they were treated with an initial dose of fluconazole (3-6 mg/kg/day), amphotericin B (0.25 mg/kg/day), micafungin (100-150 mg/day) or flucytosine (100 mg/day). Routine surveillance cultures after transplantation were obtained from pharyngeal swabs, nasal swabs, urine, central venous lines, abdominal drains, nasogasrtic tubes, endotracheal tubes and stool. Venous and urinary catheters were routinely changed when colonization of an indwelling tube was suspected. Serum level of *Candida*, *Aspergillus* and *Cryptococcus* antigens were also measured for the diagnosis. The culture medium used was fungus specific. *Candida* antigen was detected by latex agglutination and the positive value was more than two times higher compared to control. *Asperguillus* antigen was detected by latex agglutination and this is qualitative analysis. *Cryptococcus* antigen was detected by latex agglutination and the positive value was more than eight times higher compared to control. β-D glucan was determined by test kits (Wako, Inc., Tokyo), and the cut-off value was 11 pg/ml. The timing of sampling for these parameters was twice before transplantation and twice per week for one or two months after transplantation. The value was the maximal one.

The immunosuppressive regimen consisted of cyclosporine (CsA), tacrolimus (TAC), azathioprine (AZ), mycophenolate mofetil (MMF) and methylprednisolone (MP).

Definitions of invasive fungal infection

Fungal colonization was defined as the presence of a fungus in one or more surveillance superficial cultures in the absence of any clinical symptoms or signs of infection. Invasive fungal infections were divided into definite and probable infections according to the following criteria : definite invasive fungal infection was diagnosed by one of these criteria as follows: 1) presence of fungus in the blood, cerebrospinal fluid, ascites and biopsy specimens; 2) ophthalmologic evidence; 3) isolation of fungus from bronchoalveolar lavage with radiological evidence of pneumonitis. Probable invasive fungal infection was defined as isolation of a fungus from one superficial site and more than 11 pg/ml serum level of β-D glucan or one of these two criteria together with febrile symptom above 38°C which was not responded by antibiotics for more than one week or radiological evidence of pneumonitis or esophagitis.

Clinical data analysis

Clinical data were analyzed to determine the risk factors that might contribute to the fungal infections. Preoperative factors such as gender, age at transplantation, body weight, serum total bilirubin and creatinine levels, number of pre-transplanat abdominal surgery, pre-transplanat plasmapheresis and pre-transplant fungal colonization were compiled. Intraoperative variables included duration of operation, blood loss, graft vs recipient ratio and the type of biliary reconstruction. Postoperative variables were collected until present time. This included the number of laparotomies and plasma exchange, total number of days in intensive care unit (ICU), total number of days intubated, vascular and biliary complications, number of viral and methicillin-resistant staphylococcus aureus (MRSA) infections, episodes of biopsy proven rejections, graft-recipient weight ratio. Respiratory failure was defined as requiring intubation for more than 2 weeks or re-intubation, renal failure was defined as continuous hemodiafiltration (CHDF), continuous hemofiltration (CHF) or hemodialysis (HD) was initiated.

Statistical analyses were performed using Stat View 4.5 (Abacus Concepts, Inc., Berkeley, CA, USA). Univariate statistical analysis for invasive fungal infection vs control group was performed by means of the Fisher's exact test for categorical variables and Mann-Whitney's U-test for continuous variables. *P* value less than 0.05 was considered statistically significant. The factors identified on univariate analysis to be associated with *p* value less than 0.20 were then entered into a stepwise logistic regression analysis to identify independent risk factors for invasive fungal infection.

Results

During the study period, mycoses were detected in 48 patients (50.0%) before LDLT, in 63 (65.6%) after LDLT, and in 38 (39.6%) patients during both periods (Table 1). Before LDLT *C. albicans* was isolated from 72.9% of the recipients followed by *C. glabrata* (18.8%), and others (8.3%). After LDLT, *C. albicans* was isolated from 71.4% of the recipients followed by *C. glabrata* (31.7%), yeast like fungus (15.9%), *Candida spp* (12.7%), *C. guilliermondii* (7.9%) and *C. tropicalis* (7.9%). The intestines and pharynx were the most frequent colonized sites followed by urine and stomach. Forty-eight recipients who had fungal colonization before LDLT had no symptoms of infection. Sera from 8

TABLE 1. Isolates by fungal species, site of colonization.

	Pre-Tx ($n = 48$)	Post-Tx ($n = 63$)
Species		
C. albicans	72.9% (35/48)	71.4% (45/63)
C. glabrata	18.8% (9/48)	31.7% (20/63)
Yeast like fungus	4.1% (2/48)	15.9% (10/63)
Candida spp.	10.4% (5/48)	12.7% (8/63)
C. guilliermondii	0	7.9% (5/63)
C. tropicalis	0	7.9% (5/63)
C. parapsilosis	6.3% (3/48)	4.8% (3/63)
C. krusei	4.1% (2/48)	4.8% (3/63)
Trichosporon beigelli	0	3.2% (2/63)
Aspergillus spp.	2.1% (1/48)	1.6% (1/63)
Cryptococcus luteolus	2.1% (1/48)	0
Others	8.3% (4/48)	1.6% (1/63)
Site		
Stool	64.6% (31/48)	71.4% (45/63)
Pharynx	70.8% (34/48)	63.5% (40/63)
Urine	18.8% (9/48)	28.6% (18/63)
Gastric fluid	0	14.3% (9/63)
Abdominal drain	0	12.7% (8/63)
Bile juice	0	9.5% (6/63)
Sputum	8.3% (4/48)	7.9% (5/63)
Nasal cavity	4.1% (2/48)	7.9% (5/63)
Jejunum	0	4.8% (3/63)
Blood	0	1.6% (1/63)

patients were positive for *Candida* antigens before LDLT and from 18 after LDLT. *Aspergillus* antigen was not detected in serum from any patient in our institution and *Aspergillus spp* was isolated from two recipients but it was not responsible for fungal infection. There was no patient with pneumonia or meningitis caused by *Aspergillus* species. *Cryptococcus luteolus* was isolated once before LDLT and serum *Cryptococcus* antigen was detected in one recipient after LDLT. None of the patients had severe infection caused by *Cryptococcus* species.

We experienced 2 definite and 6 probable cases of invasive fungal infections (Table 2). Most of the invasive fungal infections occurred within 2 months after LDLT. Seven recipients were diagnosed as having pneumonia by chest x-ray or CT scan and one recipient was diagnosed sepsis based on the results of an arterial blood culture. *C. albicans* was isolated from sputa of 4 out of eight recipients with a body temperature of 38°C or more and radiological evidence of pneumonia. Five out of eight recipients with invasive fungal infections died because of graft failure or multiple organ failure. No fungus was isolated from two of 6 patients with probable fungal infections who had high serum levels of β-D glucan (86, 38 pg/ml), a body temperature of 38°C or more and radiological evidence of pneumonia. Two recipients who showed an improvement of

TABLE 2. Clinical spectrum of definite and probable invasive fungal infection ($n = 8$).

	Patient No.	Diagnosis	Species	β-D glucan* (pg/ml)	Outcome
Definite invasive fungal infection					
	68	Pneumonia	*P. crinii*	169	Alive
	87	Sepsis Pneumonia	*C. albicans*	29	Dead
Probable invasive fungal infection					
	12	Pneumonia	*C. albicans* *C. glabrata*	NA	Dead
	22	Pneumonia	*Candida spp* *C. glabrata*	57	Dead
	37	Pneumonia	-	86	Dead
	39	Pneumonia	-	38	Alive
	40	Pneumonia	*C. albicans*	39	Alive
	48	Pneumonia	*C. albicans* *C. glabrata*	122	Dead

* Peak value. NA, not available.

their fungal pneumonia were treated with fluconazole and antibiotics. One recipient who was diagnosed as having *Pneumocystis carinii* pneumonia had a quite high serum level of β-D glucan (169 pg/ml) and his clinical symptoms correlated with the level of β-D glucan. He was found to be positive for *Pneumocystis carinii* in his sputa from bronchoalveolar lavage (BAL) by polymerase chain reaction (PCR) and x-ray findings revealed pneumonia; thus, he was treated with trimethoprim-sulfamethoxazole.

Twenty-six factors, including 7 pretransplantation, 4 intraoperative, and 15 posttransplantation variables, were examined by univariate analysis to determine the risk factors that predispose to invasive fungal infection (Table 3). We did not show β-D glucan as a factor, because it was not measured in the first 21 patients. But there was a significant difference between recipients with invasive fungal infections and those with noninvasive fungal infections ($p = 0.001$). The statistically significant factors were the number of posttransplant episodes of acute blood purification (plasma exchange with or without CHDF) ($p = 0.031$), hepatic vein complications ($p = 0.014$), renal failure ($p = 0.004$) and respiratory failure ($p = 0.005$), etc. Multivariate analysis showed an independent association of the development of invasive fungal infection with hepatic vein complications (OR, 26.1 [95% CI, 1.23-553.19]) and respiratory failure (OR, 52.3 [95% CI, 1.51-1,811.85]) (Table 4). Among the patients of invasive fungal infections patient no. 37 and 40 had hepatic vein complication, and no. 12, 22, 39, 40, 48, 87 had respiratory failure. Overall mortality was significantly higher in the group of recipients with invasive fungal infections than in the group of those without invasive fungal infections (62.5% [5 of 8] vs 15.9% [14 of 88]; $p = 0.0015$).

DISCUSSION

Invasive fungal infection is one of the fatal complications and influences the survival of LDLT recipients. In this report we investigated the risk factors for invasive fungal infection and the usefulness of β-D glucan as an indicator of

TABLE 3. Univariate analysis of risk factors.

	Invasive fungal infection ($n = 8$)	No invasive fungal infection ($n = 88$)	p
Pretransplantation variables			
No. of male recipients	2	37	0.466
Mean age (yr)	28.3	17.8	0.106
Mean body weight (kg)	46.1	29.9	0.047
Mean total bilirubin (mg/dl)	15.8	10.2	0.070
Mean serum creatinine (mg/dl)	0.6	0.4	0.064
Mean no. of acute blood purification	0.8	0.6	0.404
Mean no. of laparotomies	0.5	1.2	0.074
No. of patients with pre-LDLT fungal colonization	5	43	0.715
Intraoperative variables			
Mean operation time (min)	882	902	0.551
Mean blood loss (ml)	9,350	9,705	0.223
Mean graft/recipient ratio	1.153	1.906	0.015
Biliary reconstruction (duct-to-duct)	0	13	0.593
Posttransplantation variables			
Mean no. of rejection	1.3	0.7	0.573
Mean no. of laparotomies	1.1	0.8	0.446
Mean no. of acute blood purification	16.8	3.3	0.031
Mean no. of days in ICU	23.1	17.5	0.989
Mean no. of days intubated	28.1	14.2	0.011
Biliary complication	2	19	0.999
Hepatic artery thrombosis	1	7	0.515
Portal vein complication	0	6	0.999
Hepatic vein complication	2	5	0.014
CMV infection	1	13	0.999
EBV infection	0	10	0.595
MRSA infection	3	21	0.408
Renal failure	5	12	0.004
Respiratory failure	6	20	0.005
Mean no. of days in hospital	131.5	78.6	0.448

TABLE 4. Multivariate analysis of risk factors for invasive fungal infection.

Variable	Odds ratio (95% CI)	p
Hepatic vein complications	26.1 (1.23-553.19)	0.036
Respiratory failure*	52.3 (1.51-1,811.85)	0.029

*More than 2 weeks intubation.
CI, confidence interval.

this type of infection in LDLT recipients.

In our institution *Candida* spp. was the species most frequently isolated pre- and post- transplantation, and among them *C. albicans* accounted for over 70% of the isolates. This result derived from the high level of gastrointestinal colonization with *Candida* spp., manipulation of the gastrointestinal tract during surgery with increased translocation across the gut mucosal barrier, and impaired hepatic reticuloendothelial function (Matsumoto et al. 2004), as well as prolonged use of an indwelling catheter which promotes fungal colonization by providing a vascular portal of entry.

In deceased donor liver transplantation (DDLT) *Candida* spp. constitute the most frequent cause of invasive fungal infection followed by *Aspergillus* spp., and the same tendency is observed in LDLT. Among *Candida* spp., *C. albicans* was the most predominant followed by *C. glabrata, C. tropicalis, C. parapsilosis, C. krusei* (Gladdy et al. 1999; Kawagishi et al. 2004; Takakura et al. 2004). *Candida* overgrowth in the lumen of the gastrointestinal tract is secondary to changes in the bacterial flora. It has been clearly established that the presence of anaerobic bacteria in the gut conveys a protective effect as they neutralize the overgrowth of *Candida* (van der Waaij 1982). This has been the basis for the use of selective bowel decontamination regimens, the main goal of which is to maintain a healthy anaerobic flora (Arnow et al. 1996; van der Waaij et al. 1982; Wiesner et al. 1988). Some selective bowel decontamination regimens have included oral amphotericin preparations to further enhance the elimination of *Candida* from the bowel. The clinical effectiveness of this technique in reducing systemic *Candida* infection, however, remains unknown. In our experience drug resistant *C. glabrata* meight be increased in the invasive fungal infection through the intensive use of prophylactic agents with our protocol.

In our series there were 8 recipients with invasive fungal infections, including 6 probable cases, and of them 5 died. Other authors also reported that the development of invasive fungal infection after liver transplantation was associated with a high mortality rate of up to 80% due to the difficulty in making an early diagnosis of the disease (Nieto-Rodriguez et al. 1996; Gladdy et al. 1999; Fortun et al. 2002). Particularly, invasive aspergillosis was quite fatal (Fortun et al. 2002). Diagnosis of invasive fungal infection by conventional superficial cultures and fungal isolation from tissues, bronchoalveolar lavage or cerebrospinal fluid from patients with clinical symptoms has some limitations. But recently, diagnostic testing in the field of medical mycology has improved allowing an earlier diagnosis of infection and the possibility to monitor the response to therapy in a noninvasive manner. An assay that measures 1-3 β-D glucan, a panfungal cell wall component, as a potentially promising indicator of invasive fungal infection has been developed (Obayashi et al. 1995, 2004; Ostrosky-Zeichner et al. 2005; Pazos et al. 2005). Although some false positives may result from hemodialysis using certain cellulose membranes, gauzes and albumin products (Nakao et al. 1997; Sakai et al. 2000; Kato et al. 2001), we think that this method is useful for the early diagnosis of invasive fungal infection. In our experience the specificity of β-D glucan was 100%. Besides, it was very useful to monitor the results of therapy for *Pneumocystis carinii* pneumonia. However it should also be recognized that β-D glucan is not expected to detect cryptoccocosis due to the low content of 1-3-β-D-glucan in the cell wall of *Cryptoccocus* species (Miyazaki et al. 1995).

The risk factors for invasive fungal infection in liver transplantation have been reported by many authors (Gladdy et al. 1999; Avery et al. 2001; Paya 2001; Fortun et al. 2002; Singh et al. 2002; Takakura et al. 2004). But these were identified mainly from their experiences with DDLT. Therefore, we think that risk factors in LDLT recipients should be discussed. In Japan, we have experienced more than 3,000 cases of LDLT so far (Japan Liver Transplantation Society, personal communication), fungal infection is one of the fatal complications (Suzuki et al. 2000). In DDLT, bile leakage, hepatic artery thrombosis, preoperative steroids, blood transfusion during the operation, respiratory failure, hyperglycemia,

retransplantation, and renal failure were reported as independent risk factors (Nieto-Rodriguez et al. 1996; Gladdy et al 1999; Fortun et al. 2002). Some other series identified thrombocytopenia, bacterial and viral infections and pre-transplant fungal colonization as risk factors (Avery et al. 2001; Verma et al. 2005). In our series, there were no pre- nor intra-operative variables identified as risk factors (except body weight and graft/recipient ratio) by univariate analysis. This may derive from the fact that LDLT is usually set up under the condition of elective surgery and prophylaxis for fungal infection is provided before transplantation, contrary to what happens in case of DDLT. Moreover, donor-transmitted infection does not happen in LDLT. Hepatic vein complications and respiratory failure were identified as risk factors by multivariate analysis in our series, these results were comparable to the reported risk factors in DDLT. But we consider that hepatic vein complication is a characteristic risk factor for LDLT, particularly when the left lobe is used as the graft. Because in such cases once hepatic vein complications occur, liver damage becomes more serious unless proper therapy is provided. This kind of serious liver damage is an important risk for the development of invasive fungal infection.

In conclusion, invasive fungal infection is an important cause of death after LDLT. We identified two factors associated with the development of invasive fungal infection : hepatic vein complications and respiratory failure. For the prevention and treatment of invasive fungal infection in LDLT, it is important to notify these risk factors, and we need more studies on early non-invasive quantitative markers like β-D glucan.

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Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes

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See Editorial, pages 1–5

***Background/Aims*: The development of an efficient in vitro infection system for HCV is important in order to develop new anti-HCV strategy. Only Huh7 hepatocyte cell lines were shown to be infected with JFH-1 fulminant HCV-2a strain and its chimeras. Here we aimed to establish a primary hepatocyte cell line that could be infected by HCV particles from patients' sera.**

***Methods*: We transduced primary human hepatocytes with human telomerase reverse transcriptase together with human papilloma virus 18/E6E7 (HPV18/E6E7) genes or simian virus large T gene (SV40 T) to immortalize cells. We also established the HPV18/E6E7-immortalized hepatocytes in which interferon regulatory factor-7 was inactivated. Finally we analyzed HCV infectivity in these cells.**

***Results*: Even after prolonged culture HPV18/E6E7-immortalized hepatocytes exhibited hepatocyte functions and marker expression and were more prone to HCV infection than SV40 T-immortalized hepatocytes. The susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infection was further improved, in particular, by impairing signaling through interferon regulatory factor-7.**

***Conclusions*: HPV18/E6E7-immortalized hepatocytes are useful for the analysis of HCV infection, anti-HCV innate immune response, and screening of antiviral agents with a variety of HCV strains.**

***Keywords*: Immortalization; Primary hepatocytes; HCV infection; IRF-7; IRF-3; HPV18/E6E7; Innate immune response**

1. Introduction

Infection with Hepatitis C virus (HCV) is a serious problem worldwide since 3% of the world's population is chronically infected [1]. Chronic HCV may lead to liver cirrhosis and hepatocellular carcinoma. Current standard therapy utilizes the combination of pegylated interferon-α and ribavirin, which results in a sustained response in only 30–60% of patients [2–5]. Many patients, however, do not qualify for or tolerate standard therapy [6]. Thus, it is important to develop an efficient in vitro infection system for HCV to facilitate the discovery of new anti-HCV strategies. Only Huh7 cell line is permissive for replication, infection and release of the fulminant hepatitis-derived HCV-2a (JFH-1) *strain* and its chimeric derivatives [7–9]. No other hepatocyte cell lines are able to support HCV replication efficiently.

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Normal human hepatocytes are the ideal system in which to study HCV infectivity. When cultured in vitro, however, they proliferate poorly and divide only a few times [10]. Continuous proliferation could be achieved however by introducing oncogenes, such as Simian virus large tumor antigen (SV40 T) [11]. This often resulted in tumor development [12] together with numerical (aneuploidy) and structural (aberrations) chromosome abnormalities [13]. The human papilloma virus E6E7 genes (HPV/E6E7) immortalized multiple cell types that were phenotypically and functionally similar to the parental cells [14–20]. As yet, no human hepatocytes have been immortalized with HPV18/E6E7.

We established a human primary non-neoplastic hepatocyte cell line transduced with the HPV18/E6E7 that retained primary hepatocyte characteristics even after prolonged culture, and were more prone to HCV infection than those cells immortalized with SV40 T antigen. We further improved the susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infectivity by impairing interferon regulatory factor-7 (IRF-7) expression. These cells are useful to assay infectivity of HCV strains other than JFH-1, HCV replication, innate immune system engagement of HCV, and screening of anti-HCV agents. This infection system using non-neoplastic cells also suggested that IRF-7 plays an important role in eliminating HCV infection.

2. Materials and methods

2.1. Cell cultures

We obtained the approval of the Ethical Committee of Kyoto University for the use of human hepatocytes and sera obtained from HCV-positive patients. Informed consent was obtained from both the hepatocyte donor and HCV-positive patients. Primary hepatocytes (P.H.) were cultured as described [21]. HeLa, 293, Huh-7.5, and PH5CH8 cells were cultured as previously described [22]. For three-dimensional (3D) cultures, Mebiol Gel (Mebiol Inc.) was prepared according to the manufacturer's instructions.

2.2. Plasmids construction

The SV40 T, hTERT and HPV/E6E7 fragments from pAct-SVT, PCX4neo/hTERT, and pLXSN-E6E7 plasmids were inserted into pCSII-EF-RFA plasmid creating the pCSII-EF-SVT, pCSII-EF-hTERT, and pCSII-EF-E6E7 plasmids, respectively. The full-length IRF-3 and IRF-7 genes were cloned by RT-PCR using total RNA isolated from 293 cells as a template and were inserted into pcDNA3 vector. Dominant-negative forms of IRF-3 (DNIRF-3) and IRF-7 (DNIRF-7) were constructed by PCR amplification of the coding region for amino acid residues 108–427 of IRF-3 and 237-514 of IRF-7, respectively. The amplified IRF-3 fragment was cloned into pcDNA3 in frame with a FLAG epitope tag generating pcFLAG-DNIRF-3. The amplified IRF-7 fragment was cloned into pLXSH in frame with HA epitope tag generating pLXSH-HA-DNIRF-7. The pIFNβ promoter-luc and pIFNα promoter-luc plasmids were gifts from Dr. Taniguchi of the Tokyo University. The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were purchased from Invivogen (USA).

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously [22]. We used anti-SV40 T (Santa Cruz), anti-HPV18/E7 (Santa Cruz), anti-tubulin (Sigma), anti-FLAG (Sigma), and anti-HA (Sigma) antibodies.

2.4. Transfection, small interfering RNA silencing and luciferase assays

Transfection of plasmid DNA was performed using Effectene transfection reagent (Qiagen) as recommended by the manufacturer. The pLXSH-HA-DNIRF-7 plasmid was transfected into the HuS-E/2 clone; transfectants were selected in 100 μg/ml hygromycin B (Gibco). The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were separately transfected into HuS-E/2 cells followed by Zeocin (250 μg/ml) selection. After two weeks of continuous selection, cells were infected with HCV. Luciferase assays were conducted as previously described [22]. The results are presented as relative light units (RLU) normalized to the total content of protein in the cell lysates.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

Using 250 ng of total RNA as a template, we performed RT-PCR with a one-step RNA PCR kit (Takara) according to the manufacturer's instructions. The primer sets and reaction conditions used are detailed in Table 1. To measure HCV-RNA titers by real-time RT-PCR, we collected RNA from infected wells. Five hundred nanograms of total cellular RNA was analyzed for the quantity of HCV-RNA as previously described [23].

2.6. HCV infection experiment

HCV infection experiment from serum was done as mentioned before [22]. HCV-infected-serums were titrated and 1×10^5 HCV-RNA copies/ml were used for each infection experiment. Concentrated culture medium for HCV/JFH1-producing cells was prepared as previously described [7]. HCV titer in the concentrated medium was measured, adjusted and added to the cells as mentioned above.

2.7. Blocking of HCV infectivity by anti-CD81

Inhibition of HCV infectivity was performed by blocking CD81 as previously described [7].

3. Results

3.1. Establishment of immortalized primary human hepatocytes

Primary hepatocytes were isolated from liver tissue obtained from a 9-year-old male patient with Primary Hyperoxaluria who had undergone liver transplantation. Hepatocytes were left unmanipulated or transduced with CSII-EF-hTERT alone or in combination with CSII-EF-SVT or CSII-EF-E6E7 to enhance the efficiency of immortalization. After six weeks only cells transduced by the combination of hTERT and either LT or HPV18/E6E7 continued to proliferate. Initially appearing colonies with a growth advantage were picked up and expanded. SV40 T-immortalized cell clones were named HuS-T cells and given numbers from 1 to 7,

Table 1
Primer sequences and RT-PCR parameters

Genes	Primer sequence 5′–3′	PCR parameters[a]
HGF	F: AGGAGCCAGCCTGAATGATGA R: CCCTCTGATGTCCCAAGATTAGC	95, 56, 72 1 min, 45 s, 1 min
TGFα	F: ATGGTCCCCTCGGCTGGA R: GGCCTGCTTCTTCTGGCTGGCA	95, 59, 72 45 s, 30 s, 1 min
TGFβ1	F: GCCCTGGACACCAACTATTGCT R: AGGCTCCAAATGTAGGGGCAGG	95, 58, 72 45 s, 30 s, 1 min
TGFβ2	F: GATTTCCATCTACAAGACCACGAGGGACTTGC R: CAGCATCAGTTACATCGAAGGAGAGCCATTCG	95, 58, 72 45 s, 30 s, 1 min
HGFR	F: TGGTCCTTGGCGTCGTCCTC R: CTCATCATCAGCGTTATCTTC	95, 54, 72 30 s, 45 s, 1 min
EGFR	F: CTACCACCACTCTTTGAACTGGACCAAGG R: TCTATGCTCTCACCCCGTTCCAAGTATCG	95, 58, 72 45 s, 30 s, 1 min
TGFβ1R	F: CGTGCTGACATCTATGCAAT R: AGCTGCTCCATTGGCATAC	95 s, 54, 72 30 s, 45 s, 1 min
TGFβ2R	F: TGCACATCGTCCTGTGGAC R: GTCTCAAACTGCTCTGAAGTGTTC	95, 58, 72 45 s, 30 s, 1 min
FGFR	F: ATGTGGAGCTGGAAGTGCCTC R: GGTGTTATCTGTTTCTTTCTCC	95, 54, 72 30 s, 45 s, 1 min
IGF-1R	F: ACCCGGAGTACTTCAGCGCT R: CACAGAAGCTTCGTTGAGAA	95, 54, 72 30 s, 45 s, 1 min
HNF1α	F: GTGTCTACAACTGGTTTGCC R: TGTAGACACTGTCACTAAGG	95, 52, 72 45 s, 30 s, 1 min
HNF1β	F: GAAACAATGAGATCACTTCCTCC R: CTTTGTGCAATTGCCATGACTCC	95, 52, 72 1 m, 45 s, 1 min
HNF3β	F: CACCCTACGCCTTAACCAC R: GGTAGTAGGAGGTATCTGCGG	95, 56, 72 1 m, 45 s, 1 min
HNF4	F: CTGCTCGGAGCCACAAAGAGATCCATG R: ATCATCTGCCACGTGATGCTCTGCA	95, 58, 72 45 s, 30 s, 1 min
Albumin	F: AGTTTGCAGAAGTTTCCAAGTTAGTG R: AGGTCCGCCCTGTCATCAG	95, 55, 72 45 s, 30 s, 1 min
Apolipoprotein-a	F:AGGCTCGGCATTTCTGGCAG R: TATCCCAGAACTCCTGGGTC	95, 55, 72 45 s, 30 s, 1 min
HTF	F: TCGCTACAGCCTTTGCAATG R: TTGAGGGTACGGAGGAGTTCC	95, 55, 72 45 s, 30 s, 1 min
E-cadherin	F: TCCATTTCTTGGTCTACGCC R: TTTGTCCTACCGACTTCCAC	95, 55, 72 45 s, 30 s, 1 min
CYP 1B1	F: CACCAAGGCTGAGACAGTGA R: GCCAGGTAAACTCCAAGCAC	94, 57, 72 30 s, 30 s, 1 min
CYP 2C9	F: GGACAGAGACGACAAGCACA R: TGGTGGGGAGAAGGTCAAT	94, 57, 72 30 s, 30 s, 1 min
CYP 2B	F: GGCACACAGCCAAGTTTACA R: CCAGCAAAGAAGAGCGAGAG	94, 57, 72 30 s, 30 s, 1 min
CYP 3A4	F: TGTGCCTGAGAACACCAGAG R: GCAGAGGAGCCAAATCTACC	94, 57, 72 30 s, 30 s, 1 min
CYP 2E1	F: CCGCAAGCATTTTGACTACA R: GCTCCTTCACCCTTTCAGAC	94, 57, 72 30 s, 30 s, 1 min
CYP 1A1	F: AGGCTTTTACATCCCCAAGG R: GCAATGGTCTCACCGATACA	94, 57, 72 30 s, 30 s, 1 min
GAPDH	F: CCATGGAGAAGGCTGGGG R: CAAAGTTGTCATGGATGACC	95, 8, 72 45 s, 30 s, 1 min

Table 1 (*continued*)

Genes	Primer sequence 5′–3′	PCR parameters[a]
CD81	F: CTCAACTGTTGTGGCTCCAAC R: CCAATGAGGTACAGCTTCCC	95, 55, 72 45 s, 30 s, 1 min
TLR3	F: GATCTGTCTCATAATGGCTTG R: GACAGATTCCGAATGCTTGTG	95, 55, 72 45 s, 30 s, 1 min
TLR7	F: CCAGACATCTCCCCAGCGTC R: GGCAAAACAGTAGGGACGGC	95, 55, 72 45 s, 30 s, 1 min
TLR8	F: CTGTGAGTTATGCGCCGAAG R: CGGGATTTCCGTTCTGGTGC	95, 55, 72 45 s, 30 s, 1 min
Myd88	F: GGTCTCCTCCACATCCTCCC R: CCAGCTTGGTAAGCAGCTCG	95, 55, 72 45 s, 30 s, 1 min
IRF3	F: GAACCCCAAAGCCACGGATC R: CCTCCCGGGAACATATGCAC	95, 55, 72 45 s, 30 s, 1 min
IRF7	F: GTGCTGTTCGGAGAGTGGCTC R: CAGCCCAGGCCTTGAAGATG	95, 55, 72 45 s, 30 s, 1 min

CYP, cytochrome P450; EGFR, epidermal growth factor receptor; F, forward primer; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde phosphate dehydrogenase; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor; HNF, hepatocyte nuclear factor; HTF, human transferrin; IGF-1R, insulin-like growth factor-type I receptor; IRF, interferon regulatory factor; R, reverse primer; TGF, transforming growth factor; TGFR, transforming growth factor receptor; TLR, toll like receptor.

[a] Temperatures are tabulated in the first lane in degrees celsius and the corresponding times in the second lane. Performing one-step RT-PCR, reverse transcription was carried out at 42 °C for 20 min with a pre-PCR denaturation at 95 °C for 10 min.

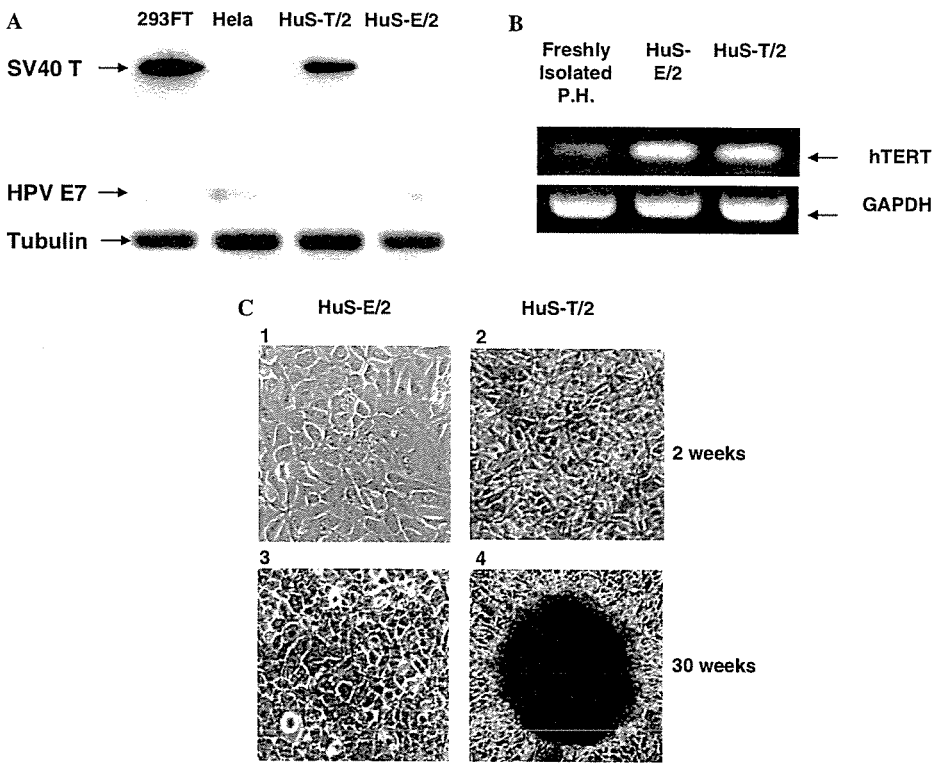

Fig. 1. (A) Immunoblot detection of SV40 T and HPV E7 expression in HuS-T/2 and HuS-E/2 cells, respectively. 293-FT and HeLa cells were used as positive controls for SV40 T and HPV E7 expression, respectively. The specific bands representing the targets are indicated. Detection of tubulin expression in all cells served as an internal control. (B) Human Telomerase Reverse Transcriptase (hTERT) expression was examined by RT-PCR in freshly isolated hepatocytes and the HuS-E/2 and HuS-T/2 cell lines. GAPDH expression was used as an internal control. The hTERT-specific bands are shown. (C) Morphological characteristics of HuS-E/2 and HuS-T/2 cells after two (panels 1 and 2) and 30 (panels 3 and 4) weeks in culture. [This figure appears in colour on the web.]

while the HPV18/E6E7-immortalized clones were named HuS-E cells and given numbers from 1 to 4. Expression of SV40 T and HPV E7 proteins was detected in the appropriate cells by immunoblot analysis (Fig. 1A). In both immortalized cell lines, expression of hTERT-mRNA was enhanced in comparison to non-transduced, freshly isolated hepatocytes as determined by RT-PCR (Fig. 1B). HuS-E cells were larger in size and exhibited slower growth than HuS-T cells (Fig. 1C).

3.2. Characterization of HuS-E and HuS-T immortalized hepatocytes

The HuS-E/2 and HuS-T/2 clones demonstrated the highest expression of hepatocyte-specific markers and transcription factors by RT-PCR (data not shown); these cells were used as representative for each group in this study. To address if HuS-E/2 and HuS-T/2 maintained similar characteristics as primary hepatocytes, they were both cultured continuously for 30 weeks and the expression profiles of a variety of growth factors (Fig. 2A), growth factor receptors (Fig. 2B), hepatocyte-specific nuclear factors (Fig. 2C), albumin, apolipoprotein-A1, transferrin (Fig. 2D), cytochrome p450 (CYP) genes (Fig. 2E), and GAPDH were compared with freshly isolated primary hepatocytes after isolation or two weeks of culture, Huh-7.5 cells, and 293 cells. After two weeks in culture, the expression of nearly all examined genes was similar between freshly isolated hepatocytes and the HuS-E/2 cell line. HuS-E/2 cells, however, exhibited higher expression of TGFβ2 (Fig. 2A), TGFβ2R, and HGFR (Fig. 2B) and lower expression of CYP 3A4 and 2C9 (Fig. 2E) in comparison to freshly isolated hepatocytes. Primary hepatocytes displayed reduced expression of TGFβ1 and TGFβ2 (Fig. 2A) and a loss of CYP1A1 expression (Fig. 2E) after two weeks of culture. HuS-E/2 cells exhibited higher expression of HGF (Fig. 2A), HGF receptor (Fig. 2B), HNF-4, (Fig. 2C), albumin, apolipoprotein-A1, HTF, and E-cadherin (Fig. 2D) in comparison to HuS-T/2 cells. Expression of CYP 3A4 (Fig. 2E) was lost from both HuS-T/2 and HuS-E/2 cells, while HuS-T/2 cells also lost the expression of HNF-1α (Fig. 2D), and CYPs 2B, 2E1 (Fig. 2E).

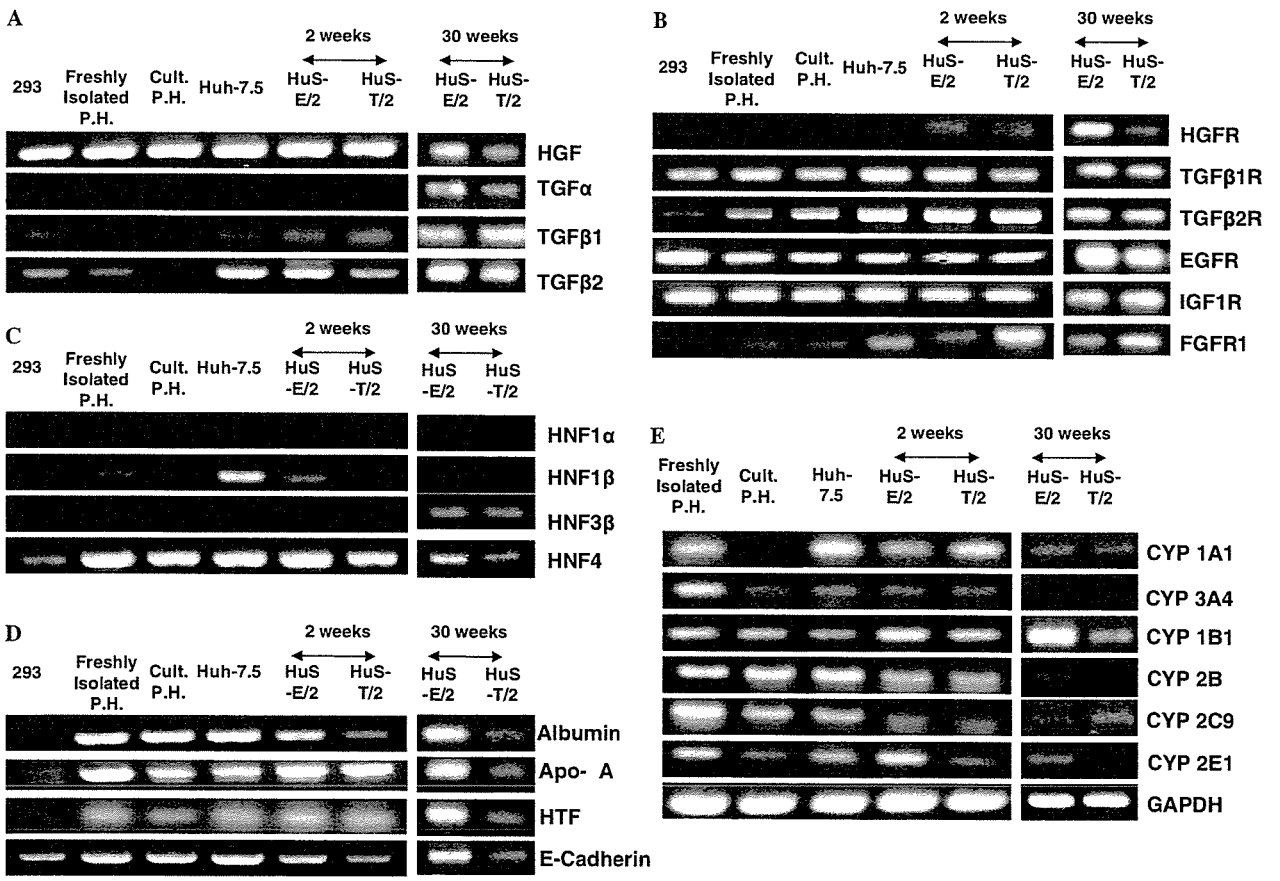

Fig. 2. Expression of the genes encoding growth factors (A), growth factor receptors (B), hepatocyte-specific nuclear factors (C), hepatocyte differentiation and functional markers (D), and CYP enzymes (E) in 293 cells, freshly isolated primary hepatocytes (P.H.), primary hepatocytes cultured for two weeks (Cult. P.H.), Huh-7.5 cells, and HuS-E/2 and HuS-T/2 cells cultured for two and 30 weeks were investigated by RT-PCR. The bands representing specific targets are indicated in the representative reactions.

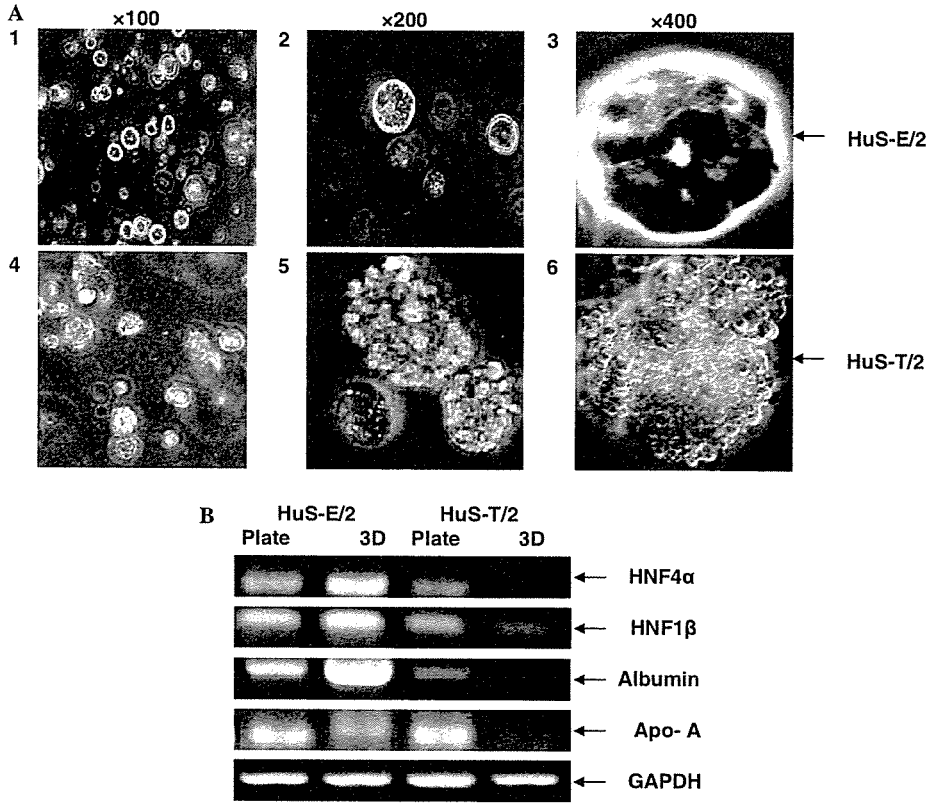

Fig. 3. (A) The morphology of HuS-E/2 and HuS-T/2 cells in 3D culture. HuSE/2 and HuS-T/2 cells were cultured in Mebiol Gel in 12-well plates at a concentration of 5×10^5 cells/well. The microscopic characteristics of these cells after one week of 3D culture are shown. (B) The expressions of HNF4α, HNF1β, albumin, and apo-A by HuS-E/2 and HuS-T/2 cells in both flat and 3D cultures are detailed. After one week of culture of HuS-E/2 and HuS-T/2 cells in flat and 3D cultures, the expressions of HNF4α, HNF1β, albumin, and apo-A were measured by RT-PCR in 250 ng total RNA.

HuS-T/2 but not in HuS-E/2 cells showed a transformed-like character starting from the 13th week of culture. This was demonstrated by continuing proliferation after confluence, pile-up formations (Fig. 1C), and proliferating in serum-deprevied condition. However, HuS-E/2 cells did not show any transformed-like characters even after 30 weeks of culture.

3.3. The characteristics of HuS-E and HuS-T immortalized hepatocytes in 3D culture

After one week in 3D culture, HuS-E/2 (Fig. 3A, panels 1, 2, and 3) cells adopted a donut-shaped structure with a central pore, while HuS-T/2 cells (Fig. 3A, panels 4, 5, and 6) displayed irregular mass formations (similar to the growth pattern of Huh-7.5 cells in 3D culture (data not shown)). In 3D culture, while the expression of HNF4, HNF1β, and albumin was enhanced in HuS-E/2, it was decreased in HuS-T/2 cells (Fig. 3B).

3.4. HCV infection to HuS-E/2

We further assessed the HCV infectivity of HuS-E- and HuS-T-derived clones by infection with HCV-1b-infected serum. Of the three HuS-E clones examined, HuS-E/2 clone demonstrated the highest infectability with HCV genotype 1b in comparison to Huh-7.5, PH5CH8 (Fig. 4A), and HuS-T cells (data not shown), which were excluded from further experiments.

3.5. Anti-CD81 blocked HCV infectivity

CD81 is involved in the entry of HCV pseudoparticles [24] and in vitro-synthesized JFH-1 [7]. To determine if authentic viral particles follow the same route of entry when infecting HuS-E/2 cells, we first examined the CD81 expression by RT-PCR. Both HuS-E/2 and HuS-T/2 cells expressed similar amounts of CD81 as freshly isolated hepatocytes and Huh-7.5 cells (Fig. 4B). Antibodies against CD81 reduced HCV infectivity of HuS-E/2 cells from the levels seen using a non-specific control antibody, confirming the importance of CD81 in HCV infectivity (Fig. 4C).

3.6. IFNα blocked HCV infectivity

We treated HuS-E/2 cells with HCV-containing serum. Cells were then cultured in fresh medium supplemented

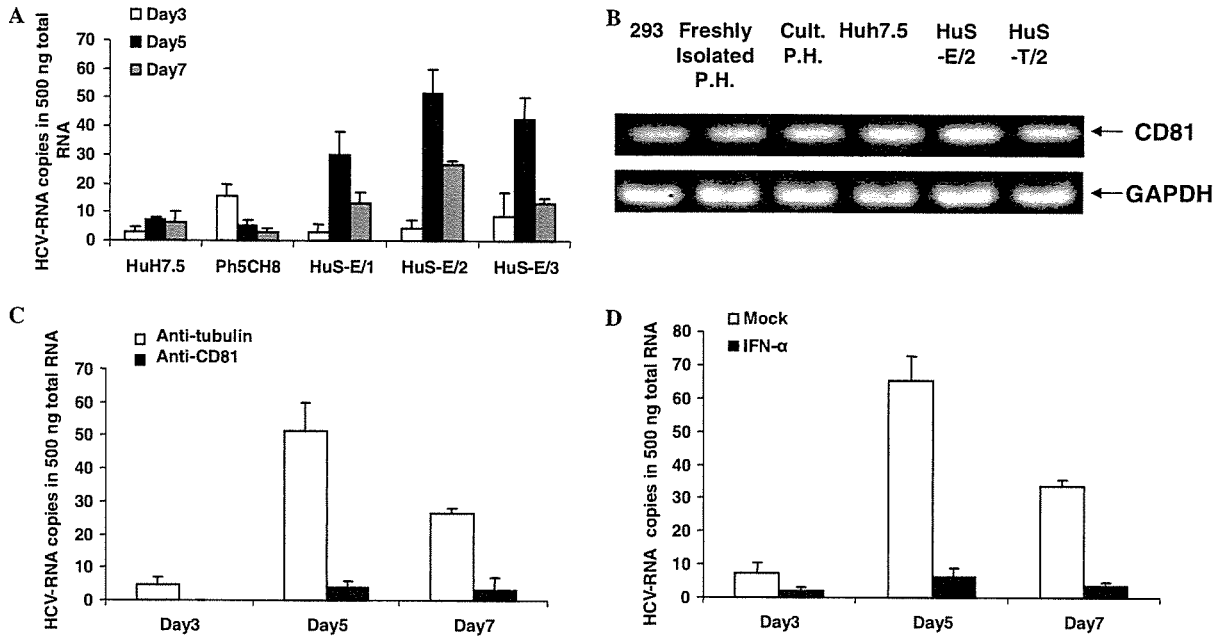

Fig. 4. (A) Serum from an HCV patient was used to infect Huh-7.5 cells, PH5CH8 cells, and three HPV E6E7-immortalized clones (HuS-E/1-3) for 24 h. After washing three times in phosphate-buffered saline (PBS), cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (B) HuS-E/2 and HuS-T/2 cells both expressed CD81. Expression of CD81 (upper panel) and GAPDH as an internal control (lower panel) in 293 cells, freshly isolated P.H., cultured P.H., and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (C) Anti-CD81 antibodies blocked HCV infectivity. HCV infection was performed as described in (A) with the addition of CD81-specific (black bar) or anti-tubulin antibodies (control, white bar). (D) IFNα inhibits HCV multiplication in HuS-E/2 cells infected with HCV-containing serum. HuS-E/2 cells were infected with HCV as described in (A). After washing three times with PBS, cells were cultured in fresh medium supplemented with (black bar) or without (white bar) 100 U/ml IFNα.

without or with 100 U/ml IFNα. The enhancement of the HCV-RNA genome titers on the fifth day (about 10-fold) was not observed in cells treated continuously with IFNα (Fig. 4D). This result suggests that IFNα inhibited HCV replication in infected HuS-E/2 cells.

3.7. The effect of blocking IRF-3 and IRF-7 signaling on HCV infectivity

Production of interferon-alpha (IFNα) and interferon-beta (IFNβ) limits viral replication and spread, providing one of the most effective innate antiviral responses [25]. Signaling through IRF-3 and IRF-7 plays important roles in the stimulation of IFN-α/β production [25]. To determined which molecules (IRF-3 or IRF-7) play an important role in modulation of the innate immune response against HCV infection in these cells, we first detected intrinsic expression of double-stranded RNA-stimulated Toll-like receptor (TLR) 3, the downstream effector IRF-3, single-stranded RNA-stimulated TLR7, and 8, and the downstream effectors MyD88 and IRF-7 by RT-PCR. TLR3 exhibited very low expression in freshly isolated hepatocytes, Huh-7.5, HuS-E/2, and HuS-T/2 cells, while TLR7, TLR8, MyD88, and IRF-7 were easily detectable in both freshly isolated and immortalized cell lines (Fig. 5A).

The abilities of DNIRF-3 and DNIRF-7 to inhibit IFNβ and IFNα production by HuS-E/2 cells infected with Sendai virus were confirmed using assays of IFNβ or IFNα promoter-driven luciferase reporters. DNIRF-3 exhibited strong inhibition of IFNβ production (Fig. 5B) and weaker inhibition of IFNα transcription (Fig. 5C), while DNIRF-7 strongly inhibited IFNα production (Fig. 5C) and only weakly inhibited IFNβ production (Fig. 5B).

We then assessed the inhibition of HCV infectivity by DNIRF-3 and DNIRF-7. Transient transfection with DNIRF-3, DNIRF-7, or an empty vector was performed prior to HCV infection. Using Effectene reagent, the efficiency of plasmid transfection into HuS-E/2 cells was approximately 70% (data not shown). While there was no significant effect of DNIRF-3 on HCV infectivity, DNIRF-7 demonstrated a marked increase in HCV titers on days 3 and 5 after infection in comparison to control cells (Fig. 5D). To confirm that the enhancement of HCV replication by DNIRF-7 is not mediated by the impairment of IRF-3 signaling by heterodimeric interactions between IRF-3 and DNIRF-7, we performed siRNA inhibition of IRF-3 and IRF-7. The reduction of IRF-3 and IRF-7 expression by siRNA was obvious by RT-PCR (Fig. 5E). siRNA-mediated suppression of either IRF-3 or IRF-7 inhibited IFNβ and IFNα production

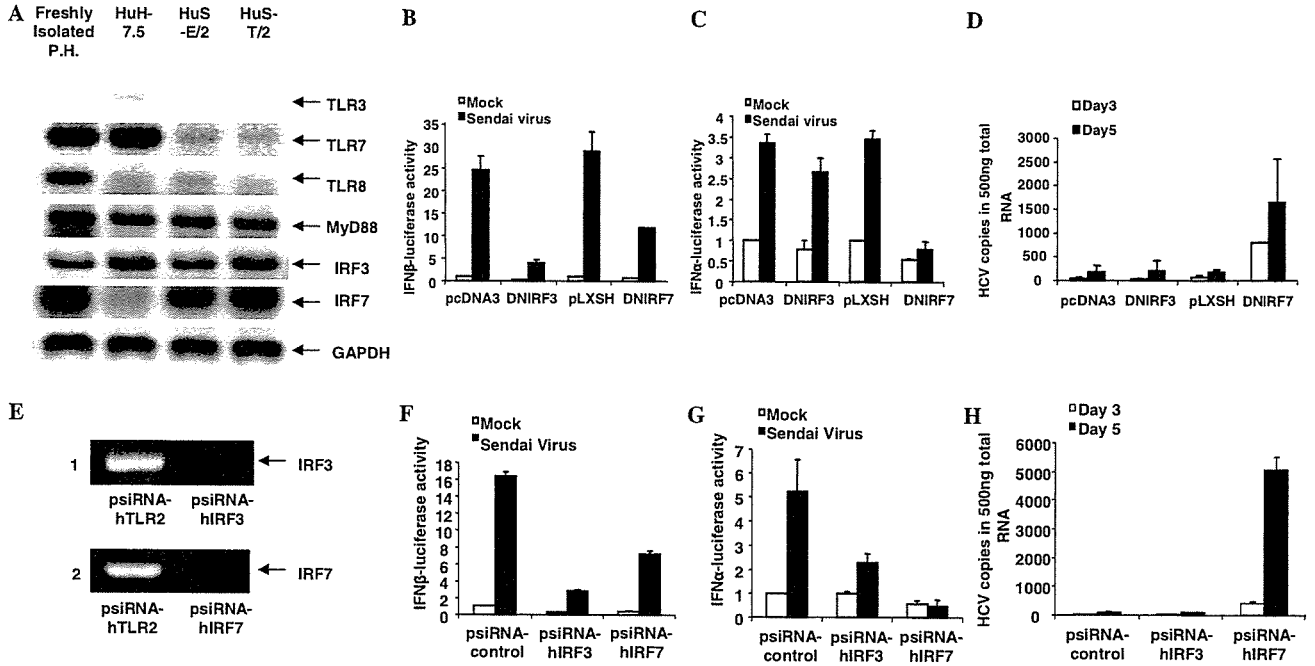

Fig. 5. (A) We examined the expression of TLR3, TLR7, TLR8, MyD88, IRF-3, and IRF-7, as well as GAPDH as an internal control in freshly isolated primary hepatocytes and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (B and C) HuS-E/2 cells were cotransfected with pIFNβ-luc (B) or pIFNα-luc (C) with an expression plasmid encoding DNIRF-3, DNIRF-7, or the appropriate empty vector (pcDNA3 and PLXSH, respectively). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (D) IRF-7, but not IRF-3, suppression enhanced HCV infectivity of HuS-E/2 cells. HuS-E/2 cells were transiently transfected with empty pcDNA3, DNIRF-3, empty pLXSH, or DNIRF-7 plasmids. Twenty-four hours later, serum from a patient with HCV was used to infect transfected cells for 24 h. After washing, cells were cultured in fresh medium. The cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (E) IRF-3 and IRF-7 levels were suppressed by specific siRNAs. HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, then selected with Zeocin at 250 μg/ml. Two weeks later, cells were harvested and assessed for the expression of IRF-3 and IRF-7 by RT-PCR. (F and G) HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, followed by selection in Zeocin at 250 μg/ml. Two weeks later, cells were cotransfected with pIFNβ-luc (F) or pIFNα-luc (G). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (H) Transfected cells were infected with serum from HCV patient; HCV infectivity was assessed as described above.

in HuS-E/2 cells infected with Sendai virus in patterns similar to the effects seen following DNIRF-3 and DNIRF-7 expression, respectively (Figs. 5F and G). Blockade of IRF-7 expression resulted in a significantly higher titer of HCV after infection, while IRF-3 down-regulation did not have any significant effect on HCV titers (Fig. 5H). The enhancement of IRF-7 silencing by siRNA improved the infectivity of HCV (data not shown). These results suggest that IRF-7 plays the major role in the innate immune response to HCV in HuS-E/2 cells.

3.8. Establishment of stable DNIRF-7 expressing clones derived from HuS-E/2 cells

Since DNIRF-7 enhanced HCV infectivity, we transduced the plasmid encoding DNIRF-7 and a hygromycin-B resistance gene, into HuS-E/2 cells. Following selection with hygromycin-B, we obtained the HuS-E7/DN22 and HuS-E7/DN24 clones. As detected by RT-PCR, both clones demonstrated similar expression levels of albumin, apolipoprotein-A1, and HNF4 as the parental HuS-E/2 cells (Fig. 6A). The HuS-E7/DN24 clone exhibited stronger expression of DNIRF-7 than the HuS-E7/DN22 clone by immunoblotting (Fig. 6B). The induction of IFNα in HuS-E7/DN24 in response to infection with an RNA virus (Sendai virus) was low in comparison to the parental HuS-E/2 and HuS-E7/DN22 clones, as detected by IFNα-luciferase reporter assay (Fig. 6C). HuS-E7/DN24 also exhibited a higher HCV infectability in comparison to parental HuS-E/2 cells and the HuS-E7/DN22 clone (Fig. 6D).

3.9. Infection of HuS-E7/DN24 cells with different HCV genotypes

Huh7.5 and HuS-E7/DN24 cells were separately infected with serums derived from 3 different HCV-patients or by JFH-1 concentrated medium (HCV-2a). Two serums were infected by HCV-1b, while the third by HCV-2b. Inoculated virus titer was adjusted to be the same in all cases. Except for JFH-1, which efficiently

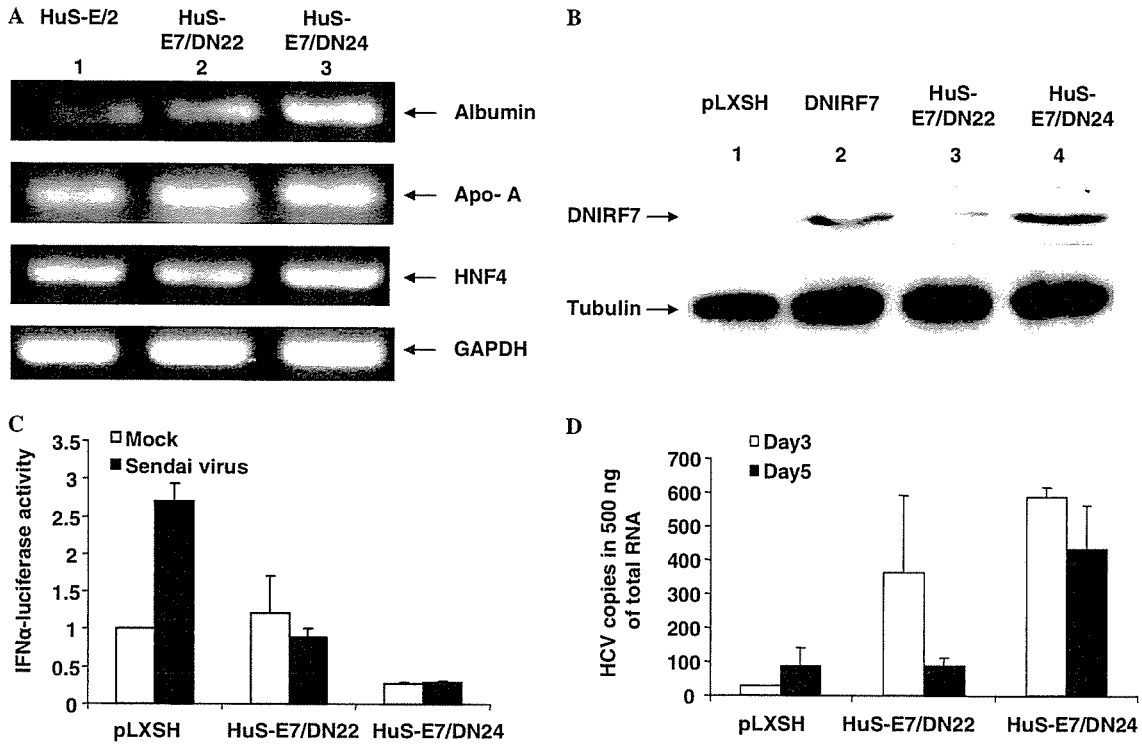

Fig. 6. (A) The pLXSH-HA-DNIRF-7 plasmid was transfected into HuS-E/2 cells, followed by selection in 100 μg/ml Hygromycin B. Two clones, HuS-E7/DN22 (lane 2) and HuS-E7/DN24 (lane 3), were obtained. We investigated the expression of albumin, apo-A, HNF4, and GAPDH as an internal control in parental HuS-E/2, HuS-E7/DN22, and HuS-E7/DN24 hepatocytes cultured for two weeks by RT-PCR. (B) Expression of HA-tagged DNIRF-7 (upper panel) and tubulin (control, lower panel) was detected by immunoblotting analysis. HuS-E/2 cells transiently transfected with either empty pLXSH vector (lane 1) or pLXSH-HA-DNIRF-7 (lane 2) were used as negative and positive controls, respectively, after 48 h. (C) HuS-E/2, HuS-E7/DN24, and HuS-E7/DN22 cells were transfected with IFNα-luc. HuS-E/2 cells were also cotransfected with pLXSH. All of these cells were then infected (black bar) or with Sendai virus or mock-infected, then analyzed for luciferase activity after 12 h. (D) HuS-E7/DN24 cells exhibited high infectivity to HCV samples derived from patient serum. HuS-E/2 cells were transiently transfected with empty pLXSH. Twenty-four hours later, serum from a recurrently transplanted HCV patient was used to infect transfected cells and HuS-E7/DN22 and HuS-E7/DN24 cells for 24 h. After washing three times, cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points.

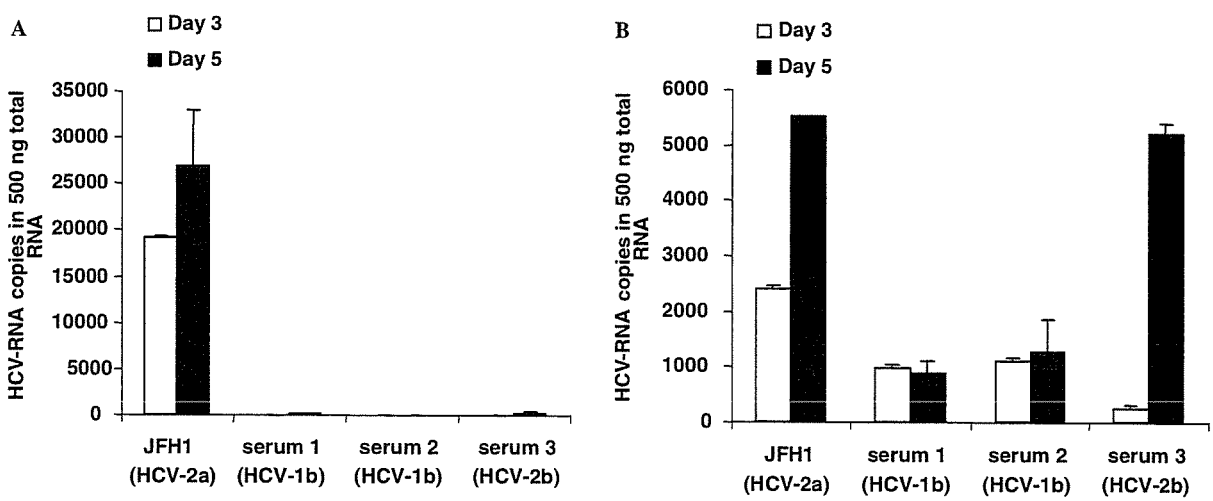

Fig. 7. The infectability of Huh-7.5 and HuS-E7/DN24 cells to different HCV genotypes. Huh-7.5 (A) and HuS-E7/DN24 (B) cells were infected with same titer of JFH1 (HCV-2a), two different HCV-1b serums and one HCV-2b serum. After removing the infected medium, the cells were washed in PBS and recultured in fresh medium. Cells were harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng RNA was detected by real-time RT-PCR analysis.

replicated in Huh7.5 cells (Fig. 7A), HuS-E7/DN24 cells showed a higher and reproducible infectability for the different HCV strains than Huh7.5 cells (Fig. 7B). Similar higher infectability of HuS-E7/DN24 cells was observed with HCV-4a genotype (unpublished data). These results suggest that the high infectability of Huh-7.5 with JFH-1 is specific among the combinations of HCV strains and cell lines; while HuS-E7/DN24 cells were generally permissive to HCV-infected serum independent of HCV strains.

4. Discussion

This study demonstrates that ectopic expression of the HPV18/E6E7 genes in combination with hTERT could efficiently immortalize mature human hepatocytes, generating a cell line with stable expression of hepatocyte markers and functions for more than 30 weeks in culture. HuS-E/2 cells continuously exhibited higher expression of both HGF and HGFR than HuS-T/2 cells. This result suggests that HPV18/E6E7-immortalized hepatocytes maintain responsiveness to paracrine signals capable of inducing cell differentiation to a greater extent than SV40 T-immortalized hepatocytes. This conclusion is further supported by the increased expression of HNF4 in HuS-E/2 cells in comparison to HuS-T/2 cells. HNF4 is a major hepatocyte transcription factor, required for hepatocyte differentiation and liver-specific gene expression [26]. HNF4 drives hepatocytes differentiation by acting upstream in a transcription factor cascade that included HNF1α [27]. HuS-E/2 cells continued to express HNF1α throughout prolonged culture, while HuS-T/2 cells lost expression completely. Maintenance of hepatocellular functions was demonstrated by continuous and high expression of albumin, apolipoprotein-A, human transferrin, and E-cadherin by HuS-E/2 in comparison to HuS-T/2 cells. These differences became more pronounced in the late passages. In a similar manner, HuS-E/2 cells continued to express all of the examined CYP genes, with the exception of CYP 3A4, while HuS-T/2 cells lost expression of CYP 3A4, 1B, and 2E1 completely and displayed markedly lower expression of CYP 1B1 than HuS-E/2 cells. Thus, human hepatocytes immortalized by HPV E6/E7 transfection are phenotypically similar to primary hepatocytes, even during extended cultures.

Recently, it was reported that the JFH-1 strain and derived chimeras could only infect and propagate efficiently in Huh7.5.1 and Huh7.5 cells, both of which are subclones of Huh7 cells [7–9]. This limitation, however, may be specific to the JFH-1 **strain**, which may not accurately reflect the course of other HCV strains' infection. Thus, usage of HCV particles isolated from patient serum could be more useful to study authentic HCV infection. Using sera from HCV patients as a source of infective virus, HPV18/E6E7-immortalized cell lines exhibited higher reproducible susceptibility to HCV infection than HuS-T, PH5CH8, and Huh-7.5 cell lines.

IRF3 and IRF7 play an important role in the activation of interferon signaling [28]. We suppressed the functions of IRF-3 or IRF-7 to assess their role in HCV infectivity. In fact, we observed significant increase of HCV replication in HuS-E/2 cells bearing dominant-negative IRF7 that impaired IFN signaling. The suppression of IRF-3, however, did not have any significant effect on HCV infectivity or replication in this cell line. This may result from the blockade of IRF-3 activation by an HCV NS3/4A serine protease [29] through at least two independent pathways that inhibit the TLR3-dependent and RIG-I-dependent signaling pathways [29–33]. Although HCV was shown to inhibit basal expression levels of IRF-7 at both mRNA and protein levels and it was shown that NS5A suppresses IRF-7-induced IFNα promoter activation [34], Stimulation of TLR7 was shown to activate IRF-7 and induce suppression of HCV replicon levels in Huh-7 cells [35]. This suggests that the inhibition of IRF7 by HCV is not complete. Using IRF-7-deficient (IRF-7−/−) mice, Honda [36] demonstrated that the transcription factor IRF-7 is essential for the induction of IFNα/β genes. We established a clone stably expressing DNIRF-7 (HuS-7E/DN24), which demonstrated higher infectivity with different HCV strains than the parental HuS-E/2 clone.

In summary, we have established a human hepatocyte-derived cell line that maintains the characteristic features of primary hepatocytes by transduction with HPV18/E6E7. This cell line is highly infectable by HCV, which suggests that these cells may be useful to characterize the molecular mechanisms involved with HCV infection and to develop novel HCV treatment modalities.

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Clinical Outcomes of Living Donor Liver Transplantation for Hepatitis C Virus (HCV)-Positive Patients

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Background. Whether hepatitis C virus recurrence occurs earlier and with greater severity for living donor liver transplantation (LDLT) than for deceased donor liver transplantation (DDLT) has recently become a subject of debate.
Methods. We retrospectively evaluated clinical outcomes for a cohort of 91 HCV-positive patients who underwent LDLT at Kyoto University with a median follow-up period of 25 months.
Results. Overall 5-year patient survival for HCV patients was similar to that for non-HCV patients (n=209) who underwent right-lobe LDLT at our institute (69% vs. 71%). Survival rate of patients without HCC (n=34) tended to be better than that of patients with HCC (n=57) (82% vs. 60%, $P=0.069$). According to annual liver biopsy, rate of fibrosis progression to stage 2 or more (representing significant fibrosis) was 39% at 2 years after LDLT. Univariate analysis showed that female recipient and male donor represented significant risk factors for significant fibrosis. Progression to severe recurrence (defined as the presence of liver cirrhosis (F4) in a liver biopsy and/or the development of clinical decompensation) was observed in five patients.
Conclusions. Postoperative patient survival was similar for HCV-positive and -negative recipients in our adult LDLT series. Rates of progression to severe disease due to HCV recurrence seemed comparable between our LDLT recipients and DDLT recipients described in the literature. Although longer-term follow-up is required, our results suggest that LDLT can produce acceptable outcomes also for patients suffering from HCV-related cirrhosis.

Keywords: Hepatitis C virus, Living donor, Recurrence.

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Chronic hepatitis C virus (HCV) has become a global epidemic, with an estimated 200 million people currently infected worldwide. Nowadays, HCV-related cirrhosis is the most common indication for liver transplantation. However, recurrence of HCV infection is universal and often occurs immediately after transplantation (*1*). The prevalence of chronic hepatitis C in HCV-positive liver transplant recipients is 70–90% after 1 year, and rate of fibrosis progression is accelerated so that 20–40% of patients progress to allograft cirrhosis within 5 years (*2–6*). As a result, graft and patient survival is significantly reduced for HCV-positive recipients compared with HCV-negative recipients (*6–7*).

In Japan, too, HCV-related cirrhosis and hepatocellular carcinoma (HCC) represent the most prevalent liver diseases, and living donor liver transplantation (LDLT) has become a treatment option for patients with these diseases. However, a warning was recently issued by some Western transplant centers that HCV recurrence may occur earlier and with greater severity, and graft loss caused by recurrent HCV may be more frequent for LDLT than for deceased donor liver transplantation (DDLT) (*8–11*). Some suggestions have been offered for mechanisms that could increase graft damage in HCV-infected LDLT recipients (*12*). First, because the right hepatic lobe graft undergoes intense regeneration immediately after LDLT, specific cellular changes occurring during this vigorous proliferative response may facilitate entry of HCV into hepatocytes or promote HCV replication. Second, since most living donors are primary relatives of the recipient, increased genetic similarity and a higher degree of HLA matching between donor and recipient compared with DDLT may affect the severity of recurrent HCV infection. Conversely, more recent studies have reported comparable results between LDLT and DDLT (*13–15*). Such discrepancies may be explained in part by the small numbers of LDLT patients included in these studies, or learning curve effects on recent data associated with increased experience (*16*).

This issue has attracted worldwide attention because, given the shortage of deceased donor organs, increasing numbers of patients are choosing to undergo LDLT. The matter is of critical importance in Japan and countries where almost all liver transplantations use living donor grafts. The present study retrospectively evaluated clinical outcomes for a comparatively large cohort of 91 patients who underwent LDLT for HCV-related cirrhosis at our institute. We investigated the frequency and severity of posttransplant recurrence of chronic HCV hepatitis and examined risk factors in order to clarify the role of LDLT in the treatment of patients with HCV cirrhosis.

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PATIENTS AND METHODS

Patients

Between March 1999 and April 2005, LDLT was performed at Kyoto University on 105 patients with HCV cirrhosis. Of these, 91 patients (61 men, 30 women) who had undergone LDLT by June 2004 and had been followed up for >12 months were included in this study (Table 1). Median age of subjects was 55 years (range, 30–69 years). Median model for end-stage liver disease (MELD) score was 16 (range, 4–33). HCV cirrhosis was accompanied by HCC in 57 patients (63%), including 25 who exceeded Milan criteria. Median MELD scores in groups with and without HCC were 15 (range, 4–33) and 18 (range, 9–33), respectively (P=0.015, Mann-Whitney U test).

All patients were positive for anti-HCV antibody before the operation. Preoperative HCV RNA load, measured using the polymerase chain reaction (PCR) method with an Amplicor HCV assay (Roche Molecular Systems, Pleasanton, CA), was obtained for 74 patients, with a median value of 260 kIU/ml (range: <0.5–2400). Patients treated during the early period, in whom viral load was measured only using DNA probe methods, were considered to lack relevant data. HCV genotype, determined using a system based on PCR with genotype-specific primers (*17*), was: 1b (n=52); 2a (n=6); 2b (n=3); others (n=2); not determined (low viral load, n=3); or not examined (n=25).

LDLT using a right-lobe graft was performed on all except two patients who received left lobe grafts. Operative procedures for donor and recipient surgery have been described elsewhere (*18*, *19*). Donors were 52 men and 39 women, with a median age of 40 years (range, 19–64 years). Relationship to the recipient was: child (n=36); spouse (n=34); sibling (n=17); parent (n=1); or other (n=3). ABO blood-type matching was incompatible in 15 cases.

After discharge, patients were scheduled for monthly visits to the outpatient clinic for the first year. Median duration of follow-up was 25 months (range, 1–72 months).

TABLE 1. Preoperative profile and clinical characteristics

Characteristic	Data
n	91
Recipient sex (male/female)	61/30
Median recipient age, years (range)	55 (30–67)
Child-Pugh grade (A/B/C)	2/26/63
Median MELD score (range)	16 (4–33)
Pretransplant HCC (yes/no)	57/34
HCV genotype (1b/2a/2b/others)	52/6/3/2
Median HCV-RNA, kIU/mL (range)	260 (<0.5–2400)
Pretransplant interferon therapy (yes/no)	30/61
Median donor age, years (range)	40 (19–64)
Donor gender (male/female)	52/39
Relation to recipient (related/unrelated)	57/34
ABO blood-type mismatch (yes/no)	15/76
HLA-A,B mismatch (≤2/≥3)	70/21
HLA-DR 2 mismatch (yes/no)	26/65
GRWR ≥1.0% (yes/no)	54/37
Immunosuppression (FK/CyA)	83/6
Steroid-free induction (yes/no)	19/72
Methylprednisolone boluses for rejection (yes/no)	32/59

Immunosuppression

The standard immunosuppression protocol comprised tacrolimus and low-dose steroid (*20*). The target whole-blood trough level for tacrolimus was 10–15 ng/ml during the first 2 weeks, approximately 10 ng/ml thereafter, and 5–8 ng/ml from the second month. Cyclosporine microemulsion was administered instead of tacrolimus for induction immunosuppression in six patients. Steroid therapy was initiated at a dose of 10 mg/kg before graft reperfusion, then tapered from 1 mg/kg/day on day 1 to 0.3 mg/kg/day until the end of the first month, followed by 0.1 mg/kg/day until the end of the third month. After this time, steroid administration was terminated. As an exception, 13 patients received steroid-free tacrolimus monotherapy as an induction procedure in an attempt to reduce HCC recurrence. In addition, four patients were assigned to a tacrolimus plus mycophenolate mofetil (MMF) (without steroid) group in a prospective comparative study started in March 2004 to evaluate the effects of steroid-free immunosuppression on recurrence of HCV. Another two patients transplanted with grafts from an identical twin did not receive any immunosuppressive treatment.

Patients who received ABO blood-type incompatible transplants were treated with preoperative plasma exchange or double-filtration plasmapheresis in order to reduce anti-A or B antibody titers. During the first 3 weeks postoperatively, prostaglandin E1 and additional steroids were administered via the portal vein or hepatic artery (*21*). Cyclophosphamide was also given intravenously for the first 2 weeks, and then orally.

Acute rejection episodes were documented by means of liver histology (*22*) and treated with methylprednisolone boluses if moderate or severe. OKT-3 was used for only one patient. MMF or azathioprine was added for patients who experienced refractory rejections or required reduction of tacrolimus dose due to adverse effects.

Antiviral Therapy

Prophylactic antiviral therapy for HCV was not administered. As a rule, antiviral treatment was used for patients with recurrent chronic hepatitis C. The treatment protocol consisted of interferon α2b ($3–6\times10^6$ units 3 times/week) plus ribavirin (400-800 mg/day orally for the first 6 months), followed by interferon monotherapy for 6 months.

Histological Assessment

A total of 398 liver biopsies were evaluated when patients displayed liver enzyme levels elevated more than two to three times the normal upper limit, or at yearly intervals when informed consent was obtained. Annual follow-up biopsies were obtained from 60 patients at 1 year after LDLT, 34 patients at 2 years, 14 patients at 3 years, 6 patients at 4 years, and 1 patient at 5 years. Biopsy specimens were evaluated by a single pathologist (H.H.) with extensive experience in the pa-