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

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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,

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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.