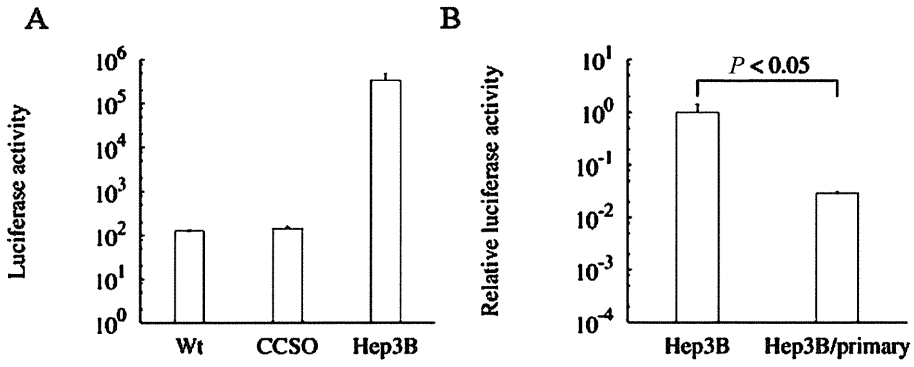

Fig. 3 HCVpp infection of primary hepatocytes from Alb-CCSO mice and fused cells. (A) The luciferase activity of cell extracts from primary hepatocytes isolated from wild-type (Wt) and CCSO mice and Hep3B cells infected with HCVpp. (B) The luciferase activity of cell extracts from Hep3B cells and Hep3B-mouse primary hepatocytes fused cells (Hep3B/primary) infected with HCVpp (n = 3, experiment in triplicate).

infected with HCVpp overnight and the luciferase activity of the cell extracts measured 72 h after infection (Fig. 3A). HCVpp infected the Hep3B cells efficiently, and the Hep3B cell extract exhibited high luciferase activity. However, the luciferase activity of the cell extracts of primary hepatocytes from Alb-CCSO mice was negligibly low, as was that of primary hepatocytes from wild-type mice (Fig. 3A). Based on our data on *in vivo* and *in vitro* infection, we concluded that the expression of the four human proteins in the mouse hepatocytes does not confer susceptibility to HCV entry into hepatocytes.

Finally, we tried to determine whether the mouse hepatocytes expressed inhibitory factor(s) to HCV entry. The Hep3B and primary hepatocyte fused cells were sorted by FACS. Mouse hepatocytes have autofluorescence; therefore, the EGFP and Ds-Red double-positive population was collected and the autofluorescent population excluded. We infected the fused cells with HCVpp. The luciferase activity of the fused cells was 35-fold lower than that of Hep3B cells (Fig. 3B), suggesting that the mouse hepatocytes have inhibitory factor(s) to HCV entry.

DISCUSSION

We succeeded in making transgenic mice in which the four human proteins were expressed in the mouse hepatocytes. The E2 protein of HCV highly bound to the liver sections of transgenic mice comparing to wild-type mice. We also demonstrated that the E2-binding site was similar to those of HCV receptors (CD81 and SR-BI) in the transgenic mice and interpreted that the E2-receptor complex would be near CLDN1 and OCLN at the tight junction.

The experiments presented here demonstrated that CD81, CLDN1, SR-BI, and OCLN expression in the hepatocytes of transgenic mice does not confer susceptibility to HCV infection *in vivo* and *in vitro*. The expression of these four factors in a non-permissive mouse embryonic fibroblast cell line, NIH3T3, makes the cells permissive to HCV entry. However, the infectivity is quite low in a mouse hepatocellular carcinoma cell line, Hepa1.6, expressing these four factors (22). The result in the latter cells and our results that the primary mouse hepatocytes expressing the four factors were non-permissive to HCV entry might indicate that a mouse hepatocyte character is not suitable for HCV entry.

There are two possibilities for no susceptibility to HCV entry; additional factor(s) are necessary for HCV entry or dominant negative restriction factor(s) are present in the mouse hepatocytes, but not in NIH3T3 cells. To evaluate the possibility of dominant negative factors, we performed the cell fusion experiment. HCV entry into the fused cells was 35-fold less than that into Hep3B cells. As this result suggests the possibility of dominant negative factor(s) in the mouse hepatocytes, further analysis is required to identify the dominant restriction factor(s). However, our results did not deny the presence of additional essential factor(s) in the human hepatocytes.

As expected, transgenic expression of CD81 and SR-BI surely enhanced the binding of E2 protein to the liver sections in our experiments. The key factors of human origin for post-binding events, including caveolin and Rab34 in Coxsackievirus entry, are thought to be absent in mouse primary hepatocytes (7). One study described that transferrin receptor 2 (TfR2) is associated with CD81 protein, and that

TfR2 is responsible for an endosomal pathway (4). To examine whether these factors interact with HCV might be important.

Many papers have reported the various receptors or factors and the susceptibility to HCV in the established or transfected cells. Comparing the expression and interaction of receptors/factors between permissive and non-permissive cells may provide a breakthrough in the molecular understanding of HCV entry. Our data demonstrated that the expression of CD81, CLDN1, SR-BI, and OCLN in the transgenic liver was not enough to confer susceptibility to HCV entry. This report provides an insight into the generation of an HCV-infectible mouse.

Acknowledgements

We are grateful to Dr. Yoshiharu Matsuura for the gift of pCAGGSc60-p7 and Dr. Thomas Pietschmann for pcDNAΔC-E1-E2. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, Culture and Technology and by a grant from the Mitsui Life Social Welfare Foundation.

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Biochemical and Biophysical Research Communications 416 (2011) 119–124

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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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ARTICLE INFO

Article history:
Received 31 October 2011
Available online 10 November 2011

Keywords:
Hepatitis C virus
Induced pluripotent stem cell
Hepatocyte
Infection
Replication
Experimental model

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.

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 nonstructural 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-4α, hepatocyte nuclear factor-4α; 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-α8; ES cells, embryonic stem cells.

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doi:10.1016/j.bbrc.2011.11.007

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 (Dotcom) 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 4α (HNF-4α) 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′-tcccggagaagaggtcatcgat-3′), scavenger receptor class B type I (SR-BI; 5′-attccgatcagtgcaa catga-3′ and 5′-cagttttgcttcctgcagcacag-3′), claudin-1 (5′-tcagcact gccctgccccagt-3′ and 5′-tggtgttgggtaagaggttgt-3′), occludin (5′-tca gggaatatccacctatcacttcag-3′ and 5′-catcagcagcagccatgtactcttcac-3′), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-tct tcaccaccatggagaag-3′ and 5′-accacctggtgctcagtgta-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$_i$235-HCV), a replication-incompetent HCV subgenomic replicon containing renilla luciferase (AdP$_i$235-ΔGDD), tet-responsive trans-activator (Ad-tTA) or a tet-controllable RNA pol-I driven firefly luciferase (AdP$_i$235-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$_i$235-HCV or AdP$_i$235-ΔGDD at multiplicity of infection (MOI; infectious unit per cell) of 3, and Ad-tTA at MOI of 15. After 24 h, the cells were treated with 10 μg/ml of doxycycline (Dox) for 48 h. Renilla luciferase activities in the lysates were then measured with the use of the Renilla Luciferase Assay System (Promega, Madison, WI). To normalize for the infectivity of Ad vector, iPS, iPS-Hep and Huh7 cells were co-infected with AdP$_i$235-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$_i$235-HCV or AdP$_i$235-ΔGDD (3 MOI), and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/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′-gtctagccatggcgttagta-3′; and RC21 primer, 5′-ctcccggggcactcgcaagc-3′), HCV minus-strand RNA (tag primer, 5′-ggccgtcatggtggcgaataa-3′; and RC21 primer), and GAPDH (5′-ggtggtctcctctgacttcaaca-3′ and 5′-gtggtcgttgagggcaatg-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-α8

iPS-Hep and Huh7 cells were infected with AdP$_i$235-HCV (3 MOI) and Ad-tTA (15 MOI). After 24 h of infection, the cells were treated with 10 μg/ml of Dox and recombinant human interferon-α8 (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 μg/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$_i$235-HCV [replication competent], and AdP$_i$235-ΔGDD [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.

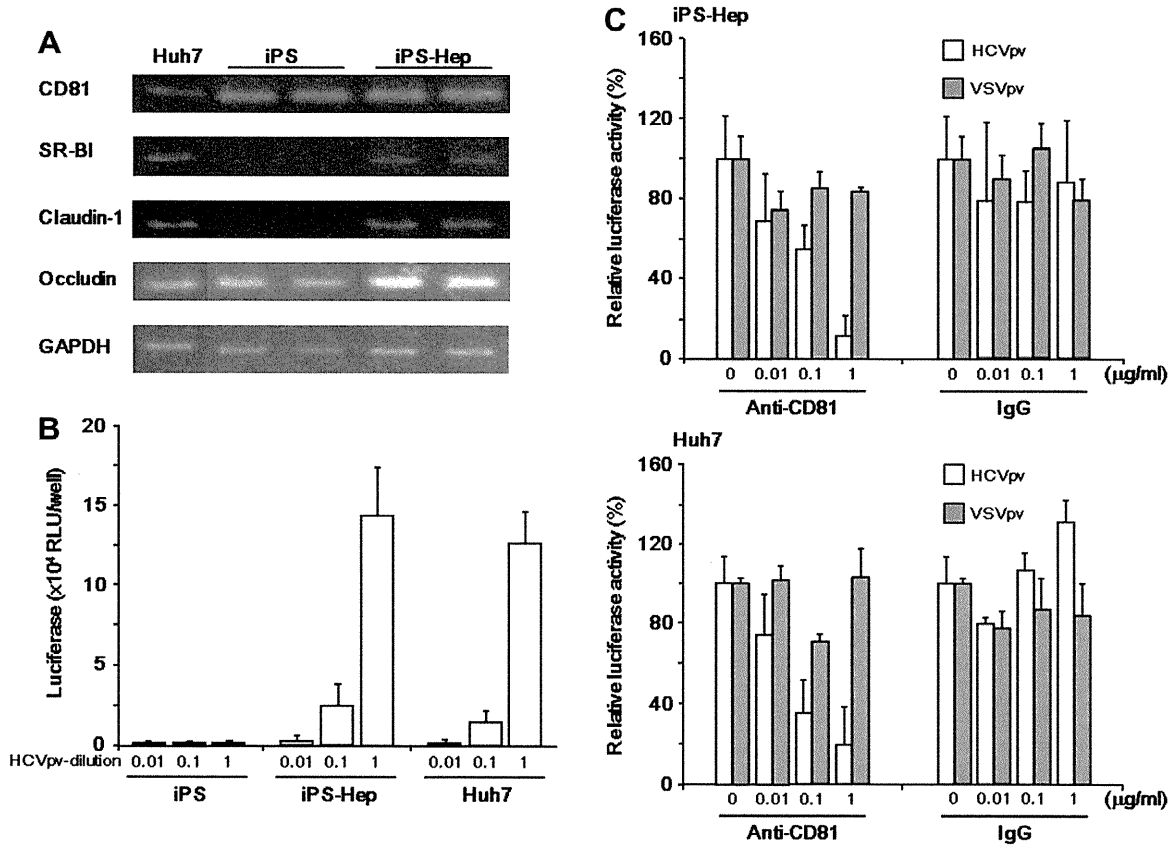

Fig. 1. HCV infection assay in iPS-Hep cells. (A) Expression of HCV receptors in iPS-Hep cells. Total RNA samples from Huh7, iPS, and iPS-Hep cells were subjected to RT-PCR analysis as described in the Section 2. The PCR products were separated on 2% agarose gels, followed by staining with ethidium bromide. (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 ± 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 ± SD (n = 3).

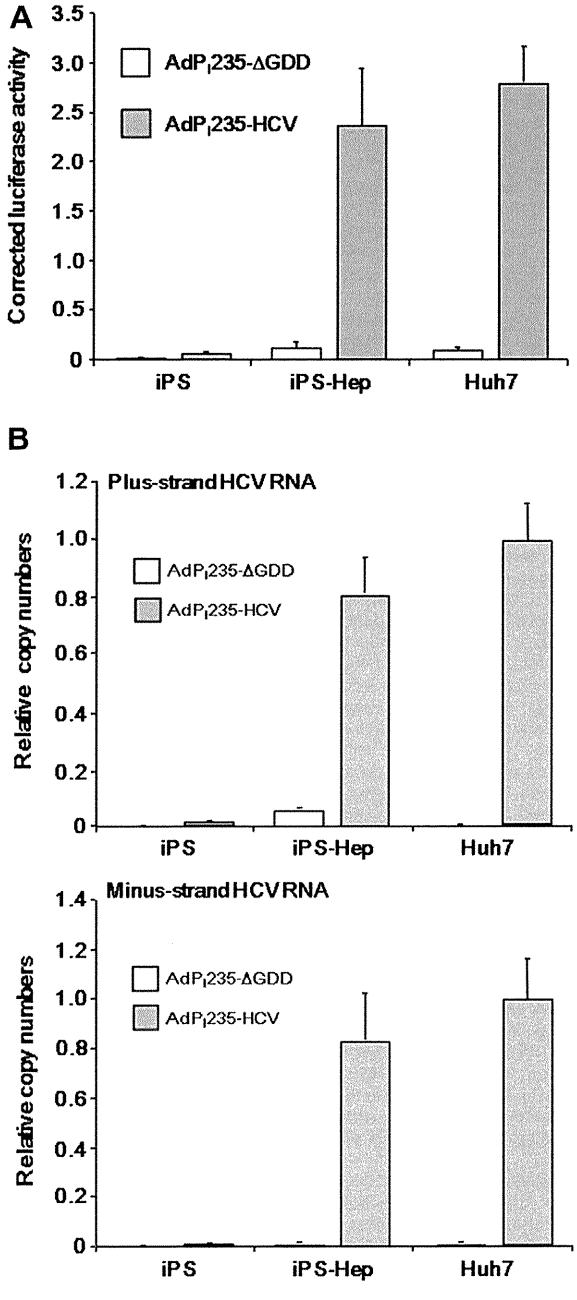

Fig. 2. HCV replication assay in iPS-Hep cells. (A) Comparison of replication of HCV subgenomic replicons, AdP$_i$235-HCV (gray column) and AdP$_i$235-ΔGDD (open column), in iPS, iPS-Hep and Huh7 cells. The cells were infected with replicons, treated with Dox, and renilla luciferase activity was measured, as described in the Section 2. To normalize for infectivity of Ad vector, cells were co-infected with AdP$_i$235-fluc and Ad-tTA. 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 ± SD (n = 3).

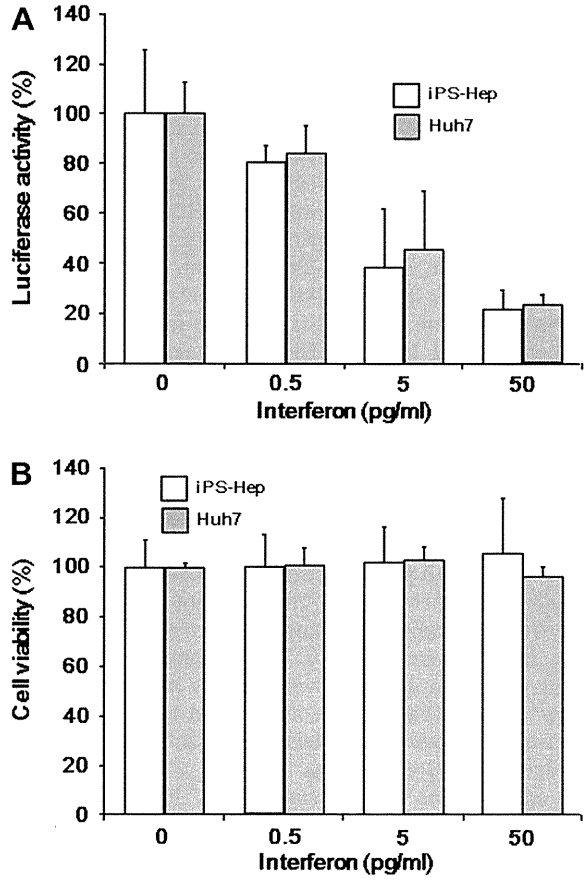

Fig. 3. Effect of interferon on HCV replication in iPS-Hep cells. iPS-Hep (open column) and Huh7 (gray column) cells were infected with AdP$_i$235-HCV and Ad-tTA. After 24 h, the cells were treated with Dox and the indicated concentration of interferon for 48 h. Luciferase activities (A) and cell viabilities (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 ± 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.

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-4α [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-4α, 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-4α-deficient mice [32]. HNF-4α 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-4α 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.

Acknowledgments

This work was supported by a Health and Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan (HM, FS and KY), by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (23659039, MK) by the Japan Research Foundation for Clinical Pharmacology (HM), and by the Uehara Memorial Foundation (HM).

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Promising Targets for Anti-Hepatitis C Virus Agents

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Abstract: Hepatitis C virus (HCV) infection is a serious global health problem, with 3-4 million new cases reported each year. Chronic HCV infection places 170 million people at risk of developing liver cirrhosis and hepatocellular carcinoma. However, difficulties in preparing HCV particles *in vitro* have delayed development of effective anti-HCV therapies. In 2005, Wakita *et al.* developed an *in vitro* method to prepare HCV particles, thereby enabling researchers to better understand the mechanism of HCV infection. Other recent advances include development of a virus-free system for evaluating HCV replication and the identification of HCV receptors, such as claudin-1 and occludin, that may serve as targets for anti-HCV drugs. In this review, we discuss recent findings in HCV infection research, including discovery of new potential targets for anti-HCV therapy.

Keywords: Hepatitis c virus, CD81, claudin-1, NS3 helicase, cyclophilin, miRNA122.

INTRODUCTION

It is estimated that approximately 170 million people worldwide are infected with hepatitis C virus (HCV). Chronic HCV infection induces cirrhosis of the liver or hepatocellular carcinoma. Currently, no vaccines or inhibitors that block HCV entry into cells are approved for clinical use. Standard therapy for chronic HCV infection is the combination of pegylated interferon (IFN) and ribavirin (RBV); however, only 50% to 60% of infected patients get a sustained anti-viral response by this therapy. In addition, the severe side effects typical of IFN and RBV treatment often lead patients to stop treatment, and development of novel treatments with fewer serious side effects are therefore necessary.

Hepatitis C virus is a single-stranded RNA virus belonging in the family *Flaviviridae*. The viral genome is approximately 9,600 nucleotides, containing a 5'untranslated region (5' UTR), a region encoding a polyprotein of about 3,000 amino acids, and a 3' UTR. An internal ribosome-entry site (IRES) in the 5' UTR induces cap-independent translation. Once translated, the viral polyprotein is proteolytically processed by cellular signal peptidases and viral proteases into at least 10 mature viral proteins. Three of these proteins (Core, E1, and E2) are the structural proteins included in virions. It is unclear whether protein p7 is included in virions. Non-structural proteins (NS) include NS2, NS3, NS4A, NS4B, NS5A, and NS5B, and all except NS2 are necessary for formation of the complex associated with viral replication. In this review, we summarize recent developments in anti-HCV agents and discuss potent targets for anti-HCV agents.

INHIBITORS OF HCV ENTRY INTO CELLS

HCV Receptors

Hepatitis C virus contains two glycosylated envelope proteins, E1 and E2. While the role of E1 in infection is poorly understood, E2 is known to play a critical role through binding to the cell surface receptor and facilitating virus entry. Several receptors and co-receptors are involved in HCV infection, including CD81, scavenger receptor class B type I (SR-BI), low-density lipoprotein receptor (LDLR), claudin-1, and occludin [1-5]. Although it has been demonstrated that both CD81 and SR-BI directly bind to E2, there is no evidence that claudin-1 and occludin bind the HCV envelope, suggesting that claudin-1 and occludin may interact with other co-receptors to induce HCV entry.

Development of inhibitors that block envelope protein E2 from interacting with cellular receptors is an important area of anti-HCV research. One such class of inhibitors, (ssDNA) aptamers that recognize the HCV E2 protein, was isolated using a living cell surface technique (Systematic Evolution of Ligands by Expotential Enrichment). The ssDNA aptamer ZE2 binds to E2 with high affinity and inhibits its interaction with CD81, and was shown to block HCV infection *in vitro* [6].

Other inhibitors of HCV infection include proteins that bind to or modulate the activity of CD81 and prevent its interaction with CD81 with E2. Salicylate derivatives identified through virtual screening inhibit HCV infection by binding to the open conformation of the large extracellular loop (LEL) of CD81 and preventing its binding to E2. Benzyl salicylate inhibits the interaction of CD81-LEL with E2 by 25% at 50 μM [7]. Another modulator of CD81 activity is PSCK9, a regulator protein of membrane-bound receptors such as LDLR, ApoER2, and very low-density lipoprotein receptor. A recent study showed that PSCK9 deregulates the cell surface localization of CD81. Soluble PCSK9 inhibits HCV infection *in vitro* in a dose-dependent manner [8].

Claudin-1 has been identified as a co-receptor involved in HCV entry into cells, and its interaction with CD81 may help facilitate the early and late stages of HCV entry [4]. Claudin-1 is estimated to be a co-receptor that interacts with CD81. Recently, a claudin-1 antibody was developed that helped elucidate the role of claudin-1 in HCV infection. Anti-claudin-1 inhibited HCV infection at the same stage of HCV entry at which an anti-CD81 antibody did [9]. Since there is no evidence that claudin-1 binds directly to any HCV envelope proteins, it is believed that claudin-1 interacts with CD81 to form a complex that enables HCV cell entry, and may thus serve as a target for development of new HCV entry inhibitors.

Several HCV entry inhibitors that target neither the HCV envelope proteins nor cellular receptors have also been developed. One such inhibitor is C5A, an amphipathic α-helical peptide derived from the membrane anchor domain of HCV NS5A. C5A prevents initiation and spread of HCV infection by destabilizing virions, and has been shown to destroy the integrity of other viral particles, including other *Flaviviridae* (West Nile virus and dengue virus), some paramyxoviruses, and human immunodeficiency virus [10, 11]. C5A might recognize lipid composition of virus membranes, leading to the antiviral activity of C5A to the other viruses [11].

Arbidol is a broad-spectrum antiviral agent that inhibits virus-induced membrane fusion [12-14]. Arbidol is an effective inhibitor of both hepatitis B and C, as well as a wide range of other viruses, including influenza A and B, parainfluenza virus 3, respiratory syncytial virus, and rhinovirus 14. Other agents that block viral entry into host cells include Peptide 75, a peptide derived from the HCV E2 protein transmembrane domain [15], and the Lamiridosins, compounds extracted from *Lamium album* [16]. While the mechanisms through which these agents act to inhibit viral infectivity are poorly understood, continued research may lead to development of additional novel series of inhibitors.

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Protein p7 Inhibitors

Viroporin protein p7 has two membrane-spanning helices and oligomerizes to form a cation-selective ion channel or pore [17-19]. However, the function of p7 and its importance in the HCV infection cycle is unclear. Recent studies revealed that p7 is critical for HCV entry as well as the release of infectious virions both *in vitro* and *in vivo* [20-23]. While p7 is not required for HCV RNA replication [24, 25], the protein is necessary for assembly of the viral particle [22], suggesting that p7 may be a virion component.

Amantadine is an inhibitor of the influenza A virus M2 protein, which is also a viriporin protein. Amantadine also blocks HCV p7 ion channel activity [26, 27]. Several clinical trials showed that amantadine treatment may be effective in patients with chronic hepatitis C infection [28-33]. Other inhibitors of protein p7 have been also identified, including iminosugar derivatives, hexamethylene amiloride, rimantadine, and GSK1-3 [20, 34-37]. More importantly, three-dimensional structure and functional amino acids of protein p7 have been determined [38, 39]. These findings will facilitate the development of new inhibitors against this important HCV protein.

INHIBITORS OF REPLICATION-ASSOCIATED VIRAL PROTEINS AND THE VIRAL GENOME

IRES Inhibitors

The internal ribosome-entry site (IRES) is a well-defined structure of about 340 nucleotides in the 5' UTR of the HCV genome [40]. The host 40S ribosomal subunit and eukaryotic initiation factor 3 (eIF3) recognize the HCV IRES and synthesize viral polyproteins in a cap-independent manner.

The IRES consisting of three domains (domain II-IV) is an attractive target for antiviral drugs because the sequences of subdomains IIIe and IIId are well conserved in all HCV sub-types. Oligodeoxynucleotides targeting IRES domain IIId had antiviral effects [41]. The structures of sub-domain IIa and IIIb are also highly conserved among many HCV sub-types [42, 43]. The L-shaped conformation of sub-domain IIa directs the apical hairpin loop of domain IIb towards the ribosomal E site in the proximity of the active site [44, 45]. The L-shaped architecture of domain II is essential for binding of the 40S ribosome to the IRES. Benzimidazole targets domain IIa and inhibits HCV replication by inducing a widening of the RNA interhelical angle in sub-domain IIa, thereby leading to inhibition of IRES-driven translation [46]. Sub-domain IIIb consists of a hairpin loop, an internal loop and two mismatched cytosine bases. The sequence is not well conserved, but the three-dimensional structure is well conserved. Recognition of the structure of sub-domain IIIb by eIF3 is essential for IRES-dependent translation, indicating that modulation of sub-domain IIIb conformation may inhibit the HCV replication [47]. IRES domain IV contains the HCV translation start codon. The GCAC sequence near the initiator AUG codon is also essential for ribosome assembly [48, 49]. Several inhibitors, including shRNA, siRNA, and hammerhead ribozyme, target the GCAC sequence and efficiently inhibit HCV replication by blocking the initiation of translation [50-52].

NS3 Helicase Inhibitors

Helicase NS3 possesses multifunctional enzymatic activities and plays an essential role in HCV replication [53]. The N-terminus of NS3 is a serine protease involved in viral polyprotein processing, while the C-terminus is an RNA helicase/nucleotide triphosphatase [54]. A number of inhibitors of NS3 protease activity, such as boceprevir, telaprevir, SCH-900518 and VX-813, have been clinically used as inhibitors of HCV replication [55, 56]. NS3 helicase unwinds RNA in a 3' to 5' direction on a 3' overhang region, using any NTPs or dNTPs as an energy source [57-60]. There are a variety of known NS3 helicase inhibitors with diverse modes of action. Benzimidazole and benzotriazole derivatives, acridone-4-carboxylic acid derivatives, triphenylmethane derivatives, QU663, and NS3 peptide (p14) have all been identified as inhibitors of the NS3 helicase [55, 61-65]. Acridone-4-carboxylic acid derivatives intercalate into RNA and inhibit both NS3 helicase and NS5B polymerase activities, while triphenylmethane derivatives inhibit NS3 helicase by preventing NTPase hydrolysis and RNA substrate binding. QU663 is a nucleotide-mimicking compound that inhibits NS3 helicase activity by competing with the enzyme for nucleic acid substrates. Finally, peptide p14, a highly conserved arginine-rich sequence of NS3 helicase, inhibits the enzyme by binding to domain I.

NS4A

NS4A forms a stable heterodimeric complex with NS3 and has serine protease activity. The NS3/4A heterodimer cleaves viral polyprotein into mature viral proteins. An important inhibitor of NS4A is ACH-806 [1-(4-pentyloxy-3-trifluoromethylphenyl)- 3-(pyridine-3-carbonyl)thiourea] (ACH-806). EC50 values for ACH-806 were 30 and 14 nM in genotype 1a and 1b replicon systems, respectively [66]. ACH-806 has synergistic activity with the NS3 protease inhibitor as well as the NS5B polymerase inhibitor, and has no cross-resistance to either inhibitor [67, 68]. A clinical study has revealed that ACH-806 is an effective antiviral agent against HCV genotype 1 [69].

NS4B

NS4B is believed to induce the formation of intracellular membrane structures termed the membranous web [70]. HCV replication complex consisting of NS4A, 5B and other NS proteins is co-localized with HCV RNA in the membranous web. An amphipathic N-terminal helix in NS4B mediates membrane association and forms the replication complex [71]. An arginine-rich motif in the C-terminus of NS4B specifically binds the 3' terminus of the negative HCV RNA strand, which is essential for HCV replication. Clemizole hydrochloride inhibits binding of NS4B to the negative RNA strand and thereby disrupts HCV replication [72]. However, the underlying mechanism has not been currently understood.

NS5A

NS5A is a 56- to 58-kDa membrane-associated phosphoprotein consisting of three domains (domains I, II, and III). In its basally phosphorylated form (p56), NS5A is active in viral replication, whereas the hyperphosphorylated form (p58) is active in viral packaging [73, 74]. Domain I, located in the N-terminus of NS5A, contains a membrane anchoring helix and zinc- and RNA-binding motifs. Domain I has multiple functions, including promoting membrane association of the replication complex, zinc-binding, RNA-binding, and dimerization of NS5A. NS5A is localized in the replication complex on endoplasmic reticulum *via* the domain I, and NS5A interacts with 3'-ends of HCV plus and minus RNA strands. A class of compounds with a thiazolidinone core structure (BMS-824, -858, and -665) inhibits HCV replication *in vitro* by interfering with one or more of the functions of NS5A domain I. These compounds target 76 N-terminal amino acids of NS5A, and they may interfere with RNA-binding or NS5A dimerization. The resultant inhibition of hyperphosphorylation of NS5A might inhibit HCV replication [75]. Another class of NS5A inhibitors, the piperazinyl-N-phenylbenzamides, prevents HCV replication by blocking dimerization of NS5A [76].

Cyclophilin

The immunosuppressant compound cyclosporin A (CsA) is one of the most well known HCV inhibitors. Cyclosporin A acts by

targeting cellular proteins involved in HCV replication [77, 78]. Several sub-types of cyclophilin (CyP), CyPA and CyPB, have been reported to be CsA targets [79-81]. A recent study involving the knockdown of individual CyP sub-types revealed that CyP40 is a novel target of CsA [82]. CyPB facilitates HCV replication *via* the regulation of the RNA binding ability of NS5B [81]. CyPA and CyPB are likely to play different roles in HCV replication than does CyPA. Non-immunosuppressive CsA analogs, such as NIM811, Debio-025, and SCY635, inhibit both CyPA and CyPB [83-85], while CyPB and CyP40 facilitate HCV replication in CsA-resistant cells. There is thus considerable interest in development of CyPB or CyP40 inhibitors [82].

Lupus Autoantigen (La)

Many cellular proteins that interact with IRES elements and stimulate IRES-driven translation have been reported, including the lupus autoantigen (La), polypyrimidine tract binding protein (PTB), poly rC binding protein 2 (PCBP2), C23 nucleolin, and NS1-associated protein 1 [86-94]. These transacting proteins are termed IRES-transacting factors (ITAFs). Granzyme H interferes with La-mediated HCV-IRES translational activity by cleaving the La protein [95]. A synthetic peptide (named LAP) that corresponds to the 18 N-terminal amino acids of La efficiently blocks HCV replication [96]. It is believed that LAP competitively blocks La from interacting with the ITAFs PTB and PCBP2, suggesting the possibility that ITAFs would be suitable targets for inhibition of HCV replication [97].

microRNAs

A few microRNAs (miRNAs) associated with HCV replication have been reported. A liver-specific miRNA, miR-122, facilitates HCV RNA replication by binding the 5' UTR of the viral genome [98]. It has been suggested that down-regulation of miR122 is involved in the anti-HCV activity of IFN [99]. Recently, development of a novel therapeutic agent targeting miR122 was reported. Silencing miR-122 with a locked nucleic acid (LAN)-modified phosphorothioate oligonucleotide (SPC3649) efficiently blocked HCV RNA replication in chronically infected chimpanzees [100].

Another miRNA, miR-199a, has anti-HCV activity that is independent of the IFN pathway. The target sequence of miR-199a is a

Table 1. Targets for HCV Entry

Targets	Anti-HCV agents	Mechanisms
E2	ssDNA aptamers (ZE2)	Inhibit the interaction of E2 with CD81 by binding to E2
CD81	Salicylate derivatives	Inhibit the interaction of E2 with CD81 by binding to LEL of CD81
	Soluble PSCK9	Deregulate the cell surface localization of CD81
Claudin-1	Claudin-1 antibody	Inhibit cell entry of HCV by blocking cladin-1
Viral membrane	C5A	Destabilize virions
Cellular membrane	Arbidol	Inhibit virus-induced membrane fusion
Unknown	Peptide 75	Not understood
	Lamiridosins	Not understood
p7	Amantadine	Inhibit p7 ion channel activity
	Amino sugar derivatives	Inhibit p7 ion channel activity

Table 2. Targets for HCV Replication

Targets	Anti-HCV agents	Mechanisms
IRES	Benzimidazole	Widen the RNA interhelical angle in sub-domain IIa
	shRNA targeting 322-340 of the 5'UTR	Inhibit ribosome assembly
	siRNA targeting 331-350 of the 5'UTR	Inhibit ribosome assembly
NS3	Boceprevir, Telaprevir, SCH-900518, VX-813	Inhibit protease activity
	Benzimidazole derivatives	Inhibit NS3 helicase activity
	Benzotriazole derivatives, Acridone-4-carboxylic acid derivatives	Intercalate into RNA and inhibit NS3 helicase activity
	Triphenylmethane derivatives (QU663)	Inhibit NTPase hydrolysis
	NS3 peptide (p14)	Bind to NS3 domain I
NS4A	ACH-806	Inhibit NS3/NS4A protease activity
NS4B	Clemizole hydrochloride	Inhibit binding of NS4B to HCV negative RNA strand
NS5A	Thiazolidinone	Inhibit a function of NS5A domain I
	Piperazinyl-N-phenylbenzamides	Inhibit dimerization of NS5A
CyP	CsA	Inhibit PPIase activity of CyPs
	NIM811, Debio-025, SCY635	Inhibit PPIase activity of CyPs
La	Granzyme H	Cleave La protein
	LAP	Competitively inhibit binding of La protein to ITAFs
miR-122	SPC3649	Silence miR-122

highly conserved region among HCV sub-types located in domain II of the HCV IRES. Thus, miRNAs are also attractive targets for development of new HCV inhibitors [101].

CONCLUSIONS

The development of an *in vitro* amplification system for HCV by the Wakita group in 2005 has had a profound impact on studies of this important virus [102]. This amplification system has enabled researchers to produce viral particles in sufficient quantities to obtain a better understanding of the molecular mechanism underlying HCV infection, and has aided in the development of inhibitors of a variety of viral target molecules. The targets for anti-HCV therapeutic agents that have been discussed here can be classified into molecules involved in HCV entry (HCV receptors and p7) and in HCV replication (HCV and host cellular components) (Tables **1** and **2**). Inhibitors of NS3/4A protease or NS5B polymerase are promising anti-HCV agents among them. However, frequent mutation of HCV during proliferation has led to the emergence of drug-resistant viruses. To address this issue, numerous efforts have been paid on identification of cellular factors involved in viral replication and infection. One such promising anti-HCV agent is the LAN-modified oligonucleotide that targets the liver specific miRNA associated with HCV replication. This agent showed anti-HCV activity in chronically infected chimpanzees without apparent side effects for an extended period [100]. Very recently, monoclonal antibodies against claudin-1 prevented infection of highly variable HCV quasispecies [103]. We believe that recent progress in understanding the biology of HCV combined with advances in medicinal chemistry will lead to additional breakthroughs in anti-HCV therapy.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (20659012), a Health and Labor Sciences Research Grant from the Ministry of Health, labor and Welfare of Japan, and by Sasagawa Science Grants from the Japan Science Society.

ABBREVIATIONS

HCV	=	hepatitis C virus
IFN	=	interferon
RBV	=	ribavirin
UTR	=	untranslated region
IRES	=	internal ribosome-entry site
NS	=	nonstructural protein
SR-BI	=	scavenger receptor class B type I
LDLR	=	low density lipoprotein receptor
LEL	=	large extracellular loop
eIF3	=	eukaryotic initiation factor 3
CsA	=	Cyclosporin A
CyP	=	cyclophilin
La	=	lupus autoantigen
PTB	=	polypyrimidine tract binding protein
PCBP2	=	poly rC binding protein 2
ITAFs	=	IRES-transacting factors
miRNA	=	microRNA
LNA	=	locked nucleic acid

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Nucleic Acids Research Advance Access published February 8, 2011

Nucleic Acids Research, 2011, 1–8
doi:10.1093/nar/gkr047

Adenovirus vector-mediated assay system for hepatitis C virus replication

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Received April 9, 2010; Revised January 17, 2011; Accepted January 18, 2011

ABSTRACT

The efficient delivery of the hepatitis C virus (HCV) RNA subgenomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.

INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6-kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

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RNA has been developed for the generation of vaccine seed strains and for basic influenza virus studies (15). These findings indicate that the RNA pol I Ad vector system can be a promising tool for basic and pharmaceutical studies on HCV. However, the development of an RNA pol I-driven vector system expressing the HCV RNA genome has never been reported.

In the present study, we developed an RNA pol I-driven vector system to monitor HCV replication using an HCV replicon in which structural genes were replaced by the luciferase gene. We prepared an Ad vector containing a tetracycline (tet)-regulated RNA pol I-expression cassette consisting of an RNA pol I-driven responsive vector and a *trans*-activator vector, and we successfully developed an Ad vector-mediated HCV replication system.

MATERIALS AND METHODS

Cell culture

Huh7.5.1 1bFeo [genotype 1b HCV replicon cell line, (8)] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and G418 (Nacalai Tesque, Kyoto, Japan) at 500 μg/ml. Huh7 and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were maintained in a 5% CO_2 atmosphere at 37°C.

Preparation of RNA pol I-driven plasmid vectors

An RNA pol I expression-cassette was subcloned as follows: pHH21 (kindly provided by Dr Kawaoka, Tokyo, Japan) containing RNA pol I expression cassette was digested with *Afl*III, blunted by the Klenow fragment of DNA polymerase, ligated with *Eco*RI linker and digested with *Eco*RI/*Nhe*I, resulting in a fragment of the RNA pol I expression-cassette. The RNA pol I cassette was inserted into the *Eco*RI-*Xba*I site of pHM5 (16), generating pPol I. A fusion gene of enhanced green fluorescent protein and firefly luciferase (EGFPLuc, Clontech, Mountain View, CA, USA) was inserted into pPol I, resulting in pP_IWT-EL.

The subgenomic HCV sequence and the replication-incompetent subgenomic HCV sequence deleting GDD motif (MLVNGDDLVV) in NS5B were amplified by polymerase chain reaction (PCR) using pRepFeo as a template (8). The PCR fragments were inserted into pPol I, generating pPol I-1bFeo and pPol I-1bFeoΔGDD. The Feo fragment in pPol I-1bFeo or pPol I-1bFeoΔGDD was replaced with firefly luciferase, generating pPol I-HCV or pPol I-ΔGDD coding firefly luciferase reporter, HCV NS3, NS4A, NS5A and NS5B or mutated NS5B, respectively. A plasmid expressing β-galactosidase, pCMVβ, was purchased from Marker Gene Inc. (Eugene, OR, USA).

Preparation of tet-controllable RNA pol I-driven plasmid vectors

To develop the tet-controllable RNA pol I promoter expression system, the minimal cytomegalovirus promoter was replaced by fragments of RNA pol I promoters (from −235 to −1, from −311 to −1 or from −412 to −1) in pHM5-TREL2 (17), generating pP_I235, pP_I311 or pP_I412. These RNA pol I plasmid vectors were used for optimization of the tetracycline responsive element (TRE)/RNA pol I chimeric promoter. pHM5-tTA, pHM5-rtTA and pHM5-TREL2 were used in tet-regulated experiments (17).

Preparation of Ad vector expressing HCV replicon

The HCV replicon fragments cloned from pPol I-HCV or pPol I-ΔGDD were inserted into pP_I235, and then the firefly luciferase was replaced by the renilla luciferase to form pP_I235-HCV or pP_I235-ΔGDD. Ad vectors were constructed by an improved *in vitro* ligation method (18). Briefly, pP_I235-EL, pP_I235-HCV and pP_I235-ΔGDD were digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I/PI-*Sce*I-digested pAdHM4 and pAdHM36, respectively. The resulting plasmids were digested with *Pac*I and transfected into 293 cells with SuperFect (Qiagen, Valencia, CA, USA). AdP_I235-EL, AdP_I235-HCV and AdP_I235-ΔGDD were purified by $CsCl_2$ gradient centrifugation and dialyzed with a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol. The multiplicity of infection (MOI) of Ad vectors was measured using an Adeno-X rapid titer kit (Clontech). Ad-tTA vectors were prepared as previously described (17).

Expression of plasmid-based HCV replicon

Huh7 cells were transfected with 0.8 μg of pPol I-HCV. After 24 h of incubation, the cells were lysed in LCβ (Toyo Ink, Tokyo, Japan). The cell lysates were frozen-thawed and centrifuged at 32 000 *g* for 5 min. The luciferase activity in the resulting supernatant was measured using a commercially available kit (PicaGene; Toyo Ink).

Inhibition assays of HCV replication in plasmid- or Ad-based RNA pol I HCV system

Huh7 cells were transfected with 0.8 μg of pPol I-HCV and 0.2 μg of pCMVβ or infected with AdP_I235-HCV (10 MOI) and Ad-tTA (50 MOI). After 2.5 or 1.5 h of transfection, the cells were treated with recombinant human interferon-α8 (IFN-α8) at the indicated concentration. After an additional 72 h of incubation, the cells were lysed in LCβ. Luciferase activity and β-galactosidase activity in the lysates was measured with PicaGene and a Luminescent β-gal Kit (Takara Bio Inc., Shiga, Japan), respectively. The cell viability was measured with a WST-8 kit according to the manufacturer's instruction (Nacalai Tesque).

Evaluation of tetracycline-controllable promoters in plasmid vector

Huh7 cells were co-transfected with 0.1 μg of reporter plasmid (pP_I235-EL, pP_I311-EL, pP_I412-EL or pP_IWT-EL), 0.8 μg of tet-responsive *trans*-activator plasmid (pHM5-rtTA in the tet-on system or pHM5-tTA in the tet-off system) and 0.1 μg of pCMVβ. After 2.5 h, the cells were treated with doxycycline (Dox) at the indicated

concentration for 48 h. Then, luciferase and β-galactosidase activities in the lysates were measured.

Expression of Ad vector containing tetracycline-controllable promoter system

Huh7 cells were transfected with a reporter Ad vector (AdP_I235-EL or AdP_I235-HCV at MOI of 5 or 10) and a *trans*-activator vector (Ad-tTA at MOI of 10 and 50). After an additional 48 h of incubation, luciferase activity in the cell lysates was measured.

Western blotting

Huh7 cells were co-infected with AdP_I235-HCV at 10 MOI and Ad-tTA at 50 MOI. The cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA] containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The cell lysates (30 μg of protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% skim milk, the filter was incubated with mouse anti-NS5A (Meridian Life Science, Sacao, ME, USA) or anti-β-actin Ab (Sigma). Then, the peroxidase-labeled secondary antibodies were added. The immunoreactive bands were visualized by chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

Evaluation of NS5B-dependent replication

Huh7 cells were transfected with AdP_I235-HCV or AdP_I235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, luciferase activities in the lysates were measured.

Detection of a fragment of the HCV negative strand RNA

Huh7 cells were co-infected with AdP_I235-HCV or AdP_I235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. The total RNAs were purified with High Pure RNA Isolation kit (Roche, Mannheim, Germany). The RNAs were reverse-transcribed to cDNA using a commercial available kit [TaKaRa RNA PCR Kit (AMV) Ver. 3.0] and a primer for the HCV negative strand RNA (5′-GCCAGCCCCCGATTGGGG-3′) or a primer for GAPDH (5′-TCTACATGGCAACTGTGA-3′), respectively. The transcription products of NS3 and GAPDH were amplified by PCR using paired primers (5′-ATGG CGCCTATTACGGCC-3′ and 5′-TGGTCTACATTAG TGTAC-3′) and (5′-GGTGGTCTCCTCTGACTTCAA CA-3′ and 5′-GTGGTCGTTGAGGGCAATG-3′), respectively. The putative sizes of the PCR products were 242-bp for NS3 and 89-bp for GAPDH. The PCR products were separated on 2% agarose gel.

RESULTS

RNA pol I-driven plasmid vector

First, we constructed an RNA pol I-driven plasmid coding an HCV replicon in which structural coding genes were replaced by the luciferase gene (Figure 1A). To investigate the expression of the HCV replicon from the RNA pol I plasmid vector, we transfected the plasmid vector into Huh7 cells. As shown in Figure 1B, the luciferase activity was observed in the RNA pol I vector-transfected cells. IFN is the most popular agent used to inhibit HCV replication. To examine whether the RNA pol I plasmid vector functions as an assay system for anti-HCV activity, we investigated the effect of IFN on the expression of the HCV replicon in the RNA pol I plasmid-transfected Huh7 cells. IFN dose-dependently reduced the replication of the HCV genome (Figure 1C), reaching 29.2% of the control at 5 pg/ml. IFN treatment did not cause any cytotoxicity (Figure 1D). These data suggest that the RNA pol I plasmid coding the HCV replicon works as an assay system for HCV replication.

RNA pol I-driven Ad5 vector

The Ad vector is the most efficient gene transfer vector for a variety of mammalian cells *in vitro* and *in vivo* (13,14,19,20). There are more than 51 serotypes of Ad. The Ad type 5 (Ad5) vector has been frequently used in basic research and clinical studies (21). Ad5 vectors are 100- and 1000-fold more efficient at mediating gene transduction than cationic lipids, an effective non-viral vector (22). A reverse genetics system for the generation of influenza virus using RNA pol I-driven Ad5 vector produced 1000-fold the virus titer of the RNA pol I plasmid system (15). These findings indicate that the Ad5 vector may have advantages for the preparation of an HCV replicon system. We prepared RNA pol I-driven Ad5 vectors and confirmed the expression of a reporter gene from the Ad5 vectors coding luciferase (Supplementary Figure S1). However, we did not succeed in preparing Ad5 vector particles coding the HCV replicon. Indeed, there have been no previous reports of the preparation of Ad5 vector expressing the HCV RNA genome.

We think that two problems must be solved in order to develop Ad5 vectors coding the HCV RNA genome. These problems are the influence of the HCV replicon on the preparation of Ad5 particles and the packaging limit of Ad5 vectors.

Preparation of the TRE/RNA pol I chimeric promoter

The tet-regulated system comprises a regulator vector that expresses tet-controlled *trans*-activators and a response vector consisting of TRE within the promoter that controls expression of the gene of interest. The tet-controlled *trans*-activators are classified into tTA and rtTA that binds to the TRE promoter and activates expression from the TRE promoter in the absence and presence of Dox, respectively (23,24). We speculated that a tet-regulated vector system would minimize the influence of the HCV replicon on the preparation of Ad vector

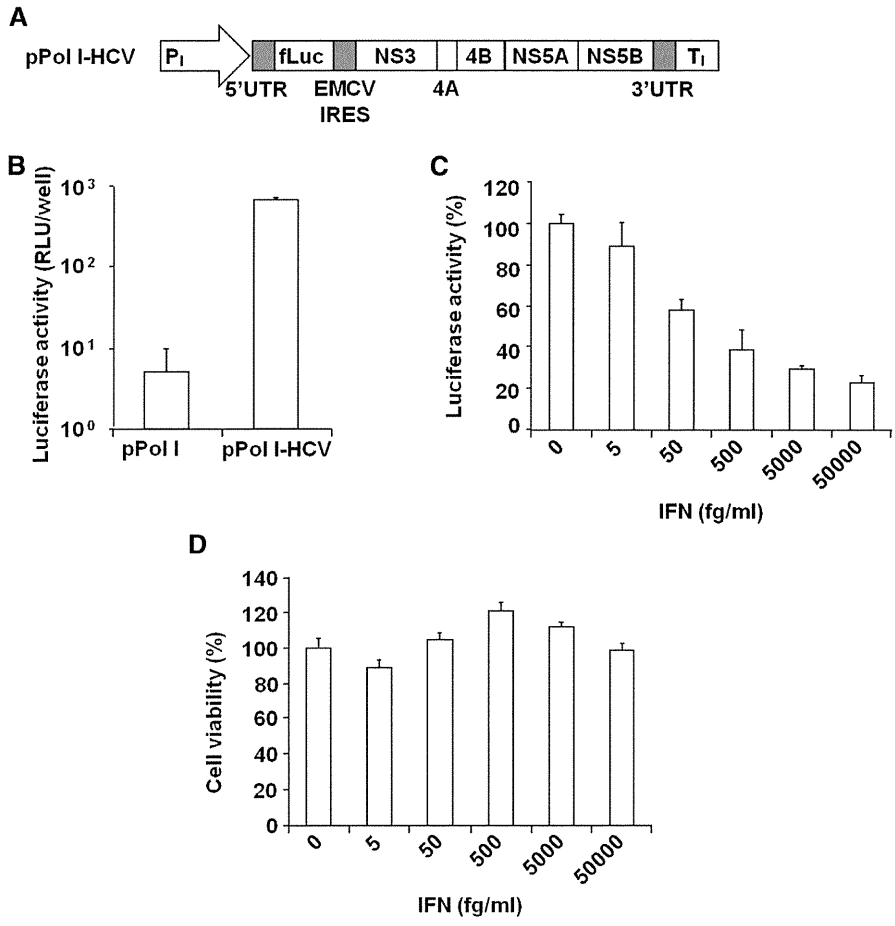

Figure 1. Preparation of plasmid expressing HCV replicon driven by RNA pol I promoter. (**A**) Schematic construct of HCV replicon-expression cassette. The HCV replicon gene was driven by the RNA pol I promoter (P_I) and terminator (T_I). (**B**) Transgene expression in Huh7 cells. Cells were transfected with pPol I-HCV. After 24 h of transfection, the luciferase activities were measured. Data are mean ± SD (n = 3). (**C** and **D**) Effect of IFN on HCV replication in RNA pol I vector-transfected cells. Huh7 cells were transfected with pPol I-HCV. After 2.5 h of transfection, the cells were treated with IFN at the indicated concentration. After an additional 72 h of incubation, the luciferase activity (C) and the cell viability (D) were measured. The luciferase activity (%) was calculated as a percentage of that in the vehicle-treated cells. Data are mean ± SD (n = 3).

particles. First, we optimized the chimeric promoter of TRE and the RNA pol I promoter. As shown in Figure 2A, the RNA pol I promoter is a 412-bp fragment containing an upstream control element (UCE) and the binding site of a transcription factor (Core). We constructed three chimeric promoter-driven plasmid vectors and checked the expression profiles using luciferase as a reporter gene. The chimeric vector was co-transfected into Huh7 cells with response vectors coding tTA or rtTA (23,24). As shown in Figure 2B and C, co-transfection with tTA exhibited a higher expression level than that of rtTA. The P_I235 promoter had the lowest luciferase expression in the absence of response vectors (Supplementary Figure S2). We used tTA and the P_I235 promoter in further studies. To investigate whether the chimeric RNA pol I promoter works in the Ad vector, we prepared Ad5 vector coding the chimeric RNA pol I-driven luciferase gene. AdP_I235-EL (MOI of 5) was co-transduced with Ad-tTA at MOI of 10 and 50. As shown in Figure 2D, the luciferase expression was increased in an Ad-tTA concentration-dependent manner.

Expression of the HCV replicon from Ad vector

The packaging limit of a foreign gene in the conventional Ad5 vector has been estimated to be 8.1–8.2-kb (25). The HCV replicon is ~8.9-kb and contains a 1.7-kb firefly luciferase gene and sequence derived from the HCV genome. Thus, another reason for no previous reports regarding the preparation of Ad5 vector coding the HCV replicon appears to be the packaging limit of the Ad5 vector. Mizuguchi and Hayakawa found that Ad5/35 vector containing chimeric fibers of Ad5 and Ad35 increased the size limit of foreign genes to 8.8-kb (26). We were successful in preparing Ad5/35 vector particles (9.53×10^8 IFU/ml) coding the TRE/RNA pol I chimeric promoter-driven HCV replicon containing the 1.0-kb renilla luciferase gene and sequence derived from the HCV genome (Figure 3A). To investigate the expression of the HCV replicon, Huh7 cells were transfected with the Ad vector coding the HCV replicon and Ad-tTA at MOI of 10 and 50, respectively. As shown in Figure 3B, western blot analysis showed that NS5A was expressed in Huh7 cells transfected with the vectors in the absence of Dox.

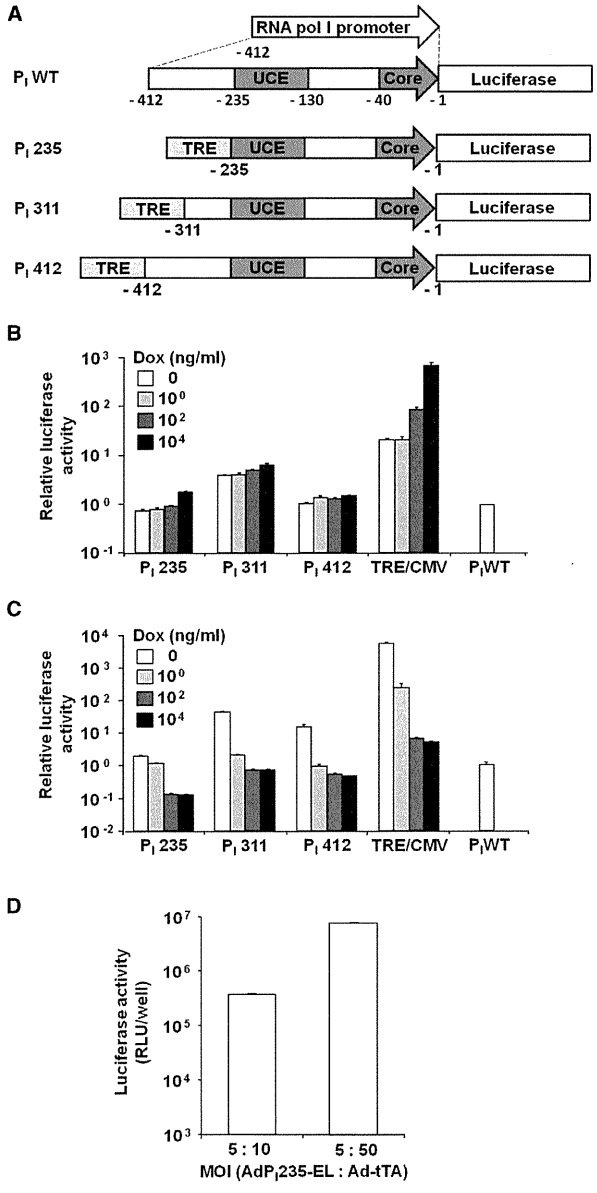

Figure 2. Development of tet-controllable RNA pol I promoter. (**A**) Construct of the chimeric RNA pol I promoter. The 412-bp human RNA pol I promoter contains Core (from −40 to −1), the binding site of the transcription factor, and UCE (from −235 to −130). TRE is connected to the full or partial RNA pol I promoter at the indicated sites, resulting in P_I235, P_I311 and P_I412. (**B** and **C**) Promoter activities of the chimeric promoter in Huh7 cells. Huh7 cells were co-transfected with the chimeric RNA pol I plasmid coding EGFPLuc, pCMVβ and *trans*-activator plasmid [rtTA (B) or tTA (C)]. After 2.5 h of transfection, the cells were treated with Dox at the indicated dose. After an additional 48 h of incubation, the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized by the β-galactosidase activity and expressed relative to that of pP$_I$WT-EL-transfected cells. Data are mean ± SD (n = 3). (**D**) Transgene activity of Ad vector coding the chimeric promoter construct. Huh7 cells were co-infected with AdP$_I$235-EL and Ad-tTA. The MOI ratio of AdP$_I$235-EL to Ad-tTA was 5:10 or 5:50. After an additional 48 h of incubation, the luciferase activity was measured. Data are the mean ± SD (n = 3).

Luciferase was also expressed (Figure 3C). Dox dose-dependently attenuated expression of luciferase (Supplementary Figure S3). To discriminate between translation of the RNA pol I-transribed HCV RNA derived from the vector DNA and translation of HCV RNA derived from autonomous HCV replication in the transcribed cells, we prepared replication-incompetent HCV replicon deleting GDD motif in NS5B. Luciferase expression was attenuated in the cells transfected with the GDD-deleted Ad vector (AdP$_I$235-ΔGDD) (Figure 3D). A fragment of the HCV negative strand RNA, an essential replication intermediate, amplified by RT-PCR has been detected in the cells transfected with AdP$_I$235-HCV but not AdP$_I$235-ΔGDD (Figure 3E). Autonomous replication of the HCV RNA may occur in this system. To evaluate whether the Ad vector systems could be used to evaluate inhibitors of HCV replication, we investigated the effect of IFN on luciferase expression from HCV replicon in the Ad vector. As shown in Figure 3F, treatment of cells with 5 pg/ml of IFN reduced luciferase expression (33.3% of vehicle-treated cells). Cell viability was not affected by IFN treatment (Figure 3G). These findings indicate that the tet-controllable RNA pol I Ad vector may be useful for evaluation of anti-HCV activity.

DISCUSSION

HCV is an RNA virus containing the positive strand of a 9.6-kb RNA genome. A technique to transfer all or part of the HCV RNA genome to cells could be widely applicable for basic studies on HCV and pharmaceutical therapy against HCV. However, efficient and convenient methods to transduce the HCV RNA genome have never been fully developed. Electroporation of *in vitro* translated HCV RNA genome into cells is the most popular method. In the present study, we used a tet-controllable expression system to successfully develop an Ad vector system expressing the HCV RNA genome.

To our knowledge, development of Ad vector expressing HCV subgenome or genome has never been succeeded. The NS3 protease is essential for processing most of the NS proteins from the HCV polyprotein (27–30). The cleavage site of the NS3 protease is estimated to be between the P1 and P1′ position of an acidic amino acid at the P6 position, a Cys or Thr residue at the P1 position, and a Ser or Ala residue at the P1′ position (31). E1A, pIIIa, pol and V proteins of Ad have the cleavage site of the NS3 protease. The lack of previous success in generating Ad vectors coding the HCV genome and subgenome might be partly due to the degradation of Ad components by the NS3 protease during the preparation of Ad particles. In the tet-regulated system, when Ad vectors coding foreign genes driven by the TRE hybrid promoter are co-transfected with tTA or rtTA vector, the foreign gene can be expressed. Expression of the foreign gene could be suppressed during amplification of Ad vector particles in 293 cells, resulting in the preparation of Ad vector particles. The critical factor in the HCV replicon must be determined in a future study.