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Cre/loxP-Based Reversible Immortalization of Human Hepatocytes[1]

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An ideal alternative to the primary human hepatocytes for hepatocyte transplantation would be to use a clonal cell line that grows economically in culture and exhibits the characteristics of differentiated, nontransformed hepatocytes following transplantation. The purpose of the present studies was to establish a reversibly immortalized human hepatocyte cell line. Human hepatocytes were immortalized with a retroviral vector SSR#69 expressing simian virus 40 large T antigen (SV40Tag) gene flanked by a pair of loxP recombination targets. One of the resulting clones, NKNT-3, showed morphological characteristics of liver parenchymal cells and expressed the genes of differentiated liver functions. NKNT-3 cells offered unlimited availability. After an adenoviral delivery of Cre recombinase and subsequent differential selection, efficient removal of SV40Tag from NKNT-3 cells was performed. Here we represent that elimination of the retrovirally transferred SV40Tag gene can be excised by adenovirus-mediated site-specific recombination.

Key words: Reversible immortalization; Human hepatocytes; Cre/loxP system; Simian virus 40 large T antigen

INTRODUCTION

Recent clinical reports have demonstrated that transplantation of hepatocytes can provide metabolic support for patients with acute liver failure and correct liver-based metabolic diseases (1,7). One of the major limitations of hepatocyte transplantation (HTX) is the present inability to isolate an adequate number of transplantable hepatocytes for clinical use. Only cadaver donor livers with traumatic damage, excess macrovesicular fat, and residual segments from reduced liver transplants can provide a source of hepatocytes for transplantation. A potential alternative source of liver cells for transplantation would be a clonal cell line. A hepatocyte cell line that could be engineered to revert from a transformed to a normal phenotype after transplantation would produce hepatocytes that could be grown in unlimited quantity and at far less cost than isolated hepatocytes. Cells derived from such a cell line would also offer the advantages of uniformity, unlimited availability, and freedom from infectious pathogens (3).

In the present work, we focused on a gene transfer strategy referred to as reversible immortalization, which allowed temporary expansion of primary cell populations by transfer of oncogenes that were subsequently excised (8). To test this feasibility, we utilized retroviral gene transfer of simian virus 40 large T antigen (SV40-Tag), flanked by a pair of loxP recombination targets, and subsequent adenovirus-mediated Cre/loxP recombination.

MATERIALS AND METHODS

Recombinant Retroviral Vector SSR#69

Ÿ Crip packaging cell line producing a recombinant retrovirus, SSR#69, provides a viral titer of 5×10^4 hygromycin-resistant colony-forming units (8). As shown in Figure 1, SSR#69 comprises the following from 5′ to 3′: (i) a long terminal repeat (LTR) with packaging signal, (ii) an initiation codon followed by a recombination target (loxP), whose overlapping open reading frame was fused to a hygromycin resistance (HygroR)/herpes simplex virus-thymidine kinase (HSV-TK) fusion gene; (iii) the encephalomyocarditis virus internal ribosomal entry site, which allows internal initiation of translation; (iv) the supertransforming U19 mutant of simian virus 40 large T antigen (SV40Tag); (v) the second recombination target in direct orientation followed in frame by the neomycin resistance (NeoR) gene, but, importantly, lacking an initiation codon; and (vi) another LTR pre-

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[1]A more comprehensive review of the work can be found in Science 287:1258–1262; 2000.

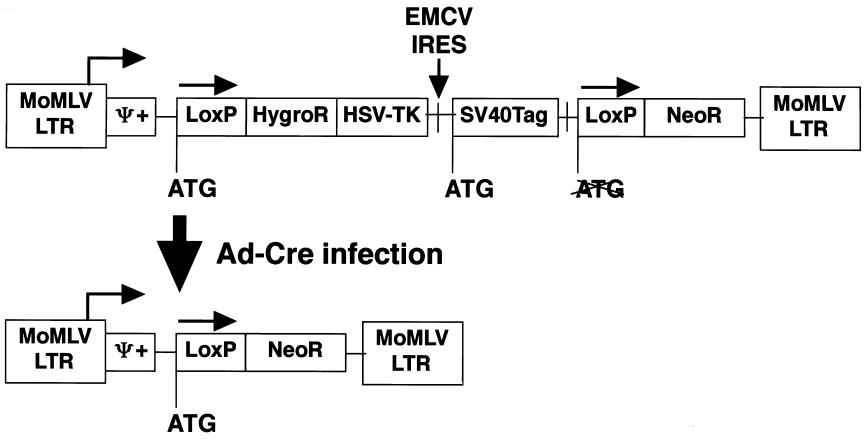

Figure 1. Schematic drawings of the strategy for reversible cell immortalization using the Cre/loxP system. The genes of HygroR/HSV-TK and SV40Tag are expressed in the transduced cells with SSR#69. After expression of Cre recombinase, the sequences flanked by a pair of loxP recombination targets are permanently excised from the genome of the transduced cells. LTR, long terminal repeat; SV40Tag, simian virus 40 large T antigen; HSV-TK, herpes simplex virus-thymidine kinase; HygroR, hygromycin resistance gene; NeoR, neomycin resistance gene; IRES, internal ribosomal entry site; ECMV, encephalomyocarditis virus.

ceded by its polypurine track. Only HygroR/HSV-TK and SV40Tag should be expressed in transduced cells in the absence of recombinase. Following Cre/loxP recombination, the intervening DNA segment between the two recombination targets would be excised.

Transduction of Human Hepatocytes With SSR#69

Human hepatocytes (purchase from Cell Systems Co., Seattle, WA) were immortalized with a retroviral vector SSR#69 expressing SV40Tag, as previously reported (4). One of the immortal clones, NKNT-3, was used for the present study. NKNT-3 cells were cultured with the chemically defined serum-free CS-C medium (purchased from Cell Systems Co.).

Infection Efficiency of a Recombinant Adenovirus Vector Expressing E. coli *lacZ Gene (Ad-lacZ) in NKNT-3 Cells*

Ad-lacZ (obtained from Riken Gene Bank, Ibaragi, Japan) was used to evaluate the efficacy of adenoviral gene delivery to NKNT-3 cells. Ad-lacZ expressed *E. coli* lacZ reporter gene under the control of CAG promoter consisting of cytomegalovirus IE enhancer, chicken β-actin promoter, and rabbit β-globin polyadenylation signal and provided a viral titer of 3.3×10^8 pfu/ml (2). NKNT-3 cells (2×10^6) were plated on six-well plates and infected with Ad-lacZ at the multiplicity of infection (MOI) 1, 5, 10, and 25. For cell staining, 24 h after infection of Ad-lacZ, cells were washed with PBS twice, fixed with 0.25% glutaraldehyde, and stained with 0.1% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). The cells expressing lacZ gene were identified using phase-contrast microscopy.

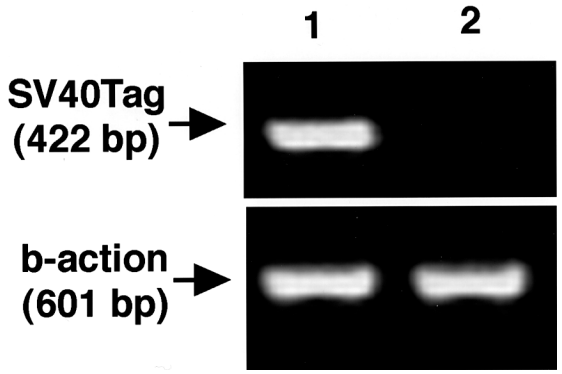

Figure 2. The expression of SV40Tag mRNA in NKNT-3 cells. We examined the expression of SV40Tag mRNA in NKNT-3 cells after infection of Ad-Cre at MOI 5 and following differential selection with G418 and ganciclovir. Elimination of SV40Tag gene was performed. The amounts of RNA used in the assay were verified by the detection of the human β-actin mRNA. The data were obtained from three independent experiments. Lanes are as follows: no Ad-Cre infection (lane 1), Ad-Cre at an MOI of 5 (lane 2).

Expression of SV40Tag mRNA in NKNT-3 Cells After Infection With a Recombinant Adenovirus Vector Expressing Nuclear Localizing Signal (NLS)-Tagged Cre Recombinase Gene (Ad-Cre) and Subsequent Differential Selection

An Ad-Cre (obtained from Riken Gene Bank, Ibaragi, Japan) produced a nuclear localization signal (NLS)-

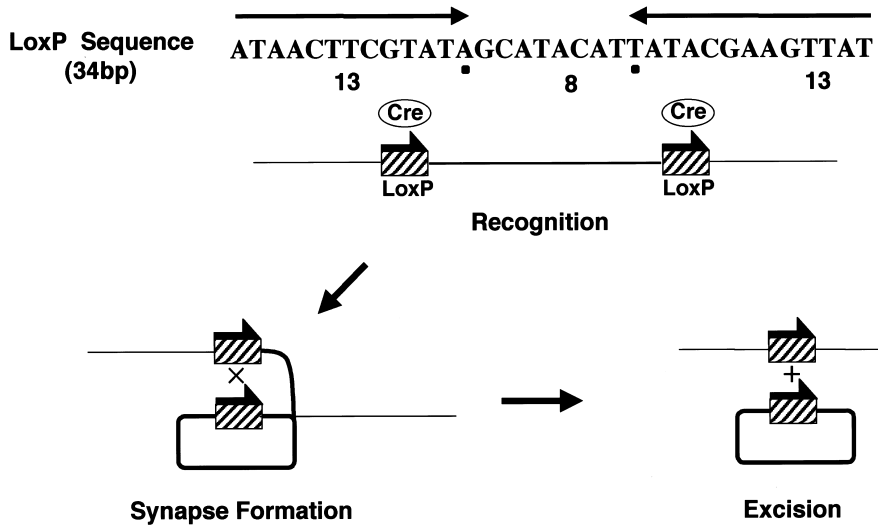

Figure 3. Schematic drawings of the Cre/loxP system. The Cre recombinase catalyzes site-specific recombination between two specific 34-base pair direct repeat called loxP. Binding of Cre to loxP results in the formation of a synapse between both loxP sites. The intervening sequences are permanently removed from the genome.

tagged Cre recombinase under the control of the CAG promoter and provided a viral titer of 3.3×10^8 pfu/ml (2). The samples of 6×10^6 NKNT-3 were inoculated on a T75-flask. One day after plating the cells were infected with Ad-Cre at an MOI of 5.Two days after Ad-Cre infection, the cells were maintained with the medium containing 100 μg/ml neomycin and 5 μM ganciclovir for 5 days and then the cells were subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for SV40Tag. Total RNA was isolated from the samples by RNAzol (Cinna/BioTecx, Friendswood, TX) in a single-step phenol extraction method and used as templates. Reverse transcription was performed at 22°C for 10 min and then 42°C for 20 min using 1.0 μg of RNA per reaction to ensure that the amount of amplified DNA was proportional to that of specific mRNA in the original samples. PCR was performed with specific primers in volumes of 50 μl containing 2.0 μg RT products according to the manufacture's protocol (PCR kit; Perkin-Elmer/Cetus, Norwalk, CT). The amplification reaction involved denaturation at 92°C for 1 min, annealing at 58°C for 1 min, and 72°C for 1 min using a thermal cycler (Perkin-Elmer, Foster City, CA). The PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. The human β-actin gene served as an internal control for the efficiency of mRNA isolation and cDNA synthesis. Primers used were as follows: SV40Tag (422 bp), 5′ primer: CAGGCATA GAGTGTCTGC, 3′ primer: CAACAGCCTGTTGGCA TATG; human β-actin (610 bp), 5′ primer: TGACG GGGTCACCCACACTGTGCCCATCTA, 3′ primer: CTAGAAGCATTTGCGGTGGACGATGGAGGG.

RESULTS

Establishment of an Immortalized Human Hepatocyte Cell Line

One of the human hepatocytes transduced with SSR#69, NKNT-3, displayed the morphological characteristics of liver parenchymal cells and showed the gene expression of differentiated liver functions (4).

Infection Efficiency of Ad-lacZ in NKNT-3 Cells

To examine the potential of Ad-lacZ infection, 2×10^6 NKNT-3 cells were stained with X-gal solution 2 days after the transduction of Ad-lacZ at MOI 1, 5, 10, and 25. Nearly 100% of the cells were stained by infection with Ad-lacZ at MOI ≥ 5.

Expression of SV40Tag mRNA in NKNT-3 Cells After Ad-Cre Transduction Followed by the Differentiated Selection

We examined the expression of SV40Tag mRNA in NKNT-3 cells 7 days after Ad-Cre infection at an MOI of 5 and subsequent selection of 100 μg/ml neomycin and 5 μM ganciclovir. As shown in Figure 2, the retrovirally transferred SV40Tag gene was removed by the treatment of Ad-Cre and following selection.

DISCUSSION

Several systems for site-specific recombination have been developed as tools for genetic engineering. These

systems include the Cre/loxP system from the bacteriophage P1, the FLP/FRT system from *Saccharomyces cerevisiae*, and the R/R5 system from *Xygosaccharomyces rouxii* (5). The comparison of the Cre/loxP system and FLP/FRT system has shown the clear superiority of Cre/loxP (5). The bacteriophage recombinase Cre catalyses site-specific recombination by recognizing a 34-base pair, unique sequence loxP (6). When two loxP sites are oriented as direct repeats, the intervening sequence can be spliced in eukaryotic cells on transient expression of Cre recombinase and are permanently removed from the cell genome, as shown in Figure 3. An adenoviral delivery of Cre recombinase has been reported to remove the DNA flanked by a pair of loxP from cell chromosomes of nearly 100% of the cultured cell population (2).

The shortage of donor livers for hepatocyte isolation limits the clinical application of hepatocyte transplantation (HTX). An attractive solution would be to use a cell line that could provide consistent and uniform cells in sufficient quantity and adequate quality. In an effort to make this idea clinically useful, we focused on Cre/loxP site-specific recombination. In this study, we investigated the efficacy of a reversible immortalization system in human hepatocytes by retroviral gene transfer of SV-40Tag and adenovirus-mediated Cre/loxP recombination (2,8).

We immortalized human hepatocytes with a retroviral vector SSR#69. One of the immortal clones, NKNT-3, showed the gene expression of differentiated liver functions and provided unlimited availability (4). After expanding the population of NKNT-3 cells in culture, a recombinant adenovirus producing Cre recombinase, Ad-Cre, was used for Cre/loxP recombination. RT-PCR analysis demonstrated that the SV40Tag gene transferred into NKNT-3 cells was efficiently excised by transduction with Ad-Cre at an MOI of 5 and subsequent selection of neomycin and ganciclovir. Then the reverted NKNT-3 cells lost the property of unlimited proliferation.

In conclusion, we demonstrate the feasibility of reversible immortalization of human hepatocytes, and this system could provide the need for immediate availability of consistent and functionally uniform cells.

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