



## Commentary

## Murine models of hepatitis C: What can we look forward to?



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## ABSTRACT

The study of interactions between hepatitis C virus (HCV) with its mammalian host, along with the development of more effective therapeutics and vaccines has been delayed by the lack of a suitable small animal model. HCV readily infects only humans and chimpanzees, which poses logistic, economic and ethical challenges with analyzing HCV infection in vivo. Progress has been made in understanding the determinants that dictate HCV's narrow host range providing a blueprint for constructing a mouse model with inheritable susceptibility to HCV infection. Indeed, genetically humanized mice were generated that support viral uptake, replication and production of infectious virions – albeit at low levels. These efforts are complemented with attempts to select for viral variants that are inherently more capable of replicating in non-human species. In parallel, engraftment of relevant human tissues into improved xenorecipients is being continuously refined. Incorporating advances in stem-cell-biology and tissue engineering may allow the generation of patient-specific humanized mice. Construction of such mouse “avatars” may allow analyzing functionally patient-specific differences with respect to susceptibility to infection, disease progression and responses to treatment. In this review, we discuss the three, before mentioned approaches to overcome current species barriers and generate a small animal model for HCV infection, i.e. genetic modification of mice to increase their susceptibility to the virus; genetic modification of HCV, to increase its pathogenicity for mice; and the introduction of human liver and immune cells into immunodeficient mice, to create “humanized” mice. Although in the foreseeable future there will not be a single model that perfectly mimics the natural course of HCV in humans there is reason for optimism. The spectrum of murine animal models for hepatitis C provides a broad arsenal for analyzing the disease. These models may play an important role by prioritizing vaccine candidates and possibly refining combination anti-viral drug therapies. This article forms part of a symposium in *Anti-viral Research* on “Hepatitis C: next steps toward global eradication.”

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## 1. Do we still need a small animal model for hepatitis C?

Hepatitis C remains a major medical problem affecting at least 150 million chronic carriers who are at risk of developing serious liver diseases, including fibrosis, cirrhosis and hepatocellular carcinoma. Until recently curative therapies were poorly tolerated and ineffective in the majority of patients (National Institutes of Health consensus development conference statement, 2002; Jesudian et al., 2013). However, therapeutic regimens are emerging that hold promise of reliably eradicating HCV in the majority of patients. This optimistic view is blurred by the risk of emerging resistance, unfavorable drug-drug interactions and treatment side effects. These potential complications paired with the projected high cost of future therapies will limit access to those in developed countries with adequate medical infrastructure. Additionally, even in developed countries, a significant number of patients is not diagnosed (Edlin, 2011). Thus, the impact of combinations of directly

acting anti-virals (DAAs) and host targeting anti-virals (HTAs) on a global scale, including in developing countries remains to be seen.

Although proof of concept for numerous novel therapeutic modalities against HCV (Olsen et al., 2011; Lanford et al., 2010) has been shown in chimpanzees, most emerging therapeutics were not routinely assessed for treatment efficacy in preclinical animal models. Thus, it appears that HCV animal models are not essential for the process. However, development of novel therapeutics could have possibly been sped up and complications of initially very promising drug candidates (Lamarre et al., 2003) could have potentially been predicted (Vanwolleghem et al., 2007) if a predictive (small) animal model(s) had been available. The rapidly evolving field of anti-HCV DAAs and HTAs could still benefit from predictive animal models for refinements of future combination therapies guiding the design of costly clinical trials. Beyond evaluating drug efficacy, HCV animal models could also be employed to analyze potentially interfering interactions between anti-HCV DAAs and other commonly administered drugs and may even serve to predict immune mediated, dose-independent toxicities.

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While the DAA/HTA pipeline looks promising, development of therapeutic or preventative vaccines, which may arguably have the broadest impact on reducing the global HCV burden, remains a formidable challenge. Few vaccination approaches are being tested in clinical trials (Feinstone et al., 2012). The complexity of the mammalian immune system, HCV's astounding genetic and antigenic diversity, our limited understanding of correlates of protection, and immune exhaustion in chronic HCV carriers are confounding problems for developing an effective HCV vaccine. Presumably, a vast number of permutations of antigens, adjuvants, vaccine vectors and administration regimens can only be systematically evaluated in small animal models and results will likely provide important guiding cues for prioritizing candidates for clinical development.

Beyond the practical applications a small animal model would be of great utility for basic research applications. Study of HCV has been hampered by the lack of infection systems that mimic accurately the unique host environment of the liver (Sheahan et al., 2010). Only a few human cell lines support efficiently HCV replication but their transformed nature does not reflect normal hepatocyte physiology (reviewed in Sheahan et al., 2010). Primary human hepatocytes replicate HCV only inefficiently, in part due to the difficulty of maintaining their highly differentiated phenotype in cell culture. Study of HCV in the physiological three dimensional context of an intact liver would be highly desirable. This would be facilitated by the use of an animal model (Billerbeck et al., 2013).

## 2. Desired features of a mouse model for hepatitis C?

As holds true for any experimental system for a human disease a mouse model for hepatitis C should optimally mimic closely as many, if not all relevant clinical features observed in patients. A mouse model for HCV should be readily susceptible to all HCV genotypes resulting in persistent viremia in the majority of exposed animals. In order to dissect mechanistically correlates of protective immunity, persistence and immune-mediated pathogenesis the model should be fully immune-competent and amenable to genetic manipulations. Since it can often take decades in humans between the acute phase of infection and development of severe liver pathologies it is impractical, and perhaps even unrealistic, to aim to wait for the natural disease progression in laboratory mice whose life-span is limited to less than three years. To overcome this challenge mouse strains may be used that are more prone to liver disease development possibly accelerating and exacerbating development of clinically relevant symptoms. From a practical perspective an animal model for hepatitis C should be highly reproducible, easy to propagate, high in throughput and cheap in production.

The chimpanzee model fulfills many of these requirements. Research using chimpanzees has been instrumental in the discovery and analysis of HCV infection and it remains the gold standard for all other animal models. However, their very high costs and scarcity have limited cohort sizes in experimental studies, which – in combination with the inter-individual variability of an outbred species – limits the reproducibility and thus their practical utility. Furthermore, use of chimpanzee in biomedical research is banned in many countries and growing ethical concerns in the US have led to an NIH memorandum that severely restricts all federally-funded (HCV) research involving chimpanzees (NIH 2011, posting date: <http://grants.nih.gov/grants/guide/notice-files/NOT-OD-12-025.html>). This creates a pressing need for alternative models to bridge the gap between currently available *in vitro* models for HCV infection and the complex situation in an (chronically) infected patient.

## 3. Approaches to generate a small animal model for HCV infection

Several independent but possibly complementary approaches have been taken to overcome current species barriers and to generate a small animal model for HCV infection, immunity and pathogenesis (reviewed in Billerbeck et al., 2013):

1. Expression of HCV proteins in transgenic mice.
2. Use of potential surrogates for HCV, i.e. viruses genetically related to HCV, which replicate more readily in non-human species. While original efforts focused on GB virus B transmission to new world monkeys (Simons et al., 1995; Lanford et al., 2003; Karayianis et al., 1989; Schaluder et al., 1995; Bukh et al., 2001), more recently viruses even more closely related to HCV have been found in a number of other species, including dogs (Kapoor et al., 2011), horses (Burbelo et al., 2012) and rodents (Kapoor et al., 2013). However, it remains to be analyzed how similar the life-cycles of these viruses are to HCV's and whether they would cause hepatitis in their respective hosts.

3. Humanization of the mouse liver and immune system by transplanting human hematopoietic stem cells and hepatocytes into the same murine recipient, thus allowing studies of pathology, immune correlates, and mechanisms of pathogen persistence.

4. Systematic screens to identify and overcome species restrictions allowing for genetic host adaptation and thus creating inbred murine models for HCV. Likewise, HCV could be adapted to infect hepatocytes of non-human origin, such as mice (Bitzegeio et al., 2010) and/or smaller non-human primates (Sourisseau et al., 2013).

Our discussions in this article will primarily focus on the latter two approaches as these focus on the virus that actually causes disease in humans and are aimed at modeling clinically relevant histopathology as a consequence of the possibly unique inflammatory milieu induced during the replicative cycle of HCV.

## 4. Genetically humanized mouse models for HCV infection – immunity and pathogenesis?

Mice are usually resistant to HCV infection but progress has been made towards a better understanding of the determinants restricting HCV's replicative cycle to humans and chimpanzees. Mouse cells do not support HCV uptake, which creates a first barrier for a broader host range. HCV – complexed with host lipoproteins in lipoviro particles – engages a large number of cellular factors to enter human hepatocytes. The exact mechanism of HCV uptake has not been completely elucidated but is a multistep process (reviewed in Zeisel et al., 2013) initiated by attachment to glycosaminoglycans (Koutsoudakis et al., 2006; Barth et al., 2003), binding to low-density lipoprotein receptor (LDLR) (Molina et al., 2007; Agnello et al., 1999; Monazahian et al., 1999; Owen et al., 2009), the scavenger receptor class B type I (SCARB1; Scarselli et al., 2002) and the tetraspanin CD81 (Pileri et al., 1998) on the hepatocyte surface. HCV subsequently engages the tight junction proteins, claudin-1 (CLDN1; Evans et al., 2007) and occludin (OCLN; Liu et al., 2009; Ploss et al., 2009), ultimately resulting in uptake via receptor mediated endocytosis. Signaling through the receptor tyrosine kinases epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2; Lupberger et al., 2011) appears to lead to the formation of CD81–CLDN1 complexes required for HCV entry.

More recently the cholesterol uptake receptor Niemann Pick C1 like 1 (NPC1L1, Sainz et al., 2012) and transferrin receptor (TfR, Martin and Uprichard, 2013) have been implicated in HCV uptake, but their exact role has yet to be determined. Although there is sequence divergence between the murine and human orthologs of

most of these molecules (reviewed in Sandmann and Ploss, 2013), only a few seem to account for the block at the level of entry. Expression of CD81, SCARB1, CLDN1 and OCLN are all required, but only CD81 and OCLN constitute the minimal set of human factors needed to facilitate viral uptake in mouse or hamster cells (Ploss et al., 2009) (Fig. 1a). These *in vitro* data also hold up in mice as adenoviral delivery (Dorner et al., 2011) or transgenic expression (Dorner et al., 2013) of human CD81 and OCLN in mice (Fig. 1b and c) is sufficient to allow for HCV entry into murine hepatocytes via an HCV glycoprotein mediated uptake process. Proof of concept was established that a mouse model for HCV uptake can be employed to assess the efficacy of entry inhibitors (Dorner et al., 2011; Giang et al., 2012; Anggakusuma et al., 2013) and also vaccine candidates (Dorner et al., 2011) which are based primarily on antibody-mediated protection.

Alternatively to the genetic host adaptation approach species barriers at the level of entry can also be overcome through viral adaptation. Using an *in vitro* selection approach, mutations within the HCV envelope proteins E1 and E2 were identified which allow HCV to enter cell lines expressing only mouse CD81, SCARB1, CLDN1 and OCLN (Bitzegeio et al., 2010) (Fig. 1d). Whether this murine tropic strain of HCV is indeed capable of entering mouse primary hepatocytes *in vitro* or *in vivo* has yet to be shown.

Previously, it was demonstrated that HCV replicons can be propagated in mouse cell lines albeit at low efficiency (Uprichard et al., 2006; Zhu et al., 2003). These data and subsequent work in human-mouse heterokaryons (Frentzen et al., 2011) suggested that dominant negative inhibitors putatively interfering with HCV RNA replication do not exist. Mutations were identified during replication of antibiotic-resistant HCV replicons in mouse cells, but those seemed to be random rather than adaptive (Uprichard et al., 2006), as none substantially enhanced replication efficiency in the murine 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, their mouse orthologs might not optimally interact with virally encoded components of HCV replication machinery. Consequently, expression of human replication co-factors may increase HCV RNA replication in mouse cells. In search for a mouse cellular environment that is more conducive to HCV's replicative cycle, mouse embryonic fibroblasts (MEFs) with targeted deletions in genes critical for type I interferon (IFN) signaling were tested for their ability to replicate subgenomic and full-length HCV replicons (Nandakumar et al., 2013; Lin et al., 2010; Chang et al., 2006).

Cumulatively, these studies demonstrated that type I IFN dependent and independent pathways restrict HCV replication in mouse cells. Follow-up studies in cell lines derived from mice with defective innate immune signaling that were engineered to express human CD81 and OCLN and were supplemented with the liver specific microRNA 122 provided evidence that the entire HCV life-cycle can be recapitulated in a murine cellular environment (Vogt et al., 2013; Frentzen et al., 2014). MiR-122 forms a complex at the very 5'-end of the HCV genome, which affects HCV RNA replication and translation (reviewed in Conrad and Niepmann, 2013). The mature form of mouse miR-122 is identical to the human sequence, and is abundantly expressed in mouse and human hepatocytes *in vivo* (Chang et al., 2004). In contrast, many immortalized hepatocyte derived lines irrespective of species origin dedifferentiate during culture resulting in a loss of endogenous expression of miR-122 and conceivably other hepatocyte specific factors and thus have to be ectopically supplied (Frentzen et al., 2014 and reviewed in Sheahan et al., 2010). This work in engineered mouse cell lines extended previous studies that showed mouse cells can produce infectious HCV particles (Long et al., 2011).

These lines of investigations culminated in the construction of a genetically humanized mouse model. Mice transgenically

expressing human CD81 and OCLN are capable of sustaining all steps of the viral life-cycle when crossed to e.g. STAT1 or IFN $\alpha\beta$  receptor deficient backgrounds, which are profoundly impaired in type I and III IFN signaling (Dorner et al., 2013) (Fig. 1c). It is unclear why HCV's sophisticated arsenal of anti-viral response evasion mechanisms does not seem to suffice to establish chronicity in human entry factor transgenic mice with intact anti-viral defenses. Conceivably, more rapid induction, greater magnitude and/or simply a different quality of anti-viral immunity in mice as compared to man may control more efficient HCV infection.

This inbred model already shows utility for dissecting genetically HCV infection (Dorner et al., 2013) offering the additional advantage of studying the virus in the 3D context of the liver, which e.g. takes better into account hepatocyte heterogeneity influenced by liver zonation or circadian rhythms. Hepatocytes along the porto-central axis of the liver are heterogenous dictated by environmental factors such as oxygen and nutrients levels, which form zonal gradients around the arteries and veins. These translate at the transcriptional level to specific pericentral versus periportal transcriptomic profiles (reviewed in Torre et al., 2010). Liver-specific transcription is further governed by organ-specific circadian rhythms (reviewed in Tong and Yin, 2013). The influence of these spatio-temporal expression patterns on physiological processes such as liver metabolism is well appreciated but the impact on hepatic inflammation, specifically HCV infection is not understood. Furthermore, additional precedence was established for assessing preclinically the efficacy of anti-HCV drug candidates in genetically humanized mice (Dorner et al., 2013).

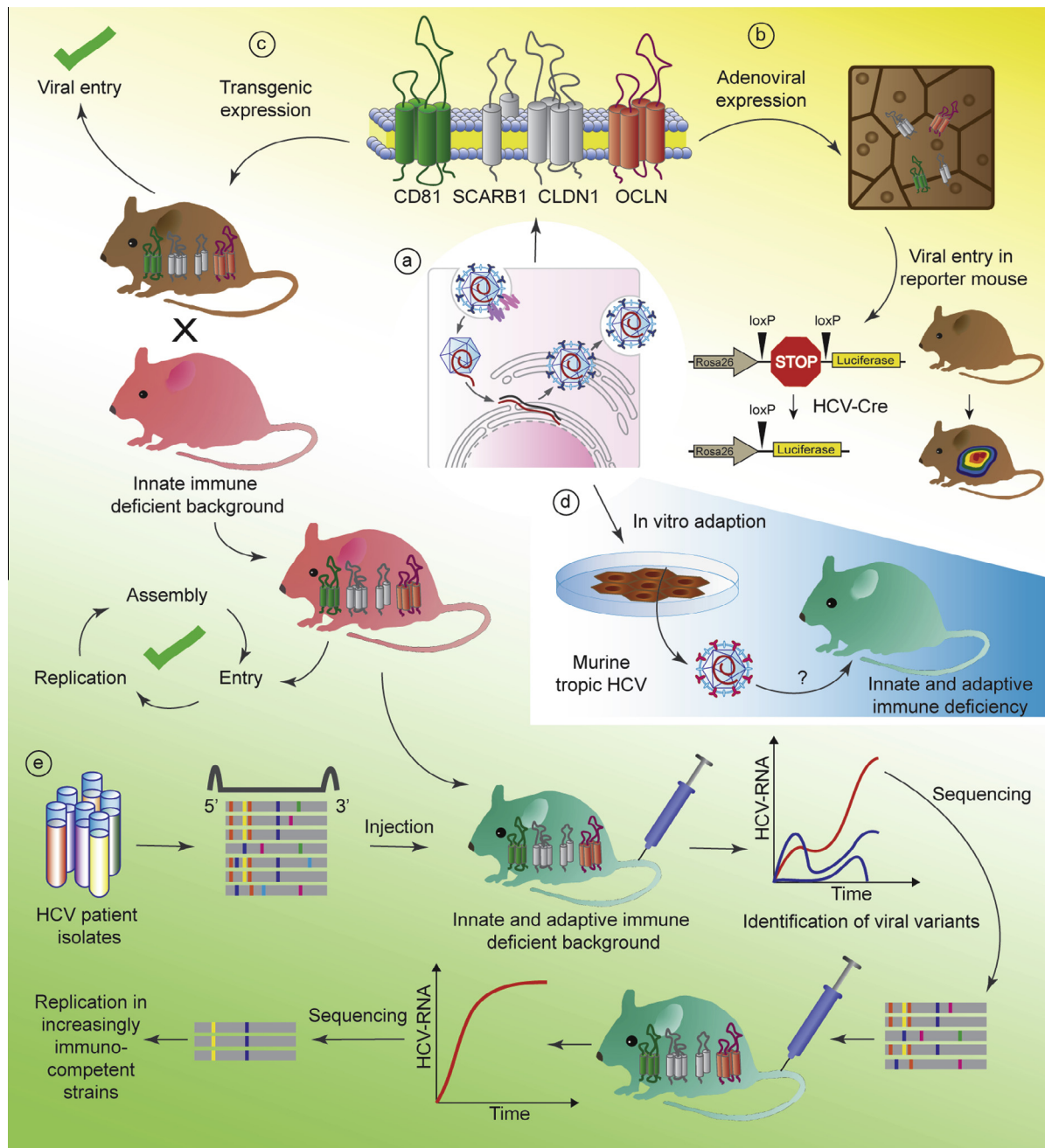
To study unperturbed host responses to HCV it will be necessary to establish persistent HCV infection in fully immunocompetent mice. Efforts are ongoing to select for viral variants that replicate more robustly in sufficiently immunocompromised rodent strains harnessing the remarkable genetic plasticity of HCV (Fig. 1e). Passage of high titer sera through progressively more immunocompetent hosts may produce this outcome. Different, genetically diverse HCV isolates and genotypes may be distinct in their ability to establish chronicity in genetically humanized mice. A fully immunocompetent, inbred mouse model would enable testing and prioritizing vaccine candidates and may present with liver pathologies reminiscent of the clinical phenotype in patients which could be exacerbated on different genetic backgrounds.

## 5. Humanized mouse avatars to dissect patient-specific (immune) responses to HCV infection

Inbred mice are the most widely used species in infectious disease research and reflect many aspects of human biology remarkably well, which is partly rooted in the considerable genetic similarity between the species. However, humans and mice, whose lineages have diverged approximately 70 million years ago, differ tremendously in size and life-span and have evolved in distinct ecological niches which translates into differences with respect to immune activation and response to challenge (Mestas and Hughes, 2004). Thus, although genetically humanized mice offer tremendous opportunities for studying HCV infection *in vivo*, caution is warranted when extrapolating data to humans. Consequently, and given the fact, that the opportunity of conducting clinical studies in patients is limited, alternative animal models need to be considered optimally allowing the study of human host (immune) responses to HCV replicating in human hepatocytes.

Engraftment of human hepatocytes into suitable xenorecipients has proven to be one solution to study HCV in its native environment (reviewed in Billerbeck et al., 2013; Meuleman and Leroux-Roels, 2008; de Jong et al., 2010). To facilitate engraftment, human hepatocytes are usually injected into immunodeficient recipients





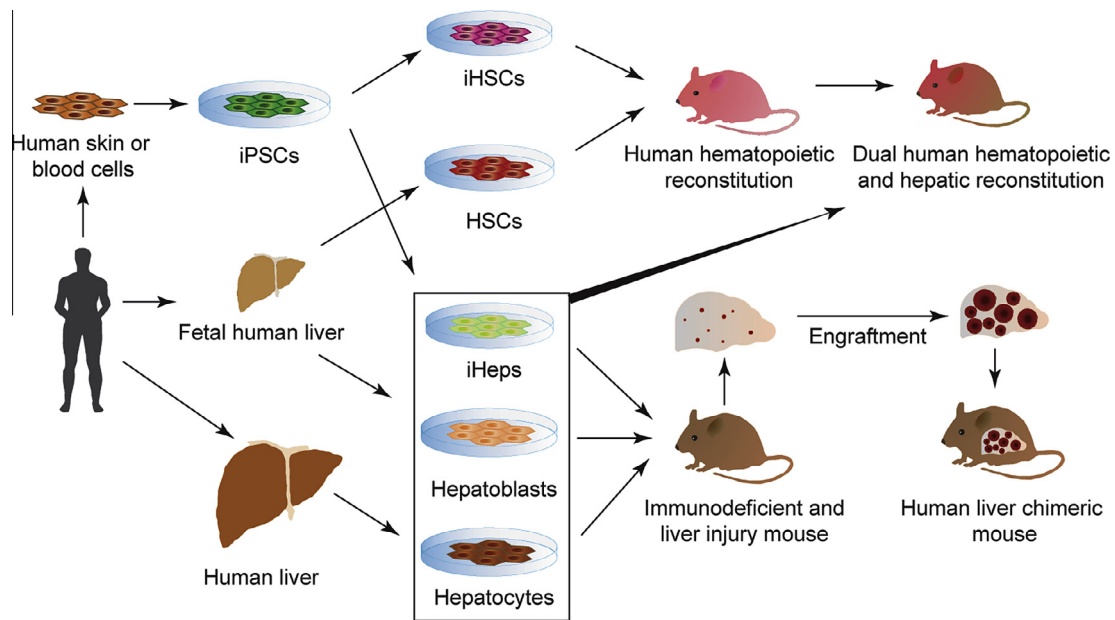
**Fig. 1.** Genetic approaches to overcome HCV species barriers. (a) Identification and expression of human specific factors and/or ablation of murine inhibitory pathways. (b) adenoviral expression of human CD81 (green) and OCLN (red) allows to visualize HCV entry using a sensitive Cre-activatable reporter. (c) Transgenic mice support HCV entry and when crossed to a mouse background blunted in innate anti-viral responses support the entire HCV life cycle. (d) Adaptation of HCV to infection of non-human cells *in vitro*. (e) Identification and characterization adaptive mutations within (patient-derived) viral populations capable of replicating in increasingly or fully immunocompetent mouse strains.

suffering from liver injury (Fig. 2). Suppression of the murine immune system is necessary to prevent graft rejection. The liver injury provides the expansion stimulus to the usually quiescent hepatocytes and gives the transplanted human liver cells a competitive growth advantage over mouse hepatocytes. Liver injury can be inflicted surgically, e.g. by partial hepatectomy, by treatment with hepatotoxins, such as retrorsine or carbon tetrachloride, or with genetic approaches. The latter are most widely used for stable engraftment as they provide a more selective control over the severity of the liver injury, and hepatotoxicity is usually limited to mouse hepatocytes.

Susceptibility to HCV upon engraftment of human hepatocytes was shown in a number of immunodeficient liver injury models,

including Alb-uPA (Meuleman et al., 2005; Mercer et al., 2001), FAH<sup>-/-</sup> (Bissig et al., 2010), AFC8 (Washburn et al., 2011) and more recently MUP-uPA (Tesfaye et al., 2013) and HSV-TK (Kosaka et al., 2013) mice. Human liver chimeric mice have proven their utility for analyzing HCV infection and testing the efficacy of various treatment modalities (reviewed in Meuleman and Leroux-Roels, 2008). The human hepatic graft offers the additional benefit of obtaining simultaneously human-like metabolic and toxicologic profiles.

Robust human hepatic chimerism can routinely be achieved with adult hepatocytes, which limits the analysis of host responses to few, often randomly selected donor lots. To enable the systematic investigation of human host genetics on HCV infection



**Fig. 2.** Current and future approaches for the generation of humanized xenotransplantation models engrafted with patient-specific cells. Depicted are sources of primary human hepatocytes and hematopoietic stem cells isolated directly from fetal or adult organs or generated via directed differentiation of human pluripotent stem cells. Human cells are used to humanized liver and immune system in the same xenorecipient. iPSC = induced pluripotent stem cell, HSC = hematopoietic stem cell, iHSC = HSC generated through directed differentiation from iPSCs, iHeps = hepatocyte-like cells derived from iPSCs.

specifically or human liver disease in general it would be desirable to utilize patient specific hepatocytes. Induced pluripotent stem cells (iPSCs) can be routinely generated from any patient starting off with easily accessible cells such as lymphocytes or skin fibroblasts in a process of cellular reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). iPSCs are also amenable to genome engineering thereby allowing the creation of basically any desired genotype and control cells on an isogenic background (Hockemeyer et al., 2011). iPSCs can be differentiated efficiently towards the hepatocyte lineage (Touboul et al., 2010; Si-Tayeb et al., 2010), yielding hepatocyte-like cells (iHeps), that not only express hepatocyte-specific markers but also have hepatocyte function and are susceptible to human hepatotropic pathogens (Schwartz et al., 2012; Wu et al., 2012; Roelandt et al., 2012) (Fig. 2). However, iHeps, similar to fetal hepatoblasts (Haridass et al., 2009), do not seem to respond to the proliferative signals in the injured liver in contrast to adult hepatocytes. Efforts are ongoing to systematically define the parameters influencing the engraftment of human iHeps with the goal of generating iHep-derived liver chimeric mice. Improvements in differentiation protocols leading to a more faithful hepatocyte phenotype (Shan et al., 2013) may need to be coupled with alternative engraftment strategies including the use of engineered tissue organoids, i.e. ex vivo produced structures mimicking liver function and architecture (Stevens et al., 2013; Chen et al., in press) implanted into improved xenorecipient strains.

A considerable shortcoming of currently available human liver chimeric mouse models is their lack of cellular and humoral immune response due to their highly immunocompromised status which is necessary to prevent graft rejection. To study HCV-specific immune responses, which counteract the infection but are also thought to contribute to the progression of liver pathogenesis, attempts have been made to co-engage mice with both human hepatocytes and components of a human immune system in a single recipient (Fig. 2). Proof of concept for this approach was established in a study demonstrating that co-injection with a mixture of human fetal hepatoblasts, non-parenchymal cells and hematopoietic stem cells yielded dually engrafted animals. Although the hepatic chimerism was very low, mice were susceptible to HCV infection, mounted antigen-specific immune responses and

developed signs of liver fibrosis (Washburn et al., 2011) additional refinements will be necessary: To increase the utility of the system, first, protocols need to be devised that reliably produce a more robust, i.e. high level dual chimerism. Furthermore, a considerable logistical challenge for such dually engrafted animals will be sourcing of donor-matched hepatocytes, non-parenchymal cells and HSCs. These populations can be isolated from fetal livers but given the usually small organ size only small cohorts of humanized cells could be generated for a single donor.

Here, progress in the generation of engraftable iHSCs from iPSCs (reviewed in Chou et al., 2013) in combination with iHeps derived from the same iPSC donor may solve this issue. Additionally, since human immune responses are generally weak in HSC-transplanted mice, further modifications will be needed to improve both the cellular complexity and functionality of the engrafted human immune system. Those include but are not limited to the expression of human MHC in the absence of mouse MHC to ensure faithful presentation of self- and virally derived peptides to human T cells and to reduced graft-versus-host-disease; co-transplantation of HSC donor-matched human thymic cortical epithelium to facilitate proper T cell selection; the expression of human orthologs of non-redundant cytokines which exhibit limited biological cross-reactivity to foster development of underrepresented human immune – in particular erythro-myeloid – cell lineages; the improvement the organization of lymphoid architecture, especially in spleen and lymph nodes, to allow for adequate T and B cell priming; genetically replacing non-compatible immune cell receptors and chemokines expressed on non-hematopoietically derived cells to improve e.g. immune cell trafficking; the introduction of a human microbiome to account for effects of species-specific commensals on the immune system (reviewed in Rongvaux et al., 2013).

## 6. A multipronged approach towards the most suitable animal model for hepatitis C

To gain a mechanistic understanding of HCV biology and host-responses in the physiological context of the liver and to address the medical need for more effective interventions, in particular

for vaccine development, animal models are needed. The issue is particularly pressing in light of the now highly restricted access to the chimpanzee model, which – is the only non-human species readily permissive to HCV infection.

To create more tractable animal models a number of distinct but likely complementary approaches are being pursued. The identification of new viruses, which are genetically related to HCV in a variety of species may shed light on the evolutionary origins of HCV but may also open opportunities to establish surrogate models. Over the last few years we have gained a better understanding about the determinants restricting HCV's host tropism which has allowed to overcome species barriers through genetic host humanization and possibly also through viral adaptation. In current models the entire HCV life-cycle can be recapitulated opening unprecedented opportunities to dissect an HCV infection in vivo. However, in order to increase the utility of the system, particularly to study HCV immunity and pathogenesis it will be necessary to establish a more robust HCV RNA replication, optimally in fully immunocompetent mice.

In parallel, humanized xenotransplantation models are continuously being refined. Human liver chimeric mice engrafted with human adult hepatocytes have a proven track record for studying HCV infection. With continuous advances in stem cell biology and improvements in directed differentiation protocols it may become possible to create humanized mouse avatars engrafted with patient specific cells. Such models would have great utility to dissect the hepatocyte-intrinsic host genetic factors influencing HCV infection. Infection with HCV can cause clinical phenotypes ranging from apparent resistance to fulminant infection. Undoubtedly, host genetics have a dramatic impact on the outcome of infection. For example, genome-wide association studies have revealed a strong association between single nucleotide polymorphisms (SNP) located within the interleukin 28B (IL28B) locus and clearance of HCV infection and response to interferon-based therapy (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Thomas et al., 2009). Subsequently, it was demonstrated that some of these variants upstream of IL28B create a new interferon gene IFNL4, which is associated with impaired HCV clearance (Prokunina-Olserson et al., 2013). However, the functional consequences of the presence of less favorable alleles have been impossible to decipher due to the lack of adequate model systems. Likewise, some individuals exhibit hyper-, hypo- or null alleles of genes implicated in the HCV life cycle (van Zelm et al., 2010; Hadj-Rabia et al., 2004; Feldmeyer et al., 2006; Vergeer et al., 2011; Innerarity et al., 1987; Mahley, 1988; Zhang et al., 2008).

Infection of humanized mice engrafted with stem cell derived hepatocytes, into which any specific gene alteration could be introduced, would allow to characterize functionally the impact of these variants on an isogenic background. Beyond hepatitis C humanized avatar mice could also be employed to dissect genetic variations affecting for example the life-cycles of other hepatotropic pathogens, non-virally associated liver inflammation (e.g. non-alcoholic fatty liver disease (Romeo et al., 2008), metabolic disorders (e.g.  $\alpha$ 1-antitrypsin deficiency, familial hypercholesterolemia, or glycogen storage diseases)) or hepatocellular carcinoma (Zhang, 2012).

Co-engraftment of a donor-matched human liver and a hematopoietic system in a single xenorecipient will provide means to study human immune response, possibly enabling analysis of HCV associated pathogenesis and to study clinically relevant co-infections with hepatitis B virus and/or human immunodeficiency virus.

This multipronged approach will yield a plethora of complementary models each with their own unique strengths and weaknesses and utility for specific applications when studying HCV vivo. To ensure relevance, the development efforts need to ensure that

new and refined models accurately reflect important hallmarks of HCV infection observed in patients.

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