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.

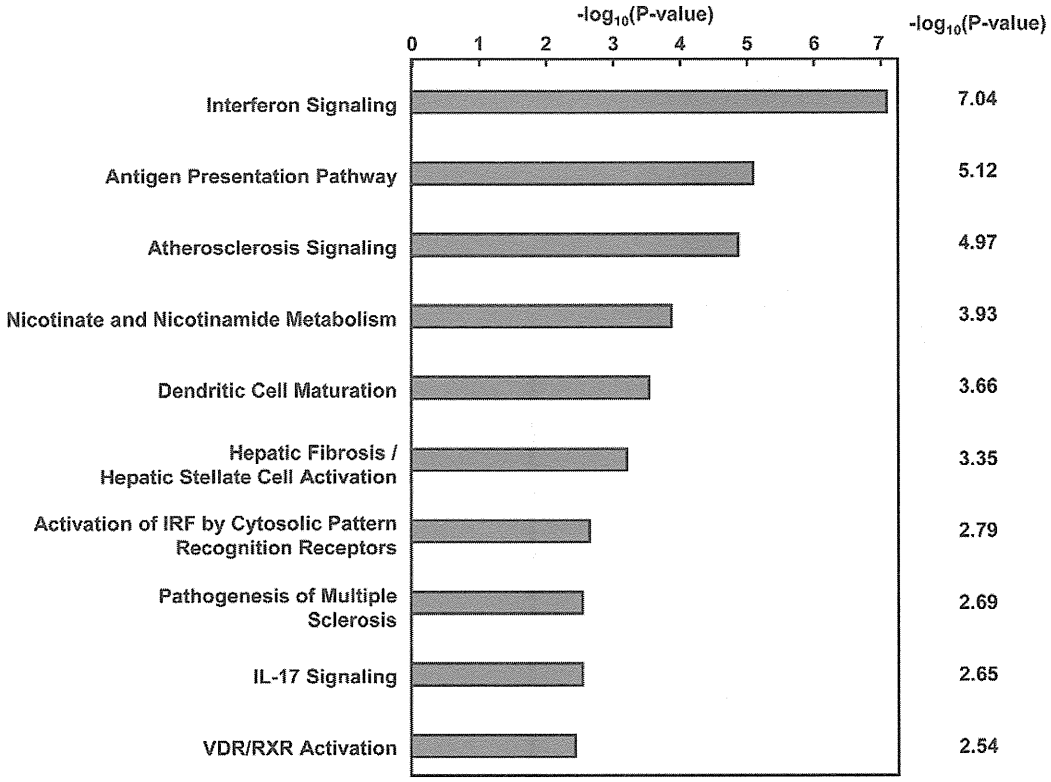


Figure 3. The effects of HCV infection on canonical pathways. To analyze the effects of HCV infection on canonical pathways, pathway analysis was performed using the 181 genes identified to be significantly up- or down-regulated following HCV infection. The IFN signaling pathway was the most significantly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

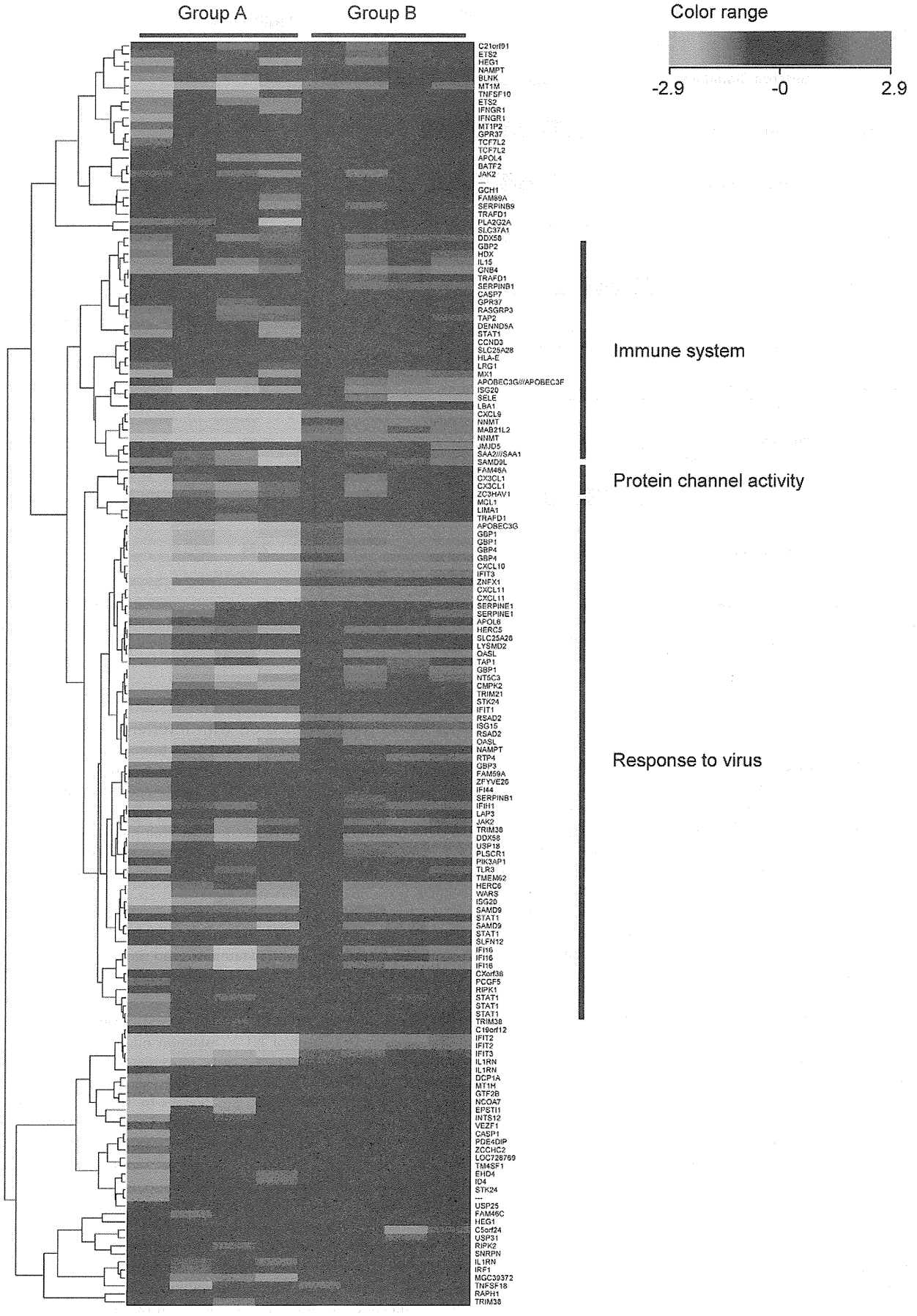
It is well known that chronic HCV infection triggers multiple biological responses. To analyze biological significance and regulatory pathways involved in the changes observed, we performed network analysis with the 181 genes using Ingenuity™ Pathway Analysis (IPA). As shown in Table 3, most of the 181 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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C21orf91
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BLNK
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IFNGR1
IFNGR1
MT1P2
GPR37
TCF7L2
TCF7L2
APOL4
BATF2
JAK2

GCH1
FAM89A
SERPINB9
TRAFD1
PLA2G2A
SLC37A1
DDX58
GBP2
HDX
IL15
GNB4
TRAFD1
SERPINB1
CASP7
GPR37
RASGRP3
TAP2
DENND5A
STAT1
CCND3
SLC25A28
HLA-E
LRG1
MX1
APOBEC3G///APOBEC3F
ISG20
SELE
LBA1
CXCL9
NNMT
MAB21L2
NNMT
JMJD5
SAA2///SAA1
SAMD9L
FAM46A
CX3CL1
CX3CL1
ZC3HAV1
MCL1
LIMA1
TRAFD1
APOBEC3G
GBP1
GBP1
GBP4
GBP4
CXCL10
IFIT3
ZNFX1
CXCL11
CXCL11
SERPINE1
SERPINE1
APOL6
HERC5
SLC25A28
LYSMD2
OASL
TAP1
GBP1
NT5C3
CMPK2
TRIM21
STK24
IFIT1
RSAD2
ISG15
RSAD2
OASL
NAMPT
RTP4
GBP3
FAM59A
ZFYVE26
IFI44
SERPINB1
IFIH1
LAP3
JAK2
TRIM38
DDX58
USP18
PLSCR1
PIK3AP1
TLR3
TMEM62
HERC6
IARS
ISG20
SAMD9
STAT1
SAMD9
STAT1
SLFN12
IFI16
IFI16
IFI16
CXorf38
PCGF5
RIPK1
STAT1
STAT1
STAT1
TRIM38
C19orf12
IFIT2
IFIT2
IFIT3
IL1RN
IL1RN
DCP1A
MT1H
GTF2B
NCOA7
EPSTI1
INTS12
VEZF1
CASP1
PDE4DIP
ZCCHC2
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USP25
FAM46C
HEG1
C5orf24
USP31
RIPK2
SNRPN
IL1RN
IRF1
MGC39372
TNFSF18
RAPH1
TRIM38
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 ($P = 5.22 \times 10^{-10} \sim 1.95 \times 10^{-2}$). 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

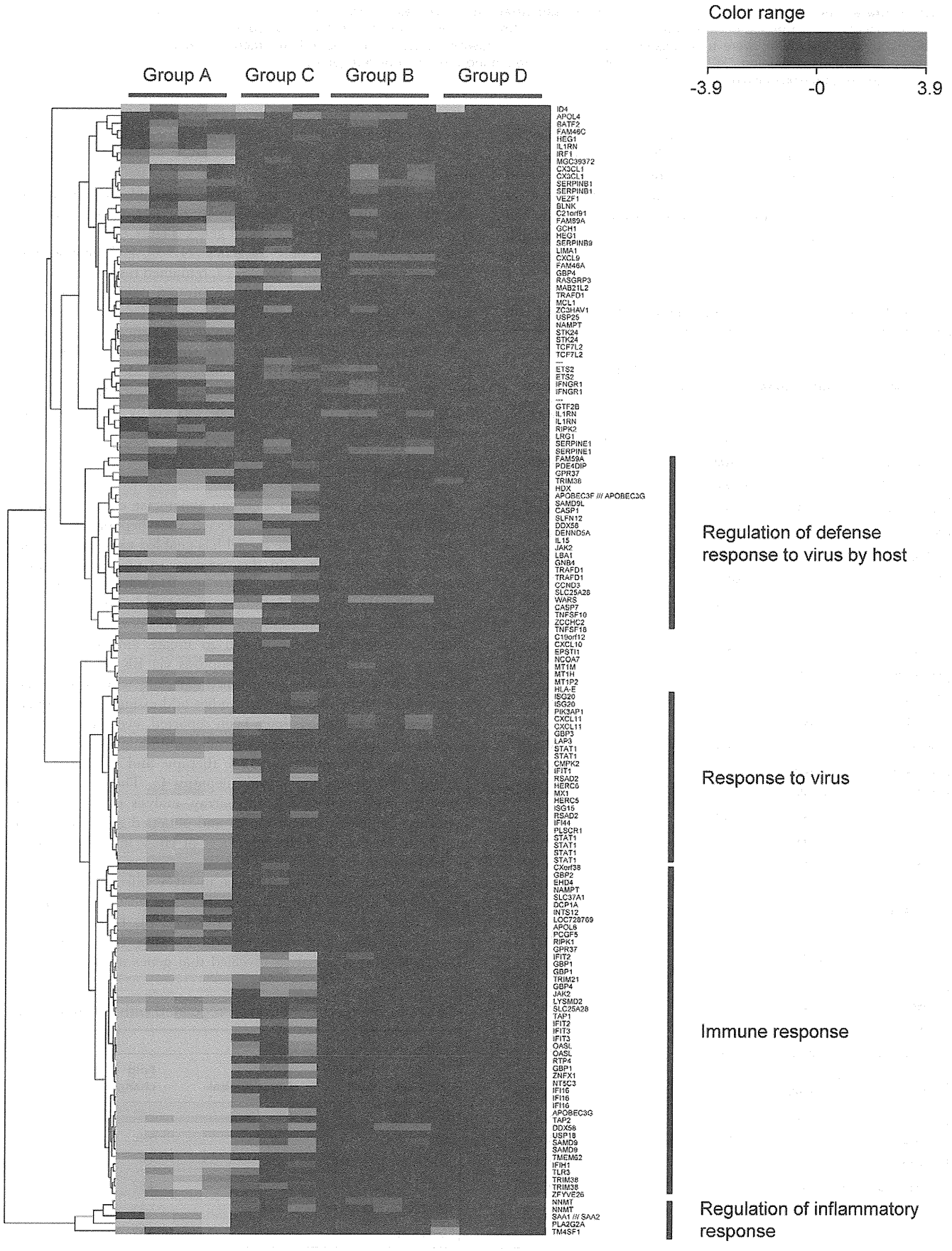
We previously developed a human hepatocyte chimeric mouse model that can be chronically infected with hepatitis B and C viruses [29–31]. This mouse model has enabled us to analyze the effect of viral infection and the response to medication under immunodeficient conditions. Microarray analyses using 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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-0
3.9
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Group C
Group B
Group D
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APOL4
BATF2
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HEG1
IL1RN
IRF1
MGC39372
CX3CL1
CX3CL1
SERPINB1
SERPINB1
VEZF1
BLNK
C21orf91
FAM89A
GCH1
HEG1
SERPINB9
LIMA1
CXCL9
FAM46A
GBP4
RASGRP3
MAB21L2
TRAFD1
MCL1
ZC3HAV1
USP25
NAMPT
STK24
STK24
TCF7L2
TCF7L2

ETS2
ETS2
IFNGR1
IFNGR1

GTF2B
IL1RN
IL1RN
RIPK2
LRG1
SERPINE1
SERPINE1
FAM59A
PDE4DIP
GPR37
TRIM38
HDX
APOBEC3F /// APOBEC3G
SAMD9L
CASP1
SLFN12
DDX58
DENND5A
IL15
JAK2
LBA1
GNB4
TRAFD1
TRAFD1
CCND3
SLC25A28
WARS
CASP7
TNFSF10
ZCCHC2
TNFSF18
C19orf12
CXCL10
EPSTI1
NCOA7
MT1M
MT1H
MT1P2
HLA-E
ISG20
ISG20
PIK3AP1
CXCL11
CXCL11
GBP3
LAP3
STAT1
STAT1
CMPK2
IFIT1
RSAD2
HERC6
MX1
HERC5
ISG15
RSAD2
IFI44
PLSCR1
STAT1
STAT1
STAT1
STAT1
CXorf38
GBP2
EHD4
NAMPT
SLC37A1
DCP1A
INTS12
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APOL6
PCGF5
RIPK1
GPR37
IFIT2
GBP1
GBP1
TRIM21
GBP4
JAK2
LYSMD2
SLC25A28
TAP1
IFIT2
IFIT3
IFIT3
OASL
OASL
RTP4
GBP1
ZNFX1
NT5C3
IFI16
IFI16
IFI16
APOBEC3G
TAP2
DDX58
USP18
SAMD9
SAMD9
TMEM62
IFIH1
TLR3
TRIM38
TRIM38
ZFYVE26
NNMT
NNMT
SAA1 /// SAA2
PLA2G2A
TM4SF1
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

Table 7. The top 5 canonical pathways associated with the 106 genes in which IFN response is suppressed by HCV infection.

Category	P value	Ratio	Associated genes
Interferon Signaling	4.41E-11	7/30 genes	IFIT1, IFIT3, IFNGR1, IRF1, MX1, STAT1, TAP1
Type I Diabetes Mellitus Signaling	1.08E-04	5/119 genes	HLA-E, IFNGR1, IRF1, RIPK1, STAT1
Antigen Presentation Pathway	5.29E-04	3/39 genes	HLA-E, TAP1, TAP2
Primary Immunodeficiency Signaling	1.64E-03	3/63 genes	BLNK, TAP1, TAP2
Activation of IRF by Cytosolic Pattern Recognition Receptors	2.95E-03	3/73 genes	ISG15, RIPK1, STAT1

doi:10.1371/journal.pone.0023856.t007

treatment in the presence of HCV infection, 7 genes in the IFN Signaling pathway became unresponsive (Table 6). Reduction in IFN responsiveness was also observed for *STAT1* (4.27 fold in the absence of HCV to 2.29 fold in the presence of HCV, $P = 4.04 \times 10^{-4}$), as well as 5 of 7 genes downstream of *STAT1* (*IFIT1*, *IFIT3*, *IRF1*, *MX1*, and *TAP1*). As shown in Figure 3, IFN

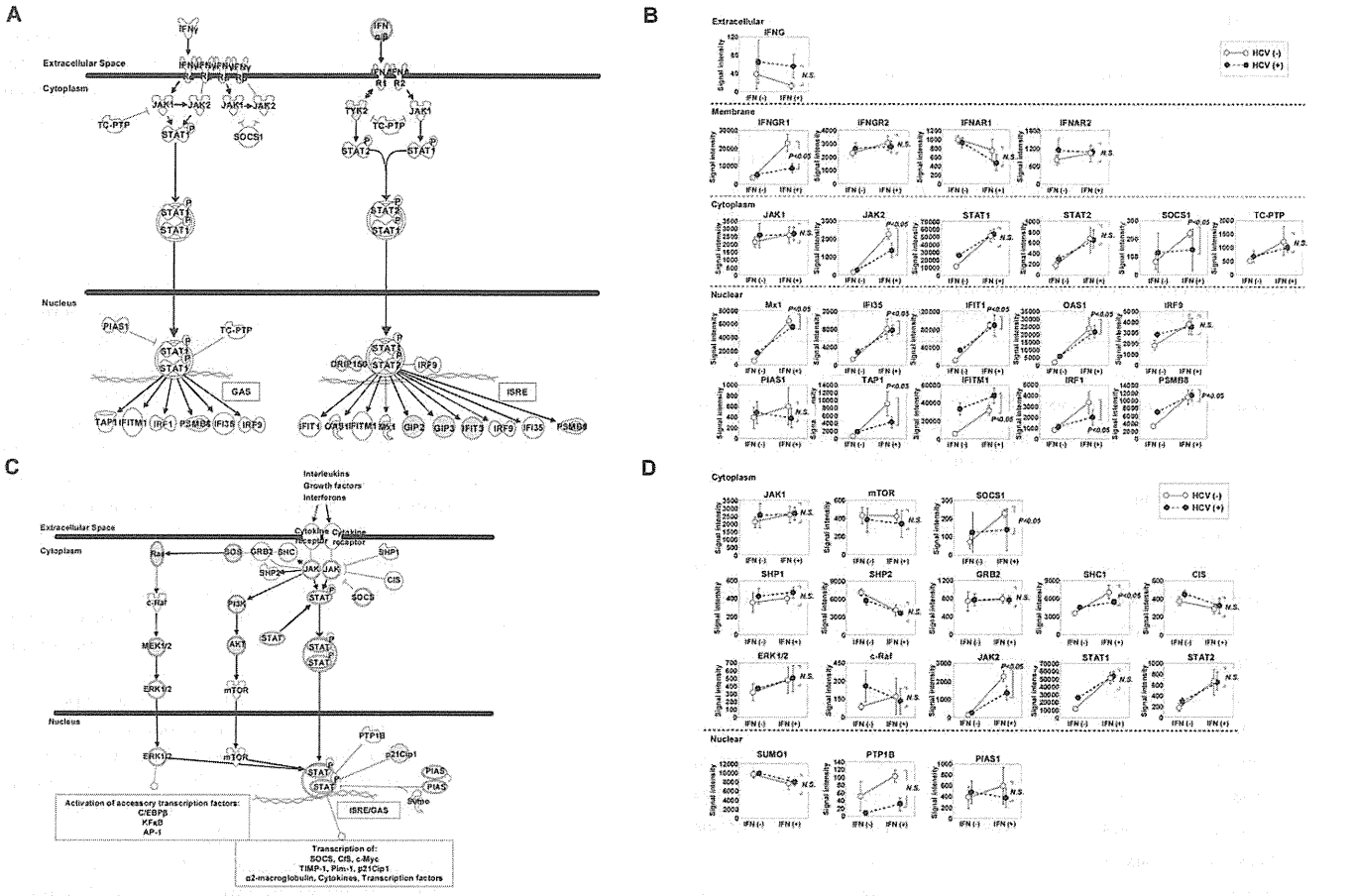

Figure 6. Changes in expression of genes in the IFN and JAK-STAT signaling pathways under HCV infection and/or IFN administration. A) An overview of the IFN signaling pathway consisting of 26 representative genes is shown. Genes illustrated as gray shapes were not included in this study. B) Relative expression levels of genes with/without HCV infection and/or IFN administration were plotted (closed dots: with HCV infection; open dots: without HCV infection) using microarray data. The slopes of the dashed and solid lines represent IFN responsiveness with and without HCV infection, respectively. In 21 of the 22 examined genes in the IFN signaling pathway, signal intensities increased and IFN responsiveness was repressed following HCV infection. Student's *t*-test was used for statistical analysis. C) An overview of the JAK-STAT signaling pathway consisting of 22 representative gene products is shown. Genes illustrated as gray shapes were not included in this study. D) Relative expression levels of genes with/without HCV infection and/or IFN administration were plotted using microarray data (closed dots: with HCV infection; open dots: without HCV infection). Signal intensities increased following HCV infection in 16 of 22 genes in the JAK-STAT signaling pathway, and IFN response was suppressed in 9 genes. Statistical analysis was performed using Student's *t*-test.
doi:10.1371/journal.pone.0023856.g006

Table 8. The 33 genes that remained more than 3-fold up-regulated following IFN treatment in HCV-infected mice.

ID	Symbol	Location	Type(s)
214995_s_at	APOBEC3F	unknown	enzyme
204205_at	APOBEC3G	Nucleus	enzyme
206011_at	CASP1	Cytoplasm	peptidase
204533_at	CXCL10	Extracellular Space	cytokine
210163_at	CXCL11	Extracellular Space	cytokine
203915_at	CXCL9	Extracellular Space	cytokine
218943_s_at	DDX58	Cytoplasm	enzyme
231577_s_at	GBP1 (includes EG:2633)	Cytoplasm	enzyme
235175_at	GBP4 (includes EG:115361)	Cytoplasm	enzyme
225710_at	GNB4	Plasma Membrane	enzyme
1553646_at	HDX	unknown	other
213069_at	HEG1	unknown	other
206332_s_at	IFI16	Nucleus	transcription regulator
219209_at	IFIH1	Nucleus	enzyme
217502_at	IFIT2	unknown	other
204747_at	IFIT3	Cytoplasm	other
205992_s_at	IL15	Extracellular Space	cytokine
204698_at	ISG20	Nucleus	enzyme
205841_at	JAK2	Cytoplasm	kinase
210302_s_at	MAB21L2	unknown	other
223298_s_at	NT5C3	Cytoplasm	phosphatase
205660_at	OASL	unknown	enzyme
205801_s_at	RASGRP3	Cytoplasm	other
242625_at	RSAD2	unknown	enzyme
228531_at	SAMD9	unknown	other
230036_at	SAMD9L	unknown	other
219885_at	SLFN12	Nucleus	enzyme
206271_at	TLR3	Plasma Membrane	transmembrane receptor
214329_x_at	TNFSF10	Extracellular Space	cytokine
221371_at	TNFSF18	Extracellular Space	cytokine
203610_s_at	TRIM38	unknown	other
219211_at	USP18	Cytoplasm	peptidase
200629_at	WARS	Cytoplasm	enzyme

doi:10.1371/journal.pone.0023856.t008

signaling was activated in the presence of HCV, and the expression of *STAT1* was more than 3.0 fold up-regulated by HCV infection (data not shown). *STAT1* expression was highest in mice with both HCV infection and IFN treatment, but downstream genes such as *MX1*, *IFIT1* and *IFIT3* showed reduced IFN response. Sarasin-Filipowicz et al. reported that IFN-induced *STAT1* phosphorylation was stronger in rapid responders than in non-rapid responders [61]. Reduced induction of genes downstream of *STAT1* by IFN under HCV infection might reflect reduced phosphorylation of *STAT1*, although we did not quantify *STAT1* phosphorylation in this study.

Recently, an *IL-28B* genetic polymorphism strongly associated with response to IFN-α plus ribavirin combination therapy [12], as well as with hepatic ISG expression [62], was identified. Further studies using chimeric mice transplanted with hepatocytes carrying different genotypes of candidate genes such as *IL-28B* will be important in order to elucidate possible mechanisms underlying host-specific responses.

In conclusion, we performed cDNA microarray analysis using HCV-infected human hepatocyte chimeric mice, which allowed us to analyze the direct effects of IFN treatment and HCV infection without the confounding effects of the lymphocytic immunological response. These results might provide molecular insights into possible mechanisms used by HCV to evade IFN-induced immune responses, as well as suggest novel therapeutic targets and a potential new indication for interferon therapy. Further analysis of the genes identified in our study would be worthwhile in order to improve efficacy of the therapy for chronic hepatitis C.

Acknowledgments

This study was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University and the Analysis Center of Life Science, Hiroshima University. The authors thank Rie Akiyama and Ruri Mikami for their excellent technical assistance, and Aya Furukawa for clerical assistance.

Author Contributions

Conceived and designed the experiments: MT YF NH MI ST KC. Performed the experiments: MT YF NH FM TT. Analyzed the data: MT YF YZ MO TK HA DM. Contributed reagents/materials/analysis tools: MT NH MI FM TT KC. Wrote the paper: MT YF ST HO CNH KC.

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Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2010.06470.x

MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Animal model for study of human hepatitis viruses

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Key words
hepatitis B virus, hepatitis C virus, uPA/scid mouse model.

Accepted for publication 26 July 2010.

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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

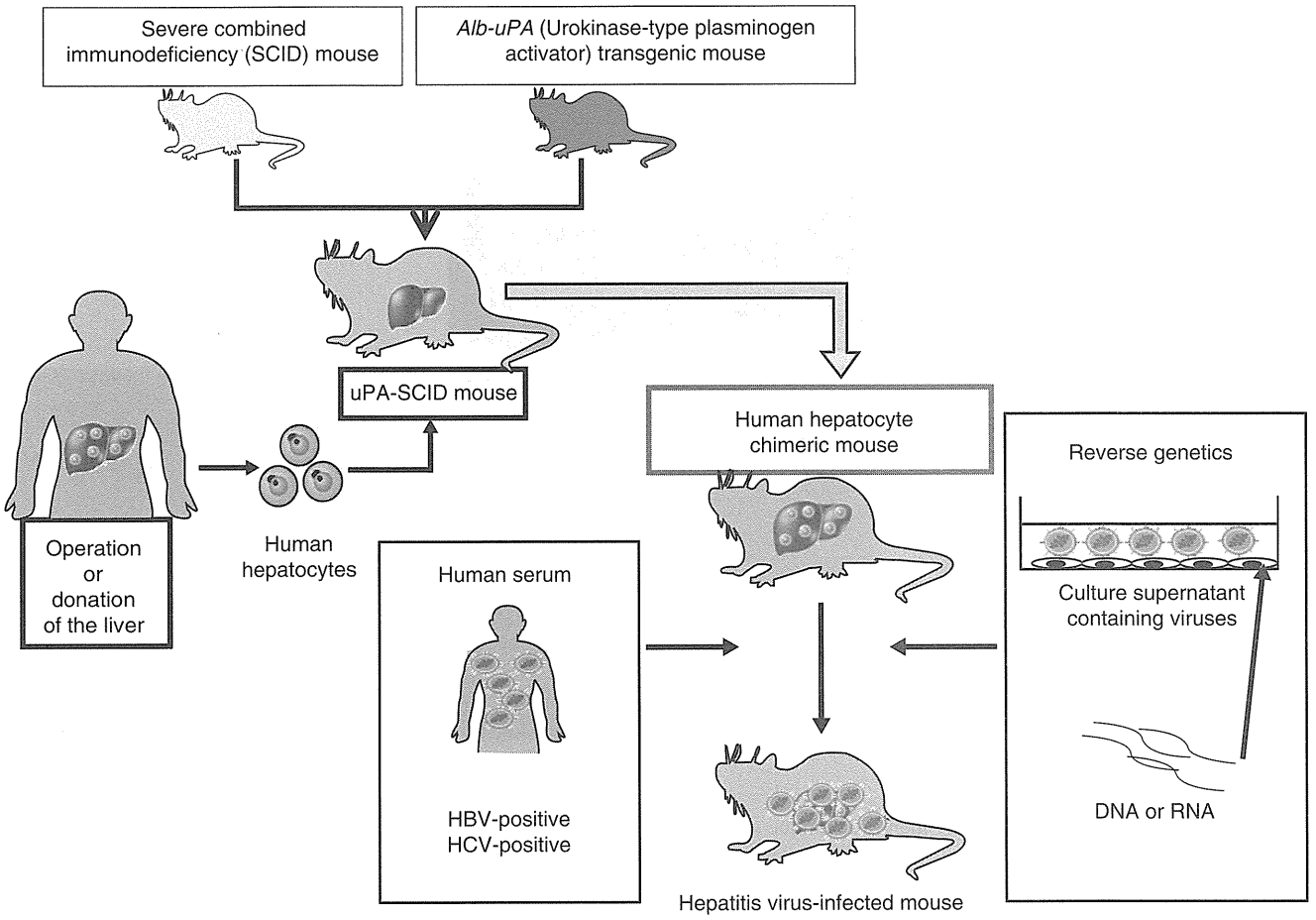

Figure 1 Generation of human hepatocyte chimeric mice and hepatitis virus infection model. A uPA/scid mouse was created by mating uPA transgenic mouse and scid mouse. Human hepatocytes obtained by surgical resection or donation were transplanted to newborn mice. The chimeric mice can be infected with 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.

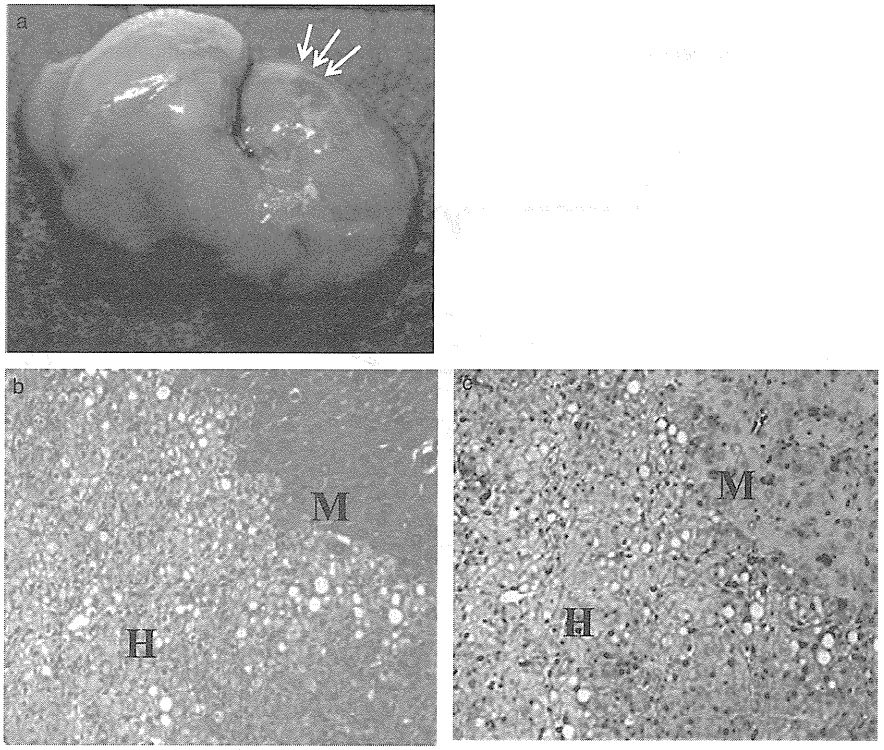

Figure 2 Representative uPA/scid mouse livers repopulated by human hepatocytes. (a) Mouse liver almost completely repopulated by human hepatocytes. Only a small portion of mouse hepatocytes are shown by arrows. (b) Microscopic figure of the mouse liver. M and H indicate regions consisting of mouse and 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]

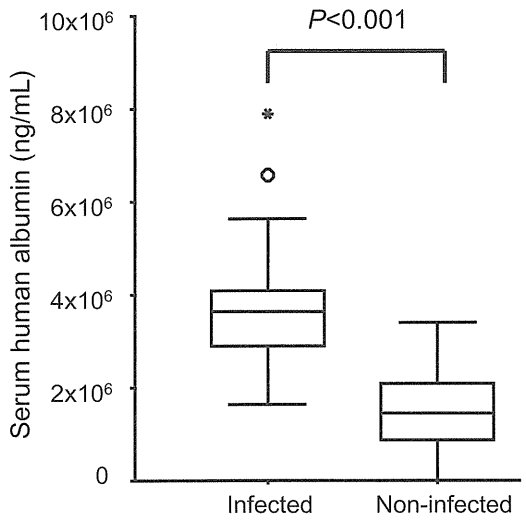

Figure 3 Human albumin levels in mice used in the hepatitis C virus (HCV) infection experiments. A total of 54 mice were injected with HCV positive serum samples containing 5×10^5 virus particles. A total of 24 mice became persistently positive for HCV-RNA, but 30 mice did not. Serum human albumin levels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

Acknowledgments

This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare, Government of Japan.

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Variation in the *DEPDC5* locus is associated with progression to hepatocellular carcinoma in chronic hepatitis C virus carriers

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Chronic viral hepatitis is the most important risk factor for progression to hepatocellular carcinoma (HCC). To identify genetic risk factors for progression to HCC in individuals with chronic hepatitis C virus (HCV), we analyzed 467,538 SNPs in 212 Japanese individuals with chronic HCV with HCC and 765 individuals with chronic HCV without HCC. We identified one intronic SNP in the *DEPDC5* locus on chromosome 22 associated with HCC risk and confirmed the association using an independent case-control population (710 cases and 1,625 controls). The association was highly significant when we analyzed the stages separately as well as together (rs1012068, $P_{combined} = 1.27 \times 10^{-13}$, odds ratio = 1.75). The significance level of the association further increased after adjustment for gender, age and platelet count ($P = 1.35 \times 10^{-14}$, odds ratio = 1.96). Our findings suggest that common variants within the *DEPDC5* locus affect susceptibility to HCC in Japanese individuals with chronic HCV infection.

HCC is the third leading cancer-related cause of death and the seventh most common form of cancer worldwide[1]. In many western countries and Japan, HCV infection is the most common risk factor for HCC[2,3]. Chronic hepatitis caused by HCV often leads to fibrosis and cirrhosis (stage F4 fibrosis), which markedly increase the risk of developing HCC. The annual incidence of HCC correlates with the severity of liver fibrosis, from 0.5% among individuals with stage F0 or F1 fibrosis to 7.9% among individuals with stage F4 fibrosis[4]. Recently, age at initial diagnosis of HCV-related HCC has been increasing in Japan, and most affected individuals are diagnosed at age 55 or older[5–8]. To date, many studies have examined individuals with HCV and identified several predictive factors for HCC in addition to fibrosis and age, including male gender, alcohol consumption, diabetes mellitus, obesity, ethnicity and co-infection with hepatitis B virus (HBV)[1,5,7,9]. In spite of recent progress in anti-HCV therapy, it remains difficult to achieve complete eradication of the virus[10]. Particularly among individuals with HCV who are unable to clear the virus, screening of any SNPs associated with susceptibility to HCC may help improve prognosis and target surveillance efforts more efficiently to high-risk individuals. Researchers from another study[11] recently identified a SNP within the *KIF1B* locus associated with progression to HCC among chronic HBV carriers; however, the virological effects of HBV and HCV are entirely different[12], and so far, SNPs associated with risk of HCC among individuals with chronic HCV have not been fully investigated. To identify genetic markers associated with risk of HCV-related HCC development in the Japanese population, we conducted a two-phase case-control study consisting of a genome-wide association study (GWAS) and a replication study using a total of 3,312 Japanese individuals over the age of 55 with chronic HCV infection. In the GWAS phase, we performed SNP genotyping using the Illumina HumanHap610-Quad BeadChip. We analyzed 467,538 SNPs that passed quality control filters using an additive model for genotype-phenotype association in 212 chronic HCV carriers with HCC (cases) and 765 chronic HCV carriers without HCC (controls). Principal component analysis revealed no population substructure in our population. In addition, a quantile-quantile plot using the results of the Cochran-Armitage trend test showed that the inflation factor, λ, was 1.00, indicating a low probability of false-positive associations resulting from population stratification (**Supplementary Fig. 1a**). Using the additive model, one intronic SNP, rs1012068, within isoform 1 of the *DEPDC5* locus on chromosome 22, showed strong association with HCC ($P = 8.05 \times 10^{-8}$) with odds ratio (OR) = 2.20 (95% confidence interval (CI) 1.64–2.97) (**Table 1**). We also found that rs1012068 showed a statistically significant association

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Received 4 January; accepted 7 June; published online 3 July 2011; doi:10.1038/ng.876

Table 1 Summary of GWAS and replication study

SNP	Gene	Study	Allele (1/2)	Case 11	Case 12	Case 22	Control 11	Control 12	Control 22	MAF Case	MAF Control	OR	95% CI[a]	P[b]	P^c_{het}
rs1012068	*DEPDC5*	GWAS	T/G	138	68	6	624	136	5	0.189	0.095	2.20	1.64–2.97	8.05×10^{-8}	
		Replication		470	221	19	1262	334	29	0.182	0.121	1.63	1.37–1.93	2.41×10^{-8}	
		Combined studies[d]										1.75	1.51–2.03	1.27×10^{-13}	0.082

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.
[a]Odds ratios of risk allele from two-by-two allele frequency table. [b]P value of Cochran-Armitage trend test. [c]Result of Breslow-Day test. [d]Combined meta-analysis was performed using the Mantel-Haenszel method.

with HCV-related HCC after Bonferroni correction for multiple testing (calculated as $P < 0.05/467{,}538 = 1.07 \times 10^{-7}$)[13,14]. No other SNPs reached genome-wide significance (**Supplementary Table 1** and **Supplementary Fig. 1b**). As shown in **Table 1**, we next performed a replication study using 710 cases and 1,625 controls and again found that rs1012068 was strongly associated with HCC ($P = 2.41 \times 10^{-8}$, OR = 1.63). The association between rs1012068 and HCC remained highly significant when we combined results of the GWAS and replication sets using the Mantel-Haenszel method (combined $P = 1.27 \times 10^{-13}$, OR = 1.75). We observed no heterogeneity across the two studies (heterogeneity test $P = 0.082$).

On the other hand, platelet count is known to correlate significantly with the stage of liver fibrosis in individuals with HCV, and a platelet count of $<10 \times 10^4/\mu l$ has also been used as a marker for cirrhosis[4,15–19]. After adjusting for age, gender and platelet count using multiple logistic regression analysis, the significance level of rs1012068 increased ($P = 1.35 \times 10^{-14}$, OR = 1.96) (**Supplementary Table 2**). Other predictive factors for HCV-related HCC have been reported, including alcohol consumption, diabetes mellitus, obesity, ethnicity and co-infection with HBV[1,5,7,9]. As all subjects enrolled were of Japanese ethnicity, and there were no HBV co-infected subjects, the effect of the SNP was reevaluated using only 994 subjects (480 cases and 514 controls) for whom data was fully available for other factors (**Supplementary Table 3**). After adjusting for each of these six factors using multiple logistic regression analysis, rs1012068 remained highly significant with an OR = 1.87 (95% CI 1.39–2.52) (**Supplementary Table 4**). However, we cannot rule out the possibility that other confounding factors influenced the results. In addition to examining the effect of rs1012068 on HCC independently of other predictive factors, we performed stratified analysis using gender, age and platelet count (**Supplementary Table 5**). Notably, this SNP tended to show a greater effect in males (OR = 1.99 (95% CI 1.63–2.42)) than females (OR = 1.51 (95% CI 1.18–1.93)), as well as in elderly subjets (age ≥65 years: OR = 1.84 (95% CI 1.52–2.24) compared to age <65: OR = 1.73 (95% CI 1.36–2.19)) and in subjects with low platelet counts ($<10 \times 10^4/\mu l$: OR = 2.35 (95% CI 1.67–3.31) compared to $\geq 10 \times 10^4/\mu l$: OR = 1.71 (95% CI 1.42–2.05)). Each of these factors (male gender, older age and lower platelet count) are well known risk factors for HCV-related HCC. rs1012068 seems to more strongly affect individuals with multiple risk factors for HCC, but we detected no heterogeneity among subgroups stratified by gender, age and platelet count (heterogeneity test $P = 0.086$, $P = 0.675$ and $P = 0.103$, respectively, for each factor).

To examine whether rs1012068 genotypes are associated with any specific HCC phenotypes, we analyzed clinical phenotypes of cases with HCC with regard to rs1012068 genotype. We observed no differences between individuals with TT and TG+GG genotypes (**Supplementary Table 6**), but when we evaluated the case to control ratio of each 5-year age group with respect to rs1012068 genotype (TT compared to TG+GG), we found that the ratio was higher among subjects with the TG+GG genotype over all 5-year age ranges, and the slope of the ratio with increasing age was steeper among these individuals (**Supplementary Fig. 2**).

In order to explore the region around the landmark SNP rs1012068 in more detail, we performed fine mapping in the GWAS-stage subjects of a 350-kb genomic region between 22q12.2 and 22q12.3 upstream and downstream of the *DEPDC5* locus, including the neighboring genes *C22orf30* and *YWHAH* (**Fig. 1** and **Supplementary Fig. 3**). We successfully genotyped 43 tagging SNPs and identified another intronic SNP, rs5998152, located about 2.7 kb upstream of the landmark SNP (rs1012068) that is in strong linkage disequilibrium (LD) with rs1012068 ($r^2 = 0.99$). However, no SNPs showed stronger association than rs1012068 (**Supplementary Fig. 3** and **Supplementary Table 7**). To investigate the existence of any functional coding SNPs linked to rs1012068, we resequenced all 42 exons of *DEPDC5* using genomic DNA from 48 individuals

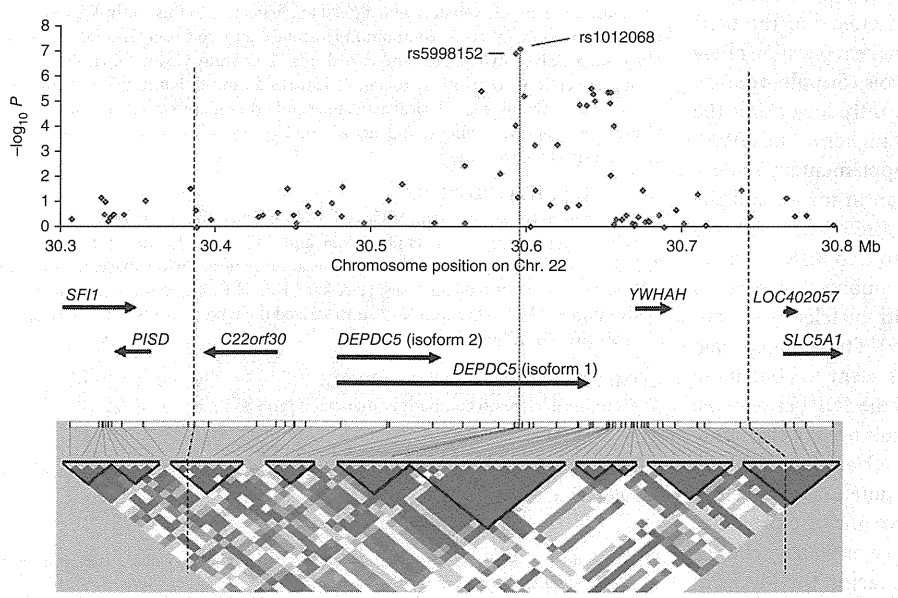

Figure 1 Case-control association plots and linkage disequilibrium (LD) map and genomic structure of the *DEPDC5* region in chromosome 22q12.2–3. The candidate region is indicated by two black dashed lines. We performed fine mapping in the region from 30.39–30.74 Mb. Blue diamonds represent $-\log_{10} P$ obtained from the GWAS and fine mapping using GWAS samples. We drew the LD map based on D' values using the genotype data of the cases and controls in the GWAS samples. The landmark SNP (rs1012068) is indicated by the red dotted line.

with HCV. We identified two new SNPs that were not registered in the dbSNP database (**Supplementary Table 8**). However, both SNPs had low minor allele frequencies (MAF) of 0.010 and were not linked to rs1012068 ($r^2 = 0.00$) nor were they significantly associated with HCC. We also performed haplotype analysis to investigate the effect of combinations of SNPs that were strongly associated with HCC susceptibility; however, no haplotype showed stronger association than the single-marker association of rs1012068 (**Supplementary Fig. 4**). Finally, rs1012068 had the strongest independent association with HCV-related HCC in our study.

Then we investigated the association between rs1012068 genotype and *DEPDC5* mRNA expression using paired tumor (HCC) and adjacent non-tumor liver tissues from 43 individuals with HCV. As shown in **Supplementary Figure 5**, real-time quantitative PCR assays revealed a significantly higher level of *DEPDC5* mRNA expression in tumor tissues than non-tumor tissues ($P = 0.025$), but we observed no significant difference with regard to rs1012068 genotype in tumor tissues as well as in non-tumor tissues ($P = 0.610$ and $P = 0.400$, respectively). On the other hand, we also evaluated *DEPDC5* expression using paired tumor and adjacent non-tumor tissues and calculated the tumor to non-tumor ratio as the *DEPDC5* expression level in tumor tissue divided by the expression level in paired non-tumor tissue from the same subject. As shown in **Supplementary Table 9**, we found that the frequency of the risk allele (G) was significantly higher in subjects with a tumor to non-tumor ratio ≥5 as well as a ratio of ≥1 in males ($P = 0.014$ and $P = 0.036$, respectively) but not in females (a ratio of ≥5: $P = 0.500$ and a ratio of ≥1: $P = 0.226$). This finding may suggest a differential effect of the SNP on *DEPDC5* expression due to gender. Although there is insufficient data to show a direct functional effect of rs1012068 on *DEPDC5* expression and HCV-related hepatocarcinogenesis, the data suggest a possible genetic association between a polymorphism within the *DEPDC5* locus and HCV-related HCC that requires further functional analysis.

In this study, we identified a common SNP associated with HCV-related HCC, and the effect of the SNP remained highly significant even after adjusting for other predictive factors. We observed no significant heterogeneity between the GWAS and replication studies, but the odds ratios for each study differed somewhat, and the 95% CIs for one phase of the study did not include the odds ratio for the other. In addition, the MAFs for the controls differed between the GWAS and replication phases (**Table 1**). We speculate that the differences between the two phases partially explain the different observed effects of the SNP. The female to male ratio was significantly higher in the replication phase than the GWAS phase for both cases and controls (**Supplementary Table 10**). The ages of the cases were significantly lower and the platelet counts of the controls were significantly higher in the GWAS phase than in the replication phase. As shown in **Supplementary Table 5**, rs1012068 showed a weaker risk in females than in males, and the frequency of the risk allele among older cases (≥65 years old) was lower than among younger cases (0.183 compared to 0.186, respectively). The risk allele frequency was also higher among controls with platelet count ≥10 ($\times 10^4/\mu l$) than in controls with platelet count <10 ($\times 10^4/\mu l$) (0.116 compared to 0.088, respectively). These unexpected differences between subjects in the two phases seem to contribute jointly to the observed differences in the effect of the SNP between the two phases. It is important to note that the controls used in this study were not healthy controls (MAF = 0.116 based on HapMap JPT data) but are chronic HCV carriers who still have the potential of developing HCC in the future, especially those who have one or more other strong predictive factors (for example, gender, age or platelet count). When we stratified samples by each predictive factor, the MAFs in the controls were varied (**Supplementary Table 5**). We speculate that after HCV infection becomes chronic, individuals with risk alleles may more easily develop HCC, and conversely, those without risk alleles are relatively less likely to progress to HCC (**Supplementary Fig. 2**), but these other risk factors influence the ultimate course of the disease. Consideration of the genetic background of subjects will likely play a role in personalized medicine, and understanding the mechanism underlying the association may suggest new therapeutic targets.

On the other hand, given the relatively small number of cases in the GWAS phase, we calculated the statistical power to detect an effect caused by rs1012068 to be only 50%, compared to the 80% recommended to detect an association of the expected effect size (**Supplementary Fig. 6**). It remains to be determined whether other SNPs influence susceptibility to HCV-related HCC in the Japanese population. The question also remains whether this susceptibility locus within *DEPDC5* is associated with HCV-related HCC in other ethnic groups, as allele frequencies of rs1012068 vary among ethnic groups, even among those with Asian ethnicities (**Supplementary Table 11**).

DEPDC5 has not been previously reported in association with HCC, but deletion of the region containing *DEPDC* has been reported in malignant brain glioblastomas[20]. Although the function of this gene is still unknown[21], it is noteworthy that DEPDC1, which contains a DEP domain similar to DEPDC5, has been reported to affect bladder carcinogenesis[22,23].

In summary, we conducted a GWAS followed by an independent replication study and fine mapping to detect polymorphisms associated with HCC in Japanese individuals with HCV. We report a common SNP within the *DEPDC5* locus associated with a twofold increased risk of HCC. Further research is required to determine the role of this gene in development of HCV-related HCC.

URLs. PLINK1.03, http://pngu.mgh.harvard.edu/~purcell/plink/; R statistical environment, http://www.cran.r-project.org/; EIGENSOFT, http://genepath.med.harvard.edu/~reich/Software.htm.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

The authors thank the subjects who agreed to participate in this study. We also thank the team members at Toranomon Hospital, Sapporo Kosei General Hospital, Hiroshima University Hospital and Hiroshima Liver Study Group for clinical sample collection. We thank T. Yokogi, Y. Hayashida and K. Izumoto for technical assistance, J. Sakamiya for clerical assistance and other members of the RIKEN Center for Genomic Medicine and Hiroshima University for assistance with various aspects of this study.

AUTHOR CONTRIBUTIONS

K.C. conceived the study. D.M., H.O. and K.C. designed the study. D.M. and H.O. performed genotyping. D.M., H.O., C.N.H. and K.C. wrote the manuscript. T.M., T.T., M.K. and N.K. performed data analysis at the genome-wide phase. H. Abe and T.Y. performed functional analyses. H. Aikata, K.I., H.K., J.T. and K.C. managed DNA samples. D.M., H.O. and K.C. summarized the whole results. Y.N., N.K. and K.C. obtained funding for the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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