

Rapid Emergence of Telaprevir Resistant Hepatitis C Virus Strain from Wildtype Clone *In Vivo*

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Telaprevir is a potent inhibitor of hepatitis C virus (HCV) NS3-4A protease. However, the emergence of drug-resistant strains during therapy is a serious problem, and the susceptibility of resistant strains to interferon (IFN), as well as the details of the emergence of mutant strains *in vivo*, is not known. We previously established an infectious model of HCV using human hepatocyte chimeric mice. Using this system we investigated the biological properties and mode of emergence of mutants by ultra-deep sequencing technology. Chimeric mice were injected with serum samples obtained from a patient who had developed viral breakthrough during telaprevir monotherapy with strong selection for resistance mutations (A156F [92.6%]). Mice infected with the resistant strain (A156F [99.9%]) developed only low-level viremia and the virus was successfully eliminated with interferon therapy. As observed in patients, telaprevir monotherapy in viremic mice resulted in breakthrough, with selection for mutations that confer resistance to telaprevir (e.g., a high frequency of V36A [52.2%]). Mice were injected intrahepatically with HCV genotype 1b clone KT-9 with or without an introduced resistance mutation, A156S, in the NS3 region, and treated with telaprevir. Mice infected with the A156S strain developed lower-level viremia compared to the wildtype strain but showed strong resistance to telaprevir treatment. Although mice injected with wildtype HCV showed a rapid decline in viremia at the beginning of therapy, a high frequency (11%) of telaprevir-resistant NS3 V36A variants emerged 2 weeks after the start of treatment. **Conclusion:** Using deep sequencing technology and a genetically engineered HCV infection system, we showed that the rapid emergence of telaprevir-resistant HCV was induced by mutation from the wildtype strain of HCV *in vivo*. (HEPATOLOGY 2011;54:781-788)

Chronic hepatitis C virus (HCV) infection is a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma.^{1,2} The current standard treatment for patients chronically infected with HCV is the combination of peg-interferon (PEG-IFN) and

ribavirin (RBV).³⁻⁵ However, this treatment results in sustained viral response (SVR), defined as negative for HCV RNA 24 weeks after cessation of the therapy, in only about 50% of patients with genotype 1 HCV infection with high viral loads.³⁻⁵ Given the low

Abbreviations: HCV, hepatitis C virus; HSA, human serum albumin; PEG-IFN, peg-interferon; RBV, ribavirin; RT-PCR, reverse transcript-polymerase chain reaction; SCID, severe combined immunodeficiency; SVR, sustained viral response; uPA, urokinase-type plasminogen activator.

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effectiveness of the current therapy, many molecules have been screened for antiviral activity against HCV for use in development of novel anti-HCV therapies. A number of new selective inhibitors of HCV proteins, the so-called STAT-C (specifically targeted antiviral therapy for HCV) inhibitors, are currently under development. Telaprevir is a reversible, selective, specific inhibitor of the HCV NS3-4A protease that has shown potent antiviral activity in HCV replicon assays.⁶ Although the antiviral effect of telaprevir is quite potent, monotherapy using these drugs results in rapid emergence of drug-resistant strains.^{7,8} Accordingly, these drugs are used in combination with pegylated-IFN and ribavirin for chronic hepatitis C patients. Because the HCV virus replicates rapidly and RNA polymerase lacks a proofreading system, HCV viral quasispecies can emerge *de novo*, and some of these variants may confer resistance. Although a resistant variant is initially present at low frequency, it may quickly emerge as the dominant species during antiviral treatment.^{9,10} Resistant clones against HCV NS3-4A protease inhibitors have reportedly been induced in replicon systems.

The immunodeficient urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes, resulting in human hepatocyte chimeric mice that are able to develop HCV viremia after injection of serum samples positive for the virus.¹¹ We and other groups have reported that the human hepatocyte chimeric mouse is useful for evaluating the effect of NS3-4A protease inhibitor.^{12,13} Using this mouse model, we developed a reverse genetics systems for HCV.^{14,15} This system is useful to study characteristics of HCV strains with various substitutions of interest because the confounding effects of quasispecies can be minimized. Using ultra-deep sequencing technology, we demonstrate the rapid emergence of telaprevir resistance in HCV as a result of mutation from wildtype strain using genetically engineered HCV-infected human hepatocyte chimeric mice.

Materials and Methods

Animal Treatment. Generation of the uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described recently by our group.¹⁶ All mice were transplanted with frozen human hepatocytes obtained from the same donor. Mice received humane care and all animal protocols were performed in accordance with the guidelines of the local committee for animal experiments. Infection, extraction of serum samples, and sacrifice were per-

formed under ether anesthesia. Mice were injected either intravenously with HCV-positive human serum samples or intrahepatically with *in vitro*-transcribed genotype 1b HCV RNA. HCV-infected mice were administered either perorally with 200-300 mg/kg of telaprevir (VX950; MP424; Mitsubishi Tanabe Pharma, Osaka, Japan) twice a day or intramuscularly with 1,500 IU/g of IFN-alpha (Dainippon Sumitomo Pharma, Tokyo). The telaprevir dose was determined in a previous study in which this dosage range was found to yield serum concentrations equivalent to treated human patients.¹³

Human Serum Samples. After obtaining written informed consent, human serum samples containing genotype 1b HCV were obtained from two patients with chronic hepatitis. The individual serum samples were divided into aliquots and stored separately in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the Institutional Review Committee.

HCV RNA Transcription and Inoculation into Chimeric Mice. We have previously established an infectious genotype 1b HCV clone HCV-KT9 derived from a Japanese patient with severe acute hepatitis (GenBank access. no. AB435162).¹⁵ We cloned this HCV complementary DNA (cDNA) into plasmid pBR322 under a T7 RNA promoter to create the plasmid pHCV-KT9. Ten μ g of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, were transcribed in a 100 μ L reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μ L of phosphate-buffered saline (PBS) and injected into the livers of chimeric mice.¹⁵ The QuikChange site-directed mutagenesis kit (Stratagene, Foster City, CA) was used to introduce a substitution at amino acid 156 of the NS3 region (A156S).

RNA Extraction and Amplification. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyak, Tokyo), dissolved in 8.8 μ L RNase-free H₂O, and reverse transcribed using a random primer (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) in a 20- μ L reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostic, Japan, Tokyo) were performed as reported.¹⁵

Ultra-Deep Sequencing. We adapted multiplex sequencing-by-synthesis to simultaneously sequence

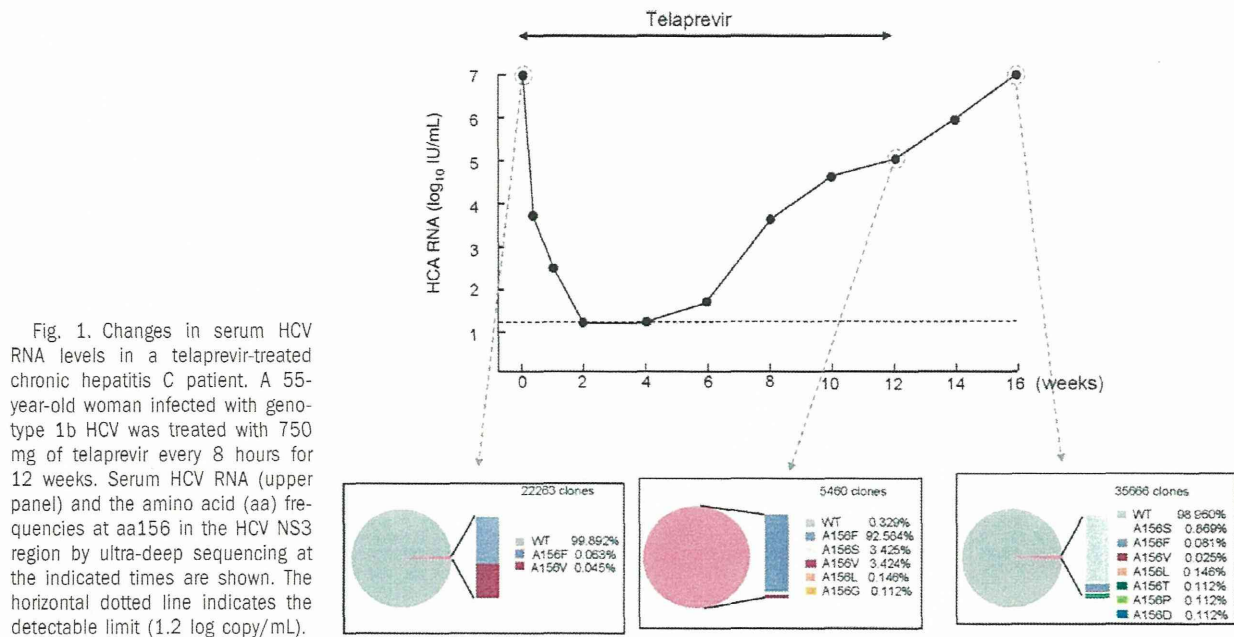


Fig. 1. Changes in serum HCV RNA levels in a telaprevir-treated chronic hepatitis C patient. A 55-year-old woman infected with genotype 1b HCV was treated with 750 mg of telaprevir every 8 hours for 12 weeks. Serum HCV RNA (upper panel) and the amino acid (aa) frequencies at aa156 in the HCV NS3 region by ultra-deep sequencing at the indicated times are shown. The horizontal dotted line indicates the detectable limit (1.2 log copy/mL).

multiple genomes using the Illumina Genome Analyzer. Briefly, cDNA was fragmented using sonication and the resultant fragment distribution was assessed using the Agilent BioAnalyzer 2100 platform. A library was prepared using the Multiplexing Sample Preparation Kit (Illumina, CA). Imaging analysis and base calling were performed using Illumina Pipeline software with default settings.¹⁷⁻²³ The N-terminal 543 nucleotides of NS3 protease were analyzed. This technique revealed an average coverage depth of over 1,000 sequence reads per basepair in the unique regions of the genome. Read mapping to a reference sequence was performed using Bowtie.²⁴ Because of the short 36 nucleotide read length, mapping hyper-variable regions with multiple closely spaced variants against a reference sequence yields poor coverage. Therefore, common variants were identified by relaxing the mismatch settings as well as using *de novo* assembly using ABySS.²⁵ Multiple alternative reference sequences were included to improve coverage in variable regions. Codon counts were merged and analyzed using R v. 2.12.

Results

Emergence of a Telaprevir-Resistant Variant in a Hepatitis C Patient Treated with Telaprevir and Analysis of the A156F Mutation. A 55-year-old woman infected with genotype 1b HCV was treated with 750 mg of telaprevir every 8 hours for 12 weeks (Fig. 1). After 1 weeks of treatment, serum HCV

RNA titer decreased below the detectable limit (1.2 log copy/mL). However, HCV RNA titer became positive by week 4. By week 12, HCV RNA titer had increased to 4.8 log copy/mL and telaprevir treatment was discontinued. Because direct sequence analysis showed an A156F mutation in the NS3 region in the serum samples at 12 weeks, we performed ultra-deep sequence analysis and confirmed the high frequency (92.5%) of A156F mutation. Four weeks after cessation of treatment (at 16 weeks), sequence analysis revealed that the major strain had reverted to wildtype (99%). To analyze the replication ability and the susceptibility of the A156F mutation to telaprevir, 100 μ L serum samples containing 10^4 copies of HCV obtained at week 12 were injected into human hepatocyte chimeric mice. Two wildtype HCV-inoculated mice became positive for HCV RNA 2 weeks after inoculation and serum HCV RNA titer increased to high levels (7.6 and 7.8 log copy/mL, respectively) at 6 weeks after inoculation (Fig. 2). In contrast to wildtype HCV-infected mice, a mouse inoculated with serum containing the A156F mutant developed measurable viremia at 4 weeks postinoculation, although serum HCV RNA titer remained low at 6 weeks (5.2 log copy/mL). Eight weeks after inoculation ultra-deep sequence analysis showed a high frequency (99.9%) of A156F mutation. From this point the mouse was administered 200 mg/kg of telaprevir perorally twice a day for 4 weeks. However, this treatment resulted in no reduction in serum HCV RNA level. During the observation period the A156F mutation remained at

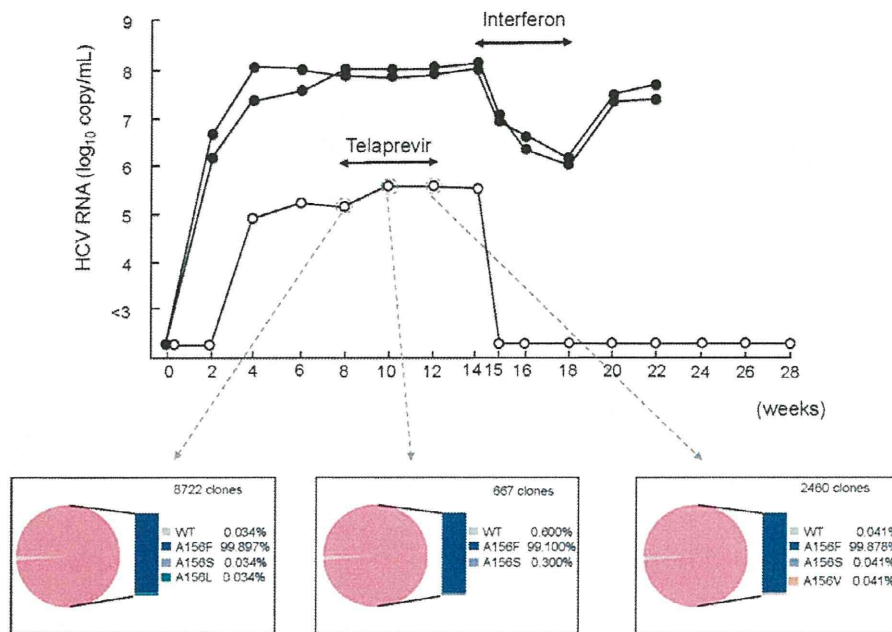


Fig. 2. Changes in serum virus titers in HCV-infected mice. Mice were injected with either wildtype (closed circles) or A156F-mutated HCV serum samples (obtained from an HCV-infected patient who received telaprevir monotherapy for 12 weeks; see Fig. 1) (open circles). Six weeks after injection the A156F mutant mouse was treated with 200 mg/kg of telaprevir orally twice a day for 4 weeks and injected intramuscularly with 1,500 IU/g/day of interferon-alpha for 4 weeks. Serum HCV RNA (upper panel) and amino acid (aa) frequencies at aa156 in the HCV NS3 region by ultra-deep sequencing at the indicated times are shown.

high frequency (>99%). To analyze the susceptibility of the A156F mutation to IFN, wildtype or A156F-mutated HCV-infected mice were treated with 1,500 IU/g/day of IFN-alpha for 4 weeks. Treatment resulted in only a two log reduction in HCV RNA level in wildtype HCV-infected mice. In contrast, serum HCV RNA titer decreased below the detectable limit 1 week after treatment in an A156F-infected mouse. Ten weeks after cessation of IFN-treatment (at week 28), HCV RNA in the mouse serum remained undetectable, suggesting that HCV RNA was eliminated. These results demonstrate that the A156F variant is associated with telaprevir-resistance, but the mutant has low replication ability and a high susceptibility to IFN.

Effect of Telaprevir on HCV-Infected Mice and Sequence Analysis of NS3 Region. Next we investigated the effect of telaprevir on wildtype HCV-infected mice. Two chimeric mice were inoculated intravenously with serum samples containing 10^5 copies of HCV obtained from an HCV-positive patient (Fig. 3). Six weeks after inoculation both mice were administered 200 mg/kg of telaprevir perorally twice a day for 4 weeks. Serum HCV RNA titer in both mice rapidly decreased; however, in one of the mice HCV RNA titer increased again 3 weeks after the start of treatment. Ultra-deep sequence analysis of the NS3 region showed that following the start of telaprevir administration the frequency of the V36A mutation increased from 18% at 2 weeks to 52% at 4 weeks, at which point it was accompanied by an increase in the HCV RNA titer. Two weeks after cessation of telapre-

vir treatment (at week 12), ultra-deep sequence analysis revealed that the frequency of the V36A mutant had decreased to 13% and the frequency of the wildtype HCV had increased to 84%, although the HCV RNA titer increased only slightly.

Intrahepatic Injection of HCV-KT9-Wild RNA and KT9-NS3-A156S RNA into Human Hepatocyte Chimeric Mice. We previously established an infectious genotype 1b HCV clone, HCV-KT9 (HCV-KT9-wild).¹⁵ We created a telaprevir-resistant HCV clone by introducing an A156S amino acid substitution in the NS3 region of HCV-KT9 (KT9-NS3-A156S) (Fig. 4A). Using wildtype and telaprevir-resistant clones we investigated the replication ability *in vivo*. Mice were injected intrahepatically with 30 μ g of *in vitro*-transcribed HCV-KT9-wild RNA or KT9-NS3-A156S RNA. Mice injected with HCV-KT9-wild developed measurable viremia at 2 weeks postinoculation and by 4 weeks postinoculation HCV RNA had reached 10^7 copy/mL (Fig. 4B). On the other hand, mice injected with KT9-NS3-A156S developed measurable viremia at 4 weeks postinoculation but maintained only low levels of viremia. These results suggest that the telaprevir-resistant HCV clone has a lowered replication ability compared to the wildtype HCV clone *in vivo*.

Treatment with Telaprevir and Analysis of Mutagenesis in Mice. Two mice infected with HCV-KT9-wild and one mouse infected with KT9-NS3-A156S were treated with 200 mg/kg of telaprevir twice a day for 2 weeks (Fig. 5A), resulting in 1.4 and 2.7 log

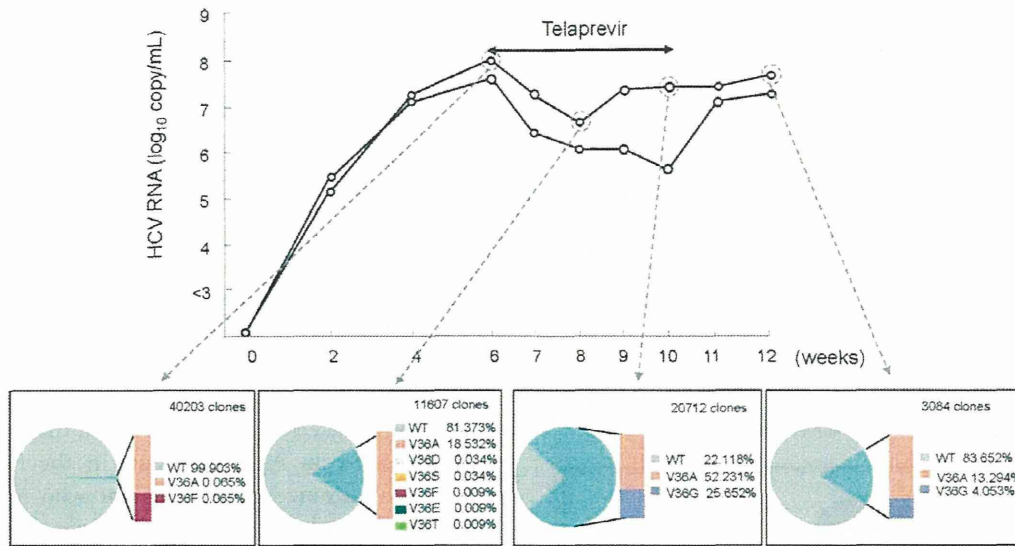


Fig. 3. Treatment with telaprevir in wildtype HCV-infected mice. Two mice were injected intravenously with 50 μ L of HCV-positive human serum samples. Six weeks after HCV injection mice were treated with 200 mg/kg of telaprevir orally twice a day for 4 weeks. Serum HCV RNA (upper panel) and amino acid (aa) frequencies at aa36 in the HCV NS3 region by ultra-deep sequencing at the indicated times are shown.

reductions in HCV RNA level in the two wildtype HCV-infected mice. In contrast, only a 0.6 log reduction was observed in the KT9-NS3-A156S-infected mouse. These results demonstrate that our human hepatocyte chimeric mouse model infected with *in vitro*-transcribed HCV RNA provides an effective system for analysis of the susceptibility of HCV mutants to antiviral drugs. Interestingly, ultra-deep sequence analysis showed a rapid emergence of a V36A variant in the NS3 region in mouse serum 2 weeks after treatment (Fig. 5B). Four weeks after cessation of treatment (at week 6) the frequency of the V36A variant had decreased. Mice were then treated with 300 mg/kg of telaprevir twice a day for 4 weeks, which resulted in an elevated frequency of V36A variants at 1 (at week 7, 5.4%) and 4 weeks (at 10 week, 41.8%) after treatment and no reduction in serum HCV RNA level. These results suggest that telaprevir-resistant mutations emerged *de novo* from the wildtype strain of HCV, presumably through error-prone replication and potent selection for telaprevir escape mutants. During the telaprevir treatment period no increases of HCV RNA titers in these mice were observed, probably due to the low frequency of the resistant strain.

Discussion

Telaprevir is a peptidomimetic inhibitor of the NS3-4A serine protease that is currently undergoing clinical evaluation. Despite its effectiveness against HCV, some patients have shown a rapid viral break-

through during the first 14 days of treatment.²⁶ Population sequencing of the viral NS3 region identified a number of mutations near the NS3 protease catalytic

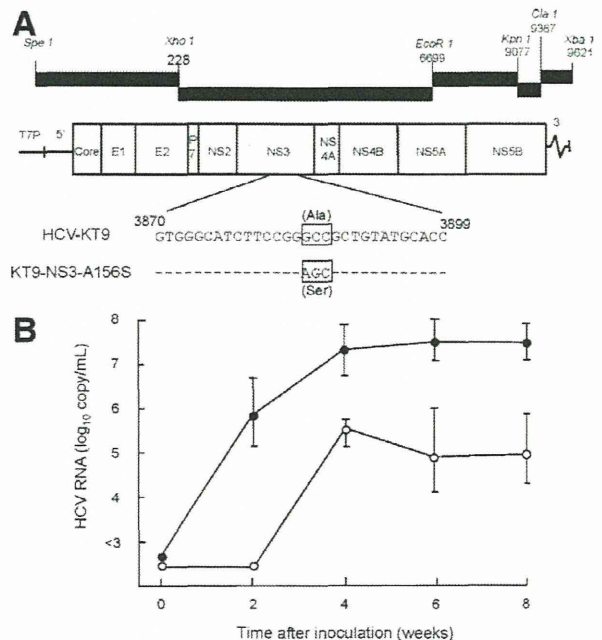


Fig. 4. Intrahepatic injection of *in vitro* transcribed HCV-KT9 RNA and KT9-NS3-A156S RNA into human hepatocyte chimeric mice. (A) The schematic of infectious genotype 1b HCV clones, HCV-KT9 and KT9-NS3-A156S. Boxes indicate codons at amino acid 156 in HCV NS3 region. Ala, alanine; Ser, serine. (B) Changes in serum levels of HCV RNA in mice intrahepatically injected with either HCV-KT9 RNA (closed circles) or KT9-NS3-A156S RNA (open circles). Data are represented as the mean \pm SD of three mice.

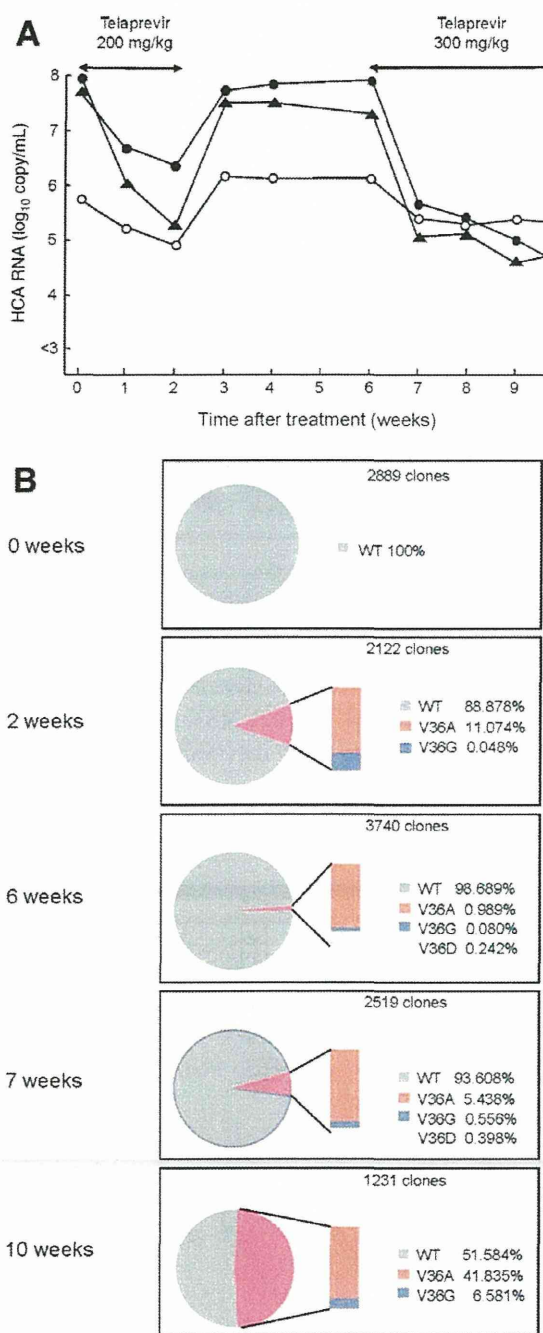


Fig. 5. The effect of telaprevir on mice infected with *in vitro*-transcribed HCV. Mice were injected with *in vitro*-transcribed HCV-KT9 RNA (closed circles and closed triangles) or KT9-NS3-A156S RNA (open circles). Six weeks after HCV RNA injection, mice were treated perorally with 200 mg/kg of telaprevir twice a day for 2 weeks. Four weeks after cessation of treatment mice were treated with 300 mg/kg of telaprevir twice a day for 4 weeks. (A) Mice serum HCV RNA titers at the indicated times are shown. Serum samples obtained from one of two HCV-KT9-infected mice (closed triangles) were used for ultra-deep sequencing. (B) Amino acid (aa) frequencies at aa36 in the HCV NS3 region based on ultra-deep sequencing are shown.

domain.²⁶ In particular, variants at NS3 residues 36, 54, 155, and 156 were shown to confer reduced sensitivity to telaprevir.²⁷

In this study we analyzed the association between the antiviral efficacy of telaprevir and sequence variants within the NS3 region using chimeric mice infected with serum samples obtained from an HCV genotype 1b-infected patient. One of two HCV-infected mice had a viral breakthrough during the dosing period (Fig. 3). Ultra-deep sequence analysis of the NS3 region showed an increase of the V36A mutant, which has been reported to confer telaprevir resistance.²⁶ Consequently, our results show evidence of emergence of a telaprevir-resistant variant previously detected in human clinical trials.

We detected an A156F mutant in the HCV NS3 region in a chronic hepatitis patient who had experienced viral breakthrough during telaprevir monotherapy (Fig. 1). Likewise, HCV RNA titer in mice infected with the A156F variant showed no reduction following 2 weeks of telaprevir treatment (Fig. 2). However, 2 weeks of treatment with IFN- α rapidly suppressed serum HCV RNA titer below the detectable limit. These results demonstrate that A156F is telaprevir-resistant but has a high susceptibility to IFN.

Interestingly, ultra-deep sequencing revealed that the wildtype strain was present at low frequency (0.3%) in the serum inoculum (Fig. 2). However, the frequency of the wildtype failed to increase over time (Fig. 3), suggesting that the very small number of wildtype viral RNA (about 30 copies) may be incomplete or defective, as a large proportion of viral genomes are thought to be defective due to the virus's high replication and mutation rates.⁹ Further analysis is necessary in order to interpret the significance of the presence of very low frequency variants detected by ultra-deep sequencing.

The short read lengths used in next generation sequencing also complicates the detection of rare variants, especially when variants are clustered within a region smaller than an individual read length (e.g., 36 basepairs). Relaxing the matching criteria allows mapping of more diverse reads but increases the error rate, whereas default settings may be geared toward more genetically homogenous haploid or diploid genomes. In this study we used *de novo* assembly to identify more diverse variants that failed to map to the reference sequence. Examining the variation in codon frequencies among samples, we created alternative reference sequences containing a sufficient range of variants to provide more uniform coverage of variable regions.

Using our previously established infectious HCV-KT9 genotype 1b HCV clone, we investigated the antiviral efficacy of telaprevir and the effect of

resistance mutations on viral replication. HCV RNA titer in mice infected with the telaprevir-resistant strain KT9-NS3-A156S was lower than in mice infected with the wildtype strain HCV-KT9-wild (Fig. 4B). HCV NS proteins include proteases for sequential processing of the polyprotein and are thought to be important in viral replication.²⁸ Our results suggest that differences in viral fitness underlie the differences in viral replication capacity. We analyzed the antiviral efficacy of telaprevir and the sequence of the NS3 region using HCV-infected mice treated with telaprevir. Although telaprevir treatment suppressed serum HCV RNA titer in mice infected with HCV-KT9, the decline of HCV RNA titer was only 0.6 log copy/mL in a mouse infected with KT9-NS3-A156S under the same treatment (Fig. 5A). These results suggest that our genetically engineered HCV-infected mouse model is useful for analyzing HCV escape mutants associated with antiviral drugs. Interestingly, treatment with telaprevir resulted in selection for V36A variants in the NS3 region in an HCV-KT9-infected mouse (Fig. 5B). There are a few controversial reports proposing that resistant variants may already be present at low frequency (<1%) within the quasispecies population in treatment-naïve patients,²⁹ consistent with their rapid emergence only days after treatment initiation.^{26,30} This might well occur, due to the large number of mutated HCV clones. However, our results provide evidence in support of *de novo* emergence of telaprevir resistance induced by viral mutation followed by selection. HCV has both a high replication rate (10^{12} particles per day) and a high mutation rate (10^{-3} to 10^{-4}),^{9,10} suggesting that the viral quasispecies population is likely to represent a large and genetically diverse substrate for immune selection.

In summary, we established an infection model of a genotype 1b HCV clone using the human hepatocyte chimeric mouse model. Using this model we demonstrate rapid emergence of *de novo* telaprevir-resistant HCV quasispecies from wildtype HCV.

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References

- Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *HEPATOLOGY* 1990;12:671-675.
- Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurrer D, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *HEPATOLOGY* 1998;28:1687-1695.
- Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958-965.
- Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncalves FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975-982.
- Hoofnagle JH, Ghany MG, Kleiner DE, Doo E, Heller T, Promrat K, et al. Maintenance therapy with ribavirin in patients with chronic hepatitis C who fail to respond to combination therapy with interferon alfa and ribavirin. *HEPATOLOGY* 2003;38:66-74.
- Perni RB, Almquist SJ, Byrn RA, Chandorkar G, Chaturvedi PR, Courtney LF, et al. Preclinical profile of VX-950, a potent, selective, and orally bioavailable inhibitor of hepatitis C virus NS3-4A serine protease. *Antimicrob Agents Chemother* 2006;50:899-909.
- Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, et al. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J Biol Chem* 2005;280:36784-36791.
- Mo H, Lu L, Pilor-Matias T, Pithawalla R, Mondal R, Masse S, et al. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor in vitro. *Antimicrob Agents Chemother* 2005;49:4305-4314.
- Bartenschlager R, Lohmann V. Replication of hepatitis C virus. *J Gen Virol* 2000;81:1631-1648.
- Rong L, Dahari H, Ribeiro RM, Perelson AS. Rapid emergence of protease inhibitor resistance in hepatitis C virus. *Sci Transl Med* 2010;2:30ra32.
- Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;7:927-933.
- Kneteman NM, Weiner AJ, O'Connell J, Collett M, Gao T, Aukerman L, et al. Anti-HCV therapies in chimeric scid-Ab/ulPA mice parallel outcomes in human clinical application. *HEPATOLOGY* 2006;43:1346-1353.
- Kamiya N, Iwao E, Hiraga N, Tsuge M, Imamura M, Takahashi S, et al. Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3-4A protease inhibitor. *J Gen Virol* 2010;91:1668-1677.
- Hiraga N, Imamura M, Tsuge M, Noguchi C, Takahashi S, Iwao E, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett* 2007;581:1983-1987.
- Kimura T, Imamura M, Hiraga N, Hatakeyama T, Miki D, Noguchi C, et al. Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. *J Gen Virol* 2008;89:2108-2113.
- Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;165:901-912.
- Cronn R, Liston A, Parks M, Gernandt DS, Shen R, Mockler T. Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology. *Nucleic Acids Res* 2008;36:e122.
- Mitsuya Y, Varghese V, Wang C, Liu TF, Holmes SP, Jayakumar B, et al. Minority human immunodeficiency virus type 1 variants in anti-retroviral-naïve persons with reverse transcriptase codon 215 revertant mutations. *J Virol* 2008;82:10747-10755.
- Margeridon-Thermet S, Shulman NS, Ahmed A, Shahriar R, Liu T, Wang C, et al. Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naïve patients. *J Infect Dis* 2009;199:1275-1285.
- Szpara ML, Parsons L, Enquist LW. Sequence variability in clinical and laboratory isolates of herpes simplex virus 1 reveals new mutations. *J Virol* 2010;84:5303-5313.

21. Wright CF, Morelli MJ, Thebaud G, Knowles NJ, Herzyk R, Paton DJ, et al. Beyond the consensus: dissecting within-host viral population diversity of foot-and-mouth disease virus by using next-generation genome sequencing. *J Virol* 2011;85:2266-2275.
22. Verbinnen T, Van Marck H, Vandenbroucke I, Vijgen L, Claes M, Lin TI, et al. Tracking the evolution of multiple in vitro hepatitis C virus replicon variants under protease inhibitor selection pressure by 454 deep sequencing. *J Virol* 2010;84:11124-11133.
23. Wang GP, Sherrill-Mix SA, Chang KM, Quince C, Bushman FD. Hepatitis C virus transmission bottlenecks analyzed by deep sequencing. *J Virol* 2010;84:6218-6228.
24. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 2009;10:R25.
25. Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I. ABySS: a parallel assembler for short read sequence data. *Genome Res* 2009;19:1117-1123.
26. Sarrazin C, Kieffer TL, Bartels D, Hanzelka B, Muh U, Welker M, et al. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* 2007;132:1767-1777.
27. Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *HEPATOLOGY* 2008;48:1769-1778.
28. Hijikata M, Mizushima H, Tanji Y, Komoda Y, Hirowatari Y, Akagi T, et al. Proteolytic processing and membrane association of putative non-structural proteins of hepatitis C virus. *Proc Natl Acad Sci U S A* 1993;90:10773-10777.
29. Lu L, Mo H, Pilot-Matias TJ, Molla A. Evolution of resistant M414T mutants among hepatitis C virus replicon cells treated with polymerase inhibitor A-782759. *Antimicrob Agents Chemother* 2007;51:1889-1896.
30. Kieffer TL, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, et al. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *HEPATOLOGY* 2007;46:631-639.

MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Animal model for study of human hepatitis virusesKazuaki Chayama,^{*,†} C Nelson Hayes,^{*,†} Nobuhiko Hiraga,^{*,†} Hiromi Abe,^{*,†} Masataka Tsuge^{*,†}
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Abstract

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) infect only chimpanzees and humans. Analysis of both viruses has long been hampered by the absence of a small animal model. The recent development of human hepatocyte chimeric mice has enabled us to carry out studies on viral replication and cellular changes induced by replication of human hepatitis viruses. Various therapeutic agents have also been tested using this model. In the present review, we summarize published studies using chimeric mice and discuss the merits and shortcomings of this model.

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are pathogens that cause chronic infection in humans. There are 360 million and 170 million people infected worldwide with HBV or HCV, respectively.^{1,2} Infected individuals develop acute hepatitis, chronic hepatitis and liver cirrhosis. The viruses are also important causative agents of hepatocellular carcinoma, especially in the Asia-Pacific region.³ Study of the biology and development of therapies for each virus has long been hampered by the lack of a small animal model that supports hepatitis virus infection. This is probably as a result of the lack of receptor molecules necessary for viral infection in animal liver cells.

Transgenic mice that express over-length HBV-DNA export viral particles into the serum,⁴ and such animals can be used to evaluate antiviral agents,⁵⁻⁷ as well as HBV-targeted siRNA⁸. However, the virus life cycle is not established in this model, and it is inappropriate for studying drug-resistant HBV strains. Accordingly, researchers attempted to transplant human hepatocytes into mice. The development of the trimera mouse was one such attempt, in which human hepatocytes were transplanted under the kidney capsule of immune-deficient mice after lethal irradiation.^{9,10} However, the number of hepatocytes that could survive on the kidney capsule was small, and normal liver architecture was not present. Although 85% of HBV-inoculated animals developed HBV viremia, the titer was less than 10³ virus particles or IU/mL.⁹ Similarly, 85% of HCV-inoculated animals also developed viremia,¹⁰ but the level of the viremia only reached 10⁵/mL.

Thus, the advent of human hepatocyte transplanted uPA/scid mice has provided the first really useful model for acute and chronic infections of human hepatitis virus.

Human liver cell transplanted uPA/scid mice

Transgenic mice in which the urokinase gene is driven by the human albumin promoter/enhancer were developed and shown to have accelerated hepatocyte death and consequent chronic stimulation of hepatocyte growth.¹¹ Transplanted rat hepatocytes proliferated and repopulated injured livers in immunodeficient uPA mice, which were produced by mating uPA transgenic mice with scid mice.¹² Human hepatocytes were then transplanted into uPA/scid mice; these cells proliferated and replaced the apoptotic mice liver cells (Fig. 1).

Such human hepatocyte chimeric mice have been shown to be susceptible to both HBV¹⁶ and HCV¹⁷ infections. Repopulation levels by human hepatocytes have been estimated by measuring human albumin levels in mouse serum. Replication levels of both HBV¹³ and HCV¹⁷ were higher in mice in which the repopulation index was higher. A unique attempt to remove mouse residual liver cells with the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) system failed to result in a higher repopulation rate as a result of damage to the transplanted human hepatocyte caused by bystander effects.¹⁸ Despite this, mice with livers that have been highly repopulated with human hepatocytes

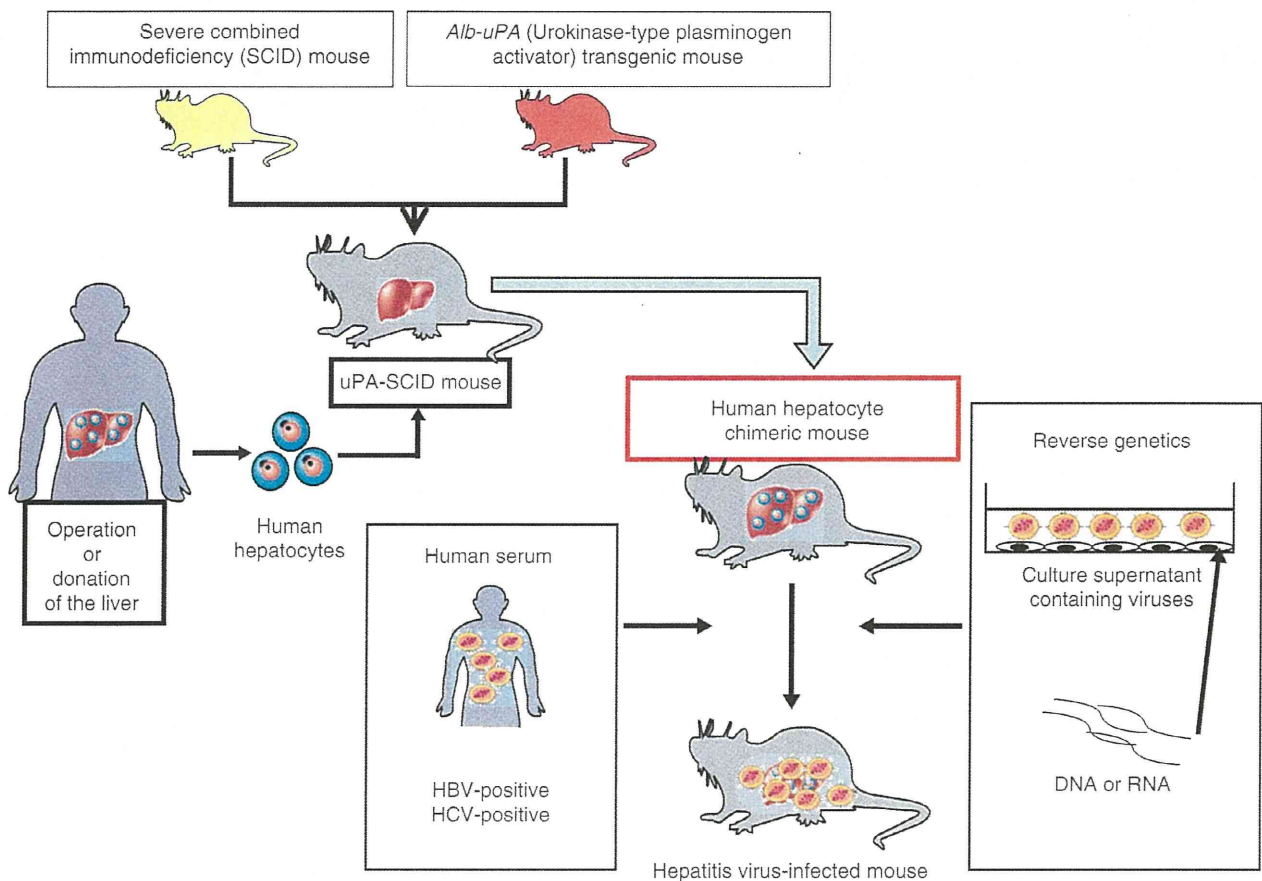


Figure 1 Generation of human hepatocyte chimeric mice and hepatitis virus infection model. A uPA/scid mouse was created by mating uPA transgenic mouse and scid mouse. Human hepatocytes obtained by surgical resection or donation were transplanted to newborn mice. The chimeric mice can be infected with hepatitis B virus (HBV) or hepatitis C (HCV) virus by injecting human serum containing these viruses. Alternatively, the mice can be infected by HBV¹³ or HCV¹⁴ created in cell culture or by injecting HCV RNA into the mouse liver.¹⁵

are susceptible to infection with both HBV and HCV, and as such comprised the most effective small animal model for chronic hepatitis so far developed.^{19,20} An example of a highly repopulated mouse liver that we are using in experiments is shown in Figure 2.

Highly repopulated mice have been shown to be a valuable model for the study of drug metabolism.^{21–29} Advances in technology for human hepatocyte transplantation have enabled serial passage of human hepatocytes in uPA/scid mice and have been shown to retain infectivity for HBV.³⁰

This mouse model and other animal models for the study of hepatitis viruses have been summarized in reviews by Meuleman and Leroux-Roels,³¹ Dandri *et al.*,^{32,33} Barth *et al.*,³⁴ and Kneteman and Toso.³⁵ The present review will focus on key issues and updated information.

Study of hepatitis B virus infection using human hepatocyte chimeric mice

Since the initial reports of successful transmission of HBV to human hepatocyte chimeric mice in 2001 and 2004,^{16,27} several researchers have reported transmission of HBV into similar

mice.^{13,36,37} In these studies, passage experiments studies show that HBV replicating in mice retain infectivity.^{13,36} Further, the presence of viral proteins has been shown immunohistochemically in human hepatocytes transplanted into mouse livers, but these are not present in mouse hepatocytes.^{13,36,37} Formation of viral particles in infected mouse livers can be shown by electron microscopy.^{36,37} Genetically engineered viruses lacking HBe-antigen have also been shown to infect chimeric mice, proving that e antigen is dispensable for viral infection and replication.¹³ In contrast, HBx protein has been shown to be indispensable for viral replication.³⁸ Transcomplementation of HBx protein with hydrodynamic injection restored HBV infectivity in mice. Interestingly, all revertant viruses show a restored ability to express HBx.³⁸

By infecting chimeric mice with genotype A, B and C, differing proliferative capacity has been shown between HBV genotypes.³⁷ In mice infected for a relatively short time, there are no morphological changes in HBV infected mice livers in studies.^{13,36} In contrast, the occurrence of liver cell damage has been reported after long-term infection of chimeric mice with HBV³⁹ or with specific strains of HBV;⁴⁰ these findings are consistent with direct cytopathic effects of HBV under certain conditions.

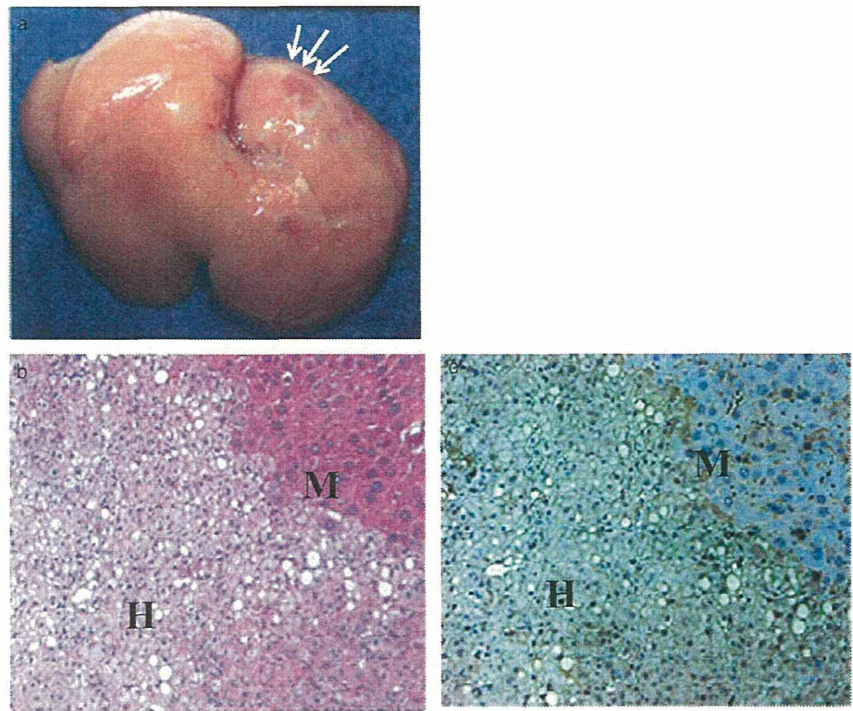


Figure 2 Representative uPA/scid mouse livers repopulated by human hepatocytes. (a) Mouse liver almost completely repopulated by human hepatocytes. Only a small portion of mouse hepatocytes are shown by arrows. (b) Microscopic figure of the mouse liver. M and H indicate regions consisting of mouse and human hepatocytes, respectively (Hematoxylin–eosin staining, magnification: $\times 100$). (c) Microscopic figure of the mouse liver stained with antibody directed against human serum albumin.

The biological properties of a newly identified unique strain of HBV, genotype G, which replicates only in the presence of another genotype, were confirmed using the chimeric mouse.⁴¹ Infectivity of another novel HBV strain, identified from a Japanese patient, that is divergent from known human and ape HBV has also been confirmed.⁴² Titration of HBV infectivity, which previously could only be carried out using chimpanzees, can be carried out effectively using chimeric mice.⁴³

Taking advantage of the absence of human immune cells in the chimeric mice, Noguchi *et al.*⁴⁴ showed that hypermutation of HBV increases in human hepatocytes under interferon treatment. Dandri *et al.* measured viral half-life in human and chimeric mice repopulated with woolly monkey hepatocytes.⁴⁵ The results clearly showed that viral half-life is shortened by immunological mechanisms in humans with low viral levels, but not in chimeric mice where functional immunity is absent. Hiraga *et al.*⁴⁶ showed an absence of interference between HBV and HCV.

Evaluation of therapeutic agents is the most important role for this mouse model. Tsuge *et al.*¹³ assessed the effect of interferon and lamivudine using chimeric mice. Similarly, Dandri *et al.*⁴⁷ showed the effects of adefovir using uPA/scid mice repopulated with tupaia hepatocytes, which also support replication of human HBV. Oga *et al.*⁴⁸ identified a novel lamivudine-resistant variant that has an amino acid substitution outside of the YMDD motif. They showed that lamivudine was ineffective against the novel mutant strain. It is thus apparent that this mouse/human liver chimeric model is ideal to study the susceptibility of mutant strains to various drugs, because mutant viruses can easily be made and infected into chimeric mice.¹³ The model has also been utilized to evaluate viral entry inhibitors derived from the large envelope protein.⁴⁹

Study of hepatitis C virus using human hepatocyte chimeric mice

As observed in studies on HBV, HCV infection efficiency was poor and levels of viremia were low in mice where the repopulation rate of the mouse liver with human hepatocyte was low.^{17,50} As shown in Figure 3, human albumin levels in mouse serum were significantly higher in mice in which measurable viremia developed (Hiraga *et al.* unpublished data). Recent studies have therefore been carried out using highly repopulated mice. The usefulness of a newly developed HCV assay,⁵¹ and infectivity of a newly identified intergenotypic recombinant strain,⁵² have been reported using the chimeric mice.

Using the remarkable replication ability of the JFH1 genotype 2a strain,⁵³ infectivity of JFH1 or intergenotypic chimeric viral particles, previously shown in cell culture, has now been shown to be infectious in chimeric mice.^{54–56} Infectivity of viruses that were replicated in chimeric mice in cell culture has also been shown, and virus fitness has been studied.^{55,56} The role of the HCV core+1 open reading frame and core *cis*-acting RNA elements has also been examined using the chimeric virus.⁵⁷ These elegant studies have the limitation that the non-structural part of the virus is limited to that of JFH1. Hiraga *et al.*¹⁴ have shown that infectious clones of genotype 1a and JFH1 can be infected with direct injection of *in vitro* transcribed RNA into the mouse liver.¹⁴ Similarly, Kimura *et al.*¹⁵ reported the establishment of infectious clones of genotype 1b and ablation of RNA polymerase by site-directed mutagenesis abolish infectivity. These infectious clones will be useful for the study of drug-resistant strains.

The model of HCV infection has also been used to show that infection of the virus can be prevented by antibodies against

Table 1 New therapeutic strategies tested by human hepatocyte chimeric mice

<i>n</i>	Drug or cell	Strategy	Reference
1	Interferon alpha 2b BILN-2061 HCV371	Activation of antiviral genes NS3-4A protease inhibition NS5B polymerase inhibition	Kneteman <i>et al.</i> ⁶⁵
2	Modified BID	Induction of apoptosis	Hsu <i>et al.</i> ⁶⁶
3	Serine palmitoyltransferase inhibitor	Disruption of lipid raft	Umehara <i>et al.</i> ⁶⁷
4	Lymphoblastoid interferon alpha	Activation of antiviral genes	Hiraga <i>et al.</i> ¹⁴
5	Amphipathic DNA polymers	Blocking viral entry	Matsumura <i>et al.</i> ⁶⁰
6	Sec-butyl-analogue of HCV-371	NS5B polymerase inhibition	LaPorte <i>et al.</i> ⁶⁶
7	HCV796	NS5B polymerase inhibition	Kneteman <i>et al.</i> ⁶⁵
8	Liver allograft-derived lymphocyte	Adoptive immunotherapy	Ohira <i>et al.</i> ⁷⁰
9	Telaprevir	NS3-4A protease inhibition	Kamiya <i>et al.</i> ⁷

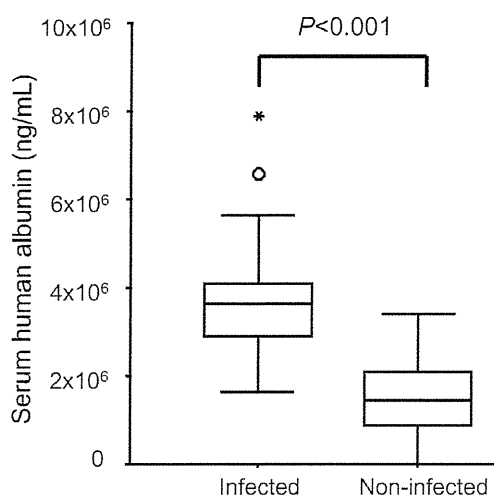


Figure 3 Human albumin levels in mice used in the hepatitis C virus (HCV) infection experiments. A total of 54 mice were injected with HCV positive serum samples containing 5×10^5 virus particles. A total of 24 mice became persistently positive for HCV-RNA, but 30 mice did not. Serum human albumin levels 2 weeks after human hepatocyte transplantation were compared between infected and non-infected mice.

CD81,⁵⁸ polyclonal human immunoglobulin directed to a similar strain,⁵⁹ and amphipathic DNA polymers.⁶⁰ Notably, the presence of broadly neutralizing antibodies to HCV that protect against heterologous viral infection has been reported, suggesting the possibility of a prophylactic vaccine against HCV.⁶¹

With respect to evasion of the virus against the innate immune response, altered intrahepatic expression profiles in the early phase of infection is of particular interest. The chimeric mice model is ideal for such studies: cross-hybridization of mouse and human can be avoided by careful experimental procedures.⁶² Microarray analysis of livers of HCV infected and non-infected mice showed transcriptional activation of genes related to innate immune response, lipid metabolism, endoplasmic reticulum (ER) stress and apoptosis in HCV-infected mice.^{63,64} The HCV infected mouse model is particularly useful for the study of newly developed HCV agents. The effect of recently developed chemicals and a unique therapy using intrahepatic lymphocytes have been shown using

this model (Table 1). However, none of these therapies have yet been able to completely eradicate HCV from mice. It is noteworthy that ultra-rapid cardiotoxicity has been reported with the protease inhibitor BILN 2061 in the uPA/scid mice, but not in scid mice, implicating involvement of the uPA transgene.⁷² Care should therefore be taken in interpreting the results obtained by this model.

Conclusion

Development of a small animal model using human hepatocyte chimeric mice has enabled us to study key aspects of HBV and HCV biology. The characteristic feature of the absence of human immune cells is suitable for studying viral replication and observing changes occurring in liver cells during viral infection, such as the innate immune response and cellular stress and metabolic responses. The model is also useful for studying the effect of drugs without the influence of cytokines and cytotoxic T lymphocytes. Nonetheless, the model is insufficient to study carcinogenesis of hepatitis viruses, because non-parenchymal cells in mouse liver are of mouse origin and do not support inflammation and fibrosis, which are probably closely related to carcinogenesis. The lack of human immune cells also limits the study of inflammation and immunity. Furthermore, the availability of human hepatocytes is limited. Despite these limitations, the current model shows great potential as a mouse model for the study of hepatitis viruses. Development of a small animal model with or without human immunity using stem cells or iPS cells would be an ideal model in the future.

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References

- Shepard CW, Simard EP, Finelli L, Flore AE, Bell BP. Hepatitis B virus infection: epidemiology and vaccination. *Epidemiol. Rev.* 2006; 28: 112–25.

- 2 Sy T, Jamal MM. Epidemiology of Hepatitis C Virus (HCV) infection. *Int. J. Med. Sci.* 2006; **3**: 41–6.
- 3 Yuen MF, Hou JL, Chutaputti A, Prevent APWP. Hepatocellular carcinoma in the Asia Pacific region. *J. Gastroenterol. Hepatol.* 2009; **24**: 346–53.
- 4 Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level Hepatitis-B Virus-replication in transgenic mice. *J. Virol.* 1995; **69**: 6158–69.
- 5 Weber O, Schlenumer KH, Hartmann E *et al.* Inhibition of human hepatitis B virus (HBV) by a novel non-nucleosidic compound in a transgenic mouse model. *Antiviral Res.* 2002; **54**: 69–78.
- 6 Julander JG, Sidwell RW, Morrey JD. Characterizing antiviral activity of adefovir dipivoxil in transgenic mice expressing hepatitis B virus. *Antiviral Res.* 2002; **55**: 27–40.
- 7 Julander JG, Colonno RJ, Sidwell RW, Morrey JD. Characterization of antiviral activity of entecavir in transgenic mice expressing hepatitis B virus. *Antiviral Res.* 2003; **59**: 155–61.
- 8 Uprichard SL, Boyd B, Althage A, Chisari FV. Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNA. *Proc. Natl. Acad. Sci U S A* 2005; **102**: 773–8.
- 9 Ilan E, Burakova T, Dagan S *et al.* The hepatitis B virus-trimera mouse: a model for human HBV infection and evaluation of Anti-HBV therapeutic agents. *Hepatology* 1999; **29**: 553–62.
- 10 Ilan E, Arazi J, Nussbaum O *et al.* The hepatitis C virus (HCV)-Trimera mouse: a model for evaluation of agents against HCV. *J. Infect. Dis.* 2002; **185**: 153–61.
- 11 Heckel JL, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen-activator. *Cell* 1990; **62**: 447–56.
- 12 Rhim JA, Sandgren EP, Palmiter RD, Brinster RL. Complete reconstitution of mouse-liver with xenogeneic hepatocytes. *Proc. Natl. Acad. Sci U S A* 1995; **92**: 4942–6.
- 13 Tsuge M, Hiraga N, Takaishi H *et al.* Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005; **42**: 1046–54.
- 14 Hiraga N, Imamura M, Tsuge M *et al.* Infection of human hepatocyte chimeric mouse with genetically engineered Hepatitis C Virus and its susceptibility to interferon. *FEBS Lett.* 2007; **581**: 1983–7.
- 15 Kimura T, Imamura M, Hiraga N *et al.* Establishment of an infectious genotype 1b Hepatitis C Virus clone in human hepatocyte chimeric mice. *J. Gen. Virol.* 2008; **89**: 2108–13.
- 16 Dandri M, Burda MR, Torok E *et al.* Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology* 2001; **33**: 981–8.
- 17 Mercer DF, Schiller DE, Elliott JF *et al.* Hepatitis C virus replication in mice with chimeric human livers. *Nat. Med.* 2001; **7**: 927–33.
- 18 Douglas DN, Kawahara T, Sis B *et al.* therapeutic efficacy of human hepatocyte transplantation in a SCID/uPA mouse model with inducible liver disease. *PLoS ONE* 2010; **5**: e9209.
- 19 Tateno C, Yoshizane Y, Saito N *et al.* Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.* 2004; **165**: 901–12.
- 20 Bissig KD, Wieland SF, Tran P *et al.* Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. *J. Clin. Invest.* 2010; **120**: 924–30.
- 21 Yu AM, Idle JR, Gonzalez FJ. Polymorphic cytochrome p450 2D6: humanized mouse model and endogenous substrates. *Drug. Metab. Rev.* 2004; **36**: 243–77.
- 22 Katoh M, Sawada T, Soeno Y *et al.* In vivo drug metabolism model for human cytochrome P450 enzyme using chimeric mice with humanized liver. *J. Pharm. Sci.-Us.* 2007; **96**: 428–37.
- 23 Katoh M, Matsui T, Nakajima M *et al.* In vivo induction of human cytochrome P450 enzymes expressed in chimeric mice with humanized liver. *Drug. Metab. Dispos.* 2005; **33**: 754–63.
- 24 Katoh M, Matsui T, Okumura H *et al.* Expression of human phase II enzymes in chimeric mice with humanized liver. *Drug. Metab. Dispos.* 2005; **33**: 1333–40.
- 25 Okumura H, Katoh M, Sawada T *et al.* Humanization of excretory pathway in chimeric mice with humanized liver. *Toxicol. Sci.* 2007; **97**: 533–8.
- 26 Shoda J, Okada K, Inada Y *et al.* Bezafibrate induces multidrug-resistance P-Glycoprotein 3 expression in cultured human hepatocytes and humanized livers of chimeric mice. *Hepatology Res.* 2007; **37**: 548–56.
- 27 Petersen J, Burda MR, Dandri M, Rogler CE. Transplantation of human hepatocytes in immunodeficient UPA mice: a model for the study of hepatitis B virus. *Methods Mol. Med.* 2004; **96**: 253–60.
- 28 Yoshizato K, Tateno C. A human hepatocyte-bearing mouse: an animal model to predict drug metabolism and effectiveness in humans. *PPAR Res.* 2009; **2009**: 476217.
- 29 Yoshizato K, Tateno C, Utoh R. The mechanism of liver size control in mammals: a novel animal study. *Int. J. Design & Nature Ecodynamics* 2009; **4**: 123–42.
- 30 Utoh R, Tateno C, Yamasaki C *et al.* Susceptibility of chimeric mice with livers repopulated by serially subcultured human hepatocytes to hepatitis B virus. *Hepatology* 2008; **47**: 435–46.
- 31 Meuleman P, Leroux-Roels G. The human liver-uPA-SCID mouse: a model for the evaluation of antiviral compounds against HBV and HCV. *Antiviral Res.* 2008; **80**: 231–8.
- 32 Dandri M, Lutgehetmann M, Volz T, Petersen J. Small animal model systems for studying Hepatitis B Virus replication and pathogenesis. *Semin. Liver Dis.* 2006; **26**: 181–91.
- 33 Dandri M, Volz TK, Lutgehetmann M, Petersen J. Animal models for the study of HBV replication and its variants. *J. Clin. Virol.* 2005; **34** (Suppl. 1): S54–62.
- 34 Barth H, Robinet E, Liang TJ, Baumert TF. Mouse models for the study of HCV infection and virus-host interactions. *J. Hepatol.* 2008; **49**: 134–42.
- 35 Kneteman NM, Toso C. In vivo study of HCV in mice with chimeric human livers. *Methods Mol. Biol.* 2009; **510**: 383–99.
- 36 Meuleman P, Libbrecht L, De Vos R *et al.* Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* 2005; **41**: 847–56.
- 37 Sugiyama M, Tanaka Y, Kato T *et al.* Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* 2006; **44**: 915–24.
- 38 Tsuge M, Hiraga N, Akiyama R *et al.* HBx protein is indispensable for development of viremia in human hepatocyte chimeric mice. *J. Gen. Virol.* 2010.
- 39 Meuleman P, Libbrecht L, Wieland S *et al.* Immune suppression uncovers endogenous cytopathic effects of the hepatitis B virus. *J. Virol.* 2006; **80**: 2797–807.
- 40 Sugiyama M, Tanaka Y, Kurbanov F *et al.* Direct cytopathic effects of particular hepatitis B virus genotypes in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mouse with human hepatocytes. *Gastroenterology* 2009; **136**: 652–62.
- 41 Tanaka Y, Sanchez LV, Sugiyama M *et al.* Characteristics of Hepatitis B Virus genotype G coinfecting with genotype H in chimeric mice carrying human hepatocytes. *Virology* 2008; **376**: 408–15.
- 42 Tatematsu K, Tanaka Y, Kurbanov F *et al.* A genetic variant of Hepatitis B Virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *J. Virol.* 2009; **83**: 10538–47.
- 43 Tabuchi A, Tanaka J, Katayama K *et al.* Titration of Hepatitis B Virus infectivity in the sera of pre-acute and late acute phases of HBV infection: transmission experiments to chimeric mice with

- human liver repopulated hepatocytes. *J. Med. Virol.* 2008; **80**: 2064–8.
- 44 Noguchi C, Imamura M, Tsuge M *et al.* G-to-A Hypermutation in Hepatitis B Virus (HBV) and clinical course of patients with chronic HBV infection. *J. Infect. Dis.* 2009; **199**: 1599–607.
 - 45 Dandri M, Murray JM, Lutgehetmann M, Volz T, Lohse AW, Petersen J. Virion half-life in chronic hepatitis B infection is strongly correlated with levels of viremia. *Hepatology* 2008; **48**: 1079–86.
 - 46 Hiraga N, Imamura M, Hatakeyama T *et al.* Absence of viral interference and different susceptibility to interferon between Hepatitis B Virus and Hepatitis C Virus in human hepatocyte chimeric mice. *J. Hepatol.* 2009; **51**: 1046–54.
 - 47 Dandri M, Burda MR, Zuckerman DM *et al.* Chronic infection with Hepatitis B Viruses and antiviral drug evaluation in uPA mice after liver repopulation with tupaia hepatocytes. *J. Hepatol.* 2005; **42**: 54–60.
 - 48 Yatsuji H, Noguchi C, Hiraga N *et al.* Emergence of a novel lamivudine-resistant hepatitis B virus variant with a substitution outside the YMDD motif. *Antimicrob. Agents Chemother.* 2006; **50**: 3867–74.
 - 49 Petersen J, Dandri M, Mier W *et al.* Prevention of Hepatitis B Virus infection in vivo by entry inhibitors derived from the large envelope protein. *Nat. Biotechnol.* 2008; **26**: 335–41.
 - 50 Turrini P, Sasso R, Germoni S *et al.* Development of humanized mice for the study of hepatitis C virus infection. *Transplant. Proc.* 2006; **38**: 1181–4.
 - 51 Cagnon L, Wagaman P, Bartenschlager R *et al.* Application of the trak-C (TM) HCV core assay for monitoring antiviral activity in HCV replication systems. *J. Virol. Methods* 2004; **118**: 23–31.
 - 52 Kurbanov F, Tanaka Y, Chub E *et al.* Molecular epidemiology and interferon susceptibility of the natural recombinant Hepatitis C Virus Strain RF1-2k/1b. *J. Infect. Dis.* 2008; **198**: 1448–56.
 - 53 Wakita T, Pietschmann T, Kato T *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 2005; **11**: 791–6.
 - 54 Grove J, Huby T, Stamatakis Z *et al.* Scavenger receptor BI and BII expression levels modulate Hepatitis C Virus infectivity. *J. Virol.* 2007; **81**: 3162–9.
 - 55 Lindenbach BD, Meuleman P, Ploss A *et al.* Cell culture-grown Hepatitis C Virus is infectious in vivo and can be recultured in vitro. *Proc. Natl. Acad. Sci. U S A* 2006; **103**: 3805–9.
 - 56 Kaul A, Woerz I, Meuleman P, Leroux-Roels G, Bartenschlager R. Cell culture adaptation of Hepatitis C Virus and in vivo viability of an adapted variant. *J. Virol.* 2007; **81**: 13168–79.
 - 57 Vassilaki N, Friebe P, Meuleman P *et al.* Role of the Hepatitis C Virus Core+1 open reading frame and core cis-acting RNA Elements in Viral RNA translation and replication. *J. Virol.* 2008; **82**: 11503–15.
 - 58 Meuleman P, Hesselgesser J, Paulson M *et al.* Anti-CD81 antibodies can prevent a Hepatitis C Virus infection in vivo. *Hepatology* 2008; **48**: 1761–8.
 - 59 Vanwolleghem T, Bukh J, Meuleman P *et al.* Polyclonal immunoglobulins from a chronic Hepatitis C Virus patient protect human liver-chimeric mice from infection with a homologous Hepatitis C Virus strain. *Hepatology* 2008; **47**: 1846–55.
 - 60 Matsumura T, Hu ZY, Kato T *et al.* Amphipathic DNA polymers inhibit Hepatitis C Virus infection by blocking viral entry. *Gastroenterology* 2009; **137**: 673–81.
 - 61 Law M, Maruyama T, Lewis J *et al.* Broadly neutralizing antibodies protect against Hepatitis C Virus quasispecies challenge. *Nat. Med.* 2008; **14**: 25–7.
 - 62 Walters KA, Joyce MA, Thompson JC *et al.* Application of functional genomics to the chimeric mouse model of HCV infection: optimization of microarray protocols and genomics analysis. *Virol. J.* 2006; **3**: 37–44.
 - 63 Walters KA, Joyce MA, Thompson JC *et al.* Host-specific response to HCV infection in the chimeric SCID-beige/Alb-uPA mouse model: role of the innate antiviral immune response. *PLoS Pathog.* 2006; **2**: 591–602.
 - 64 Joyce MA, Walters KA, Lamb SE *et al.* HCV Induces Oxidative and ER Stress, and Sensitizes Infected Cells to Apoptosis in SCID/Alb-uPA Mice. *PLoS Pathog.* 2009; **5**: e10000291.
 - 65 Kneteman NM, Weiner AJ, O'Connell J *et al.* Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* 2006; **43**: 1346–53.
 - 66 Hsu EC, Hsi B, Hirota-Tsuchihara M *et al.* Modified apoptotic molecule (BID) reduces hepatitis C virus infection in mice with chimeric human livers. *Nat. Biotechnol.* 2003; **21**: 519–25.
 - 67 Umehara T, Sudoh M, Yasui F *et al.* Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. *Biochem. Biophys. Res. Commun.* 2006; **346**: 67–73.
 - 68 Laporte MG, Jackson RW, Draper TL *et al.* The discovery of pyrano[3,4-b]indole-based allosteric inhibitors of HCV NS5B polymerase with in vivo activity. *Med. Chem.* 2008; **3**: 1508–15.
 - 69 Kneteman NM, Howe AYM, Gao TJ *et al.* HCV796: a selective nonstructural protein 5B polymerase inhibitor with potent Anti-Hepatitis C Virus activity in vitro, in mice with chimeric human livers, and in humans infected with Hepatitis C Virus. *Hepatology* 2009; **49**: 745–52.
 - 70 Ohira M, Ishiyama K, Tanaka Y *et al.* Adoptive immunotherapy with liver allograft-derived lymphocytes induces anti-HCV activity after liver transplantation in humans and humanized mice. *J. Clin. Invest.* 2009; **119**: 3226–35.
 - 71 Kamiya N, Iwao E, Hiraga N *et al.* Practical Evaluation of a Mouse with Chimeric Human Liver Model for Hepatitis C Virus Infection Using an NS3-4A Protease Inhibitor. *J. Gen. Virol.* 2010; **91**: 1668–77.
 - 72 Vanwolleghem T, Meuleman P, Libbrecht L *et al.* Ultra-rapid cardiotoxicity of the hepatitis C virus protease inhibitor BILN 2061 in the urokinase-type plasminogen activator mouse. *Gastroenterology* 2007; **133**: 1144–55.

Hepatitis C Virus Infection Suppresses the Interferon Response in the Liver of the Human Hepatocyte Chimeric Mouse

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Abstract

Background and Aims: Recent studies indicate that hepatitis C virus (HCV) can modulate the expression of various genes including those involved in interferon signaling, and up-regulation of interferon-stimulated genes by HCV was reported to be strongly associated with treatment outcome. To expand our understanding of the molecular mechanism underlying treatment resistance, we analyzed the direct effects of interferon and/or HCV infection under immunodeficient conditions using cDNA microarray analysis of human hepatocyte chimeric mice.

Methods: Human serum containing HCV genotype 1b was injected into human hepatocyte chimeric mice. IFN- α was administered 8 weeks after inoculation, and 6 hours later human hepatocytes in the mouse livers were collected for microarray analysis.

Results: HCV infection induced a more than 3-fold change in the expression of 181 genes, especially genes related to Organismal Injury and Abnormalities, such as fibrosis or injury of the liver ($P=5.90E-16 \sim 3.66E-03$). IFN administration induced more than 3-fold up-regulation in the expression of 152 genes. Marked induction was observed in the anti-fibrotic chemokines such as *CXCL9*, suggesting that IFN treatment might lead not only to HCV eradication but also prevention and repair of liver fibrosis. HCV infection appeared to suppress interferon signaling via significant reduction in interferon-induced gene expression in several genes of the IFN signaling pathway, including *Mx1*, *STAT1*, and several members of the *CXCL* and *IFI* families ($P=6.0E-12$). Genes associated with Antimicrobial Response and Inflammatory Response were also significantly repressed ($P=5.22 \times 10^{-10} \sim 1.95 \times 10^{-2}$).

Conclusions: These results provide molecular insights into possible mechanisms used by HCV to evade innate immune responses, as well as novel therapeutic targets and a potential new indication for interferon therapy.

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Introduction

Chronic hepatitis C virus (HCV) infection is one of the most serious global health threats, affecting more than 170 million people worldwide [1–3]. Interferon is administered to chronic hepatitis C patients to attempt to eradicate the virus and to prevent the development of advanced liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), with limited success. While the overall eradication rate of HCV has improved since the introduction of pegylated-interferon (PEG-IFN) and ribavirin (RBV) combination therapy, the sustained viral

response (SVR) rate of genotype 1b with high viral load still remains only 40–50% [4–6]. Viral and host factors, such as HCV RNA titer, viral substitutions in HCV core or NS5A region, age, gender, liver fibrosis, and SNPs in *IL-28B* locus, are significantly associated with the effects of PEG-IFN and RBV combination therapy [7–15], but the precise molecular mechanisms remained unclear.

Recently, some HCV-related structural as well as non-structural proteins have been reported to be associated with host proteins and affect innate immunity or lipid metabolism. RIG-I (retinoic acid inducible gene I) and Mda5 (melanoma differentiation-

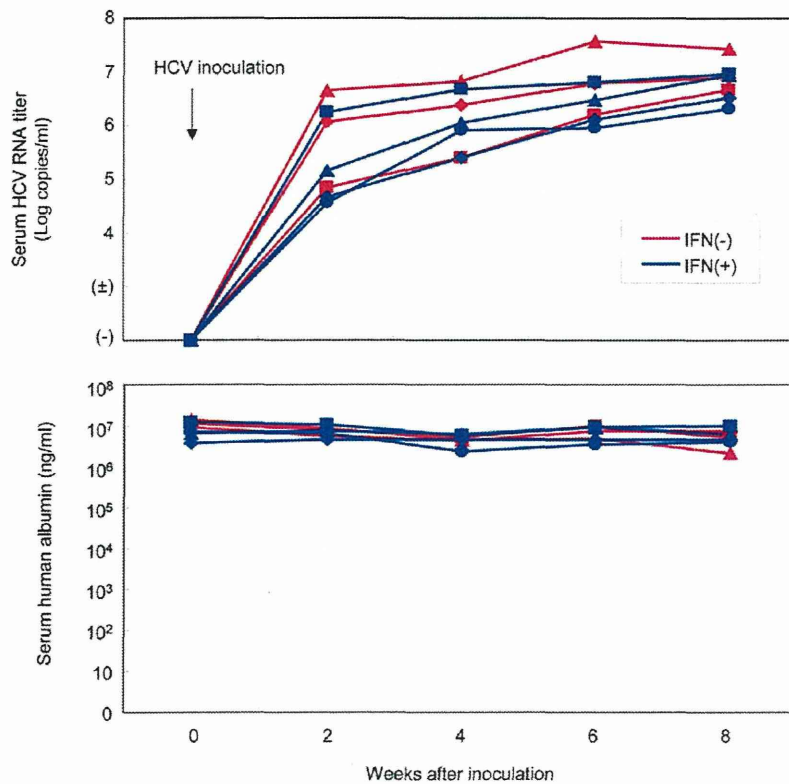


Figure 1. Change in HCV titers and human albumin levels in mouse serum. HCV RNA titers (upper panel) and human albumin levels (lower panel) in chimeric mouse sera after inoculation are shown. The horizontal axis indicates weeks after inoculation. Mouse sera were collected every two weeks after inoculation, and serum HCV RNA and human albumin levels were measured. Results were similar for all mice. doi:10.1371/journal.pone.0023856.g001

associated gene 5) are known to activate the type I interferon signaling pathway by interacting with adaptor protein IPS-1/MAVS/VISA/Cardif [16–18]. In the presence of HCV infection, the viral non-structural protein NS3/4A, which has serine protease activity, can cleave and inactivate IPS-1 [19]. TLR (Toll like receptor) is a sensor of RNA or DNA and is known to play various roles in viral infection. Abe et al. demonstrated that HCV non-structural protein NS5A inhibits the recruitment of interleukin-1 receptor-associated kinase 1 by interacting with MyD88 and impairs cytokine production in response to TLR ligands [20]. HCV core protein is also known to interact with host proteins. The core protein promotes hepatic steatosis, insulin resistance and hepatocarcinogenesis through activation of host proteins such as PPAR α and MAPK [21–26]. However, these reports were based on *in vitro* analysis of cell lines or used human liver tissues in which results were complicated by adaptive immune responses, and it has been difficult to evaluate the direct impact of HCV infection and interferon administration on human hepatocytes.

Mercer and colleagues developed a human hepatocyte chimeric mouse [27] derived from the severely immunocompromised SCID mouse, in which mouse liver cells were extensively replaced with human hepatocytes [27,28]. This mouse model facilitates continuous HCV infection and makes it possible to analyze the effects of drugs and viral infection on human hepatocytes under immunodeficient conditions [29,30]. To analyze the putative effects of HCV infection or IFN administration without the adaptive immune response, we constructed an HCV carrier mouse model using the human hepatocyte chimeric mouse and

performed cDNA microarray analysis using human hepatocytes dissected from the mouse livers. The results are intended to reflect the direct impacts of HCV infection and IFN administration on human hepatocytes and may help in elucidating HCV immune evasion mechanisms.

Materials and Methods

Human Serum Samples

Serum samples were obtained from HCV carriers after obtaining written informed consent for the donation and evaluation of blood samples. Inocula contained high viral loads of genotype 1b HCV RNA (6.9 log copies/ml). The experimental protocol met the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hiroshima University Ethical Committee.

Human Hepatocyte Chimeric Mice Experiments

The uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described previously [28]. All mice were transplanted with hepatocytes from the same donor. Human hepatocyte chimeric mice, in which liver cells were largely (>90%) replaced with human hepatocytes, were used to reduce potential influence by mouse-derived mRNA. The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

A total of 15 chimeric mice were prepared and assigned to four experimental groups. Group A contained four mice that

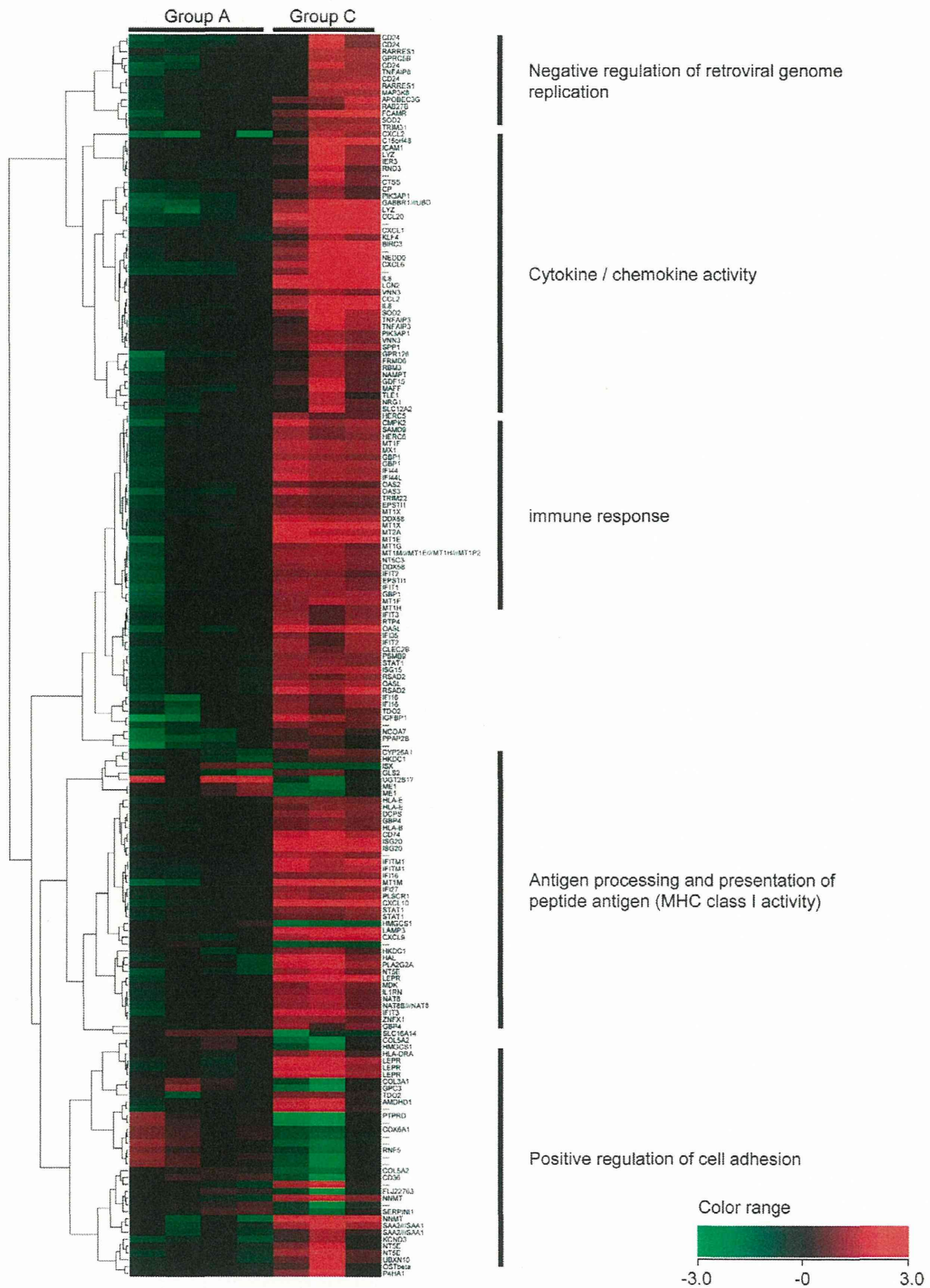


Figure 2. Hierarchical clustering analysis of 181 genes associated with HCV infection. To analyze the influence of HCV infection on human hepatocytes, clustering analysis on gene expression was performed between Group A (without HCV infection; 4 columns on the left side) and Group C (with HCV infection; 3 columns on the right side). 157 genes were up-regulated following HCV infection, including interferon-stimulated genes (ISGs) such as *MX1* and genes in the *CXCL* and *IFI* families, and 24 genes were down-regulated, including *ME1* and *HMGCS1*. doi:10.1371/journal.pone.0023856.g002

were neither infected with HCV nor treated with IFN. Group B consisted of four uninfected mice that were administered IFN- α (7,000 IU/g body weight) 6 h before sacrifice. Groups C and D were inoculated via the mouse tail vein with human serum containing 4×10^5 copies of HCV particles, and Group D was administered IFN- α at the same time as Group B. After inoculation, we collected mouse sera every two weeks and analyzed serum HCV RNA levels by real time PCR. All seven mice developed measurable viremia 4 weeks after inoculation. The levels of the virus titer reached over 6 Log_{10} copies/ml 8 weeks after inoculation (Figure 1). Conversely, serum human albumin levels remained more than 2×10^6 ng/ml in each mouse during 6 weeks after inoculation (Figure 1). Eight weeks after inoculation, when serum HCV RNA levels had plateaued, IFN- α (7,000 IU/g body weight) was administered to the four mice in Group D as well as the four uninfected mice in Group B. Six hours after IFN administration all 15 mice were sacrificed. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia as described previously [29–31]. Human albumin levels in mouse serum were measured with a Human Albumin enzyme-linked immunosorbent assay (ELISA) Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX) according to the instructions provided by the manufacturer. Serum samples obtained from mice were aliquoted and stored in liquid nitrogen until use.

Analysis of HCV markers

For quantitative analysis of HCV RNA, 10 μ l samples of mouse serum were used. Total RNA was extracted using Sepa Gene RV-R (Sanko Junyaku Co., Ltd., Tokyo, Japan) and dissolved with 8.8 μ l of RNase free water and reverse transcribed (RT). RT reactions were performed with 20 μ l of the reaction mixtures, containing random primer (Takara Bio Inc., Shiga, Japan), RT buffer and M-MLV reverse transcriptase (ReverTra Acc, TOYOBO Co., Osaka, Japan) according to the instructions provided by the manufacturer. After the RT reaction, HCV RNA was quantified by real-time PCR using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification was performed as described previously [29,30]. The lower detection limit of this assay is 300 copies. For detection of small amounts of HCV RNA, we also performed nested PCR. Amplification conditions were as described previously [29,30].

Dissection of mouse livers and total RNA extraction from human hepatocytes in the mouse livers

All 15 chimeric mice were sacrificed by anesthesia with diethyl ether. Human hepatocytes were finely dissected from mouse livers, submerged in RNA *later*[®] solution (Applied Biosystems), and stored in liquid nitrogen. Total RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacturer protocol (Qiagen Inc., Valencia, CA). RNA quality was assessed using ultraviolet

Table 1. The top 20 genes up-regulated with HCV infection.

Probe set	Unigene code	Gene symbol	Fold change	P value
202237_at	Hs.503911	NNMT	33.16	1.66E-03
205476_at	Hs.75498	CCL20	30.23	1.59E-04
202859_x_at	Hs.551925	IL8	30.16	4.42E-04
206336_at	Hs.164021	CXCL6	25.52	1.86E-03
217546_at	Hs.647370	MT1M	24.69	2.46E-04
212531_at	Hs.204238	LCN2	24.17	9.19E-04
209894_at	Hs.705413	LEPR	23.77	5.83E-04
204533_at	Hs.632586	CXCL10	23.61	1.47E-05
213797_at	Hs.17518	RSAD2	20.43	7.31E-05
204439_at	Hs.715563	IFI44L	17.92	9.73E-04
213975_s_at	Hs.706744	LYZ	15.22	1.10E-03
206643_at	Hs.190783	HAL	14.88	3.98E-03
216598_s_at	Hs.303649	CCL2	14.76	6.99E-03
235229_at	Hs.332649		13.93	6.22E-04
205890_s_at	Hs.714406	GABBR1///UBD	13.67	1.46E-03
33304_at	Hs.459265	ISG20	13.58	5.61E-05
205569_at	Hs.518448	LAMP3	10.96	3.58E-05
204470_at	Hs.789	CXCL1	10.90	9.06E-03
208607_s_at	Hs.632144	SAA1///SAA2	10.40	4.56E-03
205302_at	Hs.642938	IGFBP1	9.55	1.10E-02

doi:10.1371/journal.pone.0023856.t001

Table 2. The top 20 genes down-regulated with HCV infection.

Probe set	Unigene code	Gene symbol	Fold change	P value
207245_at	Hs.575083	UGT2B17	20.04	2.15E-02
214043_at	Hs.446083	PTPRD	6.81	2.57E-02
214416_at	Hs.702961		6.36	3.51E-02
209220_at	Hs.713537	GPC3	5.40	4.40E-02
238029_s_at	Hs.504317	SLC16A14	4.90	1.16E-02
231594_at			4.59	1.87E-02
1556824_at	Hs.702604		4.40	2.89E-02
232707_at	Hs.567637	ISX	4.30	6.52E-03
205822_s_at	Hs.397729	HMGCS1	4.23	1.95E-04
204058_at	Hs.211160	ME1	4.06	2.15E-02
1555084_at			3.95	4.10E-02
215076_s_at	Hs.443625	COL3A1	3.92	2.99E-02
209555_s_at	Hs.120949	CD36	3.91	4.49E-02
221729_at	Hs.445827	COL5A2	3.89	2.60E-02
217676_at	Hs.696837		3.86	8.73E-03
233604_at	Hs.280892	FLJ22763	3.82	2.44E-02
1563298_at	Hs.352254		3.64	1.97E-02
224344_at	Hs.497118	COX6A1	3.34	1.68E-02
237031_at	Hs.146276		3.22	4.62E-04
216018_at	Hs.534342	RNF5	3.19	3.13E-02

doi:10.1371/journal.pone.0023856.t002

Table 3. The effect of HCV infection on biological functions by category.

Category	P value	Up-regulated genes in network		Down-regulated genes in network	
		Number of genes	Representative genes	Number of genes	Representative genes
Organismal Injury and Abnormalities	5.90E-16–3.66E-03	27	CXCL1, CXCL6, CXCL9, CXCL10, IFIT1, IFIT3, MX1, etc.	1	SERPINI1
Cancer	1.81E-13–5.73E-03	54	BIRC3, CXCL9, CXCL10, GBP1, IFIT3, IGFBP1, ISG20, MAP3K8, etc.	4	CD36, COL3A1, GPC3, RNFS
Inflammatory Response	9.31E-13–5.89E-03	39	APOBEC3G, CCL2, CXCL9, CXCL10, IL8, MX1, STAT1, TRIM22, etc.	2	CD36, COL3A1
Cell-To-Cell Signaling and Interaction	4.95E-10–4.99E-03	30	CCL2, CD74, CXCL1, CXCL2, CXCL9, ICAM1, IL8, NRG1, STAT1, etc.	3	CD36, SERPINI1, GPC3
Hematological System Development and Function	4.95E-10–5.95E-03	36	CCL2, CCL20, CXCL9, CXCL10, IL8, IL1RN, TNFAIP3, etc.	1	CD36
Immune Cell Trafficking	4.95E-10–5.73E-03	26	CCL2, CCL20, CTSS, CXCL6, CXCL9, CXCL10, MDK, NEDD9, etc.	1	CD36
Infection Mechanism	5.03E-10–3.66E-03	16	CCL2, CXCL9, CXCL10, DDX58, IFIT1, IL8, ISG20, MX1, RSAD2, STAT1, etc.	0	
Infectious Disease	5.03E-10–5.46E-03	26	APOBEC3G, CXCL9, CXCL10, DDX58, MT1X, STAT1, TNFAIP3, etc.	2	CD36, HMGCS1
Reproductive System Disease	6.43E-10–1.37E-03	42	CCL2, CXCL1, CXCL2, IFIT1, IGFBP1, KLF4, MAP3K8, NEDD9, SPP1, etc.	1	RNFS
Cellular Movement	6.64E-10–5.91E-03	31	IGFBP1, IL8, KLF4, MDK, NEDD9, NRG1, RARRES1, SOD2, TNFAIP8, etc.	2	CD36, RNFS

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absorption at 260 nm/280 nm (NanoDrop Technologies, Wilmington, DE) and agarose gel electrophoresis. Microarray analysis was performed using the Affymetrix GeneChip Human Gene U133Plus2.0 Array, which interrogates 38,500 genes across 54,675 distinct probes (Affymetrix, Santa Clara, CA). The Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay Manual Version 4 was used for complementary DNA (cDNA) generation, hybridization, and array processing. Briefly, 300 ng of total RNA underwent first-strand and second-strand cDNA synthesis. Complementary RNA was generated and used to produce sense-strand cDNA, which was fragmented and end-labeled with biotin. Biotin-labeled cDNA was hybridized to the Human Gene 1.0 ST Array for 16 hours at 45°C using the GeneChip Hybridization Oven 640 (Affymetrix). Washing and staining with streptavidin-phycoerythrin was performed using the GeneChip Fluidics Station 450, and images were acquired using the Affymetrix Scanner 3000 (Affymetrix).

Microarray Data Analysis and Hierarchical Clustering

Fluorescence intensities captured by the Affymetrix GeneChip Scanner were converted to numerical values using the Affymetrix GeneChip Operating Software, were log₂ transformed, and were standardized using quantile normalization with the Robust Multiarray Analysis (RMA) algorithm [32,33]; this method normalizes the distribution of probe intensities for all the gene arrays in a given set.

Obtained gene expression profiles were analyzed using GeneSpring GX 10.0.2 software (Tomy Digital Biology, Tokyo, Japan). Expression ratios were calculated and normalized per chip to the 50th percentile and finally normalized per gene to medians. We worked on a pre-screened list of 32,885 probes obtained after filtering the data for outliers, negative and positive controls, and on the quality flag Cy3 signals being “well above background.” To pass

this last flag, Cy3 net signals needed to be positive and significant, with $g(r)BG_{SubSignal}$ greater than $2.6 g(r)BG_{SD}$. To determine if there were genes differentially expressed among samples, we performed two Welch's t-tests ($P < 0.01$) on this prescreened list of genes: one without correction and one with Benjamini and Hochberg's correction. Complete linkage hierarchical clustering analysis was applied using Euclidean distance, and differentially expressed genes were annotated using the information from the Gene Ontology Consortium. Global molecular networks and comparisons of canonical pathways were generated using Ingenuity™ Pathway Analysis 8.6 (Ingenuity™ Systems, CA, USA).

Real time PCR for analyzing the mRNA expression in the human hepatocytes

Total RNA was extracted from the implanted human hepatocytes in the mouse livers using RNeasy Mini Kit (Qiagen) and reverse-transcribed using ReverTra Ace (TOYOBO, Osaka, Japan) with random primer in accordance with the instructions supplied by the manufacturer. The selected cDNA were quantified by real-time PCR using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA), and the expression of GAPDH served as a control. Amplification was performed in a 25 µl reaction mixture containing 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 5 pmol of forward primer, 5 pmol of reverse primer, and 1 µl of cDNA solution. After incubation for 2 min at 50°C, the sample was denatured for 10 min at 95°C, followed by a PCR cycling program consisting of 40 cycles of 15 s at 95°C, 30 s at 55°C, and 60 s at 60°C.

Statistical analysis

Differences between groups were examined for statistical significance using the Student's *t*-test.

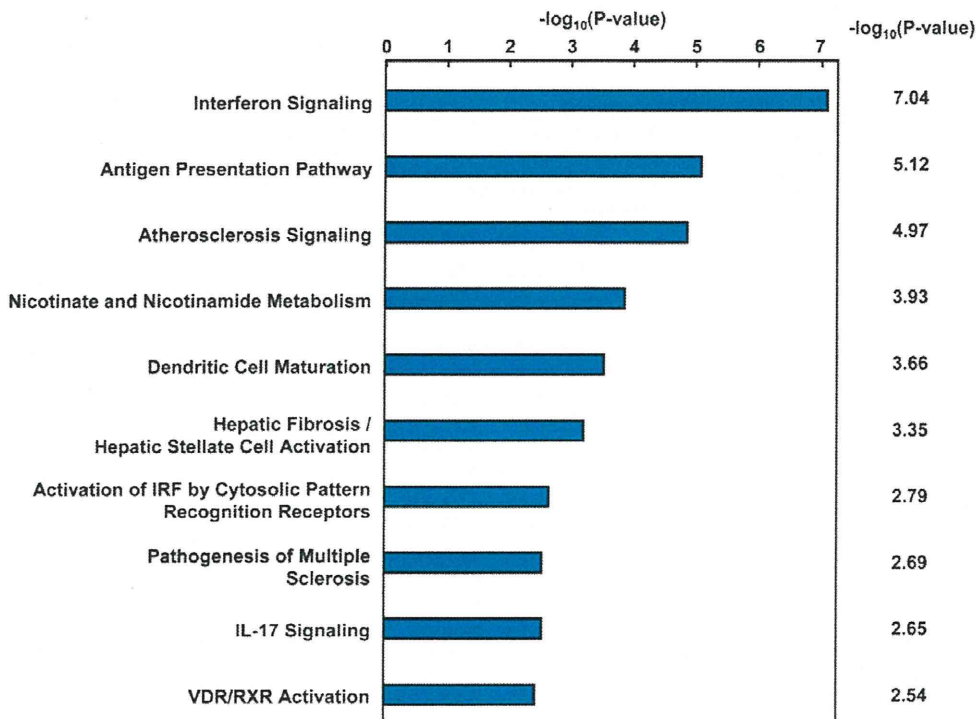


Figure 3. The effects of HCV infection on canonical pathways. To analyze the effects of HCV infection on canonical pathways, pathway analysis was performed using the 181 genes identified to be significantly up- or down-regulated following HCV infection. The IFN signaling pathway was the most significantly affected by HCV infection. Statistical analysis was performed using Fisher's exact test. doi:10.1371/journal.pone.0023856.g003

Results

Change of gene expression with HCV infection

To analyze the effect of HCV infection on gene expression in human hepatocytes, we compared the gene expression profiles between Group A (without HCV infection) and Group C (with HCV infection). Among the 2,519 genes that remained significant after screening by Welch's t-test, more than 3.0-fold expression changes between groups were observed in 181 genes. 157 of these 181 genes were up-regulated following HCV infection, and the other 24 were down-regulated. Cluster analysis of the 181 genes is shown in Figure 2, and the top 20 up-/down-regulated genes by HCV infection are listed in Tables 1 and 2, respectively.

It is well known that chronic HCV infection triggers multiple biological responses. To analyze biological significance and regulatory pathways involved in the changes observed, we performed network analysis with the 181 genes using Ingenuity™ Pathway Analysis (IPA). As shown in Table 3, most of the 181 genes (e.g. *CXCL9*, *CXCL10*, *IFIT3* and *Mx1*, which are well known interferon-stimulated genes (ISGs)) belonged to categories such as Organismal Injury and Abnormalities, Inflammatory Response, and Cell-To-Cell Signaling and Interaction. Through canonical pathway analysis of the 181 genes using Ingenuity Pathways Analysis, 10 canonical pathways significantly affected by HCV infection were identified, with interferon signaling as the most significant (Figure 3). These results indicate that the intrahepatic innate immune response was strongly activated by HCV infection in human hepatocytes.

Change of gene expression with interferon treatment

To analyze the direct effects of IFN in human hepatocytes, we compared gene expression profiles between Group A (without IFN treatment) and Group B (with IFN treatment). Out of the 218 genes that remained significant after screening by Welch's t-tests and Benjamini-Hochberg correction for multiple testing, 158 had a greater than 3.0-fold change between groups. 152 of the 158 genes were up-regulated following IFN administration, and the other 6 were down-regulated. Cluster analysis of the 158 selected genes is shown in Figure 4. The top 35 up-regulated genes (>10.0-fold changes), which include many well-known ISGs (e.g., members of the *CXCL* and *IFI* families), and the 6 down-regulated genes are listed in Tables 4 and 5, respectively.

The effect of HCV infection on IFN response

To analyze the effect of HCV infection on IFN response, we focused on the 152 genes that were up-regulated following IFN administration and compared gene expression ratios between Groups A and B (gene expression changes by IFN without HCV infection) and between Groups C and D (gene expression changes by IFN with HCV infection). In 69.7% (106/152) of the IFN-induced genes, IFN responsiveness was significantly reduced following HCV infection (Figure 5). The top 20 genes are shown in Table 6. Although viral titers differed among mice, we found no correlation between IFN responsiveness and HCV RNA titer. We performed pathway analysis to identify significant associations with canonical pathways, and the top 5 associated pathways are shown in Table 7. IFN responsiveness was significantly reduced following HCV infection in several canonical pathways, and the IFN