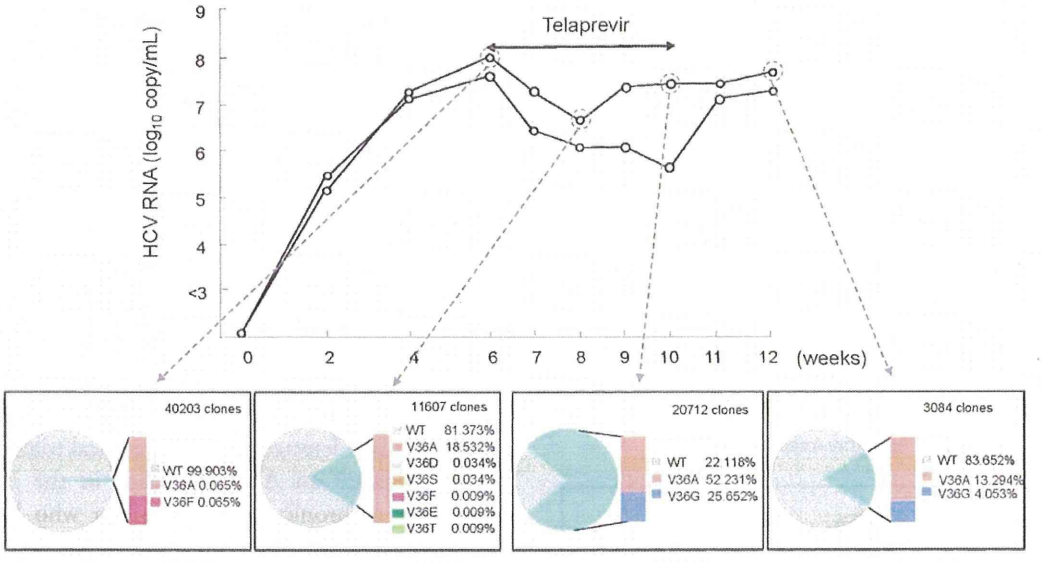

Fig. 3. Treatment with telaprevir in wildtype HCV-infected mice. Two mice were injected intravenously with 50 μL of HCV-positive human serum samples. Six weeks after HCV injection mice were treated with 200 mg/kg of telaprevir orally twice a day for 4 weeks. Serum HCV RNA (upper panel) and amino acid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

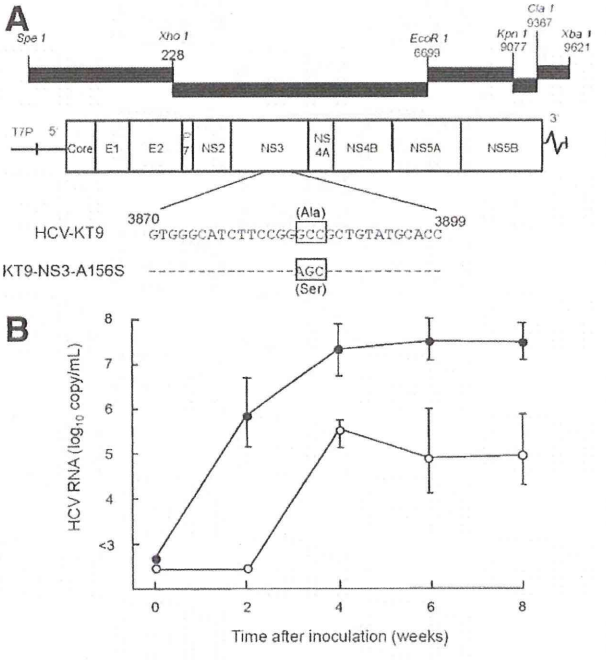

Fig. 4. Intrahepatic injection of *in vitro* transcribed HCV-KT9 RNA and KT9-NS3-A156S RNA into human hepatocyte chimeric mice. (A) The schematic of infectious genotype 1b HCV clones, HCV-KT9 and KT9-NS3-A156S. Boxes indicate codons at amino acid 156 in HCV NS3 region. Ala, alanine; Ser, serine. (B)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

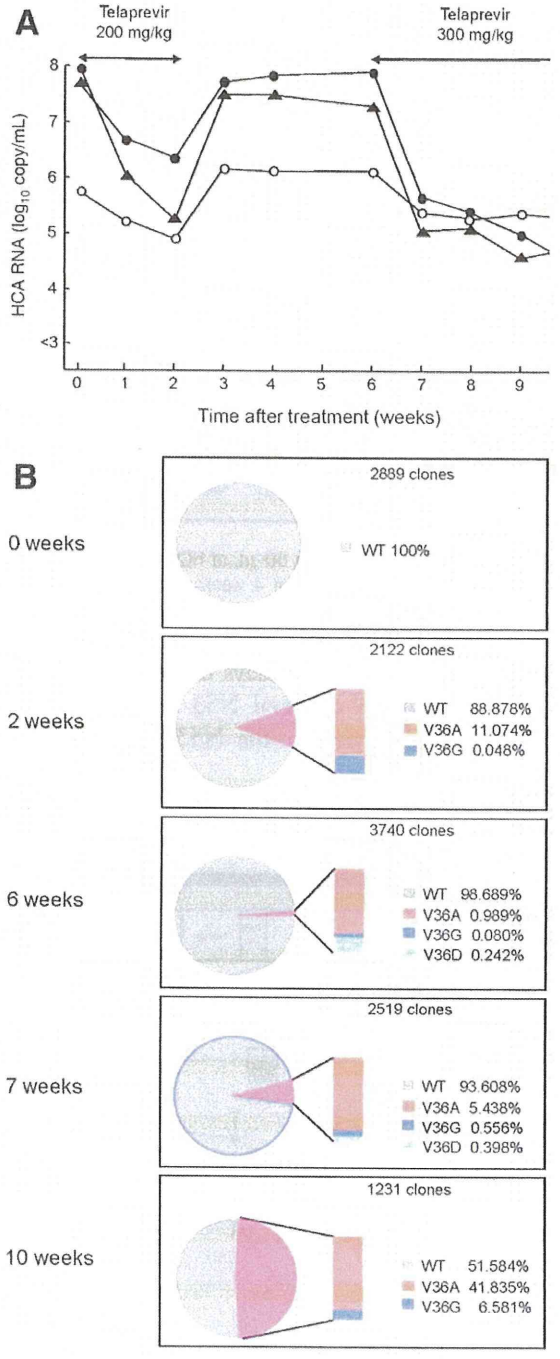

Fig. 5. The effect of telaprevir on mice infected with *in vitro*-transcribed HCV. Mice were injected with *in vitro*-transcribed HCV-KT9 RNA (closed circles and closed triangles) or KT9-NS3-A156S RNA (open circles). Six weeks after HCV RNA injection, mice were treated perorally with 200 mg/kg of telaprevir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

Animal model for study of human hepatitis viruses

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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 siRNA[8]. 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 10^5 virus particles or IU/mL.[9] Similarly, 85% of HCV-inoculated animals also developed viremia,[10] but the level of the viremia only reached 10^5/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 HBV[16] and HCV[17] infections. Repopulation levels by human hepatocytes have been estimated by measuring human albumin levels in mouse serum. Replication levels of both HBV[13] and HCV[17] 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

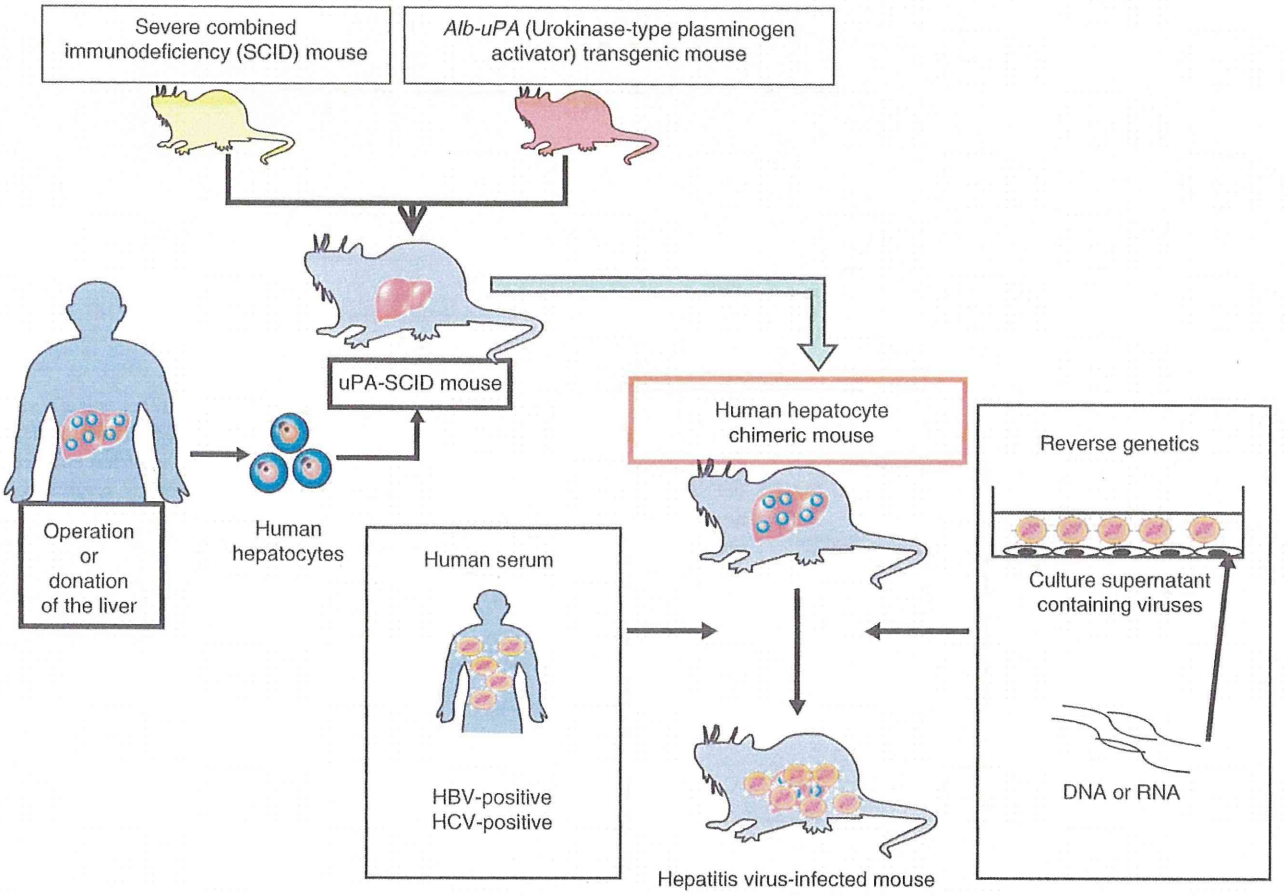

Figure 1 Generation of human hepatocyte chimeric mice and hepatitis virus infection model. A uPA/scid mouse was created by mating uPA transgenic mouse and scid mouse. Human hepatocytes obtained by surgical resection or donation were transplanted to newborn mice. The chimeric mice can be infected with hepatitis B virus (HBV) or hepatitis C (HBC) virus by injecting human serum containing these viruses. Alternatively, the mice can be infected by HBV[13] or HCV[14] 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 HBV[39] or with specific strains of HBV;[40] these findings are consistent with direct cytopathic effects of HBV under certain conditions.

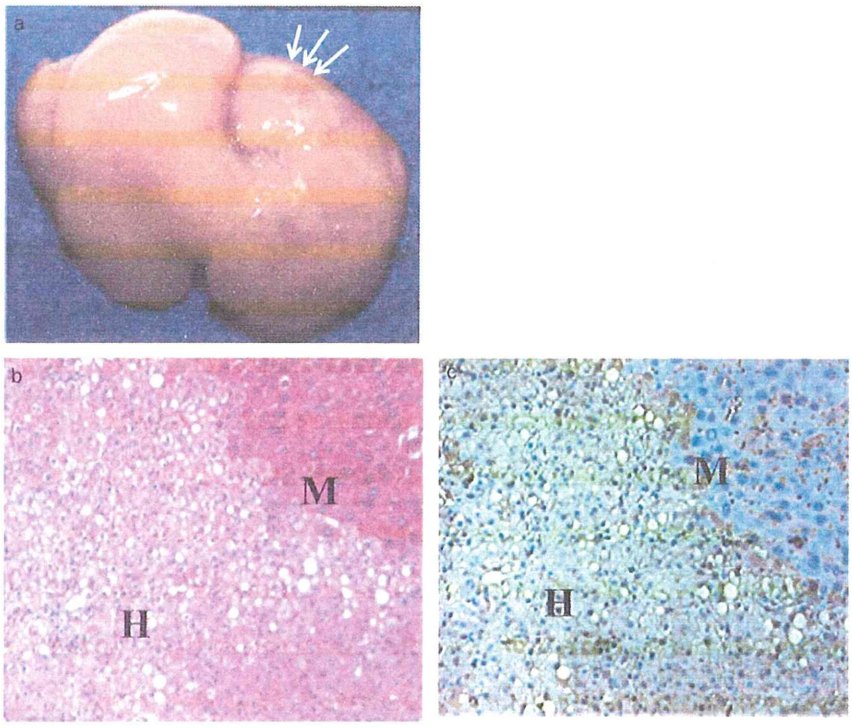

Figure 2 Representative uPA/scid mouse livers repopulated by human hepatocytes. (a) Mouse liver almost completely repopulated by human hepatocytes. Only a small portion of mouse hepatocytes are shown by arrows. (b) Microscopic figure of the mouse liver. M and H indicate regions consisting of mouse and human hepatocytes, respectively (Hematoxylin–eosin staining, magnification: ×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 wooly 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

n	Drug or cell	Strategy	Reference
1	Interferon alpha 2b	Activation of antiviral genes	Kneteman *et al.*[65]
	BILN-2061	NS3-4A protease inhibition	
	HCV371	NS5B polymerase inhibition	
2	Modified BID	Induction of apoptosis	Hsu *et al.*[66]
3	Serine palmitoyltransferase inhibitor	Disruption of lipid raft	Umehara *et al.*[67]
4	Lymphoblastoid interferon alpha	Activation of antiviral genes	Hiraga *et al.*[14]
5	Amphipathic DNA polymers	Blocking viral entry	Matsumura *et al.*[60]
6	Sec-butyl-analogue of HCV-371	NS5B polymerase inhibition	LaPorte *et al.*[68]
7	HCV796	NS5B polymerase inhibition	Kneteman *et al.*[69]
8	Liver allograft-derived lymphocyte	Adoptive immunotherapy	Ohira *et al.*[70]
9	Telaprevir	NS3-4A protease inhibition	Kamiya *et al.*[71]

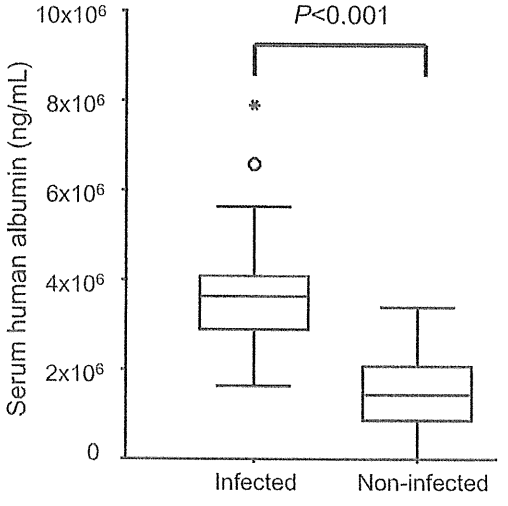

Figure 3 Human albumin levels in mice used in the hepatitis C virus (HCV) infection experiments. A total of 54 mice were injected with HCV positive serum samples containing 5×10^5 virus particles. A total of 24 mice became persistently positive for HCV-RNA, but 30 mice did not. Serum human albumin levels 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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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 ($P = 5.90E\text{-}16 \sim 3.66E\text{-}03$). 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 ($P = 6.0E\text{-}12$). 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-

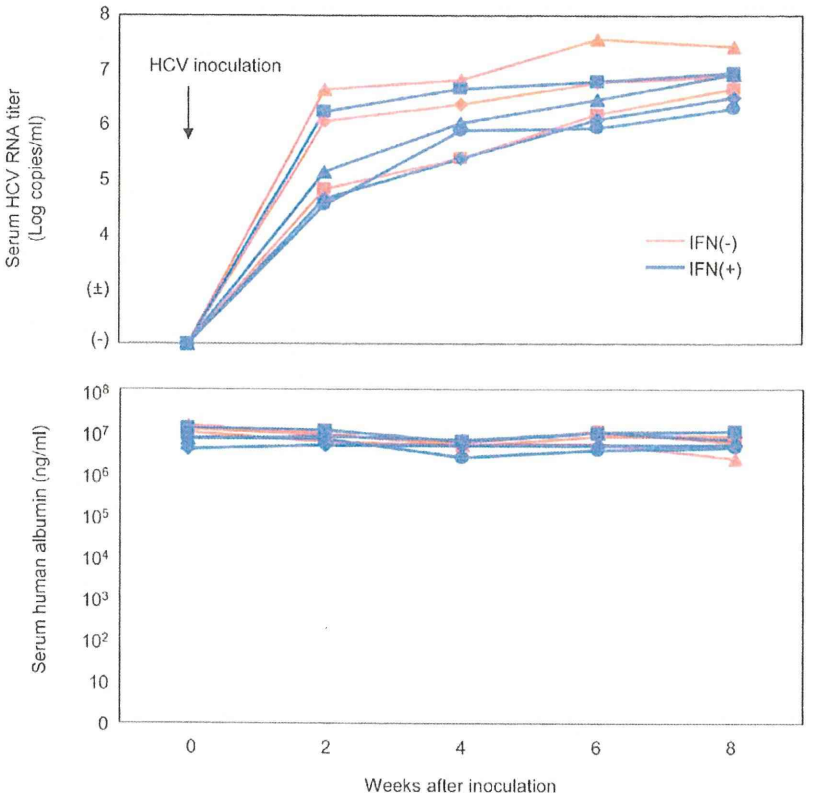

Figure 1. Change in HCV titers and human albumin levels in mouse serum. HCV RNA titers (upper panel) and human albumin levels (lower panel) in chimeric mouse sera after inoculation are shown. The horizontal axis indicates weeks after inoculation. Mouse sera were collected every two weeks after inoculation,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

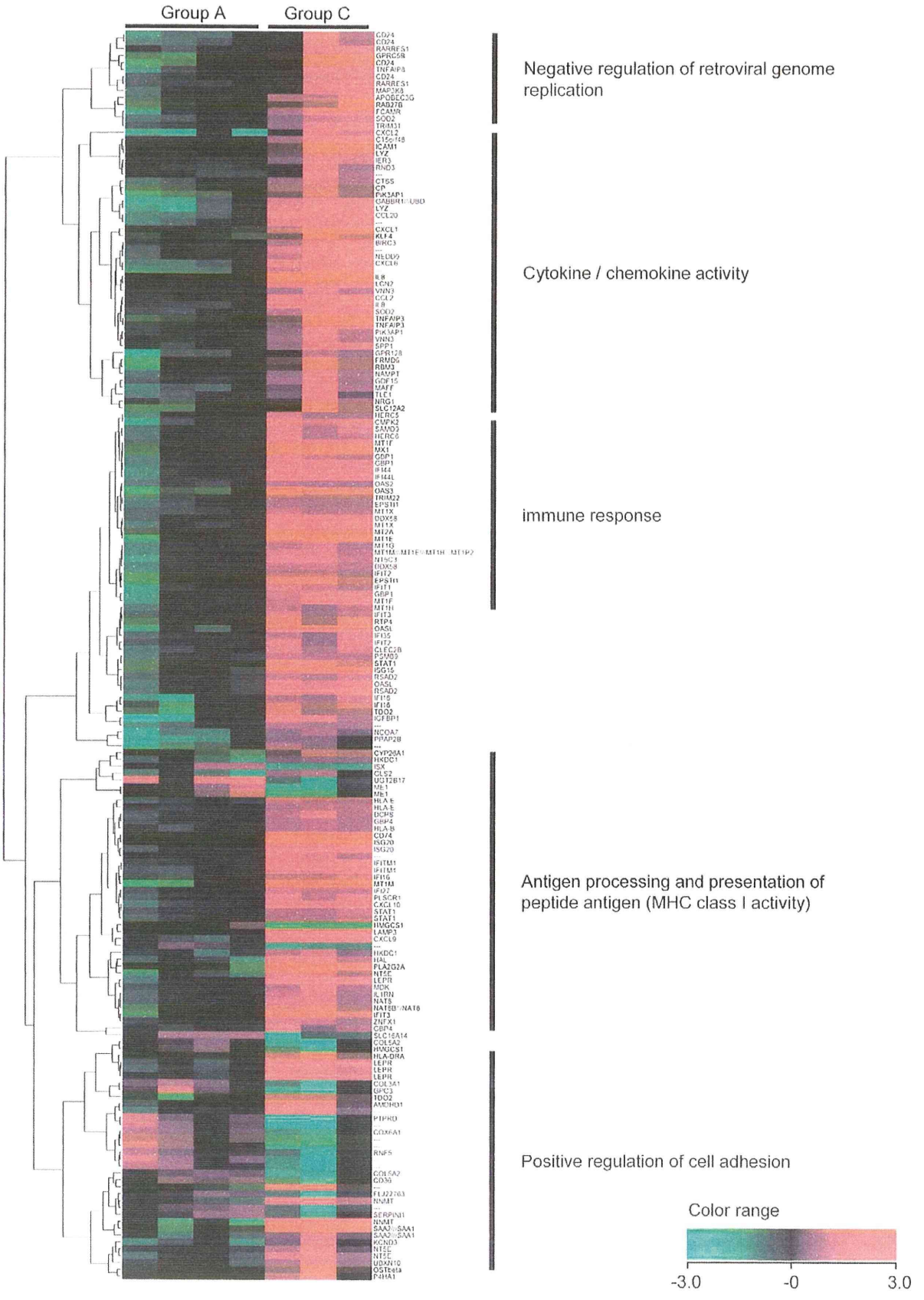
Group A
Group C
Negative regulation of retroviral genome replication
Cytokine / chemokine activity
immune response
Antigen processing and presentation of peptide antigen (MHC class I activity)
Positive regulation of cell adhesion
Color range
-3.0
-0
3.0

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*.
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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.

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

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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 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^{TM} Pathway Analysis 8.6 (Ingenuity^{TM} 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.

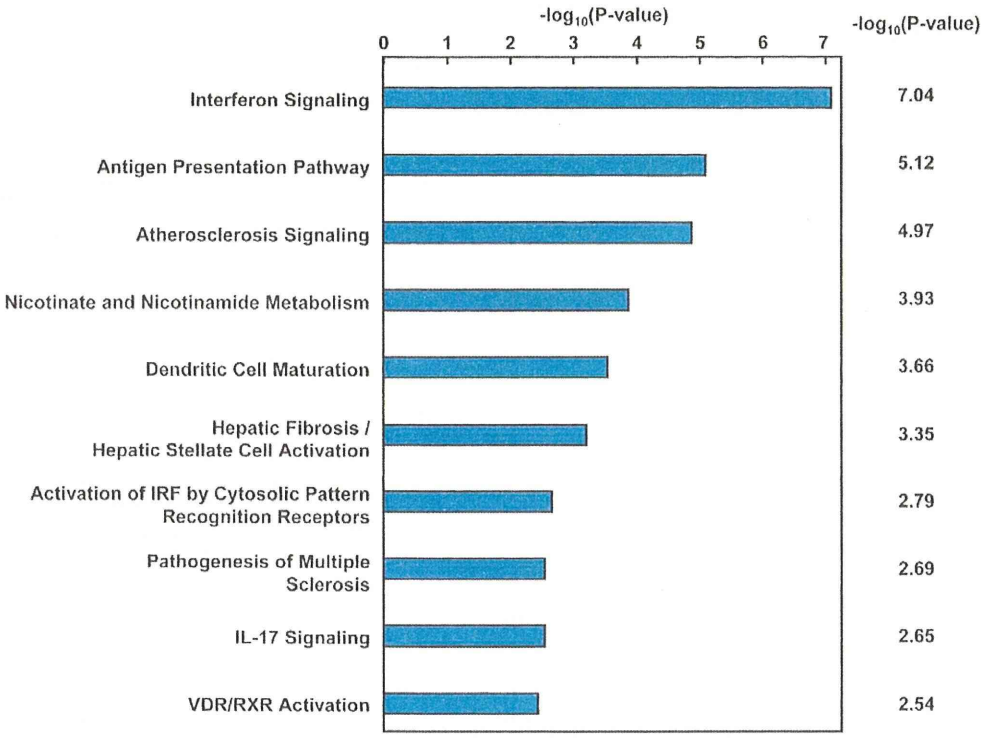

Figure 3. The effects of HCV infection on canonical pathways. To analyze the effects of HCV infection on canonical pathways, pathway analysis was performed using the 181 genes identified to be significantly up- or down-regulated following HCV infection. The IFN signaling pathway was the most significantly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

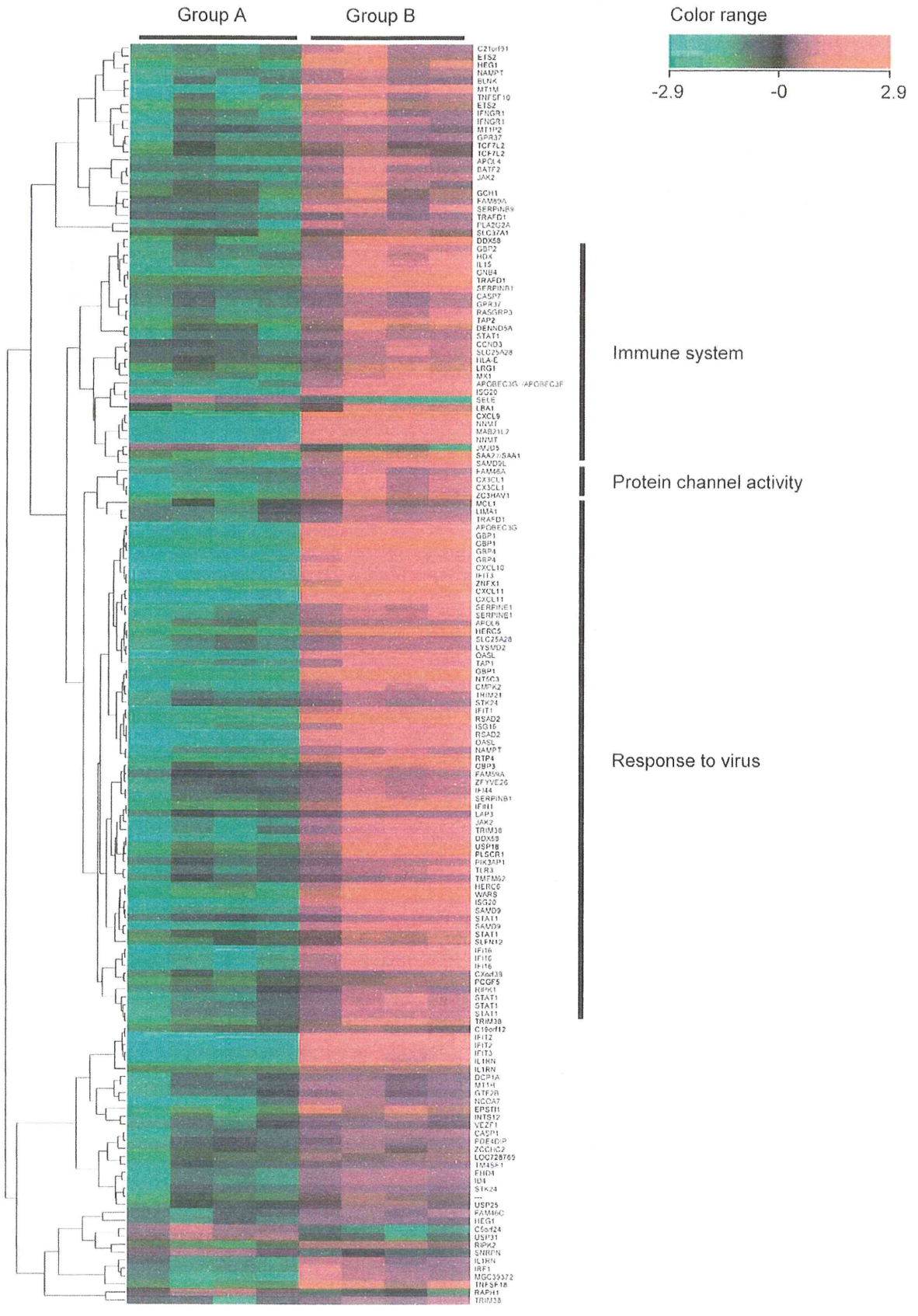
Group A
Group B
Color range
-2.9
-0
2.9
Immune system
Protein channel activity
Response to virus

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.
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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

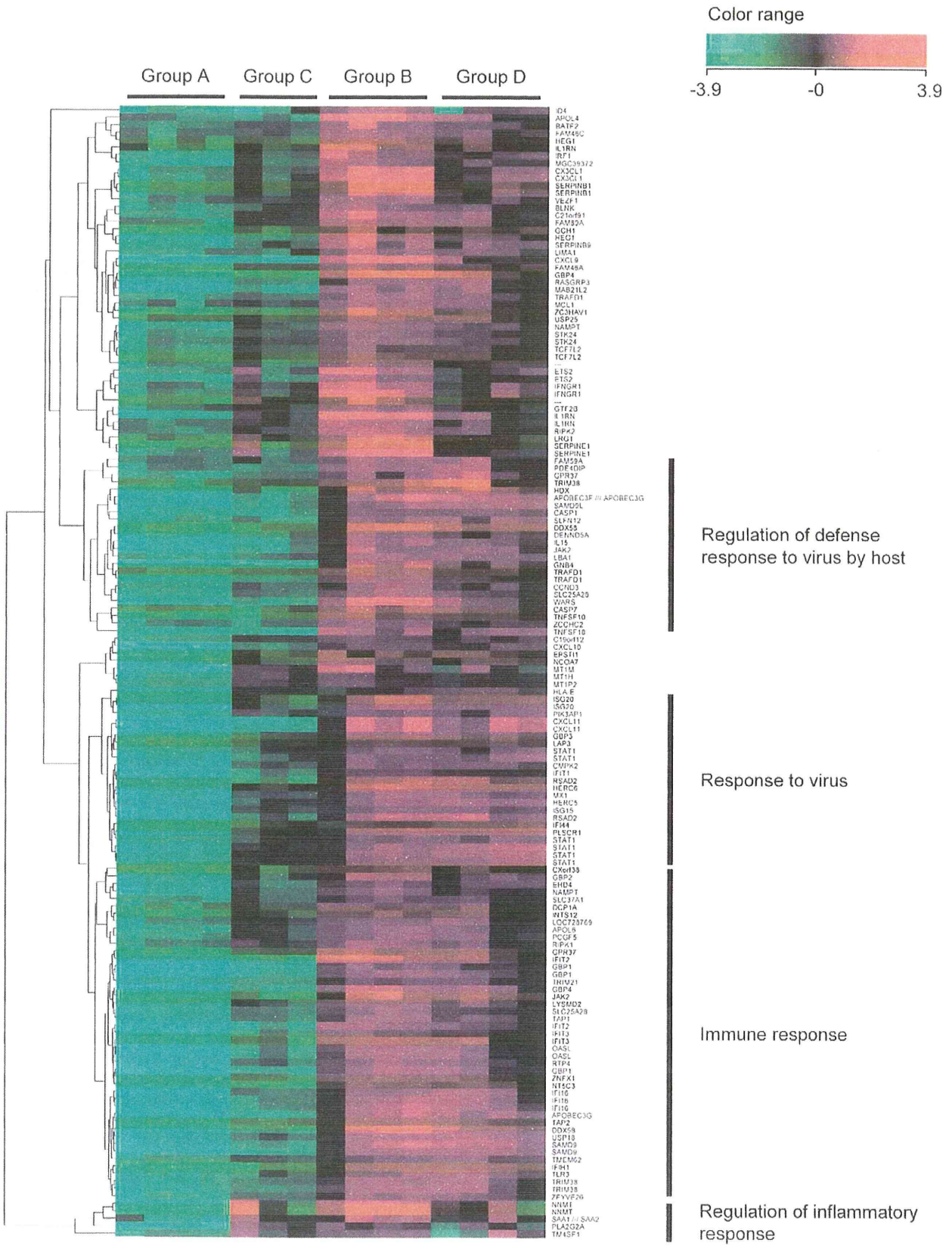
Color range
-3.9
-0
3.9
Group A
Group C
Group B
Group D
Regulation of defense response to virus by host
Response to virus
Immune response
Regulation of inflammatory response

Figure 5. Hierarchical clustering analysis of 152 genes associated with IFN administration with or without HCV infection. To analyze the effect of HCV infection on IFN response, gene expression ratios between Groups A and B (gene expression changes by IFN without HCV infection) and those between Groups C and D (gene expression changes by IFN with HCV infection) were compared in the 152 IFN-induced genes. 69.7% of the selected genes showed reduced IFN responsiveness following HCV infection.
doi:10.1371/journal.pone.0023856.g005

signaling were the most strongly up-regulated following HCV infection (Figure 3). These findings are mostly consistent with previous studies [36,37]. On the other hand, while no genes involved in lipid metabolism showed any significant induction by HCV infection in this study, Walters et al. reported that HCV-infected chimeric mice exhibited host-specific induction in the expression of lipid metabolism genes [35]. However, we used hepatocytes from a single donor, whereas Walters et al. used hepatocytes from multiple donors, so our results are not necessarily inconsistent with their findings that HCV infection causes induction of lipid metabolism genes in a host-specific manner.

Although several cDNA microarray analyses have also been performed using human liver tissues obtained after hepatic resection, the largest difference between human and chimeric mouse livers is the presence or absence of human lymphocytes. According to the previous report using human liver tissues, genes involved in the innate immune response, as well as cell cycle, growth and communication, were up-regulated by HCV infection [38]. In the present study using SCID-derived mice, genes involved in immune response (e.g. *OAS2*, *Mx1*, *IFI27* and *IFI44L*), cell cycle and growth (e.g. *HERC5*) and cell communication (e.g. *HLA-B*) were similarly up-regulated by HCV infection. However, *Apolipoprotein L*, *Cold autoinflammatory syndrome 1*, *CD97 antigen*, and *HLA-DQ*, which are mainly expressed in lymphocytes, were not observed to be up-regulated by HCV infection in the chimeric mice. These results demonstrate that the chimeric mouse model accurately reflects intracellular responses to HCV infection without the lymphocytic immune response.

Table 6. The top 20 genes in which IFN-induced up-regulation is inhibited following HCV infection.

Probe Set ID	Gene symbol	Fold change		P value
		HCV infection (-)	HCV infection (+)	
235175_at	GBP4	44.94	5.50	2.93E-07
231577_s_at	GBP1	24.60	4.89	6.15E-07
218943_s_at	DDX58	32.19	5.56	1.26E-05
226702_at	CMPK2	12.50	2.13	2.35E-05
225973_at	TAP2	7.07	2.84	3.31E-05
229450_at	IFIT3	20.66	2.74	6.37E-05
217739_s_at	NAMPT	5.91	1.95	6.51E-05
213797_at	RSAD2	69.70	4.50	7.01E-05
210797_s_at	OASL	31.59	3.53	1.02E-04
218508_at	DCP1A	3.56	1.65	1.36E-04
228531_at	SAMD9	18.65	5.87	1.45E-04
204804_at	TRIM21	5.37	2.69	1.47E-04
219209_at	IFIH1	10.05	4.69	1.67E-04
219684_at	RTP4	16.38	2.55	1.98E-04
239186_at	MGC39372	7.61	2.57	2.27E-04
219211_at	USP18	8.72	3.74	2.40E-04
225076_s_at	ZNFX1	10.40	2.91	2.91E-04
204698_at	ISG20	31.29	3.33	3.00E-04
223192_at	SLC25A28	5.05	2.20	3.25E-04
228439_at	BATF2	4.59	1.83	3.28E-04

doi:10.1371/journal.pone.0023856.t006

To verify the microarray results, expression data were compared with previously published microarray data on the GEO website (http://www.ncbi.nlm.nih.gov/geo/). Previously published microarray data showed up-regulation of *IGFBP7*, *IFI27*, *HLA-B*, and *CD74* in HCV-infected liver tissues compared to non-infected liver tissues (fold changes were 2.1, 2.2, 2.1 and 2.3, respectively) [39]. Likewise, we found that *IFI27*, *HLA-B*, and *CD74* were up-regulated following HCV infection (fold changes were 3.6, 3.3, and 6.6, respectively). These three genes are associated with MHC class I activity, suggesting that intra-cellular immunity in human hepatocytes was activated following HCV infection both in human subjects and in chimeric mouse livers. Metallothionein 1G (*MT1G*) expression was also found to be up-regulated by HCV infection in both the current and published studies [39,40]. Although metallothionein isoforms are associated with collagen deposition [41], members of the metallothionein family may be up-regulated and induce liver fibrosis in response to HCV infection.

In this study, genes associated with Organismal Injury and Abnormalities were found to be up-regulated in response HCV infection (Table 3), and some genes in this category, such as *CXCL9*, *CXCL10* and *IFIT3*, maintained high IFN responsiveness under HCV infection (Table 8). These results suggest that protective responses to fibrosis or hepatic injury were activated at the start of HCV infection and remained activated until complete eradication of HCV from hepatocytes was achieved.

Secondly, we compared gene expression profiles between groups A (without IFN treatment) and B (with IFN treatment) to evaluate IFN response without HCV infection. IFN-α stimulates the intracellular IFN-signaling cascade after binding to the IFN-α receptor and mediates the transcriptional activation of IFN-stimulated genes [42–47]. More than 3.0-fold up-regulation was observed 6hrs after IFN treatment in 152 genes. Known ISGs such as those in the *CXCL* family (*CXCL9*, *CXCL10* and *CXCL11*), the *IFIT* family (*IFIT2* and *IFIT3*) and the *APOBEC* family (*APOBEC3G*) were included among the top 20 genes up-regulated following IFN treatment (Table 4). The *APOBEC* family is well known to have anti-viral effects by inducing genomic hypermutation in human immunodeficiency virus and hepatitis B virus [48–57]. *APOBEC3G* expression has been reported to be elevated in patients infected with HCV [58], although it is not clear whether *APOBEC3G* can block HCV replication. On the other hand, *CXCL9* and *IFIT3* were reported to relate to liver fibrosis in chronic hepatitis C patients. Serum *CXCL9* concentrations correlated with the levels of fibrosis in chronic hepatitis C patients, and *CXCL9* has been shown to exert anti-fibrotic effects *in vitro* and *in vivo* [59]. *IFIT3* expression is also reportedly up-regulated in the transition from mild to moderate fibrosis [60]. The results of this study suggest that IFN treatment might lead not only to HCV eradication but also help to prevent and repair liver fibrosis by inducing these key molecules.

We focused on the 152 genes up-regulated (> 3.0 fold) as a result of IFN administration and evaluated the effect of HCV infection on IFN response among these genes. As shown in Table 8, although several ISGs still showed high response to IFN