Original Article

Construction and characterization of human bone marrow-derived mesenchymal stem cells by overexpressing human telomerase gene

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Abstract

Introduction: Currently, bone marrow-derived human mesenchymal stem cells (BM-hMSCs) have been considered a standard source of hMSCs for research and clinical applications. However, the number of BM-hMSCs in bone marrow decline with age and the isolated BM-hMSCs have limited proliferative capacity which are major hurdles for most research and clinical uses which require large number of cells, therefore development of the novel technique for enhancing BM-hMSC expansion in culture is critical.

Methods: This study, the genetic-engineering technique was employed to enhance the expression level of human telomerase gene (*hTERT*) in BM-hMSCs (hTERT-BM-hMSCs). The hTERT-BM-hMSCs were characterized in term of their morphology, gene expression profile, as well as their osteogenic and adipogenic differentiation potentials in comparison to the non-transfected BM-hMSCs (NT-BM-hMSCs)

Results: The hTERT-BM-hMSCs could be expanded in culture for a longer period of time compared with their non-transfected counterparts and retained the typical characteristics and properties of BM-hMSCs, such as morphology, surface marker expression profile, adipogenic and osteogenic differentiation capacity.

Conclusion: The *hTERT* overexpression enhanced the expansion potential of BM-hMSCs without altering their properties. The hTERT-BM-hMSCs established in this study is beneficial for genetically manipulation of BM-hMSCs which is usually difficult to perform due to their limited proliferative capability.

Key words: human mesenchymal stem cells, bone marrow, telomerase

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Introduction

Human mesenchymal stem cells (hMSCs) are multipotent adult stem cells that present in bone marrow in very small number (approximately 1 in 200,000 bone marrow cells)[1 - 3]. hMSCs can be expanded in culture and have an ability to differentiate to several mesodermal derivatives, such as osteocytes, chondrocytes and adipocytes[4].

The hMSCs possess several clinically useful properties, including 1) migration into injured and inflamed tissues after intravenous administration, that called "tissue tropism"[5 - 7], 2) differentiation to osteocytes and chondrocytes to repair damaged bones and cartilages[4, 8, 9], 3) Secreting many cytokines that induce neovascularization, increase cell proliferation and enhance cell viability in the injured tissues[8, 9] and 4) modulating immune responses by releasing cytokines that regulated functions of several immune cells, such as T-lymphocytes, B-lymphocytes, dendritic cells and macrophages and alleviate tissue inflammation[9, 10]. Hence, the hMSCs have been used to treat patients in several clinical trials involving cell transplantation therapy.

Currently, the standard source of hMSCs for most research and clinical applications are bone marrow-derived hMSCs (BM-hMSCs). However, the derivation of BM-hMSCs has some important limitations. Firstly, the isolation of BM-hMSCs require bone marrow aspiration which is invasive and painful procedure, 2) The number of BM-hMSCs in bone marrow decline with age, making it more difficult to isolate BM-hMSCs from elderly donors and 3) the isolated BM-hMSCs have limited proliferative capacity and can be expanded in culture for only 7 - 10 passages which is insufficient for most research and clinical uses that usually require large number of cells[10 - 13]. Due to those limitations, new batches of BM-hMSCs have to be repeatedly harvested from donors which is not only inconvenient but also significantly raising cost and time. Moreover, the BM-hMSCs derived from different donors usually exhibited distinct properties, in term of gene expression, differentiation potentials, and the amounts of secreted cytokines, causing the direct comparison between the effects of BM-hMSCs across studies challenging [2, 3].

Distinct from pluripotent stem cells, such as embryonic stem cells (ESCs) and induce pluripotent stem cells (iPSCs) which can be expanded indefinitely in culture, BM-hMSCs do not produce telomerase enzyme to maintain the length of telomeres at the end of chromosome and preventing the cells from reaching replicative senescence (Kheolamai P, unpublished data)[14 - 18].

Hence, the up-regulation of human telomerase enzyme production in BM-hMSCs might enhance their expansion potential in culture while maintaining their unique stem cell properties. Herein, the genetic-engineering technique was employed to enhance the expression level of human telomerase *(hTERT)* gene in BM-hMSCs and characterized those cells (named hTERT-BM-hMSCs) in term of their morphology, gene expression profile, as well as their osteogenic and adipogenic differentiation in comparison to the non-transfected BM-hMSCs (NT-BM-hMSCs).

Methods

Subjects

This study was approved by the Institutional Review Board, Faculty of Medicine, Thammasat University (project number: MTU-EC-DS-1-015/60), which was in accordance with the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines, and ICH-GCP. Human bone marrow samples were obtained from five healthy volunteers (3 males, 2 females, age 18 - 60 years old) after giving written informed consents.

Isolation and culture of BM-hMSCs

Mononuclear cells from human bone marrow were isolated using IsoPrep® (Robbins Scientific Corporation, USA) density gradient centrifugation, washed twice with Phosphate Buffer Saline (PBS) (Invitrogen, USA), and re-suspended in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Merck, USA), 100 U/ml penicillin (Invitrogen, USA), and 100 µg/ml streptomycin (Invitrogen, USA). Cell suspensions were plated in culture flask (Corning, USA) at a density of 2 x 10^5 cells/cm^2. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and medium was replaced every 3 days throughout the entire culture period. The cell morphology was determined by light microscopy.

Generation of *hTERT* overexpressing BM-hMSCs (hTERT-BM-hMSCs)

The whole coding sequence of *hTERT* gene was inserted into pENTR™/D-TOPO vector (Invitrogen, USA) prior to transferring to the expression vector, pLenti6/UbC/V5-DEST (Invitrogen, USA). The resulting recombinant DNA was named hTERT/pLenti6/UbC/V5-DEST vector (Figure 1). The transduction of BM-hMSCs by hTERT/pLenti6/UbC/V5-DEST (hTERT-BM-hMSCs) was performed using lipofectamine (Invitrogen, USA) as described in our previous study[19].

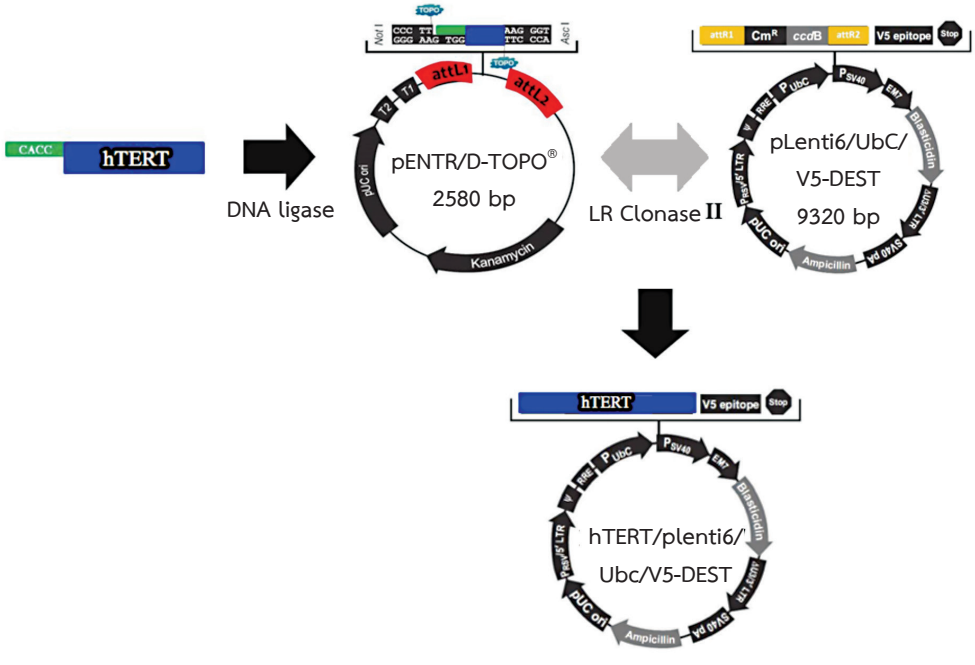

Figure 1 Schematic diagram of hTERT/pLenti6/UbC/V5-DEST vector.

The *hTERT* gene was selected for immortalizing BM-hMSCs since it has successfully been used to immortalize several human cells, such as mammary epithelial cells, keratinocytes, esophageal squamous cells, fibroblasts and retinal pigment epithelial cells without causing any oncogenic transformation[20 - 26]. The hTERT-BM-hMSCs were harvested to determine the expression level of *hTERT* gene by using quantitative realtime PCR (qRT-PCR) prior to further study.

Characterization of surface marker expression profile of BM-hMSCs by using flow cytometry

The 3rd - 5th passage of hMSCs were characterized for hMSC surface markers by incubation with the following mouse anti-human antibodies: anti-CD45-FITC (BD Pharmingen, USA), anti-CD34-PE (Biolegend, USA), anti-CD90-FITC (AbDSerotec, USA), anti-CD73-PE (BD Pharmingen, USA), and anti-CD105-PE (Miltenyi Biotec, Germany) for 30 minutes at 4°C in the dark. After incubation, cell pellets were washed twice with PBS and fixed with 1% (w/v) paraformaldehyde in PBS. Flow cytometry was performed by using FACS calibur™ Flow cytometer (Becton Dickinson, USA) and CellQuest™ software (Becton Dickinson, USA).

Osteogenic and adipogenic differentiation of cultured hMSCs

The 3rd - 5th passage of hMSCs were used to assess their adipogenic and osteogenic differentiation potentials. For adipogenic differentiation, 5 x 10^4 cells were cultured in NH AdipoDiff® Medium (Miltenyi Biotec, Germany). Medium was replaced every 3 days according to the manufacturer's instruction. After culture for 2 weeks, cells were stained with 0.5% (w/v) Oil Red O (Sigma Aldrich, USA) in Isopropanol for 20 minutes at room temperature to determine the number of hMSC-derived adipocytes in culture.

For osteogenic differentiation, 5 x 10^4 cells were cultured in NH OsteoDiff® Medium (Miltenyi Biotec, Germany). Medium was replaced every 3 days according to the manufacturer's instruction. After culture for 2 weeks, cells were stained with 40 mM Alizarin Red S (Sigma Aldrich, USA) for 20 minutes at room temperature to determine the number of hMSC-derived osteocytes in culture.

Determination of the expression levels of adipogenic and osteogenic genes in BM-hMSCs by using qRT-PCR

To study the effect of *hTERT* overexpression on the expression of osteogenic and adipogenic genes in BM-hMSCs, hTERT-BM-hMSCs and NT-BM-hMSCs were separated into three groups. The first group was cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS for 14 days while the second and third group were cultured in NH Osteodiff® medium and NH Adipodiff® medium for 2 weeks to induce their osteogenic and adipogenic differentiation, respectively.

At the end of culture, the cells were harvested for RNA isolation and the expression of three adipogenic genes, including Peroxisome proliferator-activated receptor-gamma *(PPARγ)*, Glucose transporter 4 *(GLUT4)*, and Sterol regulatory element-binding protein 1c *(SREBP1C)*, and three osteogenic genes, including Runt-related transcription factor 2 *(RUNX2)*, Osterix *(OSX)* and Osteocalcin *(OCN)* were determined by qRT-PCR.

The total RNAs were isolated from BM-hMSCs using TRIzol® reagent (Invitrogen, USA). Each cDNA was then synthesized from 2µg RNA using SuperScript™ III Reverse Trancriptase (Invitrogen, USA). MicroAmp® fast optical 96-well reaction plate (BioRad, USA) was

used for qRT-PCR. Each well contained 3 µl cDNA, 1 µl of 10 µM forward and reverse primer mix, and 10 µl SYBR® Green PCR Mastermix (Biorad, USA). Plates were sealed with MicroAmp® clear adhesive film (Biorad, USA) to prevent evaporation of the reactant. PCR was performed using 7500 Fast Real-time PCR system (Applied Biosystem, USA) under the following protocol: 95°C initial denaturation for 10 minutes, followed by 40 cycles of denaturation (95°C, 10 seconds), annealing (60°C, 10 seconds), and extension (72°C, 40 seconds). The relative quantity of the target gene was calculated by normalization with glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*, using the 7500 software version 2.0.5 (Applied Biosystem, USA). The primer sequences were listed in Table 1.

Table 1 The sequences of primers for qRT-PCR

Gene	Forward primer	Reverse primer
GLUT4	5'-CTTCGAGACAGCAGGGGTAG-3'	5'-ACAGTCATCAGGATGGCACA-3'
PPARγ	5'-GACCACTCCCACTCCTTTGA-3'	5'-AGGCTCCACTTTGATTGCAC-3'
SREBP1C	5'-TTCTCACCTCCCAGCTCTGT-3'	5'-GGAGGCTTCTTTGCTGTGAG-3'
RUNX2	5'-GACAGCCCCAACTTCCTGT-3'	5'-CCGGAGCTCAGCAGAATAAT-3'
OCN	5'-CTCACACTCCTCGCCCTATT-3'	5'-TCAGCCAACTCGTCACAGTC-3'
OSX	5'-TGCTTGAGGAGGAAGTTCAC-3'	5'-CTGCTTTGCCCAGAGTTGTT-3'
GAPDH	5'-CAATGACCCCTTCATTGACC-3'	5'-TTGATTTTGGAGGGATCTCG-3'

Abbreviation: *GLUT4* = glucose transporter type 4, *PPARγ* = peroxisome proliferator-activated receptor gamma, *SREBP1C* = sterol regulatory element-binding protein 1C, *RUNX2* = runt-related transcription factor 2, *OCN* = osteocalcin, *OSX* = osterix, *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). The unpaired t-test was used to assess the significance of differences between the observed data. $P < 0.05$ was considered to be statistically significant.

Results

The genetic engineering technique successfully enhances the expression level of *hTERT* gene in hTERT-BM-hMSCs

BM-hMSCs derived in this study exhibited spindle-shaped morphology and rapidly proliferated to cover all culture surface within 2 weeks (Figure 2A). Those BM-hMSCs differentiated to osteocytes (Figure 2B) and adipocytes (Figure 2C) and expressed typical hMSC surface marker profile, positive for mesenchymal stem cell surface markers CD73, CD90 and CD105 and negative for hematopoietic cell surface markers, CD34 and CD45 (Figure 2D)

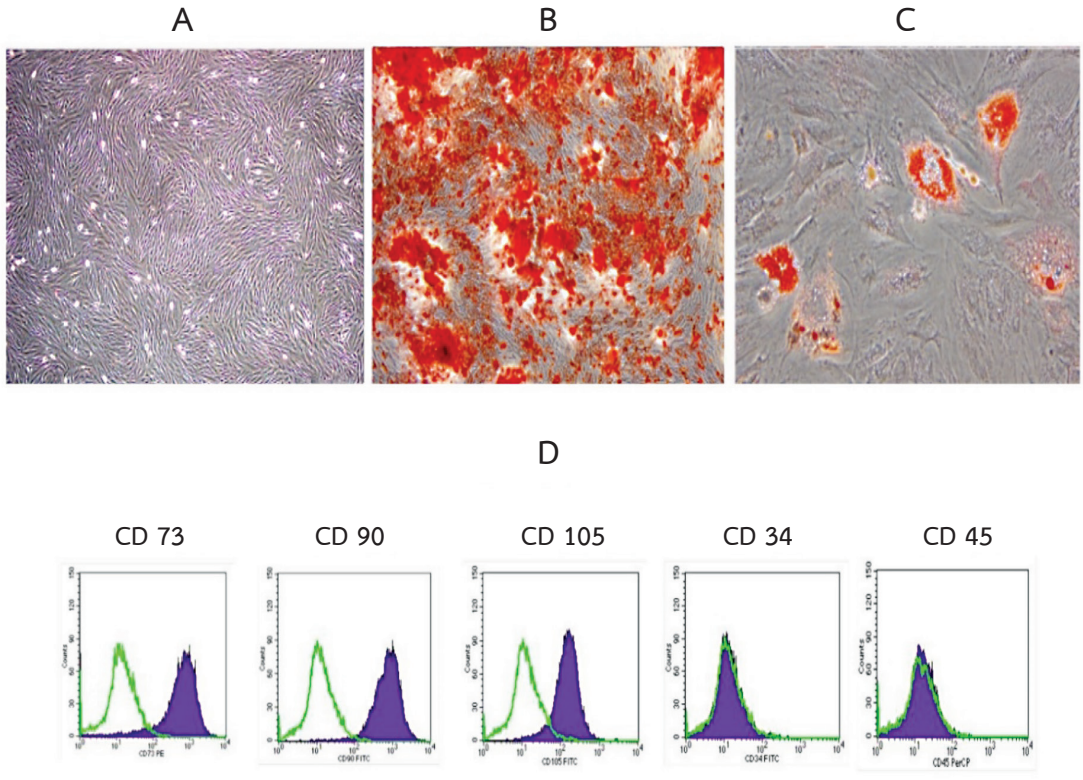

Figure 2 Characterization of BM-hMSCs.

(A) Morphology of BM-hMSCs, **(B)** The osteogenic differentiation potentials of BM-hMSCs as determined by Alizarin Red S Staining, **(C)** The adipogenic differentiation potentials of BM-hMSCs as determined by Oil Red O Staining, **(D)** The expression profiles of typical mesenchymal and hematopoietic surface markers of BM-hMSCs as determined by flow cytometry.

To enhance the expression level of *hTERT* in BM-hMSCs, the hTERT/pLenti6/UbC/V5-DEST vector containing green fluorescent protein (GFP) was transfected into BM-hMSCs using lipofectamine. The succesfully transfected BM-hMSCs were detected by fluorescent microscopy.

The results showed that GFP expression was detected in hTERT-BM-hMSCs even after the cells were expanded for 9 and 11 passages (Figure 3A) suggesting that the hTERT/pLenti6/UbC/V5-DEST vector was stably integrated into BM-hMSC genome. The expression of *hTERT* in hTERT-BM-hMSCs as determined by qRT-PCR also confirmed significantly higher level than those of NT-BM-hMSCs (Figure 3B). These results demonstrated that genetic engineering technique successfully enhanced the expression level of *hTERT* in hTERT-BM-hMSCs.

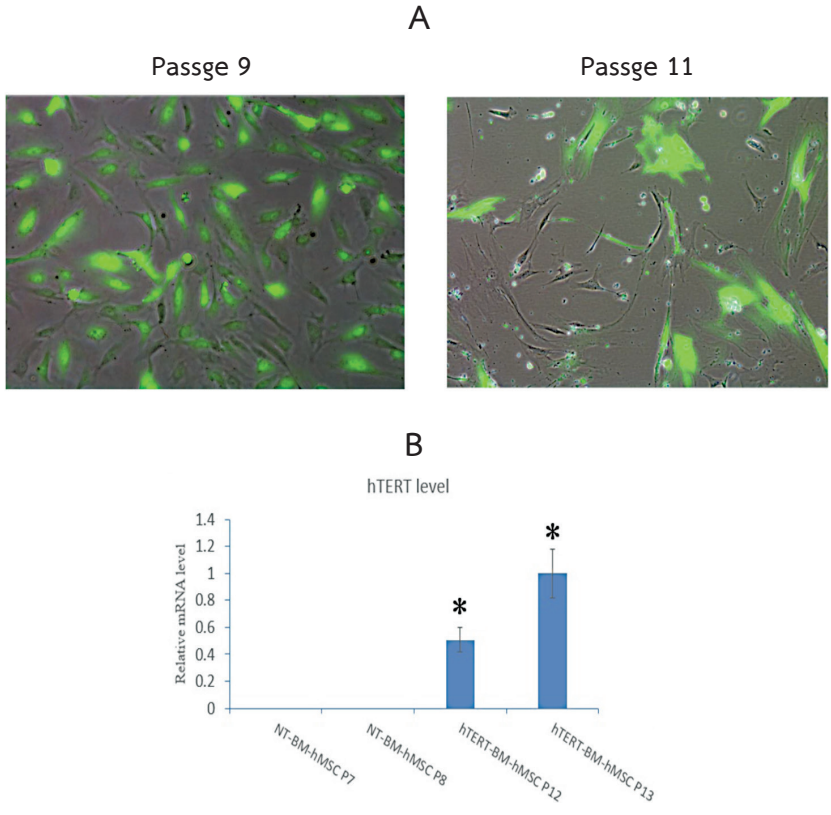

Figure 3 The expression levels of *hTERT* in hTERT-BM-hMSCs.

(A) Representative fluorescence micrographs showed the expression of GFP in hTERT-BM-hMSCs which were expanded in culture for 9 and 11 passages. **(B)** The expression levels of *hTERT* gene in the hTERT-BM-hMSCs which were expanded in culture for 12 and 13 passages in comparison to NT-BM-hMSCs. Data are presented as mean ± SEM from three experiments. *P < 0.05 vs. NT-BM-hMSCs.

The effects of *hTERT* gene overexpression on the proliferation, osteogenic differentiation and adipogenic differentiation of BM-hMSCs

To study the effects of *hTERT* gene overexpression on the properties of BM-hMSCs, the hTERT-BM-hMSCs were characterized in term of their morphology, osteogenic differentiation and adipogenic differentiation in comparison to NT-BM-hMSCs. The results showed that hTERT-BM-hMSCs retained their spindle-shaped morphology, as well as their osteogenic and adipogenic differentiation which were indistinguishable from their non-transfected counterparts (NT-BM-hMSCs) (Figure 4). Moreover, hTERT-BM-hMSCs can be expanded in culture for a longer period of time (16 passages vs.10 passages).

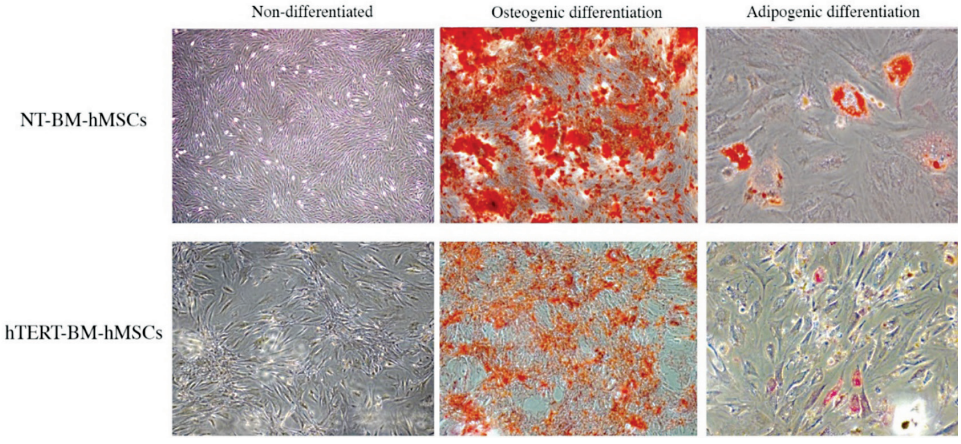

Figure 4 The adipogenic and osteogenic differentiation potentials of hTERT-BM-hMSCs

Representative micrographs showed the osteogenic and adipogenic differentiation potentials of hTERT-BM-hMSCs as determined by Alizarin Red S staining and Oil-Red O staining, respectively. The non-transduced BM-hMSCs (NT- BM-hMSCs) serve as controls.

Furthermore, the hTERT-BM-hMSCs also maintained their surface marker expression profiles similar to those of NT-BM-hMSCs (Figure 5).

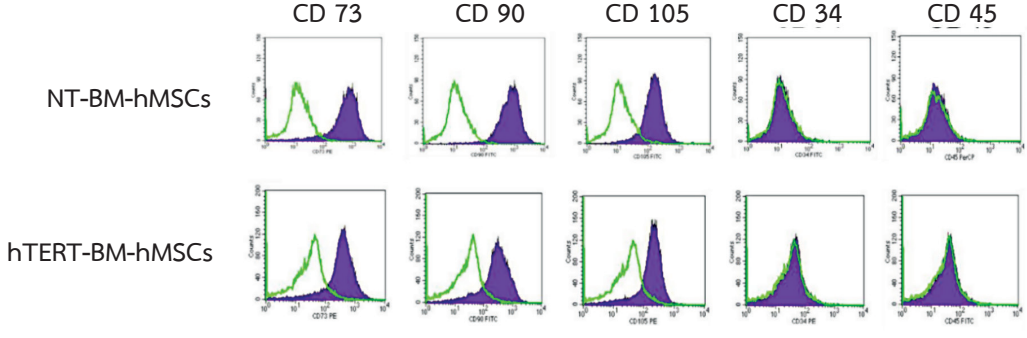

Figure 5 The surface marker expression profiles of hTERT-BM-hMSCs and NT-BM-hMSCs

The expression profile of typical mesenchymal surface markers (CD73, CD90, CD105) and hematopoietic surface markers (CD34, CD45) of hTERT-BM-hMSCs as determined by flow cytometry. The non-transduced BM-hMSCs (NT- BM-hMSCs) serve as controls.

The effect of *hTERT* overexpression on the expression levels of osteogenic and adipogenic genes in BM-hMSCs

The effect of *hTERT* overexpression revealed that the expression levels of adipogenic gene *(GLUT4, PPARγ, SREBP1C)* and osteogenic gene *(RUNX2, OSX, OCN)* in hTERT-BM-hMSCs cultured in NH Adipodiff® and NH Osteodiff® media were not different from those of NT-BM-hMSCs cultured under the same conditions (Figure 6).

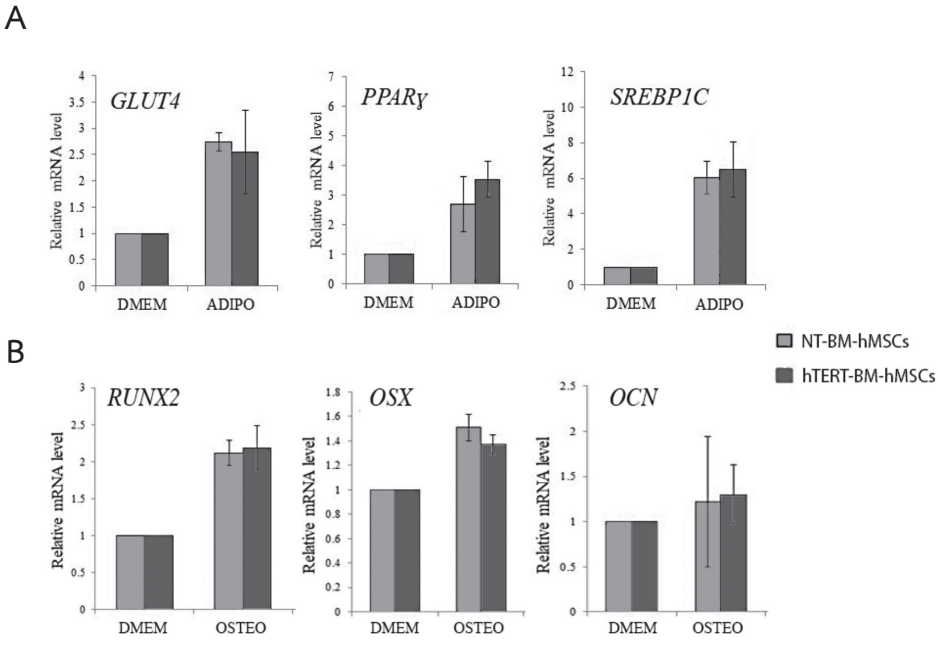

Figure 6 The expression levels of adipogenic and osteogenic genes of hTERT-BM-hMSCs during their adipogenic and osteogenic differentiation.

(A) Graph shows relative mRNA levels of adipogenic genes, *GLUT4, PPARγ* and *SREBP1C*, of hTERT-BM-hMSCs and NT-BM-hMSCs after cultured in NH Adipodiff® medium (ADIPO) for 2 weeks.
(B) Graph shows relative mRNA levels of osteogenic genes, *RUNX2, OSX* and *OCN*, of hTERT-BM-hMSCs and NT-BM-hMSCs after cultured in NH Osteodiff® medium (OSTEO) for 2 weeks The hTERT-BM-hMSCs and NT-BM-hMSCs cultured in the expansion medium (DMEM) serve as controls. Data were presented as mean ± standard error of the mean (SEM) of three independent experiments.

Discussion

Currently, BM-hMSCs have been the standard source of hMSCs for most research and clinical applications. However, the number of BM-hMSCs decline with age and their expansion potential in culture is limited to only 7 - 10 passages that is unsuitable for most research and clinical uses which require large number of cells.

Our previous study showed that the lack of human telomerase enzyme might be one of the reason that restricted BM-hMSC expansion in culture (Kheolamai P, unpublished data). Human telomerase enzyme is responsible for the maintenance of telomere length at the end of chromosome and preventing cells from entering replicative senescent stage, therefore the pluripotent stem cells, such as ESCs and iPSCs which produce high level of telomerase enzyme have unlimited expansion potential in culture[14 - 18].

Herein, the hTERT-BM-hMSCs were successfully developed to enhance the expression level of *hTERT* gene in those cells by stably transfecting hTERT/pLenti6/UbC/V5-DEST vector into their genome. The overexpression of *hTERT* in hTERT-BM-hMSCs was maintained even after the cells were expanded for 13 passages. This suggested that hTERT/pLenti6/UbC/V5-DEST vector was stably integrated into hTERT-BM-hMSC genome and the UbC promotor which promoted the expression of *hTERT* gene in hTERT/pLenti6/UbC/V5-DEST vector was not

suppressed by hTERT-BM-hMSCs. Despite its modest ability to drive gene expression , UbC promotor could enhance gene expression in several human cell types for a long period of time without being silenced [27, 28].

In agreement with the previous studies in both human and rhesus monkey BM-MSCs[29, 30], the present study suggested that the overexpression of *hTERT* enhance the expansion potential of hTERT-BM-hMSCs by preventing them from reaching replicative senescence. Comparing with those studies, the present study also provides additional information by showing that the overexpression of *hTERT* did not affect the expression profiles of both adipogenic *(GLUT4, PPARγ and SREBP1C)* and osteogenic genes *(RUNX2, OSX and OCN)* during the adipogenic and osteogenic differentiation of BM-hMSCs.

However, the effects of *hTERT* overexpression on other important aspects of BM-hMSC properties, such as their immunomodulatory property, cytokine production and tumorigenicity after transplantation have yet to be investigated by the present study. Those problems should be addressed before the hTERT-BM-hMSCs can be used for further research and clinical applications.

In conclusion, the hTERT-BM-hMSCs is a potential model for stem cell researchers, especially for genetically manipulate BM-hMSCs which is usually inefficient due to the limited proliferative capacity of those cells.

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Conflict of interest

All authors report no conflict of interest relevant to this article.

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บทคัดย่อ

การสร้างและศึกษาคุณสมบัติของเซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกมนุษย์โดยการเพิ่มระดับการแสดงออกของยีนเทโลเมอเรส

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บทนำ: ในปัจจุบันเซลล์ต้นกำเนิดมีเซ็นไคม์มนุษย์ (hMSCs) ชนิดที่เป็นมาตรฐานสำหรับใช้ในการศึกษาวิจัยและการประยุกต์ใช้ทางคลินิก คือ hMSCs ที่แยกจากไขกระดูก (BM-hMSCs) อย่างไรก็ตามนอกจากจำนวนของ BM-hMSCs ในไขกระดูกจะลดลงตามอายุของผู้บริจาคที่เพิ่มขึ้นแล้ว BM-hMSCs ที่แยกได้ในการศึกษาเกือบทั้งหมดมีความสามารถในการแบ่งตัวเพิ่มจำนวนภายนอกร่างกายที่จำกัด เพียง 7 - 10 passage ซึ่งนับเป็นอุปสรรคสำคัญต่อการนำ BM-hMSCs มาใช้ในการศึกษาวิจัยและการประยุกต์ใช้ทางคลินิกซึ่งจำเป็นต้องใช้เซลล์จำนวนมาก ทำให้การพัฒนาวิธีการเพิ่มจำนวน BM-hMSCs ภายนอกร่างกายให้มีประสิทธิภาพมากขึ้นจึงมีความจำเป็น

วิธีการศึกษา: การศึกษานี้ได้ใช้เทคนิคทางพันธุวิศวกรรมทำการเพิ่มระดับการแสดงออกของยีน human telomerase (*hTERT*) ในเซลล์ BM-hMSCs ที่แยกจากไขกระดูกของผู้บริจาค ก่อนจะนำ BM-hMSCs ที่ผ่านการเพิ่มระดับการแสดงออกของยีน *hTERT* ดังกล่าว (hTERT-BM-hMSCs) มาศึกษารูปแบบการแสดงออกของโมเลกุลบนผิวเซลล์ รูปแบบการแสดงออกของยีน ความสามารถในการเจริญพัฒนาไปเป็นเซลล์กระดูกและเซลล์ไขมันเปรียบเทียบกับเซลล์ BM-hMSCs ปรกติที่ไม่ได้ผ่านการเพิ่มระดับการแสดงออกของยีน (NT-BM-hMSCs)

ผลการศึกษา: hTERT-BM-hMSCs ที่ผ่านการเพิ่มระดับการแสดงออกของยีน *hTERT* สามารถแบ่งตัวเพิ่มจำนวนได้มากขึ้นกว่า BM-hMSCs ปรกติ ในขณะที่ยังคงคุณสมบัติเดิมของ BM-hMSC ทั้งในด้านรูปร่าง ความสามารถในการพัฒนาไปเป็นเซลล์กระดูกและเซลล์ไขมันและรูปแบบการแสดงออกของโมเลกุลบนผิวเซลล์

สรุปผลการศึกษา: การเพิ่มระดับการแสดงออกของยีนเทโลเมอเรสในเซลล์ต้นกำเนิดมีเซ็นไคม์มนุษย์ที่แยกได้จากไขกระดูกทำให้เซลล์ดังกล่าวมีความสามารถในการแบ่งตัวเพิ่มขึ้นโดยที่ยังคงคุณสมบัติเดิม hTERT-BM-hMSCs ที่สร้างขึ้นในการศึกษานี้จะช่วยให้นักวิจัยทำการศึกษา hMSCs ด้วยเทคนิคใหม่ๆ เช่น การดัดแปลงพันธุกรรมของ hMSCs ซึ่งเดิมทำได้ยากเนื่องจากความสามารถในการแบ่งตัวเพิ่มจำนวนที่จำกัดของ BM-hMSCs

คำสำคัญ: เซลล์ต้นกำเนิดมีเซ็นไคม์มนุษย์, ไขกระดูก, ยีนเทโลเมอเรส