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Hepatocytes transduced with human TERT gene acquire a prolonged lifespan in culture and retain permissiveness to hepatitis B virus

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Summary. – Hepatitis B virus (HBV) can be propagated *in vitro* in primary cultures of human hepatocytes and some stable hepatoma cell lines maintained under specific conditions. The lack of simple and non-neoplastic cell culture systems for HBV has hampered the analysis of virus life cycle and development of antiviral compounds. In this study, we succeeded in prolonging the lifespan of human hepatocytes in primary culture by transducing them with human telomerase reverse transcriptase (hTERT) gene. The transgenic cells expressed hTERT constitutively and propagated HBV up to $5x10^5$ DNA copies/ml for 28 days.

Keywords: hepatitis B virus; hepatocyte; TERT; transduction, primary

Introduction

HBV (the species *Hepatitis B virus*, the genus *Orthohepadnavirus*, the family *Hepadnaviridae*) is a small, enveloped DNA virus that causes acute and chronic viral hepatitis, as well as other liver diseases, including cirrhosis and hepatocellular carcinoma (HCC) (Seeger *et al*., 2000). It is estimated that HBV chronically infects 360 million people worldwide (Te and Jensen, 2010). In the past decade, a substantial progress has been made in treating these chronic infections, although many problems exist, including the occurrence of side effects associated with the treatment and the emergence of variants resistant to the treatment (Zoulim, 2006). Therefore, the development of new antiviral compounds that are more efficacious and better tolerated, and that produce fewer side effects, is urgently needed (Petersen *et al.,* 2008). The delay in developing such compounds has largely been due to the lack of human cell culture systems that support HBV replication and infection, in addition to the animal models for testing of antiviral compounds.

Normal human adult or fetal hepatocytes infected with serum-derived HBV are the ideal system to study HBV virus infectivity. However, the susceptibility of hepatocytes to viral infection is dependent on their stage of differentiation (Gripon *et al.,* 2002). Accordingly, it was determined that only primary cultures of human hepatocytes were susceptible to HBV infection, which facilitated systematic investigation of early events in viral infection and, subsequently, the innate immune response (Rabe *et al.,* 2006). Because human hepatocytes are well-differentiated epithelial cells, they proliferate poorly and divide only a few times when cultured *in vitro* before growth cessation and death (Delgado *et al.,* 2005). Our group is interested in whether continuous proliferation in immortalized human hepatocytes can be achieved. It is well known that cell survival is directly related to telomere length and telomerase activity. The core subunit of telomerase, telomerase reverse

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Abbreviations: AAT = α-1-antitrypsin; AFP = a-fetoprotein; ALB = albumin; CK18 = cytokeratin 18; GS = glutamine synthetase; HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; HGF = hepatocyte growth factor; IFA = immunofluorescence assay; hTERT = human telomerase reverse transcriptase; TERT = telomerase reverse transcriptase; TF = transferring

transcriptase (TERT), is involved in strict regulation of telomerase activity (Blackburn, 1991).

Human somatic cells transfected with the gene encoding human TERT (hTERT) can escape the p53-mediated cell-senescence pathway or other cell-cycle checkpoint responses, thus prolonging the *in vitro* cell-culture survival (Jiang *et al.,* 1999; Morales *et al.,* 1999).

In this study we attempted to genetically modify human hepatocytes so that they would acquire the ability to survive a prolonged cultivation *in vitro* and permissivity to HBV. This goal was achieved by transducing these cells with the hTERT gene. The transgenic HC-T cells expressed the hTERT gene constitutively and replicated HBV up to $5x10^5$ DNA copies/ml for 28 days in culture. The hTERT transgenic human hepatocytes represent a new and simple cell culture system for HBV that may facilitate all investigations into this virus (Lv *et al.,* 2009).

Materials and Methods

Cells. The PT67 Cell Line is derived from a mouse fibroblast (NIH 3T3) cell line designed for stably producing high-titer retrovirus. Human hepatocytes were isolated by collagenase perfusion with a typical two-step protocol from the resected liver tissue as previously described (Katsura *et al.*, 2002; Baccarani *et al.*, 2003; LeCluyse *et al.*, 2005). Isolated hepatocytes were seeded in collagen-coated 6-well plates (2×10^5 cells per well) and cultured in hepatocyte medium. Endothelial hepatocyte cell line QSG 7701 was cultured in RPMI 1640 containing 10% FBS. Carcinoma cell line HepG2 was maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. All cell lines were incubated at 37°C under 5% CO_2.

Table 1. PCR primers

Gene	Sequence	PCR product (bp)
AAT	F: 5′- ACAGAAGGTCTGCCAGCTTC -3′	182
	R: 5′- GATGGTCAGCACAGCCTTAT -3′	
ALB	F: 5′- TGAGCAGCTTGGAGAGTACA -3′	190
	R: 5′- GTTCAGGACCACGGATAGAT-3′	
AFP	F: 5′- TGCCAACAGGAGGCCATGC -3′	308
	R: 5′- CCCAAAGCAGCACGAGTTTT -3′	
CK18	F: 5′-TGGGGTTCAGAGGACTGTTAA-3′	261
	R: 5′-TGGAGTGAGTGGTGAAGCTCA-3′	
GS	F: 5′- GCCTGCTTGTATGCTGGAGTC -3′	419
	R: 5′- GGCGCTACGATTGGCTACAC -3′	
TF	F: 5′- GAAGGACCTGCTGTTTAAGG -3′	310
	R: 5′- CTCCATCCAAGCTCATGGC -3′	
HGF	F: 5′- AGGAGCCAGCCTGAATGATGA -3′	380
	R: 5′-CCCTCTGATGTCCCAAGATTAGC -3′	
β-actin	F: 5′- ATCATGTTTGAGACCTTCAACA -3′	302
	R: 5′- CATCTCTTGCTCGAAGTC -3′	

Transgenic HC-T cells. pLXSN-hTERT retroviral vector (a generous gift from Drs. Xiaofei Zheng and Hong Jiang, Beijing Institute of Radiation Medicine) was employed in these experiments. Viral supernatant containing hTERT was generated by packaging cell line PT67. Briefly, 2×10^5 PT67 cells were plated on a 6-well dish 12–24 hrs before transfection at 80% confluency and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. PT67 cells transfected with pLEGFP-C1 plasmid, which contains a reporter gene downstream from the CMV promoter, were used as a negative control. Stably transfected PT67 cells producing virus were screened using 600 μg/ml G418 and amplified. After collecting and purifying the viral supernatant through a 0.45μm cellulose acetate filter, viral titer was determined on NIH 3T3 cells as previously described (Kwon *et al.,* 2003). 1×10^5 primary hepatocytes in a 6-well plate were exposed to viral supernatant containing hTERT in the presence of 8 μg/ml polybrene (Sigma, USA) for 24 hours. Subsequently, the viral supernatant was replaced with fresh medium. Transgenic cells expressing *hTERT* were verified and termed transgenic HC-T cells.

HBV infection of HC-T cells. HBV-positive sera used for infection experiments were obtained from the Beijing 302 hospital. The pooled serum samples contained 1.85×10^8 copies/ml of HBV DNA as determined by Light Cycler TM (Roche). HC-T cells were infected with HBV-positive serum at 37°C for 24 hrs. The cells were then washed 6 times and cultured in standard media with FBS. After 7 days, the cell culture supernatants were harvested weekly. HBV antigens and HBV DNA were analyzed, respectively.

ELISA of HBsAg. Hepatitis B surface antigen (HBsAg) in the medium was detected by an ELISA kit obtained from Kinghawk Pharmaceutical Co., Ltd. using the protocols described in instructions.

RT-PCR for hepatocyte-specific genes. Total RNA was isolated from hepatic cells with Trizol Reagent (Invitrogen) according to the manufacturer′s instructions, followed by treatment with DNase I (TaKaRa), and extraction with phenol:chloroform. First-strand cDNA synthesis was performed with MMLV reverse transcriptase (TaKaRa), using an oligo $(dT)_{15}$ primer (TaKaRa). Reverse transcription (RT) was performed according to the manufacturer′s instructions. Cycling conditions for PCR amplification were as follows: 94°C for 1 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and final elongation at 72°C for 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis. Hepatocyte-specific genes and primers are shown in Table 1 (Poyck *et al.*, 2008).

Detection of albumin. To assess the albumin secretion activity of primary hepatocytes, albumin from cell culture supernatant was detected using albumin detection kits (Zhongkong). In brief, the collected culture supernatants were incubated with buffer solution followed by goat anti-human albumin antibody in a working solution. The absorption value was measured after each incubation

step using a UV spectrophotometer (SHIMADZU). The albumin concentration in the samples was calculated using the standard curve.

Western blot analysis of albumin. Secreted proteins in culture media were assayed every 4 days by Western blotting. Primary antibody directed against human albumin (Sigma-Aldrich) was used at a 1:1,000 dilution. Detection of the chemiluminescent signal of anti-mouse horseradish peroxidase-conjugated antibodies (1:10,000) was performed with the Super ECL Western blotting detection kit (Applygen Technologies Inc).

Glycogen detection. Cells were oxidized in 0.5% periodic acid solution for 5 min after fixation. Following rinsing in distilled water, the cells were placed in Schiff's reagent for 15 min. After washing in tap water for 5 min, the cells were observed under a DMI3000 microscope (Leica).

Immunofluorescence assay (IFA) of CK18. HC-T cells were grown on coverslips overnight, washed twice with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 30 min. The first incubation was performed with a mouse monoclonal antibody against human CK18 at a dilution of 1:100 (Zhongshan); the FITC-labeled goat anti-mouse immunoglobulin, diluted 1:1000, was added after washing. Following three washes with PBS, cells were counterstained with DAPI (Invitrogen) for nuclear visualization. After washing and mounting, the slides were observed under a DMI3000 fluorescent microscope (Leica).

IFA of HBcAg. Infected cells were incubated with polyclonal rabbit antisera to HBcAg (Dako) following cell fixation and nonspecific binding. HBcAg was detected by fluorescence microscopy after the cells were incubated with anti-rabbit antibody.

Real-time PCR for HBV DNA detection. The HBV DNA from the selected culture supernatants was extracted. The primers targeted to HBV S region were as follows: Forward, 5′-ATGTGTCTGCG GCGTTTTATCA-3′, and reverse, 5′-AGGGTCCCGTGCTGGTT GTT -3′. Real-time PCRs were run on a LightCycler (Eppendorf) using the SYBR Premix Ex Taq kit (TaKaRa). For standard curves, a series of 10-fold dilutions was prepared, ranging from 10^8 to 10^0 copies of a constructed plasmid containing the target gene (139 bp). The program used for amplification was 95°C for 2 min followed by 40 cycles of 95°C for 5 sec, 56°C for 20 sec, and 70°C for 15 sec. The melting curve was determined by heating to 95°C. The number of HBV DNA copies was calculated according to the standard curve created by the constructed plasmid. Both positive and negative controls were included in each experiment. Determinations were

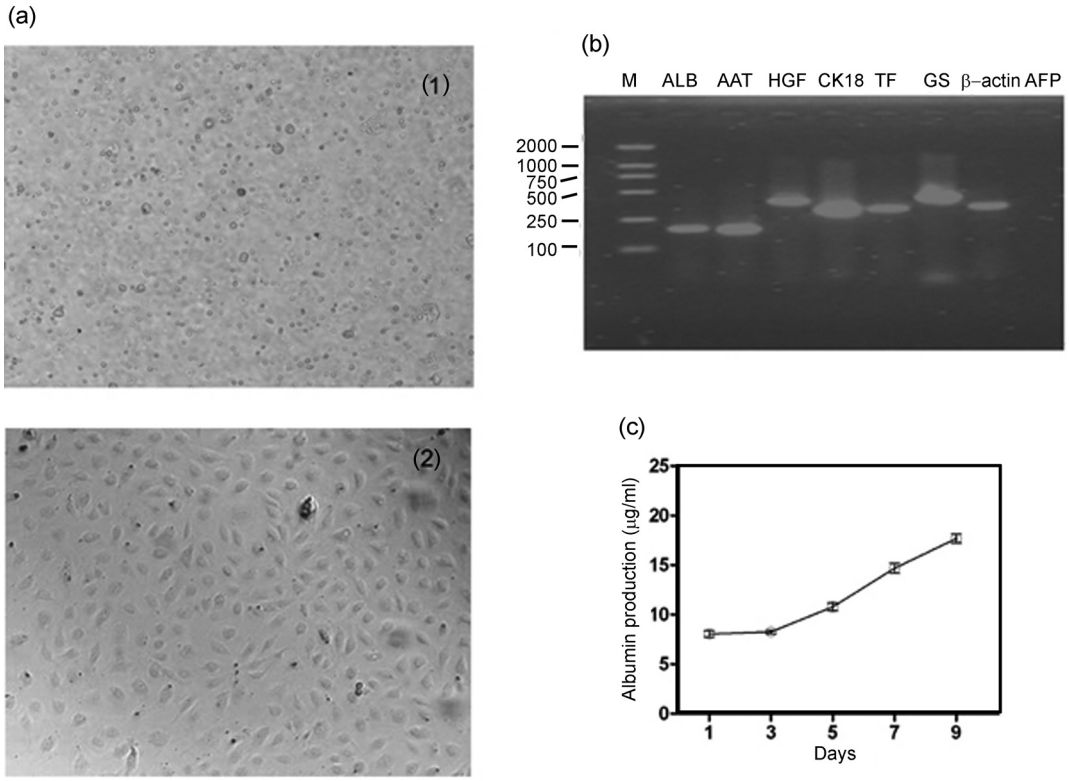

Fig. 1

Characteristics of primary hepatocyte culture

(a) Morphology on days 1 (1) and 20 (2) post seeding. (b) Expression of hepatocyte-specific genes. (c) Kinetics of albumin expression.

made in triplicate and are representative of at least three independent experiments.

Southern blot analysis of HBV DNA. Infected cells were washed and lysed, and HBV DNA packaged in core particles was isolated as previously described (Zheng *et al.*, 2005). The DNA was then subjected to Southern blot analysis using the digoxin-labeled HBV DNA probe.

Results

Establishment of a transgenic hepatocyte HC-T cell line

In order to obtain fresh human hepatocytes for successful gene transduction, we performed human hepatocyte isolation from resected liver tissue using a modified "two-step" collagenase protocol. Fig. 1a shows representative images of primary human hepatocytes in culture on day 1 and day 20 after isolation. The primary hepatocytes isolated by our method showed the same morphology as adult human hepatocytes (Fig. 1a). The transcription of hepatocyte-specific genes was analyzed by RT-PCR. The isolated primary hepatocytes expressed *ALB*, *AAT*, *HGF*, *CK18*, *TF*, and *GS*, but not *AFP* (Fig. 1b). Albumin secretion increased from day 1 to day 9, while the accumulation of albumin was a more consistent trend (Fig. 1c).

Transgenic human primary hepatocytes expressing *hTERT* were established using a retroviral infection protocol and were termed transgenic HC-T cells. The transgenic cells expressed *ALB*, *TF*, and *GS*, but not *AFP* (Fig. 2a). Liver regeneration can activate the expression of *AFP*; however, *AFP*

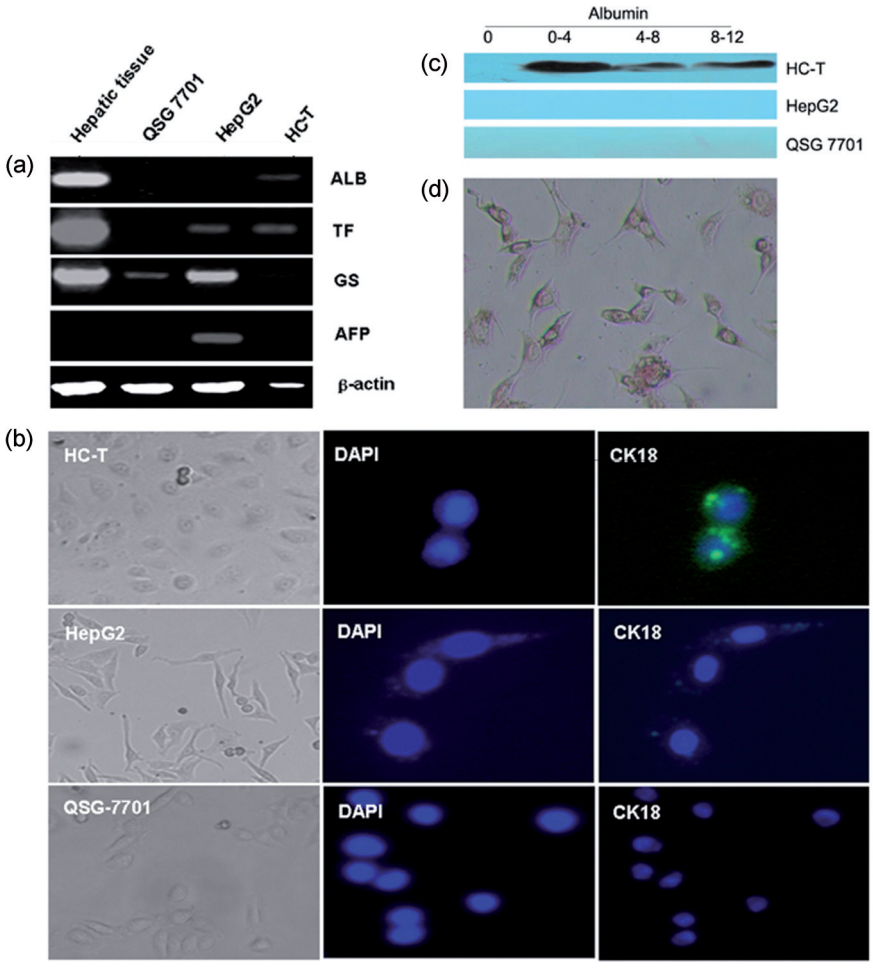

Fig. 2

Characteristics of transgenic HC-T cells in comparison with hepatoma cell lines and hepatic tissue

(a) Expression of hepatocyte-specific (ALB, TF and GS) and hepatoma-specific (AFP) genes assayed by RT-PCR. (b) Detection of CK18 by IFA. (c) Expression of albumin assayed by Western blot analysis. (d) Glycogen detected by light microscopy following staining.

expression in the transgenic HC-T cells was too low to detect using PCR. Expression of the hepatocyte-specific gene *CK18* was determined in transgenic HC-T cells at the same time. IFA showed that CK18 localized in the cytoplasm (Fig. 2b). The albumins were then synthesized (Fig. 2c) and glycogen storage detection indicated plentiful glycogenic granules in the cytoplasm (Fig. 2d). Therefore, morphologic, phenotypic, and functional analysis proved that the transgenic human primary hepatocytes possessed the same features as freshly isolated primary hepatocytes.

HBV replication in HC-T cells

In order to mimic the natural process of HBV infection, both serum-derived HBV and direct incubation protocol were utilized. Our observations suggest that HC-T cells were susceptible to HBV infection and remained infected for at least 4 weeks under natural conditions.

In order to monitor extracellular virus, we detected the HBsAg concentration and HBV DNA levels in culture supernatants by ELISA and quantitative PCR, respectively. On day seven, the expression of HBsAg reached a maximum and then decreased, but remained at a steady level throughout the infection (Fig. 3a). HBsAg level in HepG2 cells was too low and it was used as a baseline for that in infected HC-T cells. Detected by quantitative RT-PCR, using the standard curve, the HBV DNA amount remained at a steady state between $5x10^4$ and $5x10^5$ copies/ml. Though at a low level, the HBV DNA remained steady. However, this trend was not seen in the control cells (Fig. 3b).

Because extracellular viral DNA and antigen were not sufficient to definitively establish that the HC-T cells were susceptible to HBV infection, we next analyzed viral replication in the infected cells by Southern blotting. The HBV DNA packaged in core particles was detected in HBV-infected HC-T cells, showing the presence of both the RC (HBV relaxed circular DNA) and RI (HBV replicative intermediates) (Fig. 3c). Moreover, in agreement with Southern blotting results, immunofluorescence staining indicated that the core antigen was located in the cytoplasm of HC-T cells and occasionally in the nuclei (Fig. 3d). These results indicated infection of at least 10% of the HC-T cells.

Based on the above results, we ascertained that the HC-T cells were susceptible to HBV infection.

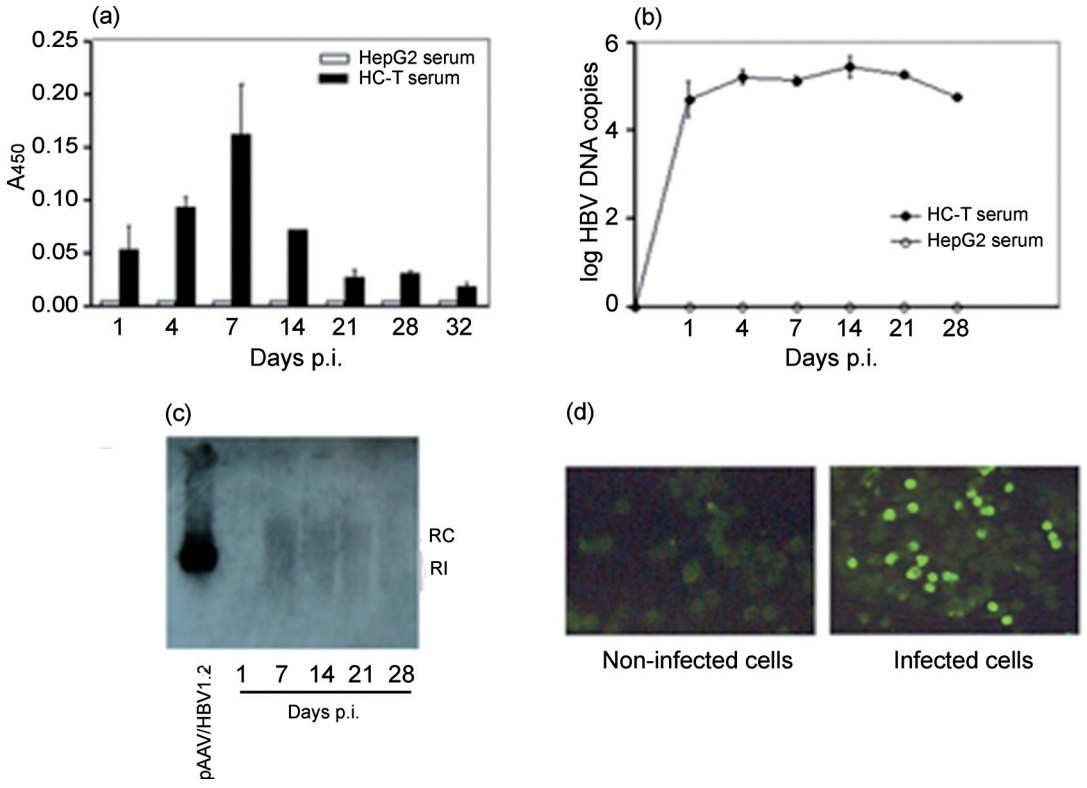

Fig. 3

HBV infection of HC-T and HepG2 cells

(a) HBsAg (A_{450}) was assayed by ELISA. (b) HBV DNA assayed by RT-PCR. (c) HBV DNA detected by Southern blot analysis. (d) HBcAg detected by IFA.

Discussion

Mature human hepatocytes immortalized with constitutively expressed exogenous *hTERT* alone can be infected with HBV derived from human positive serum. The infection of HC-T cells was robust and consistent for at least 4 weeks. The HBV DNA amplification in culture supernatants achieved in these experiments was almost $5\log_{10}$.

HBV infection was validated by several different approaches: i) Time-dependent amplification of HBV DNA in culture supernatants (as detected by RT-PCR); ii) The detection of HBsAg in culture supernatants (as detected by ELISA); iii) Viral replication in the infected cells (as detected by Southern blotting); and iv) Localization of the core antigen in the cytoplasm of infected cells (as detected by immunofluorescence staining).

Valuable studies by Gripon and his coworkers have reported that HepaRG cells were susceptible to HBV. Because the HepaRG cell line is derived from a hepatoma, the infection of HepaRG cells with HBV could not exactly mimic the natural process. In order to maintain the differentiation and virus susceptibility, utilization of HepaRG cells required the addition of corticoids and dimethyl sulfoxide (Gripon *et al.*, 2002). The main differences between the HepaRG cell line and our HC-T cell line are as follows: i) Our cells are more mature human hepatocytes; and ii) No substances were added during the infection in our culture system.

The HepG2.2.15 cells usually used are simple, transgenic cells and cannot be used to study the complete life history of virus. In contrast, the HC-T cells are permissive to naturally occurring HBV and are capable of reproducing the virus. Compared to the endothelial hepatocyte cell line QSG-7701 and carcinoma cell line HepG2, the HC-T cells share the same features of parenchymal cells, but not endothelial or carcinoma cells.

In conclusion, the establishment of transgenic human primary hepatocytes provides the foundation for studying new antiviral agents. Transgenic human primary hepatocytes are very promising for many other applications, in particular the development of certain types of antiviral drugs that interfere with early steps of viral infection.

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References

Baccarani U, Sanna A, Cariani A, Sainz-Barriga M, Adani GL, Zambito AM, Piccolo G, Risaliti A, Nanni-Costa A, Ridolfi L, Scalamogna M, Bresadola F, Donini A (2003): Isolation of human hepatocytes from livers rejected for liver transplantation on a national basis: results of a 2-year experience. Liver Transpl. 9, 506–512. http://dx.doi.org/10.1053/jlts.2003.50087

Blackburn EH (1991): Structure and function of telomeres. Nature 350, 569–573. http://dx.doi.org/10.1038/350569a0

Gripon P, Diot C, Thézé N, Fourel I, Loreal O, Brechot C, Guguen-Guillouzo C(1988): Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. J. Virol. 62, 4136–4143.

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C (2002): Infection of a human hepatoma cell line by hepatitis B virus. Proc. Natl. Acad. Sci. USA 99, 15655–15660. http://dx.doi.org/10.1073/pnas.232137699

Jiang XR, Jimenez G, Chang E, Frolkis M, Kusler B, Sage M, Beeche M, Bodnar AG, Wahl GM, Tlsty TD, Chiu CP (1999): Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. Nat. Gene21, 111–114. http://dx.doi.org/10.1038/5056

Katsura N, Ikai I, Mitaka T, Shiotani T, Yamanokuchi S, Sugimoto S, Kanazawa A, Terajima H, Mochizuki Y, Yamaoka Y (2002): Long-term culture of primary human hepatocytes with preservation of proliferative capacity and differentiated functions. J. Surg. Res. 106, 115–123. http://dx.doi.org/10.1006/jsre.2002.6446

Kwon SY, Jo SH, Lee OS, Choi SM, Kwak SS, Lee HS (2003): Transgenic ginseng cell lines that produce high levels of a human lactoferrin. Planta Med. 69, 1005–1008. http://dx.doi.org/10.1055/s-2003-45146

LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, Richert L(2005): Isolation and culture of primary human hepatocytes. Methods Mol. Biol. 290, 207–229.

Leistner CM, Gruen-Bernhard S, Glebe D (2008): Role of glycosaminoglycans for binding and infection of hepatitis B virus. Cell Microbiol. 10, 122–133.

Lv LP, Jia SZ, Wang QL, Zhang YY, Zhou HJ, Xu JB(2009): Hepatitis C virus infection of mouse hepatoma cell expressing human CD81 or LDLR. Acta Virol. 53, 185–189. http://dx.doi.org/10.4149/av_2009_03_185

Morales CP, Holt SE, Ouellette M, Kaur KJ, Yan Y, Wilson KS, White MA, Wright WE, Shay JW (1999): Absence of cancer-associated changes in human fibroblastsimmortalized with telomerase. Nat. Genet. 21, 115–118. http://dx.doi.org/10.1038/5063

Petersen J, Dandri M, Mier W, Lütgehetmann M, Volz T, von Weizsäcker F, Haberkorn U, Fischer L, Pollok JM, Erbes B, Seitz S, Urban S (2008): Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nat. Biotechnol. 26, 335–341. http://dx.doi.org/10.1038/nbt1389

Poyck PP, van Wijk AC, van der Hoeven TV, de Waart DR, Chamuleau RA, van Gulik TM, Oude Elferink RP, Hoekstra R(2008): Evaluation of a new immortalized human fetal liver cell line (cBAL111) for application in bioartificial

liver. J. Hepatol. 48, 266–275. http://dx.doi.org/10.1016/j.jhep.2007.09.018

Rabe B, Glebe D, Kann M (2006): Lipid-Mediated Introduction of Hepatitis B Virus Capsids into Nonsusceptible Cells Allows Highly Efficient Replication and Facilitates the Study of Early Infection Events. J. Virol. 80, 5465–5473. http://dx.doi.org/10.1128/JVI.02303-05

Seeger C, Mason WS (2000): Hepatitis B virus biology. Microbiol. Mol. Biol. Rev. 64, 51–68. http://dx.doi.org/10.1128/MMBR.64.1.51-68.2000

Te HS, Jensen DM (2010): Epidemiology of hepatitis B and C viruses: a global overview. Clin Liver Dis. 14, 1–21. http://dx.doi.org/10.1016/j.cld.2009.11.009

Zoulim F (2006): Antiviral therapy of chronic hepatitis B. Antiviral Res.71, 206–215. http://dx.doi.org/10.1016/j.antiviral.2006.04.003

Zheng Y, Li J, Johnson DL, Ou JH (2003): Regulation of hepatitis B virus replication bythe ras-mitogen-activated protein kinase signaling pathway. J. Virol. 77, 7707–7712. http://dx.doi.org/10.1128/JVI.77.14.7707-7712.2003