Viral persistence, liver disease and host response in Hepatitis C-like virus rat model.

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ABSTRACT

The lack of a relevant, tractable, and immunocompetent animal model for hepatitis C virus (HCV) has severely impeded investigations of viral persistence, immunity and pathogenesis. In the absence of immunocompetent models with robust HCV infection, homolog hepaciviruses in their natural host could potentially provide useful surrogate models. We isolated a rodent hepacivirus (RHV) from wild rats (*Rattus norvegicus*), RHV-rn1, acquired the complete viral genome sequence and developed an infectious reverse genetics system. RHV-rn1 resembles HCV in genomic features including the pattern of polyprotein cleavage sites and secondary structures in the viral 5’ and 3’ UTRs. We used site-directed and random mutagenesis to determine that only the first of the two miR-122 seed sites in viral 5’UTR is required for viral replication and persistence in rats. Next, we used the clone derived virus progeny to infect several inbred and outbred rat strains. Our results determined that RHV-rn1 possesses several HCV-defining hallmarks: hepatotropism, propensity to persist, and the ability of induce gradual liver damage. Histological examination of liver samples revealed the presence of lymphoid aggregates, parenchymal inflammation and macro/micro vesicular steatosis in chronically infected rats. Gene expression analysis demonstrated that the intrahepatic response during RHV-rn1 infection in rats mirrors that of HCV infection, including persistent activation of interferon signaling pathways. Finally, we determined that the backbone drug of HCV direct acting antiviral (DAA) therapy, Sofosbuvir, effectively suppresses chronic RHV-rn1 infection in rats. Taken together, we developed RHV-rn1 infected rats as a fully immunocompetent and informative surrogate model to delineate the mechanisms of HCV-related viral persistence, immunity and pathogenesis.
INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic RNA virus classified within the genus Hepacivirus of the family Flaviviridae and a leading cause of chronic liver disease globally. Following acute infection, HCV establishes lifelong persistence in 60-80% of individuals, significantly elevating the risk for developing liver cirrhosis and hepatocellular carcinoma(1). The host and viral determinants governing this acute to chronic transition of HCV infection are mostly unknown, in large part due to the absence of immunocompetent animal models for its in vivo characterization(2). Beside humans, HCV only infects chimpanzees, and therefore, unlike the in-depth in vitro characterization, most of our knowledge of HCV pathogenesis and immunity is gained through studies of infected humans or experimentally infected chimpanzees, limited by either the lack of appropriate human samples or an insufficient number of animals to enable an unambiguous analysis. Furthermore, the use of chimpanzees in the elucidation of host response patterns against HCV and for the testing of preclinical therapeutic and vaccine modalities has been stopped or restricted in most countries because of ethical considerations. Thus, a relevant, tractable, and immunocompetent animal model is urgently needed if the mechanisms behind HCV unique pathogenesis in humans are to be deciphered (3, 4).

GBV-B virus, a hepacivirus of mysterious origin, is a well-recognized HCV surrogate but unlike HCV, it usually causes only acute resolving infections in tamarins (3, 5). Nonetheless, GBV-B infection in tamarins helped in elucidating the functional importance of several viral genome features including the relevance of miR-122 binding sites in the viral 5’ UTR and the importance of different sequence elements of the 3’ UTR (6, 7). Over the years several genetically engineered mouse models of HCV have been described, but in addition of being technically cumbersome, these platforms suffer from an underlying requirement to blunt innate and/or adaptive immune responses to support even mediocre levels of viral replication, precluding detailed analysis of HCV associated immunity(8, 9).

Recently, we and others have identified multiple hepacivirus species in wild rodents (10). These rodent hepaciviruses (RHV) share common genetic and evolutionary features with HCV and therefore, with their natural hosts, could provide relevant surrogate models for the study of HCV (11). The animal host of an ideal surrogate, however, should be an accessible and well-characterized laboratory model with numerous molecular and genetic tools available for examining the mechanisms...
of viral pathogenesis. For this reason, we chose to establish a model system using a variant of a hepacivirus species identified during a metagenomics survey of pathogens in Norway rats (Rattus norvegicus) (12). We determined that this rat hepacivirus isolate, RHV-rn1 fails to establish persistent infection in immunocompetent laboratory mice (13). Here, we describe that RHV-rn1 infection in laboratory rats mirror HCV infection in humans and therefore provide an informative, tractable and fully immunocompetent surrogate model for studies of HCV persistence, immunity and liver pathogenesis.

RESULTS

Isolation, genome sequencing and reverse genetic system for RHV-rn1

Since only a partial genome of Rat hepacivirus was previously sequenced (12), we first infected four Sprague Dawley (SD) rats with a serum sample from an RHV-rn1 infected wild rat. All infected rats developed high-titer viremia at one week post-infection (p.i.) and remained viremic for the entire duration of the study (80 days p.i.) (Fig.1A). A terminal serum sample of a chronically infected female was then used to acquire the complete viral genome using next generation sequencing (Illumina HiSeq). After de novo assembly of the viral genome, the polyprotein coding sequence was re-confirmed by Sanger dideoxy sequencing. Thereafter, both genomic termini were acquired using a rapid amplification of cDNA ends and a phosphorylated oligonucleotide adapter ligation approach for 5’ and 3’ ends, respectively (14, 15). The resulting viral genome was henceforth termed RHV-rn1 as it shared 8450/8975 (94%) nucleotide and 2936/2991 (98%) amino acid identities with the published partial genome sequence of the Norway rat hepacivirus (GenBank accession no. KJ950938).

The complete genome of RHV-rn1 contains 9656 nucleotides and encodes a single 2991 amino acid long polyprotein (GenBank accession no. KX905133, Fig.1B). Cleavage sites documented for HCV are conserved in RHV-rn1 and allowed a robust prediction of the sizes of the replication-associated proteins; a separate analysis using signalaseP (16) predicted cleavage sites that would produce core, E1, E2 and p7 proteins comparable in size to those of HCV and other hepaciviruses (Fig.1B). The 5’ UTR is 485 bases long and terminates with a methionine codon in the optimal Kozak context. The 3’ end of the 5’ UTR is predicted to form a type IV internal ribosomal entry site despite a high degree of sequence divergence with HCV and other hepaciviruses in this
region (Fig. 1C). Furthermore, the 5’UTR of RHV-rn1 contains two miR-122 seed sites (CACUCC), which are important for HCV replication and hepatotropism (17, 18). The 3’UTR is 297 bases long, contains a short poly-C region followed by three stem loops (SLI to SLIII) and a U as terminal base, similar to the HCV 3’ X region (Fig. S1) (19).

Next, we constructed a complete genomic clone of RHV-rn1 using the consensus sequence described above (Fig. 2A). To test the infectivity of clone-derived transcripts, we injected 5µg of RHV-rn1 RNA directly into the livers of immunocompetent Brown Norway (BN) rats. All inoculated rats developed high-titer viremia at three days p.i. and remained viremic throughout the study period (> 17 wk p.i.) (Fig. 2B). Injected rats developed anti-NS3 IgG within 3-4 weeks p.i. No significant fluctuations in serum ALT levels were observed. In situ hybridization using a RHV-rn1 helicase specific probe showed presence of viral genomes in the cytoplasm of hepatocytes (Fig. 2C). Comparison of viral titers in different organs and a negative strand RNA assay further confirmed the hepatotropic nature of the virus (Fig. 2D and E). Sequencing of serum virus from infected rats showed 100% nucleotide identity to the plasmid clone. A serum sample from one rat with high-titer viremia was then used to infect eight naïve rats, all of which developed persistent infection (data not shown). Together, these results confirmed that the RHV-rn1 clone contains all necessary genomic elements to produce infectious virus.

**Importance of the two miR-122 seed sites in the viral 5’UTR**

The RHV-rn1 reverse genetics system provides an unlimited resource to generate natural and mutant viruses for the functional and biological characterization of different genomic features. As a proof of principle, we next focused our studies on the functional role of the two miR-122 binding sites within the viral 5’UTR. First, we created two pools of RHV-rn1 mutant plasmids, RM-1 and RM-2, using random mutagenesis of either of the two miR-122 seed sites (Fig. 3A). RNA transcripts of these pools were sequenced to confirm an approximately equimolar presence of all four ribonucleotides at the six base positions theoretically generating 4,095 unique mutants in each pool. Then, 5 µg of RM-1 and RM-2 transcripts were separately injected into the liver of individual BN rats. Both rats developed a high-titer viremia three days p.i. and remained persistently infected for the duration of the study period (82 days p.i.). All serial serum samples of the RM-1 injected rat, including the earliest sample (3 days p.i.), showed RHV-rn1 variants with the natural miR-122 seed
site sequence (CACUCC), indicating the strong selection for this seed site (Fig.3B). However, all the serial serum samples of RM-2 injected rat showed selection of an RHV-rn1 variant containing a unique sequence (ACAGUG-U) in place of the canonical miR-122 seed site sequence (Fig.3B). Even the earliest appearing serum virus, 3 days p.i., had this unique sequence, indicating rapid selection of this novel variant. Interestingly, this new sequence remained unchanged till the end of the study (90 days p.i.), indicating the dispensability of the second miR-122 seed site for viral replication and persistence. To further confirm, we infected two more rats with RM-2. Both these rats developed viremia, but, interestingly, the sequencing of serum virus showed a swarm of RM-2 variants from 3-13 days p.i. in one rat and up to 23 days p.i. in the other, after that only the wild type RHV-rn1 (CACUCC) was present in both rats. Although these results indicated the dispensability of the second miR-122 seed site for RHV-rn1 infection, this seed site apparently also conferred a replication advantage to the virus.

Next, we used site-directed mutagenesis to confirm the requirement of the first miR-122 seed site sequence (Fig.3A). Rats infected with SM-3 (auxiliary pairing mutant) and SM-12 (seed site mutant) viruses showed an early appearance of viremia that persisted for the duration of the study period (90 days p.i., Fig.3C). While the SM-3 virus did not revert back to the wild type sequence, the SM-12 virus reverted back in all three infected animals after 10 days. The SM-3 viruses maintained an intact binding site 1 for miR-122. To confirm that the SM-12 reversion is not due to the disruption of a small predicted stem loop structure, we created a double mutant virus where position 21 was changed to pair with the mutated SM-12 site. Interestingly, this double mutant failed to induce infection or seroconversion in the two injected rats. Taken together, these results indicate that only the first canonical miR-122 seed site is crucial for the virus in vivo; however, the cytidine residue at the third position in the RHV-rn1 genome, predicted to bind the 3’ region of rat miR-122, has no effect on viral replication and persistence. Interestingly, mutations at the SM-3 site in HCV can confer resistance against the miR-122 antagonir in humans (20).

**Infection outcomes and liver diseases in laboratory rats.**

While BN rats provide an exceptional in vivo platform for evaluating the role of viral genomic features, we failed to observe even partial control of RHV-rn1 infection in this inbred strain. To evaluate whether host heterogeneity influences RHV-rn1 infection outcome, we infected four
different outbred lines of rats: Sprague-Dawley (SD), Holtzman (HTZ), Long Evans (LE), and Wistar Han (WH). While LE, WH, and SD rats showed limited suppression of viral replication, HTZ rats exhibited clear differences in viral titers between individuals within the first 80 days of infection (Fig. 4). Since HTZ rats displayed the largest suppression of viremia, four rats of this strain were kept for a longer period to study the viral persistence and development of liver diseases. All six infected rats also showed elevation of ALT that fluctuated overtime similar to HCV infection in humans (Fig.5A) (21). One of the four rats started suppressing the viremia around day 150 and achieved complete virus clearance by day 275. This rat no. 52 remained clear of virus for the duration of the study (325 days), indicating complete and spontaneous resolution of viral infection (Fig.5A).

To study the histopathological changes in the livers of infected rats, two serial liver biopsies were obtained from four infected rats (no. 49, 50, 52 and 53) at days 85 and 235. A third liver biopsy was collected from one rat (no. 49) on day 287 to confirm continued progression of liver diseases. At day 85 p.i., all four infected rats exhibited moderate hepatic inflammation characterized by a consistent pattern of dense lymphocytic aggregates focused on the portal tracts. At this stage there was focal evidence of extension of the inflammation beyond the margins of the limiting plate, indicative of mild piecemeal necrosis (Fig.5B). At day 235 p.i. the histologic features of the livers had changed with evident, but diminished portal tract lymphoid infiltrates. At this point there was more parenchymal damage with hepatic plate disarray and isolated lymphocytic foci within the hepatic parenchyma and often associated with apoptotic hepatocytes. Another feature evident in the later biopsies and characteristic of human HCV infection was the presence of both macrovesicular and micro-vesicular steatosis in hepatocytes. Bile ducts were indistinct in scattered portal tracts, suggesting injury, but clearly evident injury to the ducts was rare. Next, we used in situ hybridization to co-localize virus and liver pathological changes. Interestingly, we found steatosis in the hepatocytes infected with the virus suggesting that the virus replication can directly triggers steatosis in the hepatocytes (Fig.5C).

**Intrahepatic responses during RHV-rn1 infection**

HCV infection is known to induce potent liver responses during acute as well as chronic infection (22-25). We used RNA-seq analysis of liver samples collected on days 20, 85 and 235 p.i, from HTZ rats of two different class-I haplotypes, $RT-1^d$ and $RT-1^u$. Comparison of differentially expressed genes (DEG) between infected and uninfected rats indicated an increased number of DEG.
in the later phase of infection (Fig. 6A and B). Uninfected rats with a different MHC class I molecule shared 200-285 of the DEG; most of these were involved in metabolism and physiological processes. Interestingly, we identified 416 and 457 DEG unique to infected rats with RT-1\(^l\) and RT-1\(^u\) genotypes, respectively. Analysis of the predicted biological functions of these DEG revealed their primary role in cellular and host responses against viral infection, including higher expression of several DEG involved in the interferon signaling pathway (Fig. 6C). We further identified 123 DEG that were exclusive to the infected rats of both genotypes. These DEG include genes primarily involved in leukocyte and interleukin signaling. Upstream regulators included IFN-\(\gamma\), TNF, CSF2, IL6, MYD88, STAT1 and IL2. Noticeably, while MHC class II molecules were consistently overexpressed after RHV-rn1 infection, the class I molecules were significantly repressed, albeit with an inconsistent pattern (Fig. 6C).

Notably, the magnitude of RHV-rn1 induced DEG of IFN pathways increased during the late phase of infection indicating the persistent nature of the antiviral host responses (Fig. 7 A and B). DEG shared among class I, II and III IFNs were most significantly overexpressed including OASL, OAS3, CXCL10, TNFRSF14, STAT1, STAT4, IRF7 and IFITM3. Increased IL-8 signaling was noticed in the liver at day 85 and 235 p.i. indicating an increase in angiogenesis and inflammation. There was a significant increase in the expression of genes that are known to play a role in pattern recognition receptor signaling like TLR7, TLR8, MDA-5, NALP3, PKC, IRF7, NFKB and RANTES. Suppressed biological pathways included RhoGDI signaling and Liver X-receptor activation. HCV infection in vitro leads to sequestration of cellular miR-122 (26). Our RNA-seq data of rats with the RT-1\(^l\) haplotype indicated de-repression of a number of miR-122 regulated genes (ACTR1A, CCNG1, CREB1, CYP2C9, GIT1, GNPDA1, IQGAP, KHDRBS1, LAMC1, MAP4, MTHFD2, PKM, PTPN1, RBM3, SEMA4D, SEPT2, SH3GLRL3, VAV3 and VIM). However, de-repression of DEGs was also observed for genes regulated by miR-21 and let-7 cluster in the livers of infected rats. While there were no significant differences in the expression levels of known HCV entry factors (SCRAB1, CD81, CLD1 and OCLN), the expression of a recently identified entry factor Cadherin was consistently increased in the livers of all infected rats (27).

RHV-rn1 infected rats as model for HCV Direct Acting Antiviral (DAA) therapy.
To assess the feasibility of our RHV infected rat model to study changes in the viral population and virus-specific T cell immunity after DAA cure, we treated three rats with established chronic RHV-rn1 infection with Sofosbuvir and ribavirin (Fig. 8). Daily doses of Sofosbuvir (40 mg/kg/day) and ribavirin (70 mg/kg/day in two doses each day) were comparable to the recommended human doses adjusted to rat pharmacokinetics. As controls, three chronically infected rats were treated with only ribavirin and one rat was put on a low dose Sofosbuvir (5 mg/kg/day) and ribavirin. Our results unambiguously showed that the high-dose sofosbuvir and ribavirin combination rapidly suppressed RHV-rn1 replication to below the detection threshold in all three treated rats. Low dose sofosbuvir with ribavirin and ribavirin alone, failed to suppress the viremia in treated rats. Interestingly, the viremia rapidly rebounded in all three rats a week after the drugs were discontinued (Fig. 8), suggesting that longer period of high-dose sofosbuvir and ribavirin treatment is required to achieve a sustained viral response in the rats.

DISCUSSIONS

We here isolated RHV-rn1 from samples of a wild rat, developed an infectious clone and produced homogenous virus stocks to infect several strains of laboratory rats, expecting to develop an informative model to study HCV-like viral persistence and liver diseases. Our results show that infected rats spontaneously develop chronic hepacivirus infections making this model of hepacivirus infection in its natural host of great relevance for understanding human HCV infection and immunity. We further determined that RHV-rn1 is hepatotropic and the chronic viral infection leads to slow progressive liver diseases (Fig.2).

Development of an infectious clone for RHV-rn1 will allow studies of virus mutagenesis and facilitate the development of in vitro systems. Similar developments for HCV took several years, mainly due to failure to identify the 3’ end of the viral genome and lack of a tractable model (28). We recently established a similar system for the equine non-primate hepacivirus in horses (15). We used our reverse genetic system to study the biological importance of miR-122 sites in the 5’UTR of RHV-rn1. Our results indicate that only the first of the two miR-122 sites is indispensable for RHV-rn1 replication and persistence. We also used a novel random mutagenesis approach that allowed identification of a RHV-rn1 variant with a novel sequence in place of the second miR-122 seed site.
Further studies using these different viruses, with one or both the wild type miR-122 sites may shed new light on their role in virus infection outcomes and pathogenesis. Recently, miR-122 “sponging” by HCV genomes has been suggested to play a role in regulating the gene expression of miR-122 target genes in hepatocytes, including oncogenes, thereby providing an environment fertile for end stage liver diseases such as hepatocellular carcinoma (26). Here, we showed de-repression of selected miR-122 targets in infected rats. RHV-rn1 miR-122 mutants will be useful to study the de-repression of miR-122 regulated genes in vivo, and any effect this may have on virus induced pathogenesis.

Host factors play an important role in determining the infection and the pathogenic outcomes of HCV infections in humans (2, 29). While 20-30% of infected individuals clear the virus within the first year of infection, the remaining 70-80% of individuals develop a life-long chronic virus infection (1, 30-32). The acute phase of HCV infection in humans is generally asymptomatic with the exception of mild elevations in serum ALT levels indicating liver injury (33). After several years of chronic infection, some of these patients develop liver diseases including chronic hepatitis, steatosis, fibrosis, cirrhosis and hepatocellular carcinoma (34). The variables that can dictate or contribute to these differences remain unknown, largely due to lack of a fully immunocompetent tractable model. Here, we used homogenous virus stock derived from our reverse genetics system, yet the infected HTZ rats differed in the course of viremia, infection outcomes and liver pathology (Fig.5), resembling HCV infection in humans. Thus, we believe that RHV-rn1 infection in outbred or genetically heterogeneous rats provides a unique model to identify host determinants of hepacivirus infection outcomes and pathogenesis.

HCV liver disease is characterized by the histologic triad of lymphoid aggregates in portal tracts, epithelial damage of small bile ducts and micro- and macro- vesicular steatosis of hepatocytes (35). We determined that RHV-rn1 infected rats develop 2 of these 3 features with a suggestion of biliary epithelial injury as well within 32 weeks post infection. The presence of macro- and micro-vesicular steatosis in livers of RHV-rn1 infected rats is a finding of major significance. We used in situ hybridization to determine that several virus infected hepatocytes had steatosis, suggesting direct induction by virus infection (Fig.5C). Uninfected rats and one of the infected rats did not develop steatosis. More interestingly, in rat no. 52, virus clearance lead to reversion of inflammation, confirming the direct link with the virus. Many human patients with chronic HCV also develop a
degree of hepatic steatosis. Moreover, several clinical studies have now confirmed that hepatic steatosis is an independent risk factor of HCC in patients with chronic HCV (36, 37). Development of parenchymal inflammation, hepatitis, necrosis and steatosis within eight months of RHV-rn1 infection indicates that some chronically infected rats could consequently progress to cirrhosis and HCC.

*In vitro* studies of HCV suggest that the viral NS3/4A protease can block IRF3-mediated transcriptional activation of IFNβ by cleavage and inactivation of the mitochondrial antiviral signaling (MAVS) and TIR-domain-containing adapter (TRIF) proteins (38, 39). However, strong innate immune responses are observed in humans and chimpanzees infected with HCV (22, 25, 40, 41). Notably, HCV is known to induce stronger innate immune responses than the other MAVS cleaving hepatotropic RNA virus, hepatitis A virus (22-25). Moreover, chronic liver inflammation during HCV infection contributes towards a procarcinogenic microenvironment and paves the way for development of fibrosis/cirrhosis and HCC (42). Our liver transcriptomics of RHV-rn1 infected rats during acute and chronic phase shows significant over-expression of genes associated with inflammatory responses. Interestingly, the magnitude of interferon stimulated gene (ISG) expression increased during the chronic phase compared to the early infection phase indicating persistent activation of interferon and innate immune responses during RHV-rn1 infection. Additionally, several signaling pathways of the adaptive immunity were also persistently activated. Differentiation of naïve T-cells requires antigenic stimulation mediated by the T-cell receptor and co-receptor CD4 with the antigen-MHC-II complex. IL-2 and IFN-γ are the two most important cytokines for Th1 differentiation (43). Our results indicate persistent activation of the Th1 pathway during RHV-rn1 infection in rats despite their failure to clear the viral persistence. Virus specific functional T cell responses against Core, NS3 and NS4 proteins were also detected using IFN-γ-ELISpot assays in RHV-rn1 infected HTZ rats (data not shown). MHC class-II molecules were found significantly overexpressed. Interestingly, our data showed repression of class I molecules, the expression of which is necessary for effective targeting of infected hepatocytes by cytotoxic CD8+ T cells. Overall, these results confirm that RHV-rn1 infected rats offer a suitable *in vivo* platform to elucidate the role of various ISGs and immune responses in hepacivirus pathogenesis. Notably, RNA-seq analysis of outbred animals is challenging due to inherent differences in host genetics and therefore our data needs to be confirmed using a lager cohort of infected rats and MHC matched controls.
An interferon-free regimen of DAA can achieve sustained virologic response (SVR) in most HCV infected subjects (44). Considering that persistent antigenic stimulation drives T cell exhaustion, DAA cure has the potential to reverse defects and restore protective antiviral functions (43, 45). However, recovery of T cell immunity after antiviral therapy and protection from reinfection are the critical unknowns for translational research in the post-DAA era. The rat model is suitable to characterize the function and fate of T cells during hepacivirus infection and after DAA mediated cure. Most importantly, availability of RHV-rn1 and related viruses will allow us to precisely determine if the cured rats develop protection against homologous or heterologous challenge virus infection.

Considering the strict species tropism of HCV, identification of animal hepaciviruses promised the development of a fully immunocompetent and informative surrogate model useful to delineate the mechanisms of viral persistence and immunity (10). The non-primate hepaciviruses (NPHV) or equine hepaciviruses are interesting given that they remain the closest genetic relatives of HCV, and share many features of its natural history (46). However, they mostly cause acute resolving infection with no clinical disease in horses (15, 47) and studies are limited by the number of animals, expenses and lack of species specific research reagents. We therefore focused our efforts on developing a tractable lab rodent surrogate model for HCV, and selected the rat hepacivirus RHV-rn1. We recently determined that RHV-rn1 infection is rapidly cleared by immunocompetent mice (13) and that the duration of viremia was shorter than that observed for NPHV and GBV-B infections in horses and marmosets, receptively (5, 48). However, the availability of numerous genetic variants and immunological reagents for lab mice makes this model extremely useful for characterizing the nature of host responses associated with acute virus clearance and protective immunity (13). Additionally, it is plausible that the rat virus can be further adapted to establish a fully immunocompetent mouse model for persistent hepacivirus infections. Importantly, RHV-rn1 infection in inbred and outbred rats closely resembles HCV infection outcomes since most infected animals develop life-long persistent hepatotropic infection associated with chronic inflammation and liver diseases. As with human HCV infection, full susceptibility to viral persistence probably reflects hundreds of years of co-evolution between RHV-rn1 and its natural rat host. Thus, rats being an immunocompetent and fully susceptible host of RHV-rn1 could allow optimization of effective vaccine designs to prevent hepaciviral persistence in the natural context. Overall, we conclude that
laboratory rats infected with RHV-rn1 can serve as an informative, fully immunocompetent surrogate to study the mechanisms of HCV persistence, immunity, and pathogenesis.

**Materials and Methods**

**Animals.** Brown Norway, Sprague Dawley, Wistar Han, Holtzman, and Long Evans rats were obtained from commercial suppliers (Charles River or Envigo). All animal studies were performed at North Carolina State University and The Research Institute at Nationwide Children’s Hospital using protocols approved by respective Institutional Animal Care and Use Committees.

**Viral genome, infectious clone, mutagenesis and RNA transcription.** The complete genome of RHV-rn1 was sequenced as reported by us previously for other rodent hepaciviruses(14, 15). The RHV-rn1 molecular clone was synthesized and modified to introduce desired restriction sites using standard molecular techniques (Fig.2). Site-directed and random mutagenesis was performed using commercial PCR mutagenesis kits (New England Biolabs). After linearization of the plasmid, viral transcripts were synthesized using Hi-Script Transcription kit (Promega). After a DNAase-I digestion step, purified RNA was used for intrahepatic injection into rats. Complete genome of RHV-rn1 is deposited in GenBank (accession no. KX905133).

**RHV-rn1 infection, viral titers, and antibody detection.** Rats were infected intravenously with $10^5$-$10^7$ VGE of RHV-rn1 by tail vein injection. For experiments involving the RHV-rn1 molecular clone, animals were injected intrahepatically with 5 µg of purified RNA.

Viral RNA from animal sera was extracted using the QIAamp Viral RNA Mini Kit (Qiagen). After reverse transcription, quantitative PCR was done using AmpliTaq Gold 360 Master Mix (Applied Biosystems) and following cycling conditions: 50º C for 2 min, 95º C for 2 min and 40 cycles of 95º C for 15 sec, 53º C for 45 sec, and 60º C for 45 sec. Primers and probe sequences used for this protocol were TACATGGCTAAGCAATACGG (sense), AAGCGCACCAATTCC (antisense), and [6-FAM]CTCACGTACATGACGTACGGCATG[BHQ1a-6FAM] (probe). To detect negative-strand RNA replicative intermediates, we used a novel RNA polyuridylation approach. Briefly, total RNA was polyuridylated via poly-U polymerase (NEB) followed by cDNA generation using Superscript II Reverse Transcriptase and a unique primer containing a polyadenylated 3’ end
(GAATCGAGCACCAGTTACGCATGCCGAGGTCGACTTCCTAGAAAAAAAAA - AAACCA). PCR was then performed using a truncated form of the unique RT primer (GAATCGAGCACCAGTTACG) and an RHV-rn1 specific negative-strand primer (CCATGTAGGAGGATAAAGTCC).

To detect antibody responses in infected animals, we used a sensitive luciferase immunoprecipitation system (LIPS) assay to screen serum samples for the presence of antibodies against the RHV-rn1 NS3 helicase protein. A 240 amino acid fragment (positions 1139 to 1379) of the RHV-rn1 helicase protein (NS3) was subcloned downstream of Renilla luciferase (Rluc) using the pREN2 vector to express Rluc-fused viral antigen. LIPS assays were performed as described in our previous publications (46).

**Tissue histology.** Liver sections (6µm) were fixed with 10% neutral buffered formaldehyde, processed routinely and stained using hematoxylin and eosin. In situ hybridization was performed using RNAscope and probe targeting RHV-rn1 helicase sequence.

**Serum ALT quantification.** Serum alanine aminotransferase (ALT) levels in rats were determined using an ALT assay kit (BiooScientific) as per manufacturer’s instructions.

**RNA-seq analysis of infected liver samples.** Liver sections and biopsies were immediately immersed in RNAlater (Ambion) and kept at -80°C until RNA extraction by RNeasy kit (Qiagen). DNA and rRNA from total RNA was removed using DNase-I and Ribo-zero (Illumina) and then samples with RNA integrity value of >7 were used for RNA-seq. We used Illumina Scriptseq (stranded RNAseq) for library prep and paired end 150 bp SBS Chemistry to generate sequences using the Illumina HiSeq4000 platform. Sixty million reads were generated for each liver sample. Quality filtering of reads was done using FastQC (v0.11.3) and then sequences were mapped to reference genome of *Rattus norvegicus*, NCBI Rnor_6.0 ([ftp://igenome:G3nom3s4u@ussd-ftp.illumina.com/Rattus_norvegicus/NCBI/Rnor_6.0/Rattus_norvegicus_NCBI_Rnor_6.0.tar.gz](ftp://igenome:G3nom3s4u@ussd-ftp.illumina.com/Rattus_norvegicus/NCBI/Rnor_6.0/Rattus_norvegicus_NCBI_Rnor_6.0.tar.gz)) using TopHat2 (v2.1.1). The Cufflinks v2.2.1 package was used for transcript assembly and calculation of differential gene expression. Cuffmerge (v1.0.0) tool was used to merge and generate a combined transcript. Merged transcript and reference genome fasta was used by Cuffdiff (v2.2.1) tool.
to generate differential gene expression for any two different sample conditions. RNA-Seq analysis was performed using a previously described protocol (49). Gene function and pathway analysis was performed by Ingenuity Pathway Analysis software (Qiagen Inc.). Cutoff parameters used to identify differentially expressed genes were p-value <0.05, false discovery rate q<0.05 and fold change >0.5849, (14). We used custom in house developed R-scripts and R-packages, Limma for vennDiagram and gplots for making heatmaps.

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Authors Contributions: S.T., J.C. and A.K. conceived and led the study. All authors participated in the analysis and interpretation of data. S.T., S.M., A.S.H., P.S., T.K.H.S., J.C. and A.K. wrote the paper.
REFERENCES

FIGURE LEGENDS

Fig.1. Genetic characterization of RHV-rn1. (A). Isolation and infection outcome of RHV-rn1 in Sprague Dawley rats. The serum sample pointed by red arrow was used to acquire complete RHV-rn1 genome and for construction of the genome clone (B). Genome diagram of RHV-rn1 depicting cleavage sites predicted by alignment and homology to sites in the non-structural genes previously characterized in other hepaciviruses (HCV, NPHV and GBV-B). Cleavage sites in structural gene regions were independently predicted using the SignalP 4.1 server (16). Predicted N-linked glycosylation sites (Nx[S/T]) in envelope proteins are depicted by vertical arrows. (C). The predicted secondary structure of RHV-rn1 5’ UTR. The location and sequence of the two miR-122 sites are shown as filled circles.

Fig.2. The reverse genetic system and tissue tropism of RHV-rn1. (A). Schematics and restriction map of the RHV-rn1 infectious clone. (B). Kinetics of viremia in the four rats injected with RHV-rn1 transcripts. Anti-NS3 IgG titers for two rats are shown in shaded areas (C). In situ hybridization of uninfected (left image) and infected (right image) rat liver sections. Images were captured at 400X resolution and antiviral probe was labeled with Fast-Red dye. (D). Mean viral titers in different organs of four infected HTZ rats. Two different liver sections were used for each of the four rats. (E). Results of negative strand RNA assay to detect RHV-rn1 replicative forms in different organs of the infected rats.

Fig. 3. Mutagenesis of miR-122 bindings sites, infection outcomes and evolution of RHV-rn1 mutants. (A). Schematics of mutagenesis targeting the two miR-122 sites and the names of different mutants. The blue circle represent a mixture of all four nucleotides confirmed by sequencing of pooled RNA shown below as the electropherograms of dideoxy sequencing. (B). Kinetics of viremia and evolution of mutated sites in vivo. The RM-1 infected rat rapidly selected wild type virus (CACUCC). One of the three rats infected with RM-2 selected a unique variant (ACAGUGU, green color line) that established persistent infection. The other two rats, shown with black lines, selected the wild type virus. (C). In the three SM-3 infected rats, the mutant virus remain mutant type while in the three SM12 infected rats, the mutant virus reverted back to wild type after 10 days p.i.. The double mutant with complementary SM12 and SM21 mutations failed to initiate infection in the two injected rats (pink line).
Fig. 4. RHV-rn1 infection and outcomes in outbred rat strains. Six rats of each strain were infected with $10^6$ genomic copies the clone derived RHV-rn1.

Fig. 5. RHV-rn1 infection and disease outcomes outbred HTZ rats. (A). Long-term follow-up study of four HTZ rats. Viral titers and ALT are shown. All rats seroconverted 20-24 days post infection (anti-NS3 IgG). (B) Histopathological examination of liver biopsies from these four rats collected at day 85 and 235 p.i. Portal lymphocytic aggregates are shown by the black arrow in top-left panel (rat no. 50), hepatocellular steatosis is shown by the back arrow in the middle-left panel (rat no. 50). Top and middle right side panels are liver sections from rat no. 53. Bottom panels (rat no. 50) show an apoptotic hepatocyte (left) and higher magnification of microvesicular steatosis (right), indicated by black arrows. (C) In situ hybridization of rat no. 49 liver sections serially obtained at day 85, 235 and 287 days p.i.. A large lymphocytic aggregate in shown by a black arrow in the middle panel. Microvesicular and macrovesicular steatotis is shown by red and pink arrows, respectively in the bottom panel.

Fig. 6. RNA-seq analysis of rat livers after RHV-rn1 infection. (A). Venn diagrams of liver DEG (p value <0.05 and q value <0.05) between control and infected rats of different class I haplotypes. RT-1$^l$ and RT-1$^u$ are shown as letters L and U, respectively. Days post infection are shown as numbers as suffix. (B). Dynamics of 8,201 DEG significantly changed at different times p.i. in the livers of RHV-rn1 infected rats. (C). Changes in 62 DEG known to be associated with anti-viral responses at different times p.i.

Fig. 7. Dynamics of DEG associated with IFN-γ and IFN-λ1 responses during RHV-rn1 infection. Log2 fold difference in expression of DEGs compared to the uninfected control rats is shown. All gene expression data was normalized using fragment per kilo base per million reads (FPKM) values.

Fig. 8. DAA therapy of chronically infected rats. HTZ rats infected with RHV-rn1 were treated with different doses of sofosbuvir and ribavirin and also ribavirin alone. The treatment periods is indicated by broken black arrows.
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