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Stabilization of Cellular Properties and Differentiation Mutilpotential of Human Mesenchymal Stem Cells Transduced With hTERT Gene in a Long-Term Culture

Guoping Huang,[1] Qiang Zheng,[2] Jie Sun,[1] Chunjuan Guo,[1] Jinfeng Yang,[1] Rui Chen,[1] Yuling Xu,[1] Guozhong Wang,[1] Dan Shen,[3] Zhijun Pan,[2] Jie Jin,[3] and Jinfu Wang[1]*

[1]College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, P.R. China
[2]The Second Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang 310006, P.R. China
[3]The first Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang 310009, P.R. China

Abstract Human bone marrow mesenchymal stem cells (hMSCs) are promising candidates for cell therapy and tissue engineering. The life span of hMSCs during in vitro culture is limited. Human telomerase catalytic subunit (hTERT) gene transduction can prolong the life span of hMSCs and maintain their potential of osteogenic differentiation. We established a line of hMSCs transduced with exogenous hTERT (hTERT-hMSCs) and investigated its sustaining cellular properties in a long-term culture. This line of hTERT-hMSCs was cultured for 290 population doublings (PDs) without loss of contact inhibition. Under adipogenic, chondrogenic and osteogenic induction, hTERT-hMSCs at PD 95 and PD 275 could differentiate respectively into adipocytes, chondrocytes, and osteocytes. hTERT-hMSCs at these PDs showed no transforming activity through both in vitro assay of cell growth in soft agar and in vivo assay of tumorigenicity in NOD-SCID mice. Karyotype analyses showed no significant chromosomal abnormalities in hTERT-hMSCs at these PDs. These results suggested that the hTERT-hMSCs at lower population doubling levels (PDLs) should be considered as a cell model for studies of cellular senescence, differentiation and in vitro tissue engineering experiment because of its prolonged life span and normal cellular properties. J. Cell. Biochem. 103: 1256–1269, 2008.

Key words: human MSCs; hTERT; life span; differentiation; tumorigenicity

Human mesenchymal stem cells (hMSCs) can be easily isolated from iliac crest aspiration and rapidly expanded in vitro [Liechty et al., 2000; Woodbury et al., 2000; Mackenzie and Flake, 2001; Reyes et al., 2002; Wiedswang et al., 2003]. hMSCs have the capability of self-renewing and the multipotentials of differentiation into a variety of mesenchymal lineages such as osteoblasts, chondrocytes, and adipocytes under a special culture condition [Jaiswal et al., 1997; Mackay et al., 1998; Pittenger et al., 1999; Jiang et al., 2002]. Therefore, hMSCs are a useful source of cell transplantation involved in no ethical or immunological problems [Caplan, 1991; Prockop, 1997; Caplan and Bruder, 2001]. hMSCs have been used in the clinic and full of promise for cell therapy and tissue engineering as a source of regenerating cells and for local gene delivery in genetic and acquired disease as vehicles [Wakitani et al., 1995; Dennis and Caplan, 1996; Javazon et al., 2001; Gimeno et al., 2005; Bernardo et al., 2006].

However, hMSCs isolated from human bone marrow have a limited life span [Stenderup et al., 2003]. As normal somatic cells, hMSCs enter cellular senescence and stop dividing after several cell divisions in ex vivo culture [Hayflick, 1976; Campisi, 1997; Shi et al., 2002; Simonsen et al., 2002]. Telomeres are essential structures to keep the chromosome steady in eukaryotic cells and play a key role in sustaining cell division [Counter et al., 1992; Sandell and Zakian, 1993; Blasco et al., 1997; Van Steensel et al., 1998]. Because of replication

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*Correspondence to: Jinfu Wang, College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, P.R. China. E-mail: wjfu@zju.edu.cn

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problem in linear chromosomes, telomeric DNA is not completely replicated and telomeres are progressively shortened with each successive cell division. When telomeres are attenuated to a certain extent, the normal cells enter cellular senescence and do not divide again [Harley et al., 1990; Allsopp et al., 1992; Counter et al., 1992; Vaziri et al., 1993; Broccoli et al., 1995; Lingner et al., 1997]. After every round of DNA replication, the telomeres need to be extended through telomerase to counter the normal attrition of chromosome ends. The ex vivo proliferation potential of primary hMSCs is limited [about 10–25 population doubling [PD]; Qiu et al., 2004], implying that it would be difficult to obtain enough cells for cell experiment and in vitro tissue engineering. Shi et al. [2002] and Simonsen et al. [2002] demonstrated that human telomerase catalytic subunit (hTERT) gene transduction prolonged the life span of hMSCs and maintain their potential of osteogenic differentiation. However, with a commendable lack of complacency, Serakinci et al. [2004] and Burns et al. [2005] in the same group as Simonsen et al. [2002] has found the neoplastic potential of the hTERT-transduced hMSCs at different population doubling levels (PDLs).

In the present study, we also established hMSCs transduced with human TERT (hTERT-hMSCs), analyzed the differentiation multipotential and the cellular properties of hTERT-hMSCs at PDs lower than 300 in comparison with those of primary MSCs. hTERT-hMSCs with a prolonged life span, especially for those at lower PDs, should become a good model for studies of cellular senescence, differentiation and in vitro tissue engineering experiment.

MATERIALS AND METHODS

Isolation and Culture Expansion of hMSCs

Human bone marrow samples were collected from healthy human donors (18- to 46-year old) under a protocol approved by Institutional Review Board. Each sample was washed twice and suspended with phosphate-buffered saline (PBS). The cell suspension was centrifuged over a Ficoll step gradient with a density of 1.077 g/ml (Ficoll-Histopaque 1077, Sigma, Shanghai, China) at 1,800*g* for 20 min. The mononuclear cell layer at the Ficoll/plasma interface was aspirated, washed with PBS twice, and then suspended with hMSCs medium in a flask at a density of 2×10^7 cells per 75-cm^2. Cells were cultured at 37°C in a 95% air-5% CO_2 atmosphere. hMSC medium consisted of minimal essential medium α (MEM-α; HyClone, Shanghai, China) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Hangzhou, China), 100 U/ml ampicillin and 100 U/ml streptomycin (Life Technologies, Beijing, China). In 14 days, the well-spread and attached hMSCs reached 90% confluence. After removal of non-adherent cells by changing medium, hMSCs were detached by trypsin-EDTA (Life Technologies) and seeded in a flask at a density of 5×10^5 cells per 75-cm^2 as passage 1.

For analysis of proliferation rate of cells, cells from subconfluent cultures were detached by trypsin and counted using a hemacytometer. The viability of cells was determined using the trypan blue-dye exclusion. PDs were calculated using the formula: PD = log [(n cells in)/(n cells out)]/log 2. PD times were calculated from the average of two consecutive passages.

Generation of Recombinant Retrovirus and Transduction of hMSCs

The vectors pHy-hTERT, pHy-EGFP, and pHy-hTERT-EGFP were employed in this experiment (Fig. 1). The retroviral vector pHy-hTERT was constructed by subcloning the human telomerase reverse transcriptase (hTERT) cDNA from pGRN145 (generously provided by Wu, Institute of Burn Research, Southwest Hospital, Third Military Medical University) into pHy (between Hpa I and Not I sites). The vector pHy-EGFP was constructed by subcloning the EGFP gene from pEGFP-N1 into pHy (between Sal I and Not I sites). The vector pHy-hTERT-EGFP was constructed by subcloning the EGFP gene into pHy-hTERT (between Sal I and Not I sites). The plasmid pHy-hTERT was transduced into the packaging cell line PA317 to produce viral supernatant using LipofectAMINE transfection reagent (Life Technologies). Transduced cells were selected with 200 μg/ml hygromycin for 7 days. The titers of pHy-hTERT-derived retroviruses were analyzed using NIH 3T3 target cells with varied dilutions of retroviral supernatants. Primary hMSCs (2.0×10^5) with 80% confluent at passage 3 in a 10-cm dish were exposed to viral supernatant (containing retrovirus at an approximate multiplicity of infection (MOI) of

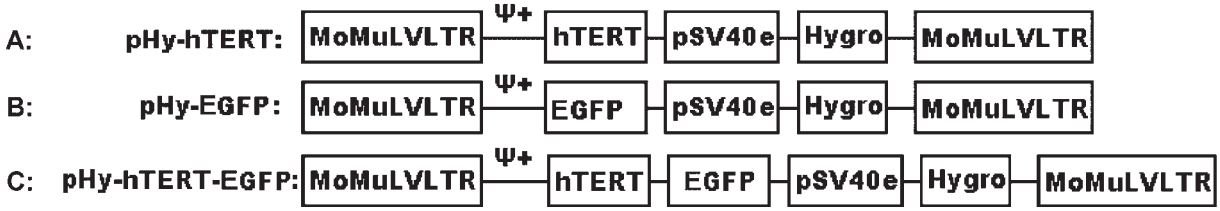

Fig. 1. Schematic representation of the retrovirus vector constructions. **A**: pHy-hTERT; (**B**) pHy-EGFP; (**C**) pHy-hTERT-EGFP. The construction of vectors used in this study is described in detail in Materials and Methods Section. MoMuLVLTR: Moloney murine leukemia virus long terminal repeat; ψ+: extended viral packaging signal; Hygro: hygromycin phosphotransferase gene; PSV40e: early SV40 promoter; EGFP: enhanced green fluorescent protein gene.

300) for 8 h in the presence of 8 μg/ml polybrene (Sigma). The transduced cells were washed with PBS and incubated in hMSC medium for 48 h, and then selected with 20 μg/ml hygromycin for 7 days. The surviving cells were noted as the first passage of hTERT-hMSCs. For the localization analysis of ectogenic hTERT gene, the plasmid pHy-hTERT-EGFP and pHy-EGFP were transduced into the primary hMSCs with 80% confluent at passage 3 using Lipofect-AMINE transfection reagent (Life Technologies) for the transient transfection according to the manufacturer's protocol. The fluorescence of transduced cells was photographed under fluorescence microscopy.

Analysis of Expression of hTERT by RT-PCR and Northern Blot Analysis

Total cellular RNA was extracted from cells using Trizol reagent (Invitrogen, Beijing, China) according to the manufacturer's instructions. RT-PCR was performed using Access-Quick™ RT-PCR System (Invitrogen). The primers for β-actin were: forward 5′-CAT CTC TTG CTC GAA GTC CA-3′; reverse 5′-ATC ATG TTT GAG ACC TTC AAC A-3′ with a 318 bp of expected band. The primers specific for MoMuLVhTERT were: forward, 5′-CTC TCC CCC TTG AAC CTC CTC GTT C-3′; reverse, 5′-AGG ACA CCT GGC GGA AGG AG-3′ with a 408 bp of expected band. PCR was for 35 cycles and each consisted of 95C for 45 s, 60C for 45 s, and 72C for 90 s after 2-min initial denaturation at 95C. PCR products were electrophoresed in a 1% agarose/ethidium bromide gel and photographed under UV light. For Northern blotting analysis, RNAs were denatured with glyoxal, electrophoresed in 1% agarose gels (5 μg RNA per lane), and transferred onto a nylon membrane overnight (Hybond-N, Amersham International PLC, Buckinghamshire, UK). Hybridization was performed with ^{32}P-labeled cRNA riboprobes. As an internal control for determination of the amount of RNA loaded, the filters were hybridized simultaneously with a ^{32}P-labeled human β-actin. ^{32}P-labelled cRNA probes were prepared using [α-^{32}P] UTP (Amersham) and Ambion's MAXIscript in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer's instruction. The intensity of the signals was quantified with a densitometric scanning apparatus (Molecular Dynamics, Beijing, China) by using ImageQuant software, and normalized relative to the internal control.

Western Blot Analysis

Western blot analysis was performed to measure expression of hTERT protein. Cells were washed twice with PBS, and nuclear proteins were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Beijing, China). Fifty micrograms of nuclear extracts were separated on a 12.5% SDS–PAGE and transferred to nitrocellulose membranes. Mouse anti-hTERT (Novus Biologicals, Littleton, CO) was used as the primary antibody. Horseradish-peroxidase conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech (Hong Kong, China). Anti-β-actin monoclonal antibody was purchased from Sigma. Proteins were visualized by means of enhanced chemiluminescence (ECL) (Sigma).

Telomeric Repeat Amplification Protocol (TRAP) Assay

Telomerase activity was assayed with a telomeric repeat amplification protocol (TRAP) using PCR-based methodology (TRAPeze telomerase detection kit; Chemicon International, Wuhan, China) on 1×10^6 cells. The manufacturer's instructions were strictly followed. The TRAP reaction products were separated on a 10% non-denaturing polyacrylamide gel. After

electrophoresis, the gel is stained with ethidium bromide and photographed under UV light.

RT-PCR Analysis of DBCCR1 Gene Expression

Total RNA from cultured cells was prepared using Total RNA Isolation Reagent (Advanced Biotechnologies). *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) RT-PCR was used as a control for RNA quality and reverse-transcription reaction. RT-PCR was performed as described by Habuchi et al. (1998) with a little modification. Briefly, approximately 2 μg of total RNA was reverse-transcribed with an oligo (dT) primer using the SuperScript Preamplification System (Life Technologies) according to the manufacturer's protocol. First-strand cDNA was diluted with 1 × TE (pH 8.0), and cDNA equivalent to 100 or 25 ng of total RNA was used as a template for PCR amplification of *DBCCR1* or *GAPDH* in 25-μl reaction volumes. The primers for *DBCCR1* were designed as: 5′-CAA CGC ACT GCC CGC AAG CTT (sense) and 5′-TGT TCC CGC CTA TCA CGC AGG (antisense), giving a 232 bp fragment from the cDNA template. Conditions for *DBCCR1* RT-PCR were 95°C for 4 min, 35 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 90 s, followed by incubation at 72°C for 5 min in 1× *Taq* polymerase buffer (Life Technologies) with 1.2 mM $MgCl_2$, 200 μM dNTPs, and 2 units *Taq* polymerase (Life Technologies). Primers for the *GAPDH* gene were designed as: 5′-CGA GCC ACA TCG CTC AGA CA (sense) and 5′-TGA GGC TGT TGT CAT ACT TCT C (antisense), giving a 455 bp fragment. Conditions for *GAPDH* amplification were 95°C for 4 min, 30 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 120 s, followed by incubation at 72°C for 5 min in 1× *Taq* polymerase buffer with 1.0 mM $MgCl_2$, 200 μM dNTPs, and 1 unit *Taq* polymerase. PCR products were electrophoresed in 2% agarose gels and photographed under UV light.

Flow Cytometry Analysis

hTERT-hMSCs at PD 95 and PD 275 as well as primary hMSCs at PD 12 were incubated with mouse anti-CD monoclonal antibody (Ab), followed by staining with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated rat anti-mouse IgG Ab specific for CD166, CD45, CD34, CD117, HLA-DR (BD Biosciences, Shanghai, China), CD105, CD29 (Serotec, Kidlington, Oxford, UK), and CD44 (Immunotech, Marseille, France) to phenotypically characterize hTERT-hMSCs and to define their purity. Appropriate isotype-matched, non-reactive fluorocrome-conjugated antibodies were employed as controls. Analysis of cell populations was performed by means of direct immunofluorescence on a FACScalibur flow cytometer (BD Biosciences) and data calculated using CellQuest software (BD Biosciences).

Adipogenesis, Chondrogenesis, and Osteogenesis of hTERT-hMSCs

For adipogenesis, hTERT-hMSCs with nearly 90% confluence were exposed to the hMSC medium supplemented with 10 ng/ml insulin-like growth factor I (IGF-I), 100 μmol/L indomethacin, 1 μmol/L dexamethasone and 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX) (Sigma) for 21 days. For control, hTERT-hMSCs at PD 95 were cultured in the hMSC medium. Medium was changed every 3 or 4 days. After induction for 3 weeks, cells were washed with PBS, stained with Oil Red O, and then observed under a light microscope. The Oil Red O solution was prepared by mixing three parts of stock solution (Sigma) with two parts of water and filtering through a 0.2-μm filter.

For chondrogenesis, hTERT-hMSCs were cultured to 90% confluence in the hMSC medium and then were replaced with chondrogenic induction medium consisting of the hMSC supplemented with transforming growth factor β1 (TGF-β1; 10 ng/ml), IGF-I (10 ng/ml), Vitamin C (50 μg/ml; Invitrogen). For histochemical analysis of induced cells, we performed the pellet culture as Johnstone et al. [1998] with a little modification. Briefly, 2×10^5 cells were centrifuged in a 15-ml polypropylene tube, and the pellets were cultured in chondrogenic induction medium for three weeks. For control, hTERT-hMSCs at PD 95 were cultured in the hMSC medium. The medium was replaced every 2–3 days. Then, the pellets were embedded in paraffin, cut into 5 μm sections and stained with alcian blue 8GX to show the cartilage proteoglycans. The expression of type II collagen was detected using EnVision immunohistochemical kit (Dako, Beijing, China).

For osteogenesis, hTERT-hMSCs with nearly 90% confluence were exposed to the hMSC medium supplemented with 0.1 μM dexamethasone, 10 mM β-glycerophosphate and 52 mg/L Vitamin C for 14 days. For control, hTERT-hMSCs were cultured in the hMSC medium. Medium was changed every 3 days. After

induction for 2 weeks, cells were washed with PBS, and then assessed the calcium phosphate using a Von Kossa staining and the alkaline phosphatase using alkaline phosphatase (ALP) staining [Jiang et al., 2002].

Karyotype Analysis

Cells at exponential phase in metaphase were arrested with 0.05 μg/ml colcemid (Gibco BRL) for 1 h and then harvested according to the standard methods. Slides for chromosomes painting were dehydrated sequentially with 70%, 90%, and 100% ethanol (10 min each) at room temperature and were then stored at −20°C until use. Cytogenetic analysis was performed using Giemsa-banding (G-banding). Interpretation of karyotypes was based on the ISCN 1995 standard.

Soft Agar Anchor-Independent Assay and Tumorigenicity Assay

To test the anchorage-independent growth, we suspended hTERT-hMSCs at PD 95 or PD 275 or primary hMSCs at PD 12 in hMSC medium with 0.35% low-melting-point agar (Sigma) at a density of 5×10^4/3 ml and poured cell suspension on a solidified layer of 0.5% agar medium in a 60 mm dish. In 1, 4, and 10 days, cell colonies forming in soft agar were photographed. NIH-3T3 cells were used as a positive control.

In vivo tumorigenicity was assessed by cell transplantation into NOD-SCID mice. 8-week-old NOD/LtSz-scid/scid (NOD/SCID) mice were bred from breeding pairs originally obtained from the Central Institute for Experimental Animals, Shanghai Institutes for Biological Sciences, CAS, and maintained in the defined flora animal facility located at Zhejiang Academy of Medical Sciences. All animals were handled under sterile conditions. 15 NOD-SCID mice per cell line were subcutaneously injected with 2×10^6 cells at the flank of abdomen and observed for tumor growth at the injection site. NIH-3T3 cells were used as a positive control.

Statistical Analysis

Statistical significance between groups was determined using mean ± SEM, and statistical comparisons were performed using the Student's *t*-test. A level of $P < 0.05$ was accepted as significant.

RESULTS

Localization of Transiently Expressed hTERT-EGFP Protein and Prolonged Life Span of the hTERT-hMSCs

For analysis of exogenous hTERT protein localization, we constructed a plasmid encoding hTERT fused with Enhanced Green Fluorescent protein (pHy-hTERT-EGFP) and a plasmid encoding EGFP alone (pHy-EGFP; Fig. 1). The hMSCs transduced with pHy-EGFP (Fig. 2A–C) or pHy-hTERT-EGFP (Fig. 2D–F) and the primary hMSCs at PD 12 without transduction (Fig. 2G–I) were photographed under a microscope (Fig. 2A,D,G), a fluorescent microscope (Fig. 2B,E,H) or a microscope after Hematoxylin–Eosin staining that showed the nucleolus of cells (Fig. 2C,F,I). We found that EGFP was localized all over the cell (Fig. 2B), and that hTERT-EGFP was localized only in the nucleus (Fig. 2E). The result indicated that hTERT was a nucleus resident protein.

For permanent exogenous hTERT protein expression in hMSCs, we constructed a retroviral vector encoding hTERT (pHy-hTERT; Fig. 1) and transduced it into the packaging cell line PA317 to produce viral supernatant. Primary hMSCs at passage 3 were exposed to viral supernatant to construct hTERT-hMSCs. Up to now, hTERT-hMSCs have been undergone 290 PDs without loss of contact inhibition while primary hMSCs undergoing senescence-associated proliferation arrest after 25 PDs (Fig. 3A). The growth curve of hTERT-hMSCs compared with that of primary hMSCs showed the effect of hTERT expression on the life span of hTERT-hMSCs. RT-PCR was performed to confirm the expression of exogenous hTERT in hTERT-hMSCs at PD 95 and PD 275 (Fig. 3B). The expression of exogenous hTERT in hTERT-hMSCs from these two passages was identical, and the exogenous hTERT was not detected in the primary hMSCs at PD 12. Northern blotting analyses and Western blotting analyses also showed that exogenous hTERT was strongly expressed in hTERT-hMSCs at PD 95 and PD 275 while no exogenous hTERT was detected in primary hMSCs (Fig. 3C,D). The TRAP assay was performed to examine the telomerase activity in primary hMSCs and hTERT-hMSCs. Figure 3E showed a high TRAP activity in hTERT-hMSCs at PD 95 and PD 275, although their levels of TRAP activity were less than that of NIH-3T3 cells as a positive control. The

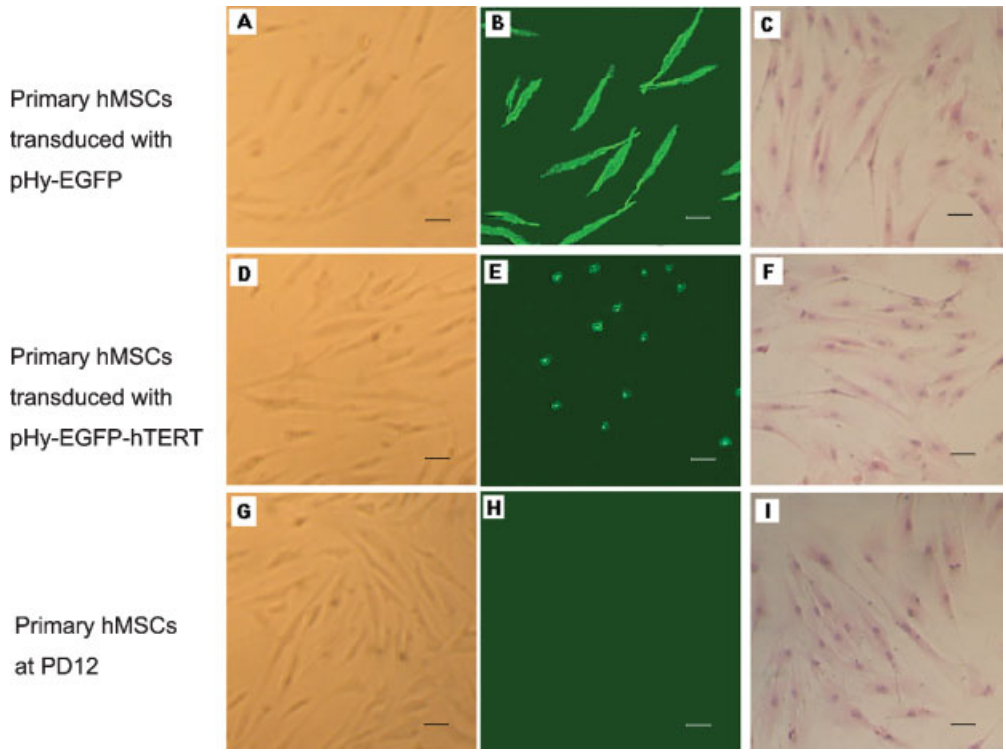

Fig. 2. The localization of exogenous expressed hTERT-EGFP in the nucleolus (scale bar: 100 μm). The pHy-EGFP transduced hMSCs were photographed under microscopy (**A**), under fluorescence microscopy (**B**), and photographed under microscopy after being stained with H.E. staining (**C**). The pHy-hTERT-EGFP transduced hMSCs were photographed under microscopy (**D**), under fluorescence microscopy (**E**), and photographed under microscopy after being stained by H.E. staining (**F**). Primary hMSCs at PD 12 were photographed under microscopy (**G**), under fluorescence microscopy (**H**), and photographed under microscopy after being stained by H.E. staining (**I**).

primary hMSCs showed no detectable TRAP activity. Therefore, it demonstrated that the exogenous hTERT gene was successfully transduced into hMSCs, and the expression of exogenous hTERT protein in hTERT-hMSCs was sustained during proliferating process.

Cell Surface Antigen and Karyotype Analysis of hTERT-hMSCs

Immunophenotypic analysis of surface antigens in hTERT-hMSCs at PD 95 and PD 275 as well as primary hMSCs at PD 12 by FACS showed that hTERT-hMSCs and primary hMSCs were all negative for hematopoietic markers (CD117, CD34, HLA-DR, and CD45) and positive for markers present in mesenchymal stem cells (CD29, CD44, CD105, and CD166) (Fig. 4A–C). We found that the pattern of hTERT-hMSCs surface antigens had no immunological difference with that of primary hMSCs ($P < 0.05$), which implied that hTERT-hMSCs maintained their surface antigens of parental hMSCs.

Karyotypic analysis was performed for primary hMSCs and hTERT-hMSCs at PD 95 and PD 275. All cells were diploid and no significant chromosomal abnormalities (Fig. 5). The chromosome number was 46. No cells containing abnormal numbers of chromosome were detected. The sex chromosomes were found to be XX, indicating that the donor was female.

Sustaining Differentiation Multipotentials of hTERT-hMSCs

hTERT-hMSCs at PD 95 and PD 275 were induced in the adipogenic induction medium containing IGF-I, indomethacin, dexamethasone, and IBMX for 3 weeks, and then stained with Oil Red O. The result showed that hTERT-hMSCs at these two passages had developed lipid vacuoles after induced with adipogenic induction medium (Fig. 6A,B), and the cells cultured in hMSC medium showed no detectable lipid vacuoles (Fig. 6C). It indicated that the differentiation potential of hTERT-hMSCs

into adipoblast was sustained during their proliferation.

hTERT-hMSCs cultured in chondrogenic induction medium containing TGF-β1, IGF-I, and Vitamin C for 21 days underwent a significant increase in type II collagen production. The matrix of the induced cells showed a strong alcian blue 8GX staining that was

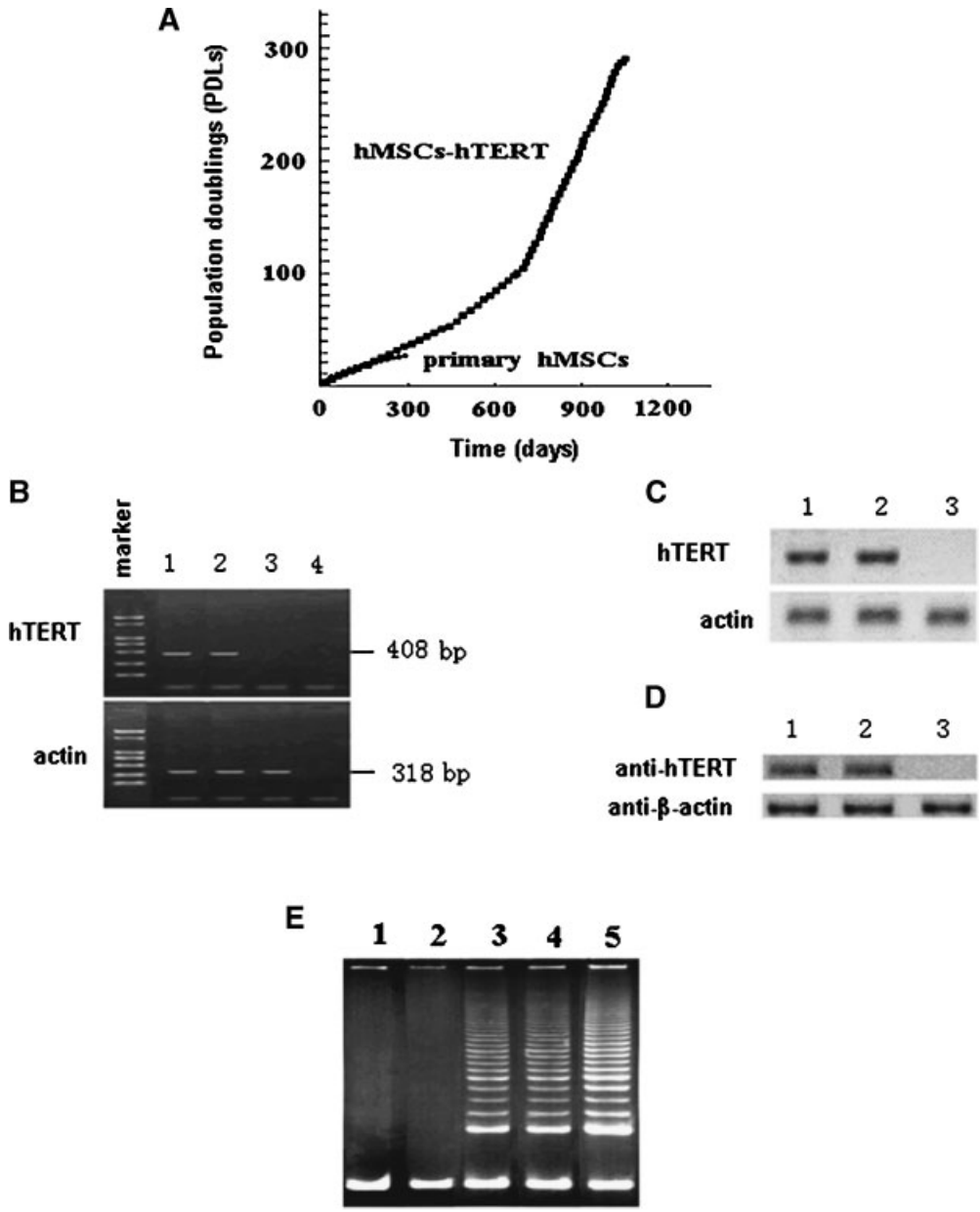

Fig. 3. The life span, hTERT expression and telomerase activity in primary hMSCs and hTERThMSCs. hTERT-hMSCs have proliferated for 290 PDs (lozenges) and primary hMSCs for 25 PDs (circular dots) (**A**). Expression of hTERT was detected using RT-PCR (**B**), Northern blotting (**C**), and Western blotting (**D**). B: The expected 408 bp for hTERT and 318 bp for β-actin bands are indicated. The four lanes are marker (3-kb ladder, Invitrogen), hTERThMSCs cells at PD 95 (**lane 1**), hTERT-hMSCs cells at PD 275 (**lane 2**), primary hMSCs at PD 12 (negative control, **lane 3**), H_2O control (**lane 4**). C: Northern blotting shows positive expression of exogenous hTERT in the hTERT-hMSCs cells at PD 95 (lane 1), the hTERThMSCs cells at PD 275 (lane 2) and negative expression in the primary hMSCs at PD 12 (negative control, lane 3). D: Western blotting shows positive expression of exogenous hTERT proteins in the hTERT-hMSCs cells at PD 95 (lane 1), the hTERT-hMSCs cells at PD 275 (lane 2) and negative expression in primary hMSCs at PD 12 (negative control, lane 3). **E**: The telomerase activity was analyzed by TRAP assay. No telomerase activity was detected in the primary hMSCs at PD 12 (lane 1) and the CHAPS buffer alone (lane 2). The hTERT-hMSCs cells at PD 95 or 275 exhibited significant telomerase activity (lanes 3 and 4, respectively) compared with that of the NIH-3T3 cells (**lane 5**) as a positive control.

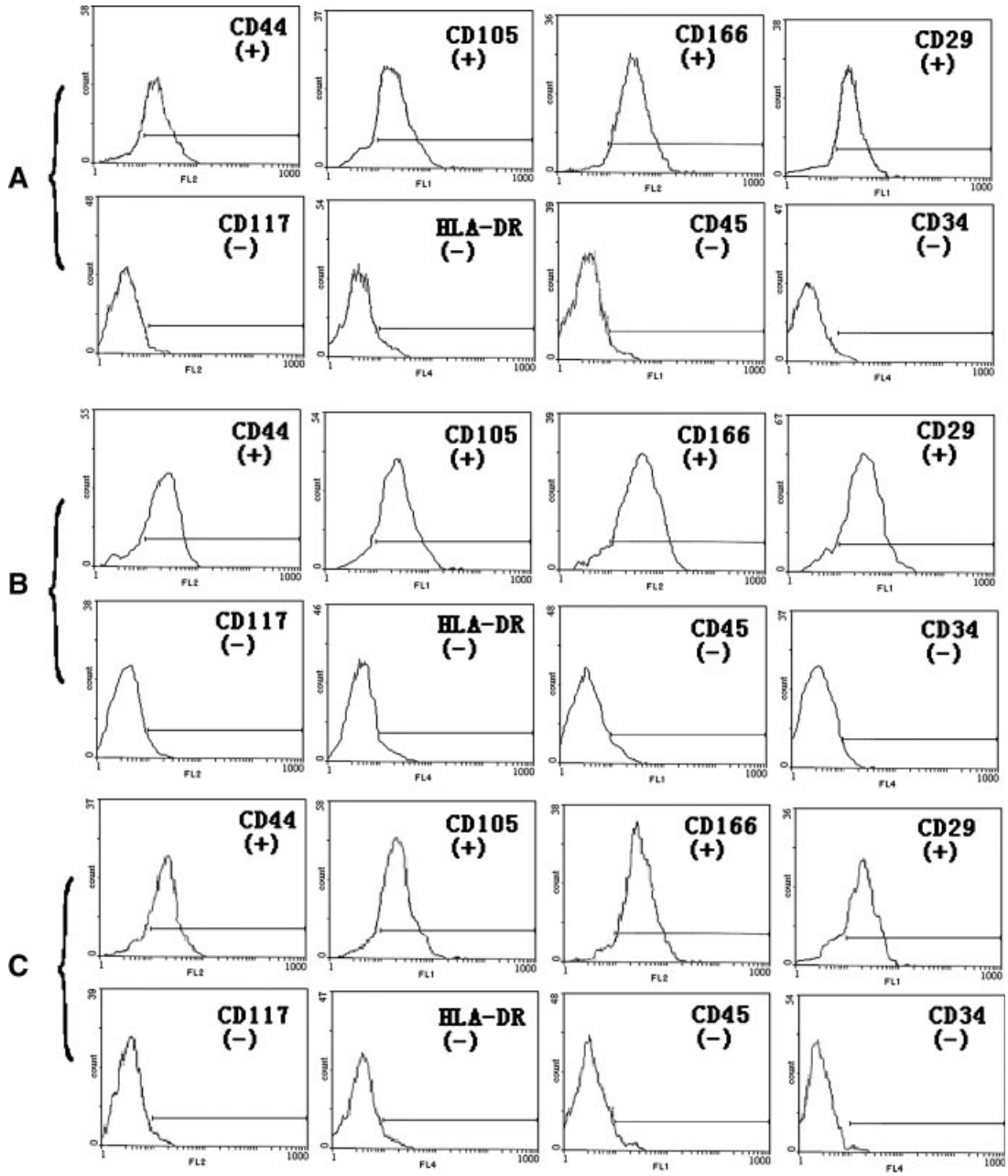

Fig. 4. Flow cytometric analysis of surface antigens on hTERT-hMSCs cells. Representative data of flow cytometric analysis of the hTERT-hMSCs cells at PD 95 (**A**), at PD 275 (**B**), and primary hMSC at PD 12 (**C**) using antihuman CD44, CD105, CD166, CD29, CD117, CD45, CD34, HLA-DR antibody. All the three cells are positive for CD29, CD105, CD44, and CD166 and negative for CD34, CD45, CD117, and HLA-DR.

specific for cartilage proteoglycans (Fig. 6D,E), and cells cultured in normal medium only showed a red nuclei staining (Fig. 6F). The expression of type II collagen was detected using EnVision two-step immunohistochemical staining technique. The matrix of induced hTERT-hMSCs at PD 95 and PD 275 showed red staining (Fig. 6G,H) and the cells cultured in primary medium only showed a blue nuclei staining (Fig. 6I).

For osteogenic differentiation, hTERT-hMSCs were cultured for 2 weeks in osteogenic induction medium containing dexamethasone, β-glycerophosphate and Vitamin C and assessed using a Von Kossa staining as an indicator of calcium phosphate and alkaline

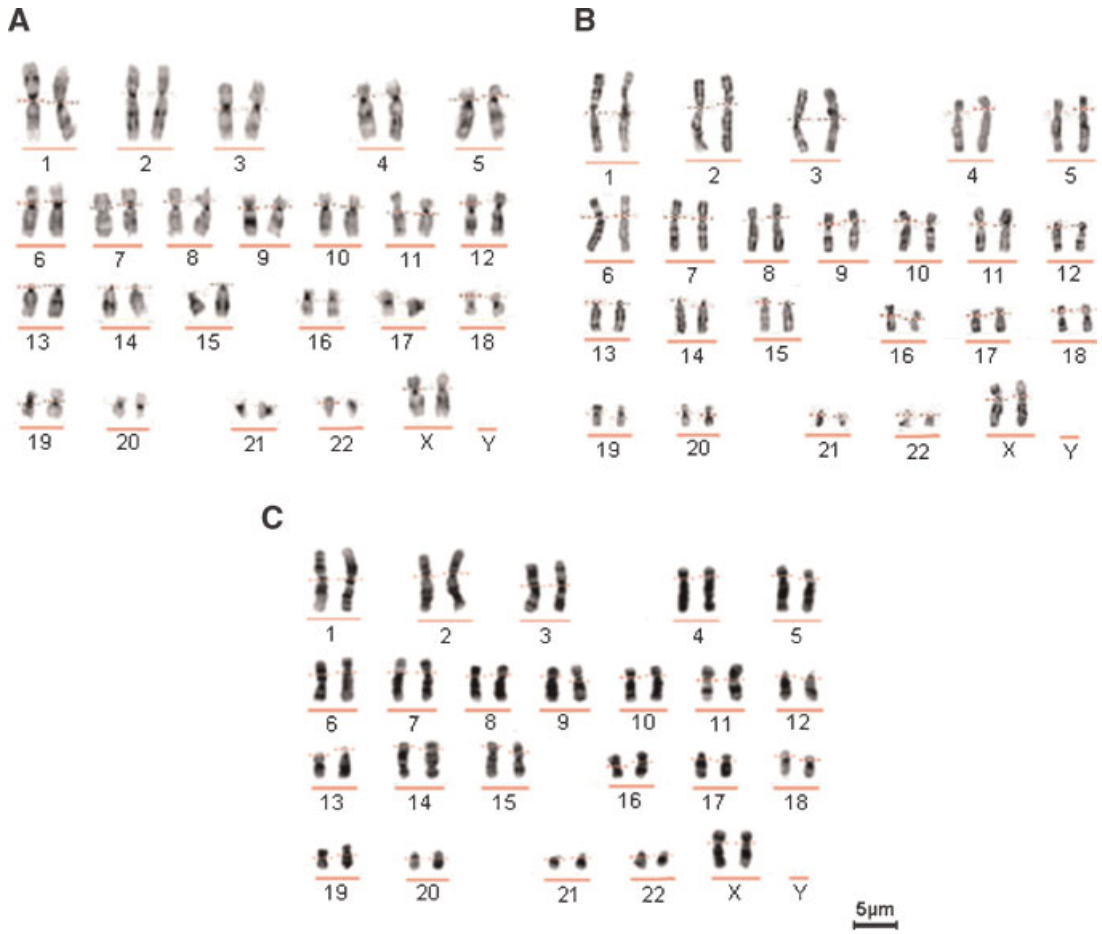

Fig. 5. G-banded karyotypes (46, XX) of primary hMSCs at PD 12 (**A**), hTERT-hMSCs at PD 95 (**B**), and PD 275 (**C**); (scale bar: 5 μm).

phosphatase (ALP) staining as an indicator of alkaline phosphatase. The induced hTERT-hMSCs at PD 95 and PD 275 showed black and blue staining respectively through Von Kossa staining and ALP staining. It indicated the existence of calcium phosphate (Fig. 6J,K) and alkaline phosphatase (Fig. 6M,N). The hTERT-hMSCs at PD 95 cultured in normal medium showed no detectable calcium phosphate (Fig. 6L) and calcium phosphate (Fig. 6O). All the results suggested that the hTERT-hMSCs sustain the multipotential of differentiation into adipocytes, chondrocytes, and osteoblasts during their proliferation.

Tumorigenicity Assay

We used both assays of in vitro cell growth in soft agar and in vivo tumorigenicity in NOD-SCID mice to examine the tumorigenicity of hTERT-hMSCs. In the assays of in vitro cell growth in soft agar, primary hMSCs (Fig. 7A1–A3), hTERT-hMSCs at PD 95 (Fig. 7A4–A6) and PD 275 (Fig. 7A7–A9) were unable to form colonies in soft-agar medium in comparison with the positive NIH-3T3 cells (Fig. 7A10–A12), indicating that they did not gain the anchorage-independent growth property. In the assays of in vivo tumorigenicity in NOD-SCID mice, 2×10^6 hTERT-hMSCs per site were injected into 15 NOD-SCID mice, no tumor formation was found at the injection site after 6 months (Fig. 7B). While 2×10^6 NIH-3T3 per site was injected into 15 NOD-SCID mice, the tumors were well vascularized within 10–14 days and caused 5 hosts death in 4–6 weeks. The size of tumors is 1.75 ± 0.9 cm in diameter (Fig. 7C). The result showed that hTERT-hMSCs has no tumorigenicity potential.

The *DBCCR1* gene at chromosome 9q33 has been identified as a candidate tumor suppressor. The loss of *DBCCR1* gene expression due to its promoter hypermethylation is correlated with acquisition of the tumorigenic phenotype in hTERT-hMSCs. We

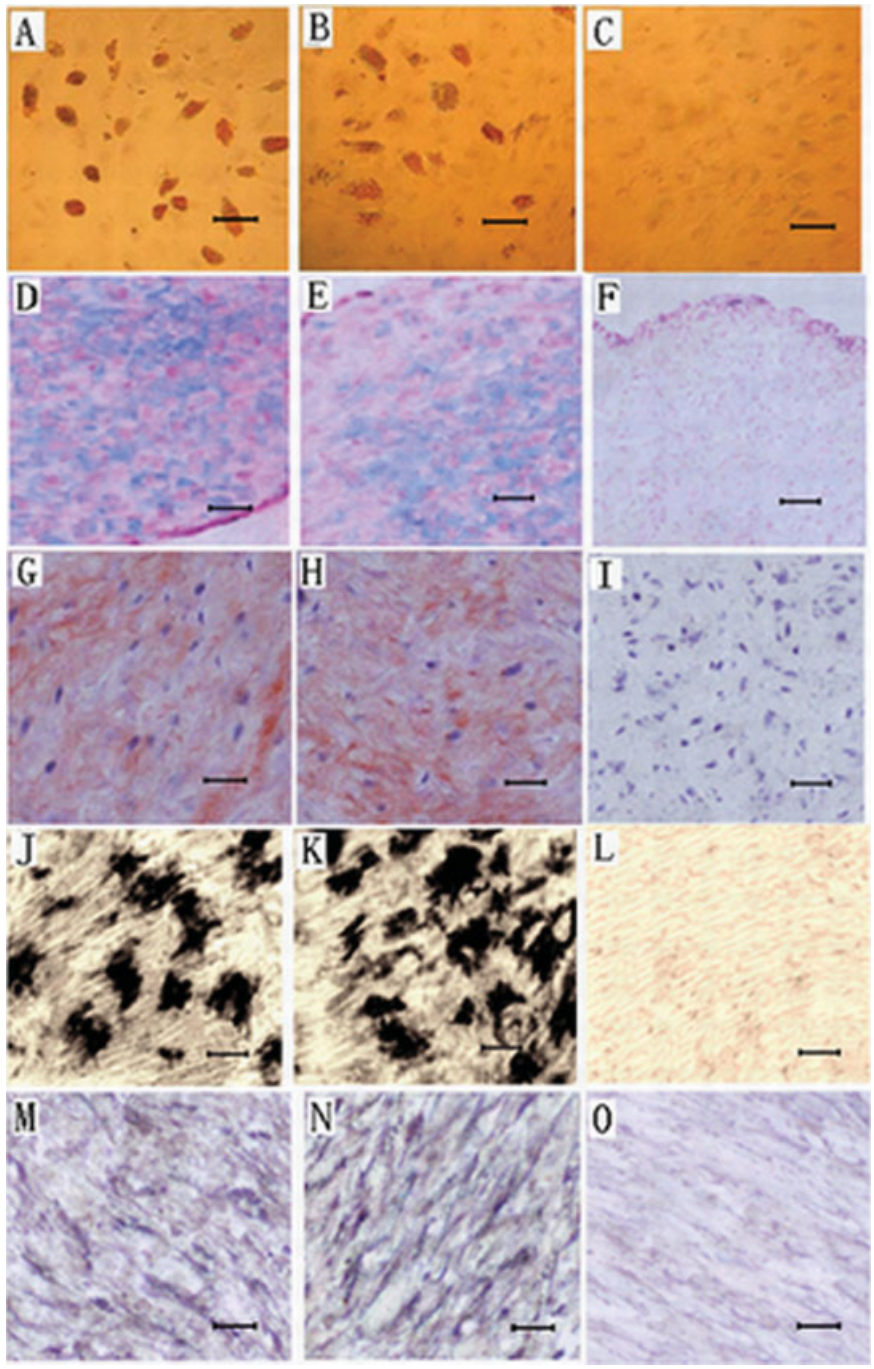

Fig. 6. Adipogenic, chondrogenic, osteogenic induction of hTERT-hMSCs (scale bar: 100 μm). Cells stained with Oil Red O (red) for fat: (**A**) hTERT-hMSCs cells at PD 95 with adipogenic induction, (**B**) hTERT-hMSCs cells at PD 275 with adipogenic induction, (**C**) hTERT-hMSCs cells at PD 95 without adipogenic induction. Chondrogenesis was documented by alcian blue staining of cartilage proteoglycans, (**D**) hTERT-hMSCs cells at PD 95 with chondrogenic induction, (**E**) hTERT-hMSCs cells at PD 275 with chondrogenic induction, (**F**) hTERT-hMSCs cells at PD 95 without chondrogenic induction, and by immunohistochemistry using type II collagen specific antibodies, (**G**) hTERT-hMSCs cells at PD 95 with chondrogenic induction, (**H**) hTERT-hMSCs cells at PD 275 with chondrogenic induction, (**I**) hTERT-hMSCs cells at PD 95 without chondrogenic induction. Osteogenesis was documented by matrix deposition of calcium phosphate demonstrated by Von Kossa staining, (**J**) hTERT-hMSCs cells at PD 95 with osteogenic induction, (**K**) hTERT-hMSCs cells at PD 275 with osteogenic induction, (**L**) hTERT-hMSCs cells at PD 95 without osteogenic induction, and by visualization of alkaline phosphatase (AP) activity, (**M**) hTERT-hMSCs cells at PD 95 with osteogenic induction, (**N**) hTERT-hMSCs cells at PD 275 with osteogenic induction, (**O**) hTERT-hMSCs cells at PD 95 without osteogenic induction.

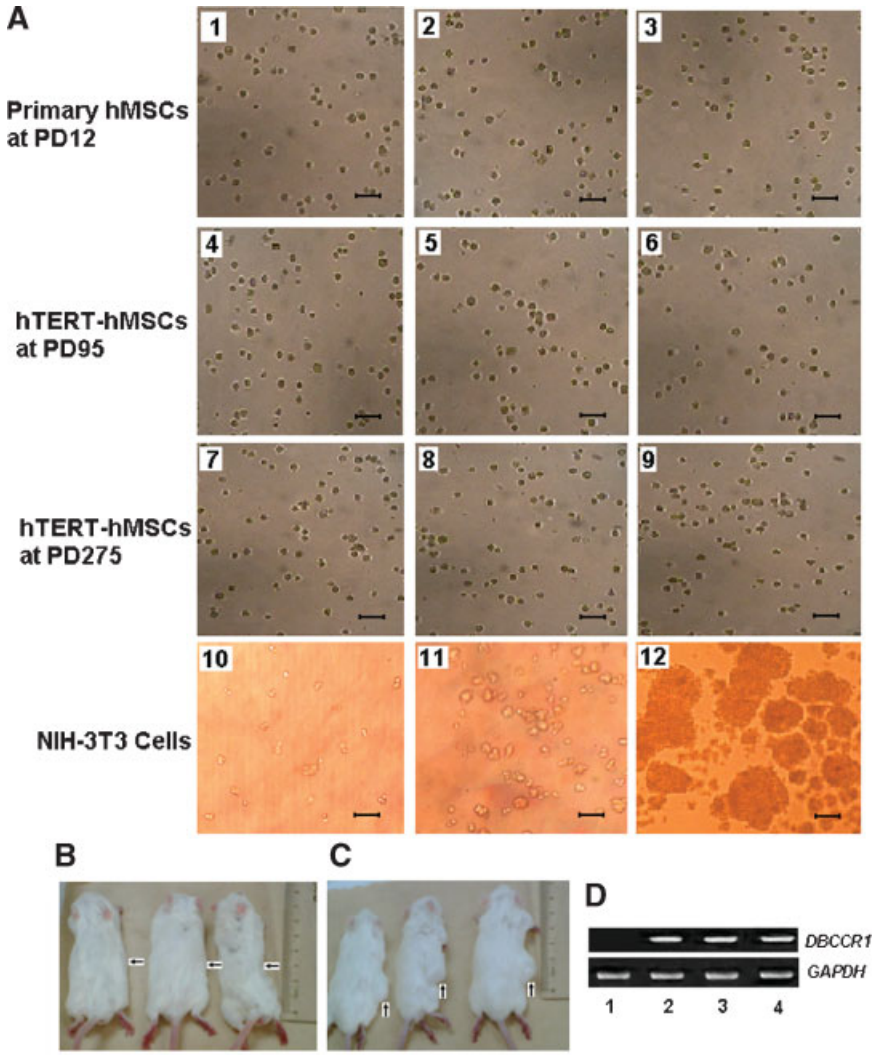

Fig. 7. Analysis of tumorigenicity of hTERT-hMSCs (scale bar: 100 μm). **A**: Soft agar anchor-independent assays of hTERT-hMSCs. Primary hMSC at PD 12 were seeded into soft agar and incubated for 1 day (A1), 4 days (A2), and 10 days (A3), hTERT-hMSCs at PD 95 were seeded into soft agar and incubated for 1 day (A4), 4 days (A5), and 10 days (A6). hTERThMSCs at PD 275 were seeded into soft agar and incubated for 1 day (A7), 4 days (A8), and 10 days (A9). NIH-3T3 were seeded into soft agar and incubated for 1 day (A10), 4 days (A11), and 10 days (A12). **B**: 2iÁ106 hTERT-hMSCs per site were injected into 15 NOD-SCID mice, no tumor formation was found at the injection site, 3 of which were indicated by arrows after 6 months of observation. **C**: 2iÁ106 NIH-3T3 (positive control) per site was injected into 15 NOD-SCID mice, tumor formation was found at the injection site, 3 of which were indicated by arrows after 60 days of observation. **D**: The expression analysis of DBCCR1 mRNA. The expression of DBCCR1 mRNA was analyzed by RT-PCR. The expression of GAPDH was used as a control for RNA quality and reverse transcription reaction. Bands (232 bp) in hTERT-hMSCs at these PDs were detected in primary hMSCs (**lane 4**), hTERT-hMSCs at PD 95 (**lane 3**) and at PD 275 (**lane 2**), while no product was identified in NIH-3T3 as a negative control (**lane 1**).

examined mRNA expression in primary hMSCs, hTERT-hMSCs at PD 95 and PD 275. 232 bp bands in hTERT-hMSCs at these PDs were detected as that of primary hMSCs (Fig. 7D), indicating that *DBCCR1* is expressed in hTERT-hMSCs. We could not detect any obvious bands in NIH-3T3 as a negative control.

DISCUSSION

SV40 large T antigen, HPV16 E6/E7 and telomerase reverse transcriptase (TERT) are able to immortalize cells [Bodnar et al., 1998; Counter et al., 1998; Cotsiki et al., 2004; Hung et al., 2004]. A number of groups have used the forced expression of telomerase to extend the

replicative capacity of hMSCs [Shi et al., 2002; Simonsen et al., 2002]. hMSCs are rendered replicatively immortal by the exogenous expression of telomerase and retain the functional characteristics and differentiation potential of the hMSC, and on transplantation into immunodeficient mice they formed bone tissue more effectively than their normal counterparts, but no tumors [Simonsen et al., 2002]. In terms of stem cell therapeutics, these results were very encouraging. However, with a commendable lack of complacency, this same group has investigated the neoplastic potential of the hTERT-transduced hMSC at different PDLs more exhaustively. It showed that hTERT-transduced hMSC could accumulate premalignant changes on continued division in culture, in some cases to the extent of acquiring the ability to form tumors in immunodeficient mice [Serakinci et al., 2004; Burns et al., 2005]. One of major purposes for the present study was to establish a line of hMSCs not only with a prolonged life span in vitro but also with cellular properties as their parental. In the present study, an hMSC line not only with a sustaining activity of telomerase and an extending life span but also with biological properties similar to those of primary hMSCs is established through the transduction of retrovirus containing the coding region of hTERT and the selection with a low dose of antibiotic. The exogenous hTERT localized and expressed in the nucleolus of hMSCs drove the proliferation of hTERT-hMSCs for approximate 300 PDs without loss of contact inhibition while the primary hMSCs stopped dividing after about 25 PDs. Meanwhile, the analysis of cell surface antigen demonstrated that hTERT-hMSCs had a similar phenotype of cell surface antigens to that of primary hMSCs, that is, positive for CD29, CD105, CD44, and CD166 and negative for CD34, CD45, CD117, and HLA-DR. Karyotype analysis for hTERT-hMSCs showed a normal karyotype and no evidence of abnormalities. The in vitro assay of cell growth in soft agar and the in vivo assay of tumorigenicity in NOD-SCID as well as RT-PCR analysis of *DBCCR1* mRNA expression confirmed no signs of transformation of hTERT-hMSCs. Another major propose for the present study was to maintain the multipotential of differentiation of TERT-hMSCs into terminal cell lineages. The results showed that hTERT-hMSCs at PD 95 and PD 275 have the identical multipotential of differentiation into osteoblasts, chondrocytes and adipocytes respectively under their corresponding induction medium. It indicated the potential of differentiation of hTERT-hMSCs have little variation during their proliferation. The sustaining of differentiation multipotential of hTERT-hMSCs is more important for studies of differentiation mechanism of hMSCs. Meanwhile, the immortalized hMSCs will also provide much more cells for in vitro tissue engineering experiment.

However, we could not deduce a conclusion from above results that this line of hTERT-hMSCs could be used in the transplantation therapy. The telomerase activity has been associated with neoplastic transformation in several models in vitro and in vivo, although it lengthens the life span of cells [Wang et al., 2000; Hahn and Meyersion, 2001; Hamad et al., 2002]. Some of hTERT-transduced hMSC lines at different PDLs showed loss of contact inhibition, anchorage independence and formed tumors in NOD/SCID mice [Serakinci et al., 2004; Burns et al., 2005]. These lines have a normal karyotype but show deletion of the Ink4a/ARF locus and loss of expression of the cell cycle-associated gene, *DBCCR1* due to promoter hypermethylation. The tumorigenicity of hTERT-transduced hMSCs seems to determine their doomsday. Many factors, such as antibiotic selection, inherent qualities of different cell types, vector integration site, levels of exogenous gene expression and culture condition, can cause genetic alterations of cells [Valera et al., 1994; Sanchez-Carbayo et al., 2003; Keith, 2004]. For hTERT-hMSCs with tumorigenicity, the former three factors should not be attributed as major reasons for tumorigenicity. The latter two factors will inevitably vary during continual culture. Since it is so, the effect of exogenous telomerase expression in long-term cultures on the genetic properties of hTERT-hMSCs is likely to remain unpredictable. For the hTERT-hMSCs line in the present study, it is remained for further study whether and when this cell line, during its proliferation process, would loss the contact inhibition of its growth, exhibit anchorage independence and finally develop tumorigenicity, although it is untumorigenicity up to now. However, it should not be impeded to use this line for in vitro tissue engineering experiment or for cell model of cellular senescence, self-new and differentiation.

In conclusion, our results demonstrated that hTERT-hMSCs kept their proliferation and differentiation multipotential similar to primary hMSCs even if they are propagated for many passages. Although we could not conclude whether or not this line would develop genetic alteration, it should be used in some experiments on stem cells. We will follow its proliferation process. Even if it developed the tumorigenicity, it would provide us a cellular model to study the mechanism balancing immortality and neoplasia from stem cells.

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