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(2013) Hepatology.

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Cell Entry, Efficient RNA Replication, and Production of Infectious Hepatitis C Virus Progeny in Mouse Liver-Derived Cells

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Only humans and chimpanzees are susceptible to chronic infection by hepatitis C virus (HCV). The restricted species tropism of HCV is determined by distinct host factor requirements at different steps of the viral life cycle. In addition, effective innate immune targeting precludes efficient propagation of HCV in nonhuman cells. Species-specificity of HCV host factor usage for cell entry and virus release has been explored. However, the reason for inefficient HCV RNA replication efficiency in mouse liver cells remains elusive. To address this, we generated novel mouse liver-derived cell lines with specific lesions in mitochondrial antiviral signaling protein (MAVS), interferon regulatory factor 3 (IRF3), or Interferon-α/β receptor (IFNAR) by *in vivo* immortalization. Blunted innate immune responses in these cells modestly increased HCV RNA replication. However, ectopic expression of liver-specific human microRNA 122 (miR-122) further boosted RNA replication in all knockout cell lines. Remarkably, MAVS$^{-/-}$miR-122 cells sustained vigorous HCV RNA replication, attaining levels comparable to the highly permissive human hepatoma cell line Huh-7.5. RNA replication was dependent on mouse cyclophilin and phosphatidylinositol-4 kinase III alpha (PI4KIIIα) and was also observed after transfection of full-length viral RNA. Additionally, ectopic expression of either human or mouse apolipoprotein E (ApoE) was sufficient to permit release of infectious particles. Finally, expression of human entry cofactors rendered these cells permissive to HCV infection, thus confirming that all steps of the HCV replication cycle can be reconstituted in mouse liver-derived cells. *Conclusion*: Blunted innate immunity, abundant miR-122, and HCV entry factor expression permits propagation of HCV in mouse liver-derived cell lines. (HEPATOLOGY 2014;59:78-88)

Persistent hepatitis C virus (HCV) infection is associated with severe liver disease including chronically active hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[1] Current treatment options are limited by side effects and suboptimal response rates and vaccines are not available. Access to permissive and predictive animal models is crucial for analysis of HCV pathophysiology, immune control,

Abbreviations: ApoE, apolipoprotein E; CD81, cluster of differentiation 81; CLDN1, claudin1; CsA, cyclosporinA; CypA, cyclophilinA; FACS, fluorescence-activated cell sorting; h, human; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcc, HCV cell culture; IFN, interferon; IFNAR, interferon alpha receptor; IRF, interferon regulatory factor; ISG, interferon stimulated gene; m, murine; MAVS, mitochondrial antiviral signaling protein; MEF, murine embryonic fibroblast; MLT, mouse liver tumor; miR-122, microRNA-122; OCLN, occludin; PMH, primary mouse hepatocytes; SCARB1, scavenger receptor class B type I; TCID50, tissue culture 50% infectious dose; WT, wild-type.

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Received May 14, 2013; accepted July 3, 2013.

Supported by a grant from European Research Council ERC-2011-StG_281473-(VIRAFRONT) and by a grant from the Helmholtz Association SO-024 to T.P.

and for vaccine development. HCV, a plus strand RNA virus of the family *Flaviviridae*, has a narrow host range and efficiently replicates only in humans and chimpanzees.

Viral adaptation and genetic manipulation of mice have emerged as attractive approaches for development of immune-competent HCV small animal models.[2,3] HCV propagation in mouse cells is likely inefficient due to genetic incompatibility of mouse cofactors and/or due to suppression of HCV replication by mouse innate immune defenses. Thus, engineering mice expressing the relevant human genes and/or with deleted mouse restriction factors may permit HCV propagation. Alternatively, adaptation of HCV to use mouse cofactors and evade mouse restriction factors may allow HCV replication in immune-competent mice.

Recent reports have highlighted that SCARB1, CD81, claudin-1 (CLDN1), and occludin (OCLN) represent the minimal cell-type-specific factors required for HCV cell entry.[4] Of these, OCLN and CD81 are used in a species-specific fashion as mouse orthologs do not sustain HCV entry.[4] Remarkably, ectopic expression of human CD81 and OCLN together with mouse SCARB1 and CLDN1 was sufficient to permit HCV cell entry into immune-competent mice.[3] However, these animals do not sustain HCV replication and chronic infection. HCV RNA replication is generally low in mouse cells. Yet which specific host factors determine the low permissiveness of mouse cells to HCV RNA replication and how these determine HCV species-tropism is poorly understood.

Numerous human factors contribute to HCV RNA replication in human cells.[5] Among these, miR-122, a liver-specific human microRNA, has emerged as an important determinant of HCV tissue tropism.[6] In fact, ectopic overexpression of miR-122 in mouse embryonic fibroblasts (MEFs) enhanced replication of subgenomic HCV replicons which was further increased in MEFs with lesions in innate immune signaling pathways.[7] Therefore, lack of human cofactors and innate immune responses apparently limited amplification of HCV replicons in these cells. Finally, Long et al.[8] recently reported that a mouse liver cell line with a selectable HCV replicon, ectopically expressing HCV structural proteins, and either mouse or human apolipoprotein E (ApoE) produced infectious HCV transcomplemented particles (HCV_{TCP}). This indicates that these mouse cells are permissive to the late steps of the HCV replication cycle. Therefore, in this work we explored determinants for complete replication of HCV in mouse liver-derived cells.

Materials and Methods

In Vivo *Immortalization and Generation of Tumor Cell Lines.* Intrahepatic tumors were induced in wild-type (WT), *MAVS*$^{-/-}$, *IRF3*$^{-/-}$, and *IFNAR*$^{-/-}$ mice by retrograde liver transduction with oncogenic plasmids using hydrodynamic injection. For this purpose, mice received 30 μg total plasmid DNA (8 μg pT/KRas-G12V, 8 μg pT3/EF1α-myrAkt1, pT3/EF1α-shRp53, and 6 μg pPGK-SB13) in 0.9% saline at a final volume of 10% of the animal's body weight by tail vein injection within 5 seconds. At the time of tumor development (palpable tumors occurred at 6-10 weeks postinjection), mice were sacrificed and tumors were harvested for subsequent isolation of immortalized cell lines. Isolated tumors were dissected and incubated for 30 minutes at 37°C in RPMI1640+Glutamax (Life Technologies) supplemented with 200 μg/mL of collagenase IA, collagenase IV, and hyaluronidase IV, 300 μg/mL dispase, and 50 μg/mL DNase I (Sigma). Separated cells were purified using a 40-μm strainer, washed once, and were then cultured in DMEM+Glutamax with 10% FCS (Life Technologies) and penicillin/streptomycin (Seromed) at 37°C in 5% CO_2. Cells were cultivated for at least 4 weeks (eight passages) to reduce the content of fibroblasts and subsequently subcloned by limiting dilution. After 2-3 weeks of cultivation, several single-cell clones showing epithelial morphology were selected and expanded for subsequent classification and functional experimentation. Deletions of *MAVS*, *IRF3*, and *IFNAR* genes were confirmed by polymerase chain reaction (PCR). For detailed information on plasmids, see the Supporting Materials and Methods. All mouse

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DOI 10.1002/hep.26626

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

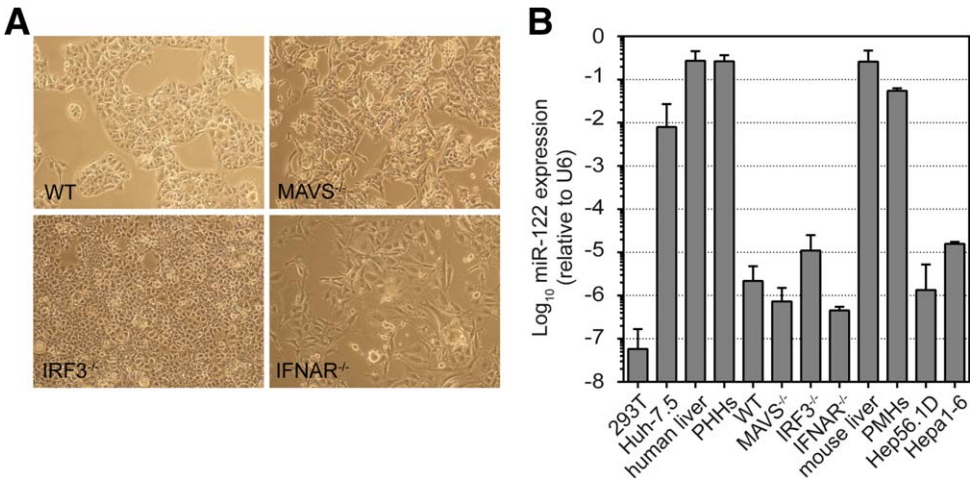

Fig. 1. Expression of miR-122 in immortalized mouse-liver derived cell lines. (A) Cell morphology of subcloned tumor cell lines originating from given mice. (B) Quantification of mature miR-122 expression, relative to U6 small RNA. Mean values and standard deviations of at least three independent experiments are given. Expression of miR-122 in liver biopsies was determined from 15 chronically HCV-infected patients and in liver resections of seven C57BL/6 mice. Mean values ± SD are given.

experimental work was performed at TWINCORE in compliance with animal welfare regulations.

Transient HCV Replication and Infectivity Assay. Methods for *in vitro* transcription and electroporation of HCV RNA are described elsewhere[2] and were employed with the minor modification that 4×10^6 cells were transfected and 4×10^5 cells were seeded onto 6-well culture plates. To inhibit HCV replication or to analyze dependence on cyclophilin A, medium was supplemented with 500 U/mL mouse IFNα-1 (PBL Interferon Source, Piscataway, NJ), 5 μg/mL of the HCV polymerase inhibitor 2′-*C*-methyladenosine (2′CMA) or increasing doses of the cyclophilin inhibitor cyclosporine A (CsA, Sigma) 4 hours posttransfection. Cells and supernatants were harvested at 4, 24, 48, and 72 hours postelectroporation. In order to determine particle release, supernatants were used to infect naive Huh-7.5 cells. Luciferase activity of reporter viruses was analyzed as described elsewhere.[2] Infectivity of WT HCV particles was titrated by a limiting dilution assay ($TCID_{50}$) as described previously.[2] Additional methods are posted as online Supporting Information.

Results

Generation of Novel Mouse Liver-Derived Cell Lines With Distinct Lesions in Innate Immune Signaling by* In Vivo *Immortalization. In human liver cells, HCV replication is sensed by host-derived pattern recognition receptors. These include retinoic acid inducible gene I-like (RIG-I), which signals by way of MAVS to induce translocation of IRF-3 into the nucleus and activation of the IFN-beta promoter. In turn, secreted IFN-beta binds to the type I interferon receptor (IFNAR) and downstream signaling induces gene expression of numerous interferon stimulated genes (ISGs) which establish an antiviral state.[9] Importantly, in human cells HCV interferes with this signaling cascade by way of cleavage of MAVS by the HCV NS3-4A protease.[10] To explore the relevance of this signaling pathway for control of HCV RNA replication in mouse liver-derived cells, we generated stable liver cell lines of WT mice and knockout animals with targeted disruption of *MAVS,*$^{-/-}$, *IRF3,*$^{-/-}$, or *IFNAR*$^{-/-}$ by *in vivo* immortalization as described in detail in the Materials and Methods section. In brief, animals were subjected to hydrodynamic tail vein injection of transposon plasmids for expression of constitutively active Akt1 (myrAkt1), for mutated Kras (Kras-G12V), and a short hairpin RNA (shRNA) targeting mouse p53 (shRp53) together with a plasmid encoding a sleeping beauty transposase (pPGK-SB13) to facilitate genomic integration of the transferred transposons. This treatment led to the growth of palpable liver tumors ~6-10 weeks postinjection. At this timepoint, animals were sacrificed and liver tumors were collected to establish individual cell lines by limiting dilution subcloning. Established mouse liver tumor (MLT) cell lines exhibited robust and sustained cell growth in cell culture (Fig. 1A). Genetic disruption of cognate innate immune signaling molecules was confirmed by PCR (data not shown). Overexpression of myrAkt1 and Kras-G12V induces HCC as well as cholangiocellular carcinomas (CCC), which may originate from hepatocytes.[11,12] Although

hydrodynamic injection mainly targets hepatocytes,[13] we characterized the MLT-MAVS$^{-/-}$ cell line by subcutaneous implantation and subsequent immunohistochemical analysis of induced tumors growing in recipient mice. Using this approach, we confirmed expression of HCC markers cytokeratine 8 (CK8) and CK18, whereas CK19, a marker of cholangiocarcinoma cells, was not expressed (Supporting Fig. S1).

Since miR-122 is an important determinant of HCV tissue tropism and enhances HCV RNA-translation/replication in MEFs,[6,7] we determined endogenous levels of mouse miR-122 in these novel liver cell-derived cell lines (Fig. 1B). The abundance of miR-122 was more than 1,000-fold lower in all generated cell lines compared with primary mouse hepatocytes (PMHs), which expressed high endogenous levels of miR-122 comparable to that observed in primary human hepatocytes and ~3-5-fold lower compared with the level in mouse and human liver.

Disruption of Innate Immune Signaling Permits Efficient miR-122-Dependent HCV RNA Replication in Mouse Liver-Derived Cells. Next we explored the relevance of innate immune signaling and miR-122 expression for HCV RNA replication in these cells by transfecting them with a JFH1 luciferase reporter replicon (Pol +). A defective replicon with an inactivating mutation of the NS5B RNA-dependent RNA-polymerase (Pol −) served as negative control. As expected, we observed efficient amplification of the replication competent replicon in the highly permissive human hepatocarcinoma cell line Huh-7.5, which is evidenced by a rapid increase in luciferase activity from 4 to 24 hours posttransfection and by more than a 5,000-fold higher luciferase activity in cells transfected with the replication-competent replicon compared with those cells receiving the inactive viral RNA (Fig. 2A). In contrast, luciferase activity was low irrespective of which replicon was transfected into the MLT-WT cells (Fig. 2A), indicating that these cells were not permissive for HCV. However, the replication-competent HCV RNA yielded slightly elevated luciferase activity compared with the Pol − viral RNA upon transfection of MLT-IFNAR$^{-/-}$, MLT-IRF3$^{-/-}$, and most notably the MLT-MAVS$^{-/-}$ mouse liver cells (Fig. 2A), suggesting that these cell lines sustain low-level HCV RNA replication.

Remarkably, reconstitution of miR-122 expression within these mouse liver cell lines to a level comparable to PMHs (Fig. 2B) using a lentiviral vector encoding human miR-122[14] greatly enhanced permissiveness of all these mouse liver cell lines to HCV RNA replication. Specifically, peak luciferase activity was increased by more than two orders of magnitude compared to the cognate parental mouse liver cell line (Fig. 2C). Moreover, maximal luciferase activity observed upon transfection of the MLT-WTmiR-122 mouse liver cell line was only ~30-fold lower than in transfected Huh-7.5 cells. Notably, disruption of innate immune signaling further increased permissiveness, since MLT-IFNAR$^{-/-}$miR-122 and MLT-IRF3$^{-/-}$miR-122 cells sustained peak luciferase levels only 5- and 2-fold lower than Huh-7.5 cells, respectively, and as MLT-MAVS$^{-/-}$miR-122 cells displayed comparable luciferase activity to Huh-7.5 cells (Fig. 2C). Consequently, numerous HCV NS5A-expressing cells were detected by immunofluorescence 48 hours after transfection of miR-122-expressing mouse liver cell lines (Fig. 2D). Collectively, these observations indicate that innate immune signaling limits HCV RNA replication in mouse liver-derived cell lines and that reconstitution of miR-122 expression is necessary and in some cells sufficient to permit HCV replicon amplification comparable to the highly permissive human Huh-7.5 cells.

The mature miR-122 sequence is conserved between humans and mice although adjacent RNA sequences are polymorphic (Fig. 3A). Since such polymorphisms may influence processing of microRNA, we constructed retroviral vectors transducing either the human or mouse miR-122 genomic locus including flanking sequences and used these to create MLT-MAVS$^{-/-}$ cell lines expressing human or mouse miR-122. Transduction of MLT-MAVS$^{-/-}$ cells with both vectors resulted in expression of comparable levels of mature miR-122 (Fig. 3B) and enhanced HCV RNA replication to a similar degree (Fig. 3C). Thus, mouse pre miR-122 is capable of sustaining vigorous HCV RNA replication in mouse liver-derived MLT-MAVS$^{-/-}$ cells in the absence of any human cofactors.

HCV RNA Replication in Mouse Liver Cells Depends on Mouse Cyclophilin and Phosphatidylinositol 4-Kinase III alpha. HCV RNA replication depends on numerous human host cell factors.[5] To test if HCV replicates in mouse liver cells using homologous mouse factors, thus reflecting authentic HCV RNA replication pathways, we explored dependence of HCV RNA replication on mouse cyclophilin (mCyp) and mouse phosphatidylinositol 4-kinase III alpha (mPI4Kα), the orthologs of which are crucial for HCV RNA replication in human cells.[15,16] Knockdown of mPI4Kα mRNA in MLT-MAVS$^{-/-}$miR-122 cells reduced HCV RNA replication (Fig. 4A) and treatment of these cells with CsA, an inhibitor of cyclophilins, reduced HCV RNA replication in a dose-dependent fashion (Fig. 4B) in the absence of

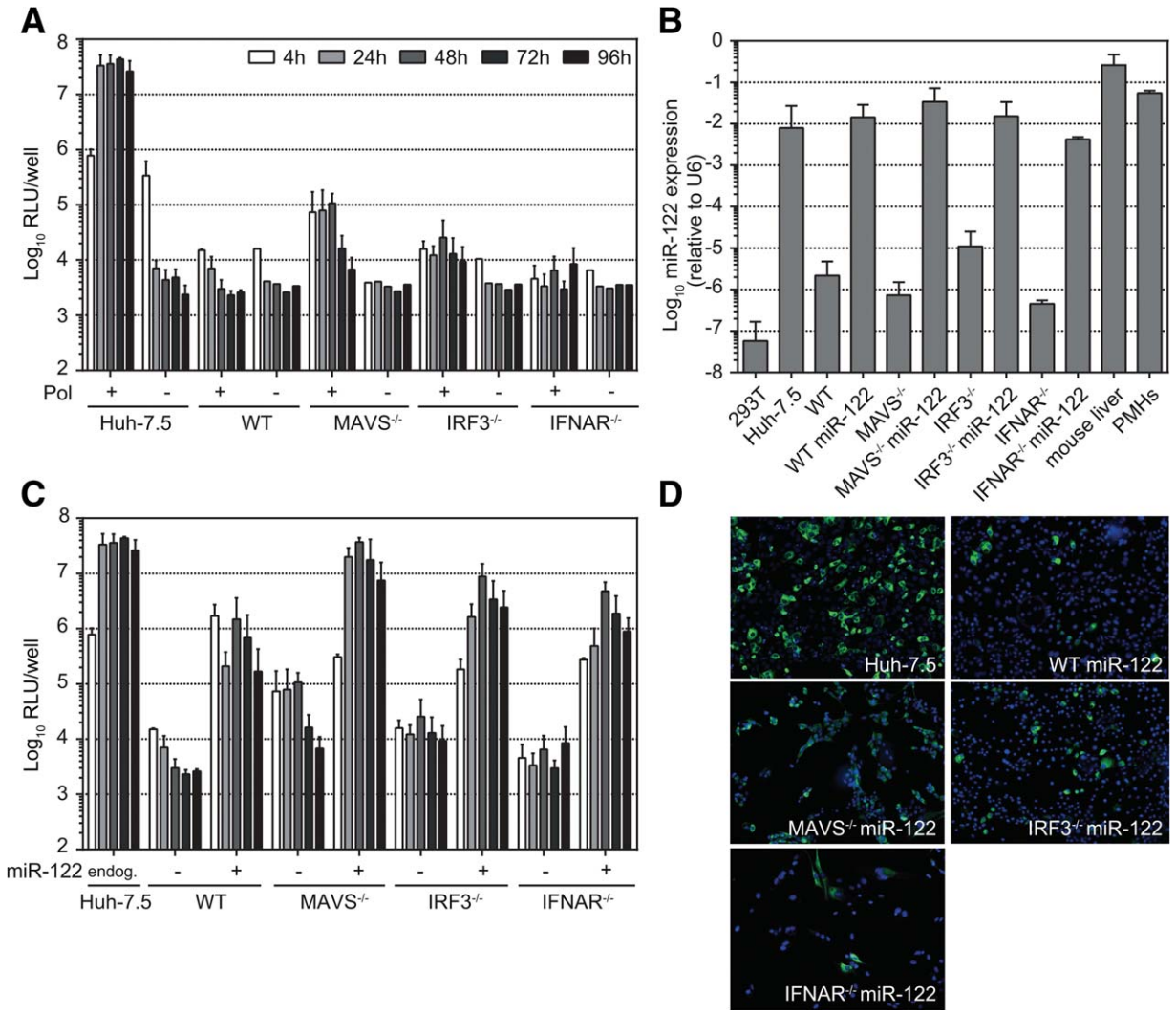

Fig. 2. Disruption of genes involved in innate immune signaling facilitates miR-122-dependent HCV RNA replication. (A) Transient transfection of given cell lines with a subgenomic HCV reporter replicon (Pol +) or a mutant replicon with inactivated NS5B polymerase (Pol −). Transfected cells were lysed at given timepoints and luciferase expression was determined. Mean values ± SD of at least three independent experiments are given. RLU, relative light units. (B) Quantification of mature miR-122 levels in given cell lines. (C) Transient transfection of given cell lines with a subgenomic HCV reporter replicon. (D) Detection of HCV NS5A expression 48 hours posttransfection. Cell nuclei were stained with DAPI. NS5A was detected with the 9E10 monoclonal antibody. Images shown are representatives of three independent experiments.

cytotoxic effects as monitored by an MTT assay (data not shown). Congruently, the number of HCV NS5A expressing cells was reduced dose-dependently (Fig. 4C). Finally, we treated HCV transfected MLT-MAVS$^{-/-}$miR-122 cells with mouse IFNα or the HCV RNA polymerase inhibitor 2′CMA, both of which efficiently repressed HCV RNA replication (Fig. S2). Taken together, these findings indicate that HCV RNA replication depends on mouse orthologs of PI4Kα and Cyp, arguing for a species-independent role of these cofactors for HCV and highlighting that HCV replicates by way of authentic, IFNα and polymerase-inhibitor sensitive pathways in these mouse liver cells.

Mouse Liver-Derived Cells Secrete Infectious HCV Upon Expression of Human or Mouse ApoE. Previous studies have established the importance of SCARB1, CD81, CLDN1, and OCLN for HCV cell entry and of ApoE for release of infectious HCV.[3,4,8] Among these factors at least CD81 and OCLN are used in a species-specific fashion.[4] To test the importance of these factors for HCV cell entry and virus production from MLT-MAVS$^{-/-}$miR-122 cells, we determined their endogenous expression. Moreover, we used lentiviral gene transfer and fluorescent-activated cell sorting (FACS) to create cell populations that express either only human cofactors (i.e., hApoE, hCD81, hOCLN, hSCARB1, hCLDN1 [MLT-

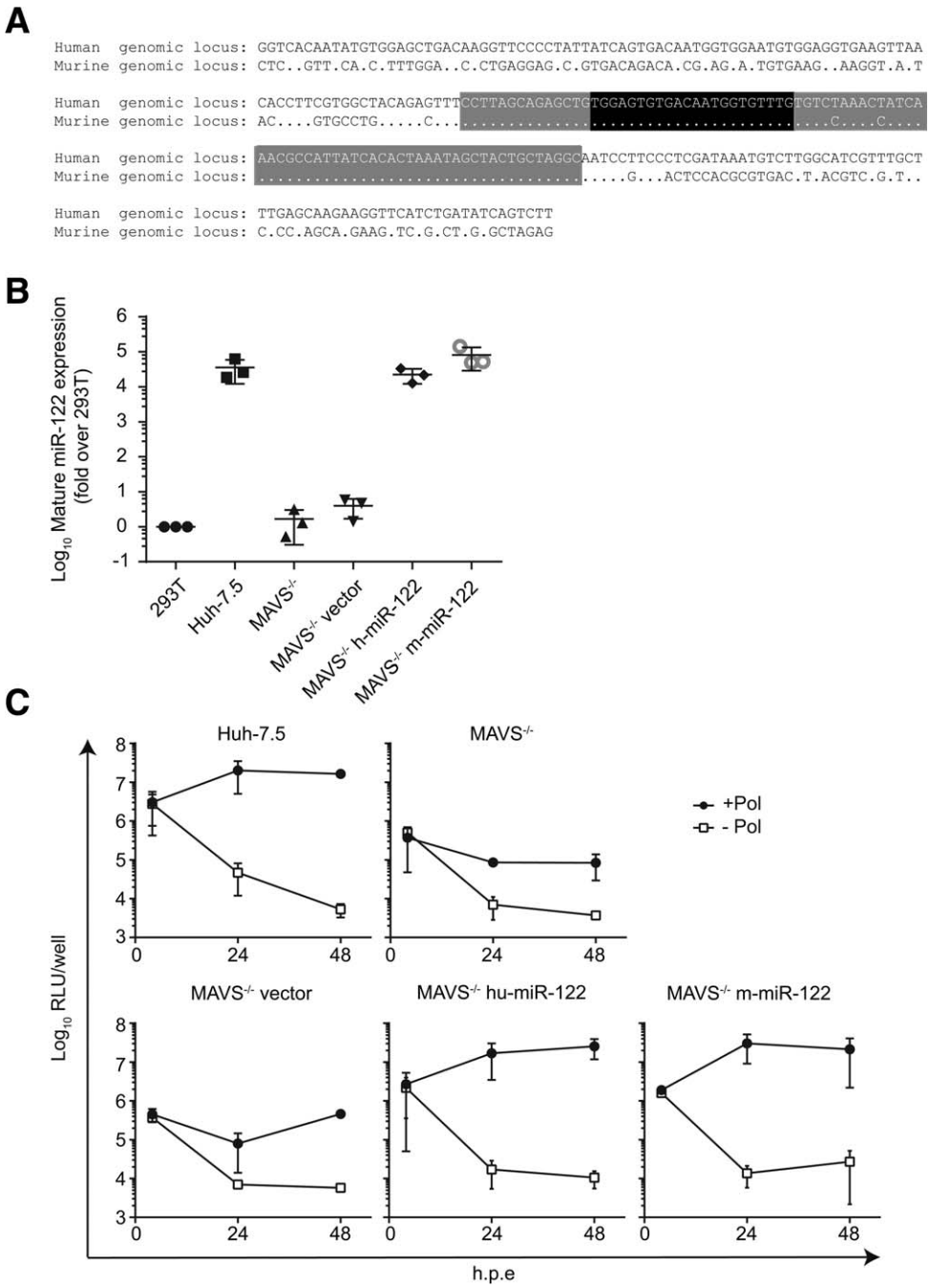

Fig. 3. Human and mouse miR-122 both sustain efficient HCV RNA replication in mouse liver-derived cells. (A) The genomic loci of human and mouse miR-122 are given. Black and gray shading indicate the mature miR-122 and the pre-miR-122 sequence, respectively. The region shown is equivalent to the fragment expressed in the retroviral vector. (B) Expression of mature miR-122 in given cell lines. (C) Transient replication of a subgenomic HCV reporter replicon with or without inactivating mutation in the NS5B polymerase. Mean values ± SD of three independent experiments are given.

MAVS$^{-/-}$miR-122 hhhhh]), or combinations of mouse and human cofactors (i.e., hApoE, hCD81, hOCLN and mSCARB1, mCLDN1 [MLT-MAVS$^{-/-}$miR-122 hhhmm] or hApoE, mCD81, mOCLN, mSCARB1, mCLDN1 [MLT-MAVS$^{-/-}$miR-122 hmmmm]) (Table S1). Finally, we created a mouse liver cell line that expressed miR-122 and only mouse cofactors (i.e., mApoE, mCD81, mOCLN, mSCARB1, mCLDN1 [MLT-MAVS$^{-/-}$miR-122 mmmmm]). Protein expression of these factors was monitored by western blot and FACS assays (Fig. 5A; and Fig. S3), whereas high permissiveness to HCV RNA replication was confirmed by transfection of a subgenomic luciferase replicon (Fig. S4). Since MLT-MAVS$^{-/-}$miR-122

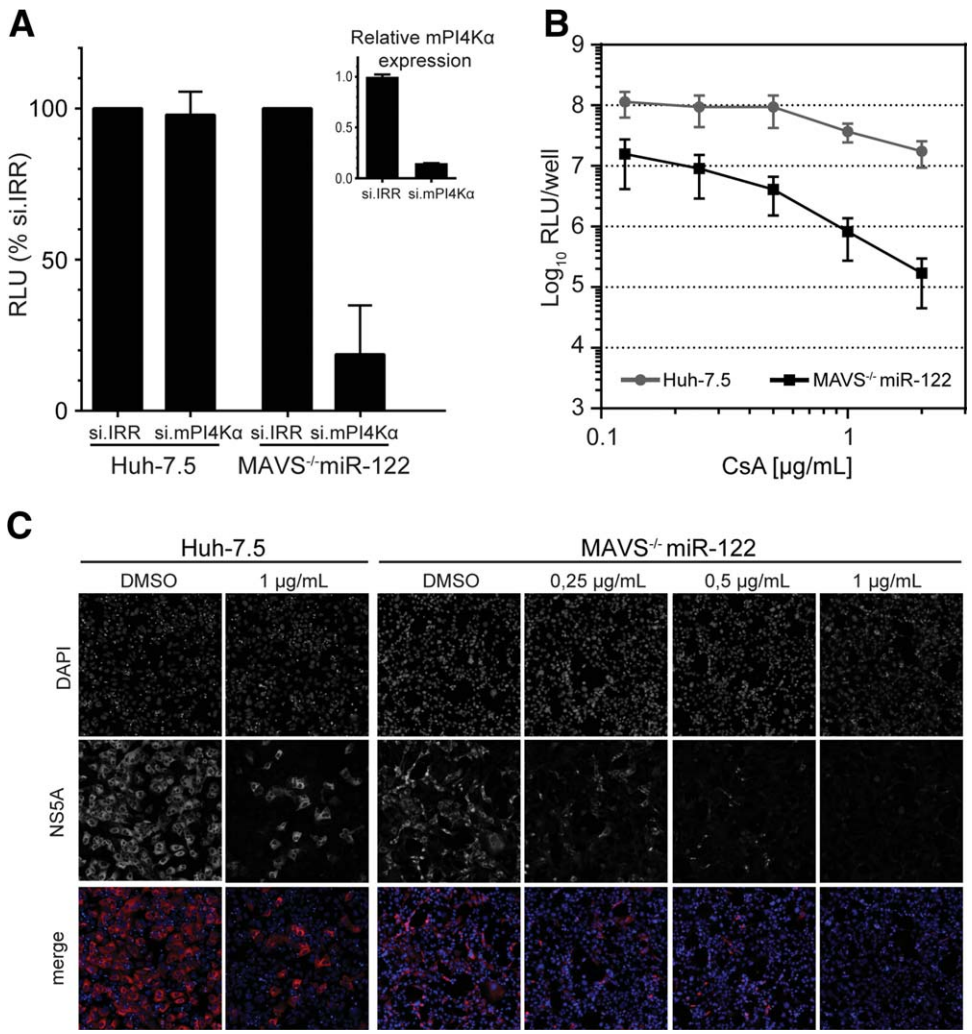

Fig. 4. HCV RNA replication in mouse liver cells depends on mouse cyclophilin and PI4Kα. (A) MLT-MAVS$^{-/-}$miR-122 or Huh-7.5 cells were transfected with a subgenomic reporter replicon and 48 hours postelectroporation cells were reverse-transfected with either control siRNA (si.IRR) or mouse-specific mPI4Kα siRNA. Forty-eight hours after knockdown, RNA replication was assessed by luciferase assay and displayed as percentage of replication with control siRNA. Inset: Knockdown of mPI4Kα in transiently transfected MLT-MAVS$^{-/-}$miR-122 cells was confirmed by determination of relative mRNA levels of murine PI4Kα 48 hours after silencing. Data shown were normalized to control siRNA and mean values ± SD deduced from three independent experiments. (B) MLT-MAVS$^{-/-}$miR-122 or Huh-7.5 cells were transfected with a subgenomic reporter replicon and 4 hours postelectroporation cells were treated with CsA at indicated concentrations. Luciferase activity was assessed 48 hours later. Mean values ± SD were calculated from three independent experiments. (C) Immunofluorescence analysis of cells from (B), using NS5A-specific antibody (9E10).

cells did not express endogenous mApoE (Fig. 5A), we also created derivatives that only restored ApoE expression with either the human or the mouse ortholog in order to examine if ApoE is necessary and sufficient for release of infectious HCV from these cells.

To this end, the above-mentioned cell lines were transfected with a full-length Jc1 luciferase reporter virus RNA,[17] and replication was monitored by luciferase assay (Fig. 5B). Production of infectious viral progeny was quantified by transfer of culture fluid of the transfected cells to naïve Huh-7.5 cells and subsequent determination of luciferase activity in the inoculated cells (Fig. 5C). All tested cell lines sustained efficient HCV RNA replication, although peak luciferase activity was consistently ∼10-fold lower compared to Huh-7.5 cells (Fig. 5B). Therefore, in contrast to subgenomic luciferase replicons (Fig. 2; Fig. S4) RNA replication from full-length reporter virus genomes is less efficient in these mouse liver cells compared to the highly permissive Huh-7.5 cell line. Importantly, once ApoE was expressed, all MLT-MAVS$^{-/-}$miR-122-derived cell lines tested sustained production of infectious reporter virus particles, as evidenced by transduction of luciferase activity to naïve Huh-7.5 cells (Fig. 5C). Moreover, when MLT-MAVS$^{-/-}$miR-122-derived cell lines were transfected with authentic Jc1

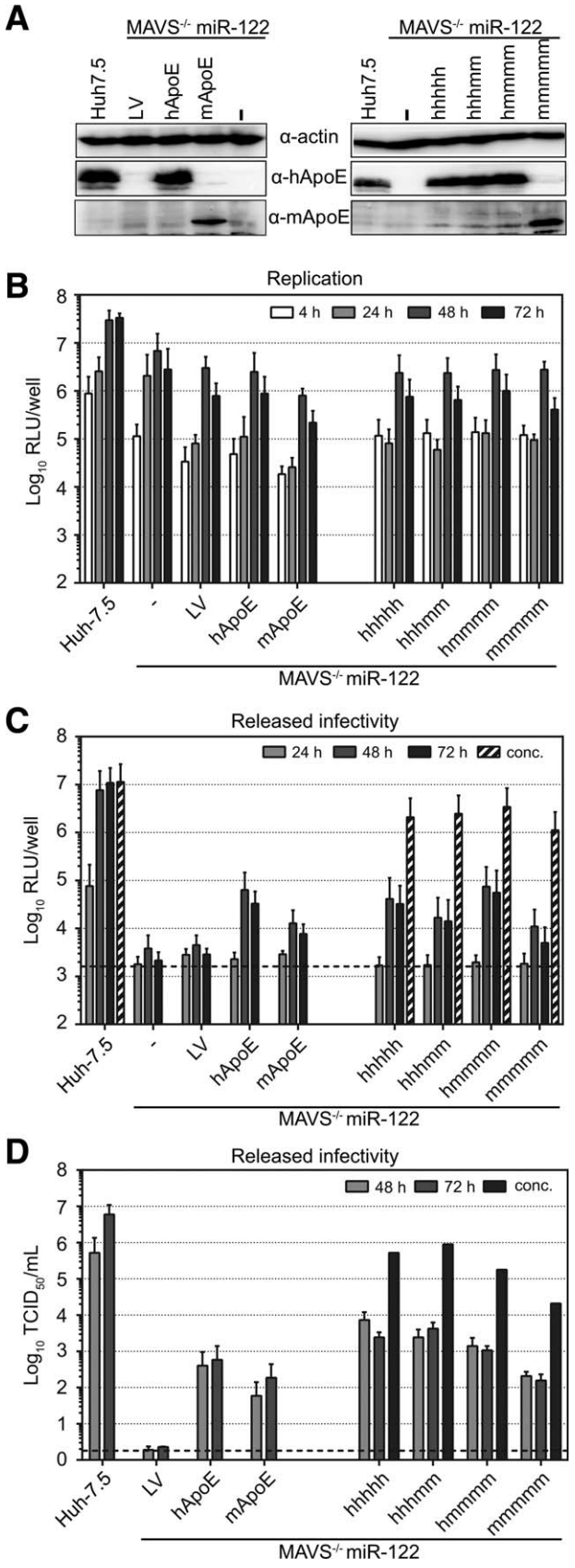

Fig. 5.

RNA, again expression of ApoE was necessary and sufficient for production of infectious progeny (Fig. 5D). Therefore, full-length HCV genomes efficiently replicate in MLT-MAVS$^{-/-}$miR-122-derived cell lines and produce infectious progeny, provided that mouse or human ApoE is expressed.

Expression of Human or Mouse Entry Factors Permits HCV Infection of MAVS$^{-/-}$miR-122/ApoE Cells. We were not able to infect MLT-MAVS$^{-/-}$miR-122/ApoE cells with mouse CD81-adapted HCVcc (Luc-Jc1mCD81;[2]), which may be due to modest endogenous expression of mCD81, mOCLN, and mCLDN1 (Fig. S3 and data not shown). Thus, we stably expressed either complete or minimal sets of human or mouse entry factors (Table S1). Enhanced receptor expression was confirmed by FACS (Fig. S3A,B) and immunoblotting (Fig. S3C). Next, we challenged these cells with Luc-Jc1 or mouse CD81-tropic Luc-Jc1mCD81.[2] Overall, we observed variable efficiencies of infection. Cells expressing complete or minimal sets of human entry receptors (hhhhh and hhhmm) were permissive to both Luc-Jc1 and Luc-Jc1mCD81 (Fig. 6A). Moreover, while Luc-Jc1 was unable to enter cells expressing only mouse receptors (hmmmm or mmmmm), we observed a significant increase in luciferase activity after inoculation of these cells with Luc-Jc1mCD81, suggesting that mouse-tropic HCVcc particles are able to infect MLT-MAVS$^{-/-}$miR-122-derived cells in the absence of human entry factors (Fig. 6A). In line with previous observations, Luc-Jc1mCD81 virus entered hhhhh and hhhmm cells more efficiently than Luc-Jc1, indicating a more potent usage of SCARB1, OCLN, and CD81.[2] Of note, MLT-MAVS$^{-/-}$miR-122/hhhmm cells were more permissive to Luc-Jc1 than MLT-MAVS$^{-/-}$miR-122/hhhhh cells, which may be due to differential expression of CD81 or SCARB1. Importantly, addition of boceprevir during infection reduced luciferase activity to background levels, indicating that

Fig. 5. Expression of human or mouse ApoE permits production of infectious progeny from full-length HCV RNA. (A) Western blot analysis of endogenous or ectopic ApoE expression in cell lysates of given cell lines (compare also Table S1). (−) Nontransduced cells; LV, cells transduced with empty vector. (B) Cells described in (A) were transfected with a full-length HCV reporter virus genome (Luc-Jc1[17]) and replication was monitored by luciferase assay. (C) Supernatants were harvested at given timepoints and used to inoculate naïve Huh-7.5 cells. Viral infectivity was assessed by luciferase assay 72 hours post-infection. Infectivity could be increased by concentrating virus-containing supernatants 20× before inoculation. Mean values ± SD from three independent experiments are shown. (D) Infectious virus titer of culture fluids from cells in (A) transfected with Jc1, determined by limiting dilution ($TCID_{50}$). One of two similar experiments is shown.

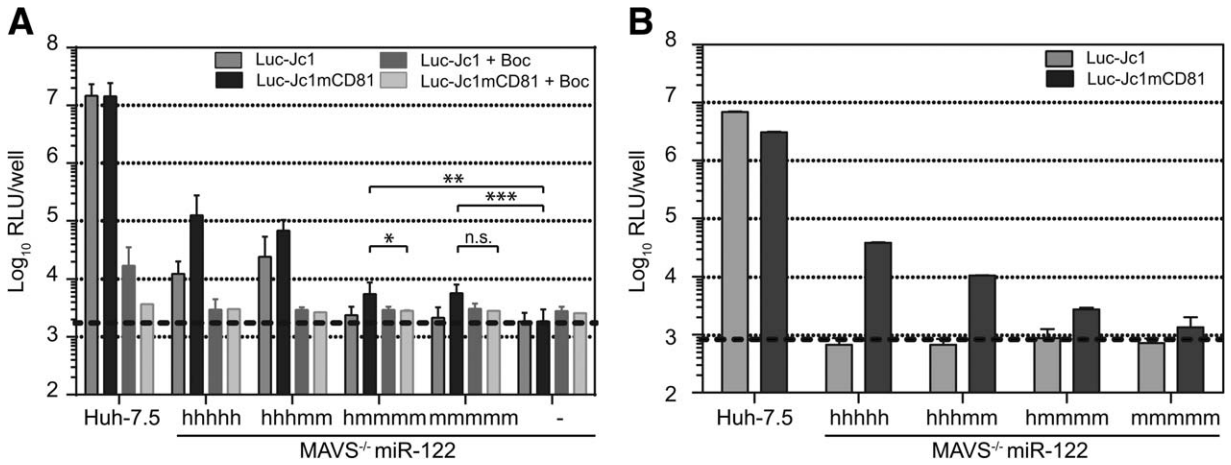

Fig. 6. Expression of human or mouse HCV entry factors permits HCV infection and the entire viral replication cycle for mouse CD81-tropic HCV. (A) Infection of Huh-7.5 or MLT-MAVS$^{-/-}$miR-122 cells stably expressing h/mApoE and human and/or mouse entry factors (compare Table S1) with Luc-Jc1 or mouse CD81-tropic Luc-Jc1mCD81.[2] Virus stocks were normalized to equal infectivity ($TCID_{50}$) prior to inoculation. Boceprevir was added at a dose of 2 μM as indicated. Luciferase activity was determined 48 hours later. For statistical analysis, graphs were plotted to show mean values $\pm$ SD of at least three independent experiments. Statistical analyses were performed using two-sample *t* tests. $P < 0.1$ considered marginally significant (*), $P < 0.05$ considered statistically significant (**), and $P < 0.01$ considered highly significant (***). (B) Release of infectious particles after infection of given cells with Luc-Jc1 or Luc-Jc1mCD81. Following a 6-hour inoculation, cells were washed and cell culture supernatants were harvested 48 hours later to inoculate naive Huh-7.5 cells; infectivity was measured by luciferase assay. A representative of three independent experiments is depicted.

transduction of luciferase reflects authentic HCV cell entry and *de novo* HCV RNA replication. To test if the complete replication cycle can be sustained in these cells, we collected supernatants from these HCV-infected mouse liver-derived cells and used them to inoculate naïve Huh-7.5 cells. Production of infectious particles could not be observed after infection with Luc-Jc1, presumably due to low entry efficiency into mouse liver-derived cells (Fig. 6B). However, infection with Luc-Jc1mCD81 virus led to production of detectable levels of infectious virus as evidenced by transduction of luciferase to naïve Huh-7.5 cells (Fig. 6B). Collectively, these data confirm that MLT-MAVS$^{-/-}$miR-122-derived cells sustain infection by HCVcc and that mouse-tropic HCVcc completes its entire replication cycle in these cells. To explore why HCVcc infection of these mouse liver cells was less efficient compared with Huh-7.5 cells, we challenged these cells with HCVpp harboring GT1a or GT2a glycoproteins or with HCV_{TCP} encasing a subgenomic luciferase replicon.[18] Interestingly, infection of hhhhh and hhhmm cells by HCVpp was only 10-fold lower compared to infection of Huh-7.5 cells, indicating that cell entry was somewhat less efficient in these mouse cells (Fig. S5A). Remarkably, infection by HCV_{TCP} was also only 20-fold lower in the mouse cells compared with Huh-7.5 cells (Fig. S5B). Thus, both experiments highlight that cell entry is somewhat less effective in the mouse liver cells, suggesting that additional entry cofactors are lacking or are not efficiently used. Since infection of the mouse liver cells by full-length HCVcc is lower compared with HCV_{TCP} which carry a subgenomic replicon only, at least for full-length viral RNAs additional replication cofactors may be needed for highly efficient infection and replication.

Discussion

It has been noted previously that inactivation of innate immune signaling facilitates propagation of HCV replicons in MEFs.[7] Our work highlights the relevance of innate immune signaling for restriction of HCV RNA replication in mouse liver-derived cells. This conclusion is based on two pieces of evidence. First, transient RNA replication of replicons is modestly elevated in IRF3$^{-/-}$ and MAVS$^{-/-}$ mouse liver cells compared to cells originating from WT animals (Fig. 2). Second, after reconstitution of miR-122 expression the replication level of HCV was consistently higher in all cell lines from knockout animals compared to the cells from WT mice (Fig. 2). Although HCV interferes with innate immune signaling in human cells by way of cleavage of MAVS[10], and it was reported that also mouse MAVS can be cleaved by the HCV protease[19], it is unclear if the efficiency and kinetics of MAVS cleavage are comparable. Thus, reduced MAVS cleavage by HCV in mouse liver cells may be responsible for restricted HCV RNA

replication in these cells. The novel mouse liver cell lines described in this work offer the opportunity to test if differential cleavage of MAVS orthologs contributes to HCV species tropism. Moreover, our results suggest that mice with targeted lesions of innate immune signaling in liver cells should provide a favorable environment for HCV propagation.

Remarkably, reconstitution of miR-122 expression was sufficient to render MLT-MAVS$^{-/-}$ cells highly permissive to HCV RNA replication. In fact, permissiveness to an HCV JFH1-replicon was indistinguishable from one of the highly HCV permissive human Huh-7.5 cells (Fig. 2; Fig. S4). We cannot exclude that the specific immortalization approach involving transformation through constitutively active Akt1 and Kras combined with knock-down of p53 is in part responsible for this. Furthermore, *in vivo* immortalization and *in vitro* cultivation presumably led to a dedifferentiated phenotype, characterized by low miR-122 and ApoE levels. Nevertheless, these results indicate that mouse liver cells can support vigorous HCV RNA replication in the absence of any human cofactors (Fig. 3C). Given that mature mouse miR-122 is highly expressed in mouse livers (Fig. 2), and since the mouse miR-122 supported HCV replication in mouse liver cells as efficiently as the human ortholog (Fig. 3), we consider it unlikely that HCV replication in mouse liver is limited by availability of miR-122. Collectively, these findings raise the hope that establishment of robust HCV RNA replication *in vivo* may require only little genetic manipulation of mice, possibly not involving ectopic expression of human replication cofactors.

Clearly, for construction of fully HCV permissive mice it is crucial that mouse liver cells not only permit efficient RNA replication but also virus production and cell entry. Using the MLT-MAVS$^{-/-}$miR-122 cells we show that reconstitution of ApoE expression is necessary and sufficient to allow production of infectious HCV progeny from full-length genomes (Fig. 5). This observation underscores the important role of ApoE during virus production and extends the findings of Long et al.,[8] who recently reported that trans-complemented HCV particles can be produced in a stable mouse replicon cell line. Similar to those authors, we did not find a striking difference between HCV usage of human or mouse ApoE, suggesting that endogenous ApoE expression in mouse liver should sustain HCV assembly. However, the efficiency of virus production from MLT-MAVS$^{-/-}$miR-122-derived cells was generally lower compared to human Huh-7.5 cells. While this may suggest that other mouse assembly cofactors are not efficiently used by HCV, it is also possible that attenuated replication of full-length HCV in mouse liver cells indirectly reduced virus production. In fact, human liver cells that are also less permissive for HCV RNA replication than Huh-7.5 cells (e.g., HepG2 and HuH6 cells) produce much lower levels of infectious virus.[14,20]

Regarding cell entry, expression of the complete or minimal set of absolutely essential human HCV entry cofactors rendered MLT-MAVS$^{-/-}$miR-122 cells permissive to HCVcc infection (Fig. 6). Notably, infection of these mouse cells was more efficient for the mouse-tropic Jc1 variant[2] although both viruses displayed comparable infectiousness on Huh-7.5 cells (Fig. 6). However, since upon dilution of these virus stocks Luc-Jc1mCD81 was also more infectious than Luc-Jc1 in Huh-7.5 cells (data not shown) we do not believe that this difference is due to increased use of additional mouse-derived entry cofactors by the mouse-tropic Luc-Jc1mCD81. Rather, this difference is likely due to the overall increased efficiency of CD81 usage of the adapted HCV variant.[2] It is currently unclear why HCVcc propagation is less efficient in the mouse liver-derived cell lines compared to Huh-7.5. However, our HCVpp and HCV$_{TCP}$ experiments suggest that efficiency of cell entry is somewhat lower in the mouse liver cells. Thus, other known HCV entry cofactors like the LDL receptor, epidermal growth factor receptor (EGFR), or Niemann-Pick C1-Like-1 (NPC1L1)[21] may contribute to species-specific HCV cell entry or may be expressed at only low levels. Moreover, at least for HCVcc particles carrying a full-length viral RNA, the somewhat lower permissiveness of the MLT-MAVS$^{-/-}$miR-122 derived cells for full-length HCV RNA replication is likely also responsible, as infection by HCV$_{TCP}$ which encase a subgenomic replicon was much more robust. Notably, in the case of Luc-Jc1mCD81 we observed a low level of luciferase expression upon inoculation of MAVS$^{-/-}$miR-122 cells expressing only mouse-derived HCV entry factors (Fig. 6). It is currently unclear if the comparatively low infection rate is due to insufficient adaptation to mouse receptor usage or due to insufficient abundance of the key HCV entry factors in these cells. Nevertheless, these results suggest that HCVcc particles with these three mouse-adaptive changes[2] may indeed enter mouse liver cells in the absence of human entry factors *in vivo*. Finally, we observed that the highly efficient mouse-tropic Luc-Jc1mCD81 virus completed the entire replication cycle including cell entry, RNA replication, and virus assembly in MLT-MAVS$^{-/-}$miR-122-derived cells. This observation raises the hope that

these cells could be used to further adapt HCV to more efficiently propagate in mouse liver cells. Ultimately, this approach or genetic manipulation may help to develop an urgently needed immune-competent and predictive small animal model for HCV.

Acknowledgment: We thank Takaji Wakita for the gift of the JFH1 isolate, Jens Bukh for the J6 strain, Charles Rice for Huh-7.5 cells and 9E10 antibody, Matthew Evans for the miR-122 expression construct, and Timothy Tellinghuisen for providing 2′CMA. We also thank Alex Schambach for providing retroviral vectors and all members of the Institute for Experimental Virology at TWINCORE for helpful comments and discussions.

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