Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Construction and expression of retroviral vector pLEGFP-N1-TERT in preparation of seed cells for skin tissue engineering

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ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 15 October 2013
Accepted 15 November 2013
Available online 20 December 2013

Keywords:
Telomerase reverse transcriptase
Retroviral vector
Hypodermal cells

ABSTRACT

Objective: To construct the retroviral vector pLEGFP-N1-telomerase reverse transcriptase (TERT) and to investigate the expression of TERT in neonatal mouse hypodermal cells. **Methods:** The polymerase chain reaction (PCR)-amplified *TERT* gene was inserted into plasmid pLEGFP-N1. The positive clone was identified by restriction enzyme digestion and sequencing, then was transfected into packaging cells to produce retrovirus particles. Neonatal mouse hypodermal cells were infected with the virus to generate a stable cell line. The *TERT* mRNA expression level, telomerase activity, and enhanced green fluorescent protein (EGFP) expression level were analyzed. **Results:** Retroviral vector pLEGFP-N1-TERT was constructed successfully, and a stable cell line of neonatal mouse hypodermal cells expressing EGFP was established. Western blot and immunohistochemical assay showed that the expression level of TERT was significantly elevated in the neonatal mouse hypodermal cells. **Conclusions:** A high titer of retrovirus pLEGFP-N1-TERT mediates high-level expression of the exogenous *TERT* gene in the neonatal mouse hypodermal cells. This protocol has potential applications for skin tissue engineering and cell transplantation therapy.

1. Introduction

Telomerase is a reverse transcriptase that uses RNA as a template to synthesize DNA repeats in order to maintain telomere length[1-3]. Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase, and the TERT expression level has a strong correlation with the activity of telomerase[4-7]. Introduction of the TERT gene into transfected cells leads to stable expression of telomerase, maintenance of chromosomal integrity, and long-term sustainability of cell growth[8-10]. We constructed the retroviral vector pLEGFP-N1-TERT and transfected 293FT packaging cells with the vector by calcium phosphate coprecipitation to obtain high-titer virus. Neonatal mouse hypodermal cells were infected with the virus and screened for positive clones showing stable expression of TERT and green fluorescence protein under a fluorescent microscope. This primary study provides the foundation for a potential new method of labeling seed cells for skin tissue engineering.

2. Materials and methods

2.1. Reagents

All animal experiments were carried out according to

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Foundation project: It is supported by National Natural Science Foundation of China (No 31170949), Natural Science Foundation of Fujian Province (No. 2012J05059).

the guidelines of the Institutional Animal Care and Use Committee. Neonatal C57BL/6 mice were provided by the Experimental Animal Center of Southern Medical University. Plasmid pLEGFP-N1, packaging plasmid PIK, competent cell line STBL3, and packaging cell line 293FT were purchased from Wuhan Bozhou Biotechnology Co., Ltd., China. Type I collagenase and dispase were from Gibco, USA. DNA fragment purification kit, agarose gel DNA purification kit, MiniBEST Plasmid Purification Kit, NucleoBond Plasmid Maxi Kit, *Hind* Ⅲ, *Sal* Ⅰ, *Bam*H Ⅰ, T4 DNA ligase, and DNA ligation kit were from TaKaRa, Japan. Lipofectamine 2000 was from Invitrogen, USA. Anti-TERT antibody was from Epitomics, USA. Primers were synthesized by Invitrogen Co., Shanghai, China.

2.2. Primer design for gene cloning

Primers were designed on the basis of pLEGFP-N1 expression vector map (Figure 1). The sequence of the *TERT* gene was obtained from NCBI (NM: 009354), and primers were designed as follows:

TERT-F: 5′ CCCAAGCTTATGACCCGCGCTCCTCGTTG 3′;

TERT-R: 5′ ACGCGTCGACCGGTCCAAAATGGTCTGAAAGTC TGT 3′.

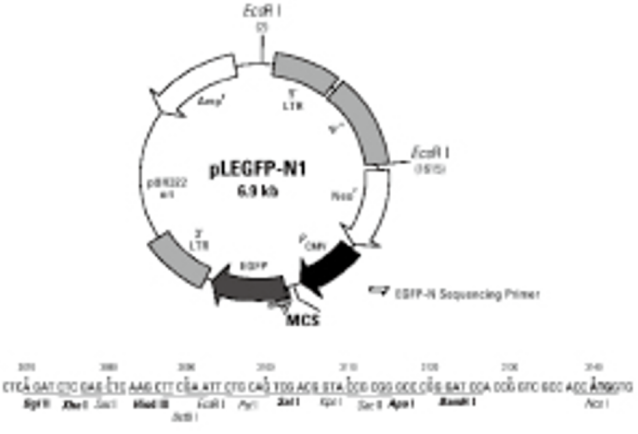

Figure 1. Map of expression vector pLEGFP-N1.

2.3. Construction of retroviral vector pLEGFP-N1-TERT

Total RNA was isolated from livers of neonatal mice, cDNA was synthesized, and the PCR product was detected by agarose gel electrophoresis. Subsequently, 3 369 bp DNA fragments were recovered from the gel. These fragments and the pLEGFP-N1 vector were digested with *Hind* Ⅲ and *Sal* Ⅰ and then ligated with T4 DNA ligase to generate pLEGFP-N1-TERT. The ligation product was transformed into STBL3 competent cells. Colonies were identified by colony PCR and restriction enzyme digestion and finally confirmed by DNA sequencing of double strands (Invitrogen Co., Shanghai, China). Positive colonies were cultured in Lysogeny broth, and plasmids were purified and verified by *Bam*H Ⅰ digestion.

2.4. Retrovirus packaging

Plasmids pLEGFP-N1 (used as a control) and pLEGFP-N1-TERT were transformed into separate STBL3 cell cultures. Amplified recombinant plasmids were extracted by CsCl density gradient centrifugation and co-precipitated with calcium phosphate for transfection into 293FT packaging cells.

2.5. Isolation and culture of hypodermal cells

Skin from the backs of newborn C57 mice was dissociated, cut into 0.5 cm ×0.5 cm tissue masses, and digested with 0.1% neutral protease overnight at 4 ℃. Derma and epiderma were separated with dissecting forceps. Derma were cut into pieces, digested with 0.2% collagenase at 37 ℃ for 30 min, and then filtrated through a 200-mesh sieve. Single cells were collected and cultured in Dulbecco′s modified Eagle′s medium, a high glucose complete medium, containing 10% fetal bovine serum. The cells after 3 passages were used for subsequent experiments.

2.6. Titer determination and retroviral infection

Supernatants containing pLEGFP-N1- or pLEGFP-N1-TERT-carrying retroviruses were filtered by using 0.45-μm filters. Neonatal mouse hypodermal cells were infected with retrovirus plus polybrene (2 μg/mL). The transfected cells were selected by resistance to the antibiotic G418, and stable cell lines were subcultured after a 7-day selection. Retrovirus titer was calculated by using the following formula: titer (PFU/mL) = number of GFP positive cells × dilution factor/0.01 mL.

2.7. Expression of TERT in neonatal mouse hypodermal cells

After infection, stable cell lines were selected for resistance to G418. Fluorescence in cells was observed under a fluorescence microscope. Western blot and immunohistochemical assay were performed to test the expression levels of TERT protein. The primary antibody was diluted 1:100.

3. Results

3.1. Identification of construct

Analysis of PCR products by agarose gel electrophoresis showed a 3 369 bp DNA fragment, which was consistent with the size of the target gene (Figure 2).

Extracted plasmid digested with *Bam*H Ⅰ produced a fragment of approximately 1 800 bp (Figure 3), indicating that TERT has been inserted into the pLEGFP-N1 vector. DNA sequencing verified that the sequence was correct.

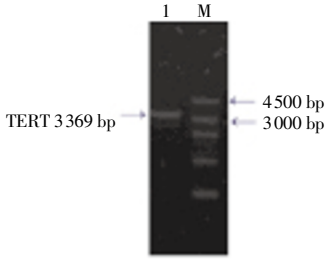

Figure 2. Electrophoresis of PCR-amplified *TERT* fragment.
Lane 1, TERT fragment (3 369 bp), indicating successful amplification of the target sequence, and lane M, Marker (bands from top to bottom are 4 500 bp, 3 000 bp, 2 250 bp, 1 500 bp, and 1 000 bp).

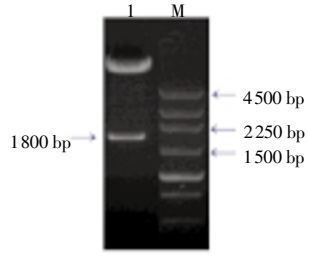

Figure 3. Identification of recombinant plasmid pLEGFP-N1-TERT by *Bam*H Ⅰ digestion.
Lane 1, the shorter fragment of approximately 1 800 bp is of the expected size, and lane M, Marker (bands from top to bottom are 4 500 bp, 3 000 bp, 2 250 bp, 1500 bp, and 1 000 bp).

3.2. Exogenous expression of TERT in mouse hypodermal cells infected with retrovirus

According to our calculations, the titer of retrovirus was 1.0×10^9 PFU/mL. Cells stably expressing GFP attached within 24 h after split. The cells first appeared round and non-uniform in size, with high nuclear/cytoplasmic ratios. The cells then gradually extended to be short spindle-shaped, polygonal, or spindle-shaped. Green fluorescence was observed under a fluorescent microscope (Figure 4). Seven days after split, the primary cultured cells reached 70%-80% confluency.

Expression of TERT was detected by western blot and immunohistochemical analysis, both of which showed significantly increased expression of TERT after infection (Figures 5 and 6, respectively).

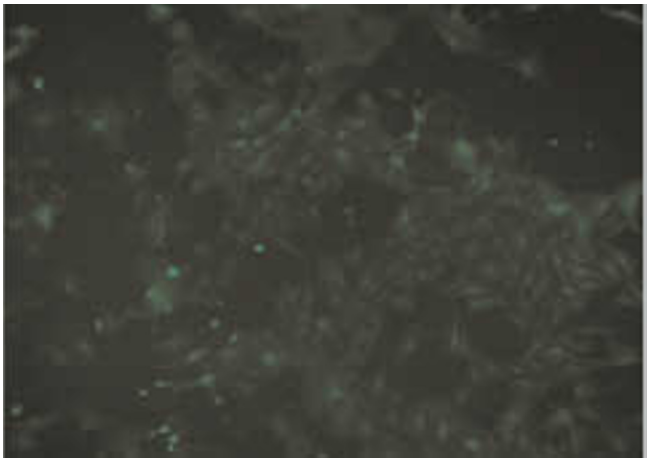

Figure 4. Expression of EGFP in hypodermal cells 4 days after infection with retrovirus (100×).

Figure 5. Comparison of TERT expression levels between pLEGFP-N1- and pLEGFP-N1-TERT-infected cells by western blot.
pLEGFP-N1 (left) and pLEGFP-N1-TERT (right).

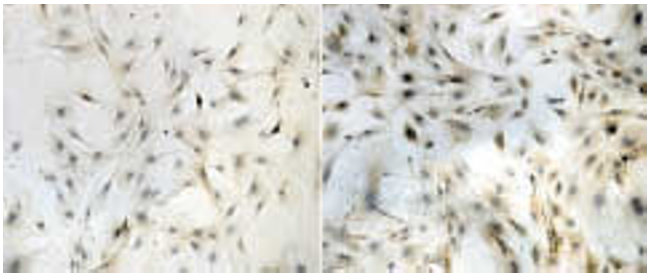

Figure 6. Comparison of TERT expression levels between pLEGFP-N1- and pLEGFP-N1-TERT-infected cells by immunohistochemical assay.
pLEGFP-N1 (left) and pLEGFP-N1-TERT (right) (200×).

4. Discussion

The seed cell is the most basic and most important part of tissue engineering. Adult cells usually reach senescence and lose normal cell functions after several passages under culture conditions. This restricts the application of adult

cells as the source of seed cells[11-15]. Homayoun et al introduced TERT into human fibroblasts, which activated the expression of telomerase and extended the telomere length and cell lifespan, but did not affect the growth, differentiation, and other biological characteristics of cells[16-20]. These findings suggested that immortalized cells can be used as seed cells for tissue engineering.

In this study, we successfully constructed the retroviral vector pLEGFP-N1-TERT. After transfection into 293FT cells, virus with a titer of up to 1.0×10^9 PFU/mL was produced. After infection of mouse hypodermal cells, expression of TERT increased significantly, and expression of green fluorescent protein facilitated cell labeling. This will not only meet the requirements of long-term and large-scale expansion of seed cells, but will also facilitate cell tracking and detection of the results of tissue engineering. Overall, our study provides a potential new method for producing and tracking seed cells for skin tissue engineering.

Conflict of interest statement

We declare that we have no conflict of interest.

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