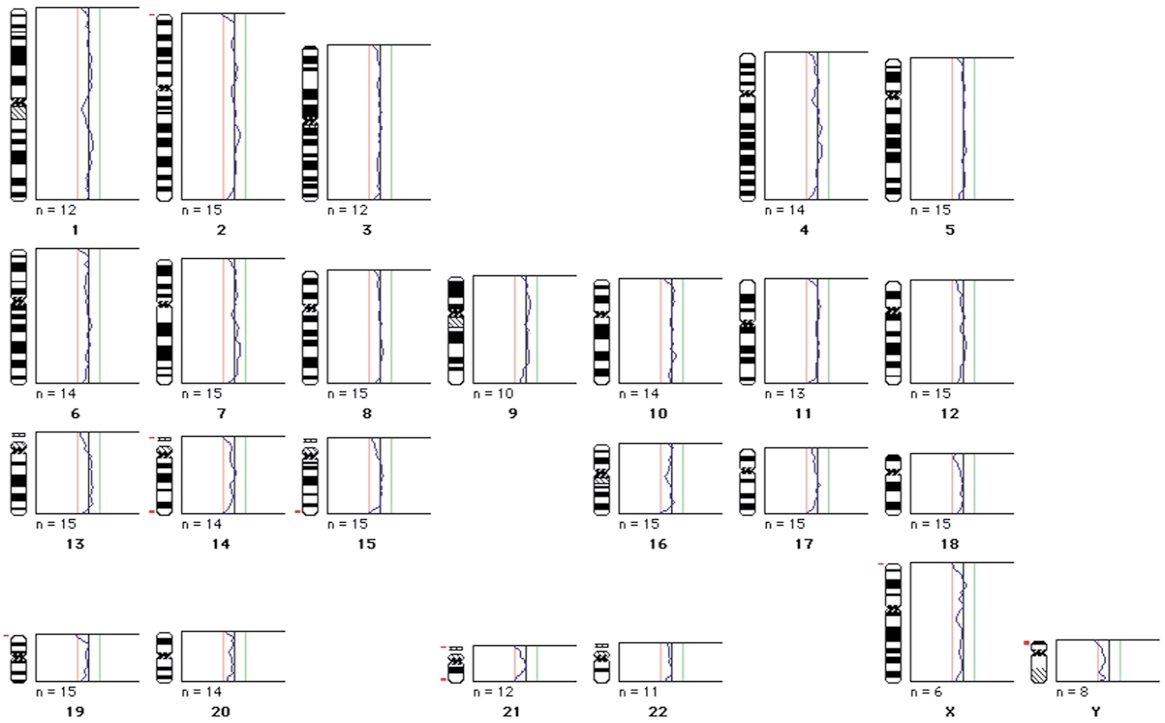

Figure 4. Comparative genomic hybridization on hMSC-TERTs. Comparative genomic hybridization identified no gains or losses at PDL 96. Interpretation profiles compiled from ten metaphases are shown. Blue lines, average profile; red lines, 95% confidence interval. Extreme ends of the chromosomes were excluded from the evaluations because of the technical difficulty of assessing them.

for 48 h, or in medium inducing adipocyte formation (MEM, 15% normal horse serum, 10^{-7} M dexamethasone, 0.45 mM isobutyl methyl xanthine, and 2×10^{-6} M insulin) for three weeks. The chondrogenic potential of hMSC-TERT cells was tested in pellet cultures as described[30]. Col I was measured by a pro-collagen type I N-terminal pro-peptide (PINP) radioimmunoassay kit (Orion Diagnostica, Espoo, Finland) and OC was measured using a N-MID osteocalcin kit (Roche). Enzymatic activity of AP was measured in the cell layers with alkaline phosphatase kit (Sigma Diagnostics, Copenhagen, Denmark). Cell number was determined using methylene blue assay as described previously[27]. Production of Col I, OC, and AP was corrected for variations in cell numbers.

RNA was extracted from confluent cell cultures with Trizol (Invitrogen, Taastrup, Denmark). cDNA synthesis and primers have been described elsewhere[4,26], except for Cbfa1/Runx2 primers: 5′-TCTTCACAAATCCTCCCC-3′ (sense) and 5′-TGGATTAAAAGGACTTGGTG-3′ (antisense). Real-time PCR was performed using an iCycler IQ Real-Time detection System (Bio-Rad, Herlev, Denmark) employing SYBR-Green.

SA β-gal and alkaline phosphatase stainings were performed as described[3,31]. Col I immunocytochemical stainings of cells and implants were performed using an anti-human Col I primary antibody (Silenus, Victoria, Australia).

In vivo bone formation and tumorigenicity assays. Normal hMSCs (pooled from two healthy donors, age 22 and 46, at PDL 13) and hMSC-TERT cells at PDL 41 were mixed with HA/TCP (Zimmer Scandinavia, Broendby, Denmark) and implanted subcutaneously into NOD/LtSz-*Prkdc*^{scid} mice for eight weeks[32]. The amount of bone formed was quantified by point counting according to Cavalieri's principle[33].

hMSC-TERT or HT1080 cells (ATCC) were mixed with Matrigel (Biosciences, Broendby, Denmark) and implanted subcutaneously in NOD/LtSz-*Prkdc*^{scid} mice for one, two, or six months. The implants were paraffin embedded and sections were stained with hematoxylin–eosin. Animal studies were approved by the Danish Experimental Animal Inspectorate (Copenhagen, Denmark).

Karyotypic evaluation. Comparative genomic hybridization was performed as described[34].

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Competing interests statement

The authors declare that they have no competing financial interests.

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