Saudi Journal of Biological Sciences (2017) 24, 519–525

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Derivation and characterization of sheep bone marrow-derived mesenchymal stem cells induced with telomerase reverse transcriptase

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Received 3 November 2016; revised 23 December 2016; accepted 6 January 2017
Available online 23 January 2017

KEYWORDS

Sheep BMSCs;
Stem cells;
TERT;
Multi-directional differentiation

Abstract Bone marrow mesenchymal stem cells (BMSCs) are a type of adult stem cells with a wide range of potential applications. However, BMSCs have a limited life cycle under normal culturing conditions, which has hindered further study and application. Many studies have confirmed that cells modified by telomerase reverse transcriptase (TERT) can maintain the ability to proliferate *in vitro* over a long period of time. In this study, we constructed a gene expression vector to transfer TERT into sheep BMSCs, and evaluated whether the TERT cell strain was successfully transferred. The abilities of cell proliferation and differentiation were evaluated using the methods including growth curve determination, inheritance stability analysis, multi-directional induction and so on, and the results showed that the cell strain can be cultured to 40 generations, with a normal karyotype rate maintained at 88.24%, and that the cell strain can be transferred and differentiated into neurocytes and lipocytes, proving that it retains the multi-directional transdifferentiation ability.

1. Introduction

The bone marrow mesenchymal stem cell (BMSC) is a kind of multi-potent adult stem cell originating from the bone marrow stromal, and is a type of adult stem cell with a wide range of potential applications in the fields of tissue engineering, and cell and gene therapy (Augello et al., 2010; Austin-Page et al., 2010; Dai et al., 2014; Machado et al., 2009; Nakahara et al., 2009; Tögel et al., 2009; Yang et al., 2011). In the present study, we found that the ability to proliferate decreases along with the number of *in vitro* passage cultures in BMSCs, which limits the application of BMSCs to a certain extent (Bonab et al., 2006; Estrada et al., 2013). In recent years, different kinds of immortalized cells have been obtained by different methods, but there is no safe way to obtain immortalized cells.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2017.01.022
1319-562X

Telomere is an important structure in maintaining chromosome stability and the life span of cells. Telomere length is inversely proportional to the number of chromosome copies. If the telomere length decreases to an extreme value, it will no longer maintain its function of ensuring chromosome stability, which leads to cell death However, telomere contains a reverse transcriptase known as telomerase reverse transcriptase (TERT), which can catalyze reverse transcription of the telomerase into telomere DNA, which is then synthesized into chromosome ends and added to the length of the telomere, thus resulting in continuous cell growth (Kim et al., 2009). Many studies show that exogenous telomerase reverse transcriptase does not produce canceration, and can maintain stem cell self-renewal and multilineage differentiation potential. Therefore, it is of great theoretical significance to study the effect of TERT on the stable passage and differentiation of MSCs.

Therefore, through introduction of the exogenously expressed TERT gene, we further studied the life cycle and biological characteristics of BMSCs as a basis for further application of mesenchymal stems cells in disease treatment and tissue repair technology.

2. Materials and methods

All chemicals and culture media used in this study were of cell culture grade and obtained from Sigma Chemicals Co., (St. Louis, US) unless otherwise indicated. The plastic ware was from Nunc (Roskilde, Denmark).

2.1. Tissue materials and cell culture

Sheep renal tissue was harvested from 12 month old small-tailed Hen sheep which were provided by a slaughterhouse. BMSCs were provided by the Experimental Center of the College of Animal Science and Technology. Cells were inoculated at a density of 2×10^4 cells/ml in DMEM containing 10% FBS, and cultured at 37 °C in a 5% CO_2 humidified incubator after thawing at 37 °C. The culture medium was replaced after 24 h, and every 3 days afterward. When cells had grown to a fusion of 80-90%, subculturing of the cells was performed at a ratio of 1:3 with digestion by 0.25% trypsin.

2.2. Construction of eukaryotic expression vector pcDNA 3.1-EGFP-TERT

Total RNA was extracted from the sheep renal tissue, and reversely transcribed into cDNA which was used as a template. A TERT primer was designed containing the Hind III and EcoR I restriction enzyme cutting site, Fwd: CCCAAGCTTGCCAC CATGA AGGTGCAGGACTGCG (Hind III), Rev: CGGAATTCTG TCCAAGATGGTCTTGAAGTCT (EcoR I). PCR amplification conditions: 94 °C, 8 min; 94 °C, 40 s; 56 °C, 30 s; 72 °C, 2 min; 35 cycles. The amplified bands were extracted and sequenced after the reaction was terminated. The recombinant plasmid containing the TERT gene and the plasmid pcDNA3.1-EGFP were cut by Hind III and EcoRI restriction enzymes, respectively, and the enzyme fragments were added into T4 DNA ligase to perform the overnight ligation. Double enzyme cutting and sequence identification of the fragments ligated by Hind III and EcoR I were carried out.

2.3. Liposome transfection and Screening of TERT-BMSCs

0.8 μg of normally sequenced plasmids was mixed with 3 μl of liposome in 100 μl of serum-free DMEM culture medium. Then, the mixture was slowly added into a culture containing 70–80% fused cells after 20 min of incubation at room temperature (RT). After the cells were transfected for 24 h, fluorescence was observed under a fluorescence microscope, and the cells were screened by adding G418 with a final concentration of 300 μg/ml. After 7 d, the G418 concentration was reduced by half and cells continued culturing.

2.4. Determination of growth curve

P5 and P40 TERT-BMSCs as well as BMSCs were selected and inoculated at a concentration of 2×10^4 in 24-well plates. The growth curve was determined by calculating the number of cells in 3 wells per day for 9 consecutive days.

2.5. Inheritance stability analysis

Numerous metaphase cells were selected from P10, P20 and P40 TERT-BMSCs and BMSCs. Then, using BEION chromosome karyotype analysis software, the number of chromosomes was analyzed, and the chromosome number and structural stability of the TERT-BMSCs during subculturing was measured.

2.6. RT-PCR analysis

Total RNA was extracted and reversely transcribed into cDNA for use as a template. A primer for the study gene was designed (Table 1). The targeted band was amplified by PCR, and the amplified band was extracted and sequenced after the reaction was terminated.

2.7. Multi-directional induction and differentiation

The P30 TERT-BMSCs were selected and inoculated at 2×10^5 cells/ml in 4-well plates. The culture medium supernatant was discarded and replaced with an adipogenic induction culture medium (DMEM-F12 + 10% FBS + 1 μM of dexamethasone + 17 μM of pantothenic acid + 5 mM of indometacin + 1 μM of insulin + 0.5 mM of IBMX) when cells had grown to a fusion of 70–80%, and the culture medium was replaced every 3 d. Cells were cultured for two weeks. For neuroblast induction, the pre-induction medium (DMEM-F12 + 10%FBS + 1 mM BME) was first added, and was then replaced with induction medium (DMEM-F12 + 5 mM BME) after 12 h of induction. The induction continued for 24 h, and changes were observed under a microscope.

2.8. Identification of induction differentiation

Identification of adipogenic induction: The culture medium was discarded after two weeks of cell induction. Cells were then rinsed three times with PBS, and then rinsed three times with distilled water after 20 min of fixation with 10% formaldehyde, then stained with Oil-Red O for 20 min at RT. The results were observed under a microscope. RT-PCR