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Animal models for the study of HCV

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1 Summary

The development and evaluation of effective therapies and vaccines for the hepatitis C virus (HCV) and the study of its interactions with the mammalian host have been hindered for a long time by the absence of suitable small animal models. Immune compromised mouse models that recapitulate the complete HCV life cycle have been useful to investigate many aspects of the HCV life cycle including antiviral interventions. However, HCV has a high propensity to establish persistence and associated histopathological manifestations such as steatosis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Better understanding of these processes requires the development of a permissive and fully immunocompetent small animal model. In this review we summarize the *in vivo* models that are available for the study of HCV.

Keywords

viral hepatitis; antiviral therapy; animal models; pathology

2 Introduction to experimental models for HCV research

After its discovery 26 years ago [1], it quickly became apparent that chimpanzees were the only other species, besides humans, to be susceptible to HCV infection. Over the years several landmark advances have been achieved including the replicon system in 1999 [2,3] and the identification of a full-length isolate of HCV (JFH1) in 2005 [4-6] that could recapitulate the full viral life cycle in the human hepatoma cell line Huh7, so called cell culture HCV (HCVcc). Together with the (sub)genomic replicon, this HCVcc system has been the working horse for screening and identification of new antiviral drugs [7]. HCV infects and replicates in hepatocytes, which *in vivo* are characterized by a highly-polarized morphology and reside in a complex liver architecture [8]. Advancing research tools beyond

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HCVcc and human hepatoma cells has remained challenging. Primary HCV isolates show a very limited ability to replicate in tissue culture and the use of primary hepatocytes in culture is complicated by rapid loss of differentiation and poor reproducibility [9,10]. Some advances have been made in the use of primary hepatocyte culture systems [11-14] and more recently with induced pluripotent stem cell (iPSC) derived hepatocyte-like cells [15-17], but only HCVcc reproducibly infect such models and no reliable *in vitro* system is able to robustly support primary HCV isolates of different genotypes.

3 Animal models to study HCV infection

Besides humans, the experimental infection of chimpanzees has played a pivotal role in the discovery of HCV and has proven very valuable for deciphering host-virus interactions, particularly of cellular immunity, and preclinical analysis of antiviral strategies [18]. Genomes that acquired cell culture adaptive mutations were found to be highly attenuated in chimpanzees or reverted to the wild type sequence once injected in mice with humanized liver, again underscoring the discrepancies between these models and restrictions to the biological relevance of *in vitro* systems [19,20]. However, even between chimpanzees and humans subtle differences in HCV became apparent. Whereas a minority of humans spontaneously clears the infection, few chimpanzees evolve to chronicity and to date no fibrosis and only one hepatocellular carcinoma (HCC) has been observed.

Because of growing ethical constraints, limited availability and the high costs associated with chimpanzee studies other animals have been tested in their ability to support HCV infection. Although HCV can infect iPSC derived hepatocyte-like cells from pigtail macaques [21], to date no viremia has been shown in macaques or other primates besides chimpanzees [22]. In addition to primates, several other species have been evaluated for HCV susceptibility but most showed to be resistant to infection. One exception is the tree shrew (*Tupaia belangeri*), a non-rodent squirrel-like mammal that was found to be permissive for persistent low-level HCV viremia including HCV-related liver disorders in some animals [23].

Due to this narrow host tropism of HCV, the development of practical small animal models for HCV, e.g. laboratory mice and rats, has been challenging [24]. Whilst rodents are naturally resistant to HCV infection different approaches have been undertaken to enable the study of the virus in mice. Four different approaches will be highlighted in this review: the study of novel HCV homologs that naturally infect rodents, the adaptation of HCV to the murine environment, humanization of the mouse through genetic approaches, and by xenotransplantation methods.

3.1 HCV HOMOLOGS

Alternatives to human and chimpanzee samples for the study of HCV biology *in vivo* could be found in HCV-related viruses that infect other animal species (Figure 1, first panel). Until 2011, the only known homolog of HCV was GBV-B, named after a surgeon (initials GB) suffering from acute hepatitis whose serum was used to infect tamarins that subsequently developed acute hepatitis [25,26]. While GBV-B infection of New World monkeys could be used as an HCV surrogate model, persistent infection is rare [27]. Indeed, an ideal surrogate

model should not only resemble HCV's hepatotropism, but essentially also its ability to establish persistent infection, associated immune responses and ultimately pathogenesis. Recent deep sequencing virome analyses have led to the identification of a number of previously unknown HCV-related hepaci- and pegiviruses in dogs, horses, rodents, bats and non-human primates, thereby greatly broadening the hepacivirus genus [28]. The equine non-primate hepaciviruses (NPHV) is the best studied of the novel *hepaciviridae*. While NPHV infection can indeed be persistent, mildly elevated liver enzymes, infiltration of lymphocytes into the liver and even mild hepatitis have been observed in horses that clear infection [29,30]. In addition, a novel equine pegivirus (EPgV) has been identified that is linked to liver disease in horses, designated Theiler's disease associated virus (TDAV) [31].

Obviously, the large size and associated animal care costs of horses, compared to conventional laboratory animal models, is a huge drawback. Therefore, novel rodent hepaciviruses are of particular interest. Infections of these viruses in their natural host, or eventually immune competent laboratory inbred mouse strains, warrant further investigation. Methodical searches for hepaciviruses in several species of wild rodents have led to the identification of potential small animal models for HCV, such as deer mice (*Peromyscus* spp.), the hispid pocket mouse (*Chaetodipus hispidus*), the desert wood rat (*Neotoma lepida*), bank voles (*Myodes glareolus*), South African four-striped mice (*Rhabdomys pumilio*) and rats (*Rattus norvegicus*) from New York City [32-34]. Importantly, hepatotropism and histopathological signs of liver inflammation have been observed in some of these rodents, e.g. in bank voles and rats. However, to confirm the utility of rodent hepacivirus infections as surrogate HCV model systems, it remains to be examined whether the pathogenesis of these viruses parallels that seen for HCV in humans.

3.2 VIRAL ADAPTATION

Using the error-prone replication of most RNA viruses, one approach to infect murine cells is to adapt HCV to the mouse environment. Long-term *in vitro* cultivation of HCV in the presence of mouse cells or cells expressing murine entry proteins could allow the virus to adapt to these murine factors (Figure 1, second panel). Indeed, using this approach Bitzegeio and colleagues discovered adaptive mutations in the viral envelope proteins E1 and E2 enabling the virus to utilize murine CD81 and OCLN for cell entry [35]. This is important because residues in both mouse CD81 and OCLN were previously shown to form the species barrier of HCV infection of mouse cells [36]. Recently, the same group reported that this mouse adapted virus was able to infect, replicate and produce new infectious viral particles in immortalized mouse liver cell lines with defective innate immunity in the absence of any human cofactors [37]. Whether this variant needs additional adaptations to productively infect primary mouse hepatocytes *in vivo* and persist in the animals is subject to further investigation.

3.3 THE GENETICALLY HUMANIZED MOUSE MODEL

Complementing the strategy of adapting HCV to the mouse environment, genetic manipulation of the host can be used to knock down murine factors that hamper productive HCV infection; or to complement the mouse with human factors that are essential for this process. Therefore, knowledge of the barriers that determine the human tropism of HCV is

essential. This approach has most efficiently been performed with the HCV entry factors, where transient expression of the human restriction factors CD81 and OCLN by adenoviral delivery supported HCVcc entry into the mouse hepatocytes and allowed evaluation of entry inhibitors and vaccine candidates (Figure 1, third panel) [38-40]. Building on this work, mice that were transgenic for four human entry factors (4hEF) and deficient in several innate immune signaling pathways not only allowed HCVcc entry but also low level replication, with infectious virus being recovered from the mouse serum [41]. Using a similar CD81/OCLN transgenic approach this was recently expanded to HCV isolates beyond HCVcc and in mice that have an intact innate immune system, albeit with very low level of viremia [42]. Differences between the ICR mouse background in this latter study and C57BL/6 mice used in the 4hEF model suggest murine genetic differences may be important for replication of patient-derived HCV isolates. Apolipoprotein E, known to enhance HCV production in mouse hepatoma cells [43], was higher expressed and the liver-specific facilitator for HCV replication, miR-122 [44], was much better induced during HCV infection in the ICR vs. C57BL/6 background upon human CD81/OCLN expression [42]. These and potentially other unique hepatic components critical for HCV replication and assembly partially explain the observed HCV persistent infections. Further work is needed to confirm the utility of these models to study adaptive immune responses, immune evasion and vaccination strategies. It will be interesting to see if long-term infection in these models leads to fibrosis and/or HCC development.

3.4 THE HUMAN-LIVER XENOGRAFT MOUSE MODEL

The introduction of mice of which the liver was xenografted with human hepatocytes was an important innovation for the study of HCV, and hepatotropic pathogens in general. Liver chimeric mice are generated by intrasplenically infusing primary human hepatocytes into mice with a genetic immune deficiency combined with a constitutive or inducible liver injury (Figure 1, fourth panel) [45-50]. While the liver injury provides a niche for hepatocellular engraftment, the immunodeficiency is needed to avoid rejection of xenogeneic grafts. Hepatocytes have a remarkable regenerative capacity that, after migration to and integration into the liver, allows them to extensively and sometimes nearly completely repopulate the diseased liver parenchyma [51-55]. Although two different recipient lines have predominantly been used for generating human-liver chimeric mice: the uPA-SCID mouse and the FRG mouse [56], susceptibility to HCV infection has also been shown in alternative immunodeficient liver injury models such as MUP-uPA [57] and TK-NOG mice [58]. While subtle differences exist, they are essentially very similar and allow robust and reproducible infection of both natural virus isolates and cell-culture produced HCV [50,59-61]. These humanized mice have been very useful for the study of the basic aspects of the HCV life cycle [20,62-70], the evaluation of passive immunization strategies [60,71-74] and the assessment of novel antiviral therapies [50,75-86].

Since the primary human hepatocytes that reside in the mouse liver have retained many of their functions, human-liver chimeric mice have also been extensively used for the study of other hepatotropic microorganisms such as HBV [46,87-92], HDV [93,94] and *Plasmodium falciparum*, the causative agent of malaria [95-101]. Their applications extend beyond infectious diseases to the evaluation of the human-type metabolism and potential toxicity of

medicinal compounds [102-107]; and the basic cell biology of the human hepatocyte [108,109].

3.5 IMMUNOCOMPETENT XENOGRAFT MOUSE MODELS

In order to prevent rejection of engrafted human hepatocytes, human-liver chimeric mouse models require an immune deficiency. However, studies on HCV immunopathogenesis, primary (human) adaptive immune response and vaccine efficacy in mice would require both a human liver graft and a functional (human) immune system in one and the same recipient animal. This has recently been achieved by combining adult human hepatocytes and human CD34⁺ hematopoietic stem cells (HSCs) from different human donors [110,111]. Although the authors did not show HCV infection, the high human engraftment levels suggest that these mice could become viremic upon HCV challenge. In addition to this allogeneic system, a syngeneic double reconstituted mouse model was generated by intrahepatic injection of human hepatoblasts (hepatocyte progenitor cells) and CD34⁺ HSCs from a single fetal donor (Figure 1, fourth panel) [112]. Importantly, HCV challenged mice developed a human anti-HCV T cell response and liver fibrosis. Although HCV RNA was present in liver extracts, the low level of human liver reconstitution in this model did not support detectable viremia. More recently the same group published another double syngeneic reconstituted model, which they used to study HBV immunopathogenesis [113]. Yet again, the rather low level of human hepatocyte engraftment will probably hamper similar studies on HCV infections. In parallel, fine-tuning of human immune cell repopulation and stimulation of specific immune subset development including myeloid and natural killer cells [114], could eventually be used to investigate involvement of these immune cells in HCV infections.

4 Conclusions and future perspectives

Recently approved direct-acting antivirals have dramatically changed the clinical HCV landscape. From a chronic disease that, even after prolonged harsh therapies, could be cured in only a subset of patients, HCV now can be eradicated in almost all patients with minimal side effects. Nevertheless, HCV animal models remain relevant, particularly for two reasons. To this date we have a limited understanding why only a subset of patients develop fibrosis and HCC, nor how to regress fibrosis and prevent HCC after curing HCV. Second, given its widespread prevalence a prophylactic vaccine remains desirable to prevent ongoing HCV spread in certain high-risk populations and geographical areas. Over the past decade several independent approaches have led to small HCV animal models that can complement or replace studies in chimpanzees. These model systems, together with the recent identification of several HCV homologs, including rodent *hepaciviridae*, open the possibility to further these two goals in mice. Given their inherent limitations all lines of HCV mouse models will probably serve their own usefulness for specific scientific questions. For example, vaccine studies using HCV homologs in wild type mice may become more relevant than HCV adapted to entry factor transgenic animals that lack essential innate immune pathways. Human-liver chimeric mice in their current state completely lack or contain only very limited cellular immunity, and therefore are not yet useful for vaccine studies. However, if human HCC could be induced or engrafted in human liver chimeric mice, such models may

become the preferred model to study various aspects of HCV-HCC interactions that may be more translationally relevant to patient tumors than murine HCC. Therefore further development and characterization of HCV animal models will remain useful until HCV has been eradicated worldwide.

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Highlights

- Ethical restraints on the use of chimpanzees have opened a quest for alternative animal models to study HCV
- Mice require humanization of the liver to become susceptible to HCV infection
- Alternatively the virus can be adapted to the mouse environment
- Immunocompetent mice with (genetically) humanized liver are available but entail further optimization
- Recently identified non-primate hepaciviruses may provide novel surrogate animal models

Figure 1. Different approaches to study HCV in animal models

First panel: HCV-related viruses that infect animal species such as wild mice, rats, tamarins, bats and horses. These infections can be studied in their natural host, or eventually immune competent laboratory inbred mouse strains. Second panel: in vitro adaptation of HCV to mouse hepatoma cells may allow the isolation of viral variants that can establish an infection in wild type mice. Third panel: transient or stable expression of human factors that are essential to support infection of wild type HCV. Fourth panel: in xenotransplantation models, the genetic background of the host permits repopulation of the liver upon transplantation of human hepatocytes. Additional transplantation of HLA-compatible hematopoietic stem cells results in dually reconstituted mice.

Table 1
Characteristics of different animal models for the study of HCV

Animal model	Detectable virus production	Assessment of antiviral efficacy	Liver disease	Adaptive immune response	Availability/throughput
CHIMPANZEE	Persistent viremia or acute self-limited	Vaccination, passive immunization [115,116], antiviral therapies [117,118]	+/-	+	Very low
TUPAIA	Intermittent viremia [23]	–	+	–	low
VIRAL ADAPTATION	–	–	–	–	high
GENETICALLY HUMANIZED MOUSE MODEL	No viremia [38] Persistent viremia [41,42]	DAA [41,42] Entry inhibitors and vaccine candidates [38] (indirect assesment)	–	+	high
HUMAN-LIVER CHIMERIC MOUSE MODEL	Persistent viremia	Passive immunization [39,60,71,72], DAAs [77,79,80], Entry inhibitors [76,78,81,82] Other antiviral therapies [50,84]	–	–	Low
HCV HOMOLOGS IN NATURAL HOST	Persistent viremia [29,30]	–	+/-	+	Low (NPHV)/ high (rodent hepacivirus)
HUMAN IMMUNOCOMPETENT LIVER MOUSE MODEL	In liver extracts [112]	–	+	+	low