学会，２０１２年１２月７日，東京

H. 知的財産権の出願・登録状況

1. 特許取得

出願名称：　ラット胚性幹細胞を用いたキメララットの作製法

出願人：国立がん研究センター；DSファーマバイオメディカル株式会社

発明者　落谷　孝広、川又　理樹

出願日時　　平成22年7月23日

出願番号　　特願 2010-166571

2. 実用新案登録

なし

2. その他

なし

厚生労働科学研究費補助金（B 型肝炎創薬実用化等研究事業）
分担研究報告書

HBV 感染を阻害する低分子化合物のスクリーニング

長田 裕之（理研 ケミカルバイオロジー研究基盤施設 施設長）

研究要旨：微生物や植物の二次代謝産物、およびその誘導体を中心に収集した化合物ライブラリーを用いて抗 HBV 薬を探索する。この探索においては、ウイルスの感染初期過程を高感度に検出できる感染モデル系を利用し、従来の治療薬（インターフェロンや核酸アナログ）とは異なる新たなタイプの HBV 治療薬の開発を目的とする。計画前期（H24-25）は微生物生合成遺伝子改変技術やフラクションライブラリー、表現型スクリーニング基盤を利用し、新規天然化合物の創製を行う。研究中期（H26-）から HBV 様蛍光粒子を用いたスクリーニングを実施し、阻害活性を示す化合物をバイオプローブとしたケミカルバイオロジー研究を展開する。これら一連の研究を通じて、HBV 治療に資する候補化合物の創製と新規標的（HBV 受容体など）の発見を目指す。

A．研究目的

HBV 感染阻害物質の探索を実施するためには、多様性に富んだケミカルライブラリーが必要である。またそのようなライブラリー構築には、①質の高い化合物群と②構造や活性情報が充実したデータベースやデータマイニング技術、などの創薬基盤の整備が不可欠である。本研究では、微生物生合成遺伝子改変技術やフラクションライブラリーを用い、天然化合物の収集を進める。また候補化合物の作用機序を簡便に解析する方法を開発し、HBV 感染阻害薬探索に資する化合物ライブラリーの整備・拡充を行う。

B．研究方法

化合物ライブラリーの整備を目的とし、①微生物由来天然化合物の網羅的な収集と②表現型スクリーニング基盤の構築を計画した。①について、生合成遺伝子改変微生物やフラクションライブラリー、天然化合物データベース NPPlot を用いて新規微生物二次代謝産物を探索した。②について、細胞形態変化などの表現型を指標に薬剤作用を簡便に予測するシステム構築を試みた。

（倫理面への配慮）

遺伝子組み換え生物等の使用に際しては、理化学研究所の定める細則や指針を遵守した。

C．研究結果

① 微生物由来天然化合物の網羅的な収集

ポリケチド生合成遺伝子（PKS）改変微生物からリベロマイシンなどポリケチド系新規代謝産物を単離した（Nogawa et al）。また微生物代謝産物を系統的に収集したフラクションライブラリーから、様々な新規物質を取得した。

②表現型スクリーニング基盤の構築

200 種類の標的既知薬剤ががん細胞に対して誘導する形態変化を網羅的に収集した細胞形態変化データベース「モルフォベース」を構築し、形態変化を指標に薬剤作用を予測するモルフォベースプロファイリング法を開発した（Futamura et al）。

D．考察

研究前期に HBV 感染阻害薬の探索源に資する化合物を３万種類以上整えることをマイルストーンとしている。今年度は様々な新規微生物代謝産物を見い出すと

共に植物エキスのフラクションライブラリー作製に着手しており、当初の目標をクリアーするべく研究は順調に進んでいる。また今回構築したモルフォベースプロファイリング法は、HBV 感染アッセイ系でヒットした化合物の作用評価に役立つことが期待される。

E. 結論

抗 HBV 薬のスクリーニングに資する化合物ライブラリーを拡充するため、遺伝子改変微生物から新規代謝産物を単離した。細胞形態変化を指標に薬剤作用を予測するモルフォベースプロファイリング法を開発した。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得：なし
2. 実用新案登録：なし
3. その他：なし

Ⅲ　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Futamura Y, Kawatani M, Kazami S, Tanaka K, Muroi M, Shimizu T, Tomita K, Watanabe N, Osada H	Morphobase, an encyclopedic cell morphology database, and its use for drug target identification.	Chem Biol,	19	1620-1630	2012

Ⅳ　研究成果の刊行物・別刷り

Microbiology and Immunology

Microbiol Immunol 2012; **56**: 1–9
doi:10.1111/j.1348-0421.2011.00403.x

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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Received 8 September 2011; revised 23 October 2011; accepted 3 November 2011.

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.

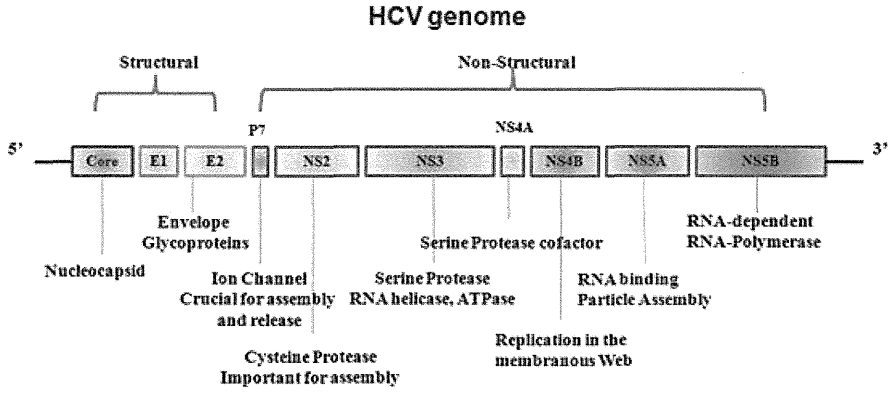

Fig. 1. Genomic structure of HCV. Genomic organization of wild-type HCV. The HCV-RNA genome consists of a major open reading frame, encoding a single polyprotein, and an alternative reading frame encoding F-proteins with unknown functions. The cleavage of the polyprotein by viral and host cell proteases 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.

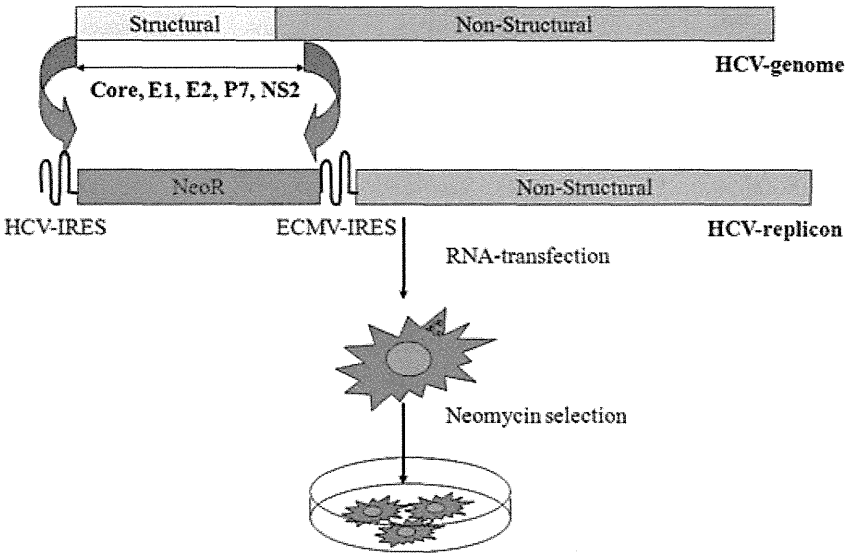

Fig. 2. HCV replicon system. The structural sequences (C, E1, E2, and p7) together with NS2 were replaced by a neomycin antibiotic-resistance gene, and an ECMV-IRES was introduced to drive translation of the remaining *non-structural proteins. Neomycin selection of* these double cistron (bicistronic) 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

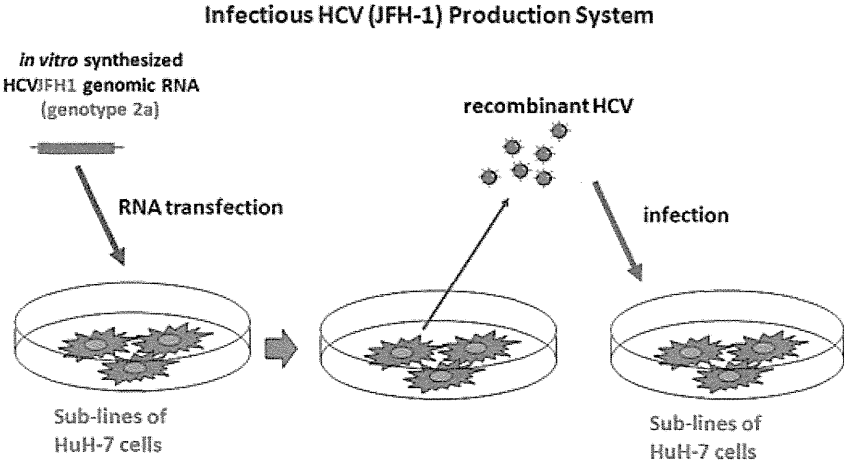

Fig. 3. JFH1 infectious system. Full-length JFH1-RNA is transcribed *in vitro*, and transfected to HuH-7-derived cell lines. JFH1 replicates in these cells, and produce infectious virions in the medium. The medium is collected, concentrated, and used to infect naive cells. Hence, the entire HCV life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

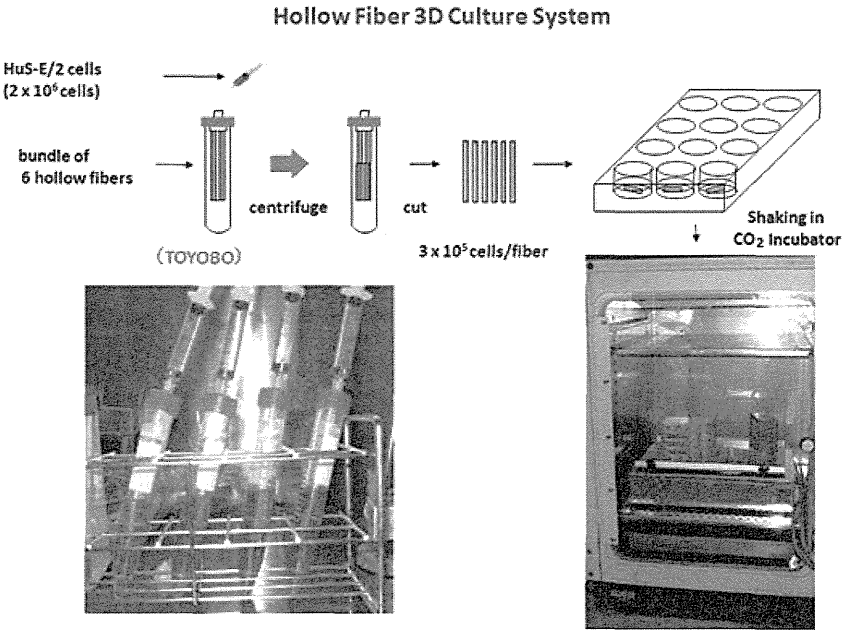

Fig. 4. 3-D hollow fiber culture. HuS-E/2 suspension was injected into the lumen of the hollow fiber system (HF; Toyobo Co., Osaka, Japan). The bundles were centrifuged to induce organoid formation. The lower 1.5 cm containing the organoid formation was then cut and cultured in 12-well plates (two capillary 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

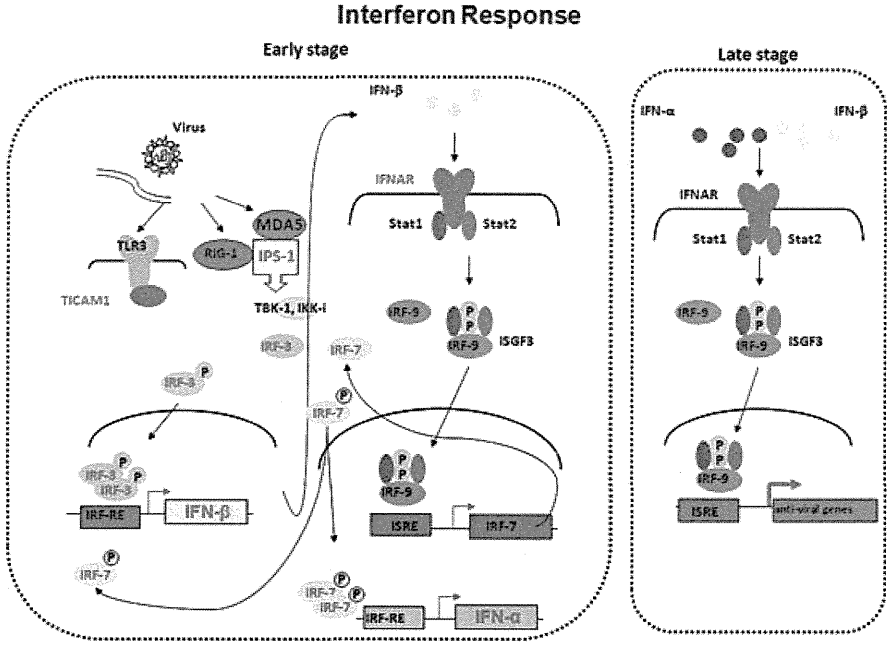

Fig. 5. Induction of interferon response by viral RNA. The cell detects viral RNA through the endosomal RNA sensor TLR3, and the cytoplasmic RNA sensors RIG-I and MDA5. Both pathways will lead to the activation of TBK-1 and IKK-I kinases, through the TICAM-1 adaptor molecule in the case of TLR3, or IPS-1 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

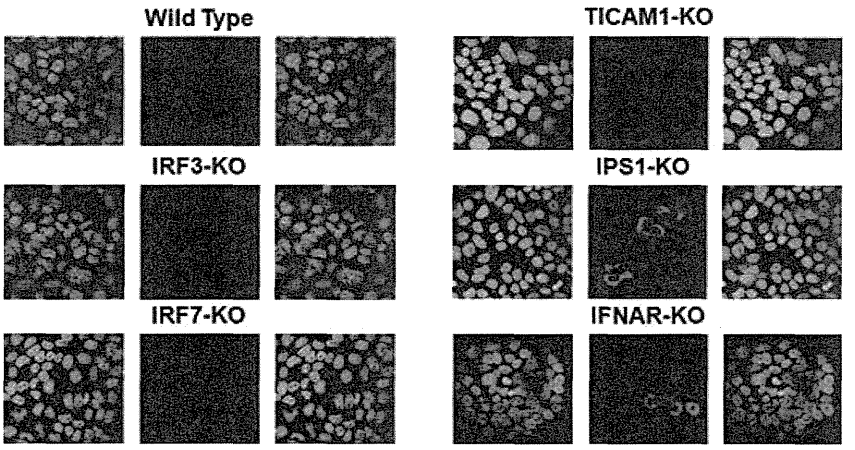

Fig. 6. Establishment of mouse hepatocyte lines permissive to J6/JFH1.
Immunofluorescence detection of J6JFH1 proteins' expression 5 days after transfection of J6JFH1-RNA through electroporation into wild-type, IRF-3-ko, IRF-7-ko, TICAM1-ko, IPS-1-ko, and IFNAR-ko, freshly isolated primary hepatocytes. 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.

ACKNOWLEDGMENT

The author wish to thank Dr. Jin Takahashi from the Japanese NIH, NIID for his help designing figure 5 in this paper.

DISCLOSURE

The authors declare no financial or commercial conflict of interest.

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Virus Research 163 (2012) 390–395

Contents lists available at SciVerse ScienceDirect

Virus Research

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Short communication

The interaction between human initiation factor eIF3 subunit c and heat-shock protein 90: A necessary factor for translation mediated by the hepatitis C virus internal ribosome entry site

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ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 4 October 2011
Accepted 6 October 2011
Available online 14 October 2011

Keywords:
eIF3c
Hsp90
HCV IRES
17-AAG
Translation initiation

ABSTRACT

Heat-shock protein 90 (Hsp90) is a molecular chaperone that plays a key role in the conformational maturation of various transcription factors and protein kinases in signal transduction. The hepatitis C virus (HCV) internal ribosome entry site (IRES) RNA drives translation by directly recruiting the 40S ribosomal subunits that bind to eukaryotic initiation factor 3 (eIF3). Our data indicate that Hsp90 binds indirectly to eIF3 subunit c by interacting with it through the HCV IRES RNA, and the functional consequence of this Hsp90–eIF3c–HCV-IRES RNA interaction is the prevention of ubiquitination and the proteasome-dependent degradation of eIF3c. Hsp90 activity interference by Hsp90 inhibitors appears to be the result of the dissociation of eIF3c from Hsp90 in the presence of HCV IRES RNA and the resultant induction of the degradation of the free forms of eIF3c. Moreover, the interaction between Hsp90 and eIF3c is dependent on HCV IRES RNA binding. Furthermore, we demonstrate, by knockdown of eIF3c, that the silencing of eIF3c results in inhibitory effects on translation of HCV-derived RNA but does not affect cap-dependent translation. These results indicate that the interaction between Hsp90 and eIF3c may play an important role in HCV IRES-mediated translation.

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a positive-strand RNA genome (Taylor et al., 1999; Bartenschlager and Lohmann, 2001) encoding a large precursor polyprotein that is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Grakoui et al., 1993; Hijikata et al., 1993). Ishii et al. identified an HCV replicon system in which the full HCV genomic RNA autonomously replicates in the Huh-7 human hepatoma cell line (Fig. 1A) (Ishii et al., 2006). This HCV replicon system allows researchers to study HCV genome replication in cell culture. HCV protein synthesis is initiated by the HCV RNA genome. This genome contains a conserved structure in its 5′-untranslated region (5′-UTR) that acts as an internal ribosome entry site (IRES) (Lukavsky, 2008). Briefly, the small ribosomal subunit (40S) and the eukaryotic initiation factor eIF3 bind specifically to the HCV IRES RNA, allowing for direct recognition of the start codon present in the 5′-UTR of the viral mRNA (Spahn et al., 2001; Collier et al., 2002; Kieft et al., 2002; Fraser and Doudna, 2007; Julien et al., 2009). Consistent with its diverse functions, eIF3 is the largest and most complex initiation factor. The mammalian version, for example, contains 13 nonidentical subunits designated eIF3a to eIF3m. The eIF3 core subunit (eIF3a–c, g, and i) is essential for translation (Kieft et al., 2002; Hinnebusch, 2006; Masutani et al., 2007; Zhou et al., 2008), and eIF3 specifically associates with the apical half of domain III of the HCV IRES (Kieft et al., 2001, 2002; Siridechadilok et al., 2005; Fraser and Doudna, 2007).

Hsp90 is a heat-shock protein that is abundant in the cytosol of eukaryotes and prokaryotes. In contrast to other chaperones, a number of substrates are known to contain Hsp90 (Schulte et al., 1995). Studies of eukaryotes have revealed that these Hsp90 client proteins include a variety of transcription factors (Coumailleau et al., 1995; Garcia-Cardena et al., 1998; Nagata et al., 1999; Sato et al., 2000; Richter and Buchner, 2001; Xu et al., 2001; Waza et al., 2005). Recently, many studies have reported that Hsp90 is involved with not only HCV RNA replication and viral protein but also HCV IRES-mediated translation (Waxman et al., 2001; Kim et al., 2006; Okamoto et al., 2006; Nakagawa et al., 2007; Ujino et al., 2009). In the present study, we demonstrate that eIF3 forms a complex with Hsp90 that is critical for HCV IRES-mediated translation.

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doi:10.1016/j.virusres.2011.10.003

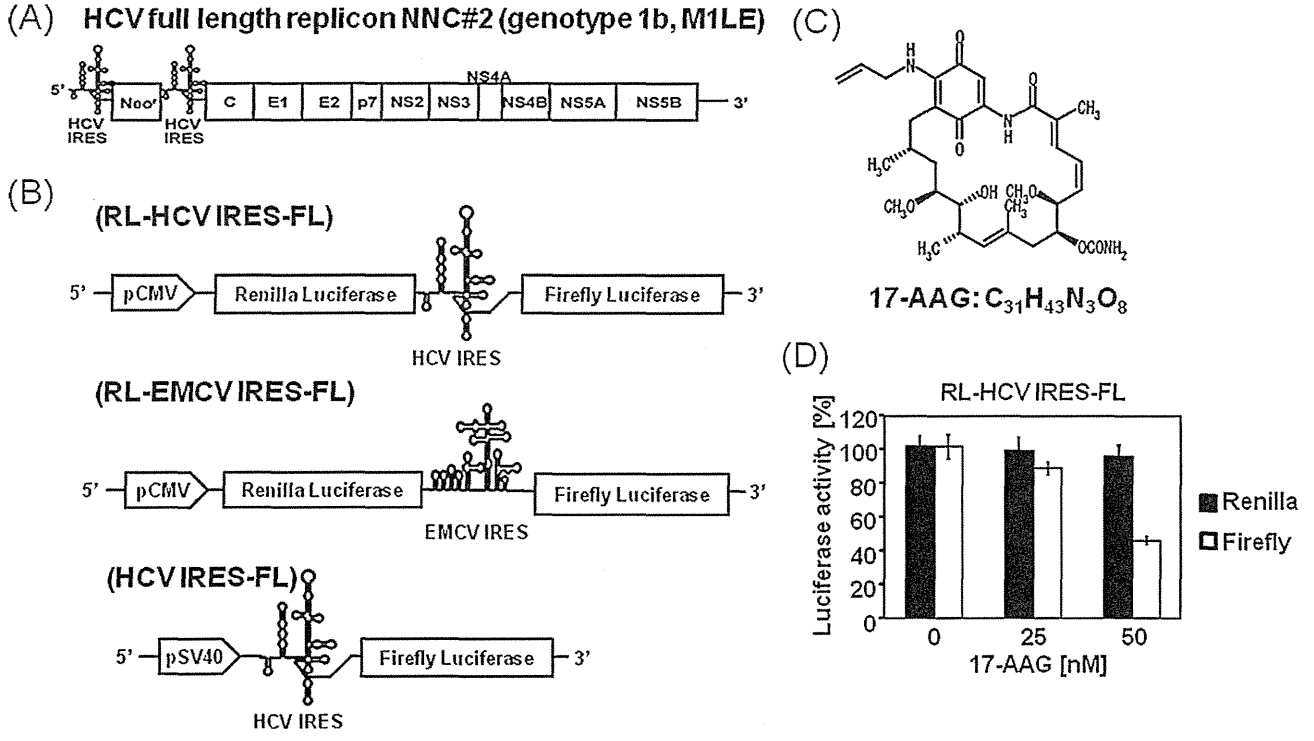

Fig. 1. *Inhibition of IRES-mediated translation by an Hsp90 inhibitor.* (A) *The structure of the HCV replicon RNA molecules comprising the HCV 5′-UTR, including the HCV* IRES, the neomycin phosphotransferase gene (Neo^r), and the coding region for the HCV proteins core to NS5B (in the HCV full-length replicon). (B) A schematic representation of the bicistronic HCV IRES or EMCV IRES reporter construct pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL) or pRenilla-EMCV IRES-firefly luciferase (RL-EMCV IRES-FL) driven by the CMV promoter to direct cap-dependent translation of renilla luciferase (RL) and HCV IRES or EMCV IRES-dependent translation of firefly luciferase (FL). The vector construct for HCV IRES-mediated translation of firefly luciferase, pHCV IRES-firefly luciferase (HCV IRES-FL) (Ujino et al., 2010). (C) The structure of the Hsp90 inhibitor 17-AAG (17-allylamino-17-demethoxygeldanamycin, Sigma–Aldrich Chemical Co.). (D) Inhibition of IRES-mediated translation by 17-AAG. Huh-7 cells (1×10^5 cells/well on 12-well plates) treated with 17-AAG (25 and 50 nM) and DMSO as a control for 24 h, and were then transfected with pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL) using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega). The data represent the mean ± standard deviations (SDs) from the experiments performed in triplicate.

To investigate the effects of the *Hsp90* inhibitor 17-AAG on HCV IRES translation, a bicistronic reporter system was used that consisted of an upstream reporter, Renilla luciferase (RL), expressed by cap-dependent translation and a downstream reporter, firefly luciferase (FL), which is under the translational control of the HCV IRES. To construct pcDNA-HCV IRES-firefly Luc, pHCV IRES-firefly Luc (HCV IRES-FL) (Ujino et al., 2010) (Fig. 1B) was digested with BamHI and SalI. The IRES-firefly Luc fragments were inserted into the BamHI-XhoI site of pcDNA3.1 (Invitrogen, Carlsbad, CA). To construct pRenilla-HCV IRES-firefly luciferase (RL-HCV IRES-FL), Renilla luciferase fragments were amplified by PCR from a pFN11A Flexi vector (Promega, Madison, WI), and the PCR products were inserted into the BamHI site of pcDNA-HCV IRES-firefly Luc. The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS). The benzoquinone ansamycin, the antibiotic geldanamycin (GA) and its less toxic analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) (Fig. 1C) (Sigma–Aldrich Chemical Co., St Louis, MO) directly bind to the ATP/ADP binding pocket of Hsp90, thus preventing ATP binding and the completion of client protein refolding (Neckers, 2003). The client proteins of Hsp90 appear to shift the role of the primary chaperone from Hsp90 to Hsp70 in cells treated with Hsp90 inhibitors (Doong et al., 2003). It is also well known that 17-AAG causes a modest increase in Hsp70 levels (Morimoto, 1998; Bagatell et al., 2000; Guo et al., 2005). In our previous report, a significant induction of Hsp70 was detected (Ujino et al., 2009).

For the reporter gene assay, Huh-7 cells were treated with different concentrations of the Hsp90 inhibitor, 17-AAG, or DMSO as a control for 24 h. They were then transfected with the bicistronic reporter construct RL-HCV IRES-FL using Lipofectamine 2000 (Invitrogen), which directs cap-dependent translation of the RL gene and HCV IRES-dependent translation of FL genes (Invitrogen). At 24 h post-transfection, the Renilla luciferase (cap-dependent translation) and firefly luciferase (HCV IRES-dependent translation) activities were measured with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). In cells treated with 50 nM 17-AAG, firefly luciferase activity was reduced by 55% with RL-HCV IRES-FL, whereas Renilla luciferase activity was mostly maintained (Fig. 1D). The inhibition of HCV IRES-mediated translation occurred in a dose-dependent manner. Recently, Kim et al. (2006) demonstrated that Hsp90 regulates ribosomal function by maintaining the stability of 40S ribosomal proteins such as rpS3 and rpS6. The interaction between the 40S ribosomal proteins and Hsp90 has also been associated with ribosomal activities such as protein synthesis. We also found that the Hsp90 inhibitor 17-AAG influences HCV IRES-mediated luciferase activity, suggesting that 17-AAG inhibited HCV RNA replication and HCV IRES-mediated translation.

The HCV IRES is recognized specifically by the small ribosomal subunit and eIF3 before the initiation of viral translation. Although the degradation of rpS3, a component of the small ribosomal subunit, has been shown to occur in the presence of the Hsp90 inhibitor (Kim et al., 2006), the influence of Hsp90 inhibition on eIF3 is not understood. To determine whether 17-AAG affects the expression of the eIF3 subunit, we analyzed eIF3a, eIF3b, eIF3c, eIF3g and eIF3i protein expression by western blot analysis. The HCV replicon cell line NNC#2 (NN/1b/FL), which carries a full genome replicon, was cultured in DMEM with 10% FBS, nonessential amino acids,

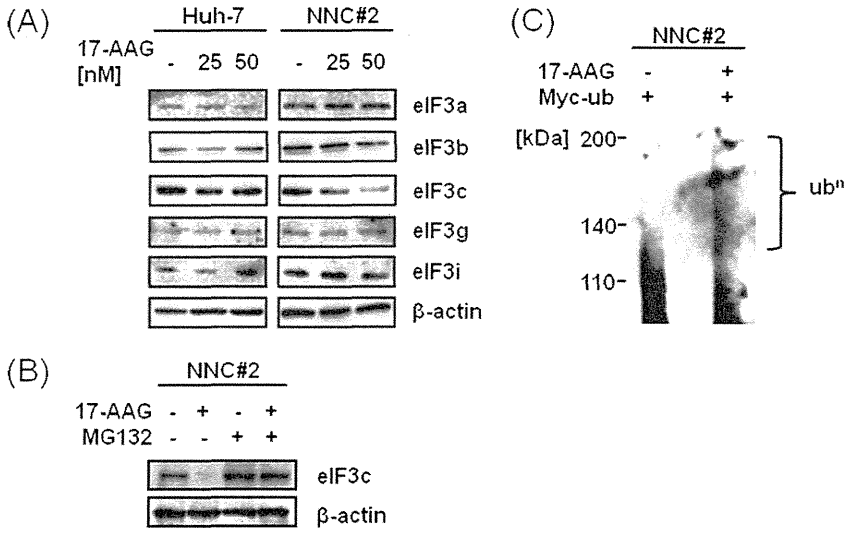

Fig. 2. Effect of 17-AAG treatment on eIF3 expression. (A) Western blot analysis of eIF3 protein expression in Huh-7 or NNC#2 cells treated with 17-AAG (25 nM and 50 nM). The cell lysates were analyzed by western blot 48 h after treatment. The primary antibodies used were monoclonal or polyclonal antibodies against eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (B) The reduction of eIF3c expression was prevented by proteasome inhibitor treatment. NNC#2 cells treated with 17-AAG (50 nM) or DMSO as a control. After 8 h treatment, the cells were treated with the proteasome inhibitor MG-132 (5 μM) or DMSO as a control. The cell lysates were analyzed by western blot 40 h after treatment. The primary antibody used was the eIF3c or β-actin (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. (C) eIF3c degradation is mediated by the ubiquitin-dependent protease pathway. NNC#2 cells were transfected with pCMV-Myc-Ubi using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were treated with 17-AAG (50 nM) or DMSO as a control for 8 h and were then treated with the proteasome inhibitor MG-132 (5 μM) for 16 h. The cell lysates were subjected to an immunoprecipitation assay using an anti-αMyc antibody (Cell Signaling) followed by an immunoblot analysis using anti-eIF3c antibody.

L-glutamine, penicillin/streptomycin, and 1 mg/mL G418 (Invitrogen) at 37 °C in 5% CO_2 (Ishii et al., 2006). For western blot analysis, NNC#2 cells and Huh-7 cells were lysed in 1× chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay buffer (Roche, Basel, Switzerland). The cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked with 5% skimmed milk. The primary antibodies used were monoclonal or polyclonal antibodies against FLAG-M2 (Sigma–Aldrich Chemical Co.), Hsp90 (Cell Signaling Tech., Beverly, MA), eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit antibody (Sigma–Aldrich Chemical Co.) was used as the secondary antibody. When the HCV replicon cell line NNC#2 (NN/1b/FL) and Huh-7 cells were treated with increasing doses of 17-AAG, the expression of the eIF3c subunit was markedly reduced in NNC#2 cells, but the expression in Huh-7 cells was unaffected (Fig. 2A). These results suggest that Hsp90 is involved in eIF3c stability through a physical interaction in the presence of HCV IRES RNA.

Protein degradation in cells is mediated by several protease systems; however, the stability of most proteins is regulated by Hsp90, and they appear to be degraded by proteasomes. To investigate whether the reduction of eIF3c was due to proteasomal degradation, we treated NNC#2 cells with a proteasome inhibitor, MG132, to prevent the 17-AAG-induced degradation of eIF3c. Our results indicated that 17-AAG-induced eIF3c degradation can be blocked by proteasome inhibitors (Fig. 2B). Proteasome inhibitors substantially prevented the degradation of eIF3c in cells treated with 17-AAG. This is most likely because the disruption of Hsp90 by the Hsp90 inhibitor treatment destabilized the eIF3c protein. Therefore, it is clear that proteasome-dependent degradation results in the decreased level of eIF3c protein. This indicates that the stability of eIF3c was supported by Hsp90, and unstable eIF3c was removed by proteasomes. Furthermore, we investigated whether the ubiquitination of eIF3c was affected by the Hsp90 inhibitor, 17-AAG. We transfected pCMV-Myc-Ubi (provided by Dr. A. Ryo) using Lipofectamine 2000 (Invitrogen) into NNC#2 cells, which were then treated with 17-AAG (50 μM). After treatment, the cells were then treated with 5 μM MG132 and subjected to immunoprecipitation with an anti-αMyc antibody (Cell Signaling) followed by an immunoblot analysis using an anti-eIF3c antibody. Notably, polyubiquitinated forms of eiF3c was detected in cells treated with 17-AAG (Fig. 2C). These results suggest that the destabilized eIF3c protein is degraded by proteasome-dependent proteolysis mediated by ubiquitin conjugation, and Hsp90 plays an important role in maintaining the stable form of the eIF3c protein in vivo.

To investigate the influence of eIF3c silencing on HCV IRES-mediated translation, Huh-7 cells were transfected with siRNA targeted to eIF3c at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen), and they were then transfected with RL-HCV IRES-FL. Control small interference RNA (siRNA) and eIF3 p110 (eukaryotic translation initiation factor 3, subunit 8, 110 kDa) siRNA were purchased from Santa Cruz Biotechnology. The protein levels of eIF3c were examined by western blot analysis, and HCV IRES-mediated translation was analyzed with a Dual-Luciferase Assay. As demonstrated in Fig. 3A, when compared to Huh-7 cells treated with control siRNA, the eIF3c protein level was markedly reduced in Huh-7 cells transfected with eIF3 p110 siRNA targeting eIF3c. Furthermore, firefly luciferase activity in RL-HCV IRES-FL was also reduced by approximately 63% in the cells treated with siRNA targeted to eIF3c, whereas Renilla luciferase activity was mostly maintained (Fig. 3B). The inhibition of HCV IRES-mediated translation by siRNA against eIF3c indicates that the suppression of HCV IRES-mediated translation by Hsp90 inhibition leads to a reduction in eIF3c. To further characterize the HCV IRES inhibitory effect of siRNA targeting eIF3c, we used additional bicistronic reporter plasmids for transient transfection assays with Huh-7 cells. Since we were mainly interested in viral IRESs, we chose to investigate the effect of the luciferase activities on translation derived from the IRES of EMCV in place of the HCV IRES (Fig. 1B). To generate pcDNA-EMCV IRES-firefly Luc, EMCV IRES fragments were created by PCR using the following primers: 5′-GAC TGG ATC CCC CCC CCT AAC-3′ and 5′-CAG TGG GCC CTA TTA TCG TGT TTT TCA AAG GAA AAC C-3′. The PCR products were inserted into the BamHI and