[15] M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, L. Peterson, Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation, N. Engl. J. Med. 331 (1994) 889–895.
[16] M.S. Rao, M.P. Mattson, Stem cells and aging: expanding the possibilities, Mech. Ageing Dev. 122 (2001) 713–734.
[17] K. Igura, X. Zhang, K. Takahashi, A. Mitsuru, S. Yamaguchi, T.A. Takahashi, Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta, Cytotherapy 6 (2004) 543–553.
[18] Y. Zhang, C. Li, X. Jiang, S. Zhang, Y. Wu, B. Liu, P. Tang, N. Mao, Human placenta-derived mesenchymal progenitor cells support culture expression of long-term culture-initiating cells from cord blood $CD34^+$ cells, Exp. Hematol. 32 (2004) 657–664.
[19] B. Johnstone, T.M. Hering, A.I. Caplan, V.M. Goldberg, J.U. Yoo, In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells, Exp. Cell Res. 238 (1998) 265–272.
[20] T. Nishida, S. Kubota, S. Kojima, T. Kuboki, K. Nakao, T. Kushibiki, Y. Tabata, M. Takagawa, Regeneration of defects in articular cartilage in rat knee joints by CCN2 (connective tissue growth factor), J. Bone Miner. Res. 19 (2004) 1308–1319.
[21] K.W. Liechty, T.C. Mackenzie, A.F. Shaaban, A. Radu, A.B. Moseley, R. Deans, D.R. Marshak, A.W. Flak, Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep, Nat. Med. 6 (2000) 1282–1286.
[22] P.A. Conget, J.J. Minguell, Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells, J. Cell. Physiol. 181 (1999) 67–73.
[23] S. Gronthos, D.M. Franklin, H.A. Leddy, P.G. Robey, R.W. storms, J.M. Gimble, Surface protein characterization of human adipose tissue-derived stromal cells, J. Cell. Physiol. 189 (2001) 54–63.
[24] I. Sekiya, J.T. Vuoristo, B.L. Larson, D.J. Prockop, In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis, Proc. Natl. Acad. Sci. USA 99 (2002) 4397–4402.
[25] S. Shirasawa, I. Sekiya, Y. Sakaguchi, K. Yagisha, S. Ichinose, T. Muneta, In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells, J. Cell. Biochem. (2005). Aug 8 [Epub ahead of print].
[26] K. Nishimura, L.A. Solchaga, A.I. Caplan, J.U. Yoo, B. Johnstone, Chondroprogenitor cells of synovial tissue, Arthritis Rheum. 42 (1999) 2631–2637.
[27] C. De Bari, F. Dell' Accio, P. Tylzanowski, F.P. Luyten, Mulitpotent mesenchymal stem cells from adult human synovial membrane, Arthritis Rheum. 44 (2001) 1928–1942.
[28] M. Ochi, Y. Uchio, K. Kawasaki, S. Wakitani, J. Iwasa, Transplantation of cartilage-like tissue made by tissue engineering in treatment of cartilage defect of the knee, J. Bone Joint Surg. 84 (2002) 571–578.
[29] S. Wakitani, K. Imoto, T. Yamamoto, M. Saito, N. Murata, M. Yoneda, Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees, Osteoarthritis Cartilage 10 (2002) 199–206.
[30] M. Radice, P. Brum, R. Cortive, R. Scapinelli, C. Battaliard, G. Abatangelo, Hyaluronan-based biopolymers as delivery vehicles for bone-marrow-derived mesenchymal progenitors, J. Biomed. Mater. Res. 50 (2000) 101–109.
[31] O.N. Koc, J. Day, M. Nieder, S.L. Gerson, H.M. Lazarus, W. Krivit, Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH), Bone Marrow Transplant. 30 (2002) 215–222.
[32] E.M. Horwitz, P.L. Gordon, W.K. Koo, J.C. Marx, M.D. Neel, R.Y. McNall, L. Muul, T. Hofman, Isolated allogeneic bone marrow-derived mesenchyal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone, Proc. Natl. Acad. Sci. USA 99 (2002) 8932–8937.
[33] K. Le Blanc, I. Rasmusson, B. Sundberg, C. Gotherstrom, M. Hassan, M. Uzuuel, O. Ringden, Treatment of severe acute graft-versus host disease with third party haploidentical mesenchymal stem cells, Lancet 363 (2004) 1439–1441.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 351 (2006) 853–859
BBRC
www.elsevier.com/locate/ybbrc

Successful immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells

Xiaohong Zhang [a], Yasushi Soda [b], Kenji Takahashi [a], Yuansong Bai [b], Ayako Mitsuru [a], Koichi Igura [a], Hitoshi Satoh [c], Satoru Yamaguchi [d], Kenzaburo Tani [e], Arinobu Tojo [b], Tsuneo A. Takahashi [a,*]

[a] *Division of Cell Processing, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*
[b] *Division of Molecular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan*
[c] *Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, Japan*
[d] *Yamaguchi Hospital, Nishi-Funa, Funabashi, Chiba, Japan*
[e] *Division of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan*

Received 3 October 2006
Available online 2 November 2006

Abstract

We reported previously that mesenchymal progenitor cells derived from chorionic villi of the human placenta could differentiate into osteoblasts, adipocytes, and chondrocytes under proper induction conditions and that these cells should be useful for allogeneic regenerative medicine, including cartilage tissue engineering. However, similar to human mesenchymal stem cells (hMSCs), though these placental cells can be isolated easily, they are difficult to study in detail because of their limited life span in vitro. To overcome this problem, we attempted to prolong the life span of human placenta-derived mesenchymal cells (hPDMCs) by modifying hTERT and Bmi-1, and investigated whether these modified hPDMCs retained their differentiation capability and multipotency. Our results indicated that the combination of hTERT and Bmi-1 was highly efficient in prolonging the life span of hPDMCs with differentiation capability to osteogenic, adipogenic, and chondrogenic cells in vitro. Clonal cell lines with directional differentiation ability were established from the immortalized parental hPDMC/hTERT + Bmi-1. Interestingly, hPDMC/Bmi-1 showed extended proliferation after long-term growth arrest and telomerase was activated in the immortal hPDMC/Bmi-1 cells. However, the differentiation potential was lost in these cells. This study reports a method to extend the life span of hPDMCs with hTERT and Bmi-1 that should become a useful tool for the study of mesenchymal stem cells.

Keywords: Placenta; Mesenchymal cells; Immortalization; Differentiation; Telomerase; Bmi-1

Human mesenchymal stem cells (hMSCs) from various sources are able to differentiate into different cell lineages under specific culture conditions [1,2], and have generated a great deal of interest because of their potential use in regenerative medicine. Recently the human placenta, umbilical cord, and amnion have received attention as possible sources of hMSCs because of their easy acquisition with few ethical problems compared to other types of cells [3–6]. Since information necessary for cord blood transplantation (i.e., HLA typing, viral screening, contamination by microorganisms, and examination of diseases in donors and their families) is routinely obtained by cord blood banks, the placenta and cord blood should be two of the safest sources of allogeneic mesenchymal cells for regenerative medicine. In this study, we chose chorionic villi from the fetal part of the human placenta as the mesenchymal cell source.

We have reported that mesenchymal progenitor cells derived from the chorionic villi of the human placenta can differentiate into osteoblasts, adipocytes, chondrocytes,

* Corresponding author. Fax: +81 3 5449 5452.
E-mail address: takahasi@ims.u-tokyo.ac.jp (T.A. Takahashi).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.10.125

and neural cells under specific induction conditions [6]. These cells can be used for chondrogenic tissue engineering [7], suggesting that hPDMCs could potentially represent a useful cell source for regenerative medicine. However, like hMSCs, hPDMCs proliferate slowly with an average life span of 21 population doublings, which makes the cells difficult to study in detail for possible clinical use. Normal human cells undergo limited cell division in culture and then enter a nondividing state called "senescence" [8]. It is generally accepted that normal human cells senesce because they acquire one or more short dysfunctional telomeres and lack telomerase expression [9]. It has also become clear that other factors such as DNA damage and oxidative stress cause cell growth arrest with a senescent phenotype, independent of telomere length and structure [10]. However, through a process known as ex vivo immortalization, it has become possible to induce primary cells to grow indefinitely in vitro by genetic manipulation [11]. Those processes represent an attractive means of producing large quantities of cells for experimental and therapeutic purposes.

In this study, we investigated life span extension of hPDMCs by lentiviral-mediated hTERT and Bmi-1 transduction. The results indicated that immortalization of hPDMCs required both activation of telomerase and down-regulation of p16 INK4a expression. The hPDMCs with an extended life span could differentiate into osteogenic, adipogenic, and chondrogenic cells in vitro. Eight clonal cell lines were established from immortalized parental hPDMC/hTERT + Bmi-1 and their capabilities for directional differentiation were examined. Interestingly, hPDMC/Bmi-1 showed an extended period of proliferation after long-term growth arrest and telomerase was activated in the immortal hPDMC/Bmi-1 cells; however, the differentiation ability was lost in those cells. Our data suggest that hPDMCs have multipotential differentiation capability and also that transduction with hTERT + Bmi-1 provides a useful method to overcome the short life span of hPDMCs.

Materials and methods

Production of lentiviral vector. To obtain the hTERT-coding lentiviral plasmid pHIV-TERT, a fragment containing hTERT cDNA was excised from PGRN145b (Geron Corp, Menlo Park, CA) with *Kpn*I and *Sal*I, and subcloned into *Nhe*I and *Xho*I sites of pCS-CDF-ChG, which coding humanized the R. reniformis GFP (hrGFP) gene. Next, the full-length Bmi-1 cDNA was generated by RT-PCR using primers (forward): 5′-*GCTAGC*AGAAATGCATCGAACAACGAGAATC-3′ (underlined: *Nhe*I site) and (reverse): 5′-*CTCGAG*TATCAACCAGAAGAAGTTGC TGA-3′ (underlined: *Xho*I site) from total RNA extracted from WI-38 cells. The Bmi-1 PCR product was cloned into pCR4Bblunt-TOPO vector using a Zero blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA). After confirming the Bmi-1 sequence, the fragment excised with *Nhe*I and *Xho*I was ligated to pCS-CDF-ChG-PRE digested with *Nhe*I and *Xho*I (pHIV-Bmi-1). The lentiviral vector stock was produced and the titer was measured as described previously [12].

Isolation, culture, and vector transduction of hPDMCs. The study was approved by the Internal Review Board of our institute. Mesenchymal cells were isolated from chorionic villi by the explant culture method described previously [6]. The migrated cells were regarded as population doubling 0 (PD 0). The hPDMCs were isolated without contamination by maternal cells, which was confirmed by XY chromosome analysis using fluorescence in situ hybridization (FISH) as described previously [6]. hPDMCs at PD 8 were inoculated with the hTERT expression lentiviral vector (hTERT-LV) or Bmi-1 expression lentiviral vector (Bmi-1-LV), or a combination of these two vectors at a multiplicity of infection (MOI) of 2.8 for 3 h to generate hPDMC/hTERT, hPDMC/Bmi-1 or hPDMC/hTERT + Bmi-1 cells.

Telomerase activity and telomere length assay. The telomerase activity was assessed by using a telomere repeat amplification protocol (TRAP) kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The telomere length was determined by using a Telo TAGGG Telomere Length Assay (Roche) as per the manufacturer's instructions.

Immunohistochemical staining of Bmi-1. The control and transduced cells at PD 10 were fixed with 4% paraformaldehyde and incubated with anti-Bmi-1 monoclonal antibody F6 (Upstate Inc., Lake Placid, NY) (1:200) overnight at 4 °C and Bmi-1 was detected using a diaminobenzidine tetrahydrochloride (DAB) substrate kit (Dako, Kyoto, Japan).

Western blot analysis. Western blotting was performed as described previously [11]. Equal amounts of protein (30 μg) were used for detection. The primary antibodies used were anti-Bmi-1 F6 and anti-β-actin (loading control) (Santa Cruz Biotech, Santa Cruz, CA).

Quantitative PCR. Total RNA was isolated using an RNAeasy mini kit (Qiagen, Tokyo, Japan). Following the manufacturer's protocol, cDNAs were synthesized from 1 μg aliquots of total RNA with Superscript II Reverse Transcriptase (Invitrogen) using the oligo (dT) primer (Invitrogen) in a total volume of 20 μl. Quantitative PCR was done as follows: 95 °C for 10 min, 50 cycles of PCR (95 °C for 15 s and 58 °C for 2 min) using 12.5 μl of 2× TaqMan Master (Roche), each primer at 0.6 μM, a 0.6 μM probe, 1 μl of the RT product, and H_2O to 25 μl. The level of mRNA was normalized by using GAPDH as an internal control. Every reaction was performed in duplicate. The results were analyzed using ABI PRISM 7700 program EDTECTOR 1.6. PCR. Sequences of primers and probes were as follows: hTERT (forward): 5′-ACGGCGACATGGAGA ACAA-3′, (reverse): 5′-CACTGTCTTCCGCAAGTTCAC-3′, probe: CTCCTGCCTTTGGTGGATGATTTCTTGTTG; p16 (forward): 5′-G CCCAACGCACCGAATAGT-3′, (reverse): 5′-CGCTGCCCATCATC ATGC-3′, probe: ACGGTCGGAGGCCGATCCA; p14 (forward): 5′-CC TCGTGCTGATGCTACT-3′, (reverse): 5′-CGCTGCCCATCATCA TGC-3′, probe: TCTAGGGCAGCAGCCGCTTC; GAPDH (forward): 5′-GAAGGTGAAGGTCGGAGTC-3′, (reverse): 5′-GAAGATGGTG ATGGGATTC-3′, probe: GGCTGAGAACGGGAAGCTTG.

Culture of immortalized clonal cells. One hundred hPDMC/hTERT + Bmi-1 cells at PD 20 were plated in a 100-mm diameter dish, and the cultures were maintained in the culture medium until well-defined clones were formed. Then the clones were harvested using sterile cloning rings and replated in a 100-mm diameter dish to form clones again. The clones were harvested and expended for analysis. Eight clonal cell lines were analyzed for osteogenic, chondrogenic, and adipogenic potential.

Osteogenic, chondrogenic, and adipogenic differentiation of hPDMCs. The differentiation potential of prolonged-culture cells was examined using the differentiation-induction protocol and differentiation assay described previously [6].

Results

Characteristics of hPDMCs transfected with hTERT, Bmi-1, or hTERT + Bmi-1

As described previously [6], the hPDMCs had fibroblast-like morphology as shown in Fig. 1Aa. By the FISH assay, 100% XY and 0% XX signals were detected in 500 migrated cells from the placenta of a male baby, indicating that these cells derived from the fetal part of the placenta, i.e., the chorionic villi, without contamination by the maternal part.

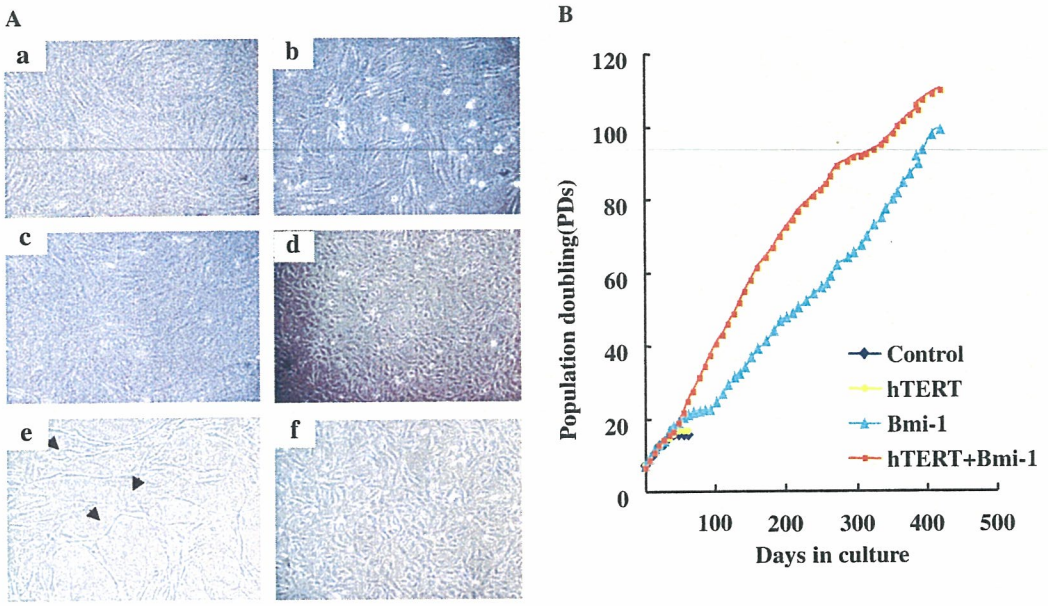

Fig. 1. Morphology and growth change. (A) Morphology of hPDMCs. (a) The control cells at PD 8. (b) The control cells at PD 15. (c) hPDMC/hTERT at PD 15. (d) hPDMC/hTERT+Bmi-1 at PD 20. (e) hPDMC/Bmi-1 at PD 22. Arrowheads indicate smaller, mitotically active cells among large cells. (f) hPDMC/Bmi-1 at PD 40. Magnification 100×. (B) Proliferation curves of control and transduced cells. Control cells (blue), hPDMC/hTERT (yellow), hPDMC/Bmi-1 (green), and hPDMC/hTERT+Bmi-1 (red) are shown. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

At PD 8, the hPDMCs were inoculated with hTERT-LV and/or Bmi-1-LV at an MOI of 2.8. The transduction efficiency of hrGFP-LV under the same conditions was shown to be almost 100% by FCM. Until PD 15, the growth rates of these transductants and untransduced hPDMCs were almost the same (Fig. 1B). The control and hPDMC/hTERT cells became broad, flat and stopped replicating (Fig. 1Ab and Ac) indicating that these cells had entered senescence. On the other hand, the cells transduced with hTERT + Bmi-1 and Bmi-1 escaped the replication crisis and proliferated continuously. During the following culture, the proliferation rate of hPDMC/hTERT + Bmi-1 accelerated and the cells became smaller (Fig. 1Ad). The proliferation rate of hPDMC/Bmi-1 became slow at approximately PD 20. However, smaller, mitotically active cells that existed among large cells proliferated when the hPDMCs/Bmi-1 were cultured continuously (Fig. 1Ae). The shape of hPDMC/Bmi-1 became uniform with increasing numbers of passages (Fig. 1Af), indicating that these cells had bypassed the replication crisis. The same results were obtained when the transduced cells at the 1st passage were cultured repeatedly to exclude the possibility that the results were caused by contamination by other types of cells during cell processing. Both hPDMC/hTERT + Bmi-1 and hPDMC/Bmi-1 were maintained in culture for more than 1 yr and continued to proliferate to more than PD 100, suggesting that these cells were immortal (Fig. 1B).

Telomerase activity and telomere length in transduced cells

Telomerase activity was undetectable in control and hPDMC/Bmi-1 cells at PD 15 (Fig. 2A). In contrast, telomerase activity was observed in hPDMC/hTERT at PD 15, and in hPDMC/hTERT + Bmi-1 at PD 15 and 30. Telomerase activity was detected in hPDMC/Bmi-1 at PD 30, indicating that telomerase was induced in hPDMC/Bmi-1 cells after they bypassed growth arrest (Fig. 2B). Telomere length in the control cells decreased with increases of PD, whereas it remained the same in the hPDMC/hTERT, regardless of the increase of PD. Telomeres in hPDMC/Bmi-1 at PD 20 were shorter than in control cells at PD 15, but became longer at PD 30 after bypassing the growth crisis. In hPDMC/hTERT + Bmi-1 telomeres were longer at PD 20 and 30, than at PD 10. Quantitative PCR of telomerase gene expression agreed with the results of the TRAP assay (Fig. 2C). That is, the control cells and hPDMC/Bmi-1 at PD 15 and 20 did not express the hTERT gene, but it was detected in hPDMC/hTERT at PD15 and in hPDMC/hTERT + Bmi-1 at all PDs. In hPDMC/Bmi-1, the hTERT gene was detected at PD 30 and continued to appear at PD 50 (Fig. 2C). These data indicated that activation of telomerase was by activating hTERT transcription in hPDMC/Bmi-1.

Overexpression of Bmi-1 in transduced cells

Overexpression of Bmi-1 was examined by immunohistochemical staining and Western blotting. The nuclei of cells transduced with hTERT + Bmi-1 and Bmi-1 were stained intensely with monoclonal antibody F6 against Bmi-1 (Fig. 3Aa and Ab). Slight nuclear staining in the control cells and hTERT-transduced cells at PD 10 was attributed to endogenous protein (Fig. 3Ac and Ad). The results of Western blotting showed overexpression of

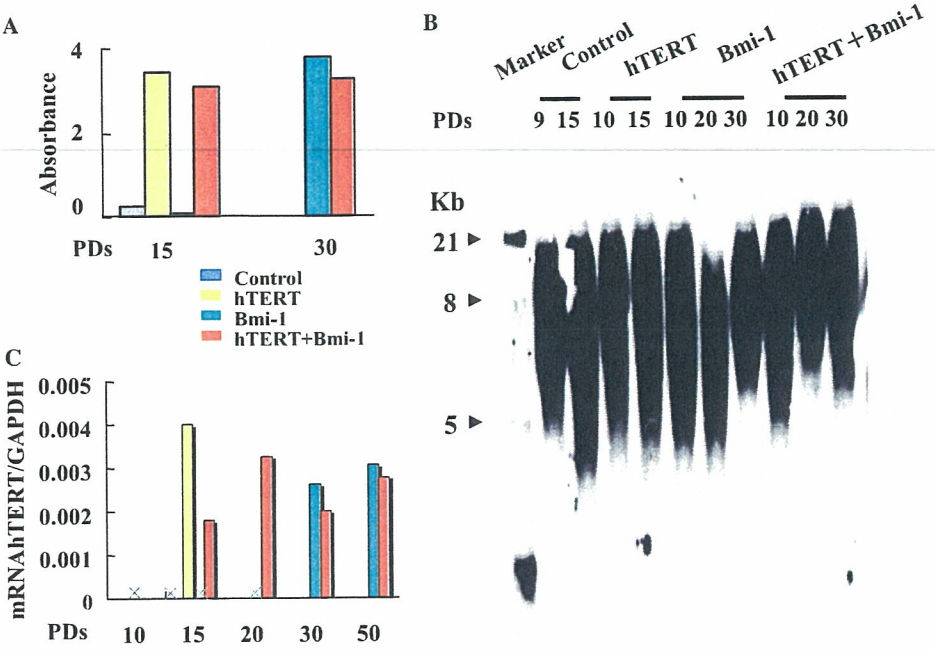

Fig. 2. Detection of telomerase activity. (A) Telomerase activity detected by TRAP assay. No control cells or hPDMC/hTERT cells were harvested at the stage of PD 30. (B) Telomere lengths of control and transduced cells. (C) Quantitative PCR assay shows the expression of hTERT. ×, not detected.

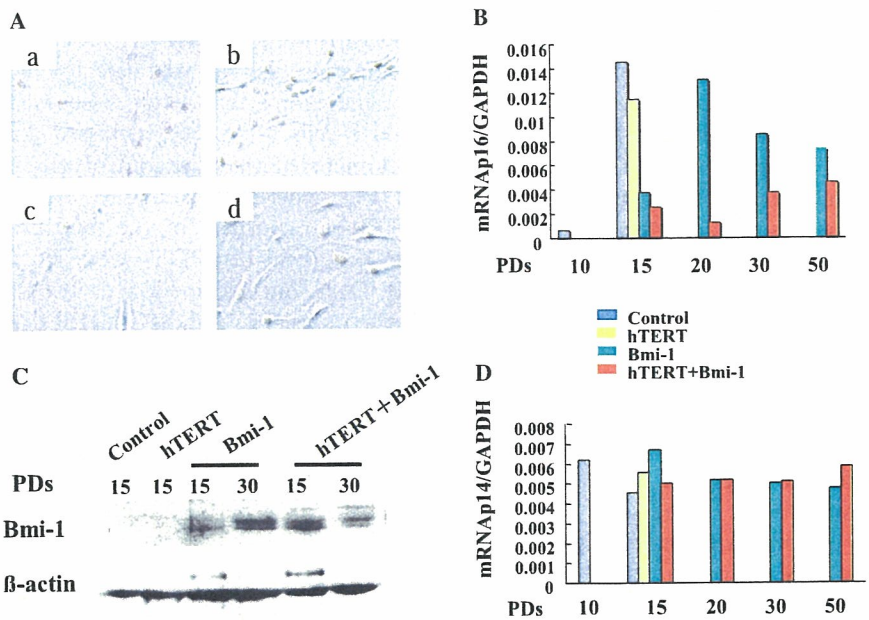

Fig. 3. Detection of overexpressed Bmi-1 and expression of p16 and p14 in hPDMCs. (A) Localization of Bmi-1. Cells at PD 10 were stained with mouse monoclonal antibody. hPDMC/hTERT+Bmi-1 (a) and hPDMC/Bmi-1 (b) were strongly stained in nuclei. Slight nuclear staining in control cells (c) and hPDMC/hTERT (d) was attributable to endogenous protein. Magnification 100×. (B) Western blotting of Bmi-1. No clear band is seen for control cells or hPDMC/hTERT at the senescent stage of PD 15. (C) Expression of p16 detected by quantitative PCR. (D) Expression of p14 detected by quantitative PCR. GAPDH served as an internal control.

Bmi-1 in hPDMC/Bmi-1 and hPDMC/hTERT + Bmi-1 at different PDs. No clear band was observed in the control cells or hPDMC/hTERT at PD 15, which was the senescent stage (Fig. 3B), indicating that expression of Bmi-1 declined in senescent cells. A similar result was shown for WI-38 fibroblasts in a previous report [13].

Down-regulation of p16 extended the life span of hPDMCs, but p14 did not

Bmi-1 is known to down-regulate the expression of two key tumor suppressors, p16 and p14 (p19 in the mouse), which are encoded by a single locus, INK4a [14]. By quan-

titative PCR analysis, we found that p16 expression was up-regulated in the control cells and hPDMC/hTERT at the senescence stage of PD 15 compared to that in the proliferating and young control cells at PD 10. In hPDMC/Bmi-1, the expression of p16 was low at PD 15 but increased at PD 20, then decreased at PD 30 and continued to be down-regulated at PD 50. On the other hand, the expression of p16 was low in hPDMC/hTERT + Bmi-1 at all PDs. However, the level of p16 expression increased as the PD increased (Fig. 3C). The expression level of p14 was the same in the control and transduced cells (Fig. 3D) indicating that extension of the life span of hPDMCs by transduction with Bmi-1 was caused by suppression of the expression of p16, but not p14.

Differentiation potentials of immortalized hPDMCs

Differentiation potentials of hPDMC/hTERT + Bmi-1 and hPDMC/Bmi-1 after prolonged culture were examined. The pellets of hPDMC/hTERT + Bmi-1 cultured for 3 weeks in chondrogenic induction conditions became larger, white, and opaque with glistening and transparency compared to those cultured without induction medium (Fig. 4A, top). The pellets of hPDMCs were positive for toluidine blue and type II collagen (Fig. 4Ba and Bb). Osteogenic differentiation was confirmed by mineralization (Fig. 4Bc) and adipogenic differentiation by lipid vesicles (Fig. 4Bd). However, hPDMC/Bmi-1 showed minimal differentiation potential after bypassing replicative senescence as demonstrated by the pellet size at each PD (Fig. 4A bottom), and no distinct osteogenic or adipogenic differentiation was observed at PD 25 or 30 (data not shown), indicating that the differentiation potential was lost in the cells with extended life spans.

Among eight clonal cell lines, there were four cell lines that showed bi-directional differentiation potential (Fig. 4D), one cell line showed only chondrogenic differentiation, and three cell lines showed no differentiation potential. However, no clonal cell lines differentiated into an adipocyte.

Discussion

In this study, we found that telomerase activity was not sufficient to immortalize hPDMCs. The hPDMCs at PD 15, the senescent stage, showed high expression of p16 compared to younger cells at PD 9. Combination of hTERT and Bmi-1 was shown to be highly effective to extend the life span of hPDMCs (Fig. 1B), indicating that the down-regulation of p16 was the first step necessary for the immortalization of the cells. hPDMC/Bmi-1 went through a long-term growth crisis, after which proliferation started (Fig. 1B). Activation of telomerase was observed in hPDMC/Bmi-1 cells that escaped from the replicative crisis

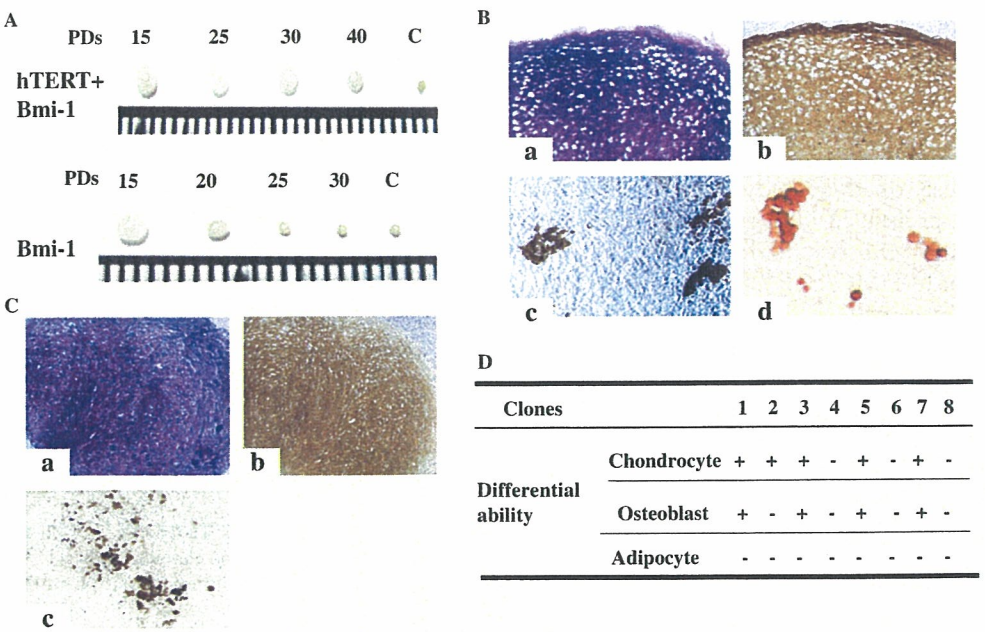

Fig. 4. Pellet culture of immortalized cells at 3 weeks of induction. (A) Top: pellet cultures of hPDMC/hTERT+Bmi-1 at PD 15, 25, 30, and 40, and the induction control (hPDMC/hTERT + Bmi-1 at PD 15). Bottom: pellet cultures of hPDMC/Bmi-1 at PD 15, 20, 25, and 30, and the induction control (hPDMC/Bmi-1 at PD 15). A 1-mm scale ruler is shown. (B) Differentiation potential of hPDMC/hTERT + Bmi-1 at PD 40. Histochemical evidence for chondrogenic ((a) toluidine blue staining, (b) type II collagen immunohistochemical staining), osteogenic ((c) von Kossa staining), and adipogenic ((d) Oil-red-O staining) differentiation. Magnification 100×. (C) Differentiation potential of hPDMC/hTERT + Bmi-1 clone 1. Histochemical evidence for differentiation in two directions, chondrogenic ((a) toluidine blue staining, (b) type II collagen immunohistochemical staining) and osteogenic differentiation ((c) von Kossa staining). Magnification 100×. (D) Differentiation ability of eight clonal cell lines derived from hPDMC/hTERT + Bmi-1 at PD 20.

and were immortalized. Such immortalization may occur in the growth arrest period, probably through the selection of cells that escape from the growth crisis, as reported previously for MRC-5 human lung fibroblasts [15].

We found that hPDMC/hTERT at PD 15 maintained telomere length compared to that in the control cells (PD 10 and 15). It is known that telomerase contributes to the stabilization and/or elongation of telomeres in immortal cells and cancer cells [16]. Telomerase function is regulated by TERT-associated protein and extension cannot be easily achieved if longer telomeres exist in cells [17]. Why telomere length was maintained but not elongated in hPDMC/hTERT at PD 15 may be explained by the long telomere or low function of telomerase in those cells. The expression of p16 was dramatically increased in hPDMC/Bmi-1 at PD 20 and then decreased at PD 30. We assume that many of the cells went into replicative senescence and crisis even though the cells were transfected with Bmi-1; but some infected cells escaped from the crisis, and in these cells the expression of p16 was down-regulated. There are many possible reasons why p16 increased at the crisis stage of hPDMC/Bmi-1, including overexpression of specific genes to induce p16, or no function of Bmi-1 for down-regulation of p16 during the replicative senescence and growth crisis, but we do not have evidence of the cause. A similar phenomenon of p16 down-regulation in immortal hPDMC/Bmi-1 cells was reported for MRC-5 fibroblasts [15]. There was no difference in cell-surface markers of hPDMCs transduced with Bmi-1 and those with a combination of hTERT and Bmi-1 (data not shown). Regarding tumorigenicity, transduced hPDMCs with an extended life span did not form any foci in vitro, and cell division stopped after they reached confluence. They were in the normal cell cycle and had diploid karyotypes (data not shown) indicating that malignant transformation did not occur.

The other purpose of this study was to determine whether cells with a prolonged life span could retain their differentiation potential. Our results showed that the differentiation potential of hPDMC/hTERT + Bmi-1 was maintained after prolonged culture (Fig. 4A, top and B). However, a gradual decrease of differentiation potential, particularly after prolonged culture (>PD 40), was observed in the pellets, i.e., decreases of size and alkaline phosphatase activity under conditions for chondrogenic and osteogenic differentiation, respectively (data not shown). There is a possibility that the decrease was caused by the long-term culture itself reported previously [18,19]. hPDMC/Bmi-1 did not differentiate after bypassing replicative senescence without any change in cell-surface marker of the mesenchymal cells (data not shown). The reason for this limitation is not known, but the possibility that a subtle mutation might have occurred in these cells and inactivated some critical master regulators of differentiation should be considered. Another possibility is that hPDMCs are heterogeneous in their potential for differentiation and only precursor hPDMCs/Bmi-1 cells with low differentiation ability were able to bypass replicative senescence.

Clonal analysis revealed that the differentiation potential was different in the clonal cell lines of hPDMC/hTERT + Bmi-1 (Fig. 4C). The existence of clonal cell lines with directional differentiation proved that the hPDMCs were likely mesenchymal stem cells. However, no cell lines could differentiate into adipocytes, and this was likely caused by long-term culture, similar to extensively cultured bone marrow-derived MSC that lost the ability to differentiate into adipocytes [20].

Our results show that extension of the life span of hPDMCs requires both activation of hTERT and down-regulation of p16. hPDMCs with an extended life span should be useful to study the use of cells for tissue engineering, as well as the mechanism of differentiation.

Acknowledgments

This work was partially supported by a Research Grant on Human Genome, Tissue Engineering (HP-D14, H17-022) from The Japanese Ministry of Health, Labor and Welfare, and Grant-in-Aid from The Japanese Ministry of Education, Culture, Sports, Science and Technology (16390429).

References

[1] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.

[2] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human adipose tissue is a source of multipotent stem cells, Mol. Biol. Cell. 13 (2002) 4279–4295.

[3] G. Kogler, S. Sensken, J.A. Airey, T. Trapp, M. Muschen, N. Feldhahn, S. Liedtke, R. Sorg, J. Fischer, C. Rosenbaum, S. Greschat, A. Knipper, J. Bender, O. Degistirici, J. Gao, A. Caplan, E.J. Colletti, G. Almeida-Porada, H.W. Muller, E. Zanjani, P. Wernet, A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential, J. Exp. Med. 200 (2004) 123–135.

[4] N. Ochsenbein-Kolble, G. Bilic, H. Hall, R. Huch, R. Zimmermann, Inducing proliferation of human amnion epithelial and mesenchymal cells for prospective engineering of membrane repair, J. Perinat. Med. 31 (2003) 287–294.

[5] Y.S. Fu, Y.C. Cheng, M.Y. Lin, H. Cheng, P.M. Chu, S.C. Chou, Y.H. Shih, M.H. Ko, M.S. Sung, Conversion of human umbilical mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: potential therapeutic application for Parkinsonism, Stem Cells 24 (2005) 115–124.

[6] K. Igura, X. Zhang, K. Takahashi, A. Mitsuru, S. Yamaguchi, T.A. Takahashi, Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta, Cytotherapy 6 (2004) 543–553.

[7] X. Zhang, A. Mitsuru, K. Igura, K. Takahashi, S. Ichinose, S. Yamaguchi, T.A. Takahashi, Mesenchymal progenitor cells derived from chorionic villi of human placenta for cartilage tissue engineering, Biochem. Biophys. Res. Commun. 340 (2004) 944–952.

[8] J. Campisi, Replicative senescencese: an old lives' tale? Cell 84 (1996) 497–500.

[9] C.B. Harley, A.B. Futcher, C.W. Greider, Telomeres shorten during ageing of human fibroblasts, Nature 345 (1990) 458–460.

[10] R.D. Ramirez, C.P. Morales, B.S. Herbert, J.M. Rohde, C. Passons, J.W. Shay, W.E. Wright, Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions, Genes Dev. 15 (2001) 398–403.
[11] T. Okamoto, T. Aoyama, T. Nakayama, T. Nakamata, T. Hosaka, K. Nishijo, T. Nakamura, T. Kiyono, J. Toguchida, Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells, Biochem. Biophys. Res. Commun. 295 (2002) 354–361.
[12] Y. Bai, Y. Soda, K. Izawa, T. Tanabe, X. Kang, A. Tojo, H. Hoshino, H. Miyoshi, S. Asano, Effective transduction and stable transgene expression in human blood cells by a third-generation lentiviral vector, Gene Ther. 10 (2003) 1446–1457.
[13] K. Itahana, Y. Zou, Y. Itahana, J.I. Matinez, C. Beausejour, J.J. Jacobs, M. van Lohuizen, V. Band, J. Campisi, Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1, Mol. Cell Biol. 23 (2003) 389–401.
[14] J.J. Jacobs, K. Kieboom, S. Marino, R.A. DePinho, M. Van Lohuizen, The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus, Nature 397 (1999) 164–168.
[15] L.M. Taylor, A. James, C.E. Schuller, J. Brce, R.B. Lock, Inactivation of p16INK4α, with retention of pRB and p53/21*cip1* function, in human MRC5 fibroblasts that overcome a telomere-independent crisis during immortalization, J. Biol. Chem. 279 (2004) 43634–43645.
[16] N.W. Kim, M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L.C. Ho, G.M. Coviello, W.E. Wright, S.L. Weinrich, J.W. Shay, Specific association of human telomerase activity with immortal cells and cancer, Science 266 (1994) 2011–2014.
[17] C. Autexier, N.F. Lue, The structure and function of telomerase reverse transcriptase, Annu. Rev. Biochem. 75 (2006) 493–518.
[18] S. Tsutsumi, A. Shimazu, K. Miyazaki, H. Pan, C. Koike, E. Yoshida, K. Takagishi, Y. Kato, Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF, Biochem. Biophys. Res. Commun. 288 (2001) 413–419.
[19] S. Shi, S. Gronthos, S. Chen, A. Reddi, C.M. Counter, P.G. Robey, C.Y. Wang, Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression, Nat. Biotechnol. 20 (2002) 587–591.
[20] P.A. Conget, J.J. Minguell, Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells, J. Cell Physiol. 181 (1999) 67–73.