

**Figure 5.** Effect of BCAA on HCV replication in cells in low-amino-acid medium. (A) Effect of BCAA on H77S.3/GLuc2A replication in Huh-7.5 cells. (B) Mx-1 expression in H77S.3/GLuc2A-transfected Huh-7.5 cells supplemented with BCAA. (C) Viability of Huh-7.5 cells. (D) Effect of BCAA on JFH-1 replication continuously infecting Huh-7 cells. (E) Mx-1 expression in continuously JFH-1-infecting Huh-7 cells supplemented with BCAA. (F) Viability of Huh-7 cells.

to these metabolic aspects, recent reports have shown that mTORC1 participates in IFN signaling and antiviral defense responses,<sup>9,10</sup> although the precise signaling pathway has not yet been clarified. In the present study, we evaluated mTORC1 signaling in CH-C livers using gene expression profiling of 91 patients (Figure 1, Supplementary Table 1). We observed a significant negative correlation between plasma Fischer's ratio and hepatic expression of BCAT1, an important catalytic enzyme of BCAA (Figure 1A). Moreover, BCAT1 expression was correlated positively with PDCD4 expression, which in turn is regulated negatively by pS6K at the transcriptional level (Figure 1D).<sup>16</sup> Thus, the expression of BCAT1 appears to be a negative indicator of mTORC1 signaling in the liver, and the plasma Fischer's ratio is partially reflected by mTORC1 signaling in the liver and muscle.

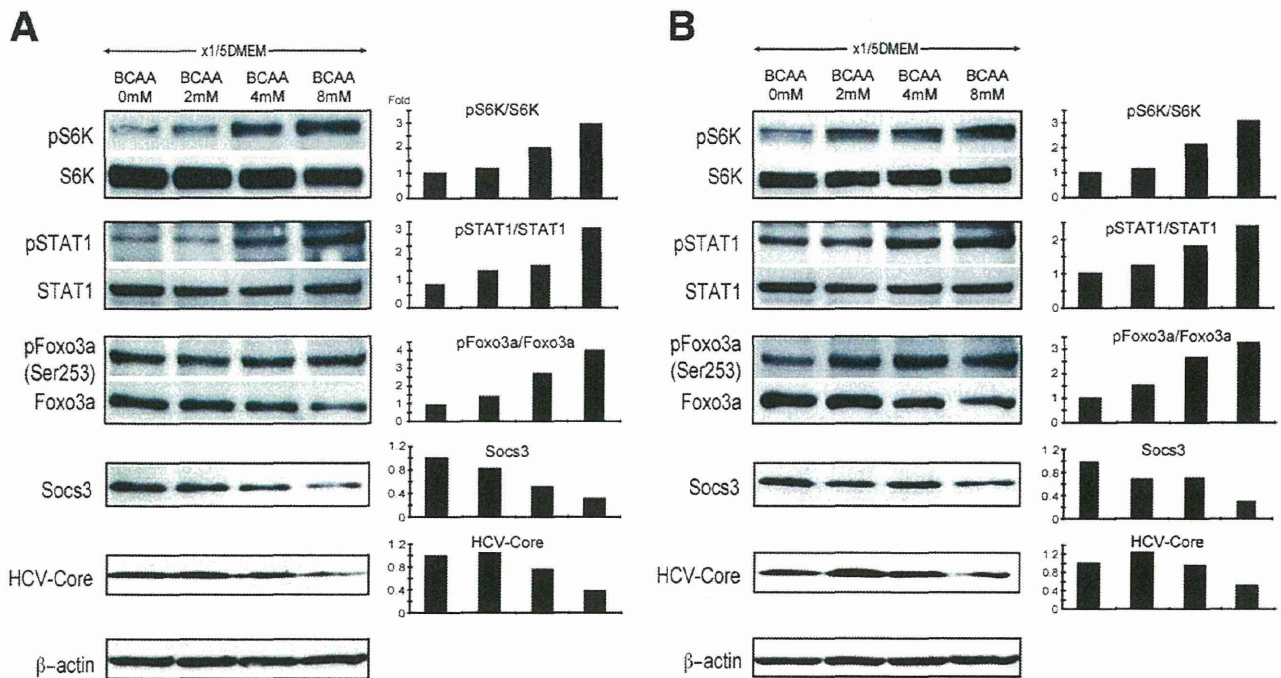
Interestingly, the expression of c-myc was correlated significantly with BCAT1 (Figure 1C) as reported previously.<sup>15</sup> Several studies observed up-regulated c-myc expression in advanced stages of CH-C<sup>19</sup> but, on the other hand, c-myc recently was shown to be a target of

mTORC1 in hepatic cells.<sup>17</sup> The existence of a feedback mechanism between c-myc and mTORC1 signaling to maintain liver homeostasis (Figure 1E) is plausible, although the precise mechanisms need to be confirmed.

Impaired mTORC1 signaling is suggested to affect the IFN- $\alpha$ -induced signaling pathway. To address this, the relationship between mTORC1 and IFN signaling was assessed using a cell culture system. In low-amino-acid medium ( $\times 1/5$ ,  $\times 1/30$ , and  $\times 1/100$  DMEM), expression of pSTAT1 was decreased substantially, correlating with the impaired mTORC1 signaling represented by decreased p-mTOR and pS6K expression in Huh-7 cells (Figure 2A).

The relationship between mTORC1 and IFN signaling was confirmed further by the knock-down experiment of Raptor, a specific subunit of mTORC1 (Figure 2B), although a more precise analysis should be performed to confirm this relationship. Importantly, when Huh-7 cells were stimulated by IFN- $\alpha$ , pSTAT1 induction was repressed significantly in low-amino-acid medium ( $\times 1/5$  DMEM) or in Raptor knocked-down conditions (Figure 2C). It therefore could be speculated that IFN treat-

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**Figure 6.** Expression of S6K, STAT1, Foxo3a, Socs3, and HCV core in H77S.3/GLuc2A-transfected Huh-7.5 cells or continuously JFH-1-infected Huh-7 cells supplemented with BCAA.

ment of patients with liver malnutrition and impaired mTORC1 signaling would lead to reduced induction of ISGs. Importantly, BCAA was able to restore impaired IFN signaling through increased binding of ISGF3 $\gamma$  to its targets (Figure 2D–F).

Besides cross-talk of mTORC1 and IFN signaling, we revealed that Foxo3a also is involved in the IFN inhibitory pathway. In low-amino-acid medium, expression of pFoxo3a (ser253) was decreased substantially whereas that of Socs3 was increased. A decreased pFoxo3a/Foxo3a ratio indicates nuclear accumulation of Foxo3a before activation of its target genes, and this was confirmed by immunofluorescent staining (Figure 3C). The expression of Foxo3a was significantly positively correlated with that of Socs3 in CH-C liver (Figure 3F). These findings prompted us to identify a putative FBE in the Socs3 promoter region (Figure 4A). In fact, Socs3 promoter reporter activity was activated by overexpression of Foxo3a, and mutation of FBE impaired Foxo3a-dependent Socs3 promoter activation. Conversely, induction of Socs3 was not observed when expression of Foxo3a was knocked down by siRNA in low-amino-acid medium. Socs3 induction in low-amino-acid medium was owing to increased binding of Foxo3a to the FBE, which was confirmed by ChIP (Figure 4D). Therefore, in addition to impaired mTORC1 signaling, the Foxo3a-mediated Socs3 IFN inhibitory pathway might be involved in impaired IFN signaling in patients with liver malnutrition (Figure 4E).

Finally, we examined whether BCAA could restore impaired IFN signaling and inhibit HCV replication in cells

under conditions of malnutrition. Importantly, BCAA could repress replication of the recombinant genotype 1a-derived HCV, H77S.3/GLuc2A, in a dose-dependent manner (Figure 5A). H77S.3/GLuc2A RNA produces infectious virus<sup>14</sup> and, therefore, the results indicate that BCAA might act on a naive HCV infection. Moreover, BCAA inhibited JFH-1-infected Huh-7 cells in which JFH-1 continuously was infecting in a dose-dependent manner. These results indicate that BCAA had an inhibitory effect on either naive or persistent HCV infection irrespective of genotypes (1a and 2a). Consistent with these results, BCAA induced the expression of pSTAT1 and Mx protein in a dose-dependent manner, and repressed Socs3 expression through increasing the ratio of pFoxo3a (ser243) to Foxo3a in a dose-dependent manner (Figures 5 and 6). Therefore, BCAA potentially could restore impaired IFN signaling and inhibit HCV replication in a CH-C state of malnutrition.

In conclusion, we addressed the clinical significance of the nutritional state of the liver on the treatment response of Peg-IFN and RBV combination therapy for CH-C. Although further studies are required to fully define the precise mechanisms underlying mTOR and IFN signaling, we showed that plasma values of Fischer's ratio are a useful nutritional parameter associated with treatment response. Fischer's ratio reflects mTORC1 signaling in the liver, which is correlated with IFN signaling and related to Socs3 IFN inhibitory signaling through Foxo3a. The potential usefulness of BCAA for the augmentation of IFN signaling could suggest a new therapeutic application for advanced-stage CH-C.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: 10.1053/j.gastro.2011.03.051.

## Appendix A

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Received October 19, 2010. Accepted March 18, 2011.

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*Acknowledgments*

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The authors thank Mina Nishiyama and Yuki Hatayama for excellent technical assistance.

*Conflicts of interest*

The authors disclose no conflicts.



oxycholate, 1.0 mmol/L EDTA, 1.0 mmol/L Tris-HCl [pH 8.1]) and Tris-EDTA buffer. Immunoprecipitated chromatin fragments were eluted with elution buffer (1% sodium dodecyl sulfate, 100 mmol/L NaHCO<sub>3</sub>, 10 mmol/L dithiothreitol), and reverse cross-linked by incubating for 6 hours at 65°C in elution buffer containing 200 mmol/L NaCl. DNA fragments were purified and quantified by real-time detection PCR with primers for putative ISRE in the 2'5'OAS promoter region (forward, 5'-AAA TGC ATT TCC AGA GCA GAG TTC AGA G-3', reverse, 5'-GGG TAT TTC TGA GAT CCA TCA TTG ACA GG-3') or putative FBE in the Socs3 promoter region (forward, 5'-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG -3', reverse, 5'-AGC GGA GCA GGG AGT CCA AGT C -3'). Values were normalized by the measurement of input DNA.

#### *pH77S.3/GLuc2A*

pH77S.2 is a modification of pH77S<sup>2</sup> containing an additional mutation within the E2 protein (N476D in the polyprotein) that promotes infectious virus yields from RNA-transfected cells (Yi et al, unpublished data). To monitor replication, the GLuc sequence, fused at its C terminus to the foot-and-mouth disease virus 2A autoprotease, was inserted between p7 and NS2 of pH77S.2 (Supplementary Figure 4). To insert the GLuc-coding sequence between p7 and NS2 in pH77S.2, followed by the foot-and-mouth disease virus 2A protein-coding sequence, Mlu I, EcoR V, and Spe I restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the Mlu I and EcoR V sites of the modified plasmid after PCR amplification using the primers: 5'-ATA ATA TTA *CGC GTA TGG GAG TCA AAG TTC TGT TTG CC-3'* (sequence corresponding to the N-terminal GLuc is italicized and that corresponding to Mlu I is underlined) and 5'-ATA AAT AGAT ATC GTC ACC ACC GGC CCC CTT GAT CTT-3' (C terminal GLuc is italicized and EcoR V is underlined). A DNA fragment encoding the 17 amino acids of the foot-and-mouth disease virus 2A protein was generated by annealing the following complementary oligonucleotides: 5'-ATA TGA TAT CAA CTT TGA CCT TCT CAA GTT GGC CGG CGA CGT

CGA GTC CAA CCC AGG GCC CAC TAG CAT AT-3' and 5'-ATA TGC TAG TGG GCC CTG GGT TGG ACT CGA CGT CGC CGG CCA ACT TGA GAA GGT CAA AGT TGA TAT CAT AT-3' (underlined sequences indicate EcoR V and Spe I sites). The annealed oligonucleotides were digested by both restriction enzymes and the product inserted into the corresponding sites of pH77S.2 containing GLuc to generate pH77S.2/GLuc2A. Q41R is a cell-culture adaptive mutation within the NS3 protease domain of pH77S. Because it is not essential for production of infectious virus (Yi et al, unpublished data), pH77S.2 and pH77S.2/GLuc2A constructs underwent this mutation by site-directed mutagenesis of a PCR fragment spanning the Afe I and BsrG I sites to replace Gln<sub>41</sub> with wild-type Arg. The resulting plasmids (pH77S.2/R41Q and pH77S.2/GLuc2A/R41Q) were redesignated pH77S.3 and pH77S.3/GLuc2A, respectively.<sup>3,4</sup> GLuc has several advantages over other luciferase reporter enzymes in that it is smaller and allows more sensitive detection than either firefly or Renilla luciferase.<sup>3,4</sup> In addition, a signal sequence directs its secretion into cell-culture media, allowing real-time dynamic measurements of GLuc expression without the need for cell lysis. H77S.3/GLuc2A RNA produces infectious virus, although with lower efficiency than H77S.3 RNA (10-fold less).

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# Development of Mouse Hepatocyte Lines Permissive for Hepatitis C Virus (HCV)

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## Abstract

The lack of a suitable small animal model for the analysis of hepatitis C virus (HCV) infection has hampered elucidation of the HCV life cycle and the development of both protective and therapeutic strategies against HCV infection. Human and mouse harbor a comparable system for antiviral type I interferon (IFN) induction and amplification, which regulates viral infection and replication. Using hepatocytes from knockout (ko) mice, we determined the critical step of the IFN-inducing/amplification pathways regulating HCV replication in mouse. The results infer that interferon-beta promoter stimulator (IPS-1) or interferon A receptor (IFNAR) were a crucial barrier to HCV replication in mouse hepatocytes. Although both IFNARko and IPS-1ko hepatocytes showed a reduced induction of type I interferons in response to viral infection, only IPS-1<sup>-/-</sup> cells circumvented cell death from HCV cytopathic effect and significantly improved J6JFH1 replication, suggesting IPS-1 to be a key player regulating HCV replication in mouse hepatocytes. We then established mouse hepatocyte lines lacking IPS-1 or IFNAR through immortalization with SV40T antigen. Expression of human (h)CD81 on these hepatocyte lines rendered both lines HCV-permissive. We also found that the chimeric J6JFH1 construct, having the structure region from J6 isolate enhanced HCV replication in mouse hepatocytes rather than the full length original JFH1 construct, a new finding that suggests the possible role of the HCV structural region in HCV replication. This is the first report on the entry and replication of HCV infectious particles in mouse hepatocytes. These mouse hepatocyte lines will facilitate establishing a mouse HCV infection model with multifarious applications.

**Citation:** Aly HH, Oshiumi H, Shime H, Matsumoto M, Wakita T, et al. (2011) Development of Mouse Hepatocyte Lines Permissive for Hepatitis C Virus (HCV). *PLoS ONE* 6(6): e21284. doi:10.1371/journal.pone.0021284

**Editor:** Jacques Zimmer, Centre de Recherche Public de la Santé (CRP-Santé), Luxembourg

**Received:** May 13, 2011; **Accepted:** May 24, 2011; **Published:** June 22, 2011

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**Funding:** This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research), the Ministry of Health, Labor, and Welfare of Japan, and the Hokkaido University Leader Development System in the Basic Interdisciplinary Research Areas (L station). Supports from Mitsubishi Foundation, Mochida Foundation, NorthTec Foundation Waxman Foundation and Yakult Foundation are gratefully acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of mortality and morbidity throughout the world infecting around 3.1% of the world's population [1]. The development of much needed specific antiviral therapies and an effective vaccine has been hampered by the lack of a suitable small animal model. The determinants restricting HCV tropism to human and chimpanzee hosts are unknown. Replication of HCV strain JFH1 has been demonstrated in mouse cells only upon antibody selection [2], highlighting the very limited replication efficiency. Human CD81 and occludin have been implicated as important entry receptors for retrovirus particles bearing HCV glycoproteins, HCV pseudoparticles (HCVpp), into NIH3T3 murine cells [3]. However, HCV infection, spontaneous replication and particle production by mouse cells have not yet been reported.

In mammalian cells, the host detects and responds to infection by RNA-viruses, including HCV, by primarily recognizing viral RNA through several distinct pathogen recognition receptors (PRRs), including the cell surface and endosomal RNA sensors Toll-like receptors 3 and 7 (TLR3 and TLR7), and the cytoplasmic RNA sensors retinoic acid-inducible gene I (RIG-I

and melanoma differentiation associated gene 5 (MDA5) [4]. The detection of virus infection by these receptors leads to the induction of interferons (IFNs) and their downstream IFN-inducible anti-viral genes through distinct signaling pathways [5]. Type I IFN is an important regulator of viral infections in the innate immune system [6]. Another type of IFN, IFN-lambda, affects the prognosis of HCV infection, and its response to antiviral therapy [7,8].

Mutations impairing the function of the RIG-I gene and the induction of IFN were essential in establishing HCV infectivity in human HuH7.5 cells [9]. Similarly, the HCV-NS3/4a protease is known to cleave IPS-1 adaptor molecule, inducing further downstream blocking of the IFN-inducing signaling pathway [10]. These data clearly demonstrate that the host RIG-I pathway is crucial for suppressing HCV proliferation in human hepatocytes. Using a similar strategy, we investigated whether suppressing the antiviral host innate immune system conferred any advantage on HCV proliferation in mouse hepatocytes. We examined the possibility of HCV replication in mice lacking the expression of key factors that modulate the type I IFN-inducing pathways. Only gene silencing of the IFN receptor (IFNAR) or IPS-1 was sufficient to establish spontaneous HCV replication in



mouse hepatocytes. To establish a cell line permissive for HCV replication, which is required for further *in vitro* studies of the HCV life cycle in mouse hepatocytes, we immortalized IFNAR- and IPS-1-knockout (ko) mice hepatocytes with SV40 T antigen. Upon expression of the human (h)CD81 gene, these newly established cell lines were able to support HCV infection for the first time in mouse hepatocytes. Viral factors required for HCV replication in mouse hepatocytes were also analyzed.

**Results**

**IPS-1-mediated IFN signaling is important for HCV replication in mouse hepatocytes**

As a first step in establishing HCV infection in mice, we tested the susceptibility of mouse hepatocytes to persistent expression of HCV proteins after RNA transfection. *In vitro* transcribed chimeric J6JFH1 RNA, in which the HCV structural and non-structural regions were from J6 and JFH1 isolates respectively, was transfected into hepatocytes from wild-type mice. We used a highly sensitive polyclonal antibody derived from HCV-patient serum for the detection of HCV proteins. No HCV proteins were detected five days after transfection (Fig. 1 A), suggesting that wild-type mouse hepatocytes were unable to maintain HCV replication. We then tried to find and block the pathway used by mouse hepatocytes for the detection of viral-RNA and the induction of IFN response. Mouse hepatocytes did not show the expression of either TLR3 or TLR7 as detected by RT-PCR, unlike IPS-1 and RIG-I which was fairly detected (Fig. S1), suggesting that the cytoplasmic RIG-I/IPS-1 pathway is the main pathway utilized by mouse hepatocytes for the detection of RNA viruses. We then checked the susceptibility of hepatocytes from TICAM-1ko, IPS-1ko and IFNARko mice to the prolonged expression of HCV proteins (Fig. 1B–D). Only IPS-1- and IFNARko mouse hepatocytes showed expression of J6JFH1 proteins five days after transfection (Fig. 1), indicating the importance of impaired IPS-1 and/or IFNAR receptors for HCV persistence. Similarly, the detection of the J6JFH1-RNA in transfected hepatocyte lines from various knockout mice showed higher levels in IPS-1 or IFNAR knockout cells compared to TICAM-1knockout cells in which a rapid decline of J6JFH1-RNA levels was noticed similar to the non-replicating control JFH1GND construct (Fig. S2). These data

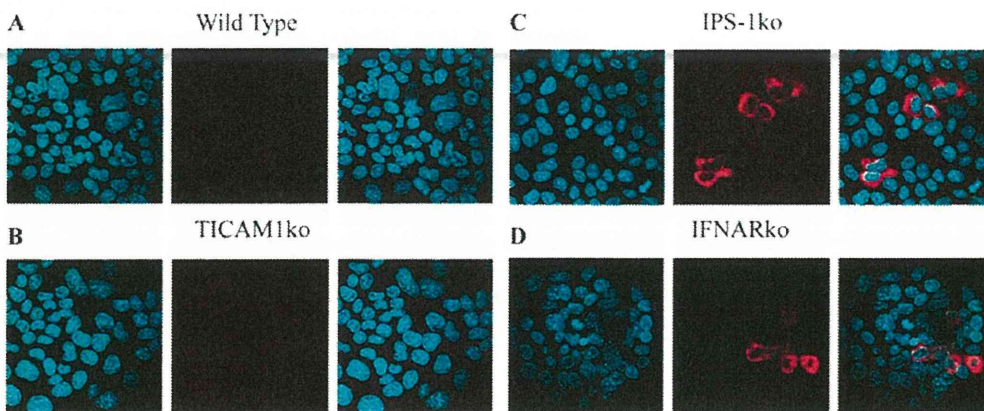
clearly suggest that the RIG-I/IPS-1 but not TLR3/TICAM-1 is the main pathway utilized for the detection of HCV-RNA and the induction of anti-viral immune response in mouse hepatocytes. Its suppression significantly improves HCV replication in mouse hepatocytes.

**Establishment and characterization of immortalized mouse hepatocyte cell lines lacking expression of the IFNAR or IPS-1 gene**

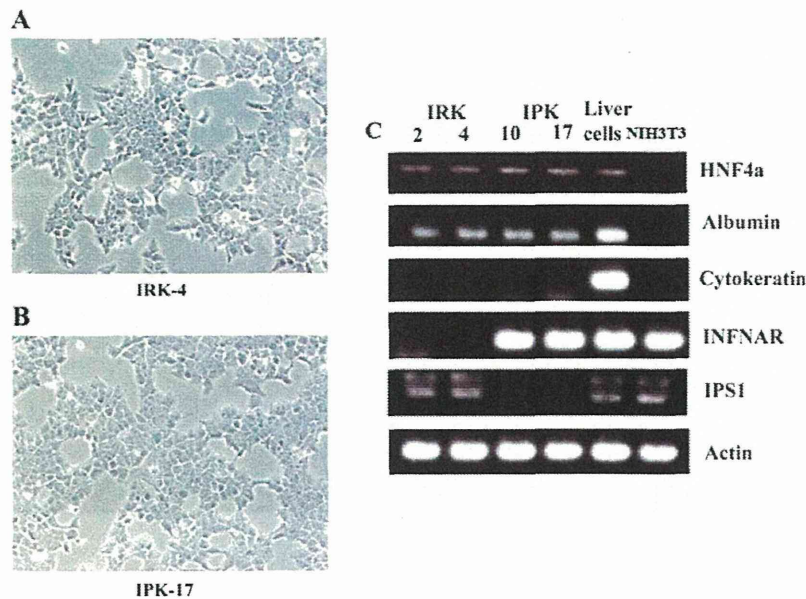
We further established mouse hepatocyte lines with disrupted IFNAR or IPS-1 genes through immortalization with SV40T antigen, and used these cell lines to study factors required for the HCV life cycle. Hepatocytes were transduced with SV40T-expressing lentivirus vectors. Six weeks after transduction, hepatocytes transduced with SV40T showed continuous proliferation and clonally proliferating hepatocyte lines were selected. SV40T-immortalized IFNARko and IPS-1ko clones were designated IRK (Fig. 2 A) and IPK (Fig. 2 B), respectively. 20 IRK and 19 IPK clones were picked up, of which IRK clones 2 and 4 (IRK2 and IRK4) and IPK clones 10 and 17 (IPK10 and IPK17) were most closely related to primary mouse hepatocytes in term of differentiation (Fig. 2 C) and were used in the following experiments. Expression of SV40T was confirmed by RT-PCR analysis (data not shown). IRK2, IRK4, IPK10 and IPK17, but not the non-hepatocytic NIH3T3 cells, displayed albumin and hepatocyte nuclear factor 4 (HNF4) expression similar to that observed in liver tissue, but did not express the bile duct marker, cytokeratin. IRK and IPK cells did not show expression of IFNAR and IPS-1 respectively (Fig. 2 C).

**Replication of the HCV genome in IRK and IPK cells**

To assess the permissiveness of the established cell lines to HCV replication, we transduced IRK4 and IPK17 cells with J6JFH1 RNA and monitored the HCV protein and RNA levels by IF (Fig. 3 A) and real time RT-PCR (Fig. 3 B). The number of cells expressing HCV proteins, as detected by IF, increased over time, indicating the continuous proliferation of J6JFH1 in these cells. However, the ratio between infected and non-infected cells did not significantly change over time for 7 days after transfection. Similarly, the amount of total J6JFH1 RNA in 1 µg of total cellular RNA was reasonably constant. By contrast, the level of



**Figure 1. IF detection of J6JFH1 proteins' expression 5 days after transfection of J6JFH1-RNA through electroporation into wild type (A), TICAM-1ko (B), IPS-1ko (C), and IFNARko (D), freshly isolated primary hepatocytes.** A highly sensitive polyclonal antibody extracted from HCV-patient serum (AbS3) was used for the detection. Staining of the uninfected hepatocytes from different Ko mice was also performed and they showed negative for HCV proteins (data not shown). doi:10.1371/journal.pone.0021284.g001



**Figure 2. Morphological characteristics of IRK-4 (A) and IPK-17 (B) cells.** (C) RT analysis for the expression of albumin, HNF4, cytoke­ratin, interferon A receptor, and IPS-1 in 2 IFNAR-KO cell lines (IRK2 and 4), 2 IPS-1-KO cell lines (IPK-10 and 17), total liver, and NIH3T3 cells. doi:10.1371/journal.pone.0021284.g002

JFH1GND RNA carrying a mutation in NS5B hampering HCV replication, rapidly declined, indicating the requirement of continuous HCV replication for the maintenance of HCV positivity in the transfected mouse hepatocytes. Similar data were obtained from IRK2 and IPK10 cells (data not shown).

**IPS-1-dependent/Interferon-independent pathway is responsible for HCV's cytopathic effect**

In comparison to IPS-1ko hepatocytes, J6JFH1-RNA in IFNARko were lower and decreased further after its transfection, while higher stable levels of J6JFH1-RNA were maintained in IPS-1ko cells (Fig. 3 B and Fig. S2). Similarly, larger numbers of HCV-positive cells were detected in IPS-1ko hepatocytes compared with their IFNARko counterparts (Fig. 3 A), suggesting that the IPS-1 disruption benefits HCV replication in a distinct manner from IFNAR disruption. To measure the interferon induction after RNA virus infection in those cells, we used a highly infectious RNA-Virus (VSV) and measured the induction of interferon after its infection. All the interferons measured showed similar suppression of induction in IFNARko and IPS-1ko hepatocytes (Fig. 4). Surprisingly, cellular cytopathic effect that was monitored after transfection of J6JFH1-RNA was markedly reduced in IPS-1ko but not in IFNARko hepatocytes after transfection (Fig. 5A). This suppression was accompanied by an increase of J6JFH1-RNA levels in IPS-1ko cells, suggesting that minimal cellular damage induced by HCV replication in IPS-1<sup>-/-</sup> cells led to the improvement of HCV proliferation in mouse hepatocytes (Fig. 5B). Reduction of HCV-induced cellular cytotoxicity (Fig. 5C), and improvement of HCV replication (Fig. 5D) in wild type, and IFNAR-KO cells were found when we cultured the cells with a pan-caspase inhibitor, zVAD-fmk, 2 days before and after HCV-RNA transfection. We reasoned that the IPS-1 pathway rather than the IFNAR pathway capacitates hepatocytes to induce HCV-derived apoptotic cell death and its disruption resulted in the circumvention of cell death.

**Human CD81 is required for HCV infection of mouse hepatocytes**

Similar to the primary mouse hepatocytes, immortalized mouse hepatocytes showed the expression of all the mouse counterparts of human HCV entry receptors (Fig. S3). Human CD81 and hOccludin, but not other human HCV receptors such as SR-B1 or claudin1, have previously been reported to be essential for HCVpp entry into NIH3T3 mouse cells [3]. We then expressed hCD81 and/or hOccludin in IRK2 and IRK4 cells using lentivirus vectors. Using a MOI of 10, 95% transfection efficiency was achieved (Fig. S4) with lentivirus vector. We next tested the effect of these proteins on HCV particle (HCVcc) infection. Human CD81 alone was found to be required for J6JFH1 infection into all IRK and IPK cells tested (Fig. S5 and Fig. 6 A, and B). For the first time in mouse hepatocytes, HCV proteins were detected in nearly 1% of the cells used for infection. These data demonstrated the importance of hCD81 in establishing HCVcc infection in mouse hepatocytes.

**Viral factors affecting HCV replication in mouse hepatocytes**

After successfully establishing J6JFH1 infection in mouse hepatocytes, we attempted to infect these cells with other strains of HCV. Human CD81-expressing IPK17 cells were infected with full-length JFH1FL, however, no infection was detected (data not shown). This might be due to a problem in infection and/or replication. We further examined the replication efficiency of JFH1FL, the subgenomic JFH1 replicon and the J6JFH1 chimera in two different mouse hepatocyte lines and the HuH7.5.1 cell line. The persistent expression of HCV proteins was detected seven days after RNA transfection. Although HCV proteins were detected in HuH7.5.1 cells in all cases (Fig. 7 C), only J6JFH1 proteins were detected in the mouse hepatocyte lines, suggesting for the first time the importance of the J6 structural region for the replication of HCV in mouse hepatocytes (Fig. 7 A, and B).