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Towards a small animal model for hepatitis C

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Hepatitis C virus (HCV) causes chronic liver disease and affects an estimated 3% of the world's population. Options for the prevention or therapy of HCV infection are limited; there is no vaccine and the nonspecific, interferon-based treatments now in use are frequently ineffective and have significant side effects. A small-animal model for HCV infection would significantly expedite antiviral compound development and preclinical testing, as well as open new avenues to decipher the mechanisms that underlie viral pathogenesis. The natural species tropism of HCV is, however, limited to humans and chimpanzees. Here, we discuss the prospects of developing a mouse model for HCV infection, taking into consideration recent results on HCV entry and replication, and new prospects in xenotransplantation biology. We highlight three independent, but possibly complementary, approaches towards overcoming current species barriers and generating a small-animal model for HCV pathogenesis.

Keywords: hepatitis C virus; humanized mice; virus adaptation; entry; pathogenesis
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See Glossary for abbreviations used in this article.

The need for a small animal model

Hepatitis C virus (HCV) is a hepatotropic pathogen of significant importance to public health. An estimated 130 million people worldwide are chronically infected and at risk of progression to cirrhosis, hepatocellular carcinoma and end-stage liver disease. These sequelae make HCV infection the most common cause of liver transplantation (Brown, 2005). There is no HCV vaccine available, and the current treatment—which is a combination of PEGylated interferon (IFN)-α and ribavirin—is often ineffective and poorly tolerated (Zeuzem *et al*, 2009).

The development of additional preventive and therapeutic alternatives has been severely hampered by the lack of suitable animal models, a deficit resulting from the limited species tropism of HCV. Chimpanzees are the only available immunocompetent *in vivo* experimental system, but their use is limited by ethical concerns, restricted availability and prohibitively high costs (Bukh, 2004). An amenable small-animal model with exogenously introduced HCV susceptibility traits could significantly accelerate the preclinical testing of vaccine and drug candidates, as well as facilitate *in vivo* studies of HCV pathogenesis. Two alternative and not necessarily mutually exclusive approaches can be proposed to achieve this: the virus could be adapted to infect non-human cells, or rodent tissues could be humanized (Fig 1). The latter might be achieved either by xenotransplantation of human tissues, or by genetic manipulation to express or ablate key genes. Here, we discuss the progress and prospects towards developing small-animal models for HCV pathogenesis, with particular emphasis on the creation of an inbred mouse model.

HCV life cycle

HCV is an enveloped virus with a positive-strand RNA genome. It was first discovered as the causative agent of non-A, non-B hepatitis in 1989 (Choo *et al*, 1989), and has since become amenable to cell culture studies of RNA replication—using subgenomic replicons—and entry—with HCV pseudoparticles (HCVpp). Most recently, it has become possible to study the entire viral life cycle in an HCV cell culture system (HCVcc; Bartenschlager & Sparacio, 2007; Tellinghuisen *et al*, 2007). HCV uses several host proteins to enter its target cell, the human hepatocyte (Fig 2); the minimal set of cell-specific uptake factors includes CD81 (Pileri *et al*, 1998), SCARB1 (Scarselli *et al*, 2002) and the tight junction molecules CLDN1 (Evans *et al*, 2007) and OCLN (Liu *et al*, 2009; Ploss *et al*, 2009). The internalization of the virion through receptor-mediated endocytosis is followed by the initiation of genome translation through an internal ribosome entry site. The virus encodes one long open reading frame, which generates a polyprotein that is processed into ten individual gene products by host-encoded and virally-encoded proteases (Moradpour *et al*, 2007).

In addition, the HCV genome functions as the template for RNA-dependent RNA replication, which is a highly error-prone process. The final steps of the HCV life cycle are the least understood, as these have only recently become amenable to systematic study. The structural components of the virus—which are the core protein (C) and the envelope glycoproteins (E1 and E2)—and a growing number of non-structural proteins, including p7, NS2, NS3 and NS5A, have been implicated in the production of infectious virions (Murray *et al*, 2008). Infectious particles are thought to form by budding into the lumen of the endoplasmic reticulum, followed by egress through the cellular secretory pathway. Many host factors

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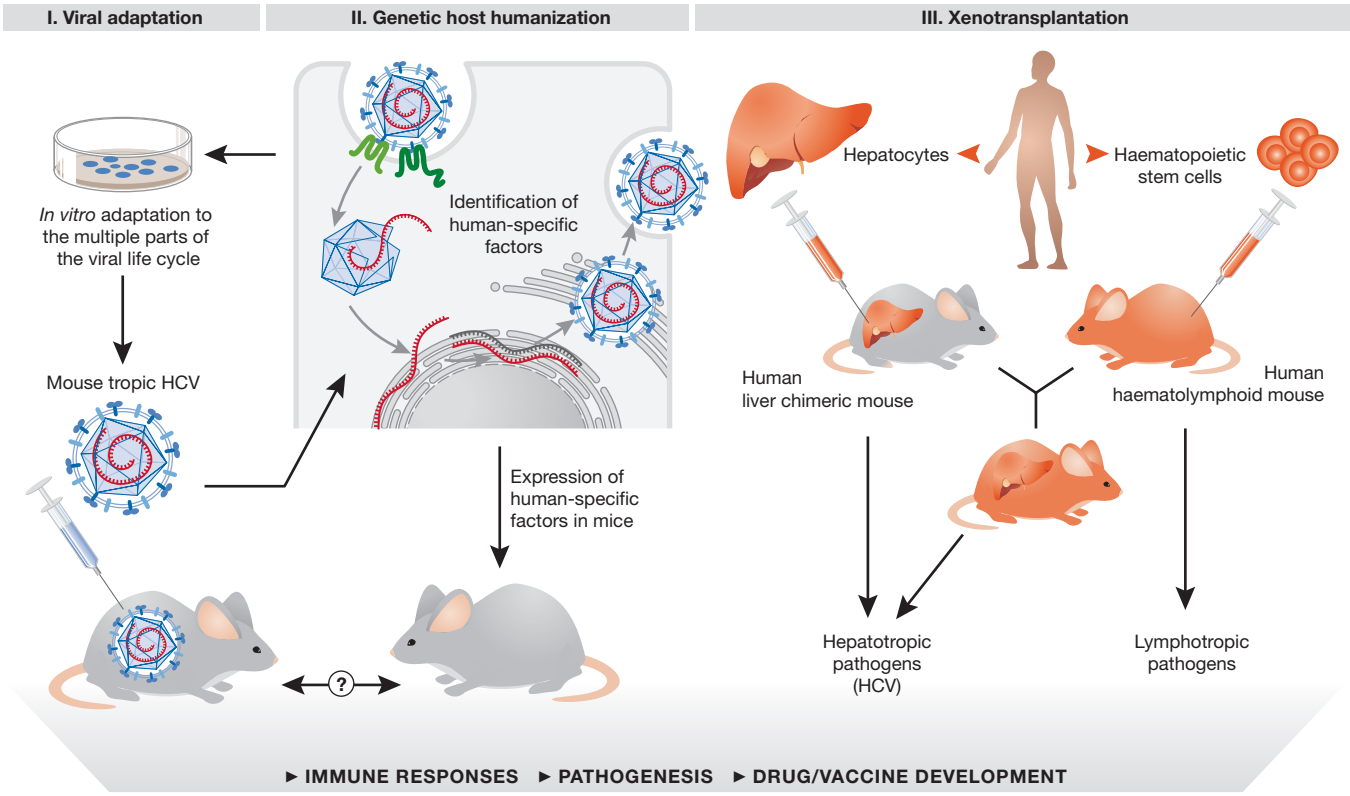

Fig 1 | Strategies to create mouse models for HCV. Strategy I, viral adaptation; Strategy II, genetic host humanization; Strategy III, humanization by xenotransplantation. Refer to text for further details. HCV, hepatitis C virus.

Glossary

CD	cluster of differentiation
CLDN1	claudin 1
HBV	hepatitis B virus
FAH	fumaryl acetoacetate hydrolase
miRNA	microRNA
OCLN	occludin
PKR	protein kinase R
SCARB1	scavenger receptor class B member 1
Sip-L	submergence induced protein-like factor

might be required for these processes, and proteins involved in lipid metabolism are emerging as crucial players (Kapadia & Chisari, 2005; Miyanari *et al*, 2007; Ye, 2007).

Viral adaptation: generation of murine-tropic HCV

The inoculation of sera from HCV-infected individuals or of tissue-culture-derived virus into rodents—even into highly immunodeficient mice—does not result in detectable infection (A.P. and C.M.R., unpublished data). This resistance phenotype is probably multifactorial, but is at least partly attributable to a block in HCV entry. Although mouse SCARB1 and CLDN1 can mediate efficient HCVpp uptake, CD81 and OCLN must be of human origin to render mouse cells permissive to HCV infection (Ploss *et al*, 2009). The expression of mouse CD81 in human hepatoma cells (HepG2)—which lack endogenous expression of human CD81—can support HCV entry. However, the levels of infection are substantially lower (<15%) than in cells expressing primate and human CD81 orthologues, probably due to a reduced affinity between the HCV glycoproteins and mouse CD81 (Flint *et al*, 2006). Although mouse CD81 is more than 90% identical to the human protein, four positions that are crucial for the interaction with HCV E2—Ile 182, Asn 184, Phe 186 and Asp 196 (Higginbottom *et al*, 2000)—are not conserved in the murine sequence. HCV can, however, be adapted *in vitro* to use mouse CD81 (Bitzegeio & Pietschmann, 15th International Symposium on Hepatitis C Virus and Related Viruses, 2008, Abstract 24). The HCV glycoproteins have remarkable plasticity, as shown by the continuous escape of the virus from neutralizing antibodies over the course of chronic infection (von Hahn *et al*, 2007). This flexibility allowed the selection of three mutations in E1 and E2 after the serial passage of HCV on human cells that express only mouse CD81. Together, these changes enhanced mouse CD81-dependent uptake to levels comparable with infection using the human orthologue. A similar approach could be envisioned for the adaptation of HCV to entry through mouse OCLN, although it might not be so straightforward. Unlike the well-documented binding of E2 to CD81 (Pileri *et al*, 1998), it is not clear whether HCV interacts physically with OCLN. In addition, the adaptive mutations required for the use of mouse CD81 might not be compatible with the changes needed

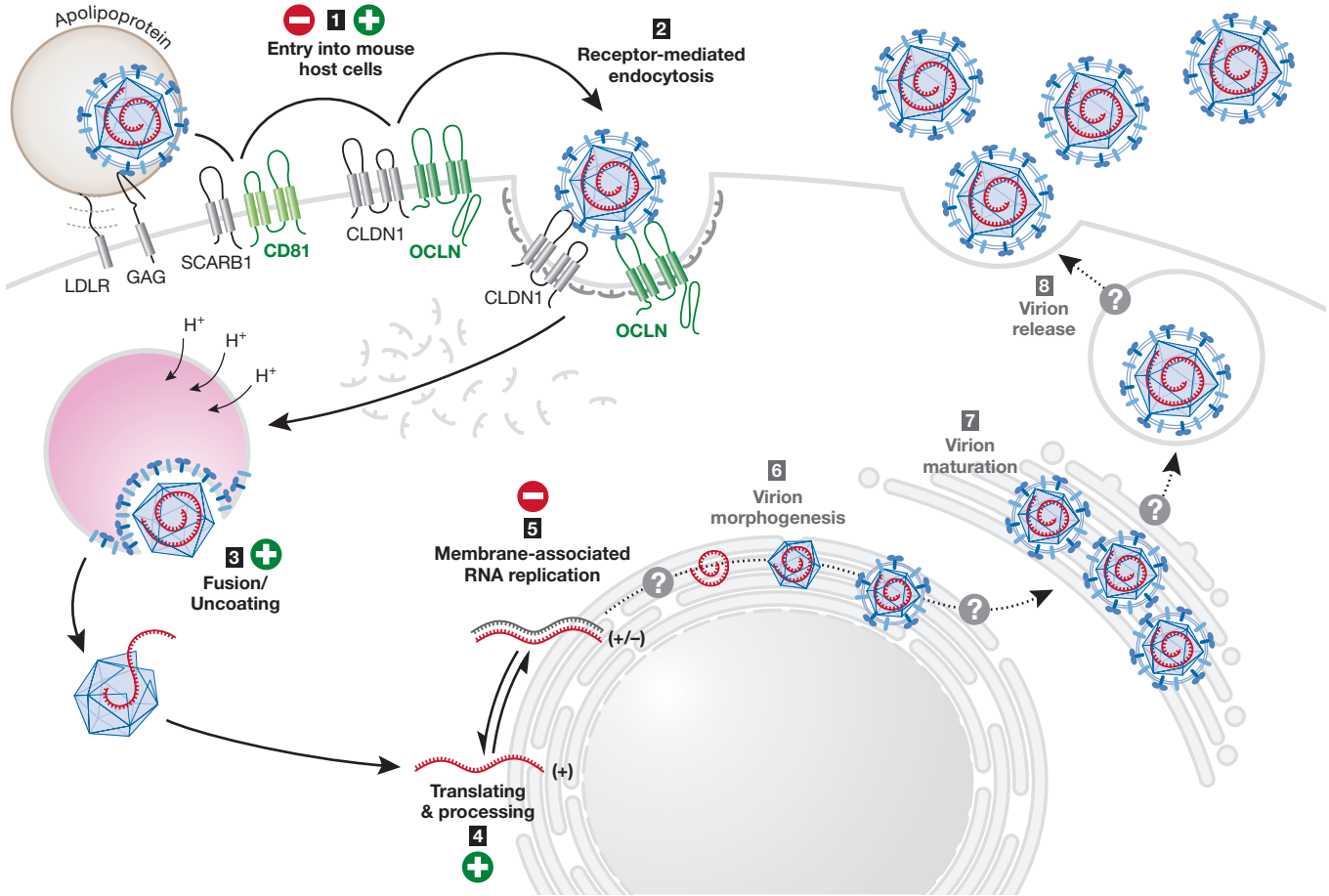

Fig 2 | Blocks in HCV species tropism. Mouse cells inefficiently support the HCV life cycle. The expression of human CD81 and OCLN can overcome the block in entry, and HCV RNA translation is supported, whereas HCV RNA replication can only occur under selective pressure. Whether the late stages of the HCV life cycle (virion assembly and release) can take place in mouse cells is unclear. CD, cluster of differentiation; CLDN1, claudin 1; GAG, glycosaminoglycan; HCV, hepatitis C virus; LDLR, low density lipoprotein receptor; OCLN, occludin.

to allow the engagement of mouse OCLN. It is uncertain whether murine-tropic HCV would infect mouse hepatocytes as efficiently as human cells expressing the mouse entry factors on which it was selected, as mouse cells might lack necessary intracellular factors. However, 'murinizing' the HCV glycoproteins could, in principle, produce a virus that efficiently enters mouse cells, thereby overcoming the first block to the viral life cycle (Fig 1).

The hydrodynamic transfection of HCV genomic RNA into the cytoplasm of mouse hepatocytes fails to initiate viral replication, indicating that an additional, post-entry restriction might exist (McCaffrey *et al*, 2002). Real-time imaging of firefly luciferase expression in living mice has shown that the HCV internal ribosome entry site is functional in mouse liver cells, indicating that this restriction occurs after initial translation (McCaffrey *et al*, 2002). HCV genomes that express antibiotic-selectable markers such as neomycin phosphotransferase can replicate in mouse hepatic cell lines (Uprichard *et al*, 2006; Zhu *et al*, 2003). The selection of similar HCV antibiotic-resistant replicons in human cell lines results in the appearance of adaptive mutations, which often significantly increase replication efficiency when re-engineered into the parental genome (Bartenschlager & Sparacio, 2007; Moradpour *et al*, 2007). Although mutations also occurred during replication of antibiotic-resistant HCV replicons in mouse cells, they seemed to be random rather than adaptive (Uprichard *et al*, 2006), as none significantly enhanced replication efficiency in the mouse cell environment when reintroduced into the parental subgenome (Zhu *et al*, 2003). This suggests that, although all essential cellular factors that support HCV replication are probably present in mouse cells, the virally encoded replication machinery might not mesh efficiently with the murine counterparts.

Drug selection is a proven strategy to establish HCV RNA replication in cells that are naturally less permissive, such as non-hepatic human and mouse cells; however, this approach cannot be translated easily to living animals due to broad toxicity. A possible solution could be the expression of a cDNA transgene that encodes the entire HCV genome, as is the case in HBV transgenic mice, which replicate and secrete HBV and have been crucial for the study of HBV immunobiology and pathogenesis (Chisari *et al*, 1985). The production of infectious HCV from stably HCV cDNA-transfected HepG2 has been reported (Cai *et al*, 2005).

In this case, the generation of proper 5′ and 3′ ends of the HCV RNA genome is crucial to ensure efficient replication initiation. Although this approach is appealing, simply producing a greater abundance of replication-competent HCV RNA might not be sufficient to initiate and sustain replication in a mouse liver. To create a system for *in vivo* selection, an HCV genome expressing a heterologous selectable marker such as FAH could be used to complement a hepatotoxic FAH deficiency in engineered mice (Grompe *et al*, 1993), thereby taking advantage of the selective pressure that would be applied *in vivo* for HCV replication in $FAH^{-/-}$ mouse hepatocytes.

As highlighted above, previous studies have shown that HCV entry and replication can occur in an appropriate murine environment, but whether HCV virions can be assembled and released from mouse cells remains unknown. Mouse hepatoma cells harbouring a full-length HCV genome were found not to release infectious virus (Uprichard *et al*, 2006). However, this lack of virion production might be due to low levels of RNA accumulation, and it is possible that increasing viral replication will also allow the detection of infectious virus. If not, additional adaptation steps might be necessary to select for genomes with assembly competence in a mouse cell environment. Ideally, viral adaptation would culminate in a genome that recapitulates most or all of the viral life cycle in mouse cells, and would represent a significant step towards a readily available small-animal model for HCV (Fig 2). It should be considered, however, that any modifications affecting species tropism might also have significant effects on immune responses and pathogenesis, and that it is not clear which of the clinical features observed in humans would be recapitulated in such a model. Nonetheless, an easily accessible, cost-effective model would be useful for investigating at least some of the aspects of viral growth *in vivo*.

Host adaptation: xenotransplantation models

Adapting the murine environment to support the replication of wild-type HCV is an alternative approach that has already met with success. Chimeric mice that harbour HCV-permissive tissue can be obtained by transplanting human hepatocytes into mouse recipients with liver injury and severe immunodeficiency (Fig 1; Meuleman & Leroux-Roels, 2008). The most commonly used recipient strain is a urokinase-type plasminogen activator (uPA) transgenic mouse, in which transgene expression in the liver is driven by an albumin (Alb) promoter. The Alb-uPA transgene overexpression is hepatotoxic and results in homozygous mice with severe liver damage (Heckel *et al*, 1990), which can be rescued by transplanting non-transgenic (human) hepatocytes. These chimeric-liver mice are susceptible to human hepatotropic pathogens, including HBV and HCV, as well as the malaria parasite (Dandri *et al*, 2001; Mercer *et al*, 2001; Morosan *et al*, 2006). The inoculation of HCVcc or sera from HCV-positive patients into these mice leads to a rapid increase in viraemia, which is sustained over several weeks (Lindenbach *et al*, 2006; Mercer *et al*, 2001; Meuleman *et al*, 2005).

Xenotransplanted humanized mice hold great potential as a means of assessing drug and vaccine efficacy (Meuleman & Leroux-Roels, 2008). For example, they were used to show that *in vivo* HCV infection can be prevented with antibodies directed against CD81 (Meuleman *et al*, 2008), as well as with polyclonal antibodies obtained from a chronically infected patient (Vanwolleghem *et al*, 2008). Such chimeric mice have also been used for the study of pharmacokinetics and drug toxicity (Katoh *et al*, 2008) and, in fact, accurately reproduce the toxicological features of the HCV protease inhibitor BILN 2061 (Vanwolleghem *et al*, 2007), a compound that was withdrawn from clinical development because of the occurrence of cardiotoxic side effects in primates.

Unfortunately, chimeric-liver mice can be produced only in small numbers, and their use is limited by logistical constraints and substantial variability. It might be possible to overcome some of these issues by using alternative liver injury models, such as animals with a targeted FAH disruption, which have already been successfully engrafted with human hepatocytes (Azuma *et al*, 2007; Bissig *et al*, 2007) but have not yet been shown to be susceptible to HCV or HBV. Pathogenesis and immunity studies are also limited in liver chimeric mice, as the animals lack a functional immune system—which is required to avoid the rejection of the xenograft. To overcome this problem, substantial efforts are under way to combine humanized liver models with mice harbouring a human haematolymphoid system (Fig 2; Legrand *et al*, 2009). These mice are generated by engrafting suspensions of human haematopoietic progenitor stem cells into immunodeficient animals; after successful human immune reconstitution, these animals can elicit virus-specific immune responses (Legrand *et al*, 2009; Strowig *et al*, 2009). Merging hepatic and haematolymphoid reconstitution in a single recipient will allow studies of pathogenesis, immune correlates and mechanisms of persistence of HCV and other human hepatotropic pathogens (Fig 2). These xenograft models provide a unique opportunity to study HCV biology in human hepatocytes and to monitor human immune responses *in vivo*. However, the generation of these types of humanized mice requires substantial infrastructure and advanced technical skills. The scarcity of adequate human primary material remains a significant logistical challenge. Therefore, it will be crucial to improve the distribution of available material and to develop reproducible and robust protocols for the *in vitro* expansion or differentiation of hepatocytes and haematopoietic progenitor stem cells from renewable sources, such as embryonic or induced pluripotent stem cells.

Host adaptation: a genetic approach

An inbred mouse model with inheritable susceptibility to HCV would overcome the technical difficulties of the xenotransplantation model. The challenge is to systematically identify and overcome any restrictions to HCV growth in mouse cells. At the level of pathogen entry, there are precedents for the successful genetic humanization of receptors and/or co-receptors in mouse cells for other human pathogens, such as poliovirus (CD155; Racaniello & Ren, 1994), measles virus (CD46/CD150; Sellin & Horvat, 2009), human coronavirus (CD13; Lassnig *et al*, 2005), human immunodeficiency virus (CD4/CCR5/CXCR4; Klotman & Notkins, 1996) and *Listeria monocytogenes* (Lecuit *et al*, 2001). After the discovery of CD81 as an essential HCV entry factor (Pileri *et al*, 1998), transgenic mice expressing the human protein in a wide variety of tissues were produced (Masciopinto *et al*, 2002). However, although the binding of recombinant E2 to liver, thymocytes and splenic lymphocytes was increased in comparison to non-transgenic controls, human CD81-transgenic mice were resistant to HCV infection. This led to the conclusion that the expression of human CD81 alone is insufficient to confer susceptibility to HCV infection in mice. Enthusiasm for creating an inbred mouse model for HCV has recently been rekindled by the identification of OCLN as the second human factor that is essential to overcome the cross-species barrier at the level of entry (Ploss *et al*, 2009). However, to accurately

Table 1 | Cellular factors with potential roles in hepatitis C virus replication

Gene	Protein	Approximate reduction of HCV RNA replication (%)	Sequence identity (%)	Reference
HAMP	Hepcidin antimicrobial peptide	80	58.3	Tai *et al*, 2009
LTB	Lymphotoxin-β	80	60.0	Ng *et al*, 2007
TBXA2R	Thromboxane A2 receptor	80	75.1	Ng *et al*, 2007
VAPA	Vesicle-associated membrane protein-associated protein A	n/a	82.3	Gao *et al*, 2004
NUAK2	NUAK family, SNF1-like kinase, 2	61	85.7	Ng *et al*, 2007
TRAF2	TNF receptor-associated factor 2	65	86.8	Ng *et al*, 2007
VRK1	Vaccinia-related kinase 1	50	87.1	Supekova *et al*, 2008
MAP2K7	Mitogen-activated protein kinase kinase 7	80	88.0	Ng *et al*, 2007
RelA	V-rel reticuloendotheliosis viral oncogene homologue A	80	88.6	Ng *et al*, 2007
VAPB	Vesicle-associated membrane protein-associated protein B and C	n/a	90.1	Hamamoto *et al*, 2005; Kukihara *et al*, 2009
NFKB2	Nuclear factor of κ light polypeptide gene enhancer in B cells 2 (p49/p100)	80	91.8	Ng *et al*, 2007
DICER1	Endoribonuclease Dicer	>85	92.0	Randall *et al*, 2007
JAK1	Janus kinase 1	50	94.7	Supekova *et al*, 2008
SLC12A5	Solute carrier family 12 (potassium/chloride transporters), member 5	>90	95.3	Ng *et al*, 2007
FBXL2	F-box and leucine-rich repeat protein 2	65	95.7	Wang *et al*, 2005
SLC12A4	Solute carrier family 12 (potassium/chloride transporters), member 4	>90	96.2	Ng *et al*, 2007
PI4KA	Phosphatidylinositol 4-kinase, catalytic, α	>80	97.7	Vaillancourt *et al*, 2009; Tai *et al*, 2009
DDX3X	DEAD box protein 3, X-chromosomal	>95	98.0	Randall *et al*, 2007
PPIA	Peptidylprolyl isomerase A; cyclophilin A	>99	98.2	Kaul *et al*, 2009; Yang *et al*, 2008
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3; eukaryotic translation initiation factor 2 subunit γ (eIF-2-γ)	>95	99.0	Randall *et al*, 2007
CSK	c-src tyrosine kinase	60	99.1	Supekova *et al*, 2008
CDC42	Cell division cycle 42 (GTP-binding protein, 25 kDa)	>60	100.0	Berger *et al*, 2009
COPZ1	Coatomer protein complex, subunit ζ 1	*ca.* 90	100.0	Tai *et al*, 2009

These studies have provided quantitative information about the impact of loss-of-function experiments on HCV replication. The reduction of replication was generally achieved by silencing the cellular transcripts with short interfering RNA. The factors included here are those that the authors of each study have highlighted as their top hits. Genes are placed in inverse order of similarity between the human and mouse orthologues. HCV, hepatitis C virus.

reproduce the complex process of HCV entry *in vivo*, it will be important to achieve native expression patterns of the human HCV entry factor orthologues. Advances in mouse genetics, including bacterial artificial chromosome transgenics and knock-in approaches, will undoubtedly be crucial in achieving this. Such a model would allow HCV-glycoprotein-mediated entry in an inbred mouse strain, and would be an invaluable tool for analysing HCV entry *in vivo* and for preclinical testing of new intervention strategies (Fig 2).

Although the minimal human factors that are crucial for viral uptake have been defined, the essential host factors required for replication are less clear. Human Sip-L has been reported to increase HCV replication in otherwise non-permissive cell lines, such as HepG2 and HEK293T (Yeh *et al*, 2001), and was subsequently shown to slightly enhance replication in a mouse hepatoma cell line (Hepa1.6) that expresses human CD81 (Yeh *et al*, 2008). However, the list of cellular components that are implicated in HCV replication is still growing (Table 1; Ng *et al*, 2007; Randall *et al*, 2007; Supekova *et al*, 2008; Tai *et al*, 2009; Vaillancourt *et al*, 2009; Wang *et al*, 2005; Watashi *et al*, 2005). Unfortunately, independent studies often have minimal overlap and the relevance of many of these interactions to HCV biology remains to be demonstrated. Although the amino-acid sequences of most of these proteins are conserved between mice and humans, it is possible that divergence of crucial functional

domains will reduce the capacity of certain murine factors to support HCV replication. A comprehensive gain-of-function analysis of individual human genes in mouse cells has not yet been completed. Alternatively, a functional cDNA complementation screen could be a less biased approach to identify specific human factors that are required for efficient HCV RNA replication in mouse cells. However, such a screen would be particularly challenging if more than one gene is required to establish efficient replication.

Cellular miRNAs can also profoundly influence virus replication and pathogenesis (Gottwein & Cullen, 2008). Two liver-specific miRNAs—miR122 and miR199a*—have been shown to affect HCV RNA translation and replication (Jopling *et al*, 2005, 2008; Murakami *et al*, 2009). Mouse miR122 and miR199* are identical in sequence and show similar expression levels to their human counterparts (Chang *et al*, 2004); however, slight sequence divergence or a differential abundance of other miRNAs with seed sites in the HCV genome might affect tropism (Pedersen *et al*, 2007).

In addition to missing or incompatible positive regulators of HCV replication, dominant-negative restriction factors might be present in mouse cells. Altered or exacerbated innate antiviral responses, the inability of HCV proteins to overcome murine defences, or mouse-specific restriction factors similar to those that control retroviral infection—such as Fv1, TRIM5α or APOBEC3 cytidine deaminases—could impair HCV replication in mouse cells. Variations in the type or intensity of the antiviral response between hosts is known to restrict the tropism of certain viruses, such as myxoma virus, which is only permissive in mouse cells that have impaired IFN responses (Werden *et al*, 2008). The induction of type I IFNs is also extremely important for cellular defence against HCV, and is counteracted by the cleavage of several host proteins by the viral serine protease (Keller *et al*, 2007). In agreement with this, subgenomic HCV RNA was shown to replicate more efficiently in PKR-deficient mouse embryonic fibroblasts than in wild-type cells (Chang *et al*, 2006).

In summary, due to the limited capacity of mouse cells to support the HCV life cycle, several genetic adaptations to humanize the murine host are required to create an inbred mouse model for HCV infection. In addition, combining these humanizations with mouse strains that harbour a targeted downregulation of key molecules involved in antiviral signalling could create an environment that is more suitable to initiate and sustain viral replication (Fig 2). Although genetic disruption of the molecules that are critical for mediating antiviral responses probably alters immunity and pathogenesis, an inbred mouse model of sustained HCV infection could be a platform for further improvements designed to mimic more closely the clinical features of human hepatitis.

Conclusions

Hepatitis C is a complex medical problem that is aggravated by the insufficient efficacy and global accessibility of current standard-of-care medications and by the lack of a vaccine to achieve widespread protection. A robust animal model that can be easily propagated and that recapitulates all or part of the viral life cycle would not only be instrumental in improving our understanding of HCV pathogenesis, but would also help to guide drug and vaccine development (Sidebar A). Furthermore, the ability to model liver disease progression is crucial for devising new intervention strategies that are able to slow down or even reverse liver cirrhosis and fibrosis. It is probable that deciphering the barriers to HCV replication in mouse cells will provide rich insight into virus–host interactions and create a blueprint for a robust mouse model of HCV infection.

Sidebar A | In need of answers

(i) What is the composition of an infectious virion (structure, viral and host proteins)?
(ii) Does HCV have regulated gene expression? If so, what steps in the HCV life cycle are subject to regulation?
(iii) Is there a particular spatiotemporal engagement of cellular HCV entry factors?
(iv) Which cellular factors participate in HCV RNA replication and virus assembly?
(v) What determines HCV tissue and species tropism?
(vi) What host and viral mechanisms are involved in viral clearance and maintaining viral persistence? What are the mechanisms of disease progression?
(vii) Can immune responses be primed or stimulated in naive or chronically infected individuals to eliminate the virus? If so, can this information be used to create a therapeutic or preventive vaccine? How would an HCV vaccine be deployed and who would receive it?
(viii) Is the liver the only natural reservoir for HCV replication? How does HCV spread in the liver? What is the phenotype of HCV-infected hepatocytes compared with uninfected bystander cells?
(ix) Can drugs targeting viral proteins and/or cellular cofactors required for replication lead to an effective treatment for all HCV genotypes?

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