

Figure 3 Antiviral effects of NS5A replication complex inhibitor combinations with either an NS3 protease inhibitor or an NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 1b. The four mice were treated with 10 mg/kg of BMS-788329 and either 75 mg/kg twice daily of BMS-605339 (A) or 100 mg/kg of BMS-821095 (B) for 4 weeks (closed circles). Mice without treatment were also analysed (open circles). Serum samples were obtained at the indicated times, and HCV RNA titres were measured. The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml). (C) Nested PCR of HCV RNA, human serum albumin and GAPDH in mouse livers. Livers from mice treated with BMS-788329 and either BMS-605339 (lane 2) or BMS-821095 (lane 3) were obtained. Mouse livers with (lane 1) or without (lane 4) HCV infection were also analysed.

DISCUSSION

DAA-only therapy may offer a promising option to eradicate HCV without incurring the severe side effects of Peg-alfa. However, the emergence of drug-resistant variants is expected for all DAA²¹ and has already been observed in combination therapies with two DAA.^{5 23 24} If the exposure of the drugs can be safely increased, as we recently reported for a two-drug combination administered to human hepatocyte chimeric mice,¹² eradication of virus is still possible. In this study, we tested the ability of different two-DAA combination therapies to eradicate HCV. Although DAA monotherapies resulted in a viral breakthrough due to the development of a high prevalence of drug-resistant variants (figure 2A–D), DAA combination therapies with the NS5A RCI and either the NS3 PI or NS5B NNI were shown to eradicate virus successfully from HCV genotype 1b-infected mice with only 4 weeks of treatment (figure 3). These two-DAA combination treatments resulted in more rapid, robust declines within the first week of treatment

when compared with the suboptimal antiviral responses from each of their respective monotherapies. Furthermore, regimens containing NS5A RCI appeared equally effective in treating mice chronically infected with hepatitis C genotype 1b.

In contrast to the rapid decrease in HCV RNA in mice infected with HCV genotype 1b, HCV genotype 2a and 2b-infected mice either did not respond or responded poorly to treatment with the NS5A RCI combined with either the NS3 PI or NS5B NNI (figure 4A,B). In this study, NS3 PI and NS5B NNI IC₅₀ values against genotype 1b were markedly more potent than against genotype 2a in cell culture systems (table 1). These findings are consistent with previous experimental results that reported reduced activity of these drug classes against genotype 2.^{25–28} In clinical trials, telaprevir monotherapy was found to result in a rapid decrease in serum HCV RNA levels in patients infected with HCV genotype 2; however, another protease inhibitor, BILN-2061, was less effective in patients with HCV genotype 2 compared to genotype 1.²⁹ Sequence analysis revealed a pre-existing A156G variant in the NS3 region, a L31M variant in the NS5A region and a I482L variant in the NS5B region in both HCV genotypes 2a and 2b infecting strains used in this study (data not shown). These NS3-A156G and NS5A-L31M variants confer resistance to inhibitors with similar chemical structures to BMS-605339 and BMS-788329, respectively, in genotype 2a replicon cell culture assays.^{30–32} Although BMS-788329 was very potent against the genotype-2a JFH-1 replicon (IC₅₀ 0.014 nM; table 1), its activity was significantly less against other genotype 2a and 2b viruses, such as genotype 2a HC-J6CF. The loss in potency observed in these viruses is not surprising because these viruses have a methionine at NS5A amino acid residue 31. The IC₅₀ of a genotype 2a hybrid replicon containing HC-J6CF NS5A with L31M substitution is approximately 10 nM (data not shown). The minimal antiviral response in mice infected with genotypes 2a and 2b receiving treatments containing BMS-788329 with either BMS-605339 or BMS-821095 can therefore be explained by pre-existing NS3, NS5A and NS5B resistance variants. Nevertheless, it is possible that mice infected with wild-type genotype 2 viruses and subsequently treated with higher doses of each of these DAA in dual or even triple combination therapy may have resulted in more robust reductions in viral load. The human hepatocyte chimeric mouse model offers a viable approach for identifying effective DAA-only combinations that not only act against HCV genotype 1 but against all HCV genotypes.

In summary, we demonstrated that an NS5A RCI can be effectively combined with different inhibitor classes to cure human hepatocyte chimeric mice infected with HCV genotype 1b after 4 weeks of treatment. However, these treatment combinations were not effective against HCV genotype 2. Oral combinations incorporating an NS5A RCI might offer Peg-alfa-free treatment options for genotype 1b chronic hepatitis C patients.

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Competing interests MG and FM are employees of Bristol-Myers Squibb. All other authors declare no competing interests.

Ethics approval The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care.

Patient consent Obtained.

Viral hepatitis

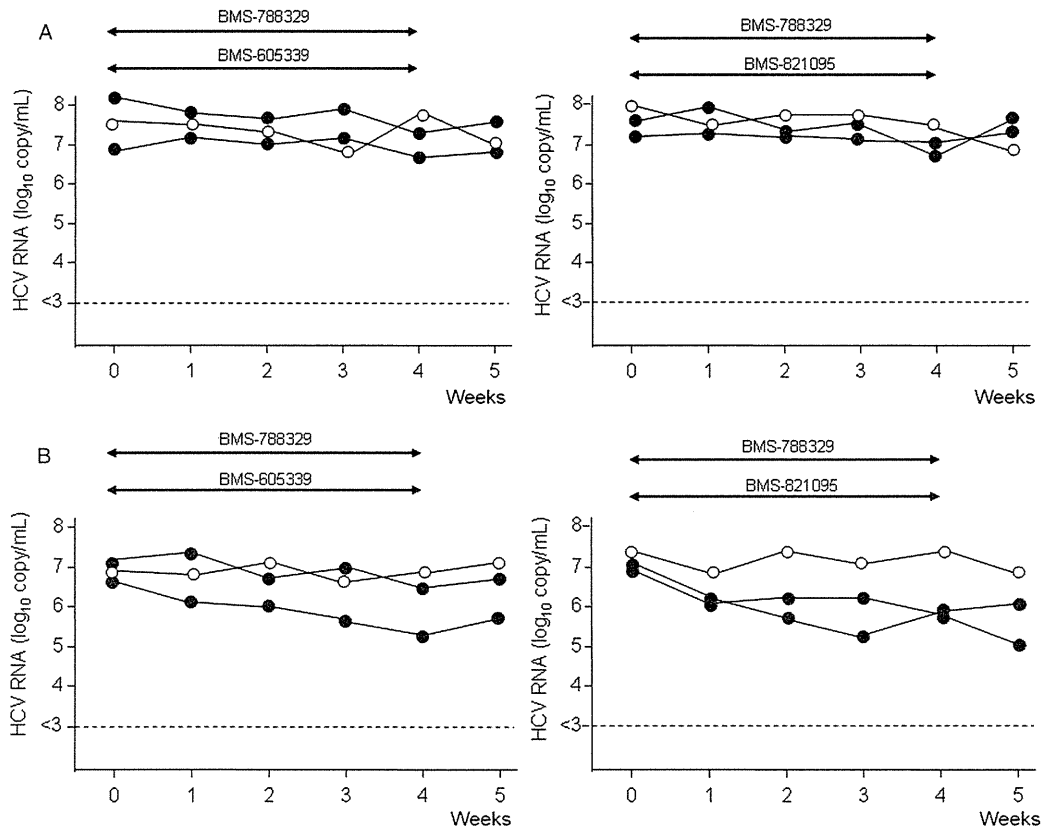


Figure 4 Antiviral effects of NS5A replication complex inhibitor combinations with either NS3 protease inhibitor or NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 2. Each of the four HCV genotype 2a (A) or 2b (B) infected mice were treated with 10 mg/kg of BMS-788329 combined with either 75 mg/kg twice daily of BMS-605339 (left panel) or 100 mg/kg of BMS-821095 (right panel) for 4 weeks (closed circles). Mice without treatment were also analysed (open circles). Serum samples were obtained at the indicated times, and HCV RNA titres were measured. The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml).

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Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice

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Impact of Viral Amino Acid Substitutions and Host Interleukin-28B Polymorphism on Replication and Susceptibility to Interferon of Hepatitis C Virus

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Amino acid (aa) substitutions of core 70 and 91 and in the NS5A (nonstructural protein 5A) interferon sensitivity determining region (ISDR) as well as genetic polymorphisms in the host interleukin-28B (IL28B) locus affect the outcome of interferon (IFN)-based therapies for patients with chronic hepatitis C. The combination of these factors and the quasi-species nature of the virus complicate understanding of the underlying mechanism. Using infectious hepatitis C virus (HCV) genotype 1b clone HCV-KT9, we introduced substitutions at both core aa70 (Arg to Gln) and aa91 (Leu to Met). We also introduced four and nine ISDR aa substitutions into core mutant HCV-KT9. Using human hepatocyte chimeric mice with different IL28B genotypes, we examined the infectivity, replication ability, and susceptibility to IFN of these clones. Although aa substitutions in the ISDR significantly impaired infectivity and replication ability of the virus, core aa70 and 91 substitutions did not. The effect of IFN treatment was similar in core wild-type and mutant viruses. Interestingly, virus titer was significantly higher in mice with the favorable IL28B allele (rs8099917 TT and rs12979860 CC) in the transplanted hepatocytes than in mice with hepatocytes from rs8099917 TG and rs12979860 TT donors ($P < 0.001$). However, the effect of IFN was significantly greater, and intrahepatic expression levels of IFN-stimulated genes were significantly higher in mice with the favorable IL28B allele. **Conclusion:** Our data suggest that HCV replication levels and response to IFN are affected by human hepatocyte IL28B single-nucleotide polymorphism genotype and mutations in the ISDR. The mechanism underlying the clinically observed association of wild-type core protein in eradication-favorable host cells should be investigated further. (HEPATOLOGY 2011;54:764-771)

Hronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma.^{1,2} Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection, and the most effective currently available therapy is combination therapy with pegylated (PEG)-IFN and ribavirin (RBV).³⁻⁵ Among HCV genotypes, genotype 1 is the most resistant to

Abbreviations: aa, amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL28B, interleukin-28B; ISDR, interferon-sensitivity-determining region; ISG, interferon-stimulated gene; MxA, myxovirus resistance protein A; NVR, nonvirological response; OAS, oligoadenylate synthetase; PBS, phosphate-buffered saline; PEG, pegylated; PKR, RNA-dependent protein kinase; RBV, ribavirin; RT-PCR, reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SNP, single-nucleotide polymorphism; SVR, sustained virological response; uPA, urokinase-type plasminogen activator.

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IFN therapy.⁶ The limited success of combination therapy for genotype 1 HCV infection is because of the low response rate during therapy and high relapse rate after therapy.⁷

Recent studies have identified both viral and host factors predictive of IFN therapy. Among the viral factors, amino acid (aa) substitutions in the IFN-sensitivity-determining region (ISDR) (nucleotides 2209-2248 or aa positions 237-276 within the NS5A region) are associated with sustained virological response (SVR) after IFN treatment in HCV genotype 1b patients.^{8,9} Akuta et al. reported that substitution of aa70 or 91 in the HCV core region are independent predictors of SVR and nonvirological response (NVR).¹⁰⁻¹² Recently, we¹³ and another group¹⁴ also reported that wild-type HCV core aa70 and two or more aa substitutions in the ISDR are effective predictors of SVR in patients with HCV genotype 1b.

Among host factors associated with SVR, many common genetic polymorphisms in the human genome have been identified, including single-nucleotide polymorphisms (SNPs).¹⁵⁻¹⁹ More recently, an association between several linked SNPs in the interleukin-28B (IL28B) locus and the effect of combination therapy has been reported.²⁰⁻²²

We recently reported that the core aa wild type is significantly more likely to be found in patients with the eradication-favorable IL28B SNP genotype.²³⁻²⁵ The underlying mechanism of this association as well as the reason for the differential response to therapy by viruses with core aa substitutions are unknown. This is partly because of the presence of HCV quasi-species in human serum samples and the difficulty of performing infection experiments in a small animal model.

The severe combined immunodeficient (SCID) urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes, resulting in human hepatocyte chimeric mice able to develop HCV viremia after injection of serum samples positive for the virus.²⁶ We and other groups have reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs, such as IFN- α and NS3-4A protease inhibitor.^{27,28} We have further improved the replacement levels of the human hepatocytes in this mouse model,²⁹ which enabled us to perform infection experiments more easily because highly repopulated mice (defined as human serum albumin [HSA] levels well above 1 mg/mL) successfully develop viremia more often than poorly repopulated mice.³⁰ Using this mouse model, we developed a reverse genetics system for HCV.^{31,32} This system is

Table 1. Characteristics of Donors for Transplanted Human Hepatocytes

Donor	A	B	C	D
Sex	Female	Male	Female	Male
Age	10	2	5	2
Ethnic group	Caucasian	Caucasian	African American	Hispanic
rs8099917	TG	TT	TG	TT
rs8109886	AA	CC	AA	CC
rs12979860	TT	CC	TT	CC
rs11882871	GG	AA	GG	AA
rs73930703	TT	CC	TT	CC
rs8107030	AG	AA	AG	AA
rs28416813	GG	CC	GG	CC
rs8103142	CC	TT	CC	TT
rs11881222	GG	AA	GG	AA
rs4803217	AA	CC	AA	CC

useful for studying characteristics of HCV strains with various substitutions of interest, because the effects of quasi-species can be minimized. Furthermore, as there is no adaptive immune system in this mouse model, we are able to examine the replication of HCV and the effect of therapy while avoiding the influence of the immunological response. In the present study, we investigated effects of viral and host factors on HCV infectivity, replication ability, and IFN susceptibility using genetically engineered genotype 1b HCV-infected mice that underwent transplantation with hepatocytes having eradication-favorable or eradication-unfavorable IL28B SNP genotypes.

Materials and Methods

Animal Treatment. Generation of the uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described previously.²⁹ All animal protocols described in this study were performed in accord with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of HSA, which serve as useful markers of the extent of repopulation, were measured as previously described.²⁹ Mice underwent transplantation with frozen human hepatocytes obtained from four different human donors (Table 1). Genotyping of IL28B SNPs of human hepatocytes was performed using the Invader assay as described previously.^{33,34} We used 1000 IU/g/day of IFN- α (Dainippon Sumitomo Pharma Co., Tokyo, Japan) for 2 weeks. This dosage was selected based on a previous report showing that this regimen reduced mouse serum

Consensus (Core aa 61-100)	RRQPIPKARRPEGRAWAQPGYPWPPLYGNEGLGWAGWLLSP
Core-Wild	-----
Core-Mutant	-----Q-----M-----
HCV-J (ISDR)	PSLKATCTTHHDSFPDADLIEANLLWRQEMGGNITRVESEN
ISDR0	-----
ISDR4	-----N--R-----W--K-----
ISDR9	---R---P-N--A--I--AQ-----Q-----T-----

Fig. 1. The aa sequences of infectious genotype 1b HCV clones, Core-Wild, Core-Mutant (substitutions at aa70 and aa91), and ISDR variants (with 0, 4, and 9 substitutions).

HCV RNA levels by 0.5-2 log copies/mL during therapy.³¹

HCV RNA Transcription and Inoculation into Mice. We previously established an infectious genotype 1b HCV clone, HCV-KT9, that was obtained from a Japanese patient with severe acute hepatitis (GenBank accession no. AB435162).³² Ten micrograms of plasmid DNA, linearized by digestion with *Xba*I (Promega, Madison, WI), was transcribed in a 100- μ L reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours and then analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μ L of phosphate-buffered saline (PBS) and injected into the livers of chimeric mice.³² The HCV-KT9 clone has aa substitutions at aa70 and 91 (arginine to glutamine and leucine to methionine, respectively) in the core region (Core-Mutant), compared to the consensus sequence,¹⁰⁻¹² and no aa substitutions in the ISDR (ISDR0),⁸ relative to the prototype sequence (HCV-J).³⁵ Using the original HCV-KT9 clone, we created two additional HCV clones having wild-type core aa70 and 91 (Core-Wild) and four (ISDR4) and nine (ISDR9) aa substitutions in the ISDR, respectively (Fig. 1). To introduce the aa substitutions, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Human Serum Samples. Human serum samples containing a high titer of genotype 1b HCV (2.2×10^6 copies/mL) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots of serum were stored in liquid nitrogen until use. Core 70 and 91 aas were Gln and Leu, respectively, and only one aa substitution was present in the ISDR. The study protocol involving human subjects conformed to the ethical guidelines of the

1975 Declaration of Helsinki and was approved by the institutional review committee.

Quantitation of HCV RNA and IFN-stimulated gene-expression levels. RNA was extracted from mice serum and liver samples by Sepa Gene RV-R (Sankojun-yaku, Tokyo, Japan), dissolved in 8.8 μ L of ribonuclease-free H₂O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in 20 μ L of reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostics, Tokyo, Japan) were performed as previously described.³² Quantitation of IFN-stimulated genes (ISGs) (myxovirus resistance protein A [MxA], oligoadenylate synthetase [OAS], and RNA-dependent protein kinase [PKR]) was performed using real-time PCR Master Mix (Toyobo, Kyoto, Japan) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a pre-cycling period of 1 minute at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. ISG messenger RNA expression levels were expressed relative to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis. The HCV infectious ratio of chimeric mice was assessed using the chi-square test. Mice serum HCV RNA titers, HSA concentrations, and ISG expression levels were compared using the Mann-Whitney U test. A *P* value less than 0.05 was considered statistically significant.

Results

Influence of aa Substitutions in the HCV Core Region and ISDR on HCV Infectivity and Replication Ability. We investigated the influence of aa substitutions in the core region and ISDR on HCV infectivity and replication ability in mice that underwent transplantation with human hepatocytes obtained from donor A (Table 1). Each 30 μ g of *in vitro*-transcribed RNA was inoculated into the livers of mice. Six weeks after inoculation, serum HCV RNA titers increased above the detectable limit (1000 copies/mL) in 11 of 12 (92%) mice infected with Core-Wild-ISDR0 and in 14 of 16 (88%) mice with Core-Mutant-ISDR0 (Fig. 2A). HCV RNA titers in Core-Wild-ISDR0- and Core-Mutant-ISDR0-infected mice increased to the same levels (Fig. 2B). In contrast, serum HCV

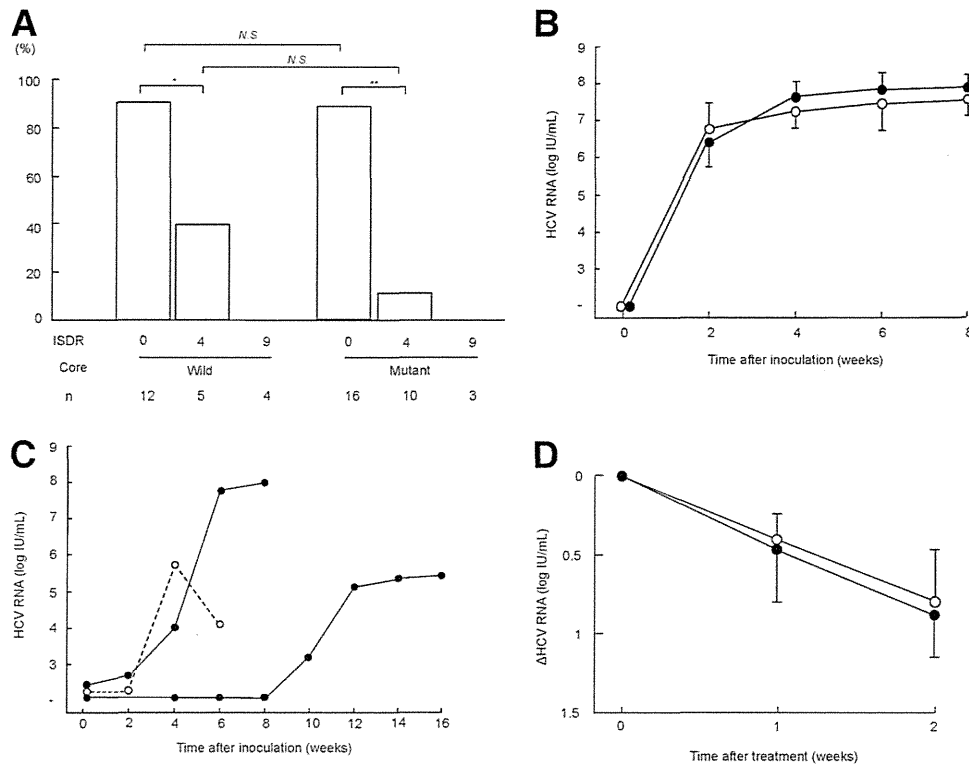


Fig. 2. Infectivity and replication ability of HCV clones. Mice that underwent transplantation with hepatocytes obtained from donor A were inoculated with 30 μ g of *in vitro*-transcribed RNAs of indicated clones. (A) Proportion of HCV-infected mice. Infection was defined as serum HCV RNA titer above the detection limit (1000 copies/mL) 6 weeks after inoculation. aa sequences of the core (Wild or Mutant) and number of substitutions in the ISDR are noted below the graph. (B) Time course of serum HCV RNA levels in mice inoculated with either Core-Wild-ISDR0 (closed circles, n = 11) or Core-Mutant-ISDR0 (open circles, n = 14) HCV clones. Data are represented as mean \pm standard deviation. (C) Time course of serum HCV RNA levels in two Core-Wild-ISDR4-infected mice (closed circles) and a Core-Mutant-ISDR4-infected mouse (open circles). Serum HCV RNA levels were measured until the mice died. (D) Core-Wild-ISDR0- (closed circles, n = 8) and Core-Mutant-ISDR0 (open circles, n = 4)-infected mice were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Mice serum HCV RNA titers were measured at the indicated times. *P < 0.05, **P < 0.01; NS, not significant.

RNA titer increased above the detection limit in only two of five (40%) Core-Wild-ISDR4 mice and in only 1 of 10 (10%) Core-Mutant-ISDR4 mice, and the titers in these mice were lower than in mice with ISDR0 (Fig. 2C). HCV RNA titers failed to increase above the detection limit in mice with Core-Wild-ISDR9 and Core-Mutant-ISDR9 (Fig. 2A).

Influence of Core aa Substitutions on the Effect of IFN. To investigate the influence of aa substitutions in the core region on the effect of IFN, Core-Wild-ISDR0- and Core-Mutant-ISDR0-infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in a 0.84 ± 0.3 log IU/mL reduction of HCV RNA titer in Core-Wild-ISDR0-infected mice and a 0.79 ± 0.34 log IU/mL reduction in Core-Mutant-ISDR0-infected mice (Fig. 2D).

We also investigated the influence of aa substitutions in the core region on the effect of IFN plus RBV combination therapy. Core-Wild-ISDR0- and Core-Mutant-ISDR0-infected mice were treated with 1000 IU/

g of human IFN-alpha and 20 mg/kg of RBV daily for 2 weeks. The treatment resulted in similar HCV RNA reductions in all treated mice. However, as with IFN monotherapy, there were no significant differences in HCV reductions among mice with different aa substitutions in the core region (data not shown). The dose of ribavirin used was relatively small, however, because of the drug's toxicity in mice.

HCV Infectivity, Replication Levels, and IFN Susceptibility by Core aa Substitutions and Genetic Variation in the IL28B Locus. We investigated the influence of IL28B genotypes on HCV infectivity, replication ability, and IFN susceptibility. *In vitro*-transcribed RNA (30 μ g) was inoculated into the livers of mice with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) or donor B (rs8099917 TT and rs12979860 CC). Eight weeks after inoculation, serum HCV RNA titers increased above the detection limit in 22 of 25 (88%) mice with hepatocytes from donor A and in 20 of 23 (87%) mice with hepatocytes from donor B (Fig. 3A). Serum HCV RNA levels were

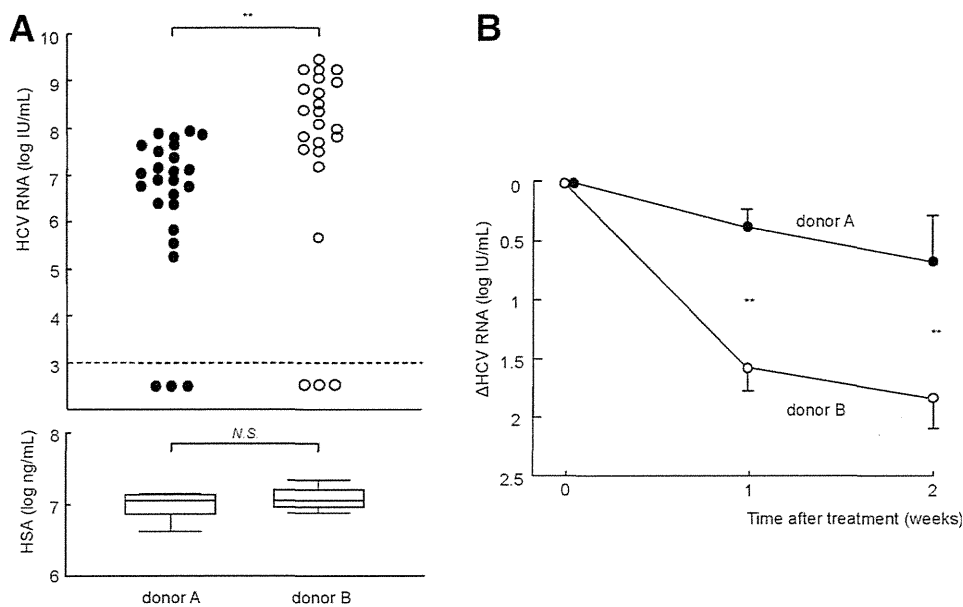


Fig. 3. HCV infectivity, replication ability, and IFN susceptibility in HCV-KT9-injected mice. Mice that underwent transplantation with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) (closed circles, $n = 25$) or B (rs8099917 TT and rs12979860 CC) (open circles, $n = 23$) were intrahepatically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

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

To confirm an association between IL28B SNP genotype and HCV RNA titer, we compared HCV RNA titers using mice with hepatocytes from an additional pair of donors with the favorable (donor C) and unfavorable (donor D) SNP genotypes. To determine whether results obtained by clonal infection would be comparable to results obtained using the more natural serum injection, which should have contained more complex viral species, mice were injected with genotype 1b HCV obtained from a human patient with core and ISDR substitutions, as described above. Mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) or donor D (rs8099917 TT and rs12979860 CC) were inoculated intravenously with

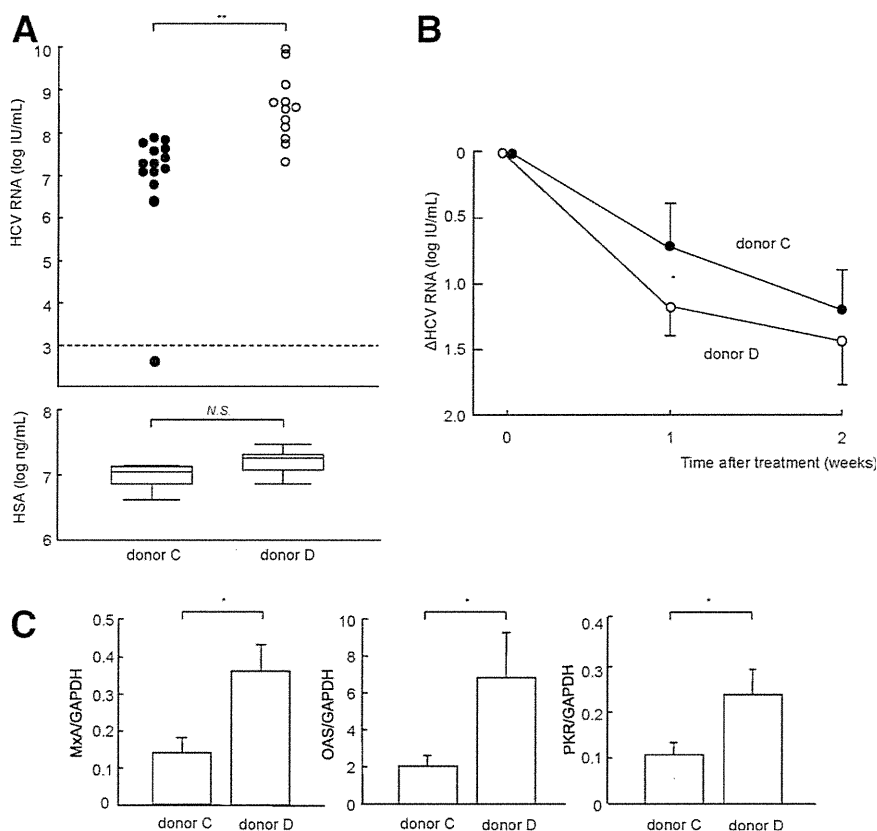
10^5 copies of HCV. Eight weeks after inoculation, serum HCV RNA titer increased above the detection limit in 13 of 14 (93%) mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) and in 12 of 12 (100%) mice with hepatocytes from donor D (rs8099917 TT and rs12979860 CC) (Fig. 4A). With results similar to those found for the mice inoculated with transcribed HCV RNA, serum HCV RNA levels were significantly higher in mice with hepatocytes from donor D than from donor C ($P < 0.001$), and the effect of IFN was also greater in donor D mice than in donor C mice (Fig. 4B); however, statistical significance using these donors was only achieved at week 1, probably resulting from fluctuation of HCV RNA titers and the small number of animals analyzed.

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

Discussion

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

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



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

minicore protein and its effect on IFN therapy should be further investigated using the chimeric mouse model.

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

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

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

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

In summary, we demonstrated that viral infectivity and replication ability are associated with hepatocyte IL28B genotype and are not associated with viral sub-

stitutions in the core protein or ISDR. Understanding the mechanism underlying the higher, more prolonged expression of antiviral genes in response-favorable hepatocytes will help us to develop improved therapeutic regimens to eradicate HCV more effectively.

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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

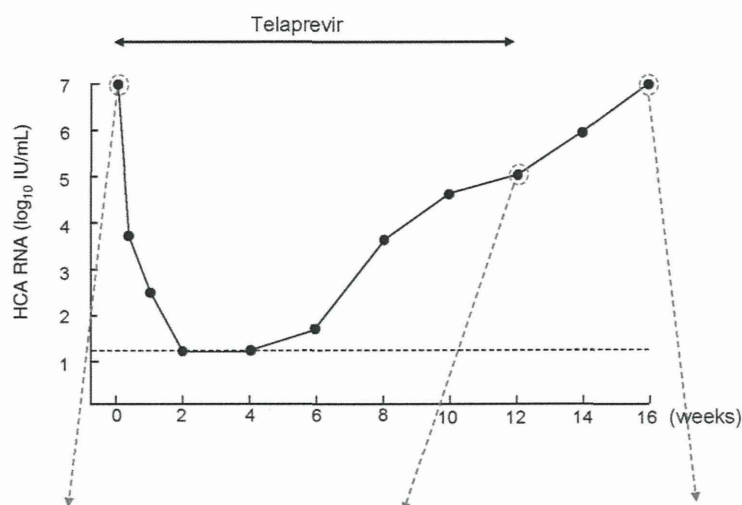
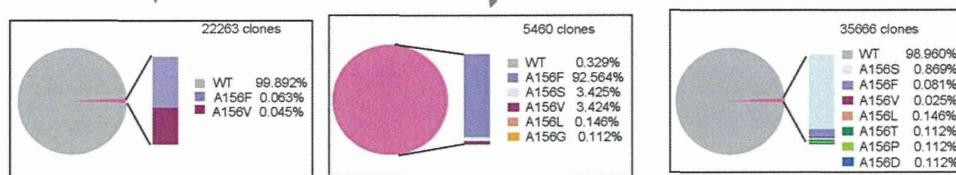


Fig. 1. Changes in serum HCV RNA levels in a telaprevir-treated chronic hepatitis C patient. A 55-year-old woman infected with genotype 1b HCV was treated with 750 mg of telaprevir every 8 hours for 12 weeks. Serum HCV RNA (upper panel) and the amino acid (aa) frequencies at aa156 in the HCV NS3 region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



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

Results

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

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

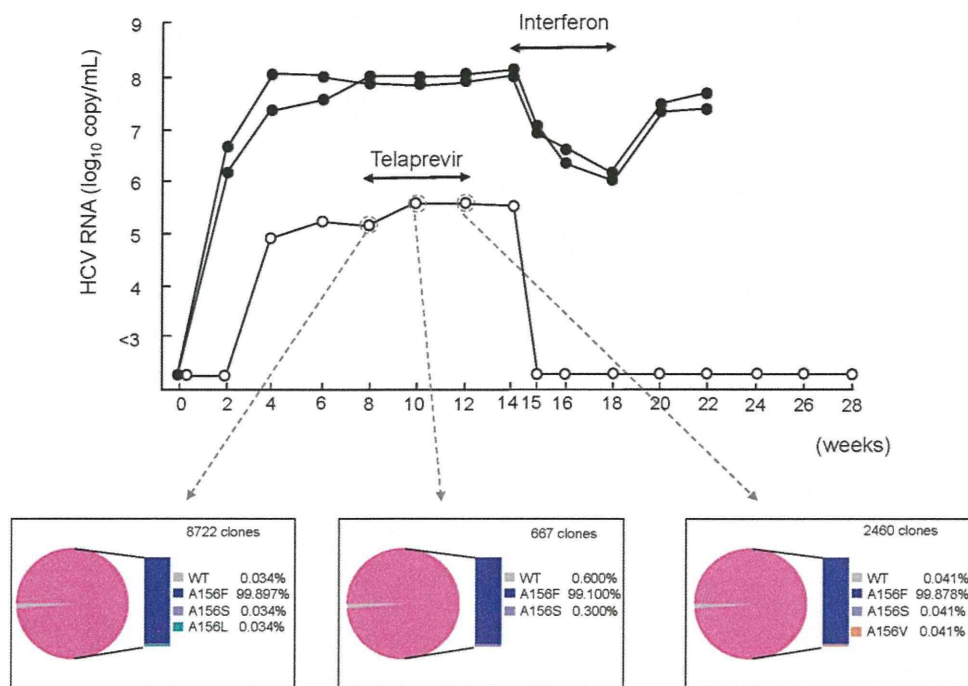


Fig. 2. Changes in serum virus titers in HCV-infected mice. Mice were injected with either wildtype (closed circles) or A156F-mutated HCV serum samples (obtained from an HCV-infected patient who received telaprevir monotherapy for 12 weeks; see Fig. 1) (open circles). Six weeks after injection the A156F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

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

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

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

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

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

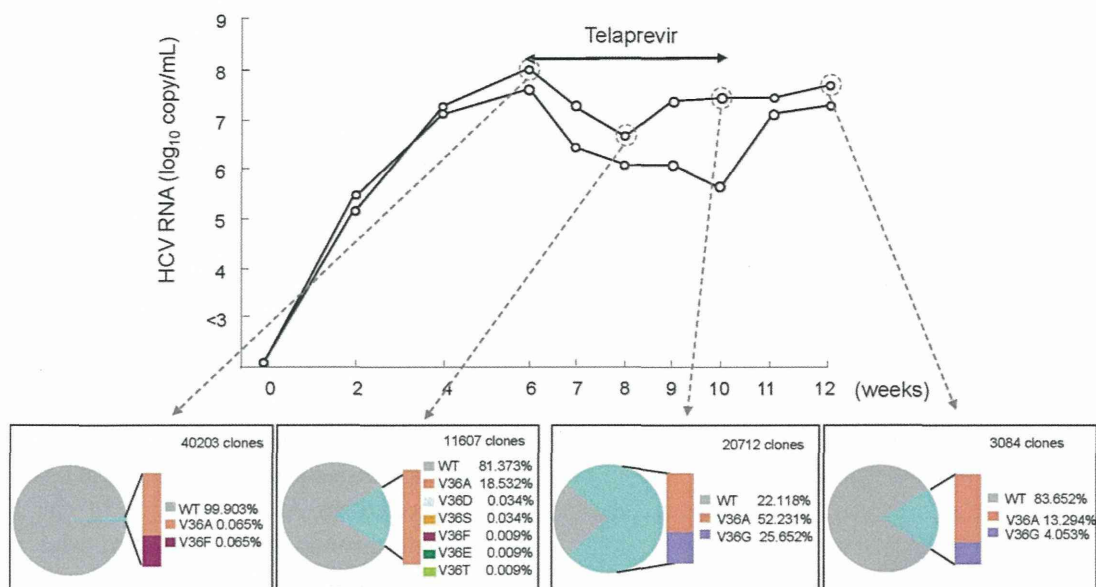


Fig. 3. Treatment with telaprevir in wildtype HCV-infected mice. Two mice were injected intravenously with 50 μ L of HCV-positive human serum samples. Six weeks after HCV injection mice were treated with 200 mg/kg of telaprevir orally twice a day for 4 weeks. Serum HCV RNA (upper panel) and amino acid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

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

Discussion

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

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

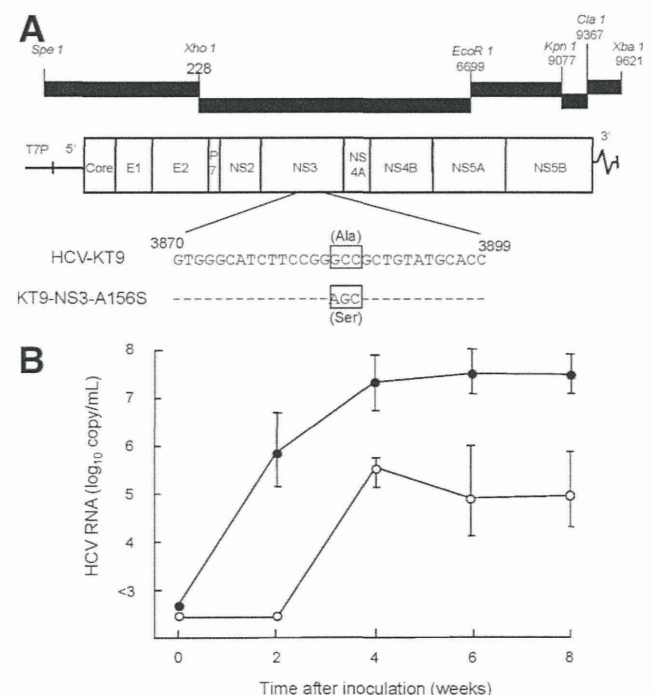


Fig. 4. Intrahepatic injection of *in vitro* transcribed HCV-KT9 RNA and KT9-NS3-A156S RNA into human hepatocyte chimeric mice. (A) The schematic of infectious genotype 1b HCV clones, HCV-KT9 and KT9-NS3-A156S. Boxes indicate codons at amino acid 156 in HCV NS3 region. Ala, alanine; Ser, serine. (B)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

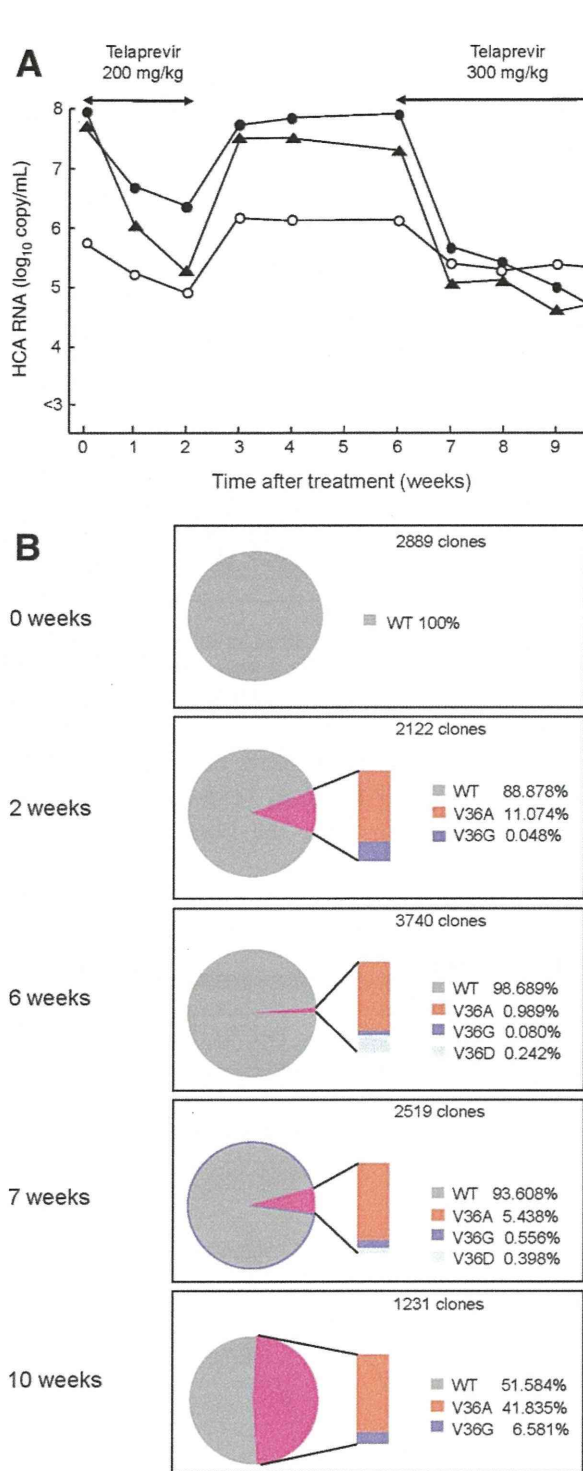


Fig. 5. The effect of telaprevir on mice infected with *in vitro*-transcribed HCV. Mice were injected with *in vitro*-transcribed HCV-KT9 RNA (closed circles and closed triangles) or KT9-NS3-A156S RNA (open circles). Six weeks after HCV RNA injection, mice were treated perorally with 200 mg/kg of telaprevir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

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

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

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

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

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

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

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

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

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