Transfection of human bone marrow mesenchymal stem cells with hTERT and SV40LT genes☆

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Abstract

Objective. To investigate the transduction of human bone marrow mesenchymal stem cell (MSC) lines by transfection with simian virus40 large T-antigen (SV40L Tag) derived from simian virus40 and human telomerase reverse transcriptase (hTERT). *Methods.* Two different vectors that contained hTERT/SV40LT fragment and drug resistance gene were used to transfect normal human bone marrow MSCs. The transfected cells were screened with 0.5 μg/ml puromycin and 450 μg/ml G418. The expression of SV40Tag and hTERT in all transfected cell lines was verified by PCR and immunocytochemistry. The morphology and growth of the cells were observed under inverted microscope. *Results.* The transfected MSCs were spindle-shape fibroblast and resembled marrow MSCs. The chromosome number in the transfected cells was 46, same as the normal human cell's. The cells could be subcultured successively and maintained the most morphologic characteristics of primary cells, such as monolayer, anchorage dependent. *Conclusions.* hTERT and SV40Tag genes can successfully induce the transformation of human bone marrow MSCs. [Life Science Journal. 2008; 5(1): 30 – 34] (ISSN: 1097 – 8135).

Keywords: mesenchymal stem cells; transfection; hTERT; SV40L Tag

1 Introduction

Mesenchymal stem cells (MSCs), the un-haemopoietic stem cells which are isolated from bone marrow, can be differentiated into neuron-like cells under given conditions. MSCs are such focused not only for their characteristics of general stem cells, but also for their easy access, applicable self-transplantation, convenient transcription and so on. It can also provide seed cell for biological study, especially play an important role in tissue engineering. However, MSCs have a finite capacity to replicate *in vitro* and eventually enter a state of irreversible growth arrest for about 20 generations, and then become aging. These cells were the important primary cells in tissue engineering and can be differentiated into many kinds of mature cells in suitable conditions *in vivo* or *in vitro*, such as cartilage, nerves, muscles, cutaneous covering, liver and bone[1–3]. However, the life period of MSCs is limited and it's hard for further research and the application to MSCs. The establishment of human normal marrow mesenchymal stem cell lines is the premise to implement the application. In recent years, some methods have been used to induce the cells to immortalization, through virus, radioactive factors, oncogenes as well as proto-oncogene, and telomerase. Recently, hTERT and simian virus40 large T-antigen (SV40L Tag) have been used to immortalize the mammalian cells[4–5]. The single use of hTERT or SV40Tag is of low efficiency. This research tried to combined the hTERT and SV40Tag genes together to transform cells, so as to establish a reliable and lasting cell lines.

2 Materials and Methods

2.1 MSCs preparation and culture

The MSCs were isolated from thoracic bones of 20-year-old healthy male volunteers. They were all informed consent on institutional review board-approved protocols.

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We drew 3 ml marrows aseptically, anticoagulation with heparin and mixed fully with PBS (0.1 mol/L, pH 7.4). The mixture was dropped on the surface of lymphocyte separating medium (TBD, Tianjin, China) (1 : 1, v/v), centrifuged at 2000 rpm for 15 minutes. We got middle layer carefully, the cells on which lay like white membrane in mixture, and mixed with PBS. The dispersed cells were centrifuged at 1500 rpm for 5 minutes. The cells were counted under the light microscope and inoculated in a 50 ml culture flask (1×10^6 cells/ml). The culture medium was dulbecco's minimum essential medium (DMEM, Gibco-BRL, USA) supplemented with 50 μg/ml streptomycin, 50 U/ml penicillin (Gibco-BRL). Primary cultures lasted for 21 days in modular incubator chambers, with changing medium every other day. When the cells outgrowths were visible, we subcultured the cells at 1 : 4 ratio with the DMEM. All cultures were maintained at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air. All the procedures in this study were performed in compliance with the regulations of Zhengzhou University. We identified the third untransfected and transfected cells by the cell surface antigen (CSA): CD44, CD45, CD29 and CD34 with immunocytochemistry.

2.2 Transfection

All MSCs (5×10^5 cells/ml) were transfected with 4 μg plasmid pCDNA3.1-hTERT including a neomycin-resistant gene and 3 μg plasmid pCDNA3.1-SV40LT including a puromycin-resistant gene (constructed by our Laboratories) by using Fugene6 (Roche, USA) according to the manufacturer's instructions. These cells were exposed to 10% FBS/DMEM containing 450 μg/ml G418 (Gibco-BRL) for 11 – 14 days, and then to 10% FBS/DMEM containing 0.5 μg/ml puromycin (Gibco-BRL) for 13 – 15 days. Four parallel control groups were: transfected with hTERT, transfected with SV40L Tag, transfected with purified water, and normal group. The survival cells were expanded and cultured in this study.

2.3 Gene and CSA detection

2.3.1 PCR. The genome DNA of hTERT-SV40-MSC was extracted and primer was designed according to the gene sequence of SV40 large T-antigen and hTERT. PCR products were separated on 1.5% agarose gels by electrophoresis and stained by ethidium-bromide. The primer sequences, product sizes, and annealing temperatures for each gene were shown as follows. SV40Tag: forward, 5'-TGTG2GTATGGCTGATTATGA-3', and reverse, 5'-CGCAGT2GAGTTTTTGTTAGA-3'; hTERT: forward, 5'-CGGAAGAGTGTCTGGAGCAA-3', and reverse, 5'-GGATGAAGCGGAGTCTGGA-3'. Amplification was carried out as follows: SV40Tag, 99 ºC force-degeneration for 7 minutes, then 30 cycles, 95 ºC for 45 seconds, 55 ºC for 45 seconds, 72 ºC for 60 seconds, and 72 ºC, elongation for 7 minutes; hTERT, 94 ºC force-degeneration for 5 minutes, then 33 cycles, 95 ºC for 30 seconds, 58 ºC for 45 seconds, 72 ºC for 40 seconds, and at 72 ºC elongation for 7 minutes.

2.3.2 Immunocytochemistry. The transfected cells were inoculated onto the coverslip, washed by PBS (0.1 mol/L, pH 7.4), fixed by cool acetonum. The coverslip was dropped onto anti-hTERT and anti-SV40LT monoclonal antibody (Mab). The cells were observed under the light microscope after 3, 3'-diaminobenzidine (DAB) staining, dehydration, cleaning, and fix.

2.4 Characterization of cells

The transfected and untransfected cells were cultured according to the routine methods, and subcultured at the ratio 1 : 3 every 3 – 4 days. Morphologic characteristics were observed under the light microscope. The cells of 4th generation were inoculated onto the 24-well plates (3×10^4 cells/well), with changes of medium once every 3 – 4 days. Outgrowths were trypsinized, resuspended as a single cell suspension, and counted with blood cell counting plate every 24 hours, three times/well. Population doubling (PD) was calculated at each passage by using the following equation: $PD = \{\lg 2/(\lg N_t - \lg N_0)\} \times t$. Where N_t was the number of cells harvested at the end of the growth period, and N_0 was the number of cells inoculated. Gained cell populations at each passage were depicted in the growth curve figures.

2.5 Karyotyping

Cells were incubated with colcemid (Invitrogen, USA) for 4 hours and harvested with 0.25% trypsin. Cells were then treated with hypotonic KCl solution (0.075 mol/L) for 30 minutes at 37 ºC, fixed and washed three times with methanol/acetic acid (3 : 1), resuspended, and dropped onto slides. GTG-banded chromosome were prepared with standard methods. At least 24 cells were analyzed from each culture, and chromosome images were captured and analyzed.

2.6 Statistical analysis

The results were expressed as mean ± SD. Differences between groups were analyzed using one-way ANOVA on ranks with the least significant difference (LSD) tests. P value of 0.05 was regarded as significant. All analysis were performed using SPSS10.0 software.

3 Results

3.1 Morphological appearance and growth curve

Figure 1 showed that MSCs ceased dividing on 15 – 20th generation of culture. The major mononuclear cell began to adhere wall after 24 – 48 hours. 72 hours later the cells began to form cell colonies. The hTERT-SV40-MSC cells were spindle-shape fibroblast and resembled MSCs. Some cells were triangle or polygon (Figures 2A – 2D). By contrast, the growth rates of hTERT-MSC (PD: 67.8 ± 0.52 hours) and SV40-MSC (PD: 64.9 ± 0.19 hours) were higher than those of MSC (PD: 94.3 ± 0.26 hours) ($P < 0.05$). The growth rates of hTERT-SV40-MSC (PD: 43.5 ± 0.24 hours) were higher than hTERT-MSC and SV40-MSC ($P < 0.05$). There was no statistical significance for the difference of growth rate between hTERT-MSC and SV40-MSC ($P > 0.05$). The CSA showed that the cells were positive for CD29, CD44 and negative for CD34, CD45 (Figures 3A, 3B).

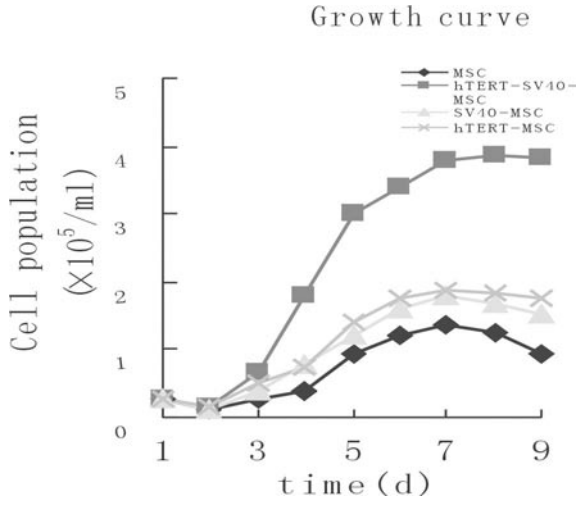

Figure 1. Cell population (× 10^5 cells/L) of MSCs and the transfected cells.

3.2 Expression of SV40L Tag and hTERT in transfected MSCs

It was showed that the gene integration of SV40L Tag and hTERT in transfected cells from the 3rd generation. Both genes were detected only in the transfected group but not in untransfected (negative control). Idio-strap of SV40L Tag and hTERT were observed on 1.5% agarose gels by electrophoresis (Figures 4 and 5). Many orange drops appeared in the transfected cells, which indicated that the transfected cells had stably expressed the protein (Figures 3 C & D).

3.3 The analysis of karyomorphism

The transfected cells maintained the normal chromosome number of human being's: 23 pairs, 46 (22, XY). There were no fragmentation, perversion and other structural abnormalities of chromosome (Figure 6).

4 Discussion

Telomerase is activated by the introduction of exogenous hTERT gene in many kinds of cells and the length of chromosome maintains stable[6]. A major advantage of using hTERT alone is that the telomerase immortalizes the primary human cell without causing cancer-associated changes or altering phenotypic properties[7,8]. Since Bodnar[4] immortalized normal human cells by transfecting the plasmid containing hTERT gene, plenty of immortalized cell lines have been established. Some studies indicated that the introduction of hTERT immortalized the cells by inactivating pRb or p16[9]. However, SV40 large T gene may reduce the growth inhibition, at least on three kinds of proteins (pRb, p53 and SEN6)[11]. Other studies showed transducing the two kinds of genes to same cells didn't change their characteristics[9–10]. So we transduced gene hTERT and SV40LT together to ensure the establishment.

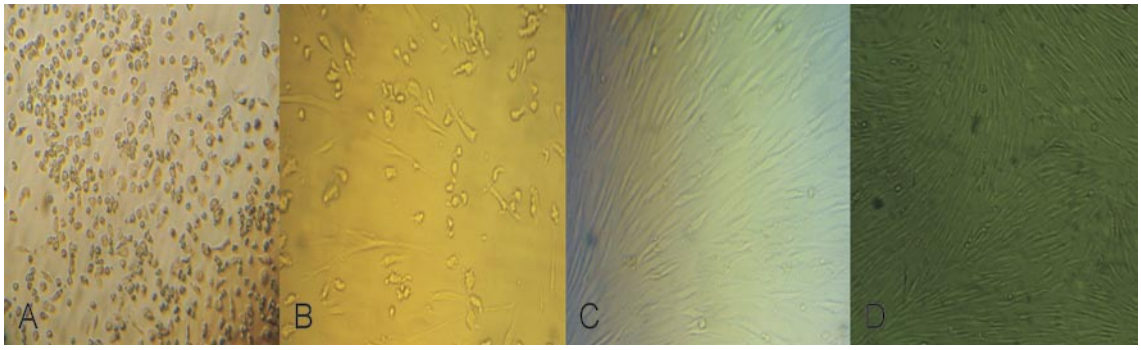

Figure 2. Morphologic appearance of MSC and hTERT-SV40-MSC. A: MSCs after 24 – 48 hours; B: MSCs after 72 hours; C: 14 generations of TERT-SV40-MSC; D: 35 generations of hTERT-SV40-MSC.

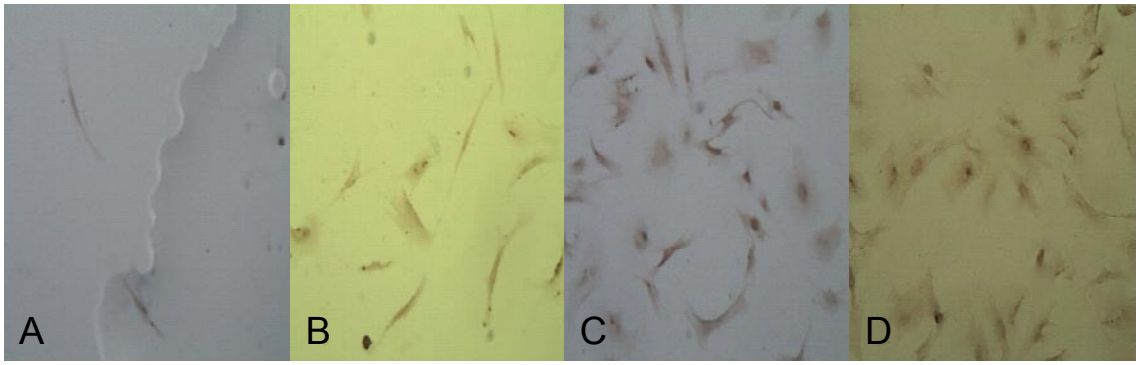

Figure 3. Characteristics of the hTERT-SV40-MSC line. A: The expression of CD29 of the transfected cells of the third generation; B: The expression of CD44 of the transfected cells of the third generation; C: The expression of SV40L Tag of the transfected cells, and the orange yellow drops appeared in the cytoblast and cell membrane; D: Expression of hTERT of the transfected cells.

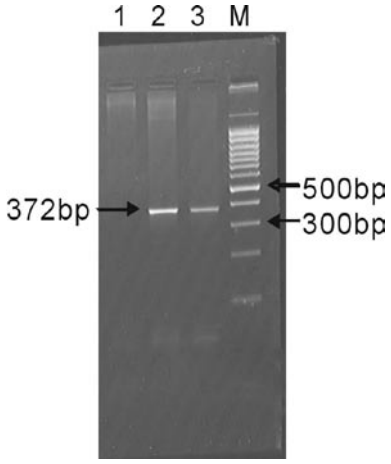

Figure 4. The ectopic expression of SV40LT (372 bp). Lane M: Marker (100 bp DNA ladder); Lane 1: MSC; Lane 2: Plasmid pDNA3.1-SV40; Lane 3: hTERT-SV40-MSC.

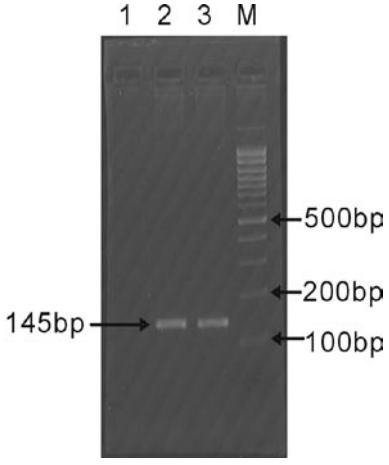

Figure 5. The ectopic expression of hTERT (145 bp). Lane M: Marker (100 bp DNA ladder); Lane 1: MSC; Lane 2: Plasmid pDNA3.1-hTERT; Lane 3: hTERT-SV40-MSC.

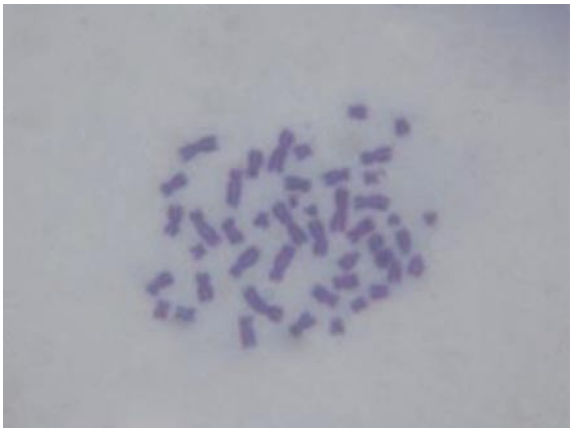

Figure 6. The karyotype of hTERT-SV40-MSC.

In our studies, doubling time of the untransfected human bone MSCs began to increase and there were many particles or kinds of vacuoles in 6th – 7th generations in serial subcultivation; doubling time obviously increased after 11 – 12 generations and the speed of proliferation remarkably descended. At the same time, figure of cell body began to change. But doubling time of the transfected cell kept stable under the same condition and the cells were cultured for more than 40 generations. The phenotypes of MSCs weren't single but appeared the characteristics of endodermis, desmohemoblast and muscle cells. Up to now, specific surface marker of MSCs has not been found. At present, morphological appearance and cultural characteristics have played an important role in identifying MSCs[11–12]. General surface marker of MSC were: SH2, SH3, CD90, CD44, CD29, CD71, CD106, CD120a, CD124 and MSCs can not express the cell surface antigens, such as CD11b, CD45, CD14, CD34, CD31[13]. So we identified MSCs by CD44, CD29, CD34 and CD45.

The results showed that the cultured cells were positive of CD29, CD44 and negative of CD34, CD45, consistent with surface marker of MSCs.

5 Conclusion

We have established an apparent "immortalization" of human marrow MSCs by transfecting SV40L Tag and hTERT into primary MSCs. The phenotypes of the hTERT-SV40-MSC were similar to those of MSCs. The life of transfected MSCs was obviously extended, which made them to be an ideal tool for applications in tissue engineering and for advanced researches.

References

1. Joanna EG, Emanuela AB, Diane SK. Plasticity of bone- derived stem cells. Stem Cells 2004; 22: 487 – 500.
2. Jiang Y, Jahagirdar BN, Reinhardt RL, *et al*. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 13: 1185 – 8.
3. Bonnet D. Biology of human bone marrow stem cells. Clin Exp Med 2003; 3: 140 – 9.
4. Bodnar AG, Ouellette M, Frolkis M, *et al.* Extension of life-span by introduction of telomerase into normal human cells. Science 1998; 279: 349 – 52.
5. Kirchhoff C, Araki Y, Huhtaniemi I, *et al*. Immortalization by large T-antigen of the adult epididymal duct epithelium. Mol Cell Endocrinol 2004; 216: 83 – 94.
6. Greider CW, Blackburn EH. Telomeres, telomerase and cancer. Sci Am 1996; 274: 92 – 7.
7. Jiang XR, Jimenez G, Chang E, *et al*. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. Nat Genet 1999; 21: 111 – 4.
8. Morales CP, Holt SE, Ouellette M, *et al*. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nat Genet 1999; 21: 115 – 8.
9. Kiyono T, Foster SA, Koop JI, *et al*. Both Rb/p16 INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 1998; 396: 84 – 8.
10. Jha KK, Banga S, Palejwala V, *et al*. SV40-mediated immortalization. Exp Cell Res 1998; 245: 1 – 7.
11. Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 2001; 98(14): 7841 – 5.
12. Kang TJ, Yeom JE, Lee HJ, *et al*. Growth kinetics of human mesenchymal stem cells from bone marrow and umbilical cord blood. Acta Haematol 2004; 112(4): 230 – 3.
13. Wexler SA, Donaldson C, Denn NG, *et al*. Adult bone marrow is arichsource of human mesenchymal stem cells but umbilical cord and mobilized adult blood are not. Br J Haematol 2003; 121: 368 – 74.