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A genetically humanized mouse model for hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) remains a major medical problem. Antiviral treatment is only partially effective and a vaccine does not exist. Development of more effective therapies has been hampered by the lack of a suitable small animal model. While xenotransplantation of immunodeficient mice with human hepatocytes has shown promise, these models are subject to important challenges. Building on the previous observation that CD81 and occludin (OCLN) comprise the minimal human factors required to render mouse cells permissive to HCV entry in vitro, we attempted murine humanization via a genetic approach. Here we show that expression of two human genes is sufficient to allow HCV infection of fully immunocompetent inbred mice. We establish a precedent for applying mouse genetics to dissect viral entry and validate the role of SCARB1 for HCV uptake. We demonstrate that HCV can be blocked by passive immunization, as well as show that a recombinant vaccinia virus (rVV) vector induces humoral immunity and confers partial protection against heterologous challenge. This system recapitulates a portion of the HCV life cycle in an immunocompetent rodent for the first time, opening opportunities for studying viral pathogenesis and immunity and comprising an effective platform for testing HCV entry inhibitors in vivo.

Humans and chimpanzees are the only species permissive to HCV infection. The basis for this highly restricted tropism is not completely understood, but may result from viral dependence on host factors present in only a few cell types. Murine cells are resistant to

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Author contributions M.D., C.M.R. and A.P. designed the project, analyzed results and wrote the manuscript. M.D., J.A.H., J.B.R., W.B., Q.F., K.M, M.T.C. and M.L. performed the experimental work, J.W.S., C.T.J, and D.R.B. provided reagents.

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HCV entry, show inefficient viral replication, and may be blocked at later life cycle steps. HCV enters hepatocytes through the combined action of at least four host molecules: CD81¹, scavenger receptor type B class I (SCARB1)², claudin 1 (CLDN1)³ and occludin (OCLN)⁴. We have previously shown that CD81 and OCLN comprise the minimal human factors required for HCV uptake by rodent cells⁴. This led to the hypothesis that expression of these human orthologs could render mice susceptible to HCV infection *in vivo*. We therefore constructed recombinant adenoviruses encoding human CD81, SCARB1, CLDN1 and/or OCLN. Intravenous delivery of these vectors resulted in 100 to 1000-fold overexpression of the corresponding mRNA in the murine liver and strong expression of all four proteins with the expected subcellular distribution (Supplementary Fig. 1). We determined that 18–25% of murine hepatocytes expressed human CD81 and OCLN together, while approximately 5% of cells expressed all four heterologous genes (Supplementary Fig. 2b–d). These results encouraged us to investigate infection of these animals. Unfortunately, HCV replication in mouse cells is inefficient *in vitro* and *in vivo*^{5,6,7,8,9}. Consistent with this, challenge of mice expressing all four human factors with a firefly luciferase (Fluc)-encoding HCV genome [Jc1FLAG2(p7Fluc2A)] did not yield bioluminescent signal above background (Supplementary Fig. 3a). Direct measurement of Jc1FLAG2(p7Fluc2A) genome levels by quantitative reverse transcription (qRT)-PCR demonstrated a slight increase in HCV RNA in the serum (at 3h) and liver (at 3 and 24h); at 72h, however, the signal was reduced to background (Supplementary Fig. 3b–d). These data highlight the difficulty of detecting HCV infection in cell types that do not support robust replication. In mouse cells, this defect may result from incompatibility between the viral replication machinery and murine factors and/or from exacerbated murine innate antiviral responses. Furthermore, adenoviral gene delivery strongly induces interferon-stimulated genes, including viperin, IFI44, Mx1, 2'OAS, IP-10 and PKR, creating an environment that mimics recombinant IFN α treatment (Supplementary Fig. 4) and may antagonize HCV replication¹⁰.

As an alternate approach, we constructed a bicistronic HCV genome expressing CRE recombinase (Bi-nlsCre-Jc1FLAG2, abbreviated HCV-CRE), which activates a loxP-flanked luciferase reporter in the genome of the Gt(ROSA)26Sor^{tm1(Luc)Kaelin} (Rosa26-Fluc) mouse¹¹. Hydrodynamic delivery of HCV-CRE RNA into Rosa26-Fluc mice led to reporter signal in the liver, indicating that CRE recombinase is active in the context of the HCV genome (Supplementary Fig. 5). Delivery of a polymerase-defective HCV-CRE RNA produced similar results, suggesting significant CRE production was derived from initial translation without the need for replication (Supplementary Fig. 5). To test whether mice could be infected by authentic HCV particles, we generated Rosa26-Fluc animals expressing human CD81 and OCLN, or all four human entry factors, and inoculated these mice with cell culture-derived HCV-CRE. In mice expressing all four transgenes, luciferase signals increased longitudinally, peaked at approximately 72 h post-infection, and decreased thereafter; mice lacking the transgenes did not show significant reporter activity (Fig. 1b and Supplementary Fig. 6). All animals expressing at least human CD81 and OCLN could be successfully infected. Loss of signal after 72h was likely due to strong anti-vector immunity, as evidenced by the increased frequencies of natural killer (NK) cells (Supplementary Fig 7a); depletion of NK cells prior to adenovirus injection prolonged luminescence activity

(Supplementary Fig. 7c). Bioluminescent signals were dependent on the doses of both adenovirus and HCV-CRE (Supplementary Fig. 6), and infection was observed across a panel of chimeras expressing the structural proteins of diverse HCV genotypes (Fig. 3a). HCV core, NS3 or NS5A could not be detected (data not shown) probably owing to inefficient viral replication in murine hepatocytes. To estimate the number of HCV-infected liver cells, we therefore used an indicator mouse strain in which CRE leads to activation of a nuclear-localized green fluorescent protein/ β -galactosidase (GNZ) reporter (B6.129-Gt(ROSA)26Sor^{tm1Joc}/J, abbreviated Rosa26-GNZ)¹². We observed HCV-CRE infection in approximately 4–5% of murine hepatocytes expressing human CD81 and OCLN (Fig. 1c and Supplementary Fig. 2h) and up to 20% expressing all four factors (Fig 1c). We did not observe HCV uptake in non-parenchymal liver cells. These frequencies are consistent with previous estimates from primary human hepatocyte cultures^{13,14} and HCV-positive patient tissues¹⁵, although our system represents a single cycle infection and does not fully reflect the natural equilibrium. Taken together, these data provide, for the first time, evidence that mice can be rendered susceptible to HCV infection by a defined set of human genes.

Previous studies in Chinese hamster ovary (CHO) and mouse fibroblast (NIH3T3) cells have shown that CD81 and OCLN are the primary determinants of HCV tropism at the level of entry⁴. To investigate the *in vivo* determinants of species restriction, we delivered combinations of human or mouse CD81, SCARB1, CLDN1 and OCLN to Rosa26-Fluc mice. In accordance with the *in vitro* data, HCV permissivity increased 6–10 fold in mice expressing human CD81 and OCLN, compared to those expressing only murine factors. Notably, addition of exogenous human or mouse SCARB1/CLDN1 (Fig. 1d) boosted permissivity compared to human CD81/OCLN alone (Fig. 1b), suggesting that low endogenous entry factor levels may limit infection. While overexpression of mouse CD81 or OCLN in combination with the other human entry factors permits low-level uptake, human CD81 and OCLN are required for optimal infection. To verify authentic co-receptor function, we delivered entry factors bearing mutations previously shown to disrupt their activities. CD81 mutations F186L and E188K are known to abrogate soluble E2 binding and to reduce HCV uptake by human hepatocytes¹⁶. Indeed, expression of mutant human CD81 reduced *in vivo* entry efficiency by about 50% (Fig. 1e). A human OCLN chimera in which the second extracellular loop is replaced with the murine sequence has been shown to impair HCV entry *in vitro* by approximately four-fold⁴. Concordantly, HCV susceptibility of mice expressing the chimeric molecule was reduced by approximately 50% (Fig. 1e). These data indicate that infection of murine hepatocytes *in vivo* is dependent on the native functions of human CD81 and OCLN.

Unlike the human-restricted entry factors, mouse SCARB1 appears to support HCV uptake *in vitro*⁴ and *in vivo* (Fig. 1b). To validate the importance of this endogenous factor, we crossed mice with a disruption in *Scarb1* (SCARB1^{-/-})¹⁷ to the Rosa26-Fluc background. Expression of human CD81, CLDN1 and OCLN in mSCARB1^{-/-} Rosa26-Fluc mice reduced HCV-CRE infection efficiency by ~90%, as compared to SCARB1^{+/+}, and ~50% compared to SCARB1^{+/-} mice (Fig. 1f). This reduction appears to be a direct consequence of murine SCARB1 deficiency, and not transduction efficiency, as expression levels of CD81, CLDN1 and OCLN were similar in wild-type (Supplementary Fig. 1g) and SCARB1

knockout mice (Supplementary Fig. 8). Complementing mSCARB1^{-/-} Rosa26-Fluc mice with human CD81, CLDN1 and OCLN and SCARB1 of either human or mouse origin fully restored infectivity (Fig. 1f). These data provide the first direct evidence that SCARB1 is a bona fide HCV entry factor in vivo. Taken together, these results suggest that infection of humanized mice occurs via authentic entry pathways, and highlight the value of this system for studying HCV co-receptor biology in vivo.

Despite considerable efforts, the lack of preventative or therapeutic HCV vaccines remains a major clinical challenge. Delivery of antibodies via passive immunization might be particularly suitable to block HCV entry, possibly limiting graft re-infection during liver transplantation and potentially boosting treatment success. Antibodies against E2 and CD81 have been shown to block HCV infection in cell culture^{13,18} and human liver chimeric mice^{19,20}. We investigated passive immunization in the genetically humanized model. Delivery of anti-CD81 antibodies resulted in a dose-dependent inhibition of HCV-CRE infection, whereas isotype control immunoglobulins had no effect (Fig. 2a,d). Similarly, pre-incubation of HCV-CRE with antibodies directed against E2 (clone 3/11), but not an isotype control, significantly inhibited infection (Fig. 2b,e). These data further affirm that HCV is taken up in a glycoprotein-specific fashion in vivo and suggest the utility of this model for evaluation of passive immunization strategies.

Induction of broad-spectrum adaptive immunity is a key goal of HCV vaccine research. A promising candidate is a recombinant vaccinia virus (rVV) vector expressing HCV proteins, which has been shown to induce adaptive responses in rodents and chimpanzees²¹. To evaluate this candidate in genetically humanized animals, Rosa26-Fluc mice were immunized intraperitoneally with 1×10^7 plaque forming units (PFU) of rVV expressing the HCV proteins C-E1-E2-p7-NS2 (strain HCV-1, genotype 1a)²². Robust titers of anti-E2 antibodies (Fig 3b) and decreased susceptibility to heterologous challenge with HCV-CRE expressing the structural proteins of genotypes 1b, 2a, or 4a (Fig. 3c) were observed. Protection directly correlated with the levels of anti-E2 antibodies in the serum. Challenge with genotype 1b (strain Con1) HCV-CRE virus, whose envelope proteins are closely related to those of HCV-1, resulted in complete protection in two out of 10 animals (Fig. 3d, e, f). Pooled sera of immunized, but not non-immune, mice neutralized diverse HCV genotypes in vitro (Supplementary Fig. 9) and reduced infection by 50% when transferred passively (Fig. 2c,f). These data demonstrate the value of the genetically humanized mouse as the first immunocompetent small animal model amenable to HCV-challenge.

This study represents an important step forward in developing a robust small animal model for HCV infection and immunity. To our knowledge, this is the first time that any step in the viral life cycle has been recapitulated in a rodent simply by the expression of human genes. Previously developed liver chimeric mice are susceptible to HCV infection and support the entire viral life cycle²³⁻²⁵. Despite recent improvements²³, however, these models are hampered by low throughput, intraexperimental variability, and high costs²⁶. Furthermore, the lack of a functional immune system negates their value for vaccination studies. The inbred mouse model presented here is not only susceptible to infection by diverse HCV genotypes, but it is also fully immunocompetent, providing the first small animal platform suitable for combined immunization and challenge studies. While it may eventually be

desirable to transgenically express the HCV entry factors, adenoviral expression allows rapid testing of various mutant proteins on diverse backgrounds. The genetically humanized mouse model provides proof-of-principle for such applications and holds promise for assessing the immunogenicity and efficacy of HCV vaccines.

Methods summary

For more detailed methods see Supplementary Methods.

Mice

Gt(ROSA)26Sor^{tm1}(Luc)Kaelin11, B6;129-Gt(ROSA)26Sor^{tm1}Joe/J²⁷ and B6;129S2-Scarb1^{tm1}Kri/J¹⁷ and FVB/NJ wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained at the Comparative Bioscience Center of The Rockefeller University according to guidelines established by the Institutional Animal Committee.

Recombinant adenoviruses

Adenoviral constructs encoding human and murine homologues of the four HCV entry factors (CD81, SCARB1, CLDN1 and OCLN) and mutants thereof were created using the AdEasyTM Adenoviral Vector System (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions.

Hepatitis C virus

Plasmids encoding chimeric HCV genomes, including Jc1FLAG2(p7Fluc2A) and bicistronic HCV genomes expressing CRE, were linearized with XbaI and transcribed using MEGAscript T7 (Ambion, Austin, Texas). RNA was electroporated into Huh-7.5 cells using a ECM 830 electroporator (BTX Genetronics) and infectious virus was collected from supernatants 48–72 h after transfection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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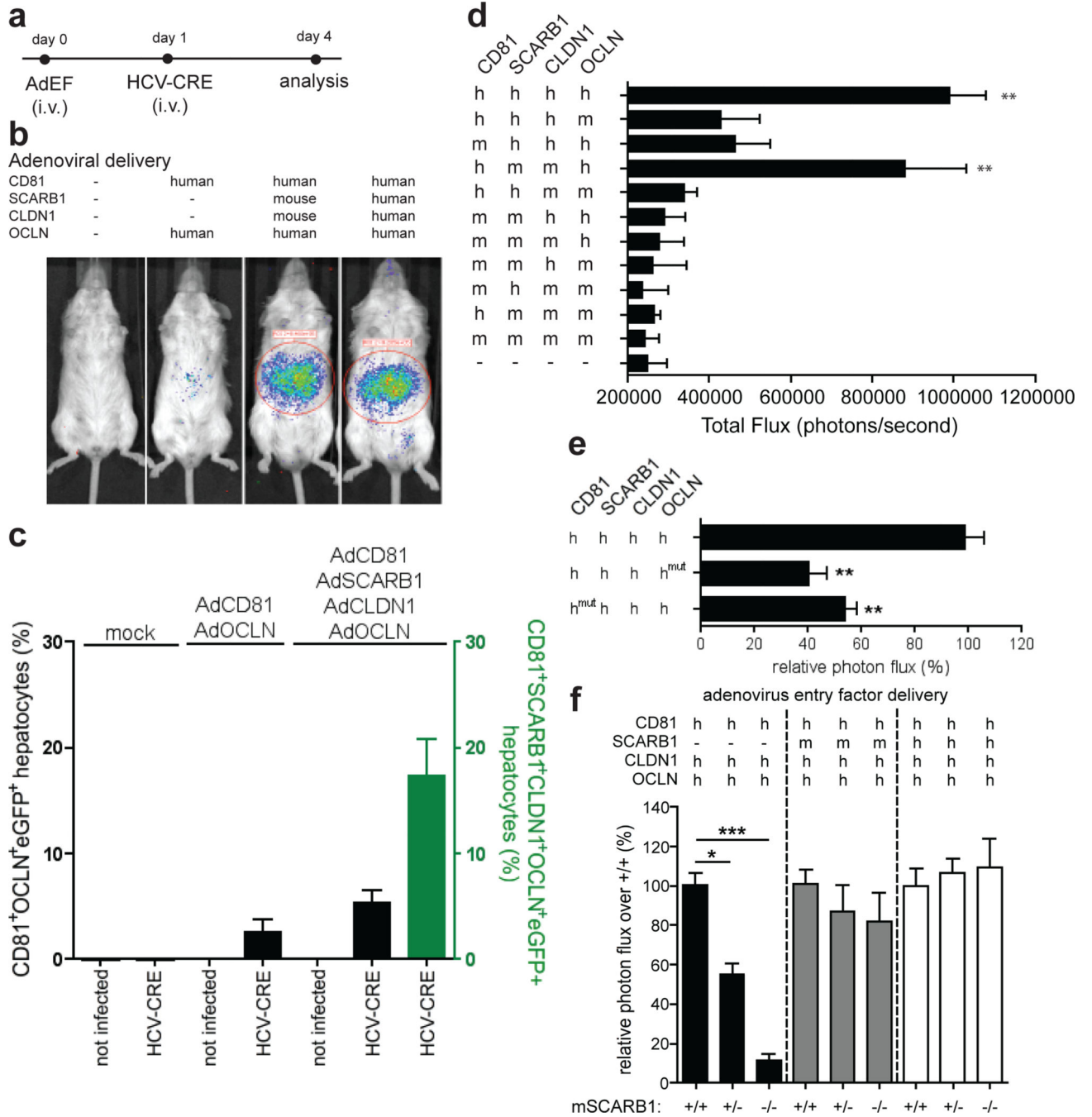


Figure 1. Genetic requirements for HCV entry in vivo
a, Timeline for administration of adenovirus and HCV-CRE. **b**, HCV entry into Rosa26-Fluc mice 96h post-injection of medium (n=20), 10¹¹ adenovirus encoding CD81 and OCLN (n=10) or 10¹¹ particles encoding CD81, SCARB1, CLDN and OCLN of the indicated species (n=20 each). Bioluminescence was measured at 72h post-injection of 2×10⁷ 50%-tissue culture infectious doses (TCID₅₀) HCV-CRE. **c**, Frequency of HCV-infected hepatocytes. Rosa26-GNZ mice were injected with medium or adenovirus encoding human CD81, mKate-SCARB1, Cerulean-CLDN1 and Venus/YFP-OCLN. 72h post-

injection of 2×10^7 TCID₅₀ HCV-CRE, the frequency of infected hepatocytes was determined by flow cytometry. Data are percent infected (eGFP⁺) cells relative to either CD81/OCLN transduced (left axis) or CD81/SCARB1/CLDN1/OCLN transduced hepatocytes (right axis) **d.** Rosa26-Fluc mice were injected with human (h) and murine (m) entry factors 24h prior to injection of HCV-CRE (n = 4). **e.** Entry factor mutants reduce infection in vivo. Combined delivery of human (h) and mutant human (h^{mut}), hCD81/F186L/E188K or hOCLN/mEL2 (n=3). **f.** mSCARB1 is an essential HCV entry factor in vivo. Rosa26-Fluc mice were crossed with mSCARB1^{-/-} mice offspring were injected with adenoviruses encoding human CD81, CLDN1, and OCLN, and 24h later with 2×10^7 TCID₅₀ HCV-CRE (n=5-7). Data represent mean \pm standard deviation (SD). Statistical significance was calculated by Kruskal-Wallis one way analysis of variance (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

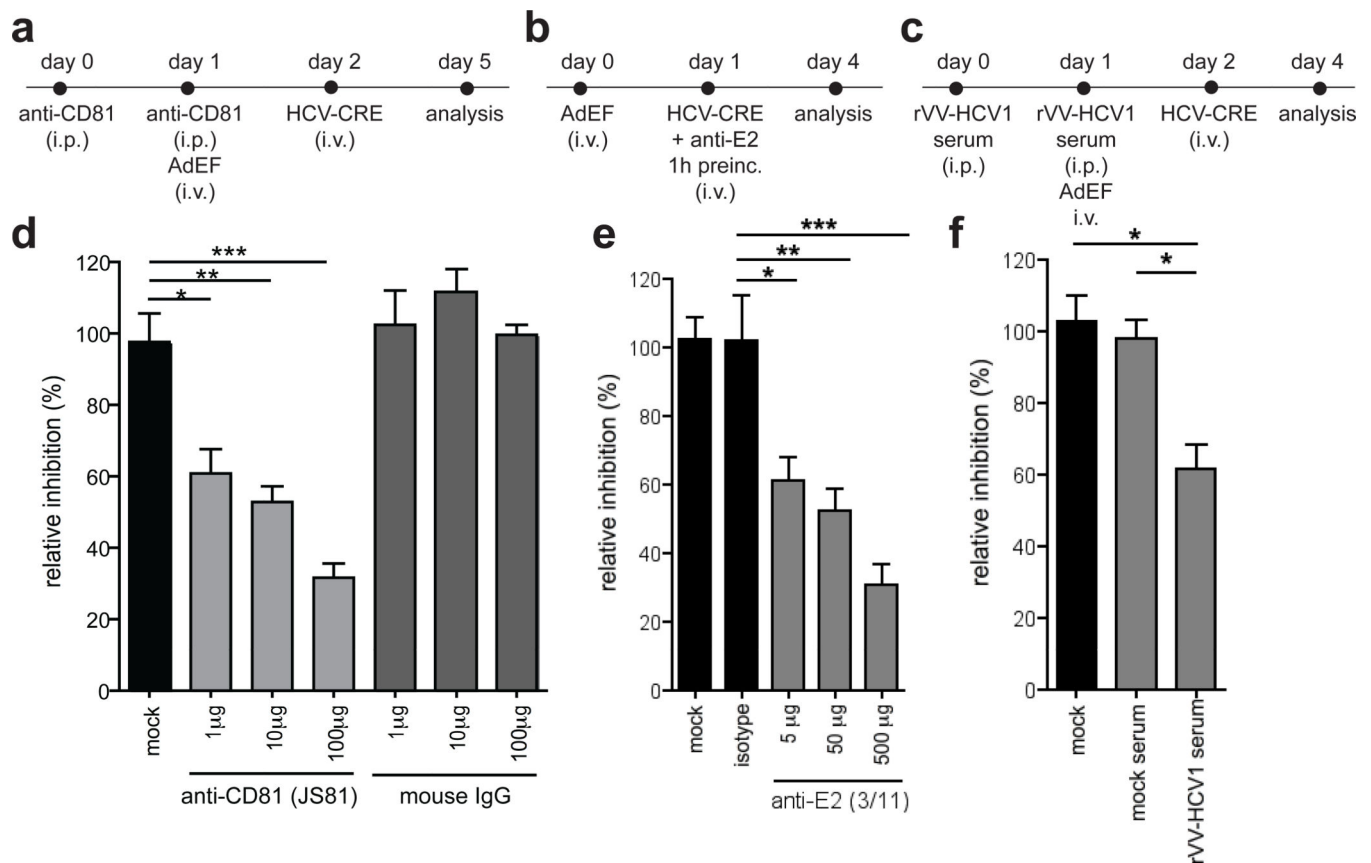


Figure 2. HCV entry into murine hepatocytes *in vivo* can be blocked by antibodies or passive transfer of vaccine induced antiserum

a–c, Experimental layout of **a**, CD81-blocking, **b**, virus pre-neutralization with anti-E2 (3/11) and **c**, *in vivo* neutralization with pooled sera from immunized mice. **d**, Rosa26-Fluc mice were injected with anti-CD81 antibodies (two doses of 1, 10 or 100 µg/animal at 24h prior to and with injection of HCV-CRE, n=4) or isotype control (n=3). **e**, HCV-CRE was incubated with 5, 50 or 500µg of anti-HCV E2 (clone 3/11) for 1h prior to injection into Rosa26-Fluc mice expressing all four human entry factors (n=3). **f**, Rosa26-Fluc mice were injected with 200µl pooled serum from wild-type (FVB/nJ) mice immunized with rVV-HCV1 or naïve control (2 doses 24h prior to and with injection of HCV-CRE [n=4]). Data shown are mean ± SD. Statistical significance was calculated by Kruskal-Wallis one way analysis of variance (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

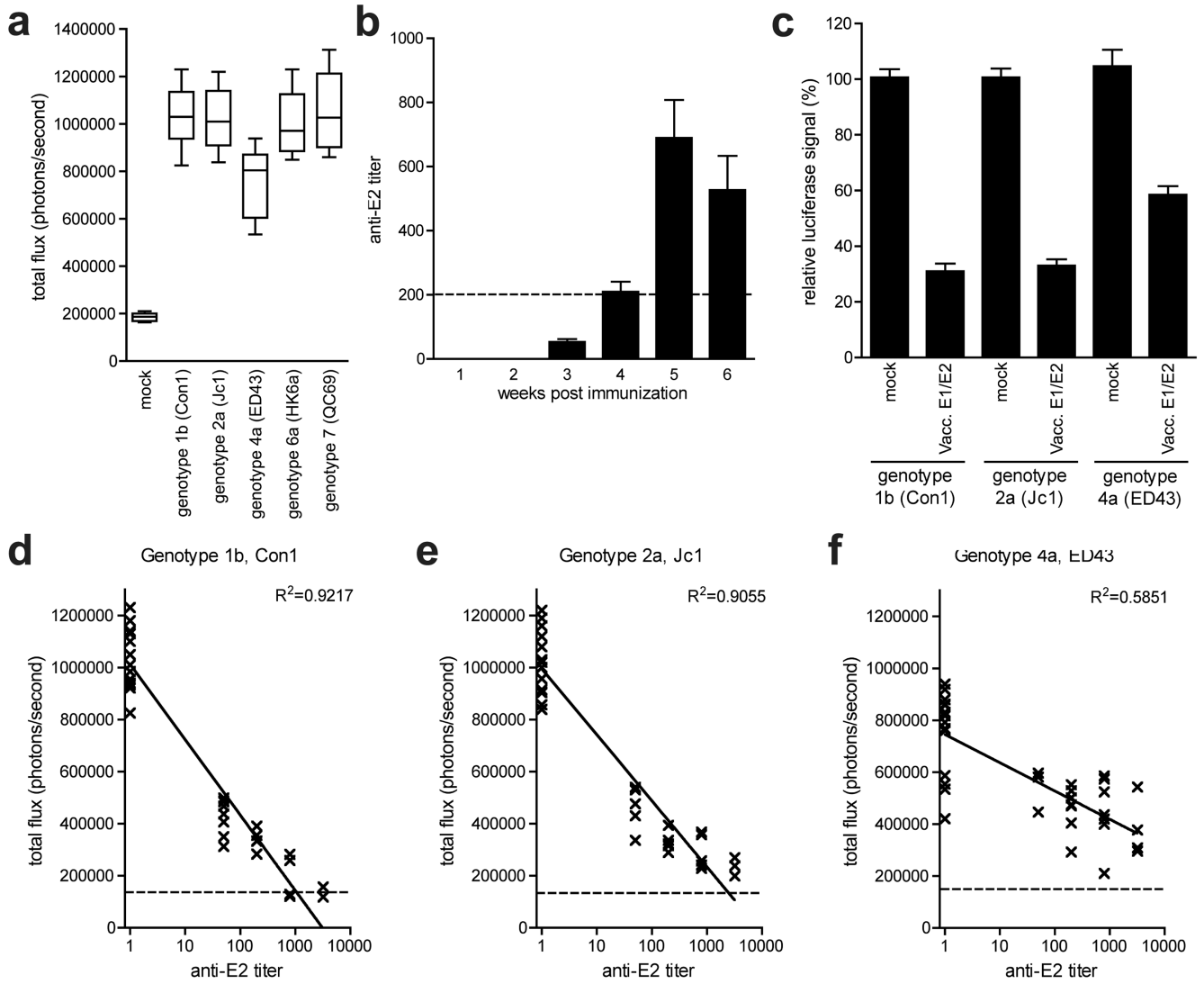


Figure 3. Use of genetically humanized mouse model to evaluate vaccines against multiple HCV genotypes

a. Genetic humanization supports HCV entry mediated by structural proteins of various genotypes. Rosa26-Fluc mice expressing all four human entry factors were infected with intergenotypic chimeras (Con1 genotype 1b, Jc1 genotype 2a, ED43 genotype 4a, HK6a genotype 6a and QC69 genotype 7a, 2×10^7 TCID₅₀/animal, n=3). **b.** Priming of humoral immune responses with recombinant vaccinia virus (rVV). Rosa26-Fluc mice were injected intraperitoneally with rVV encoding the HCV-1 (genotype 1a) structural genes (10^7 PFU/animal, n=10) and anti-HCV E2 antibody titers were determined by ELISA. **c-f.** Protection of rVV-immunized mice against challenge. Immunized mice were challenged with heterologous HCV strains Con1 (1b), Jc1 (2a) and ED43 (4a) and infection was quantified by bioluminescence imaging 72h later (n=5). Data represent mean \pm SD.