



Animal Models to Study Hepatitis C Virus Infection

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With more than 71 million chronically infected people, the hepatitis C virus (HCV) is a major global health concern. Although new direct acting antivirals have significantly improved the rate of HCV cure, high therapy cost, potential emergence of drug-resistant viral variants, and unavailability of a protective vaccine represent challenges for complete HCV eradication. Relevant animal models are required, and additional development remains necessary, to effectively study HCV biology, virus–host interactions and for the evaluation of new antiviral approaches and prophylactic vaccines. The chimpanzee, the only non-human primate susceptible to experimental HCV infection, has been used extensively to study HCV infection, particularly to analyze the innate and adaptive immune response upon infection. However, financial, practical, and especially ethical constraints have urged the exploration of alternative small animal models. These include different types of transgenic mice, immunodeficient mice of which the liver is engrafted with human hepatocytes (humanized mice) and, more recently, immunocompetent rodents that are susceptible to infection with viruses that are closely related to HCV. In this review, we provide an overview of the currently available animal models that have proven valuable for the study of HCV, and discuss their main benefits and weaknesses.

Keywords: hepatitis C virus, animal models, humanized mice, homologs, vaccine, antiviral therapy

INTRODUCTION

The worldwide prevalence of hepatitis C virus (HCV) infection is 3% with an estimated 71 million people who are persistently infected. The severity of HCV infection ranges from mild symptoms to serious illness with chronic hepatitis. Chronic infection may lead to liver cirrhosis and eventually hepatocellular carcinoma (HCC) (1). In recent years, new direct acting antivirals (DAAs) have first supplemented the treatment combination of ribavirin and pegylated interferon alpha (IFN α), reaching cure rates of up to 90% in genotype 1 infected patients. The latest DAA combinations are even more effective and do not require additional ribavirin or interferon administration. Despite these recent advances, significant concerns remain about drug resistance, high cost, and worldwide accessibility of these new antivirals. Besides, DAAs do not necessarily ameliorate the long-term effects of chronic infection and predisposition for liver disease (2). In addition, since therapy-induced HCV clearance does not provide immunity to a new infection, an effective preventive vaccine remains an important need (3).

The first accessible system to study HCV replication in cell culture was the sub-genomic replicon system (4). This approach allows efficient viral replication in human hepatoma (Huh7) cells, transfected with sub-genomes that contain a selectable marker linked to the non-structural region (NS2-NS5B) of HCV (4, 5). Using this system, HCV RNA replication and cellular immunity (6, 7) can be studied

and novel antiviral compounds (8) can be evaluated. Important to note is that no infectious viral particles are produced using this sub-genomic replicon system. In parallel, *in vitro* systems for the study of viral entry were developed. Virus-like particles, produced in a baculovirus system and containing the structural proteins core, E1 and E2, resemble HCV virions and are capable of inducing humoral immune responses against HCV (9). However, these particles are not secreted and have no infectious potential. The first infectious systems consisted of pseudotyped vesicular stomatitis virus or influenza virus containing chimeric E1 and/or E2 glycoproteins (10–13). However, due to modifications that allow assembly at the cell surface, the conformation and functions of the E1/E2 complexes are disturbed (13). The development of infectious HCV pseudo-particles (HCVpp), which consist of defective retroviral particles expressing HCV E1 and E2 glycoproteins on their surface, represented a major breakthrough for investigating the HCV entry process (14–16). More specifically, the role of putative HCV (co-) receptors, the host range, and the E1 and E2 glycoproteins can be examined. This system also allows screening of potential entry inhibitors. In this way, the HCVpp are shown to be hepatotropic and can specifically be neutralized by anti-E2 monoclonal antibodies and HCV-infected patient sera (15). Further steps in the HCV life cycle are not supported by HCVpp and can, therefore, not be explored using HCVpp (15). In 2005, transfection of *in vitro* transcribed full-length genotype 2a HCV (JFH1) isolate and chimeric derivatives thereof into Huh7 cells was described, showing RNA replication and secretion of infectious viral particles (17–20). In contrast to the HCVpp system, this cell culture-derived HCV (HCVcc) system allows the study of all aspects of the viral life cycle and still plays a major role in the identification and evaluation of novel antivirals (19, 20).

Cell culture systems are very useful for initial studies of different aspects of HCV. However, culture conditions are artificial; hence, *in vivo* studies are required to more closely mimic the natural situation. Due to the narrow tropism of HCV, *in vivo* studies were long restricted to chimpanzees. Over the years, other animal species have been evaluated for their susceptibility to HCV infection, although most of them seemed resistant. Therefore, several modified models have been developed in recent years, which allow either complete or partial study of HCV infection. In this review, we provide an overview of currently existing *in vivo* models for HCV infection. We will also discuss their applicability, major advantages, and limitations (Table 1; Figure 1).

HOST SYSTEM REQUIREMENTS FOR HCV REPLICATION

As with any experimental system for human disease, a model for HCV infection should mimic as many, if not all, relevant clinical features as observed in human patients. Desirably, the model should be susceptible to all HCV genotypes with resulting persistent viremia in the majority of exposed animals. The ideal model should also be fully immunocompetent in order to study protective immunity, persistence, and immune-mediated pathogenesis. From a practical point of view, the animal model for HCV infection should be cheap, highly reproducible, easy to

propagate and high in throughput (21). Finally, the ethical impact should be as minimal as possible. Up to this day, no such model exists.

Since the number of unmodified hosts perceptive to HCV infection is limited, extensive research is performed to create a suitable model by modifying existing models. From all animal models used in research, rodents are currently the most popular species for genetic modifications and are therefore highly explored, also in the field of HCV research. Genetic manipulation of the host can be applied to knock down certain host factors that interfere with viral replication or on the other hand, to complement the host with human factors that are essential for this process. The propagation of HCV in rodent cells is inefficient, presumably due to genetic incompatibility of rodent cofactors and/or due to suppression of HCV replication by rodent innate immune defenses. Thus, engineering mice expressing the relevant human genes and/or with deleted mouse restriction factors may permit HCV propagation (22).

A large number of human factors have been determined to be involved in the uptake of HCV into human hepatocytes: glycosaminoglycans (23), low density lipoprotein receptor (24), CD81 (25), scavenger receptor class B type 1 (SR-BI) (26), tight junction proteins claudin-1 (CLDN1) (27) and occludin (OCLN) (28, 29), the receptor tyrosine kinases epidermal growth factor receptor and ephrin receptor A2 (30), the cholesterol transporter Niemann-Pick C1-like 1 (31), transferrin receptor 1 (32), cell death-inducing DFFA-like effector b (33), and E-cadherin (34). The entry of HCV into primary hepatocytes is mediated by CD81, OCLN, CLDN1, and SR-BI. To our current knowledge, CD81 and OCLN comprise the minimal human factors required for HCV uptake by rodent cells (35). However, these animals do not sustain viral replication and chronic infection. Finally, it is still not entirely clear which host factors should be humanized, because there is little knowledge about the specific host factors that cause inhibition of HCV RNA replication or host factors that determine species tropism.

NON-RODENT MODELS

The chimpanzee (*Pan troglodytes*) played an important role in the discovery of HCV. In fact, the viral genome of HCV was cloned from a chimpanzee that was experimentally infected with non-A, non-B hepatitis (36). For a long time, the chimpanzee was the only available model to study HCV, and their use has greatly advanced our knowledge on this virus. Humans and chimpanzees share more than 98% of their genome sequence. Despite this high genomic homology, there are some clear differences between the two which makes that the disease pattern and outcome in chimpanzees does not necessarily reflect that in humans. Whereas only a minority of humans spontaneously clear an acute infection (15%), few chimpanzees evolve to chronicity (30–40%) (37), and to date, no fibrosis and only one HCC case has been observed in this model (38). Nevertheless, the chimpanzee proved very valuable for the study of the molecular, immunological, and clinical aspects of HCV infection. Furthermore, while it is very difficult to study the acute phase of HCV infection in humans because specific symptoms are usually absent during that phase, experimental infection of chimpanzees

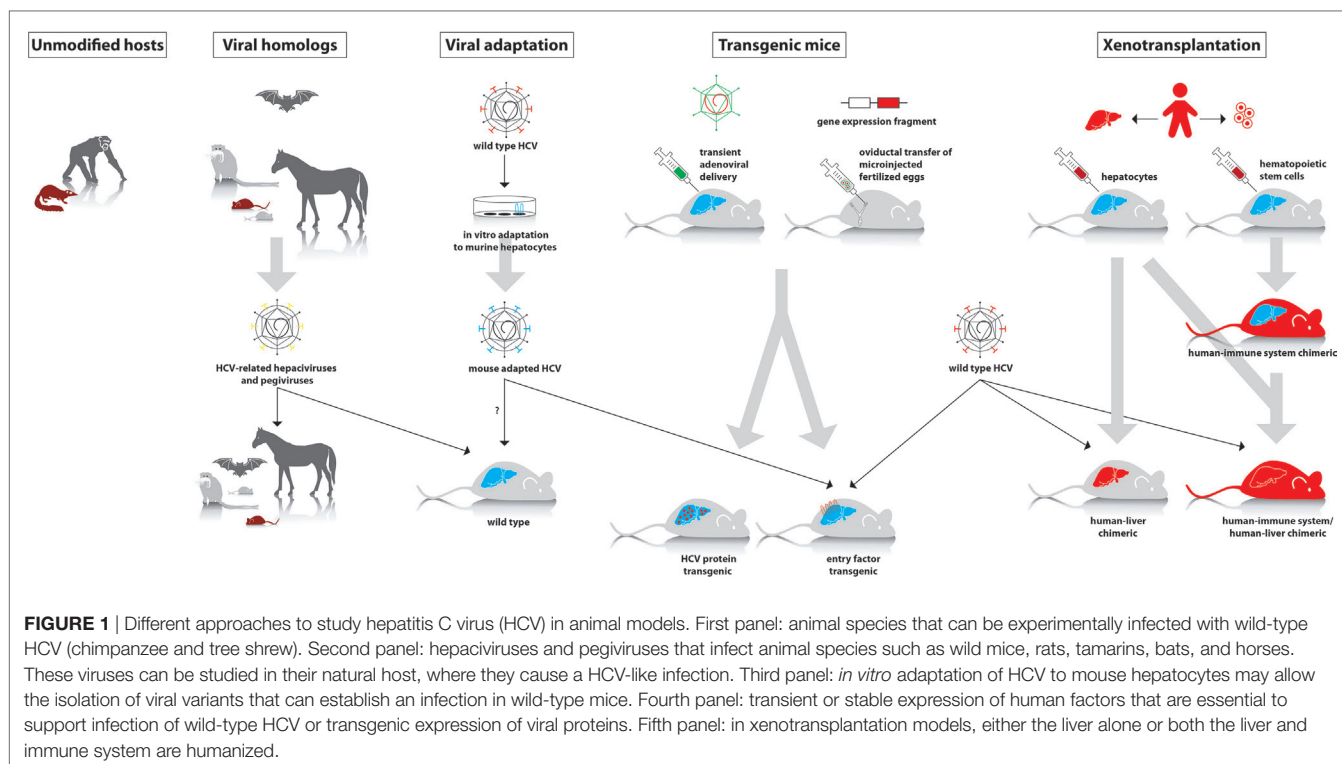
TABLE 1 | Characteristics of hepatitis C virus (HCV) animal models and HCV homologs.

Animal model	Complete viral life cycle	Viremia	Liver disease	DAA testing	Passive immunization	Vaccine development	Availability	Reference
Non-rodent models								
Chimpanzee	Yes	High	Acute, chronic ^a	Yes	Yes	Yes	Very low	(37, 39, 45)
Tree shrew	Yes	Low	Fibrosis, cirrhosis	No	Yes	No	Low	(54, 55)
Zebrafish	Replication	Not relevant	Virus–host interaction	Yes	No	No	High	(56)
Viral protein transgenic mouse models								
Inducible transgene expression	Not relevant	Not relevant	Virus–host interaction	Not relevant	Not relevant	No	High	(58, 67, 68)
Full HCV genome	Not relevant	Not relevant	Fibrosis, HCC	Not relevant	Not relevant	No	High	(70)
Immunocompromised human liver xenograft mouse models								
Trimera mouse	Yes	Low	No	Yes	Yes	No	Low	(78, 80)
Alb-uPA-SCID mouse with humanized liver	Yes	High	No	Yes	Yes	No	Low	(79, 83, 88, 90–92)
FRG mouse	Yes	High	No	Yes	Yes	No	Low	(96, 130, 133, 134, 136, 137)
Immunocompetent xenograft mouse models								
Tolerized rat	Yes	Very low	No	Yes	Yes	Yes	Very low	(139, 141)
AFC8-hu HSC/Hep mouse	Yes	Only in liver	Inflammation, fibrosis	No	No	Yes	Very low	(142)
HIL mouse	Yes	Very low	Inflammation, fibrosis	No	No	Yes	Very low	(155, 156)
Viral adaptation								
	Entry	No	No	No	Yes	No	High	(157, 158)
Genetically humanized mouse models								
Rosa26-Fluc mouse	Yes	Persistent viremia	No	Yes	Yes	Yes	High	(159, 160)
JCR-C/OTg mouse	Yes	Persistent viremia	Fibrosis	Yes	Yes	Yes	High	(161)
HCV homologs in natural host								
GB-virus	Yes	Acute	No	Yes ^b	Yes ^b	Yes ^b	Low	(165)
NPHV in horses	Yes	Persistent viremia, acute	Inflammation	Yes ^b	Yes ^b	Yes ^b	Low	(168, 169)
NHIV in rats	Yes	Acute, chronic	Inflammation	Yes ^b	Yes ^b	Yes ^b	High	(172–174)

DAA, direct-acting antiviral; HCC, hepatocellular carcinoma; uPa, urokinase-type plasminogen activator; HSC, hematopoietic stem cell; HIL, human immune system and liver; NPHV, non-primate hepatitis virus; NHV, Norway rat hepatitis virus.

^aChimpanzees are not necessarily good models of chronic liver disease. They do not develop fibrosis or HCC.

^bThese models can be used to evaluate the efficacy of DAAs and vaccine candidates against the homolog virus, not HCV itself.



allows close monitoring of viral kinetics, host immune response, disease manifestation, and outcome in a highly controlled manner (39–43). Immunological studies in chimpanzees have also led to the development and evaluation of several candidate vaccines (44, 45). Moreover, in the context of antiviral efficacy studies, they have been successfully used to track resistance associated with the use of entry (46), protease, NS5A (47), and polymerase (48, 49) inhibitors and combinations thereof (50).

The chimpanzee model fulfills many of the requirements for a good animal model. However, limited availability and ethical and financial constraints associated with these studies are major drawbacks. Recently, the National Institute of Health of the United States Department of Health and Human Services decided to effectively end its support for invasive research on chimpanzees. Other primates have been tested for their susceptibility to HCV infection, with little success. Although HCV can infect induced pluripotent stem cells derived from hepatocyte-like cells from pigtail macaques (51, 52), HCV does not seem to be able to establish persistent infection in non-human primates except for chimpanzees (53).

In addition, several other non-primate species have been tested for their susceptibility to HCV. The tree shrew (*Tupaia belangeri*) is for example a non-rodent squirrel-like mammal that is permissive for persistent low-level HCV viremia, including HCV-related liver disorders (54, 55). Still, limited availability and incompatibility of the *Tupaia* host environment with robust HCV replication limits the use of this animal for the study of HCV pathogenesis and vaccine development.

Recently, Ding et al. (56) developed a zebrafish model for sub-genomic HCV replication. The zebrafish is often used as a model

organism for human diseases, including liver disease (57). The sub-replicon is created using two vectors: one containing HCV NS5B and the other containing the minus strand of HCV 5'UTR, core, and 3'UTR, under the control of the mouse hepatocyte nuclear factor 4 promoter. These vectors are then co-injected into zebrafish zygotes. The sub-replicon is able to replicate in the liver and causes alterations in the expression of certain genes, which is similar to HCV infection in human liver cells. Administration of ribavirin and oxymatrine significantly inhibits the replication of the HCV sub-replicon in the larvae (56). To conclude, the zebrafish is small, easy to handle experimentally, and useful for investigating mechanisms of HCV replication and liver pathology *in vivo*. Also, this model may aid in drug evaluation studies and thus the discovery of new anti-HCV drugs.

VIRAL PROTEIN TRANSGENIC MICE

Mice that transgenically express viral proteins have been created to study the *in vivo* interactions between these viral proteins and the host cell. Transgenic mice, containing the genetic code for HCV structural proteins E1, E2 or core (or combinations thereof); or the non-structural NS3/4A protein, show conflicting results in the development of liver pathologies. Some reports do not show any evidence of hepatocellular damage (58–61), while other groups describe progressive hepatic steatosis and HCC (62–65). These discrepancies may be explained by the relationship between inflammation-associated hepatocarcinogenesis and the host genetic background (66). A major drawback of these HCV-transgenic mice is that the transgene integrates randomly and in high copy numbers. Consequently, the viral proteins are highly

overexpressed, often in an uncontrolled manner. Certain aspects of the HCV-transgenic mouse phenotype may be attributed to the artificial overexpression and/or interference with the regulation of genes located near the integration site. If the expression of viral proteins can be controlled and fine-tuned, the limitations of these models may be overcome. The Cre/Lox system (67) or hydrodynamic injection (68) allows inducible expression of the transgene. Using the murine major urinary protein (MUP)-promoter, the expression can be delayed (58). The immune system of this model closely resembles that of a chronically infected patient. Hence, it allows the evaluation of potential therapeutic vaccine strategies (69). Lerat et al. (70) created a transgenic FL-N/35 mouse model expressing the full HCV genome at levels corresponding to natural human infection (70, 71). The FL-N/35 mouse model is certainly the most relevant transgenic mouse model available at this time, especially for investigating hepatic steatosis, fibrosis, and HCC.

IMMUNOCOMPROMISED (HUMAN) LIVER XENOGRAFT MOUSE MODELS

Because mice are naturally not susceptible to HCV infection, an interesting approach to overcome the species barrier is by humanizing the liver *via* transplantation of primary human hepatocytes. In this way, mice can not only be infected with HCV but also with other human hepatotropic pathogens. However, if immunocompetent rodents are transplanted with xenogeneic hepatocytes, rejection of donor cells by the host cellular immune system is observed (72–74). In order to prevent this rejection, mice need to be immunocompromised. In addition, recipients must suffer from some type of liver disease to ablate murine hepatocytes and to allow proliferation of donor hepatocytes in the mouse liver parenchyma. This liver injury can be generated in three ways: chemically, surgically, or genetically (75). Several humanized mouse models have been developed and explored for HCV infection during the past 20 years.

The Trimer Mouse Model

The Trimer mouse was the first chimeric model and is composed of three genetically disparate sources of tissue (i.e., recipient mouse, bone marrow donor mouse, and human liver tissue), hence its name (76). After the recipient mouse is preconditioned by lethal total body irradiation, it is radioprotected by immediate injection of bone marrow cells from an immunodeficient SCID mouse (76). Then, human liver fragments, infected *ex vivo* with hepatotropic virus, are transplanted in ectopic sites of the recipient mouse such as the ear pinna or under the kidney capsule (76–78). Using this method, Ilan et al. (77, 78) were able to generate mice that can be infected with HBV and HCV. Higher serum HCV loads are obtained when pre-infected liver fragments from HCV-positive patients are employed compared to *ex vivo* infected liver fragments (78). HCV viremia persists for approximately 1 month and declines thereafter as a result of fibrosis and necrosis of the human graft (78). These observed histological abnormalities of the transplant can be attributed to their transplantation at an extrahepatic location (79). Also, *de novo* infection of Trimer

mice, transplanted with healthy liver grafts, has so far not been achieved. This means that viral entry or neutralization studies cannot be performed using this model (79). Nevertheless, the 1-month time window may be sufficient for the evaluation of certain anti-HCV therapeutics or HCV vaccines (78). In fact, an HCV internal ribosomal entry site inhibitor was successfully tested in the Trimer mouse model (78, 80).

The Alb-uPA-SCID Mouse Model With Humanized Liver and Variants

The Alb-uPA mouse model was initially designed to study the pathophysiology of plasminogen hyperactivation and to evaluate new therapy regimens for bleeding disorders (81). These transgenic mice carry a tandem repeat of four murine urokinase-type plasminogen activator (uPA) genes under the control of a mouse albumin (Alb) promoter/enhancer (Alb-uPA mice) (81, 82). The hepatic uPA transgene overexpression results in elevated uPA plasma levels, but also leads to accelerated hepatocyte death, hypofibrinogenemia, and serious hemorrhagic events such as intra-abdominal and intestinal bleedings in neonatal transgenic mice (81–83). However, the high uPA concentration gradually returns to normal levels by the age of 2 months (82). This is probably due to somatic deletions of (parts of) the transgene construct within hepatocytes (82). Consequently, these transgene-deficient cells can selectively proliferate and regenerate the diseased liver tissue (82). On the other hand, when newborn Alb-uPA mice are transplanted with healthy donor hepatocytes, their functional liver deficit is also restored by the transplanted cells that repopulate the diseased liver (82, 84). In order to prevent rejection of hepatocyte transplants of xenogeneic origin, Alb-uPA mice should be backcrossed to an immunotolerant genetic background (84).

Mouse, rat, and woodchuck hepatocytes can be successfully transplanted into immunodeficient Alb-uPA mice using intrasplenic injection (84–86). Mouse livers are chimerically composed of both donor-derived and host-derived cells, the latter having a survival advantage by deletion of (parts of) the transgene (84–86). This transgene inactivation occurs less frequently in homozygous uPA animals compared to their hemizygous counterparts, because in the former two transgene arrays must be inactivated which is less likely to occur (82, 86). Accordingly, liver chimerism can be sustained for a much longer period and at higher levels in homozygous mice (86). Up to 90% of the liver may be reconstituted with donor hepatocytes and initially these cells appear to grow in a nodular fashion (84, 85).

By extension, this model is suitable for evaluating the susceptibility of donor hepatocytes to liver infections with a specific tropism for the donor species. Petersen et al. (85) were able to detect persistent non-cytotoxic woodchuck hepatitis virus infection in chimeric livers of uPA/recombination activating gene 2 (RAG2) mice transplanted with woodchuck hepatocytes (85). Accordingly, the same group (87) was able to transplant adult human hepatocytes early after birth in immunotolerant uPA/RAG2 mice. Up to 15% of the livers consist of human hepatocytes and homozygosity of the Alb-uPA transgene is also required to ensure sustained human engraftment (83, 87). The human hepatocytes seem to repopulate the liver in a well-organized fashion

with preservation of normal cell function and pharmacological responses (88, 89). In addition, human albumin, which indicates the functionality of the chimeric liver, is detected in plasma for at least 2 months after transplantation (87). Besides mature human hepatocytes, also hepatic progenitor cells are observed in these livers (90). Better humanization is obtained using commercially available, cryopreserved human hepatocytes (91). Remarkably, after inoculation with human HBV infectious serum, productive infection is initiated (87). Mercer et al. (83) showed for the first time that chimeric immunotolerant uPA-SCID mice were susceptible to HCV infection, thereby permitting the *in vivo* study of HCV biology and the evaluation of different antivirals. Efficient infection is independent from HCV genotype, but human albumin plasma levels exceeding 1 mg/ml are required for a consistent HCV infection in chimeric mice, whereas infectivity criteria for HBV infection are clearly less stringent (91, 92).

However, several shortcomings can be highlighted regarding the uPA-SCID mouse model: high neonatal lethality, a tendency to develop kidney disorders, lower body size, reduction of donor hepatocytes (even in homozygotes), less efficient breeding, technically challenging surgical manipulation in young and fragile mice, and finally the inability to expand engrafted hepatocytes (83, 88, 93–96). Tateno et al. (93) hypothesized that the first four mentioned limitations are caused by inadequate transgene structure and/or very high expression levels of the uPA gene before or after birth. Therefore, they produced chimeric mice using embryonic stem cell techniques in order to generate a number of transgenic lines. In addition, transgenic lines with the most appropriate uPA expression for a damaged, but not a detrimental liver were selected (93). This variant is called the hemizygous cDNA-uPA-SCID mouse model (93). More albumin-positive human hepatocytes are present compared to the original model, potentially due to an overgrowth of mouse hepatocytes in the uPA-SCID mouse by somatic deletion of uPA genes (97). After HBV infection, high titers of HBV viremia that persisted for at least 34 weeks are found in cDNA-uPA-SCID mice, but entecavir treatment results in a similar viremia decline in both models (97). HCV viremia is significantly more observed in cDNA-uPA-SCID mice in comparison with uPA-SCID mice, but not one mouse remains HCV-positive 8 weeks post-inoculation (97). Finally, fewer kidney disorders, higher body weight, and a higher survival rate are observed in the cDNA-uPA-SCID model (93, 97). Taken together, the cDNA-uPA-SCID mouse model may be preferred over the original uPA-SCID model for the study of HBV and HCV biology and by extent for the evaluation of anti-HBV/HCV drugs.

A second variant consists of transgenic mice carrying the uPA gene driven by the MUP promoter (98). These mice can be backcrossed onto a SCID/Beige background, resulting in the MUP-uPA SCID/Bg mouse model (99). The initial purpose of this model was to study liver regeneration after repopulation of the diseased liver, but Tesfaye et al. (100) were able to show that, upon humanization, these mice are susceptible to infection with HBV, genotypes 1–6 of HCV and tissue culture-derived virus (98, 99). Interestingly, these mice are in better health compared to the classical uPA-SCID mouse model and they offer a longer time window (up to 4–12 months of age) for transplantation

of human hepatocytes (100). The same group (101) reported successful HCV infection after engraftment with hepatocyte-like cells, generated from both human embryonic stem cells and patient-specific human-induced pluripotent stem cells. Finally, this model is also valuable for the study of HCV-associated HCC and for the analysis of tumor-promoting factors in liver cancer (102).

As a third uPA-based variant, the non-obese diabetic (NOD)/Shi-scid IL2Rg^{null} (NOG) background is employed, resulting in the uPA/NOG mouse model (94). Donor hepatocytes can be transplanted in 6-week-old uPA/NOG mice which enable easier surgical manipulation and moreover an improved survival rate of the transplanted mice (94). In addition, absence of neonatal lethality increases the efficacy of homozygote production by mating and finally, the severely immunocompromised NOG background allows higher xenogeneic cell engraftment (94). Another advantage is that a relatively low frequency of physical loss of the transgene is observed (94). However, HCV infection is not reported in this model yet. Importantly, Hasegawa et al. (103) generated another model by using an alternative strategy for the endogenous liver injury: targeting the expression of herpes simplex virus type 1 thymidine kinase to the liver of the NOG mice. Hepatocytes that express this transgene can be ablated after brief exposure of a non-toxic dose of ganciclovir (103). Thereby, mouse livers can be stably replaced with mature and functional human liver tissue at a chosen time (103). This model can be successfully infected with HBV and HCV and is therefore useful to test different antiviral agents (104).

Taken together, the chimeric human liver uPA-SCID mouse model or discussed variants thereof have proven valuable for *in vivo* metabolism studies, basic biology research of HCV infection, and the evaluation of different antiviral therapies and passive immunization strategies (79, 105–113). Our group also contributed by demonstrating the prophylactic effect of monoclonal and polyclonal antibodies, isolated from a chronically infected patient, against challenge with different HCV genotypes (107, 108, 114). In addition, anti-receptor antibodies are shown to protect these mice from a subsequent challenge with HCV of different genotypes (106, 115–117). Next to chimpanzees, this human liver chimeric mouse model is also attractive for monitoring HCV drug resistance (118). Our laboratory has particular interest in this matter and showed that the combination of DAAs with entry inhibitors restricts the breakthrough of DAA-resistant viruses (119, 120). Finally, the uPA-SCID mouse model is also applicable for studies concerning malaria, which is caused by the parasite *Plasmodium falciparum*, and the study of the hepatitis E virus (HEV) (121–125).

The FRG Mouse Model With Humanized Liver

In the original uPA-SCID mouse model, hepatocyte transplantation needs to be performed very shortly after birth (i.e., in very fragile and small animals) (126). Because of this practical inconvenience, other models were explored in which liver injury can be induced at a later age, such as in certain earlier discussed variants of the uPA-SCID model. Another example is based on mice

that have a genetic knockout for fumarylacetoacetate hydrolase (Fah^{-/-}), a metabolic enzyme that catalyzes the last step of the tyrosine catabolism pathway (127, 128). This knockout results in an accumulation of toxic compounds (e.g., fumarylacetoacetate and maleylacetoacetate), which in turn leads to liver dysfunction and lethality, unless mice are rescued by the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) (127–129). NTBC blocks the enzyme hydroxyphenylpyruvate dioxygenase upstream of FAH, thus preventing the accumulation of hepatotoxic metabolites (130). Using this approach, Grompe et al. (129) showed that liver injury can be induced at any desired time point when NTBC is withdrawn. The resulting prolonged lifespan of these Fah^{-/-} mice resembles a phenotype of humans suffering with hereditary tyrosinaemia type I (HT1), which is an inborn error of metabolism caused by deficiency of the Fah enzyme (127–129). As a result, the adult Fah^{-/-} mouse, when removed from NTBC treatment, is a valuable model for studying the pathophysiology and evaluating the treatment options of HT1 and by extension hepatic cancer (128, 129). In the immunodeficient FRG mouse, the aforementioned Fah, RAG2, and common γ -chain of the interleukin receptor (Il2rg) are knocked out. The livers of these mice can be successfully repopulated with human hepatocytes after NTBC withdrawal (95, 130). In support of enhanced engraftment, Azuma et al. (95) administered an uPA-expressing adenovirus before transplantation which induces cell-autonomous hepatotoxicity rendering a more favorable niche for transplantation (131). In later experiments, Bissig et al. (96) showed higher transplantation rates (up to 95%) using an increased human hepatocyte dose per mouse. They also demonstrated successful infection of the FRG mouse with HBV and HCV, however, infection with HCV is only achieved in mice with a high human liver chimerism (96).

The FRG mouse model is in some ways favorable over the uPA-SCID mouse model. First, the deficiency caused by the Fah deletion cannot revert back to its wild type form, as seen in the uPA-SCID model (95). As a result, serial transplantations are possible and transplantation can be performed in adult animals (at any age) which simplifies surgery (95). Second, mutant breeders are completely viable and finally, there is no renal disease observed (95). Unfortunately, there are also drawbacks for such a model. First, primary engraftment does not occur in 100% of the recipients, even when the aforementioned urokinase-expressing adenovirus is administered (95). Second, the growth disadvantage of mouse hepatocytes in the FRG mouse depends on the absorbed tyrosine and the use of NTBC, whereas the growth advantage in uPA-SCID mice is sustained as long as the transgene is expressed (130).

Applications of this FRG mouse model with humanized liver are wide-ranging. First, human lipid and bile acid metabolism can be studied, next to the metabolism of candidate pharmaceuticals or toxicity of drug metabolites (95, 132). Second, after inoculation with pathogens that are dependent on human liver cells for replication such as HBV, HCV, and HEV, the life cycle can be studied, but also experimental treatment options can be evaluated (95, 133–137). Finally, because the FRG mouse model also supports complete *P. falciparum* liver stage development, this model is suitable for evaluating existing drugs and screening of candidate antimalarials (138).

IMMUNOCOMPETENT XENOGRAFT MOUSE MODELS

The human liver xenograft mouse models are very valuable as challenge models for HCV or other human hepatotropic pathogens, but their major drawback is the lack of a functional immune system. As a consequence, they cannot be used for the study of HCV-specific immune responses or HCV immunopathogenesis after infection, nor for HCV vaccine studies (79). Second, histopathology such as fibrosis, cirrhosis, or HCC has not been reported, in contrast to what is seen in humans that are chronically infected with HCV (75). In human patients, an ongoing inflammatory response is probably responsible for disease progression, so the presence of a functional human immune system in HCV mouse models is highly demanded and explored (75).

The Tolerized Rat Model

Another way to avoid rejection of allogeneic transplants, in addition to generalized immunosuppression or the use of genetically immunodeficient animals, is by induction of immunological tolerance to transplanted cells in immunocompetent animals (76, 77, 85, 139, 140). Therefore, Huh7 cells can be injected *in utero* into the peritoneal cavity of fetal rats (139). In this time frame, the immune system is still in development, so tolerance toward engrafted hepatocytes can be established (139, 140). Corresponding cells are then intrasplenically injected into the newborn rats within the first 24 h after birth (139). The major benefit of this model is that there is no need for genetic or pharmacological immunosuppression (139). However, engraftment rates are much lower compared to the uPA-SCID model for example, because there is no mechanism for host hepatocyte depletion (141). The use of hepatoma cells instead of primary hepatocytes also limits further applications. Another drawback is the mismatch between the human major histocompatibility complex (MHC) antigens on the transplanted cells and the rat immune system, so there will be no recognition of HCV antigens by the immune cells of the rat (141). Despite these limitations, HCV gene expression, viral replication, and hepatitis symptoms can be observed when these tolerized immunocompetent rats are intrasplenically injected with HCV inocula 1 week after transplantation (141). Unfortunately, HCV viremia is low (peak at 22,500 copies/ml) and the observed inflammation is probably due to cytokine-mediated effects (141).

The Dually (Immune System and Hepatocytes) Engrafted Mouse Models

To overcome the human/rodent MHC mismatch as in the tolerized rat model, it would be favorable to introduce both human hepatocytes and human immune cells from the same donor into the same recipient animal. The first mouse model that supported this double engraftment was generated in 2011 (142). A fusion protein of the FK506 binding protein (FKBP) and caspase 8 under the control of the albumin promoter (AFC8) is therefore transgenically expressed in hepatocytes of immunodeficient Balb/C Rag2- γ C^{null} mice. After administration of an FKBP dimerizer, hepatocytes that expressed the transgene are depleted (142, 143).

This induced liver-specific cell death provides a niche for engraftment with human hepatocyte progenitors (142). Moreover, after irradiation, these mice are transplanted with human CD34⁺ hematopoietic stem cells (HSCs) from the same human fetal liver tissue, providing these AFC8-hu HSC/Hep mice with a, to some extent, functional human immune system (142, 144). Following inoculation with primary HCV isolates, HCV infection can be observed that in turn induces infiltration of human immune cells in the livers with liver inflammation and fibrosis as a result (142, 145). This model enables the study of HCV-specific immune responses (i.e., T-cell responses) and HCV immunopathogenesis (142, 145). However, HCV RNA could only be detected in the liver and not in plasma, probably due to the low level of human liver engraftment (~15%) in this model (126, 142). Another limitation is the suboptimal activity of the immune system inherent in human CD34⁺ HSC transplanted mice and also the lack of functional B-lymphocytes that hampers the study of antibody responses and vaccine development (126, 142, 146).

A second immunocompetent model was launched by Gutti et al. (147) who used non-myeloablative conditioning with treosulfan as a safe and well-tolerated alternative to total body irradiation for HSC transplantation. Long-term dual reconstitution is achieved in uPA/NOG mice with HSCs and allogeneic mature hepatocytes (not fetal hepatoblasts) (147). Even MHC mismatched transplantation is sustained without any evidence of hepatocyte rejection by the human immune system (147). Wilson et al. (148) also accomplished double humanization of mice. Following preconditioning with a DNA-damaging chemical for enhanced HSC engraftment and an uPA-expressing adenovirus for enhanced hepatocyte engraftment, they co-transplanted adult human hepatocytes and HSCs in immunodeficient FRG mice on a NOD-strain background (FRGN mice) (95, 148). Another variant is achieved in BALB/c RAG2^{-/-} IL-2R γ ^{-/-} NOD.sirp α (BRGS) mice that harbor the uPA transgene (uPA/BRGS mice) (149). Irradiated newborn pups are injected with human HSCs and later implanted with human hepatocytes to generate dually engrafted mice that are not haplo-type matched. Engraftment (~20–50% of chimerism) is stable for at least 5 months and is similar as observed in the uPA/NOG and FRGN host, but higher than in the AFC8 host (142, 147–149). Interestingly, a complete viral life cycle can be observed after HBV infection in this model (150). This enables the evaluation of experimental anti-HBV therapies, but also the study of anti-HBV immune responses (150). Bility et al. (151) also reported successful HBV infection in a similar human liver progenitor cell and human CD34⁺ HSC cell engraftment model using mice on a NOD-SCID IL2r γ ^{-/-} background (HLA-A2/NSG mice). These mice carry the human HLA-A2 transgene that enhances the development of human MHC-restricted T-lymphocytes (151, 152). To promote efficient hepatocyte repopulation, mice are first treated with an anti-Fas agonistic antibody (151, 153). Chen et al. (154) performed one-step engraftment of hepatoblasts and a matching human immune system using fetal liver-derived HSC cells in the same NSG mouse (human immune system and liver or HIL mice) and this without the need for transgenic modification or drug treatment. HIL mice support HCV infection, liver inflammation, human

HCV-specific immune responses, as well as liver fibrosis, however, in a low number of hepatocytes (154, 155). This can be explained by the low human chimerism rate (<10%) (154, 155). Antiviral treatment using IFN α -2a is able to block the progression of the HCV-associated liver pathogenesis (154, 155). These HCV-infected mice also show expansion of monocytes/macrophages and (especially CD4⁺) T-cells, suggesting exhaustion of immune cell phenotypes as seen in HCV patients (156). Unfortunately, HCV infection is not reported in every discussed dually engrafted model and this will also be challenging, especially due to the very low engraftment rates.

VIRAL ADAPTATION

Hepatitis C virus exhibits a narrow species tropism which is incompletely understood. Resistance of mice to HCV infection is determined to be at the level of viral entry and/or replication. There are two ways of surmounting this barrier: either the host can be adapted to the virus or the virus can be adapted to the host. First, utilizing the error-prone replication of RNA viruses, the HCV virus can be adapted to the murine environment. More specifically, long-term cultivation in the presence of mouse cells could allow the virus to adapt to murine entry factors (CD81, OCLN, CLDN1, and SR-BI). Bitzegeio et al. (157) attempted to adapt an HCV genotype 2a strain (Jc1) to the murine CD81. They identified three adaptive mutations in the HCV envelope proteins E1 and E2. This Jc1/mCD81 virus has increased affinity for the extracellular loops of human CD81, indicating a more accessible binding site for human CD81 (157). The uptake of this murine-tropic HCV in mouse primary hepatocytes *in vitro* and *in vivo* is rather inefficient and more modifications are required to increase efficiency. There is unfortunately no persistent infection observed, even in mice with impaired innate and adaptive immune system. To conclude, additional barriers may exist in the replication and post-entry steps (158). In addition, the applicability of such systems for the study of entry processes might be affected by the influence of the adaptive mutations on the envelope conformation and receptor usage.

GENETICALLY HUMANIZED MOUSE MODEL

Rather than to adapt the virus to a new host, an alternative strategy could be to genetically adapt the host to natural HCV isolates. Despite differences to the natural human host of HCV, an immunocompetent animal model can be generated in this way. Transient expression of the minimal human factors (CD81, OCLN, CLDN1, and SR-BI) by adenoviral delivery in Rosa26-Fluc mice allows entry of HCVcc in mouse hepatocytes (35). Furthermore, mice transgenic for these four human receptors (4hEF-mice), but with deficiencies in several innate immune signaling pathways (STAT1^{-/-}), support not only viral entry of HCVcc but also low-level replication and sustained HCV infection for 90 days. The infection elicits antiviral cellular and humoral responses, but does not result in development of liver disease (159).

However, these models express non-physiologically high levels of the entry factors and impair tight junction formation and B-cell development (160). Interestingly, by selectively humanizing the second extracellular loops of CD81 and OCLN, required for HCVcc entry, the chimeric alleles are expressed at physiological levels and mice support HCV uptake at similar levels as mice expressing HCV receptors using transgenic or adenoviral methods. Also, tight junctions are formed normally and the defects in B-cell development are absent (160).

However, since replication in immunocompetent mice is inefficient, the latter model does not allow a profound study of all complex virus–host interactions. Viral RNA replication in mouse cells appears to be the final hurdle to overcome in order to reconstitute the entire viral life cycle in mice. Chen et al. (161) described an immunocompetent animal model permissive for HCV infection and ensuing development of liver disease. They created transgenic mice expressing OCLN and CD81 on an outbred ICR (CD-1) background (C/OTg). These mice can be infected with serum- or cell culture-derived HCV and sustain this infection for over 12 months. Moderate hepatic inflammation, micro- and macro-vesicular steatosis, and fibrosis are observed in some of the infected animals. However, none of the animals develop HCC (161). It is rather striking that HCV can establish a persistent infection in ICR-C/OTg mice, whereas a similar approach on a C57BL/6 background fails to show sustained HCV replication. Backcrossing C/OTg to a C57BL/6 background (B6-C/OTg) significantly reduces the RNA copy number in serum and liver. ICR hepatocytes express higher levels of apolipoprotein E, which is shown to improve HCV production (162). Also, miR-122 is not upregulated upon HCV infection in B6-C/OTg (161). In conclusion, the ICR-C/OTg mouse model appears to fulfill to a large extent the criteria for a suitable HCV animal model and is therefore a valuable addition to the current pool of animal models.

HCV HOMOLOGS

Alternative models are based on the use of HCV homologs. These HCV-related viruses infect either rodents, horses or dogs and can therefore be used to study viral biology, pathogenesis, and host immune responses in an immunocompetent setting. The GB virus B has long been the only known homolog to HCV. This virus was first discovered in tamarins experimentally infected with serum of a surgeon (G.B.) suffering from acute hepatitis (163). The infected tamarins developed acute hepatitis (164) and are used together with marmosets as a surrogate model for the study of protective immunity (165) and evaluation of antivirals (166). However, persistence is rare in these animals and the natural host is yet to be identified (163, 167, 168).

By using deep sequencing virome analyses, novel HCV-related hepaciviruses and pegiviruses have been identified in dogs, horses, bats, rodents, and non-human primates (168). Several of these viruses have the potential to serve as a surrogate model for HCV. However, not all are hepatotropic or mimic the natural course of HCV infection. The non-primate hepacivirus (NPHV) was first discovered in dogs and therefore termed canine hepacivirus (169), but subsequent studies revealed that horses are

the natural host for this virus (168). NPHV infection in horses greatly resembles HCV infection in humans. It is a hepatotropic virus that is able to establish a persistent infection, although the chronicity rate is considerably lower than for HCV infection. The host immune response is similar to that in humans, including the delayed seroconversion and immune-related liver pathology (168). These characteristics allow NPHV to be a valuable animal model for HCV, especially since it is immunocompetent. Drawbacks, however, are the large size and animal care costs that accompany this model. Conceivably, rodents are still the desired animal model, due to their small size, easy handling, and possibility to be genetically manipulated. Therefore, the newly discovered rodent hepaciviruses (RHV) are of particular interest (170–172). Infections of these viruses in their natural host, or possibly in immunocompetent laboratory inbred mouse strains, require further investigation.

Methodical searches for hepaciviruses in several wild rodent species have led to the identification of potential small animal models for HCV. Some of these rodents, including bank voles (*Myodes glareolus*) and rats (*Rattus norvegicus*), experience signs of liver inflammation after infection with a RHV. During a metagenomics survey in commensal Norway rats (Nr) in New York city, Firth et al. (172) also discovered some new viruses, including two novel hepaciviruses (NrHV-1 and NrHV-2) and one novel pegivirus (NrPgv). These hepaciviruses were demonstrated to be hepatotropic and are consequently the first small-mammal hepaciviruses known to replicate in the liver (172). Although rats are the natural hosts of NrHV, Billerbeck et al. (173) aimed to develop a mouse model of NrHV infection. NrHV is able to establish a persistent infection in immunocompromised mice lacking type I interferon and adaptive immunity (A129, AG129, and NRG). On the other hand, immunocompetent mice (C57BL/6J and BALB/c) clear the virus in a few weeks (173). NrHV, passaged through NRG mice, is cleared significantly slower than NrHV derived from rats, indicating possible adaptation to the mouse host. The developed immunocompetent inbred mouse model can potentially help to unravel certain mechanisms of hepacivirus host adaptation, immune activation and evasion, and development of liver disease (173). Because this inbred mouse model only results in an acute, self-limiting infection, Trivedi et al. (174) searched for a fully immunocompetent surrogate model in which a persistent infection can be established. The natural host of NrHV, the rat, was therefore further investigated. Inbred Brown Norway rats fail to even partially control the infection, while different outbred lines [Sprague–Dawley, Holtzman (HTZ), Long Evan, and Wistar Han] show limited suppression of viral replication. HTZ rats display the largest suppression of viremia and were explored in more detail. The rats exhibit hepatic inflammation characterized by dense lymphocytic aggregates focused on the portal tracts, parenchymal damage, associated with apoptotic hepatocytes and macro- and micro-vesicular steatosis, characteristic for human HCV infection (174). This model is also suitable to study the role of various interferon stimulating genes and immune responses in hepacivirus pathogenesis. Thus, NrHV infected rats can serve as an informative, fully immunocompetent surrogate to study the mechanisms of HCV persistence, immunity, and pathogenesis.

CONCLUSION

Despite extensive research, there is still no vaccine available for the prevention of HCV infection. In order to design and test new vaccines, the immunocompetent human liver xenograft mouse models are very promising. Next to the study of HCV immune responses, these models also allow investigation of disease progression. Contrary to this, the immunocompromised human liver xenograft mouse models only allow passive immunization. In this way, antibodies targeting different genotypes of HCV can be evaluated. Vaccine studies are not relevant, because these mice lack or only show limited cellular immunity. Furthermore, during the past decade, especially the uPA-based mouse with humanized liver has considerably contributed to our understanding of the HCV life cycle and the development of antiviral strategies. Alternatively, for studying the basic aspects of HCV biology, viral replication or for the evaluation of certain antiviral strategies, it may not be necessary to establish complicated dual-chimeric models. The genetically humanized models are adequate to study viral biology. However, they can only be used to evaluate prophylactic vaccines, not therapeutic vaccines. Finally, the HCV homologs, more specifically NrHV, can be used for vaccination studies and for the evaluation of both humoral and cellular immune responses. The knowledge that is built from this model can be partially transferred to the existing HCV models, but it is important to consider that HCV and hepaciviruses comprise different viruses. In conclusion,

it is clear that the HCV model of choice is highly dependent upon the specific research question. The development and characterization of new HCV animal models or the improvement of existing models, especially those with a human immune system, is highly demanded to develop a potent HCV vaccine. An effective vaccine is probably the most essential key for eradication of HCV.

AUTHOR CONTRIBUTIONS

RB and LC wrote the manuscript. AM and PM revised the manuscript.

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