Ectopic hTERT gene expression in human bone marrow mesenchymal stem cell☆

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Abstract

Objective. To investigate how mesenchymal stem cell (MSC) telomerase activity and life span changes after introducing the human telomerase reverse transcriptase (hTERT) gene. *Methods*. Human MSCs were isolated from healthy volunteers and transfected with a pEGFP-hTERT plasmid. hTERT mRNA expression in MSCs was verified, telomerase activity quantitated and cell passages recorded. *Results*. The hTERT mRNA was detected in hTERT-transfeced MSCs but not in the untransfected cells. The untransfected human MSCs remained telomerase-negative but the hTERT-transfected cells showed robust telomerase activity. The telomerase-negative MSCs entered a non-dividing state after about 20 to 25 passages and senesced in contrast, telomerase-positive MSCs underwent at least 35 passages. *Conclusions*. Ectopic expression of the hTERT gene in human MSCs can reconstitute their telomerase activity and extend their replicative lifespans. [Life Science Journal. 2007; 4(4): 21 – 24] (ISSN: 1097 – 8135).

Keywords: mesenchymal stem cell; hTERT; transfection

1 Introduction

Human mesenchymal stem cell (hMSC) is the cell of early mesoblast that have multipotent differentiation capacity. It is capable to differentiate to both mesodermal cells such as osteoblast, cartilage cells, adipocyte, sarcoblastcadiocyte[1,2], and other blastodermic cells such as neurocyte, hepatocyte, endotheliocyte and so on[3–6]. However, there is only one MSC among 10^4 – 10^6 cells in nucleated cells of the marrow, and moreover, the MSCs in human bone marrow have the same characteristics that MSCs have a limited life, gradually lose their differentiation potential after several passages and will be senescent and die as the systemic cells[7–12], which restricts the scientific and clinical application. So it is crucial to maintain the MSCs' proliferation and multipotent differentiation *in vitro*.

Some investigation have shown that telomere has close correlation with life span of the cells, and activation of telomerase can maintain the length of the telomere.

Telomerase is a ribonucleoprotein that consist of RNA and protein and has action of reverse transcription. Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase and it is of vital importance in activating telomerase, so its expression decided the activity of telomerase[13].

We introduced the hTERT gene to hMSCs, hoping that ectopic expression of human telomerase reverse transcriptase (hTERT) gene can induce telomerase activity, maintain telomere length, and extend the replicative life span of MSCs while maintaining their normal characteristics.

2 Materials and Methods

2.1 Isolation, purification and expansion of human MSCs *in vitro*

Human bone marrow was obtained from healthy volunteers after informed consent. After dilution with PBS (1 : 1, v/v), low-density (below 1.077 g/ml) mononuclear cells (MNCs) were separated by Ficoll-Paque Plus (Pharmacia). Then MNCs were inoculated at the density of 2 – 5 × 10^6 cells/ml in high-Dulbecco's modified eagle medium (DMEM, Gibco, USA) supplemented with 15% heat-inactived fetal bovine serum (FBS), 2 mM gluta-

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mine, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37 ºC). Nonadherent cells were discarded at 72 hours and every 3 days thereafter. After 10 to 14 days, when the cells reached about 90% confluence, adherent cells were dissociated by 0.25% trypsinase containing 0.02% ethylenediaminetetraacetic acid (EDTA), washed twice with PBS, and subcultured into 24 multiwell plates.

2.2 pEGFP-hTERT plasmid transduced into human MSC

Human MSCs (hMSCs) were transfected with four groups (including pEGFP-C1 plasmid group, pEGFP-hTERT plasmid group, lipofectamine group and untransfected MSCs group) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The pEGFP-C1 plasmid and pEGFP-hTERT plasmid were kindly presented by Dr. Chantal Autexier (Canada). The former contained the enhanced green fluorescent protein (EGFP) and neomycin resistance genes, the latter contained the hTERT, EGFP and neomycin resistance genes. The 5th passage cells which 90% – 95% confluented were treated for 4 hours with 100 μl of DNA-Lipofectamine 2000 complexs in a 24-well culture plates. Dilute the cells (1 : 10, v/v) with fresh growth medium 24 hours after transfection. Twenty-four hours after inoculation, added 200 μg/ml G418 into the medium. Fourteen days later, the concentration of G418 was decreased to 100 μg/ml. The transfected cells were observed under fluorescent microscope to determine EGFP gene expression 24 hours after transfection.

2.3 RT-PCR analysis of hTERT gene expressed in transduced and untransfected MSCs

For RT-PCR analysis, total cellular RNA was prepared from cells of the Trizon total RNA Isolation System (Sangon, China). The RNA isolated (1 μg) was reverse transcribed to cDNA in a 20 μl reaction mixture with the use of the AMV First Strand cDNA Synthesis Kit (Sangon, China). PCR was performed with primers specific for retrovirally encoded hTERT (5' - TCTGGATTTGCAGGTGAACAG - 3' and 5'- GTAGGTGACACGGTGTCGAGT - 3') and primers specific for retrovirally encoded beta-actin (5' - GGCATGGGTCAGAAGGATTCC - 3' and 5' - GTAGGTGACACGGTGTCGAGT - 3'). The PCR reaction was performed at 94 ºC for 5 minutes, followed by amplification cycles consisting of 94 ºC for 30 seconds (denaturation), 65 ºC for 50 seconds (annealing), and 72 ºC for 60 seconds (extension), 35 cycles. Expected lengths of the amplified products for hTERT and beta-actin were 310 bp and 500 bp, respectively. The PCR products electrophoresis were on the agarose gel and visualized by staining with ethidium bromide.

2.4 Detection of telomerase activity in transfected and untransfected MSCs

Telomerase activity in EGFP-transfected MSCs, untransfected MSCs and hTERT-transfected MSCs was detected using the Telo TAGGG Telomerase PCR ELISA kit (Roche, Germany) according to the manufacturer's instructions. The result above 0.2 was considered positive.

2.5 Statistical analysis

The results were expressed as means ± SE. All data were satistically analysed by one-factor analysis of variance. Significance was set as 0.05.

3 Results

3.1 Isolation, purification and expansion of human MSCs *in vitro*

Freshly isolated MSCs were small and round. During culture, cells turned spindle-like. After cultured for 2 – 3 weeks, cell colonies were formed. Growth rate of the cell became stable from the second passage.

3.2 Immortalization of human MSCs after transfected with ectopic hTERT gene

Green fluorescence appeared 48 hours after transfection in the pEGFP-C1 group and the pEGFP-hTERT group and disappeared one month later (Figure 1). Positive colonies of the pEGFP-C1 and the pEGFP-hTERT group were selected by G418 within 1 – 2 weeks, and well separated colonies were isolated by trypsinization and subcultured into 6 multiwell plates. Another 10 to 15 days later, when the cells reached about 90% confluence, the transduced cell were dissociated by 0.25% trypsinase containing 0.02% EDTA and subcultured into 6 multiwell plates. The hTERT-transduced cells underwent 35 passages and continued to proliferate, but the EGFP-transfected cells and normal human MSCs senesced and entered the nondividing stage which was followed by cell death after 20 to 25 passages in culture. We didn't observe any gross phenopypic, morphological characteristics (such as loss of contact inhibition or growth in low serum) or anchorage in hTERT-transduced cells. Thus ectopic expression of the hTERT gene extended the life span of hTERT-transduced cells.

3.3 Detection of hTERT mRNA in transfected and untransfected MSCs

We confirmed the integration and mRNA expression of

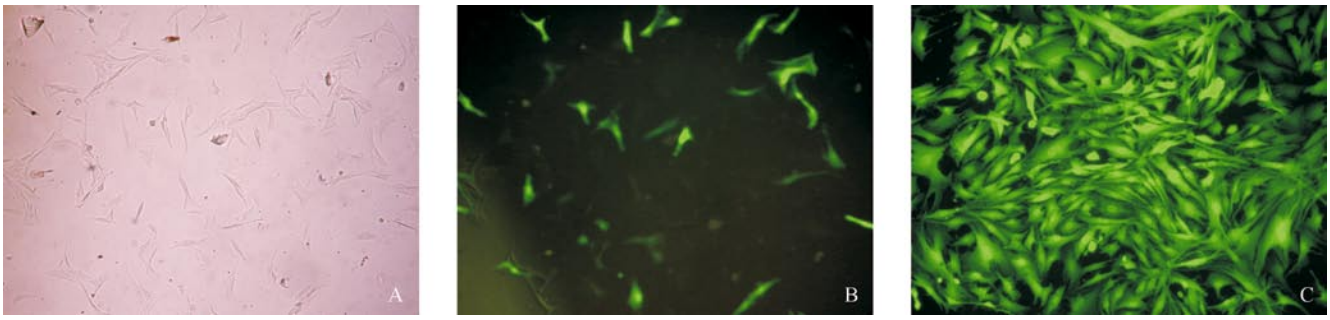

Figure 1. Detection of EGFP expression of the hTERT-transfected MSCs after transfection. A: under light microscope 48 hours after transfection (size bars 100); B: under fluorescent microscope 48 hours after transfection (size bars 100); C: under fluorescent microscope 1 month after transfection (size bars 100)

the exogenous hTERT in hTERT-MSC by RT-PCR. As shown in Figure 2, 310 bp fragment was observed in the hTERT-transduced cells and K562 cells (a kind of tumor cell) but not in the EGFP-transduced and untransfected hMSCs, which demonstated the integration and mRNA expression of the exogenous hTERT gene in hTERT-transduced cells.

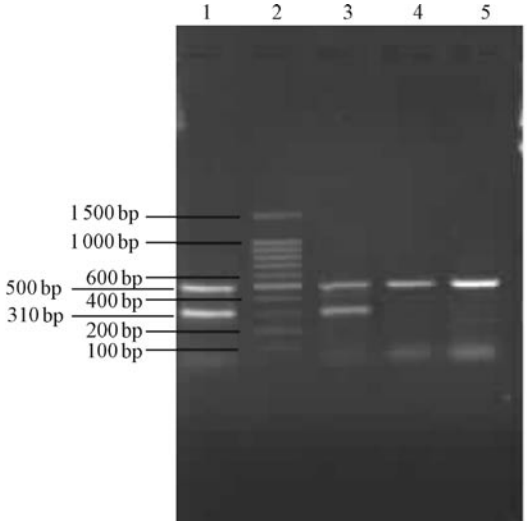

Figure 2. RT-PCR analysis of hTERT gene transcripts in transfected and untransfected MSCs. 1: K562 cells (positive control); 2: Marker; 3: hTERT-transfected MSCs; 4: the EGFP-transfected MSCs; 5: untransfected MSCs.

3.4 Detection of telomerase activity in transfected and untransfected MSCs

As shown in Figure 3, the absorbance (A450nm – A690nm) of the hTERT-transfected cells was lower than that of positive control (provided by the Telo TAGGG Telomerase PCR ELISA kit) but obviously higher than that of the EGFP-transfected cells and untransfected human MSCs. As samples regarded as telomerase-positive if the difference in absorbance (A450 nm – A690 nm) was above 0.2 A450 nm – A690 nm units, we concluded that the EGFP-transfected cells and untransfected human MSCs remained telomerase-negative but the hTERT-transfected cells showed robust telomerase activity.

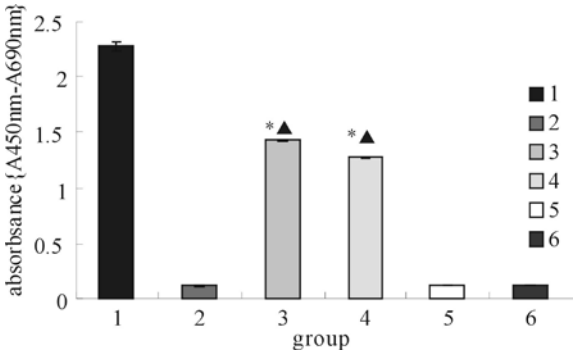

Figure 3. Comparison of telomerase activity in transfected and untransfected MSCs. 1: positive control; 2: negative control; 3: the hTERT-transfected MSCs at passage 5; 4: the hTERT-transfected MSCs at passage 30; 5: the pEGFP-C1-transfected MSCs at passage 5; 6: the untransfected MSCs at passage 10. *: *vs.* group 2, 5, 6, $P < 0.05$; ▲: *vs.* group 2, 5, 6; $P < 0.05$.

4 Discussion

The activity of telomerase is decided by the expression of hTERT[14], and telomerase was important in the immortalization of cells. In this research, we transfected hTERT gene into hMSCs, activated telomerase, maintained the telomere length, and finally got in the immortalization of cells[15–19].

Reverse transcriptase PCR was used to detect whether the exogenous hTERT gene was ectopic expressed in hMSCs. It indicated that the expression of hTERT mRNA was positive in hTERT-transfected hMSCs but negative in EGFP-transfected and untransfected hMSCs,

which demonstated the integration and expression of the exogenous hTERT gene in hTERT-transfected cells.

Telomerase activity was examined by TRAP (PCR)-ELISA to determine whether the exogenous hTERT gene affected the activity of hMSCs[20,21]. It showed that telomerase activity was positive in hTERT-transfected hMSCs but negative in EGFP-transfected and untransfected hMSCs. Together with the results of PCR, it can be concluded that the exogenous expression of hTERT gene could induce the activity of hMSCs.

Five cell colonies of hTERT-transfected were obtained by drug selection (G418). Among the five cell colonies, one colony had been cultured for nearly 1 year and had undergone 35 generations, meanwhile, the parallel groups, both the untransfected and transfected with a control plasmid senesced and died. As shown in our report, functional expression of hTERT in hMSCs with reconstitution of telomerase activity is sufficient to prolong their proliferative life span. Whether the cells can immortalize depends on the future research.

As mentioned above, the aim of this study was to establish an immortal human MSCs line without losing multipotent differentiation capacity. So EGF and bFGF[22] will be used to induced telomerase-positive human MSCs in the following study to assess if they maintain their multipotent differentiation capacity.

5 Conclusion

Ectopic expression of the hTERT gene in human MSCs can reconstitute their telomerase activity and extend their replicative life-spans. This study provides a new approach for obtaining unlimited quantities of normal phenotypic and homogeneous human MSC *in vitro.*

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