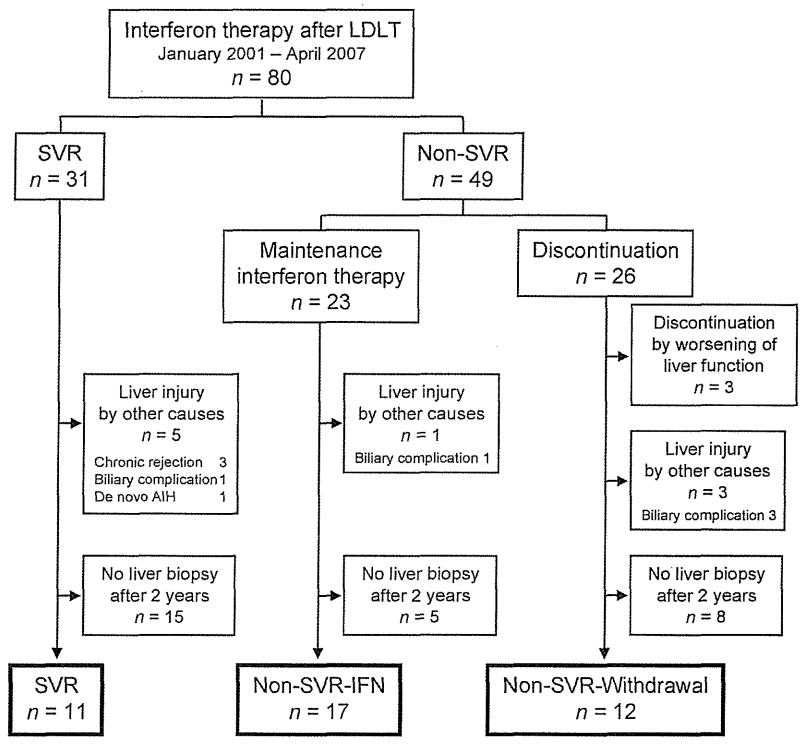

Fig. 1 Flow diagram showing the outcome of interferon therapy for patients with recurrent hepatitis C after living donor liver transplantation and indicating the classification of patients in this study.

23 (47%) received the low-dose peginterferon maintenance therapy, while 26 (53%) discontinued treatment within 12 months and did not receive low-dose peginterferon maintenance therapy as this was the patients' wish (n = 4), because of general fatigue (n = 4), recurrent hepatocellular carcinoma (n = 4), worsening of liver function (n = 3), biliary complications (n = 3), heart failure (n = 2), brain haemorrhage (n = 1), dementia (n = 1), sinusitis (n = 1), anaemia (n = 1), neutropenia (n = 1), and haemosputum (n = 1).

Of the 31 SVR patients, five were excluded because of chronic rejection (n = 3), biliary complications (n = 1) and *de novo* AIH (n = 1). Fifteen patients did not have liver biopsies more than 2 years after the initiation of the interferon therapy, mainly because liver function tests were normal. The remaining 11 patients were classified as the SVR group for analysis in this study. Among the 23 patients who received maintenance therapy, one patient with biliary complications and five patients who did not have liver biopsy more than 2 years after the initiation of therapy were excluded from the study. The remaining 17 patients were classified into the non-SVR-IFN group. Among the 26 patients who discontinued treatment within 12 months, three patients who initially experienced worsening of liver function were excluded because of the rapid progression of HCV; an additional three patients were excluded because of biliary complications. Eight patients were excluded because they had no liver biopsies taken more than 2 years after the initiation of the treatment. The remaining 12 patients were classified into the non-SVR-Withdrawal group. Cumulatively, we analysed the long-term histological changes of 40 patients: 11 in the SVR group (27.5% of the total), 17 in the non-SVR-IFN group (42.5% of the total) and 12 in the non-SVR-Withdrawal group (30% of the total).

There were no significant differences in the baseline characteristics among patients in the SVR, non-SVR-IFN, and non-SVR-Withdrawal groups (Table 1). The median age of patients at the beginning of therapy was 56.5 years (range, 15–70 years). The treatment started at a median of 9.5 months (range, 1.1–85.3 months) after LDLT. Thirty-five patients (88%) were infected with HCV genotype 1b. HCV genotypes of the remaining patients were 2a (n = 3), 2b (n = 1) and undetermined (n = 1). Median serum HCV RNA load was 2290 kIU/mL (range, 73.7–5000 kIU/mL); i.e. most patients had an extremely high viral load. Before the treatment, the necroinflammatory activity of all patients was A1 or greater, and 33 patients (83%) had a fibrosis score of F1 or greater. Among patients receiving tacrolimus for immunosuppression, the median serum trough level was 5.95 ng/mL (range, 3.3–10.9).

Effect of maintenance interferon therapy on liver histology

To evaluate the efficacy of long-term peginterferon therapy on histological changes, we compared scores between final biopsy samples (median, 44.0 months; range, 24.0–81.3 months) and those taken prior to treatment. Five patients in the non-SVR-IFN group discontinued maintenance

therapy between 26.5 and 53.1 months after the initiation of the treatment because of the adverse events. For these patients, the biopsies taken just before or within 3 months after discontinuation of the treatment were analysed as final biopsies. Despite the variation in time between pretreatment and final biopsy sample collection, there were no significant differences in the duration among the three groups ($P = 0.547$). Median duration from initiation of interferon therapy to final liver biopsy was 41.9 months (range, 24.0–81.3 months) in the SVR group, 41.7 months (range, 26.5–68.4 months) in the non-SVR-IFN group and 46.5 months (range, 30.4–79.6 months) in the non-SVR-Withdrawal group.

There were no significant differences in baseline activity grades or fibrosis stages of patients in the three treatment groups when they were first diagnosed with recurrent hepatitis C (Table 1). However, there were noticeable differences among the three groups by the end of treatment (Fig. 2a). The activity grade of all patients in the SVR and non-SVR-IFN groups improved or remained stable, whereas it deteriorated in 6 (50%) of 12 patients in the non-SVR-Withdrawal group. The fibrosis stage deteriorated in all patients in the non-SVR-Withdrawal group; nine of these patients (75%) deteriorated by more than one stage. In contrast, only four patients (24%) in the non-SVR-IFN group deteriorated, all by only a single stage. Furthermore, three patients actually improved. In the SVR group, fibrosis stage decreased or remained stable in 10 of 11 patients (91%).

In patients in the SVR and non-SVR-IFN groups, the mean activity grade was markedly reduced in the final biopsy, compared to the pretreatment biopsy (Fig. 2b). In contrast, patients in the non-SVR-Withdrawal group experienced an increase in activity grade. The differences between the non-SVR-Withdrawal group and both the SVR and the non-SVR-IFN groups were statistically significant ($P < 0.001$). The mean changes in fibrosis stage in the SVR and non-SVR-IFN groups were −0.18 and +0.06, respectively, suggesting that fibrosis did not change during the follow-up period. However, there was an obvious increase (+2.2) among patients in the non-SVR-Withdrawal group, indicating marked progression of fibrosis.

The Kaplan–Meier analysis allowed us to investigate whether patients in the three treatment groups experienced different progression rates to late-stage fibrosis (Fig. 2c). No patient in the SVR group and only 1 patient (6%) in the non-SVR-IFN group developed fibrosis stage F3 or F4, whereas nine patients (75%) in the non-SVR-Withdrawal group progressed to these stages. The rates of fibrosis progression were significantly higher in the non-SVR-Withdrawal group than in the non-SVR-IFN and SVR groups ($P = 0.0049$ and $P = 0.0086$, respectively). There was no significant difference between the SVR group and the non-SVR-IFN group ($P = 0.3980$). Five-year progression rates to F3 or F4 were 0% in the SVR group, 14% in the non-SVR-IFN group and 54% in the non-SVR-Withdrawal group.

Safety and tolerability of maintenance interferon therapy

Five of 17 patients (29%) who received low-dose maintenance peginterferon treatment discontinued interferon therapy because of biliary complications ($n = 2$), neutropenia ($n = 1$), anaemia ($n = 1$) and *de novo* AIH ($n = 1$), between 26.5 and 53.1 months after its initiation. The biliary complications were not related to interferon therapy. Patients with neutropenia and anaemia recovered after discontinuing interferon therapy and were able to resume therapy within months (3 and 10, respectively). Steroid therapy alleviated the *de novo* AIH, but the patients did not resume interferon therapy.

DISCUSSION

Studies have repeatedly shown the benefits of achieving SVR via interferon therapy after liver transplantation. For instance, the durability of the SVR is associated with improvements in hepatic inflammation and histological regression of fibrosis over the long-term [18–23]. In contrast, efficacy of interferon therapy for non-SVR patients after liver transplantation had not previously been investigated. Here, we have demonstrated that long-term peginterferon maintenance therapy suppresses histological progression of recurrent hepatitis C after LDLT.

Maintenance interferon therapy was recently shown to have no influence on either histological or clinical outcomes in patients with nontransplant hepatitis C [24]. This conclusion was drawn after observing that the rate of fibrosis progression was similar between treatment and control groups following a 3.5-year randomized controlled trial of low-dose peginterferon. As a large number of patients with advanced fibrosis were enrolled in the randomized controlled trial, it is difficult to compare with our study in which the number of patients studied is much smaller and patients with advanced fibrosis were not enrolled. In the current study after liver transplantation, however, we demonstrated that low-dose maintenance interferon therapy reduced necroinflammatory activity and fibrosis scores in non-SVR patients to levels similar to those in SVR patients. Furthermore, we found that non-SVR patients who discontinued treatment had significantly worse scores once no longer receiving therapy.

Although these results clearly suggest that low-dose peginterferon maintenance therapy is beneficial for non-SVR patients with recurrent hepatitis C after liver transplantation, the mechanism behind this positive response is unknown. Progression of hepatitis C and development of fibrosis after discontinuation of interferon treatment has been shown to proceed more rapidly in patients who have undergone liver transplantation [20,21]. Our results, indicating that activity grade and fibrosis stage markedly deteriorated in non-SVR patients who discontinued maintenance treatment, support these previous findings. Thus, such a

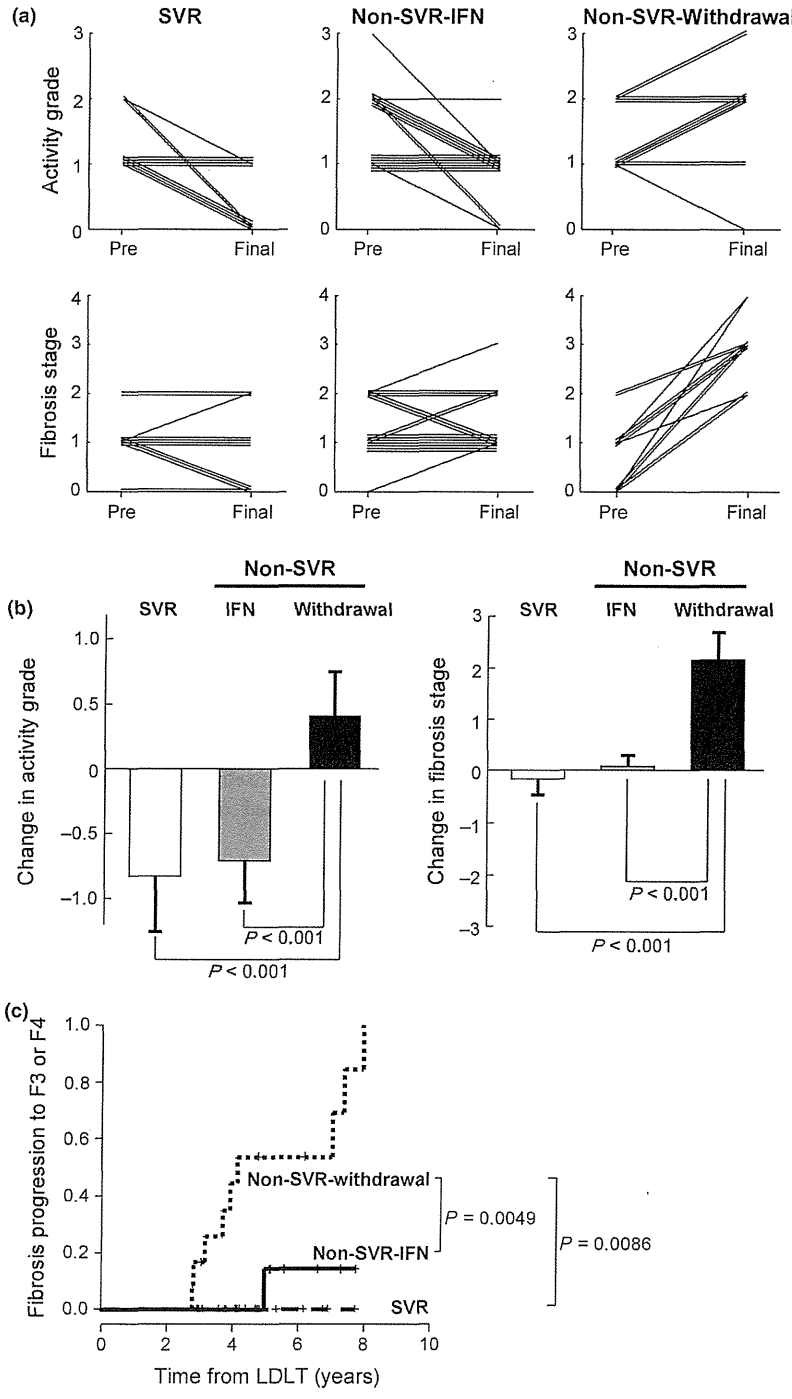

Fig. 2 Effect of maintenance interferon therapy on liver histology: (a) Changes in activity grade (upper) and fibrosis score (lower) of individual patients before interferon therapy (Pre) and at final biopsy (final). (b) Mean changes of liver activity grade (left) and fibrosis stage (right) between pretreatment liver biopsy and the final liver biopsy in each of the three treatment groups. The error bars represent 2 SEs. (c) Kaplan–Meier estimates of the progression rates among patients whose fibrosis advanced to F3 or F4. The dashed line indicates the sustained virological response (SVR) group, the solid line indicates the non-SVR-IFN group and the dotted line indicates the non-SVR-Withdrawal group.

rapid progression of recurrent hepatitis C in patients who discontinued interferon therapy may have highlighted the beneficial effect of the low-dose peginterferon maintenance therapy.

Another issue is the tolerability and safety of long-term peginterferon maintenance treatment. In this study, five patients (29%) discontinued the treatment during the peginterferon maintenance treatment, but only three did so for reasons directly related to the treatment. While two of these patients recovered simply by discontinuing the treatment, the third did require steroid pulse therapy to treat *de novo* AIH. Overall, however, the maintenance therapy did not result in the incidence of major adverse events, suggesting that it is both a tolerable and a safe treatment method.

Our work shows that long-term, low-dose peginterferon administration is an effective method for inhibiting the

progression of liver damage for recurrent hepatitis C after liver transplantation. Unfortunately, this was not a randomized control study, and only a small number of patients were eligible for research. Therefore, we recommend further work to more fully explore the effects of this treatment and to improve the outcomes for patients who do not achieve SVR.

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REVIEW

In vitro models for analysis of the hepatitis C virus life cycle

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ABSTRACT

Chronic hepatitis C virus (HCV) infection affects approximately 170 million people worldwide. HCV infection is a major global health problem as it can be complicated with liver cirrhosis and hepatocellular carcinoma. So far, there is no vaccine available and the non-specific, interferon (IFN)-based treatments now in use have significant side-effects and are frequently ineffective, as only approximately 50% of treated patients with genotypes 1 and 4 demonstrate HCV clearance. The lack of suitable *in vitro* and *in vivo* models for the analysis of HCV infection has hampered elucidation of the HCV life cycle and the development of both protective and therapeutic strategies against HCV infection. The present review focuses on the progress made towards the establishment of such models.

Key words hepatitis C virus, HuH-7 cell, knockout mice, type I interferon.

Chronic HCV infection is a major cause of mortality and morbidity throughout the world, infecting approximately 3.1% of the world's population (1). Only a fraction of acutely infected individuals are able to clear the infection spontaneously, whereas approximately 80% of infected individuals develop a chronic infection (2, 3). Patients with chronic HCV are at increased risk for developing liver fibrosis, cirrhosis, and/or hepatocellular carcinoma. Currently, these long-term complications of chronic HCV infection are the leading indication for liver transplantation (4, 5). Because of the high incidence of new infections by blood transfusions in the 1980s before the discovery of the virus, and because morbidity associated with chronic HCV infection generally takes decades to develop, it is expected that the burden of disease in the near future will rise dramatically.

HCV is an enveloped flavivirus, with a positive-stranded RNA genome of approximately 9600 nucleotides. The coding region is flanked by 5′ and 3′ non-coding regions, which are important for the initiation of translation and regulation of genomic duplication, respectively. The coding region itself is composed of a single open reading frame, which encodes a polyprotein precursor of approximately 3000 amino acids. This polyprotein is cleaved by host and viral proteases into structural and NS proteins (Fig. 1). Replication of the HCV genome involves the synthesis of a full-length negative-stranded RNA intermediate, which in turn provides a template for the de novo production of positive-stranded RNA. Both these synthesis steps are mediated by the viral RNA-dependent RNA polymerase NS5B (6–8). NS5B lacks proofreading abilities, and this leads to a high mutation rate and the

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List of Abbreviations: 3-D, three-dimensional; 3-D/HF, three-dimensional hollow fiber system; bbHCV, blood borne hepatitis C virus; HCV, hepatitis C virus; HPV/E6E7, human papilloma virus E6/E7 genes; IFN, interferon; IFNAR, interferon A receptor; IRES, internal ribosome entry site; ko, knockout; MDA-5, melanoma differentiation associated gene 5; MEF, mouse embryo fibroblasts; mir199, micro RNA 199; NS proteins, non-structural proteins; PPAR, peroxisome proliferator-activated receptor; RFB, radial flow bioreactor; RIG-I, retinoic acid-inducible gene I; TLR, Toll-like receptor; uPA, urokinase plasminogen activator.

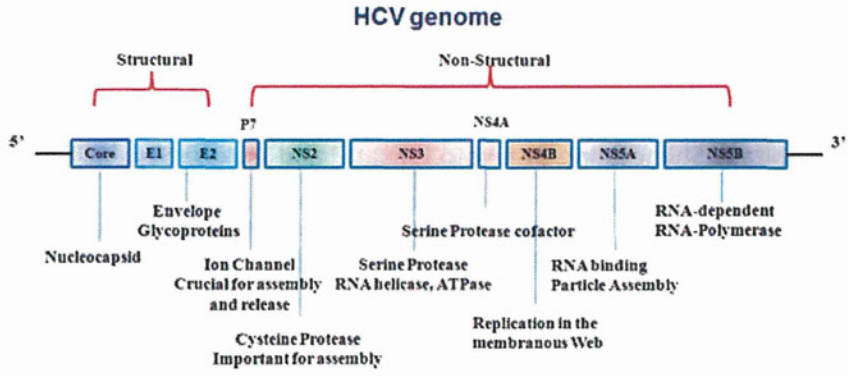

Fig. 1. Genomic structure of HCV. Genomic organization of wild-type HCV. The HCV-RNA genome consists of a major open reading frame, encoding a single polyprotein, and an alternative reading frame encoding F-proteins with unknown functions. The cleavage of the polyprotein by viral and host cell proteases gives rise to the mature structural (core, envelope proteins E1 and E2, and p7) and NS viral proteins (NS2 through NS5B). The putative activities and functions of viral proteins are indicated. The IRES located in the 5′ non-coding region initiates ribosome binding and translation. Both the 5′ and 3′ non-coding regions are essential for viral RNA replication involving the RNA-dependent RNA polymerase NS5B. NTPase, nucleotide triphosphatase.

generation of numerous quasispecies. HCV isolates can be classified into seven major genotypes, which vary in sequence by more than 30%. In addition to the distinct prevalence and global spread of the virus, the genotype is an important factor determining disease progression and responses to antiviral therapy (9).

Currently, the only licensed treatment for HCV is the combination of (pegylated)-interferon-alpha (IFN-α) and ribavirin. Although the success rate of treatment has improved substantially, standard therapy is not effective in all patients. Moreover, severe adverse effects and high costs limit the compliance and global application of this treatment. The development of prophylaxis and novel therapeutics to treat HCV infection has been hampered by the lack of suitable *in vitro* and *in vivo* culture systems. In this review, we describe the development of *in vitro* culture systems for HCV.

Tissue culture-adapted HCV (sub-)genomic replicons

Dr Bartenschlager's group was the first to establish a convenient reproducible *in vitro* cell culture system for the study of HCV replication (10). They created antibiotic-resistant HCV genomes to select replication-competent viral clones by conveying antibiotic resistance to cells. This was achieved by replacing the structural protein-coding sequences, as well as p7 of the consensus genome Con1, by the neomycin resistance gene. In addition, a second IRES was introduced to promote translation of the non-structural protein-coding sequences important for viral replication (Fig. 2). Upon transfection of these so-called subgenomic replicons in specific cell lines, drug-resistant cell colonies were isolated in which high levels of viral replication occurred. Subsequent analysis confirmed that these HCV replicons indeed were capable of self-amplification through synthesis of a negative-strand replication intermediate, and could be stably propagated in cell culture for many years (10, 11).

HCV replication was supported by several cell types such as HuH6 (12), HepG2 (13), Li23 (14), and 293 cells (15), with the human hepatoma cell line HuH-7 being the most permissive (16). Interestingly, removal of replicon RNA from these cell clones by treatment with type 1 IFN rendered the cells more permissive to reintroduction of replicons, resulting in higher replication rates. Examples of these highly permissive cells are HuH-7.5 and HuH-7-Lunet cells (16, 17). The efficient replication in the replicon systems was found to depend on tissue-culture-adaptive mutations. Introduction of these specific mutations in the wild-type consensus sequence significantly enhanced viral replication *in vitro* (18–22). Mutational hot spots were found clustered primarily in the NS3, NS4B, and NS5A regions. The mechanisms behind the enhanced replication caused by these tissue-culture-adaptive mutations are still largely unknown, and the interesting fact that these mutations are not commonly found in patients suggests that these may have a toll on the viral fitness.

HCV replicons have proven to be extremely valuable for studies on the process of HCV replication, as well as for testing novel antiviral compounds that specifically target the protease activity of NS3 or the polymerase activity of NS5 (23).

Cell culture-derived infectious HCV

Studies using HCV replicons have provided detailed knowledge on the mechanisms of replication of HCV.

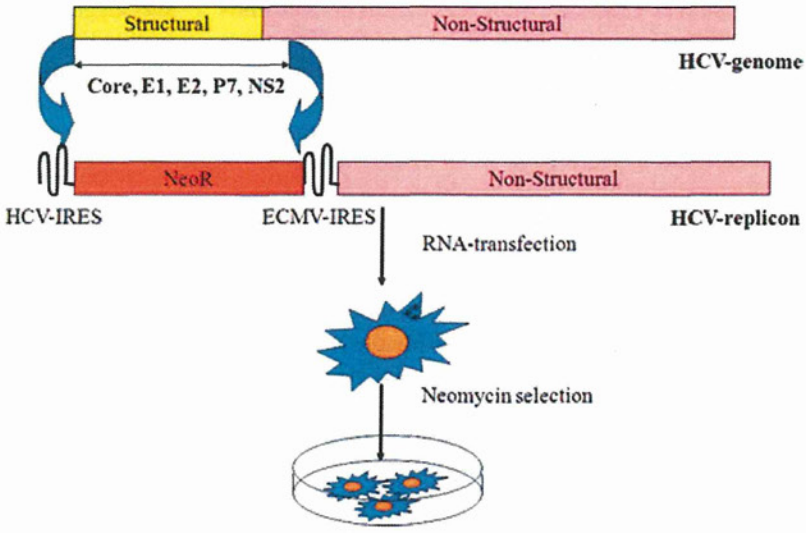

Fig. 2. HCV replicon system. The structural sequences (C, E1, E2, and p7) together with NS2 were replaced by a neomycin antibiotic-resistance gene, and an ECMV-IRES was introduced to drive translation of the remaining non-structural proteins. Neomycin selection of these double cistron (bicistronic) replicons in the hepatoma cell line Huh7 resulted in high-level HCV-RNA replication, depending on the gain of so-called 'tissue-culture' adaptive mutations mostly confined to the NS3, NS4B, and NS5A regions.

However, an apparent shortcoming of these models was that stable cell clones containing self-replicating replicons and expressing all viral proteins remained unable to release infectious HCV particles. The inability to secrete viral particles may be the consequence of adaptive mutations, which are needed to enhance viral replication rates, but at the same time may block viral assembly. Indeed, replicons without adaptive mutations show very low replication rates (16, 24). A different situation emerged when the first genotype 2a consensus genome was established (25, 26).

A subgenomic replicon constructed from a clone called JFH-1, isolated from a Japanese patient with fulminant hepatitis C, replicated up to 20-fold higher in HuH-7 cells as compared to Con1 replicons, and did not require adaptive mutations for efficient replication *in vitro* (26). Transfection of HuH-7 and HuH-7.5.1 cells with the *in vitro*-transcribed full-length JFH-1 genome or a recombinant chimeric genome with another genotype 2a isolate, J6, resulted in the secretion of viral particles that were infectious in cultured cells (Fig. 3), in chimeric mice, and in chimpanzees (27–29).

The infectivity of cells could be neutralized with antibodies against the HCV entry receptor CD81, antibodies against E2, or immunoglobulins from chronically infected patients. Importantly, the replication of cell-cultured HCV in this system was inhibited by IFN-α as well as by several HCV-specific antiviral compounds (29). Since 2005, chimeric JFH-1-based genomes have been constructed of all seven known HCV genotypes. Similar to the J6-JFH-1 chimera, in these so-called intergenotypic recombinants, the structural genes (core, E1, and E2), p7, and NS2 of JFH-1 were replaced by genotype-specific sequences which often resulted in lower infectious virion production than

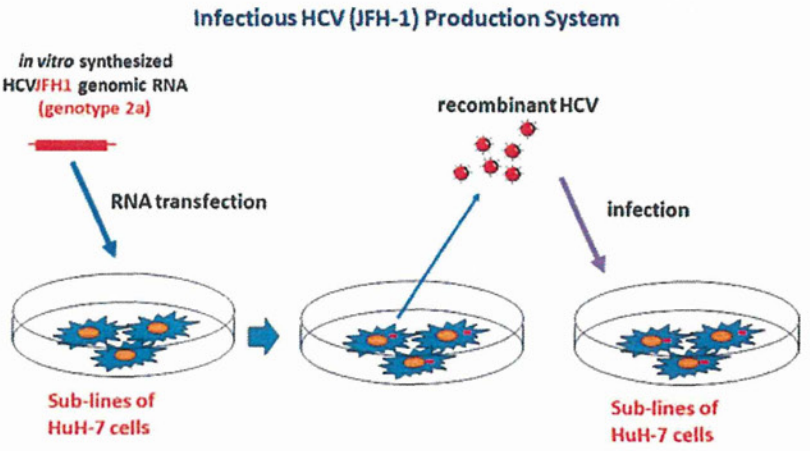

Fig. 3. JFH1 infectious system. Full-length JFH1-RNA is transcribed *in vitro*, and transfected to HuH-7-derived cell lines. JFH1 replicates in these cells, and produce infectious virions in the medium. The medium is collected, concentrated, and used to infect naive cells. Hence, the entire HCV life cycle was reproduced for the first time *in vitro*.

wild-type JFH-1 (30–32). Most NS proteins of intergenotypic chimeras originate from JFH-1, and therefore these genomes are unlikely to reflect genotype-specific characteristics of replication. However, these intergenotypic chimeras may become critically important in the study of differences in HCV entry or to assess the efficacy of HCV entry inhibitors. Interestingly, production of infectious genotype 1a HCV in cells transfected with synthetic RNA (H77-S) derived from a prototype virus (H77-C) was also reported (33). H77-S carries adaptive mutations that promote efficient viral RNA replication in HuH-7.5 cells. These mutations are located within the NS3/4A protease complex, and the NS5A protein (34) H77-S showed similar replication efficiency to JFH-1 isolate; however, it showed lower expression of HCV core protein, and lower production of infectious HCV particles (33).

Serum-derived HCV infection

The previously mentioned models used to study HCV infection are based on subclones of HuH-7 cells infected with JFH1 recombinant virus or its derivatives (27). HuH-7 cells and its subclones, however, do not support the entire life cycle of the bbHCV present in the blood of patients (35). Moreover, HCV has considerable diversity and variability. It is generally classified into six major genotypes and more than 100 subtypes (36). JFH1, however, is a single isolate of HCV genotype 2a that was originally derived from a patient with rare fulminant hepatitis (27). Thus, usage of HCV particles isolated from patient serum could be more useful to study authentic HCV infection.

Many researchers have attempted to develop an *in vitro* system for bbHCV (37–39). These current systems, however, are still insufficient due to their low efficiency for infectivity and replication of bbHCV. Normal human hepatocytes are the ideal system in which to study HCV infectivity. When cultured *in vitro*, however, they proliferate poorly and divide only a few times (40). Continuous proliferation could be achieved by introducing oncogenes, the HPV/E6E7 immortalized multiple cell types that were phenotypically and functionally similar to the parental cells (41–45). We established a human primary non-neoplastic hepatocyte cell line transduced with the HPV18/E6E7 that retained primary hepatocyte characteristics even after prolonged culture (35). We further improved the susceptibility of HPV18/E6E7-immortalized hepatocytes (HuS-E/2 cells) to bbHCV infectivity by impairing the innate immune response of these cells through suppression of interferon regulatory factor-7 (IRF-7) expression. These cells were useful to assay infectivity of HCV strains other than JFH-1, HCV replication, innate immune system engagement of HCV, and screening of anti-HCV agents. This infection system using non-neoplastic cells also suggested that IRF-7 plays an important role in eliminating HCV infection. Using this system, the suppressive effect of tamoxifen and mir199 on HCV replication was reported (46, 47).

Three-dimensional culture

A major limitation of the immortalized hepatocytes infection system was the failure to produce infectious HCV particles. Because the 3-D cell culture condition more closely reproduces the *in vivo* environment of hepatocytes (48), culturing these cells in this manner may support the entire HCV life cycle. Similarly, a previous report showed the production of HCV particles from the FLC4 hepatocyte line transfected with HCV-RNA and cultured in a 3-D radial-flow bioreactor (RFB). The RFB system is composed of a dedicated device containing 1×10^9 FLC4 cells with a culture area of 2.7 m^2. A more convenient, smaller and easy to use 3-D culture system is required for the study of the several aspects of bbHCV infection. (49). A hybrid artificial liver support system was developed using animal hepatocytes cultured in a 3-D/HF. This bioartificial liver showed several characteristic features of liver tissue for more than 4 months (50–52).

By growing our HuSE/2 cells in a similar 3-D culture (53) the gene expression profile was improved to more closely match that of human primary hepatocytes. We used this small 3-D culture system and showed it to be ideal for culturing HuS-E/2 cells for the study of bbHCV infection (Fig. 4) (54). Using this system we observed not only the enhancement of HCV replication, but also the production of infectious HCV particles in the medium using the 3-D/HF system. The cell mass formed by the 3-D culture system, most likely the polar character, was essential for the life cycle of bbHCV. Using microarray comparison of gene expression between 2-D and 3-D cultured cells, we found a higher activation of the PPAR-α signaling pathway which was shown to be important for the improvement of HCV replication in 3-D culture. Suppression of the PPAR-α signaling pathway using its antagonist MK886 markedly suppressed HCV replication in two different cell lines (53). A recent study showed that the induction of PPAR-α or PPAR-γ led to the suppression or enhancement of HCV replication, respectively, in HuH-7 cells (55). Using HuH-7-derived clones, three different independent studies confirmed our data, showing the suppression of HCV replication by PPAR-α blockers such as (MK886) (56, 57) or 2-chloro-5-nitro-*N*-(pyridyl) benzamide (BA) (58). Furthermore, no effect of PPAR-γ was observed on HCV replication (58).

Delayed production of infectious particles was also observed in cells infected with some HCV strains after prolonged culture (54). It is likely that mutation of the HCV

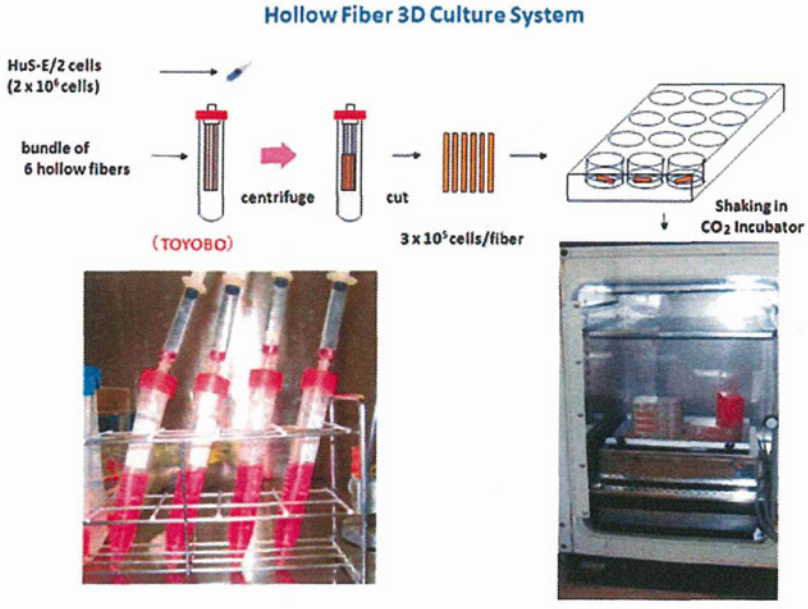

Fig. 4. 3-D hollow fiber culture. HuS-E/2 suspension was injected into the lumen of the hollow fiber system (HF; Toyobo Co., Osaka, Japan). The bundles were centrifuged to induce organoid formation. The lower 1.5 cm containing the organoid formation was then cut and cultured in 12-well plates (two capillary bundles per well) with gentle rotation using serum-free medium (Toyobo Co.) in a CO_2 incubator at 37°C. The number of cells was adjusted to 3×10^5 cells per two-capillary bundle at the start of each experiment.

genome and/or selection of clones during prolonged culture improved the productivity of infectious particles. This lack of production of infectious particles soon after infection may serve to avoid an early strong response from the host immune system, and demonstrates a novel mechanism of latent infection by HCV. Similarly, fluctuation in HCV proliferation was observed during the prolonged culture of 3-D-HuS-E/2 cells infected with bbHCV (54); this fluctuation was associated with a change in viral quasispecies, suggesting that an HCV strain having a growth advantage proliferates selectively and dominantly in these culture conditions. Because the progressive emergence of each dominant strain was only temporary, it is highly likely that the infection and proliferation of such an HCV strain is suppressed by cellular mechanism(s). Our results showed two cellular mechanisms functioning to do this. The first is the involvement of the innate immune system, as evidenced by the secretion of IFN-α during the first week of infection. The second mechanism is HCV-induced apoptosis. Although HCV-induced apoptosis was not found when HCV-1b was used for infection, it was found in all cases where HCV-2a was used, suggesting a higher cytopathic tendency of the HCV-2a genotype.

Mouse cells permissible to HCV infection

The development of prophylaxis and novel therapeutics to treat HCV infection has been hampered by the lack of suitable animal models, a deficit resulting from the limited species tropism of HCV. Chimpanzees are the only available immunocompetent *in vivo* experimental system, but their use is limited by ethical concerns, restricted availability and prohibitively high costs (59).

A convenient small-animal model supporting the HCV life cycle could significantly accelerate the preclinical testing of vaccine and drug candidates, as well as facilitate *in vivo* studies of HCV pathogenesis. A murine model was described in which overexpression of a uPA transgene resulted not only in neonatal bleeding disorders, but also in severe liver toxicity (60). Importantly, the diseased liver could be replaced by donor hepatocytes of murine origin, as well as by hepatocytes from rats, woodchucks, and humans once the uPA transgenic mice were backcrossed on an immunodeficient background. Mice with chimeric human livers that were inoculated with serum from HCV-positive donors developed prolonged HCV infections with high viral titers and evidence for active replication of the virus in chimeric human livers (61). At present, the chimeric human liver uPA/SCID mouse model is physiologically closest to a natural human infection and therefore represents the most successful small-animal model for HCV infection. Several shortcomings, however, limit its widespread use and application. Most importantly, the immunodeficiency required to allow successful xenotransplantation precludes studies on the adaptive immune response, immunopathology, and active immunization strategies (vaccine development). Second, only a few laboratories have reported successful generation of these chimeras, because this model requires high-quality human donor hepatocytes and the actual transplantation is difficult to carry out in small animals with a tendency to bleed. Finally, the efficacy of human hepatocyte engraftment is highly variable

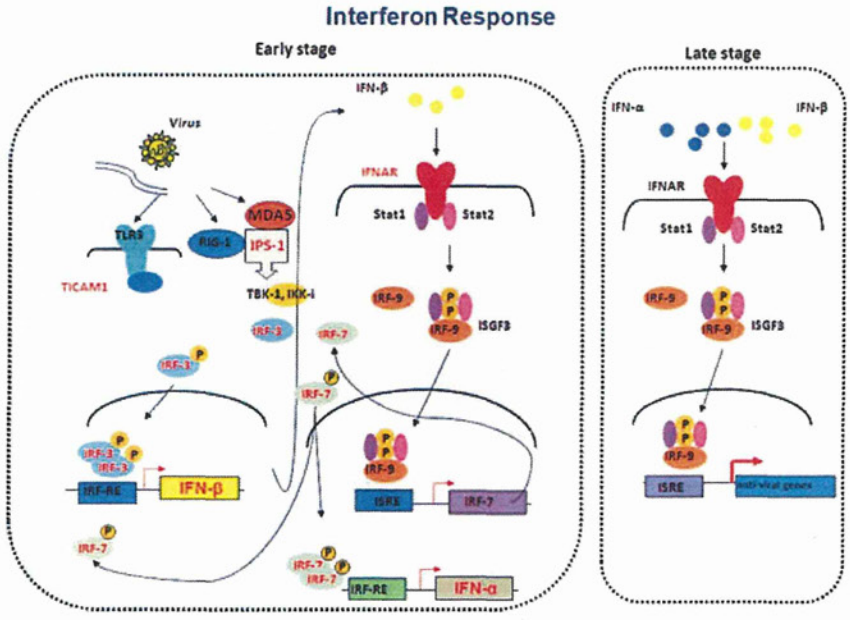

Fig. 5. Induction of interferon response by viral RNA. The cell detects viral RNA through the endosomal RNA sensor TLR3, and the cytoplasmic RNA sensors RIG-I and MDA5. Both pathways will lead to the activation of TBK-1 and IKK-I kinases, through the TICAM-1 adaptor molecule in the case of TLR3, or IPS-1 in the case of RIG-I and MDA5. These kinases will induce phosphorilization of interferon regulatory factor (IRF)-3, which will then dimerize and translocate to the nucleus. IRF-3 will then bind to the IRF response elements (IRF-RE) of IFN-β and lead to the induction of IFN-β expression. The IFN-β that is produced and secreted binds to the IFN receptor in an autocrine or paracrine manner to direct Janus Kinase Signal Transducer and Activator of Transcription (JAK-STAT) signaling and the interferon-stimulated gene factor 3 (ISGF3)-dependent expression of IRF-7 and other interferon-stimulated genes (ISG). IRF-7 will be phosphorylated by the activated TBK-1 and IKKε kinases, and form homo, or hetero-dimers with IRF-3, leading to further induction of IFN-β and -α genes. This signaling serves to amplify the IFN response by increasing the expression of IFN-β, IFN-α subtypes and ISG in a positive feedback loop.

in these animals, ranging from approximately 2% to 92% after additional treatment with an antibody to asialo- GM-1 (62).

The successful establishment of the HCV life cycle in mouse hepatocytes is another tempting alternative to overcome these problems. In addition to missing or incompatible positive regulators of HCV replication, dominant-negative restriction factors might be present in mouse hepatocytes. Altered or exacerbated innate antiviral responses, the inability of HCV proteins to overcome murine defenses, or mouse-specific restriction factors similar to those that control retroviral infection, such as Fv1, TRIM5α or APOBEC3 cytidine deaminases, could impair HCV replication in mouse cells.

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 (PRR), including the cell surface and endosomal RNA sensors TLR3 and TLR7, and the cytoplasmic RNA sensors RIG-I and MDA5 (Fig. 5) (63). The detection of virus infection by these receptors leads to the induction of IFN and their downstream IFN-inducible anti-viral genes through distinct signaling pathways (64). Type I IFN is an important regulator of viral infections in the innate immune system (65). Another type of IFN, IFN-lambda, affects the prognosis of HCV infection, and its response to antiviral therapy (66,67). Variations in the type or intensity of the antiviral response between hosts are known to restrict the tropism of certain viruses, such as myxoma virus, which is only permissive in mouse cells that have impaired IFN responses. Similarly, we previously reported that the impairment of IRF-7, and suppression of the interferon response improved HCV replication in immortalized primary human hepatocytes. (35)

Mutations impairing the function of the RIG-I gene and the induction of IFN were essential in establishing HCV infectivity in human HuH-7.5 cells (68). Similarly, the HCV-NS3/4a protease is known to cleave the IPS-1 adaptor molecule, inducing further downstream blocking of the IFN-inducing signaling pathway (69). 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 (70). We examined the possibility of HCV replication

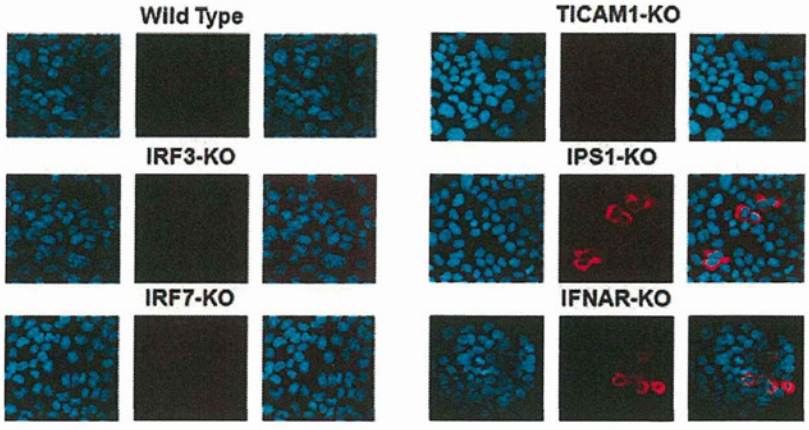

Fig. 6. Establishment of mouse hepatocyte lines permissive to J6/JFH1.
Immunofluorescence detection of J6JFH1 proteins' expression 5 days after transfection of J6JFH1-RNA through electroporation into wild-type, IRF-3-ko, IRF-7-ko, TICAM1-ko, IPS-1-ko, and IFNAR-ko, freshly isolated primary hepatocytes. A highly sensitive polyclonal antibody extracted from HCV-patient serum (AbS3) was used for the detection.

in mice lacking the expression of key factors that modulate the type I IFN-inducing pathways (Fig. 6). Only gene silencing of 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-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 and replication for the first time in mouse hepatocytes. Using these cell lines, we demonstrated that the suppression of IPS-1 enhances HCV infection and replication in mouse hepatocytes through the suppression of both IFN induction and an IFN-independent J6JFH1-induced cytopathic effect. We also showed for the first time the importance of the HCV structural region for viral replication, as JFH1 chimera containing the J6 structure region showed a privilege for spontaneous replication over full-length JFH1 or the subgenomic JFH1 replicon. IRF-3-ko MEF were previously shown to support HCV replication more efficiently than wild MEF (71). As the knockout of IPS-1 mainly suppresses signaling in response to virus RNA detection, and maintains an intact IFN response and induction to other stimulants, it may result in minimum interference to adaptive immune responses as compared to IRF-3 or IFNAR-ko.

Conclusion

We have established an *in vitro* culture system that can support the entire life cycle of a variety of HCV isolates and genotypes. Although this *in vitro* model system may not completely reproduce the *in vivo* situation, we believe it is the first *in vitro* system showing HCV strain-dependent virus/cell interaction including induction of cellular apoptosis and/or evasion from the cellular innate immune response, which may make it a good tool for the analysis of virus/host interaction, together with the development of new anti-HCV strategies for the different bbHCV strains. We have also established hepatocyte lines from IPS-1-ko mice that support HCV replication and infection. These cell lines will be very useful in identifying other species' restriction factors and viral determinants required for the further establishment of a robust and efficient HCV life cycle in mouse hepatocytes. Further development of hCD81-transgenic IPS-1-ko mice may serve as a good model for the study of immunological responses against HCV infection. This mouse model can be used as a backbone for any further future models supporting robust HCV infectivity for the study of HCV pathogenesis, propagation and vaccine development.

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DISCLOSURE

The authors declare no financial or commercial conflict of interest.

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REVIEW

Multi-Step Regulation of Interferon Induction by Hepatitis C Virus

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Abstract Acute hepatitis C virus (HCV) infection evokes several distinct innate immune responses in host, but the virus usually propagates by circumventing these responses. Although a replication intermediate double-stranded RNA is produced in infected cells, type I interferon (IFN) induction and immediate cell death are largely blocked in infected cells. Type I and III IFNs are mainly produced in HCV-infected hepatocytes if the MAVS pathway is functional, and dysfunction of this pathway may lead to cellular permissiveness to HCV replication and production. Cellular immunity, including natural killer cell activation and antigen-specific CD8 T-cell proliferation, occurs following innate immune activation in response to HCV, but is often insufficient for eradication of HCV. Although type I IFN therapy has been an authentic therapy for patients with HCV, host innate immune responses to HCV RNA/proteins may be associated with progressive hepatic fibrosis and carcinogenesis once persistent HCV infection is established in opposition to the IFN system. Hence, innate RNA sensing exerts pivotal functions against HCV genome replication and host pathogenesis through modulation of the IFN system. Molecules participating in the RIG-I and Toll-like receptor 3 pathways are the main targets for HCV, disabling the anti-viral functions of these IFN-inducing molecules. We discuss the mechanisms that abolish type I and type III IFN production in HCV-infected cells, which may contribute to understanding the mechanism of virus persistence and resistance to the IFN therapy.

Keywords Hepatitis C virus · TLR3 · TICAM-1 (TRIF) · MAVS (IPS-1, Cardif, VISA) · Interferon-inducing pathway · Double-stranded RNA

Abbreviations

BMDC	Bone marrow-derived dendritic cells
CTL	Cytotoxic T lymphocytes
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
dsRNA	Double-stranded RNA
IFN	Interferon
LD	Lipid droplet
MAM	Mitochondrial-associated endoplasmic reticulum membranes
MAVS	Mitochondrial antiviral signaling protein
Mf	Macrophages
mRNA	Messenger RNA
NK	Natural killer
NS	Non-structural
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
STING	Stimulator of IFN genes
TICAM-1	Toll-IL-1-homology domain-containing adaptor molecule-1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR1	TNF-α receptor 1

MAVS has been identified as the adaptor for RIG-I and MDA5 by four independent groups, and then also known as IPS-1, Cardif or VISA (Kawai and Akira 2009). TICAM-1 has been identified as the adaptor for TLR3 and TLR4 by two independent groups, and thus also described as TRIF (Oshiumi et al. 2003). In accordance with the HUGO Gene Nomenclature Committee-approved nomenclature, here we refer to these adaptor molecules as MAVS and TICAM-1, respectively.

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Introduction

Hepatitis C virus (HCV) mainly infects human hepatocytes, and triggers induction of cytokines and type I (IFN-α/β) and type III interferons (IFN-λ) (Fig. 1). Although cells expressing IFN receptors respond to the released IFN and amplify type I IFN production, IFN induction is not always robust in the infected cells due to the fact that HCV proteins inhibit host IFN-inducing pathways. IFN-stimulated genes (ISGs), such as IRF-7, MAP3K14, RIG-I, IRF-2, and IRF-1 are known to inhibit HCV replication (Schoggins et al. 2011). In particular, type III IFNs are more produced than IFN-α/β in HCV-infected hepatocytes via the mitochondrial antiviral signaling protein (MAVS) pathway to induce a set of ISGs (Thomas et al. 2012). Cytokines and chemokines are released from infected hepatocytes and myeloid cells in the liver. These mediators affect the formation of inflammatory environments and modify homeostasis of the host cell community, including the recruited bystander cells. Although these scenarios generally reflect the signs of patients with HCV, what occurs following initial virus entry into host cells remains obscure at the molecular level. HCV genome RNA is internalized via fusion and a portion of 3′-polyU/UC or 5′-triphosphate-short stem RNA acts directly as a ligand for RIG-I (Saito et al. 2008). The HCV genome functions as a messenger (m)RNA for HCV polyprotein production and, at the same time, HCV genome replicates in the cytoplasm (Lindenbach et al. 2007). Double-stranded (ds)RNA accumulating in infected cells is the main pattern molecule (PAMP) and, once liberated, provokes activation of innate immunity in myeloid cells. How host cells sense HCV RNA or dsRNA during infection and replicon transfection has been investigated, and has led to an understanding of the importance of the cytoplasmic RNA recognition pathways (Fig. 1), particularly, the MAVS pathway (Cheng et al. 2006; Li et al. 2005a, b). The current concept is that MAVS signals the kinases, TANK-binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε), to phosphorylate IFN regulatory factors (IRF)-3 and IRF-7, resulting in the induction of type I IFN (Kawai and Akira 2009). Likewise, IRF-3 and nuclear factor (NF)-κB appear to participate in the induction of IFN-λ (Ding

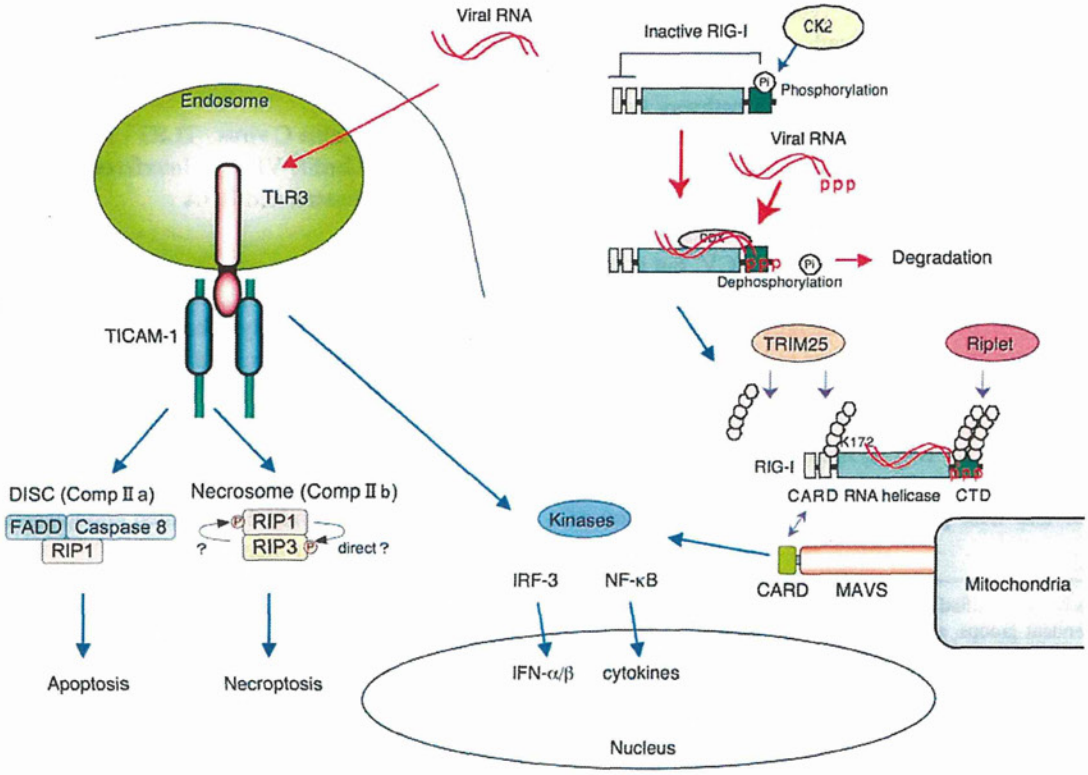

Fig. 1 Cytoplasmic and endosomal sensors for virus dsRNA in HCV infection. Live signal (*right*) and cell death signal (*left*) in response to viral dsRNA are illustrated. The live signal occurs with stimulation of TLR3 or RIG-I-like receptors and essentially induces activation of NF-κB to support induction of pro-inflammatory cytokines and type I/type III interferons (IFNs) (*right*). This live signal may be amplified by the function of a small amount of IFN-β that is required for the maintenance of homeostasis of the cellular microenvironment. In contrast, death signal occurs with TLR3: caspase 8 is a key molecule for discriminating between apoptosis and necroptosis, and its functional absence sustains the RIP1/RIP3 necrosome signal leading to necroptosis (*left*). Viral dsRNA recognition by RIG-I is induced by RIG-I ubiquitination (*right*). The two modes of K63 polyubiquitination activate RIG-I

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et al. 2012). Because RIG-I-like receptors are IFN-inducible genes, only trace levels are found in resting cells or those in the early stage of virus entry. Thus, how RIG-I/MDA5 captures internalized or replicating virus RNA to evoke an antiviral response in such situations remains unexplained.

There are many reports suggesting that Toll-like receptor 3 (TLR3) participates in the response to HCV dsRNA (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012) (Fig. 1). Most of the relevant studies have been performed with hepatoma cell lines due to the lack of proper systems for reproducing the HCV life cycle in culture as well as the in vivo animal model to examine the HCV immune responses. Cell death accompanied with a cytopathic effect is another phenotype of infected hepatocytes (Lim et al. 2012). Hepatocyte death is characterized as apoptosis, but the possible involvement of the pathway with receptor-interacting protein (RIP) kinases in infection-induced cell death has not been strictly ruled out (Fig. 1). Necrosis-like cell death (necroptosis) might cause a source of infectious virions and lead to the pathogenesis of HCV-associated liver damage. Ligands of the death receptor family, including FasL and TRAIL, are likely to associate with hepatocyte death induced by HCV infection (Bantel and Schulze-Osthoff 2003; Saeed et al. 2011; Zhu et al. 2007); however, what triggers the induction of the effector cells is still undetermined. Apart from these cell death family proteins, it is accepted that TLR3 is an activator of the RIP1 pathway (Meylan et al. 2004), which clearly participates in macrophage necroptosis (He et al. 2011). TLR3 is up-regulated in macrophages/dendritic cells (Mf/DC) in an IFN-dependent manner (Tanabe et al. 2003) and recognizes internalized virus dsRNA in the endosome of these phagocytes (Matsumoto et al. 2011). TLR3 has been characterized as an inducer of cellular immune effectors (Matsumoto et al. 2011; Seya and Matsumoto 2009). In accordance with the current dogma, natural killer (NK)-ligand up-regulation or cross-presentation of DCs that occurs with the internalization of dead cell-derived dsRNA may bridge the missing link between HCV dsRNA and TLR3-derived DC maturation (Ebihara et al. 2008).

Dead cells are a source of damage-associated molecular pattern (DAMP) (Kono and Rock 2008). DAMP refers to an intracellular molecule with inflammation-inducing capacities when it is released out of the cell. DAMP does not belong to the cytokine family, but resembles PAMP in its functional properties toward activation of myeloid DCs and macrophages (Kono and Rock 2008). Its function may be associated with physiological responses related to HCV immune response in a broad sense, including regeneration and tumorigenesis. Recently, necrotic or necroptotic cell death has been closely connected with innate immune responses involving pattern sensing (Kono and Rock 2008; Nace et al. 2012). How HCV patterns are sensed and linked to the cellular immunity will be an intriguing issue. DAMP contains a number of cytosolic or nucleic molecules, as in Table 1, and in particular nucleic acids from infected cells. Thus, DAMP and dsRNA of viral origin are extrinsic patterns for sensors to evoke unique features of inflammation during HCV infection.

Table 1 Sensors for nucleic acid PAMPs and DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids (PAMP)	
Cytosolic long dsRNA	MDA5
Cytosolic 5′-PPP-RNA	RIG-I
Endosomal >140 bp dsRNA	TLR3
Nonmethylated CpG DNA	TLR9
Cytosolic dsDNA	DNA sensors[a]
Self-molecular patterns (DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4[b]
S100 proteins	RAGE
Self-nucleic acids (DAMP)	
Self-DNA	DNA sensors[a]
Self-mRNA	TLR3

HMGB1 High-mobility group box 1, *HSPs* heat shock proteins

[a] See Table 2

[b] Scavenger receptors: D40, CD91, etc.

Herein, we discuss the interrelationship between these recent findings on innate immunity and HCV infection.

Blocking IFN Induction by HCV Proteins

Proteolytic Control of the IFN-Inducing Pathways by NS3/4A

HCV genome RNA also serves as a single mRNA that encodes ~3,000 amino acids, consisting of 10 virus proteins (Lindenbach et al. 2007). Structural proteins (core, E1, and E2) are situated at the N-terminal region of this polyprotein (Fig. 2a). The HCV polyprotein is first cleaved between 191A and 192Y by signal peptidase to separate the core protein from E1 protein and the core is retained on the endoplasmic reticulum (ER) membrane (McLauchlan et al. 2002). Then, signal peptide peptidase scissors out the core protein by cleavage at 177F and 178L from the ER membrane (Okamoto et al. 2004). E1 and E2 are also released from the remaining structural protein complex by the proteolytic function of signal peptidase (McLauchlan et al. 2002). Non-structural proteins of HCV are fragmented into functional units by NS2 and NS3/4A proteases. Hence, the release of the structural proteins precedes the mature

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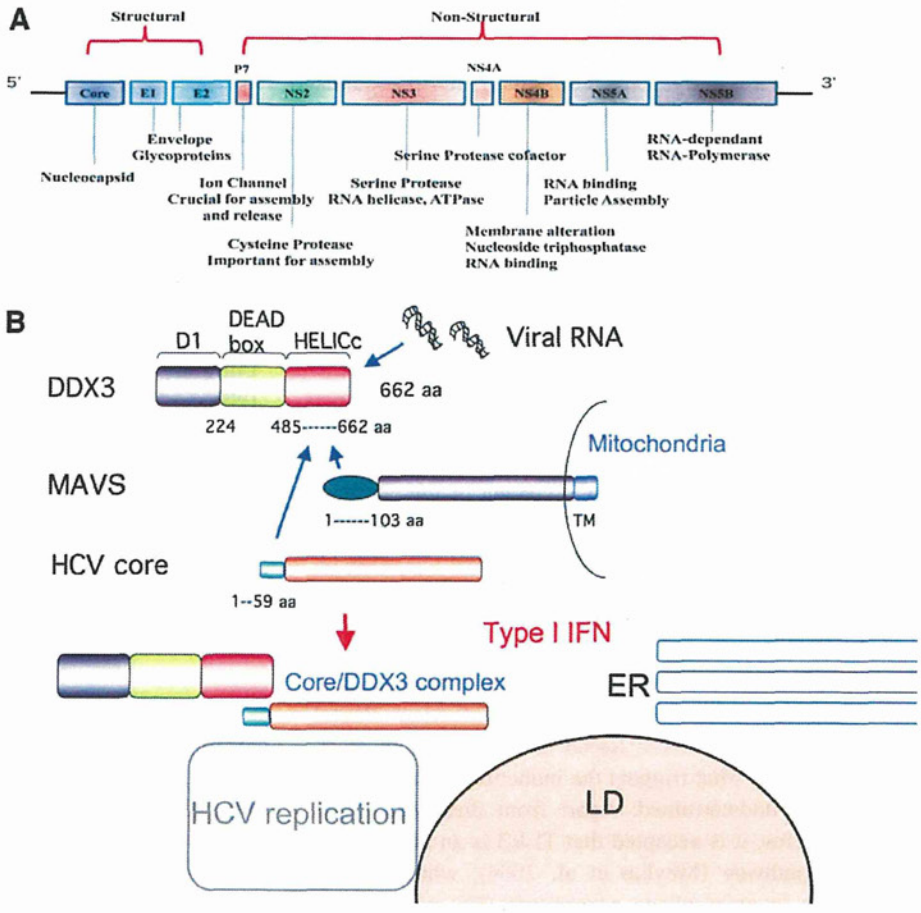

Fig. 2 Two different functions of HCV core protein. HCV genome and the functions of each HCV protein. HCV core is first clipped out from the polyprotein of HCV, and later NS proteins are generated (**a**). HCV core protein retracts DDX3 from the MAVS–DDX3 signal complex on the mitochondria (**b**). DDX3 usually couples with MAVS on mitochondria and directly binds overwhelmed virus dsRNA in virus-infected cells. When the HCV core protein is produced, DDX3 binds core protein with high affinity and moves from the mitochondria to the HCV replication apparatus, where the core is recruited. The HCV replication apparatus is situated near the lipid droplet (LD) in ER. DDX3 supports HCV replication in the apparatus

processing of non-structural (NS) proteins during the HCV polyprotein processing (Lindenbach et al. 2007). Notably, two structural proteins, core and E2, exhibit regulatory functions against type I IFN induction (Florentin et al. 2012; Mulhern and Bowie 2010).

NS3/4A protease is reported to be crucial, not only for the liberation of HCV NS proteins, but also for the regulation of host anti-viral reactions by proteolytic inactivation of host cytosolic proteins, which also interfere with homeostasis of live cells. It has been reported that NS3/4A proteolytically degrades MAVS (Cheng et al. 2006; Li et al. 2005b; Loo et al. 2006). In addition, NS4B protein has been reported to target STING to repress RIG-I-mediated type I IFN induction in hepatocytes (Nitta et al. 2012). Preceding the generation of these NS proteins, HCV core (Oshiumi et al. 2010a) and E2 proteins (Florentin et al. 2012) can suppress RIG-I-mediated type I IFN production in hepatocytes and plasmacytoid DC, respectively. In particular, the generation of core protein and NS3/4A are closely associated with suppression of the HCV-mediated host IFN biological response and the promotion of HCV replication. Since core protein is produced prior to NS3/4A in HCV-infected cells, many other functions of the core are expressed just before the proteolytic processing of HCV NS proteins within the cells.

NS3/4A cleaves MAVS (Cheng et al. 2006; Li et al. 2005b; Oshiumi et al. 2010a) and TICAM-1 (Li et al. 2005a). Hence, NS3/4A proteolytically controls at least two adaptor proteins as its substrates. In addition, Riplet is reduced in response to HCV replication (Oshiumi et al. 2010c). Whether or not Riplet is a substrate for NS3/4A is still under investigation.

MAVS Inactivation by NS3/4A

The current assumption is that the RIG-I family proteins, RIG-I and MDA5, sense viral RNA to induce type I IFN and pro-inflammatory cytokines, which in turn suppress viral infection. RIG-I and MDA5 possess the N-terminal caspase activation and recruitment domain (CARD), the central DExD/H-box helicase domain, and the C-terminal RNA-binding domain (CTD) (Fig. 1). According to crystal structure analysis, the basic region of CTD binds virus dsRNA irrespective of the presence of 5′-triphosphate (Yoneyama et al. 2004), while the CARD domain participates in interaction with the adaptor.

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RIG-I recognizes HCV RNA, as well as its replication intermediate, dsRNA (Saito et al. 2008). Although MDA5 recognizes long dsRNA patterns, the role of MDA5 in HCV RNA recognition is unknown. MAVS is the key adaptor for RIG-I/MDA5-mediated IFN induction in HCV infection, although it is localized in the mitochondrial outer membrane apart from intact RIG-I molecules (Seth et al. 2005). RIG-I is not quantitatively sufficient at the protein level to capture the abundant dsRNA replicating in infected cells during the early stage; other molecules have to accept the overwhelmed dsRNA in other cytoplasmic regions (Oshiumi et al. 2010b). RIG-I is initially involved in a molecular complex (RIG-I granule), which contains many other molecules that make up a nucleocapture complex. E3 ubiquitin ligases are involved in the RIG-I granule. Ubiquitin ligases, TRIM25 (Gack et al. 2007) and Riplet (Oshiumi et al. 2009), are also situated in the RIG-I granule together with RIG-I and confer RNA-binding capacity on RIG-I through RIG-I ubiquitination (Fig. 1). TRIM25 ubiquitinates N-terminal lysines of RIG-I (Gack et al. 2007), while Riplet ubiquitinates C-terminal lysines of RIG-I (Oshiumi et al. 2009), either or both of these molecules enable RIG-I to interact with MAVS and confer mobility on mitochondria (Fig. 3). A recent report

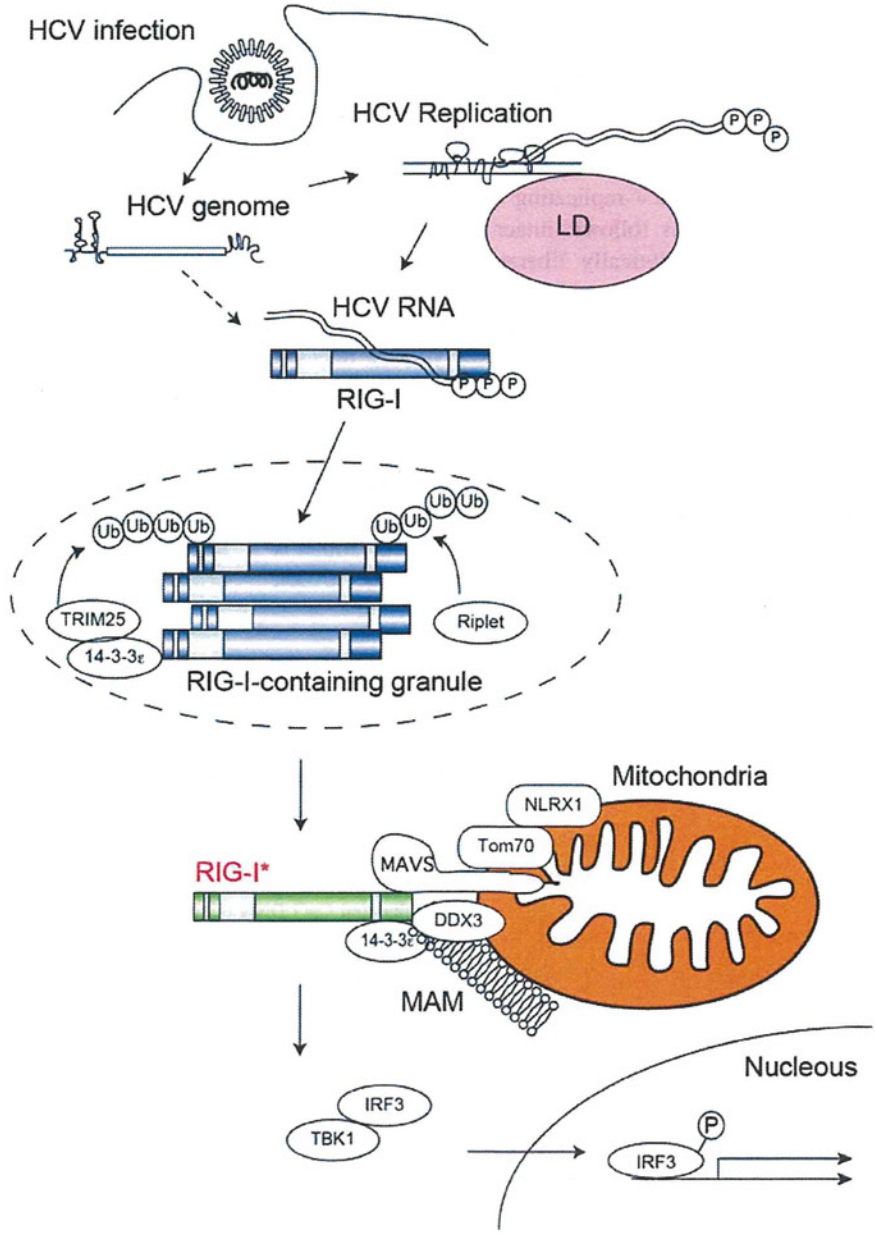

Fig. 3 Translocation of RIG-I from the cytoplasm to the mitochondria. RIG-I is diffusely distributed in the cytoplasm. When minute quantities of dsRNA enter the cytoplasm (*dashed line*), the RIG-I granule is formed with many other molecules to sense dsRNA. Once RIG-I molecules are polyubiquitinated, they form a complex with dsRNA and becomes mobile (RIG-I*). RIG-I* is recruited to the mitochondria to couple with MAVS. There are many other molecules associated with mitochondrial signaling. Because DDX3 captures overwhelmed dsRNA, the RIG-I–DDX3–MAVS complex allows robust IFN production in conjunction with dsRNA/DDX3. Whether DDX3 participates in IFN-λ induction remains undetermined

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speculated that after RIG-I is up-regulated and ubiquitinated in the RIG-I granule, the 14-3-3ε is coupled with newly ubiquitinated RIG-I (Liu et al. 2012). Then, RIG-I moves from the granule to the mitochondrial membrane, a distinct membrane compartment linked to the ER, which is referred to as mitochondrial-associated endoplasmic reticulum membranes (MAM) (Horner et al. 2011). MAM accumulates MAVS and may coordinate MAVS signaling of innate immunity from peroxisomes (Dixit et al. 2010) and mitochondria (Seth et al. 2005), while MAVS localized to MAM serves as a molecular platform for the IFN-inducing signal. MAVS is constitutively complexed with DDX3, which serves as an acceptor of dsRNA in resting (RIG-I-insufficient) cells (Oshiumi et al. 2010b). If this is the case, the location for RIG-I ubiquitination (i.e., RIG-I granule) may differ from the site at which RIG-I interacts with the MAVS–DDX3 complex for signaling. Validating this issue will be of great interest in understanding initial viral RNA recognition.

Our previous data suggested that three forms of MAVS are detected in HCV-replicating hepatocyte lines by imaging analysis, as follows: intact MAVS, sequestered MAVS, and proteolytically liberated MAVS (Oshiumi et al. 2010a). These forms of MAVS simultaneously exist in hepatocytes expressing the HCV replicon or those infected with HCV. The MAVS proteolytically released from mitochondria appear to have decreased ability to activate IRF-3. MAVS is also diminished in some HCV-infected cells to lose its IFN-inducing function (Oshiumi et al. 2010a), suggesting that NS3/4A is a protease that determines the inactivation state of MAVS in HCV-replicating hepatocytes. A recent report suggested that Riplet is depleted during HCV replication (Oshiumi et al. 2010c), indicating the possibility that participation of the expressed NS3/4A protease in degrading other molecules upstream of MAVS is more important for IFN regulation than clipping out of MAVS in infected cells (Fig. 1). Similarly, other factors independent of proteolytic control may be critical for dsRNA-mediated IFN inducibility, as demonstrated by Cheng et al. (2006).

Blocking of the DDX3-Augmented IFN Production by Core Protein

Three reports have independently showed that DEAD/H Box 3 (DDX3, also known as DBX) acts as a positive regulator for MAVS-mediated type I IFN induction (Table 2; Fig. 2b). Elevation of MAVS pathway-mediated type I IFN production by DDX3 is modally different in these three reports (Mulhern and Bowie 2010). Like RIG-I and MDA5, DDX3 is a member of the DExD/H-box family of RNA helicases and is ubiquitously expressed in a variety of cells (Kim et al. 2001). The DExD/H motif of the members in this family of proteins is predictive of a role in RNA-binding and RNA-dependent cellular processing (Schroder 2009). Schroder et al. (2008) showed that the vaccinia virus protein K7 binds DDX3 and inhibits pattern-recognition receptors-induced IFN-β promoter activation. They suggested that DDX3 interacts with IKKε to enhance IRF-3 activation, while K7 counters DDX3 activity of MAVS-mediated IFN-β induction. This IFN-enhancing function of DDX3 in IRF-3 activation is located at the N-terminus of DDX3, which is the same region of the protein targeted by K7 for IRF-3 inhibition. Structure analysis of K7 complexed with a peptide from the N-terminus of DDX3 (Oda et al. 2009) has confirmed this finding.

Table 2 Nucleic acid sensors related to IFN induction in innate immunity

Pattern-recognition receptors	Adaptors	Agonists (references)	Origin
MDA5	MAVS	Cytosolic long dsRNA (Yoneyama et al. 2008)	RNA viruses
RIG-I	MAVS	Cytosolic 5′-PPP-RNA (Yoneyama et al. 2008)	RNA viruses
NOD2	MAVS	Cytosolic ssRNA (Morosky et al. 2011)	RNA viruses
TLR3	TICAM-1	Endosomal >140 bp dsRNA viruses, host (Matsumoto and Seya 2008)	DNA/RNA
TLR7/8	MyD88	Endosomal ssRNA (Uematsu and Akira 2007)	RNA viruses, bacteria
TLR9	MyD88	Nonmethylated CpG DNA (Uematsu and Akira 2007)	RNA viruses, bacteria
DDX3	MAVS	dsRNA, ssRNA (Oshiumi et al. 2013; this review)	Viruses, host
DDX1/21, DHX36	TICAM-1	dsRNA (Rathinam and Fitzgerald 2011)	Viruses?
DDX60	MAVS	dsRNA, ssRNA, dsDNA (Oshiumi et al. 2013; this review)	Viruses, host
DHX9/DHX36 MyD88	STING	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DDX41	TBK1	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
DAI (ZBP1)	STING	dsDNA (Takaoka and Taniguchi 2008)	DNA viruses
IFI16	β-Catenin	dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses
LRRFIP1		dsDNA (Rathinam and Fitzgerald 2011)	DNA viruses

ssRNA single-stranded RNA

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The second study indicated that DDX3 constitutively interacts with MAVS via the C-terminal region of DDX3 (Oshiumi et al. 2010b). The binding of DDX3 to MAVS is constitutive and not through the N-terminus, in contrast to the case of the virus-dependent interaction between DDX3 and IKKε (Schroder et al. 2008). RIG-I-induced IFN-β promoter reporter gene activity is inhibited by DDX3 small interfering RNA and enhanced by overexpression of DDX3 (Oshiumi et al. 2010b). Thus, DDX3 synergistically activates the IFN-β promoter together with MAVS. The C- and N-terminal regions of the DDX3 regulate MAVS-mediated IFN induction (Hogbom et al. 2007) (Fig. 1).

In contrast, Soulat et al. (2008) demonstrated a positive role for DDX3 in IFN-β promoter induction in another distinct manner. Specifically, DDX3 is shown to be a kinase substrate for TBK1 to synergistically enhance IFN-β promoter activation by TBK1. Furthermore, they demonstrated that DDX3 is recruited to the IFN-β promoter via its N-terminal region (Soulat et al. 2008). Together, these findings show that DDX3 is a positive regulator targeting the multiple sites of the RLR-induced IFN pathway. A role for DDX3 in cell cycle control and apoptosis has also been proposed in response to dsRNA (Schroder et al. 2008).

DDX3 facilitates viral replication in a variety of viruses, including HCV. The N-terminus of HCV core protein binds the C-terminus of DDX3 (Owsianka and Patel 1999). According to a recent finding, the HCV core protein participates in the suppression of DDX3-augmented MAVS signaling for IFN-β induction (Fig. 2b), which may also be related to the function of DDX3 described in the second study mentioned before (Oshiumi et al. 2010b). Unlike the DExD/H-box helicases, such as RIG-I and MDA5, DDX3 is constitutively expressed and co-localized with MAVS around mitochondria (Table 2). However, HCV core protein interferes with DDX3 function to enhance MAVS signaling by coupling with DDX3 to dissociate it from MAVS in the mitochondria. In hepatocytes with the HCV replicon, DDX3/MAVS-enhanced IFN-β-induction is largely abrogated, even when DDX3 is co-expressed. Whether DDX3 enhances IFN-λ induction like RIG-I remain untested. DDX3 is spotted with minimal merging with MAVS in confocal analysis, and partly assembles in the HCV core protein located near the lipid droplet (LD) in replicon-positive hepatocytes, although in some cells MAVS is diminished or disseminated apart from mitochondria. Thus, HCV core retracts DDX3 from MAM, where RIG-I moves from the RIG-I granule to assemble together with MAVS (Figs. 2b, 3).

Our consensus is that the binding of HCV core protein to DDX3 and suppressing DDX3–MAVS complex formation are crucial for inhibition of the MAVS pathway. However, multiple functions of DDX3 may be reflected in other functional aspects of core protein; specifically, the DDX3–core interaction is required for HCV replication (Ariumi et al. 2007). Although DDX3 promotes efficient HCV infection by accelerating HCV RNA replication, the processes appear independent of its interaction with the viral core protein (Angus et al. 2010). In addition, the association between DDX3 and core protein implicates DDX3 in HCV-related hepatocellular carcinoma progression (Chang et al. 2006). Based on its core protein association and MAVS-regulatory properties, DDX3 appears to be switched by the core protein from an HCV-suppressing (i.e., IFN-inducing) mode to an HCV replication-supporting mode (Fig. 2b). The results enable us to conclude that HCV infection is promoted by modulating the dual function of DDX3.

Evidence is accumulating that HCV assesses many steps in the IFN-inducing pathway throughout the early and late infection stages, and suppresses IFN production by multiple means. Disruption of MAVS function by NS3/4A and core protein may be crucial in HCV-infected Huh7.5 cells, even though the cells harbor dysfunctional RIG-I (Binder et al. 2007). Type I IFN suppresses tumor progression by causing expression of p53 and other tumor-suppressing agents (Takaoka et al. 2005). E2 and NS4B may affect tumor progression by controlling type III IFN induction. These unique features of the HCV proteins require further confirmation and should be in the focus of investigation regarding HCV persistence, chronic infection, and progression to cirrhosis and carcinoma.

Inactivation of the TICAM-1 Pathway by NS3/4A

TICAM-1 pathway has been associated with chemokine production, apoptosis, necroptosis, and IL-12p40 production in hepatoma cell lines expressing TLR3 (Li et al. 2012); however, such immune responses are predominantly absent in primary cultured cells. This might be explained by the TLR3 signaling that is likely to be shut off in most normal hepatocytes, but executed in hepatocytes of the infectious liver during chronic states of HCV infection or exposure to dsRNA stimulation. Despite the constitutive expression of the adaptor molecule TICAM-1 in human hepatocytes, only trace amount of TLR3 is being expressed in comparison to RIG-I that is commonly expressed. This gives us an insight of the role played by other cytoplasmic dsRNA sensors such as DDX1/DDX21/DHX36 (Table 2), in the activation of TICAM-1 pathway (Zhang et al. 2011). It remains unknown whether these cytoplasmic dsRNA sensors participate in IFN-λ induction.

Although there are positive findings supporting the importance of TLR3 in the pathogenesis of HCV infection (Eksioglu et al. 2011; Khvalevsky et al. 2007; Li et al. 2012), the expression level of TLR3 is still a contentious issue. TLR3 protein is undetectable by immunostaining with monoclonal antibody (TLR3.7) against huTLR3 in

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