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Construction of retroviral vector pLEGFP-N1-TERT for skin tissue engineering

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Abstract: Background: The seed cell is the most basic and important factor of tissue engineering. **Objective:** To construct the retroviral telomerase reverse transcriptase (TERT) and investigate the expression of TERT in neonatal mouse hypodermal cells. **Methods:** The TERT gene amplified by polymerase chain reaction (PCR) was inserted into plasmid pLEGFP-N1. The positive clone identified by restriction enzyme digestion and sequencing 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 expression of TERT mRNA and enhanced green fluorescent protein (EGFP) was detected by western blot. **Results:** Retroviral vector pLEGFP-N1-TERT was constructed successfully, and a stable cell line of neonatal mouse hypodermal cells which express EGFP was established. Western blot and immunohistochemical assay showed that the expression level of TERT was significantly elevated in the neonatal mouse hypodermal cells transfection. **Conclusion:** A high titer of retrovirus TERT mediates high-level expression of the exogenous TERT gene in the neonatal mouse hypodermal cells. Our study has important significance for skin tissue engineering.
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Keywords: telomerase reverse transcriptase; retroviral vector; hypodermal cells

1. Introduction

In order to maintain telomere length, telomerase, a reverse transcriptase uses RNA as a template to synthesize DNA repeats(Robertson et al., 2005; Counter 1996; Steinert et al., 2000). Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase, and the TERT expression level has a strong correlation with the activity of telomerase (Steinert et al., 2000). Transfection of the TERT gene into cells would lead to stable expression of telomerase, maintenance of chromosomal integrity, and long-term sustainability of cell growth (Wang et al., 2000). In this study, we constructed the retroviral vector pLEGFP-N1-TERT, transfected 293FT packaging cells with the vector, and used calcium phosphate coprecipitation to obtain high-titer virus. Neonatal mouse hypodermal cells showed stable expression of TERT after infection with virus. Our study provides a potential strategy for skin tissue engineering.

2. Materials and methods

2.1 Reagents

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. 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′-ACGCGTCGACCGGTCCAAAATGGTCTGAAA GTCTGT-3 ′.

2.3 Construction of retrovirus

Total RNA was isolated from liver of neonatal mice, RNA was reverse transcribed into cDNA, and the PCR product was detected by agarose gel electrophoresis. 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 vector. Then the vector was transformed into STBL3 competent cells. Colonies were identified by colony PCR and restriction enzyme digestion, and finally confirmed by DNA sequencing (Invitrogen Co., Shanghai, China). Positive colonies were cultured in Lysogeny broth (LB) medium, and plasmids were purified and verified by *Bam*H I digestion.

2.4 Retrovirus purification and packaging

The control plasmids pLEGFP-N1 and pLEGFP-N1-TERT were transfected into separate STBL3 cells. Amplified recombinant plasmids were purified by CsCl density gradient centrifugation and co-precipitated with calcium phosphate to transfect into 293FT packaging cells.

2.5 Isolation and culture of hypodermal cells

Neonatal C57BL/6 mice were provided by the Experimental Animal Center of Southern Medical University. The back skin of newborn C57BL/6 mice was dissociated, cut into 0.5 cm × 0.5 cm tissue mass, and digested with 0.1% neutral protease overnight at 4°C. Derma and epidermis were separated with dissecting forceps. Derma were cut into pieces, digested with 0.2% collagenase at 37°C for 30 min, and then filtrated through a 200-mesh sieve. Single cells were collected and cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep solution. The cells more than 3 passages were used for subsequent experiments. All animal experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee.

2.6 Retroviral infection and titer determination

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

2.7 Expression of TERT in hypodermal cells

After selection, the green 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 pLEGFP-N1-TERT

Analysis of PCR products by agarose gel electrophoresis showed a 3369 bp DNA fragment, which was consistent with the size of the target gene (Figure 1). Extracted plasmid which was digested with *Bam*H I produced a fragment of approximately 1800 bp (Figure 2), indicating that TERT has been inserted into the pLEGFP-N1 vector. DNA sequencing confirmed that the construction was successful.

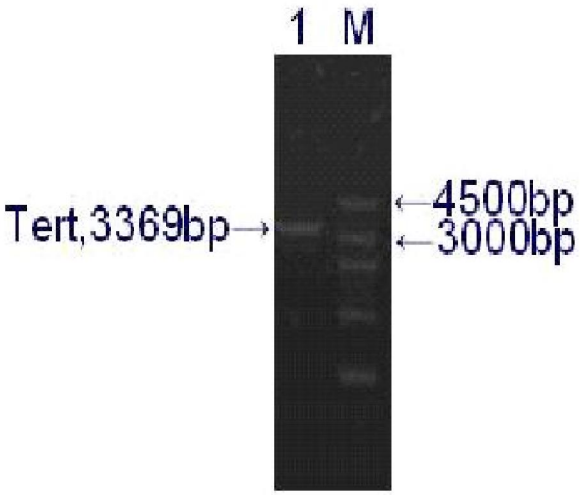

Figure 1. Electrophoresis of PCR-amplified TERT fragment. Lane 1, TERT fragment (3369 bp), indicating successful amplification of the target sequence, and lane M, Marker (bands from top to bottom are 4500 bp, 3000 bp, 2250 bp, 1500 bp, and 1000 bp).

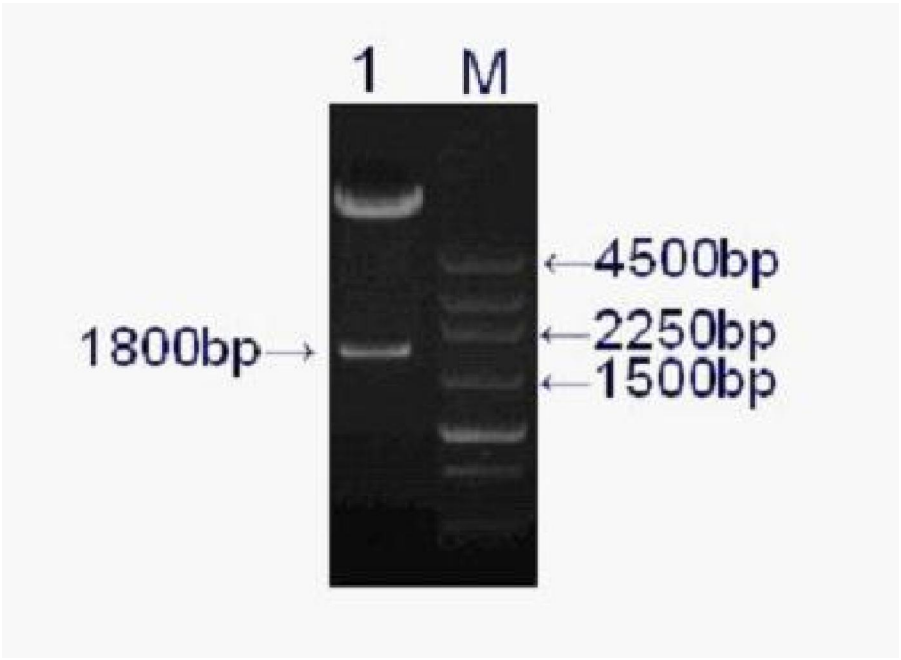

Figure 2. Identification of recombinant plasmid pLEGFP-N1- TERT by *Bam*H I digestion. Lane 1, the shorter fragment of approximately 1800 bp is of the expected size, and lane M, Marker (bands from top to bottom are 4500 bp, 3000 bp, 2250 bp, 1500 bp, and 1000 bp).

3.2 Exogenous expression of TERT in hypodermal cells

The titer of retrovirus was 1.0×10^9 PFU/mL. Firstly, the cells which could stably express GFP appeared round and non-uniform in size, with high nucleus/cytoplasm ratios. Then cells gradually extended to be short spindle-shaped and polygonal, or spindle-shaped. Green fluorescence was observed under a fluorescent microscope (Figure 3). Seven days after split, the primary cultured cells reached 70%– 80% confluence.

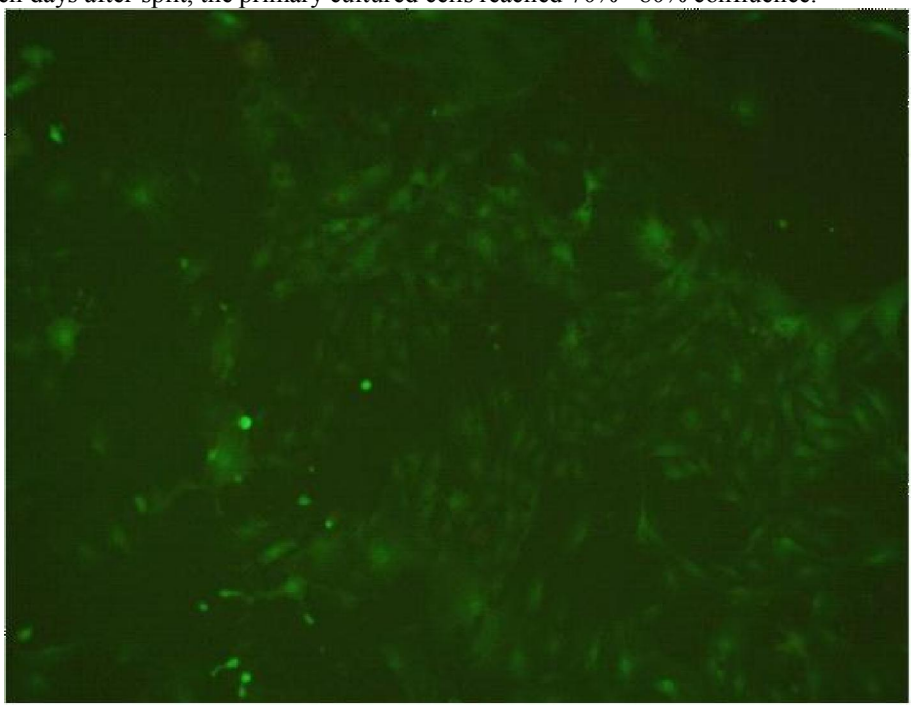

Figure 3. The expression of EGFP in hypodermal cells 4 days after infection with retrovirus (100×).

Expression of TERT was detected by western blot and immunohistochemical analysis, both of which showed significantly increased expression of TERT after infection (Figures 4 and 5, respectively).

Figure 4. Comparison of TERT expression levels between pLEGFP-N1- (left) and pLEGFP-N1-TERT-infected cells (right) by western blot.

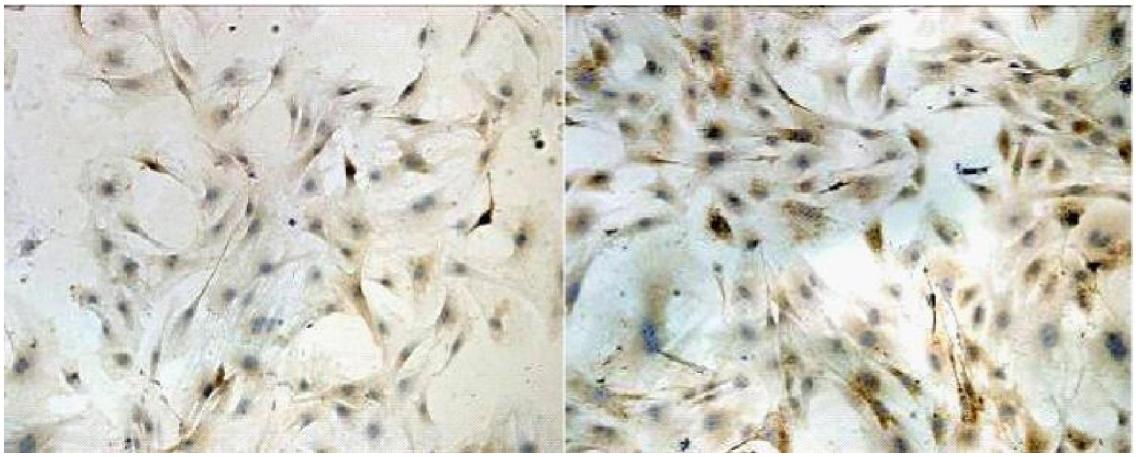

Figure 5. Comparison of TERT expression levels between pLEGFP-N1- (left) and pLEGFP-N1-TERT-infected cells (right) by immunohistochemical assay (200×).

4. Discussion

The seed cell is the most basic and important factor of tissue engineering. Adult cells usually appear senescence and lose normal cell functions after several passages under culture conditions, which restricted the application of adult cells as the source of seed cells(Artandi and DePinho 2010). Vaziri *et al* (1998) 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 (Kang and Park 2007; Utikal et al., 2009). 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 transfecting into 293FT cells, virus with a titer of up to 1.0×10^9 PFU/mL was produced. After infection of mouse hypodermal cells, green fluorescent could be observed in cells, and the expression of TERT was increased significantly. This strategy meets the requirements of long-term and large-scale expansion of seed cells, but also facilitates cell tracking and detection of the results in tissue engineering. Overall, our study provides a potential method for producing and tracking seed cells for skin tissue engineering.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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