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Reversible immortalization of rat pancreatic β cells with a novel immortalizing and tamoxifen-mediated self-recombination tricistronic vector

Hui-Ling Wu[a], Yu Wang[b], Ping Zhang[c], Shu-Fa Li[a], Xiuping Chen[d], Yao-Kai Chen[e], Jun-Gang Li[e], Shi-Ming Yang[f], Yong-Ping Su[g], Jun-Ping Wang[g], Bing Chen[a,*]

[a] Department of Endocrinology, Southwest Hospital, Third Military Medical University, No. 29 Gaotanyan Street, Chongqing 400038, China
[b] Department of Orthopaedics, Research Institute of Surgery, Daping Hospital, Third Military Medical University, Chongqing 400042, China
[c] Department of Ultrasound, the Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China
[d] Institute of Chinese Medical Sciences, University of Macau, China
[e] Department of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
[f] Department of Gastroenterology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
[g] State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Combined Injury, College of Preventive Medicine, Third Military Medical University, Chongqing 400038, China

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ABSTRACT

Although the strategy of "Cre/LoxP-based reversible immortalization" holds great promise to overcome the cellular senescence of primary cell cultures for their further use, a secondary gene transfer for Cre expression is usually utilized to trigger the excision of the immortalizing genes in a large number of cells, thus presenting a formidable hurdle for large-scale application. We modified the strategy by utilizing a tricistronic retroviral vector pLCRSTP, in which Cre-ER, simian virus 40 large T antigen (SV40LTAg) oncogene, and a reporter gene were flanked by the same pair of LoxA sites. Five immortalized rat pancreatic β cell clones transduced with pLCRSTP, and six immortalized rat pancreatic β cell clones co-transduced with pLCRSTP and another vector encoding the human telomerase reverse transcriptase (hTERT) gene, were obtained, respectively. The Cre-ER protein could be induced to translocate from the cytoplasm to the nucleus by 4-hydroxytamoxifen to make SV40LTAg, hTERT and the Cre-ER gene itself excise without a secondary gene transfer. Our studies suggest that this system is useful to expand rat β cells and may allow for large-scale production due to its simpler manipulation.

1. Introduction

Many fundamental *in vitro* studies on primary pancreatic β cells are hampered by the limited life span and the poor replicative ability of β cells. Also, cell-based therapy using islet transplantation, a potentially promising therapy to cure diabetes (Fiorina and Secchi, 2007; Marzorati et al., 2007; Meloche, 2007), is hindered by the very important constraint of the critical paucity of pancreatic β cells. A number of alternative approaches have been pursued for the generation of insulin-producing cell lines using gene therapy (Limbert et al., 2008), stem cells (Baetge, 2008; Moritoh et al., 2003), and the expansion of primary β cells with growth factors (Schuit and Drucker, 2008; Vasavada et al., 2006). However, these approaches are limited by cellular senescence and the partial loss of differentiated function. The transfer of specific oncogenes into primary cells can enable the generation of cell populations, a process known as cell immortalization (Counter et al., 1992; Kobayashi et al., 2000; Matsumura et al., 2004; Totsugawa et al., 2007). Primary cells that are transduced by simian virus 40 large T antigen (SV40LTAg) have a higher replicative ability than their parent cells. Although few cell lines transduced by SV40LTAg alone become truly immortalized, cells at early stages of immortalization may provide sufficient populations for use. SV40LTAg has been proven to be an efficient immortalizing gene for some types of cells (Kobayashi et al., 2000; Noguchi et al., 2002; Ying et al., 2007). However, co-expression of SV40LTAg and human telomerase reverse transcriptase (hTERT) has been utilized to enhance the immortalization potential of other types of cells (Matsumura et al., 2004). For pancreatic β cells, the transduction of hTERT alone was insufficient to immortalize them (Halvorsen et al., 2000). According to this, we tried to immortalize rat pancreatic β cells with two strategies (SV40LTAg vs. SV40LTAg and hTERT).

A number of concerns have been raised regarding the safety and the differentiated function of the transfected cells because of the integration of SV40LTAg or hTERT into the genome. To recover the functions of the cells and remove the potential tumorigenic

* Corresponding author. Tel.: +86 23 68754138; fax: +86 23 68754138.
E-mail address: chenbing3@medmail.com.cn (B. Chen).

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dangers of the immortalizing genes, a "Cre/LoxP-based reversible immortalization" procedure has been applied in a series of studies to obtain "reverted cells" (i.e., cells after the removal of the immortalizing genes) (Kobayashi et al., 2000; Totsugawa et al., 2007). However, the requirement for the expression of the Cre recombinase at the completion of the expansion phase in order to trigger the excision of the integrated immortalizing genes necessitates a secondary virus-mediated gene transfer in a large number of cells. An excessive selection of cells, whose cytotoxicity induces considerable cell loss, has to be undergone. This technical requirement has presented a formidable hurdle to making this approach practical for large-scale application. The use of a TAT-Cre recombinase fusion protein capable of penetrating cell membranes without the need for gene transfer, is also limited by its low recombination efficiency (Matsumura et al., 2004).

To address these issues, we modified the strategy of the reversible immortalization by utilizing a tricistronic retroviral vector pLCRSTP with the aid of two autocleavable foot-and-mouth disease virus 2A (FMDV-2A) sequences to connect the three target genes. An inducible recombinase gene, the Cre-ER gene, the SV40LTAg oncogene, and a reporter gene, a herpes simplex virus thymidine kinase (HSV-TK)/puromycin resistance fusion gene, were flanked by the same pair of LoxA DNA sequences. For our studies, we also extensively constructed a retroviral vector phTERT-I-GTKlox, expressing hTERT cDNA and an enhanced green fluorescent protein (EGFP)/HSV-TK fusion gene, flanked by a pair of loxB DNA sequences. Five immortalized rat pancreatic β cell clones transduced with pLCRSTP, LT-β (1–5), and six immortalized rat pancreatic β cell clones co-transduced with pLCRSTP and phTERT-I-GTKlox, LT-hTERT-β (1–6), were obtained, respectively. We demonstrated the feasibility of the immortalizing and tamoxifen-mediated self-recombination system in the LT-β cells and LT-hTERT-β cells. Here, we also preliminarily examined the differentiated function of the reverted β cells at the early stages of immortalization.

2. Materials and methods

2.1. Vector construction and virus production

The constructions were made according to standard techniques. The retroviral vectors, pLCRSTP and pBTGTKlox, both derived from the retroviral vector pBABE-puro (Geron Corp., Menlo Park, CA), were kindly provided by Dr. Yao-kai Chen. The wild type LoxP site (as LoxA) and the mutant LoxP site (as LoxB) were inserted in the NheI site of the 3′ long terminal repeat (LTR) U3 region of the pLCRSTP and the pBTGTKlox, respectively. The sequences of LoxA and LoxB sites are as follows, respectively: ATAACTTCGTATA ATGTAT**G**C TATACGAAGTTAT, ATAACTTCGTATA ATGTAT**A**C TATACGAAGTTAT. LoxA and LoxB differ by a G → A mutation in their spacer regions (Bouhassira et al., 1997; Hoess et al., 1986). The plasmid pGRN145 encoding the hTERT gene (GenBank accession no. AF015950) was provided by Geron Corp. The plasmid pIRES2-EGFP was kindly provided by Dr Tao Wang. The complete details of the construction steps for phTERT-I-GTKlox are as follows. The encephalomyocarditis virus internal ribosome entry site (IRES) amplified from pIRES2-EGFP was inserted into the EcoRI and SalI sites of the vector pBTGTKlox, obtaining a recombinant vector pI-GTKlox. The hTERT cDNA from the plasmid pGRN145 was inserted into the EcoRI site of the vector pI-GTKlox. The sense hTERT expression vector was our targeting vector, designated as phTERT-I-GTKlox. The Phoenix amphotropic packaging cell line, NIH3T3 cells, and the HeLa cell line were kindly provided by Dr. Zhe Wang, Dr. Nan Su, and Dr. Fang Chen, respectively. The stable viral producers were generated, and the viral supernatants were harvested and filtered as described previously (Danos and Mulligan, 1988; Pear et al., 1993). The virus titers were estimated by the infection and selection of NIH 3T3 cells using standard calculations as previously described (Danos and Mulligan, 1988; Pear et al., 1993).

2.2. Primary rat islet cell culture and transduction

Pancreatic islets were isolated from Sprague–Dawley rats weighing 200 g to 250 g by collagenase (Sigma, St. Louis, MO) infusion digestion, and purified with Ficoll400 (GE Healthcare, Uppsala, Sweden) density gradient centrifugation as previously described (Chen et al., 2007; Parnaud et al., 2008; Sutton et al., 1986), then assessed by dithizone (DTZ; Sigma, St. Louis, MO) staining and seeded in culture dishes in RPMI1640 medium containing 10% fetal calf serum, 10 mM HEPES, 500 ng/ml human growth hormone (GeneScience Pharmaceuticals Co., Ltd, Changchun, China), penicillin-G (100 U/ml), and streptomycin sulfate (100 μg/ml). After culturing for 3–4 days, the monolayer-cultured islet cells were infected with the pLCRSTP retroviral particles at multiplicity of infection (MOI) 10. Six days after the pLCRSTP infection, puromycin (Sigma, St. Louis, MO) selection (1.6 μg/ml) was applied and continued for two weeks. The monoclones were obtained by a limiting dilution and selected by DTZ staining. One of the DTZ-staining-positive monoclones was designated as LT-β1, cultured continuously, and chosen for further experiments. The LT-β1 cells at passage 4 (P4) were infected with the phTERT-I-GTKlox retroviral particles at an MOI of 0.5. Three days after the phTERT-I-GTKlox infection, the cells were cloned by a limiting dilution. GFP-positive cell monoclones were selected. One of them was designated as LT-hTERT-β1, cultured continuously, and chosen for further experiments. All of the procedures were approved by the institutional animal care and usage committee of the Southwest Hospital of Third Military Medical University.

2.3. Reversion of immortalized β cells

The culture dishes were treated with 1 μg/ml of 4-hydroxytamoxifen (4-OHT, Sigma, St. Louis, MO) for 10 days. Ganciclovir (Sigma, St. Louis, MO) treatment (5 μM) began 5 days after the 4-OHT treatment had been finished and continued for 10 days.

2.4. Detection of expression of Cre-ER, SV40LTAg, hTERT, EGFP in immortalized and reverted β cells

The integration and expression of Cre-ER, SV40LTAg, or/and hTERT genes were evaluated by PCR, RT-PCR, Western blot, and immunocytochemistry analyses. Genomic DNA was extracted from the immortalized and the reverted β cells for PCR analysis. Primary rat islets served as a negative control. RNA was isolated for RT-PCR analysis. β-actin was analyzed as an internal control. Primary rat islets and HeLa cells served as a negative and positive control, respectively. Touch-down PCR was used. The annealing temperature was gradually decreased in two degree increments from 64 °C to 52 °C with one cycle at each temperature. The final 20 cycles were completed at 50 °C. The primers used were: Cre-ER (458 bp for PCR and RT-PCR), sense strand, 5′-AATGCTTCTGTCCGTTTG-3′, antisense strand, 5′-GGATTAACATTCTCCCACC-3′; SV40LTAg (422 bp for PCR and RT-PCR), sense strand, 5′-CAGGCATAGAGTGTCTGC-3′, antisense strand, 5′-CAACAGCCTGTTGGCATATG-3′; hTERT (644 bp for PCR), sense strand, 5′-CCCGGACCTCCATCAGAGC-3′, antisense strand, 5′-CACCGGCCTTATTCCAAGC-3′; hTERT (362 bp for RT-PCR), sense strand, 5′-GTCCCTGGGACACGCCTTG-3′, antisense strand, 5′-CACAGAGCCCTGGGGCTTC-3′; β-actin (587 bp), sense strand, 5′-CTAAGGCCAACCGTGAAAAGATGAC-3′, antisense strand, 5′-TGGGTACATGGTGGTGCCACCAGAC-3′. To avoid the non-specific