Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Responses of Nontransformed Human Hepatocytes to Conditional Expression of Full-Length Hepatitis C Virus Open Reading Frame

Weiliang Tang,* Catherine A. Lázaro,*
Jean S. Campbell,* W. Tony Parks,*
Michael G. Katze,[†] and Nelson Fausto*

From the Departments of Pathology,* and Microbiology,[†]
University of Washington School of Medicine, Seattle, Washington

Hepatitis C virus (HCV) is a major cause of chronic hepatitis that can lead to cirrhosis and hepatocellular carcinoma. To study the effects of HCV protein expression on host cells, we established conditional expression of the full-length open reading frame (ORF) of an infectious cDNA clone of HCV (genotype 1a, H77 strain) in the nontransformed human hepatocyte line cell HH4 using the ecdysone receptor regulatory system. Treatment with the ecdysone analog ponasterone-A induced tightly regulated and dose-dependent full-length HCV ORF expression and properly processed HCV proteins. HCV Core, NS3, and NS5A colocalized in perinuclear regions and associated with the early endosomal protein EEA1. HCV ORF expression caused marked growth inhibition, increased intracellular reactive oxygen species, up-regulation of glutamate- γ -cysteine ligase activity, increased glutathione level, and activation of nuclear factor κ B. Although it was not directly cytotoxic, HCV ORF expression sensitized HH4 cells to Fas at certain concentrations but not to tumor necrosis factor-related apoptosis-inducing ligand. HCV ORF expression in HH4 cells up-regulated genes involved in innate immune response/inflammation and oxidative stress responses and down-regulated cell growth-related genes. Expression of HCV ORF in host cells may contribute to HCV pathogenesis by producing oxidative stress and increasing the expression of genes related to the innate immune response and inflammation. (*Am J Pathol* 2007, 171:1831–1846; DOI: 10.2353/ajpath.2007.070413)

Hepatitis C virus (HCV) infects an estimated 4 million people in the United States and around 170 million worldwide.¹ For reasons that are still unclear, the majority of

HCV-infected individuals are unable to clear the virus and become chronically infected. Chronic HCV infection causes liver injury, resulting in varying degrees of liver inflammation and associated fibrosis. In 10 to 30 years, chronically infected patients may develop liver cirrhosis and hepatocellular carcinoma. HCV infection is a serious public health problem, and HCV-induced liver disease is the leading indication for liver transplantation in the U.S.^{2–4}

A major obstacle toward understanding the mechanisms of HCV-induced liver disease has been the lack of suitable cell culture systems and small animal models for HCV replication. The development of HCV subgenomic and genomic replicon systems in 1998 greatly advanced studies on HCV replication.⁵ A breakthrough in 2005 achieved the efficient propagation of HCV JFH-1 virus, an HCV genotype 2a, isolated from a Japanese patient with fulminant hepatitis, in the human hepatoma line Huh-7 cells.^{6–8} This system has been widely used for HCV studies, particularly for those involving the virus life cycle and antiviral defense mechanisms. However, these studies use a transformed cell line as host cells and rely on the infection of an unusual HCV strain. Recently, our laboratory reported the development of a human fetal hepatocyte system in which HCV replication was achieved after transfection of unmodified virus of different genotypes and after infection by serum of patients with chronic HCV infection.⁹ To understand better the interactions between unmodified genotype 1a HCV, the most common type of HCV, and nontransformed human hepatocytes, we designed a system in which the expression of

Supported by National Institute of Health grants U19 AI 148214 and CA 074131 (to N.F.) and CA074131, U19 AI148214, and P30 DA01562501 (to M.G.K.).

Accepted for publication August 21, 2007.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Nelson Fausto, M.D., Department of Pathology, University of Washington School of Medicine, K078 Health Sciences Building, Box 357705, Seattle, WA 98195-7705. E-mail: nfausto@u.washington.edu.

viral proteins would be controlled through a conditional mechanism.

In this study, we report the development of conditional expression of the open reading frame (ORF) of an infectious cDNA clone of genotype 1a HCV (H77 strain) in HH4 cells, using the ecdysone-inducible system. HH4 cells, a nontransformed hepatocyte line derived from HPV E6/E7 immortalized adult human hepatocytes, express hepatocytic markers, such as albumin, α -antitrypsin, and transferrin and exhibit a nontransformed phenotype, as assessed by soft-agar assay and nude mouse transplantation. Our results demonstrate that HCV ORF expression in cultured human hepatocytes induced oxidative stress, activated nuclear factor κ B (NF- κ B), inhibited cell growth, sensitized cells to Fas-mediated apoptosis, and up-regulated genes involved in the innate immune response and inflammation.

Materials and Methods

Cell Culture and Reagents

HH4 human hepatocyte cell line^{10,11} (see description under Results) was cultivated in William's E medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), ITS premix (BD Biosciences, Bedford, MA), 50 μ g/ml gentamicin, 0.2 mmol/L L-ascorbic acid-2-phosphate, 10 mmol/L nicotinamide, 2 mmol/L L-glutamine, 0.1 μ mol/L dexamethasone, 20 mmol/L HEPES, 1 mmol/L sodium pyruvate, 17 mmol/L sodium carbonate, 14 mmol/L glucose, and 10 mg/L ciprofloxacin. For detection of hepatocytic markers, HH4 cells were cultivated in the above medium except that 10% fetal bovine serum was replaced by human epidermal growth factor (20 ng/ml, BD Biosciences). Phenix-Ampho (PNXA) cells (gift from Dr. Gary Nolan, Stanford University, Stanford, CA) were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and penicillin (100IU/ml)/streptomycin (100 μ g/ml). Zeocin was pur-

chased from Clontech (Mountain View, CA), and hygromycin-B and ponasterone-A (ponA) were obtained from Stratagene (La Jolla, CA). Sources for antibodies used for immunoblot, immunohistochemistry (IHC), and immunofluorescence (IF) are listed in Table 1.

Plasmids

The 5.0-kb PstI/NotI fragment from plasmid pER3 (Stratagene) containing a CMV immediate early promoter driving the ecdysone receptor coding sequence was subcloned into PstI/NotI sites of plasmid pBSIIKS (Stratagene). The 5.0-kb EcoRI/NotI fragment from the resulting plasmid was then ligated into the EcoRI/NotI site of plasmid pLXSZ, derived from pLXSN (Clontech) by replacing the neomycin resistance gene with the zeocin selection gene, to generate the retroviral vector pLCERSZ. The ponA-inducible expression vector for the full-length HCV ORF, pE-HCV, was also constructed in multiple steps. The N-terminal portion of the HCV ORF was generated by PCR as a 259-bp fragment containing newly created XhoI and EcoRI sites at the 5' end of the coding sequence for the first 80 amino acids of HCV core protein (nucleotides 342 to 580). The C-terminal part of the HCV ORF was a PCR-derived 174-bp fragment containing newly created BamHI sites at the 3' end, and the coding sequence for the C terminus of NS5B protein (nucleotides 9220 to 9400). The 259-bp XhoI-EcoRI/KpnI fragment and 174-kb BamHI/NotI fragment were sequentially inserted into plasmid pCEP4 (Invitrogen) to make pCEP4-HCV Δ . The NotI site in the ecdysone-inducible expression vector pEGSH (Stratagene) was eliminated by NotI digestion followed by Klenow fill-in to make pEGSH2. The 0.43-kb EcoRI/BamHI fragment from pCEP4-HCV Δ was ligated into the MfeI/BamHI sites of pEGSH2 to make pE-HCV Δ . Finally, the 8.74-kb Acc65I/NotI fragment of p90/HCV-FLpU (a gift of Dr. Charles Rice, Rockefeller University, New York) was inserted into Acc65I/NotI sites of pE-HCV Δ to complete the construction of pE-HCV. The plasmid p90/HCV-FLpU contains an infectious HCV cDNA (genotype 1a;

Table 1. Sources and Concentrations of Antibodies Used in This Study

Antigen	Source	Dilutions
HCV core	Affinity Bioreagents	AIHC, 1:100; IF, 1:100; Immunoblot, 1:3000
HCV E1	BioDesign (Saco, ME)	IF, 1:25; Immunoblot, 1:1000
HCV E2	BioDesign	Immunoblot, 1:400
HCV NS3	Affinity Bioreagents	IF, 1:50; Immunoblot, 1:8000
HCV NS5A	Austral Biologicals (San Ramon, CA)	IF, 1:50
HCV NS5A	BioDesign	Immunoblot, 1:3000
Albumin	ICN Pharmaceuticals (Aurora, OH)	Immunoblot, 1:1000
α 1-Antitrypsin	Sigma	Immunoblot, 1:2000
α 1-Fetoprotein	DAKO Corp. (Carpinteria, CA)	Immunoblot, 1:1000
β -Actin	Sigma	Immunoblot, 1:10,000
Caveolin2	Santa Cruz Biotechnology	IF, 1:100
EEA1	Santa Cruz Biotechnology	IF, 1:100
Golgi58K	Sigma	IF, 1:100
P450 1A1	Santa Cruz Biotechnology	Immunoblot, 1:500
P450 3A4	Santa Cruz Biotechnology	Immunoblot, 1:500
Rab-8	Pharmingen	IF, 1:125
SARA	Santa Cruz Biotechnology	IF, 1:100
Transferrin	Rockland (Gilbertsville, PA)	Immunoblot, 1:1000

GenBank access no. AF009606), and it was used as template for generating PCR fragments for pE-HCV. All of the PCR-generated fragments were verified by sequencing.

Control vectors pE-GFP and pE-Core allow ponA-inducible expression of green fluorescent protein (GFP) and HCV core protein, respectively. The pE-GFP was constructed by ligation of 0.7-kb Acc65I/XhoI fragment of pCEP4-GFP (in which a 0.7-kb NheI/XhoI fragment of pEGFP-C1 was inserted into the NheI/XhoI sites of pCEP4) into the Acc65I/XhoI sites of pEGSH2. pE-Core was made by ligation of a 0.6-kb EcoRI/SalI fragment containing the HCV core coding region into the MfeI/XhoI sites of pEGSH2.

Retrovirus Production and Transduction

Retrovirus production and subsequent transduction of HH4 cells were performed according to a protocol developed by Gary Nolan (Stanford University). In brief, PNXA packaging cells were seeded at 5×10^6 in a 10-cm tissue culture dish and transfected with 25 μ g of pL-CERSZ plasmid DNA 16 to 24 hours later, using CalPhos Mammalian Transfection kit (Clontech). Cell culture medium was replaced 8 hours after transfection; culture supernatant containing retroviruses was collected 24 hours later and filtered through a syringe-top filter unit with 0.45- μ m polyvinylidene difluoride membrane (Millipore, Bedford, MA). Retroviral transduction was performed by incubating recipient cells in culture medium containing 4 μ g/ml Polybrene (Sigma, St. Louis, MO) and the retrovirus for 8 hours.

Northern Blot Analysis

Total cellular RNA was extracted from cell monolayers using acid-guanidinium thiocyanate-phenol-chloroform method as previously described.¹² Probes used were antisense riboprobe (NS5B; nucleotides 8187 to 9220), for the detection of full-length HCV transcript, and a HCV core cDNA fragment (core; nucleotides 602 to 730), for the detection of truncated HCV transcript. The adequacy of RNA sample loading and efficiency of transfer were determined by ethidium bromide staining.

Immunoblot Analysis

Protein samples (20 μ g) were resolved on a 12% SDS-polyacrylamide gel, transferred to Immuno-PVDF membrane (Millipore, Bedford, MA), blocked with 5% nonfat milk in 1 $\times$ PBS plus 0.05% Tween 20, and probed with primary antibodies (Table 1) diluted in 1 $\times$ PBS plus 0.05% Tween 20 containing 1% bovine serum albumin overnight at 4°C followed by incubation with secondary antibody conjugated with horseradish peroxidase (1:5000; Amersham, Piscataway, NJ) for 1 hour at room temperature. Membrane-bound antibodies were detected with the Pierce Chemiluminescent ECL Detection System (Pierce Biotechnology, Rockford, IL). Even sample loading and efficiency of transfers were routinely assessed via Ponceau-S staining and probing with β -actin antibody.

Densitometry Analysis

Autoradiographies obtained from Northern blot or immunoblot analysis were scanned with a flatbed scanner and analyzed using NIH Image software (Wayne Rasband; <http://rsb.info.nih.gov/ni-image/Default.html>). Blots exposed to phosphor screens were scanned using the Storm PhosphorImager System (Molecular Dynamics, Piscataway, NJ) and analyzed with the accompanied ImageQuant software.

IHC

PonA-treated or untreated cells were fixed in cold methanol:acetone (1:1, v/v) for 25 minutes, washed in PBS, blocked in PBS containing 5% normal horse serum, and incubated with anti-core antibody (1:100; Affinity Bioreagents, Golden, CO) in blocking solution overnight at 4°C. The presence of anti-core primary antibody was detected using the Vectastain ABC System (Vector Laboratories, Burlingame, CA) in accordance with manufacturer's instructions.

IF Microscopy

Cells were fixed in cold PBS containing 4% paraformaldehyde for 25 minutes, permeabilized in 0.1% Triton X-100 for 5 minutes, and rinsed in cold PBS containing 5% glycine for 15 minutes. Fixed cells were washed in PBS, blocked in PBS containing 10% horse serum for 1 hour at room temperature, and stained with primary antibody diluted in blocking solution overnight at 4°C. After three washes in PBS, cells were then incubated with Alexa Fluor488 or Alexa Fluor594-conjugated secondary antibodies (1:2000; Molecular Probes, Eugene, OR) for 1 hour at room temperature, washed in PBS, and mounted in Immunomount with 4,6-diamidino-2-phenylindole counterstaining (Vector Laboratories). Subcellular organelle markers used are Ds2Red-ER (Clontech) for endoplasmic reticulum, MitotrackerRed (Molecular Probes) for mitochondria, 4,6-diamidino-2-phenylindole (Vector Laboratories) for nucleus, Golgi58k (Sigma) for Golgi apparatus, Rab-8 (Pharmingen, San Diego, CA) for *trans*-Golgi apparatus/basolateral plasma membrane, SARA and caveolin2 (Santa Cruz Biotechnology, Santa Cruz, CA) for plasma membrane periphery, and Hsp27 (Stressgen, Victoria, BC, Canada) for microtubules. IF microscopy was performed using a Nikon Eclipse E600 fluorescence microscope with a QImaging Retigra EX CCD camera and a $\times 60$ objective.

Cell Proliferation and Cell Growth Rate Assays

Cell proliferation was measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay as previously described.¹³ For cell growth rate assay, cell numbers were determined using a crystal violet assay as previously described.¹⁴

Cell Viability Assays

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay to measure apoptosis was performed using the *In Situ* Cell Death Detection kit (Roche Diagnosis, Indianapolis, IN) in accordance with manufacturer's instructions. After hematoxylin counterstaining, labeled nuclei were scored in 1000 cells on each slide. Cell viability was also assessed using trypan blue exclusion assay.¹⁵ The number of trypan blue-stained (dead) and unstained (live) cells were counted, and percent cell death was expressed as (number of blue cells $\times$ 100)/number of blue and unstained cells.

Induction of Apoptosis by Fas and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Subconfluent HCV ORF- and GFP-inducible cells were treated with or without 10 μ mol/L ponA for 24 hours followed by incubation with protein A-crosslinked APO-1-3 (Kamiya Biomedical, Seattle, WA), a Fas agonist, at various concentrations (0 to 250 ng/ml) for 36 hours and subjected to the trypan blue exclusion cell death assay. Similarly, a leucine zipper form of human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that induces apoptosis in human hepatocytes¹⁶ was added at various concentrations (0 to 200 ng/ml) for 8 hours to induce cell death in untreated and ponA-treated (10 μ mol/L, 24 hours) HCV ORF and GFP cells.

Measurement of Intracellular Reactive Oxygen Species Levels

Untreated or ponA-treated (5 μ mol/L, 2 or 6 days) subconfluent HCV ORF and GFP cells were stained with or without 2.5 μ mol/L RedoxSensorRed for 10 minutes and analyzed immediately by flow cytometry using a FACS-can (BD Biosciences). The difference in geometric means of the red fluorescence (600 nm wavelength) measurement in stained and unstained cells for each experimental condition was used as measurement of intracellular reactive oxygen species (ROS) level.

Measurement of Total Intracellular Glutathione Levels and Glutamate-L-Cysteine Ligase Activity Assay

Total intracellular glutathione level (GSH, reduced form, plus GSSG, oxidized form) was determined by a modification of the Tietze assay as described previously.¹⁷ For glutamate-L-cysteine ligase (GCL) activity measurements, cells were harvested, cell pellets were washed in PBS before lysis to remove any traces of cysteine remaining from the medium, and GCL-specific activity in the cell lysate was determined as described previously.¹⁸

Electrophoretic Mobility Shift Assay

Binding of the transcription factors NF- κ B to oligonucleotide probes containing consensus NF- κ B sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3'; Santa Cruz Biotechnology) was assessed by electrophoretic mobility shift assay (EMSA) as previously described,¹⁹ using 4 μ g of nuclear extract prepared from HH4 cells inducible for HCV ORF or GFP.

RNA Extraction and Analysis with High-Density Oligonucleotide Microarrays

HCV ORF cells were cultured, in quadruplicate, in the presence or absence of 5 μ mol/L ponA for 2 or 6 days with medium replacement every 2 days. One set of these cells was used to confirm ponA-inducible expression of HCV proteins by immunoblot. Total RNA samples were isolated from cultured cells using an acid-guanidinium thiocyanate-phenol-chloroform method as previously described.¹² Microarray analysis of a single experiment comparing two samples using the Cy3/Cy5 dye reverse technique with an oligonucleotide microarray of approximately 20,000 unique probes was performed in triplicate for each time point as previously described.²⁰ Mean ratios between the expression levels of each gene in the analyzed sample pair, SD, and *P* values were obtained and analyzed using Rosetta Resolver System 3.0 (Rosetta Biosoftware, Seattle, WA). Gene function information was obtained from Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>) and GeneCards (<http://thr.cit.nih.gov/cards/index.shtml>).

Quantitative Real-Time Reverse Transcription-PCR

Differential gene expression detected by microarray was validated using quantitative real-time reverse transcription-PCR (QPCR). Total RNA samples that were used in the microarray analysis were treated with DNase using DNA-free DNase Treatment and Removal Reagents (Ambion, Inc., Austin, TX) to remove residual DNA contamination. Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Primer and probe sets for each of selected five target sequences [interleukin 1-a (IL1A), CD14, toll-like receptor 2 (TLR2), colony stimulating factor 2 (CSF2), and E-cadherin (CDH1)] were chosen from the Applied Biosystems Assays-on-Demand product list. QPCR was performed on an ABI 7500 Real Time PCR System, using TaqMan chemistry (Applied Biosystems). Each target was run in triplicate, with 20- μ l reaction volumes of TaqMan 2 $\times$ PCR Universal Master Mix (Applied Biosystems). The quantification of each target was normalized to the 18S endogenous control.

Statistical Analysis

Values are expressed as mean $\pm$ SD. Group means calculated from 3 to 4 independent data sets were compared with the Student's two-tailed unpaired *t*-test using Prism software (version 4.0 for Macintosh; GraphPad Software, San Diego, CA). Differences with a *P* value less than 0.05 were considered significant.

Results

Characterization of HH4 Human Hepatocyte Line

The HH4 cell line was created by the introduction of the HPV E6 and E7 genes into hepatocytes isolated from a normal adult liver, followed by derivation of a clonal, immortalized cell line. The transduction techniques used to create the HH4 line were similar to those reported for the development of immortalized cell lines from human prostate, pancreas, and melanocytes.^{21–23} Immunoblot analysis revealed that HH4 cells expressed hepatocytic markers such as albumin, α 1-antitrypsin, and transferrin but not the fetal hepatocyte marker α -fetoprotein (Figure 1A). Exposure of HH4 cells to β -naphthoflavone or phenobarbital resulted in the induction of cytochrome P450 enzymes 1A1 and 3A4, respectively (Figure 1B). These results indicate that cultured HH4 cells retain the expression of adult hepatocytic markers. The growth properties of HH4 cells were analyzed by counting cell numbers of HH4 cultures over a period of 15 days. The average doubling time was 49 hours (58 hours for doubling at low cell density and 34 hours for doubling at high cell density). HH4 cells did not form anchorage-independent colonies in soft-agar assay (data not shown). Furthermore, whereas transplantation of HepG2 human hepatoma cells at 10^7 cells per mouse led to the formation of large tumors in nude mice by about 56 days, no tumors were detected for at least 111 days in mice transplanted with HH4 cells (at 10^7 per mouse).

Figure 1. Expression of hepatocyte markers by HH4 human hepatocytes. **A:** HH4 cells were cultured in William's E medium with epidermal growth factor for 4 days, and protein lysates were prepared and analyzed by immunoblot for albumin, α 1-antitrypsin, transferrin, and α -fetoprotein as previously described.⁷³ F, fibroblasts; AH, isolated human adult hepatocytes; FH, isolated human fetal hepatocytes. **B:** Induction of cytochrome P450 proteins. HH4 cells were cultured in the presence or absence of 0.025 mmol/L β -naphthoflavone (for 1A1 induction) or 0.5 mmol/L phenobarbital (for 3A4 induction) for 48 hours. Cell lysates were then prepared and analyzed by immunoblot.

Generation of HCV ORF-Inducible Stable Clones

HH4 cells transduced with pLCERSZ-derived retrovirus were selected with zeocin for 10 to 14 days. About 20 of the resulting zeocin-resistant clones were each transfected with pEGSH-luc, and their luciferase activities in the presence or absence of ponA were measured. Among all, clone 4E2 exhibited the highest ponA inducibility ($\sim$ 204-fold) and was chosen as the founder clone. The 4E2 cells were then transfected with pE-HCV, pE-Core, or pE-GFP and selected with hygromycin-B for 10 to 14 days. Stable hygromycin-resistant clones were screened for ponA-induced protein expression using various assays. The HCV ORF- and HCV C-inducible candidates were screened using IHC for HCV core protein, and clones that stained positive for HCV core were further tested for inducible expression of HCV proteins (C, E1, E2, NS3, and NS5A) by immunoblot analysis. For GFP-inducible clones, the resistant cells were cultured in the presence and absence of 10 μ mol/L ponA for 24 hours and evaluated for the expression of GFP under a fluorescence microscope. Nearly one-half of 40 clones transfected with either pE-core or pE-GFP expressed these proteins on ponA induction, indicating that the system was fully functional. By contrast, we obtained only 2 full-length HCV ORF-inducible clones after screening 169 clones transfected with pE-HCV. These clones were designated as clones 16 and 50 and will be referred to as HCV-ORF. The low yield of full-length HCV ORF-inducible clones may have been due to the relatively large size of the HCV ORF sequence (approximately 9 kb) in the pE-HCV vector or perhaps because the HCV ORF is unstable in DNA form before integration into host chromosome. Interestingly, about 11% of pE-HCV-derived stable clones that expressed the core protein were inducible for core, E1, and E2 but not NS3 and NS5A proteins. These cells, which expressed part of the HCV ORF encoding the structural proteins, will be referred as HCV Δ cells.

Induction of HCV RNA Transcripts in HH4 Cells

To verify that full-length HCV ORF transcript was induced, we performed Northern blot analysis on total RNA samples prepared from HCV ORF-inducible cells of clones 16 and 50. A single RNA transcript of 9.5 kb in length was detected in ponA-treated HCV-ORF cells when probed with 32 P-labeled antisense riboprobe corresponding to the NS5B region of HCV ORF (Figure 2A), indicating the induction of full-length HCV ORF in the HCV-ORF cells. The HCV-specific RNA transcript was first detectable at 1 day, reached peak expression level at 2 days, and remained at maximal level for at least 4 days after 5 μ mol/L ponA treatment. No RNA transcript was seen in HCV Δ cells when the NS5B riboprobe was used, demonstrating the lack of coding sequences for the C terminus of the HCV ORF. When probed with a 32 P-labeled core probe (located at 5'-end of HCV ORF), two HCV-specific RNA transcripts (about 3.2 and 3.8 kb in length) were detected in the HCV Δ cells with induction kinetics similar to that of

Figure 2. Time course of HCV RNA induction in HH4 human hepatocytes. Detection of full-length HCV ORF cells and the truncated form of HCV ORF (HCV Δ cells) after induction with 5 μ mol/L ponA from 1 to 6 days. **A:** Detection of the full-length HCV ORF transcript ($\sim$ 9.5 kb) in ponA-induced HCV ORF cells. **B:** The full-length HCV ORF transcript was not detected in ponA-induced HCV Δ cells. Instead, two smaller RNA transcripts ($\sim$ 3.2 and 3.8 kb) were detected in ponA-induced HCV Δ cells when HCV C region probe was used, indicating truncation of HCV ORF expression in these cells. Relative RNA levels were calculated using densitometry, as indicated under each autoradiograph. Equal RNA loading is demonstrated by ethidium bromide staining (**bottom panels in A and B**).

full-length HCV ORF transcript (Figure 2B). Based on sizes of the HCV transcripts detected in HCVΔ cells, it is likely that truncations of HCV ORF were located between NS2 and NS3 regions.

Inducible Expression and Proper Processing of HCV Polyprotein in HH4 Cells

To determine whether the HCV polyprotein was induced and faithfully processed, we investigated the expression of HCV proteins by immunoblot analysis in the two HCV ORF-inducible clones (16 and 50), using a panel of antibodies against HCV C, E1, E2, NS3, and NS5A proteins. We detected HCV proteins only in ponA-treated HCV-ORF cells (Figure 3A), confirming the high inducibility and low basal level of ponA-regulated HCV ORF expression in these cells. In the absence of SDS in the cell lysis buffer, HCV core, NS3, and NS5A proteins were detected almost exclusively in the insoluble portion of the cell lysates, suggesting that these HCV proteins were membrane associated. The molecular weights of HCV C, NS3, and NS5A proteins detected by immunoblot matched those predicted from the HCV ORF sequence (GenBank access no. AF009606), demonstrating that HCV proteins

Figure 3. Immunoblot detection of inducible expression of HCV proteins in HH4 cells. **A:** Two stable inducible clones for the full-length HCV ORF (16 and 50) and a GFP control clone were treated with 10 $\mu\text{mol/L}$ ponA for 24 hours. Cell lysates were prepared, and levels of HCV C, E1, E2, NS3, NS5A, and loading control β -actin were evaluated by immunoblotting. Note the tightly regulated expression and proper translation/processing of HCV proteins in these cells. Immunoblotting for multiple HCV proteins was also performed to characterize time course (**B**) and dose response (**C**) of ponA-induced HCV protein expression in HH4 cells. Results from both clones 16 and 50 of HCV ORF cells were almost identical and those from clone 16 are shown here (**B** and **C**).

were faithfully translated and processed in the HCV ORF cells. The E1 and E2 proteins were detected as 31- and 70-kDa bands, respectively, in agreement with previous reports,^{24–26} and consistent with the highly glycosylated nature of these two HCV envelope proteins.²⁷

The efficient and tightly regulated HCV ORF induction in HH4 cells was further demonstrated by IHC for HCV C protein. The vast majority of ponA-induced cells (85% HCV ORF, 94% HCV C, and 95% HCV Δ) stained positive for HCV C, whereas only less than 1% of uninduced cells showed very weak core staining (data not shown).

We examined the kinetics of HCV protein expression by immunoblot in HCV ORF cells treated with 10 $\mu\text{mol/L}$ ponA for various times. Induction of HCV proteins was detectable at 2 hours and peaked 24 to 48 hours after ponA treatment (Figure 3B). HCV protein expression was stable in these cells because peak-level expression was maintained for at least 6 days when fresh culture medium containing 10 $\mu\text{mol/L}$ ponA was replaced every 2 days (data not shown). The dose-dependent induction of HCV protein expression was also examined in the HCV ORF cells (Figure 3C). By densitometry analysis, we calculated that as little as 0.3 $\mu\text{mol/L}$ ponA could induce detectable HCV expression whereas maximal induction was achieved at 5 $\mu\text{mol/L}$, and 1 $\mu\text{mol/L}$ ponA was needed to induce half-maximal level of HCV protein expression.

Subcellular Localization of HCV Proteins in HH4 Cells

Next, we investigated subcellular localization of HCV proteins in HCV ORF cells by IF staining. As shown in Figure 4, A and B, HCV C, NS3, and NS5A displayed similar punctate cytoplasmic staining in the perinuclear area and overlapped with each other, suggesting that they colo-

Figure 4. Colocalization of HCV core, NS3, and NS5A proteins in HH4 cell. HCV ORF-inducible cells were cultured in the presence of 10 $\mu\text{mol/L}$ ponA for 24 hours and fixed to IF staining. **A:** HCV core is shown in green (**top**), and NS3 is shown in red (**middle**). **B:** HCV NS5A is shown in green (**top**), and NS3 is shown in red (**middle**). Regions with colocalization appear yellow (**bottom**). **C:** HCV E1 is shown in green. Note the diffused cytoplasmic staining pattern of HCV E1 in ponA-induced HCV ORF cells (**right**) that is distinct from those of C, NS3, and NS5A seen in **A** and **B**. UI, uninduced. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue).

calized in HH4 cells. Colocalization of HCV C, NS3, and NS5A was further confirmed by confocal microscopy (data not shown). In contrast, HCV E1 exhibited a diffuse cytoplasmic staining pattern similar to that of endoplasmic reticulum distribution (Figure 4C) and was distinct from those of C, NS3, and NS5A (Figure 4, A and B). IF staining with the E2 antibody was unsuccessful, although the same antibody worked well for immunoblot.

Double IF staining using C as a marker for the HCV protein complex, and various subcellular organelle markers, revealed that HCV core colocalized with early endosome antigen-1 (EEA1), an early endosomal marker, in HCV-ORF but not HCV-C cells (Figure 5A). We have observed distinct HCV C staining patterns in cells ex-

Figure 5. Colocalization of HCV C protein with EEA1 in ponA-induced HCV ORF but not HCV C cells. HCV ORF and HCV C cells were cultured in the presence of 10 $\mu\text{mol/L}$ ponA for 24 hours and fixed for IF staining. **A:** HCV C is shown in green (**top**), and EEA1 is shown in red (**middle**). Colocalization of HCV core and EEA1 is seen only in ponA-induced HCV ORF cells (**bottom**). **B:** Distinct staining patterns of HCV C protein (shown in green) in ponA-induced HCV ORF and HCV C cells. In contrast to the punctate perinuclear staining seen in HCV ORF cells, HCV C protein in HCV C cells was associated with bubble-like structures indicative of lipid droplets. The white arrows point out some of the ring-shaped C staining in HCV C cells. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue).

pressing C in the context of full-length HCV ORF (HCV-ORF cells) as opposed to C alone (HCV-C cells). In contrast to the punctate perinuclear staining in HCV-ORF cells, HCV C staining in HCV-C cells was almost exclusively associated with small bubble-like structures, a characteristic of lipid droplets (Figure 5B). Therefore, the association of HCV protein complex with EEA1 is likely mediated by HCV NS protein(s). We detected no colocalization between HCV C and markers for nucleus, endoplasmic reticulum, Golgi apparatus, *trans*-Golgi apparatus/basolateral plasma membrane, mitochondria, plasma membrane periphery, and microtubules (data not shown). However, we observed that HCV C was localized very close to the Golgi apparatus in HCV-ORF cells (data not shown).

Figure 6. Effects of HCV ORF expression on HH4 cell proliferation. **A:** Expression of HCV ORF but not GFP inhibited HH4 cell proliferation. HCV-ORF and GFP cells were cultured in the presence or absence of 5 $\mu\text{mol/L}$ ponA for 2 and 6 days and subjected to BrdU incorporation assay as described in Materials and Methods. Percentage of BrdU-positive cells is used here as measurement of cell proliferation. **B:** Growth rate analysis of uninduced and ponA-induced HCV ORF cells. Data were shown as mean $\pm$ SD in both **A** and **B**. They are representative of at least three independent experiments (* $P < 0.01$).

Effects of Regulated Expression of HCV ORF on Proliferation and Cell Viability

We investigated the effect of HCV expression on cell proliferation and viability using various assays. BrdU incorporation was performed to determine whether HCV proteins altered the replication of host cells. Both short-term (2 days) and prolonged (6 days) ponA treatments significantly reduced BrdU incorporation in HCV-ORF but not in the control GFP cells (Figure 6A), demonstrating that expression of HCV proteins inhibited DNA replication in human hepatocytes. A growth rate assay to measure the overall effects of HCV ORF expression on human hepatocyte growth over 10 days showed (Figure 6B) that HH4 cells induced to express HCV proteins grew at a slower rate compared with uninduced cells. However, HCV ORF expression did not significantly alter the saturation densities of the cultures.

Next, we examined whether HCV ORF expression affected host cell viability. PonA treatment over a period of 3 months did not cause any noticeable morphological changes indicative of cell death, such as detachment/floating, cytolysis, or condensed nuclei. The effect of

Table 2. TUNEL Measurement of Cell Death in HH4 Human Hepatocytes Induced to Express Full-Length HCV ORF or GFP

	Two-day induction		Six-day induction	
	No ponA	5 $\mu\text{mol/L}$ ponA	No ponA	5 $\mu\text{mol/L}$ ponA
HCV-ORF	6.4 $\pm$ 1.2	6.5 $\pm$ 1.3	6.3 $\pm$ 1.2	6.5 $\pm$ 1.5
GFP	5.2 $\pm$ 0.5	5.9 $\pm$ 0.9	5.5 $\pm$ 0.9	6.1 $\pm$ 0.6

Differences in mean percentage of TUNEL-positive cells between no ponA- and 5 $\mu\text{mol/L}$ ponA-treated cells were not significant at both 2- and 6-day time points ($P > 0.05$).

ponA-induced HCV protein expression on HH4 cell viability was further analyzed quantitatively using the TUNEL assay. As summarized in Table 2, the percentage of TUNEL-positive cells, a measurement of apoptotic cell death, was not significantly altered when HCV-ORF and GFP cells were induced with 5 $\mu\text{mol/L}$ ponA for 2 and 6 days, indicating that expression of all HCV proteins was not cytotoxic to human hepatocytes under normal culture condition. Similar results were also obtained when cell death was measured by the trypan blue exclusion assay (data not shown).

HCV ORF Expression Sensitizes Human Hepatocytes to Fas-Induced Apoptosis

Fas-mediated apoptosis is believed to play a role in viral persistence and liver injury in HCV infection. Therefore, we examined the effects of HCV protein expression on Fas-induced apoptosis in HH4 human hepatocytes. Shown in Figure 7A (left), significantly more cell death was observed in HH4 human hepatocytes expressing HCV ORF when treated with 31 or 63 ng/ml of Apo-1-3, and maximal cell death was reached at higher doses of Apo-1-3 (125 and 250 ng/ml), regardless of their induction status. In contrast, expression of GFP did not alter the sensitivity of HH4 cells to Fas-induced apoptosis at every dose of Apo-1-3 treatment (Figure 7A, right). Furthermore, expression of HCV ORF and GFP in HH4 cells had no effect on cell death induced by TRAIL, a member of TNF/TNFR superfamily that includes FasL/Fas (Figure 7B). Our data suggest that HCV ORF expression specifically sensitizes HH4 human hepatocytes to Fas-induced cell death.

HCV ORF Expression Induces Oxidative Stress and Activated Protective GSH Antioxidant System

We investigated the effect of HCV protein expression on intracellular levels of ROS in HH4 cells using RedoxSensorRed as previously described.²⁸ At both 2- and 6-day time points, increased oxidation of Redox SensorRed, was detected in HCV ORF cells induced to express HCV proteins (Figure 8A), suggesting elevated oxidative stress in these cells. By contrast, no significant changes in RedoxSensorRed staining were seen in ponA-induced GFP cells at either 2- or 6-day time points.

Figure 7. Effects of HCV ORF expression on Fas- and TRAIL-mediated apoptosis in HH4 cells. **A:** HCV ORF expression sensitized HH4 cells to Fas-induced cell death. The HCV ORF- and GFP-inducible cells were treated with or without 10 μ M ponA for 24 hours followed by incubation with indicated doses of Fas agonist for 36 hours. Cell death was then assessed by trypan blue exclusion assay. **B:** HCV ORF expression did not affect TRAIL-induced apoptosis in HH4 cells. The HCV ORF and GFP cells were cultured in the presence or absence of 10 μ M ponA for 24 hours, treated with indicated doses of recombinant human TRAIL for 8 hours, and subjected to trypan blue exclusion assay. Results are shown here as mean $\pm$ SD. They are representative of at least three independent experiments (* P < 0.01).

To test whether HH4 cells activated the protective GSH antioxidant system in response to HCV ORF-induced oxidative stress, total cellular GSH contents and GCL activity, a rate-limiting enzyme in GSH synthesis, were measured in HCV ORF cells treated with or without 5 μ M ponA for 2 days. PonA treatment of HCV ORF cells caused a 36% increase in total intracellular GSH level and a 135% increase in GCL activity, whereas similar treatment in GFP-inducible cells caused only a 7% increase in total GSH level and a 14% increase in GCL activity (Figure 8, B and C). Taken together, our results suggest that HCV ORF expression in human hepatocytes induces oxidative stress and subsequently activates the GSH-mediated protective antioxidant system.

HCV ORF Expression Activates NF- κ B

Oxidative stress is known to activate the transcription factors NF- κ B and AP1 in different types of cells.^{29–31} To examine whether HCV ORF expression-induced oxidative stress in HH4 human hepatocytes activates NF- κ B, nuclear extracts prepared from uninduced or ponA-induced (5 μ M/L, 2 and 6 days) HCV-ORF cells were subjected to EMSA using a ³²P-labeled NF- κ B consensus probe. A representative of three independent EMSA experiments is presented in Figure 9A (top), showing enhanced NF- κ B binding activity in ponA-induced HCV-

Figure 8. HCV ORF expression induced intracellular ROS elevation and increased intracellular GSH level and GCL activity in HH4 cells. **A:** Geometric mean of single-cell fluorescence in cells stained with RedoxSensorRed was measured by flow cytometry to assess intracellular ROS levels. Results from three independent experiments were represented here as relative fluorescence and normalized to untreated control cells. HCV ORF and GFP cells were seeded at semiconfluence and induced with or without 5 μ M ponA for 2 days and harvested for measurement of total intracellular glutathione levels (**B**) and GCL activity (**C**) as described in Materials and Methods. Results of three experiments are represented here as mean $\pm$ SD (* P < 0.01, ** P < 0.05).

ORF cells. Densitometry analysis revealed a nearly 100% increase in NF- κ B activity in HCV-ORF cells in their induced state over the uninduced cells compared with GFP cells (<20% increase) at both 2- and 6-day time points (Figure 9A, bottom). NF- κ B activation by HCV proteins was much weaker than that caused by TNF α treatment (10 ng/ml, 30 minutes) (Figure 9B). TNF α -induced NF- κ B activation in HH4 cells was not significantly altered by HCV ORF or GFP overexpression.

Figure 9. HCV ORF expression induced basal level NF- κ B activation but did not alter TNF α -induced NF- κ B activation in HH4 cells. **A:** Nuclear extracts were prepared from untreated and ponA-treated (2 and 6 days) HCV ORF and GFP cells, and NF- κ B-binding activity was measured by EMSA as described in Materials and Methods. **B:** EMSA measuring NF- κ B-binding activities in the nuclear extracts from untreated and ponA-treated (2 days) HCV ORF and GFP cells in the presence and absence of TNF α (10 ng/ml, 15 min). Both figures are representative of three independent experiments.

Effects of Regulated HCV ORF Expression on Global Gene Expression in HH4 Cells

To gain better insight into the effect of ponA-induced HCV ORF expression on cellular mRNA abundance, we analyzed and compared global gene expression profiles in uninduced and ponA-induced HCV-ORF cells using oligonucleotide microarrays. To study the effects of short-term and long-term HCV ORF expression on host cells, we chose to analyze gene expression profiles at 2 or 6 days after ponA induction, with three independent experiments performed for each time point. Another set of cultured HCV-ORF cells was treated in parallel and analyzed for ponA-induced HCV protein expression by immunoblot. Immunoblot analysis confirmed that HCV core protein expression was induced at very similar levels in both 2- and 6-day ponA-induced HCV-ORF cells (data not shown). The ratios of expression levels for about 20,000 genes were obtained in each microarray assay comparing two paired mRNA samples from ponA-induced and uninduced HCV-ORF cells. Differentially regulated genes were identified using the following selection criteria: 1) $\geq 95\%$ probability of being differentially expressed ($P \leq 0.05$) and 2) greater than twofold up- or down-regulation (mean ratio of expression level ≥ 2 and ≤ -2). According to these criteria, 167 genes from 2-day ponA induction and 211 genes from 6-day ponA induction were identified as differentially regulated. About 106 up- and 11 down-regulated after 2 days and 140 up- and 71 down-regulated after 6 days of ponA induction were found to have available functional information and analyzed. A selected list of these genes is listed in Table 3. A complete list of differentially regulated genes is available in Supplemental Table 1 (see <http://ajp.amjpathol.org>). We summarize here the data regarding genes involved in the innate immune response and inflammation, genes involved in cell cycle progression, and genes involved in oxidative stress and its consequences.

Genes Involved in the Innate Immune Response, Adhesion Molecules, and Extracellular Matrix

Many genes involved in the innate immune response and inflammation were up-regulated by ponA induction at both 2- and 6-day time points. These genes include TLR2, CD14 (component of TLR4 or TLR2), IL1A (a pro-inflammatory cytokine), CSF2 (also called GM-CSF; a potent cytokine that stimulates proliferation and differentiation of hematopoietic precursor cells from various lineages, including granulocytes, macrophages, eosinophils, and erythrocytes), CXCL1 (chemokine and adhesion molecule for T cells and monocytes but not neutrophils), TNFSF4 (cytokine that costimulates T-cell proliferation and cytokine production), and DPP4 (also called CD26; another T-cell activating molecule). The expression of defensin- β 1; growth differentiation factor 15; orosomucoid 1; spondin 2; SPARC (secreted protein acidic and rich in cysteine)-related modular calcium binding 2; serine/cysteine proteinase inhibitor, clade B, member 2; and tissue factor pathway inhibitor 2 was

up-regulated by ponA induction in HH4 cells. These proteins play various roles during the innate immune response, and their expression is usually increased in infection and tissue injury. Only one gene, CXCL1 (one of the chemokines for neutrophils) was down-regulated at the 2-day time point.

Consistent with the activation of innate immune response in an inflammatory response, a number of adhesion molecule genes (intercellular adhesion molecule 1; intercellular adhesion molecule 5; vascular cell adhesion molecule 1; CDH1; CD44 antigen, hyaluronate receptor; claudin 5; integrin- α 3; integrin- α 7; integrin- α V; LIM and senescent cell antigen-like domains 2; and nidogen 2) and extracellular matrix genes (collagen 5A1; collagen 6A1; collagen 9A3; laminin- γ 2; microfibrillar-associated protein 4; microfibrillar-associated protein 5; and transforming growth factor- β -induced) were also up-regulated. SPARCL1, a negative regulator of cell adhesion, was down-regulated. In addition, mRNA levels for ADAMTS2 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif-2), which cleaves propeptides of collagen type I and II before collagen assembly, lumican, which regulates collagen assembly, and tissue inhibitor of metalloproteinase 4, which irreversibly inactivates metalloproteinases that degrade extracellular matrix, were all increased by ponA induction in HH4 cells. These changes suggest increased extracellular matrix deposition and enhanced cell adhesion, which are both important events in inflammation. It is noteworthy that E-cadherin was markedly up-regulated (17.1-fold at 2 days and 19.7-fold at 6 days) by HCV ORF expression. E-cadherin-mediated adhesion may sequester β -catenin, an essential molecule for the canonical WNT signaling pathway, and also inhibit receptor tyrosine kinase activity, therefore, negatively regulating cell proliferation.

Genes Involved in Cell Proliferation and Cell Cycle Progression

After 2 days of ponA induction, genes for LAG1 longevity homolog 2, Cdk5 and Abl enzyme substrate 1, inhibitor of DNA binding 4, and transgelin (an actin cross-linking protein that is believed to contribute to replicative senescence), which are all negative regulators of cell growth, were up-regulated. ASPM (abnormal-spindle-like microcephaly associated), which plays role in mitotic spindle regulation thus required for mitosis, was down-regulated. Therefore, these changes indicate a decrease in cell proliferation and cell cycle progression in 2-day ponA-induced HCV-ORF cells. In RNA samples isolated from 6-day ponA-induced HCV-ORF cells, 36 genes related to cell growth were differentially expressed. The up-regulation of LAG1 longevity homolog 2, transgelin, and inhibitor of DNA binding 4 and the down-regulation of inner centromere protein antigens 135/155 kDa, MK167 (antigen identified by monoclonal antibody Ki-67), neuroregulin 2, and tumor-associated calcium signal transducer 2 suggest decreased cell proliferation. Furthermore, the down-regulation of mRNA levels for 22 histone genes (listed in Supplemental Table 1, see <http://ajp.amjpathol.org>), which are tightly regulated during DNA

Table 3. Selected List of Differentially Regulated Genes by HCV-ORF Expression

Accession no.	Description	Mean fold change	
		2 days	6 days
	Innate immune response		
NM_000575	Interleukin 1 α	1.5	4
NM_003264	Toll-like receptor 2	2	3.4
NM_000591	CD14 antigen	2.6	3.2
NM_000758	Colony stimulating factor 2 (granulocyte-macrophage)	2.2	4.6
NM_002996	Chemokine (C-X3-C motif) ligand 1	2.2	2.2
NM_003326	Tumor necrosis factor (ligand) superfamily, member 4	2.1	2
NM_001935	Dipeptidylpeptidase 4 (CD26)	3	6
NM_001511	Chemokine (C-X-C motif) ligand 1	-2.2	-1.3
NM_005218	Defensin- β 1	2	1.9
NM_000607	Orosomucoid 1	1.7	2.2
NM_002349	Lymphocyte antigen 75	2.2	2.4
NM_004428	Growth differentiation factor 15	3.2	4.1
NM_012445	Spondin 2, extracellular matrix protein	3.3	1.8
NM_022138	SPARC-related modular calcium binding 2	2.3	2.2
SERPINB2	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	-1.6	2
AK092499	Tissue factor pathway inhibitor 2	9.4	10.3
	Adhesion molecules and extracellular matrix proteins		
NM_002210	Integrin- α V (vitronectin receptor, α polypeptide, antigen CD51)	0.6	2
NM_000610	CD44 antigen (homing function and Indian blood group system)	1.3	2.1
NM_007361	Nidogen 2 (osteonidogen)	1.5	2.2
NM_003259	Intercellular adhesion molecule 5, telencephalin	2.1	1.7
NM_002206	Integrin- α 7	2.5	2.6
NM_002204	Integrin- α 3 (antigen CD49C, α 3-subunit of VLA-3 receptor)	2.5	2.7
NM_017980	LIM and senescent cell antigen-like domains 2	2.6	3
NM_003277	Claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)	2.8	1.8
NM_000201	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	3.1	5.7
NM_001078	Vascular cell adhesion molecule 1	3.4	5.9
NM_004360	Cadherin 1, type 1, E-cadherin (epithelial)	17.1	19.7
NM_001848	Collagen, type VI, alpha 1	1.5	2.1
NM_000358	Transforming growth factor- β -induced, 68 kDa	1.6	2.6
NM_005562	Laminin- γ 2	1.9	2.3
NM_000093	Collagen, type V, α 1	2.1	1.7
NM_003480	Microfibrillar-associated protein 5	2	2.4
NM_002404	Microfibrillar-associated protein 4	2.1	2
NM_001853	Collagen, type IX, α 3	6	3.2
NM_014244	Disintegrin-like and metalloprotease (repolysin type) with thrombospondin type 1 motif, 2	2	1.6
NM_002345	Lumican	3	4.5
NM_003256	Tissue inhibitor of metalloproteinase 4	4	7.2
NM_004684	SPARC-like 1 (mast9, hevin)	-1.3	-2.5
	Cell proliferation/cell cycle progression		
NM_018136	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	-2.9	-1.8
NM_000076	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	-1.8	-4.8
NM_020238	Inner centromere protein antigens 135/155 kDa	-1.2	-2.3
NM_002417	Antigen identified by monoclonal antibody Ki-67	-1.8	-2.2
NM_013982	Neuregulin 2	-1.6	-3.6
NM_002353	Tumor-associated calcium signal transducer 2	-1.6	-2.4
NM_139211	Homeodomain-only protein	-1.5	-2
NM_007329	Deleted in malignant brain tumors 1	-1.3	-2.1
NM_003236	Transforming growth factor- α	1.3	2
NM_019617	Gastrophilin 1	1.3	2.1
NM_001432	Epiregulin	1.6	2.2
NM_015714	Putative lymphocyte G0/G1 switch gene	1.7	2.1
NM_181746	LAG1 longevity assurance homolog 2 (<i>Saccharomyces cerevisiae</i>)	2	2.3
NM_138375	Cdk5 and Abl enzyme substrate 1	2.3	1.9
NM_015996	Transgelin	2.3	2.1
U90551	Human histone 2A-like protein (H2A/L) mRNA, complete cds.	-1.3	-2.9
NM_017445	H2B histone family, member S	-0.4	-2.1
NM_005319	Histone 1, H1c	0.4	-2.9
NM_005321	Histone 1, H1e	0.4	-2.5
NM_021052	Histone 1, H2ae	1.2	-2.5
NM_021062	<i>Homo sapiens</i> histone 1, H2bb (HIST1H2BB), mRNA	-0.3	-2.4
NM_003526	Histone 1, H2bc	-0.4	-2.3
NM_021063	Histone 1, H2bd	-1	-2.2

(table continues)

Table 3. *Continued*

Accession no.	Description	Mean fold change	
		2 days	6 days
NM_003523	<i>H. sapiens</i> histone 1, H2be (HIST1H2BE), mRNA	−0.4	−2.3
NM_003518	Histone 1, H2bg	0.4	−3.6
NM_003524	Histone 1, H2bh	−0.4	−2.1
NM_003525	<i>H. sapiens</i> histone 1, H2bi (HIST1H2BI), mRNA	−0.4	−2.4
NM_021058	Histone 1, H2bj	−0.4	−2.4
NM_003519	Histone 1, H2bl	−0.4	−2.4
NM_003520	Histone 1, H2bn	−0.4	−2.3
NM_003527	Histone 1, H2bo	−0.4	−2
NM_003537	Histone 1, H3b	−0.3	−2.2
NM_021065	Histone 1, H3 days	1.1	−2
BC052981	Histone 1, H3e	1.1	−2.2
BC007518	Histone 1, H3 hours	1.1	−2.3
NM_003516	Histone 2, H2aa	1.2	−2.5
NM_175055	Histone 3, H2bb	−0.4	−2.1
	Genetic stability		
NM_000135	Fanconi anemia, complementation group A	1.2	2
S62138	Fusion [involved in t(12;16) in malignant liposarcoma]	1.8	2.9
NM_016821	8-oxoguanine DNA glycosylase	2	1.7
NM_002912	REV3-like, catalytic subunit of DNA polymerase ζ (yeast)	−2.2	−2.4
	Oxidative stress response		
NM_004528	Microsomal glutathione S-transferase 3	2	1.6
NM_005949	Metallothionein 1F (functional)	3.5	4.9
NM_004881	Tumor protein p53-inducible protein 3	3.1	2.9
NM_006472	Thioredoxin interacting protein	−1.6	−5.4

replication,³² indicates that cell proliferation and cell cycle progression were likely inhibited in 6-day ponA-induced HCV-ORF cells.

Genes Involved in Oxidative Stress and Maintenance of Genetic Integrity

Microarray indicated that HCV ORF expression up-regulated microsomal glutathione S-transferase 3 at 2 days, down-regulated thioredoxin-interacting protein (an inhibitor of thioredoxin) at 6 days, and up-regulated metallothionein 1F (an antioxidant protein) at both 2 and 6 days of ponA induction. Microsomal glutathione S-transferase 3, metallothionein 1F, and thioredoxin all play a protective role against ROS. Thus, the differentially regulated expression of microsomal glutathione S-transferase 3, MT1F, and thioredoxin-interacting protein in HH4 cells expressing HCV ORF is likely to reflect an activated cellular response to oxidative stress in these cells. Furthermore, 8-oxoguanine DNA glycosylase (an enzyme that specifically repairs oxidative DNA damage) was up-regulated after 2 days of ponA induction. Modulated genes involved in genetic integrity maintenance also include up-regulation of Fanconi anemia complementation group A (a DNA repair enzyme that is responsible for postreplication repair and cell cycle checkpoint function) and fusin (a protein thought to play a role in maintenance of genomic integrity), after 2 days of ponA induction. REV3-like (the catalytic subunit of error-prone DNA polymerase ζ that performs translation DNA synthesis and thus plays a crucial role in mutagenesis) was down-regulated at both 2- and 6-day time points. Collectively, these changes suggest that ponA-induced HCV ORF expression may induce oxidative DNA damage and conse-

quently activate a protective response to maintain genomic integrity.

Validation of Microarray Data by QPCR

The validity of the microarray data was determined by quantitative real-time reverse transcription-PCR on five selected genes (TLR2, CD14, CSF2, CDH1, and IL1A). As shown in Figure 10, the QPCR results agreed well with the microarray data at both 2- and 6-day time points. The QPCR results showed a consistently higher level of changes in gene expression than the microarray data, most likely because of the high sensitivity of the assay.

Discussion

Using the ecdysone-regulated gene expression system, we have established stable clones of HH4 nontransformed human hepatocytes conditionally expressing the full-length HCV ORF. In addition, we have developed stable HH4 clones expressing only the HCV structural genes (HCV Δ), HCV C alone, and GFP control. We show that HCV C, E1, E2, NS3, and NS5A proteins were induced and faithfully processed in ponA-treated HCV-ORF cells. Because the full-length HCV ORF transcript was detected and the host- and virus-derived proteases required for faithful processing of HCV polypeptide were functional, as demonstrated by the proper translation of HCV C, E1, E2, NS3, and NS5A proteins, we believe that all mature HCV proteins were expressed and properly processed in ponA-induced HCV-ORF cells. Because the 5' and 3' untranslated regions were not included in the HCV plasmids introduced in HH4 cells, no HCV replica-

Figure 10. Validation of microarray assay data by QPCR. QPCR was performed in triplicate on total RNA samples isolated from HCV ORF-inducible cells treated with or without 5 μ mol/L ponA for 2 and 6 days (same samples used for microarray analysis) using TaqMan Real Time PCR System as described in Materials and Methods. Endogenous control 18S was used to normalize quantification of target gene. QPCR results for the five selected target genes (IL1A, TLR2, CD14, CSF2, and CDH1) were consistent with microarray data at both 2-day (A) and 6-day (B) time points. A $\log_{10}$ (ratio of changes) value of 0.3 indicates a ratio of 2 in quantification of gene expression in ponA-induced over uninduced cells.

tion is expected to occur in these cells, despite the production of the entire HCV coding region. It is of great interest to determine whether the inclusion of the untranslated regions would result in the regulated induction of infectious HCV in this system.

Tet-regulated expression of HCV ORF in a human osteosarcoma line (U-2-OS) has been described, and attempts to extend this inducible system to hepatocytic cells were not successful.²⁴ Tet-regulated expression of HCV ORF (genotype-1b, S1 strain^{33,34}) in Huh7 and HepG2 human hepatoma cell lines has been reported.²⁵ The transformed nature of cells used in these studies bring difficulties for studying HCV/host cell interactions because, among many genetic defects, both Huh7 and HepG2 cells are deficient in producing and responding to type I IFNs³⁵ and responding to Fas.³⁶ By contrast, the many cellular responses that are implicated in HCV/host cell interactions such as IFN induction, IFN (both type I and II) signaling, Fas- and TRAIL-mediated apoptosis, and cytokine [interleukin (IL)-6 type] signaling are intact in the nontransformed HH4 human hepatocytes (W. Tang and N. Fausto, unpublished observations). To our knowledge, the ponA-inducible expression system described here is the first system in which expression of all mature HCV proteins can be regulated in nontransformed human hepatocytes. Because expression of the HCV viral

polyprotein is an essential early step in the HCV life cycle and current systems for the study of HCV protein and host cells interactions during early phase of viral infection are limited, the regulated HCV ORF expression in HH4 cells provides a reasonable model to study the direct effects of HCV proteins on host cells. However, it is unclear why the frequency of clones that expressed full-length HCV ORF was much lower than that of clones expressing the core protein alone or GFP. Also unclear is why the majority of stable clones expressed truncated HCV ORF containing only structural genes. Transfection efficiency is usually low in HH4 cells (5 to 10% for GFP or core protein vectors and less than 1% for HCV ORF in transient transfection assays), but even in HEK293 cells, which have an overall transfection efficiency of more than 80%, the efficiency of HCV ORF transfection was about 2%. Thus, it seems unlikely that HH4 clones that express the entire HCV ORF have a permissible mutation that allows ORF expression. Nevertheless, because of the low overall transfection frequency of HH4 cells, we have not been able to determine in transient or stable transfection assays whether the expression of nonstructural proteins is toxic to the cells.

We have observed that HCV C, NS3, and NS5A colocalized in perinuclear structures in the HH4 cells. The HCV nonstructural proteins have been reported to assemble on detergent-resistant membranes called lipid rafts as part of a functional replication complex^{37–42} in the perinuclear region.^{39,43} Our finding suggests that in HH4 human hepatocytes, HCV nonstructural proteins may also assemble into structure similar to HCV replication complex. Moreover, HCV C is almost exclusively associated with HCV nonstructural proteins. Although direct interaction among HCV nonstructural proteins has been demonstrated,⁴⁴ no interaction between HCV C and NS proteins has been reported. It is likely that HCV C associates with HCV replication complex via host protein(s). Double IF staining revealed colocalization of HCV C with EEA1 but not with markers for other structures. Because HCV C is associated with EEA1 only in the context of full-length HCV ORF expression, we believe that EEA1 may be associated with the HCV replication complex. EEA1, effector of ras-associated protein 5, plays a critical role in the endosome pathway of membrane trafficking.⁴⁵ It is possible that an interaction between HCV core/NS proteins and EEA1/ras-associated protein 5 may inhibit normal endosomal membrane trafficking and contribute to the formation of membranous web structures harboring the HCV replication complex.

Although ponA-induced HCV-ORF cells grow slower initially, both uninduced and ponA-induced HCV ORF cells reached similar saturation densities. Furthermore, no significant decrease in cell viability, as assessed by TUNEL assay and trypan blue exclusion assay, was observed in HH4 cells induced to express HCV ORF. These results indicate that HCV ORF expression is not cytotoxic in HH4 human hepatocytes under normal culture conditions. The lack of detectable cytotoxicity may be due to the moderate level of HCV ORF expression in these cells and to protective mechanisms against oxidative stress. These mechanisms may be defective or less functional in

some transformed cells, such as U2-OS and HepG2 cells, used in previous studies.^{24,46}

We have observed that ponA-induced HCV ORF expression increased cellular ROS levels, as measured by RedoxSensorRed staining. Hepatocytes are equipped with multiple protective systems to combat the accumulation of ROS and prevent oxidative stress. These systems include ROS scavenger enzymes, such as catalase, superoxide dismutases, and glutathione peroxidase, as well as many nonenzymatic molecules, such as GSH and vitamins A, C, and E. Hepatocytes are the major source of GSH in the body, and the GSH antioxidant system plays an important role against oxidative stress in these cells. PonA treatment of HCV-ORF cells for 2 days resulted into a much larger increase in total intracellular GSH level and an almost 10-fold higher increase in GCL activity than PonA-induced GFP-expressing cells. These results suggest that the GSH-mediated protective system is activated and could be responsible for the lack of direct cytotoxicity in HH4 cells expressing HCV polyprotein. Oxidative stress and activation of antioxidant cellular responses have been described in hepatoma cells that conditionally express HCV core protein.⁴⁷

NF- κ B is a ubiquitous transcription factor involved in regulation of the immune response, inflammation, and apoptosis. Tai et al⁴⁸ reported that NF- κ B is activated in HCV-infected liver tissue and suggested that the activation had an anti-apoptotic effect that favors viral persistence. Both HCV core and NS5A have been implicated in NF- κ B activation. Although contradictory effects of HCV core protein on NF- κ B activation have been reported,^{49–62} activation of NF- κ B by NS5A protein is more consistently observed.^{34,63,64} In HH4 HCV ORF cells, moderate levels of regulated HCV polyprotein expression activated NF- κ B, suggesting that HCV can activate NF- κ B in its host cells in the absence of inflammatory cells. We did not detect any significant change in TNF α -activated NF- κ B DNA-binding activity by EMSA in ponA-induced HCV ORF cells, although some studies have suggested that HCV C protein may influence TNF α -mediated NF- κ B activation.^{49–62} The discrepancies about these data may be due to the differences in the cell response to the expression of the complete HCV ORF as opposed to the massive expression of transfected single HCV protein or partial HCV ORF.

Death receptor Fas- and TRAIL-mediated apoptosis pathways play important roles in immune surveillance against virus-infected and transformed host cells^{65,66} and in HCV-induced liver injury.^{67–71} Although many previous studies focused on effects of individual HCV proteins on cellular apoptosis pathways, such effects in the context of polyprotein expression have not been adequately investigated. Our results showed that although ponA-induced HCV ORF had no detectable effect on apoptosis induced by TRAIL, it sensitized HH4 cells to Fas-mediated apoptosis. However the sensitization was evident only at certain concentrations of the ligand. Both cross-linked Apo-1-3, a Fas agonist, and the Lucien-zipper form of human TRAIL efficiently induced apoptosis in 50 to 80% of HH4 cells without the use of any inhibitors of RNA or protein synthesis. Sensitization of HH4 cells to

Fas-induced apoptosis may be related to elevated ROS levels, and it is of interest that it occurred directly on hepatocytes in the absence of cells of the immune system or nonparenchymal cells.

Analysis of gene expression profiles indicates that ponA-induced HCV ORF expression in HH4 cells significantly affected RNA levels of several groups of genes that may be important for HCV/host cell interactions. Of particular interest is the observation that genes for the pro-inflammatory cytokines IL1A and CSF2 (granulocyte-macrophage colony stimulating factor) were unregulated by HCV ORF expression. These genes are usually regulated at the mRNA level; therefore, it is likely that the protein expression level and biological activity of these proteins are also activated by HCV ORF expression. IL1A and CSF2 could strongly induce production of inflammatory mediators (IL-6, TNF α , IL-1, IL-8, and IL-12) by liver resident macrophages, the Kupffer cells, thus initiating the inflammatory response in the liver. Consistent with the notion that HCV ORF expression activates the innate immune response in HH4 cells, up-regulation of Toll-like receptor 2; CD14 (the lipopolysaccharide-binding molecule of the Toll-like receptor 4 signaling complex); adhesion molecules (ICAM1 and VCAM1); a chemokine that promotes growth and maturation of neutrophils, macrophages, and dendritic cells (CSF2); and cytokines that activate homing and proliferation/differentiation of T lymphocytes (CX3CL1 and TNFSF4) was observed in HH4 cells expressing HCV polyprotein.

In addition to the protective GSH antioxidant system, HH4 human hepatocytes may activate other antioxidant mechanisms in response to HCV ORF expression. We found that HCV ORF expression up-regulated antioxidant proteins microsomal glutathione S-transferase 3 and MT1F and down-regulated thioredoxin-interacting protein, an inhibitor of antioxidant protein thioredoxin, in HH4 cells. Furthermore, up-regulation of 8-oxoguanine DNA glycosylase (a DNA repair enzyme that repairs oxidative DNA damage), Fanconi anemia complementation group A, and FUB (both are implicated in maintaining genetic integrity) and down-regulation of REV3-like (an error-prone DNA polymerase) suggest that the cellular responses to maintain genetic integrity may also be activated in these cells.

In summary, HCV ORF expression in HH4 human hepatocytes triggers innate immune and inflammatory responses. It is not cytotoxic but inhibits cell proliferation and activates the cellular response to oxidative stress and ROS-related DNA damage. Activation of the innate immune response and the subsequent inflammatory response plays a critical role in early host defense against bacterial and viral infections. Here, we show that some of these responses occur in hepatocytes, without participation of nonparenchymal or immune cells. It may be argued that in the absence of an appropriate immune response, HCV-infected cells might persist, as has been reported in transgenic mice expressing the HCV polyprotein.⁷² Prolonged activation of the inflammatory response in hepatocytes could conceivably contribute to HCV liver injury, either directly or through the involvement of other cell types.

Acknowledgments

We thank Dr. Charles Rice (Rockefeller University) for the gift of HCV cDNA construct p90/HCVFLpU; Dr. Rolf Carlson (Rhode Island Hospital, Providence, RI) for providing the C7-50 anti-core monoclonal antibody; and Melissa Johnson, James Thompson, and Jill Thompson for their excellent technical assistance.

References

- Lauer GM, Walker BD: Hepatitis C virus infection. *N Engl J Med* 2001, 345:41–52
- Hoofnagle JH: Hepatitis C: the clinical spectrum of disease. *Hepatology* 1997, 26:15S–20S
- Di Bisceglie AM: Hepatitis C and hepatocellular carcinoma. *Hepatology* 1997, 26:34S–38S
- Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS: The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N Engl J Med* 1999, 341:556–562
- Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999, 285:110–113
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ: Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005, 11:791–796
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM: Complete replication of hepatitis C virus in cell culture. *Science* 2005, 309:623–626
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV: Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005, 102:9294–9299
- Lázaro CA, Chang M, Tang W, Campbell J, Sullivan DG, Gretsch DR, Corey L, Coombs RW, Fausto N: Hepatitis C virus replication in transfected and serum-infected cultured human fetal hepatocytes. *Am J Pathol* 2007, 170:478–489
- Yan W, Lee H, Deutsch EW, Lazaro CA, Tang W, Chen E, Fausto N, Katze MG, Aebersold R: A dataset of human liver proteins identified by protein profiling via isotope-coded affinity tag (ICAT) and tandem mass spectrometry. *Mol Cell Proteomics* 2004, 3:1039–1041
- Wang HC, Chang WT, Chang WW, Wu HC, Huang W, Lei HY, Lai MD, Fausto N, Su IJ: Hepatitis B virus pre-S2 mutant upregulates cyclin A expression and induces nodular proliferation of hepatocytes. *Hepatology* 2005, 41:761–770
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162:156–159
- Kirillova I, Chaisson M, Fausto N: Tumor necrosis factor induces DNA replication in hepatic cells through nuclear factor kappaB activation. *Cell Growth Differ* 1999, 10:819–828
- Orsulic S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE: Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. *Cancer Cell* 2002, 1:53–62
- Freshney RI: *Culture of Animal Cells: A Manual of Basic Technique*. New York, Wiley Liss, 1994
- Walczak H, Miller RE, Arai K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 1999, 5:157–163
- Franklin CC, Krejsa CM, Pierce RH, White CC, Fausto N, Kavanagh TJ: Caspase-3-dependent cleavage of the glutamate-L-cysteine ligase catalytic subunit during apoptotic cell death. *Am J Pathol* 2002, 160:1887–1894
- Thompson SA, White CC, Krejsa CM, Diaz D, Woods JS, Eaton DL, Kavanagh TJ: Induction of glutamate-cysteine ligase (gamma-glutamylcysteine synthetase) in the brains of adult female mice subchronically exposed to methylmercury. *Toxicol Lett* 1999, 110:1–9
- Chaisson ML, Brooling JT, Ladiges W, Tsai S, Fausto N: Hepatocyte-specific inhibition of NF-kappaB leads to apoptosis after TNF treatment, but not after partial hepatectomy. *J Clin Invest* 2002, 110:193–202
- Smith MW, Yue ZN, Korth MJ, Do HA, Boix L, Fausto N, Bruix J, Carithers RL Jr, Katze MG: Hepatitis C virus and liver disease: global transcriptional profiling and identification of potential markers. *Hepatology* 2003, 38:1458–1467
- Furukawa T, Duguid WP, Rosenberg L, Viallet J, Galloway DA, Tsao MS: Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. *Am J Pathol* 1996, 148:1763–1770
- Le Poole IC, van den Berg FM, van den Wijngaard RM, Galloway DA, van Amstel PJ, Buffing AA, Smits HL, Westerhof W, Das PK: Generation of a human melanocyte cell line by introduction of HPV16 E6 and E7 genes. *In Vitro Cell Dev Biol Anim* 1997, 33:42–49
- Schwab TS, Stewart T, Lehr J, Pienta KJ, Rhim JS, Macoska JA: Phenotypic characterization of immortalized normal and primary tumor-derived human prostate epithelial cell cultures. *Prostate* 2000, 44:164–171
- Moradpour D, Kary P, Rice CM, Blum HE: Continuous human cell lines inducibly expressing hepatitis C virus structural and nonstructural proteins. *Hepatology* 1998, 28:192–201
- Lim SP, Soo HM, Tan YH, Brenner S, Horstmann H, MacKenzie JM, Ng ML, Lim SG, Hong W: Inducible system in human hepatoma cell lines for hepatitis C virus production. *Virology* 2002, 303:79–99
- Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, Bartenschlager R: Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* 2002, 76:4008–4021
- Miyamura T, Matsuura Y: Structural proteins of hepatitis C virus. *Trends Microbiol* 1993, 1:229–231
- Chen CS, Gee KR: Redox-dependent trafficking of 2,3,4,5,6-pentafluorodihydroxytetramethylrosamine, a novel fluorogenic indicator of cellular oxidative activity. *Free Radic Biol Med* 2000, 28:1266–1278
- Michiels C, Minet E, Mottet D, Raes M: Regulation of gene expression by oxygen: NF-kappaB and HIF-1, two extremes. *Free Radic Biol Med* 2002, 33:1231–1242
- Haddad JJ: Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cell Signal* 2002, 14: 879–897
- Martindale JL, Holbrook NJ: Cellular response to oxidative stress: signaling for suicide and survival. *J Cell Physiol* 2002, 192:1–15
- Gunjan A, Paik J, Verreault A: Regulation of histone synthesis and nucleosome assembly. *Biochimie* 2005, 87:625–635
- Lim SP, Khu YL, Hong WJ, Tay A, Ting AE, Lim SG, Tan YH: Identification and molecular characterisation of the complete genome of a Singapore isolate of hepatitis C virus: sequence comparison with other strains and phylogenetic analysis. *Virus Genes* 2001, 23:89–95
- Soo HM, Garzino-Demo A, Hong W, Tan YH, Tan YJ, Goh PY, Lim SG, Lim SP: Expression of a full-length hepatitis C virus cDNA up-regulates the expression of CC chemokines MCP-1 and RANTES. *Virology* 2002, 303:253–277
- Keskinen P, Nyqvist M, Sareneva T, Pirhonen J, Melen K, Julkunen I: Impaired antiviral response in human hepatoma cells. *Virology* 1999, 263:364–375
- Natoli G, Ianni A, Costanzo A, De Petrillo G, Ilari I, Chirillo P, Balsano C, Leverro M: Resistance to Fas-mediated apoptosis in human hepatoma cells. *Oncogene* 1995, 11:1157–1164
- Gao L, Aizaki H, He JW, Lai MM: Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J Virol* 2004, 78:3480–3488
- Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM: Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 2004, 324:450–461
- Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, Bienz K, Moradpour D: Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J Virol* 2003, 77:5487–5492
- Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K: Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 2002, 76:5974–5984

41. Egger D, Gosert R, Bienz K: Role of cellular structures in viral RNA replication. Role of Cellular Structures in Viral RNA Replication. Edited by B Semler, E Wimmer. Washington, DC, ASM Press, 2002, pp 247–253
42. Schwartz M, Chen J, Janda M, Sullivan M, den Boon J, Ahlquist P: A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol Cell* 2002, 9:505–514
43. Matto M, Rice CM, Aroeti B, Glenn JS: Hepatitis C virus core protein associates with detergent-resistant membranes distinct from classical plasma membrane rafts. *J Virol* 2004, 78:12047–12053
44. Dimitrova M, Imbert I, Kieny MP, Schuster C: Protein-protein interactions between hepatitis C virus nonstructural proteins. *J Virol* 2003, 77:5401–5414
45. Mills IG, Jones AT, Clague MJ: Regulation of endosome fusion. *Mol Membr Biol* 1999, 16:73–79
46. Kalkeri G, Khalap N, Garry RF, Fermin CD, Dash S: Hepatitis C virus protein expression induces apoptosis in HepG2 cells. *Virology* 2001, 282:26–37
47. Li K, Prow T, Lemon SM, Beard MR: Cellular response to conditional expression of hepatitis C virus core protein in Huh7 cultured human hepatoma cells. *Hepatology* 2002, 35:1237–1246
48. Tai DI, Tsai SL, Chen YM, Chuang YL, Peng CY, Sheen IS, Yeh CT, Chang KS, Huang SN, Kuo GC, Liaw YF: Activation of nuclear factor kappaB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. *Hepatology* 2000, 31:656–664
49. Joo M, Hahn YS, Kwon M, Sadikot RT, Blackwell TS, Christman JW: Hepatitis C virus core protein suppresses NF-kappaB activation and cyclooxygenase-2 expression by direct interaction with IkappaB kinase beta. *J Virol* 2005, 79:7648–7657
50. Lasarte JJ, Sarobe P, Boya P, Casares N, Arribillaga L, de Cerio AL, Gorraiz M, Borras-Cuesta F, Prieto J: A recombinant adenovirus encoding hepatitis C virus core and E1 proteins protects mice against cytokine-induced liver damage. *Hepatology* 2003, 37:461–470
51. Shrivastava A, Manna SK, Ray R, Aggarwal BB: Ectopic expression of hepatitis C virus core protein differentially regulates nuclear transcription factors. *J Virol* 1998, 72:9722–9728
52. Zhu N, Khoshnan A, Schneider R, Matsumoto M, Dennert G, Ware C, Lai MM: Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J Virol* 1998, 72:3691–3697
53. Balasubramanian A, Munshi N, Koziel MJ, Hu Z, Liang TJ, Groopman JE, Ganju RK: Structural proteins of hepatitis C virus induce interleukin 8 production and apoptosis in human endothelial cells. *J Gen Virol* 2005, 86:3291–3301
54. Kim WH, Hong F, Jaruga B, Hu Z, Fan S, Liang TJ, Gao B: Additive activation of hepatic NF-kappaB by ethanol and hepatitis B protein X (HBX) or HCV core protein: involvement of TNF-alpha receptor 1-independent and -dependent mechanisms. *FASEB J* 2001, 15:2551–2553
55. de Lucas S, Bartolome J, Amaro MJ, Carreno V: Hepatitis C virus core protein transactivates the inducible nitric oxide synthase promoter via NF-kappaB activation. *Antiviral Res* 2003, 60:117–124
56. Marusawa H, Hijikata M, Chiba T, Shimotohno K: Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. *J Virol* 1999, 73:4713–4720
57. You LR, Chen CM, Lee YH: Hepatitis C virus core protein enhances NF-kappaB signal pathway triggering by lymphotoxin-beta receptor ligand and tumor necrosis factor alpha. *J Virol* 1999, 73:1672–1681
58. Chung YM, Park KJ, Choi SY, Hwang SB, Lee SY: Hepatitis C virus core protein potentiates TNF-alpha-induced NF-kappaB activation through TRAF2-IKKbeta-dependent pathway. *Biochem Biophys Res Commun* 2001, 284:15–19
59. Yoshida H, Kato N, Shiratori Y, Otsuka M, Maeda S, Kato J, Omata M: Hepatitis C virus core protein activates nuclear factor kappa B-dependent signaling through tumor necrosis factor receptor-associated factor. *J Biol Chem* 2001, 276:16399–16405
60. Kato N, Yoshida H, Kioko Ono-Nita S, Kato J, Goto T, Otsuka M, Lan K, Matsushima K, Shiratori Y, Omata M: Activation of intracellular signaling by hepatitis B and C viruses: c-viral core is the most potent signal inducer. *Hepatology* 2000, 32:405–412
61. Heim MH, Moradpour D, Blum HE: Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *J Virol* 1999, 73:8469–8475
62. Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Matsuura Y, Kimura S, Koike K, Miyamura T: Alteration of intrahepatic cytokine expression and AP-1 activation in transgenic mice expressing hepatitis C virus core protein. *Virology* 2002, 304:415–424
63. Waris G, Livolsi A, Imbert V, Peyron JF, Siddiqui A: Hepatitis C virus NS5A and subgenomic replicon activate NF-kappaB via tyrosine phosphorylation of IkappaBalpha and its degradation by calpain protease. *J Biol Chem* 2003, 278:40778–40787
64. Girard S, Vossman E, Misk DE, Podevin P, Hanash S, Brechot C, Beretta L: Hepatitis C virus NS5A-regulated gene expression and signaling revealed via microarray and comparative promoter analyses. *Hepatology* 2004, 40:708–718
65. Barber GN: Host defense, viruses and apoptosis. *Cell Death Differ* 2001, 8:113–126
66. Sträter J, Moller P: TRAIL and viral infection. *Vitam Horm* 2004, 67:257–274
67. Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, Krammer PH, Runkel L: Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. *J Exp Med* 1995, 182:1223–1230
68. Bantel H, Schulze-Osthoff K: Apoptosis in hepatitis C virus infection. *Cell Death Differ* 2003, 10(Suppl 1):S48–S58
69. Mundt B, Wirth T, Zender L, Waltemathe M, Trautwein C, Manns MP, Kuhnel F, Kubicka S: Tumour necrosis factor related apoptosis inducing ligand (TRAIL) induces hepatic steatosis in viral hepatitis and after alcohol intake. *Gut* 2005, 54:1590–1596
70. Yin XM, Ding WX: Death receptor activation-induced hepatocyte apoptosis and liver injury. *Curr Mol Med* 2003, 3:491–508
71. Kountouras J, Zavos C, Chatzopoulos D: Apoptosis in hepatitis C. *J Viral Hepat* 2003, 10:335–342
72. Disson O, Haouzi D, Desagher S, Loesch K, Hahne M, Kremer EJ, Jacquet C, Lemon SM, Hibner U, Lerat H: Impaired clearance of virus-infected hepatocytes in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2004, 126:859–872
73. Lázaro CA, Croager EJ, Mitchell C, Campbell JS, Yu C, Foraker J, Rhim JA, Yeoh GC, Fausto N: Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. *Hepatology* 2003, 38:1095–1106