Recent findings have shown that PGC-1α-dependent myokine irisin plays a role in brown fat-like development in white adipocytes (Bostrom *et al.* 2012). Thus, the possibility of a link between the role of 4E-BP1 in white adipocyte differentiation and irisin should be addressed in a future study.

Experimental procedures

Animal experiments

The 4E-BP1$^{-/-}$ mice (Tsukiyama-Kohara *et al.* 2001) and 4E-BP1$^{+/+}$ mice were anesthetized, and their adipose tissues were characterized. Histological analysis of adipose tissues was carried out as described previously (Tsukiyama-Kohara *et al.* 2001) using hematoxylin and eosin staining. All animal experiments were carried out according to the guidelines of the local ethics committee. The protocol was approved by an institutional review board.

Cell culture

Mouse embryonic fibroblasts (MEFs) were established as described previously (Tsukiyama-Kohara *et al.* 2001) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HB and HW cells were established from brown and white adipocytes, respectively (Irie *et al.* 1999), and both lines were cultured in DMEM supplemented with 10% fetal calf serum (FCS). To induce differentiation into adipocytes, HB and HW cells were cultured until confluency (2 days) in DMEM-10% FCS containing 0.5 mM 3-isobutyl-1 methylxanthine (IBMX, Sigma) and 1 μM dexamethasone (Dex; Sigma). After reaching confluency, the medium was changed to fresh DMEM-10% FCS containing 10 μg/mL insulin (Sigma), 50 nM 3-3′-5-triiodo-L-thyronine (T3, Sigma) and 1 μM norepinephrine (Sigma) for 5–7 days (Fig. 4C).

Plasmids

Cells were transfected with pCALN-4E-BP1 (Tsukiyama-Kohara *et al.* 1992) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Immunoblot analysis

In the immunoblot analysis, 4E-BP1 was detected as described previously (Tsukiyama-Kohara *et al.* 2001), whereas PGC-1α was detected using a specific polyclonal antibody (SC-13067, Santa Cruz).

Statistical analysis

The Student's *t*-test was used to determine statistical significance. Data were considered statistically significant if the *P*-value was <0.05.

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References

Bostrom, P., Wu, J., Jedrychowski, M.P., *et al.* (2012) A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* **481**, 463–468.

Brunn, G.J., Fadden, P., Haystead, T.A. & Lawrence, J.C. Jr (1997a) The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-Pro motif and is activated by antibodies to a region near its COOH terminus. *J. Biol. Chem.* **272**, 32547–32550.

Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C. Jr & Abraham, R.T. (1997b) Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* **277**, 99–101.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H. & Sabatini, D.M. (1998) RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl Acad. Sci. USA* **95**, 1432–1437.

Dufner, A., Andjelkovic, M., Burgering, B.M., Hemmings, B.A. & Thomas, G. (1999) Protein kinase B localization and activation differentially affect S6 kinase 1 activity and eukaryotic translation initiation factor 4E-binding protein 1 phosphorylation. *Mol. Cell. Biol.* **19**, 4525–4534.

Fadden, P., Haystead, T.A. & Lawrence, J.C. Jr (1997) Identification of phosphorylation sites in the translational regulator, PHAS-I, that are controlled by insulin and rapamycin in rat adipocytes. *J. Biol. Chem.* **272**, 10240–10247.

Gingras, A.C., Kennedy, S.G., O'Leary, M.A., Sonenberg, N. & Hay, N. (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev.* **12**, 502–513.

Gomez-Ambrosi, J., Fruhbeck, G. & Martinez, J.A. (2001) Rapid in vivo PGC-1 mRNA upregulation in brown adipose tissue of Wistar rats by a beta(3)-adrenergic agonist and lack of effect of leptin. *Mol. Cell. Endocrinol.* **176**, 85–90.

Haghighat, A., Mader, S., Pause, A. & Sonenberg, N. (1995) Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.* **14**, 5701–5709.

Hara, K., Yonezawa, K., Kozlowski, M.T., Sugimoto, T., Andrabi, K., Weng, Q.P., Kasuga, M., Nishimoto, I. & Avruch, J. (1997) Regulation of eIF-4E BP1 phosphorylation by mTOR. *J. Biol. Chem.* **272**, 26457–26463.

Irie, Y., Asano, A., Canas, X., Nikami, H., Aizawa, S. & Saito, M. (1999) Immortal brown adipocytes from p53-

knockout mice: differentiation and expression of uncoupling proteins. *Biochem. Biophys. Res. Commun.* **255**, 221–225.

Kohn, A.D., Barthel, A., Kovacina, K.S., Boge, A., Wallach, B., Summers, S.A., Birnbaum, M.J., Scott, P.H., Lawrence, J.C. Jr & Roth, R.A. (1998) Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J. Biol. Chem.* **273**, 11937–11943.

Lin, T.A., Kong, X., Haystead, T.A., Pause, A., Belsham, G., Sonenberg, N. & Lawrence, J.C. Jr (1994) PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science* **266**, 653–656.

Mader, S., Lee, H., Pause, A. & Sonenberg, N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Mol. Cell. Biol.* **15**, 4990–4997.

von Manteuffel, S.R., Dennis, P.B., Pullen, N., Gingras, A.C., Sonenberg, N. & Thomas, G. (1997) The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70s6k. *Mol. Cell. Biol.* **17**, 5426–5436.

Mendez, R., Myers, M.G. Jr, White, M.F. & Rhoads, R.E. (1996) Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Mol. Cell. Biol.* **16**, 2857–2864.

Pause, A., Belsham, G.J., Gingras, A.C., Donze, O., Lin, T.A., Lawrence, J.C. Jr & Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* **371**, 762–767.

Poulin, F., Gingras, A.C., Olsen, H., Chevalier, S. & Sonenberg, N. (1998) 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. *J. Biol. Chem.* **273**, 14002–14007.

Proud, C.G. (2004) Role of mTOR signalling in the control of translation initiation and elongation by nutrients. *Curr. Top. Microbiol. Immunol.* **279**, 215–244.

Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M. & Spiegelman, B.M. (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**, 829–839.

Takata, M., Ogawa, W., Kitamura, T., Hino, Y., Kuroda, S., Kotani, K., Klip, A., Gingras, A.C., Sonenberg, N. & Kasuga, M. (1999) Requirement for Akt (protein kinase B) in insulin-induced activation of glycogen synthase and phosphorylation of 4E-BP1 (PHAS-1). *J. Biol. Chem.* **274**, 20611–20618.

Tiraby, C., Tavernier, G., Lefort, C., Larrouy, D., Bouillaud, F., Ricquier, D. & Langin, D. (2003) Acquirement of brown fat cell features by human white adipocytes. *J. Biol. Chem.* **278**, 33370–33376.

Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. & Nomoto, A. (1992) Internal ribosome entry site within hepatitis C virus RNA. *J. Virol.* **66**, 1476–1483.

Tsukiyama-Kohara, K., Poulin, F., Kohara, M., DeMaria, C.T., Cheng, A., Wu, Z., Gingras, A.C., Katsume, A., Elchebly, M., Spiegelman, B.M., Harper, M.E., Tremblay, M.L. & Sonenberg, N. (2001) Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. *Nat. Med.* **7**, 1128–1132.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C. & Spiegelman, B.M. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115–124.

Yamaguchi, S., Ishihara, H., Yamada, T., Tamura, A., Usui, M., Tominaga, R., Munakata, Y., Satake, C., Katagiri, H., Tashiro, F., Aburatani, H., Tsukiyama-Kohara, K., Miyazaki, J., Sonenberg, N. & Oka, Y. (2008) ATF4-mediated induction of 4E-BP1 contributes to pancreatic beta cell survival under endoplasmic reticulum stress. *Cell Metab.* **7**, 269–276.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1 Microarray analysis of expression profiles of the host factors whose expression is higher in HW cells (a white preadipocyte cell line) than in HB cells (a brown preadipocyte cell line) (top 20).

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Targeted Induction of Interferon-λ in Humanized Chimeric Mouse Liver Abrogates Hepatotropic Virus Infection

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Abstract

Background & Aims: The interferon (IFN) system plays a critical role in innate antiviral response. We presume that targeted induction of IFN in human liver shows robust antiviral effects on hepatitis C virus (HCV) and hepatitis B virus (HBV).

Methods: This study used chimeric mice harboring humanized livers and infected with HCV or HBV. This mouse model permitted simultaneous analysis of immune responses by human and mouse hepatocytes in the same liver and exploration of the mechanism of antiviral effect against these viruses. Targeted expression of IFN was induced by treating the animals with a complex comprising a hepatotropic cationic liposome and a synthetic double-stranded RNA analog, pIC (LIC-pIC). Viral replication, IFN gene expression, IFN protein production, and IFN antiviral activity were analyzed (for type I, II and III IFNs) in the livers and sera of these humanized chimeric mice.

Results: Following treatment with LIC-pIC, the humanized livers of chimeric mice exhibited increased expression (at the mRNA and protein level) of human IFN-λs, resulting in strong antiviral effect on HBV and HCV. Similar increases were not seen for human IFN-α or IFN-β in these animals. Strong induction of IFN-λs by LIC-pIC occurred only in human hepatocytes, and not in mouse hepatocytes nor in human cell lines derived from other (non-hepatic) tissues. LIC-pIC-induced IFN-λ production was mediated by the immune sensor adaptor molecules mitochondrial antiviral signaling protein (MAVS) and Toll/IL-1R domain-containing adaptor molecule-1 (TICAM-1), suggesting dual recognition of LIC-pIC by both sensor adaptor pathways.

Conclusions: These findings demonstrate that the expression and function of various IFNs differ depending on the animal species and tissues under investigation. Chimeric mice harboring humanized livers demonstrate that IFN-λs play an important role in the defense against human hepatic virus infection.

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Introduction

The interferon (IFN) family of cytokines are important mediators of the innate immune response and contribute to the first line of defense against viral infection. The IFNs are classified as type I (IFN-α and IFN-β), type II (IFN-γ), or type III (IFN-λ), based on receptor complex recognition and protein structure.

Innate immune responses, such as IFN induction, contribute to defenses against microbial pathogens. Pathogens are recognized by four types of receptors associated with innate immunity: Toll-like

receptors (TLRs), Nod-like receptors, retinoic acid-inducible gene-I (RIG-I) -like receptors and C-type lectins [1]. The initial recognition of pathogens by these receptors induces inflammatory reactions at the infected site, and also triggers adaptive immunity against the pathogens. Thus, activation of the innate immune response exerts antiviral effects. Immunity-associated receptor agonists are therefore good candidates for antiviral drugs and adjuvants [2].

IFN-α and IFN-β are currently employed therapeutically. The most noteworthy example is in the treatment of chronic hepatitis C virus (HCV) infection with pegylated IFN-α (PegIFN-α) [3]. These IFNs also are used against chronic hepatitis B virus (HBV) infection [4]. Some groups recently have reported that HCV infection results in expression of IFN-λs in primary human hepatocytes [5] and in the livers of chimpanzees [6]. Additionally, variation near the IFN-λ3 (IL-28B) -encoding gene is strongly associated with treatment response to pegylated IFN and ribavirin for chronic HCV infection and spontaneous eradication [7,8,9,10].

To target induction of the innate immune system of liver without inducing systemic immune activation, we developed a complex of cationic liposome (LIC) and the synthetic double-stranded RNA analog (polyinosinic-polycytidylic acid; pIC), termed LIC-pIC. We have reported that LIC is a safe and effective delivery tool for oligonucleotides [11,12]. Furthermore, we have shown, using administration in animal models, that RNA complexed with LIC can be delivered in large quantities to the liver (**Figure S1**). pIC is a well-known inducer of IFN-α and IFN-β production, a role mediated through pIC's recognition by TLR3 [13] or RIG-I-like receptors [14].

In the present study, we demonstrate, using administration of LIC-pIC, the distinct innate immune responses of human and mouse hepatocytes in chimeric mice harboring human/mouse livers infected with HCV or HBV [15,16]. The animals used here were transgenic severe combined immunodeficient (SCID) mice that carried additional copies of the urokinase plasminogen activator-encoding gene, resulting in the apoptosis of endogenous mouse hepatocytes, which then were replaced with human hepatocytes. This animal model provided robust HBV or HCV infection in chimeric mice harboring humanized livers. Because these rodents were T- and B-cell deficient, the mice were not appropriate for studies of adaptive immunity. Nonetheless, this model provided new insights into HCV innate host responses and therapeutic approaches. Furthermore, these models permitted analyses of human/mouse hepatocyte immune responses in the same liver. These analyses indicated that IFN-λs play a critical role in the antiviral response of human hepatocytes, and that IFN-β is induced in the response of mouse hepatocytes. Analysis of several cell lines showed that this robust IFN-λ induction was limited to the human hepatocytes. These results suggest that the function of IFN-λs, IFN-α, and IFN-β differ depending on the animal species and on the tissue under study.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with both the *Guidelines for Animal Experimentation* of the Japanese Association for Laboratory Animal Science and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All protocols were approved by the ethics committee of Tokyo Metropolitan Institute of Medical Science. The HCV-infected patient who served as the source of the serum samples provided written informed consent prior to blood collection.

Nucleic Acids and Complexes

pIC and LIC were prepared as previously described [11]. In brief, the distribution of the chain lengths of polyinosinic and polycytidylic acids (Yamasa, Chiba, Japan) was adjusted by heating to give an apparent maximum of 200–400 bases, as determined by gel filtration high-performance liquid chromatography. Liposomes composed of the cationic lipid analogue 2-*O*-(2-diethylaminoethyl)-carbamoyl-1,3-*O*-dioleoylglycerol (synthesized at Nippon Shinyaku Co., Ltd., Kyoto, Japan) and egg phosphatidylcholine (NOF Corp., Tokyo, Japan) were lyophilized and formulated by Nippon Shinyaku Co., Ltd. A short dsRNA, a synthetic siRNA against the firefly luciferase gene, was purchased from Hokkaido System Science (Sapporo, Japan). The sequence of this dsRNA was 5′-GCUAUGAAAC-GAUAUGGGC-dTdT-3′ (sense) and 5′-GCCCAUAUCGUUU-CAUAGC-dTdT-3′ (antisense). Atelocollagen, a highly purified type I collagen prepared by pepsin treatment of calf dermis, was obtained from Koken (Tokyo, Japan). Each nucleic acid complex with LIC or atelocollagen was freshly prepared for each experiment.

Generation of Chimeric Mice Infected with HCV or HBV

Chimeric mice infected with HCV or HBV were prepared as previously described [17,18]. In brief, uPA$^{+/+}$/SCID mice transplanted with human hepatocytes were obtained from PhoenixBio (Hiroshima, Japan) [19]. Six weeks after transplantation, we injected each chimeric mouse intravenously (IV) with HCV (10^6 copies per dose) -containing serum or HBV-containing culture supernatant. The serum was obtained from a HCV-infected patient harboring HCV genotype 1b (HCR6; accession number AY045702) or 1a (HCG9; accession number AB520610); the supernatant was concentrated from a culture containing HBV genotype C. By the time of the first drug administration, the serum HCV genomic RNA levels had reached 6.6×10^5 to 2.7×10^7 copies/ml (genotype 1b) or 2.9×10^6 to 2.8×10^8 copies/ml (genotype 1a); the serum HBV genomic DNA levels had reached 2.4×10^7 to 1.2×10^9 copies/ml.

Treatment of Chimeric Mice

Virus-infected chimeric mice with humanized livers were randomly allocated to treatment groups of 3–5 mice each. Starting on Day 0, the chimeras received once- or three-times-daily IV injections (via the tail or orbital vein) of saline, LIC-pIC, atelocollagen-pIC, LIC-short dsRNA, pIC, human IFN-λ1, human IFN-λ2, or human IFN-λ3 (the human IFN-λs were from R&D Systems, Minneapolis, MN, USA) and once-weekly (Days 0 and 7) IV injections of α-galactosylceramide (KRN7000; Kirin Brewery, Gunma, Japan); twice-weekly (Days 0, 3, 7 and 10) subcutaneous (SC) injections of 30 μg/kg PegIFN-α (PegIFNα-2a; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan); or once-daily peroral entecavir (ETV) (Bristol-Myers Squibb, New York, NY, USA). The IV injections were performed under gentle pressure to avoid non-specific delivery effects. All drugs were administered at a dosing volume of 5–10 μl/g of body weight.

Quantification of HCV Genomic RNA Levels by qRT-PCR

Total RNA was purified from the serum or liver tissue of chimeric mice with humanized livers by the acid guanidinium-phenol-chloroform method. HCV genomic RNA levels were quantified by qRT-PCR with an ABI7700 sequence detector system (Applied Biosystems, Foster City, CA, USA) as previously described [20].

Quantification of HBV Genomic DNA by qPCR

DNA extraction from the serum and liver tissue, and quantification of HBV genomic DNA, were performed as previously described [21].

Immunofluorescence Analysis

Liver tissues obtained from mice were embedded in OCT compound. The frozen tissues were cut into thin sections (5–8 μm) and placed on glass slides. After fixation, mouse liver sections were probed with an anti-HCV core protein monoclonal antibody (5E3) labeled with biotin [22] as the primary antibody, followed by streptavidin Alexa-488 conjugate (Invitrogen Corp., Carlsbad, CA, USA). To detect human hepatocytes, liver sections were probed with anti-human hepatocyte monoclonal antibody (Dako, Glostrup, Denmark), followed by anti-mouse IgG-Alexa 546 (Molecular Probes). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Cells

HepG2, Huh-7, and HEK293T cells were obtained from the American Type Culture Collection. MRC-5 (RCB0218) cells were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the MEXT, Japan. All cells were cultured as described [23]. Two types of human hepatoma HuH-7 cells carrying an HCV sub-genomic replicon (FLR 3-1 (genotype 1b, Con-1) and R6FLR-N (genotype 1b, strain HCR6 and N)) were used and cultured as described [24], [25].

RNA-mediated Interference

Chemically synthesized 21-nucleotide siRNA, including control siRNA (siPerfect Negative control), was obtained from Sigma. The sequences of these RNAs are 5′-GCCAUAGACCACUCAGCUU-3′ (siTICAM-1) and 5′-CCACCUUGAUGCCUGUGAA-3′ (siMAVS) (only the sense strands are shown). Cells were transfected with 50 nM siRNA in 2.0 μl Lipofectamine RNAiMAX (Invitrogen), and then were used 48 h later for further experiments.

Microarray Analysis

Total RNA was extracted from chimeric mouse livers using ISOGEN (Nippon Gene, Tokyo, Japan), and was purified using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). RNA integrity was assessed with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cRNA was prepared and the microarray (Agilent Technologies) was hybridized and scanned according to the manufacturer's instructions. Whole human genome 4×44 K format microarrays (G4112F; Agilent Technologies) were used.

Gene Expression Analysis

Total RNA or cDNA, which was synthesized from total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), was used for qRT-PCR or qPCR performed using TaqMan Gene Expression Assays and the ABI7700 sequence detector system. The TaqMan Gene Expression Assays for *IFNB1*, *Ifnb1*, *IFNA2*, *Ifna2*, *IFNG*, *Ifng*, *IFNL1* (also known as *IL29*), *Ifnl2/3* (also known as *Il28a/b*), *IFNAR1*, *Ifnar1*, *IFNAR2*, *Ifnar2*, *IFNLR1* (also known as *IL28RA*), and *Ifnlr1* (also known as *Il28ra*) were obtained from Applied Biosystems. The primers and probes for the *IFNL2* and *IFNL3* (also known as *IL28A* and *IL28B*, respectively) genes were obtained from Takara Bio, Inc. (Shiga, Japan). The primers and probes for the *IFNL2* gene consisted of a forward primer, IL28A-207-S21 (nt 207–227; 5′-GCTGAAGGACTGCAGGTGCCA-3′); a reverse primer, IL28A-378-R20 (nt 359–378; 5′-GGGCTGGTCCAAGACGTCCA-3′); and a TaqMan probe, IL28A-286-S24FT (nt 286–309; 5′-ATGGCTTTGGAGGCTGAGCTGGCC-3′). The primers and probes for the *IFNL3* gene consisted of a forward primer, IL28B-207-S21 (nt 207–227; 5′-GCTGAAGGACTGCAAGTGCCG-3′); a reverse primer, IL28B-379-R21 (nt 359–379; 5′-GGGGCTGGTCCAAGACATCCC-3′); and a TaqMan probe, IL28B-286-R20FT (nt 267–286; 5′-CGGGGCGCTCCCTCACCTGC-3′). A reporter dye, 6-carboxy-fluorescein, was covalently attached to the 5′-end of the probe sequence and a quencher dye, 6-carboxytetramethylrhodamine, was attached to its 3′-end.

Calibration curves for quantification of the IFN-α- or IFN-β-encoding genes and the IFN-λs-encoding genes were prepared by using a series of ten-fold dilutions of human *IFNB1* and *IFNA2* RNAs (synthesized in our laboratory), and human *IFNL1*, *IFNL2*, and *IFNL3* cDNA clones (Open Biosystems, Inc., Huntsville, AL, USA). Each assay specifically detected its own target.

Quantification of *IFNAR1/2*, *Ifnar1/2*, *IFNLR1*, and *Ifnlr1* mRNA was estimated using the calibration curves for the *IFNL1* cDNA.

IFN-λ Neutralization Studies

IFN-λ1 to IFN-λ3 in the chimeric mice were neutralized by daily IV injection of 3 mg/kg anti-human IFN-λ1 (R&D Systems) and 1 mg/kg anti-human IFN-λ2 (R&D Systems) antibodies.

Statistical Analysis

The data were analyzed with SAS System version 8.2 (SAS Institute, Inc., Cary, NC, USA). P values of ≤ 0.05 were considered significant.

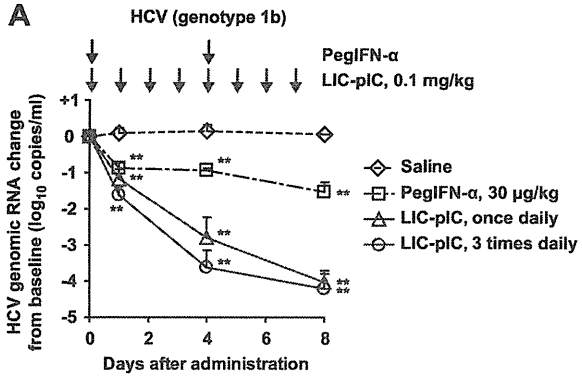
Results

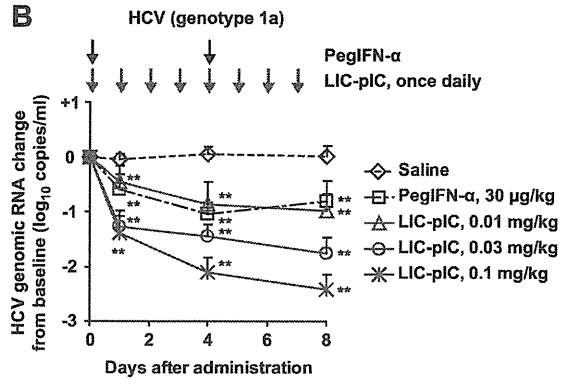
Antiviral Responses Elicited by LIC-pIC in HCV- or HBV-infected Chimeric Mice with Humanized Livers

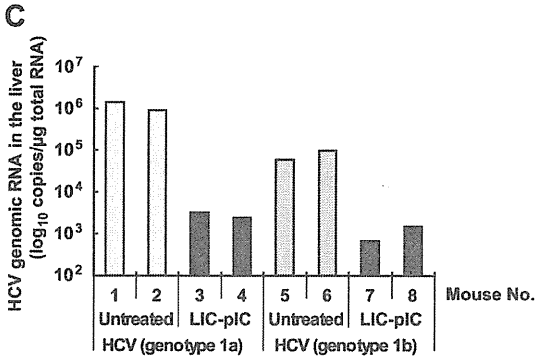
To confirm that HCV and HBV can infect humanized chimeric mice, we measured HCV RNA or HBV DNA levels in serum weekly after inoculation with the respective virus (**Figure S2A and S2B**). At 3 weeks post-infection, HCV-RNA levels reached 10^6–10^7 copies/mL in the genotype 1b group (**Figure S2A**); at 9 weeks post-infection, HBV-DNA levels reached 10^7–10^8 copies/mL (**Figure S2B**). These results demonstrated that HCV or HBV can replicate in, and persistently infect, human hepatocytes transplanted into chimeric mice.

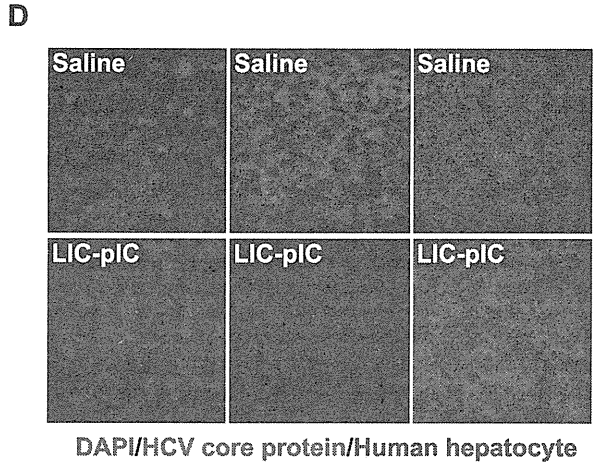
Treatment of HCV genotype 1b-infected chimeric mice with LIC-pIC (0.1 mg/kg; 1 or 3 times/day) led to a dose-dependent reduction in serum HCV RNA that was greater than the reduction induced by PegIFN-α treatment (30 μg/kg; twice/week, 20-fold the typical clinical dose) (**Figure 1A**). Treatment of HCV genotype 1a-infected mice with LIC-pIC (0.01, 0.03 or 0.1 mg/kg; once/day) had a similar antiviral effect (**Figure 1B**). Treatment of these mice with LIC-pIC also reduced liver HCV RNA levels (**Figure 1C**) and liver HCV core protein levels (**Figure 1D**).

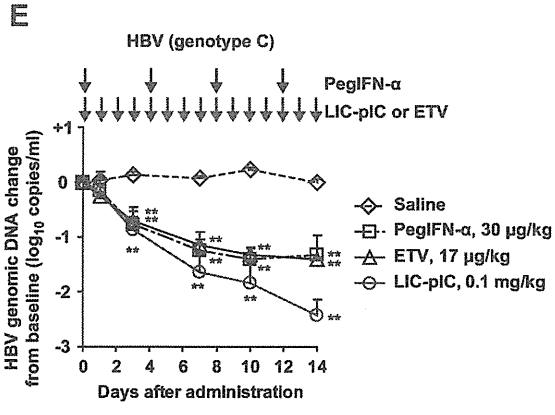
Treatment with LIC-pIC (0.1 mg/kg/day) also reduced serum HBV DNA levels in HBV-infected mice more effectively than entecavir (ETV) treatment (17 μg/kg/day; the same as the clinical dose) by Day 14 (**Figure 1E**) and additionally reduced liver HBV DNA levels (**Figure 1F**). LIC-pIC complex treatment therefore elicited stronger antiviral responses against both HCV and HBV than currently used antiviral agents without marked hepatotoxicity (**Figure S3**).

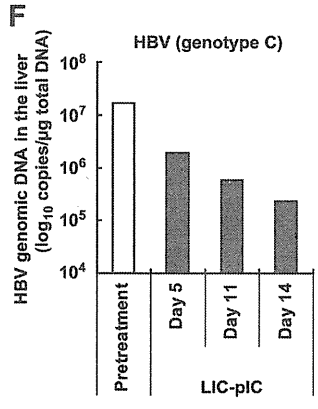

A
HCV (genotype 1b)
PegIFN-α
LIC-pIC, 0.1 mg/kg
HCV genomic RNA change from baseline (log10 copies/ml)
Days after administration
Saline
PegIFN-α, 30 μg/kg
LIC-pIC, once daily
LIC-pIC, 3 times daily


B
HCV (genotype 1a)
PegIFN-α
LIC-pIC, once daily
HCV genomic RNA change from baseline (log10 copies/ml)
Days after administration
Saline
PegIFN-α, 30 μg/kg
LIC-pIC, 0.01 mg/kg
LIC-pIC, 0.03 mg/kg
LIC-pIC, 0.1 mg/kg


C
HCV genomic RNA in the liver (log10 copies/μg total RNA)
Mouse No.
Untreated
LIC-pIC
Untreated
LIC-pIC
HCV (genotype 1a)
HCV (genotype 1b)


D
Saline
LIC-pIC
DAPI/HCV core protein/Human hepatocyte


E
HBV (genotype C)
PegIFN-α
LIC-pIC or ETV
HBV genomic DNA change from baseline (log10 copies/ml)
Days after administration
Saline
PegIFN-α, 30 μg/kg
ETV, 17 μg/kg
LIC-pIC, 0.1 mg/kg


F
HBV (genotype C)
HBV genomic DNA in the liver (log10 copies/μg total DNA)
Pretreatment
Day 5
Day 11
Day 14
LIC-pIC

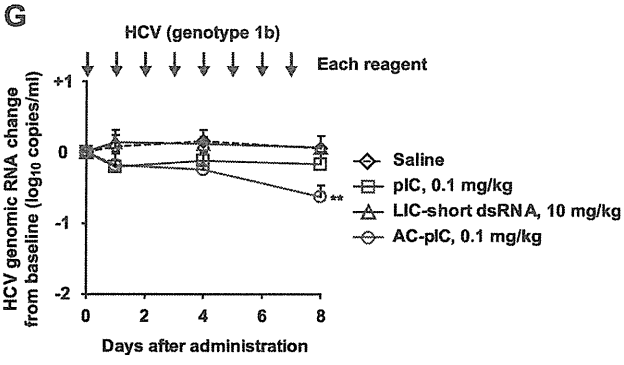

G
HCV (genotype 1b)
Each reagent
HCV genomic RNA change from baseline (log10 copies/ml)
Days after administration
Saline
pIC, 0.1 mg/kg
LIC-short dsRNA, 10 mg/kg
AC-pIC, 0.1 mg/kg

Figure 1. Antiviral responses elicited by LIC-pIC in chimeric mice with humanized livers. (**A**, **B**) Serum HCV RNA levels, relative to the baseline, in HCV genotype 1b-infected (**A**) or 1a-infected (**B**) chimeric mice following the indicated treatments, as determined by qRT-PCR ($n = 3$–5 per group). (**C**) Liver HCV RNA levels on Day 4 of LIC-pIC treatment (0.1 mg/kg/day), as determined by qRT-PCR. (**D**) Immunostained liver tissues of HCV genotype 1b-infected chimeric mice treated with saline (Upper) and LIC-pIC (0.1 mg/kg/day) (Lower) at Day 4. (**E**) Serum HBV DNA levels relative to the baseline following the indicated treatments, as determined by qPCR ($n = 3$–4 per group). (**F**) HBV DNA levels of chimeric mouse livers following LIC-pIC treatment, as determined by qPCR ($n = 1$ per each time point, the results indicates the mean of two measurements) (**G**) HCV RNA levels relative to the baseline following the indicated treatments, as determined by qRT-PCR ($n = 3$–4 per group). In all cases, bars indicate SD. $^{**}P<0.01$, treatment vs. saline control by Dunnett's multiple comparison test.
doi:10.1371/journal.pone.0059611.g001

We next analyzed the effect on serum HCV RNA levels of treatment with pIC alone; with pIC complexed with the non-hepatotropic carrier atelocollagen (AC) [26]; or with a short dsRNA complexed with LIC. Significant reductions in serum HCV RNA levels were observed only following treatment with the AC-pIC complex (**Figure 1G**), indicating that the active ingredient of LIC-pIC was pIC and that the antiviral effects of pIC were enhanced by complexing with LIC.

Expression of IFN-α, IFN-β and IFN-γ Induced by LIC-pIC in Chimeric Mice with Humanized Livers

pIC induces IFN-α and IFN-β production through recognition by TLR3 [13] or RIG-I-like receptors [14]. To examine whether the potent antiviral responses elicited by LIC-pIC were caused by its reported ability to induce IFN-α and IFN-β, we analyzed the expression levels of these IFNs after administration of 0.1 mg/kg LIC-pIC to virally infected mice. Administration and sampling were conducted according to the schedules shown in **Figure 2A** (HCV-infected mice) and **Figure S4A** (HBV-infected mice). Because the mouse livers used in this study were human-mouse chimeras (average substitution rate ≈80%) [19], we measured the induction levels of both human and mouse IFNs.

The first administration of LIC-pIC (Day 0) induced both human and mouse IFN-β in the serum, as well as *IFNB1* (human) and *Ifnb1* (mouse) mRNA in the liver (**Figure 2B and 2C**). The mRNA induction levels peaked 3 h after the first administration and decreased to the pretreatment levels within 24 h (data not shown). On repeated administration, the peak levels of both protein and mRNA fell gradually in the mouse, whereas induction of humanized liver-derived IFN products was observed only at the first administration (**Figure 2B and 2C**), although the antiviral responses elicited by LIC-pIC were sustained throughout the administration period. In addition, we did not observe induction of human or mouse IFN-α in the serum, or of *IFNA2* or *IFNG* (human) or *Ifna2* or *Ifng* (mouse) mRNA in the liver, at any point during LIC-pIC administration (**Figure 2B and 2C**, and **Figure S4B** and **S4C**). The induced mouse IFN-β that was still observed after the fifth administration was not expected to have any antiviral effect because mouse IFN-β does not suppress HCV replication in HCV replicon-containing cells (**Figure 2D**), indicating that mouse IFN-β does not activate signaling by the human receptor. Thus, the kinetics of IFN-α, IFN-β, and IFN-γ induction by LIC-pIC were inconsistent with the duration of the antiviral responses that were observed.

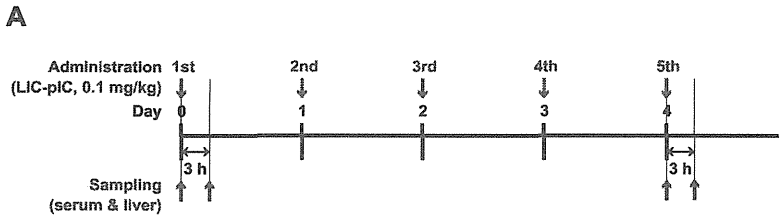
Moreover, the maximum serum concentration of human IFN-β that was induced by LIC-pIC was only 92 pg/ml (**Figure 2B**), which was 13-fold lower than the serum level of mouse IFN-β, despite the high human hepatocyte substitution rate in the liver. This poor induction of human IFN-β by LIC-pIC was independent of HCV infection (**Figure S5**). As for IFN-α, no serum IFN-α could be detected following LIC-pIC treatment, whereas a constant high concentration of human IFN-α (≈20,000 pg/ml) was observed in the serum of chimeric mice treated with PegIFN-α (**Figure 2B**). Thus, the concentration of IFN-α and IFN-β in the serum of chimeric mice treated with LIC-pIC could not explain the potency of the LIC-pIC-induced antiviral response.

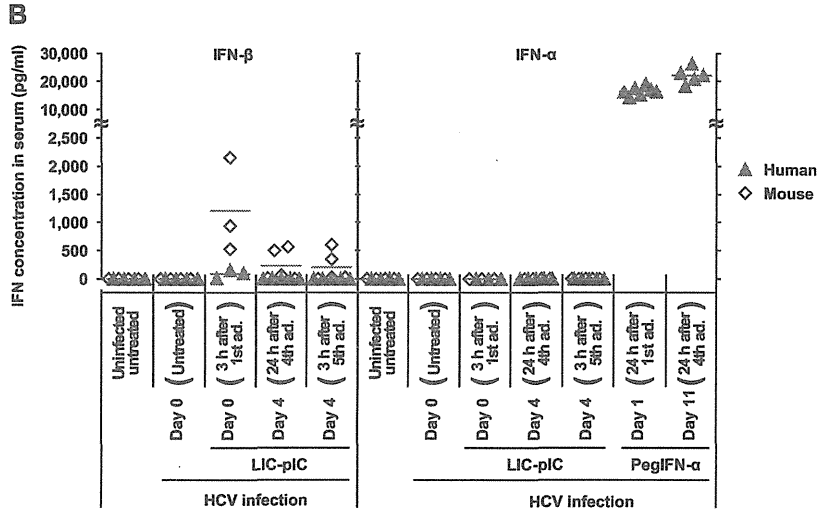
Although pIC is also known as an activator of natural killer (NK) or NKT cells [27,28], these cell types were not involved in the antiviral response elicited by LIC-pIC in the virus-infected chimeric mice of this study (**Figure S6**). These cumulative data indicated that an unknown mechanism was responsible for the observed antiviral response.

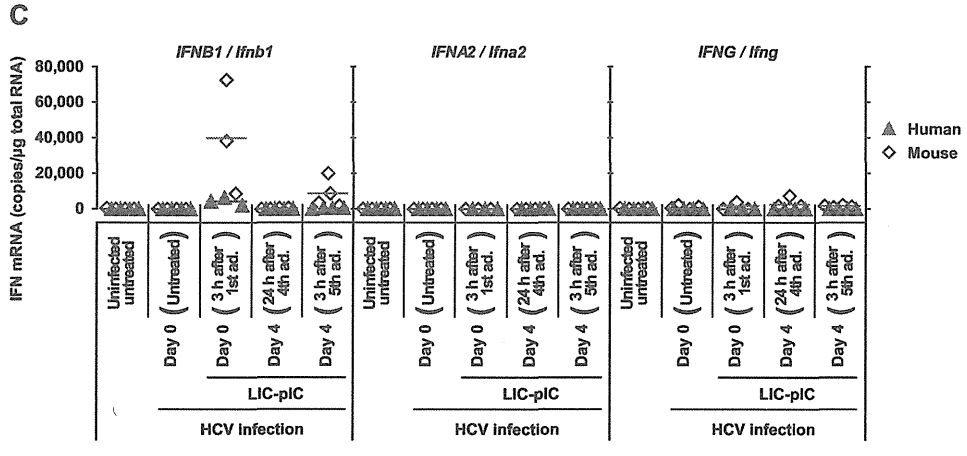
IFN-λs Mediate the Antiviral Response of Chimeric Mice with Humanized Livers to LIC-pIC

To determine the mechanism of the LIC-pIC-induced antiviral response, we performed comprehensive gene expression analysis to identify genes whose expression kinetics were consistent with the duration of this response. DNA microarray analysis of the livers of chimeric mice infected with HCV and treated, or not, with LIC-pIC identified type III IFN genes as having suitable expression kinetics (**Figure 3A and 3B**). There are three type III IFN genes in humans, *IFNL1*, *IFNL2*, and *IFNL3* (formerly *IL29*, *IL28A*, and *IL28B*, respectively), which encode IFN-λ1, IFN-λ2 and IFN-λ3 (also designated IL-29, IL-28A, and IL-28B), respectively [29,30]. The genes that were analyzed included those encoding IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-γ, and IFN-λ. Among these genes, the three IFN-λ-encoding genes were the only ones whose levels remained up-regulated 3 h after the fifth LIC-pIC administration to HCV-infected mice on Day 4 (**Figure 3B**). qRT-PCR demonstrated that, unlike *IFNB1*, the mRNA levels of human *IFNL1* (*IL29*), *IFNL2* (*IL28A*), and *IFNL3* (*IL28B*) were strongly induced in the livers of chimeric mice treated with LIC-pIC (**Figure 3C**). In addition, expression of human *IFNL1*, *IFNL2*, and *IFNL3* was induced at each LIC-pIC administration. Consistent with these gene expression results, ELISA analysis revealed that the serum levels of IFN-λ1 and IFN-λ2 were much higher than the level of IFN-β (**Figure 3D**). These results indicated that the expression of IFN-λs was strongly and persistently induced by LIC-pIC in human hepatocytes transplanted into chimeric mice.

We further investigated whether IFN-λs directly affected HCV or HBV replication. Indeed, recombinant human IFN-λ1, IFN-λ2, and IFN-λ3, when added to HCV replicon cells, suppressed virus replication in a concentration-dependent manner (**Figure S7**). Furthermore, chimeric mice dosed with IFN-λs had (by Day 8) serum HCV RNA levels that were significantly lower than those of the saline-treated control group (**Figure 3E**). Treatment with IFN-λ2 also reduced serum HBV DNA levels compared to those seen in the saline-treated control group by Day 7 (**Figure S8**). Furthermore, combination treatments with neutralizing antibodies against IFN-λ1 and IFN-λ2 (a treatment that neutralizes all three IFN-λ subtypes due to cross-reactivity (**Figure S9**)) attenuated the LIC-pIC-dependent reduction in HCV RNA levels in both serum and liver (**Figure 3F**). These results demonstrated that IFN-λs play a critical role in the antiviral response elicited in human hepatocytes by LIC-pIC.


A
Administration (LIC-pIC, 0.1 mg/kg)
1st
2nd
3rd
4th
5th
Day
0
1
2
3
4
3 h
3 h
Sampling (serum & liver)


B
IFN concentration in serum (pg/ml)
IFN-β
IFN-α
Human
Mouse
Uninfected untreated
Untreated
3 h after 1st ad.
24 h after 4th ad.
3 h after 5th ad.
24 h after 1st ad.
Day 0
Day 4
Day 1
Day 11
LIC-pIC
PegIFN-α
HCV infection


C
IFN mRNA (copies/μg total RNA)
IFNB1 / Ifnb1
IFNA2 / Ifna2
IFNG / Ifng
Human
Mouse
Uninfected untreated
Untreated
3 h after 1st ad.
24 h after 4th ad.
3 h after 5th ad.
Day 0
Day 4
LIC-pIC
HCV infection

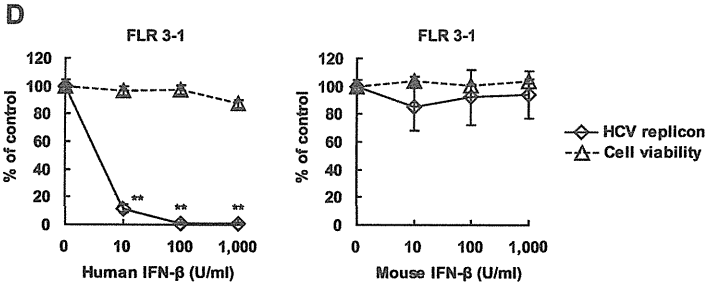

D
FLR 3-1
FLR 3-1
% of control
HCV replicon
Cell viability
Human IFN-β (U/ml)
Mouse IFN-β (U/ml)

Figure 2. Expression of genes encoding IFN-α, IFN-β, and IFN-γ following induction by LIC-pIC in chimeric mice with humanized livers. (**A**) Experimental schedule in chimeric mice infected with HCV. (**B**) Serum concentrations (in chimeric mice) of human and mouse IFN-β and IFN-α, as determined by ELISA ($n=3$–5 for no treatment or LIC-pIC group; $n=6$–8 for PegIFN-α group). Bars indicate the mean concentration of human (red) and mouse (green) IFNs averaged across the entire group. The results indicates the representative of two measurements (**C**) The liver mRNA levels of genes encoding IFN-β, IFN-α2 and IFN-γ (human genes: *IFNB1*, *IFNA2* and *IFNG*, respectively; mouse genes: *Ifnb1*, *Ifna2* and *Ifng*, respectively), as determined by qRT-PCR ($n=3$–4 per group). The bars indicate the mean mRNA levels of human (red) and mouse (green) IFN genes averaged across the entire group. The results indicates the representative of two measurements (**D**) Effectiveness of recombinant human or mouse IFN-β against HCV in FLR 3-1 HCV-replicon cells. The bars indicate SD ($n=4$ per group). $^{**}P<0.01$, treatments vs. the vehicle by Dunnett's multiple comparison test.
doi:10.1371/journal.pone.0059611.g002

The Induction of IFN-λs by LIC-pIC and Antiviral Effect in Humanized Chimeric Mice with Genetic Variants of IFNL3 (IL28B)

Patients with the G-allele of rs8099917, a SNP that is located near the *IFNL3* gene, are less likely to spontaneously clear acute HCV infection and respond to combination therapy with PegIFN-α and ribavirin [7,8,9,10]. We examined whether this genetic variation influenced the induction of IFN-λs in human hepatocytes and antiviral response to HCV. In the present study, LIC-pIC induced IFN-λ gene expression and anti-HCV effects to the same degree, even in chimeric mice harboring human hepatocytes derived from donors bearing the rs8099917 G-allele (**Figure S10A** and **10B**). These results indicate that neither induction of IFN-λs by LIC-pIC nor antiviral response was significantly influenced by this genomic polymorphism.

The Difference in Innate Immunity Induced by LIC-pIC in Human and Mouse Livers

The present study used chimeric mice with humanized livers, a model that permitted simultaneous analysis of human and mouse gene expression in the same liver. With LIC-pIC treatment, expression of IFN-λ-encoding genes was induced to levels greater than those of IFN-α- and IFN-β-encoding genes in human hepatocytes, whereas *Infb1* was induced to levels greater than expressions of IFN-λ-encoding genes in mouse hepatocytes (**Figure S11A**). Moreover, gene expression analysis indicated that the expression patterns of the genes encoding the corresponding IFN receptors differed between the human and mouse hepatocytes. Specifically, the genes encoding the IFN-α and IFN-β receptors were more strongly expressed in mouse hepatocytes (as *Ifnar1* and *Ifnar2*, respectively) than in human hepatocytes (as *IFNAR1* and *IFNAR2*, respectively), whereas the gene encoding the IFN-λ receptor was more strongly expressed in human hepatocytes (as *IFNLR1* (formerly *IL28RA*)) than in mouse hepatocytes (as *Ifnlr1* (formerly *Il28ra*)) (**Figure S11B**, **S11C**, and **S11D**). These results strongly suggest that IFN-λs play a critical role in the immune response by human hepatocytes, while IFN-α and IFN-β are important for the response by mouse hepatocytes.

IFN Expression in Human Cell Lines Following the Induction of Innate Immunity with Various Stimuli

To provide a comparison with the innate immune response of human hepatocytes, we investigated the role of IFNs in cell lines derived from other tissues. For this purpose, we examined the expression levels of the genes encoding IFN-λ and IFN-β following treatment with LIC-pIC in several human cell lines. In response to treatment with LIC-pIC, *IFNL1* mRNA was more strongly induced in HepG2 cells, a hepatocyte cell line, than in HEK293T or MRC-5 cells, kidney and fibroblast cell lines, respectively; the opposite expression pattern was observed for the induction of *IFNB1* mRNA (**Figure 4A**). Indeed, the protein level of IFN-λ1 in cell culture medium was also much higher in HepG2 cells than in MRC-5 cells (**Figure 4B**). Interestingly, whereas LIC-pIC induction of *IFNL1* mRNA and IFN-λ1 protein was weaker in MRC-5 cells than in HepG2 cells, the levels of *IFNL1* mRNA and IFN-λ1 protein in MRC-5 cells were almost comparable to those seen in HepG2 cells upon infection with Newcastle disease virus (NDV) or vesicular stomatitis virus (VSV) (**Figure 4C and 4D**). These data suggest that the *IFNL1*/IFN-λ1 expression profile induced by LIC-pIC was different from that induced upon viral infection. Using siRNA technology, we found that the expression of *IFNL1* induced by LIC-pIC was mediated by both MAVS (the adaptor molecule that links RIG-I-like receptor signaling pathways) and TICAM-1 (a TLR adaptor molecule) (**Figure 4E and 4F**). These results suggest that the innate immune response of human hepatocytes has unique characteristics in terms of IFN responses, and that LIC-pIC can induce robust production of IFN-λs by human hepatocytes through recognition by two distinct innate immune molecules (**Figure 4G**).

Discussion

In this study, we demonstrated that treatment of chimeric mice (harboring human hepatocytes) with LIC-pIC resulted in the efficient induction of innate immunity that was associated with robust production of IFN-λs and resulted in the abrogation of infection by hepatotropic viruses such as HCV and HBV. Recently, two different groups, Marukian *et al.* [5] and Thomas *et al.* [6], have reported that HCV infection results in production of IFN-λs in primary human hepatocytes and in the livers of chimpanzees, providing control of HCV replication. In the present study, we extend those analyses by showing that the activation of the intrinsic immune system in human hepatocytes induces the robust production of IFN-λs, thereby providing efficient defense against viral pathogens. Furthermore, we show that neither induction of IFN-λs by LIC-pIC nor associated antiviral effects are influenced significantly by a genomic polymorphism near the *IFNL3* gene. In contrast, the efficacy of combination therapy of PegIFN-α and ribavirin varies with genomic variations at this site. More recently, Prokunina-Olsson *et al.* have demonstrated that a dinucleotide variant, ss469415590 (TT or ΔG), located upstream of *IFNL3* is strongly associated with HCV clearance, and that its frameshift variant ss469415590[ΔG] creates a new gene, termed *IFNL4* [31]. These findings may provide a new approach to investigate whether treatment with LIC-pIC induces *IFNL4* and whether the antiviral effects of LIC-pIC are influenced by the induction of *IFNL4*.

To date, several mouse studies have indicated that IFN-λs play an important role in host defense against pathogens in vaginal, intestinal, and tracheal epithelial cells [32,33,34], but that these IFN molecules do not contribute to innate immunity against hepatotropic viruses [35]. In the present study, we showed that LIC-pIC treatment activated the innate immune response of human hepatocytes in chimeric mouse liver, providing robust expression of the IFN-λ-encoding gene without significantly inducing expression of the IFN-β-encoding gene. Moreover, we showed that human hepatocytes expressed the IFN-λ receptor-

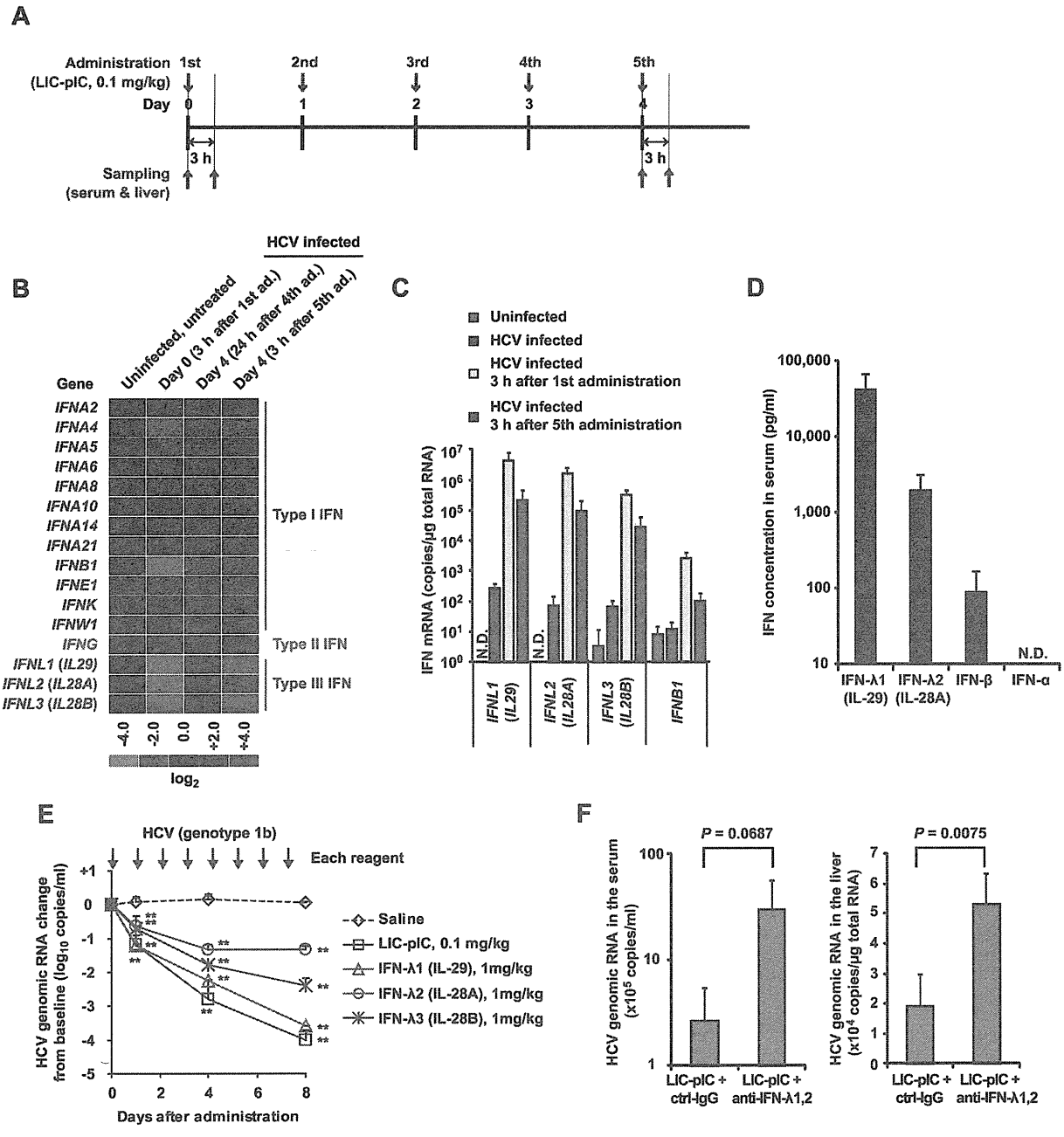


Figure 3. IFN expression following induction of innate immunity by LIC-pIC. (**A**) Experimental schedule in the chimeric mice infected with HCV. (**B**) Microarray analysis of IFN family gene expression. Log_2 ratio values of gene expression were calculated with respect to control (untreated and HCV-infected) chimeric mice. (**C**) Liver mRNA levels of human IFN genes ($n = 3–5$ per group), as determined by qRT-PCR. (**D**) Serum concentration of human IFN-λ1, IFN-λ2, IFN-β, and IFN-α ($n = 3$ per group), as determined by ELISA. (**E**) Serum HCV RNA levels, relative to the baseline, in HCV-infected chimeric mice treated with human IFN-λ, LIC-pIC, or control saline ($n = 3–5$ per group), as determined by qRT-PCR. (**F**) The HCV RNA levels in the serum and liver of HCV genotype 1a-infected chimeric mice collected on Day 4 of LIC-pIC treatment, as determined by qRT-PCR. The mice were additionally treated with either a control antibody or a neutralizing antibody against IFN-λ ($n = 3$ per group). Bars indicate SD. $^{**}P<0.01$, treatments vs. saline controls on the respective day, by Dunnett's multiple comparison test.
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encoding genes at high levels, while expression of the IFN-β receptor-encoding genes remained low. In contrast, expression of the IFN-λ receptor-encoding genes was low and expression of the IFN-β receptor-encoding genes was high in mouse hepatocytes from the same livers. Thus, human hepatocytes (in contrast to mouse hepatocytes) are rich sources of IFN-λs, while IFN-α or IFN-β are produced at lower levels in these cells. Indeed, the expression (in humanized livers) of these IFN-λ-encoding genes

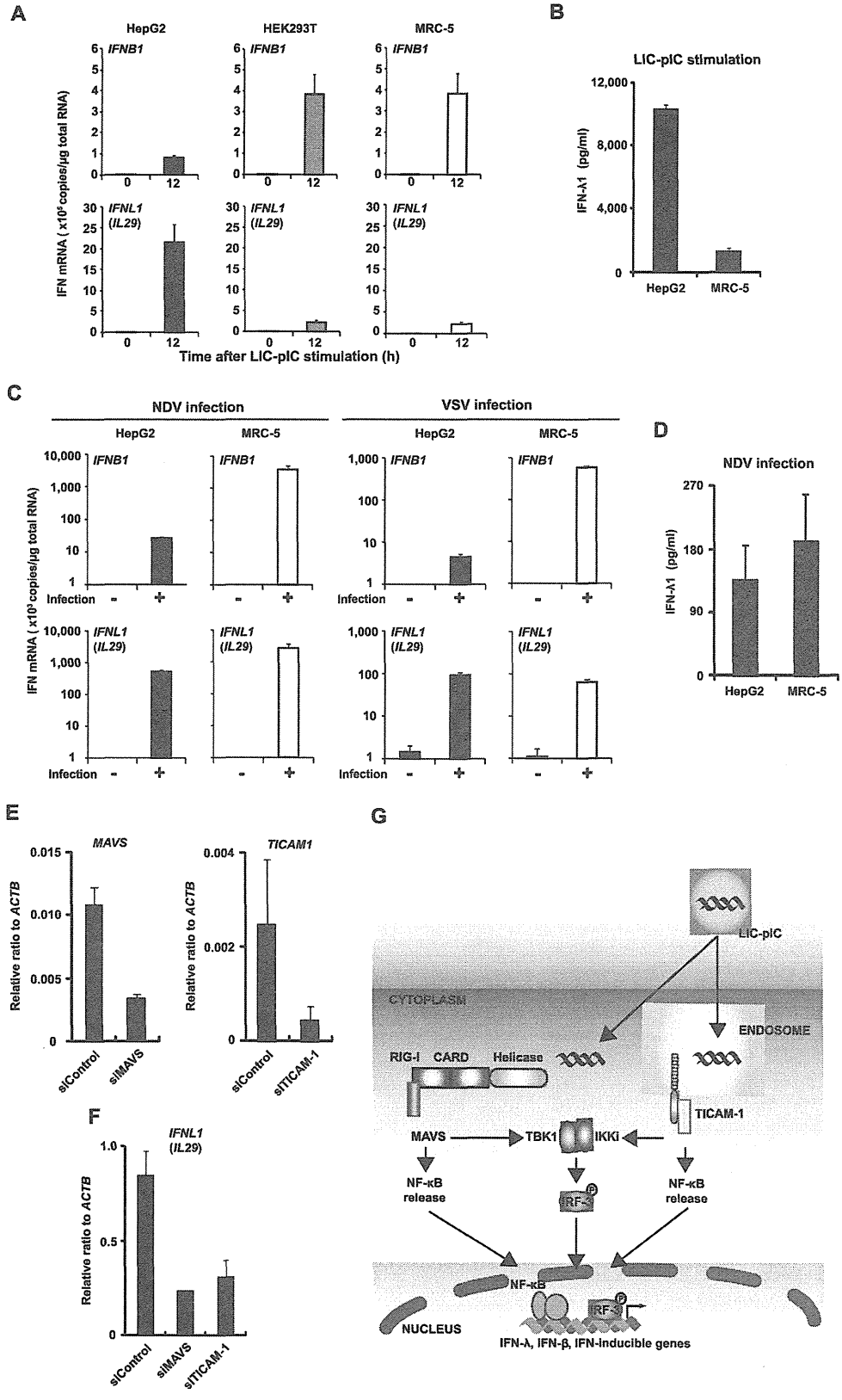
A
HepG2
HEK293T
MRC-5
IFNB1
IFNL1 (IL29)
IFN mRNA (x10³ copies/μg total RNA)
Time after LIC-pIC stimulation (h)
B
LIC-pIC stimulation
IFN-λ1 (pg/ml)
HepG2
MRC-5
C
NDV infection
VSV infection
HepG2
MRC-5
IFN mRNA (x10³ copies/μg total RNA)
Infection
D
NDV infection
IFN-λ1 (pg/ml)
E
MAVS
TICAM1
Relative ratio to ACTB
siControl
siMAVS
siTICAM-1
F
IFNL1 (IL29)
G
LIC-pIC
CYTOPLASM
ENDOSOME
RIG-I
CARD
Helicase
TICAM-1
MAVS
TBK1
IKKi
NF-κB release
IRF-3
NF-κB
NUCLEUS
IFN-λ, IFN-β, IFN-inducible genes

Figure 4. IFN expression in cell lines following the induction of innate immunity by various stimuli. (**A**, **B**) The indicated cell lines were stimulated by exposure to LIC-pIC (1 μg/ml). (**A**) The mRNA levels of IFN-β- and IFN-λ1-encoding genes (*IFNB1* and *IFNL1* (*IL29*), respectively) before and after (12 h) stimulation, as determined by qRT-PCR. (**B**) The concentration of IFN-λ1 (IL-29) in the culture medium 24 h after stimulation, as determined by ELISA. (**C**, **D**) The indicated cell lines were stimulated by viral infection with NDV or VSV. (**C**) The mRNA levels of IFN-β- and IFN-λ1-encoding genes (*IFNB1* and *IFNL1* (*IL29*), respectively) in HepG2 cells and MRC-5 cells in the absence or presence (12 h) of NDV or VSV, as determined by qRT-PCR. (**D**) The concentration of IFN-λ1 protein in the culture medium at 24 h post-infection, as determined by ELISA. (**E**, **F**) Expression in HepG2 cells of genes encoding MAVS, TICAM-1, and IFN-λ1 (*MAVS, TICAM1,* and *IFNL1* (*IL29*) respectively), as determined by qRT-PCR, after 12 h stimulation with LIC-pIC (1 μg/ml). Cells were pre-treated for 48 h with siRNAs against *MAVS, TICAM-1,* or a non-target control. Bars indicate SD ($n = 3$ per group). (**G**) Schematic outline of the proposed mechanism of the antiviral response induced by LIC-pIC.
doi:10.1371/journal.pone.0059611.g004

remained upregulated through at least five daily LIC-pIC administrations to chimeric mice infected with HCV. Consistent with this bias in IFN production, human hepatocytes were expected to be more receptive to stimulation by IFN-λs than their mouse counterparts.

We demonstrated the robust induction (by LIC-pIC) of IFN-λ rather than IFN-α or IFN-β in human hepatocytes. In several human cell lines derived from other tissues, IFN-λ1 induction by LIC-pIC stimulation was reduced in comparison to that seen in human hepatocytes, indicating that induction of IFN-based innate immunity differs among tissues and that human hepatocytes are high producers of IFN-λs. We also demonstrated that NDV and VSV, ssRNA viruses that are recognized by cytoplasmic sensors (RIG-I-like receptors) [36], could induce the expression of IFN-λs in several human cell lines. However, in contrast to LIC-pIC stimulation, the expression level of IFN-λ1 induced by these viruses was as low in HepG2 hepatocyte cells as in MRC-5 fibroblast cells. These results motivated us to investigate which sensors recognize LIC-pIC resulting in robust IFN-λ production. The presence of two distinct viral sensing innate immune pathways (TLR3 and RIG-I-like receptors) in human hepatocytes has been reported [37,38]. We showed here that the effects of LIC-pIC were mediated by both MAVS, a signaling adaptor molecule of RIG-I-like receptors, and TICAM-1, a TLR adaptor molecule. Given that both NDV and VSV effects are mediated by MAVS, we speculate that the strong induction of IFN-λs by LIC-pIC reflects dual recognition of LIC-pIC by RIG-I-like receptors and TLR3. In this context, we note that both MAVS and TICAM-1 are direct targets for proteolytic degradation by the HCV-encoded NS3/4A protease [39,40]. Related reports [39,40,41] suggest that the inhibitory effect of this viral protease may affect the induction of IFN-λs, particularly in HCV-infected hepatocytes. However, we observed significantly higher levels of IFN-λ expression in response to treatment with LIC-pIC, regardless of *in vivo* HCV infection status (Fig. 3 and data not shown), suggesting that the *in vivo* induction of IFN- λs by LIC-pIC treatment may not be affected by this viral protease in our experimental system. Further analyses will be needed to clarify the balance between viral evasion and activation of the innate immune response.

Our results demonstrate that the dominant IFN systems exhibit tissue- and animal-specific induction patterns, and that IFN-λs plays a critical role in innate immune response in human hepatocytes. These findings expand our understanding of the role of IFN-λs in the human immune system.

Supporting Information

Figure S1 Hepatotropic property of LIC. Biodistribution of RNA following administration of a complex of LIC and ^{3}H-labeled short dsRNA to normal mice.
(EPS)

Figure S2 Time course studies in 3 mice inoculated with HCV genotype 1b or HBV genotype C. (**A**) HCV RNA levels in chimeric mouse serum after inoculation. (**B**) HBV DNA in chimeric mouse serum after inoculation.
(EPS)

Figure S3 Hepatotoxicity of the LIC-pIC complex. (**A**, **B**) Chimeric mice infected with HCV genotype 1a were treated daily for 8 days (Days 0–7) with LIC-pIC at 0.01, 0.03, or 0.1 mg/kg. The sera of chimeric mice were collected on the indicated days following the initial administration of LIC-pIC and the concentration of human albumin (**A**) or alanine transferase (ALT) (**B**) was determined ($n = 3$–5 per group).
(EPS)

Figure S4 Expression of IFN-α, IFN-β, and IFN-γ in HBV-infected chimeric mice with humanized livers following treatment with LIC-pIC. (**A**) Schedule of the LIC-pIC treatments and serum or liver sampling in the HBV-infected chimeric mice. (**B**) Serum concentrations of human and mouse IFN-β and IFN-α, as determined by ELISA. (**C**) The liver mRNA levels of genes encoding IFN-β, IFN-α2 and IFN-γ (human genes: *IFNB1*, *IFNA2* and *IFNG*, respectively; mouse genes: *Ifnb1*, *Ifna2* and *Ifng*, respectively), as determined by qRT-PCR. For (**B**) and (**C**), each point indicates the value for a single chimeric mouse; groups consisted of 1–4 mice each.
(EPS)

Figure S5 Expression of genes encoding human IFN-β (*IFNB1*) and mouse IFN-β (*Ifnb1*) in HCV-infected or uninfected chimeric mice with humanized livers following treatment with LIC-pIC, as determined by qRT-PCR. The data points indicate the mRNA levels in individual chimera; the horizontal bars indicate the mean transcript concentrations for the human (red) and mouse (green) genes ($n = 3$ per group).
(EPS)

Figure S6 Mouse NK and NKT cells are not involved in the anti-HCV effects of LIC-pIC. Chimeric mice with humanized livers were infected with HCV genotype 1b and treated for 14 days (Days 0–13) with one of the following: saline, daily; 1 μg/kg α-galactosylceramide (αGalCer, a specific activator of NKT cells), weekly (Days 0 and 7); or 0.1 mg/kg LIC-pIC, daily. Half of the chimeras receiving the LIC-pIC treatment were pre-treated with a TM-β1 antibody in order to deplete NK and NKT cells. The other half were left untreated (ctrl). The serum HCV genomic RNA levels were determined using qRT-PCR on the indicated days after the initial treatment was administered. Data points indicate mean values and the bars in---dicate SD ($n = 4$–5 per group). *$P<0.05$ and **$P<0.01$, treatments vs. the saline control by Dunnett's multiple comparison test.
(EPS)

Figure S7 The anti-HCV response elicited by IFN-λ in HCV replicon cells. FLR3-1 HCV replicon cells were treated with the indicated recombinant human IFN-λs or with saline control for 72 h, and HCV replication (diamonds) and cell viability (triangles) of the cells then were assessed. Values are

expressed as a percentage of the untreated control ($n = 4$ per group). Data are mean values and the bars indicate SD. $^{**}P<0.01$, IFN treatments vs. the vehicle by Dunnett's multiple comparison test.
(EPS)

Figure S8 The anti-HBV response elicited by IFN-λ. Serum HBV genomic DNA levels were assayed in chimeric mice infected with HBV and administered daily treatments of recombinant human IFN-λ2 or LIC-pIC. HBV levels were assayed on the indicated days after the initial treatments. Values are expressed relative to the baseline value. Data points indicate mean values and the bars indicate SD ($n = 3$–5 per group). $^{**}P<0.01$, treatments vs. the saline control by Dunnett's multiple comparison test.
(EPS)

Figure S9 Neutralizing activity of anti-human IFN-λ antibodies. FLR 3-1 HCV replicon cells were pre-treated with 1 μg/ml or 10 μg/ml of anti-human IFN-λ1 or anti-human IFN-λ2 antibodies before the addition of recombinant human IFN-λ1, IFN-λ2, or IFN-λ3 to the culture medium. The replication of HCV replicons was determined by luciferase assays (red bars), and cell viability was determined using a WST-8 assay (gray bars). Values are expressed as a percentage of the untreated control. Data points are mean values and the error bars indicate SD ($n = 4$ per group).
(EPS)

Figure S10 Induction of IFN-λs by LIC-pIC and antiviral effect in human hepatocytes with genetic variants near the *IFNL3* (*IL28B*) gene. Chimeric mice were transplanted with human hepatocytes from donors bearing a T- or G-allele of the rs8099917 (*IFNL3*-proximal) SNP. Animals were infected with HCV genotype 1b and treated daily with LIC-pIC. (**A**) The liver mRNA levels of human IFN-λ-encoding genes (*IFNL1*, *IFNL2*, and *IFNL3* (also known as *IL29*, *IL28A*, and *IL28B*, respectively)) were assayed by qRT-PCR. (**B**) The serum HCV genomic RNA levels were assayed by qRT-PCR. Values are expressed relative to the baseline value. Data points are presented as mean values with SD ($n = 1$–6 per group). $^{**}P<0.01$, treatments vs. the saline control by Dunnett's multiple comparison test.
(EPS)

Figure S11 The expression of mouse IFN genes and corresponding receptors in chimeric mice with humanized livers. (**A**) The liver mRNA levels of mouse IFN-encoding genes (*Ifnl2* (*Il28a*), *Ifnl3* (*Il28b*), and *Ifnb1*) ($n = 3$–5 per group). (**B**, **C**, **D**) The liver mRNA levels of human and mouse IFN receptor-encoding genes (mouse/human genes are *IFNAR1*/*Ifnar1*, *IFNAR2*/*Ifnar2*, and *IFNLR1*/*Ifnlr1* (*IL28RA*/*Il28ra*), respectively) ($n = 8$–9 per group) in uninfected chimeric mice (HCV-) and in chimeric mice infected with HCV genotype 1a (HCV+). Bars indicate SD. *P*-values were determined using unpaired *t*-tests.
(EPS)

Text S1 Supporting materials, methods, and references. Methods for "Biodistribution of nucleic acids complexed with LIC", "Measurement of human serum albumin", "Measurement of alanine aminotransferase", "Enzyme-linked immunosorbent assay", "Luciferase and WST-8 assays of HCV replicon cells", "Depletion of NK and natural killer T cells", "Activation of NKT cells", "Neutralizing activity of the anti-human IFN-λ antibody", and "SNP genotyping of *IFNL3* (*IL28B*)", along with supporting references, are provided in the Supporting Materials section.
(DOC)

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Author Contributions

Performed the experiments that investigated the antiviral effects of the LIC-pIC complex and the molecules that it induced: SN. Performed the analyses of type-III IFNs, both in vitro and in vivo: YH. Established the system for analysis of the type-III IFNs: Y. Tokunaga. Performed Microarray analysis: YN. Performed the in vitro analyses: TK AT. Conceived and designed the experiments: MK. Analyzed the data: MK. Contributed reagents/materials/analysis tools: KH JY TO CT Y. Tanaka MM KT-K KI MY. Wrote the paper: SN YH.

References

1. Kumar H, Kawai T, Akira S (2011) Pathogen recognition by the innate immune system. Int Rev Immunol 30: 16–34.
2. Akira S (2011) Innate immunity and adjuvants. Philos Trans R Soc Lond B Biol Sci 366: 2748–2755.
3. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, et al. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975–982.
4. Yuen MF, Hui CK, Cheng CC, Wu CH, Lai YP, et al. (2001) Long-term follow-up of interferon alfa treatment in Chinese patients with chronic hepatitis B infection: The effect on hepatitis B e antigen seroconversion and the development of cirrhosis-related complications. Hepatology 34: 139–145.
5. Marukian S, Andrus L, Sheahan TP, Jones CT, Charles ED, et al. (2011) Hepatitis C virus induces interferon-lambda and interferon-stimulated genes in primary liver cultures. Hepatology 54: 1913–1923.
6. Thomas E, Gonzalez VD, Li Q, Modi AA, Chen W, et al. (2012) HCV Infection Induces a Unique Hepatic Innate Immune Response Associated with Robust Production of Type III Interferons. Gastroenterology 142: 978–988.
7. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461: 399–401.
8. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, et al. (2010) Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 138: 1338–1345, 1345 e1331–1337.
9. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41: 1100–1104.
10. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
11. Hirabayashi K, Yano J, Inoue T, Yamaguchi T, Tanigawara K, et al. (1999) Inhibition of cancer cell growth by polyinosinic-polycytidylic acid/cationic liposome complex: a new biological activity. Cancer Res 59: 4325–4333.
12. Yano J, Hirabayashi K, Nakagawa S, Yamaguchi T, Nogawa M, et al. (2004) Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. Clin Cancer Res 10: 7721–7726.
13. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413: 732–738.
14. Yoneyama M, Fujita T (2009) RNA recognition and signal transduction by RIG-I-like receptors. Immunol Rev 227: 54–65.
15. Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, et al. (2001) Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 33: 981–988.
16. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, et al. (2001) Hepatitis C virus replication in mice with chimeric human livers. Nat Med 7: 927–933.
17. Nakagawa S, Umehara T, Matsuda C, Kuge S, Sudoh M, et al. (2007) Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem Biophys Res Commun 353: 882–888.
18. Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, et al. (2006) Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44: 915–924.
19. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, et al. (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 165: 901–912.

20. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, et al. (1999) Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116: 636–642.
21. Tanaka T, Inoue K, Hayashi Y, Abe A, Tsukiyama-Kohara K, et al. (2004) Virological significance of low-level hepatitis B virus infection in patients with hepatitis C virus associated liver disease. J Med Virol 72: 223–229.
22. Kashiwakuma T, Hasegawa A, Kajita T, Takata A, Mori H, et al. (1996) Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). J Immunol Methods 190: 79–89.
23. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, et al. (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 448: 501–505.
24. Sakamoto H, Okamoto K, Aoki M, Kato H, Katsume A, et al. (2005) Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol 1: 333–337.
25. Watanabe T, Sudoh M, Miyagishi M, Akashi H, Arai M, et al. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther 13: 883–892.
26. Minakuchi Y, Takeshita F, Kosaka N, Sasaki H, Yamamoto Y, et al. (2004) Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo. Nucleic Acids Res 32: e109.
27. Gidlund M, Orn A, Wigzell H, Senik A, Gresser I (1978) Enhanced NK cell activity in mice injected with interferon and interferon inducers. Nature 273: 759–761.
28. Djeu JY, Heinbaugh JA, Holden HT, Herberman RB (1979) Role of macrophages in the augementation of mouse natural killer cell activity by poly I:C and interferon. J Immunol 122: 182–188.
29. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, et al. (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 4: 69–77.
30. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, et al. (2003) IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4: 63–68.
31. Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, et al. (2013) A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 45: 164–171.
32. Pott J, Mahlakoiv T, Mordstein M, Duerr CU, Michiels T, et al. (2011) IFN-lambda determines the intestinal epithelial antiviral host defense. Proc Natl Acad Sci U S A 108: 7944–7949.
33. Iversen MB, Ank N, Melchjorsen J, Paludan SR (2010) Expression of type III interferon (IFN) in the vaginal mucosa is mediated primarily by dendritic cells and displays stronger dependence on NF-kappaB than type I IFNs. J Virol 84: 4579–4586.
34. Mordstein M, Neugebauer E, Ditt V, Jessen B, Rieger T, et al. (2010) Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. J Virol 84: 5670–5677.
35. Mordstein M, Kochs G, Dumoutier L, Renauld JC, Paludan SR, et al. (2008) Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. PLoS Pathog 4: e1000151.
36. Kumar H, Kawai T, Kato H, Sato S, Takahashi K, et al. (2006) Essential role of IPS-1 in innate immune responses against RNA viruses. J Exp Med 203: 1795–1803.
37. Li K, Chen Z, Kato N, Gale M Jr, Lemon SM (2005) Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J Biol Chem 280: 16739–16747.
38. Wang N, Liang Y, Devaraj S, Wang J, Lemon SM, et al. (2009) Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. J Virol 83: 9824–9834.
39. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, et al. (2005) Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A 102: 2992–2997.
40. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, et al. (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 437: 1167–1172.
41. Bellecave P, Sarasin-Filipowicz M, Donze O, Kennel A, Gouttenoire J, et al. (2010) Cleavage of mitochondrial antiviral signaling protein in the liver of patients with chronic hepatitis C correlates with a reduced activation of the endogenous interferon system. Hepatology 51: 1127–1136.

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ORIGINAL ARTICLE

Genomic polymorphisms in 3β-hydroxysterol Δ24-reductase promoter sequences

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ABSTRACT

It was recently reported by the present team that 3β-hydroxysterol Δ24-reductase (DHCR24) is induced by hepatitis C virus (HCV) infection. In addition, upregulation of DHCR24 impairs p53 activity. In human hepatoma HuH-7 cells, the degree of DHCR24 expression is higher than in normal hepatic cell lines (WRL68) at the transcriptional level. The genomic promoter sequence of DHCR24 was characterized and nucleotide substitutions were observed in HuH-7 cells at nucleotide numbers −1453 (G to A), −1420 (G to T), −488 (A to C) and −200 (G to C). The mutations of these sequences from HuH-7 cell types to WRL68 cell types suppressed DHCR24 gene promoter activity. The sequences were further characterized in hepatocytes from patient tissues. Four tissues from HCV-positive patients with cirrhosis or hepatocellular carcinoma (#1, 2, 3, 5) possessed HuH-7 cell type sequences. Interestingly, one patient with liver cirrhosis (#4) possessed WRL68 cell-type sequences; this patient had been infected with HCV and was HCV negative for 17 years after interferon therapy. Next, the effect of HCV infection on these polymorphisms was examined in humanized chimeric mouse liver and HuH-7 cells. The human hepatocytes possess WRL68 cell type and did not show the nucleotide substitution after HCV infection. The HCV-replicon was removed by interferon treatment and established the cured K4 cells. These cells possess HuH-7 cell type sequences. Thus, this study showed the genomic polymorphism in DHCR24 promoter is not directly influenced by HCV infection.

Key words 3β-hydroxysterol Δ24-reductase, hepatitis C virus, promoter.

Liver cancer is one of the most prevalent forms of cancer (1). More than 80% of cases occur in developing countries; however, Japan also has a remarkably high incidence (2). Among the primary liver cancers, HCC is the most common (3). Its incidence is increasing: between 1975 and 2005, age-adjusted HCC rates tripled (4).

One crucial cause of HCC is HCV infection (5). DHCR24, which functions as an oxidoreductase during cholesterol biosynthesis (6, 7), is linked to HCV-associated hepatocarcinogenesis and development of HCC (8–10). Infection of hepatocytes with HCV results in over-expression of DHCR24. This enzyme protects cells from oxidative stress and inhibits p53 activity (8), thus

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List of Abbreviations: DHCR24, 3β-hydroxysterol Δ24-reductase; DMEM, Dulbecco's modified Eagle's medium; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; SVR, sustained viral response.

contributing to the development of HCC (5). These facts prompted us to investigate whether the molecular features of *DHCR24* are linked to HCC development. To this end, we characterized the promoter region of *DHCR24* in HCC cell lines and clinical samples.

MATERIALS AND METHODS

Cell lines and growth conditions

HuH-7 and HepG2 cells were cultured in (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (Sigma-Aldrich). WRL68 cells were cultured in DMEM supplemented with 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 0.1 mM non-essential amino acids (Invitrogen) and 10% FCS. HuH-7 cell-based HCV replicon harboring cell lines (R6FLR-N) (11) were cured off HCV by interferon treatment (12) and designated as K4 cells.

Northern and western blotting

Northern and western blotting were performed as previously described (8).

Sequencing of genomic DNA and reporter plasmid construction

Genomic DNA was extracted from HuH-7 and WRL68 cells using standard methods. DNA from the promoter region of *DHCR24* (~5 kb) was amplified using PCR (sense primer: 5′-CACTCCTGCTCACCACTGAT-3′; antisense primer: 5′-GTAGTAGATATCGAAGATAAGCGAGAGCGG-3′). These fragments were individually cloned into the upstream region of the firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI, USA) at the *Xho*I and *Nco*I sites (as we had done previously for the HepG2 cell line) (6). DNA sequences were determined using standard methods. Reporter plasmids that possessed chimeric promoters were constructed using restriction enzyme sites for *Tth111*I (position −2160) and *Bss*HII (position −1030).

Dual luciferase reporter assay

Using Lipofectamine LTX (Invitrogen), HepG2 cells (1×10^4 cells/well in a 96-well plate) were transfected with a reporter plasmid (0.25 μg/well) together with an internal control plasmid (phRL-TK; 0.025 μg/well) encoding *Renilla* luciferase (Promega). Forty-eight hours after transfection, the cells were assayed with the Dual-Glo Luciferase Assay System (Promega). Luminescence was measured using a TriStar LB941 microplate reader (Berthold Technologies GmbH, Bad Wildbad, Germany).

Liver tissue samples from chimeric mice or patients infected with hepatitis C virus

Severely combined immunodeficient mice carrying human primary hepatocytes were purchased from BD BioSciences (Franklin Lakes, NJ, USA) and African American, male, 5-year-old, HCV negative mice from PhoenixBio (Hiroshima, Japan) (13). These "human liver chimeric" mice were inoculated or mock-inoculated with plasma collected from an HCV-positive (HCR6 strain (14), GenBank accession #AY045702) patient in accordance with the requirements of the Declaration of Helsinki. HCV infection in the mice thus infected was confirmed by using quantitative PCR for HCV mRNA as previously described (9). The protocols for the animal experiments were pre-approved by the local Ethics Committee, and the animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Informed consent for this clinical study was obtained from five patients with HCV (Table 1) at the Kumamoto University Hospital (Kumamoto, Japan), in accordance with the Helsinki Declaration prior to 2003, and the protocol was approved by the Regional Ethics Committee. LiverPool 20-donor pooled cryopreserved human hepatocytes (Celsis IVT, Baltimore, MD, USA) were purchased and used as the normal human liver tissue control. HCV RNA was detected by the COBAS TaqMan HCV test (Hoffman–La Roche, Basel, Switzerland). Liver

Table 1. Summary of patients with HCC

Patient ID	Sex	Age (years)	Diagnosis	ALT (IU/mL)	Outcome of IFN treatment	HCV RNA detection[†]
#1	F	60	LC	22	NR	+
#2	M	65	LC	31	NT	+
#3	F	57	LC	24	NR	+
#4	M	61	LC, HCC	12	SVR	−[‡]
#5	M	51	LC, HCC	91	NR	+

[†]Serum was tested for HCV RNA using quantitative PCR; [‡]In 1995, #4 was diagnosed with HCV-associated LC and HCC and HCV RNA was detected in his serum. As a result, #4 was treated with IFN. Since then, no HCV RNA has been detected in this patient's serum (>17 years). ALT, alanine aminotransferase; F, female; LC, liver cirrhosis; M, male; NR, no response; NT, not treated.

tissue was obtained from either mice or patients and processed for DNA sequencing. Two DNA fragments (corresponding to positions −1600 to −1292 and −631 to −86) were amplified using PCR with Tth-Bss forward and reverse primers (5′-ATTTCAACATGTCATTAACA-3′ and 5′-TTCTAGCACGGTGCTTTGTG-3′) and Bss-Nco forward and reverse primers (5′-CCAGCCA-TAGCCTTCCATG-3′ and 5′-AATGGCGAGCCGCGC CGG-3′), respectively. The amplified fragments were directly sequenced using the same set of primers.

Statistical analysis

Student's *t*-test was used to test the statistical significance of the results. P values of < 0.05 were considered statistically significant.

RESULTS

First, we measured DHCR24 expression in cell lines of noncancerous hepatocytes (WRL68) and hepatoma cells (HuH-7 and HepG2). Compared with noncancerous hepatocytes, DHCR24 expression in the two hepatoma cell lines was considerably increased with respect to both mRNA and protein (Fig. 1a, b). In addition, the different culture media used for the WRL68 and HuH-7 cells did not significantly influence the degree of expression of DHCR24 protein (Fig. 1c).

To identify the genetic characteristic(s) that govern DHCR24 upregulation, we isolated genomic DNA from these three cell lines and sequenced the *DHCR24* promoter region (nucleotide positions −4976 to +113, where +1 indicates the transcription start site). For this analysis, we sequenced three molecular clones from each cell line. Alignments of WRL68 and HuH-7 sequences showed different nucleotides at four positions: (i) an A to G switch at −1453 (i.e., A in WRL68 and G in HuH-7); (ii) a T to G switch at −1420; (iii) a C to A switch at −488; and (iv) a C to G switch at −200 (Fig. 2). The two hepatoma cell lines (HuH-7 and HepG2) had no nucleotide differences within these regions.

Next, we investigated whether these small changes in the promoter sequence affect gene expression in a heterologous context. We constructed reporter plasmids that placed the firefly luciferase gene under the control of *DHCR24* promoter sequences (either from HuH-7 or WRL68 cells) (Fig. 3a). We measured the promoter activity of each construct in HepG2 cells with dual-luciferase assays. The *DHCR24* promoter derived from HuH-7 cells showed significantly greater activity (i.e., induced greater expression) than the WRL68 promoter (Fig. 3b). We also constructed two reporter plasmids that contained chimeric promoters. In each of these chimeras,

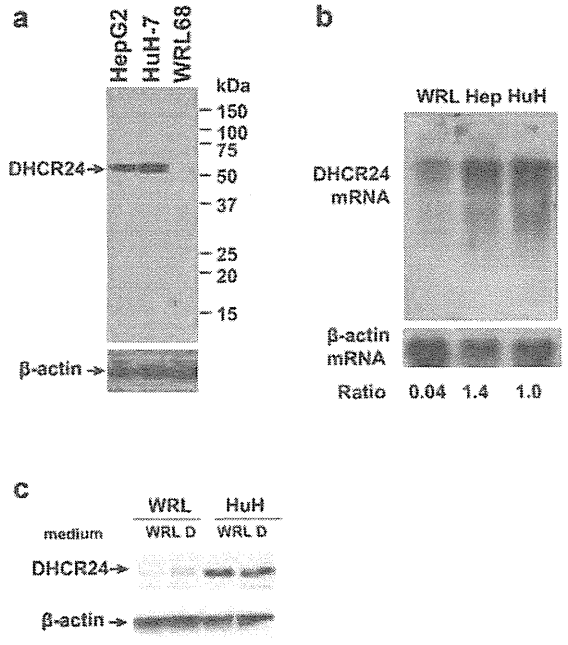

Fig. 1. Expression of DHCR24 in hepatoma cell lines. (a) Lysates from WRL68, HuH-7 and HepG2 cells were subjected to western blot analysis using antibodies directed against DHCR24 (upper panel) and β-actin (lower panel). (b) RNA was extracted from WRL68, HepG2 and HuH-7 cells and subjected to northern blot analysis using probes specific for *DHCR24* (upper panel) and β-actin (lower panel). Band intensities were quantified with a densitometer. Relative band intensity ratios (*DHCR24*/β-actin) are indicated below the gel images (the ratio for HuH-7 cells was set at 1). (c) DHCR24 protein (upper panel) and β-actin (lower panel) were detected in WRL 68 or HuH-7 cells with culture media for WRL68 cells (DMEM, 1 mM sodium pyruvate and 1 mM nonessential amino acids) or HuH-7 cells (DMEM alone).

we replaced HuH-7 fragments containing two polymorphisms with wild-type WRL68 sequences (Fig. 3a). These chimeric promoters had less activity than did intact promoters from both HuH-7 and WRL68 cells (Fig. 3b). These results indicate that the *DHCR24* promoter from HuH-7 cells contributes to the strong degree of *DHCR24* expression. In addition, all four nucleotide sequences of HuH-7 cell type in promoter fragments might be important for strong promoter activity.

Thereafter, we examined whether polymorphisms within the *DHCR24* promoter could be detected in clinical samples. We collected samples of liver tissue from five patients infected with HCV (Table 1) and sequenced the *DHCR24* promoter region (Table 2). Of the five samples tested, four (#1–3 and #5) showed all four of the polymorphisms associated with strong promoter activity (i.e., G, G, A and G nucleotides at positions −1453, −1420, −488, and −200). In contrast, promoter

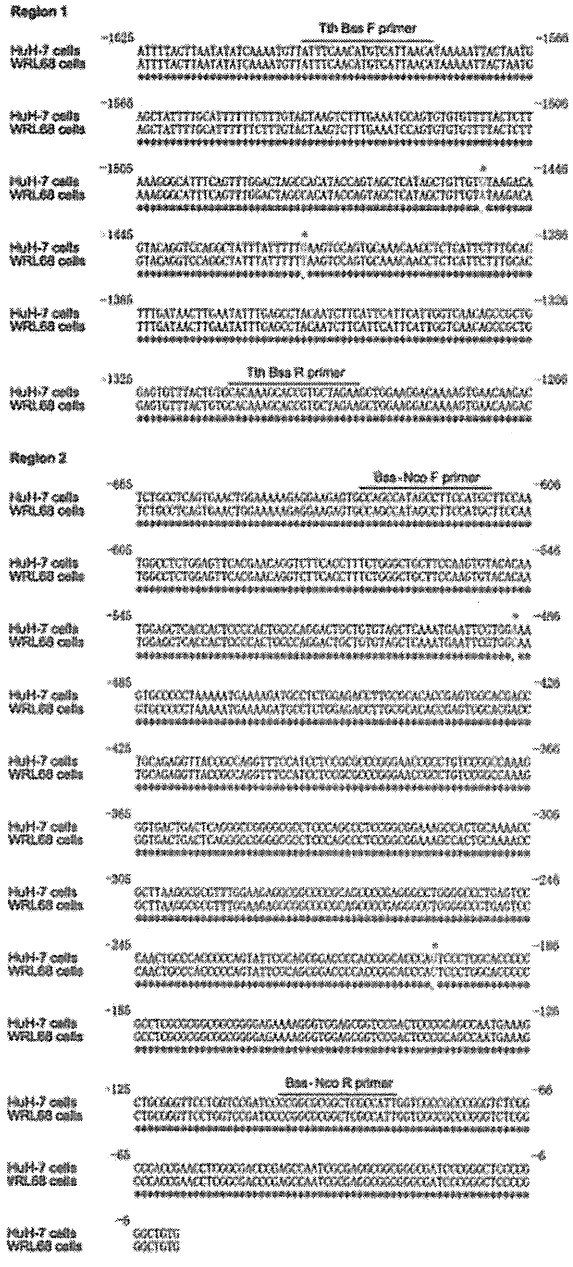

Fig. 2. Alignment of *DHCR24* promoter sequences. Nucleotide sequences from *DHCR24* promoter regions obtained from HuH-7 and WRL68 cells are shown. Cell-type-specific differences between these sequences (at positions −1453, −1420, −488, and −200) are indicated by asterisks and colors (red and blue represent nucleotides in HuH-7 and WRL68 cells, respectively). Positions of primer sequences are indicated.

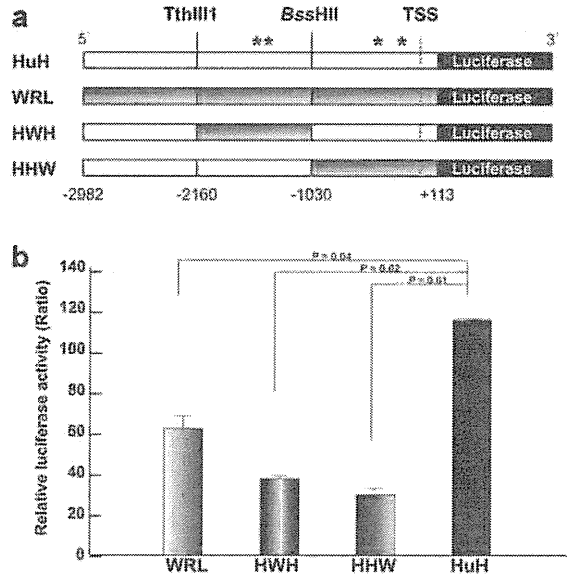

Fig. 3. Effect of nucleotide changes on *DHCR24* promoter activity. (a) Schematic diagrams of reporter constructs. Intact or chimeric *DHCR24* promoter sequences were used to drive the expression of firefly luciferase. Fragments of DNA derived from HuH-7 and WRL68 cells are colored white and grey, respectively. The asterisks indicate the position of each nucleotide polymorphism. Restriction enzyme sites (*Tth111I* and *Bss*HII) and transcription start sites are indicated. (b) Promoter activity of reporter constructs. HepG2 cells were transfected with the indicated reporter construct together with a control plasmid encoding *Renilla* luciferase. The relative ratio of firefly/*Renilla* luciferase activity is shown. Error bars indicate the standard deviation of two independent experiments. Each experiment was performed in triplicate. TSS, transcription start sites.

Table 2. Summary of nucleotide substitutions within the *DHCR24* promoter region

	Nucleotide position			
Origin of DNA sample	−1453	−1420	−488	−200
HuH-7 cells (high)†	G	G	A	G
WRL68 cells (low)‡	A	T	C	C
Patient #1	G	G	A	G
Patient #2	G	G	A	G
Patient #3	G	G	A	G
Patient #4	A	T	C	C
Patient #5 (NC)	G	G	A	G
Patient #5 (C)	G	G	A	G
20-donor pool§	A	T	C	C

†DHCR24 was expressed strongly in HuH-7 cells (Fig. 1), ‡DHCR24 was expressed weakly in WRL68 cells (Fig. 1), §Pooled normal human hepatocytes from a 20-donor pool. C, cancerous region; NC, non-cancerous region.

Table 3. Summary of nucleotide substitutions within the *DHCR24* promoter region with or without HCV infection

Origin of DNA sample	HCV	Nucleotide position			
		−1453	−1420	−488	−200
HuH-7 cells (high)	−	G	G	A	G
WRL68 cells (low)	−	A	T	C	C
Chimeric mouse liver	−	A	T	C	C
HCV infected chimeric mouse liver	+[†]	A	T	C	C
HCV replicon cells (R6FLR-N)	+	G	G	A	G
Cured K4 cells	+	G	G	A	G

[†]7.5×10^6 copies/mL of HCV in patient plasma was inoculated.

sequences from patient 4 (#4) had nucleotides associated with weak activity at these positions (i.e., A, T, C, and C). Intriguingly, only #4 exhibited an SVR, which is characterized by the absence of detectable HCV RNA in serum for >24 weeks following IFN treatment. The SVR status of #4 has persisted since 1995. In #5, promoter sequences were the same in cancerous and non-cancerous regions of the liver. These results suggest that the four polymorphisms within the *DHCR24* promoter region may influence the susceptibility to malignancy and IFN responsiveness of hepatoma cells and thus influence the fate of patients with HCC.

To assess the impact of HCV infection on genomic polymorphism in DHCR24 promoter sequences, we determined the sequences in human hepatocytes that had been transplanted into severely combined immunodeficient mice that we infected or mock-infected with HCV We detected markedly high titers of HCV only in the infected mice (Table 3). Sequencing revealed that all four polymorphic nucleotide positions were of the weak activity type. Notably, we detected no nucleotide differences between HCV- and mock-infected mice in the targeted regions (Table 3). We also established cured K4 cells by treating HCV replicon cells R6FLR-N with IFN. Analysis of the genomic sequence of these cell lines showed no nucleotide differences in R6-FLR-N and K4 cells (Table 3). These results suggest that the differences in the DHCR24 promoter sequence are ingenerate rather than induced by HCV infection.

DISCUSSION

In this study, we analyzed the promoter sequences associated with *DHCR24* in hepatocytes and identified polymorphisms that regulate the degree of expression of downstream genes (Figs. 1–3). #4 had an SVR in response to IFN treatment; thus these *DHCR24* promoter sequence polymorphisms are potential biomarkers for predicting patients' responsiveness to IFN treatment.

Genomic polymorphisms within the *DHCR24* promoter region may influence binding of transcription factors (Supplementary Fig. S1). In fact, a T-to-G nucleotide substitution at position −1420 generates a potential binding site for the protein encoded by the caudal homeobox gene (*CdxA*), a homeobox transcription factor responsible for gastrointestinal tract development and epithelial differentiation (15). A C-to-A substitution at position −488 generates potential binding sites for nuclear factor kappa-light-chain enhancer of activated B cells and STATx (16), as well as a low-affinity binding site for Nkx-2 (17). Finally, a C-to-G substitution at position −200 potentially abolishes a p300 binding site (18). These changes in transcription factor binding affinities could upregulate *DHCR24* expression, thereby promote carcinogenesis.

Previously, we discovered that DHCR24 is a host factor involved in HCV-associated development of HCC (8, 9). This protein is upregulated by HCV infection (8), and reduced degrees of expression (via siRNA knockdown) inhibit HCV replication (9). These findings are consistent with the role of DHCR24 in cholesterol biosynthesis (6, 7), which is important for HCV replication (19). Also, because the efficiency of HCV replication might have been lower in #4 than in other patients with strongly active DHCR24 promoter, the weak DHCR24 expression in this patient (Supplementary Fig. S2) might have contributed to the efficacy of IFN treatment.

In conclusion, we have discovered polymorphisms in the promoter region of DHCR24 gene that have not been induced by HCV infection. Future study will clarify their biological significance.

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DISCLOSURE

The authors have no financial relationships to disclosure.

REFERENCES

1. Wong C.M., Ng I.O. (2008) Molecular pathogenesis of hepatocellular carcinoma. *Liver Int* **28**: 160–74.
2. Center M.M., Jemal A. (2011) International trends in liver cancer incidence rates. *Cancer Epidemiol Biomarkers Prev* **20**: 2362–8.
3. Perz J.F., Armstrong G.L., Farrington L.A., Hutin Y.J., Bell B.P. (2006) The contributions of hepatitis B virus and hepatitis C

virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* **45**: 529–38.
4. Altekruse S.F., McGlynn K.A., Reichman M.E. (2009) Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J Clin Oncol* **27**: 1485–91.
5. Farazi P.A., DePinho R.A. (2006) Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* **6**: 674–87.
6. Greeve I., Hermans-Borgmeyer I., Brellinger C., Kasper D., Gomez-Isla T., Behl C., Levkau B., Nitsch R.M. (2000) The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress. *J Neurosci* **20**: 7345–52.
7. Wu C., Miloslavskaya I., Demontis S., Maestro R., Galaktionov K. (2004) Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. *Nature* **432**: 640–5.
8. Nishimura T., Kohara M., Izumi K., Kasama Y., Hirata Y., Huang Y., Shuda M., Mukaidani C., Takano T., Tokunaga Y., Nuriya H., Satoh M., Saito M., Kai C., Tsukiyama-Kohara K. (2009) Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol delta24-reductase. *J Biol Chem* **284**: 36442–52.
9. Takano T., Tsukiyama-Kohara K., Hayashi M., Hirata Y., Satoh M., Tokunaga Y., Tateno C., Hayashi Y., Hishima T., Funata N., Sudoh M., Kohara M. (2011) Augmentation of DHCR24 expression by hepatitis C virus infection facilitates viral replication in hepatocytes. *J Hepatol* **55**: 512–21.
10. Saito M., Kohara M., Tsukiyama-Kohara K. (2012) Hepatitis C virus promotes expression of the 3beta-hydroxysterol delta24-reductase through Sp1. *J Med Virol* **84**: 733–46.
11. Watanabe T., Sudoh M., Miyagishi M., Akashi H., Arai M., Inoue K., Taira K., Yoshiba M., Kohara M. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. *Gene Ther* **13**: 883–92.
12. Blight K.J., McKeating J.A., Rice C.M. (2002) Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* **76**: 13001–14.
13. Mercer D.F., Schiller D.E., Elliott J.F., Douglas D.N., Hao C., Rinfret A., Addison W.R., Fischer K.P., Churchill T.A., Lakey J.R., Tyrrell D.L., Kneteman N.M. (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**: 927–33.
14. Inoue K., Umehara T., Ruegg U.T., Yasui F., Watanabe T., Yasuda H., Dumont J.M., Scalfaro P., Yoshiba M., Kohara M. (2007) Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice *in vivo*. *Hepatology* **45**: 921–8.
15. Hecht N.B. (1995) The making of a spermatozoon: a molecular perspective. *Dev Genet* **16**: 95–103.
16. Kim J., Sharma S., Li Y., Cobos E., Palvimo J.J., Williams S.C. (2005) Repression and coactivation of CCAAT/enhancer-binding protein epsilon by sumoylation and protein inhibitor of activated STATx proteins. *J Biol Chem* **280**: 12,246–54.
17. Shiojima I., Komuro I., Mizuno T., Aikawa R., Akazawa H., Oka T., Yamazaki T., Yazaki Y. (1996) Molecular cloning and characterization of human cardiac homeobox gene CSX1. *Circ Res* **79**: 920–9.
18. Rikitake Y., Moran E. (1992) DNA-binding properties of the E1A-associated 300-kilodalton protein. *Mol Cell Biol* **12**: 2826–36.
19. Aizaki H., Lee K.J., Sung V.M., Ishiko H., Lai M.M. (2004) Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* **324**: 450–61.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1 Nucleotide differences between the two sequences of DHCR24 promoter are indicated by red asterisks and colors (red and blue represent diverged nucleotide sequences between HuH-7 and WRL68). Potential transcription factor binding sites are indicated that are: (1) common to HuH-7- and WRL68-derived sequences (black), (2) only found in HuH-7-derived sequences (red), and (3) only found in WRL68-derived sequences (blue). Arrows indicate potential binding sequences. Numbers in parentheses indicate the binding potential scores calculated with TFSEARCH.

Figure S2 DHCR24 protein (upper) in hepatocytes from patients 1–5 (P1–5) or actin (lower).