Für meine Großmutter

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Recapitulation Of The Hepatitis C Virus Life Cycle In Engineered Murine Cell Lines

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2. List of Abbreviations

Apo	Apolipoprotein
cDNA	complementary DNA
CLDN1	Claudin1
DAA	Direct Acting Antiviral (drugs)
DNA	Deoxyribonucleic Acid
E	Envelop (protein)
EMCV	Encephalomyocarditis Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HCVcc	Hepatitis C Virus cell culture
HUH	Human Hepatoma Cell
IFN	Interferon
IL	Interleukin
IRES	Internal Ribosome Entry Site
IRF	Interferon-Regulating Factor
LDL	Low-Density Lipoprotein
MAVS	Mitochondrial Antiviral-Signalling Protein
MDA 5	Melanoma Differentiation Associated gene 5
miR122	micro RNA 122
NANBH	Non-A non-B Hepatitis (Hepatitis C)
NS	Non-Structural (protein)
NTR	Non-Translating Region
OCLN	Occludin
PCR	Polymerase Chain Reaction
PKR	Protein Kinase R
RdRp	RNA-dependent RNA polymerase
RIG I	Retinoic Inducible Gene-I like receptors
RNA	Ribonucleic Acid
SRBI	Scavenger Receptor B1
SVR	Sustained Viral Response
TIR	Toll Interleukin Receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β
VLDL	Very Low-Density Lipoprotein

3. Introduction

This thesis provides conceptual proof-of-concept for recapitulating the hepatitis C virus (HCV) life cycle towards the development of a small animal model for hepatitis C. The next pages will alight why hepatitis C is still a significant health problem. It will be explained what the hepatitis C virus is and the progress that has already been made. The reader will see of what immense importance model systems have been for the study of HCV. Each new model system was entailed by an abundance of new insights into this fascinating but elusive virus. Considerable progress has been made over the last years towards more tolerable and efficient therapies. However more research has to be done to provide safe treatment with low side effects to everybody infected, not only in developed countries. The development of new drugs and especially of a vaccine would benefit highly from an in vivo model. Unfortunately the chimpanzee is the only animal model currently available, which presents obvious financial and ethical problems. A mouse model could be a solution to this dilemma.

3.1. The History of HCV

Hepatitis C, then designated as non-A non-B hepatitis (NANBH), was first discovered in 1975 in a group of 22 patient, who displayed symptoms of transfusion associated hepatitis, but were negative for the at that time known agents: Hepatitis A and B Virus, as well as Epstein-Barr-Virus and Cytomegalovirus [1]. Although the association between blood transfusions and this new hepatitis was readily made [2, 3] a causative agent had not been found. In 1985 it was shown that the NANBH virus was transmissible to Chimpanzees from filtered serum of infected patients [4]. Two year later the size of the infectious particle was determined to be about 30-60nm [5]. The chimpanzee played a key role as model for the study of hepatitis C, before this cryptic disease even had its name.

First in 1989, fourteen years after the first description of the virus, the group of Choo et al. extracted a cDNA clone from infected chimpanzees and demonstrated alignment with RNA from NANBH infected humans. They called the disease hepatitis C [6]. The same research group developed an antibody test that showed that HCV is responsible for over 70% of all cases of post-transfusional non-A non-B hepatitis as well as the majority of community acquired NANBH [7].

It took another decade until the first cell culture system was developed: Self-replicating HCV genomes called replicons. In a bi-cistronic configuration the 5′ nontranslated region (NTR) drove expression of a neomycin phosphotransferase (neo) coding sequence, while a heterologous encephalomyocarditis virus (EMCV) IRES (internal ribosome entry site) drove expression of the HCV non-structural proteins NS3 through NS5B. When transfected into human Huh7 hepatoma cells and cultured in a medium containing the cytotoxic drug G418, only cells harbouring the HCV RNA and amplifying the replicon would survive [8]. This system became an important tool for the study of replication and provided the first cell-based platform to screen for drugs interfering with the HCV replication. The major obstacle of this system was its limitation to the intracellular steps of the HCV life cycle. Neither the entry nor the assembly of the viral particles could be studied [9]. The entry of HCV into cells was first studied in a system called HCVpp: Retroviral and lentiviral core particles were generated displaying unmodified and functional HCV glycoproteins on their surface. The expression of the envelope proteins allowed the study of possible HCV receptors on host cells [10]. Only in 2005 several groups finally succeeded in

propagating HCV in cell culture (HCVcc) [11, 12]. This ultimately allowed the examination of the entire HCV life cycle in a reproducible in-vitro system and also provided a platform to test putative new drugs. The next step towards better understanding of HCV would be the development of a small animal model.

3.2. Epidemiology

Chronic hepatitis C is a major health burden in Europe, the USA and worldwide with 130 million people being chronically infected [13]. HCV poses a tremendous socio-economic impact [14]. In the United States five million people have been infected with HCV that is five times as many as with HIV [15]. In Germany this number is considerably lower with 0.63% of the population (an absolute of 510000 people) [16] being chronically infected.

Infection with HCV increases mortality, especially in young people. Patient between the age of 20 and 29 have a 18.2-fold increased risk of death [17] and the age group between 25-29 is the one with the highest incidence [13]. Unlike other viruses causing hepatitis HCV is primarily transmitted through the parenteral route and sexual transmission is rare [18], although recent data suggest that the risk of sexual transmission is significantly higher in promiscuous male homosexuals [19]. Historically blood transfusions were responsible for a significant fraction of HCV infections. The introduction of antibody and RNA testing has reduced the incidence of transfusion-associated infection to 1:4,000,000 [20]. The risk of obtaining HCV following a needle stick injury is 0.4% in Europe and 1% in the rest of the world [21]. The vertical transmission rate is low too (1-6%), mildly higher for girls than for boys and furthermore increased in HIV positive mothers [22]. The accurate epidemiology of HCV is notoriously difficult to determine as only 20-30% of patients display symptoms of acute infection [23].

The young infection age in the majority of patients subsequently raises the risk for long-term complications such as cirrhosis and hepatocellular carcinoma. That might as well explain the above-mentioned increase in HCV related death.

The HCV population is not only young, but often from a marginalized background. While an average of 2% of the American population is infected with HCV, groups at

the edge of the society show a much higher prevalence: 19% of all prisoners, 35% of homeless people, 50% of injection drug users are HCV positive. The fraction of iv drug users goes even up to 90% after 10 years of addiction [15]. In Germany 34% of all HCV cases are related to intravenous drug use [13]. An increasing risk not only in Germany, but worldwide is the co-infection of HCV with HIV especially in the gay community [24]. These populations are notoriously difficult to treat and especially at risk to develop long-term complications of chronic infection, such as liver cirrhosis and hepatocellular carcinoma (HCC).

When infected with HCV only 20% of the patients clear the virus spontaneously, while the other 80% become chronically infected [25]. HCV represents the single most important risk factor for the development of HCC. In western countries the hepatitis C virus can be detected in up to 70% of patients with HCC [26]. Rare cases of HCC in non-cirrhotic livers of patients with HCV are reported, but in the majority the stages of chronic hepatitis and cirrhosis are passed before the cancer develops. This process can take an average of 28 years [27, 28]. The long incubation period gives us the chance to treat the condition in an early stage. In order to provide effective treatment options further research on the pathogenesis and the development of new drugs is essential. A small animal model for HCV could tremendously accelerate this process.

3.3. The Hepatitis C Virus

Hepatitis C is an RNA virus and belongs to the genus Hepacivirus in the Flaviviridae family. A classification into 7 genotypes has been established with a difference in their nucleotide sequence of 30-35% [29]. In the infected individual the virus exists as a quasispecies due to the frequently occurring mutations in the genome and the highly error prone RNA polymerase. This allows the virus to adapt under selection pressure from the host immune system [30] and antiviral agents [31].

The HCV particle is made up of the viral RNA, the core proteins as well as two envelope proteins E1 and E2. HCV is strongly associated with lipoproteins. They form a complex also known as lipoviroparticle [32]. The viral RNA codes for three structural proteins, core, E1, E2 and 7 non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The core protein and the RNA form the nucleocapsid, which

is surrounded by a lipidmembrane into which the E1 and E2 proteins are anchored, forming the viral envelope. The E1 and E2 glycoproteins are responsible for the interaction with the cellular entry factors [33]. The HCV particle is a mixture of a virion and liporoteins and shows a strong resemblance with low density (LDL) and very low-density lipoproteins (VLDL). These particles can be found associated with apolipoproteins such as apoE, apoB, apoC1-C3. While the association with lipoproteins seems to play a role in the entry of HCV [34], the presence of apolipoproteins in the producing cells is crucial in order to generate infectious particles [35].

The p7 protein is a small hydrophobic polypeptide essential for the production of infectious virions [36]. It belongs to a family of proteins called viroporins, known to enhance membrane permeability [37]. The second protein is NS2, composed of a highly hydrophobic N-terminal membrane anchor domain and a C-terminal cysteine protease domain [38]. In cell culture the deletion of NS2 does not hinder the replication of HCV RNA [39]. However it appears to be necessary for the completion of the viral life cycle in vivo and in vitro being involved in the viral assembly [40]. NS3 functions both as a helicase to unwind viral RNA and in conjunction with its co-factor NS4A as a protease, essential for polyprotein processing [38]. The NS3/4A protease also contributes to the immune evasion of HCV by cleaving the host signalling proteins Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF or TICAM-1) [41] and mitochondrial antiviral signalling protein (MAVS) [42].

The NS4B protein induced a tight structure, designated membranous web, consisting of vesicles in a membranous matrix. This specialized subcellular structure forms the viral replication complex [43]. NS5A protein is believed to be key factor in viral replication and assembly [38]. NS5B is the viral RNA-dependent RNA polymerase (RdRp) [44]. The enzyme is capable to synthesize viral RNA de novo, which is thought to be the mode of action in vivo [38]. The C-terminal membrane insertion sequence, which is dispensable for RdRp activity in vitro, represents an essential element that may be involved in critical intramembrane protein-protein interactions within the HCV replication complex [45].

3.4. The Viral Life Cycle

3.4.1 Entry

The entry of the hepatitis C virus, which includes the binding of the virus to the cell membrane as well as the endocytosis and the fusion with the acidic lysosome is mediated through the E1 and E2 glycoproteins on the envelop of the virus [46].

Several host proteins are involved in the entry of HCV into the hepatocyte:

Glycosaminoglycans (GAG), low density lipoprotein receptor (LDLR), scavenger receptor B1 (SRBI), CD81, Claudin1 (CLDN1) and Occludin (OCLN) [46]. Recently epidermal growth factor receptor (EGFR), ephrin type-A receptor 2 (EphA2) and Nieman-Pick C1-like1 cholesterol absorption receptor have been shown to play a role in HCV entry [47, 48].

Glycosaminoglycans (GAG) are thought to be responsible for an initial low affinity interaction between the virus and the host cell [49]. The low-density lipoprotein receptor (LDLR) supports the cellular binding using apolipoproteins on the viral envelope [50]. While GAG seems to promote an initial affinity, the SRBI plays a key role in the binding of the viral particle [51, 52]. It was speculated that SRBI primes the viral glycoprotein for the first step of the post-binding interaction with CD81 by altering the conformation of the virion [46]. Subsequently CD81 appears to alter the confirmation of E1 and E2 again facilitating the following steps of the viral uptake [53]. CD81 is one of the know factors determining the species tropism of HCV. Murine CD81 does not interact with the viral glycoproteins. It has been shown though that providing the factor to the animals [54] as well as mutating and adapting the virus allows successful entry, provided no other crucial factor is missing [55].

Furthermore two tight junction proteins are involved in the uptake: OCLN [55] and CLDN1 [56]. Tight junction membrane proteins normally control the intracellular diffusion and regulate the exchange between the apical and distal pole of the cell [57]. It is unknown in which way these factors participate in the virus cell interaction. So far it has only been shown that cells suppressed or lacking these two proteins are unable to support entry [46]. The fact that both of them are localized at the inaccessible lateral side of the cells is another unsolved mystery. It is speculated that the CD81-HCV complex is transferred to the lateral pole by intercellular actin-depend

mechanisms [46]. Study of the polarized cell and its interaction with the virus requires a more suitable tissue culture model than the ones available. Occludin is the second entry factor that is too different in rodents to allow viral uptake [55]. But occludin, as CD81, can be provided to murine cells and animals to enable this step [55]. The two missing factors provided to animals offer the possibility to study the entry of HCV in an in vivo model [54]. The distribution of the entry factors determines not only the affinity of HCV to certain species, but is as well important for the tissue tropism of the virus [46]. Although HCV RNA has been found in other cells of the human body, for example in the brain during post-mortem examinations [58], it is to be proven that the other steps of the life cycle take place in these organs. It seems to be not only the lack of entry receptors, but also the presents of hindering proteins that determines the tissue specificity. Factors have been discovered, which inhibit the entry of HCV in non-hepatic cells such as the EWI-2wint, a CD81 partner [59]. It was shown in tissue culture that HCV can spread directly from cell to cell [60], if this can occur in polarized liver cells remains to be seen.

3.4.2. Translation

The released HCV genome is a single stranded positive sense RNA with a length of about 9.6 kB [38]. The single open reading frame (ORF) of the uncapped linear molecule is flanked by 5' and 3' noncoding regions [38]. The 5' nontranslating region (NTR) with a length of approximately 340 nucleotides contains a type 3 internal ribosome entry side (IRES) as well as a replication signal required for the replication of the negative strand replication intermediate [38]. This is also the binding side for the microRNA122. The 3' UTR is of importance for RNA replication [38]. Capped mRNA molecules are translated via a cap dependent mechanism. This is not the case for naturally uncapped viral RNA molecules, which are translated by cap-independent IRES-mediated translation. This is regulated through the direct recruitment of ribosomes [61]. The translation is initiated by direct binding of the vacant 40S ribosomal subunit to the IRES [37]. Under assistance of the eukaryotic initiation factors the 60S subunit binds to the complex in order to form the 80S complex. This step is rate-limiting [62]. It was proposed that the resulting core protein binds to the IRIS and inhibits the translation and functions therefore as a negative feedback mechanism [37].

The translation of the viral RNA leads to the production of one large polyprotein of about 3000 amino acids, which is subsequently processed by viral and cellular enzymes into at least 10 viral proteins: three structural proteins, core, E1, E2 and 7 non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [38].

The core/E1, E1/E2, E2/p7 and p7/NS2 junctions are processed by host signal peptidase [37]. Cleavage between NS2 and NS3 is a rapid intramolecular reaction mediated by a protease consisting of elements of NS2 and NS3 [37]. A serine protease in the n-terminal residue of the NS3 protein cuts all the remaining proteins: NS3/NS4 intramolecularly and subsequently intermolecularly [37].

3.4.3. Replication

It is believed that the production of new copies of the viral RNA starts with the synthesis of complementary negative stranded RNA using the genomic RNA as template. The positive stranded RNA is subsequently generated using this negative stranded model. Both of these steps are exercised by the NS5B polymerase [37]. It shows RNA dependent RNA polymerase activity, although it lacks the strict template specificity and fidelity, which is necessary for viral replication. This makes it likely that other viral and host factors are involved in the formation of the replication complex, together with NS5B [37]. This process is thought to take place in the already mentioned structure called the membranous web, a membrane formation that is seen in the presence of the HCV polyprotein [43]. Electromicroscopic pictures showing similar morphological changes in the liver of HCV infected chimpanzees reinforce this association [63]. Since all non structural proteins are associated with the endoplasmatic reticulum [64] and the membranous web is frequently observed close to the endoplasmatic reticulum it is possible that the membranous web is derived from the ER [37].

Replicon studies have shown that the greatest viral RNA levels can be seen during the growth face of the cells, with the number of copies dropping as soon as the cells reach confluence. This indicates that the viral replication is linked to the cellular metabolism [39].

3.4.4. Assembly

HCV assembly is the least understood step of the viral life cycle. Both structural and non-structural proteins are involved in the assembly process of HCV [65]. It is believed that the core protein associates with cellular lipid droplets (LD) consisting of triglycerides in order to form a functional module [66]. A second functional module involves the non-structural protein NS2, p7, the two envelop proteins E1 and E2 and maybe NS3 [67]. These form dotted structures, which are juxtaposed to the lipid droplets containing the core protein. When core LD association is prevented the dotted NS2 structures still form and there number increases significantly [67]. The arrival of the core loaded LD seems to initiate the assembly process [65]. At least some of the proteins appear to travel to the assembly side together; this includes NS2, p7 and the envelope proteins [65]. The last functional module is the replication complex with the non-structural proteins. They accumulate in the proximity of the core LD at the proposed side of the viral assembly [68]. NS5A is thought to be a crucial factor in the localization of the replication complex to the side of assembly and was found to be associated with viral RNA in the vicinity of the core LD [69]. In the presence of these three modules viral budding is induced by the pushing force of the nucleocapsid [70] together with the pulling force of the envelope proteins [71] and potentially the accumulating neutral lipids between the two leaflets of the ER. The result would be a hybrid lipoviroparticle [32], which matures further by interaction with ApoE. In cells with an efficient VLDL metabolism, the lipoprotein moiety may mature into a VLDL-like structure [65].

3.5. Diagnosis and Treatment of Chronic Hepatitis C

The natural history of HCV can be divided into acute and chronic infection. Within seven and 21 days after viral transmission HCV RNA becomes detectable in the serum. Longer incubation periods can occur in cases where small amounts of virus have been transmitted [72]. Only very few patients report clinical symptoms, which can include: fatigue, nausea, abdominal pain, loss of appetite, fever, itching or myalgia [72]. About 20-40% of patients clear the infection spontaneously, while the rest progresses to chronic hepatitis C with the associated complications. Patients with chronic disease can present with similar unspecific symptoms including nausea,

fatigue, abdominal discomfort, myalgia or arthralgia. Associated diseases like vasculitis with cryoglobulins and liver cirrhosis or raised liver enzymes can point the clinician in the direction of HCV [72].

The diagnosis of hepatitis C is based on two laboratory tests: the detection of anti-HCV antibodies by enzyme immunoassay, and the detection of HCV RNA by a sensitive molecular technique - ideally a real-time PCR assay [73]. This should be preceded by a complete medical history and a thorough clinical examination. A baseline assessment of liver function by blood tests (liver enzymes, coagulation and full blood count) should be carried out and the presence or degree of cirrhosis should be assessed by ultrasounds or biopsy for every patient. It is furthermore advised to determine the genotype of the virus [73].

Unlike HIV, the goal of any treatment of chronic infection with HCV is the clearance of the virus and the cure of the patient. This is called sustained virological response (SVR) and defined as an undetectable HCV RNA level in the serum of the patient 24 weeks after completion of treatment. Until very recently the standard treatment for chronic hepatitis C was pegylated interferon α and ribavirin. Patients with genotype 1 achieved SVR in 40-54% of cases and 65-82% if infected with genotype 2 and 3 [74]. Differences in response to this treatment, which were associated with ethnic background, inspired Dongliang et al to use a genome-wide association study to search for genetic polymorphisms predicting these different responses. The genetic polymorphism of IL28B is a strong predictor of clinical outcome [75] and an investigation into the patient's IL28B status may be warranted [74].

Failure of treatment is divided in two categories: Relapsers are patients who had undetectable HCV RNA at the end of treatment but showed a relapse before the 24-week point. Non-responders are patients who fail to achieve an at least 100 fold decline in HCV RNA during treatment. This is also called virological breakthrough [73].

Almost all patients under a treatment regime with peg-interferon and ribavarin experience adverse effects. This is the most common reason for patients to decline or interrupt treatment [73]. In trials, 10-14% of patients ended up discontinuing treatment due to adverse effects [76, 77]. The most frequent side effects include flu-like symptoms: chills, rigors, headache, fatigue and fever, which occurred in half of

the patients, as well as psychiatric symptoms such as depression, irritability and insomnia, which showed up in 22-31% of patients. In addition abnormalities in the full blood count have been observed frequently, especially neutropenia and anaemia [76, 77].

Only recently, two direct acting antiviral drugs (DAA) have been approved for the treatment of chronic hepatitis c: boceprevir and telaprevir (both inhibitors of the HCV NS3/4A protease). These have to be taken in combination with ribavarin and peg-interferon to avoid the development of resistance [78]. This triple therapy can increase the SVR rate to 70% for treatment naïve patients with genotype one infections [79-82]. Unfortunately these treatment regimes can have even more side effects than ribavarin and peg-interferon alone. The risk of anaemia is 20% higher during triple therapy than with the previous standard treatment. The incidence of other side effects such as nausea, diarrhoea, pruritus and rash is at least 10% higher with telaprevir therapy and a more than 10% increase of nausea, chills and fatigue can be seen for boceprevir [79, 80]. Other new direct acting antiviral drugs are in the pipeline. SVR rates of over 90% have been shown in clinical trials and interferon-free regimes combining several DAA and ribavarin have demonstrated SVR rates of over 70% [83].

Although a significant progress could be observed over recent years, the drugs available have numerous side effects and resistance is emerging quickly. A small animal model would facilitate the research into new drugs and provide a platform to test new medications for side effects in an efficient and cost-effective way. However, one must be mindful that these therapeutic schemes are currently only accessible to people in the first world due to their high costs. A small animal model might help to accelerate the development of a vaccine, which would be an ultimate breakthrough in tackling HCV in both developing and developed countries.

3.6. Animal models

A small animal model for hepatitis C would offer unprecedented opportunities for the research into the pathogenesis of the virus and offer a platform for drug and vaccine testing. The following paragraph will show the possible positive impact of a small

animal model on the example of the polio mouse model as well as the current state of research on the hepatitis model.

A good example for the success of small animal models is the TgPVR mouse model for poliovirus. It had been know that the poliovirus enters the human cells through the poliovirus receptor CD155 [84] a protein markedly different to his rodent homolog. After the human gene of this poliovirus receptor (PVR) was transferred into now transgenic mice the virus was able to replicate in the neuronal tissue and animals displayed symptoms of polio comparable to humans [85]. But this model was still lacking the normal entry of viral particles into the body of the animals, since only an intravenous or intracranial injection would lead to the desired infection. It was demonstrated that after blunting of the immune response in the gut of these mice by further knocking out the interferon-α/β-receptor on oral infection with subsequent viral replication and clinical symptoms could be recapitulated [86]. These models have accelerated the development and the approval of new oral vaccines immensely. They have been used not only in the phase of development, but also as a tool for safety testing reducing the need for studies in monkeys [87]. The mouse model for polio provides only example of how the hurdles of transspecies transmission can be overcome and the subsequent benefits that can be obtained from a small animal model. This process also generates immense knowledge acquired through the intensive research on the virus itself.

There are three main approaches in creating a small animal model for hepatitis C virus: 1. The adaptation of HCV to non-human hosts, 2. The xenotransplantation of human tissue into animals and 3. The genetic host adaptation [88]. As already described, HCV uses a minimal set of four entry factors to penetrate the human cell: SCARB1, CD81, hOCLN and hCLD1 [46]. Two of these, CD81 and OCLN, cannot be used by HCV to enter murine cells. The idea of the first system is to adapt the virus to the rodent factors in order to allow entry. It has been shown recently that a laboratory strain of HCV could be adapted to murine CD81 using selection pressure [53]. During this process mutations in the E1 and E2 envelope proteins appeared to change not only the behaviour towards CD81, but also towards the other entry factors. It is unclear how close this process mimics the naturally occurring entry and it remains to be shown that these viruses work *in vivo* [89]. The aims of this approach include the gain in knowledge about the entry factors and the viral glycoproteins and the

prospect of finding new therapeutic targets, as well as a system needing only little modifications in the virus. It has yet to be shown, if results obtained with an engineered virus would allow deduction to a human strain. Until now, only the process of entry has been addressed leaving the question, if the virus would overcome hurdles in replication and assembly in the murine liver [88].

The second approach is a xenotransplanted model in which mouse cells replace the human liver cells. With these models up to 99% chimerism can be achieved [90]. Two requirements have to be fulfilled to allow successful engraftment: The recipient animals must be immunocompromised and a liver injury must be induced to destroy the endogenous liver cells. Two well-characterized models are used for this purpose: the urokinase plasminogen activator transgenic mouse [91] and the fumarylacetoacetate hydrolase (FAH) gene deficient mouse of which the latter one allows a more robust model and a higher throughput [92, 93]. FAH is the last step in the tyrosine breakdown. A knockout leads to hypertyrosinemia, which is lethal in humans and leads to liver failure in mice. Treatment with 2-(2-nitro-4-trifluoromethylbenzyol)-cyclohexane-1,3-dione (NTBC) prevents liver toxicity and offers the option of inducing the liver injury at any point by withdrawing the drug (NTBC). These mice have been crossed with immunocompromised background models and could subsequently be engrafted to high levels with human hepatocytes [90]. Human liver chimeric mice are susceptible to HCV and HBV and can be used for drug testing [88]. Unfortunately the generation of these animals is expensive and complicated. Furthermore they do not display any of the long term complictions of chronic HCV infection such as cirrhosis or hepatocellular carcinoma. Another disadvantage is the lack of a working immune system, which limits its utility for the study of the pathogenisis of HCV and the inflammatory response to the virus [90]. Although there are attempts to transplant parts of the human immune system into these mice, this manipulation and the necessary surgery further decreases the survival rate of the animals [89].

The third way of rendering rodents susceptible to HCV is the generation of inbred genetically modified model with inherited susceptibility to human hepatitic C virus. To achieve this one has to identify the barriers in the murine organism that hinder HCV from successfully multiplying. This could be either human specific genes whose murine orthologs are non-functional and need to be expressed in mice or obstacles

that have to be removed e.g. by targeted gene disruption [89]. At the level of entry it has been shown that human CD81 and OCLN constitute the minimal set of genes required for viral uptake *in vitro* [55] and *in vivo* [54]. The model presented by Dorner *et al* is only limited to entry of the virus, but since these animals have a fully competent immune system they can already be used for the study of vaccines and drugs inhibiting the entry process. Research in recent years has tried to dissect the remaining life cycle of HCV in order to identify the crucial steps which are blocked in mice. Translation of viral RNA into proteins is known to be successful, but only under high selection pressure HCV is able to replicate in these mouse derived cells [94]. The replication can be further increased by blunting of interferon type 1 response [95]. This suggests that an overactivation of the cellular antiviral response plays a key role in the decreased RNA replication. It is encouraging that Frentzen et al were able to show that no contrutive or virus-induced expression of dominant restriction factors prevents propagation of HCV in murine cells [96]. The later stages of the life cycle appear to be supported in rodents if all the required apolipoproteins are provided [97].

The aim of my research work is to unravel and fit all the pieces of the puzzle together and demonstrate that the entire HCV life cycle can be recapitulated in one cell. I hope that my findings will significantly contribute to the translation and construction of an *in vivo* model.

3.7. The Immune Response to Hepatitis C Infection

The infection with hepatitis C virus is not a unidirectional process. The human body and the liver cell as principle target of the HCV infection have systems and measures in place to fight, restrain and overcome the infection. The response of the immune system to HCV has been studied intensively with the hope that a better understanding of the process would help to offer new treatment options and better outcome for patients. Only 20% of the individuals exposed to the virus are capable to clear the virus while others develop a chronic infection [38]. This is likely the consequence of differences in host genetics affecting directly the susceptibility to host infection in combination with differences in the magnitude and kinetics of innate and adaptive immune response. This has already been illustrated with the example

of the IL28B polymorphism [75]. Of particular interest for our study is the hypothesis that a difference in the innate immune activation might be responsible for the low replication in mouse cells and hence contribute to the species tropism. The next paragraph will provide deeper inside into the antiviral mechanisms in mammalian cells and their relevance for our research.

Important players in the innate immune response to HCV are the different types of interferon. There are three key steps in this process: the activation of the interferon cascade in the affected cell leading to a direct immune response and production of interferon, the stimulation of neighbouring cells and cells of the immune system, and finally the response in these neighbouring cells through interferon stimulated genes (ISG) [98].

The three types of interferon can be distinguished depending on the type of receptor they use. Type I interferon (IFN-β and IFN-α subtypes) is produced by nearly all cells in the body and receptors exist on equally many cell types [98]. Type II interferon (IFN-γ) is exclusive to natural killer cells and activated T-cells and type III interferon consists of IL29, IL28A and IL28B. Many cells produce type III interferon, but only epithelial cells like hepatocytes have the specific receptor [98]. Type I interferon receptor activation on neighbouring cells leads to a signal cascade starting with an intracellular Janus kinase which subsequently phosphorylates the transcription factors STAT 1 and STAT 2. These recruit interferon-regulating factor 9 (IRF9) to form a complex called interferon-stimulating gene complex 3. This complex binds to the IFN-stimulated response element activating various ISGs [98]. Interferon type III uses the same pathway but a different initial receptor. This type III receptor (IL29, IL28A and IL28B) appears to have a bigger role in HCV infection than previously thought [99]. This is also reflected in the significance that a polymorphism in the IL28B has on the outcome of a therapy with PEG-interferon and ribavarin [75].

As previously described, HCV is a single stranded RNA virus, but a RNA double strand is produced in the process of replication. It is this double-stranded RNA that does not occur in human cells and therefore functions as an alarm for the immune system indicating a viral infection [98]. These alarm signals are called viral pathogen associated molecular patterns (PAMP) and also include other features of viral metabolism such as single stranded RNA of untypical length or certain proteins.

These PAMPs are recognized by innate immune sensors called pattern recognition receptors (PRR) and lead to activation of the innate immune system [98].

This sensing can be crudely divided by intracellular localization of the pattern recognition. It either takes place in the cytoplasm through retinoic inducible gene-I (RIG-I) like receptors (RLRs) or in the endosomes through membrane bound toll-like receptors (TLR) [100].

The group of the RLRs consists of three kinds of receptors: the melanoma differentiation associated gene 5 (MDA 5), the laboratory of genetics and physiology 2 (LGP2) and RIG I itself. Both MDA 5 and RIG I bind their viral ligand with their c-terminal domain (CTD). In the case of RIG I they are short blunt ended double stranded RNA and single stranded RNA bearing 5' triphosphates and rich in polyuridine runs [101, 102]. RIG I-mediated signalling is defective in Huh 7.5 cells and deemed responsible for the high replication in theses cells [103]. MDA 5 is known to recognize long double stranded RNA. Although it plays a role in interferon activation in other flaviviridae there is no clear evidence for its involvement in the HCV immune response [98]. The c-terminal binding leads to a conformational change exposing the N terminal caspase activation and recruitment domain (CARD), which in turn interacts with the CARD of mitochondrial antiviral signalling protein MAVS (IPS1, Cardif, VISA). MAVS subsequently triggers an activation cascade through IRF (interferon regulatory factor) 1,3 and 7 leading to a immediate short term activation of ISG (interferon stimulated genes) [104] and a later long term activation of interferon production through other factors like TRAF3, TBK1 and NF-κB. The function of LGP2 in this interaction remains unknown for infections with HCV, albeit there are indications that it plays a regulatory role for the other two receptors (MDA5 and RIG I) [105]. In summary RIGI and MDA5 identify viral structures and activate ISG (interferon stimulated genes) and the production of interferon itself.

The other substantial categories of recognition receptors are the toll-like receptors (TLR). The role of TLR8 for HCV is not fully understood. TLR7 derives its importance from its expression in plasmocytoid dendritic cells (pDC) [106] and their infiltration into the liver tissue during HCV infection [107]. TLR3 has the most significance being expressed in hepatocytes [108]. TLR3 was also the first TLR discovered to recognize viral RNA [109] and it's binding occurs in the late endosomes and lysosomes [110]. Following the interaction with the viral RNA, TLR3 recruits the TIR-

domain-containing adaptor inducing IFN-β (TRIF or TICAM1). Through the mediator proteins TRAF3, TRAF6, RIP1 and TBK1, IRF3 and NF-κB the production of interferon and ISG is increased, representing a common final pathway with the PRR like RIG I and MDA5 [98]. Interestingly, like RIG I in HUH 7.5 TLR3 is missing in HUH 7 and is deemed accountable for the high replication of the virus, while reconstitution decreases replication significantly [111]. A special role plays TLR2, which can also sense the presence of HCV core and NS3 protein and trigger a NF-κB immune response [112, 113]. Another important enzyme with dsRNA recognition ability is the protein kinase R (PKR). PKR has a singular position, since it shuts down protein synthesis after sensing dsRNA and is involved in messaging through the NF-κB/TRAF pathway [114]. At the same time it is an ISG. PKR knockout in mouse embryonic fibroblasts has been shown to increase replication after transfection with mouse subgenomic replicons [95]. This might be due to the above-described inhibition of protein synthesis following interferon activation [115]. In this case PKR might function more as effector than as sensor for dsRNA.

After the sensing, interferon stimulated genes are activated either in the infected cell or in adjacent cells to prevent spread of the virus. There are over 300 ISG known and only a couple of them have been studied concerning their antiviral effect [116]. They interfere at different points in the cellular processes: some induce apoptosis of the targeted cell, some function as RNAse and some destroy the viral RNA or introduce substations in the viral genome during the RNA polymerization [98]. The two interferon-regulatory factors IRF1 and IRF7 fall into this group, both demonstrating a decrease in viral replication when overexpressed in normal susceptible cells [117, 118]. This was confirmed in a large-scale scan of ISGs and their effects on HCV by Schoggins et al. [119].

HCV for its part has devised means to evade the immune response by hiding from and interfering with the human immune system. Lipoproteins play a crucial role in the assembly of HCV as described above, but this might have more advantages than the usage of the LDL receptor for cellular entry. When travelling through the human body, this association protects the virus from being recognized by the immune cells [120]. The fact that the replication takes place in a rearranged part of the endoplasmatic reticulum called the membranous web may protect the virus from enzymes willing to interfere with this process [43].

But HCV also pursues more active strategies to ensure its survival in the liver. An early activation of the PKR pathway explained above with expression of a small subset of ISGs functions as negative feedback to down-regulate the detection of dsRNA by the RLR like RIG I through a protein called TRIM25 [121].

Of specific relevance for our studies is the fact that HCV tries to reduce the activation of the cytosolic and the endoplasmatic pathway of interferon induction by directly targeting the two key enzymes MAVS and TRIF. The NS3/4A protease of HCV cleaves MAVS at position Cys508 disrupting the subsequent signalling cascade and blunting the cellular immune reply through the RLR pathway [42, 122]. The same tactic applies to the proteolytic cleavage of TRIF by the NS3/4A protease at Cys 327 [41] to disrupt the toll-like-receptor pathway. Other direct interactions of viral proteins with the interferon activation cascade have been described such as the direct inhibitory effect of NS3 on TBK1 [123], an interaction between NS5A with STAT 1 leading to reduced IFN production [124] or the induction of IL8 through NS5A reducing the response to viral infection [125]. The caveat of these results is that they are all derived from cell culture or even cell free *in vitro* systems and their impact *in vivo* remains to be shown.

The role of the innate immune response is a pivotal point of our project. We hypothesized that an over-activation of the cellular immune system is responsible for the reduced replication in murine cells. To address this issue we asked two questions: Is the NS3/4A protease capable of cleaving mouse IPS1 and TRIF? We explained the important role these two proteins play in orchestrating the initial activation of the interferon cascade and how HCV manages to obviate them. So far it has not been shown if the NS3/4A protease is capable of performing this step in mice. We set out to elucidate this question.

The second question was if further blunting of the innate immune response might increase the only moderate replication in murine cells. To do this we created mouse embryonic fibroblast with knockouts of proteins from the interferon-signalling cascade, including PKR, IFN type I receptor, STAT 1, RIG I, IRF1, IRF3, IRF7, IRF9, LGP2. We then examined their ability to replicate HCV using a selectable replicon system.

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Recapitulation of the hepatitis C virus life-cycle in engineered murine cell lines

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ABSTRACT

Hepatitis C virus (HCV) remains a major medical problem. In-depth study of HCV pathogenesis and immune responses is hampered by the lack of suitable small animal models. The narrow host range of HCV remains incompletely understood. We demonstrate that the entire HCV life-cycle can be recapitulated in mouse cells. We show that antiviral signaling interferes with HCV RNA replication in mouse cells. We were able to infect mouse cells expressing human CD81 and occludin (OCLN)—the minimal set of entry factor factors required for HCV uptake into mouse cells. Infected mouse cells sustain HCV RNA replication in the presence of miR122 and release infectious particles when mouse apoE is supplied. Our data demonstrate that the barriers of HCV interspecies transmission can be overcome by engineering a suitable cellular environment and provide a blue-print towards constructing a small animal model for HCV infection.

Introduction

Hepatitis C virus (HCV), the causative agent of classically defined non-A, non-B hepatitis, is highly prevalent, with approximately 3% of the worldwide population infected. Acute HCV infection often evades immune-mediated clearance and results in chronic, life-long persistence. Chronic infections can have severe health consequences, including hepatitis, cirrhosis, liver failure, and hepatocellular carcinoma. Treatment options are limited and are often plagued with serious side effects. A preventative or therapeutic vaccine for HCV does not exist.

HCV has been notoriously difficult to study in cell culture and in vivo systems (reviewed in Dustin and Rice (2007)), which has hampered development of more tolerable and effective therapies. Few species are known to be susceptible to HCV infection, including humans, chimpanzees and tree shrews (reviewed in Bukh (2012)). The HCV life cycle is blocked or insufficiently supported at multiple steps in murine cells and the barriers for interspecies transmission remain poorly defined (reviewed in Sandmann and Ploss (2013)). To enter hepatocytes, HCV utilizes several host proteins including glycosaminoglycans (GAGs) (Barth et al., 2003; Koutsoudakis et al., 2006), the low density lipoprotein receptor (LDLR) (Agnello et al., 1999; Molina et al., 2007; Monazahian et al., 1999; Owen et al., 2009), the high density lipoprotein receptor scavenger receptor class B type I (SCARB1; Scarselli et al., 2002), tetraspanin CD81 (Pileri et al., 1998), and two tight junction (TJ) proteins, claudin-1 (CLDN1; Evans et al., 2007) and occludin (OCLN; Liu et al., 2009; Ploss et al., 2009). CD81, SCARB1, CLDN1 and OCLN comprise the minimal set of host factors required for HCV uptake into mouse cell lines, where only CD81 and OCLN must be of human origin to overcome the species barrier in mouse cell lines (Ploss et al., 2009) and genetically humanized mice (Dorner et al., 2011). More recently, additional host factors including the cholesterol absorption receptor Niemann–Pick C1-like 1 (NPC1L1; Sainz et al., 2012) and two receptor tyrosine kinases, epidermal growth factor receptor (EGFR; Lupberger et al., 2011) and EphrinA2 (Lupberger et al., 2011) have been implicated in the viral uptake pathway into human cells. Mouse and human EGFR and EphrinA2 both share approximately

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90% amino acid sequence identity, suggesting that functionality within the HCV entry pathway maybe conserved across species. Interestingly, NPC1L1 is not expressed in the mouse liver (Altmann et al., 2004). Nonetheless, it was demonstrated that mice expressing human CD81 and OCLN support HCV uptake into mouse hepatocytes (Dorner et al., 2011) suggesting that lack of NPC1L1 does not limit HCV infection of murine cells.

Following uptake into murine cells, HCV RNA is translated (Dorner et al., 2011; McCaffrey et al., 2002) but does not appear to accumulate, suggesting that viral RNA replication is impaired in mouse cells. HCV RNA replicons, which are selectable HCV RNA genomes, can replicate in murine cell lines (Frentzen et al., 2011; Uprichard et al., 2006; Zhu et al., 2003), demonstrating that interfering dominant negative inhibitors do not appear to exist. These observations also suggest that murine orthologs of host factors critical for HCV replication appear to cooperate sufficiently with the viral replication machinery.

Several reports have hinted that antiviral cellular defenses limit HCV genome propagation. For example, both human hepatoma cells (Blight et al., 2002; Sumpter et al., 2005) as well as human primary hepatocytes impaired in innate immunity are more conducive to viral replication (Andrus et al., 2011; Marukian et al., 2011). It was reported that HCV can counteract innate immune defenses in human cells, e.g. by cleavage of the mitochondrial antiviral signal protein (MAVS) (Meylan et al., 2005) or Toll/IL-1 receptor domain-containing adapter inducing IFN-beta (TRIF or TICAM; Li et al., 2005). However, these mechanisms may not work with equal efficiency in all cell types and species. In fact, it was recently shown that MAVS from multiple primates is resistant to inhibition by the HCV NS3/4A protease (Patel et al., 2012). This resistance maps to single amino acid changes within the protease cleavage site in MAVS, which protect MAVS proteolytic cleavage by the NS3/4A protease. In murine cells, the NS3/4A protease cleavage motifs of mouse MAVS and TRIF are conserved, but it has not been formally proven that the viral protease actually cleaves the murine ortholog and that this targeted proteolysis translates into increased RNA replication. Thus, it is conceivable that different kinetics and/or a greater magnitude of virally induced innate defenses prevent induction or maintenance of HCV RNA replication in mouse cells. This hypothesis is supported by the previous observation that mouse embryonic fibroblasts (MEFs) with targeted disruptions protein kinase R (PKR; (Chang et al., 2006)) or interferon regulatory factor 3 (IRF3; Lin et al., 2010b) are more conducive to HCV RNA replication. In contrast, infectious HCV particles can assemble and be released in mouse cell lines if apolipoprotein E is sufficiently expressed (Long et al., 2011b) suggesting that later stages in the HCV life cycle are not blocked in mouse cells.

In this study we attempted to recapitulate the entire HCV life-cycle in mouse cells. Specifically, we demonstrate that the HCV NS3/4A serine protease is capable of cleaving MAVS and TRIF in mouse cells, thereby creating an environment which more efficiently supports HCV RNA replication. However, this evasion mechanism is not sufficient to readily overcome host defenses interfering with HCV RNA replication in mouse cells. MEFs derived from mouse strains harboring targeted deletions in genes critically involved in type I and III interferon signaling can improve the efficiency of HCV RNA replication, especially in the presence of the liver-specific microRNA 122 (miR122), a host factor that was previously shown to be important for HCV RNA replication (Jopling et al., 2005). In accordance with a previous study (Long et al., 2011a; Scull and Ploss, 2012), expression of human or mouse apoE in mouse fibroblasts infected with a selectable infectious HCV genome with apoE results in production of infectious

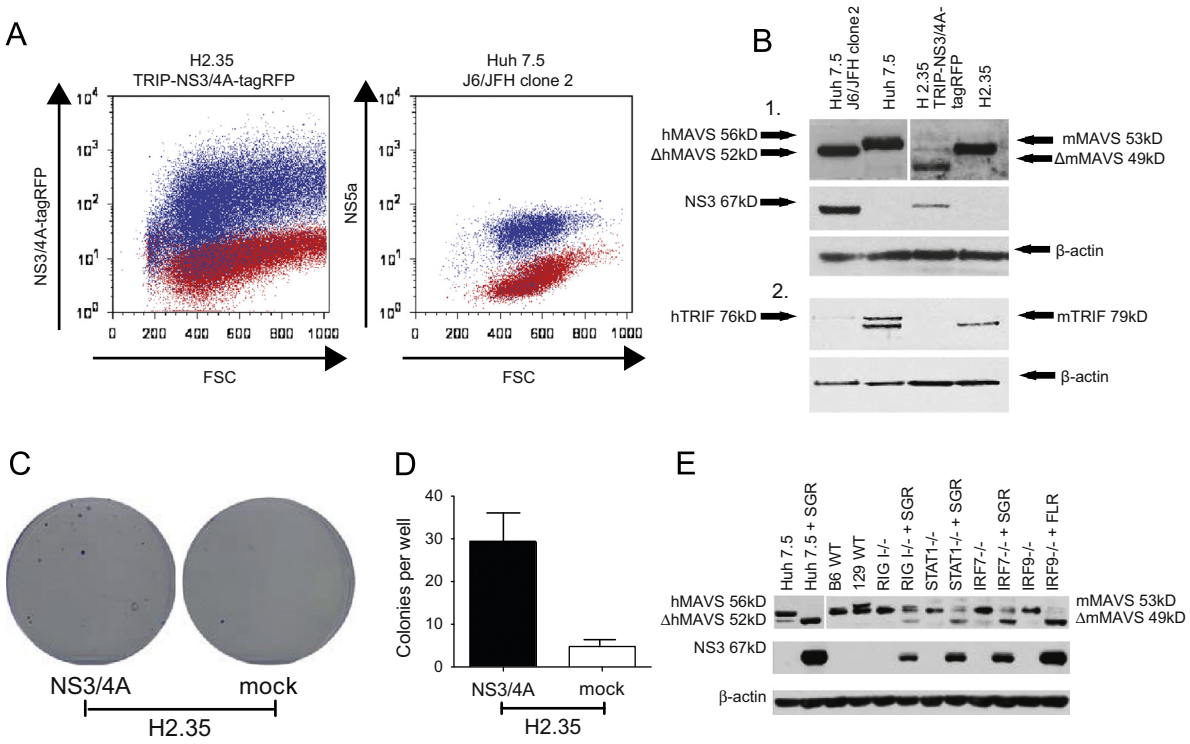

Fig. 1. The HCV NS3/4A serine protease efficiently cleaves mouse MAVS and TRIF. (A) (left) Overlay plot of naïve H2.35 cells (red) or H2.35 cells transduced with VSVg-pseudotyped TRIP-NS3/4A (JFH1)-puro2atagRFP and sorted for high expression of the viral protease (blue). (right) Overlay plot of naïve (red) or J6/JFH1 clone 2-infected (blue) Huh7.5 cells stained for HCV NS5A 72 hours following infection. (B) Western blot of lysate of naïve and transduced (mouse) and infected (human) cells detecting mouse and human MAVS, TRIF and β-actin. (C) Selection of SGR-bsd-JFH1-containing colonies in H2.35/TRIP-NS3/4A-tagRFP and the parental H2.35 cells Representative wells showing colonies of crystal violet stained cells. (D) Quantitation of crystal violet-positive colonies per well shown from three independent experiments. E. Western blot detecting mouse and human MAVS in either control or replicon-containing Huh7.5 cells and iMEFs. SGR=SGR-Bsd-JFH1; FLR=FL-Bsd-JFH1.

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particles. Taken together, these data show that all steps of the HCV life cycle can be recapitulated in murine cells, providing the framework for an inbred mouse model of HCV infection.

Results

Cleavage of MAVS and TRIF improves HCV RNA replication in mouse cells

To test whether the NS3/4A serine protease is capable of cleaving mouse MAVS and TRIF, two of the known targets of NS3/4A in human cells, we transduced H2.35 mouse hepatoma cells with a lentivirus TRIP-NS3/4A-TagRFPpuro expressing an enzymatically active HCV NS3/4A (JFH-1) with a TagRFP-puromycin fusion protein expressed in a downstream cistron. This allowed us to sort flowcytometrically a mouse cell population expressing similar levels of NS3/4A to human Huh7.5 cells infected with the robustly replicating J6/JFH1 clone 2 virus (Walters et al., 2009) (Fig. 1A and B). In the parental H2.35 and Huh7.5 cells MAVS and TRIF can be detected using specific antibodies for the respective proteins. In mouse cells overexpressing NS3/4A and Huh7.5 cells infected with HCV, the proteolytic products of mouse and human MAVS and TRIF were readily detectable (Fig. 1B), suggesting that HCV NS3/4A protease mediated immune evasion occurs in mouse cells.

To assess whether NS3/4A-mediated cleavage of mouse MAVS and TRIF would render mouse cells more permissive to HCV RNA replication, we transfected H2.35 cells with TRIP-NS3/4A-TagRFP-puro and selected for cells highly expressing the HCV protease. We then transfected NS3/4A-expressing cells and H2.35 controls with a selectable subgenomic JFH1 replicon (SGR-bsd-JFH1) encoding the blasticidin resistance gene blasticidin-S-deaminase (bsd). When selected with blasticidin, H2.35 cells expressing NS3/4A formed 8–10 times more colonies (Fig. 1C and D) than NS3/4A-negative controls, suggesting that NS3/4A expression significantly blunted innate antiviral signaling in mouse hepatoma cells, thereby boosting HCV replication.

Blunting antiviral innate defenses improves HCV RNA replication in mouse cells

HCV can replicate in mouse cells lines suggesting that murine orthologs of host factors critical for HCV replication cooperate sufficiently with the virally encoded replication machinery. HCV replication is augmented in mouse cells lacking expression of the protein kinase R (Chang et al., 2006) or interferon regulatory factor 3 (IRF3) (Lin et al., 2010b), HCV replication is augmented suggesting that antiviral immunity limits viral replication in mouse cells. Our data above indicate HCVs antiviral evasion mechanisms are functional, but not readily capable of overcoming innate cellular defenses. To identify a murine environment that is more conducive for robust HCV replication we surveyed a larger panel of MEFs deficient in genes critically involved in type I and III interferon responses, including PKR (Balachandran et al., 1998), RIG-I (Kato et al., 2005), IRF-1 (Kimura et al., 1994; Matsuyama et al., 1993), IRF-3 (Sato et al., 2000), IRF-7 (Honda et al., 2005), IRF-9 (Kimura et al., 1996), STAT1 (Durbin et al., 1996), IFNαβ receptor (Muller et al., 1994) or a knock-in strain with a dead mutation in the helicase of LGP2 (Satoh et al., 2010) for their ability to replicate HCV RNA. We generated immortalized MEFs (iMEFs) from these strains as they provide a clean knock-out background for the gene of interest. Liver-specific micro-RNA 122 (miR122) is a critical host factor regulating HCV RNA translation and replication (Henke et al., 2008; Jopling et al., 2005). It has previously been demonstrated that exogenous administration of miR122 can boost

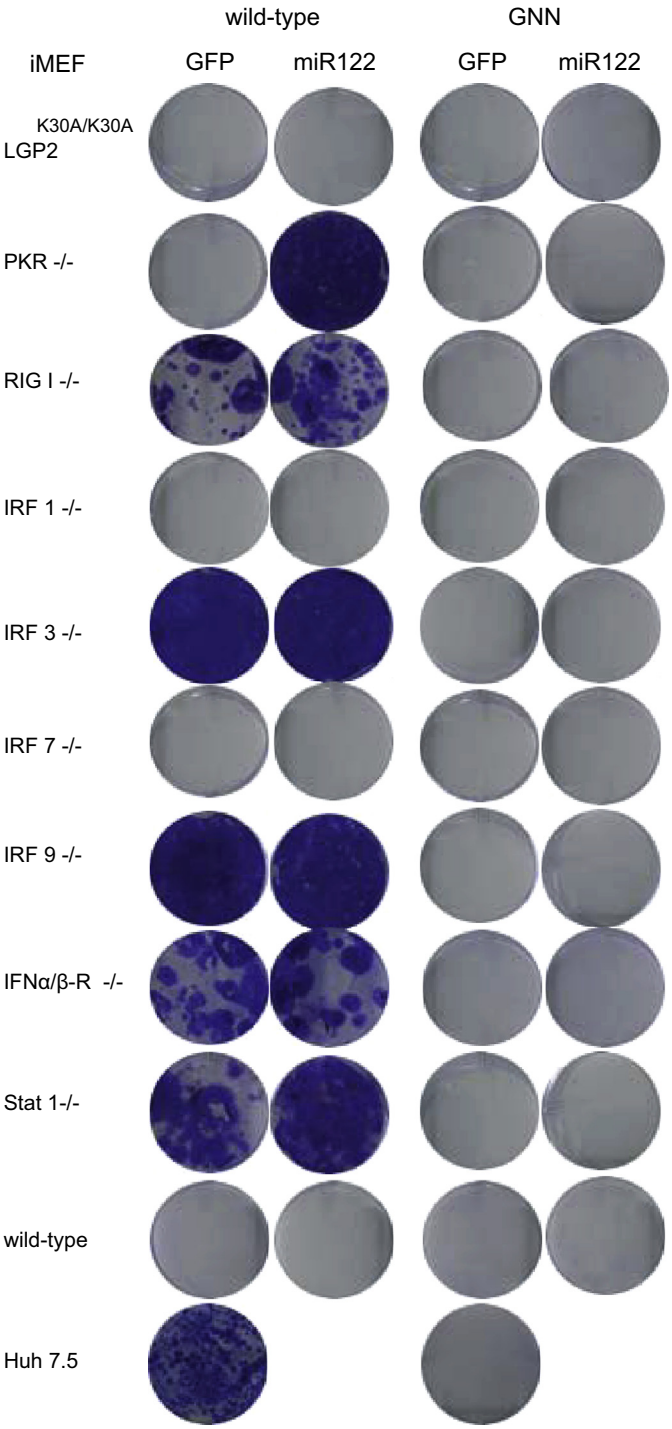

Fig. 2. Innate immune deficiencies and expression of miR122 facilitate replication of HCV replicons in mouse fibroblasts. Immortalized MEFs (iMEFs) of the indicated genotypes were lentivirally transduced with GFP or miR122 and then transfected with replication-competent (wild-type) or defective (GNN) bicistronic subgenomic (JFH1) constructs expressing blasticidin S deaminase (bsd) gene that confers resistance blasticidin selection. Cells were selected in blasticidin-containing medium and stained with crystal violet after fixation.

HCV RNA replication in non-hepatic human (Da Costa et al., 2012b) and murine cells (Lin et al., 2010a). In order to more closely mimic the murine hepatic environment, we transduced iMEF lines from the various innate immune knockout backgrounds with a lentivirus expressing miR122. This resulted in stable expression of miR122 to similar levels detectable in highly permissive Huh7.5 cells (data not shown). iMEFs expressing miR122 or control cells

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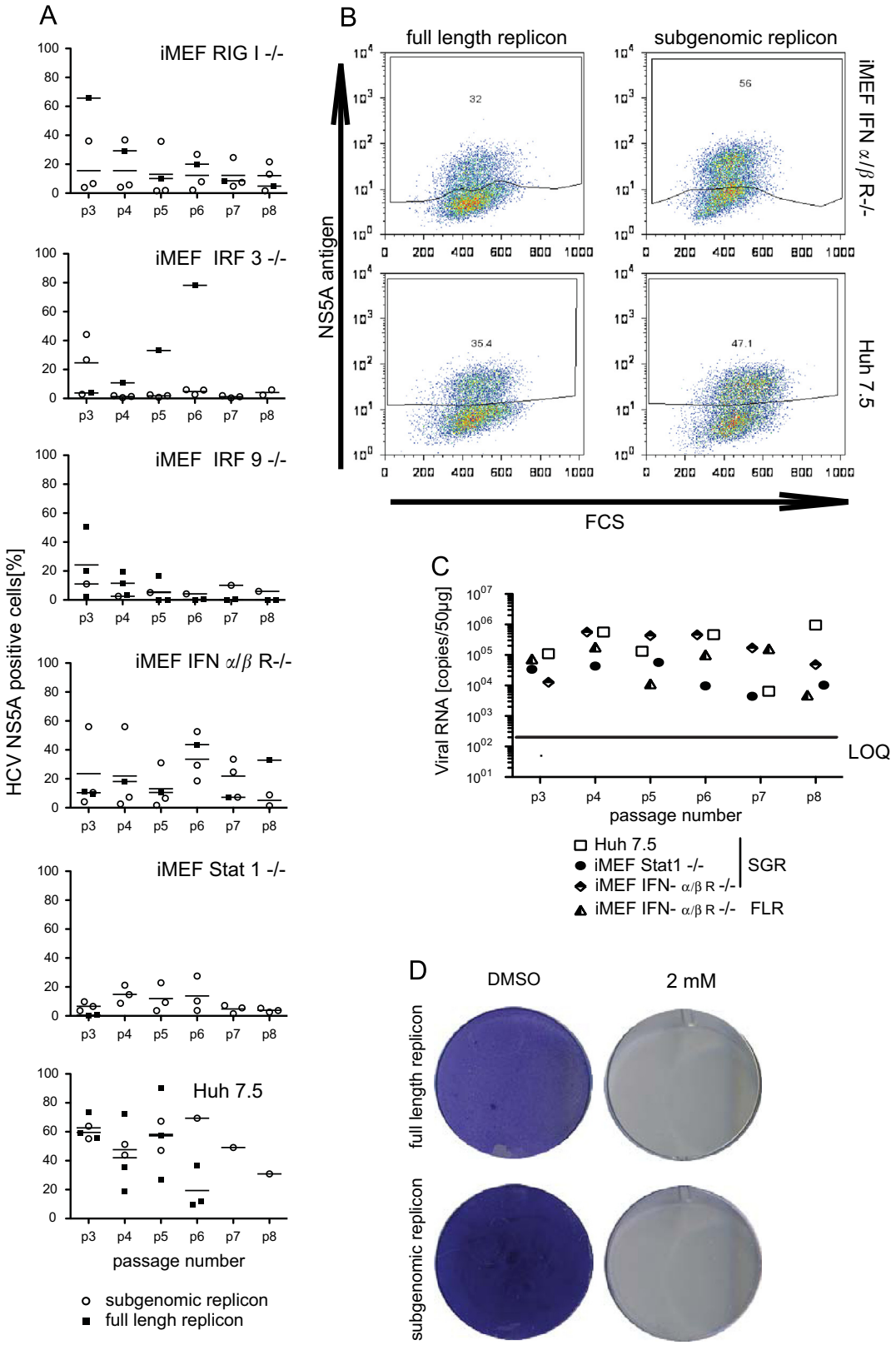

Fig. 3. Persistent HCV RNA replication in mouse fibroblasts on immunodeficient backgrounds. Immortalized MEFs of the indicated genotypes or Huh7.5 cells were transfected with full-length J6/JFH1 (FLR) or subgenomic JFH1 replicons expressing blasticidin S deaminase and cell populations selected in the presence of blasticidin. (A) Quantification of HCV NS5A antigen positive cells over six passages following HCV RNA transfection. (B) Representative flow cytometry plots of IFNαβR-/- and Huh7.5 cells stably harboring FLR- or SGR-stained for HCV NS5A antigen. **C.** Quantification of HCV RNA over several passages in Huh7.5 and STAT1-/- iMEFs stably replicating SGRs or FLRs as indicated. **D.** STAT1-/- iMEFs stably replicating SGRs or FLRs were grown in the presence or absence of 2′CMA (2mM) and stained with crystal violet after fixation. LOQ=limit of quantitation.

were transfected with SGR-bsd-JFH1 and drug selection was applied. Transfection of the SGR-bsd-JFH1 replicons into iMEFs derived from wild-type control mice resulted in negligible numbers of blasicidin resistant cells irrespective of the presence of miR122 (Fig. 2). In contrast, in the absence of RIG-I, IRF-3, IRF-9, IFNαβR, STAT1 but not IRF-1 or IRF-7 SGR-bsd-JFH1 conferred resistance to blasticidin selection, indicating that blunting specific antiviral signaling pathways improves HCV RNA replication. iMEF lines stably replicating HCV RNA expressed HCV NS3 which correlated with efficient cleavage of mouse MAVS (Fig. 1E). In order to provide additional evidence for HCV RNA replication we quantified HCV NS5A-positive cells by flow-cytometry over multiple passages in iMEF replicon lines harboring SGR-bsd-JFH1 and maintained under bsd selection (Fig. 3). HCV NS5A protein was detectable in iMEFs lacking RIG-I, IRF-3, IRF-9, IFNαβR and STAT1 harboring either subgenomic JFH1 or full-length (FL) J6-JFH1 replicons (Fig. 3A and B; and data not shown). The frequency of NS5A positive cells was expectedly lower in the iMEFs lines harboring SGR-bsd-JFH1 compared to Huh7.5 cells replicating either full-length (FL) or subgenomic replicon-based (SGR) HCV genomes. However, HCV RNA was readily detectable in STAT1-/- and IFNαβR-/- iMEFs (data not shown) harboring SGR or FL replicons at levels comparable to or only slightly lower than replicon-containing Huh7.5 cells (Fig. 3C). When treated with 2′C methyl adenosine (2′CMA), an inhibitor of the HCV NS5B polymerase, STAT1-/- iMEFs that previously stably replicated SGR or FL genomes did not withstood blasticidin selection, pointing to robust HCV replication on the STAT1-/- background. For all subsequent experiments we primarily used STAT1-/- iMEFs because of their superior stable growth under bsd selection when harboring replicating HCV RNA. Taken together, these data demonstrate that HCV replicates efficiently in non-hepatic mouse cell lines with disrupted type I/III IFN signaling pathways.

Initiation of HCV life-cycle following infection of mouse cells expressing human CD81 and OCLN with HCVcc.

Transfection is likely to introduce considerably more HCV RNA into a cell as compared to a natural infection process. Thus, HCV virion uptake via receptor-mediated endocytosis may prevent putative hyperactivation of antiviral signaling pathways. It was previously demonstrated that CD81, SCARB1, CLDN1 and OCLN are required for HCV entry into rodent cells and CD81 and OCLN constitute the minimal set of human genes required for viral uptake in vitro and in vivo (Dorner et al., 2011; Ploss et al., 2009). To determine whether the HCV life-cycle could be established in iMEFs defective in STAT1, we transduced these cells with human fluorescently-tagged CD81, SCARB1, CLDN1 and OCLN and subsequently with miR122 (Fig. 4A). Expression of human entry factors was confirmed by flow cytometry 3 days following transduction. Approximately 30% of all cells expressed both human CD81 and OCLN and a subset of those also expressed human CLDN1 and

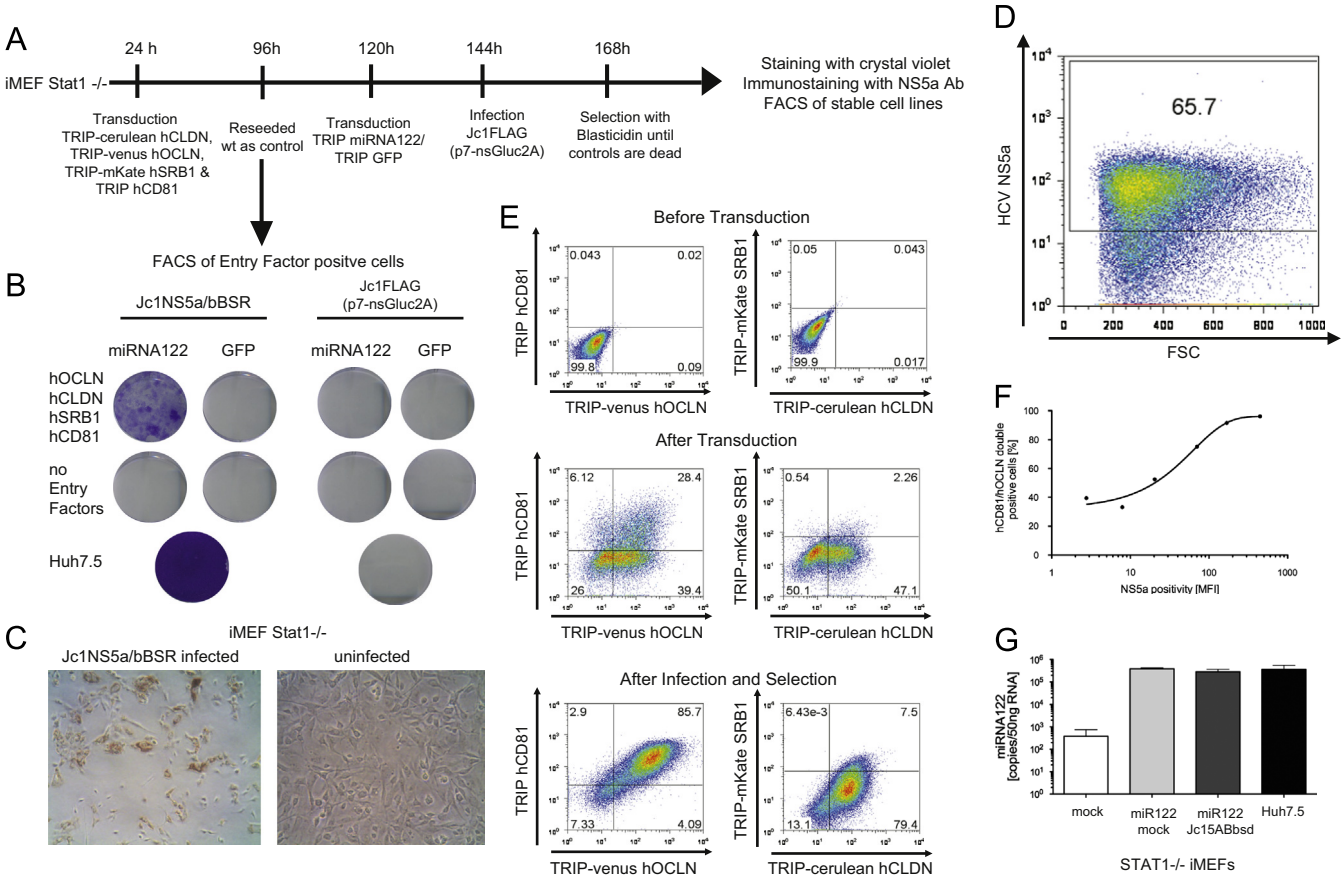

Fig. 4. Stable HCV RNA replication in mouse fibroblasts expressing HCV entry factors and miR122 following infection with Jc1-5AB bsd. (A) Schematic of the experimental set-up. (B) Crystal violet stained blasticidin-resistant colonies of the indicated cell populations infected with Jc1-5AB bsd or Jc1FLAG(p7-nsGluc2A). (C) Immunohistochemical staining of STAT1-/- iMEFs expressing human HCV entry factors stably infected with Jc1-5AB bsd or non-infected control cells. (D) Flow cytometry quantification of HCV NS5A antigen in STAT1-/- iMEFs expressing human HCV entry factors stably infected with Jc1-5AB bsd. (E) Flow cytometric analysis of ectopic expression of human CD81, OCLN, CLDN1 and SCARB1 before (upper panels) and after transduction (middle panels) and after infection and selection (lower panels). (F) Correlation between human CD81/OCLN and NS5A antigen positivity in Jc1-5AB bsd infected STAT1-/- iMEFs. (G) miR122 expression in Jc1-5AB bsd infected or non-infected STAT1-/- iMEFs transduced with miR122 and HCV entry factors, untreated iMEFs and Huh7.5 cells.

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SCARB1 (Fig. 4E middle panels). Similarly, lentiviral delivery of miR122 resulted in robust expression of the microRNA reaching levels equivalent to those in highly permissive Huh7.5 cells (Fig. 4G). To visualize HCV infection more readily we constructed a recombinant HCV genome expressing bsd between NS5A and NS5B, Jc1-5AB bsd (Fig. 5A and (Horwitz et al., 2013)). Jc1-5AB bsd replicates following electroporation in Huh7.5 cells (Fig. 5B), produces infectious particles (Fig. 5C), both albeit lower than the parental Jc1 genome and renders cells replicating the genome resistant to blasticidin selection (Fig. 5D and E).

Following infection of human entry factor- and miR122-transduced STAT1-/- iMEFs with a control virus or Jc1-5AB bsd and application of selection pressure, only cells that expressed HCV entry factors and miR122 and were infected with Jc1-5AB bsd were resistant to the antibiotic (Fig. 4B). Further analysis of the cells that, stably replicated Jc1-5AB bsd revealed that the majority of cells ($>60\%$) expressed high levels of HCV NS5A antigen—evidenced by immune-histochemical staining (Fig. 4C) and flow-cytometric quantitation (Fig. 4D). Notably, following bsd selection, expression of HCV entry factors was skewed towards substantially higher expression, in particular human CD81 and human OCLN ($>85\%$ cells) suggesting a beneficial effect presumably due to de novo infection and spread within the culture (Fig. 4E, bottom panels). Indeed, the NS5A staining intensity correlated with the frequency of human CD81/OCLN double positive cells (Fig. 4F). These data demonstrate that HCV replication can be initiated and maintained in murine cells expressing HCV entry factors following uptake of infectious HCV.

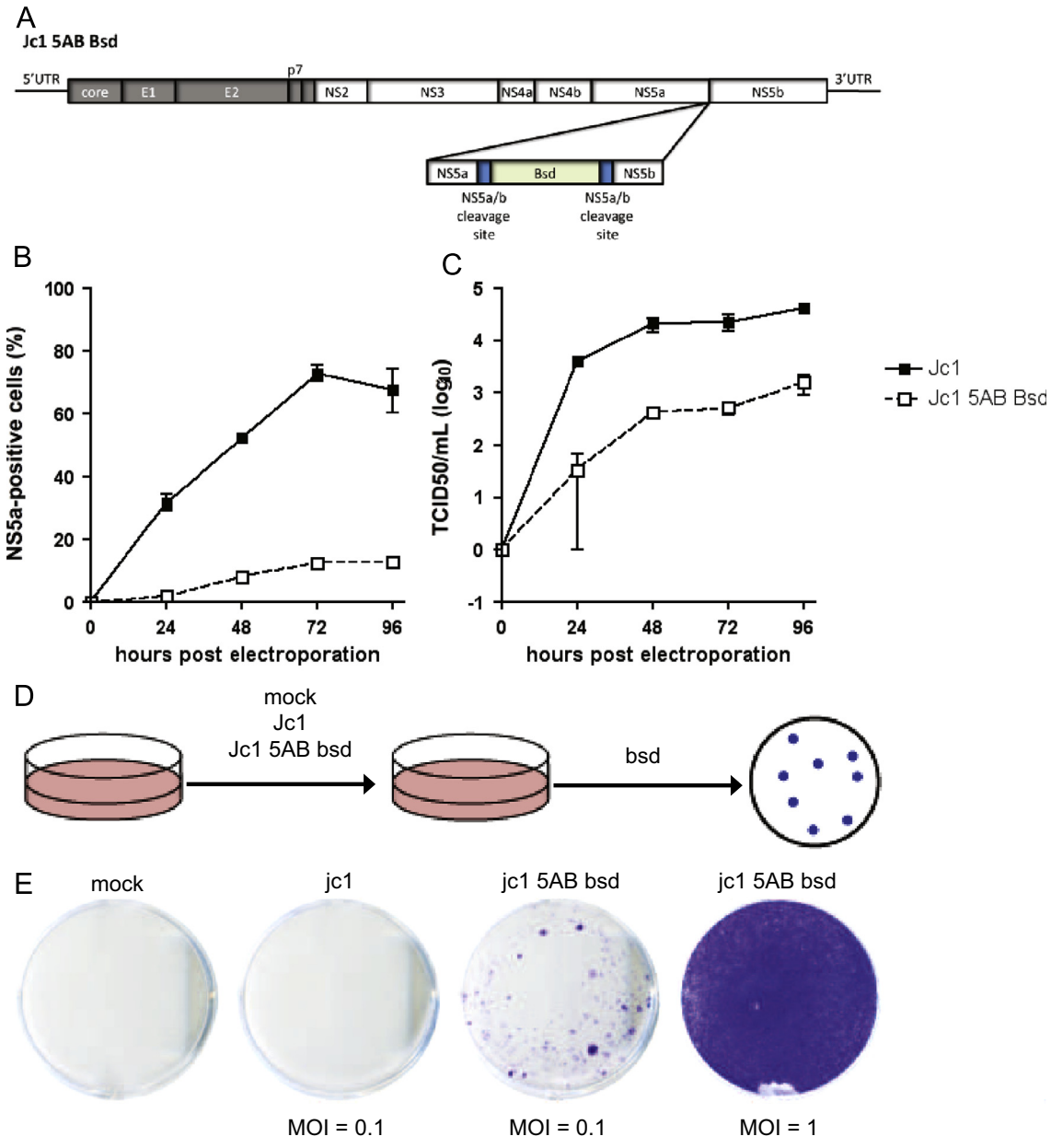

Fig. 5. Construction of selectable HCVcc by insertion of bsd between NS5A and NS5B. (A) Genome structure of Jc1-5AB bsd illustrating the insertion a blasticidin resistance gene (bsd) by duplicating the NS3/4A protease cleavage site between NS5A and NS5B (NS5A/B CS) flanking the bsd insertion. (B) HCV replication following electroporation of in vitro-transcribed RNA of Jc1 or Jc1-5AB bsd into Huh7.5 cells as measured by flow cytometry. (C) Longitudinal virus production as measured by end-point limiting dilution following electroporation of Jc1 and Js1-5AB bsd RNA into Huh7.5 cells. (D) Schematic depiction of the experimental set-up for the selection of blasticidin resistant colonies. (E) Huh7.5 cells infected with Jc1 (MOI=0.1), Jc1-5AB bsd (MOIs=0.1 or 1), selected with bsd and stained with crystal violet following fixation.

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Production of infectious virions in mouse cell lines.

It was recently demonstrated that mouse hepatoma cells stably harboring a subgenomic HCV replicon can produce infectious particles when transcomplemented with the missing HCV structural proteins (Long et al., 2011a). This further affirms that virion production is dependent on an intact VLDL pathway, in particular, expression of apoE, irrespective of human or mouse species origin. We aimed to determine whether mouse cells of non-hepatic origin supplemented with mouse apoE are capable of supporting release of infectious HCV virions. iMEFs lacking STAT1 or Huh7.5 cells were transduced with HCV entry factors, miR122 and/or mouse apoE, infected with Jc1-5AB bsd and subjected to bsd selection (Fig. 6A). To determine whether the STAT1-/- iMEFs supplemented with hCD81, hSCARB1, hCLDN1 and hOCLN, miR122 and/or mouse apoE produced infectious particles, we passed cell culture supernatants on naïve Huh7.5 cells. Only iMEFs expressing apoE released infectious particles, albeit at low levels. These data confirm that mouse cells even of non-hepatic origin can produce infectious HCV simply by overexpressing apoE (Long et al., 2011a). We demonstrate for the first time that this can be achieved with a mono-cistronic HCV genome, which expresses the HCV gene products presumably in stoichiometric ratios as opposed to mouse cells harboring a subgenomic replicon in which structural proteins are overexpressed.

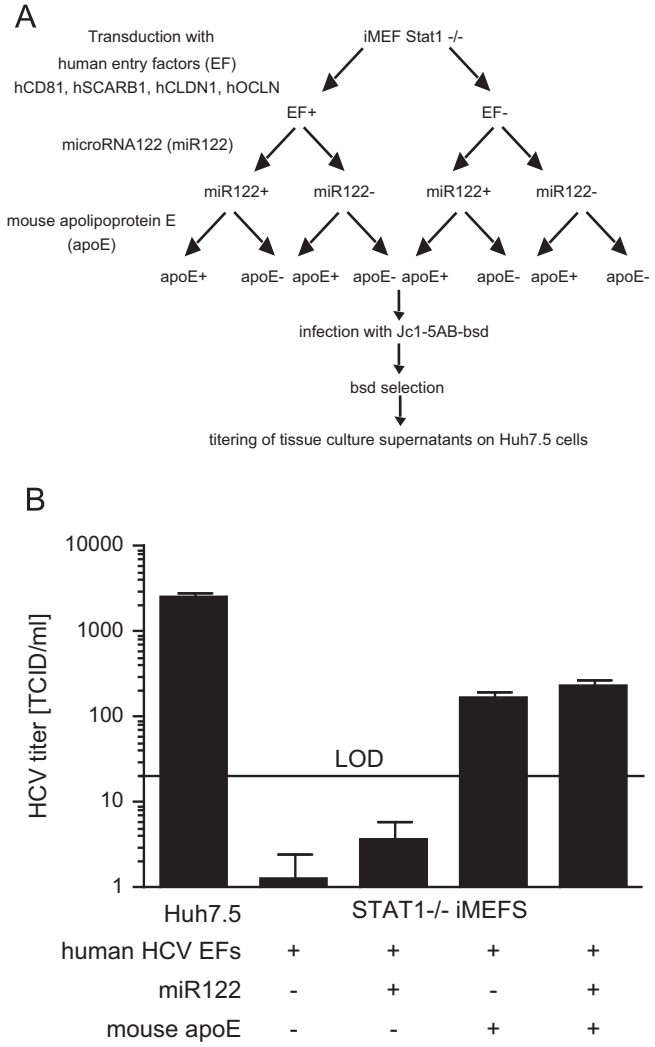

Fig. 6. Production of infectious HCV in mouse fibroblasts supplemented with apoE. (A) Schematic of the experimental set-up. (B) Infection of naïve Huh7.5 cells with supernatants from cultures of STAT1-/- iMEFs transduced with mouse apoE, eGFP, mock transduced iMEFs, and Huh7.5. NS5A staining was performed 72 h post infection and the number of infectious virions quantified in a limiting dilution assay. LOD=limit of detection.

Discussion

HCVs narrow host range, limited to humans and chimpanzees, is incompletely understood. Given the NIH moratorium on 'non-essential' chimpanzee research that will severely limit future chimpanzee HCV research, alternative animal models are urgently needed to study HCV associated pathogenesis and to aid HCV vaccine development. Different approaches have been taken to construct small animal models for HCV infection (reviewed in (Ploss and Rice, 2009)): efforts have been made to adapt HCV to infect cells of non-human origin. Alternatively, the murine host can be engineered to provide an environment that is conducive to HCV infection. One approach requires humanization of relevant human tissue compartments, in particular the liver and immune system, via xenotransplantation resulting in so called 'humanized mice.' Human liver chimeric mice, in particular, have proven useful to study HCV and to test anti-viral regimens (reviewed in Meuleman and Leroux-Roels (2008)). However, generation of xenotransplanted mice requires a high degree of technical skills, is costly and low in throughput, which limits their practical use. In an effort to more systematically define host determinants required to overcome species barriers we have identified the combination of human CD81 and OCLN as the minimal set human cellular factors required to facilitate entry into rodent cells (Ploss et al., 2009). Capitalizing on this discovery we subsequently translated these in vitro observations to a mouse model supporting viral uptake (Dorner et al., 2011, 2012). While this alternative host adaptation approach demonstrates how barriers of interspecies transmission can be overcome genetically, this particular humanized mouse model can only be used to monitor early steps in the viral life-cycle in vivo as it does not support HCV replication and virion assembly. These data are consistent with previous observations demonstrating that HCV RNA cannot readily replicate in mouse hepatocytes in vivo (McCaffrey et al., 2002). However, dominant-negative restriction factors do not seem to be present in mouse cells as HCV is generally capable, albeit at low levels, to replicate in murine hepatoma cells (Frentzen et al., 2011; Zhu et al., 2003).

Here, we attempted to shed light on restrictions of HCV species tropism in cells derived from non-permissive species. Our data demonstrate that species barriers can be overcome by engineering a suitable cellular environment. It was previously shown that the virally encoded NS3/4A protease is not only critical for processing of the HCV polyprotein but also cleaves numerous cellular targets (reviewed in Morikawa et al. (2011)). In particular, cleavage of the antiviral signal mediators MAVS and TRIF has been inferred as one way HCV interferes with innate immune responses. Here, we formally demonstrate that the NS3/4A protease does indeed degrade the murine orthologs of both MAVS and TRIF. Consequently, this cleavage moderately increases the ability of HCV to replicate in mouse cell lines. However, this antiviral evasion strategy does not seem to be sufficient for HCV to readily establish persistent RNA replication. In order to interrogate whether more severe interference with antiviral signaling would boost viral replication we surveyed cell lines derived from a number of mouse knock-out lines. In contrast to a knock-down approach which fails to completely abrogate gene expression and can have considerable off-target effects (Buehler et al., 2012; Marine et al., 2012), targeted disruptions of genes provides a clean null background for the allele of interest. We demonstrate that mouse embryonic fibroblast lines derived from mice impaired type I and/or III

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interferon responses more readily support HCV replication. These data are consistent with previous observations showing that HCV can replicate more efficiently in PKR and IRF3 deficient fibroblasts (Chang et al., 2006; Lin et al., 2010b).

Besides interfering with innate responses it is conceivable that incompatible host factors modulate HCV RNA replication in mouse cells. It was demonstrated initially in cell culture that the liver specific miRNA122 plays an important role in the HCV life-cycle (Jopling et al., 2005) which was later corroborated in vivo (Lanford et al., 2010). Two sites in the HCV 5′ non-translated region appear to interact with miR122 (Jopling et al., 2008, 2005). These interactions were shown to be critical for efficient HCV RNA replication, presumably by protecting 5′ terminal viral sequences from nucleolytic degradation or from inducing innate immune responses to the RNA terminus (Machlin et al., 2011), resulting in greater viral RNA abundance in both infected cultured cells and in the liver of infected chimpanzees (Lanford et al., 2010). Not surprisingly, ectopic expression of the liver specific microRNA 122 in MEFs slightly augments HCV RNA replication, which is supported by results from previous studies (Lin et al., 2010b). However, this pronounced effect of miRNA122 overexpression is more likely a reflection of the non-hepatic cellular background used here than a bona-fide species restriction factor. In fact, the exact sequence of miR122 is highly conserved in vertebrate species; comparatively, mouse miR122 is identical in sequence and with 50,000 copies per hepatocytes, similar in expressed level to human miR122 (Chang et al., 2004). Consequently, miR122 is not likely to contribute to the restricted host tropism in non-permissive species. Interestingly, HCV RNA replication in PKR deficient iMEFs appeared to be more dependent on the presence of miR122. It is conceivable that in PKR deficient cells, which are presumably less impaired in their ability to mount antiviral innate responses, residual antiviral signaling triggers expression of an ISG profile, which still interferes with HCV RNA replication. In contrast, in cells with more severe innate immune signaling impairments, such as IRF9-/-, IFNαβbR-/-, or STAT1-/-, early signals upstream innate immune response cascade triggered by HCV RNA terminus are not amplified and thus do not translate into induction of an antiviral program. Likewise, the 5′UTR of HCV may also be less susceptible to nucleolytic degradation. Numerous other host factors have been implicated in the HCV replication cycle (reviewed in Bartenschlager et al. (2010)). Of those, the most convincing experimental evidence has been provided for the critical role of cyclophilin A (CypA) and phosphatidylinositol 4 kinase IIIα (PI4KIIIα) in the HCV life-cycle. However, limited compatibility between the virally encoded replication machinery and these host factors is not likely since the human and murine protein sequences of CypA and PI4KIIIα are highly similar (98.2% and 98.6%, respectively). Nonetheless, it has not been shown whether ectopic expression of human CypA, PI4KIIIα or other host factors may increase HCV RNA replication in mouse cells.

We demonstrate that expression of HCV entry factors in the most permissive immunodeficient cellular backgrounds allows for initiation of replication via infection. Interestingly, we observe a strong skewing towards high expression of human CD81, SCARB1, CLDN1 and OCLN. High-level expression of HCV entry factors may be beneficial for efficient spread in the cell culture. Given the fact that we were only able to measure detectable levels of infectious particles when apoE was expressed infection of neighboring cells may via a direct cell-to-cell transfer in this culture system. Consistent with previous data (Long et al., 2011a) we show that expression of mouse apoE does support assembly of infectious virions. While Long and colleagues used a transcomplementation system (Long et al., 2011a) we show here that mouse cells can assemble and release monocistronic recombinant viruses. It is important to note that apoE, as part of the VLDL pathway, is highly expressed in the mouse liver. Thus, the need for overexpression of apoE to complete the late stages of the HCV life-cycle in cell lines as shown here and in other studies (Da Costa et al., 2012a; Long et al., 2011a) highlights the artificial nature of commonly used cell culture systems.

In summary, we define here the essential elements to recapitulate the entire HCV life-cycle in murine cell lines. Experimental evidence to validate these observations in vivo is underway. Undoubtedly, additional improvements to the genetically humanized mouse model would provide unprecedented opportunities to genetically dissect HCV infection and pathogenesis in vivo and will also serve as tractable, low-cost preclinical platform for testing and prioritizing drug and vaccine candidates.

Materials and methods

Cells and antiviral drugs

Mouse embryonic fibroblasts (MEFs) were generated from day 12.5 or 13.5 embryos from Irf1^{tm1Mak} (IRF1-/-)(Matsuyama et al., 1993) (obtained from the Jackson Laboratory, Bar Harbor, Maine, USA), Ifnar1^{tm1Agt} (IFNαβR-/-) (Muller et al., 1994) (obtained from B&K Universal Ltd (Hull, UK)) and Stat1^{tm1Dlv} (STAT1-/-) (Durbin et al., 1996) from Taconic (Hudson, NY, USA). Bcl2l12/Irf3^{tm1Ttg} (IRF3-/-) (Sato et al., 2000), Irf7^{tm1Ttg} (IRF7-/-) (Honda et al., 2005) and Irf9^{tm1Ttg} (IRF9-/-) (Kimura et al., 1996) (kindly provided by Tadatsugo Taniguchi, University of Tokyo, Tokyo, Japan), Dhx58$^{tm1(A30K)Aki}$ (LGP2$^{K30A/K30A}$) (Satoh et al., 2010) (kindly provided by Takashi Satoh and Shizuo Akira, Osaka University, Osaka, Japan), Eif2ak2^{tm1Cwe} (PKR-/-) (Yang et al., 1995) (kindly provided by Adolfo Garcia-Sastre (Mount Sinai School of Medicine, New York, NY, USA) immortalized via transduction with TRIP-SV40 large T antigen. RIG-I MEFs originating from the Akira lab were made available through Alexander Tarakhovsky (The Rockefeller University). Huh 7.5 cells, Huh 7.5.1 cells, immortalized MEFs (iMEFs), 293 T cells, and H2.35 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and penicillin/streptomycin, if not noted otherwise. Media were supplemented with blasticidin, puromycin and 2′C methyl adenosine (2′CMA) as indicated. 2′CMA was the gift of. D. Olsen and S. Carroll (Merck Research Laboratories, West Point, PA) and also was obtained from Carbosynth Limited.

Generation of recombinant HCV plasmids

HCV replicons

The full length replicon contains the J6/JFH-1 polyprotein expressed from an encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES). In an upstream cistron, the HCV 5′ untranslated region (UTR) drives expression of the first 19 amino acids of J6 core followed by blasticidin S-deaminase (bsd) containing a C-terminal STOP codon. Transfected into permissive cells, a blasticidin resistant population can be selected and infectious virus produced. The replication-impaired full-length construct contains two mutations in NS5B (GDD→GNN) that render this virus incapable of replication by deactivation of the viral polymerase. Transfected into permissive cells this replicon will be translated but no replication will take place. The other replicon used contains the subgenomic JFH-1 polyprotein including the nonstructural protein set (NS3-NS5B) expressed from an EMCV IRES. In an upstream cistron, the HCV 5′UTR drives expression of the first 19 amino acids of J6 core followed by blasticidin S-deaminase (bsd) containing a C-terminal STOP codon. Transfected into permissive cells, a blasticidin resistant population can be selected, but no infectious virus is released from the cells. Comparable to the full length a replication impaired subgenomic

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replicon was made. A mutation in NS5B (GDD→GNN) renders this construct incapable of replication by deactivation of the viral polymerase. After initial translation no replication of the viral genome occurs.

Infectious viruses

HCVcc containing bsd between NS5A and NS5B: A detailed characterization of the HCV expressing heterologous proteins flanked by NS3/4A cleavage sites within the HCV polyprotein is described elsewhere (Horwitz et al., 2013). Briefly, we generated a Gateway®-compatible destination vector (Invitrogen, Life Technologies, Carlsbad, CA) based upon the fully infectious Jc1 HCV genome, Jc1-5AB-DEST, for insertion of reporter genes between NS5A and NS5B. The 9-amino acid region spanning P7-P2′ of the NS3/4A proteolytic cleavage site between NS5A and NS5B was positioned on both ends of the destination cassette. Jc1-5AB-DEST was generated by PCR amplification of the Gateway® (Invitrogen, Life Technologies, Carlsbad, CA) destination cassette and insertion into the *Dra*III restriction site at the 3′ end of Jc1(2a) NS5A using standard molecular cloning techniques. Jc1-5AB-BSD was generated by PCR amplification of blasticidin S deaminase (bsd) with primers containing AttB sites for Gateway®-mediated insertion into pDONR™221, and subsequent BP and LR reactions were performed.

Jc1FLAG2(p7-nsGluc2A) is a fully-infectious HCVcc virus that has been previously described (Marukian et al., 2008; Pietschmann et al., 2006). J6/JFH Clone2 is a passaged derivative of J6/JFH that contains a number of adaptive mutations that increase infectious titers (Walters et al., 2009).

RNA transcription.

In vitro transcripts were generated as previously described (Lindenbach et al., 2005). Briefly, plasmid DNA was linearized by XbaI and purified by using a Minelute column (Qiagen, Valencia, CA). RNA was transcribed from 1 μg of purified template by using the T7 Megascript kit (Ambion, Austin, TX) or the T7 RNA polymerase kit (Promega, Madison, WI). Reaction mixtures were incubated at 37 °C for 3 h, followed by a 15-min digestion with 3 U of DNase I (Ambion, Austin, TX). RNA was purified by using a RNeasy Mini kit (Qiagen) with an additional on-column DNase treatment. RNA was quantified by absorbance at 260 nm and diluted to 0.5 μg/μl. Prior to storage at −80 °C, RNA integrity was determined by agarose gel electrophoresis and visualization by ethidium bromide staining.

HCVcc generation

Jc1 NS5AB bsd and Jc1FLAG(p7ns Gluc2A) were generated by electroporating in vitro transcribed RNA into Huh7.5.1 cells. After 72 h the medium was changed from 5% FBS to 1.5% FBS. The supernatants were collected every 6 h from 72 to 144 h after transfection and concentrated through a 100 kDa MWCO membrane using a Stirred Ultrafiltration Cell. Supernatant from J6/JFH1 clone 2 electroporated Huh7.5.1 cells was collected daily from 72 to 144 h but not concentrated. The virus infectivity was determined by limiting dilution as described previously (Lindenbach et al., 2005).

Pseudoparticles

Construction of proviral constructs

TRIP-HCV(JFH1)–NS3/4A-TagRFP/Puro. This TRIP-based bicistronic lentiviral vector encodes the HCV NS3/4A protease as a single-chain fusion protein driven by a CMV promoter. The downstream cistron is driven by an encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES) and encodes a fusion protein containing a puromycin resistance gene followed by a foot and mouth disease virus autoproteolytic peptide and TagRFP (Puro-2A-TagRFP). Transduction of permissive cell lines with these pseudoparticles results in stable expression of functional NS3/4a and Puro-2A-TagRFP. Transduced cell populations were selected on puromycin, and sorted using fluorescence based life cell sort.

TRIP-mApoE-TagRFP. The cDNA clone of murine apolipoprotein E was obtained in pCMV6 (Origene NM_009696). mApoE was PCR amplified from the cDNA vector and flanking attB sites added using primers RU-O-16597 and RU-O-16596, allowing us to insert the resulting product into pDONOR221 (Invitrogen, containing attP sites) using BP clonase II and Gateway cloning technology. mApoE was then shuttled into the destination vector pTRIP.CMV.IVSb.ires. TagRFP-DEST (Schoggins et al., 2011) using LR clonase II (Gateway cloning technology, Life Technologies).

TRIP-miRNA122. The proviral plasmid was generated by insertion of the miR122-generating hairpin-loop structure from pCMV-miR122 into the BamHI/XhoI digested pTRIP-GFP.

HCV entry factors. Generation of TRIP-human CD81(Flint et al., 2006), -Venus/YFP-human OCLN and -human OCLN(Ploss et al., 2009), -Cerulean/CFP-human CLDN1 (Ploss et al., 2009), human CLDN1(Evans et al., 2007) and human SCARB1 (Catanese et al., 2010) were described previously. TRIP-mKate-human SCARB1 was generated by overlapping PCR of the coding sequence of mKate, also designated as TagFP635(Shcherbo et al., 2007) (Evrogen) and human SCARB1 resulting into an N-terminal fusion protein which was subsequently cloned into the BamHI/XhoI digested pTRIP-GFP.

Pseudoparticle generation

All pseudoparticles were generated as described previously (Ploss et al., 2009) in 293 T cells by FuGENE6 (Roche Applied Science, Indianapolis)-mediated cotransfection of plasmids encoding (1) a minimal HIV provirus (TRIP) encoding a reporter gene or other transgene, (2) HIV gag-pol and (3) and VSV-G. Pseudoparticle-containing supernatants were harvested at 24, 48 and 72 h were pooled and filtered (0.45 μm mesh). Pseudoparticle infections were performed in the presence of 4 μg /ml polybrene. A minimum of 48 h elapsed between transduction and reporter gene quantification or subsequent experiments.

Transfection of HCV RNA into mouse and Human cell lines

For all replicon experiments cells were plated in tissue culture treated 6 well (2 × 10E5 cells) or 10 cm (1 × 10E6 cells) plates one day or in case of transduction with miRNA122 two days before the transfection. In the latter case, the miR122 or GFP pseudoparticles were applied on the day between seeding and transfection. Mirus TransIT transfection kit was used to transfect 0.5 μg (6-well plate) or 3 μg (10 cm dish) HCV replicon RNA into the cells. According to manufacturer's instructions the transfection medium was changed after six hours. We changed the medium to blasticidin containing selection medium the day after transfection and from there on regularly every 3 days. For all transfection experiments a concentration of 1 μg/ml blasticidin was used for ca. 2 weeks before raising the concentration to 2 μg/ml blasticidin for another two weeks. After completing this month the plates were either fixed in PFA and stained using crystal violet or resistant cells were trypsinized and passaged for further experiments. Only the H2.35 cells were immediately exposed to 2 μg/ml blasticidin and stained with crystal violet after 2 weeks.

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HCV infections

Transduced and control MEFs or Huh7.5 cells were infected with HCVcc (J6/JFH1 clone 2), Jc1 NS-5AB bsd or Jc1FLAG2 (p7nsGluc2A as indicated)-containing supernatants one day after cell seeding. For selection experiments, media was replaced the next day and switched to medium containing blasticidin, increasing the bsd concentration every 2 weeks from 1, to 2 to a final concentration of 6 μg/ml. Stably infected cells were either fixed in PFA and stained with crystal violet or resistant cells were passaged for further experiments. To quantify HCV infection, cells were fixed in paraformaldehyde, permeabilized with saponin and stained for NS5A with an Alexa488-conjugated 9E10 anti-NS5A antibody (Lindenbach et al., 2005); the signal was quantified by flow-cytometrical analysis.

Flow cytometry

Expression of lentivirally-delivered transgenes was quantified by flow cytometry. CD81 expression was detected by staining with an anti-human CD81-APC (clone JS81, Pharmingen), other proteins were directly fluorescently tagged, i.e. SCARB1 (mKate), CLDN1 (Cerulean) and OCLN (Venus), ApoE, NS3/4A (both TagRFP). All samples were acquired on a LSR2 flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Western blotting

Cells (Huh7.5, H2.35 and indicated iMEF lines) were trypsinized and the pellet was lysed using the following buffer: TRIS Base 1 M, NaCl, Triton-X 20% and Mini EDTA-free protease Inhibitor (Roche). After protein quantification using a Pierce BCA Protein Assay Kit (Thermo Scientific), equal amounts of protein were loaded onto 4–12% or 10% Bis/Tris NuPage polyacrylamide gels (Invitrogen). Proteins were transferred to a nitrocellulose membrane and detected with the respective antibodies: NS3 (Virostat #1878) 1:100, mMAVS (Cell Signaling #4983) 1:1000, hMAVS (Enzo #ALX-804-847) 1:1000, TRIF (Novus #NB120-13810) 1:1000. β-actin was detected as a loading control (Sigma #A3854) 1:200000. After incubation with the respective secondary antibody the proteins were visualized using Super Signal West Pico or Femto (Thermo Scientific).

HCV RNA quantification

After trypsinization, the total RNA from cell pellets was isolated using the RNeasy Mini kit (Qiagen) and the HCV genome copy number was quantified by one-step rtPCR using Multicode-RTx HCV RNA kit (EraGen) and a Roche LC480 light cycler, according to manufacturer's instructions.

Authorship

Contribution: A.V., B.M.D., M.D., G.G. performed research; J.A.H. and M.A.S. performed research and edited the manuscript; C.M.R. provided reagents, support and edited the manuscript. A.V. and A.P. designed research, analyzed data and A.P. wrote the paper.

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5. Discussion

Significant advances in the treatment of hepatitis C have been made during recent years, most notably the introduction of the protease inhibitors telaprevir and boceprevir. These have led to a substantial improvement in SVR. Novel therapeutic agents on the horizon might allow treatment without pegylated interferon-α in the near future [126]. However these management options still entail objective and perceptive barriers especially in regions without access to a comprehensive health care system. The long course of treatment and out of pocket expenses prevent patients in Africa and the Middle East from being treated adequately [127]. Bothersome side effects over a long period of time discourage patients from completing the required course. The high costs of the direct-acting antivirals like the protease inhibitors and the former standard therapy make treatment for HCV unaffordable for many people in the developing world. The development of more cost effective treatment options such as prophylactic or therapeutic vaccines would be desirable, but is still severely hampered by the lack of a suitable small animal model [128]. Studies are further hindered by the NIH moratorium on 'non essential' chimpanzee research [129].

Therefore alternative models are urgently needed in order to study the pathogenesis of HCV and provide a platform for drug and vaccine trials. From the three approaches pursued to create a small animal (see introduction) we focused on host adaptation by genetic modification.

On a molecular level several blocks in the HCV life cycle have been elucidated: The entry of the virus into the host cell, the replication of the viral RNA and the assembly of viral particles in the cell culture model. A minimal set of entry factors necessary for HCV entry into the liver cells has been identified. When these were introduced into engineered murine cells or into the liver cells of transgenic mice, entry of the virus into the cells was observed [54, 55]. Unfortunately only the entry and the initial translation, which was shown to work sufficiently in mice, can be studied in this particular model. In the past low-level replication of HCV in mouse cells was initiated in a cell-culture but could only be sustained if selection pressure was applied [95]. Replication *in vivo* on the other hand side appeared to be impossible [130]. The

release of infectious particles from rodent cells has been demonstrated only if apolipoprotein E is provided [97]. Furthermore the absence of dominant restriction factors in mouse liver cells has been proven by using a trans-complementation model [96].

The aim of this study was to identify factors that impede the completion of the HCV life cycle in mouse cells. We hypothesized that differences in the magnitude and kinetics of the innate immune response between different species contributes to the reduced replication in mouse cells. MAVS and TRIF are important players in the coordination of RIG-like-receptor and Toll-like-receptor signalling [131, 132]. By cleaving these two proteins HCV has established an immune evasion mechanism playing a key role in human liver cells. In a first step we established that the NS3/4A protease can successfully cleave murine MAVS and murine TRIF and subsequently increases the replication of HCV in mouse cell lines. This indicates that the immune response and especially the interferon-signalling cascade contributes to the reduced replication of viral RNA and might therefore play a pivotal role in the species tropism. For our next experiments we used mouse embryonic fibroblast lines derived from animals with targeted disruptions in the interferon type I and/or type III cascade. We were able to show that this blunting of the innate immune response increases the replication of HCV and improves survival of cells under selection pressure. This is consistent with previous studies examining the influence of PKR and IRF3 knockouts on replication [95, 133] The expression of micro RNA122 through lentiviral delivery further increased the replication in certain cell types; this is in keeping with previously mentioned role of miRNA122 [133, 134]. The different receptiveness of our various cell lines could be due to a diverse baseline expression of miRNA122. After we succeeded in establishing a model of continuous replication under selection pressure we wanted to explore whether these cells were able of producing infectious particles. A low production of infectious virions was found. This might be due to the fact that these cells are not liver cells but fibroblasts. The number of virions could be increased further through the addition of apolipoprotein E. This is consistent with previous findings [97]. In a next step we aimed to recapitulate the entire HCV life cycle. This would indicate that no further blocks exists in mouse cells and serves as a proof of principle that murine cells can support an infection with the hepatitis C virus. By supplying all the human entry factors and miRNA122 to the immune-impaired cells and infecting them with a virus harbouring a dominant selectable marker we

were able to grow cells that persistently replicated HCV. FACS analysis proved expression of viral proteins, although this expression decreased as soon as the selection pressure was withdrawn. This could again be attribute to the fact that fibroblasts instead of liver cells were used. We had decided to rely on immortalized mouse embryonic fibroblasts for our experiments since they can provide a clean null background even if experiments continue over several passages. By transferring the supernatants from these infected murine cells onto naïve highly permissive human hepatoma cells thus infecting these, we have shown that infective virions were produced. Therefore the entire HCV life cycle has been recapitulated.

This model lays the basis for further research *in vivo* and served as a proof of principle that mouse cells support HCV replication. It appears that the knockouts of IRF9, STAT 1 and IFNα/β-receptor have the strongest impact on viral replication. These three proteins are part of the same pathway in the interferon signalling cascade, being linked by the tyrosine kinase JAK (Janus kinase) [98]. The immune knockouts identified through this work have been tested *in vivo* and results will be published in the near future (Dorner *et al* manuscript, accepted).

Through this work it has also been shown that a selection marker can be inserted as heterologous protein into the NS5A/B cleavage side of the virus described by Horwitz *et al* [135]. In the future this model could prove useful for further dissection of HCV in cell culture.

It is clear that this system is an artificial representation of the *in vivo* processes. This is evidenced by the fact that replication can only be sustained if constant selection pressure is applied. Replication decreased as soon as the blasticidin medium was withdrawn.

One aspect that has not been explored was the possibility of adaptive mutations in the viral genome that might have occurred in the harbouring cells over several passages. The main focus of this study was to demonstrate evidence that the whole life cycle can be recapitulated and to provide immune knockouts that could be tested *in vivo.*

In the foreseeable future HCV infection will continue to pose major health challenges. As described above, new directly acting antiviral agents can provide higher clearance rate than ribavirin and pegylated interferon alone, but still have considerable side

effects and costs. High prevalence of HCV as seen for example in Egypt (22%), Pakistan (4.8%) and China (3.2%) [136] will pose a significant burden on the health services of developing countries. These countries would particularly benefit from new therapeutic approaches. This small animal model will provide a platform for research on the pathogenesis of HCV, serve as an immunocompetent alternative for the vaccine and drug testing *in vivo* and thus open unprecedented research opportunities for future studies of the hepatitis C virus.

6. Summary

Hepatitis C virus (HCV) is a global medical burden, infecting at least 130 million people worldwide. Despite recent advances in the development of direct-acting antiviral drugs HCV infection continues to present significant health challenges particularly in the developing world. In depth study of hepatitis C pathogenesis and immune responses is hindered by the lack of suitable small animal models. The host range of HCV is limited to humans, chimpanzees and tree shrews. The reason behind the inability of HCV to infect for example mice is poorly understood. Barriers are known to exist on levels of viral entry, replication and assembly. Recent studies have identified human CD81 and the tight junction protein occludin (OCLN) as the minimal set of entry factor factors required for HCV uptake into mouse cells. Post-entry, translation of the viral polyprotein from the incoming single-stranded positive sense RNA is supported in mouse cells. HCV can infect and replicate mouse-human cell heterokaryons suggesting that dominant restriction factors do not exist in murine liver cells. Selectable HCV genomes, so called HCV replicons, can indeed replicate in mouse hepatoma cell lines at low levels, indicating that all essential host factors aiding the HCV replication machinery are present in mouse cells. When antiviral defences are blunted HCV replicates more efficiently in mouse cells indicating that innate immunity contributes at least in part to limiting propagation of HCV in rodents. Furthermore, it was evidenced that the assembly of infectious HCV particles in certain mouse cell lines can occur as long as essential apolipoproteins (Apo) are expressed at sufficient levels. However, until now it was not unambiguously demonstrated that the HCV life cycle can be completed in murine cells, which was the focus of this work.

In human cells the viral NS3/4A protease cleaves the signalling molecules MAVS and TRIF, which allows HCV to evade antiviral defences. In a first step we were able to demonstrate that HCV NS3/4A is indeed capable of cleaving mouse MAVS and TRIF when overexpressed in mouse cells and that in turn this cleavage does augment HCV RNA replication. However, we hypothesized that following HCV RNA transfection or conceivably even after infection, faster and more robust induction of the antiviral response in mouse cells abrogates the infectious cycle before enough NS3/4A can accumulate to establish the infectious cycle.

Therefore, in order to identify a cellular environment that is more conducive to HCV RNA replication we generated SV40 T antigen immortalized mouse embryonic fibroblast (MEF) lines derived from mice with targeted disruptions of genes, which are critical for the induction of type I and/or III interferon (IFN) responses. In particular MEFs deficient in STAT1 or the IFN-α/β-receptor supported particularly well propagation of full-length and subgenomic HCV replicons under selection pressure. Increases in the frequency of drug resistant colonies correlated with higher HCV RNA loads as determined by quantitative RT-PCR and expression of the proteins as determined by flow cytometry.

After having identified this cellular background that sustained HCV RNA replication more efficiently, we aimed to recapitulate the entire HCV life cycle in these cells.. Immune-impaired MEFs were transduced with the minimal set of human entry factors and subsequently infected with a cell-culture derived virus HCV engineered to express a blasticidin resistance gene to identify cells in which HCV entered successfully and stably replicated. MEFs, which are not of hepatic origin, were further supplied with liver specific micro RNA 122 (miR122), which – as suggested by previously published data – further boosted HCV replication. Recent reports showed that HCV assembly depends on an intact very-low-density-lipoprotein (VLDL) pathway, in particular the expression of apoE. Thus, to encourage formation of infectious particles in our mouse cell culture system we lentivirally transduced the engineered MEFs with mouse apoE which led indeed to release of infectious HCV as determined in limiting dilution assays.

Taken together, our results affirm that the entire HCV life cycle can be recapitulated in murine cells, laying the foundation for developing a mouse model with inheritable susceptibility to HCV infection.

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Marcus Dorner, Joshua A. Horwitz, Bridget M. Donovan, Rachael N. Labitt, William C. Budell, Tamar Friling, Alexander Vogt, Maria Teresa Catanese, Tadatsugu Taniguchi, Takashi Satoh, Taro Kawai, Shizuo Akira, Mansun Law, Charles M. Rice, and Alexander Ploss
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2012 **'Recapitulation of the entire HCV life cycle in engineered mouse cell lines'** International Liver Congress, EASL, Barcelona April 2012
Auszeichnung: "**Best Basic Science Abstract**"

2011 '**Towards a small animal model for HCV'** IALS Forum in Boston, MA, USA März 2012

KURSE und SEMINARE

2013 **IMPACT Course,** Hull and East Yorkshire Hospitals, Hull

2013 **Red Dot Chest X-Ray Course,** Northwick Park Hospital, Harrow

2012 **Medical Ethics and Law,** MDU, London

2012 **ALS course**, Hillingdon Hospital, London

2012 **A&E Radiology Survival Course**, Northwick Park Hospital, Harrow

2011 **Scientific Methods: Survival Skills for Young Biomedical Investigators**
Tung-Tien Sun, NYU School of Medicine

2011 **Hepatitis C Seminar Series** Weill Cornell Medical College, New York

2009 **Kurs 'Tropenmedizin und International Health",**
Universitätsmedizin Charité, Berlin

10. Eidesstattliche Erklärung

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel „Recapitulation Of The Hepatitis C Virus Life Cycle In Engineered Murine Cell Lines“ in der Klinik für Gastroenterologie, Hepatologie und Endokrinologie in Kooperation mit dem Center for the Study of Hepatitis C unter Betreuung von PD Dr. med. Sandra Ciesek und Prof. Alexander Ploss, PhD ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel bisher noch nicht erworben habe.

Ergebnisse der Dissertation werden im Publikationsorgan Virology veröffentlicht.

Hannover, den _____

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(Alexander Vogt)