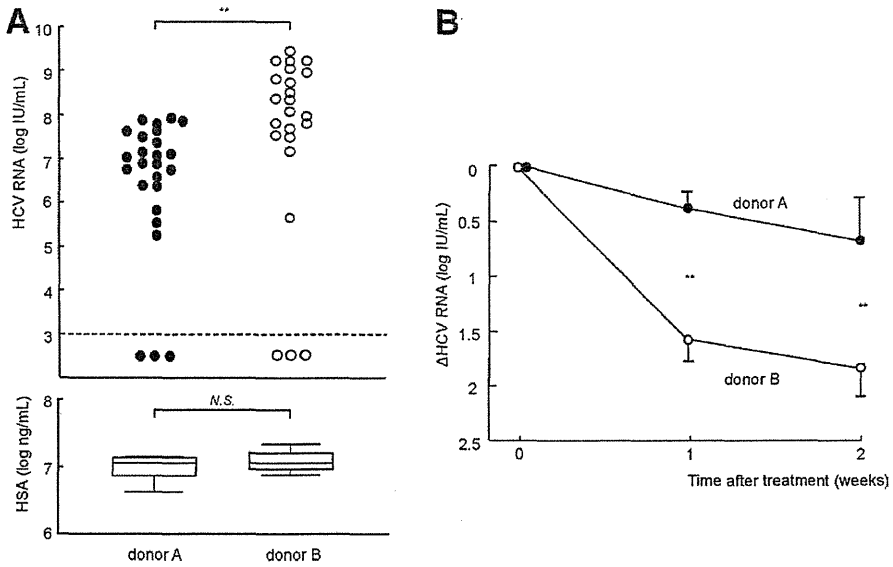

Fig. 3. HCV infectivity, replication ability, and IFN susceptibility in HCV-KT9-injected mice. Mice that underwent transplantation with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) (closed circles, n = 25) or B (rs8099917 TT and rs12979860 CC) (open circles, n = 23) were intrahepatically inoculated with RNA transcribed from either Core-Wild-ISDR0 or Core-Mutant-ISDR0 clones. (A) Eight weeks after infection, serum HCV RNA titers (upper panel) and HSA concentrations (lower panel) were measured. The horizontal dotted line indicates the HCV RNA titer detection limit (1000 copies/mL). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (B) HCV-infected mice with hepatocytes from donor A (closed circles, n = 12) or B (open circles, n = 8) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. Data are represented as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$; NS, not significant.

significantly higher in mice with hepatocytes from donor B than from donor A ($P < 0.001$). HCV-infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in 0.65 ± 0.38 and 1.84 ± 0.23 log IU/mL reductions in HCV RNA titer in mice with hepatocytes from donors A and B, respectively ($P < 0.01$) (Fig. 3B). Interestingly, despite the higher serum HCV RNA levels, reduction levels of HCV were higher in mice that underwent transplantation with hepatocytes obtained from donor B than in mice that underwent transplantation with hepatocytes obtained from donor A.

To confirm an association between IL28B SNP genotype and HCV RNA titer, we compared HCV RNA titers using mice with hepatocytes from an additional pair of donors with the favorable (donor C) and unfavorable (donor D) SNP genotypes. To determine whether results obtained by clonal infection would be comparable to results obtained using the more natural serum injection, which should have contained more complex viral species, mice were injected with genotype 1b HCV obtained from a human patient with core and ISDR substitutions, as described above. Mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) or donor D (rs8099917 TT and rs12979860 CC) were inoculated intravenously with 10^5 copies of HCV. Eight weeks after inoculation, serum HCV RNA titer increased above the detection limit in 13 of 14 (93%) mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) and in 12 of 12 (100%) mice with hepatocytes from donor D (rs8099917 TT and rs12979860 CC) (Fig. 4A). With results similar to those found for the mice inoculated with transcribed HCV RNA, serum HCV RNA levels were significantly higher in mice with hepatocytes from donor D than from donor C ($P < 0.001$), and the effect of IFN was also greater in donor D mice than in donor C mice (Fig. 4B); however, statistical significance using these donors was only achieved at week 1, probably resulting from fluctuation of HCV RNA titers and the small number of animals analyzed.

Expression Levels of ISGs in Mouse Livers. ISG expression levels in mice livers were measured after 2 weeks of IFN treatment (Fig. 4B). MxA, OAS, and PKR levels were significantly higher in mice with human hepatocytes from donor D than from donor C (Fig. 4C).

Discussion

In this study, we investigated the effect of substitutions at core protein aa70 and 91 and within the

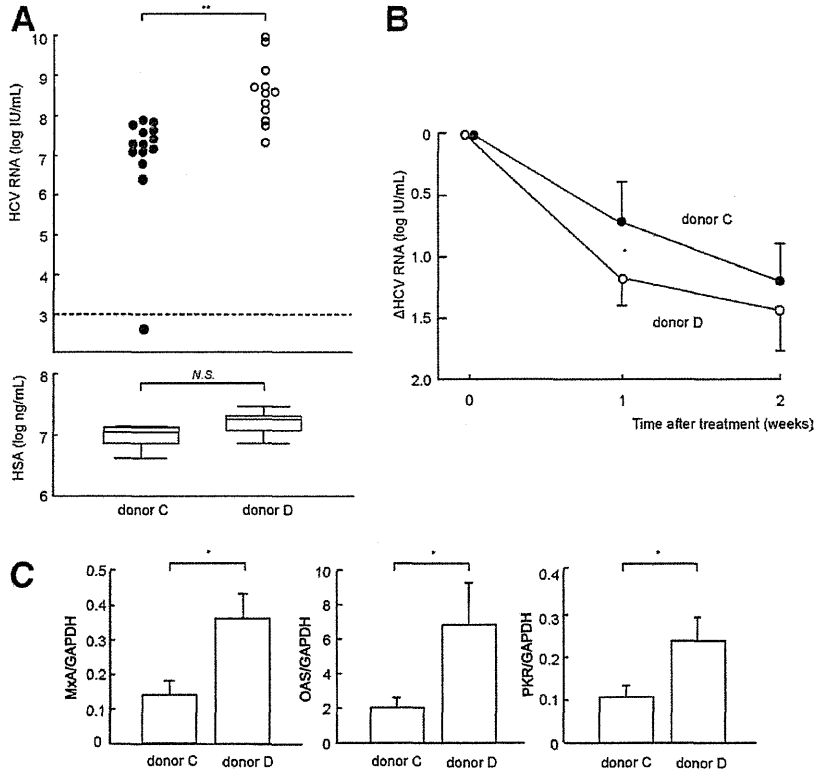

Fig. 4. HCV infectivity, replication ability, and IFN susceptibility in HCV-infected mice. Mice that underwent transplantation with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) (closes circles, n = 14) or D (*rs*8099917 TT and *rs*12979860 CC) (open circles, n = 12) were intravenously injected with HCV-infected patient serum samples. (A) Eight weeks after infection, serum HCV RNA titers (upper panel) and HSA concentrations (lower panel) were measured. The horizontal dotted line indicates the HCV RNA titer detection limit (1000 copies/mL). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. HCV-infected mice with hepatocytes from donor C (closed circles, n = 5) or D (open circles, n = 4) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. (B) Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. (C) Intrahepatic ISG expression levels in the IFN-treated mice with donor C (n = 4) or D (n = 3) were measured and expressed relative to GAPDH messenger RNA. Data are reported as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$; NS, not significant.

ISDR, which have been reported to be associated with the outcome of IFN plus ribavirin combination therapy.[8-14] Clones with core aa70 and 91 substitutions showed comparable infection and replication abilities, whereas clones with substitutions in the ISDR showed reduced infectivity and replication rates. It has been reported that patients infected with HCV strains with multiple substitutions in the ISDR have lower viral titers than those with wild-type ISDR, and that these patients respond well to IFN therapy.[8,9] We showed, in this study, that infectivity and replication ability of HCV are apparently impaired in ISDR mutants (Fig. 2A,C). This may explain, at least partially, the better effect of IFN therapy in patients with multiple ISDR mutations. However, why aa substitutions in this particular region are associated with the effect of IFN still remains to be elucidated. In contrast, aa substitutions in the core, which more profoundly affect the outcome of combination therapy,[10-13] did not influence the infectivity and replication ability of the virus (Fig. 2A,B). This suggests that aa substitutions in this region affect response to therapy in a way that is independent of the replication level of the virus. A recent report by Eng et al.[36] showed that a mutation in core aa91 results in the production of minicore protein, which might alter the effect of IFN. The presence of minicore protein and its effect on IFN therapy should be further investigated using the chimeric mouse model.

In contrast to these viral substitutions, host IL28B genotype significantly affected viral replication levels (Figs. 3A and 4A). Curiously, replication levels of the virus are higher in mice with human hepatocytes from donors with rs8099917 TT and rs12979860 CC genotypes, even though these genotypes are associated with successful response to the therapy.[20-22] This result is consistent with clinical observation of higher viral loads in patients with the rs12979860 CC genotype.[20] The favorable IL28B genotype is associated not only with successful response to IFN treatment, but also to spontaneous clearance of the virus.[37,38] However, the incidence of HCV infection was similar in mice with hepatocytes from donors with rs8099917 TT and rs8099917 TG (Figs. 3A and 4A), suggesting that spontaneous clearance was rare. The fact that our animal model was immunodeficient suggests that spontaneous clearance of HCV might require the involvement of the adaptive immune system. The wild-type core protein, aa70, is reported to be found more often in patients with the rs8099917 TT genotype,[23,24] even though patients with this genotype are more likely to be able to eradicate the virus without therapy during

the natural course of infection.[37,38] These data suggest that core aa70 wild-type virus can be eradicated more easily in the natural course of infection, especially in patients with rs8099917 TT or rs12979860 CC genotypes; but once the infection is established, core aa70 wild type replicates more effectively than core aa70 mutant strains.

The effect of IFN on reduction of the virus did not differ between core aa70 wild-type and mutant strains, which showed similar replication levels (Fig. 2D). This is in contrast to clinical observations that the effect of therapy on viral reduction is more prominent in patients with wild-type core protein.[13,25] One of the differences between the mouse model and human patients is term of infection. Long-term HCV infection results in alteration of lipid metabolism and accumulation of lipids in hepatocytes.[39] Patients with fatty change of the liver often fail to respond to therapy.[40] We observed no severe fatty change in mouse livers, suggesting that such long-term change might be absent in this mouse model (data not shown).

On the other hand, the effect of IFN was significantly greater in mice with hepatocytes with the eradication-favorable IL28B genotype (rs8099917 TT and rs12979860 CC) (Figs. 3B and 4B), despite the higher replication rate of the virus. This suggests that the IL28B genotype affects the outcome of therapy based on a different mechanism than viral replication. Because of strong linkage disequilibrium, genotypes of the SNPs around the two IL28B landmark SNPs (rs8099917 and rs12979860) were identical between donors A and C as well as between B and D (data not shown). Further study using human hepatocytes with various IL28B SNP genotypes will identify a primary SNP that directly affects the outcome of therapy. Response to IFN was associated with higher expression levels of ISGs, including MxA, OAS, and PKR (Fig. 4C). This is in agreement with previous studies showing that SVR is associated with stronger induction of ISG expression.[41] However, we observed no statistically significant differences in ISG expression levels from the IL28B SNP genotype before therapy (data not shown). This may result from lower ISG expression levels before therapy and the relatively small number of mice examined. Because there is no adaptive immune system in this mouse model, such differences primarily involve individual hepatocytes, although whether the presence of immune cells enhances this difference should be investigated further.

In summary, we demonstrated that viral infectivity and replication ability are associated with hepatocyte IL28B genotype and are not associated with viral substitutions in the core protein or ISDR. Understanding the mechanism underlying the higher, more prolonged expression of antiviral genes in response-favorable hepatocytes will help us to develop improved therapeutic regimens to eradicate HCV more effectively.

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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 performed 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 μg of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, were transcribed in a 100 μL 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 μL 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 (Sankojunyaku, Tokyo), dissolved in 8.8 μL RNase-free H_2O, and reverse transcribed using a random primer (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) in a 20-μL 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

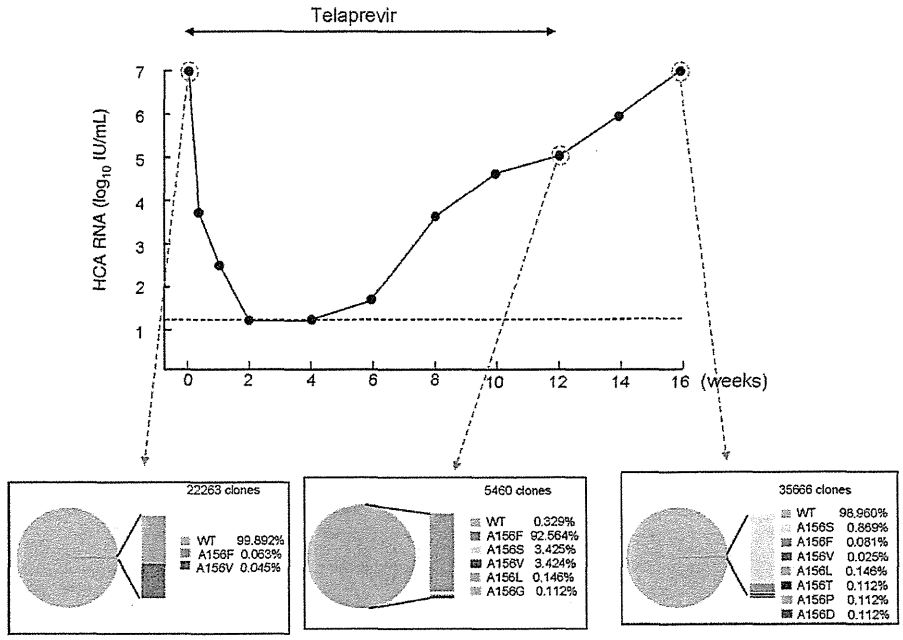

Fig. 1. Changes in serum HCV RNA levels in a telaprevir-treated chronic hepatitis C patient. A 55-year-old woman infected with genotype 1b HCV was treated with 750 mg of telaprevir every 8 hours for 12 weeks. Serum HCV RNA (upper panel) and the amino acid (aa) frequencies at aa156 in the HCV NS3 region 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 hypervariable 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 μL 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 administrated 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

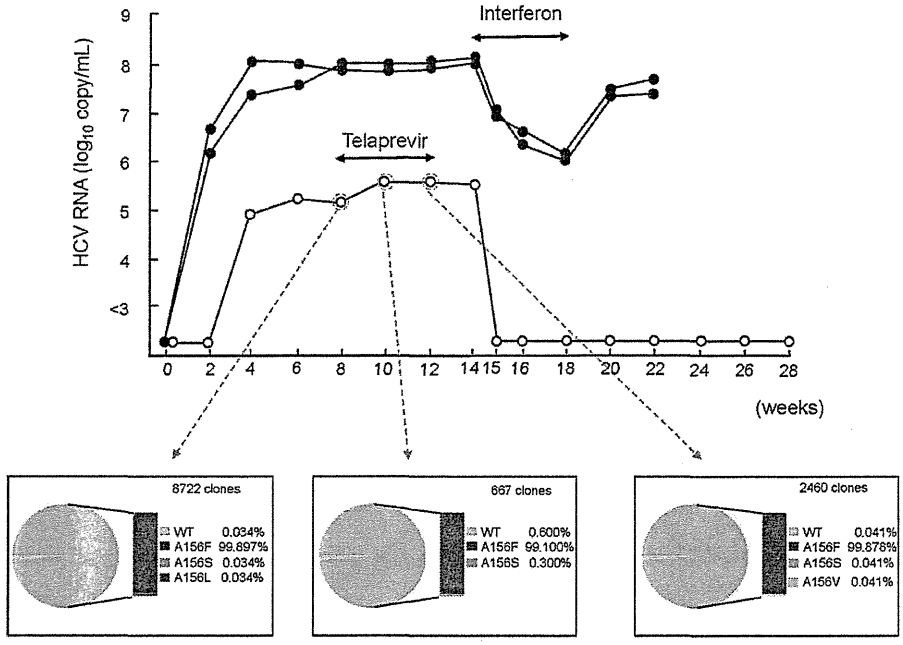

Fig. 2. Changes in serum virus titers in HCV-infected mice. Mice were injected with either wildtype (closed circles) or A156F-mutated HCV serum samples (obtained from an HCV-infected patient who received telaprevir monotherapy for 12 weeks; see Fig. 1) (open circles). Six weeks after injection the A156F 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 telaprevir 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 μg 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

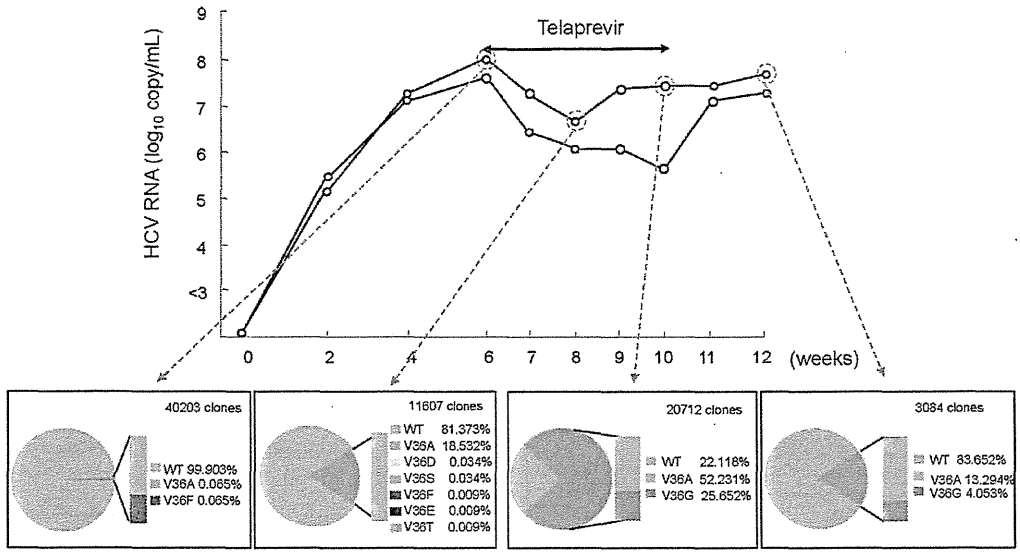

Fig. 3. Treatment with telaprevir in wildtype HCV-infected mice. Two mice were injected intravenously with 50 μL of HCV-positive human serum samples. Six weeks after HCV injection mice were treated with 200 mg/kg of telaprevir orally twice a day for 4 weeks. Serum HCV RNA (upper panel) and amino acid (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 breakthrough 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

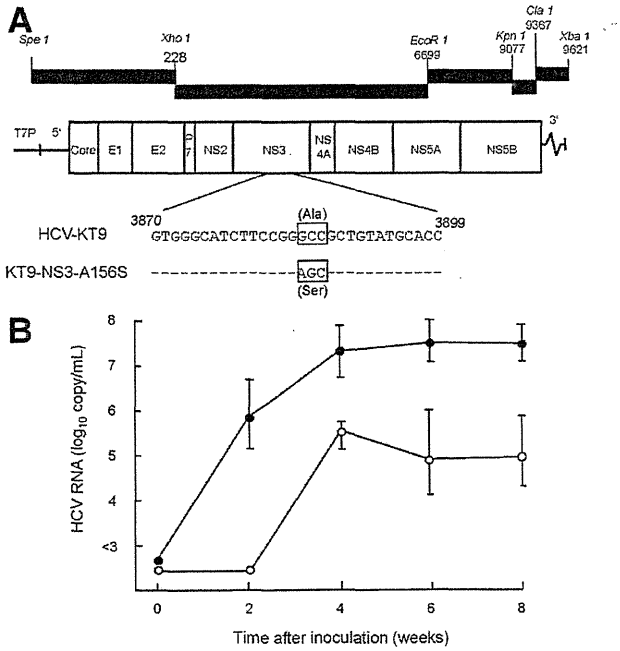

Fig. 4. Intrahepatic injection of *in vitro* transcribed HCV-KT9 RNA and KT9-NS3-A156S RNA into human hepatocyte chimeric mice. (A) The schematic of infectious genotype 1b HCV clones, HCV-KT9 and KT9-NS3-A156S. Boxes indicate codons at amino acid 156 in HCV NS3 region. Ala, alanine; Ser, serine. (B) 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 ± SD of three mice.

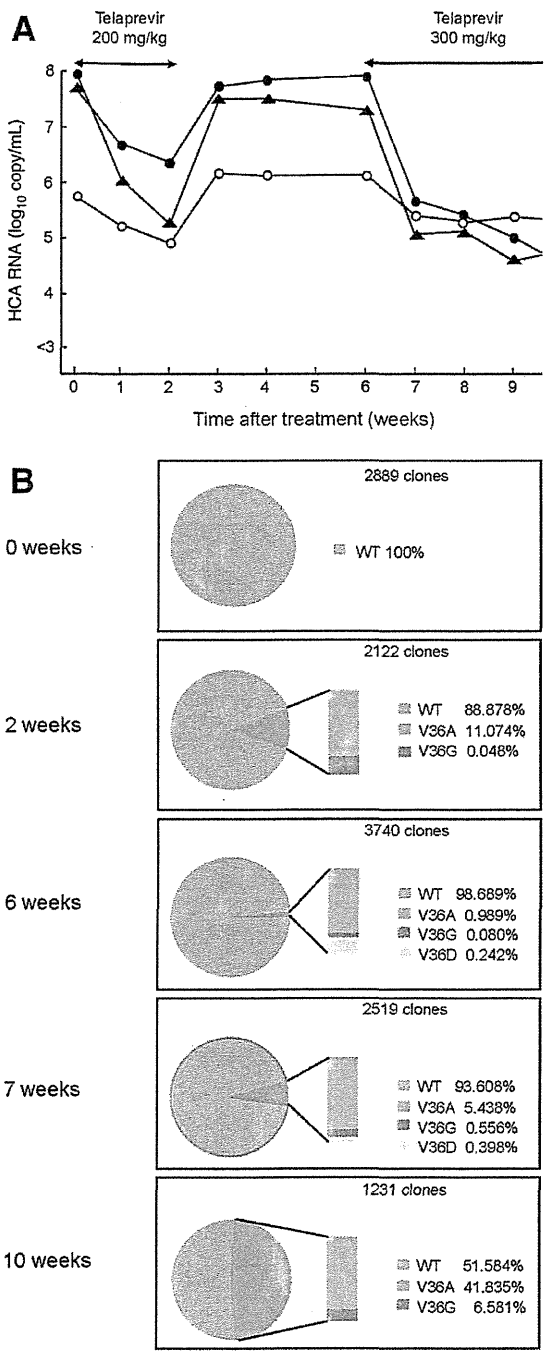

Fig. 5. The effect of telaprevir on mice infected with *in vitro*-transcribed HCV. Mice were injected with *in vitro*-transcribed HCV-KT9 RNA (closed circles and closed triangles) or KT9-NS3-A156S RNA (open circles). Six weeks after HCV RNA injection, mice were treated perorally with 200 mg/kg of telaprevir 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-alpha 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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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 ~ 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-

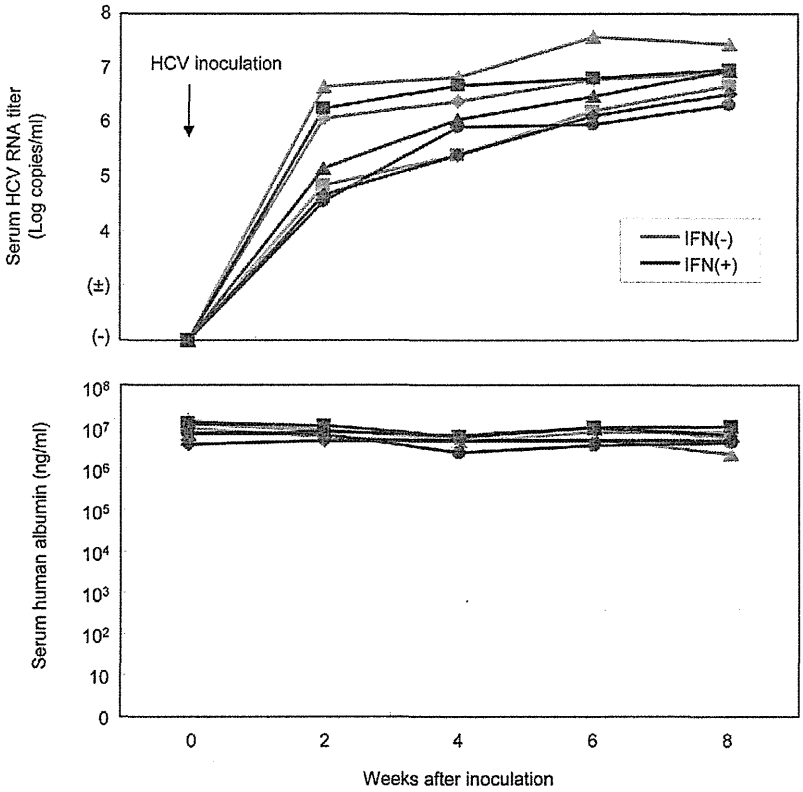

Figure 1. Change in HCV titers and human albumin levels in mouse serum. HCV RNA titers (upper panel) and human albumin levels (lower panel) in chimeric mouse sera after inoculation are shown. The horizontal axis indicates weeks after inoculation. Mouse sera were collected every two weeks after inoculation,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

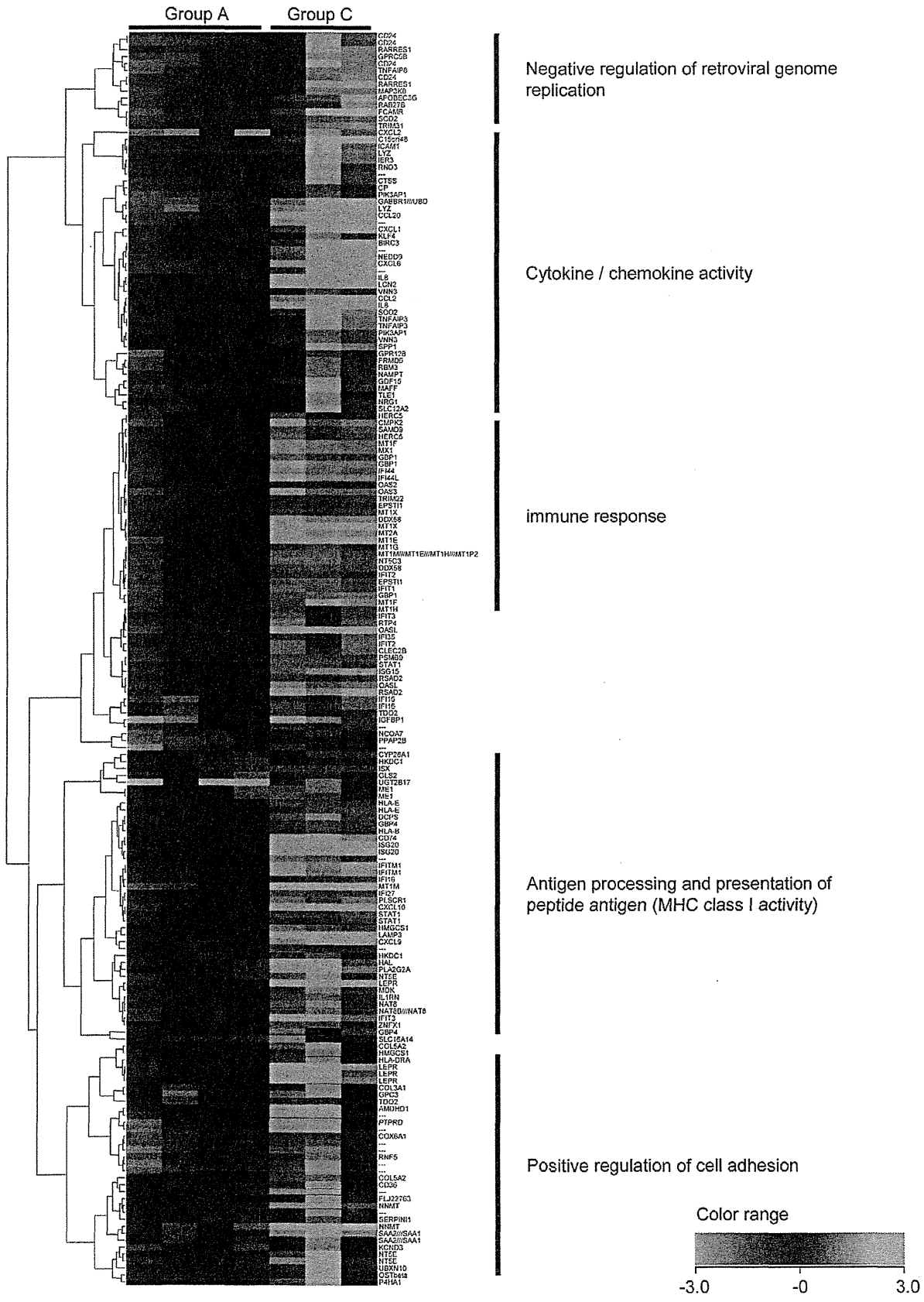
Group A
Group C
Negative regulation of retroviral genome replication
Cytokine / chemokine activity
immune response
Antigen processing and presentation of peptide antigen (MHC class I activity)
Positive regulation of cell adhesion
Color range
-3.0
-0
3.0

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 INF- 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 μl 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 μL of RNase free water and reverse transcribed (RT). RT reactions were performed with 20 μl of the reaction mixtures, containing random primer (Takara Bio Inc., Shiga, Japan), RT buffer and M-MLV reverse transcriptase (ReverTra Ace, 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 heptocytes 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.21160	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, RNF5
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	RNF5
Cellular Movement	6.64E-10–5.91E-03	31	IGFBP1, IL8, KLF4, MDK, NEDD9, NRG1, RARRES1, SOD2, TNFAIP8, etc	2	CD36, RNF5

doi:10.1371/journal.pone.0023856.t003

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)BGSubSignal 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.

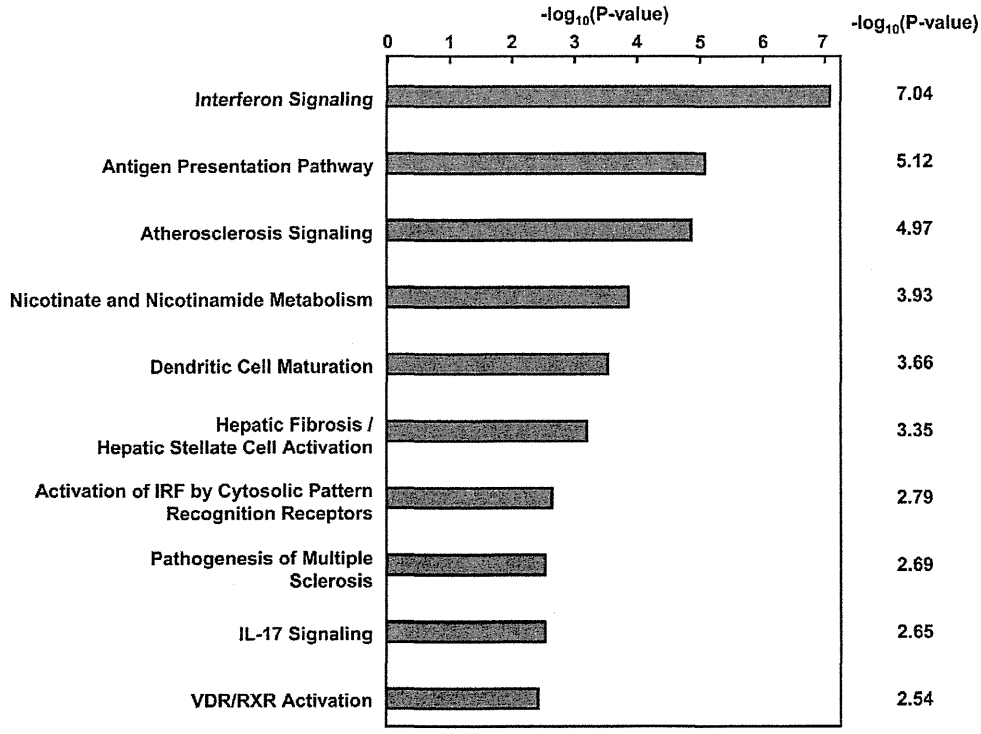

Figure 3. The effects of HCV infection on canonical pathways. To analyze the effects of HCV infection on canonical pathways, pathway analysis was performed using the 181 genes identified to be significantly up- or down-regulated following HCV infection. The IFN signaling pathway was the most significantly 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 intra-hepatic 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

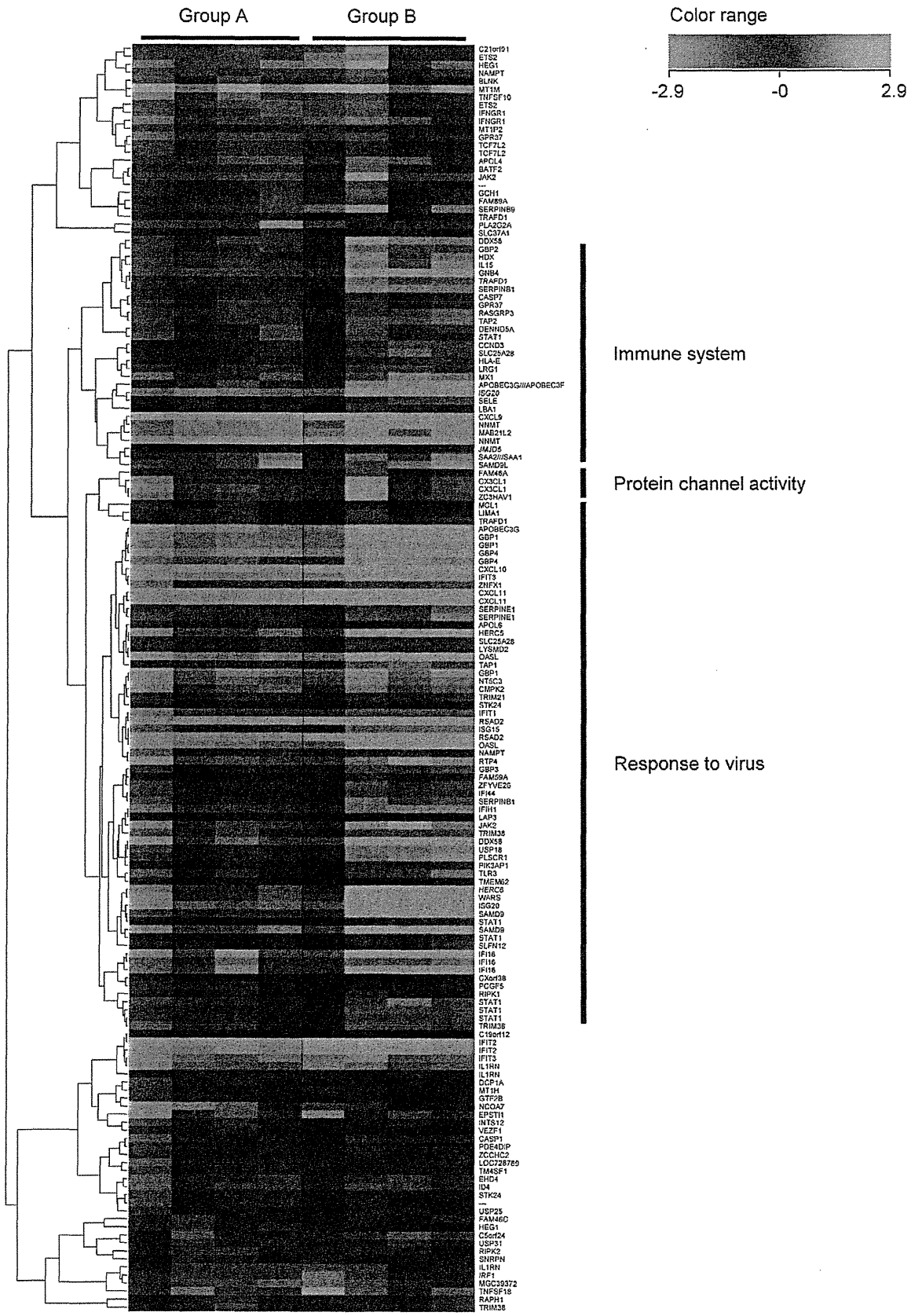
Group A
Group B
Color range
-2.9
-0
2.9
Immune system
Protein channel activity
Response to virus

Figure 4. Hierarchical clustering analysis of 158 genes associated with IFN treatment. To analyze the effects of IFN in human hepatocytes, clustering analysis was performed between Group A (without IFN treatment; 4 columns on the left side) and Group B (with IFN treatment; 4 columns on the right side). 152 genes were up-regulated, and 6 genes were down-regulated following IFN treatment. Several well-known interferon-stimulated genes (ISGs), including *CXCL9*, *Mx1*, *ISG20* and *OASL*, were among the up-regulated genes.
doi:10.1371/journal.pone.0023856.g004

signaling pathway, in particular, was strongly associated. To verify the effects of HCV infection and/or IFN treatment on gene expression, signal intensities of genes involved in the IFN and JAK-STAT signaling pathways were analyzed. As shown in Figure 6A, among 28 representative genes in the IFN signaling pathway, signal intensities of 22 genes could be analyzed through cDNA microarray analysis. In all genes except *IFNAR1*, expression was up-regulated following HCV infection, whereas IFN responsiveness was suppressed as a result of HCV infection (Figure 6B). 16 out of 22 genes in the JAK-STAT signal pathway could be analyzed via cDNA microarray analysis (Figure 6C), and 12 of the 16 genes were up-regulated following HCV infection, whereas IFN responsiveness was suppressed in 9 genes (Figure 6D).

On the other hand, only 33 genes (21.7%), including several ISGs, such as *GBP1*, *GBP4* and *IFIT3*, remained responsive to IFN in the presence of HCV and were expressed more than 3.0-fold higher in Group D compared to Group C (Table 8). Pathway analysis indicated that these 33 genes were significantly associated with Antimicrobial Response and Inflammatory Response ($P = 5.22\times10^{-10} \sim 1.95\times10^{-2}$). Changes in mRNA expression for 29 down-regulated genes, including *ISG20*, *WARS*, *Mx1*, *CXCL10*, *IFNGR1* and *IFITM1* were verified by real time PCR (data not shown).

Table 4. The top 35 genes up-regulated with IFN treatment.

Probe set	Unigene code	Gene symbol	Fold change	P values
211122_s_at	Hs.632592	CXCL11	482.47	1.30E-06
203915_at	Hs.77367	CXCL9	216.26	1.35E-07
242625_at	Hs.17518	RSAD2	101.24	1.26E-05
202237_at	Hs.503911	NNMT	86.80	6.52E-06
217502_at	Hs.437609	IFIT2	75.05	1.73E-06
204533_at	Hs.632586	CXCL10	67.43	2.72E-07
217546_at	Hs.647370	MT1M	46.69	1.12E-04
235175_at	Hs.409925	GBP4	44.94	1.03E-06
204205_at	Hs.660143	APOBEC3G	43.39	5.55E-06
204747_at	Hs.714337	IFIT3	32.73	1.17E-06
218943_s_at	Hs.190622	DDX58	32.19	1.44E-04
33304_at	Hs.459265	ISG20	31.97	3.94E-05
202269_x_at	Hs.62661	GBP1	31.73	5.30E-06
210797_s_at	Hs.118633	OASL	31.59	9.21E-06
200629_at	Hs.497599	WARS	29.65	2.28E-04
206332_s_at	Hs.380250	IFI16	26.37	3.80E-05
210302_s_at	Hs.584852	MAB21L2	24.31	5.31E-07
228531_at	Hs.65641	SAMD9	18.65	1.45E-05
223298_s_at	Hs.487933	NT5C3	17.48	6.65E-06
219863_at	Hs.26663	HERC5	17.02	2.29E-05
225710_at	Hs.173030	GNB4	16.98	3.30E-05
219684_at	Hs.43388	RTP4	16.38	2.55E-05
212657_s_at	Hs.81134	IL1RN	15.18	1.33E-06
219352_at	Hs.529317	HERC6	14.86	1.31E-04
226702_at	Hs.7155	CMPK2	12.50	1.86E-05
205842_s_at	Hs.656213	JAK2	12.49	6.16E-05
230036_at	Hs.489118	SAMD9L	11.98	7.84E-05
214995_s_at	Hs.660143	APOBEC3F/// APOBEC3G	11.62	1.58E-04
823_at	Hs.531668	CX3CL1	11.15	1.07E-04
203153_at	Hs.20315	IFIT1	10.84	5.93E-06
225076_s_at	Hs.371794	ZNFX1	10.40	1.38E-06
213069_at	Hs.477420	HEG1	10.37	3.52E-05
205483_s_at	Hs.458485	ISG15	10.34	1.29E-05
235276_at	Hs.546467	EPSTI1	10.21	2.10E-04
219209_at	Hs.163173	IFIH1	10.05	4.06E-05

doi:10.1371/journal.pone.0023856.t004

Discussion

We previously developed a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [29–31]. This mouse model has enabled us to analyze the effect of viral infection and the response to medication under immunodeficient conditions. Microarray analyses using the human hepatocyte chimeric mouse model with HCV infection have recently been reported, and HCV infection was found to affect expression of genes related to innate antiviral immune response, lipid metabolism and apoptosis via ER stress [34,35]. Whereas these reports were concerned especially with host specific responses to HCV infection, no studies addressing viral modulation of the IFN response have been reported, even though such studies might be important for understanding viral evasion mechanisms in response to IFN therapy and for improving therapy effectiveness for chronic hepatitis C. Therefore, in this study we performed cDNA microarray analysis using a human hepatocyte chimeric mouse model and obtained gene expression profiles to investigate direct influences of HCV infection on IFN responses in human hepatocytes.

First, we evaluated host response to HCV infection in human hepatocytes by comparing profiles between groups A (without HCV infection) and C (with HCV infection). 181 genes were significantly up- or down-regulated following HCV infection. Canonical pathway analysis revealed that genes involved in IFN

Table 5. The top 6 genes down-regulated with IFN treatment.

Probe set	Unigene code	Gene symbol	Fold change	P value
206211_at		SELE	5.83	6.11E-05
224875_at		C5orf24	5.46	1.11E-04
227256_at	Hs.183817	USP31	3.94	7.27E-05
220070_at	Hs.145717	JMJD5	3.87	5.04E-05
1552482_at	Hs.471162	RAPH1	3.31	1.73E-04
226587_at	Hs.592473	SNRPN	3.17	6.13E-05

doi:10.1371/journal.pone.0023856.t005