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Basic

HCV animal models and liver disease

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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. Due to the narrow host tropism of HCV, the development of mice that can be robustly engrafted with human hepatocytes was a major breakthrough since they recapitulate the complete HCV life cycle. This model has been useful to investigate many aspects of the HCV life cycle, including antiviral interventions. However, studies of cellular immunity, immunopathogenesis and resulting liver diseases have been hampered by the lack of a small animal model with a functional immune system. In this review, we summarize the evolution of *in vivo* models for the study of HCV.

Introduction to experimental models for HCV research

A breakthrough discovery that guided the development of HCV research tools was the cloning of the HCV genome 25 years ago [1]. However, early attempts to initiate viral replication *in vitro* or in various small animal models failed, restricting functional studies to humans and chimpanzees [2–5]. This remained a major roadblock for HCV research until the HCV replicon system became available in 1999 [6,7]. In this system, efficient viral replication was reached in hepatoma cells, transfected with sub-genomes that were comprised of a selectable marker, linked to the HCV non-structural region NS3 up to NS5B and the 3′ UTR, respectively under the control of the 5′ HCV-internal ribosome entry site (IRES) and the IRES of the encephalomyocarditis virus (EMCV). This system allowed the *in vitro* study of HCV RNA replication and the evaluation of novel antiviral compounds, but also led to key discoveries such as NS3/4A-mediated innate immunity interference [8,9] and growth factor signalling induction [10]. In parallel, several models to study HCV binding and entry *in vitro* were developed, such as virus-like particles (VLP), produced in a baculovirus system [11] and retroviral particles, pseudotyped with HCV envelope glycoproteins (HCVpp) [12,13]. In 2005, a full-length isolate of HCV (JFH1) [14,15] and the intragenotypic chimeric J6-JFH1 [16] were described that, besides RNA replication and viral entry, also supported infectious viral particle production *in vitro* (HCVcc). Together with the (sub)genomic replicon, this HCVcc system has been the working horse for screening and identification of new antiviral drugs [17]. Since only recently alternative full-length isolates have been described that efficiently propagate in cell culture [18–22], this system has long been restricted to one specific viral isolate, designated JFH1, and intra- or inter-genotypic chimeric derivatives thereof [23]. In addition, hepatoma cells that support robust replication of HCVcc have characteristics that differ significantly from those of primary human hepatocytes, e.g., continuous proliferation, impaired innate immunity and reduced metabolic activity [24,25]. Indeed, the *in vivo* target cell of HCV, the hepatocyte, is characterized by a highly-polarized morphology and resides in a complex liver architecture [26]. Consequently, studies on virus–host interactions have been hampered by limited *in vivo* and *ex vivo* models that more closely mimic the natural infection and environment of the liver. Primary HCV isolates show no, or only poor ability to replicate in tissue culture and the use of primary hepatocytes in culture is complicated by rapid loss of differentiation, low-level viral replication and poor reproducibility [27,28]. Some advances have been made in this area by the use of primary human hepatocytes in combination with a highly replicative JFH1-variant [28], fluorescence-based reporter systems in co-cultures of primary human hepatocytes with supportive stroma [29], primary human foetal liver cultures [30,31], a human liver slice model [32] and more recently with induced pluripotent stem cell derived hepatocyte-like cells [33–35].

Although these cell culture models have proven very useful to study different aspects of the HCV life cycle, their lack of physiological cell-cell interactions has greatly limited the generalizability of these findings. One example of such a discrepancy was the increased HCV blocking efficacy of an anti-SR-BI entry inhibitor *in vivo* compared to results generated in cell culture studies [36]. Therefore, *in vivo* studies that more closely mimic the natural situation in humans are essential. A milestone discovery, enabling such *in vivo* HCV studies, was the generation of mice that can be robustly engrafted with human hepatocytes [37].

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Key Points

- The chimpanzee has been a valuable model to study HCV-host interactions and vaccine candidates but its use is severely hampered by financial and ethical constraints
- Human-liver chimeric mice are well-characterized tools for the efficacy assessment of antiviral interventions
- The entry factor transgenic mouse is a recently developed immunocompetent mouse model, which is appropriate for the evaluation of both antivirals and murine vaccine responses
- The HIS/hep chimeric mouse combines a human liver with a human immune system and therefore holds promise to study HCV immunopathogenesis and resulting liver diseases. Changes to the mouse genetic background might further optimize human immune and liver cell engraftment

Preclinical *in vivo* models

Besides the study of natural infections that occur in 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 and preclinical analysis of antiviral strategies [3] (Table 1). Genomes that acquired cell culture adaptive mutations were found to be highly attenuated in chimpanzees, again underscoring the discrepancies between these models and restrictions to the biological relevance of *in vitro* systems [38]. Despite the genomic homology between chimpanzees and humans, few chimpanzees develop chronic HCV infection and to date no fibrosis and only one hepatocellular carcinoma (HCC) has been observed. In addition, ethical constraints, availability and cost are increasingly limiting the use of these large primates for HCV research.

Although the HCV life cycle has been recapitulated in hepatocytes derived from induced pluripotent stem cells (iPSCs) from pigtail macaques [39], HCV does not seem to be able to establish persistent infection in non-human primates other than chimpanzees [40]. In addition to primates, several other species have been evaluated for HCV susceptibility but most were resistant to infection. Tree shrews (*Tupaia belangeri*) are non-rodent squirrel-like mammals that were found to be permissive for HCV infection [41]. While long-term follow-up revealed histological progression to HCV-related liver disorders in some animals, HCV viraemia was not sustained but could only be detected intermittently. Therefore, compatibility of the Tupaia host environment with HCV replication seems rather low. Due to the narrow host tropism of HCV, the development of small animal models for HCV has been challenging [42]. Different approaches have been undertaken to enable the study of the virus in a murine environment, although mice are naturally resistant to HCV infection. For matching a human hepatotropic pathogen with a non-human liver, either the virus or the host must be adapted.

Table 1. Overview of HCV animal models and their applicability.

HCV animal model	Detectable virus production	Assessment of antiviral efficacy	Liver disease	Adaptive immune response	Availability/ throughput
Chimpanzee	Persistent viraemia or acute self-limited	• Vaccination • Passive immunization [136, 137] • Antiviral therapies [138, 139]	+/-	+	Very low
Tupaia	Intermittent viraemia	-	+	-	Low
Viral adaptation	-	-	-	-	High
Viral protein-HCV transgenic mouse	-	-	+/-	+	High
Genetically humanized mouse model	No viraemia [60] Persistent viraemia [67]	Direct: DAA [67] Indirect: Entry inhibitors and vaccine candidates [60]	-	+	High
Human-liver chimeric mouse model	Persistent viraemia	• Passive immunization [61, 85, 96, 97] • DAAs [101, 103, 104] • Entry inhibitors [100, 102, 105, 106] • Other antiviral therapies [72, 107]	-	-	Low
Human-liver tolerized rat	Viraemia (up to 16 wk)	-	+/-	n.a.	Low
Human immunocompetent liver mouse model	In liver extracts	-	+	+	Low

+, reported; –, not reported; n.a., not applicable.

Viral adaptation

Expansion of the HCV host range could be achieved by allowing the virus to adapt to a non-human environment. *In vitro* long-term cultivation of HCV in the presence of mouse cells, or cells expressing murine entry proteins, could familiarize the virus to efficiently use such factors from this potential host (Fig. 1, left panel). Using this approach, Bitzegeio and colleagues discovered adaptive mutations in E1 and E2, enabling the virus to utilize murine CD81 and OCLN for cell entry [43]. This is important because residues in both mouse CD81 and OCLN diverging from the human orthologs were shown to affect efficient HCV infection of mouse cells [44]. 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 [45]. Whether this variant needs additional adaptations for productive infection of primary mouse hepatocytes *in vivo* is subject to further investigation. Nevertheless, the applicability of such systems for the study of entry processes might be affected by the influence of the adaptive mutations on envelope conformation and receptor usage.

Viral protein-HCV transgenic mice

To study the *in vivo* interactions between the viral proteins and the host cell, mice have been created to transgenically express these proteins (Fig. 1 middle panel). Several investigations of transgenic mice, containing the genetic code for HCV structural proteins E1, and/or E2, and/or the core, or the NS3/4A protein did not provide evidence for liver pathology [46–49]. Others, however, did describe development of hepatic steatosis and HCC in mice upon transgenic expression of the HCV core protein [50,51] with E1 and E2 [52]. In addition, TNF-mediated hepatic apoptosis was affected in NS3/4A and NS5A-transgenic mice [46,53]. However, interpretation of HCV-transgenic mouse phenotypes is complicated by the random transgene integration site, its artificial expression, the inherent immune tolerance and the potential influence of the mouse genetic background. Nevertheless, immune tolerance can be (partially) avoided by using immune competent mice, in which the HCV transgene expression is delayed (MUP-promoter) [46] or inducible (using the Cre/Lox system [54] or via hydrodynamic injection [55]). The activity of the immune system of the MUP-HCV transgenic mice more closely resembles that of a chronically infected patient and hence allows the evaluation of potential therapeutic vaccine strategies [56]. Perhaps the most relevant transgenic mouse model is the FL-N/35 mouse, which expresses the complete viral polyprotein at more physiological levels [57]. Hepatic steatosis, enhanced liver fibrosis and increased risk of HCC have been observed in this model [57–59].

The genetically humanized mouse model

Genetic manipulation of the host can be used to knock down host factors that hamper productive HCV infection, or to complement the host with exogenous human factors that are essential for this process. Therefore, knowledge of the barriers that determine the human tropism of HCV is essential. HCV uses different host receptors for hepatocyte entry, among which CD81 and OCLN

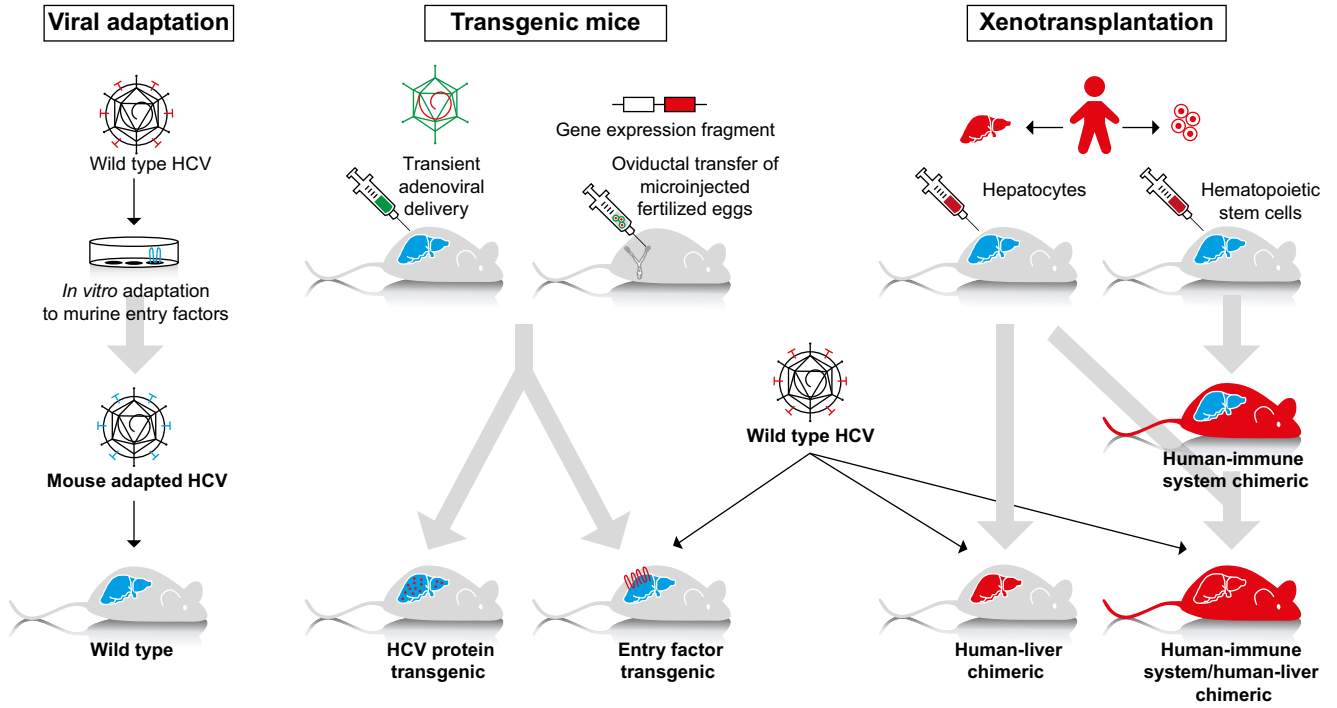

Fig. 1. Different approaches to create mouse models for the study of HCV. Left panel: *In vitro* adaptation of HCV to mouse hepatoma cells may allow the isolation of viral variants that can establish an infection in wild type mice. Middle panel: Transient or stable expression of viral genes may provide essential information on viral protein-host interactions. In addition, mice can be made transgenic for human factors that are essential to support infection of wild type HCV. Right 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 may result in dually reconstituted mice.

were identified to limit HCV mouse tropism. Indeed, both receptors had to be of human origin to allow efficient infection of HCVpp in murine cells [44]. Accordingly, a genetically humanized mouse that expresses this minimal set of human factors upon transient adenoviral delivery supports HCV entry and allows evaluation of entry inhibitors and vaccine candidates (Fig. 1, middle panel) [60–62]. Despite the observation that HCV RNA is translated in mouse hepatocytes and replicons can be sustained in murine hepatic cell lines, viral replication is not efficiently supported in these cells [63–65]. However, data generated with human–mouse heterokaryon cells and murine hepatoma cells, that ectopically express apolipoprotein E, excluded dominant mouse factors that restricted virion assembly and egress, further supporting the idea that a transgenic mouse model, supporting the complete HCV life cycle, could be achieved [63,66]. More recently Dorner and colleagues demonstrated that innate and adaptive immune responses limited HCV replication in (four) human entry factor-transgenic mice (4hEF) that stably express human CD81, SR-BI, CLDN1 and OCLN [67]. Blunting the innate immune response of 4hEF mice by crossing them with innate immune deficient mouse strains, such as STAT1 or IRF7 knockout mice, allowed persistent infection and virus production. In these mice viraemia disappeared after two months, most likely because of an adaptive immune response. Since the complete HCV life cycle is thereby recapitulated in partially immunocompetent mice, it will be interesting to determine whether these mice develop HCV-induced liver fibrosis and/or HCC.

The human-liver xenograft mouse model

A major breakthrough in the field was the development of human liver xenotransplantation models. Humanization of the mouse liver can be achieved by transplantation of primary human hepatocytes into immunodeficient mice that suffer from a constitutive or inducible liver injury (Fig. 1, right panel) [37,68–72]. The genetic immunodeficiency of such mice prevents xenograft rejection and the liver injury provides a competitive growth advantage to the human donor hepatocytes over the resident mouse hepatocytes. After intrasplenic injection, human hepatocytes migrate through the portal venous system to the diseased mouse liver where the engraftment and repopulation process is initiated. This liver reconstitution exploits the inherent capacity of hepatocytes to proliferate and regenerate an injured liver [73–77]. The resulting human-liver chimeric mouse is currently the only reproducible system that supports robust infection of non-JFH based HCV clones and is the most widely used small animal model for the study of HCV.

The uPA-SCID mouse with humanized liver

The regenerative capacity of individual liver cells was first demonstrated in a mouse model initially developed to study bleeding disorders [73]. Liver dysfunction was observed in mice overexpressing the urokinase-type plasminogen activator (*uPA*) transgene under control of the albumin promoter. Remarkably, hepatocytes that underwent somatic deletion of the transgene selectively proliferated and gradually repopulated the entire liver. Backcrossing of these mice to an immunodeficient background allowed acceptance of hepatocytes from other species [78,79]. However, in order to achieve high-level humanization of the mouse liver, primary hepatocytes of excellent quality need to be transplanted in mice that are homozygous for the uPA-transgene [80–82]. Mercer and colleagues were the first to demonstrate the susceptibility of homozygous uPA-SCID mice with human chimeric liver to HCV infection [37]. Importantly, these mice cannot only be infected with JFH1-based viruses, as in the cell culture system, but also with patient-derived viruses of all genotypes [83–85]. Although the study of host antiviral immune responses is hampered by the immunodeficiency of the xenorecipient, the chimeric uPA-SCID model has been very useful for the study of the basic aspects of the HCV life cycle [86–95], the evaluation of passive immunization strategies [85,96–98] and the assessment of novel antiviral therapies [36,99–107].

The FRG mouse with humanized liver

Pre-weaning mortality, due to the transgene-induced liver disease, necessitates hepatocyte transplantation shortly after birth in very small and fragile uPA-transgenic animals. To overcome difficulties in the robust generation of chimeric human-liver mice, modifications to induce liver disease at adulthood have been explored. An alternative xenotransplantation model that employs such an inducible liver injury is based on a genetic knockout of the fumarylacetoacetate hydrolase gene ($FAH^{-/-}$), which causes a defective tyrosine catabolic pathway, resulting in accumulation of the liver toxic metabolites fumarylacetoacetate and succinylacetone, comparable to hereditary tyrosinemia type I [108]. Because administration of the drug 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC, nitisinone) blocks the tyrosine catabolic pathway upstream of FAH, mouse hepatocellular injury can be induced by NTBC withdrawal, allowing the investigator to control the time and severity of liver disease [108]. Extensive liver humanization was supported after crossing $FAH^{-/-}$ animals with recombination activating gene 2 knockout mice ($RAG2^{-/-}$) and IL-2 receptor γ-chain knockout mice ($IL\text{-}2R\gamma^{null}$), hence the designation FRG model [70,71]. Furthermore, Bissig and colleagues demonstrated productive HCV infection of these mice that was sensitive to antiviral treatment [72].

More recently, susceptibility to HCV infection was also shown in other immunodeficient liver injury models such as the MUP-uPA [109] and HSV-TK mice [110]. Because so far no side-by-side comparison of the different human-liver chimeric mouse models has been performed, it is as of yet unclear how these models compare to human hepatocyte or HCV biology. In any case, mice in which the liver is extensively repopulated with primary human hepatocytes are now increasingly being used for the study of hepatotropic microorganisms such as HBV, HCV, HDV, and *Plasmodium falciparum*, the causative agent of malaria [68,111–123]; and for the evaluation of human-type metabolism and potential toxicity of medicinal compounds [124–129].

Immunocompetent xenograft models

One of the major drawbacks of human-liver chimeric mouse models is their requirement to be immune deficient, in order to prevent rejection of the engrafted human hepatocytes. HCV immunopathogenesis, primary (human) adaptive immune response and vaccine efficacy studies in mice would require both a human liver graft and a functional (human) immune system in one and the same recipient animal. One attempt to overcome this hurdle was the tolerized rat model, in which primary human hepatocytes and HuH7 human hepatoma cells are injected into the peritoneal cavity of foetal rats at a moment when the rat foetal immune system is still in development [130]. Subsequent intrasplenic

injection of human hepatocytes or HuH7 cells one day after birth resulted in survival of functional human cells in the absence of rejection. This model was then explored as a platform to study HCV infection [131]. Although HuH7-transplanted tolerized rats were shown to produce HCV viraemia, exceeding 10^4 copies/ml, no data are available on tolerized rats transplanted with primary hepatocytes. Biochemical evidence of hepatic inflammation was demonstrated by elevation of serum alanine aminotransferase and the infiltration of mononuclear cells in the liver. However, while immunocompetent, the mismatch between human HLA and rat MHC still hampers the study of adaptive rat immune responses against infected human hepatoma cells. Of note, the utility of this model in the context of HCV infection has never been confirmed in follow-up studies by this or other groups. Because of this mismatch between human hepatocytes and the rat immune system, an alternative approach is to engraft mice with both human hepatocytes and human immune cells. This has recently been achieved by combining adult human hepatocytes and human $CD34^+$ hematopoietic stem cells (HSCs) from different human donors [132]. Although the authors did not show HCV infection, the high human engraftment levels suggest that these mice could become viraemic when challenged with HCV. Another model was reported by Washburn and colleagues, who generated dually engrafted AFC8-hu HSC/Hep (human HSCs/hepatocyte progenitor) mice after intrahepatic injection of human hepatoblasts and $CD34^+$ HSCs isolated from a single foetal donor (Fig. 1, right panel) [133]. Importantly, upon viral challenge these mice developed a demonstrable human anti-HCV T cell response and liver fibrosis. However, HCV RNA could only be detected in liver extracts and not in the plasma of inoculated mice, possibly because of the low level of human liver engraftment in this model. Finally, because of the suboptimal activity of the immune system that resides in human $CD34^+$ HSCs transplanted mice, the utility of this model remains to be awaited.

Conclusions and future perspectives

Over the past decade different approaches have led to small HCV animal models that can complement or replace studies in chimpanzees. Further improvements are still needed since mouse models have thus far not exhibited the two most feared complications of HCV infection, namely the development of liver cirrhosis and HCC. Due to an increased understanding of key factors that determine the narrow host range of HCV, significant progress has recently been made in adapting the mouse environment to allow HCV infection. Besides blunting innate immune responses in these mice [67], additional optimisation may lead to HCV persistence and possibly development of fibrosis or HCC. In contrast to adaptation of the host, the generation of host-adapted viral particles could also overcome the species barrier. A recently described HCV variant that is adapted for the use of murine CD81 and OCLN for hepatocyte entry could be further adapted to resist the innate immune response, mounted by the infected mouse hepatocyte [43]. Separate from viral adaptation to the mouse, engraftment of human hepatocytes in xenograft models has been the most widely used model to investigate HCV *in vivo* [37]. Human-liver chimeric mouse models have gradually improved with adjustments, allowing the selection of the time point of liver disease induction [70,72]. In addition, human liver repopulated mice have also been transplanted together with human immune cells [133]. The possibility of combining a human liver with a syngeneic human immune system permits the study of human adaptive immune responses and HCV liver disease in a mouse environment. Further improvements to these models will be required to enhance liver engraftment levels and to overcome the limited development of human leukocytes, particularly non-lymphocyte subsets. These models will likely be further improved with recent advances in stem cell and tissue engineering technologies, which in the near future may allow for the creation of dually engrafted mice with hepatocyte-like cells and haematopoietic precursors, originating from iPSCs of a single donor [134,135]. Whether such improved xenograft models will develop fibrosis and/or HCC remains to be determined. Therefore, even with the pending release of highly potent direct acting antiviral treatment regimens, improved immunocompetent animal models remain essential for the development of an HCV vaccine and to elucidate certain clinical enigmas, e.g., why patients with cirrhosis require prolonged treatment or how HCV contributes to HCC development.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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