Completion of the entire hepatitis C virus life cycle in genetically humanized mice

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More than 130 million people worldwide chronically infected with hepatitis C virus (HCV) are at risk of developing severe liver disease. Antiviral treatments are only partially effective against HCV infection, and a vaccine is not available. Development of more efficient therapies has been hampered by the lack of a small animal model. Building on the observation that CD81 and occludin (OCLN) comprise the minimal set of human factors required to render mouse cells permissive to HCV entry1, we previously showed that transient expression of these two human genes is sufficient to allow viral uptake into fully immunocompetent inbred mice2. Here we demonstrate that transgenic mice stably expressing human CD81 and OCLN also support HCV entry, but innate and adaptive immune responses that transgenic mice stably expressing human CD81, SCARB1, CLDN1 and/or OCLN under the control of a liver-specific albumin promoter. Transgenic expression of the human orthologues of the HCV entry factors resulted in similar miRNA levels of the human and endogenous mouse genes in the murine liver (Supplementary Fig. 1) and expression of all four proteins (Supplementary Fig. 2) with the expected subcellular distribution in the liver (Supplementary Fig. 3). Next, we aimed to test the susceptibility of entry factor transgenic (EFT) mice to HCV infection. To identify founder lines supporting viral entry we took advantage of a previously generated, highly sensitive detection system which is based on the activation of a loxP-flanked STOP-luciferase reporter in the genome of ROSA26-Fluc mice by Cre recombinase encoded in recombinant HCV genomes. We crossed EFT mice to a ROSA26-Fluc background and challenged these animals with a bicistronic HCV genome expressing Cre (HCV-Cre). Consistent with previous data3, the bioluminescent reporter was activated in mice expressing human CD81 and OCLN (Fig. 1a and Supplementary Fig. 4a). The addition of human SCARB1 and CLDN1 (Supplementary Fig. 4b) did not increase the entry signal, demonstrating that their murine orthologues are functional for HCV entry in vivo. For subsequent experiments, founder lines Alb-hCD81/hOCLN#941 (2hEF) and Alb-hCD81/hSCARB1/hCLDN1/hOCLN#100 (4hEF), which supported the most efficient viral uptake (Supplementary Fig. 4a), were used. To estimate the number of HCV-infected liver cells, we used an indicator mouse strain in which Cre leads to activation of a nuclear-localized green fluorescent proteinβ-galactosidase (GNZ) reporter (ROSA26-GNZ)11. Similar to our previous observations2, HCV-Cre infection resulted in reporter activation in approximately 1–1.5% of murine hepatocytes in 2hEF or 4hEF mice (Fig. 1b and Supplementary Fig. 5). To provide additional evidence that viral uptake into EFT mice is mediated by the specific interaction of HCV glycoproteins with host entry factors, we administered antibodies directed against the HCV envelope glycoprotein complex E1E2 or the host entry factor CD81. Delivery of anti-human CD81 or anti-E1E2 (AR4A; ref. 12) antibodies resulted in a dose-dependent inhibition of HCV-Cre infection (Fig. 1c), whereas isotype control immunoglobulins had no effect. These data further affirm that HCV is taken up in vivo by a viral glycoprotein-specific fashion in vivo and underscore the utility of this model for evaluation of passive immunization strategies.

Direct measurement of HCV genome levels by quantitative reverse transcription (qRT)–PCR demonstrated a slight increase in HCV RNA in the serum (at 4 h) and liver (at 3 h and 24 h) of inoculated mice expressing the human entry factors; at 72 h, however, the signal was reduced to background levels (Fig. 1d, e). HCV infection resulted in the upregulation of several interferon-stimulated genes (Fig. 1f, g and Supplementary Fig. 6), infiltration of immune cells, especially natural killer (NK) cells, into the liver (Fig. 1i), and elevated proinflammatory cytokine levels in the serum (Fig. 1h and Supplementary Fig. 7), which could antagonize HCV replication. This hypothesis is further supported by the previous observation that HCV replications,
selectable HCV RNA genomes, replicate more efficiently in murine cells with impaired antiviral signalling.

To identify a murine environment more conducive to HCV replication, we crossed 4hEF ROSA26-Fluc mice to strains carrying targeted disruptions in Eif2ak2, Mavs, Ifi1, Ifr3, Ifr7, Stat1 or the Ifn-αβ receptor (Fig. 2). These strains are viable and known to be hypersusceptible to RNA viruses, due to impaired innate immune responses. The luminescent reporter signal was slightly elevated during the early phase after infection with HCV-Cre in most EFT strains impaired in innate immunity (Fig. 2a–d). Between 20 and 40 days after infection there was a marked increase in the luciferase signal, particularly in Ifr1 (eightfold), Ifr7 (16-fold) (Fig. 2c), Ifn-αβR (20-fold) and STAT1 (40-fold) deficient mice (Fig. 2d) compared to non-transgenic littermate controls. The increased reporter signal, which presumably reflects a transient burst in viral replication and spread, eventually returned to background levels, possibly marking clearance of HCV-infected cells by the murine immune system. The elevated luminescent signal correlated with increases in serum HCV RNA levels at peak time points (Fig. 2e).

To validate that the elevated signal is indeed due to increased HCV RNA replication in mice with blunted antiviral immunity, we crossed cyclophilin A (CypA)-deficient mice (Ppia–/–) to the EFT ROSA26-Fluc Stat1–/– background. CypA is a critical host factor for HCV RNA replication in human cells. In Ppia–/– 4hEF ROSA26-Fluc Stat1–/– mice RNA signal was similar (Fig. 3a), whereas reporter activity in 4hEF mice deficient in MAVS or IRF9 (Fig. 3b) was significantly lower. A more pronounced effect was observed in 4hEF mice deficient in IRF7 (Fig. 3c), providing additional evidence that HCV RNA does indeed replicate in these mice.

Unambiguous detection of HCV antigens in situ is difficult. Therefore, we constructed a transgenic mouse line expressing a modified version of a previously described cell-based fluorescent reporter system to visualize infection directly in the liver of infected mice (Supplementary Fig. 8a). This highly sensitive reporter, the activity of which directly correlates with the level of HCV RNA replication, is based on cleavage-mediated overexpression of NS3-4A activated the reporter in a majority of cells (Supplementary Fig. 9), demonstrating the functionality of the reporter in vivo. Furthermore, treatment of 4hEF Stat1–/– mice with an NS3A inhibitor for 3 weeks after infection suppressed HCV RNA loads to below the limit of detection (Fig. 3e), providing additional evidence that HCV RNA does indeed replicate in these mice.
**Figure 3** | Visualization and genetic and pharmacological interference with HCV infection. a, b, Quantification of murine hepatocytes actively replicating HCV in wild-type, 4hEF and 4hEF Stat1−/− mice as measured by the HCV NS3-4a-dependent cleavage of the TagBFP–nlsMAVS transgenic reporter construct by ImageStream X analysis. c–e, Longitudinal HCV RNA levels and luciferase signal in 4hEF Stat1−/− mice lacking PPIA (c, d), and longitudinal HCV RNA levels in 4hEF Stat1−/− mice treated with an HCV NS5A inhibitor (BMS-790052) for 20 days (e). Data shown are mean ± s.d. of n = 10–18 mice from two independent experiments. **P < 0.01.

**Figure 4** | HCV infection in 4hEF Stat1−/− mice leads to immune activation. a–d, HCV RNA copies in serum (a, c) and liver (b, d) of 4hEF Stat1−/− mice during early (a, b) or late (c, d) infection with Con1/Jc1. e, Relative frequencies of the indicated lymphocyte subsets in spleens of wild-type, 4hEF, Stat1−/− or Stat1−/− 4hEF mice isolated at the indicated time points after infection with Con1/Jc1. f, Analysis of liver-infiltrating IFN-γ-producing CD3+CD8+ T cells of wild-type, 4hEF, Stat1−/− and 4hEF Stat1−/− mice after infection with Con1/Jc1. Data shown are mean ± s.d. of three independent experiments. ***P < 0.001.
in STAT1 sufficient mice, the signal persisted at least until day 10 in mice on a Stat1−/− background (Fig. 3b). Taken together, these results indicate that HCV can replicate in immunocompromised mice expressing human CD81 and OCLN and highlight the value of the model for studying HCV replication in vivo.

We further characterized viral kinetics in mice infected with a monocistronic cell-culture-derived HCV (HCVcc). HCV RNA rose approximately tenfold over the limit of quantification in serum (Fig. 4a, c) and liver (Fig. 4b, d) of EFT Stat1−/− mice as compared to non-transgenic Stat1−/− controls. Mice remained persistently infected for most of the observation period, with HCV RNA becoming nearly undetectable after 90 days (Fig. 4c, d). Sequence analysis of HCV RNA detected at late time points in mice infected with HCV-Cre revealed mutations in some viral genomes, but none was shared among the five mice that were analysed (Supplementary Fig. 12). Whether any of these are adaptive mutations that increase viral fitness in vivo will be the subject of future studies. HCV infection caused splenomegaly in some innate immune NK and IFN-γ responses, albeit potentially confounded by the STAT1 deficiency, which may contribute to eventual viral clearance.

To determine whether primary hepatocytes in EFT mice on immunodeficient backgrounds were capable of producing infectious virions, sera collected at day 40 after infection were used to inoculate naive Huh-7.5 cells. Infectious virus was detected in sera of 4hEF mice deficient for Stat1, Irf1 and Irf7 (Fig. 5a), consistent with the increased persistency of HCV RNA from Stat1−/− mice (Fig. 2c, d) and HCV RNA load (Fig. 2e) in these strains. In HCV-infected 4hEF Stat1−/− mice the livers of 4hEF Stat1−/− mice with a skewing towards an effector memory phenotype near the end of the time course (Supplementary Fig. 10c). These data indicate that HCV infection elicits cellular immune responses, albeit potentially confounded by the STAT1 deficiency, which may contribute to eventual viral clearance.

Percentage NS5a-positive cells (log10)

Figure 5 | Evidence for production of infectious particles. a, Stat1−/−, Irf1−/−, Irf3−/−, Irf7−/−, Irf9−/− and Mavs−/− mice expressing all four human HCV entry factors were infected with BiCre-Jc1. Sera were collected 6 weeks after infection and were used to infect naive Huh-7.5 cells. NSSA staining was performed 72 h after infection and the frequency of HCV antigen-bearing cells was quantified by flow cytometry. b, HCV infectious particles released into the serum of 4hEF Stat1−/− mice, 4hEF Stat1−/− mice lacking Ppl or 4hEF Stat1−/− mice treated with BMS-790052 for 20 days as determined by limiting dilution assay. Data shown are mean ± s.d. of four independent experiments. ND, not detectable.
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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank J. Sable, E. Castillo, B. Flatley, S. Shirley, A. Webson and E. Giang for laboratory support, A. North and the Rockefeller University Bioimaging Core Facility, S. Mazel and the Rockefeller University Flowcytometry Core Facility, C. Yang and the Gene Targeting Center and R. Tolwani and the staff of the Comparative Bioscience Center provided technical support. This study was supported in part by award number RC1DK087193 (to C.M.R. and A.P.) from the National Institute of Diabetes and Digestive and Kidney Diseases, R01AI072613, R01AI099284 (to C.M.R.), R01AI079031 (to M.L.) from the National Institute for Allergy and Infectious Disease, R01CA057973 (to C.M.R.) from the National Cancer Institute, The Starr Foundation, the Greenberg Medical Research Institute, the Richard Salomon Family Foundation, the Ronald A. Shellow, M.D. Memorial Fund, the MGM Mirage Voice Foundation, Gregory F. Lloyd Memorial contributions, and anonymous donors. M.D. was supported by a postdoctoral fellowship from the German Research Foundation (Deutsche Forschungsgesellschaft). M.T.C. is a recipient of The Rockefeller University Women & Science Fellowship. A.P. is a recipient of the Astella Young Investigator Award from the Infectious Disease Society of America and a Liver Scholar Award from the American Liver Foundation. The funding sources were not involved in the study design, collection, analysis and interpretation of data or in the writing of the report.

Author Contributions M.D. and J.A.H. planned and performed experiments and contributed to writing the manuscript. B.M.D., R.N.L., W.C.B., T.F., A.V. and M.T.C. performed the experimental work; T.K., T.S., S.A. and M.L. provided reagents. C.M.R. provided laboratory infrastructure, space, reagents, advice and edited the manuscript. A.P. planned and performed experiments and wrote the manuscript.

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METHODS

Animals and cell lines. Gt(Rosa26)Sor<tm1(Luc)Kaelin (ref. 19) (Rosa26-Fluc), B6.129-Gt(Rosa26)Sor<tm1(Luc)Kaelin (ref. 20) (Rosa26-GNZ) and C57BL/6 (wild type) mice were obtained from The Jackson Laboratory. J. Colgan (University of Iowa) made the Ppia<tm1Lubn (Ppia<tm1Lubn) mice available. ROSA26-Fluc mice contain the firefly luciferase (lac) gene inserted into the Gt(Rosa26)Sor locus. Expression of the luciferase gene is blocked by a loxp-flanked STOP sequence fragment placed between the lac sequence and the Gt(Rosa26)Sor promoter. Cre-recombinase-mediated excision of the transcriptional stop cassette results in luciferase expression in Cre-expressing tissues. ROSA26-GNZ knock-in mice have widespread expression of a nuclear-localized green fluorescent protein–β-galactosidase fusion protein (nlsGFP/β-gal) once an upstream loxp-flanked STOP sequence is removed. When Cre recombinase is introduced into cells the resulting GNZ fusion protein expression allows for enhanced (single cell level) visualization. Mice were bred and maintained at the Comparative Bioscience Center of the Rockefeller University according to guidelines established by the Institutional Animal Committee. Huh-7 (ref. 22), Huh-7.5 (ref. 23) and Huh-7.5 stably expressing the TagRFP-nlsMAVS reporter8 were maintained in DMEM with 10% fetal bovine serum (FBS) and 1% nonessential amino acids (NEAA).

Mutant mice with targeted disruptions in genes involved in antiviral defences. Irf1<tm1Ap6 (Irf1<tm1Ap6) mice were obtained from the Jackson Laboratory, Irf1<tm1Ap6 (Irf1<tm1Ap6) from B&K Universal Ltd, and Irf1<tm1Ap6 (Irf1<tm1Ap6) from Taconic. B6.129-Jc1 (ref. 37), Jc1 (ref. 37), Jc1 (ref. 37), and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere.

Generation of HCV entry factor transgenic mice. cDNAs encoding human CD81, SCARB1, CLDN1 or OCLN were cloned into a vector between a chimeric intron and the 3′ flanking regions of human growth hormone (GH1), in which the mouse albumin enhancer/promoter drives gene expression34. Vector-free human eCD81, SCARB1, CLDN1 and/or OCLN expression fragments were prepared by NotI and KpnI digestion and microinjected alone or in combination into fertilized C57BL/6 mouse eggs. Transgenic offspring were mated with C57BL/6 wild-type animals to select for founder lines stably inheriting the transgene(s). In some mice that were co-injected with multiple expression constructs, transgenes did not segregate in the F1 generation, indicating separate insertions in close proximity or insertion as concatemers. The founder lines were designated as follows: C57BL/6-Tg(AcbhdClN1)976Krlf (AcbhdClN1), C57BL/6-Tg(Abc-hScarb1)Prrk (Alb-hScarb1), C57BL/6-Tg(Alb-hOcln)941Prlk (Alb-hOcln), C57BL/6-Tg(Alb-hScarb1)/hClDN1)935Prlk (Alb-hScarb1)1/1 (Alb-hScarb1), C57BL/6-Tg(Acb-hOcln)941Prlk (Alb-hOcln) and C57BL/6-Tg(Acb-hScarb1)Prrk (Alb-hScarb1). Generation of TagRFP-nlsMAVS reporter mice (C57BL/6-Tg(TagRFP-nlsMAVS)4065). The TagRFP-NLS-MAVS(WT) cassette18 was inserted into pCR2.1-TOPO (Invitrogen) and modified to contain TagRFP in place of TagRFP. The resulting TagRFP–NLS–MAVS cassettes was inserted into the pCAGGS vector (Addgene) to yield pCAGGS-TagRFP–NLS–MAVS(WT). The pCAGGS backbone drives transgene expression from a ubiquitously active chimaeric CMV/β actin promoter26 and has been used to create transgenic mice successfully (for example, ref. 36). The pCAGGS-TagRFP–NLS–MAVS(WT) internal transgene cassette was isolated by linearization with Sall/PstI enzymes and integrated into C57BL/6 pronuclei. Founder animals were identified by PCR and bred with congenic C57BL/6 animals.

HCV generation and infections. Construction of BiCre-Jc1 (ref. 2), Jc1 (ref. 37), Con1-Jc1 (ref. 38) and Jc1 5AB Ypet39 was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere. Huh-7.5.1 (ref. 23) and Jc1 (ref. 37) was described elsewhere.

Immune activation. Lymphocytes were isolated from liver and spleen by digestion with 0.1% collagenase (Sigma) for 30 min at 37°C. Lymphocytes were then isolated from the cell suspensions as well as from peripheral blood by density gradient centrifugation. Cells were stained with directly fluorochrome-conjugated antibodies against CD3, CD4, CD8, B220 (B220) and NKp46 (BD Biosciences). After cell surface staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) and stained with antibodies against IFN-γ and TNF-α. Samples were measured using a BD LSRII flow cytometer (BD Biosciences) and data were analysed using Flowjo (Treestar Software).

Western blotting. Perfused murine liver tissue was homogenized in lys buffer containing 1% Triton X-100, 50 mM Tris-HCl pH 8.5, 150 mM NaCl and Mini EDTA-free Protease Inhibitor Cocktail (Roche) for 30 min on ice. Thirty micrograms of protein lysate was separated on 4–12% Bis-Tris NuPage polyacrylamide gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and entry factors were detected using antibodies against human SCARB1 (1:500) and CLDN1 (1:200). β-actin (1:10,000) was probed as a loading control. After secondary antibody staining with HRP-conjugated anti-mouse IgG Fc (JRir, 1:10,000), western blots were visualized using SuperSignal West Pico (Thermo Scientific).

RT–PCR quantification of HCV entry factors and interferon-stimulated genes. To quantify expression of human and murine genes (entry factors and interferon-stimulated genes), the livers of FVB/N mice were collected at the indicated time points. Total liver RNA was isolated using RNeasy isolation kit (Qiagen) and cDNA was synthesized from 0.5 µg RNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative RT–PCR was performed with a light cycle LC480 (Roche Applied Science) using an Applied Biosystems SYBR Green PCR Master Mix and the following primer pairs: human CD81 forward 5′-TGTTCATTGACGCTGAGGTGGTC-3′, reverse 5′-TGGTGATGATGATGATGGCAAC-3′; human SCARB1 forward 5′-GGGATTGGCAGAGGGCTAC-3′, reverse 5′-GGGAGAGAGGCTCTTTACACATTAC-3′; human CLDN1 forward 5′-CACCTGATTCGTCTCAAGGAC-3′, reverse 5′-CTTGAGGATCTACGATCTTTG-3′; human OCLN forward 5′-GGAATAAGGGATCCTTTGG-3′, reverse 5′-GGTTCAGTATTGATGATGGCAAC-3′; mouse CD81 forward 5′-GCGCTTTGCTCGATGTGATG-3′, reverse 5′-AGAGGCTTCTTGGACCTT-3′; mouse CLDN1 forward 5′-GCGCTTTGCTCGATGTGATG-3′, reverse 5′-AGAGGCTTCTTGGACCTT-3′; mouse Ocln forward 5′-ACTAAAGAGGATCCTTTGG-3′, reverse 5′-GGTTCAGTATTGATGATGGCAAC-3′, reverse 5′-GCGCTTTGCTCGATGTGATG-3′, reverse 5′-AGAGGCTTCTTGGACCTT-3′; mouse

Biosciences (for western blotting). The HCV NS5A inhibitor BMS-790052 (ref. 41) was obtained from Sellick Chemicals.
Gapdh forward 5'-ACGGCGCATTCTCTGTGCA-3', reverse 5'-ACGGCCA AATCGTGCCACCC-3'; mouse viperin forward 5'-TGCTGCGGTGAAATAG CATTAG-3', reverse 5'-GGCTGAGTGCTGCCCTACT-3'; mouse Ifi44 forward 5'-GGTTGGTAGGGAACCTTGGTT-3', reverse 5'-GGATTGGCATTGGTAT GTGGG-3'; mouse Ifi44 forward 5'-AATCCGTTCACACC-3'; mouse Oxlid forward 5'-AGAGCTTCAACCTCCC-3' (antisense, NS4A); RU-O-15695, 5'-TGGGCAGGATGGCTCCTGTC-3' (antisense, nlsCre); RU-O-16490, 5'-GATGTTAATGGTCCCTAACCC-3' (antisense, NS5B); RU-O-15354, 5'-CAAGAAGAAGAGG AAGGGTTC-3' (sense, NS3); RU-O-14317, 5'-GACTGGGAGTTATCTCTGTA A-3' (sense, NS5B); RU-O-17119, 5'-GGCTCCCATCTGCTTATGGC-3' (sense, NS3); RU-O-16768, 5'-CCGACC CCACTACTGTTGGGGG-3' (sense, E2); RU-O-15354, 5'-CAGGCCCCAGCACCTGGAAGG-3' (antisense, core); RU-O-15356, 5'-GCAAAGTCTGTTAATGGTG-3' (sense, EMCV); RU-O-12170, 5'-AGCCCTCATAAGACCCCTC-3' (antisense, NS4A); RU-O-9028, 5'-GATGCTACCTCCCTTCG-3' (sense, NSB); RU-O-17375, 5'-CAGCAACCGGAGGTC-3' (antisense, EMCV); RU-O-10215, 5'-CATCTATG CCACTCCACCC-3' (antisense NS2); RU-O-17391, 5'-CATAGTTGGTGGAC CACACA-3' (sense, NS3); RU-O-17388, 5'-ATTGGCGAGGGGGCCTA CT-3' (antisense, 3' UTR).

The resulting PCR amplicons were cloned into the pCR2.1 vector using the TOPO TA Cloning kit (Invitrogen Life Technologies). Resulting clones were screened for proper amplicon insertion size by EcoRI digestion, and sequenced using M13 F/R primers, as well as additional internal primers for full genomic coverage (Macrogen USA).

Statistical analysis. Statistical analyses were performed using Graphpad Prism Software. Statistics were calculated using Kruskal–Wallis one-way analysis of variance. P values below 0.05 were considered statistically significant.

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