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RESEARCH ARTICLE

Telomerized presenescent osteoblasts prevent bone mass loss in vivo

K Yudoh and K Nishioka
Department of Bioregulation, Institute of Medical Science, St. Marianna University, Miyamae-ku, Kawasaki City, Japan

Previously, we showed that human osteoblasts expressing the human telomerase reverse transcriptase (hTERT) gene exhibited specific survival advantages – the result of breaching the replicative senescence barrier and maintaining the phenotypic and functional properties of primary osteoblasts in vitro over the total replicative capacity of primary osteoblasts. We postulated that rejuvenated osteoblasts may have a potential to correct bone loss or osteopenia in age-related osteoporotic diseases. In the present study, we studied whether telomerized presenescent osteoblasts prevent bone mass loss in vivo. After obtaining the informed consent from a patient with osteoarthritis who underwent the arthroplastic knee surgery, osteoblastic cells were isolated from donor bone sample. We transfected the gene encoding hTERT into human osteoblastic cells. Human bone fragments from a donor were incubated with human hTERT-transfected presenescent (in vitro aged) osteoblasts or mock-transfected presenescent osteoblasts in culture medium containing Matrigel. We subcutaneously implanted human bone fragments with telomerized presenescent osteoblasts or primary presenescent osteoblasts as three-dimensional Matrigel xenografts in severe combined immunodeficiency (SCID) mice (each group: six mice) and analyzed the grafts at 6 weeks after implantation. We also determined whether telomerized osteoblasts affect the bone-forming capacity in vivo, using a well-established mouse transplantation model in which ceramic hydroxyapatite/tricalcium phosphate particles are used as carrier vehicle. Telomerized presenescent osteoblasts were rejuvenated, and maintained the functional properties of young osteoblasts in vitro. Bone mineral content (BMC) and bone mineral density (BMD) were measured by ash weight and dual-energy X-ray absorptiometry, respectively. Whereas BMC and BMD of human bone fragments, which were inoculated with aged osteoblasts in SCID mice, decreased with time, telomerized presenescent osteoblasts maintained the BMC and BMD of human bone fragments, indicating that telomerized and rejuvenated osteoblasts may be functional to prevent bone mass loss in vivo. In xenogenic transplants, telomerized osteoblasts generated more bone tissue with lamellar bone structure and cellular components, than did control osteoblasts. These findings suggest that telomerized/rejuvenated presenescent osteoblasts may be used in the development of tissue engineering or cell-based therapy for bone regeneration and repair.
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Introduction

Most normal somatic cells from humans cannot divide indefinitely, because of the progressive loss of DNA from telomeres, the specialized ends of the linear chromosomes.[1–3] The gradual erosion of telomeres during repeated cell division results from the combined effects of the inability of normal DNA replication to completely extend telomeric DNA and the lack of expression of the reverse transcriptase component of the telomerase ribonucleoprotein complex (human telomerase reverse transcriptase (hTERT)).[4,5] Stem cells and germline cells, which normally express TERT and have telomerase activity, are able to fully replicate telomeric DNA, and thereby are able to divide indefinitely.[6] In contrast, most of the normal somatic cells that do not have telomerase activity are able to divide only a limited number of times before they senesce.[4,5] However, in cells with forced expression of TERT, the progressive shortening of telomeres is prevented, and these cells continue to proliferate rapidly at a cumulative population-doubling level greatly exceeding the population-doubling level at which they would otherwise senesce.[5–9]

These results indicate that expression of TERT is sufficient to extend the cellular lifespan of some cell types that normally undergo cellular senescence. Moreover, extension of cellular lifespan by TERT expression does not appear to be accompanied by the acquisition of characteristics of a malignant tumor cell, such as chromosomal abnormalities, anchorage-independent growth in culture, loss of normal cell cycle checkpoints, or tumorigenicity in immunodeficient mice.[10,11] Such findings encouraged speculation that cells expressing TERT could be used in cell-based therapies, such as *ex vivo* gene therapy, tissue engineering, and transplantation of cells, to correct tissue damage, metabolic or endocrine defects.

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Correspondence: Dr K Yudoh, Department of Bioregulation, Institute of Medical Science, St Marianna University, Sugoh 2-16-1, Miyamae-ku, Kawasaki City 216-8511, Japan
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Currently, there is much research activity on the use of bone marrow stromal and hematopoetic stem cells for cell-based therapy and tissue engineering. However, the occurrence of stromal stem cells is rare in bone marrow. In addition, it has been reported that human postnatal bone marrow stromal cells have a limited lifespan and progressively lose their stem cell properties during *ex vivo* expansion.[12] To succeed, tissue engineering with bone marrow stromal stem cells must rely on *ex vivo* expansion to obtain enough cells. It has been reported that telomerized stromal stem cells exhibited an extended lifespan and maintained their osteogenic potential *in vivo*.[12] These findings suggest that telomerase activation can help to maintain the osteogenic stem cell pool during *ex vivo* expansion.

Telomerase expression could overcome critical technical barriers to the *ex vivo* expansion of stem cells. Telomerase induction may be useful for the expansion of not only bone marrow stromal stem cells, but also of mature differentiated somatic cells. In addition to the expansion of cell number, forced expression of telomerase in presenescent cells could induce re-activation of cell proliferative potential and cellular activity. We have already reported that the introduction of telomerase into human osteoblasts leads to an extension of their replicative lifespan and the maintenance of the osteoblast activity pattern typical of young normal osteoblasts.[13] In the previous study, we transfected the gene expressing TERT cDNA into human osteoblasts that were less than four population doublings (less than three passages) from primary culture.[13] Therefore, it still remained unclear whether or not telomerase induction can rejuvenate aged presenescent or senescent osteoblasts. We speculated that even presenescent or senescent osteoblasts may be rejuvenated by the introduction of telomerase activity. Also, rejuvenated presenescent osteoblasts may have a potential to develop tissue engineering or cell-based therapy for bone and joint diseases.

We postulated that rejuvenated osteoblasts also have a potential for bone regeneration and repair *in vivo*, similar to telomerized bone stromal stem cells. Here, we show that TERT-modified presenescent osteoblasts maintain the functional properties of young osteoblasts *in vitro*. We also used a telomerized presenescent osteoblast-transplantation model mouse to address our hypothesis that rejuvenated osteoblasts prevent bone mass loss *in vivo*. Here, we show for the first time that telomerase activation in human aged presenescent osteoblasts results in the rejuvenation and maintenance of a young bone matrix-producing cell property *in vitro* and *in vivo*.

Results

Telomere length in hTERT-transfected presenescent osteoblasts

To determine if the hTERT-reconstituted telomerase has the potential to rejuvenate human presenescent osteoblasts, hTERT was transfected into presenescent osteoblasts (*in vitro* aged culture cells: 12 population doublings). Significant levels of telomerase activity were observed in eight of the 12 resultant stable hTERT-transfected osteoblastic cell populations from a donor, but not in mock-transfected osteoblastic cell populations (Figure 1a). Even after 30 population doublings, hTERT-transfected osteoblastic cell populations expressed both telomerase and hTERT (Figure 1b).

Telomere lengths in the hTERT-negative presenescent osteoblasts decreased with advancing population doublings in a manner comparable to the shortening seen in mass cultures at equivalent population doublings, whereas telomere lengths in the hTERT-transfected presenescent osteoblasts did not shorten up to 20 population doublings after hTERT transfection (Figure 2). After 25 population doublings after the transfection, the gradual decrease of telomere length was observed even in the hTERT-transfected presenescent osteoblasts.

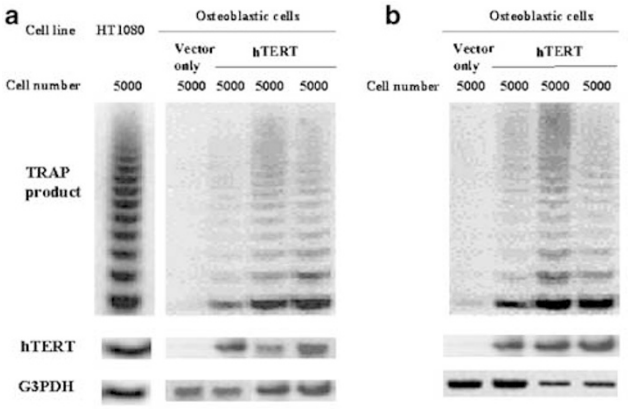

Figure 1 *Telomerase expression in human osteoblastic cell populations obtained by transfection with a vector expressing the hTERT cDNA. Telomerase expression in representative osteoblastic cell populations from patient with OA (65 years old). At population doubling 0 after transfection, telomerase activity and hTERT were expressed in hTERT-transfected osteoblastic cells, whereas the mock-transfected osteoblast cell populations did not express telomerase activity. Positive control was the telomerase activity extracted from fibroblastosarcoma HT1080 cells. Even after 30 population doublings, hTERT-transfected osteoblastic cell populations expressed both hTERT and telomerase.*

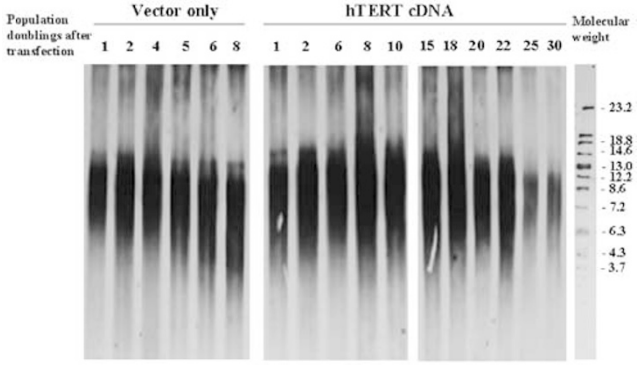

Figure 2 *Telomere length of human hTERT-transfected or mock-transfected osteoblasts. Representative TRF lengths of DNA from osteoblastic cell populations obtained from the donor. Telomere lengths in the hTERT-negative cells decreased gradually with population doublings, whereas telomeres in the hTERT-positive osteoblastic cell populations did not shorten during continuous culture for more than 20 population doublings after hTERT transfection. After 20 population doublings, telomere shortenings were observed even in the telomerized osteoblastic cell populations.*

Replicative lifespan and osteoblastic activity in hTERT-transfected presenescent osteoblasts

To investigate the effect of telomerase expression on the lifespan and cellular activity of presenescent osteoblasts, we compared the lifespan and osteoblastic activity of telomerase-positive and telomerase-negative aged osteoblasts. Telomerase-positive presenescent cells exceeded the mean lifespan of telomerase-negative presenescent cells. These cells continued to divide at the rate observed in young osteoblastic cells. The average lifespan of telomerase-positive presenescent osteoblasts was approximately 22–23 doublings after hTERT transfection beyond the average lifespan of the hTERT-negative osteoblasts, whereas most of the hTERT-negative presenescent osteoblasts senesced after 4–8 population doublings.

The alkaline phosphatase (ALP) activity and C-terminal type I procollagen (PICP) of hTERT-negative presenescent osteoblasts decreased gradually with population doublings, whereas the hTERT-positive presenescent cells maintained the ALP activity and PICP secretion for more than 15–20 population doublings after hTERT transfection (Figure 3).

Persistence of telomerized osteoblasts in vivo

We subcutaneously implanted telomerized presenescent osteoblasts or aged osteoblasts as three-dimensional Matrigel xenografts in severe combined immunodeficiency (SCID) mice (Figure 4) and analyzed the grafts at 4 and 8 weeks after implantation. Figure 5 showed representative immunofluorescent images of telomerized osteoblasts or control osteoblasts detected by GFP fluorescence 8 weeks after xenografting. On the surface of the implanted bone fragments, telomerized osteoblasts had markedly stronger GFP signal intensity (Figure 5b). In contrast, control osteoblasts-inoculated bone samples did not maintain GFP fluorescence 8 weeks after xenograft (Figure 5a). These results support our *in vitro* study that showed a survival advantage of telomerized osteoblasts relative to aged presenescent osteoblasts.[13]

In vivo bone formation

We investigated if the introduction of telomerase into presenescent osteoblasts could influence their ability to form or maintain bone tissue in a three-dimensional bone

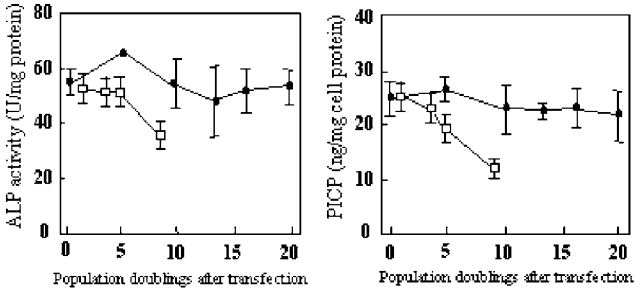

Figure 3 *ALP activity and PICP secretion in hTERT-transfected or mock-transfected osteoblastic cell populations. The ALP activity and PICP secretion in hTERT-negative osteoblastic cell populations (open square) from donors decreased with population doublings, whereas the hTERT-positive cells (closed circle) maintained the ALP activity and PICP secretion for more than 15–20 population doublings after hTERT transfection. The closed circle represents hTERT-positive cell populations. The open square corresponds to hTERT-negative cell populations.*

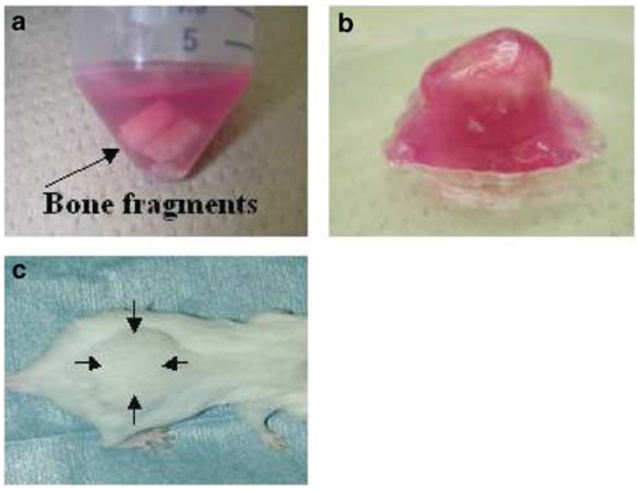

Figure 4 *Three-dimensional Matrigel xenograft in SCID mouse. Human bone fragment (1 cm^3) from the donor was incubated with human hTERT-transfected osteoblasts or mock-transfected osteoblasts in the culture medium containing Matrigel (50 mg/ml) for 1 h at 37°C (a). We subcutaneously implanted human bone fragment with telomerase-positive osteoblasts or telomerase-negative osteoblasts as three-dimensional Matrigel xenografts (b) in SCID mouse (c), and analyzed the grafts at 4 and 8 weeks after implantation.*

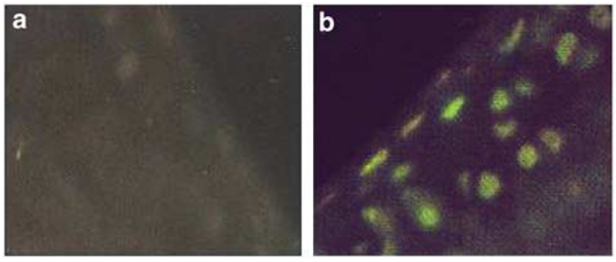

Figure 5 *Green fluorescent protein (GFP) fluorescence in bone tissue. Representative immunoflourescent images of telomerized osteoblasts and presenescent osteoblasts detected by GFP fluorescence 8 weeks after xenografting. The mock-transfected osteoblasts did not maintain GFP fluorescence 8 weeks after xenograft (a), whereas telomerized osteoblasts had markedly stronger GFP signal intensity (b).*

and Matrigel-xenografted SCID mouse model (Figure 4). Presenescent osteoblast-containing bone samples exhibited significant decreases of bone mineral density (BMD) and bone mineral content (BMC) with time, relative to telomerized osteoblast-containing bone implants. There were statistically significant differences between these xenograft groups 8 weeks after xenografts (Figure 6).

We also determined whether telomerase induction affects the bone-forming capacity of osteoblasts *in vivo*, using a mouse transplantation model in which ceramic hydroxyapatite/tricalcium phosphate (HA/TCP) particles are used as a carrier vehicle. Bone formation was evident in the sample where osteoblasts have been implanted onto HA/TCP (Figure 7a, b). Implants of osteoblasts contained matrix structures and cellular components, such as bone-lining osteoblasts, osteocytes, and lamellar bone covering the HA/TCP surfaces (Figure 7a, b). Osteocalcin-positive osteoblasts lining the edge of bone tissue can be observed (Figure 7c). Quantitative image analysis showed that telomerized

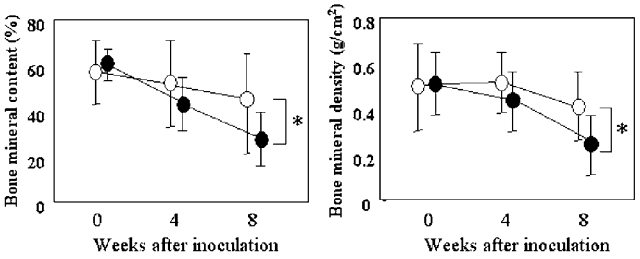

Figure 6 BMCs and BMD of presenescent osteoblast-containing bone groups (closed circle) decreased gradually with time, whereas the telomerized osteoblast-containing bone groups (open circle) maintained BMC and BMD. There were statistically significant differences in the BMC and BMD between these xenograft groups 8 weeks after xenografts, respectively (*$P<0.05$; $n=3$ each).

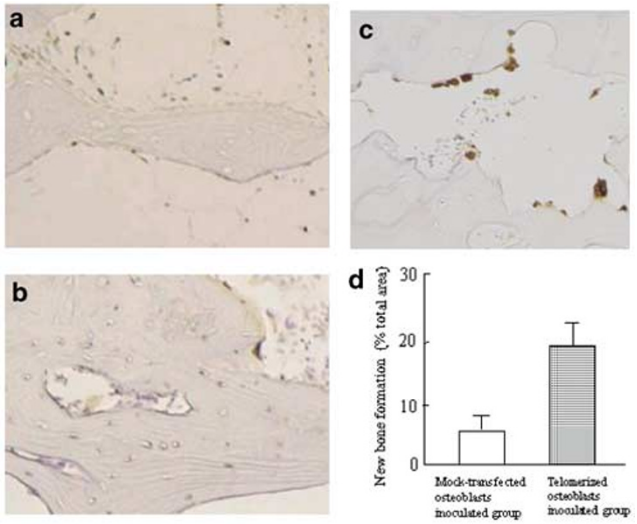

Figure 7 Representative fields of histological sections of regenerated bone by transplantation of telomerized osteoblasts. Figures are paraffin-embedded cross-sections of representative control osteoblast transplants (a) and telomerized osteoblast transplants (b) harvested 6 weeks after transplantation. (c) Bone-lining osteoblasts detected by immunohistochemical staining for osteocalcin were observed on the surfaces of regenerated bone. Telomerized osteoblasts generated more bone than did control osteoblasts in vivo (d). Five representative areas from each group were quantified and averaged using an image analyzer.

osteoblasts generated significantly more bone than did their control osteoblasts (Figure 7d, $P<0.01$).

Discussion

By directly comparing rejuvenated osteoblasts to aged osteoblasts, our results demonstrate for the first time that telomerase activation in human presenescent osteoblasts results in the maintenance of a stable bone matrix producing cell property *in vivo* as well as *in vitro*, suggesting that telomerized/rejuvenated osteoblasts prevent bone mass loss *in vivo*.

Our previous study suggested that the replicative senescence of osteoblasts in the periarticular bone occurred with aging in patients with osteoarthritis (OA).[13] Osteoblast senescence may, at least in part, contribute to the decrease of bone formation, consequently leading to periarticular osteopenia commonly observed in patients with this condition. For mature or aged osteoblasts in the osteoporotic bone tissue, the challenge of extending cellular lifespan, rejuvenating and maintaining these cells as young differentiated bone matrix-producing cells, may prove important in the development of new therapeutic strategies for bone and joint diseases.

Several reports demonstrated that telomerase expressing life-extended cells have been used to engineer functional tissues *in vivo*.[12,14,15] Shi *et al* have already reported that telomerase induction in human postnatal bone marrow stromal stem cells extended their lifespan and maintained their osteogenic potential, suggesting that telomerase expression helps to maintain the osteogenic stem cell pool during *ex vivo* expansion.[12] The occurrence of hematopoetic or mesenchymal stem cells is rare in bone marrow. If aged somatic cells are rejuvenated, the engineered and rejuvenated somatic cells are very useful for tissue engineering and cell-based therapy. Our present results demonstrate that not only osteogenic stem cells but also mature aged osteoblasts are used in therapeutic strategies to correct bone loss as telomerized and life-extended cells. Importantly, implants with telomerized osteoblasts did not result in tumor formation as long as 8 weeks after implantation, consistent with previous studies of hTERT-transduced primary cells (data not shown).

During passaging, human osteoblasts did not express both endogenous telomerase activity and hTERT. Our findings indicated that significant levels of telomerase activity were observed in stable hTERT-transfected osteoblasts, but not in mock-transfected osteoblasts. It is well known that hTERT expression is essential for telomerase expression. Our data indicate that introduction of telomerase activity by the telomerase catalytic subunit (hTERT) can lead to preservation of the telomere lengths of presenescent osteoblasts. Even after 30 population doublings, reconstituted telomerase had been maintained in hTERT-transfected osteoblastic cell populations. However, even in the hTERT-transfected osteoblastic cell populations that expressed telomerase, telomere shortenings were observed after 25 population doublings after hTERT transfection. It still remains unclear whether exogenous telomerase induction can immortally keep the telomere lengths maintaining for longer periods. It is critically important to verify whether exogenous telomerase expression leads to the immortalization of hTERT-transfected cells. Kiyono *et al*[16] reported that both Rb/p 16 INK 4a inactivation and telomerase activity are required to immortalize human epithelial cells. Furthermore, recent reports have demonstrated that telomerase reconstitution in SV40 T-antigen-transformed human somatic cells was shown to allow cells to escape from crisis and become immortal.[17–21] It is suggested that SV40T antigen and telomerase are required to obtain immortalized cells.[21] Only an introduction of telomerase into the cells may be insufficient to immortalize normal somatic cells. We think that the longer-term effects of exogenous telomerase expression on telomere maintenance and the lifespan of these cells remain to be determined in studies of longer duration.

We have already reported that cellular lifespan and telomere length of osteoblasts were elongated by the introduction of telomerase into human donor osteoblasts.[13] In the previous study, we transfected the gene expressing hTERT cDNA into young osteoblasts within

four population doublings after primary culture. It had remained unclear whether or not introduction of telomerase leads to the rejuvenation of aged/presenescent osteoblasts. In the present study, to determine if the hTERT-reconstituted telomerase has the potential to rejuvenate human presenescent osteoblasts, hTERT was transfected into presenescent osteoblasts (*in vitro* aged culture cells: 12 population doublings) and their lifespan and telomere lengths analyzed. Our previous study indicated that normal human osteoblasts exhibited senescence after 10–15 population doublings after cell isolation from bone fragment in mass cultures.[13] Therefore, in the present study, presenescent osteoblasts were prepared as *in vitro* aged osteoblasts with 12 population doublings in the mass culture. Our results demonstrate that the forced expression of the hTERT in presenescent osteoblasts results in an extension of their replicative lifespan and the maintenance of the osteoblast activity pattern typical of young normal osteoblasts. These findings provide evidence to support that rejuvenated osteoblasts may have an advantage in forming and maintaining bone tissue *in vivo*. Indeed, in the current study, our results revealed that BMC and BMD of bone fragments, which were inoculated with telomerized osteoblasts, were maintained, whereas aged osteoblast-inoculated bone samples showed significant decrease of BMC and BMD with time *in vivo* model mouse, suggesting that telomerized/rejuvenated osteoblasts prevent bone mass loss *in vivo*. Also, we observed that telomerized osteoblasts generated more bone tissue with a lamellar bone structure and associated cellular components than did control osteoblasts. Taken together, these results show that telomerase induction significantly enhances and maintains the bone-forming capacity of osteoblasts *in vivo*.

In conclusion, the ability to extend the cellular lifespan of osteoblasts may have important implications for biological research and the development of new technologies for age-related bone and joint diseases.

Materials and methods

Osteoblastic cell culture

After obtaining informed consent from donor, bone sample was obtained from juxta-articular bone marrow in the patient with OA (female, 65 years old) who underwent arthroplastic knee surgery. This patient had no clinical symptoms or history of bone metabolic disorders, and had not previously taken prednisolone or intra-articular injection therapy of corticosteroid. For *in vitro* experiments, osteoblastic cells were isolated from the outgrowth of juxta-articular bone sample, as described previously.[22–24] In brief, bone fragment was minced into 1 mm^3 pieces and washed extensively with phosphate-buffered saline (PBS) to remove adherent bone marrow cells. The fragments were seeded onto culture dishes, and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere supplemented with 5% CO_2. Osteoblasts were further isolated by immunomagnetic sorting using biotin-labeled antiparathyroid hormone (anti-PTH) receptor antibody and antibiotin magnetic beads, according to the manufacturer's instructions (Milteny Biotec GmbH, Bergisch Gladbach, Germany).[13] Anti-PTH receptor antibody was purchased from Funakoshi Co. (Tokyo, Japan).

Culture medium was changed twice weekly until subconfluence (3–4 weeks). Subconfluent cells were washed with PBS, and detached with 5.0 ml of 10% trypsin in PBS. In parallel cultures, to check the osteoblastic cell properties, we examined the expression of various osteoblastic features such as ALP activity, PICP and osteocalcin secretion, and PTH-stimulated cAMP production by these cells.[13]

ALP activity and PICP secretion of human osteoblasts

ALP activity was determined in six different wells derived from each culture after extraction with 0.1% Triton X-100 by hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol, as described previously.[25] *p*-Nitrophenol was measured spectroscopically at 410 nm using an EAR 400 multiwell spectrophotometer (SLT Labinstruments, Salzburg, Austria). Results are expressed as nmol/min (U) per mg cell protein (U/mg protein).

PICP levels were considered to be an index of osteoblastic bone matrix formation *in vitro*. PICP in the cell-conditioned medium was measured using an enzyme-linked immunosorbent assay (Metra Biosystems, Mountain View, CA, USA).[13]

Transfection of presenescent osteoblasts with hTERT cDNA-expressing plasmid

To clarify if presenescent osteoblasts were rejuvenated and the functional properties of young osteoblasts *in vitro* maintained and bone mass loss *in vivo* prevented, we compared telomerized presenescent osteoblasts to aged presenescent osteoblasts. We have previously shown that normal human osteoblasts senesced after about 15 population doublings in mass cultures.[13] From the result of our previous study, presenescent osteoblasts were prepared as *in vitro* aged cells with 12 population doublings after osteoblastic cell isolation in the mass culture.

In this study, the pcDNA 3.1/CT-GFP-TOPO® vector (Invitrogen Co., Carlsbad, CA, USA) was used to express the full-length cDNA encoding hTERT with Cycle 3 GFP fused to the C-terminus of the cDNA. The pcDNA3.1/CT-GFP is a 6141 bp control vector containing the gene for Cycle 3 GFP. The vector was constructed by cloning an *Xba*I–*Eco*RI/Klenow fragment containing Cycle 3 GFP into pcDNA3.1/V5-His B digested with *Xba*I and *pme*I. Expression and fluorescence of Cycle 3 GFP has been confirmed. The vector contains a neomycin resistance gene to allow for selection of the cells. The full-length cDNA encoding hTERT was subcloned into the mammalian expression vector pcDNA3.1/CT-GFP. The full-length cDNA encoding hTERT with GFP fused to the C terminus of hTERT cDNA was expressed in human osteoblasts under control of the CMV promoter. Subconfluent *in vitro* aged osteoblasts (population doubling level 12) were transfected with either the hTERT cDNA-expressing plasmid or the control plasmid. The full-length cDNA encoding hTERT was kindly provided by Dr R Weinberg (Whitehead Institute for Biomedical Research, MA, USA). Each plasmid preparation (5 μg) was added to 10 μg of lipofectin (GIBCO BRL, Grand Island, NY, USA), and the mixture was added to the cells,

and the cells were incubated for 2 days. The cells were washed with DMEM without serum, and were suspended in the culture medium. Cells were cultured in the presence of 600 μg/ml of G418 sulfate (GIBCO BRL), an analog of neomycin, for 2 weeks to select for stably transfected cells expressing neomycin resistance. Transfected cells were maintained into α-MEM with 10% FBS.

Expression of hTERT in human osteoblastic cells

We confirmed the stable transfection of hTERT into human osteoblasts not only at the time of population doubling 0 after transfection, but also after 30 population doublings. RT-polymerase chain reaction (PCR) for hTERT and GAPDH (to normalize for equal amounts of RNA template) was performed using RNA derived from donor osteoblastic cells that were transfected with either hTERT-expressing plasmid or control plasmid. The cell extract for each assay was isolated from primary cultures that they first reached subconfluence after the selection in G418 containing medium. RT-PCR was performed with total RNA (1.0 μg) using the GeneAmp Ez rTth RNA PCR kit (Perkin-Elmer Co., Norwalk, CT, USA). hTERT mRNA was amplified using oligonucleotide primers LT5 (CGGAAGAGTGTCTGGAGCAA) and LT6 (GGATGAAGCGG-AGTCTGGA) for 31 cycles (94°C for 45 s, 60°C for 45 s, 72°C for 90 s). GAPDH mRNA was amplified using primers K136 (CTCAGACACCATGGG-GAAGGTGA) and K137 (ATGATCTTGAGGCTGTTGT-CATA) for 16 cycles (94°C for 45 s, 55°C for 45 s, 72°C for 90 s). The amplified products were fractionated on a 2% agarose gel. Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) and analyzed using a fluorescence imaging analyser.

Determination of the lifespan of human osteoblastic cells

hTERT-transfected or mock-transfected osteoblastic cell populations were subcultured weekly. At each subculturing, the total number of cells in the dish was determined, and 2.5×10^5 cells were transferred to a new dish. The number of cells that had attached 6 h after seeding was determined. The increase in cumulative population doublings at each subculture was calculated based on the number of cells attached and the cell yield at the time of the next subcultivation. Cell cultures were considered to have achieved their proliferative limit when they did not exceed 5×10^5 cells/dish in 4 weeks. Senescence was defined as less than one population doubling in 4 weeks. The *in vitro* lifespan (remaining replicative capacity) was expressed as population doublings up to cellular senescence.[5,26]

Telomerase assay

Presenescent osteoblasts from donors, transfected with either the hTERT cDNA-expressing plasmid or control plasmid, were treated with ice-cold hypotonic detergent lysis buffer (10^6 cells/100 μl: 0.5% CHAPS (Sigma)), incubated on ice for 30 min, and centrifuged at 12 000 *g* for 20 min at 4°C. The supernatant was stored at −80°C. Cellular telomerase activity was measured by the telomeric repeat amplification protocol (TRAP), as reported by Kim *et al*.[27] Briefly, 2 μl of cell extract was assayed in reaction tubes containing 50 μl of the TRAP reaction mixture. The telomerase substrate (TS) primer (5′-AATCCGTCGAGCAGAGTT-3′) was end-labeled using T4 polynucleotide kinase (Promega, Madison, WI, USA) and 25 μCi of 3000 Ci/mM [α-^{32}P]adenosine triphosphate/μg TS. T4 polynucleotide kinase was used at 25 U/μg TS and incubated at 37°C for 20 min, followed by heat inactivation at 95°C for 5 min. Each TRAP reaction consisted of 2 μl of cell extract (equivalent to 2×10^4 cells), 5 μl of 10 × TRAP buffer, 50 μM deoxynucleotide triphosphate, 0.1 μg of the end-labeled TS, 0.1 μg of return primer (5′-GCGCGG[CTTACC]^{3}CTAACC-3′), 0.1 μg of the nontelomerase primer (5′-ATCGCTTCTCGGCCTTTT-3′), 0.01 amol of the telomerase substrate-nontelomerase (TNST) internal control (5′-AATCCGTCGAGCAGAGTT-AAAAGGCCGAGAAGC-GAT-3′), and 2 U of Taq DNA polymerase (Perkin-Elmer Corp., Branchburg, NJ, USA). TNST is the PCR internal control amplified by the primers TS and NT, which gives a 36-base pair (bp) product, running 14 bp below the smallest TRAP band. After 30 min of incubation at room temperature, the reaction mixture was amplified by 30 cycles at 94°C, 60°C, and 72°C for 30 s each. The PCR product was electrophoresed on a 15% polyacrylamide nondenaturing gel. A cell extract was considered negative if no ladder was detectable after a 10-h exposure. The telomerase-positive extracts were given a relative value of activity as a percent of the reference cell line (HT1080, a fibrosarcoma cell line), based on the mean value of two separate runs with two different exposures.

Telomere length assay

Telomere length was determined by terminal restriction fragment (TRF) Southern blot analysis, as described previously.[5,13] Genomic DNA from osteoblasts from the donor, transfected with either the hTERT cDNA-expressing plasmid or control plasmid, was digested with 400 ml of DNA extraction buffer (100 mm NaCl, 40 mm Tris (pH 8.0), 20 mm EDTA (pH 8.0), and 0.5% SDS) and proteinase K (0.1 mg/ml). Extraction was performed using phenol-chloroform. Extracted DNA (5–10 μg) was digested with 10 U of *Msp*I and *Rsa*I (Boehringer Mannheim, Indianapolis, IN, USA) for 12–24 h at 37°C. The integrity of the DNA before digestion and the completeness of digestion were monitored by gel electrophoresis. Electrophoresis of digested genomic DNA was performed in 0.5% agarose gels in 45 mM Tris-borate EDTA buffer (pH 8.0) for a total of 660–700 V/h. After electrophoresis, gels were depurinated in 0.2 N HCL, denatured in 0.5 M NaOH and 1.5 M NaCl, transferred to a nylon membrane using 20 × SSC, and dried for 1 h at 70°C. The telomeric probe (TTAGGG)^3 (Genset, La Jolla, CA, USA) was 5′ end-labeled with [α-^{32}P]ATP using T4 PNK (Boehringer Mannheim). Prehybridization and hybridization were performed at 50°C using 5 × Denhardt's solution, 5 × SSC/0.1 M Na_2HPO_4/0.01 M $Na_4P_2O_7$, and 30 μg salmon sperm DNA per ml/0.1 mM ATP. The mean TRF length was determined from densitometric analysis of autoradiograms, as described.[5]

SCID mouse xenograft and bone mass evaluation

Human bone fragment (1 cm^3) from the donor was incubated with human hTERT-transfected osteoblasts or mock-transfected osteoblasts in the culture medium containing Matrigel (50 mg/ml) for 1 h at 37°C. We

subcutaneously implanted human bone fragment with telomerase-positive osteoblasts or telomerase-negative osteoblasts, as three-dimensional Matrigel xenografts in SCID mouse (total six mice), and analyzed the grafts at 4 (three mice) and 8 weeks (three mice) after implantation.

BMCs and BMD of grafted bone were measured by the ash method and dual-energy X-ray absorptiometry (DEXA), respectively.[28] Bone sample was excised and soft tissue and Matrigel surrounding the bone were completely removed. BMD was determined using a DCR-600R instrument (Aloka Co., Japan) and expressed as mg/cm^2. After determining the wet weight of the bone sample, the samples were dried at 100°C for 48 h, and ashed samples were weighted to determine the bone mineral weight. BMC was defined as the percentage of ratio of bone mineral weight to wet weight.

To investigate the TERT-transfected or mock-transfected osteoblasts in the bone fragments, bone fragments were examined by fluorescence microscopy as follows. Inoculated bone fragments were kept in 70% ethanol for plastic embedding. Plastic sections were processed with immunofluorescent examination. Enhanced GFP signals were captured using the fluorescein isothiocyanate filter on a Zeiss Axioshop microscope.

Transplantation of telomerized osteoblasts with HA/TCP into SCID mice

Telomerized osteoblasts or mock-transfected osteoblasts (1×10^5 cells) were mixed with 10 mg of HA/TCP (Sumitomo-Osaka Cement, Osaka, Japan) and then transplanted subcutaneously in the dorsal of SCID mouse, as described by Shi *et al*[12] The transplants were recovered with buffered 10% EDTA for paraffin embedding. Paraffin sections were deparaffinized, hydrated, and then immunohistochemical staining for osteocalcin was performed using an antiosteocalcin antibody (Nichirei, Tokyo, Japan). For quantification of new bone regeneration *in vivo*, an image analyzer was used to calculate five representative areas from either telomerized osteoblasts-inoculated transplants or control osteoblasts-inoculated transplants.

Statistical analysis

Results were expressed as the mean value$\pm$standard deviation. Comparison of the means was performed by ANOVA. Analyses resulting in $P<0.05$ were considered statistically significant.

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