Conditional removal of SV40T from transformed primary human hepatocytes recovers selective CYP gene expression

Remoção condicional de SV40T de hepatócitos humanos primários transformados recupera a expressão seletiva do gene CYP

Cong Liu[1,2], Jorge A. Bezerra[1]

Research performed at Divisions of [1]Gastroenterology, Hepatology and Nutrition and of [2]Experimental Hematology at Cincinnati Children's Hospital Medical Center and the Department of Pediatrics, University of Cincinnati, Cincinnati, Ohio, USA.
Conflict of interest: None.
Correspondence address: Jorge A. Bezerra: jorge.bezerra@cchmc.org; Cong Liu: cong.liu@cchmc.org
Submitted: July 31, 2011. Accepted, after review: August 22, 2011.

ABSTRACT

Purpose: Primary cultures of human hepatocytes are common in vitro systems to study hepatic metabolism of xenobiotics. However, the sources of healthy human tissues as donors of hepatocytes are very limited. In the present study, we aimed to develop a reversible system to facilitate self-renewal of primary hepatocytes while maintaining the ability to undergo restoration of differentiated function. Our hypothesis was that the timely removal of SV40T from transformed hepatocytes renews the expression of highly specialized metabolic functions. **Methods:** We generated recombinant retroviruses with *LoxP*-flanked SV40T flagged with EGFP (RetroSV40T) and adenovirus driving the expression of Cre-recombinase flagged with DsRed (AdCreDsRed). The viruses were used sequentially to conditionally transform murine and human hepatocytes in vitro. **Results:** Infection with RetroSV40T transformed both murine and human hepatocytes. EGFP expression facilitated the monitoring of infected cells and isolation of clones of transformed cells. SV40T expression resulted in a generalized decrease in expression of CYP genes by transformed clones of human hepatocytes. Infection of one of these clones with AdCreDsRed excised SV40T and EGFP, and efficiently expressed DsRed to identify cells undergoing reversion. Analysis of gene expression in adenovirus-infected cells revealed full restoration of CYP3A4 mRNA expression, and partial recovery of CYP2C9 and CYP2D6 expression. **Conclusion:** Sequential infection of primary hepatocytes with RetroSV40T and AdCreDsRed generates self-renewing cells with the potential to restore differential gene expression upon excision of SV40T by Cre-recombinase.
Key words: Human hepatocytes. Cultures. CYP3A4 mRNA expression. CYP2D6 expression.

RESUMO

Objetivo: Culturas primárias de hepatócitos humanos são comuns em sistemas *in vitro* para estudar o metabolismo hepático de xenobióticos. No entanto, as fontes de tecidos humanos saudáveis como doadores de hepatócitos são muito limitados. No presente estudo, objetivamos desenvolver um sistema reversível para facilitar a auto-regeneração dos hepatócitos primários, mantendo a capacidade de restauração da função diferenciada. Nossa hipótese foi que a remoção temporal dos SV40T dos hepatócitos transformado renovava a expressão de funções metabólicas altamente especializadas. **Métodos:** Foram gerados retrovírus recombinantes com LoxP-ladeado SV40T sinalizados com EGFP (RetroSV40T) e adenovírus com a expressão de Cre-recombinase sinalizadas com DsRed (AdCreDsRed). Os vírus foram usados seqüencialmente para condicionalmente transformar hepatócitos murinos e humanos *in vitro*. **Resultados:** A infecção com RetroSV40T transformou tanto hepatócitos murinos quanto humanos. A expressão de EGFP facilitou a monitorização das células infectadas e isolamento de clones de células transformadas. A expressão de SV40T resultou em uma redução generalizada na expressão dos genes CYP por clones transformados de hepatócitos humanos. A infecção de um desses clones com AdCreDsRed excisou SV40T e EGFP e expressou eficientemente DsRed para identificar células em reversão. A análise da expressão gênica em células infectadas com os adenovírus revelou plena restauração da expressão de CYP3A4 RNAm e recuperação da expressão parcial de CYP2C9 e CYP2D6. **Conclusão:** A infecção sequencial dos hepatócitos primários com RetroSV40T e AdCreDsRed gera células com capacidade de auto-regeneração, com o potencial para restaurar a expressão gênica diferencial sobre a excisão de SV40T pela Cre-recombinase.

Descritores: Hepatócitos. Cultura. Regeneração. Remoção de SV40T. Expressão de CYP3A4 mRNA. Expressão de CYP2D6.

Introduction

Primary cultures of human hepatocytes are commonly used by both academic and industrial laboratories to evaluate hepatic metabolism of xenobiotics. However, there is a remarkable need for alternative in vitro hepatic model systems relevant to human biology due to the limited supply of healthy livers from human donors. In this regard, several research models have been investigated, such as the use of fetal hepatocytes and stem cells[1-3]. An alternative approach is to develop an in vitro expandable system of human hepatocytes that maintains functions of fully differentiated cells. In the adult liver, hepatocytes are fully differentiated cells performing complex biotransformation reactions on endogenous substrates, products of digestion, and exogenous pollutants and drugs[4,5]. Despite this highly differentiated state, hepatocytes maintain the innate ability to substantially proliferate

while displaying differentiated functions, as demonstrated by the proliferative and functional profiles of hepatocytes during fetal development and after a liver injury[2,6]. The ability to recapitulate these features in vitro faces remarkable biological challenges that may be overcome by a strategy that allows for the conditional immortalization of primary hepatocytes.

Conditional immortalization of mature hepatocytes has the potential to foster the proliferative expansion of hepatocytes while allowing the restoration of a quiescent state and recovery of differentiated cell function in a timely fashion[7,8]. To use this method, proliferation and differentiation of hepatocytes are separated into two steps: expansion of the cell number by a factor that facilitates proliferation and recovery of cell function by excision or inhibition of the factor. Such experimental strategy must overcome molecular barriers to cell cycle typical of primary hepatocytes, such as the retention in the G1 phase by the cell cycle regulators p53 and pRB genes[9,10]. This biological barrier can be circumvented by the expression of the simian virus 40 large T antigen (SV40T) via its ability to bind and inactivate p53. As a consequence, hepatocytes are able to proliferate, but lose many of the phenotypic properties of differentiated cells[11]. Here, we aimed to develop a reversible system of SV40T-induced self renewal of human hepatocytes that would allow for proliferation while maintaining the potential to undergo restoration of differentiated function. We hypothesized that the timely removal of SV40T from transformed human hepatocytes renews the expression of highly specialized metabolic functions. We found that the transformation of primary human hepatocytes by the expression of SV40T flanked by *loxP* sites greatly suppresses the expression of CYP3A4, CYP2C9 and CYP2D6 genes. In this system, the removal of the SV40T transgene by Cre-recombinase recovered the full expression of CYP3A4, and the partial expression of CYP2C9 and CYP2D6.

Methods

Generation of the retrovirus RetroSV40T

To generate a SV40T flanked by two *loxP* sites, the PLNCX2 vector (Clontech, Mountainview, CA) containing elements from murine retrovirus was modified for retroviral gene delivery. First, two *LoxP* sites were introduced into the vector by PCR cloning, followed by the insertion of an IRES-EGFP cassette (Clontech, Mountainview, CA) into the EcoR I site. Then, the SV40T mini-gene was cloned in-frame at the 5' end of the IRES element, and an ATG-deficient neomycin resistance gene was cloned downstream from the second *LoxP* site to complete the

construction of the pSV40T/EGFP vector (Figure 1). This strategy would allow for the removal of the SV40T-IRES-EGFP fragment by Cre/LoxP recombination in the presence of Cre-recombinase[12]. Cell-free, replication-deficient recombinant retrovirus was generated by calcium phosphate transfection of 293T-derived Phoenix-gp, as described previously[13]. Briefly, cells were co-transfected with the pSV40T/EGFP construct, a plasmid expressing the Moloney Murine Leukemia Virus (MoMLV) gag-pol, and a plasmid containing either the MoMLV ecotropic (Eco) env gene or feline leukemia virus envelope (RD114) env gene, to generate ecotropic or RD114-pseudotyped virus, respectively[14,15]. Recombinant RetroSV40T (RD114) retrovirus was concentrated prior to use through a Vivaspin ultrafiltration spin column with a 100 KD membrane (Vivascience, Inc., Edgewood, NY). Viral titers were calculated based on the percentage of cells expressing enhanced green fluorescent protein (EGFP) as determined by FACScan analysis, 48-96 hours after a 4-hour infection of a known number of NIH/3T3 cells (ATCC No. CRL-1568; for ecotropic vector) or HT1080 (ATCC No. CCL-121; for RD114 pseudotyped vector), as previously described[16]. The titer of recombinant RetroSV40T (Eco) virus ranged from 2-5 x 10^5 IU/mL, while the titer of concentrated RetroSV40T (RD114) ranged from 5-22 x 10^5 IU/mL.

Cell culture and primary hepatocyte isolation

H2.35 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium with 1.0g/L glucose and 200nM dexamethasone and 5% fetal bovine serum (FBS). Primary mouse hepatocytes were isolated from adult mouse livers after perfusion with collagenase-containing buffer followed by centrifugation at 40 *g* as described previously[17]. Primary human hepatocytes were isolated from segments of livers that were not used in reduced-size liver transplantation, as approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center, Cincinnati, OH. Liver pieces weighing 20-25 g were perfused with Hanks' Balanced Salt Solutions (HBSS) containing 2mM EDTA for 5 minutes at 42 °C. After clear flow was observed, HBSS supplemented with 0.33mM $CaCl_2$ and 1mg/ml fresh collagenase (Roche, Indianapolis, IN) was used to perfuse/digest the liver for another 5 to 10 minutes. Liver cells were obtained from the liver segments, filtered through three layers of gauze and washed with ice cold Williams' E medium containing 5% FBS, penicillin-streptomycin and glutamine [18]. Hepatocytes were isolated by centrifugation at 40 *g*, viability was checked by trypan blue exclusion, and then cultured in Williams' E medium supplemented with 5% fetal bovine serum (FBS), 2mM glutamine, 100

units/mL penicillin, 100 μg/mL streptomycin, 2.5 μM dexamethasone, and 50 IU/L human insulin (Williams' E-Plus).

Retroviral infection

For retroviral infection of murine and human hepatocytes, 10^5 hepatocytes were plated on 60 mm collagen I-coated dish (BD bioscience, Bedford, MA) in Williams' E-Plus. Five hours after plating, medium was replaced with Williams' E-Plus containing 20ng/mL recombinant human hepatocyte growth factor (hHGF). After 16 hours of incubation with hHGF, 1-5 x 10^5 infectious virus particles were added to the cell culture in the presence of polybrene (8μg/mL). After 24 hours of incubation, retrovirus-containing medium was removed, and hepatocytes were cultured in Williams' E-Plus medium. Then, cultured hepatocytes were observed daily for up to 6 weeks of incubation. To clone transformed primary hepatocytes, cloning cylinders (Fisher Scientific, Atlanta, GA) were placed around the cell colonies and attached by sterile grease. The cells were first washed with HBSS, then incubated with HBSS containing 2mM EDTA for 2 to 5 minutes or until cells detached from the dish. Each clone was propagated and cryopreserved in Williams' E-Plus medium containing 10% DMSO.

Detection of SV40T by immunostaining

SV40T-expressing hepatocytes were cultured on collagen-coated chamber slides (BD bioscience, Bedford, MA), washed with phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA) and fixed with acetone. After permeablelized with 0.1% Triton/PBS, cells were blocked in PBS with10% FBS for one hour and incubated with antibody to SV40 T (Santa Cruz, Santa Cruz, CA) at 4°C overnight, followed by washes with 0.5% Tween-20/PBS, and incubation with Texas-Red conjugated goat anti mouse antibody at 37 °C for 1 hour.

RNA extraction, reverse transcription and PCR

Total RNA was extracted from cultured cells and treated with DNase I using the Qiagen RNA extraction kit (Qiagen, Valencia, CA). About 2 ug of total RNA was used to generate cDNA template using random primers and superscript II (Invitrogen, Carlsbad, CA) as described previously [19]. Regular PCR was

performed to detect SV40T/IRES fragment and Cre/LoxP recombinant cDNA. PCR primers used to detect SV40/IRES had the following sequences 5'-TGA TGA TGA TGA AGA CAG CCA GG- 3' and 5'-CCT CAC ATT GCC AAA AGA CGG -3', indicated as red arrow heads in Figure 1. PCR primers used to detect SV40/IRES that underwent Cre/LoxP-mediated recombinantion were the following: 5'-ATC CTC CCT TTA TCC AGC CCT CAC- 3' and 5'-AGC GAG AAG CGA ACT GAT TGG- 3', indicated as green arrows in Figure 1. Real time quantitative PCR (qPCR) was performed to determine the levels of expression for albumin, CYP3A4, CYP2C9, CYP2D6 and CYP2C19 using the primers listed in Table1.

Table 1 - Primers for detecting the levels of expression of selected genes by real-time PCR
Amplification cycles consisted of initial denaturing at 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 30 seconds, 55˚C for 1 minute, and 72˚C for 30 seconds. Specificity of fluorescent signal was confirmed by a dissociation curve determined at the end of each reaction. Amplification reactions were performed in triplicate and normalized to the expression level of 18s rRNA. Primer pairs used in real-time PCR analysis:

Gene Name	Primer Sequences*
18s rRNA	5' CCCTGTAATTGGAATGAGTCCAC 3' (F)
	5' GCTGGAATTACCGCGGCT 3' (B)
Albumin	5' GCT TGA ATG TGC TGA TGA CAG GG 3' (F)
	5' GCA GTG GGA TTT TTC CAA CAG AGG 3' (B)
CYP3A4	5' CCA CCC ACC TAT GAT ACT GTG CTA C 3' (F)
	5' AGG AAC TTC TCA GGC TCT GTC CAG 3' (B)
CYP2C9	5' GAA CCT TGA CAC CAC TCC AGT TG 3' (F)
	5' GAT AAT GCC CCA GAG GAA AGA GAG 3'(B)
CYP2D6	5' TTG GTA GTG AGG CAG GTA TGG G 3' (F)
	5' GCA GTA TGG TGT GTT CTG GAA GTC C(B)
CYP2C19	5' CGG GAC TTT ATT GAT TGC TTC CTG 3' (F)
	5' GGG TTG TGC TTG TTG TCT CTG TC 3' (B)

* F, forward primer; B, backward primer.

Adenoviral Infection

Adenovirus expressing Cre-recombinase (AdCre) was purchased from Microbix Company (Toronto, Ontario, Canada). AdCre expresses high levels of Cre-recombinase containing a nuclear localization signal (NLS) driven by the murine cytomegalovirus immediate early gene promoter (MCMV). To monitor the expression of Cre-recombinase in infected cells, an IRES/DsRed gene cassette was inserted at the 3' end of the NLSCre fragment in the AdCre vector to generate the AdCreDsRed recombinant adenovirus (Figure 2). The modified adenovirus construct was plaque

purified and confirmed by capillary gene sequencing. Both AdCreDsRed and AdCre were expanded and tittered in human embryonic kidney 293 monolayers by agarose overlay. In brief, diluted virus was incubated with 293 cells at about 80-90% confluency at 37°C for 30-60 minutes, and then an overlay of 2% agarose made in culture medium was added to cover the cells. Plaques were visible and counted at about 1 week after infection. The number of plaque forming units (PFU) of virus was then calculated. Immortalized hepatocytes were infected at a multiplicity of infection of 100 PFU per cell for 2 h at 37°C in a humidified, 5% CO_2 incubator. Thereafter, the adenovirus-containing medium was removed and the cells were cultured in Williams' E-Plus for an additional 24 h to 9 days prior to treatments or harvesting.

Flow cytometry and cell sorting

H2.35 cells and immortalized hepatocytes detached by trypsin or EDTA were resuspended in PBS with 5% FBS and then subjected to cell sorting using a FACSVantage^{TM} SE DiVa high speed flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm laser (Enterprise IIC argon laser), 90 micron nozzle, with sheath pressure adjusted to 26 psi. Standard optics included a 560 SP dichroic mirror, a 530/30 nm band pass emission filter (FL1) for EGFP and a 575/26 nm band-pass filter (FL2) for DsRed. EGFP+ and DsRed+ cells were sorted in purity mode by strategically gating on forward scatter and side scatter to eliminate debris, followed by gating for cells expressing EGFP or DsRed.

Results

Expression of RetroSV40T in transformed hepatocytes

To incorporate the SV40T gene into the chromosome of target cells, we first generated the infectious but amplification-deficient Ecotropic retrovirus RetroSV40T by co-transfecting phoenix-gp cells with the pSV40T/EGFP and helper plasmids containing *gag* and *pol* and Eco *env* genes. Production of RetroSV40T was tested by incubation of virus with NIH/3T3 cells in the presence of polybrene, which showed expression of EGFP within 4 hours as determined by flow cytometric analysis (data not shown). To test the ability of RetroSV40T to successfully infect hepatocytes, we incubated Ecotropic RetroSV40T with the murine hepatocyte line H2.35, a commercially available cell line derived from murine hepatocytes by the expression of a temperature-sensitive SV40T; control cells consisted of H2.35 cells not exposed

to the virus. No morphological change was observed in the cells after RetroSV40T infection. Three days after infection, flow cytometric analysis showed the emergence of a new population of cells expressing EGFP in infected H2.35 cells indicating the successful delivery of the transgene by RetroSV40T (Figure 3 A, B). To detect the expression of SV40T, RNA was extracted from both EGFP positive H2.35 cells and control H2.35 cells, and reversely transcribed for PCR analysis. Because H2.35 cells were produced by incorporation of a SV40T mini-gene, we used a primer set that spans the SV40T and IRES transition which is specific to the RetroSV40T construct (Figure 1). Primers that specifically bind to SV40T/IRES region produced a fragment in EGFP positive cells but not in control cells, indicating that SV40T gene was actively transcribed in RetroSV40T infected cells (Figure 3C).

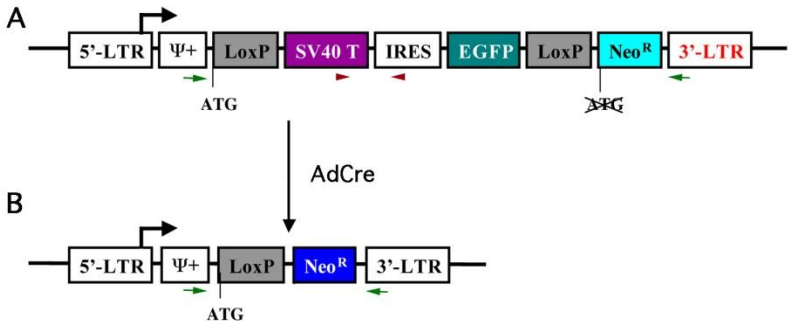

Figure 1 - Schematic drawing of the elements in RetroSV40T retrovirus. The SV40T and EGFP mini-genes flanked by two loxP elements are cloned into a recombinant retrovirus. A neomycin resistance gene (Neo^R) is cloned downstream of EGFP/loxP (A). The lack of an initiation codon in the Neo^R gene only allows the expression of neomycin if the entire SV40T/EGFP cassette is excised, which brings the 5' ATG in-frame with the Neo^R gene (B). Green arrows indicate the positions of PCR primers for detecting Cre/LoxP recombination; red arrowheads indicate the positions of PCR primers for detecting SV40T/IRES.

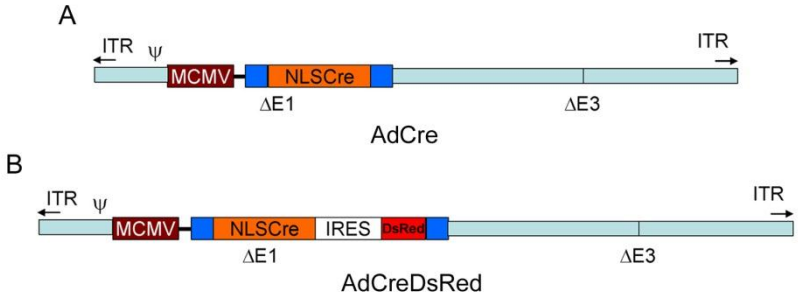

Figure 2 - Schematic drawing of the elements in AdCreDsRed adenovirus. The E1/E3 deficient Ad5 adenoviral vector contains the Cre-recombinase gene driven by MCMV promoter (A). This vector was modified by in-frame insertion of IRES element and DsRed cDNA to allow the simultaneous expression of Cre-recombinase and DsRed (AdCreDsRed, B).

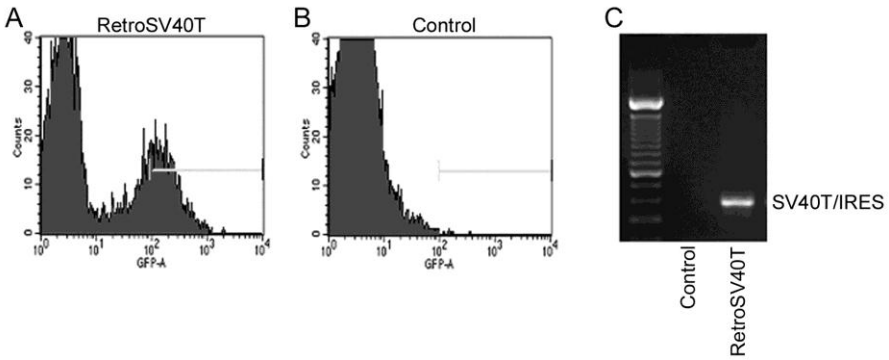

Figure 3 - Expression of EGFP and SV40T in RetroSV40T infected H2.35 cells. Flow cytometric analysis detected a new population of cells with high EGFP signal in RetroSV40T infected H2.35 cells (A) compared with non infected H2.35 control (B). In panel (C), SV40T mRNA signal was detected by PCR only in RetroSV40T infected cells that were sorted using EGFP signal.

Transformation of primary murine hepatocytes by RetroSV40T

To test the ability of RetroSV40T to immortalize primary hepatocytes, $1x10^5$ primary mouse hepatocytes isolated from an adult C57Bl/6 mouse were plated in 60 mm collagen-I coated culture dishes and infected with $5 x10^5$ IU RetroSV40T (Eco) following the same experimental protocol described for H2.35 cells with the addition of recombinant hHGF. One week after RetroSV40T infection, colonies of proliferating cells emerged; at two weeks, individual colonies of hepatocytes were detached from the plate by a brief incubation with HBSS-EDTA, and transferred to new plates for cell expansion. Individual hepatocyte clones were shown to have undergone transformation by the expression of high levels of EGFP by flow cytometric analysis (Figure 4A, B). Immunostaining of these cells localized the SV40T protein to the nucleus of transformed hepatocytes (Fig 4C, D). These data showed that RetroSV40T has the ability to transform primary mouse hepatocytes and successfully induce the expression of EGFP to facilitate enrichment of the cell population by flow cytometry-based sorting.

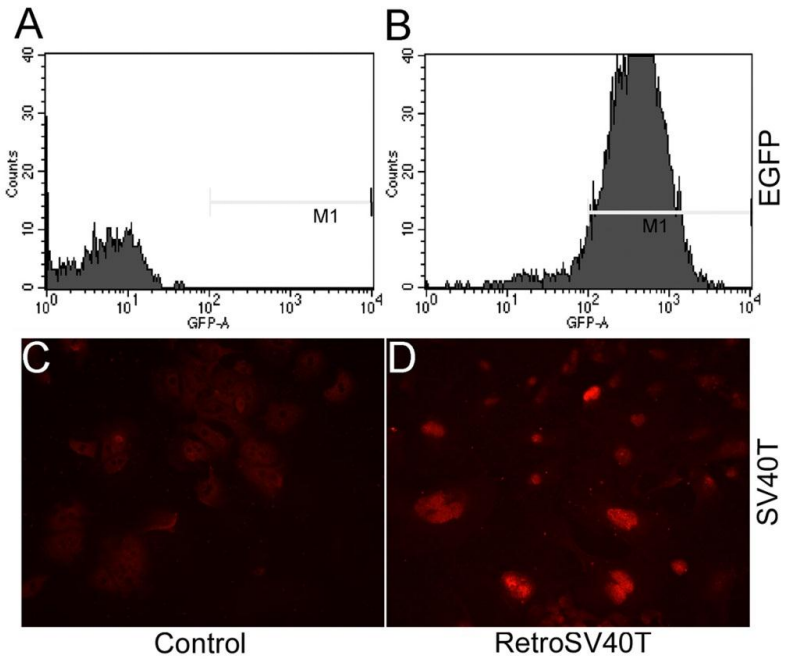

Figure 4 - Transformation of primary mouse hepatocytes. Flow cytometric analysis of primary mouse hepatocytes upon isolation showed no EGFP signal (A), but a hepatocyte clone obtained after infection with RetroSV40T retrovirus displayed a uniform population of EGFP-expressing cells (B). In the lower panels, immunostaining to detect SV40T showed no signal in control mouse hepatocytes (C), specific SV40T staining in red in RetroSV40T-infected cells (D).

Transformation of primary human hepatocytes by RetroSV40T

To generate clones of self-renewing human hepatocytes, primary human hepatocytes were isolated from unused lobe of a human liver harvested for reduced-size organ transplantation into a pediatric patient. The viability of hepatocytes was 85% as determined by trypan blue exclusion. Human hepatocytes were cultured overnight, followed by infection with RetroSV40T (RD114) in the presence of recombinant hHGF as described above for murine hepatocytes. Three weeks after RetroSV40T (RD114) infection, colonies of proliferating cells emerged and were transferred to new culture plates for further subcloning by low-density plating. A total of fifty three clones were successfully expanded, and aliquots were cryopreserved in 10% DMSO-containing medium. All clones maintained the ability to be subcultured and expanded following rapid thawing at 37 C and incubation in Williams' E Medium-Plus.

To investigate the effect of SV40T on the expression of genes encoding hepatic enzymes, we isolated RNA from each one of the 53 clones, generated cDNA pools, and used real-time PCR to test the expression of albumin, CYP3A4, CYP2C19, CYP2D6 and CYP2C9, gene products typical of fully differentiated hepatocytes. The levels of expression of the five genes were either undetectable or dramatically low in all 53 clones when compared to the levels of expression of freshly isolated human hepatocytes (data not shown). To further investigate the phenotype of these clones after several culture passages, one clone (number 619) was selected for further analysis based on a detectable but low expression of CYP genes by PCR. Successive subcultures revealed that clone 619 had a doubling time of 48-72 hours, and underwent more than 20 passages without obvious growth crisis. Upon plating, the morphology of clone 619 cells flattened in a fashion similar to primary hepatocytes. When analyzed by flow cytometric analysis after 20 passes, clone 619 cells were uniformly positive for EGFP (Figure 5, A-C).

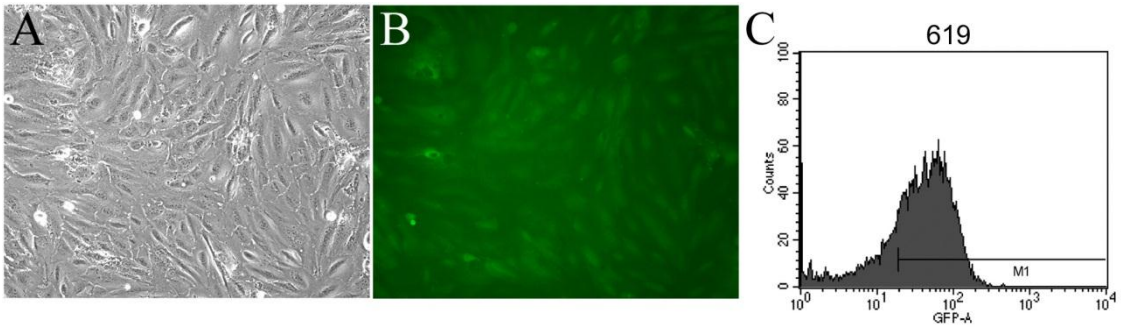

Figure 5 - Transformation of primary human hepatocytes. Representative photomicrographs of cells from clone 619 in bright field (A) and under fluorescence microscope (B). Flow cytometric analysis showed EGFP expression in clone 619 cells (C).

Conditional removal of the SV40T gene from immortalized human hepatocytes

To test whether the expression of CYP genes could be restored by the removal of the SV40T gene, clone 619 cells were incubated with the AdCreDsRed. The infection efficiency and the expression of Cre-recombinase were examined by the expression of the red fluorescent protein (DsRed). Twenty four hours after AdCreDsRed infection, DsRed-expressing cells were easily detectable by fluorescence microscopy (not shown). By flow cytometric analysis, DsRed-based sorting enabled the purification of infected cells (Figure 6A, B). To determine whether Cre-recombinase excised the SV40T gene from clone 619 cells, cDNA pools were generated from DsRed-positive sorted cells and from clone 619 cells not subjected to AdCreDsRed infection (as negative controls), and subjected to PCR to

determine the excision of SV40T in infected cells. DsRed-expressing cells underwent recombination events as demonstrated by a shortened PCR amplicon that displayed the predicted molecular weight (Figure 6C). This was further supported by the decrease in the levels of SV40T mRNA by qPCR to less than 1% of that in control 619 cells (data not shown). Collectively, these experiments clearly showed that SV40T gene can be excised from clone 619 cells within 24 hrs of the infection with adenovirus carrying Cre-recombinase gene.

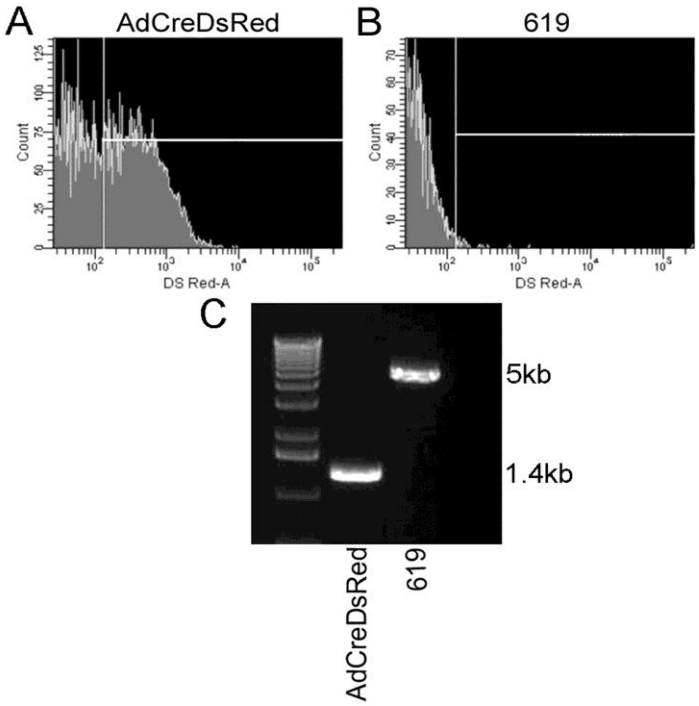

Figure 6 - DsRed expression and loss of SV40T following infection with AdCreDsRed. DsRed signal is present in cells from clone 619 three days after AdCreDsRed infection (A), while no DsRed is expressed in non-infected cells (B). PCR using the primers flanking the two LoxP sites (as shown in green arrows in figure 1) demonstrated the complete loss of SV40T/EGFP in DsRed positive cells (1.4 kb product). The same primers produced a 5 kb product in non-recombinant clone 619 cells (C).

To enrich the population of clone 619 cells undergoing Cre/LoxP recombination in culture, we added G418 (750 μg/mL) to the culture medium of AdCre-infected cells. This was possible because the excision of SV40T/EGFP by Cre-recombinase would place an initiator ATG in-frame with the neomycin resistance gene (Figure 1). Three days after infection and G418 selection, the levels of EGFP expression dramatically decreased under fluorescence microscopy

monitoring, and a new population of cells with a 10-fold lower expression of EGFP could be detected by flow cytometric analysis (Figure 7).

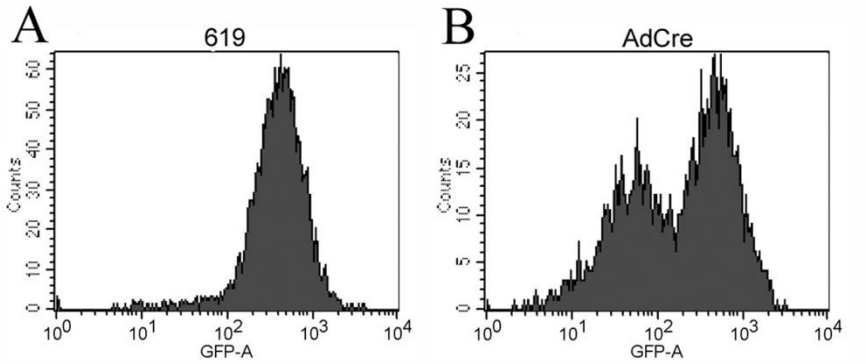

Figure 7 - Decreased expression of EGFP in AdCre infected clone 619 cells. The uniform population of EGFP-expressing cells from clone 619 cells (A) changes with the emergence of a fraction of cells that lost most of the EGFP signal 3 days after AdCre infection and G418 selection (B).

Increased expression of CYP genes after the excision of SV40T

To investigate whether the removal of SV40T restored high levels of expression to CYP genes, we analyzed their expression in AdCre-infected/G418-treated clone 619 cells. Before infection, mRNA expression of CYP3A4 in clone 619 cells approached 5% of the levels observed in the primary hepatocytes, and did not change significantly in the first 24 hrs after AdCre infection (data not shown). Notably, 7 days after AdCre infection/G418 selection, CYP3A4 mRNA levels increased significantly and reached levels comparable to the levels detected in primary hepatocytes (Figure 8A). The recovery of CYP gene expression was not uniform to all functional counterparts, with two patterns identified. In the first pattern, the levels of mRNA expression for CYP2C9 varied overtime, with an initial increase, followed by a transient drop at 5 days in culture, and then an increase by 7 days (Figure 9A). In the second pattern, levels of mRNA for CYP2D6 gradually increased in the first 7 days, but did not reach levels comparable to levels of primary hepatocytes (Figure 9B), as observed in CYP3A4 gene. The findings that the excision of SV40T allowed for the increased expression of CYP genes was not applicable to the albumin gene, as demonstrated by a modest 2-fold increase in albumin mRNA when compared to control clone 619 cells, which was ~1000 fold lower than the albumin mRNA expression of primary hepatocytes (data not shown). Collectively, these data show that the removal of SV40T enriches the level of

expression of highly specialized genes, but the degree of expression varies with individual genes.

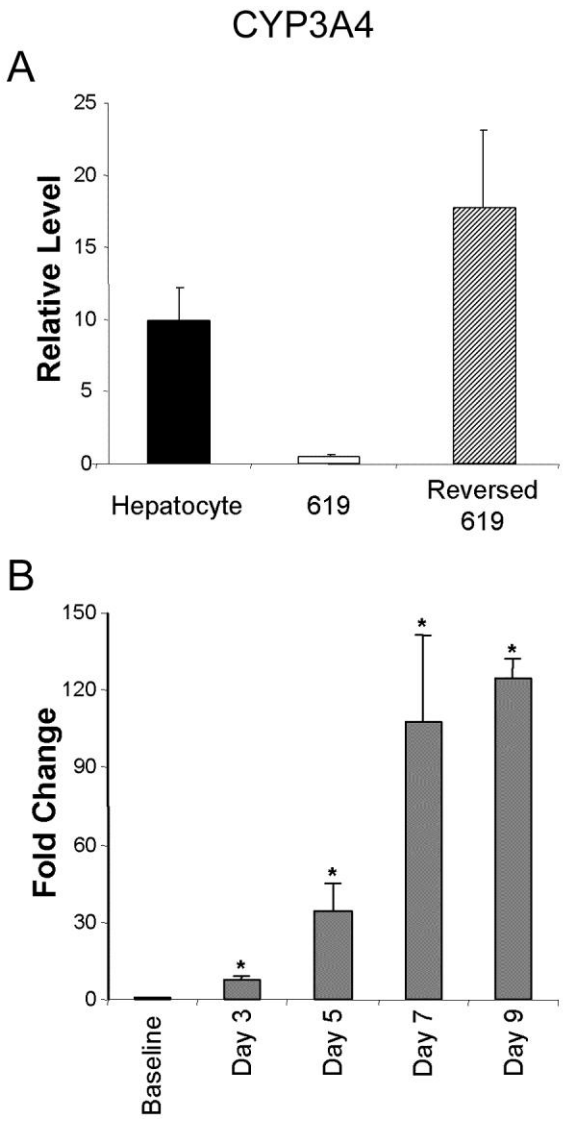

Figure 8 - CYP3A4 gene expression in reversed clone 619 cells. In panel (A), the mRNA levels of CYP3A4, in reversed clone 619 cells (stripe), after normalized to the mRNA levels of 18s rRNA, increased after 7 days of culture several fold above SV40T-expressing cells (white) and reached levels comparable to primary hepatocytes (black). Panel (B) shows the levels of CYP3A4 mRNA in cells from clone 619 after infection with AdCre as a fold change from baseline levels. (Data presented as mean and S.D.; *P<0.05 by student t-test)

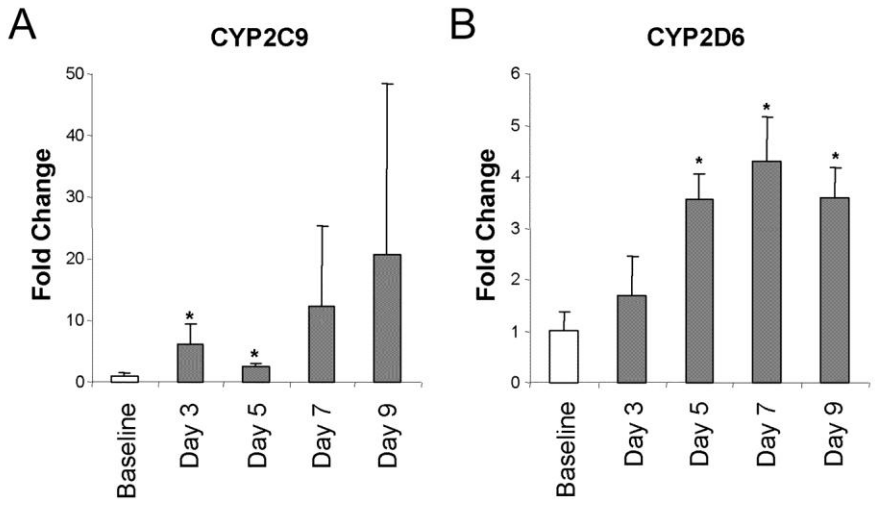

Figure 9 - Expression of CYP2C9 AND CYP2D6 following excision of SV40T in clone 619 cells. Levels of CYP2C9 (A) and CYP2D6 (B) mRNA at different days in culture following AdCre infection depicted as fold changes above baseline levels. (Data presented as mean and S.D.; *P<0.05 by student t-test).

Discussion

We produced a two-step approach to expand the lifespan of human hepatocytes while maintaining the potential for recovery of differentiated cellular function. In the first step, the retroviral-mediated expression of SV40T resulted in the emergence of self-renewing cell clones. Isolation of these clones was greatly facilitated by the simultaneous expression of EGFP; SV40T-expressing clones tolerated cryopreservation without obvious adverse impact on cellular viability. The ability to undergo self renewal, however, was accompanied by a generalized loss of expression of genes typical of hepatocytes. In the second step, the adenovirus-driven expression of Cre-recombinase by self-renewing cells successfully removed both SV40T and EGFP, restored the expression of individual genes at variable levels, and shortened the cellular lifespan. Combined, these experiments point to the validity of the two-step approach to expand the availability of human hepatocytes, and underscore the differential effect of removal of SV40T on gene expression.

The expression of SV40T in primary hepatocytes is a viable strategy to overcome retention in a phase (e.g.: G1) of the cell cycle. This release from retention in the cell cycle results from the ability of SV40T to bind and inactivate p53 and pRB[8]. To develop a system that facilitated the production of hepatocytes with the constitutive expression of SV40T in a timely fashion, we used recombinant

retrovirus with the addition of HGF to induce hepatocyte proliferation and the expression of EGFP to identify infected cells. HGF is a known potent mitogen to hepatocytes[20]; thus, the presence of HGF in the culture media at the time of incubation with RetroSV40T has the potential to optimize infection efficiency by increasing the number of actively proliferating hepatocytes. Since the expression of the EGFP gene occurs in synchrony with SV40T, monitoring EGFP expression in cultured cells enables the identification of transformed cells as early as one week after retrovirus infection. While this approach commonly generates transformed cells, our study did not determine whether SV40T/EGFP-expressing human hepatocytes are fully immortalized. In addition to overcoming the retention in the cell cycle typically seen in primary cells, the process of immortalization also requires an escape from later replication crisis induced by greatly shortened telomeres, which may be seen when transformed cells undergo greater than 20 population doublings[21]. Interestingly, although we did not include a telomerase gene in our viral vector to promote immortalization[22,23], cells from clone 619 underwent more than 20 passages without changes in cellular morphology. It is possible that this clone may be one of a few selected clones that may significantly delay (or completely overcome) the crisis stages associated with short telomeres. Alternatively, the expression of EGFP may have greatly facilitated the identification of individual clones, thus decreasing the number of passages that is commonly required to clone and expand transformants.

The timely excision of SV40T from transformed cells has the potential to restore the expression of a wide range of genes. In our system, excision of SV40T by Cre-recombinase did not restore the levels of albumin mRNA, but successfully resulted in an increased expression of CYP3A4 mRNA to the levels typically seen in untransformed hepatocytes. Although increases were also observed in CYP2D6 and CYP2C9 mRNA, they did not reach the baseline levels of primary hepatocytes. These findings are in keeping with a significant impact of transformation of primary hepatocytes in the expression of highly specialized genes, and with the ability to restore the expression of individual genes or gene networks after reversal of transformation. The degree of recovery of expression, however, is gene specific. In this setting, cells from clone 619 described herein have the potential to serve as a cellular source for studies of biotransformation reactions. The full support of this claim requires formal studies that directly investigate how their metabolism of xenobiotics compares to primary hepatocytes. In addition to the use of the two-step approach to transform hepatocytes, the sequential use of recombinant retrovirus and adenovirus that incorporate EGFP and DsRed to monitor transformation and reversion has the potential to serve as an effective experimental tool to study other primary cells and biological systems.

Conclusion

Our data demonstrates that the sequential infection of primary hepatocytes with RetroSV40T and AdCreDsRed generates self-renewing cells with the potential to restore differential gene expression upon excision of SV40T by Cre-recombinase.

Abbreviations

CYP: Cytochrome P450

IRES: Internal ribosome entry site

EGFP: Enhanced green fluorescent protein

Competing interests

'The authors declare that they have no competing interests'.

References

1. Ishii T, Yasuchika K, Fujii H, Hoppo T, Baba S, Naito M, Machimoto T, Kamo N, Suemori H, Nakatsuji N et al: In vitro differentiation and maturation of mouse embryonic stem cells into hepatocytes. Exp Cell Res. 2005; 309:68-77.
2. Nowak G, Ericzon BG, Nava S, Jaksch M, Westgren M, Sumitran-Holgersson S: Identification of expandable human hepatic progenitors which differentiate into mature hepatic cells in vivo. Gut. 2005; 54:972-9.
3. Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, Chen JR, Chen YP, Lee OK: In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology. 2004; 40:1275-84.
4. Faber KN, Muller M, Jansen PL: Drug transport proteins in the liver. Adv Drug Deliv Rev. 2003; 55:107-24.
5. Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, Sugiyama Y: Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. Pharmacol Ther. 1997; 73:147-71.
6. Oh SH, Hatch HM, Petersen BE: Hepatic oval 'stem' cell in liver regeneration. Semin Cell Dev Biol. 2002; 13:405-9.
7. Kobayashi N, Noguchi H, Westerman KA, Watanabe T, Matsumura T, Totsugawa T, Fujiwara T, Leboulch P, Tanaka N: Cre/loxP-based reversible immortalization of human hepatocytes. Cell Transplant. 2001; 10:383-6.
8. Cascio SM: Novel strategies for immortalization of human hepatocytes. Artif Organs. 2001; 25:529-38.
9. Mayhew CN, Bosco EE, Fox SR, Okaya T, Tarapore P, Schwemberger SJ, Babcock GF, Lentsch AB, Fukasawa K, Knudsen ES: Liver-specific pRB loss results in ectopic cell cycle entry and aberrant ploidy. Cancer Res. 2005; 65:4568-77.

10. Loyer P, Ilyin G, Cariou S, Glaise D, Corlu A, Guguen-Guillouzo C: Progression through G1 and S phases of adult rat hepatocytes. Prog Cell Cycle Res. 1996; 2:37-47.
11. Schippers IJ, Moshage H, Roelofsen H, Muller M, Heymans HS, Ruiters M, Kuipers F: Immortalized human hepatocytes as a tool for the study of hepatocytic (de-)differentiation. Cell Biol Toxicol. 1997; 13:375-86.
12. Van Duyne GD: A structural view of cre-loxp site-specific recombination. Annu Rev Biophys Biomol Struct. 2001; 30:87-104.
13. Pear WS, Nolan GP, Scott ML, Baltimore D: Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci U S A. 1993; 90:8392-96.
14. Cosset FL, Takeuchi Y, Battini JL, Weiss RA, Collins MK: High-titer packaging cells producing recombinant retroviruses resistant to human serum. J Virol. 1995; 69:7430-36.
15. Morita S, Kojima T, Kitamura T: Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. 2000; 7:1063-66.
16. van der Loo JC, Liu BL, Goldman AI, Buckley SM, Chrudimsky KS: Optimization of gene transfer into primitive human hematopoietic cells of granulocyte-colony stimulating factor-mobilized peripheral blood using low-dose cytokines and comparison of a gibbon ape leukemia virus versus an RD114-pseudotyped retroviral vector. Hum Gene Ther. 2002; 13:1317-30.
17. Shanmukhappa K, Mourya R, Sabla GE, Degen JL, Bezerra JA: Hepatic to pancreatic switch defines a role for hemostatic factors in cellular plasticity in mice. Proc Natl Acad Sci U S A. 2005;102:10182-7.
18. Strom SC, Jirtle RL, Jones RS, Novicki DL, Rosenberg MR, Novotny A, Irons G, McLain JR, Michalopoulos G: Isolation, culture, and transplantation of human hepatocytes. J Natl Cancer Inst. 1982;68:771-8.
19. Zhang DY, Sabla G, Shivakumar P, Tiao G, Sokol RJ, Mack C, Shneider BL, Aronow B, Bezerra JA: Coordinate expression of regulatory genes differentiates embryonic and perinatal forms of biliary atresia. Hepatology. 2004;39:954-62.
20. Lilja H, Blanc P, Demetriou AA, Rozga J: Response of cultured fetal and adult rat hepatocytes to growth factors and cyclosporine. Cell Transplant. 1998; 7:257-66.
21. Bryan TM, Reddel RR: SV40-induced immortalization of human cells. Crit Rev Oncog. 1994; 5:331-57.
22. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ: Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature. 1998; 396(6706):84-88.
23. Counter CM, Hahn WC, Wei W, Caddle SD, Beijersbergen RL, Lansdorp PM, Sedivy JM, Weinberg RA: Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. Proc Natl Acad Sci U S A. 1998; 95:14723-8.