

## ORIGINAL ARTICLE

# Neutralization and receptor use of infectious culture-derived rat hepacivirus as a model for HCV

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## Funding information

Supported by the European Research Council (Grant No. 802899 to T.K.H.S.), the Independent Research Fund Denmark (Grant No. 4004-00598 to J.B. and 1030-00426 to T.K.H.S.), the Danish Cancer Society (R204-A12639 to J.B. and T.K.H.S.), the Novo Nordisk Foundation (Grant Nos. NNF19OC0054518 and NNF19OC0054462 to J.B.), and U.S.

## Abstract

**Background and Aims:** Lack of tractable immunocompetent animal models amenable to robust experimental challenge impedes vaccine efforts for HCV. Infection with rodent hepacivirus from *Rattus norvegicus* (RHV-rn1) in rats shares HCV-defining characteristics, including liver tropism, chronicity, and pathology. RHV in vitro cultivation would facilitate genetic studies on particle production, host factor interactions, and evaluation of antibody neutralization guiding HCV vaccine approaches.

**Approach and Results:** We report an infectious reverse genetic cell culture system for RHV-rn1 using highly permissive rat hepatoma cells and adaptive mutations in the E2, NS4B, and NS5A viral proteins. Cell culture-derived RHV-rn1 particles (RHVcc) share hallmark biophysical characteristics of HCV and are infectious in mice and rats. Culture adaptive mutations attenuated RHVcc in immunocompetent rats, and the mutations reverted following prolonged infection, but not in severe combined immunodeficiency (SCID) mice, suggesting that adaptive immune pressure is a primary driver of reversion. Accordingly, sera from RHVcc-infected SCID mice or the early acute phase of immunocompetent mice and rats were infectious in culture. We further established an in vitro RHVcc neutralization assay, and observed neutralizing activity of rat sera specifically from the chronic phase of infection. Finally, we found that scavenger receptor class B type I promoted RHV-rn1 entry in vitro and in vivo.

**Conclusions:** The RHV-rn1 infectious cell culture system enables studies of humoral immune responses against hepacivirus infection. Moreover,

**Abbreviations:** CypA, cyclophilin A; dpi, days post infection; FFU, focus-forming unit; GE, genome equivalent; MHC, major histocompatibility complex; nAbs, neutralizing antibodies; REM, replication-enhancing mutation; RHVcc, cell culture-derived RHV; RHV-rn1, rodent hepacivirus from *Rattus norvegicus* 1; SCID, severe combined immunodeficiency; siRNA, small interfering RNA; SR-BI, scavenger receptor class B type I; wpi, weeks post infection; WT, wild type.

Kenn Holmbeck and Troels K. H. Scheel contributed equally to this work.

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Public Health Service/National Institutes of Health awards (R01AI137567 and R01AI151175 to A.K.; R01AI131688 to C.M.R.; and R01AI124680, R01AI126890, R01AI136533, and U19AI159819 to A.G. and ORIP/OD P51OD011132 [formerly NCRR P51RR000165] to the Yerkes National Primate Research Center [A.G.]). R.W. was supported by an Early Postdoc. Mobility Fellowship (P2BEP3\_178527) and a Postdoc. Mobility Fellowship (P400PB-183952) from the Swiss National Science Foundation.

recapitulation of the entire RHV-rn1 infectious cycle in cell culture will facilitate reverse genetic studies and the exploration of tropism and virus–host interactions.

## INTRODUCTION

Globally, an estimated 58 million people are chronically infected with HCV, which can lead to fibrosis, liver cirrhosis, and HCC.<sup>[1]</sup> Effective therapeutics have been developed with cure rates >90% and greatly improved prognosis of HCV-mediated liver disease, especially following early treatment.<sup>[2,3]</sup> Autonomously replicating HCV subgenomic replicons<sup>[4,5]</sup> have been pivotal for this development. Furthermore, the subsequent development of infectious culture systems enabled the production of infectious particles<sup>[6,7]</sup> and the improvement of HCV neutralization assays.<sup>[8]</sup> Nonetheless, a vaccine and detailed understanding of pathology and intrahepatic immune responses are still lagging. This is largely due to the lack of immune-competent animal models amenable to experimental challenge following cessation of chimpanzee use in research.<sup>[9]</sup> Advances in sequencing technology recently enabled the discovery of many HCV-related hepaciviruses, for example in bats, cows, horses, monkeys, and rodents (reviewed in Hartlage et al.<sup>[10]</sup>), with equine hepacivirus being the closest genetic relative.<sup>[11]</sup> Particularly, the discovery of rodent hepacivirus (RHV) in Norway rats (*Rattus norvegicus*) was of importance,<sup>[12]</sup> by enabling studies in a widely used and immune-competent small animal model.<sup>[13]</sup> Furthermore, RHV has been adapted to infect laboratory mice, to exploit a broader set of genetic variants and research tools.<sup>[14]</sup>

The RHV *Rattus norvegicus* isolate 1 (RHV-rn1), designated as RHV hereafter, has a positive single-stranded genomic RNA of 9656 nucleotides encoding a single polyprotein of 2958 amino acids (aa). Despite limited sequence identity (~30% at amino acid level), RHV displays key similarities with HCV in genomic organization and liver tropism. Like HCV, the RHV 5' untranslated region also possesses two binding sites for the liver-specific microRNA-122 (miR-122),<sup>[13]</sup> and binding of miR-122 is required for replication.<sup>[14,15]</sup> Moreover, RHV naturally causes chronic high-titer infection in rats and pathological manifestations reminiscent of HCV-induced liver disease, including steatosis, cirrhosis, and hepatic lymphoid infiltration. An RHV in vivo reverse genetic

system has been established in rats and derived viruses proven infectious in several inbred and outbred strains.<sup>[13]</sup> Similar to most HCV isolates, however, the RHV molecular clone did not lead to productive infection in cell culture.<sup>[15]</sup>

The RHV model has been used to assess T cell-based vaccine candidates in the absence of concomitant humoral antibody responses to viral envelope proteins.<sup>[16,17]</sup> Although preliminary data provide evidence of partial protection from persistency upon RHV challenge, application of a similar strategy failed to provide protection from chronic HCV infection in a double-blinded, randomized clinical phase 1/2 trial.<sup>[18]</sup> This suggests that induction of neutralizing antibodies (nAbs) may be an important property of future vaccine platforms. In line with this, early development of broadly neutralizing antibodies has been associated with the natural clearance of HCV infection.<sup>[19,20]</sup> We previously established selectable subgenomic RHV replicon systems, which enabled the study of RHV replication, identification of replication-enhancing mutations (REMs), and selection of rat hepatoma cells with increased permissiveness.<sup>[15]</sup> However, there is currently no infectious cell culture system available to assess virus neutralization. Here, we report the successful complete propagation of RHV in vitro using these REMs and permissive rat hepatoma cells that enable studies of viral entry and neutralization.

## METHODS

### Cells and antibodies

McA-RH7777.hi rat hepatoma cells supporting RHV replication<sup>[15]</sup> were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Pen Strep; Sigma-Aldrich) at 37°C, 5% CO<sub>2</sub>, and passaged every 2–3 days using trypsin-EDTA (Sigma-Aldrich).

RHV-specific IgG (anti-RHV) was purified from sera derived from four C57BL/6 mice at week 6 after infection with mouse-adapted RHV,<sup>[14]</sup> at a point when infection had been cleared.

RHV anti E2 monoclonal IgG was isolated from mice immunized with purified RHV E2 ectodomain (aa 418-597), which was recombinantly expressed in 293T cells. Detailed information on all antibodies, including the production of the RHV E2 monoclonal antibody, is outlined in Text S1.

## RNA in vitro transcription of RHV clones

RNA was transcribed from 2.5 µg *MluI*-linearized RHV plasmid using the RiboMAX T7 RNA polymerase kit (Promega).<sup>[13]</sup> Template DNA was degraded for 30 min at 4°C with RQ1 RNase-free DNase. For in vivo inoculation, subsequent RNA purification was carried out on RNeasy Mini columns, including an additional on-column DNase I digestion step (Qiagen). Integrity of the RNA was assessed by formaldehyde agarose gel electrophoresis and quantities measured using the Qubit RNA Broad-Range Assay Kit (Thermo Fisher Scientific). See Text S1 for RNA transfection and template DNA plasmid construction.

## Extraction, quantification, and sequencing of viral RNA

For the extraction of RHV genomic RNA, 250 µl supernatant from infected cell cultures, density gradient fractions, or rodent serum samples (25 µl diluted in 225 µl PBS) were added to a 2-ml Phasemaker tube (Thermo Fisher Scientific) and mixed with 750 µl TRIzol LS Reagent (Thermo Fisher Scientific). After addition of 200 µl chloroform, vigorous shaking for 15 s, 3 min incubation at room temperature, and centrifugation at 12,000g for 15 min at 4°C, the aqueous phase was mixed with 450 µl anhydrous ethanol and transferred to an RNA Clean & Concentrator-5 column (Zymo Research) for downstream RNA purification and concentration. The complete RHV open reading frame (ORF) was amplified and subjected to deep-sequencing analysis as previously described.<sup>[14]</sup> RHV RNA was quantified by one-step TaqMan reverse-transcription quantitative PCR, as previously described.<sup>[15]</sup> Details on isopycnic centrifugation and reverse-transcription quantitative PCR primers are specified in Text S1.

## RHV infectivity titration

For cell culture-derived RHV (RHVcc) infectivity titration assays, 96-well microplates were coated for 2 h at 37°C with 50 µl of laminin (Sigma-Aldrich) at a concentration of 10 µg/ml, corresponding to

approximately 1.5 µg/cm<sup>2</sup>. Unbound laminin was discarded, and the wells were washed three times with PBS before seeding of 13,500 permissive McA-RH7777.hi rat hepatoma cells per well. The following day, the cells were infected with serially diluted supernatant (10-fold dilutions; lowest dilution 1:2) and incubated for 48 h under standard growth conditions. The cells were fixed with methanol for 5 min at room temperature, and viral antigen was visualized by immunofluorescence staining using mouse anti-RHV IgG and Alexa Fluor 594 goat anti-mouse IgG (A-11005; Thermo Fisher Scientific). Imaging was performed at ×50 magnification using a Carl Zeiss Axio Vert.A1 microscope. Culture supernatant infectivity titers were calculated following manual enumeration of focus-forming units (FFUs). One FFU was defined as a cluster of more than one infected cell at a distance of at least two noninfected cells from any other FFU. Data points represent means ± SD from triplicates.

## RHVcc neutralization assay

RHV permissive McA-RH7777.hi rat hepatoma cells were seeded in laminin-coated 96-well microplates. The next day, RHVcc adjusted to 25,000 FFUs per milliliter was incubated for 1 h at 37°C with heat inactivated serum (56°C for 30 min). Subsequently, the culture media was discarded from the 96-well plates, and the virus-antibody suspension (100 µl) was transferred to the McA-RH7777.hi cells and incubated for 4 h at 37°C. Following removal of the virus-antibody mixture, the cells were washed twice with PBS supplemented with 10% FBS and incubated for 48 h under standard growth conditions. Infected cells were visualized as previously described. Percent neutralization was calculated by relating FFU counts to the mean of three replicate negative control samples. Pre-immune sera of the respective animal were used as a negative control.

## Immunoblotting

For immunoblotting, anti-RHV-E2 mouse monoclonal IgG (clone 3G2, 1:1000); anti-scavenger receptor class B type I (SR-BI) rabbit polyclonal IgG (1:1000, NB400-104SS; Novus Biologicals), and anti-β-tubulin mouse monoclonal IgG (1:2000, MA5-16308; Thermo Fisher Scientific) were applied in blocking buffer for 4 h at room temperature with agitation. Species-specific Alexa Fluor Plus 488 (A32731; Thermo Fisher Scientific) or Alexa Fluor Plus 647 (A32728; Thermo Fisher Scientific) conjugated antibodies were diluted in PBS supplemented with 0.1% Tween 20 and used for visualization of antigen. See Text S1 for details.

## Knockdown of SR-BI by RNA interference

SR-BI depletion was achieved by Lipofectamine RNAiMAX (Thermo Fisher Scientific) transfection of small interfering RNA (siRNA) pools (Horizon Discovery). See Text S1 for details.

## RESULTS

### Production of infectious RHV in cell culture after genomic RNA transfection

To establish infectious RHV cell culture systems, we used McA-RH7777.hi rat hepatoma cells with increased replication permissiveness.<sup>[15]</sup> Transfection with in vitro transcribed (IVT) RNA from the wild-type (WT) pRHV-rn1 clone, infectious in vivo<sup>[13]</sup> (Figure 1A), led to a slower decrease in intracellular and extracellular viral RNA compared with a replication deficient mutant [pol(-)] (Figure 1B). To increase replication and chances of adaptation to virus production, we engineered mutations coding for previously described<sup>[15]</sup> single (W2372R and D2374G) or combined (K2130Q/E2223K/D2374G, S1757A/T2373A, and T2175A/W2372R/V2560A) nonsynonymous REMs into the pRHV-rn1 clone. Early after transfection, viral RNA levels decreased for all mutants; however, from day 4 and onward, extracellular RHV RNA from cells transfected with RHV-D2374G, RHV-K2130Q/E2223K/D2374G, and RHV-S1757A/T2373A plateaued and subsequently increased. For the latter two, increases of >6-fold (cells) and >45-fold (supernatant) were observed from 16 to 24 days following transfection (Figure 1B). Supernatant from the RHV-S1757A/T2373A transfection was passaged to naïve cells, and viral antigen could be visualized using RHV-specific IgG (anti-RHV) (Figure 1C). Using a newly established FFU assay, no infectivity was observed for WT; however, infectivity titers of up to approximately 5.5 log<sub>10</sub> FFU/ml were observed in the RHV-K2130Q/E2223K/D2374G and RHV-S1757A/T2373A transfection cultures. Although RHV-W2372R efficiently enhanced RHV replication,<sup>[15]</sup> no virus was produced in this culture (Figure 1D). In aggregate, productive infection and efficient infectious particle production could be confirmed in culture.

### Adaptation of envelope protein E2 is crucial for efficient spread of RHV in cell culture

To confirm infectivity and study any further adaptation, transfection supernatant of RHV-S1757A/T2373A day 12 and 24, and RHV-K2130Q/E2223K/D2374G day 24, was serially passaged 3 times at 7–9 days post infection (dpi) (Figure 1E). In all passages, the three lines

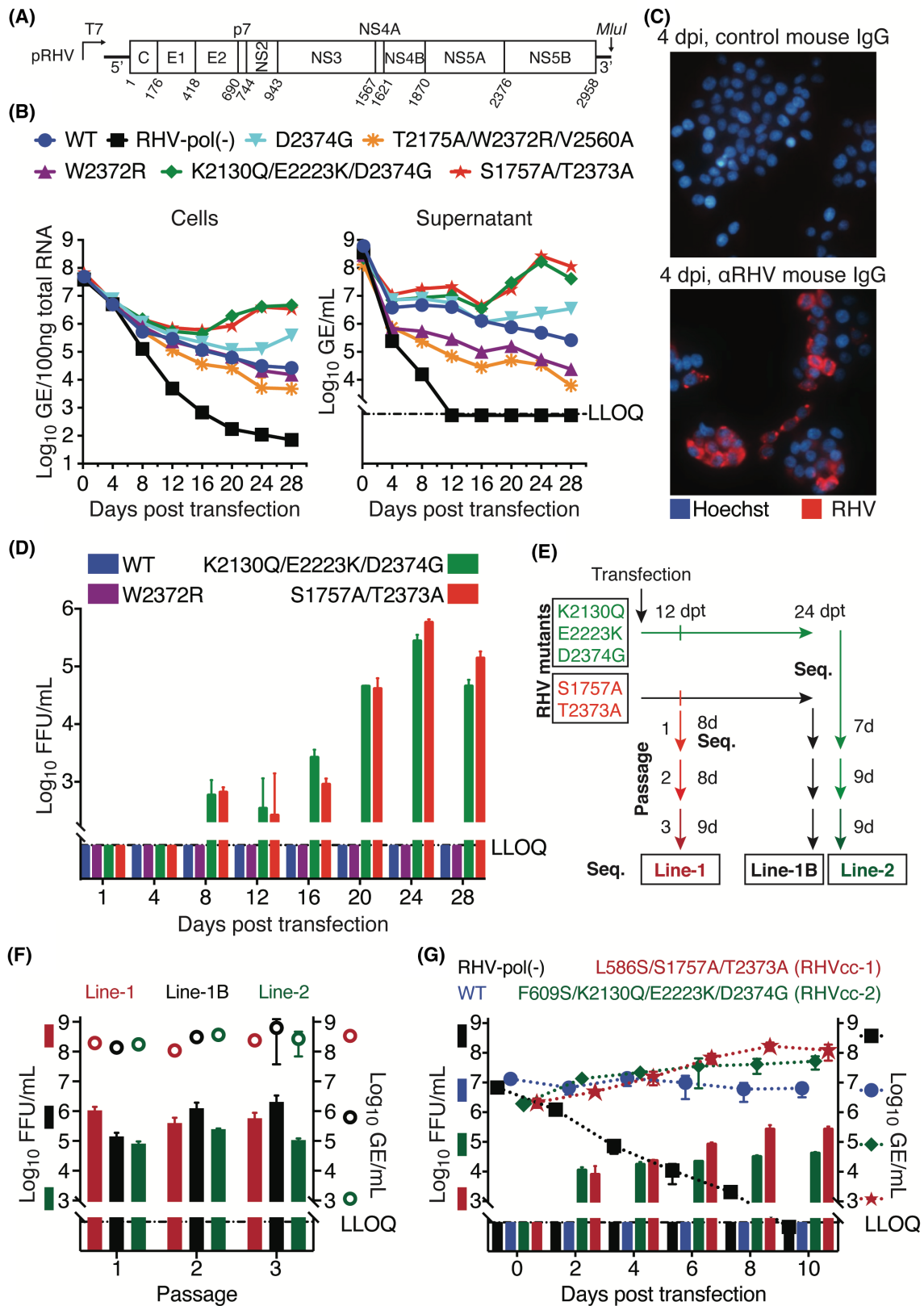
(named 1, 1B, and 2) led to viral RNA levels of >8 log<sub>10</sub> genome equivalents (GE)/ml supernatant, and infectivity titers of 5.0–6.5 log<sub>10</sub> FFU/ml (Figure 1F). In adaptation Line-1 and Line-2, the E2 substitutions L586S and F609S were acquired at passage 1 or already in the transfection culture (passage 0), respectively (Table 1). For Line-1B, the E2 substitutions N562D and N637K were found in the transfection culture, whereas L586S appeared later at passage 3. In addition to the engineered REMs, Line-1 carried only the L586S substitution, whereas Line-1B and Line-2 had accumulated eight and five additional mutations, respectively. Line-2 had low specific infectivity of approximately 1000–2000 GE per FFU, whereas for Line-1 and Line-1B it was 100–500 GE per FFU, comparable to culture-derived HCV.<sup>[6]</sup> The REMs were maintained in culture in all adaptation lines throughout the three passages, and additional E2 mutations were consistently observed.

To confirm that the RHV E2 substitutions L586S and F609S permitted infectious RHV production, we engineered these to produce RHV-L586S/S1757A/T2373A (RHVcc-1) and RHV-F609S/K2130Q/E2223K/D2374G (RHVcc-2). Following transfection, viral RNA levels and FFU titers increased to about 8 log<sub>10</sub> GE/ml and 5 log<sub>10</sub> FFU/ml within 6 days (Figure 1G). Hence, we established a robust and efficient cell culture system for RHV with only minor modification (3–4 substitutions) of the genomic viral RNA. Given its fewer mutations and high fitness in culture, RHVcc-1 was pursued for further experiments.

### Cell culture–derived RHVcc-1 is infectious in vivo

To investigate whether culture-adapted RHV mutations were compatible with infection in vivo, we inoculated IVT RNA from either RHV-rn1 (WT) or RHVcc-1 directly into the liver of inbred Lewis rats (Figure 2A,B). Compared with RHV-rn1, RHVcc-1 viremia was about 150-fold lower at 1 week post infection (wpi) but from 3 wpi and onward it was comparable, with titers peaking at about 9 log<sub>10</sub> GE/ml, indicating that culture-adaptive mutations attenuated infection in vivo. All rats remained persistently infected for >1 year.

To next assess whether culture-derived virus particles were infectious in vivo, we infected Lewis rats, C57BL/6 mice, and severe combined immunodeficiency (SCID) mice with RHVcc-1. In Lewis rats, the course of infection mimicked that of the RHVcc-1 RNA inoculation. In C57BL/6 mice, RNA levels peaked at about 6.0–6.5 log<sub>10</sub> GE/ml, and the infection was cleared within 2 weeks, similar to infection with rat serum-derived RHV.<sup>[14]</sup> In SCID mice, however, infection persisted until the end of study at 13 wpi with viremia about 10-fold lower compared with immunocompetent rats (Figure 2C). The increased RHVcc-1 fitness observed



in rats from week 3 and onward was accompanied by the loss of infectivity in culture, as inoculation with serum samples from 1 wpi but not 8 wpi led to productive infection. For the 8 wpi samples, RHV-positive cells

were observed early after culture inoculation, suggesting some level of virus uptake, but absence of robust replication and virus production. Serum samples from C57BL/6 mice 1 wpi and SCID mice 1 and 8 wpi were

**FIGURE 1** Productive rodent hepacivirus (RHV) infection in rat hepatoma cells. (A) Diagram outlining the RHV from *Rattus norvegicus* 1 (RHV-rn1) molecular consensus clone with indication of predicted protein boundaries. (B) Quantification of intracellular and extracellular RHV-RNA genome equivalents (GE) after transfection of McA-RH7777.hi cells with RHV genomic RNA harboring single and combined REMs. (C) RHV-S1757A/T2373A antigen visualized using total IgG from C57BL/6 mice that resolved RHV infection. (D) Infectivity titration given as focus-forming units (FFUs) per milliliter in culture supernatant from (B). (E) Strategy for cell culture passaging of RHV. (F) Peak RHV GE (circles) and FFU titers (bars) per milliliter supernatant for each passage. (G) Quantification of RHV GE (dashed lines) and FFUs (bars) per milliliter of supernatant after transfection of McA-RH7777.hi cells with replication-deficient mutant (RHV-pol[-]), wild-type (WT), or cell culture–adapted RHV (RHVcc) RNA. All data points in (B), (D), (F), and (G) represent mean values  $\pm$  SD from technical duplicates (reverse-transcription quantitative PCR) and triplicates (infectivity titration assay). Abbreviations: dpi, days post infection; LLOQ, lower limit of quantification

all infectious in cell culture (Figure 2D), although serum from two of three C57BL/6 mice led to delayed infection kinetics.

To compare sequence adaptation in vivo and in vitro, virus recovered from serum samples and corresponding passaged cell culture supernatant were subjected to whole ORF deep sequencing analysis. At 8 wpi, the RHVcc-1 cell culture–adaptive substitutions L586S and T2373A almost completely disappeared in all three rats analyzed, whereas S1757A remained in R16 and partially in R14. Interestingly, the culture-adaptive mutations prevailed in C57BL/6 mice (1 wpi) and during chronic infection in SCID mice (Figure 2E and Data S2). Thus, maintenance of culture-adaptive mutations negatively correlated with immune competency. Furthermore, a mutation hotspot was observed in the NS5A domain II with changes in particular at residues 2211, 2214, 2217, 2225, and 2245 in all three types of animals (Figure 2E). No other major changes were

observed for C57BL/6 mice, but in SCID mice a number of E1-E2 mutations also previously observed when adapting RHV to different immunodeficient mice<sup>[14]</sup> appeared. Except for L366I, these putative mouse-adaptive mutations were selected against following culture passage in rat cells (Figure 2E; w8+2). Hence, we demonstrate that culture-derived RHVcc-1 is infectious in vivo, and that animal-derived serum from SCID mice or early time points in immunocompetent rats and mice likewise is infectious in culture.

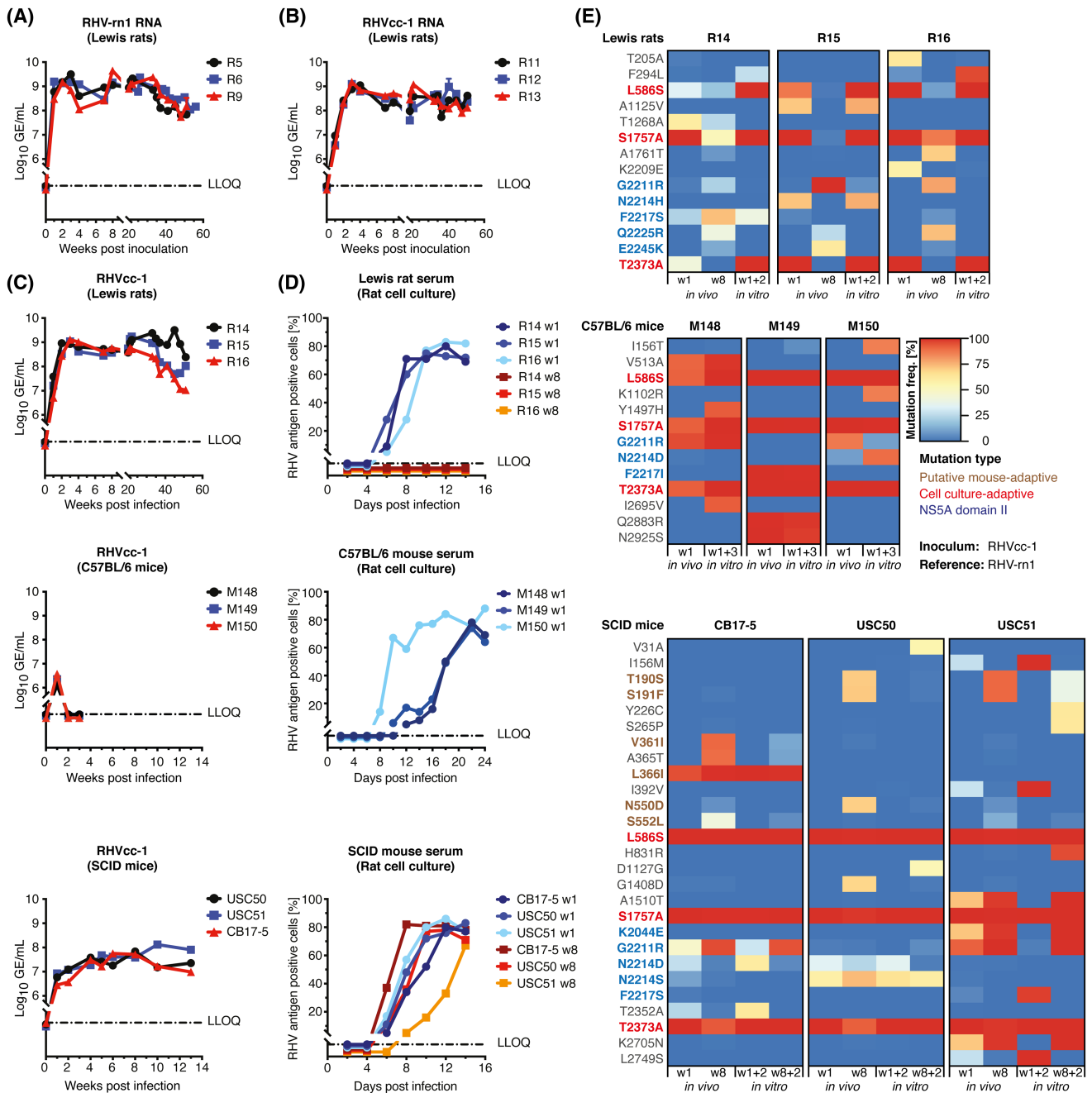
### Biophysical properties of RHVcc particles are characteristic of hepaciviruses

In patient serum, infectious HCV particles circulate as lipoviro-particles in association with VLDL.<sup>[21,22]</sup> Using isopycnic equilibrium centrifugation and reverse-transcription

**TABLE 1** Sanger sequencing of passages 1–3 during RHVcc adaptation

Protein	Passage No.			0		1	3		
	Pos	Ref	Alt	RHVcc-1B	RHVcc-2	RHVcc-1	RHVcc-1B	RHVcc-2	RHVcc-1
Core	953	A	G				I156M		
E1	1388	G	A				M301I		
	1480	A	G				N332S		
E2	2169	A	G	N562D			N562D		
	2242	T	C			L586S	L586S		L586S
	2311	T	C		F609S			F609S	
	2396	C	G	N637K			N637K		
NS4B	5754	T	G	<u>S1757A</u>		<u>S1757A</u>	<u>S1757A</u>		<u>S1757A</u>
NS5A	6322	T	A					I1946N	
	6873	A	C		K2130Q			K2130Q	
	7099	A	G					Q2205R	
	7152	G	A		E2223K			E2223K	
	7173	A	G					T2230A	
	7390	T	C					F2302S	
	7602	A	G	<u>T2373A</u>		<u>T2373A</u>	<u>T2373A</u>		<u>T2373A</u>
	7606	A	G		D2374G			D2374G	
NS5B	7660	A	G	Q2392R			Q2392R		
	8212	A	G	K2576R			K2576R		

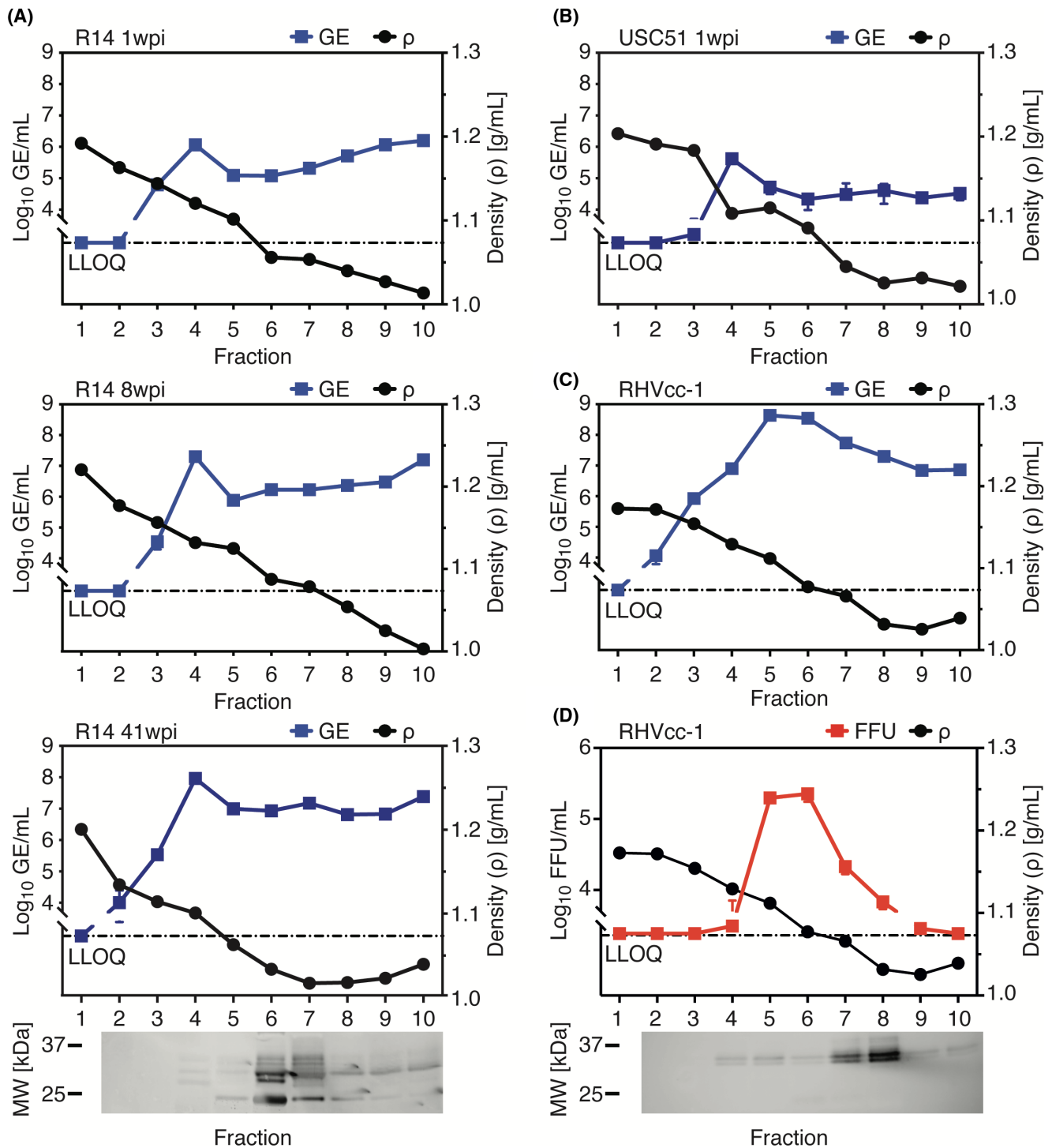
Note: Amino acid numbering according to the RHV-rn1 polyprotein (GenBank ID: AQV09561). Underlined terms indicate replication-enhancing mutations.



**FIGURE 2** RHVcc-1 is infectious in mice and rats. Intrahepatic inoculation of *in vitro*-transcribed genomic RNA of RHV-rn1 (A) and RHVcc-1 (B) in Lewis rats. (C) Infection of Lewis rats, C57BL/6, and SCID mice with RHVcc-1. (D) Infection of McA-RH7777.hi rat hepatoma cells with sera collected 1 week post infection (wpi) and/or 8 wpi of RHVcc-1-infected Lewis rats, C57BL/6, and SCID mice. (E) RHVcc-1 whole open reading frame deep-sequencing analysis in Lewis rats, C57BL/6, and SCID mice *versus* rat cell culture. Substitutions are included when the frequency was >50% in at least one condition and shown against the RHV-rn1 reference, resulting in red coloring of cell culture-adaptive mutations whenever these were kept *in vivo* and *in vitro*. For SCID mice, previously observed putative mouse-adaptive substitutions<sup>[14]</sup> are included in addition. GE, genome equivalent

quantitative PCR, we found that RHV particles collected from rat serum 1, 8, and 41 wpi also had low densities of 1.0–1.12 g/ml (Figure 3A). RHV-RNA levels peaked at 1.12 g/ml and were not detected at densities higher than 1.18 g/ml. The highest abundance of particle-associated E2 glycoprotein from chronically infected rats (41 wpi) was about 1.02–1.04 g/ml, which did not correspond with

the peak RHV-RNA titer. RHV particles derived from SCID mouse serum collected 1 wpi had a highly similar density profile (Figure 3B). Cell culture-produced particles, on the other hand, had peak RNA titers at a slightly lower density of about 1.10 g/ml. Compared with rat serum-derived particles, we observed a decline in RHV genomic RNA in very-low-density fractions (Figure 3C).



**FIGURE 3** RHVcc-1 particles have biophysical properties characteristic of hepaciviruses. (A) Rat sera derived from R14 (1, 8, and 41 wpi) were loaded on iodixanol density gradients. For each collected fraction, RNA GE per milliliter and buoyant density ( $\rho$ ) are plotted. For the 41 wpi time point, fractions 2–10 were analyzed for E2 by immunoblot (bottom). (B) Density profile for SCID-derived RHVcc-1 particles (1 wpi) is shown as in (A). (C,D) Density profile for cell culture–derived RHVcc-1 is shown as described in (A) with indication of GE per milliliter (C) and FFUs per milliliter (D). Fractions 2–10 were analyzed for E2 by immunoblot (bottom). MW, molecular weight

The highest infectivity titers for culture-produced virus corresponded with the highest RNA levels, suggesting efficient packaging of viral genomic RNA and release of infectious RHVcc-1 particles in cell culture (Figure 3D). Immunoblotting of E2 revealed more heterogenous

electrophoretic mobility of rat serum compared with cell culture–derived particles, suggestive of more heterogenous posttranslational modifications, such as glycosylation,<sup>[23]</sup> in vivo (Figure 3A,D). Enrichment of the RHVcc-1 E2 glycoprotein was observed at a low density of 1.05 g/

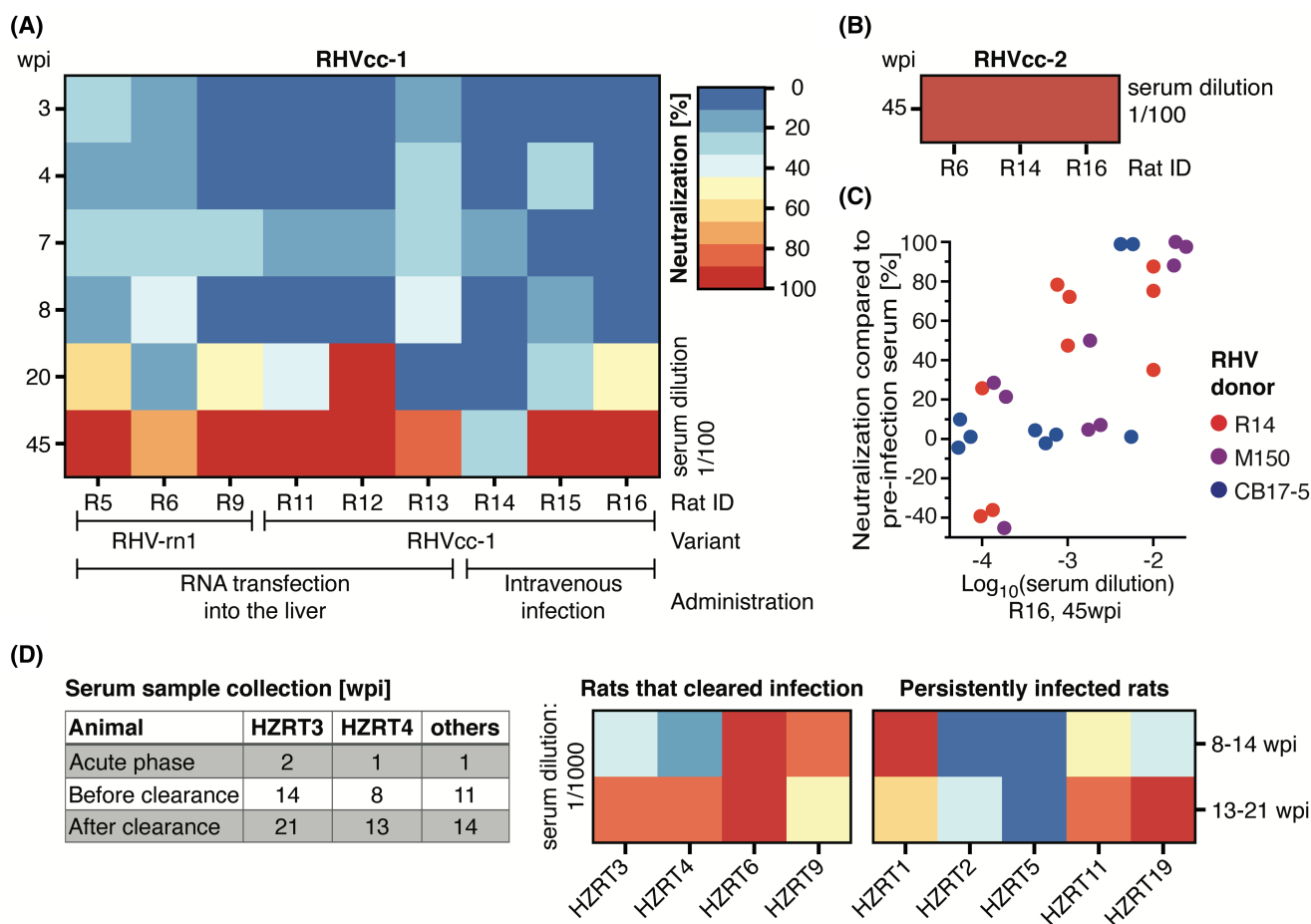


ml, similarly to what was found in vivo. Thus, increased levels of E2 glycoproteins embedded in the virus envelope were not associated with increased infectivity in vitro.

### Development of RHV neutralizing antibodies during the chronic phase of infection in persistently infected rats

To allow the assessment of nAbs, we developed an in vitro neutralization assay based on RHVcc-1 (see Methods section and Figure S3A). For up to 8 wpi, no nAbs were detected in the serum of rats inoculated intrahepatically with genomic RHV-rn1 or RHVcc-1 RNA or infected with RHVcc-1 (Figure 4A). However, from 20 wpi, neutralizing activity was detected and >95%

neutralization was observed in 6 of 9 rats at 45 wpi (Figure 4A). Low neutralization (e.g., for R11 at 20 wpi) was not due to the absence of structural protein specific antibodies, as shown by immunostaining of 293T cells expressing RHV envelope proteins E1 and E2 (Figure S3B). Because culture-adaptive mutations could affect neutralization for HCV,<sup>[24–26]</sup> we compared the ability of rat sera with low, moderate, or high nAb titers (R14, R6, and R16, respectively, all at week 45) to neutralize RHVcc-2. RHVcc-2 was highly neutralized by all sera, suggesting that its E2 mutation, F609S, increases sensitivity to nAbs (Figure 4B). In addition, highly neutralizing chronic phase R16 serum efficiently neutralized acute-phase in vivo–derived RHV from rat, C57BL/6, and SCID mice with an efficiency of >75%, ruling out that the observed neutralization was caused by culture-specific biophysical



**FIGURE 4** Delayed development of neutralizing antibodies in RHV-infected rats. (A) Lewis rats were inoculated with RHV or RHVcc-1 genomic RNA or infected with in vitro–produced RHVcc-1 particles. The percentage of neutralization compared with naïve pre-infection serum at indicated time points is shown. Each data point of the heat map represents triplicate experiments. (B) Serum from 45 wpi from Lewis rats with low (R14), moderate (R6), and high (R16) levels of nAbs against RHVcc-1 were used to neutralize cell culture–derived RHVcc-2 (percentage of neutralization as in [A]). (C) R16 week 45 serum was assayed for ability to neutralize virus present in week 1 acute phase sera (diluted 1:50) of Lewis rat R14, C57BL/6 mouse M150, and SCID mouse CB17-5. Virus spread was quantified at 8 days post infection (dpi) when >70% of the rat hepatoma cells were infected using naïve R16 pre-infection serum. Each replicate is shown. One replicate each of SCID mouse and rat serum showed low neutralization at 1:100 dilution. No putative E1-E2 escape mutations were identified in these after sequencing, suggesting that in these samples, borderline nAb titers did not prevent RHV spread. (D) Sera derived from persistent and resolving RHV-infected Holtzman rats were sampled during acute phase, shortly before clearance, and after clearance or at corresponding time points. Percentage of neutralization is shown as in (A)

properties of RHVcc (Figure 4C). Thus, inbred Lewis rats failed to clear RHV infection despite the development of nAbs (Figures 2A–C and 4A). The development of nAbs between weeks 20 and 45 in R15 and R16 (Figure 4A) was accompanied by an approximate 10-fold decrease in viremia from 33 wpi (Figure 2C), which contrasted with that observed for R14 that did not develop nAbs. A high level of sequence evolution of the envelope proteins was observed during that period for R15 and R16 compared with R14, including mutations in a previously described major histocompatibility complex (MHC) class I epitope<sup>[27]</sup> (Figure S3C). This was compatible with potential escape from adaptive immune responses.

Suppression of viremia and delayed virus clearance has been observed in outbred Holtzman rats.<sup>[13]</sup> For such rats, we observed a tendency toward higher nAb titers in resolvers, as >65% neutralization was observed for 3 of 4 animals following clearance. In comparison, only 1 of 5 chronically infected Holtzman rats had that neutralizing capacity at comparable time points (Figure 4D). Thus, nAbs may play a role in RHV clearance, although further studies will be needed.

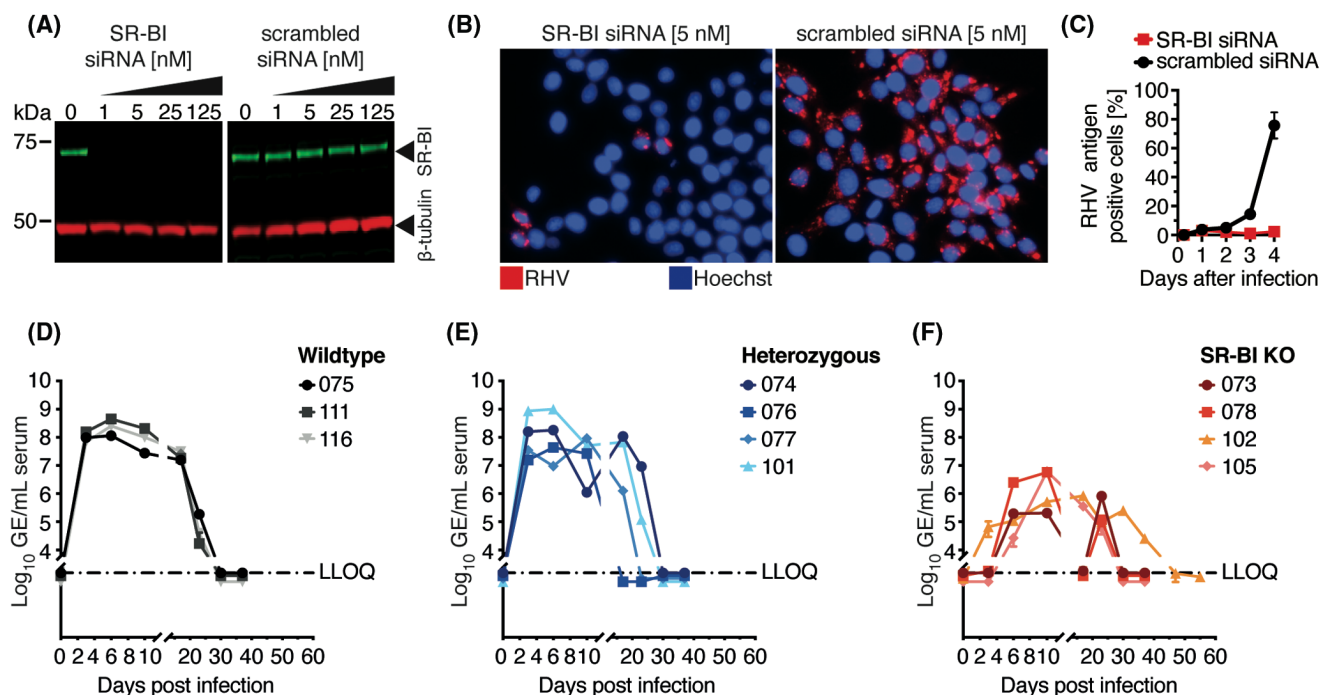
## Scavenger receptor class B type I is important for RHV entry

For HCV, resistance to nAbs has been partially ascribed to correlate with a closed conformation of the envelope

proteins. The entry factor SR-BI has been suggested to confer transition to a more open conformation, mediating virus uptake.<sup>[28]</sup> In addition, residues S112 and T175, pivotal for receptor function during HCV uptake,<sup>[29]</sup> are conserved among human, mouse, and rat SR-BI (Figure S4). We therefore set out to investigate SR-BI requirement for RHVcc-1 entry in vitro by siRNA-mediated knockdown of SR-BI (Figure 5A). RHVcc-1 entry was significantly reduced under SR-BI depletion, indicating a strong dependence on this rat entry factor (Figure 5B). At 4 dpi, less than 5% of SR-BI siRNA transfected cells were infected as compared with >75% in control cells (Figure 5C). To evaluate this observation in vivo, we used an SR-BI-deficient mouse model.<sup>[30]</sup> Infection of WT (SR-BI<sup>+/+</sup>) or heterozygous (SR-BI<sup>+/-</sup>) B6;129 mice with mouse-adapted RHV led to an approximate 4-week high-titer infection, similarly to that observed previously in C57BL/6 mice<sup>[14]</sup> (Figure 5D,E). However, SR-BI-deficient mice (SR-BI<sup>-/-</sup>) had a delay of 3–4 days in onset of viremia and about 100-fold lower peak viremia (Figure 5F). SR-BI therefore appears to be an important entry factor for RHV in vitro and in vivo.

## DISCUSSION

We report an infectious cell culture system for the efficient propagation of RHV and establishment of in vitro infectivity and neutralization assays pivotal for



**FIGURE 5** SR-BI facilitates RHV infection. (A) Immunoblot of McA-RH777.hi cells transfected with SR-BI-targeting or scrambled control small interfering RNAs (siRNAs). (B) RHVcc-1 antigen (red) was visualized at 4 dpi in siRNA-transfected McA-RH777.hi cells (blue nuclei, Hoechst dye). (C) Quantification of RHVcc-1 antigen-positive cells following siRNA transfection. All data points represent mean  $\pm$  SD from three representative microscopy images. Infection of *scarb1*<sup>+/+</sup> (D), *scarb1*<sup>+/-</sup> (E), and *scarb1*<sup>-/-</sup> mice (F) with mouse-adapted RHV.<sup>[14]</sup> KO, knockout

evaluation of natural and induced immunity. RHVcc particles have biophysical properties characteristic of hepaciviruses and are infectious in both mice and rats. Using this RHV *in vitro* system, we validated that the rodent animal models recapitulate hallmarks of chronic HCV infection, including the typical delayed development of nAbs and dependence on the entry factor SR-BI.<sup>[31,32]</sup>

Efficient RHV cell culture propagation required a minimum of three adaptive mutations in E2, NS4B, and NS5A viral proteins. This is comparable to the HCV genotype 2a strain JFH1, which readily replicates in Huh-7 human hepatoma and derived cell lines at low efficiency<sup>[7]</sup> but gains fitness by accumulating few culture-adaptive mutations,<sup>[33,34]</sup> unlike other clinical HCV isolates, which do not easily adapt to culture. RHV REMs<sup>[15]</sup> persisted during successful cell culture propagation, confirming that these substitutions mediate infectious virus production. This is in contrast to HCV, in which REMs have been described to attenuate virus replication and particle production *in vivo*<sup>[35]</sup> and *in vitro*.<sup>[36]</sup> Unfortunately, all RHV adaptation schemes led to the selection of at least one E2 mutation that greatly improved RHV spread and infectivity titers, thereby preventing neutralization assays using the native E2.

RHVcc-1 infection was attenuated at early time points in rats, but serum titers comparable to WT infection were observed 3 wpi, concomitant with the reversion of cell culture-adaptive substitutions. Similar to animal-derived RHV,<sup>[14]</sup> intravenous RHVcc-1 virus inoculation caused short-lived acute infection in C57BL/6 mice and chronic high-titer infection in immunocompromised mice. Cell culture-adaptive mutations persisted in all animals at 1 wpi, and corresponding acute phase serum-derived RHVcc-1 could be successfully recultured *in vitro*. Reversion of mutations was not observed in immunodeficient SCID mice, suggesting that adaptive immune pressure in rats was a primary driver of reversion. Accordingly, serum samples collected from SCID mice at 8 wpi, unlike those from rats, could be propagated in cell culture. It is possible that neutralizing antibodies in rats led to reversion of L586S in E2, whereas the mutations in nonstructural proteins could either be functionally linked to the E2 mutation or be targeted by T cells. Indeed, residue 1757 lies within a previously described MHC-II epitope<sup>[27]</sup> (Figure S3C). For HCV, serum from chimpanzees infected with culture-derived JFH1<sup>[37]</sup> or J6/JFH1 recombinant<sup>[38]</sup> collected after 7 wpi and 3 wpi, respectively, contained *in vitro* infectious virus. J6/JFH1 did not require culture adaptive mutations,<sup>[6,39]</sup> and could be propagated in culture after up to 20 wpi of human liver-chimeric uPA-SCID mice.<sup>[38]</sup> For JFH1, adaptations that appeared *in vivo* also led to increased particle production *in vitro*.<sup>[40]</sup> Reversion of cell culture-adaptive mutations has, however, been reported in a chimpanzee

following challenge with cell culture-derived HCV genotype 1a H77,<sup>[41]</sup> similar to what we observed for RHV in rats. During persistent RHV infection of SCID mice, mutations arose primarily in E1-E2 with residues overlapping those previously identified in immunocompromised NOD-Rag1<sup>-/-</sup>IL2R $\gamma$ <sup>-/-</sup> mice,<sup>[14]</sup> suggesting those to be generally mouse-adaptive. Furthermore, infection in all tested rodents led to mutations in NS5A domain II, which for HCV has been shown to engage in critical interactions with the host factor cyclophilin A (CypA).<sup>[42]</sup> For HCV, CypA is essential for viral replication, and its interaction with NS5A promotes binding to HCV RNA.<sup>[42,43]</sup> It remains to be investigated whether CypA is similarly required for RHV.

We found that most RHVcc-1 particles had buoyant density of 1.10 g/ml, whereas animal-derived RHV particles peaked at higher buoyant density of 1.12 g/ml. In contrast, cell culture-derived JFH1 and J6/JFH1 HCV particles (1.14–1.17 g/ml) were denser in comparison with particles isolated from chimpanzee plasma (1.10 g/ml),<sup>[7,38]</sup> but had highest specific infectivity at 1.10 g/ml.<sup>[6]</sup> The low density of RHVcc-1 therefore indicates that the highly permissive McA-RH7777.hi rat hepatoma cells<sup>[15]</sup> allow for a lipid composition more closely mimicking that *in vivo*.<sup>[44]</sup> Unlike for HCVcc,<sup>[6]</sup> the density fractions with peak genomic RHVcc-1 RNA titers correlated with highest specific infectivity.

Using the RHV animal model, previous reports showed that a T cell-targeted vaccine at least partially can prevent persistent RHV infection in rats.<sup>[16,17]</sup> However, in a clinical trial this strategy did not protect individuals at risk for HCV infection.<sup>[18]</sup> This suggests that humoral immune responses are critical for preventing chronicity. Using RHVcc-1, we confirmed the emergence of nAbs in the serum of persistently infected inbred Lewis rats in the late chronic phase of infection at >20 wpi. Sera with highly neutralizing capacity against RHVcc-1 also efficiently neutralized RHV derived from acute phase rat and mouse infections. For HCV, sensitivity to neutralization was strongly increased by certain culture-adaptive mutations.<sup>[24–26]</sup> This was also observed for the RHVcc-2 E2 substitution F609S; hence, RHVcc-2 was less suited for use in neutralization assays. In 4 of 9 outbred Holtzman rats, infection was cleared within 13–21 weeks. Similar to HCV infection, viral clearance was associated with the emergence of nAbs; however, 2 of 5 persistently infected rats failed to resolve RHV infection despite development of high levels of nAbs. This is reminiscent of chronic HCV infection, which can be established in spite of nAbs due to continuous viral escape.<sup>[45]</sup> The breadth of nAbs<sup>[46]</sup> and time of emergence<sup>[19,47]</sup> have particularly been identified as important parameters of HCV clearance. The rodent HCV surrogate model and complementary *in vitro*

neutralization assays will therefore enable in-depth studies to characterize the determinants of humoral immune responses critical in mediating hepacivirus clearance.

The HDL receptor SR-BI has been demonstrated to interact with the HCV E2 glycoprotein as an important entry factor.<sup>[31]</sup> Strikingly, RHV infection was also considerably compromised in vitro and in vivo when SR-BI was depleted or ablated, respectively. The highly enhancing yet dispensable SR-BI interaction compares well with HCV, for which SR-BI has been shown to promote uptake in vitro<sup>[48]</sup> and in vivo.<sup>[49]</sup> In a mouse model expressing human CD81 and occludin, HCV infection was successful; however, exogenous mouse or human SR-BI significantly boosted susceptibility.<sup>[49]</sup>

In conclusion, we present an infectious cell culture system for RHV that will be highly useful for production of infectious particles, reverse genetic analyses to study virus–host interactions, and in vitro RHV neutralization assays for critical evaluation of vaccine platforms. Ultimately, this model will shed light on the importance of nAbs and deepen our knowledge of the humoral adaptive immune response to hepaciviral infection. The RHV model has renewed interest in HCV vaccine development,<sup>[50]</sup> as proof-of-concept from RHV vaccine challenge studies could inform platform selection and potential analogous combinations for HCV vaccine candidates. Given the absence of HCV vaccine challenge models and the difficulties in accessing high-incidence cohorts for meaningful vaccine studies, such pilot studies could become critical for future vaccine strategies.

## ACKNOWLEDGMENTS

We thank Birgitte Børsting for the RHV E1-E2 expression construct; Elias H. Augestad, Emil Bach, Rui Costa, Andrea Galli, Emma A. Lundsgaard, and Jannick Prentoe for constructive discussions and methodological insights; Lotte Mikkelsen and Anna-Louise Sørensen (CO-HEP) and Louise B. Christensen (Department of Clinical Microbiology, Hvidovre Hospital) for the laboratory assistance; and Bjarne Ørskov Lindhardt (Department of Infectious Diseases, Hvidovre Hospital) and Carsten Geisler (University of Copenhagen) for valuable departmental support. The Department of Clinical Microbiology, Hvidovre Hospital, contributed access to miSeq equipment. We further acknowledge the Department of Experimental Medicine, University of Copenhagen, for rodent husbandry and care.

## CONFLICT OF INTEREST

Nothing to report.

## AUTHOR CONTRIBUTIONS


*Conceptualization – Ideas; formulation or evolution of overarching research goals and aims:* Raphael

Wolfisberg, Jens Bukh, Kenn Holmbeck, Troels K. H. Scheel; *Data curation – Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later re-use:* Raphael Wolfisberg, Ulrik Fahnøe; *Formal analysis – Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data:* Raphael Wolfisberg, Caroline E. Thorselius, Eduardo Salinas, Ulrik Fahnøe; *Funding acquisition – Acquisition of the financial support for the project leading to this publication:* Raphael Wolfisberg, Amit Kapoor, Arash Grakoui, Charles M. Rice, Jens Bukh, Troels K. H. Scheel; *Investigation – Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection:* Raphael Wolfisberg, Caroline E. Thorselius, Eduardo Salinas, Elizabeth Elrod, Sheetal Trivedi, Louise Nielsen, Kenn Holmbeck; *Methodology – Development or design of methodology; creation of models:* Raphael Wolfisberg, Eduardo Salinas, Jens Bukh, Kenn Holmbeck, Troels K. H. Scheel; *Project administration – Management and coordination responsibility for the research activity planning and execution:* Kenn Holmbeck, Troels K. H. Scheel; *Resources – Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools:* Eduardo Salinas, Elizabeth Elrod, Sheetal Trivedi, Amit Kapoor, Arash Grakoui; *Software – Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components:* Raphael Wolfisberg, Ulrik Fahnøe; *Supervision – Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team:* Amit Kapoor, Arash Grakoui, Charles M. Rice, Jens Bukh, Kenn Holmbeck, Troels K. H. Scheel; *Validation – Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs:* Raphael Wolfisberg, Caroline E. Thorselius, Eduardo Salinas; *Visualization – Preparation, creation and/or presentation of the published work, specifically visualization/data presentation:* Raphael Wolfisberg, Caroline E. Thorselius, Kenn Holmbeck, Troels K. H. Scheel; *Writing – original draft – Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation):* Raphael Wolfisberg, Troels K. H. Scheel; *Writing – review & editing – Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or post-publication stages:* Raphael Wolfisberg, Amit Kapoor, Arash Grakoui, Jens Bukh, Kenn Holmbeck, Troels K. H. Scheel.

## ETHICS STATEMENT

Animal RHV infection experiments were conducted under protocol 2017-15-0201-01288 approved by the Danish Veterinary and Food Administration, or protocol no. AR15-00116 approved and monitored by the Institutional Animal Care and Use Committee (IACUC) of the Nationwide Children's Hospital according to guidelines specified in the *Guide for the Care and Use of Laboratory Animals*. RHV E2 immunization procedures were approved by the Institutional IACUC number PROTO201700372 at Emory University School of Medicine. Detailed animal procedures are outlined in Text S1.

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## SUPPORTING INFORMATION

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**How to cite this article:** Wolfisberg R, Thorselius CE, Salinas E, Elrod E, Trivedi S, Nielsen L, et al. Neutralization and receptor use of infectious culture-derived rat hepacivirus as a model for HCV. *Hepatology*. 2022;76:1506–1519. <https://doi.org/10.1002/hep.32535>