# Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression

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Human postnatal bone marrow stromal stem cells (BMSSCs) have a limited life-span and progressively lose their stem cell properties during *ex vivo* expansion. Here we report that ectopic expression of human telomerase reverse transcriptase (hTERT) in BMSSCs extended their life-span and maintained their osteogenic potential. In xenogenic transplants, hTERT-expressing BMSSCs (BMSSC-Ts) generated more bone tissue, with a mineralized lamellar bone structure and associated marrow, than did control BMSSCs. The enhanced bone-forming ability of BMSSC-Ts was correlated with a higher and sustained expression of the early pre-osteogenic stem cell marker STRO-1, indicating that telomerase expression helped to maintain the osteogenic stem cell pool during *ex vivo* expansion. These results show that telomerase expression can overcome critical technical barriers to the *ex vivo* expansion of BMSSCs, and suggest that telomerase therapy may be a useful strategy for bone regeneration and repair.



In normal somatic cells, each cell division is associated with shortening of telomeres, leading to the eventual arrest of cell growth and proliferation[1–6]. The activation of telomerase, the ribonucleoprotein responsible for extending telomere length at the end of chromosomes, can prevent telomere erosion and inhibit replicative senescence *in vitro*[7–9]. Although telomerase activity is absent or present only at very low levels in most normal human somatic cells, it seems to be expressed by highly proliferating germline cells, hematopoeitic stem cells, and various types of cancer cells[5,10–17]. Telomerase contains an RNA component, which provides the template for the synthesis of TTAGGG repeats, and protein components including the catalytic subunit hTERT[4,8,18–19], which provide the reverse-transcriptase activity.

Possible mechanisms of age-dependent bone loss may be attributed, at least in part, to a deficiency of osteoblast function and/or a decrease in the number of osteogenic progenitor cells rather than to an increase in bone resorption by osteoclasts[20–24]. It has been suggested that telomere-associated cellular senescence may contribute to various age-related disorders[25]. Recent studies reported that the introduction of hTERT into osteoblasts isolated from human trabeculae induced telomerase activity and extended the life-span of these cells[26]. However, the role of telomerase in bone formation, particularly with respect to maintenance of the osteogenic precursor cell population, is largely unknown. Pluripotent human BMSSCs were originally described as progenitors of osteoblasts because of their capacity to form normal bone *in vivo*[27,28]. BMSSCs are considered new therapeutic agents for repairing critical-size bone defects that normally cannot undergo spontaneous healing[27–30].

Primary BMSSCs must be expanded *ex vivo* to obtain large numbers of BMSSCs for cell transplantation. A potential obstacle to *ex vivo* expansion, however, is that BMSSCs have a limited replicative life-span and gradually lose their osteogenic potential *in vitro*[28,29]. In this study, we investigated whether ectopic expression of the *hTERT* gene in BMSSCs extends their replicative life-span and maintains their bone-forming capability. We found that telomerase expression not only extends the life-span of BMSSCs but also enhances their bone-forming capacity *in vivo*. To the best of our knowledge, it has not been shown previously that telomerase expression can maintain the phenotypic and functional properties of adult somatic stem cells. These results suggest that telomerase therapy may be useful in the repair of human bone injuries and bone defects.

## Results

**hTERT expression extends the life-span of BMSSCs *in vitro*.** Under normal culture conditions, BMSSCs, like other somatic cells, begin to senesce after prolonged expansion. We found no detectable telomerase activity in human adult primary BMSSCs at early passages, as examined by the telomeric repeat amplification protocol (TRAP) assay (Fig. 1B; data not shown). We therefore tested whether overexpression of hTERT could extend the life-span of BMSSCs after extensive propagation *in vitro*. Primary BMSSC cultures were first established after selection with the osteogenic precursor marker STRO-1 using fluorescence-activated cell sorting. Multicolony-derived cells were transduced with either hTERT or control retroviral particles to mimic the cellular heterogeneity typically seen in normal primary BMSSC cultures. Both BMSSC-Ts and control cells (BMSSC-Cs) were assessed for hTERT

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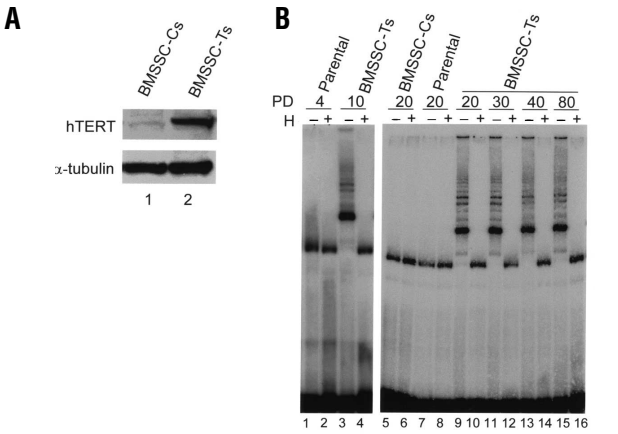


**Figure 1.** Ectopic expression of hTERT induces telomerase activity in BMSSCs. (A) For detection of hTERT protein by western blot analysis, whole-cell protein extracts prepared from PD20 BMSSC-Cs (1) and BMSSC-Ts (2) were separated by SDS–PAGE (80 μg protein/lane) and then probed with polyclonal antibodies reactive to either hTERT or human α-tubulin. (B) Assessment of telomerase activity in BMSSC-Ts and BMSSC-Cs after continuous subculture *in vitro* for the indicated number of population doublings. Cell extracts were prepared as described in the Experimental Protocol and telomerase activities were analyzed by TRAP detection kit. H, heat-inactivated cell extracts.

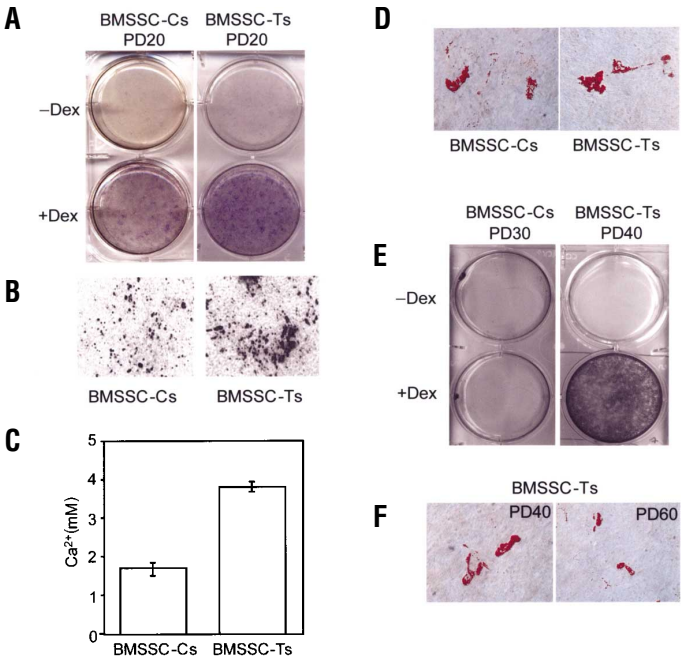


**Figure 2.** Telomerase expression enhances osteoblast differentiation *in vitro*. (A) Alkaline phosphatase activity in cultures of BMSSC-Cs and BMSSC-Ts (PD20) induced with L-ascorbate-2-phosphate, dexamethasone, and inorganic phosphate for two weeks. (B) von Kossa–staining calcified deposits in BMSSC-C and BMSSC-T (PD20) cultures after six weeks of osteoinduction. (C) Measurement of matrix calcium levels released by acid treatment in BMSSC-C and BMSSC-T (PD20) cultures after six weeks of osteoinduction. (D) Detection of oil-red O–positive, lipid-laden adipocytes in replicate cultures. (E) Staining for von Kossa–positive mineralized deposits in BMSSC-C (PD30) and BMSSC-T (PD40) cultures after six weeks of osteoinduction. (F) Detection of oil-red O–positive, lipid-laden adipocytes in BMSSC-Ts (PD40 and PD60) cultures.

protein expression by western blot analysis (Fig. 1A). Predictably, telomerase activity was detected in BMSSC-Ts, but not in BMSSC-Cs, using the TRAP assay (Fig. 1B). At early cell passages equivalent to 20 population doublings (PD20), there was no significant difference in the proliferation rate between BMSSC-Ts and BMSSC-Cs, as measured by the bromodeoxyuridine (BrdU) uptake method. With continuous expansion *in vitro*, BMSSC-Ts at PD40 maintained a rate of proliferation comparable to that seen in early passages. In contrast, proliferation rates of BMSSC-Cs at PD30 had decreased by up to 50% (see Supplementary Fig. 1 online). Moreover, BMSSC-Cs and untransduced parental cells became senescent after 32 PD, whereas BMSSC-Ts continued growing *in vitro* for over 80 PD (data not shown). These findings indicate that the replicative life-span of BMSSCs can be prolonged by ectopic expression of hTERT.

**hTERT expression enhances the osteogenic potential of BMSSCs *in vitro*.** BMSSCs are known progenitors of skeletal tissues and differentiate into osteoblast-like cells in cultures supplemented with ascorbic acid and a source of glucocorticoid[27–30]. However, BMSSCs gradually lose their osteogenic capacity during continuous subculture *in vitro*[28,29]. This may limit their therapeutic use, as effective treatment of extensive bone defects requires large numbers of *ex vivo*–expanded BMSSCs. To examine whether an increase in the life-span of telomerase-expressing BMSSCs affects their osteogenic potential, we cultured the cells under conditions previously shown to induce calcium accumulation *in vitro*[31]. After 14 days of osteogenic induction at PD20, alkaline phosphatase (ALP) activity was increased in both BMSSC-Ts and control cultures (Fig. 2A). Although not specific to the osteoblast lineage, ALP is expressed by functional osteoblasts and precedes the onset of mineralization. After four weeks of osteoinduction, BMSSC-Ts produced considerably more mineralized deposits than BMSSC-Cs as detected by von Kossa staining (Fig. 2B). Calcium levels were measured after acid treatment of extracellular matrix (Fig. 2C). BMSSC-T cultures contained approximately twofold higher calcium levels than did the corresponding BMSSC-C cultures. In addition, sporadic clusters of adipocytes that stained positive for oil-red O were also seen for both BMSSC-Ts and BMSSC-Cs under long-term osteogenic induction, confirming the pluripotency of the telomerase-expressing cells (Fig. 2D). Similar studies verified the capacity of BMSSC-Ts to accumulate lipid when cultured in the presence of an adipogenic cocktail that contains methylisobutylxanthine, hydrocortisone, and indomethacin[32] (data not shown). Finally, in contrast to BMSSC-Cs, BMSSC-Ts propagated in culture for 40 PD retained their osteogenic potential *in vitro* (Fig. 2E). BMSSC-Ts at PD40 and at PD60 were still capable of differentiating into adipocytes (Fig. 2F). Collectively, these data suggest that telomerase expression can enhance and/or maintain the differentiation potential of BMSSCs during *ex vivo* culture.

**Telomerase expression increases bone formation *in vivo*.** Next, we determined whether telomerase expression affects the bone-forming capacity of BMSSCs *in vivo* using a well established mouse transplantation model in which ceramic hydroxyapatite/tricalcium phosphate particles are used as a carrier vehicle[28,33]. First, we compared the osteogenic potential of early-passage (PD20) BMSSC-Ts and BMSSC-Cs by subcutaneous transplantation into immunocompromised mice. Implants of BMSSC-Ts, harvested two months post transplant, contained all the cellular components and matrix structures seen in normal control transplants, including lamellar bone covering the HA/TCP surfaces, bone-lining osteoblasts, encapsulated osteocytes, and fibrous tissue with associated hematopoietic marrow elements (Fig. 3A, B). Quantitative image analysis (Scion Image software) showed that BMSSC-Ts generated significantly more bone (fivefold, $P < 0.01$) than did their matched BMSSC-Cs (Fig. 3C) and parental BMSSCs (data not shown), using cell populations transplanted at PD20. Histological characterization of the BMSSC-T

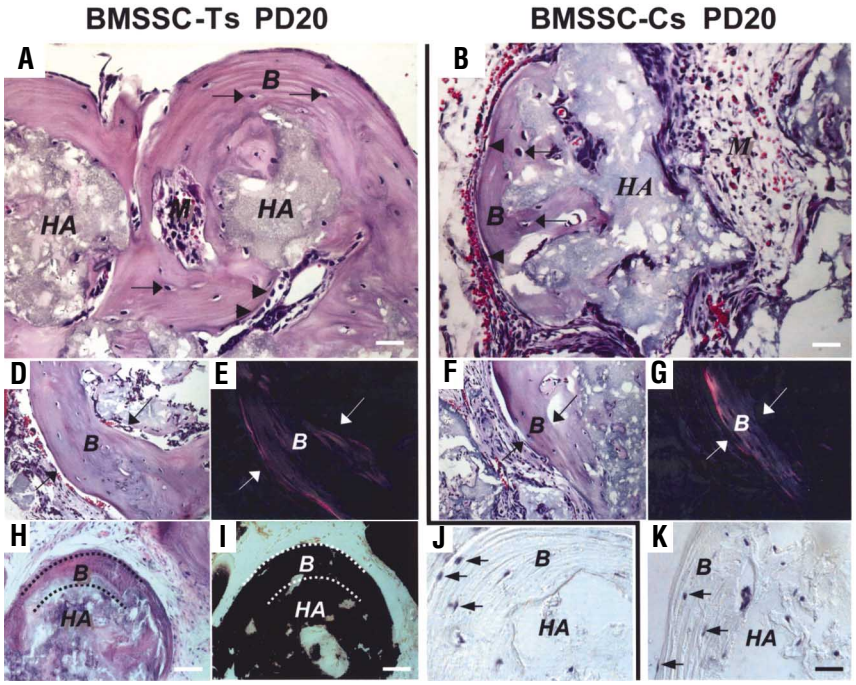


**Figure 3.** Histological characterization of regenerated bone by transplantation of BMSSC-Ts at PD20 and BMSSC-Cs at PD20 *in vivo*. Shown are paraffin-embedded cross-sections of representative BMSSC-T (A) and BMSSC-C transplants (B) harvested eight weeks after transplantation and stained with H&E. Lamellar bone (*B*) was generated on the surfaces of the HA/TCP carrier (*HA*) lined with osteoblasts (triangles) and containing entrapped osteocytes (arrows), in association with a hematopoeitic marrow element (*M*). (C) BMSSC-Ts generated over fivefold more bone than did BMSSC-Cs *in vivo.* Five representative areas from each group were quantified and averaged using Scion Image software. A magnified view of regenerated bone in BMSSC-T transplants (*B*) stained with H&E under normal (D) and under polarized light (E), showing ordered collagen fibers (arrows). Similar views were shown of regenerated bone in BMSSC-C transplants stained with H&E under normal (F) and polarized light (G). (H) A section of plastic-embedded BMSSC-T transplant showing regenerated bone (*B*) inside the dash lines on the surface of HA/TCP (*HA*) after H&E staining. (I) A serial section of the same mineralized regenerated bone is shown positive for von Kossa staining. *In situ* hybridization showing osteocytes and bone-lining osteoblasts (arrows) positive for the human-specific repetitive *Alu* DNA sequence in regenerated bone (*B*) in BMSSC-Ts (J) and BMSSC-C transplants (K). Bar, 40 μM in panels A, B, 20 μM in panels C–F, I, J, 60 μM in panels G, H.

transplants yielded an ordered matrix rich in parallel collagen fibers (Fig. 3D, E) supporting a von Kossa–positive mineralized lamellar bone structure containing osteocytes and bone-lining osteoblasts of human origin (Fig. 3H, I, J), confirming the ability of BMSSC-Ts to generate normal bone tissues *in vivo*.

We examined whether telomerase expression maintained the bone-forming capacity of BMSSCs *in vivo* after extensive propagation in culture. As shown in Figure 4A and B, the osteogenic potential of BMSSC-Cs at late cell passage (PD30) decreased markedly, whereas BMSSC-Ts (PD40) retained a strong osteogenic capacity. Quantitative analysis demonstrated that BMSSC-Ts generated ten times more bone than BMSSC-Cs at PD40 for BMSSC-Ts and PD30 for BMSSC-Cs (Fig. 4C). Moreover, BMSSC-Ts at PD80 still generated ectopic bone tissues *in vivo*, although they had less bone-forming capacity than at PD40 (Fig. 4D).

As telomerase activity has been associated with oncogenesis or transformation, we tested whether BMSSC-Ts acquired a malignant phenotype using established *in vitro* and *in vivo* assays. We found that BMSSC-Ts did not grow in soft agar and were unable to develop tumors when injected subcutaneously into nude mice (data not shown). Taken together, these results show that telomerase expression significantly enhances and maintains the bone-forming capacity of BMSSCs *in vivo* without inducing oncogenic transformation.

**Telomerase expression maintains the pre-osteogenic phenotype of BMSSCs.** To gain insight into the mechanism(s) by which telomerase expression increases the bone-forming capacity of BMSSCs, we characterized the cell populations that constituted the BMSSC-T and BMSSC-C cultures before transplantation by measuring the number of cells expressing the stromal stem cell marker STRO-1 using flow cytometric analysis. Previous studies have shown that the monoclonal antibody for STRO-1 identifies all colony-forming osteogenic precursor cells isolated from aspirates of adult human bone marrow[31]. The STRO-1 antigen is progressively lost by the progeny of BMSSCs in culture and seems to be restricted to a subpopulation of cells that maintain an immature, pre-osteoblastic phenotype[34,35]. Notably, we found that BMSSC-Ts at PD20 contained 1.7-fold more STRO-1-positive cells than did control BMSSCs (Fig. 5). As described above, because we used whole cell populations in our experiments, differences in STRO-1 expression between BMSSC-Ts and BMSSC-Cs probably did not result from clonal variation. These results suggest that telomerase expression might help to preserve an immature phenotype, indicated by STRO-1 expression, during the early stages of cell propagation and stable selection. To confirm this, we examined STRO-1 expression in later cell passages. Over successive subculture, BMSSC-Ts at PD40 maintained a similar level of STRO-1-positive cells (Fig. 5). By contrast, the number of STRO-1-positive cells in BMSSC-Cs declined rapidly during consecutive passages, with about threefold fewer at PD30 than are found in BMSSC-Ts at PD40 (Fig. 5). Our results imply that telomerase activity may help to maintain a higher proportion of primitive osteogenic BMSSCs after *ex vivo* expansion. The effect may also be related to our culture conditions, however, as STRO-1 expression in BMSSC-Ts at PD80 was significantly less than at PD40 (data not shown).

## Discussion

Human BMSSCs, also referred to as mesenchymal stem cells, are defined as pluripotent progenitor cells with the ability to differentiate into osteoblasts, chondrocytes, adipocytes, muscle cells, and neural cells[27–30,36,37]. Recent efforts have focused on using *ex vivo*–expanded BMSSCs as therapeutic agents for repairing critical-size bone defects that normally do not undergo spontaneous healing[28,38,39]. To date, preliminary studies have shown that vast numbers of *ex vivo*–expanded BMSSCs are required to repair relatively small bone fractures in animal models[38]. The efficacy of this type of cellular therapy is further complicated by the limited life-span of normal

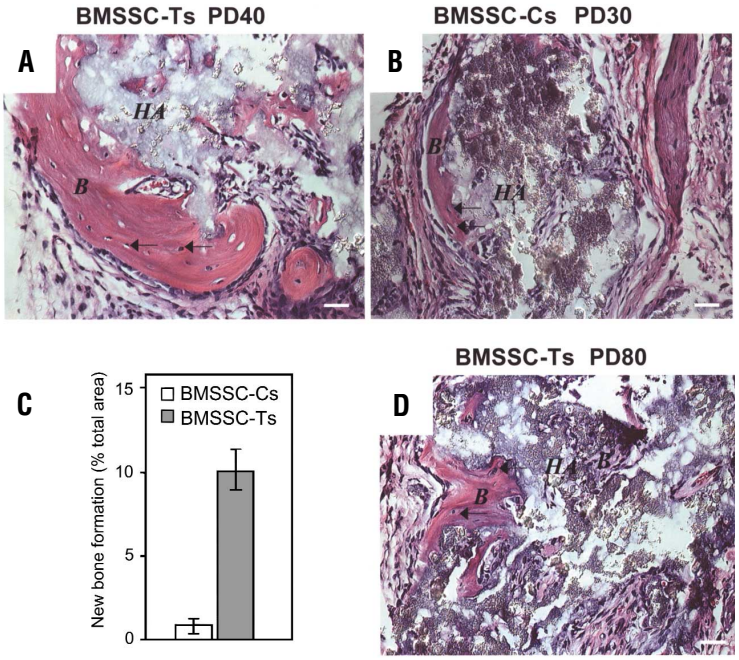


**Figure 4.** Telomerase expression extends bone-forming capacity of BMSSCs *in vivo*. Both BMSSC-Ts at PD40 (A) and BMSSC-Cs at PD30 (B) were transplanted in mice for eight weeks. Ectopic bone formation (*B*) on the surface of HA/TCP (*HA*) containing encapsulated osteocytes (arrows) was examined by H&E staining. BMSSC-Ts generated ten times more bone than did BMSSC-Cs when the same numbers of cells were transplanted *in vivo* (C), as determined by Scion Image analysis. Ectopic bone formation by BMSSC-Ts at PD80 in mice (D) was examined by H&E staining. Bar, 40 μM.

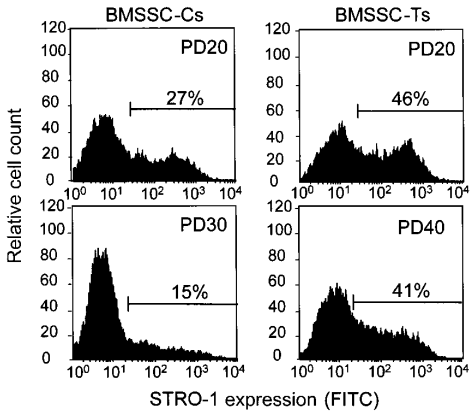


**Figure 5.** Telomerase expression enhances and maintains STRO-1 expression in BMSSCs. Both BMSSC-Ts and BMSSC-Cs at early or late cell passages were fixed and stained with FITC-conjugated STRO-1 antibody and analyzed by flow cytometry. Experiments were performed in duplicate. BMSSC-Ts had a relatively high ratio of cells expressing STRO-1: 46% at PD20 and 41% at PD40, respectively. In BMSSC-Cs, by contrast, 27% of cells expressed STRO-1 at PD20, and the population of STRO-1-positive cells decreased to 15% by PD30.

BMSSCs and by the progressive loss of their osteogenic potential after extensive propagation *in vitro*[28,29]. Consistent with previous studies on fibroblasts and pigmented epithelial cells[9], we found that ectopic expression of hTERT extended the replicative life-span of BMSSCs *in vitro*. Unexpectedly, we found that telomerase expression not only extended, but also enhanced the osteogenic potential of BMSSCs, which correlated with sustained high levels of expression of the stromal stem cell marker STRO-1. These results strongly suggest that telomerase expression may act to prevent phenotypic changes of BMSSCs during *ex vivo* expansion.

Currently, we do not know how telomerase expression is associated with STRO-1 expression. It is possible that STRO-1-positive cells have a reduced replicative life-span *in vitro* as compared with STRO-1-negative cells. Thus, STRO-1-positive cells would be gradually lost by replicative aging and progressively overgrown by STRO-1-negative cells. Overexpression of telomerase might help to promote or maintain replicative life-span and growth rate of STRO-1-positive cells *in vitro*, resulting in a greater fraction of STRO-1-positive cells in BMSSC-Ts. We did observe, however, that STRO-1 expression and the bone-forming capacity of BMSSC-Ts decreased markedly after prolonged culture (>PD80), although telomerase activity was maintained (data not shown). These results suggest that our culture conditions or other factors may play important roles in the regulation of STRO-1 expression and in BMSSC function.

Several studies have found that the yield of BMSSCs gradually declines with the age of the donor[20–24,40–42]. We found that adult BMSSCs (derived from patients age 20–35) had no detectable telomerase activity and that their life-span was extended by telomerase expression. In this regard, it is also possible that BMSSCs from older donors, like other somatic cells, may have shorter replicative life-spans than those from younger donors. It would be interesting to examine whether telomere shortening is associated with the osteogenic potential of BMSSCs, which declines with age. Telomerase plays an important role in preventing the telomere shortening that leads to replicative senescence. Thus, the prevention of proliferative failure or of conflicting DNA-damage signals from shortened telomeres is probably an important mechanism by which ectopic expression of hTERT maintains the progenitor function of BMSSCs. Recent studies show that the absence of telomerase increases mutation rates in *Saccharomyces cerevisiae*, suggesting that telomerase can inhibit chromosomal instability[43]. Thus, it is also possible that the introduction of telomerase into BMSSCs may help to maintain their genomic stability during *ex vivo* expansion.

Although telomerase activity has been associated with tumorigenesis[5,16,17], we found that telomerase expression in BMSSCs did not induce oncogenic transformation, as determined by soft agar assay and tumor growth *in vivo*. Importantly, our *in vivo* xenogenic transplantation model showed that telomerase-expressing BMSSCs generated normal bone tissues with organized collagen fibers, mineralized components, and an associated hematopoietic marrow element. Consistent with our results, several studies have found that ectopic expression of hTERT in normal human somatic cells did not induce malignant transformation[9,14,15,44]. Telomerase therapy has been proposed for diseases other than cancer, including liver cirrhosis and Werner syndrome[45,46]. We have shown that telomerase expression can potentiate bone regeneration of BMSSCs *in vivo* by maintaining their osteogenic potential. Our results suggest that the introduction of hTERT can overcome critical technical barriers in the *ex vivo* expansion of BMSSCs for transplantation and that telomerase therapy may be beneficial in the treatment of bone injuries and bone defects.

## Experimental protocol

**Cell culture and retrovirus transduction.** BMSSCs, processed from marrow aspirates of normal human adult volunteers (20–35 years of age), were purchased from Poietic Technologies (Gaithersburg, MD). About $1 \times 10^6$ STRO-1-positive cells per sample were sorted using a FACSTAR$^{PLUS}$ (Becton Dickinson, Franklin Lakes, NJ) as described elsewhere[47] and then seeded for clone formation into six-well plates containing the alpha modification of Eagle's medium supplemented with 15% fetal calf serum. About 300 colonies containing proliferative cells were pooled for experiments. BMSSCs were infected either with retroviruses expressing hTERT or with empty control

vector. Forty-eight hours after infection, cells were selected with puromycin (1 μg/ml) for 10 days and ~500–600 resistant clones were pooled. For passaging, approximately $0.5 \times 10^5$ BMSSCs were seeded into 10 cm culture dishes every three to four days. The criteria for cell senescence were that BMSSCs did not divide for a month in culture and that over 60% of the cells were stained positive for β-galactosidase[9,26]. TRAP was performed according to the manufacturer's instruction (Intergen, Purchase, NY). Alkaline phosphatase activity was assessed using a Sigma (Saint Louis, MO) *in vitro* alkaline phosphatase substrate kit. Calcium deposits were detected by using von Kossa staining as previously described[31]. Calcium concentration was measured using both the Sigma Calcium Kit and Calcium Standard Kit[31].

**Transplantation of BMSSCs into immunocompromised mice.** BMSSCs ($2.0 \times 10^6$) were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer, Warsaw, IN) and then transplanted subcutaneously into the dorsal surface of ten-week-old immunocompromised mice (NIH-bg-nu-xid, Harlan Sprague Dawley, Indianapolis, IN) as described elsewhere[28]. These procedures were performed in accordance with specifications of a small-animal protocol approved by the institutional board of the US National Institute of Dental and Craniofacial Research (#00-113). The transplants were recovered eight weeks after transplantation, fixed with 4% formalin, and either decalcified with buffered 10% EDTA for paraffin embedding or kept in 70% ethanol for plastic embedding. Paraffin sections were deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E). Plastic sections were processed with H&E and von Kossa staining (Pathology Associates, Frederick, MD). For quantification of new bone regeneration *in vivo*, Scion Image (Scion, Bethesda, MD) was used to calculate five representative areas at 5× magnification from either BMSSC-T or BMSSC-C transplants. *In situ* hybridization for human *Alu* sequences was performed as previously described[47].

**Flow cytometric analysis.** Adherent monolayers were digested with trypsin/EDTA to obtain single-cell suspensions. Cells ($2 \times 10^5$) were incubated with anti-STRO-1 monoclonal antibodies (1:100) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson) as described elsewhere[31]. Positive expression was defined as the level of fluorescence greater than 99% of corresponding isotype-matched control antibodies.

*Note: Supplementary information is available on the* Nature Biotechnology *website.*

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*Competing interests statement*

*The authors declare that they have no competing financial interests.*

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