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Original article

Non-viral transient transfection of *hTERT* gene into hBMSCs from elderly patients delays cellular aging *in vitro*

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Background: Human bone marrow stem cell (hBMSC) is a promising candidate in regenerative medicine due to its plasticity and homing ability. However, in vitro survival and therapeutic efficacy of hBMSCs decline with donor age as a result of physiological cellular aging.

Objectives: We transfected human telomerase reverse transcriptase (*hTERT*) into hBMSCs from patients more than 60 years old in the hope of extending cellular life span.

Methods: In our study on 11 donors (aged 16-74 years), no telomerase activity was detected in hBMSCs from donors ≥60 years old. We then performed transient transfection on hBMSC from these elderly donors (n = 4) with *hTERT* gene using non-viral methods.

Results: Transfection efficiency of 38.5% and 55.6% was achieved by lipofection and electroporation with viability at 83% and 44%, respectively. Telomerase was detected on day 5 for all post-transfected samples. Maximum passage number achieved after transfection was ≥13 (≈45 doublings) compared with 6 (≈15 doublings) before transfection.

Conclusion: We conclude that transient expression of *hTERT* increased cellular life span of hBMSCs from elderly patients, while maintaining a normal cell morphology and growth rate.

Keywords: Cellular life span, human bone marrow stem cell, telomerase, transfection

List of abbreviations

hBMSCs	Human bone marrow stem cells
MSC	Mesenchymal stem cell
FBS	Fetal bovine serum
hTERT	Human telomerase reverse transcriptase
RTA	Relative telomerase activities
UKMMC	Universiti Kebangsaan Malaysia Medical Centre

The evidence of the existence of multipotent stem cells within the adult bone marrow led to the surge of interest in utilizing it for regenerative medicine. However, cells from elderly patients have been associated with reduced regeneration capacity and extracellular matrix protein production [1]. In a retrospective analysis conducted on bone marrow cells in our previous study attested the association of human aging with a significant reduction on the number of osteoblastic progenitors (number of colony forming unit) [2].

Cellular aging is a physiological process that occurs both *in vivo* and *in vitro*. Cellular aging is not a real time event; rather it is determined by the number of cell divisions. Studies have confirmed that human fibroblasts in culture have life span of 50-60 doublings [3, 4]. In other study it was shown that *in vitro* cultured cells that were being cryopreserved at a known cell doubling, upon re-culturing continued to replicate until the projected cellular life span [5].

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Programmed theories imply that cellular aging process is regulated by biological clock through progressive shortening of the chromosomal ends (known as telomere) during replication. This specific sequence of the telomere confers protection to DNA from enzymatic digestion or fusion [6]. The progressive shortening of telomere is a result of incomplete replication of linear chromosomes [7, 8], and when telomere length reached to critical size, often referred to as the Hayflick limit, cells enter into senescence phase [5]. However, germ cells, stem cells and tumour cells circumvent this problem by expressing telomerase, a reverse transcriptase enzyme, which is capable of elongate or restore telomere length by adding specific DNA repeat sequences at the end of chromosome [9]. Further supporting this, the higher expression of telomerase was correlated with longer telomere length, higher proliferation rate and extended cellular life span [10-12]. Porter et al had shown that transduction of human telomerase reverse transcriptase (*hTERT)* gene can facilitate indefinite cell proliferation of human fibroblast cells [13]. Hence, the problem of limited *in vitro* expansion of hBMSCs from elderly patient can be circumvented by ectopic expression of *hTERT*.

Despite these positive results, the use of transduced hBMSCs for therapeutic use raises skepticism over certain safety issues such as the increase risk mutagenesis with long term expression of telomerase, presence of viral particles and possibilities of genetic instability upon integration of viral vector into the genome. However, these safety issues can be overcome by using non-viral and transient transfection methods. In our previous study, SOX-9 gene was transfected transiently in human chondrocytes for enhancing the expression of mature cartilage markers [14]. The objective of the current study was to proof the hypothesis that transient transfection of *hTERT* in hBMSCs of elderly patients using non-viral methods can delay cellular aging during *in vitro*.

Material and methods

hBMSC isolation and surface antigen characterization

Bone marrow aspirates were obtained from 11 consenting patients undergoing bone grafting procedure with no other underlying health conditions for corrective orthopedic indications, aging between 16-74 years old with approval and ethical clearance from the Universiti Kebangsaan Malaysia Medical Centre (UKMMC) Research Ethics Committee. hBMSCs were isolated using gradient centrifugation on Ficoll Pague. In brief, aspirated bone marrow was diluted with equivalent amount of complete culture medium (α-MEM supplemented with 10% Fetal Bovine Serum; FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine; each obtained from GIBCO, USA). The diluted bone marrow was then layered over about 10 ml of ficoll (Ficoll-Paque; Pharmacia, Sweden), and centrifugation at 2,500×*g* for 30 min. Mononuclear cells were collected at the interface, suspended in PBS, and centrifuged at 1,500×*g* for 15 min. The pellet was then suspended in 5 ml of complete medium and seeded in a 25-cm^2 culture flask (Nunc), and incubated at 37°C with 5% humidified CO_2.

To characterize the surface antigens of hBMSCs, immunostaining of CD13, CD34, CD45, CD90, and CD105 (1:20) were performed on passage 1 cells. Briefly, bone marrow stromal cells were cultured in chamber slides until 80% confluency, rinsed with buffer solution followed by fixation with absolute alcohol. The cells were then incubate with antibodies raised in mouse against the desired surface antigens (Chemicon, USA) and subsequently labeled with fluorescent dye using Envision Kit Plus (Dako, Denmark) according to the manufacturer instruction.

hBMSC differentiation potential

To determine the differentiation potential of hBMSCs, osteogenic induction was performed on passage 1 cells by the addition of 10^{-7} M dexamethasone; 0.05 mg/ml ascorbate-2-phosphate; 10 mM β-glycerophosphate into the complete culture medium on passage 1 cells. Differentiation of hBMSCs into osteogenic lineage was determined by detection of alkaline phosphatase (fast-red chromogen substrate reaction) and mineralization activity (Von Kossa staining) three weeks post-induction.

Telomerase activity assay

Quantitative determination of telomerase activity was performed by TeloTAGGG Telomerase PCR ELISA Plus kit (Roche Diagnostic GmbH, Germany) according to the manufacturer instructions. Briefly, 2×10^5 hBMSCs from passage 1 were harvested and 200-µl lysis buffer was added prior to centrifugation for telomerase extraction. Total crude protein from cell lysates was assayed using BCA Protein Assay

kit (Pierce Biotechnology, USA) according to the manufacturer protocol. Six dilution points of standard bovine serum albumin was used to plot the protein standard curve.

The telomerase repeat amplification protocol (TRAP) assay is a two-step process in which the telomerase-mediated elongation products are subsequently amplified by PCR to allow highly sensitive detection of telomerase activity. The level of telomerase activity in a given sample is determined by comparing the signal from the sample to the signal obtained using a known amount of a control template (0.001 mol/ml). The Control template used is identical to a telomerase elongation product with 8 telomeric repeats. Telomerase activity measured is expressed as absorbance at 450 nm against a reference wavelength of 690 nm.

Relative telomerase activities (RTA) within different samples in an experiment are obtained calculated according to manufacturer's recommendation.

Transfection with hTERT gene

Out of the eleven samples, four from patients aged ≥60 years were selected for subsequent transfection with *hTERT* gene using vector pcDNA3-*hTERT* n2 as shown in **Figure 1A** (a kind gift from Prof Fuyuki Ishikawa, Kyoto University, Japan). The primary bone marrow stem cells were isolated and expanded in culture at a seeding density of 5×10^3 cells/cm^2 in 6-well plates. Upon 85% confluence of passage 1 culture, standard culture medium was replaced with 2 ml of medium without antibiotics, and the transfection was performed on the following day. Two methods of transfection were performed in parallel including lipofection (Lipofectamine 2000 kit; Invitrogen, USA) and electroporation (Human Mesenchymal Stem Cells (MSC) Nucleofector kit; Amaxa, Germany). The plasmid encoding the green fluorescent protein (GFP), pMaxGFP (**Figure 1B**) was co-transfected with each reaction. It was assumed that pcDNA-*hTERT* n2 vector uptake is in concert with the pMax vector.

For lipofection, DNA (μg) and Lipofectamine 2000 (μl) complex was formed at a ratio of 1: 2.5. Briefly 10 μl of Lipofectamine 2000 was first diluted with 250 Opti-MEM I Medium and incubated for five minutes at room temperature. Subsequently, 2.0 μg of pcDNA3-*hTERT* n2 and 2.0 μg of pMaxGFP that was suspended in 250 μl OptiMEM I, and subsequently added to the Lipofectamine solution to form complexes. The two solutions were mixed gently and incubated for 20 min at room temperature. The entire complexes were then added to cells in the well plate (containing 2 ml medium without antibiotics). Lipofectamine solution without the DNA was added into the other well serving as a negative control. Cells were then incubated at 37°C in a CO_2 incubator with gentle rocking for 24 hours. The medium was then refreshed with complete culture medium.

For electroporation, cells from each well were trypsinized with 0.05% trypsin-EDTA (Invitrogen, USA), washed, pelleted and resuspended in 100 μl of Human MSC Nucleofector Solution. Two micrograms each of pcDNA3-*hTERT* n2 and pMaxGFP in Tris-chloride buffer (pH8.5) was then added to the cell suspension which was then transferred into an electroporation cuvette before insertion into the electroporator. Electroporation proceeded using the preset programme U-23 on the AMAXA Electroporator. Upon completion (within two seconds), 500 μl of pre-warmed standard culture medium was added into the sample. Subsequently, the cells were transferred using a plastic pipette into a 6-well plate. Cells with Human MSC Nucleofector Solution minus the DNA served as a negative control.

Post transfection analysis

Cell viability was monitored after 24 hours upon transfection by evaluating the proportion of cells attached to the culture wells to the original number of cells seeded. Efficiency of the two transfection procedures was evaluated after five days by manual counting of cells expressing GFP against the total adherent cells under a fluorescence microscope in three different representative regions.

Post transfected cells derived from the same patients but were transfected separately using one of the two methods were trypsinized and pooled together at day five. Half of these pooled cells were lysed for telomerase activity quantification while the rest were subjected to subsequent *in vitro* culture. They were passaged upon confluence and replated at a constant density of 5×10^3 cells/cm^2 until the onset of senescence.

Results

hBMSC characterization

Isolated bone marrow stromal cells displayed a variety of morphology in monolayer cultures

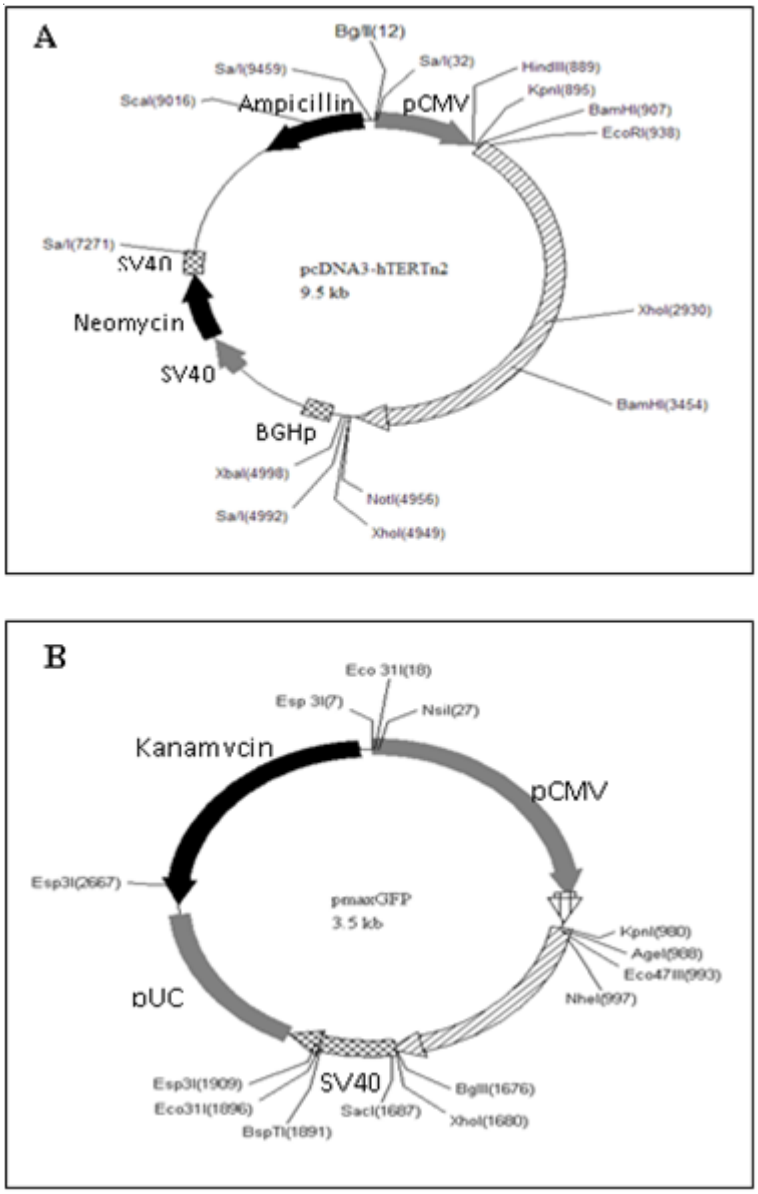

Figure 1. **A:** Plasmid map of pcDNA3-*hTERT* n2. Gene composition and restriction enzyme cutting sites of the plasmid. **B:** Plasmid map of pmaxGFP (Amaxa, USA), encoding green fluorescent protein from *Potellina sp.* Gene composition and restriction enzyme cutting sites of the plasmid.

(**Figure 2**). Clonal proliferation is not a frequent event in contrary [15]. Immunostaining with specific surface antigens displayed presence of heterogeneous populations within the hBMSC population. All cells showed consistent negativity for CD45 and positivity for CD13 and CD90 with varied expression of CD34 and CD105 (**Figure 3**). These hBMSCs can be induced toward osteogenic lineage as shown by alkaline phosphatase expression and mineralization activities (**Figure 4**).

Transfection efficiency and cell viability

Positively transfected cells emitted a green fluorescence. Representative micrographs of post transfected cell are shown in **Figure 5**. Transfection efficiency of 55.6%±4.3 and 38.5%±3.4 was achieved for electroporation and lipofection, respectively. In contrast, cell viability was significantly higher for electroporation 44%±5.1 compared to liporection 83.1%±10.5.for electroporation and lipofection was evaluated to be and, respectively.

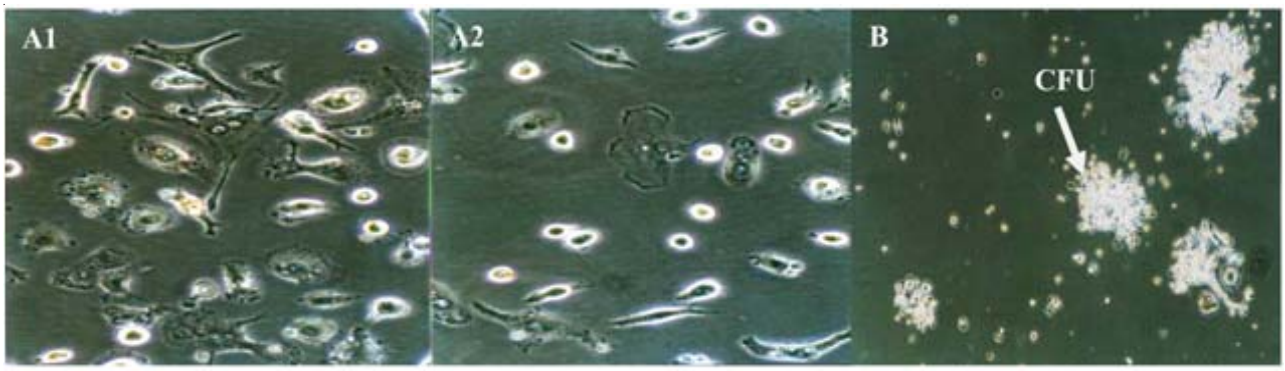

Figure 2. hBMSC cell morphology at passage 0. **A1** and **A2**: Polymorphic morphology – petal-shaped, spindle-shaped and star-shaped cells. (10x). **B:** Colony-forming-unit (CFU) expansion (40x).

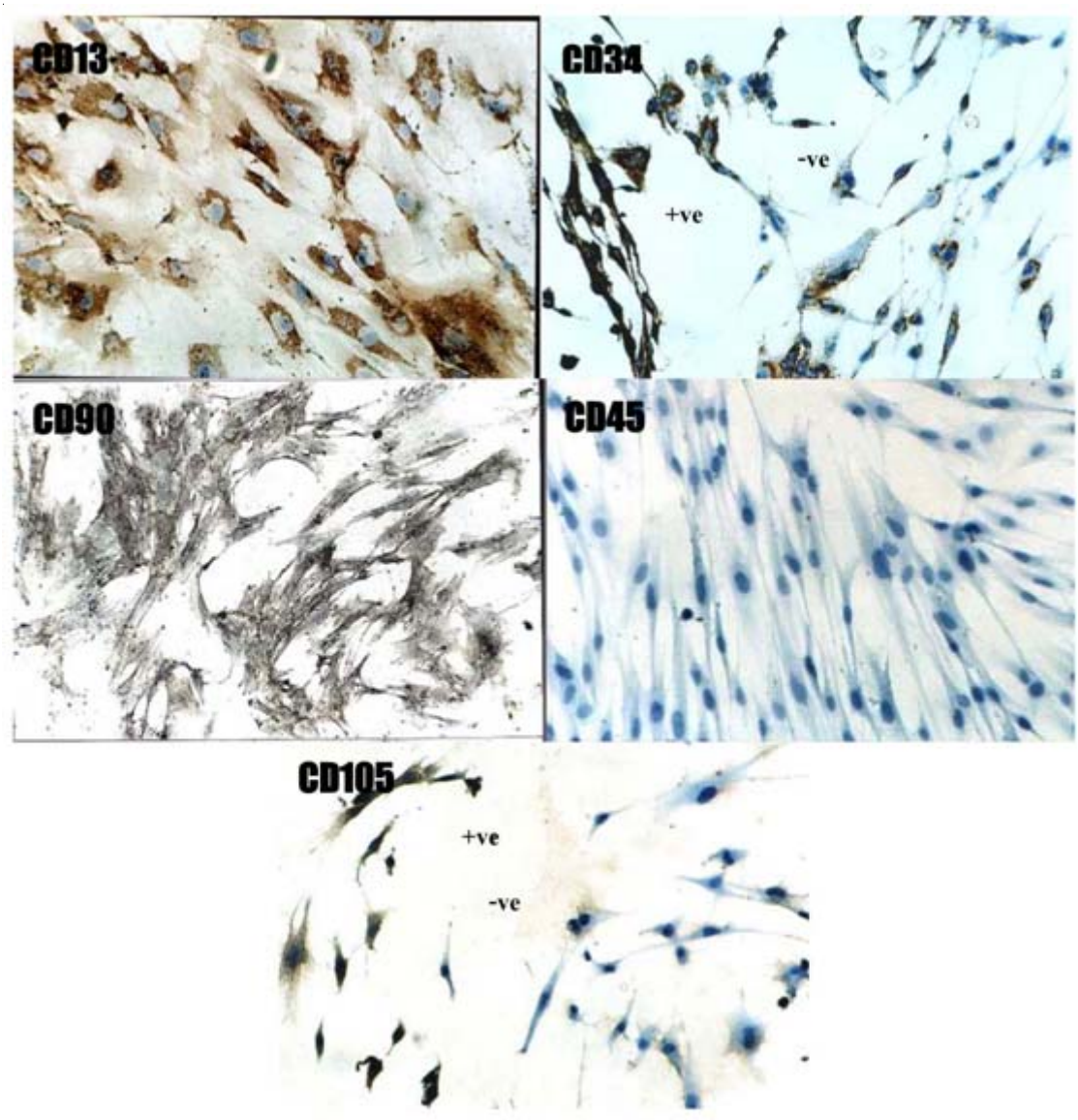

Figure 3. Photomicrographs of hBMSCs immunostaining at passage 1. Heterogeneous populations that stained either positively or negatively for CD34 and CD105 but demonstrated consistent negativity for CD45, positivity for CD13 and CD90

Figure 4. Osteogenic induction of hBMSC at passage 1. Control: No induction; Osteogenic induction for three weeks. No or minimal alkaline phosphatase and calcium deposition occurs without osteogenic induction. Substantial expression of alkaline phosphatase activity and mineralization activity occurred by week three of osteogenic induction.

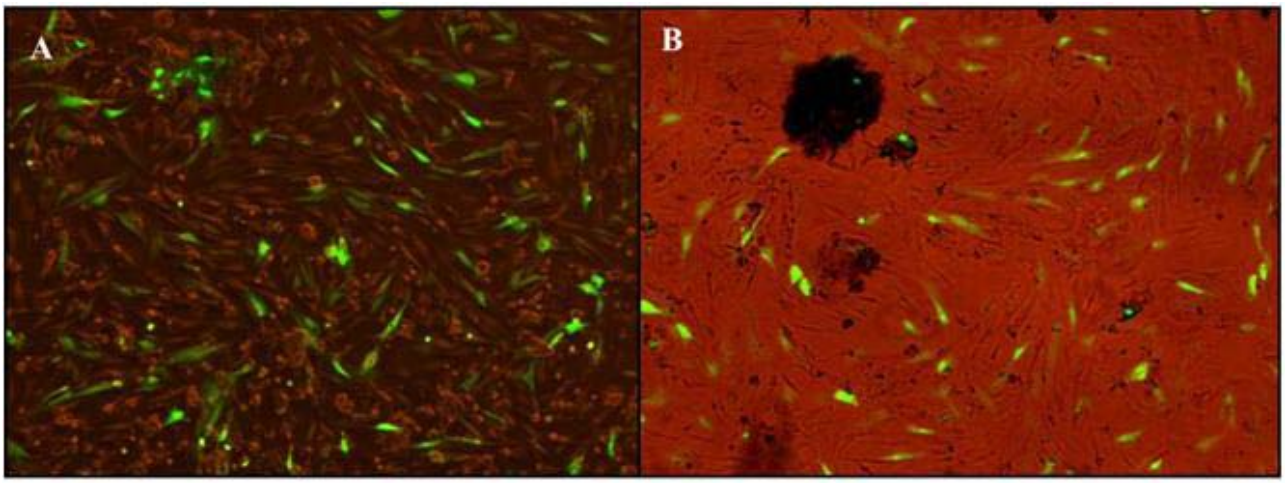

Figure 5. Photomicrograph of hBMSCs five days post transfection. **A:** Tranfection via electroporation showing an efficiency of transfection at approximately 55%, **B:** Transfection via lipofectamine with transfection efficiency of approximately 40% (viewed under phase contrast with GFP filter using both halogen and mercury light;×100).

Telomerase activity

Telomerase activity was detected in the hBMSCs from younger patients (<60 years; n = 5), while lost in older patient (≥60 year: n = 6), as shown in **Figure 6**. Telomerase activity for the pre-transfected samples from donors aged 16, 18, 18, 47, 59, 60, 60, 63, 64, 72, and 74 were 37.3, 20.1, 35, 32.5, 63.7, 0.1, 0, 0.2 and 0.4, respectively. Telomerase activity was enhanced drastically in the post transfected samples from donors aged 60, 64, 72, and 74. The values were 63.2, 34.1, 5.2, and 13.1, respectively (**Figure 7**). After nine days post transfection, telomerase activity was lost along with the GFP expression.

Passage number at the onset of cell senescence

The mean passage number achieved in non-transfected samples (collected from the patients aged ≥60 years) was six, where cells underwent approximately 15 population doublings (**Figure 8**). In contrast, post-transfected samples survived more than 13 passages, where cells underwent approximately 45 population doublings. In addition, post transfected cells displayed normal morphology, and maintained growth rate.

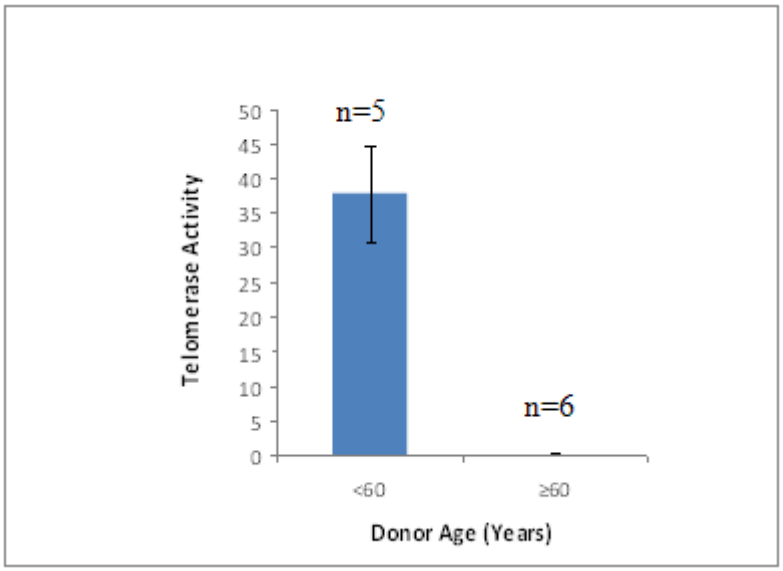

Figure 6. Correlation of RTA and donor age of hBMSC at passage 1. hBMSCs from patients at or above 60 years of age (n = 6) did not have detectable telomerase activity.

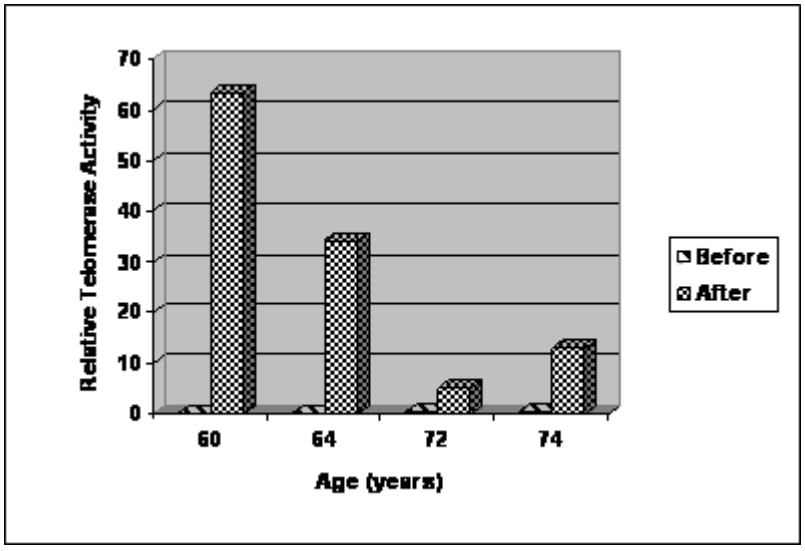

Figure 7. RTA of individual sample before and after transfection. No telomerase activity was detected before *hTERT* transfection. Substantial telomerase activity was detected in all samples that were transfected with *hTERT* (n = 4).

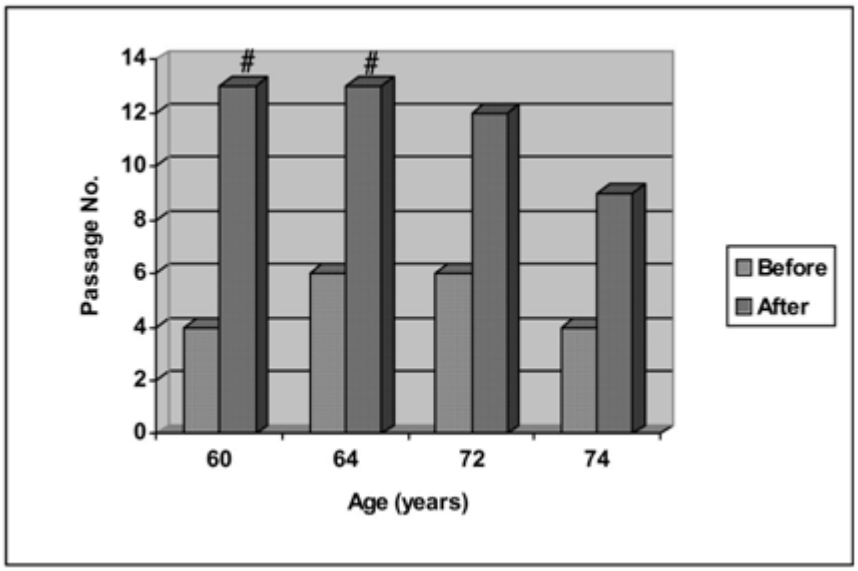

Figure 8. Maximum passage number before and after transfection. The maximum passage number achieved for cells without transfection was P6 while cells after the *hTERT* transfection displayed increased cellular life span and achieved at least P13 before senescence. Samples (#) were still amenable to subculturing but were deliberately terminated.

Discussion

Human bone marrow stem cells (hBMSCs) have been actively applied in tissue engineering because of its potentiality to differentiate into various mesenchymal cells such as chondrocyte, osteoblasts, adipocytes, etc. [16]. In this study, hBMSCs was successfully isolated from bone marrow aspirate and bone marrow scraping from patients of various ages ranging from 16 to 74 years, and these cells were characterized for their telomerase activity and life span.

Our findings showed that different populations persist in hBMSC culture. It was also illustrated that mean telomerase activity in patients of ≥60 years old was significantly lower compared to young patients. The loss of telomerase activity in hBMSCs from older patients implied its limitation to be used in tissue engineering owing to their short life span in culture unless other interventions are introduced such as transfection with telomerase gene or supplementation of growth factors. A previous study successfully demonstrated that transfection of telomerase gene in hBMSCs resulted in extended cellular life span and enhanced differentiation potential, whereas untransfected cells ceased growth [17]. This result implied that telomerase is required for both cell replication and differentiation [18].

Previous study had demonstrated that the ectopic expression of telomerase in hBMSCs through transduction of *hTERT* using lentiviral vector increased the life-span of these stem cells [19]. However, the use of viral vectors raised concerns over safety issues in cell therapy. In this work, a transient transfection of the *hTERT* gene using non-viral method was used considering the safety issues and ease of handling.

Two most common non-viral transfection technique used are lipofection and electroporation. Lipofection (or liposome transfection) is a technique used to inject genetic material into a cell through liposomes, which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer. Electroporation is based on the principle that a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field. Pores are formed when the voltage across a plasma membrane exceeds its dielectric strength. If the strength of the applied electrical field and/or duration of exposure to it are properly chosen, the pores formed by the electrical pulse reseal after a short period of time, during which extracellular compounds have a chance to enter into the cell. In this study, electroporation resulted in higher

transfection efficiency (55.6%) compared to lipofection (38.5%) but the cell viability via the former was lower (44% *vs.* 83%). Both methods can be further fined tuned such as by altering the lipofectamine dosage, electroporation voltage and duration of exposure. Electroporation has a clear advantage due to the simplicity of the procedures involved but requires specialized instrument to perform.

Post-transfected cells showed positive telomerase activity. The expression of telomerase albeit low compared to that detected in hBMSCs from donors aged <60 years was sufficient for extending the cellular life span. Total population doublings increased three folds (from 15 doublings to 45 doublings) attaining a passage number of 13 or beyond. The transient expression of telomerase activity precludes the risk or possibilities of telomerase-induced carcinogenesis. As opposed to many viral mediated transfection techniques, lipid transfection and electroporation are a safe and rapid methods that be easily adopted for clinical therapy. Furthermore, the using of viral vectors in clinical therapy may induce unexpected adverse effects such as immunogenicity and cytotoxicity [20, 21].

The mechanism by which cellular life span extension is achieved through telomerase expression is not fully understood. In our observation, post transfected cells were extremely robust showing no signs of apoptosis such as condensed nuclei and cell shrinkage even when they were subjected to nutritional and environment stress. It is hypothesized that the delayed in cell senescence is due partly to the suppression of apoptosis.

Conclusion

In conclusion, hBMSC isolated from our laboratory showed presence of a heterogeneous population with varied surface antigens and telomerase activity. Upon osteogenic induction, alkaline phosphatase and mineralization activities were observed. Lipofection and electroporation can be used to facilitate *hTERT* transfection of hBMSCs. Transient transfection of *hTERT* in hBMSCs proved to be a solution for prolonging cellular life span that would enable further cell expansion and manipulation at the *in vitro* level prior to its use in clinical therapies. In this study, the cellular life span of transient transfected hBMSCs from elderly patients is able to increase for approximately three folds. This could be the long awaited answer to the need for propagating primary cells from elderly donors. Adult stem cells derived from bone marrow holds a great promise for a myriad of medical problems such as autoimmune disorder, degenerative and traumatic-induced diseases. Thus, the ability to tap this source from elderly patients opens up a new horizon for future stem cell based therapies. However, further studies should be conducted to proof the functionality of the transfected cells (multipotency) before it can be introduced to the clinic. Safety issues associated such as genetic stability of transfected cells should be addressed further.

Acknowledgements

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References

1. Muschler GF, Nitto H, Cynthia A, Boehm CA, Easley KA. Age and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. Journal of Orthopaedic Research. 2001; 19:117-25.
2. Shamsul BS, Aminuddin BS, Ng MH, Ruszymah BH. Age and gender effect on the growth of bone marrow stromal cells in vitro. Medical Journal of Malaysia. 2004; 59(Suppl B):196-7.
3. Rajasekhar VK, Vemuri MC (eds.). Regulatory Networks in Stem Cells, Stem Cell Biology and Regenerative Medicine. Humana Press. 2009; p.123-133 doi:10.1007/978-1-60327-227-811.
4. Matsui M, Miyasaka J, Hamada K, Ogawa Y, Hiramoto M, Fujimori R, et al. Influence of aging and cell senescence on telomerase activity in keratinocytes. Journal of Dermatological Science. 2000; 22:80-7.
5. Hayflick L. The Limited In Vitro Lifetime Of Human Diploid Cell Strains. Experimental Cell Research. 1967; 37:614-36.
6. Blackburn EH, Gall JG. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. Journal of Molecular Biology. 1978; 120:33-53.
7. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990; 345: 458-60.
8. Blackburn G. Identification of a Specific Telomere

Terminal Transferase Activity in Tetrahymena Extracts. Cell. 1985; 43:405-13.
9. Wright WE, Shay JW. Telomere positional effects and the regulation of cellular senescence. Trends in Genetics. 1992; 8:193-7.
10. Wolbank S, Stadler G, Peterbauer A, Gillich A, Karbiener M, Streubel B, et al. Telomerase Immortalized Human Amnion and Adipose-Derived Mesenchymal Stem Cells: Maintenance of Differentiation and Immunomodulatory Characteristics. Tissue Engineering Part A. 2009; 15:1843-54.
11. Tsai CC, Chen CL, Liu HC, Lee YT, Wang HW, Hou LT, et al. Overexpression of hTERT increases stem-like properties and decreases spontaneous differentiation in human mesenchymal stem cell lines. Journal of Biomedical Science. 2010; 17:64.
12. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, et al. Extension of Life-Span by Introduction of Telomerase into Normal Human Cells. Science. 1998; 279:349-52.
13. Porter PC, Clark DR, McDaniel LD, McGregor WG, States JC. Telomerase-immortalized human fibroblasts retain UV-induced and p53-mediated DNA damage responses. DNA Repair. 2006; 5:61-70.
14. Munirah S, Samsudin OC, Nor Hamdan MY, Saim BS, Ruszymah BHI. Sox-9 Transient transfection enhances chondrogenic expression of osteoarthritic human articular chondrocytes in vitro: preliminary analysis. Tissue Engineering and Regenerative Medicine. 2011; 8:32-41.
15. Abdallah BM, Haack-Sorensena M, Burnsa JS, Elsnab B, Jakob F, Hokland P, et al. Maintenance of differentiation potential of human bone marrow mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene in despite of extensive proliferation. Biochemical and Biophysical Research Communications. 2005; 326:527-38.
16. Eugene See YS, Toh SL, James Goh CH. Multilineage Potential of Bone-Marrow-Derived Mesenchymal Stem Cell Cell Sheets: Implications for Tissue Engineering. Tissue Engineering Part A. 2010; 16: 1421-31.
17. Simonsen JL, Rosada C, Serakinci N, Justesen J, Stenderup K, Rattan Suresh IS, et al. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nature Biotechnology. 2002; 20: 592-6.
18. Hiyama E, Hiyama K. Telomere and telomerase in stem cells. British Journal of Cancer. 2007; 96:1020-4.
19. Boker WG, Yin ZH, Drosse I, Haasters F, Rossmann O, Wierer M, et al. Introducing a single-cell-derived human mesenchymal stem cell line expressing hTERT after lentiviral gene transfer. Journal of Cellular and Molecular Medicine. 2008; 12:1347-59.
20. Glover DJ, Lipps HJ, Jans DA. Towards save, non-viral therapeutic gene expression in humans. Nature Reviews Genetics. 2005; 6:299-310.
21. He CX, Tabatab Y, Gao JQ. Non-viral gene delivery carrier and its three-dimensional transfection system. International Journal of Pharmaceutics. 2010; 386: 232-42.