

## Development of hepatitis C virus production reporter-assay systems using two different hepatoma cell lines

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A hepatitis C virus (HCV) infection system was developed previously using the HCV JFH-1 strain (genotype 2a) and HuH-7 cells, and this cell culture is so far the only robust production system for HCV. In patients with chronic hepatitis C, the virological effects of pegylated interferon and ribavirin therapy differ depending on the HCV strain and the genetic background of the host. Recently, we reported the hepatoma-derived Li23 cell line, in which the JFH-1 life cycle is reproduced at a level almost equal to that in HuH-7-derived RSc cells. To monitor the HCV life cycle more easily, we here developed JFH-1 reporter-assay systems using both HuH-7- and Li23-derived cell lines. To identify any genetic mutations by long-term cell culture, HCV RNAs in HuH-7 cells were amplified 130 days after infection and subjected to sequence analysis to find adaptive mutation(s) for robust virus replication. We identified two mutations, H2505Q and V2995L, in the NS5B region. V2995L but not H2505Q enhanced JFH-1 RNA replication. However, we found that H2505Q but not V2995L enhanced HCV RNA replication of strain O (genotype 1b). We also selected highly permissive D7 cells by serial subcloning of Li23 cells. The expression levels of claudin-1 and Niemann–Pick C1-like 1 in D7 cells are higher than those in parental Li23 cells. In this study, we developed HCV JFH-1 reporter-assay systems using two distinct hepatoma cell lines, HuH-7 and Li23. The mutations in NS5B resulted in different effects on strains O and JFH-1 HCV RNA replication.

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## INTRODUCTION

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis and leads to liver cirrhosis and hepatocellular carcinoma. Elimination of HCV by antiviral reagents seems to be the most efficient therapy to prevent fatality.

HCV belongs to the family *Flaviviridae* and contains a positive ssRNA genome of 9.6 kb. The HCV genome encodes a single polyprotein precursor of approximately 3000 aa, which is cleaved by host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Kato, 2001; Kato *et al.*, 1990; Tanaka *et al.*, 1996).

Evaluation of anti-HCV reagents was difficult before the development of the HCV replicon system (Lohmann *et al.*, 1999). The HCV replicon system enabled investigation of anti-HCV reagents and the cellular factors involved in HCV RNA replication. Following introduction of the replicon system, genome-length HCV RNA-replication systems and reporter-assay systems were also developed (Ikeda *et al.*, 2002, 2005; Lohmann *et al.*, 2001; Pietschmann *et al.*, 2002). In 2005, an HCV infection system was developed using the genotype 2a JFH-1 strain (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The JFH-1 infection system has been used to study not only viral RNA replication, but also virus infection and release. This HCV cell-culture system was developed using the human hepatoma cell line HuH-7 and, thus far, HuH-7 is the only cell line to exhibit robust HCV production. Therefore, we intended to test the susceptibility of various other cell lines to HCV RNA replication. We reported previously that the hepatoma cell line Li23 supports robust HCV RNA replication and is also susceptible to authentic JFH-1 infection (Kato *et al.*, 2009). Microarray analysis

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## Raloxifene inhibits hepatitis C virus infection and replication

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### ABSTRACT

**Postmenopausal women with chronic hepatitis C exhibited a poor response to interferon (IFN) therapy compared to premenopausal women. Osteoporosis is the typical complication that occurs in postmenopausal women. Recently, it was reported that an osteoporotic reagent, vitamin D3, exhibited anti-hepatitis C virus (HCV) activity. Therefore, we investigated whether or not another osteoporotic reagent, raloxifene, would exhibit anti-HCV activity in cell culture systems. Here, we demonstrated that raloxifene inhibited HCV RNA replication in genotype 1b and infection in genotype 2a. Raloxifene enhanced the anti-HCV activity of IFN- $\alpha$ . These results suggest a link between the molecular biology of osteoporosis and the HCV life cycle.**

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### 1. Introduction

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family and contains a positive single-stranded RNA genome of 9.6 kb. The HCV genome encodes a single polyprotein precursor of approximately 3000 amino acid residues, which is cleaved by the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1–3].

The virological study and screening of antiviral reagents for HCV was difficult until the replicon system was developed [4–7]. In 2005, an infectious HCV production system was developed using genotype 2a HCV JFH-1 and hepatoma-derived HuH-7 cells, and the HCV life cycle was reproduced in a cell culture system [8]. We previously developed genome-length HCV reporter assay systems using HuH-7-derived OR6 cells [4]. In OR6 cells, the genotype 1b HCV-0 with renilla luciferase (RL) replicates robustly. We also developed an HCV JFH-1 reporter infection assay system [9].

HCV infection frequently causes chronic hepatitis (CH) and leads to serious liver cirrhosis and hepatocellular carcinoma. Therefore, HCV infection is a major health problem worldwide. The elimination of HCV by antiviral reagents seems to be the most efficient therapy for preventing the fatal state of the disease. PEGylated-interferon (PEG-IFN) with ribavirin (RBV) is the current standard therapy for CH-C,

but its sustained virological response (SVR) rate has remained 40–50%. Recently, a protease inhibitor, telaprevir, improved the SVR rate by up to 60–70% in combination with PEG-IFN/RBV [10]. The response to PEG-IFN/RBV therapy depends on host factors as well as viral factors. Among the host factors, age and gender are known to be associated with the outcome of IFN/RBV therapy [11,12]. Postmenopausal women with CH-C exhibited a poor response to IFN therapy compared to premenopausal women [11]. The decrease in estrogen may affect the response to IFN therapy. Dyslipidemia and osteoporosis are the typical complications in postmenopausal women. We and other groups reported that statins, which are dyslipidemia reagents, inhibited HCV proliferation in vitro and in vivo [13–17]. Recently it was reported that vitamin D3, an osteoporotic reagent, exhibited anti-HCV activity in vitro and in vivo [18–21]. It was also reported that 17 $\beta$ -estradiol inhibited the production of infectious HCV [22]. Taken together, these reports suggest an association between hepatitis C and complications due to the decrease of estrogen.

Raloxifene and tamoxifen are synthetic selective estrogen receptor modulators (SERMs) and are used for breast cancer and osteoporosis, respectively, in clinical settings. The responses of SERMs are mediated by estrogen receptors (ERs), either ER $\alpha$  or ER $\beta$ . SERMs exhibit agonistic actions in some tissues but antagonistic actions in others. Both raloxifene and tamoxifen are antagonists in breast and agonists in bone. However, only tamoxifen, and not raloxifene, exhibited agonistic activity in the uterus. It was reported that tamoxifen inhibited HCV RNA replication [23]. However, tamoxifen's agonist action leads to uterine cancer. Raloxifene belongs to an antiosteoporotic reagent and offers the advantage of safety without uterine cancer. Therefore, we decided to investigate whether or not raloxifene would exhibit anti-HCV activity in our developed cell culture systems.

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# For the future studies of Kaposi's sarcoma-associated herpesvirus

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It is 18 years since Kaposi's sarcoma-associated virus (KSHV), also called human herpesvirus 8 (HHV-8), was found from Kaposi's sarcoma (KS) by Chang et al. (1994). More than 8,000 reports have been published so far and we have learned many things from this virus. I would like to say it is about time to look back previous studies and to think what to study next on the virus, and planed a topic to think what to study next on the virus for future.

Herpesviruses have relatively big genomes and encode a 100 genes or so. Thus, the virion assembly/structure, gene expression regulation and attachment/entry are complicated and have known only an iceberg of them. Studying the details how the viruses run their life cycles and cause diseases in their processes will lead to exploring new therapeutic drugs/methods.

A viral life cycle starts from attachment on the susceptible cells and then, entry into the cells, followed by the viral gene expression, the genome replication, the particle assembly and finally the daughter viruses egress out of the cells. This process is skillfully built and all the viral genes are required for the process, though there are essential genes and non-essential ones. Viral pathogenesis could be established during this process by the interaction between viruses and host cells, and individual host systems such as immune system. In this topic, although I would like to cover all the processes, thankfully, 15 specialists in each field have contributed for this topic.

Polizzotto et al. (2012) described clinical manifestations of KSHV-associated diseases. So far, there were few reports on clinical manifestations of primary KHSV infection. In this term, KHSV inflammatory cytokine syndrome (KICS) is a new concept and we might have been looked over an important disease sign on KHSV infection. We will have to be more careful about what happens in primary KHSV infection than before.

Fukumoto et al. (2011) describe KSHV infection from a pathologist's points of view. Pathologic study is very important to know what happens in the lesions. Currently, we are able to know what is going on only in the KSHV associated lesions such as Kaposi's sarcoma, multicentric Castleman's disease and primary effusion lymphomas (PEL) of human samples suffered from KSHV infection, but once an infection model is established, chronological pathologic studies will provide a lot of information on how KSHV-associated diseases are formed.

Chakraborty et al. (2012) review the entry mechanism of KSHV into cells. In general, herpesviruses can infect various kinds of cells *in vitro* including non-human cell lines, but the infectivity to B lymphocyte originated cells is very inefficient. Their report will give us a hint why such phenomenon happens.

An immediate early gene, *RTA* (reactivation and transcription activator) is very important for the viral lytic replication induction and shows multifunctions. We still have not understood how the factor functions. Guito and Lukac (2012) and Tsai et al. (2012) review or report mechanistic regulation of this strong transactivator, respectively.

Jackson et al. (2012) describe *ORF57*, which is also an interesting and multifunctional protein. This is involved in post-translational processes of the viral gene expression as sumoylation and ubiquitination described by Campbell and Izumiya (2012) and Ashizawa et al. (2012) respectively. We had believed that *K-bZIP*, a homolog of Epstein-Barr virus *Zta* was a transactivator and origin recognition factor in the lytic replication. *K-bZIP*, however, has other important roles for KSHV lytic replication. In latency, metabolism of *LANA* (latency-associated nuclear antigen) could be critical for KSHV-induced tumor formation and/or its phenotype.

Viral particle assembly is virologically an exciting and interesting field. There have been few reports on this, Sathish et al. (2012) try to search this issue.

The detail replication mechanism of KSHV in both lytic and latent phase has been still unclear. In latency, the virus is supposed to utilize host replication machinery including pre-replication complexes (pre-RC) for the viral replication initiation in the presence of *LANA*. The viral factor, *LANA*, is an essential factor, but its necessity has not been elucidated well. *LANA* binds with *LANA*-binding sites (LBS) and recruits origin recognition complexes (ORCs) on the viral replication origin (ori-P), which cannot account for necessity of the GC-rich element followed by LBS. Ohsaki and Ueda (2012) will give us a hint about this question.

Viral immune evasion system is very tactic to maintain its latency in case of herpesviruses. The maintenance of latency is then critical for the virus to wait for reactivation to produce daughter viruses, whose transition may a step for the viral oncogenic process. Lee et al. (2012) summarize KSHV immune evasion strategy and make a comment on the future landscape.

Kaposi's sarcoma-associated virus mediated tumorigenesis including PEL and KS has been still unclear, though there are many reports on individual viral putative oncogenes. KSHV has not been reported to infect and immortalize and/or transform endothelial cells or peripheral blood mononuclear cells *in vitro*. And thus, we have not known how the viral genes with oncogenic potentials such as *vFLIP*, *vCYC*, *vGPCR* and so on in addition to *LANA* cooperate in the viral oncogenic process. DiMaio and Lagunoff (2012) address on this issue and look forward for this field.



## Kaposi's Sarcoma-Associated Herpesvirus Induced Tumorigenesis; How Viral Oncogenic Insults are Evaded

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Some viral infections in human are strongly related to cancer formation. Apart from retrovirus induced cancer formation seen in rodents and avian, virus induced cancer formation in human seems to be very complicated. In human, mainly DNA viruses such as papillomavirus, hepatitis B virus (HBV), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV) are etiological agents and some RNA viruses such as human T-cell leukemia virus and hepatitis C virus (HCV) are involved in their specific cancer formation. It takes long time for the viruses to cause cancers and we do not have good systems to observe how the viral infection leads to cancer formation.

KSHV is belonging to gamma-herpesviridae and an agent involved in the formation of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD).

The virus infection has a very strong link with these cancers. The mechanism how the virus causes such cancers is, however, still enigmatic and remains to be elucidated. KSHV latent infection should be important in terms that this type of infection provides with an origin of the related cancers. But, many genes with oncogenic activity of this virus are lytic genes, which are expressed only in the lytic phase.

As mentioned above, virus induced carcinogenesis is very complicated and is attractive to take an insight how the virus causes related cancers [1].

KSHV expresses an extremely limited number of viral genes such as latency-associated nuclear antigen (LANA), viral cyclin (v-cyc), viral FLICE inhibitory protein (vFLIP), kaposin and viral interferon regulatory factor-3 (vIRF-3) and 17 viral microRNAs in latency. The genes build an active gene locus in the KSHV genome in latency.

Among them, v-CYC, a homolog of cellular D-type cyclins, functions as an oncogene to deregulate cellular proliferation which leads to DNA damage response (DDR) and p53 induced apoptosis. Normal cells respond to oncogenic insults and cannot be easily transformed by choosing suicide pathway through p53 [2]. If the virus

survives this situation, there must be a mechanism and this is one of ways how KSHV causes cancers.

In this point a recent report from Leidal et al. [1] is attractive for an insight to link the v-CYC induced oncogenic insult with subversion of this activity by vFLIP and how the virus causes related cancers. They found that v-CYC caused autophagy induced senescence and/or apoptosis. On the other hand, vFLIP is known for an autophagy inhibitor as well as an NF- $\kappa$ B activator [3,4]. And thus, vFLIP functions to evade from v-CYC induced oncogenic insult/senescence and make a direction of KSHV induced carcinogenesis.

However, we should be careful whether such a pathway happens in the natural infection course, since this kind of experiment is usually performed in over-expression system. Actually, vFLIP expression at the protein level has not been confirmed even in KSHV infected PEL cell lines and thus it is unclear whether such vFLIP activity is seen in the native situation.

In summary, a report from Liang seems to be very important to explain how KSHV causes cancers by connecting oncogene (v-CYC in this case) induced apoptosis and/or senescence [5]. Although there is no related report about the other human virus induced carcinogenesis, similar mechanisms might be stealthing.

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## Inhibition of Hepatitis C Virus Replication and Viral Helicase by Ethyl Acetate Extract of the Marine Feather Star *Alloeocomatella polycladlia*

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# Add-on Therapy of Pitavastatin and Eicosapentaenoic Acid Improves Outcome of Peginterferon Plus Ribavirin Treatment for Chronic Hepatitis C

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Despite the use of pegylated-interferon (peg-IFN) plus ribavirin combination therapy, many patients infected with hepatitis C virus (HCV)-1b remain HCV-positive. To determine whether addition of pitavastatin and eicosapentaenoic acid (EPA) is beneficial, the “add-on” therapy option (add-on group) was compared retrospectively with unmodified peg-IFN/ribavirin therapy (standard group). Association of host- or virus-related factors with sustained virological response was assessed. In HCV replicon cells, the effects of pitavastatin and/or EPA on HCV replication and expression of innate-immunity- and lipid-metabolism-associated genes were investigated. In patients infected with HCV-1b, sustained virological response rates were significantly higher in the add-on than standard group. In both groups, sustained virological response rates were significantly higher in patients with genotype TT of IL-28B (rs8099917) than in those with non-TT genotype. Among the patients with non-TT genotype, sustained virological response rates were markedly higher in the add-on than standard group. By multivariate analysis, genome variation of IL28B but not add-on therapy remained as a predictive factor of sustained virological response. In replicon cells, pitavastatin and EPA suppressed HCV replication. Activation of innate immunity was obvious in pitavastatin-treated cells and EPA suppressed the expression of sterol regulatory element binding protein-1c and low-density lipoprotein

receptor. Addition of pitavastatin and EPA to peg-IFN/ribavirin treatment improved sustained virological response in patients infected with HCV-1b. Genotype variation of IL-28B is a strong predictive factor in add-on therapy.

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Abbreviations: EPA, eicosapentaenoic acid; HCV, hepatitis C virus; HMGCR, HMG-CoA reductase; IRF3, IFN regulatory factor 3; ISG15, IFN-stimulated gene 15; ITPA, inosine triphosphatase; LDLR, low-density lipoprotein receptor; MAVS, mitochondrial antiviral signaling; NPC1L1, Niemann-Pick C1 like 1; OR, odds ratio; PCR, polymerase chain reaction; peg-IFN, pegylated-interferon; PUFA, polyunsaturated fatty acid; RIG-I, retinoic acid inducible gene I; SNP, single nucleotide polymorphism; SREBP, sterol regulatory element binding protein; TRAF6, TNF receptor associated factor 6.

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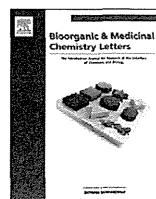
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## 2'-Fluoro-6'-methylene-carbocyclic adenosine phosphoramidate (FMCAP) prodrug: In vitro anti-HBV activity against the lamivudine–entecavir resistant triple mutant and its mechanism of action

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Drug-resistant mutants

### ABSTRACT

Novel 2'-fluoro-6'-methylene-carbocyclic adenosine (FMCA) monophosphate prodrug (FMCAP) was synthesized and evaluated for its in vitro anti-HBV potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). FMCA demonstrated significant antiviral activity against wild-type as well as lamivudine–entecavir resistant triple mutant (L180M + M204V + S202G). The monophosphate prodrug (FMCAP) demonstrated greater than 12-fold (12×) increase in anti-HBV activity without increased cellular toxicity. Mitochondrial and cellular toxicity studies of FMCA indicated that there is no significant toxicity up to 100 μM. Mode of action studies by molecular modeling indicate that the 2'-fluoro moiety by hydrogen bond as well as the Van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug-resistant mutants.

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The chronic HBV infection is strongly associated with liver diseases like chronic hepatic insufficiency, cirrhosis and hepatocellular carcinoma (HCC).<sup>1</sup> According to the World Health Organization (WHO), currently about 2 billion people world-wide have been infected with HBV and more than 350 million live with chronic infection. Acute or chronic outcomes of HBV infection are estimated to cause the deaths of 600,000 people worldwide every year.<sup>2</sup>

Currently, there are several nucleos(t)ide analogues available to treat chronic hepatitis B virus infection.<sup>3–6</sup> The major target of these drugs is to inhibit the viral reverse transcriptase (RT)/DNA polymerase, which is responsible for the synthesis of the minus-strand DNA. Although the currently used agents are well tolerated and effective in suppressing the viral replication for extended periods, the significant rate of virological relapse caused by drug resistance remains a critical issue.

Lamivudine (LVD) was first introduced as the orally active anti-HBV agent in 1998. Lamivudine profoundly suppresses HBV replication in patients with chronic hepatitis B infection; however, lamivudine-resistant HBV (LVD<sup>r</sup>) was isolated from a significant numbers of patients during the treatment with lamivudine.

Currently, there are several antiviral options exist for these patients viz., to use adefovir or high dose (1.0 mg/day) of entecavir, or more recently tenofovir. However, this resulted in also the development of resistance mutants during the long term therapy. At present, entecavir is the most prescribed drug, and is recommended for patients with the wild-type as well as for those harboring adefovir and lamivudine-resistant strains. However, recent clinical studies by Tanaka and his co-workers suggested that the entecavir mutant in the lamivudine-resistant patients (L180M + M204V + S202G) causes a viral breakthrough: 4.9% of patients at baseline increases to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.<sup>7</sup> Therefore, it is of great interest to discover novel anti-HBV agent, which is effective against lamivudine- and entecavir-resistant triple mutants (L180M + M204V + S202G).

The potency of a nucleos(t)ide analogue is determined by its ability to serve as a competitive inhibitor of the HBV polymerase relative to that of the natural substrate, the nucleotide triphosphate.<sup>8</sup> However, host cellular kinases limit the pharmacological potency of nucleoside analogues by phosphorylation to their corresponding triphosphates. Particularly, the initial kinase action on the nucleoside to the monophosphate is the rate-limiting step. However, many synthetic nucleosides are not phosphorylated or the rate of phosphorylation is very slow due to the structural requirement of the kinases, resulting in only generating a low quantity of the triphosphate. To overcome this phosphorylation issue, nucleoside phosphoramidate prodrugs have been introduced,<sup>8,9</sup> which

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## Replication of Hepatitis C Virus Genotype 3a in Cultured Cells

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see editorial on page 13.

**Hepatitis C virus (HCV) genotype 3a is widespread worldwide, but no replication system exists for its study. We describe a subgenomic replicon system for HCV genotype 3a. We determined the consensus sequence of an HCV genome isolated from a patient, and constructed a subgenomic replicon using this clone. The replicon was transfected into HuH-7 cells and RNA replication was confirmed. We identified cell culture-adaptive mutations that increased colony formation multiple-fold. We have therefore established a genotype 3a replicon system that can be used to study this HCV genotype.**

**Keywords:** Virology; Experimental Model; HCVGT3; In Vitro Culture System.

Hepatitis C virus (HCV) infection leads to chronic infection and advanced liver diseases in most infected adults.<sup>1</sup> Of the 6 major HCV genotypes, genotypes 1 and 2 are the most prevalent in North America, Europe, and Japan,<sup>2,3</sup> and are the most highly studied. However, other genotypes display specific characteristics. For example, genotype 3a infection can result in hepatic steatosis<sup>4</sup> and telaprevir and boceprevir are less effective against genotype 3a.<sup>5</sup> Therefore, the pathogenesis and inhibitor sensitivity of all HCV genotypes should be studied. Although HCV subgenomic replicons are useful for understanding viral/host factors involved in HCV replication and inhibitor sensitivity, only HCV replicons for genotypes 1a, 1b, and 2a have been established.<sup>6–9</sup> Here, we report on the robust genotype 3a replication system.

An almost complete HCV genome was recovered from the serum of a patient with post-transplantation recurrent HCV infection. This serum exhibited higher infectivity than other tested sera toward primary human hepatocytes (Supplementary Figure 1A). The isolate, named S310, contained the following structural elements: a 5'UTR (nt 1-339), an open reading frame encoding 3021 aa (nt 340-9402), and a 3'UTR (nt 9403-9654). Only the last 44 nt of the X-region (nt 9611-9654) could not be recovered. Two major virus populations were found; S310/A contained Ala, Thr, Thr, and Ile, and S310/B

contained Thr, Ala, Ala, and Thr, at the 7<sup>th</sup>, 151<sup>st</sup>, 431<sup>st</sup>, and 472<sup>nd</sup> aa of the NS3 protein, respectively. S310 was clustered into genotype 3a by phylogenetic analysis (Supplementary Figure 1B). The complexity of the virus quasi-species in the serum was analyzed by sequencing the hypervariable region. Identical amino acid sequences in all 10 hypervariable region clones indicated a very low degree of diversity. The hypervariable region sequence of the JFH-1 strain also exhibited monoclonality,<sup>10</sup> which can be important for efficient replication in cultured cells.

Subgenomic replicons SGR-S310/A and SGR-S310/B were constructed and their replication efficiency was evaluated by G418-resistant colony-formation assay. After 3 weeks, a small number of colonies were visible for both replicons (Figure 1A). Because more colonies were observed in SGR-S310/A than in SGR-S310/B, we focused on SGR-S310/A (henceforth called SGR-S310). Ten cell colonies of SGR-S310 were isolated and analyzed for HCV replication. The mean RNA titer was  $9.1 \times 10^7 \pm 4.6 \times 10^7$  copies/ $\mu$ g total RNA (Figure 1B). HCV RNA (approximately 8 kb) was detected by Northern blotting (Supplementary Figure 2A). Viral proteins in the replicon cells were detected by immunofluorescence and Western blotting (Supplementary Figure 2B and 2C). To determine whether the G418 resistance of the cells was transmissible by cellular RNA transfection, we electroporated total cellular RNA isolated from 4 replicon clones into naïve HuH-7 cells. Multiple G418-resistant colonies appeared after transfection of the RNA isolated from the replicon clones (Supplementary Figure 3A), but not from the naïve HuH-7 cells. These results indicate that the replicon RNA in the parental colonies could replicate in naïve cells. Thus, the G418-resistant colonies that were isolated from cells electroporated with SGR-S310 synthetic RNA contained replicating viral RNA.

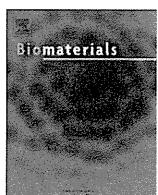
Replicating genomes have been shown to accumulate cell culture adaptive mutations, which increase their replication potential. To examine whether SGR-S310 acquired mutations, the complete HCV sequences from 10 replicon clones were sequenced. At least one nonsynonymous mutation was detected in the NS3-NS5B region of each replicon clone (Figure 1B). The following mutations were identified: T1286I in the NS3 helicase (6 of 10

**Abbreviation used in this paper:** HCV, hepatitis C virus.

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## 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing

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### ABSTRACT

Although it is expected that hepatocyte-like cells differentiated from human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells will be utilized in drug toxicity testing, the actual applicability of hepatocyte-like cells in this context has not been well examined so far. To generate mature hepatocyte-like cells that would be applicable for drug toxicity testing, we established a hepatocyte differentiation method that employs not only stage-specific transient overexpression of hepatocyte-related transcription factors but also a three-dimensional spheroid culture system using a Nanopillar Plate. We succeeded in establishing protocol that could generate more matured hepatocyte-like cells than our previous protocol. In addition, our hepatocyte-like cells could sensitively predict drug-induced hepatotoxicity, including reactive metabolite-mediated toxicity. In conclusion, our hepatocyte-like cells differentiated from human ES cells or iPS cells have potential to be applied in drug toxicity testing.

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## 1. Introduction

Hepatocyte-like cells that are generated from human embryonic stem cells (hESCs) [1] or human induced pluripotent stem cells (hiPSCs) [2] are expected to be used in drug screening instead of primary (or cryopreserved) human hepatocytes (PHs). We recently demonstrated that stage-specific transient transduction of transcription factors, in addition to treatment with optimal growth factors and cytokines, is useful for promoting hepatic differentiation [3–6]. The hepatocyte-like cells, which have many hepatocyte characteristics (the abilities to uptake low-density lipoprotein and Indocyanine green, store glycogen, and synthesize urea) and drug metabolism capacity, were generated from hESCs/hiPSCs by

combinational transduction of FOXA2 and HNF1 $\alpha$  [6]. However, further maturation of the hepatocyte-like cells is required because their hepatic characteristics, such as drug metabolism capacity, are lower than those of PHs [6].

To promote further maturation of the hepatocyte-like cells, we subjected them to three-dimensional (3D) spheroid cultures. It is known that various 3D culture conditions (such as Aligmatrix scaffolds [7], cell sheet technology [8], galactose-carrying substrata [9], and basement membrane substratum [10]) are useful for the maturation of the hepatocyte-like cells. Nanopillar Plate technology [11] used in the present study makes it easy to control the configuration of the spheroids. The Nanopillar Plate has an arrayed  $\mu$ m-scale hole structure at the bottom of each well, and nanopillars were aligned further at the bottom of the respective holes. The seeded cells evenly drop into the holes, then migrate and aggregate on top surface of the nanopillars, thus likely to form the uniform spheroids in each hole. Not only 3D spheroid cultures [12] but also Matrigel overlay cultures [13] are useful for maintaining the hepatocyte characteristics of PHs. Therefore, we employed both 3D

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