



## Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection

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### ABSTRACT

Host tropism of hepatitis C virus (HCV) is limited to human and chimpanzee. HCV infection has never been fully understood because there are few conventional models for HCV infection. Human induced pluripotent stem cell-derived hepatocyte-like (iPS-Hep) cells have been expected to use for drug discovery to predict therapeutic activities and side effects of compounds during the drug discovery process. However, the suitability of iPS-Hep cells as an experimental model for HCV research is not known. Here, we investigated the entry and genomic replication of HCV in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively. We showed that iPS-Hep cells, but not iPS cells, were susceptible to infection with HCVpv. The iPS-Hep cells expressed HCV receptors, including CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin; in contrast, the iPS cells showed no expression of SR-BI or claudin-1. HCV RNA genome replication occurred in the iPS-Hep cells. Anti-CD81 antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic replication, dose-dependently attenuated HCVpv entry and HCV subgenomic replication in iPS-Hep cells, respectively. These findings suggest that iPS-Hep cells are an appropriate model for HCV infection.

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### 1. Introduction

Hepatitis C virus (HCV), a hepatotropic member of the *Flaviviridae* family, is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. Approximately 130–200 million people are

estimated to be infected with HCV worldwide. Each year, 3–4 million people are newly infected with HCV [1]. Thus, overcoming HCV is a critical issue for the World Health Organization.

HCV contains a positive strand ~9.6 kb RNA encoding a single polyprotein (~3000 aa), which is cleaved by host and viral proteases to form structural proteins (core, E1, E2, and p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1]. These virus proteins might be potent targets for anti-HCV drugs. However, combination therapy with interferon and ribavirin, which often causes severe side-effects leading to treatment termination, has been the only therapeutic choice [2]. Very recently, new direct antiviral agents have been approved or are under clinical trials; these agents include NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors [2–4]. However, the emergence of drug resistance is a serious problem associated with the use of direct antiviral agents [5].

Host targets are alternative targets for the development of anti-HCV drugs. A liver-specific microRNA (miRNA), miR-122, facilitates the replication of the HCV RNA genome in cultured liver cells [6]. Administration of a chemically modified oligonucleotide complementary to miR-122 results in long-lasting suppression of HCV with no appearance of resistant HCV in chimpanzees [7]. Epidermal

**Abbreviations:** HCV, hepatitis C virus; iPS-Hep cells, human induced pluripotent stem cells-derived hepatocyte-like cells; HCVpv, HCV pseudotype virus; SR-BI, scavenger receptor class B type I; miRNA, microRNA; EGF-R, epidermal growth factor receptor; EphA2, ephrin factor A2; iPS cells, human induced pluripotent stem cells; FCS, fetal calf serum; Ad, adenovirus; HNF-4 $\alpha$ , hepatocyte nuclear factor-4 $\alpha$ ; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VSV, vesicular stomatitis virus; VSVpv, VSV pseudotype virus; tet, tetracycline; pol, polymerase; MOI, multiplicity of infection; Dox, doxycycline; IFN, interferon- $\alpha$ 8; ES cells, embryonic stem cells.

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growth factor receptor (EGF-R) and ephrin factor A2 (EphA2) are host cofactors for HCV entry [8]. Inhibitors of EGF-R and EphA2 attenuated HCV entry, and prevented the appearance of viral escape variants [8]. These findings strongly indicate that identification of host factors associated with infection of human liver by HCV is a potent strategy for anti-HCV drug development. Because the host tropism of HCV is limited to human and chimpanzee [9], there is no convenient model for the evaluation of HCV infections. This has led to a delay in the development of anti-HCV agents targeting host factors.

Takahashi and Yamanaka developed human induced pluripotent stem (iPS) cells from human somatic cells [10]. The stem cells can be redifferentiated *in vitro*, leading to new models for drug discovery, including iPS-based models for drug discovery, toxicity assessment, and disease modeling [11,12].

Recently, several groups reported that iPS cells can be successfully differentiated into hepatocyte-like (iPS-Hep) cells that show many functions associated with mature hepatocytes [13–19]. However, whether iPS-Hep cells are suitable as a model for HCV infection has not been fully determined. Here, we investigated HCV entry and genomic replication in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively.

## 2. Materials and methods

### 2.1. Cell culture

Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). An iPS cell-line (Dot-com) generated from the human embryonic lung fibroblast cell-line MCR5 was obtained from the Japanese Collection of Research Bioresources Cell Bank [20,21]. The iPS cells were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore, Billerica, MA) in iPSellon culture medium (Cardio, Hyogo, Japan) supplemented with 10 ng/ml fibroblast growth factor-2.

### 2.2. *In vitro* differentiation

Before the initiation of cellular differentiation, the medium of the iPS cells was replaced with a defined serum-free medium, hESF9, and the cells were cultured as previously reported [22]. The iPS cells were differentiated into iPS-Hep cells by using adenovirus (Ad) vectors expressing SOX17, the homeotic gene HEX or hepatocyte nuclear factor 4 $\alpha$  (HNF-4 $\alpha$ ) in addition to the appropriate growth factors, cytokines, and supplements, as described previously [19].

### 2.3. Reverse transcription (RT)-polymerase chain reaction (PCR) analysis of HCV receptors

Total RNA samples were reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and the resultant cDNAs were PCR amplified by using Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and specific paired-primers for CD81 (5'-cgccaaggatgtgaagcagttc-3' and 5'-tcccggagaagaggtcatc-gat-3'), scavenger receptor class B type I (SR-BI; 5'-attccgatcagtgcaacatga-3' and 5'-cagtttgcttctcagcacag-3'), claudin-1 (5'-tcagcagccctgccctag-3' and 5'-tggtgttggtgaagaggtgt-3'), occludin (5'-tca gggaaatccacctatcactcag-3' and 5'-catcagcagcagcatgactcttcac-3'), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-tct tcaccacatggagaag-3' and 5'-accactgtgtcctcagtgta-3'). The expected sizes of the PCR products were 245 bp for CD81, 788 bp for SR-BI, 521 bp for claudin-1, 189 bp for occludin, and 544 bp for GAPDH. The PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide.

### 2.4. HCVpv infection

Pseudotype vesicular stomatitis virus (VSV) bearing HCV envelope glycoproteins (HCVpv) and VSV envelope glycoproteins (VSVpv) were prepared as described previously [23]. iPS, iPS-Hep and Huh7 cells were treated with HCVpv or mixtures of HCVpv or VSVpv and anti-CD81 monoclonal antibody (JS-81; BD Biosciences, Franklin Lakes, NJ) or control mouse IgG for 2 h. After an additional 24 h of culture, the luciferase activities were measured by using a commercially available kit (PicaGene, Toyo Ink, Tokyo, Japan).

### 2.5. Preparation of Ad vector expressing the HCV replicon

Ad vectors expressing a tetracycline (tet)-controllable and RNA polymerase (pol) I promoter-driven HCV subgenomic replicon containing renilla luciferase (AdP<sub>235</sub>-HCV), a replication-incompetent HCV subgenomic replicon containing renilla luciferase (AdP<sub>235</sub>- $\Delta$ GDD), tet-responsive trans-activator (Ad-tTA) or a tet-controllable RNA pol-I driven firefly luciferase (AdP<sub>235</sub>-fluc) were prepared by using an *in vitro* ligation method as described previously [24–26]. The biological activity (infectious unit) of the Ad vectors was measured by using an Adeno-X rapid titer kit (Clontech, Mountain View, CA).

### 2.6. HCV replication assay

iPS, iPS-Hep and Huh7 cells were infected with AdP<sub>235</sub>-HCV or AdP<sub>235</sub>- $\Delta$ GDD at multiplicity of infection (MOI; infectious unit per cell) of 3, and Ad-tTA at MOI of 15. After 24 h, the cells were treated with 10  $\mu$ g/ml of doxycycline (Dox) for 48 h. Renilla luciferase activities in the lysates were then measured with the use of the Renilla Luciferase Assay System (Promega, Madison, WI). To normalize for the infectivity of Ad vector, iPS, iPS-Hep and Huh7 cells were co-infected with AdP<sub>235</sub>-fluc (3 MOI) and Ad-tTA (15 MOI). After a 72-h incubation, the firefly luciferase activities in the lysates were measured, and the renilla luciferase activities were normalized by dividing by the corresponding firefly luciferase activities.

### 2.7. Quantitative analysis of plus- and minus-strand HCV RNA

iPS, iPS-Hep and Huh7 cells were co-infected with AdP<sub>235</sub>-HCV or AdP<sub>235</sub>- $\Delta$ GDD (3 MOI), and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10  $\mu$ g/ml of Dox for 48 h. Total RNA was reverse-transcribed into cDNA by using the ThermoScript reverse transcriptase kit (Invitrogen) as described previously [27,28]. Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa Bio Inc.) by using Applied Biosystems StepOne Plus (Applied Biosystems, Foster City, CA). The transcription products of the HCV plus-strand RNA, minus-strand RNA, and GAPDH gene, were amplified by using specific primers for HCV plus-strand RNA (RC1 primer, 5'-gtctagc-catggcgttagta-3'; and RC21 primer, 5'-ctcccggggcactcgaagc-3'), HCV minus-strand RNA (tag primer, 5'-ggcgtcatggtggcgaataa-3'; and RC21 primer), and GAPDH (5'-ggtgtctcctcagctcaaca-3' and 5'-gtgtcgttgagggaatg-3'), respectively. The copy numbers of the transcription products of the HCV plus- and minus-strand RNA were normalized with those of the GAPDH gene and infectivity of Ad vector as described in the Section 2.6.

### 2.8. Inhibition of HCV replication by interferon- $\alpha$ 8

iPS-Hep and Huh7 cells were infected with AdP<sub>235</sub>-HCV (3 MOI) and Ad-tTA (15 MOI). After 24 h of infection, the cells were treated with 10  $\mu$ g/ml of Dox and recombinant human interferon- $\alpha$ 8 (IFN) at the indicated concentration. After an additional 48-h incubation, renilla luciferase activity in the lysates was measured with the use of the Renilla Luciferase Assay System. Cell

viability was measured with the use of a WST-8 kit (Nacalai Tesque, Kyoto, Japan).

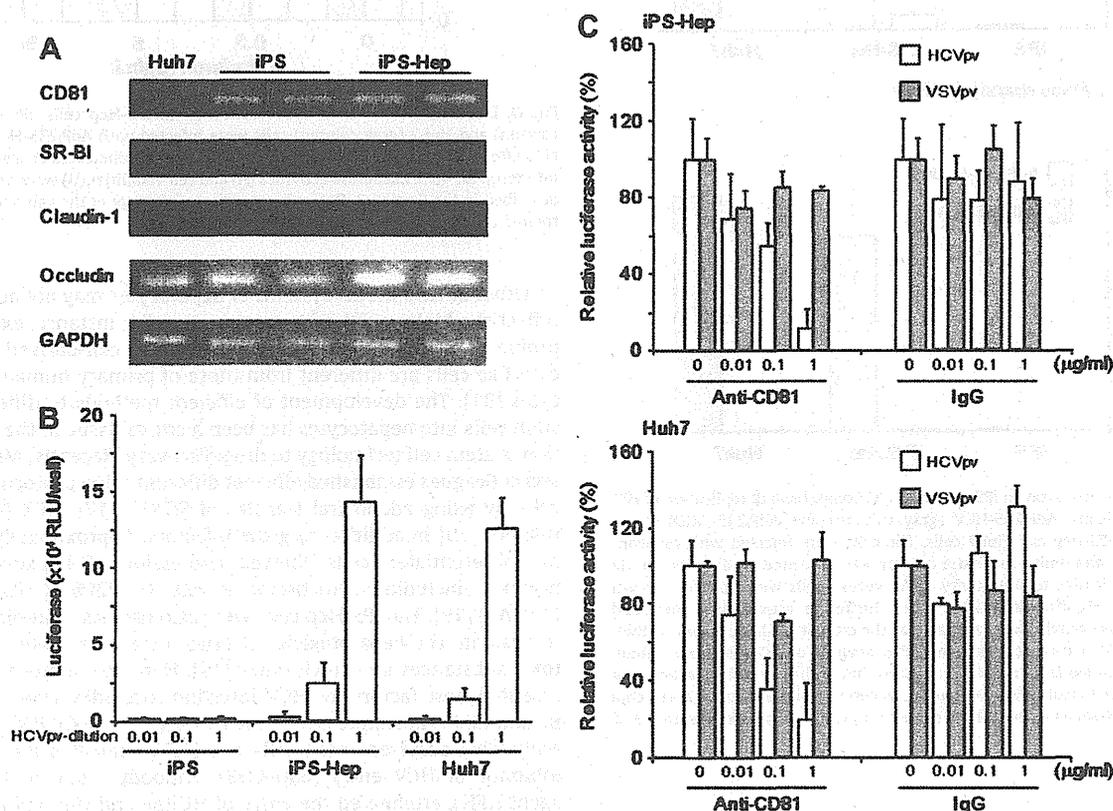
### 3. Results

#### 3.1. Infection of iPS-Hep cells with HCVpv

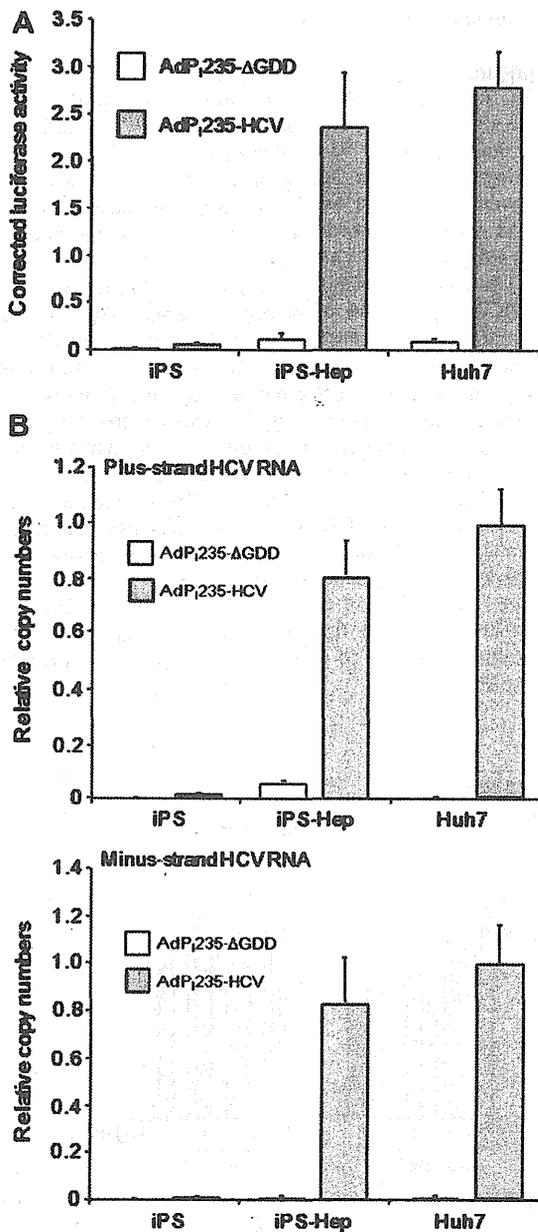
HCV entry requires sequential interaction between the envelope proteins and multiple cellular factors, including CD81, SR-BI, claudin-1, and occludin [29]. To investigate expression of these receptors in iPS-Hep cells, we performed RT-PCR analysis. iPS cells expressed CD81 and occludin, but not SR-BI and claudin-1. In contrast, iPS-Hep and Huh7 cells expressed all four receptors (Fig. 1A). HCVpv have been widely used in studies of the mechanism of HCV entry and in screens for inhibitors of HCV infection [30]. We therefore investigated HCVpv infection in iPS-Hep cells. iPS cells showed no susceptibility to HCVpv infection. In contrast, HCVpv dose-dependently infected iPS-Hep cells as well as Huh7 cells, a popular model cell line for HCV research (Fig. 1B). Treatment of the cells with IgG did not affect susceptibility of iPS-Hep or Huh7 cells to HCVpv infection, even at IgG concentrations of 1  $\mu\text{g}/\text{ml}$ . In contrast, anti-CD81 antibody dose-dependently inhibited HCVpv infection of iPS-Hep and Huh7 cells, and the antibody treatment did not affect infection of VSVpv with iPS-Hep (Fig. 1C). These findings suggest that iPS-Hep cells are a useful model for HCV infection.

#### 3.2. Replication of subgenomic HCV RNA in iPS-Hep cells

We previously developed Ad vectors containing tet-controllable and RNA pol I-driven HCV RNA subgenomic replicons (AdP<sub>235</sub>-HCV [replication competent], and AdP<sub>235</sub>- $\Delta$ GDD [replication incompetent]). The replicons encoded luciferase, and monitoring of luciferase activity in infected cells was a simple and convenient method to evaluate HCV replication [24]. Here, we found cells transduced with the replication-competent HCV replicon expressed luciferase in iPS-Hep cells, but not in iPS cells (Fig. 2A). In contrast, cells transduced with the replication-incompetent HCV replicon did not express luciferase (Fig. 2A). Taken together, these results suggest that replication of the HCV RNA genome occurred in the iPS-Hep cells. To confirm replication of the HCV genome, we investigated production of minus-strand HCV RNA from the positive-strand HCV RNA genome by performing real time-PCR analysis. The results of this analysis showed that minus-strand HCV RNA was produced in iPS-Hep cells and Huh7 cells, but not in iPS cells (Fig. 2B). To investigate whether the iPS-Hep cells could be used to screen for drugs that suppress HCV replication, we treated the cells with a suppressor of HCV replication, IFN. Treatment with IFN resulted in dose-dependent attenuated replication of the HCV genome with no cytotoxicity (Fig. 3A and B). These findings suggest that the iPS-Hep cells are a suitable system to use for monitoring the replication of the HCV RNA genome.



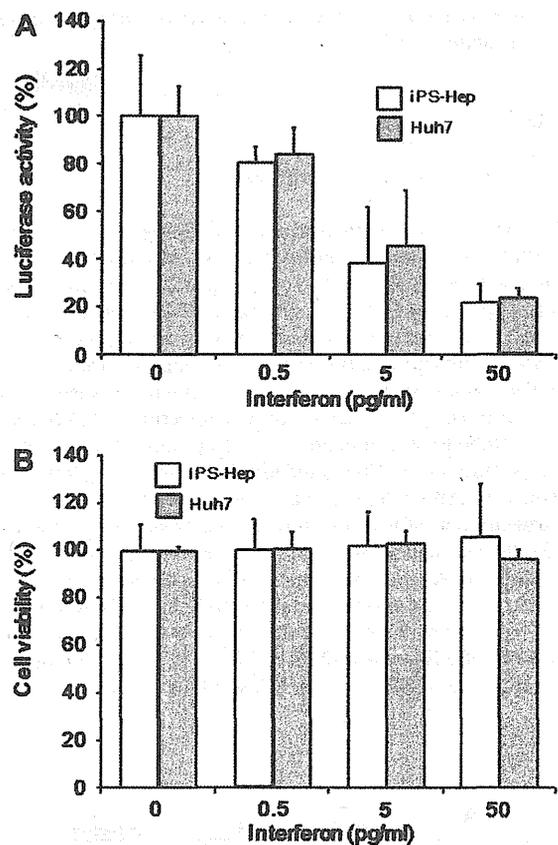
**Fig. 1.** HCV infection assay in iPS-Hep cells. (A) Expression of HCV receptors in iPS-Hep cells. Total RNA samples from Huh7, iPS, and iPS-Hep cells were subjected to RT-PCR analysis as described in the Section 2. The PCR products were separated on 2% agarose gels, followed by staining with ethidium bromide. (B) Infection of iPS-Hep cells with HCVpv. iPS, iPS-Hep and Huh7 cells were infected with HCVpv at the indicated dilution. After 2 h of infection, the cells were cultured with fresh medium for 24 h. Then, luciferase activities were measured. Data are presented as means  $\pm$  SD ( $n = 3$ ). (C) Effect of anti-CD81 antibody on infection of iPS-Hep cells with HCVpv. iPS-Hep (upper panel) and Huh7 (lower panel) cells were treated with mixtures of HCVpv (open column) or VSVpv (gray column) and anti-CD81 antibody or control mouse IgG at the indicated concentrations. After a 2-h incubation, the cells were cultured with fresh medium for 24 h. Then, the luciferase activities were measured. Data represent the percentage of vehicle-treated cells. Data are presented as means  $\pm$  SD ( $n = 3$ ).



**Fig. 2.** HCV replication assay in iPS-Hep cells. (A) Comparison of replication of HCV subgenomic replicons, AdP<sub>235</sub>-HCV (gray column) and AdP<sub>235</sub>-ΔGDD (open column), in iPS, iPS-Hep and Huh7 cells. The cells were infected with replicons, treated with Dox, and renilla luciferase activity was measured, as described in the Section 2. To normalize for infectivity of Ad vector, cells were co-infected with AdP<sub>235</sub>-fluc and Ad- $\beta$ gal. After 72 h, firefly luciferase activity was measured. Corrected luciferase activity was calculated as the ratio of renilla luciferase activity to firefly luciferase activity. (B) Real-time PCR analysis of HCV plus- and minus-strand RNA in iPS-Hep cells. iPS-Hep cells were infected with replicons, and total RNA was subjected to real-time PCR analysis, as described in the Section 2. The copy numbers were shown as ratio of those of Huh7. Data are presented as means  $\pm$  SD ( $n = 3$ ).

#### 4. Discussion

Tropism of HCV is limited to human and chimpanzee. Our understanding of HCV infection has been delayed by the lack of appropriate model systems. In the present study, we demonstrated that iPS-Hep cells are suitable *in vitro* models of hepatocytes for use in the study of HCV infection.



**Fig. 3.** Effect of interferon on HCV replication in iPS-Hep cells. iPS-Hep (open column) and Huh7 (gray column) cells were infected with AdP<sub>235</sub>-HCV and Ad- $\beta$ gal. After 24 h, the cells were treated with Dox and the indicated concentration of interferon for 48 h. Luciferase activities (A) and cell viabilities (B) were measured as described in the Section 2. Data represent the percentage of the value for vehicle-treated cells, and are presented as means  $\pm$  SD ( $n = 3$ ).

Other *in vitro* model systems of hepatocytes may not accurately reflect the biology of hepatocytes *in vivo*. For instance, expression profiles of mRNAs in embryonic stem (ES) cell-derived hepatocyte-like cells are different from those of primary human hepatocytes [31]. The development of efficient methods to differentiate stem cells into hepatocytes has been a critical issue in the application of stem cell technology to drug discovery. Recently, Mizuguchi and colleagues established efficient differentiation protocols for iPS cells by using adenoviral transfer of SOX17 [17], HEX [18], and HNF-4 $\alpha$  [19] in addition to growth factors. Approximately 80% of the differentiated cells showed expression of hepatic-specific proteins, including cytochrome P-450s (CYP2D6, CYP3A4, and CYP7A1) [19]. The iPS-Hep cells were also used as a simple system to evaluate the hepatotoxicity of drugs that are metabolized into toxic substances by cytochromes [19]. Here, we showed that the essential host factors for HCV infection (occludin, claudin-1, SR-BI, and CD81) are expressed in the iPS-Hep cells. HCV RNA genome replication occurred in the cells, and HCVpv infected the cells. An inhibitor of HCV entry (anti-CD81 antibody), and an anti-HCV agent (IFN), attenuated the entry of HCVpv and the replication of the HCV genome in the cells, respectively. These findings suggest that the iPS-Hep cells are useful for understanding HCV infection and for screening anti-HCV drugs.

We found that iPS cells express CD81 and occludin, and are not susceptible to HCV entry, whereas iPS-Hep cells express all four HCV receptors and are susceptible to HCV entry. These findings are consistent with previous studies showing that CD81, occludin,

SR-BI, and claudin-1 are key receptors for HCV [29]. HNF-4 $\alpha$ , which promotes the differentiation of iPS cells to iPS-Hep cells, is essential for the expression of a multitude of genes encoding cell junction and adhesion proteins during embryonic development of the mouse liver [32]. For instance, claudin-1 expression is not detected in the liver of HNF-4 $\alpha$ -deficient mice [32]. HNF-4 $\alpha$  enhances peroxisome proliferator-activated receptor-mediated SR-BI transcription [33]. Thus, the susceptibility to HCV entry observed in iPS-Hep cells may be the result of the additional expression of claudin-1 and SR-BI following HNF-4 $\alpha$  treatment.

miR-122 is a liver specific miRNA that constitutes 70% of the total miRNA population [34] and is essential for replication of the HCV genome in the liver [6]. ES cells do not express miR-122, whereas expression of miRNA is observed during differentiation into hepatocyte-like cells [35]. Replication of HCV subgenomic replicons was observed in iPS-Hep cells, but not iPS cells (Fig. 2A). Expression of miR-122 might be a key factor controlling the replication of the HCV RNA genome in iPS-Hep cells.

The reasons that 15–20% of people infected with HCV can clear the virus without pharmaceutical intervention, and patients vary in their sensitivity to pharmaceutical treatments, are still unclear [36]. Understanding the basis of these variable responses to infection and treatment would facilitate the discovery of potent targets for drug development for HCV. iPS-derived hepatocytes are a promising system for drug discovery for HCV infection. In the present study, we showed that the iPS-derived hepatocyte-like cells can be used with popular models of HCV infection: HCV subgenomic replicons and HCVpv. Our findings will contribute to our understanding of the mechanisms of HCV infection and to the identification of novel targets for HCV therapy by means of iPS technology.

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## Short communication

## Suppression of hepatitis C virus replicon by adenovirus vector-mediated expression of tough decoy RNA against miR-122a

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## ABSTRACT

Recent studies have demonstrated that the liver-specific microRNA (miRNA) miR-122a plays an important role in the replication of hepatitis C virus (HCV). Antisense nucleotides against miR-122a, including locked nucleic acid (LNA), have shown promising results for suppression of HCV replication; however, a liver-specific delivery system of antisense nucleotides has not been fully developed. In this study, an adenovirus (Ad) vector that expresses tough decoy (TuD)-RNA against miR-122a (TuD-122a) was developed to suppress the HCV replication in the liver hepatocytes. Ad vectors have been well established to exhibit a marked hepatotropism following systemic administration. An in vitro reporter gene expression assay demonstrated that Ad vector-mediated expression of TuD-122a efficiently blocked the miR-122a in Huh-7 cells. Furthermore, transduction with the Ad vector expressing TuD-122a in HCV replicon-expressing cells resulted in significant reduction in the HCV replicon levels. These results indicate that Ad vector-mediated expression of TuD-122a would be a promising tool for treatment of HCV infection.

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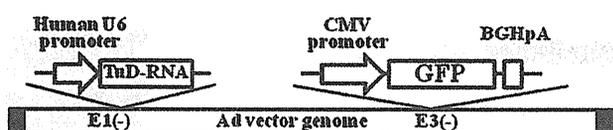
Hepatitis C virus (HCV) is a hepatotropic human virus belonging to a member of the family *Flaviviridae* and possessing a 9.6-kb positive-sense RNA genome. HCV infection causes chronic hepatic inflammation and fibrosis, leading to hepatocellular carcinoma (Hoofnagle, 2002). Currently, 170 million people worldwide are infected with HCV, and suffering from or at risk for the diseases described above. In order to suppress the replication of HCV, PEGylated interferon alpha and ribavirin, which is a nucleotide analogue, have been used as standard-of-care therapy; however, the therapeutic efficiency has been limited, in spite of relatively severe side effects, including fever and malaise (Chisari, 2005; Feld and Hoofnagle, 2005). Another therapeutic strategy should be developed to efficiently suppress the HCV infection and HCV-caused diseases.

Among several host factors involved in HCV infection, the abundant liver-specific microRNA (miRNA), miR-122a has been demonstrated to be crucial for efficient replication and/or

translation of the HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007). The HCV genome has two closely spaced miR-122a-binding sites in the 5'-untranslated region (UTR), which contains overlapping *cis*-acting signals involved in translation and RNA synthesis (Jopling et al., 2005). Although the mechanism of the miR-122a-mediated enhancement of HCV replication is controversial (Henke et al., 2008; Jopling et al., 2005; Machlin et al., 2011; Roberts et al., 2011; Wilson et al., 2011), antisense oligonucleotides complementary to miR-122a, including locked nucleic acid (LNA) oligonucleotides, have been shown to significantly inhibit miR-122a and reduce the HCV genome, and thereby to exhibit superior therapeutic effects (Henke et al., 2008; Jopling et al., 2005; Krutzfeldt et al., 2005; Lanford et al., 2010). Intravenous administration of LNA oligonucleotides against miR-122a into HCV-infected chimpanzees resulted in the long-lasting suppression of HCV viremia without viral resistance or severe side effects (Lanford et al., 2010). In addition, the 5'-UTR of the HCV genome is composed of highly conserved structural domains, suggesting that a mutant lacking the miR-122a-binding sites in the genome is unlikely to appear. These results indicate that miR-122a is a promising target for the treatment of HCV-related diseases; however, LNA oligonucleotides accumulate in the kidney immediately after intravenous administration and are excreted into the urine (Fluiter et al., 2003).

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**Fig. 1.** Structure of Ad vectors used in this study. The human U6 promoter-driven TuD-RNA expression cassette was inserted into the E1-deleted region of the Ad vector genome. The CMV promoter-driven GFP expression cassette was inserted into the E3-deleted region of the Ad vector genome.

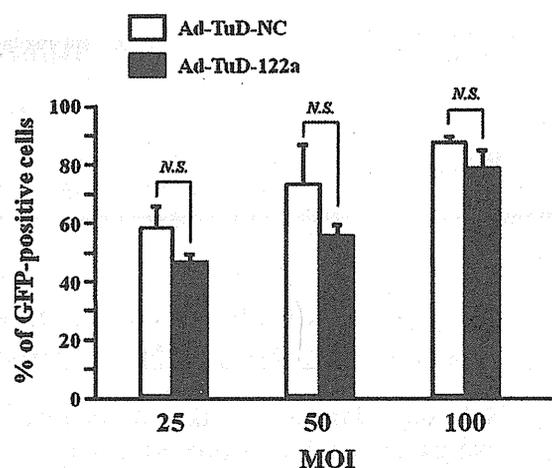
Systems which efficiently deliver or express anti-miR-122a drugs in the liver are necessary to efficiently treat HCV-related diseases.

Recently, tough decoy (TuD)-RNAs against miRNAs, which efficiently and specifically inhibit miRNAs, were developed by Haraguchi et al. (2009). TuD-RNAs are composed of two miRNA-binding sequence (MBS) regions and two stem structures with 3-nucleotide linkers. The MBS in the TuD-RNA is considered to tightly bind to miRNAs, leading to the inhibition of miRNAs. The inhibition activity of the TuD-RNA against miRNAs is higher than that of LNA oligonucleotides and miRNA sponges (Haraguchi et al., 2009). Another advantage of the TuD-RNA is that it can be expressed by viral and non-viral vectors. miRNAs can be persistently suppressed by lentivirus vector- and retrovirus vector-mediated expression of the TuD-RNA. Furthermore, liver-specific expression of the TuD-RNA is thought to be achievable by an adenovirus (Ad) vector and adeno-associated virus vector, because these vectors can express transgenes in a liver-specific manner after systemic administration. These properties of the TuD-RNA are highly promising for inhibition of miR-122a in the liver and suppression of HCV replication.

In the present study, we developed an Ad vector expressing the TuD-RNA against miR-122a (TuD-122a) to efficiently inhibit miR-122a and to suppress the HCV replication. Transduction with an Ad vector expressing TuD-122a efficiently inhibited miR-122a in vitro. In HCV replicon-expressing cells, HCV replicon levels were significantly reduced by Ad vector-mediated TuD-122a expression.

First, in order to examine the transduction efficiencies of the Ad vectors constructed in this study in the HCV replicon-expressing cells, Huh-7.5.1 1bFeo cells, which is a genotype 1b HCV replicon cell line (Yokota et al., 2003), were transduced with an Ad vector expressing TuD-122a (Ad-TuD-122a) or the control TuD-RNA (Ad-TuD-NC). Ad-TuD-122a and Ad-TuD-NC were prepared as described in Supplemental materials and methods. Structure of Ad vectors used in this study is shown in Fig. 1. The ratio of particles-to-biological titer was between 6 and 9 for each Ad vector used in this study. Both Ad-TuD-122a and Ad-TuD-NC carry the TuD-RNA expression cassette and the green fluorescence protein (GFP) expression cassette in the E1-deleted and E3-deleted region, respectively (Fig. 1). Both Ad-TuD-NC and Ad-TuD-122a efficiently transduced Huh-7.5.1 1bFeo cells (Fig. 2). More than 80% of the cells were found to be GFP-positive following transduction with Ad-TuD-122a and Ad-TuD-NC, respectively, at a multiplicity of infection (MOI) of 100. The averages of GFP-positive cells following transduction with Ad-TuD-NC were slightly higher than those with Ad-TuD-122a; however, statistically significant differences were not found for either group. Apparent cellular toxicity was not found following transduction with Ad-TuD-122a or Ad-TuD-NC (data not shown). These results indicate that Ad-TuD-122a and Ad-TuD-NC efficiently transduce Huh-7.5.1 1bFeo cells.

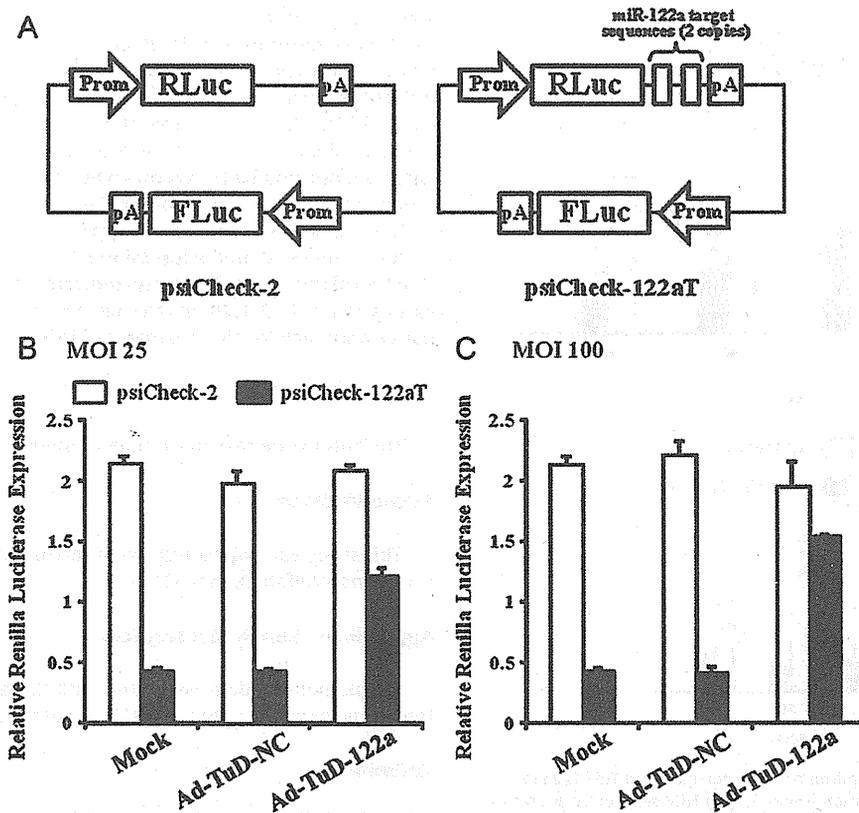
Next, in order to examine the inhibitory effects of TuD-122a expressed by the Ad vector on miR-122a, a reporter gene assay using the miR-122a complementary sequence-encoded plasmid, psiCheck-122aT, was performed in Huh-7 cells. Huh-7 cells endogenously express a high level of miR-122a (Suzuki et al., 2008). Huh-7 cells were transduced with the Ad vectors at MOIs of 25 and 100 for 1.5 h. After a 24-h incubation, the cells were transfected with



**Fig. 2.** Transduction efficiencies of Ad-TuD-122a and Ad-TuD-NC in Huh-7.5.1 1bFeo cells. The cells were transduced with Ad-TuD-122a or Ad-TuD-NC at multiplicities of infection (MOIs) of 25, 50, and 100 for 1.5 h. At 48 h after transduction, GFP expression was evaluated by flow cytometry. The data are expressed as the means  $\pm$  S.D. ( $n=3$ ). The percentage of GFP-positive cells in the mock-transduced group was less than 0.2%. N.S.: not significant.

psiCheck-2 or psiCheck-122aT. The renilla and firefly luciferase expression was evaluated 48 h after transfection with the plasmid DNA. psiCheck-122aT, plasmid DNA containing the two copies of miR-122a complementary sequences in the 3'-UTR of the renilla luciferase gene, was constructed by ligation of *NotI/XhoI*-digested psiCheck-2 (Promega, Madison, WI) with the oligonucleotides 122aT-F and 122aT-R. The sequences of the oligonucleotides 122aT-F and 122aT-R are described in the Supplemental information. In mock-transduced cells, the relative renilla luciferase expression level by psiCheck-122aT was about 5-fold lower than that by the control plasmid psiCheck-2, which does not possess miR-122a target sequences, due to the endogenous expression of miR-122a in Huh-7 cells (Fig. 3). The renilla luciferase expression profiles following transfection with psiCheck-122aT were similar in the mock-transduced cells and Ad-TuD-NC-transduced cells, indicating that expression of the control TuD-RNA does not inhibit the miR-122a. Ad-TuD-122a did not alter the renilla luciferase expression level by psiCheck-2; on the other hand, psiCheck-122aT-mediated renilla luciferase expression was significantly restored by Ad-TuD-122a. The cells transduced with Ad-TuD-122a exhibited 2.8-fold and 3.5-fold higher renilla luciferase expression at MOIs of 25 and 100, respectively, than the mock-transduced cells following transfection with psiCheck-122aT. These results indicate that miR-122a is efficiently inhibited by Ad-TuD-122a. We also performed quantitative RT-PCR analysis for miR-122a following transduction with Ad-TuD-122a and Ad-TuD-NC in Huh-7 cells. No significant differences in the miR-122a expression levels were found in the cells transduced with Ad-TuD-122a and the cells transduced with Ad-TuD-NC (data not shown), probably because TuD-RNA does not induce degradation of miRNA, although TuD-RNA tightly binds to the target miRNA (Haraguchi et al., 2009).

Next, in order to examine whether TuD-122a-mediated inhibition of miR-122a suppresses the HCV replicon, Huh-7.5.1 1bFeo cells were transduced with Ad-TuD-122a and Ad-TuD-NC at the indicated MOIs. Huh-7.5.1 1bFeo cells express an mRNA consisting of the HCV 5'-UTR and the upstream part of the core region, connected in-frame with the firefly luciferase gene, which allows the simple evaluation of the HCV replicon levels by measuring the firefly luciferase activity in the cells (Yokota et al., 2003). Huh-7.5.1 1bFeo cells were transduced with the Ad vectors at MOIs of 25, 50, and 100 for 1.5 h. After a total 48-h incubation,



**Fig. 3.** Inhibition of miR-122a by Ad vector-mediated TuD-122a expression. (A) Structure of the reporter gene-expressing plasmids psiCheck-2 and psiCheck-122aT. (B and C) Relative renilla luciferase expression levels following transduction with Ad-TuD-NC or Ad-TuD-122a at MOIs of 25 (B) and 100 (C). The data are expressed as the means  $\pm$  S.D. ( $n = 4$ ).

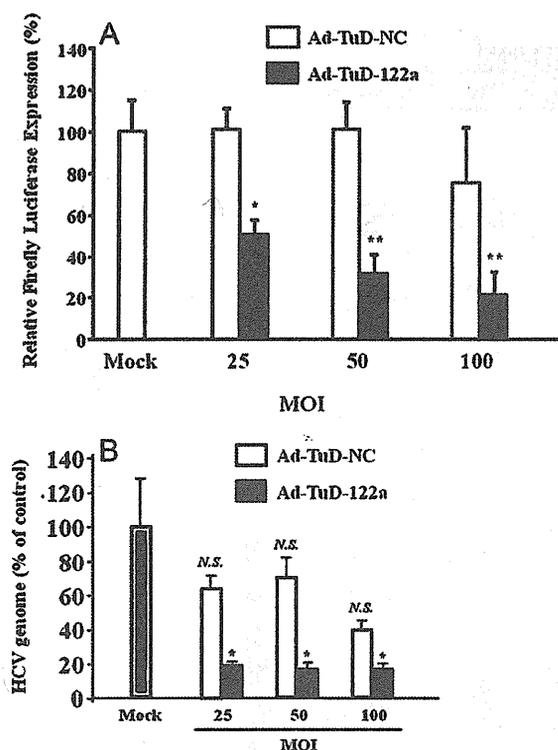
firefly luciferase expression levels were determined. Ad-TuD-122a significantly reduced the firefly luciferase expression levels in a dose-dependent manner (Fig. 4a). The firefly luciferase expression level was reduced to 29% of that in the cells transduced with Ad-TuD-NC at MOI of 100 by transduction with Ad-TuD-122a at MOI of 100. In contrast, no significant changes in the firefly luciferase expression were found by transduction with Ad-TuD-NC.

To examine whether inhibition of miR-122a by Ad vector-mediated TuD-122a expression leads to a reduction in HCV replicon RNA levels, strand-specific real-time RT-PCR analysis was performed to determine the HCV replicon RNA levels. Briefly, Huh-7.5.1 1bFeo cells were transduced with the Ad vectors as described above, and the total RNA was isolated 48 h after transduction. Real-time RT-PCR analysis for the HCV positive-strand RNA genome was performed as follows. Briefly, 2  $\mu$ g of total RNA was reverse-transcribed to cDNA using the primer specific for the HCV positive-strand genome (RC21; 5'-ctc ccg ggg cac tcg caa gc-3'). Real-time RT-PCR was performed using the primers (RC21 and RC1; 5'-gtc tag cca tgg cgt tag ta-3') and SYBR Premix Ex Taq II (Takara Bio Inc., Kyoto, Japan). Similarly to the results for the firefly luciferase expression in Fig. 4A, HCV replicon RNA levels were significantly reduced by Ad-TuD-122a (Fig. 4B). There was an approximately 2.2-fold decline in the HCV replicon RNA level in the cells transduced with Ad-TuD-122a at an MOI of 100, compared with the HCV replicon RNA level in the cells transduced with Ad-TuD-NC at an MOI of 100. Ad-TuD-NC did not apparently decrease the HCV replicon RNA levels. These results indicate that the inhibition of miR-122a by Ad vector-mediated TuD-122a expression efficiently suppresses the replication of the HCV replicon.

The present study demonstrates that Ad vector-mediated TuD-122a expression significantly inhibits the function of miR-122a and

replication of the HCV replicon. Replication of the HCV genome is promoted by the direct interaction between miR-122a and the complementary sequences in the 5'-UTR of the HCV genome (Henke et al., 2008; Jangra et al., 2010), indicating that sequestration of miR-122a leads to suppression of the HCV replication. In order to suppress the HCV replicon by inhibiting miR-122a, TuD-RNA was selected as an inhibitor of miRNA in this study, because TuD-RNA potentially inhibits miRNA by strongly binding to miRNA (Haraguchi et al., 2009). In addition, TuD-RNA can be expressed by conventional gene delivery vectors, including virus vectors. One drawback of TuD-RNA is that TuD-RNA does not discriminate miRNA members that belong to the same miRNA family (Haraguchi et al., 2009); however, miR-122a does not constitute a family of miRNA, suggesting that TuD-122a would not inhibit other miRNAs.

As described above, an Ad vector is suitable for liver-specific expression of TuD-RNA due to the strong hepatotropism. Previous studies demonstrated that Ad vectors expressing short-hairpin RNA (shRNA) or antisense RNA against the HCV genome successfully exhibited the suppressive effects on HCV infection in vivo (Gonzalez-Carmona et al., 2011; Sakamoto et al., 2008). Another advantage of using an Ad vector for treatment of HCV-related diseases is that in vivo administration of an Ad vector induces type I interferon (IFN) production via innate immune responses (Huarte et al., 2006; Zhu et al., 2007). Our group previously demonstrated that VA-RNA, which is a small non-coding RNA expressed from a replication-incompetent Ad vector as well as wild-type Ad, stimulates type I IFN production in an IFN- $\beta$  promoter stimulator-1 (IPS-1)-dependent manner (Yamaguchi et al., 2010). Ad vector-induced type I IFN would contribute to suppression of HCV infection. The anti-HCV activity of Ad-TuD-122a can also be up-regulated by insertion of an expression cassette of an



**Fig. 4.** Suppression of the HCV replicon by Ad vector-mediated TuD-122a expression. (A) Firefly luciferase expression levels and (B) HCV replicon RNA levels in Huh-7.5.1 1bFeo cells following transduction with the Ad vectors. All the data are shown as the means  $\pm$  S.D. ( $n=3$ ). N.S.: not significant. \* $P<0.05$ , \*\* $P<0.005$  between mock-transduced cells and cells transduced with Ad-TuD-122a.

anti-HCV gene, including type I IFN genes and short-hairpin RNA (shRNA) or antisense RNA against the HCV genome, into the Ad vector genome. Our group has developed various types of Ad vectors in which two or three transgene expression cassettes can be inserted into a single Ad vector genome (Mizuguchi et al., 2001, 2005, 2003).

Previous studies have demonstrated that lipid droplets, which are lipid-storage intracellular organelles, are crucial for the production of infectious HCV particles (Hinson and Cresswell, 2009; Miyanari et al., 2007). Miyanari et al. demonstrated that HCV capsid proteins recruit the non-structural proteins and the replication complex to the lipid droplet-associated membrane (Miyanari et al., 2007). miR-122a is an important factor that regulates cholesterol and fatty-acid metabolism in the hepatocytes (Esau et al., 2006; Iliopoulos et al., 2010). Intravenous administration of the antisense oligonucleotide against miR-122a resulted in a reduction in the plasma levels of cholesterol and triglycerides (Esau et al., 2006; Lanford et al., 2010). In addition to the enhancement of accumulation and translation of the HCV genome, miR-122a might up-regulate HCV infection by regulating lipid metabolism in the hepatocytes.

Almost similar levels of reduction in the HCV replicon RNA copy numbers were found for Ad-TuD-122a at MOIs of 25, 50, and 100, although there was dose-dependent reduction in the firefly luciferase expression following transduction with Ad-TuD-122a. It remains unclear why dose-dependent reduction in the HCV replicon RNA copy numbers was not found, however, miR-122a plays a crucial role in the enhancement of both translation and stability of HCV genome (Henke et al., 2008; Jopling et al., 2005; Randall et al., 2007; Shimakami et al., 2012). Stability of HCV genome might be more susceptible to inhibition of miR-122a than translation. The averages of HCV replicon RNA levels were also reduced following transduction with Ad-TuD-NC, although

statistically significant differences were not found, compared with the mock-transduced cells. Replication-incompetent Ad vectors express non-coding small RNA (VA-RNA), which forms RNA-induced silencing complex (RISC) with argonaute 2 (Ago2) (Xu et al., 2007). Ago2 is an important factor for miRNA processing (Diederichs and Haber, 2007). Processing of miR-122a might be slightly disturbed by Ad vector-expressed VA-RNA, leading to the reduction in the HCV replicon RNA levels.

In summary, we efficiently suppressed the HCV replicon levels by Ad vector-mediated expression of TuD-122a, which blocks the function of miR-122a. This study indicates that Ad vector-mediated expression of TuD-122a in liver hepatocytes would offer an alternative approach for the treatment of HCV infection.

#### Conflict of interest

The authors declare no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2012.02.003.

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## アデノウイルスベクターを利用した C 型肝炎治療薬創製基盤技術の開発

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### 研究要旨

C型肝炎ウイルス (HCV) のキャリアは、全世界で 2 億人、国内では 200 万人にものぼり、世界では年間 200~300 万人の規模で増加している。インターフェロン (IFN) 療法の進展に伴い C型肝炎の根治率は向上しているものの、依然として難治性 1b 型高ウイルス量患者に対しては奏効率が 50% にすぎず、C型肝炎克服に向けた新たな作用点を有する抗 HCV 薬の創製が急務となっている。しかしながら、新薬開発のための基盤である HCV 感染評価系の開発は立ち遅れているのが現状である。実際、HCV 長鎖 RNA ゲノム (9.6 kb) を肝臓に効率よく導入する方法が確立されていないため、依然として HCV 感染評価系には患者血清が使用されており、簡便な HCV 感染評価系の開発が C型肝炎治療薬創製における重要課題となっている。そこで本研究は、肝臓への遺伝子導入に優れたアデノウイルス (Ad) ベクターに長鎖 RNA 発現系 RNA pol I を搭載させたものを利用することで、HCV ゲノムを肝臓で発現可能なベクターを開発し、新たな抗 HCV 活性評価系の構築を試みた。

RNA polymerase I (pol I) 発現系は rRNA (13 kb) 発現に関与していることから、長鎖である HCV RNA の発現も可能であると考えられる。そこで、長鎖 RNA 発現系である RNA pol I プロモーター下に HCV サブゲノムを組み込んだ発現プラスミドベクターを作製し、本プラスミドを搭載した HCV サブゲノム発現 Ad ベクターを開発することに成功した。本 HCV サブゲノム発現 Ad ベクターは、ヒト肝臓細胞株やヒト iPS 細胞由来肝細胞などの様々な細胞へ簡便に HCV ゲノムを導入可能であり、HCV 複製阻害剤の効果や細胞の HCV 複製能を評価可能であることが明らかとなった。以上、本研究において開発した HCV サブゲノム発現 Ad ベクターは、汎用性に優れた HCV 複製解析系であると考えられる。

### A. 研究目的

C型肝炎ウイルス (HCV) は日本で 200 万人、世界中では 2 億人もの感染者が存在すると推定されている。HCV の慢性持続感染は 10~30 年かけて慢性肝炎、肝硬変を引き起こし、さらに 5~10 年で肝細胞癌に至る。本邦における肝細胞癌患者の約 8 割は HCV 感染者であり、C型肝炎は最も深刻な肝疾患である。これまで、C型肝炎に対する治療法は複製阻害薬であるインターフェロン (IFN) およびリバビリンの併用療法が用いられてきたが、奏効率は 50% に過ぎなかった。2011 年、新たにプロテアーゼ阻害剤が認可され、ポ

リメラーゼ阻害剤についても臨床試験が行われている。しかしながら、これらはいずれもウイルス側の因子を標的としており、薬剤耐性ウイルスの出現が報告されている。そのため、薬剤耐性ウイルスが出現しにくい HCV 複製に関わる宿主因子を標的とした新規治療法の開発が期待されている。

HCV は約 9.6 kb の一本鎖プラス鎖 RNA をゲノムにもつ RNA ウイルスでフラビウイルス科に分類される。HCV 感染機構は肝細胞内への侵入および複製ステップに大別され、それぞれの感染段階に関与する宿主因子の同定が耐性ウイルスの出現を回避する新

たな治療戦略として注目されている。昨今の HCV 侵入機構解析により、HCV 感染受容体として CD81、scavenger receptor class B type I (SR-BI)、claudin-1、occludin が同定され、抗 claudin-1 抗体などが HCV 感染阻害薬シーズとして注目されている。一方、HCV 複製機構については、HCV 複製解析系開発の遅延により、cyclophilin や miR-122 などしか明らかとなっていない。

HCV が感染可能な宿主はヒトとチンパンジーに限られる。そのため、*in vivo* HCV 複製解析系にはチンパンジーまたは免疫不全マウスにヒト肝細胞を移植したヒト肝臓キメラマウスが用いられている。*In vitro* ではヒト初代培養細胞やヒト肝臓由来培養細胞株 (Huh7 細胞) が用いられている。また、ヒト induced pluripotent stem cell (iPS) 細胞から肝細胞への分化誘導技術の進展に伴い、様々な患者から樹立されたヒト iPS 細胞由来肝細胞を用いることで HCV 複製能の違いや、抗 HCV 薬に対する効果の違いを解析可能であり、新たな HCV 複製解析系として注目されている。しかしながら、HCV ゲノム導入法の問題から利用には至っていない。HCV ゲノム導入法としては HCV 粒子を感染させる方法と HCV RNA をエレクトロポレーション法により導入させる方法がある。HCV 粒子は培養細胞で産生した HCV あるいは患者血清が用いられている。培養細胞で産生可能な HCV 株は、日本では感染の少ない遺伝子型 2a に属し劇症肝炎という C 型肝炎では特殊な病態を示す JFH-1 株のみである。患者血清は入手が困難であり、遺伝子工学的な解析が不可能であるなどの問題点がある。HCV RNA のエレクトロポレーション導入法は、これらの問題点を解決可能であるが、細胞障害性や導入効率の問題から *in vivo* や初代培養細胞などへは適用できない。以上のことから、汎用性に優れ、導入効率の高い HCV ゲノム導入法の確立が新たな抗 HCV 薬開発の課題であると考えられる。

そこで、本研究は、長鎖 RNA 発現系 RNA pol I を搭載した Ad ベクターを用いて、HCV ゲノムを肝臓で発現可能なベクターを開発し、新たな抗 HCV 活性評価系の構築を試みた。

## B. 研究方法

### 1. 細胞培養

Huh7 細胞、293T 細胞は 10% fetal bovine serum (FBS) 含有 Dulbecco's modified Eagle medium (DMEM) を用いて 37 °C、飽和蒸気圧、5% CO<sub>2</sub> 条件下で培養した。ヒト iPS 細胞およびヒト iPS 細胞由来肝細胞は以下の方法にて培養および分化誘導を行った。

ヒト胎児肺線維芽細胞株 MRC5 細胞から樹立された iPS 細胞 (Dotcom, JCRB Number: JCRB1327) は マイトマイシン C 処理マウス線維芽細胞上で 10 ng/ml fibroblast growth factor 2 (FGF2) 添加 iPSellon 培養液 (Cardio 社) を用いて 37 °C、飽和蒸気圧、5% CO<sub>2</sub> 条件下で培養した。分化誘導前日、ヒト iPS 細胞の培養液を hESF9 培養液 [10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, oleic acid conjugated with fatty-acid-free bovine albumin (BSA), 10 ng/ml FGF2, 100 ng/ml heparin を添加した hESF-GRO 培養液 (Cell Science & Technology Institute 社)] に交換し培養した。シングル細胞にしたヒト iPS 細胞をマトリゲル (BD 社) 上に播種し、10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, 0.5 mg/ml BSA, 100 ng/ml Activin A を添加した hESF-DIF 培養液 (Cell Science & Technology Institute 社) で 3 日間培養した。培養 3 日後、細胞に Ad-SOX17 を 3,000 VP/cell で 1.5 時間感染させ、10 μg/ml human recombinant insulin, 5 μg/ml human apotransferrin, 10 μM 2-mercaptoethanol, 10 μM ethanolamine, 10 μM sodium selenite, 0.5 mg/ml BSA, 20 ng/ml bone morphogenetic protein 4 (BMP4), 20 ng/ml FGF4 添加 hESF-DIF 培養液を用いてマトリゲル上で 3 日間培養した。3 日間培養後、細胞に Ad-HNF4 $\alpha$  を 3,000 VP/cell で 1.5 時間感染させ、8.3% tryptose phosphate broth、8.3% FBS、10 μM hydrocortisone

21-hemisuccinate、1  $\mu$  M insulin、25 mM NaHCO<sub>3</sub>、20 ng/ml hepatocyte growth factor、20 ng/ml Oncostatin M、10<sup>-6</sup> M Dexamethasone 添加 L15 培養液 (Invitrogen 社) を用いてマトリゲル上で 11 日間培養し、ヒト iPS 細胞由来肝細胞を分化誘導させた。

## 2. RNA pol I 発現系 Ad ベクターの開発

### 2-1 遺伝子組換え

ヒト RNA pol I プロモーターおよびマウス RNA pol I ターミネーターがコードされたプラスミド pHH21 (東京大学、河岡博士より譲渡) 由来の RNA pol I 発現カセットを Ad ベクターシャトルプラスミド pHM5 (大阪大学、水口博士より譲渡) に組み込み、さらにヒト RNA pol I プロモーター下にレポーター遺伝子 EGFPLuciferase (EGFP 遺伝子と Luciferase 遺伝子の融合遺伝子) を搭載した。各プラスミドの遺伝子組換えは以下の方法で行った。pHH21 の RNA Pol I プロモーターの上流に存在する AflIII サイトを切断し、酵素 Klenow fragment を用いて平滑末端化後に DNA 断片 EcoRI linker (TaKaRa) とのライゲーションを行った。ライゲーション産物を制限酵素 EcoRI で切断後、さらに RNA pol I ターミネーターの下流に存在する NheI サイトを切断することにより、RNA pol I 発現カセット DNA フラグメントを作製した。続いて、シャトルプラスミド pHM5 のマルチクローニングサイト上にある EcoRI サイトおよび NheI サイトを切断し、RNA pol I 発現カセット DNA フラグメントとライゲーションし、ライゲーション産物によりコンピテントセル DH5 $\alpha$  (TOYOBO) をトランスフォーメーションさせた。形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、制限酵素解析によりインサートを確認することで pHM5-Pol I を得た。次に、pHM5-Pol I の RNA pol I プロモーター下に存在する KpnI サイトを切断し、マルチクローニングサイト DNA フラグメントをライゲーションした。マルチクローニングサイト DNA フラグメントはセンス鎖 DNA: 5'-GCCATGGGATATCTCTAGAGGATCCGAGCTCG CGGCCGCTAC-3' およびアンチセンス鎖 DNA: 5'-GCGGCCGCGAGCTCGGATCCTCTAGAGATAT CCCATGGCGTAC-3' をハイブリダイゼーションさせ

たものを用いた。ライゲーション産物によりコンピテントセル DH5 $\alpha$  をトランスフォーメーションさせ、形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、シーケンス解析により pHM5-Pol I-MCS を得た。続いて、pCMV-EGFPLuc の EGFPLuciferase 遺伝子の上流と下流の2カ所に存在する NcoI サイトを切断し、EGFPLuciferase DNA フラグメントを作製した。pHM5-Pol I-MCS のマルチクローニングサイト上に存在する NcoI サイトを切断し、EGFPLuciferase DNA フラグメントとライゲーションし、ライゲーション産物によりコンピテントセル DH5 $\alpha$  をトランスフォーメーションさせた。形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、制限酵素解析を行うことで、ヒト RNA pol I 下に EGFPLuciferase 遺伝子を配置した DNA カセットを有した Ad ベクターシャトルプラスミドである pP<sub>1</sub>WT-EL を得た。

### 2-2 Ad ベクターの作製

RNA pol I 発現カセット搭載 5 型 Ad ベクター Ad-pol I-EGFPLuc は水口博士らが開発した *in vitro* ligation 法により作製した。pP<sub>1</sub>WT-EL を制限酵素 I-CeuI および PI-SceI により切断し、I-CeuI および PI-SceI で制限酵素処理した pAdHM4 (5 型 Ad ベクタープラスミド) とライゲーションを行った。親ベクターにのみ存在する制限酵素 SwaI で切断したライゲーション産物によりコンピテントセル DH5 $\alpha$  をトランスフォーメーションさせた。形成した独立大腸菌クローンを培養し、制限酵素 HindIII で切断することにより、目的遺伝子の挿入を確認した。続いて、組換え Ad ベクタープラスミドを PacI で処理した後、SuperFect (QIAGEN 社) を用いて 293 細胞にトランスフェクションした。10~14 日間培養した後、CPE (cytopathic effect) を起こした 293 細胞を 1,200 rpm、5 分間遠心して回収し、少量の培養液に懸濁した。3 回凍結融解を繰り返すことにより溶液中に遊離した Ad ウイルスを 2,000 rpm、10 分間遠心して cell debris を除去した後に、新たな 293 細胞に感染させた。CPE が観察されると同様の操作を繰り返し、徐々に感染させる 293 細胞を増やすことで高タイトーの CVL (crude virus lysate) を得た。

回収した CVL は CsCl 密度勾配遠心法により精製した。CVL を 5 回凍結融解を繰り返すことにより Ad を溶液中に遊離させ、DNase および RNase 処理を 1 時間行った。比重 1.25~1.40 の CsCl 密度勾配上に重層し、35,000 rpm、18°C、1 時間遠心した。遠心チューブ内にできた下方のバンドを回収し、さらに比重 1.35 の CsCl 上に重層し、35,000 rpm、18°C で 16 時間遠心した。遠心チューブ内にできた下方のバンドを回収し、以下のバッファー [10mM Tris-HCl(pH7.4), 1 mM MgCl<sub>2</sub>, 10% glycerol] を用いて 4°C で透析を行った。Ad の物理学的タイターは Ad を TE 0.1% SDS 溶液で可溶化し 14,000 rpm、10 分間遠心を行った上清の 260 nm の波長の吸光度を測定し、以下の式を用いて算出した。精製した Ad は -80°C にて保存した。

$$\text{Titer (VP/ml)} = [\text{OD}_{260} - \text{OD}_{260}(\text{blank})] \times 1.1 \times 10^{12}$$

### 2-3 Ad ベクター発現確認

Huh7 細胞、HepG2 細胞、SK HEP-1 細胞を 24-well plate に播種した。培養 24 時間後、培養液を除き Ad ベクターを 300、1000、3000 VP/cell で感染させた。感染 1.5 時間後、培養液を添加した。感染 24 時間後、培養液を除去し、細胞溶解剤 LCβ (東洋インキ社) 100 μl を用いて細胞を溶解した。回収した細胞溶解液を -80°C で凍結、37°C で融解した後に 14,000 rpm 5 min で遠心を行った。遠心後、上清 20 μl のルシフェラーゼ活性を Luciferase assay system PGL5500 (ピッカジーン、東洋インキ社) を用いて測定し、Ad ベクター遺伝子導入効率を評価した。

## 3. HCV サブゲノム発現プラスミドベクターの作製および抗 HCV 活性評価

### 3-1 遺伝子組換え

HCV サブゲノムレプリコンフラグメントは pRep-Feo (東京医科歯科大学、坂本博士より譲渡) からクローニングした。pHM5-Pol I-MCS の RNA pol I プロモーターの下流にある BsmBI サイトと RNA pol I ターミナーの上流にある BsmBI サイトを切断し、HCV サブゲノムレプリコンフラグメントとライゲーションした。ライゲーション産物によりコンピテントセル DH5α をトラ

ンスフォーメーションさせた。形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、制限酵素解析およびシーケンス解析を行うことにより pPol I-1bFeo を得た。続いて、pPol I-1bFeo のレポーター遺伝子と薬剤耐性遺伝子の融合遺伝子である Feo 遺伝子を制限酵素処理によりレポーター遺伝子 luciferase 遺伝子置き換え、pPol I-HCV を得た。

### 3-2 HCV 評価系の構築

Huh7 細胞を 24-well plate に播種し、24 時間培養した後、HCV 発現プラスミド pPol I-HCV 0.8 μg および遺伝子導入効率補正用プラスミド pCMVβ 0.2 μg を SuperFect により細胞へ導入した。遺伝子導入 2.5 時間後、各濃度のインターフェロンを含む細胞培養液に交換し、72 時間後 luciferase 活性、β-galactosidase 活性および細胞生存率を測定した。Luciferase 活性および β-galactosidase 活性は以下の方法により測定した。遺伝子導入 72 時間後、培養液を除去し、細胞溶解剤 LCβ 100 μl を用いて細胞溶解液を回収した。回収した細胞溶解液は -80°C で凍結、37°C で融解した後に 14,000 rpm、5 min で遠心を行い、Luciferase assay system PGL5500 を用いて上清 20 μl のルシフェラーゼ活性を測定した。また、Luminescent β-gal Kit (Clontech) を用いて上清 20 μl の β-galactosidase 活性を測定した。次に、細胞生存率は以下の方法によって測定した。遺伝子導入 72 時間後、細胞に WST-8 試薬 (Nacalai tesque) を添加し、0.5~1 時間培養した後に培養上清の吸光度 (450 nm) を測定した。Luciferase 活性は β-galactosidase 活性で補正し、インターフェロン非添加群を 100% として算出した。また、細胞生存率はインターフェロン非添加群を 100% として算出した。

### 3-3 HCV サブゲノム発現 Ad ベクターの作製

研究方法 2-2 と同様の方法を用いて HCV サブゲノム発現 Ad ベクターの作製を試みた。

## 4. RNA pol I 発現系の最適化

### 4-1 遺伝子組換え

テトラサイクリン (tet) *trans*-activator 結合配列

TRE と 3 種類の RNA pol I プロモーターフラグメント (-235~-1, -311~-1, -412~-1) とを融合させた tet 制御性 RNA pol I プロモーター (P<sub>235</sub>, P<sub>311</sub>, P<sub>412</sub>) を作製した。Tet 制御性 CMV プロモーターがコードされたプラスミド pHM5-TREL2 (水口博士より譲渡) から TRE フラグメントの上流にある P<sub>I</sub>-SceI サイトおよび下流にある KpnI サイトで切り出すことにより、TRE フラグメントを作製した。3 種類の RNA pol I フラグメントは pHM5-Pol I をテンプレートとして PCR 法により増幅した。pHM5-Pol I 溶液 (0.1 mg/ml) 1 μl, 10 x PCR buffer for KOD plus 5 μl, 2.5 mM MgSO<sub>4</sub> 2 μl, 2.5 mM dNTP mix 5 μl, 10 μM primers 3 μl, 滅菌精製水 30 μl, 5 U/μl Takara kod plus 1 μl を混合し PCR を行った。各 RNA pol I フラグメント用のプライマー配列は、P<sub>235</sub> Forward; 5' - GCGGTACCCGGGTCGAGACGGGCGG CCGGGAGGGCGT -3', P<sub>311</sub> Forward; 5' - GCGG TACCCGGGTCGAGGTGTGTCCCGTCTAGGAG- 3', P<sub>412</sub> Forward; 5' - GCGGTACCCGGGTCGAG TCTTTCCTGCGTTATCCCCT -3', Reverse (全フラグメント共通); 5' - GCTCTAGAGATATCGCTAGCGT -3' とした。PCR の条件は、94°C 2 min の後、94°C 30 sec, 59°C 30 sec, 68°C 30 sec を 35 サイクル。PCR 後、PCR 産物を電気泳動により分離・精製し、制限酵素 KpnI と XbaI により切断した。pHM5-Pol I-MCS の RNA pol I プロモーターの上流にある P<sub>I</sub>-SceI サイトおよび下流にある XbaI サイトを切断し、TRE フラグメントおよび RNA pol I フラグメントをライゲーションした。ライゲーション産物によりコンピテントセル DH5α をトランスフォーメーションさせた。形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、制限酵素解析およびシーケンス解析により、pP<sub>235</sub>, pP<sub>311</sub>, pP<sub>412</sub> を得た。次に、pCMV-EGFPLuc-IRES-neo の EGFPLuciferase 遺伝子の上流に存在する NheI サイトおよび下流に存在する BamHI サイトを切断することにより、EGFPLuciferase DNA フラグメントを作製した。pP<sub>235</sub>, pP<sub>311</sub>, pP<sub>412</sub> のマルチクロニングサイト上に存在する NheI サイトおよび BamHI サイトを切断し、EGFPLuciferase DNA フラグメントとライゲーションを行った。ライゲーション産物によりコ

ンピテントセル DH5α をトランスフォーメーションさせた。形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、制限酵素解析により、pP<sub>235</sub>-EL, pP<sub>311</sub>-EL, pP<sub>412</sub>-EL を得た。

#### 4-2 Tet 制御性 RNA pol I 発現系の機能評価

Huh7 細胞を 24-well plate に播種し、24 時間培養した後、Tet 制御性 RNA pol I 発現系プラスミド (pP<sub>235</sub>-EL, pP<sub>311</sub>-EL または pP<sub>412</sub>-EL) 0.1 μg、tet 制御性 *trans*-activator 発現プラスミド (pHM5-tTA または pHM5-rtTA) 0.8 μg および遺伝子導入効率補正用プラスミド pCMVβ 0.1 μg を SuperFect を用いて細胞に導入した。遺伝子導入 2.5 時間後、各濃度のドキシサイクリンを含む細胞培養液に交換し、遺伝子発現を誘導した。遺伝子発現誘導 48 時間後、luciferase 活性および β-galactosidase 活性を実験方法 3-2 と同様な方法で測定した。

#### 4-3 Ad ベクターの作製

AdP<sub>235</sub>-EL および Ad-tTA はシャトルプラスミド pP<sub>235</sub>-EL および pHM5-tTA から研究方法 2-2 と同様な方法で作製・精製した。精製した Ad ベクターの生物学的タイターは Adeno-X rapid titer kit (Clontech) を用いて測定した。

#### 4-4 Ad ベクター発現確認

Huh7 細胞を 24-well plate に播種し、Ad-tTA (10 または 50 MOI) および AdP<sub>235</sub>-EL (5 または 10 MOI) を共感染させた。感染 48 時間後に細胞溶解剤 LCB を用いて細胞溶解液を回収し、Luciferase assay system PGL5500 を用いてルシフェラーゼ活性を測定した。

### 5. HCV サブゲノム発現 Ad ベクターの作製

#### 5-1 遺伝子組換え

HCV サブゲノムレプリコンフラグメントは pPol I-1bFeo からクローニングした。pP<sub>235</sub> の RNA pol I プロモーターの下流にある BsmBI サイトと RNA pol I ターミネーターの上流にある BsmBI サイトを切断し、

HCV サブゲノムレプリコンフラグメントとライゲーションした。ライゲーション産物によりコンピテントセル DH5 $\alpha$  をトランスフォーメーションさせた。形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、制限酵素解析を行うことにより、pP<sub>1</sub>235-1bFeo を得た。続いて、pP<sub>1</sub>235-1bFeo のレポーター遺伝子と薬剤耐性遺伝子とレポーター遺伝子の融合遺伝子である Feo 遺伝子を制限酵素処理によりレポーター遺伝子 *renilla luciferase* 遺伝子に置き換え、pP<sub>1</sub>235-HCV を得た。また、複製能欠損体 HCV サブゲノムフラグメントは pP<sub>1</sub>235-HCV をテンプレートとして PCR 法により作製した。pP<sub>1</sub>235-HCV 溶液(0.1 mg/ml) 1  $\mu$ l、10 x PCR buffer for KOD plus 5  $\mu$ l、2.5 mM MgSO<sub>4</sub> 2  $\mu$ l、2.5 mM dNTP mix 5  $\mu$ l、10  $\mu$ M primer A 3  $\mu$ l、10  $\mu$ M primer B 3  $\mu$ l、滅菌精製水 30  $\mu$ l、5 U/ $\mu$ l Takara kod plus 1  $\mu$ l を混合し PCR を行うことにより PCR 産物 1 を得た。pP<sub>1</sub>235-HCV 溶液(0.1 mg/ml) 1  $\mu$ l、10 x PCR buffer for KOD plus 5  $\mu$ l、2.5 mM MgSO<sub>4</sub> 2  $\mu$ l、2.5 mM dNTP mix 5  $\mu$ l、10  $\mu$ M primer C 3  $\mu$ l、10  $\mu$ M primer D 3  $\mu$ l、滅菌精製水 30  $\mu$ l、5 U/ $\mu$ l Takara kod plus 1  $\mu$ l を混合し PCR を行うことにより PCR 産物 2 を得た。PCR 産物 1 および 2 は 1%アガロースゲルを用いて電気泳動を行った後、QIAEX II Gel Extraction Kit (QIAGEN 社) を用いて精製した。PCR 産物 1 溶液(0.1 mg/ml) 0.5  $\mu$ l、PCR 産物 2 溶液(0.1 mg/ml) 0.5  $\mu$ l、10 x PCR buffer for KOD plus 5  $\mu$ l、2.5 mM MgSO<sub>4</sub> 2  $\mu$ l、2.5 mM dNTP mix 5  $\mu$ l、10  $\mu$ M primer A 3  $\mu$ l、10  $\mu$ M primer D 3  $\mu$ l、滅菌精製水 30  $\mu$ l、5 U/ $\mu$ l Takara kod plus 1  $\mu$ l を混合し PCR を行うことにより PCR 産物 3 を得た。プライマー配列は、primer A; 5' - TTTGACAGACTGCAGGTCCTGGATGACCAT-3'、primer B; 5' - CGCGCTTTCGAGATCGTGAGTCCTGGAGCTTTGCAGCT-3'、primer C; 5' - CTC CAGGACTGCACGATCTGCGAAAGCGCGGGAACC CAGG -3'、primer D; 5' - CATCCTTGCCCATAAG GTGGGCGCATACAT -3' とした。PCR の条件は、94°C 2 min の後、94°C 30 sec、50°C 30 sec、68°C 2 min を 35 サイクル。PCR 後、PCR 産物 3 を QIAquick PCR Purification Kit (QIAGEN 社) を用いて精製し、制

限酵素 HpaI と MfeI により切断し、GDD 欠損 NS5B フラグメントを作製した。pP<sub>1</sub>235-HCV の GDD モチーフの上流にある MfeI サイトおよび下流にある HpaI サイトを切断し、GDD 欠損 NS5B フラグメントをライゲーションした。ライゲーション産物によりコンピテントセル DH5 $\alpha$  をトランスフォーメーションさせた。形成した独立大腸菌クローンを培養し、プラスミド DNA を回収した後、シーケンス解析により、pP<sub>1</sub>235- $\Delta$ GDD を得た。

## 5-2 Ad ベクターの作製

HCV サブゲノム発現 Ad ベクター AdP<sub>1</sub>235-HCV および複製能欠損体 HCV サブゲノム発現 Ad ベクター AdP<sub>1</sub>235- $\Delta$ GDD は実験方法 2-2 と同様な方法で作製した。

## 5-3 Ad ベクター発現確認

HCV サブゲノム発現 Ad ベクター AdP<sub>1</sub>235-HCV の発現を HCV NS5A タンパク質を検出することで確認した。Huh7 細胞を 24-well plate に播種し、培養 24 時間後、培養液を除き AdP<sub>1</sub>235-HCV (10 MOI) を Ad-tTA (50 MOI) と共感染させた。感染 1.5 時間後、培養液を添加した。感染 72 時間後、培養液を除去し、RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1% protease inhibitor cocktail] を用いて細胞を溶解した。回収した細胞溶解液は 14,000 rpm、5 min で遠心を行った。遠心後、上清のタンパク質濃度を BCA protein assay kit (Thermo 社) を用いて測定した。細胞溶解液は western blotting 法により、NS5A および  $\beta$ -actin の発現を確認した。細胞溶解液は最終濃度 30 mg/ml となるように 4 x sample buffer [0.5M Tris-HCl (pH 6.8), 20% 2-Mercaptoethanol, 8% SDS, 20% Sucrose, 0.008% Bromophenol blue] と混合し、100°C 5 min 処理した。細胞溶解液 30  $\mu$ g は 10% ポリアクリルアミドゲルを用いて電気泳動を行った後、polyvinylidene difluoride membrane に転写した。メンブランは 5% スキムミルクを用いて室温で 2 時間処理することによりブロッキングした。メンブランは抗 HCV

NS5A 抗体(Meridian Life Science 社)または抗 $\beta$ -actin 抗体(Sigma 社)を用いて室温で2時間処理した後に、HRP 標識二次抗体(Millipore 社)を室温で1時間処理し、ECL Western Blotting Detection System (GE Healthcare 社)を用いて検出した。

また、HCV サブゲノム発現 Ad ベクター AdP<sub>235</sub>-HCV の発現をレポータータンパク質ルシフェラーゼの活性を測定することで確認した。Huh7 細胞を24-well plate に播種し、AdP<sub>235</sub>-HCV (10 MOI) および Ad-tTA (50 MOI)を共感染させた。感染1.5時間後、ドキシサイクリン含有培養液を添加した。感染48時間後に Renilla Luciferase Assay System (Promega 社)を用いてルシフェラーゼ活性を測定した。

#### 5-4 Adベクター由来 HCV サブゲノムの複製能の検証

HCV サブゲノム発現 AdベクターAdP<sub>235</sub>-HCV 由来の HCV サブゲノム RNA が HCV NS5B (RNA 依存性 RNA polymerase) により複製されることを、複製能欠損体 HCV サブゲノム発現 Ad ベクター AdP<sub>235</sub>- $\Delta$ GDD のルシフェラーゼ活性と比較することにより検証した。Huh7 細胞を24-well plate に播種した。培養24時間後、培養液を除き AdP<sub>235</sub>-HCV または AdP<sub>235</sub>- $\Delta$ GDD (3 MOI) を Ad-tTA (15 MOI) と共感染させた。感染24時間後、最終濃度 10  $\mu$ g/ml ドキシサイクリン含有培養液を添加した。感染72時間後、培養液を除去し、Renilla Luciferase Assay System を用いてルシフェラーゼ活性を測定した。

また、一鎖 HCV RNA は HCV が NS5B により複製をした場合にのみ産生される。HCV サブゲノム発現 Ad ベクターAdP<sub>235</sub>-HCV 感染細胞において一鎖 HCV RNA が産生されることを検証した。Huh7 細胞を24-well plate に播種した。培養24時間後、培養液を除き AdP<sub>235</sub>-HCV または AdP<sub>235</sub>- $\Delta$ GDD (3 MOI) を Ad-tTA (15 MOI) と共感染させた。感染24時間後、最終濃度 10  $\mu$ g/ml ドキシサイクリン含有培養液を添加した。感染72時間後、トリプシン処理により細胞を回収しリン酸緩衝溶液に懸濁した。細胞懸濁液は

High Pure RNA Isolation Kit (Roche 社)を用いて RNA を精製した。精製 RNA は一鎖 HCV RNA 特異的 primer または GAPDH 特異的 primer を用いて逆転写反応を行うことにより cDNA を作製した。RNA 溶液(0.1 mg/ml) 2  $\mu$ l、2  $\mu$ M primers 1  $\mu$ l、滅菌精製水 7.5  $\mu$ l を混合し 65°C、5 min 処理し、すぐに氷上で 5 min 冷却した。10 x RT buffer 2  $\mu$ l、25 mM MgCl<sub>2</sub> 4  $\mu$ l、2.5 mM dNTP mix 2  $\mu$ l、40 U/ $\mu$ l RNase Inhibitor 0.5 ml、20 U/ $\mu$ l AMV Reverse Transcriptase XL 1  $\mu$ l を添加し 60°C、50 min 反応させた後に、85°C、5 min 処理した。各 primer の配列は一鎖 HCV RNA 特異的 primer: 5'-GCCAGCCCCCGATTGGGG -3'、GAPDH 特異的 primer: 5'-TCTACATGGCAACTGTGA -3' とした。cDNA は PCR 法により HCV NS3 および GAPDH を増幅し、2% アガロースゲルを用いて電気泳動を行い検出した。cDNA 溶液 1  $\mu$ l、10 x Ex Taq buffer 2  $\mu$ l、25 mM MgCl<sub>2</sub> 1.2  $\mu$ l、2.5 mM dNTP mix 1  $\mu$ l、10  $\mu$ M primers 1  $\mu$ l、滅菌精製水 12.7  $\mu$ l、5 U/ $\mu$ l TaKaRa Ex Taq 0.1  $\mu$ l を混合し PCR を行った。各 primer の配列は HCV NS3 Forward primer: 5'-ATGGCGCCTATTACGGCC -3'、HCV NS3 Reverse primer: 5'-TGGTCTACATTAGTGAC -3'、GAPDH Forward primer: 5'-GGTGGTCTCCTCTGACTTCAACA -3'、GAPDH Reverse primer: 5'-GTGGTCGTTGAGGGCAATG -3' とした。PCR の条件は、95°C 2 min の後、95°C 10 sec、55°C 30 sec、72°C 30 sec を 35 サイクル。

#### 6. HCV サブゲノム発現 Adベクターを用いた抗 HCV 薬評価

Huh7 細胞を24-well plate に播種し培養した。培養24時間後、培養液を除き AdP<sub>235</sub>-HCV または AdP<sub>235</sub>- $\Delta$ GDD (3 MOI) を Ad-tTA (15 MOI) と共感染させた。感染24時間後、Dox (最終濃度 10  $\mu$ g/ml) および各濃度の IFN を含む培養液を添加した。感染72時間後、ルシフェラーゼ活性および細胞生存率を測定した。ルシフェラーゼ活性は Renilla Luciferase Assay System を用いて、細胞障害性は WST-8 試薬を用いて測定した。ルシフェラーゼ活性および細胞障

害性は IFN 非添加群を 100%として算出した。

## 7. ヒト iPS 細胞由来肝細胞における HCV 感染受容体の発現確認

ヒト iPS 細胞、ヒト iPS 細胞由来肝細胞および Huh7 細胞はトリプシン処理により回収し PBS に懸濁した。細胞懸濁液は High Pure RNA Isolation Kit を用いて RNA を精製した。精製 RNA は SuperScript VILO cDNA Synthesis Kit (Invitrogen 社) を用いて逆転写反応を行うことにより cDNA を作製した。RNA 溶液 (0.1 mg/ml) 2  $\mu$ l、5 x VILO Reaction Mix 4  $\mu$ l、10 x SuperScript Enzyme Mix 2  $\mu$ l、滅菌精製水 12  $\mu$ l を混合し 25  $^{\circ}$ C、10 min 処理し、42  $^{\circ}$ C、60 min 反応させた後に、85  $^{\circ}$ C、5 min 加熱した。cDNA は PCR 法により CD81、SR-BI、claudin-1、occludin および GAPDH を増幅し、2%アガロースゲルを用いて電気泳動を行い検出した。cDNA 溶液 1  $\mu$ l、10 x Ex Taq buffer 2  $\mu$ l、25 mM MgCl<sub>2</sub> 1.2  $\mu$ l、2.5 mM dNTP mix 1  $\mu$ l、10  $\mu$ M primers 1  $\mu$ l、滅菌精製水 12.7  $\mu$ l、5 U/ $\mu$ l TaKaRa Ex Taq 0.1  $\mu$ l を混合し PCR を行った。各 primer の配列は CD81 Forward primer: 5' - CGCCAAGGATGTGAA GCAGTTC -3'、CD81 Reverse primer: 5' - TCCCGGAGAAGAGGTCATCGAT -3'、SR-BI Forward primer: 5' - ATTCCGATCAGTGCAACATGA -3'、SR-BI Reverse primer: 5' - CAGTTTTGCTTC CTGCAGCACAG -3'、claudin-1 Forward primer: 5' - TCAGCACTGCCCTGCCCCAGT -3'、claudin-1 Reverse primer: 5' - TGGTGTGGGTAAGAGGTTGT -3'、occludin Forward primer: 5' - TCAGGGAATAT CCACCTATCACTTCAG -3'、occludin Reverse primer: 5' - CATCAGCAGCAGCCATGTA CTCTTCAC -3'、GAPDH Forward primer: 5' - TCTTCACCACCA TGGAGAAG -3'、GAPDH Reverse primer: 5' - ACCACCTGGTGCTCAGTGTA -3' とした。PCR の条件は、CD81: 94  $^{\circ}$ C 2 min の後、94  $^{\circ}$ C 45 sec、60  $^{\circ}$ C 60 sec、72  $^{\circ}$ C 90 sec を 27 サイクル、SR-BI: 94  $^{\circ}$ C 2 min の後、94  $^{\circ}$ C 45 sec、55  $^{\circ}$ C 60 sec、72  $^{\circ}$ C 90 sec を 27 サイクル、claudin-1: 94  $^{\circ}$ C 2 min の後、94  $^{\circ}$ C 45 sec、55  $^{\circ}$ C 60 sec、72  $^{\circ}$ C 90 sec を

27 サイクル、occludin: 94  $^{\circ}$ C 2 min の後、94  $^{\circ}$ C 45 sec、55  $^{\circ}$ C 60 sec、72  $^{\circ}$ C 90 sec を 27 サイクル、GAPDH: 94  $^{\circ}$ C 2 min の後、94  $^{\circ}$ C 45 sec、55  $^{\circ}$ C 60 sec、72  $^{\circ}$ C 90 sec を 20 サイクルにて行った。予想される PCR 産物のサイズは、CD81: 245 bp、SR-BI: 788 bp、claudin-1: 521 bp、occludin: 189 bp、GAPDH: 544 bp である。

## 8. HCVpv の作製

HCV 侵入解析のために用いた VSVpv は以下の方法で増幅した。

293T 細胞を 100 mm dish に播種し 24 時間培養した後に、TransIT-LT1 (Mirus 社) を用いて VSV G タンパク質発現プラスミド pCAGVSVG をトランスフェクションした。プラスミド導入 24 時間後、エンベロープ G タンパク質をルシフェラーゼ遺伝子に置き換えた VSVpv (大阪大学微生物病研究所、松浦博士より御供与頂いた) を感染させた。感染 24-36 時間後、培養液を回収し、2,000 rpm、5 min 遠心した後の上清を VSVpv として使用した。VSVpv は -80  $^{\circ}$ C にて保存した。

HCV 侵入解析のために用いた HCVpv は以下の方法で作製した。

293T 細胞を 100 mm dish に播種し 24 時間培養した後に、TransIT-LT1 (Mirus 社) を用いて HCV E1、E2 発現プラスミド pCAG-Con1 をトランスフェクションした。プラスミド導入 24 時間後、VSVpv を 2 時間感染させ、細胞を DMEM 8 ml で 5 回洗浄した後に DMEM 10 ml を添加し培養した。VSVpv 感染 24 時間後、培養液を回収し、2,000 rpm、5 min 遠心した後の上清を HCVpv として使用した。HCVpv は -80  $^{\circ}$ C にて保存した。

## 9. HCV サブゲノム発現 Ad ベクターを用いたヒト iPS 細胞由来肝細胞での複製・抗 HCV 薬評価解析

9-1 ヒト iPS 細胞由来肝細胞における HCV 侵入解析  
48-well plate にて分化誘導した iPS 細胞由来肝細胞および 48-well plate に播種した iPS 細胞および Huh7 細胞を用意した。培養 24 時間後、培養液を除き HCVpv を添加し 2 時間感染させた。感染 2 時間後、新しい培養液に交換し、24 時間培養した。その後、ル

シフェラーゼ活性を測定した。

## 9-2 ヒト iPS 細胞由来肝細胞における感染受容体依存的な HCV 侵入解析

48-well plate にて分化誘導した iPS 細胞由来肝細胞および 48-well plate に播種した iPS 細胞および Huh7 細胞を用意した。培養 24 時間後、あらかじめ抗 CD81 抗体(JS-81; BD Biosciences 社)またはコントロールマウス IgG と 2 時間インキュベートした HCVpv または VSVpv を細胞に添加し 2 時間感染させた。感染 2 時間後、新しい培養液に交換し、24 時間培養した。その後、ルシフェラーゼ活性を測定した。

## 9-3 HCV サブゲノム発現 Ad ベクター由来 HCV サブゲノムの複製能の検証

48-well plate にて分化誘導した iPS 細胞由来肝細胞および 48-well plate に播種した iPS 細胞および Huh7 細胞を用意した。以下、実験方法 5-4 と同様な方法で行った。

## 9-4 HCV サブゲノム発現 Ad ベクターを用いた抗 HCV 薬評価

48-well plate にて分化誘導した iPS 細胞由来肝細胞および 48-well plate に播種した Huh7 細胞を用いて、B. 3 の方法に準じて実験を行った。

## 10. HCV フルゲノム polI 発現系シャトルプラスミドの作製

HCV フルゲノム polI 発現系シャトルプラスミドである pHM18-HCV は以下の方法で作製した。

まず、pHM5-TREP235-MCS よりテトラサイクリン制御 polI 発現カセットを、テトラサイクリン制御 polI プロモーター上流に MfeI サイトを付加したプライマー(5'-ATTGCAATTGTGAAAGTCGAGCTCGGTACC-3')および polI ターミネーター下流に AflII サイトを付加したプライマー(5'-ACGGCTTAAGGTAGCGAAAGCTTGATGCC-3')を用いて PCR 法により増幅した。増幅した断片に対して MfeI 消化及び AflII 消化したのち、EcoI 消化および AflII 消化を行った

pHM18-U6-GFP に、T4DNA リガーゼを用いたライゲーション反応によりテトラサイクリン制御 polI 発現カセットを挿入し、pHM18-TREP235-MCS を作製した。次に、挿入したテトラサイクリン制御 polI カセットに、以下に示す 3 段階のステップにより HCV フルゲノム cDNA(遺伝子型 2b 型)の挿入を行った。なお、用いた HCV フルゲノム cDNA は、東京医科歯科大学 坂本直哉 博士より御供与頂いた。まず、鑄型とした HCV cDNA の最下流に存在する BamHI サイトから 3' 末端までの領域を、以下に示すプライマー(5'-GCCGGATCCACTCCCCCTTC-3')、及び下流に BsmBI サイトを付加したプライマー(5'-AATTAacgtctcaGGGACATGATCTGCAGAGAGACCA-3')を用いて PCR 法により増幅した。増幅した断片に対して BamHI 消化及び BsmBI 消化したのち、BamHI 消化及び SpeI 消化した pHM18-TREP235-MCS に、T4DNA リガーゼを用いたライゲーション反応により挿入することで HCV cDNA の 3' 側の一部を挿入し、pHM18-TREP235-3' HCV を作製した。次に、鑄型とした HCV cDNA の 5' 末端から、HCV cDNA の最上流に存在する SpeI サイトまでの領域を、上流に BsmBI を付加したプライマー(5'-TTAATTCGTCTCGTATTGACCTGCCCTAATAGGGCGA-3')、及び下流に BamHI を付加したプライマー(5'-AATTAAGGATCCACCCACTAGTAGTACGACCCGCT-3')を用いて PCR 法を行うことで HCV cDNA の 5' 側の一部を増幅した。増幅した断片に対して BsmBI 消化及び BamHI 消化したのち、BamHI 消化した pHM18-TREP235-3' HCV に、T4DNA リガーゼを用いたライゲーション反応により挿入し、pHM18-TREP235-5' 3' HCV を作製した。次に、HCV cDNA に対して SpeI 消化及び BamHI 消化したのち、得られた断片を、SpeI 消化及び BamHI 消化したのちにアルカリフォスファターゼ反応により脱リン酸化処理を施した pHM18-TREP235-5' 3' HCV に、T4DNA リガーゼを用いたライゲーション反応により挿入することで pHM18-HCV を作製した。なお、遺伝子組み換えの各段階において、作製したプラスミドの挿入断片の塩基配列は、シーケンス解析(ジーンデザイン社