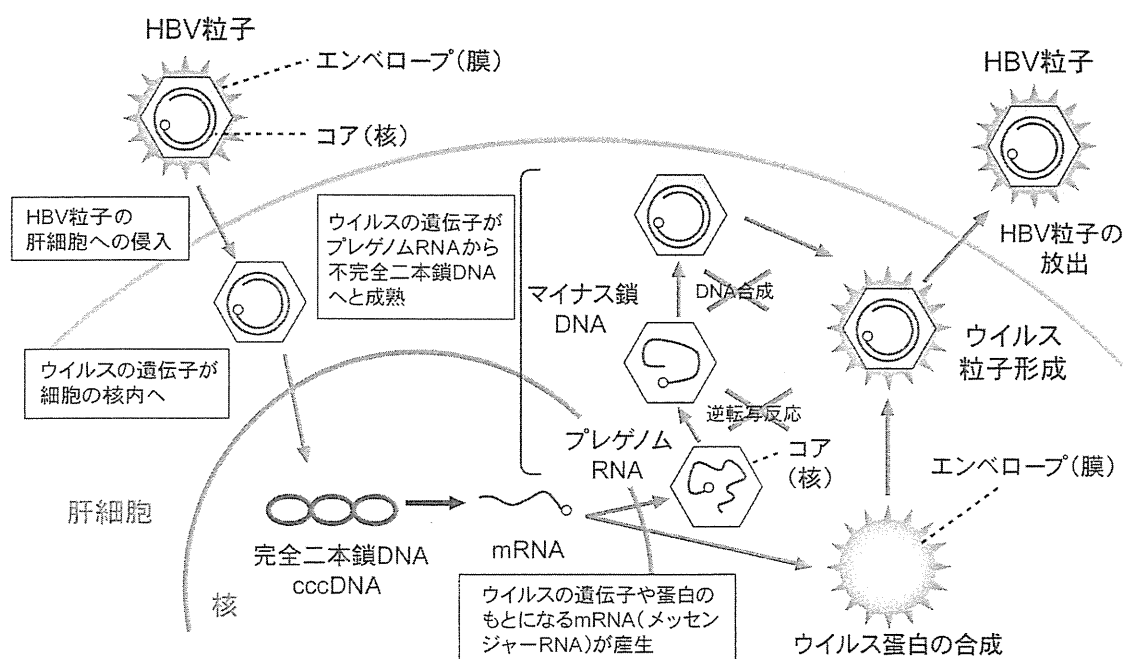


核酸アナログはどのようにして効くか？

核酸アナログとは、DNA や RNA に類似したもの（核酸＝遺伝子の構成成分，アナログ＝類似）と考えると分かりやすくなります。つまり、ウイルスの遺伝子の構成成分に似た物質を内服することによってウイルス遺伝子の複製を阻害し、ウイルスの増殖を抑えようとする物質です。HBV は、ヒトの肝細胞に感染すると、ウイルス遺伝子は核の中に運び込まれます。そこで、不完全二本鎖であったウイルスDNAは修復され完全二本鎖 DNA となり、さらに輪ゴムをよじったような形状の cccDNA となります。その後、この cccDNA 遺伝子を鋳型としてウイルスを構成する蛋白質やウイルス遺伝子が複製され、細胞内で粒子を形成しながら、放出されます。核酸アナログは、このウイルス増殖過程において、核酸と競合し、DNA 合成や逆転写反応といったウイルス遺伝子の形成に欠く事のできない過程を阻害し、HBV の増殖を強力に抑制します。



核酸アナログの作用機序

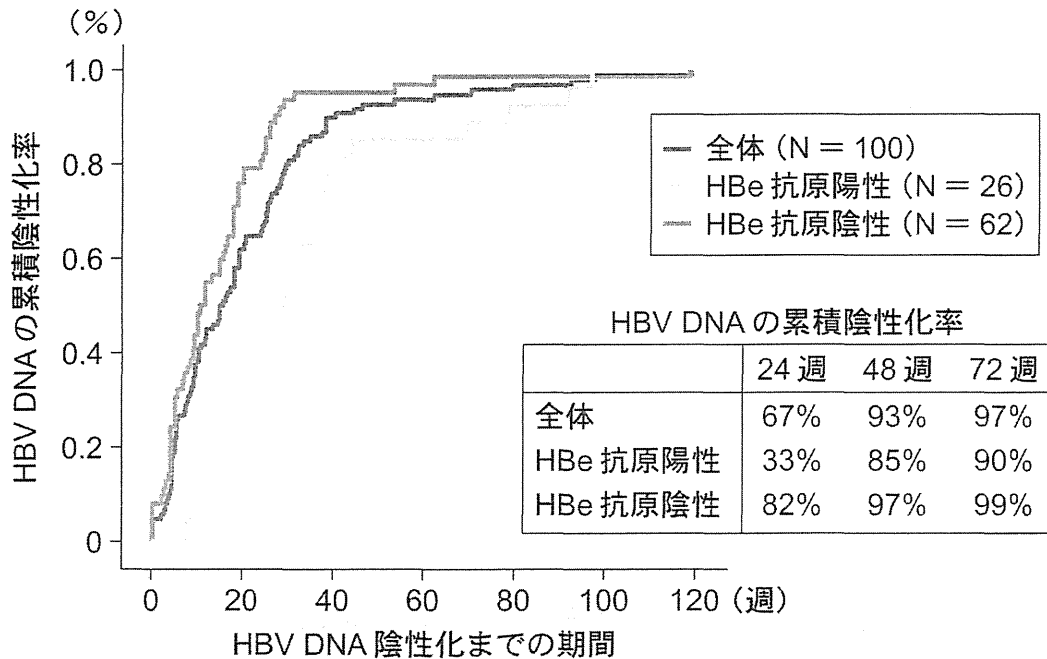
(筆者作成)

核酸アナログの治療効果は？

核酸アナログ治療では、HBV 増殖が強力に抑制されることから、血中に放出されるウイルスの量も急激に減少し、エンテカビルの内服を約 1 年間継続後には 90% 以上で、血液中の HBV DNA 量は陰性化 (< 2.6 log copies/mL) し、ALT も正常化

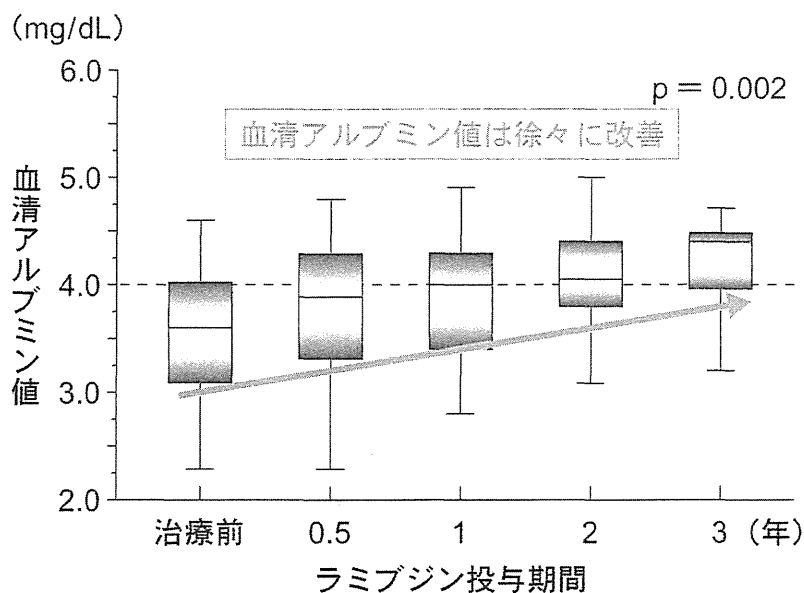
I. B型肝炎

します。緩徐ではありますが、長期間の核酸アナログ治療により、肝組織の線維化改善が期待され、肝硬変例では、アルブミン値の増加や腹水の減少が期待できます。ただし、核酸アナログ製剤は、ウイルスcccDNAを完全に肝臓から排除することは困難ですので、血液中のHBV DNAが陰性化したとしても肝臓からHBVが消えるわけではありません。



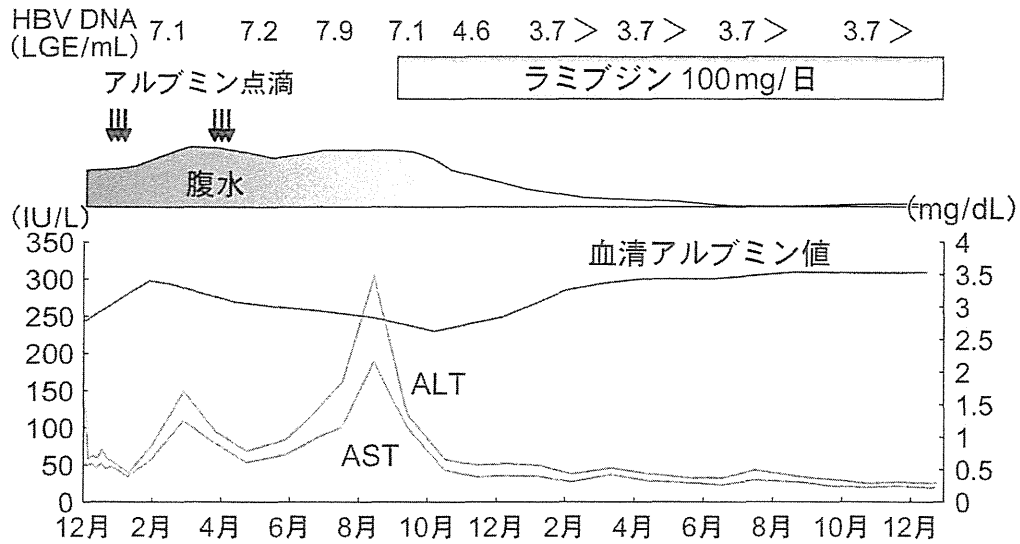
エンテカビル投与によるHBV DNAの累積陰性化率

(筆者作成)



肝硬変患者に対するラミブジン投与による血清アルブミン値の改善

(筆者作成)

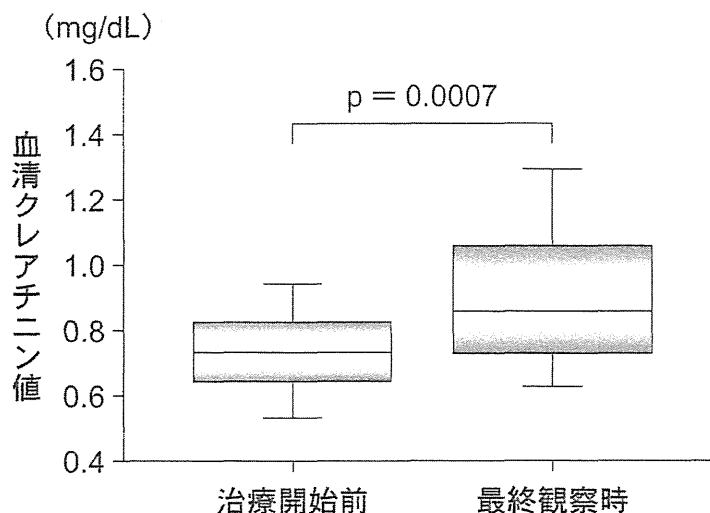


ラミブジン投与により腹水貯留が消失した肝硬変症例

55歳、男性。肝硬変に伴う腹水貯留とAST, ALTの上昇を認めます。ラミブジンの投与を行ったところ、AST, ALTは速やかに正常化し、HBV DNAも低下しました。血清アルブミンが改善するにつれ、腹水は消失し、アルブミンの点滴も不要となりました。
(筆者作成)

核酸アナログの副作用は？

核酸アナログ治療による主な副作用としては、頭痛、倦怠感、悪心、腹痛、鼻咽頭炎、下痢などが報告されていますが、軽度であることが多く、重篤な副作用は少ないとされています。ただし、アデホビルを使用する場合には、腎機能障害（血清クレアチニン値の上昇）が出現する場合があります、注意が必要です。



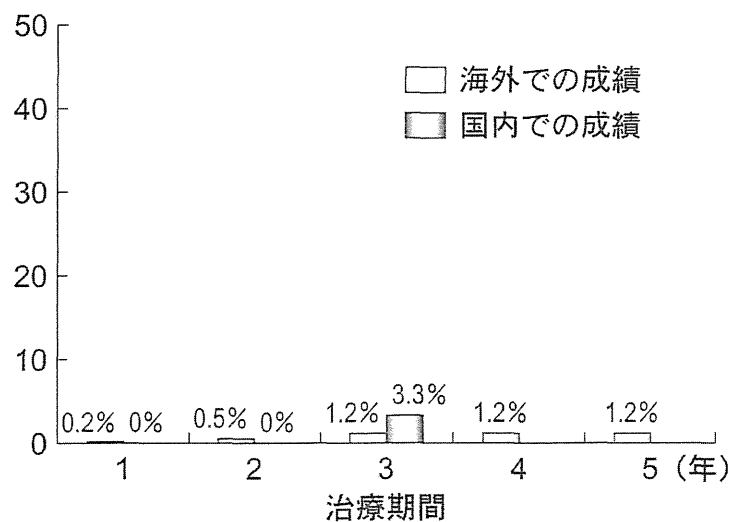
ラミブジン、アデホビル併用療法症例の血清クレアチニン値の変化

(Mitsui F, Tsuge M, et al : Antimicrob Agents Chemother 54 : 3205-3211, 2010 より引用一部改変)

核酸アナログ治療の問題点

薬剤耐性ウイルスの出現

核酸アナログ治療は劇的な治療効果が得られる反面、長期間の治療継続により、薬の効かないウイルス(薬剤耐性ウイルス)が出現する場合があります。耐性ウイルスが出現すると、徐々に HBV DNA 量が増加し、B型肝炎が増悪し、肝臓の予備能力によっては肝不全に至る場合があります。そのため、定期的に HBV DNA 量をモニターしていくことが重要であり、もし薬剤耐性ウイルスが出現した場合には、別の核酸アナログを追加するなどの早急な対応が必要となります。耐性株の出現はラミブジンやアデホビルと比較して、エンテカビルでは非常に頻度が低いことが知られています。したがって、核酸アナログを初めて投与する場合にはエンテカビルが第一選択となります。

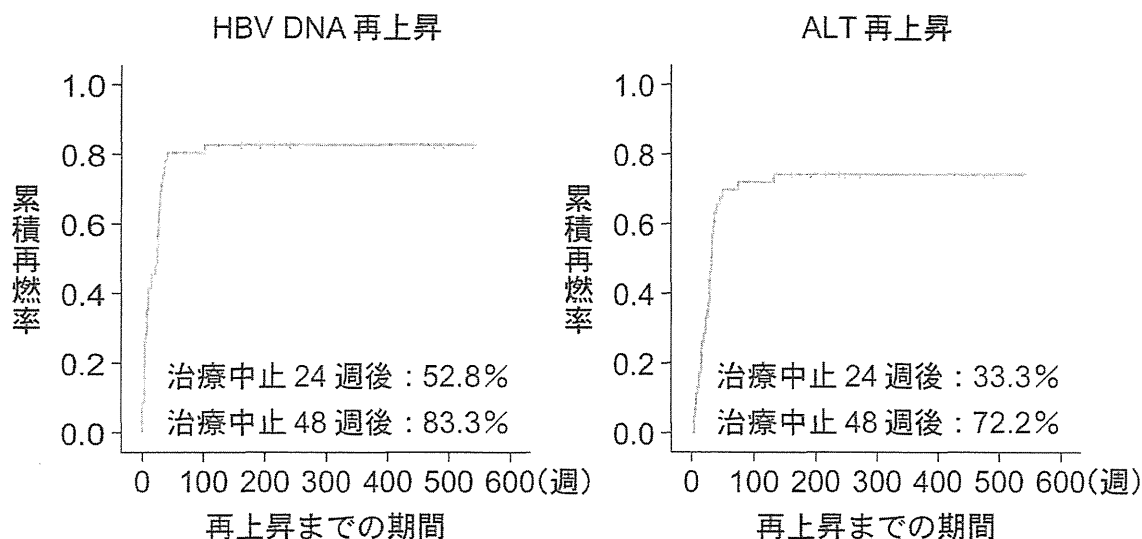


エンテカビル耐性株出現率

(Tenney DJ, et al : Hepatology 49 : 1503-1514, 2009 より引用一部改変)

核酸アナログ治療の中止

前述しましたように核酸アナログ治療は、HBVを感染した肝臓から完全に排除できる薬剤ではありません。治療を中止すると多くの症例で1年以内に肝炎が再燃してくることがわかっています。そのため、安全に治療を中止することは非常に困難であり、長期間継続加療していく必要があります。万が一、治療を中止する場合には、専門医の注意深い観察のもとで、判断する必要があります。



核酸アナログ中止後の HBV DNA, ALT の累積再燃率

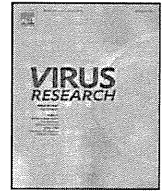
核酸アナログを中止すると、ほとんどの症例は HBV DNA が再び増加し、1 年以内に肝炎が再燃します。

(筆者作成)

今後の核酸アナログ治療

これまで、ラミブジン、アデホビル、エンテカビルという3種類の核酸アナログが保険適用となり、良好な治療効果が得られています。稀にいずれの薬剤でも十分な効果が得られない症例や、いずれにも薬剤耐性を示す症例が存在します。現在、新たな核酸アナログとしてテノホビルの治験が進行中です。テノホビルもエンテカビルと同様、強力な抗ウイルス効果を示すことがわかっており、今後、エンテカビルと並ぶ治療薬として期待されるほか、薬剤耐性ウイルスへの治療薬としても期待されています。

(柘植 雅貴・茶山 一彰)



Identification of host genes showing differential expression profiles with cell-based long-term replication of hepatitis C virus RNA

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ABSTRACT

Persistent hepatitis C virus (HCV) infection frequently causes hepatocellular carcinoma. However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are unclear. Although the human hepatoma cell line, HuH-7, has been widely used as the only cell culture system for robust HCV replication, we recently developed new human hepatoma Li23 cell line-derived OL, OL8, OL11, and OL14 cells, in which genome-length HCV RNA (O strain of genotype 1b) efficiently replicates. OL, OL8, OL11, and OL14 cells were cultured for more than 2 years. We prepared cured cells from OL8 and OL11 cells by interferon- γ treatment. The cured cells were also cultured for more than 2 years. cDNA microarray and RT-PCR analyses were performed using total RNAs prepared from these cells. We first selected several hundred highly or moderately expressed probes, the expression levels of which were upregulated or downregulated at ratios of more than 2 or less than 0.5 in each set of compared cells (e.g., parent OL8 cells versus OL8 cells cultured for 2 years). From among these probes, we next selected those whose expression levels commonly changed during a 2-year culture of genome-length HCV RNA-replicating cells, but which did not change during a 2-year culture period in cured cells. We further examined the expression levels of the selected candidate genes by RT-PCR analysis using additional specimens from the cells cultured for 3.5 years. Reproducibility of the RT-PCR analysis using specimens from recultured cells was also confirmed. Finally, we identified 5 upregulated genes and 4 downregulated genes, the expression levels of which were irreversibly altered during 3.5-year replication of HCV RNA. These genes may play roles in the optimization of the environment in HCV RNA replication, or may play key roles in the progression of HCV-associated hepatic diseases.

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1. Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (HCC) (Choo et al., 1989; Saito et al., 1990; Thomas, 2000). However,

the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are still unclear. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993; Kato et al., 1990).

The initial development of a cell culture-based replicon system (Lohmann et al., 1999) and a genome-length HCV RNA-replication system (Ikeda et al., 2002) using genotype 1b strains enabled the rapid progression of investigations into the mechanisms underlying HCV replication (Bartenschlager, 2005; Lindenbach and Rice, 2005). Furthermore, these RNA replication systems have been improved such that they have become suitable for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase (Ikeda et al., 2005; Krieger et al., 2001). Moreover, in 2005, an efficient virus production system using the JFH1 genotype 2a strain was developed using human hepatoma cell line HuH-7-derived cells (Wakita et al., 2005). However, to date, HuH-7-derived cells are used as the only cell culture

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; E1, envelope 1; EGF, epidermal growth factor; RT-PCR, reverse transcription-polymerase chain reaction; IFN, interferon; ACSM3, acyl-CoA synthetase medium-chain family member 3; ANGPT1, angiopoietin 1; CDKN2C, cyclin-dependent kinase inhibitor 2C; PLA1A, phospholipase A1 member A; SEL1L3, Sel-1 suppressor of lin-12-like 3; SLC39A4, solute carrier family 39 member 4; TBC1D4, TBC1 domain family member 4; WISP3, WNT1 inducible signaling pathway protein 3; ANXA1, annexin A1; AREG, amphiregulin; BASP1, brain abundant, membrane attached signal protein 1; CIDE, cell death activator CIDE-3; CPB2, carboxypeptidase B2; HSPA6, heat-shock 70 kDa protein B'; PI3, peptidase inhibitor 3; SLC1A3, solute carrier family 1 member 3; THSD4, thrombospondin type-1 domain-containing protein 4; ICAM-1, intercellular adhesion molecule-1; ALXR, ANXA1 receptor.

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system for robust HCV replication (Bartenschlager and Sparacio, 2007; Lindenbach and Rice, 2005). Most studies of HCV replication or anti-HCV reagents are currently carried out using a HuH-7-derived cell culture system. Therefore, it remains unclear whether or not recent advances obtained from the HuH-7-derived cell culture system reflect the general features of HCV replication or anti-HCV targets. To resolve this issue, we aimed to find a cell line other than HuH-7 that enables robust HCV replication. We recently found a new human hepatoma cell line, Li23, that enables efficient HCV RNA replication and persistent HCV production (Kato et al., 2009b). In that study, we established genome-length HCV RNA replicating cell lines, OL (polyclonal; a mixture of approximately 200 clones), OL8 (monoclonal), OL11 (monoclonal), and OL14 (monoclonal), and characterized them (Kato et al., 2009b). We further developed Li23-derived drug assay systems (ORL8 and ORL11) (Kato et al., 2009b), which are relevant to the HuH-7-derived OR6 assay system (Ikeda et al., 2005). Since we demonstrated that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells (Mori et al., 2010), we expected to find that the host factors required for HCV replication or anti-HCV targets in Li23-derived cells would also be distinct from those in HuH-7-derived cells. Indeed, we found that treatment of the cells with approximately 10 μ M (a clinically achievable concentration) of ribavirin, an anti-HCV drug, efficiently inhibited HCV RNA replication in both the Li23-derived ORL8 and ORL11 assay systems, but not in the HuH-7-derived OR6 assay system (Mori et al., 2011). We further demonstrated that more than half of the 26 anti-HCV reagents that have been reported by other groups as anti-HCV candidates using HuH-7-derived assay systems other than OR6 assay system exhibited different anti-HCV activities from those of the previous studies (Ueda et al., 2011). In addition, we observed that the anti-HCV activities evaluated by the OR6 and ORL8 assay systems were also frequently different (Ueda et al., 2011). Furthermore, Li23-derived cells showed epidermal growth factor (EGF)-dependent growth (Kato et al., 2009b)-like immortalized or primary hepatocyte cells (e.g., PH5CH8 (Ikeda et al., 1998)), whereas HuH-7-derived cells can grow in an EGF-independent manner. Our findings, when taken together, suggested that a study using Li23-derived cells might yield unexpected results, since only HuH-7-derived cells are commonly used in a wide range of HCV studies.

Moreover, our findings to date suggested that the long-term replication of HCV RNA may cause irreversible changes in the gene expression profiles of host cells, yielding an environment for facilitative viral replication or progression of a malignant phenotype. To investigate this possibility, we carried out cDNA microarray and/or reverse transcription-polymerase chain reaction (RT-PCR) analyses using Li23-derived cells (OL, OL8, OL11, and OL14) in order to identify host genes for which expression levels were irreversibly altered by the long-term replication of HCV RNA. Here we report the identification of such host genes.

2. Materials and methods

2.1. Cell culture

The Li23 cell line consists of human hepatoma cells from a Japanese male (age 56) was established and characterized in 2009 (Kato et al., 2009b). Li23 cells were maintained in modified culture medium for the PH5CH8 human immortalized hepatocyte cell line (Ikeda et al., 1998), as described previously (Kato et al., 2009b). Genome-length HCV RNA-replicating cells (Li23-derived OL, OL8, OL11, and OL14 cells) were also maintained in the medium for the Li23 cells in the presence of 0.3 mg/mL of G418 (Geneticin, Invitrogen, Carlsbad, CA). Cured cells (OL8c and OL11c cells), from which the HCV RNA had been eliminated by

interferon (IFN)- γ treatment (Abe et al., 2007), were cultured in the medium for the Li23 cells. These cells were passaged every 7 days for 3.5 years. In this study, OL, OL8, OL11, OL14, OL8c, and OL11c cells were renamed as OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells, respectively, to specify the time at which the cells were established. These “0Y” cells of passage number 3 were used in this study. Two-year cultures of OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL(2Y), OL8(2Y), OL11(2Y), OL14(2Y), OL8c(2Y), and OL11c(2Y) cells, respectively. The 3.5-year cultures of OL8(0Y), OL11(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL8(3.5Y), OL11(3.5Y), OL8c(3.5Y), and OL11c(3.5Y) cells, respectively. The cured cells obtained from OL8(2Y) and OL11(2Y) cells by IFN- γ treatment (Abe et al., 2007) were designated as OL8(2Y)c and OL11(2Y)c cells, respectively, and were maintained in the medium for the Li23 cells.

2.2. cDNA microarray analysis

OL(0Y), OL(2Y), OL8(0Y), OL8(2Y), OL11(0Y), OL11(2Y), OL8c(0Y), OL8c(2Y), OL11c(0Y), and OL11c(2Y) cells were cultured in the medium without G418 during a few passages, and then these cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 or 3 days. Total RNAs from these cells (approximately 70–80% confluency) were prepared using the RNeasy extraction kit (QIAGEN, Hilden, Germany). As previously described (Kato et al., 2009b; Mori et al., 2010), cDNA microarray analysis was performed by Dragon Genomics Center of Takara Bio. (Otsu, Japan) through an authorized Affymetrix service provider using the GeneChip Human Genome U133 Plus 2.0 Array. Differentially expressed genes were selected by comparing the arrays from the genome-length HCV RNA-replicating cells, and the selected genes were further compared with the arrays from the cured cells (see Fig. 2 for details).

2.3. RT-PCR

We performed RT-PCR in order to detect cellular mRNA as described previously (Dansako et al., 2003). Briefly, total RNA (2 μ g) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of the synthesized cDNA was used for the PCR. The primers arranged for this study are listed in Table 1.

2.4. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed using a real-time LightCycler PCR (Roche Diagnostics, Basel, Switzerland) as described previously (Ikeda et al., 2005; Kato et al., 2009b). Quantitative RT-PCR analysis for the mRNAs of the selected genes was also performed using a real-time LightCycler PCR. The primer sets used in this study are listed in Table 1.

2.5. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting analysis with a PVDF membrane were performed as previously described (Kato et al., 2003). The antibodies used for the O strain in this study were those against Core (CP9, CP11, and CP14 monoclonal antibodies [Institute of Immunology, Tokyo, Japan]; a polyclonal antibody [a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science]), E1 and NS5B (a generous gift from Dr. M. Kohara), and NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK). β -Actin antibody (Sigma, St. Louis, MO)

Table 1
Primers used for RT-PCR analysis.

Gene (accession no.)	Direction	Nucleotide sequence (5'–3')	Products (bp)	Gene (accession no.)	Direction	Nucleotide sequence (5'–3')	Products (bp)
Acyl-CoA synthetase medium-chain family member 3 (ACSM3; NM.005622)	Forward	GCATTC AAGTTCTACCCAACCGAC	258	Brain abundant, membrane attached signal protein 1 (BASP1; NM.006317)	Forward	GGATGAATGCCAGCTTCAGACAG	247
	Reverse	GGCTGCTGACAACAGCTGACTC			Reverse	ACTGGAAGTCAATGAACCGCAGAC	
Angiopoietin 1 (ANGPT1; NM.001146)	Forward	ATACAACATCGTGAAGATGGAAGTC	287	Cell death activator CIDE-3 (CIDE3; NM.022094)	Forward	GATCTGTACAAGCTGAACCCACAG	265
	Reverse	CCGTGTAAGATCAGGCTGCTCTG	Reverse		GACAGGTCGGGATAAGGGATGAG		
Cyclin-dependent kinase inhibitor 2C (CDKN2C; NM.001262)	Forward	AAGACCGAACTGGTTTCGCTGTC	246	Carboxypeptidase B2 (CPB2; NM.001872)	Forward	GGAAGTGTCTCTAGTAGCCAGTG	242
	Reverse	CATAGAGCCTGGCCAAATCACAG			Reverse	CAGCGGCAAAAGCTTCTCTACAG	
Phospholipase A1 member A (PLA1A; NM.015900)	Forward	GGAGTTTCACTGAAGGAACTGAG	292	Heat shock 70 kDa protein B' (HSPA6; NM.002155)	Forward	TGAAGCCGAGCAGTACAAGGCTG	235
	Reverse	GTTCAGTGGTTCAGGTAAGCAGAC			Reverse	CTCCCTCTCTGATGCTCATACTC	
Sel-1 suppressor of lin-12-like 3 (SEL1L3; NM.015187)	Forward	ACCTGCACTTGCGGCTTCTCTG	212	Peptidase inhibitor 3 (PI3; NM.002638)	Forward	GGTTCTAGAGGCAGCTGTCACG	276 ^a
	Reverse	AGAGGCATCTGCAGCTGGAGTC	Reverse		CCGCAAGAGCCTTCACAGCAC		
Solute carrier family 39 member 4 (SLC39A4; NM.017767)	Forward	GCCTGTTCTCTACGTAGCACTC	158	Peptidase inhibitor 3 (PI3; NM.002638)	Forward	GGTTCTAGAGGCAGCTGTCACG	241 ^b
	Reverse	GAAGGTGATGTCATCTCGTACAG			Reverse	GCACTCAGTATCTTTCAAGCAGC	
TBC1 domain family, member 4 (TBC1D4; NM.014832)	Forward	GGAGAGGGCCAATAGCCAACCTG	198	Solute carrier family member 3 (SLC1A3; NM.004172)	Forward	CAATGGCGTGGACAAGCGCTC	240
	Reverse	AGCTTCCGGAGTTGCTCCACTG			Reverse	CCGACAGATGTCAGCACAAATGAC	
WNT1 inducible signaling pathway protein 3 (WISP3; NM.003880)	Forward	AGAGATGCTGTATCCCTAATAAGTC	129	Thrombospondin type-1 domain-containing protein 4 (THSD4; NM.024817)	Forward	TGGAGTCAGTGTCCATCGAGTG	275
	Reverse	CAGGTTCTCTGCAGTTTCTCTGAC			Reverse	GGGTCACAGAGGTTACTTAGAGTC	
Annexin A1 (ANXA1; NM.000700)	Forward	GACTTGGCTGATTGATGATGCCAG	192	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM.002046)	Forward	GACTCATGACCACAGTCCATGC	334
	Reverse	AATGTACCTTTCAACTCCAGGTC			Reverse	GAGGAGACCACCTGGTGTCTCAG	
Amphiregulin (AREG; NM.001657)	Forward	CGGGAGCCGACTATGACTACTC	391				
	Reverse	AAGGCAGCTATGGCTGCTAATGC					

^a This primer set was used for RT-PCR analysis.

^b This primer set was used for quantitative RT-PCR analysis.

was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected by the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Sciences, Boston, MA).

2.6. Statistical analysis

Statistical comparison of the mRNA levels between the various time points was performed using Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Efficient replication of genome-length HCV RNA is maintained in long-term cell culture

To prepare specimens for the cDNA microarray analysis, genome-length HCV RNA-replicating OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were cultured for 2 years, and were designated as OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y) cells, respectively. OL8c(0Y) and OL11c(0Y) cells were also cultured for 2 years, and were designated as OL8c(2Y) and OL11c(2Y) cells, respectively. We observed that the growth rates of all cell lines increased in a time-dependent manner, while the appreciable changes of cell shapes were not observed. The doubling time of genome-length HCV RNA-replicating cells (OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y)) and cured cells (OL8c(0Y) and OL11c(0Y)) was approximately 41 h and 32 h, respectively. After 2-year culture, these values reduced to approximately 28 h and 23 h.

Using the total RNA specimens obtained from genome-length HCV RNA-replicating cells, the levels of genome-length HCV RNAs were examined by quantitative RT-PCR analysis. The results revealed that the levels of the genome-length HCV RNAs had increased in all cases after a 2-year period of HCV RNA replication (Fig. 1). The levels of HCV proteins (Core, E1, NS3, and NS5B) were also examined by Western blot analysis. The E1, NS3, and NS5B were detected in all specimens, except for the Li23 cells, although a little larger size of E1 was additionally detected in the specimens from 2-year culture (Fig. 1). This phenomenon may indicate the appearance of additional N-glycosylation sites by mutations caused during the 2-year replication of the HCV RNA, as observed in a previous report (Mori et al., 2008). However, genetic analysis of HCV RNAs from 2-year culture of OL8, OL11, and OL14 cell series has detected no additional N-glycosylation sites by mutations (Kato et al., unpublished results). Therefore, the mobility change of E1 may be due to the other modifications such as O-glycosylation. In addition, Core was not detected in the cultures of OL11(2Y) cells, even when polyclonal anti-Core antibody was used (Fig. 1). A similar phenomenon was observed in a previous study using HuH-7-derived genome-length HCV RNA-replicating cells (Kato et al., 2009a). In that study, we showed that the Core region was not deleted, but mutated at several positions within the epitopes of the anti-Core antibody (Kato et al., 2009a). The results of genetic analysis using Li23-derived cells as described above (Kato et al., unpublished results) were also similar with those in the previous study using HuH-7-derived cells (Kato et al., 2009a).

3.2. Selection of genes showing irreversible changes with long-term HCV RNA replication

To identify those genes whose expression levels were irreversibly altered by the long-term replication of HCV RNA, we performed a combination of cDNA microarray and RT-PCR analyses using several Li23-derived cell lines. An outline of the selection process performed in this study is provided in Fig. 2. The first microarray analysis I was carried out by the comparison of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and

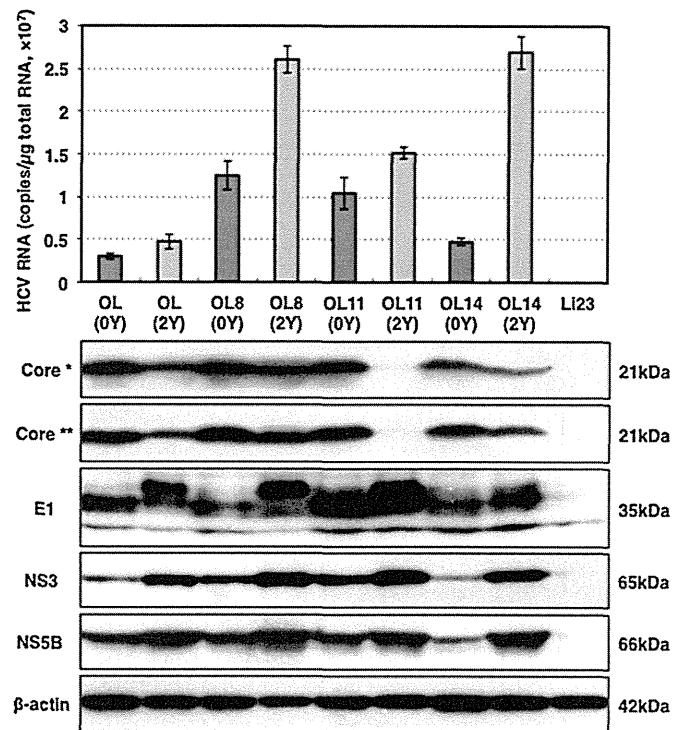


Fig. 1. Characterization of genome-length HCV RNA-replicating cells in long-term cell culture. The upper panel shows the results of a quantitative RT-PCR analysis of intracellular genome-length HCV RNA. Total RNAs from OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells after 2 years [OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y)] in culture, as well as total RNAs from the parental OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were used for the analysis. Total RNA from Li23 cells was used as a negative control. The lower panel shows the results of the Western blot analysis. Cellular lysates from cells used for quantitative RT-PCR were also used for comparison. HCV Core, E1, NS3, and NS5B were detected by Western blot analysis. β -Actin was used as a control for the amount of protein loaded per lane. A single asterisk indicates that the anti-Core polyclonal antibody was used for detection. A double asterisk indicates that a mixture of three kinds (CP9, CP11, and CP14) of anti-Core monoclonal antibodies was used for detection.

OL11(0Y) cells versus OL11(2Y) cells. In this step, we selected those genes whose expression levels commonly showed changes in at least two of three comparative analyses to avoid the bias caused by the difference of cell clonality, since OL(0Y) was a polyclonal cell line, while OL8(0Y) and OL11(0Y) were monoclonal cell lines (Kato et al., 2009b). As regards the selected genes, a microarray analysis II was performed in which OL8c(0Y) cells were compared to OL8c(2Y) cells, and OL11c(0Y) cells were compared to OL11c(2Y) cells. In this step, the genes were excluded from those selected by the microarray analysis I if their expression levels had changed during the 2-year culture of cured cells. As regards the selected genes, we next performed a RT-PCR analysis I to examine the reproducibility of changes in gene expression levels. In this step, we added the results of a new comparative series, OL14(0Y) versus OL14(2Y), to arrive at the judgment to advance to the next step of analysis. We selected genes for which expression levels had changed in more than five of six comparative series (Fig. 2). At the last step, we confirmed by RT-PCR analysis II whether or not the expression levels of the selected genes in OL8(2Y) or OL11(2Y) cells had changed by HCV RNA replication. When the gene expression levels had not changed in two comparative series (OL8(2Y) versus OL8(2Y)c and OL11(2Y) versus OL11(2Y)c), the genes were selected as the candidates exhibiting irreversible changes after 2-year HCV RNA replication.

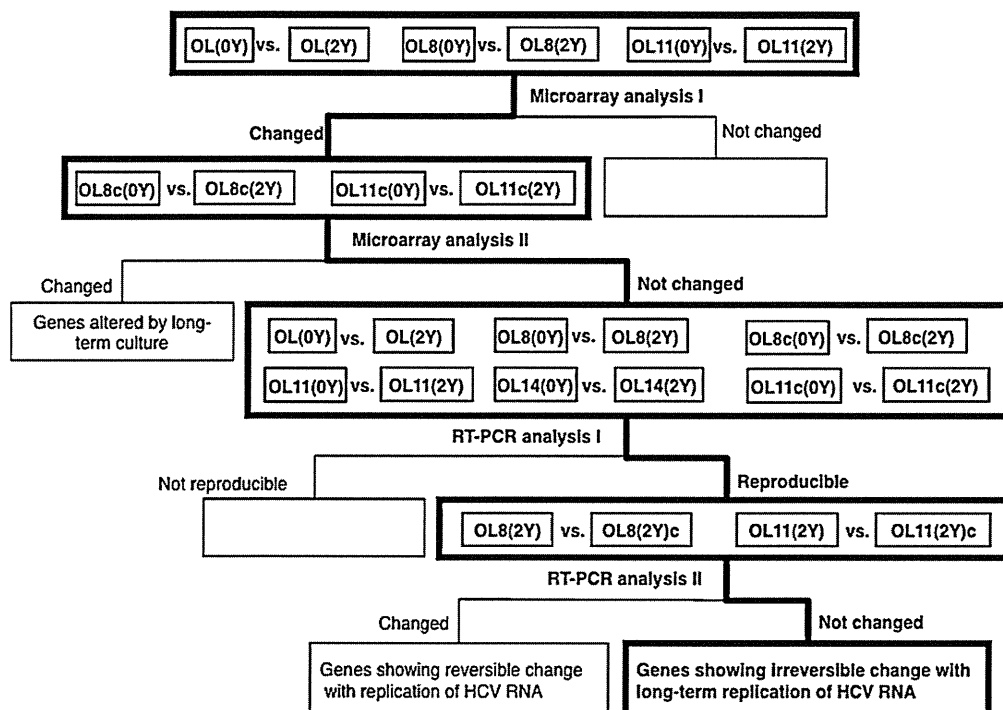


Fig. 2. Outline of selection process performed in this study. To obtain the objective genes, cDNA microarray analyses I and II were performed, and then RT-PCR analyses I and II were also performed.

3.3. Selection and expression profiles of genes showing upregulated expression during long-term HCV RNA replication

The process outlined in Fig. 2 was used to identify those genes that exhibited irreversibly upregulated expression during the 2-year replication of HCV RNA. Microarray analysis I revealed 1912, 1148, and 1633 probes, the expression levels of which were upregulated at a ratio of more than 2 in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. To avoid the possibility that the genes showing low expression level are selected, the ratios and expression values were used in combination for the selection. As the minimum expression level, more than 100 (actual value of measurement), which was detectable within 30 cycles in RT-PCR analysis, was adopted. From among these probes, we selected those showing ratios of more than 4 with an expression level of more than 100, or those showing ratios of more than 3 with an expression level of more than 200, or those showing an expression level of 1000. By this selection process, 559, 237, and 368 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 3A). At this step, we obtained 51 genes as candidates exhibiting upregulation in more than two of three comparisons. Based on the results of the subsequent microarray analysis II, we further selected 14 genes from a total of 51 genes, because the expression levels of the remaining 37 genes increased during the 2-year culture of cured cells (Fig. 3B). The list of these genes was shown in Supplemental Table 1. As regards the 14 selected genes, we performed an RT-PCR analysis I to confirm the results obtained by the cDNA microarray analysis and to examine the status of gene expression in an additional comparison of OL14(0Y) cells versus OL14(2Y) cells. This analysis revealed that the mRNA levels of 6 of 14 genes showed no enhancement in two of four comparative series (data not shown). Therefore, in this step, these 6 genes were excluded from the candidate genes. However, the mRNA levels of the remaining 8 genes (acyl-CoA synthetase

medium-chain family member 3 [ACSM3], angiopoietin 1 [ANGPT1], cyclin-dependent kinase inhibitor 2C [CDKN2C], phospholipase A1 member A [PLA1A], Sel-1 suppressor of lin-12-like 3 [SEL1L3], solute carrier family 39 member 4 [SLC39A4], TBC1 domain family member 4 [TBC1D4], and WNT1 inducible signaling pathway protein 3 [WISP3]) were enhanced in more than three of four comparative series (Fig. 3C). Furthermore, we demonstrated by RT-PCR analysis II that the expression levels of these 8 genes did not return to initial levels, even after elimination of HCV RNA from OL8(2Y) or OL11(2Y) cells (Fig. 3C). It was noteworthy that the mRNA levels of the *ANGPT1* and *PLA1A* genes were enhanced in all comparative series (Fig. 3C).

3.4. Selection and expression profiles of genes showing downregulated expression during long-term HCV RNA replication

To obtain genes showing irreversibly downregulated expression during the 2-year HCV RNA replication period, we performed a selection of genes according to the methods described for the selection of upregulated genes. The first microarray analysis I in this series revealed 1901, 2128, and 1579 probes whose expression levels were downregulated at a ratio of less than 0.5 in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. As described in Section 3.3, the ratios and expression values were used in combination for the selection. From among these probes, we selected those showing ratios of less than 0.25 with an initial expression level of more than 1000 (actual value of measurement), or those showing ratios of less than 0.33 with an initial expression level of more than 200, or those showing an initial expression level of 100. By this selection process, 828, 622, and 466 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 4A). At this step, we obtained 236 genes as candidates showing downregulation in more than two of three comparisons. Based on the results

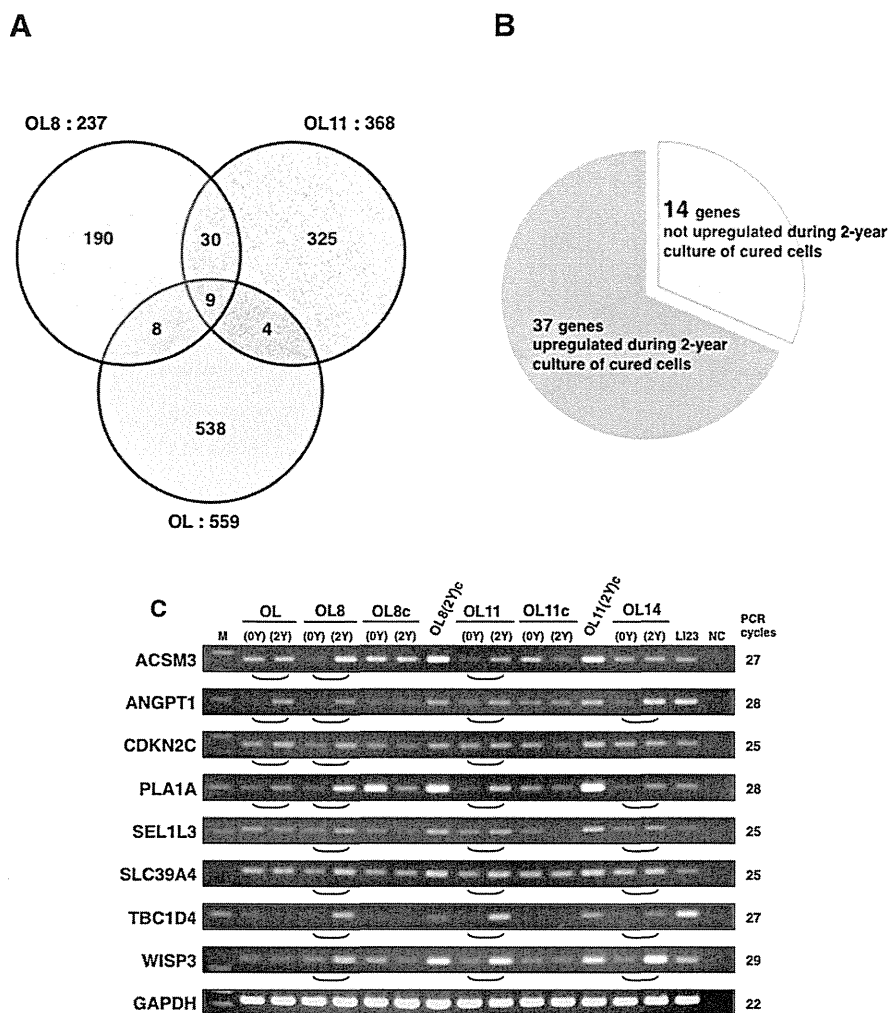


Fig. 3. Identification of genes irreversibly upregulated during 2-year replication of HCV RNA. (A) Upregulated genes obtained by microarray analysis I shown in Fig. 2. Genes whose expression levels were upregulated at ratios of more than 2 in the case of OL(0Y) versus OL(2Y) cells, OL8(0Y) versus OL8(2Y) cells, or OL11(0Y) versus OL11(2Y) cells were selected, and 51 genes upregulated in at least two of three comparisons were obtained. (B) Further selection by microarray analysis II, shown in Fig. 2. Genes whose expression levels were upregulated during 2-year culture (OL8c(2Y) or OL11c(2Y) cells) of the cured OL8c(0Y) or OL11c(0Y) cells were eliminated. (C) Expression profiles of upregulated genes. RT-PCR analyses I and II shown in Fig. 2 were performed as described in Section 2. PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. The round parenthesis indicates the comparative series showing the upregulated expression.

of the second microarray analysis II, we were able to select 17 genes from a total of 236 genes, as the expression levels of most of the genes had decreased during the 2-year culture of cured cells (Fig. 4B). The list of these genes was shown in Supplemental Table 2. As regards the 17 selected genes, we performed an initial RT-PCR analysis I to confirm the results obtained by the microarray analysis I and to examine the status of gene expression by additional comparison of OL14(0Y) cells versus OL14(2Y) cells. This analysis revealed that the mRNA levels of 8 of 17 genes showed no suppression in more than two of four comparative series (data not shown). Therefore, these 8 genes were excluded from the candidate genes in this step. However, the mRNA levels of the remaining 9 genes (annexin A1 [ANXA1], amphiregulin [AREG], brain abundant, membrane attached signal protein 1 [BASP1], cell death activator CIDE-3 [CIDE3], carboxypeptidase B2 [CPB2], heat-shock 70 kDa protein B' [HSPA6], peptidase inhibitor 3 [PI3], solute carrier family 1 member 3 [SLC1A3], and thrombospondin type-1 domain-containing protein 4 [THSD4]) were suppressed in more than three of four comparative series (Fig. 4C). Furthermore, we demonstrated by RT-PCR analysis II that the expression levels of these 9 genes did not return to initial levels, even after the elimination of HCV RNA from

OL8(2Y) or OL11(2Y) cells (Fig. 4C). It is noteworthy that the mRNA levels of *BASP1*, *CIDE3*, *HSPA6*, and *PI3* genes were suppressed in all comparative series (Fig. 4C).

3.5. Expression profiles of selected genes during 3.5-year replication of HCV RNA

As described above, we selected 8 upregulated genes and 9 downregulated genes, the expression levels of which had irreversibly changed after a 2-year period of HCV RNA replication. However, reproducibility of the RT-PCR analysis using total RNA specimens prepared from independent recultured cells would be needed or arriving at a reliable conclusion. Furthermore, in this context, it would also be important to clarify whether or not these irreversible changes in RNA expression levels remained stable or were further enhanced during HCV RNA replication if the cells were cultured for a period of more than 2 years. Since the OL8(2Y), OL8c(2Y), OL11(2Y), and OL11c(2Y) cells were continuously cultured for a period of up to 3.5 years, they were used as OL8(3.5Y), OL8c(3.5Y), OL11(3.5Y), and OL11c(3.5Y) cells with the recultured OL8(0Y), OL8(2Y), OL8c(0Y), OL8c(2Y), OL11(0Y),

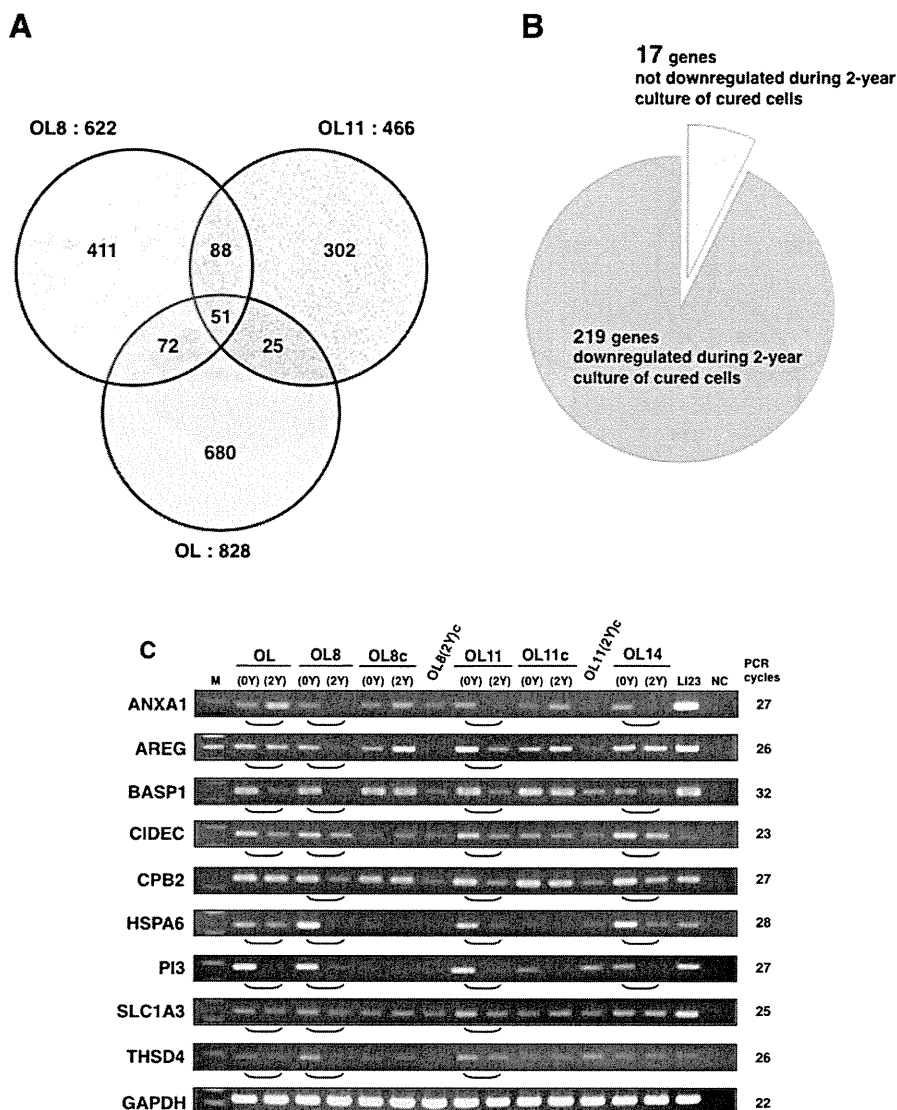


Fig. 4. Identification of genes irreversibly downregulated during 2-year replication of HCV RNA. (A) Downregulated genes obtained by microarray analysis I shown in Fig. 2. Genes were selected whose expression levels were downregulated at ratios of less than 0.5 in the case of OL(0Y) versus OL(2Y) cells, OL8(0Y) versus OL8(2Y) cells, and OL11(0Y) versus OL11(2Y) cells. A total of 236 genes were obtained that were downregulated in at least two of three comparisons. (B) Further selection by microarray analysis II shown in Fig. 2. Genes whose expression levels were downregulated during 2-year culture (OL8c(2Y) or OL11c(2Y)) of the cured OL8c(0Y) or OL11c(0Y) cells were eliminated. (C) Expression profiles of downregulated genes. RT-PCR analyses I and II, shown in Fig. 2, were performed as described in Fig. 3C. The round parenthesis indicates the comparative series showing the downregulated expression.

OL11(2Y), OL11c(0Y), and OL11c(2Y) cells, respectively, for the RT-PCR analysis in order to address the questions raised above. We first performed RT-PCR analysis of the genes indicated in Figs. 3C and 4C. The results revealed that most of the genes examined showed reproducible results, as shown in Figs. 3C and 4C (data not shown). However, no reproducible results were obtained regarding *ACSM3* selected as an upregulated gene and *HSPA6* selected as a downregulated gene (data not shown), suggesting that the mRNA levels of both genes were sensitively affected by the cell culture conditions (e.g., cell density). Regarding the remaining 7 upregulated and 8 downregulated genes, we next performed a quantitative RT-PCR analysis using the total RNA specimens prepared from OL8(0Y), OL8(2Y), OL8(3.5Y), OL11(0Y), OL11(2Y), OL11(3.5Y), OL8c(0Y), OL8c(2Y), OL8c(3.5Y), OL11c(0Y), OL11c(2Y), and OL11c(3.5Y) cells.

As regards the upregulated genes, statistically significant differences between their mRNA levels of HCV RNA-replicating cells and their cured counterparts during the culture for a period of up to 3.5 years were observed in the case of 5 genes (*WISP3*, *TBC1D4*,

ANGPT1, *SEL1L3*, and *CDKN2C*) (Fig. 5). However, such a significant difference was not maintained for a period up to 3.5 years in the case of *PLA1A* gene (OL8(3.5Y) cells versus OL8c(3.5Y) cells) and *SLC39A4* gene (OL11(3.5Y) cells versus OL11c(3.5Y) cells) (Fig. 5). These results suggest that the upregulated expression of *PLA1A* or *SLC39A4* gene is not irreversible change by long-term replication of HCV RNA. A drastic difference between mRNA levels in HCV RNA-replicating cells versus cured cells was observed in the case of the genes *WISP3* and *TBC1D4* (Fig. 5).

As for the downregulated genes, the results revealed that 4 genes (*BASP1*, *CPB2*, *ANXA1*, and *SLC1A3*) showed statistically significant differences between their mRNA levels of HCV RNA-replicating cells and their cured counterparts during the culture for a period of up to 3.5 years (Fig. 6). However, such a significant difference was not continuously observed for a period up to 3.5 years in the case of 3 genes (*AREG*, *CIDEC*, and *THSD4*) (Fig. 6), although the expression levels (except for *AREG* in the OL11 series and *CIDEC* in the OL8 series) at 2 years in cell culture showed reproducible

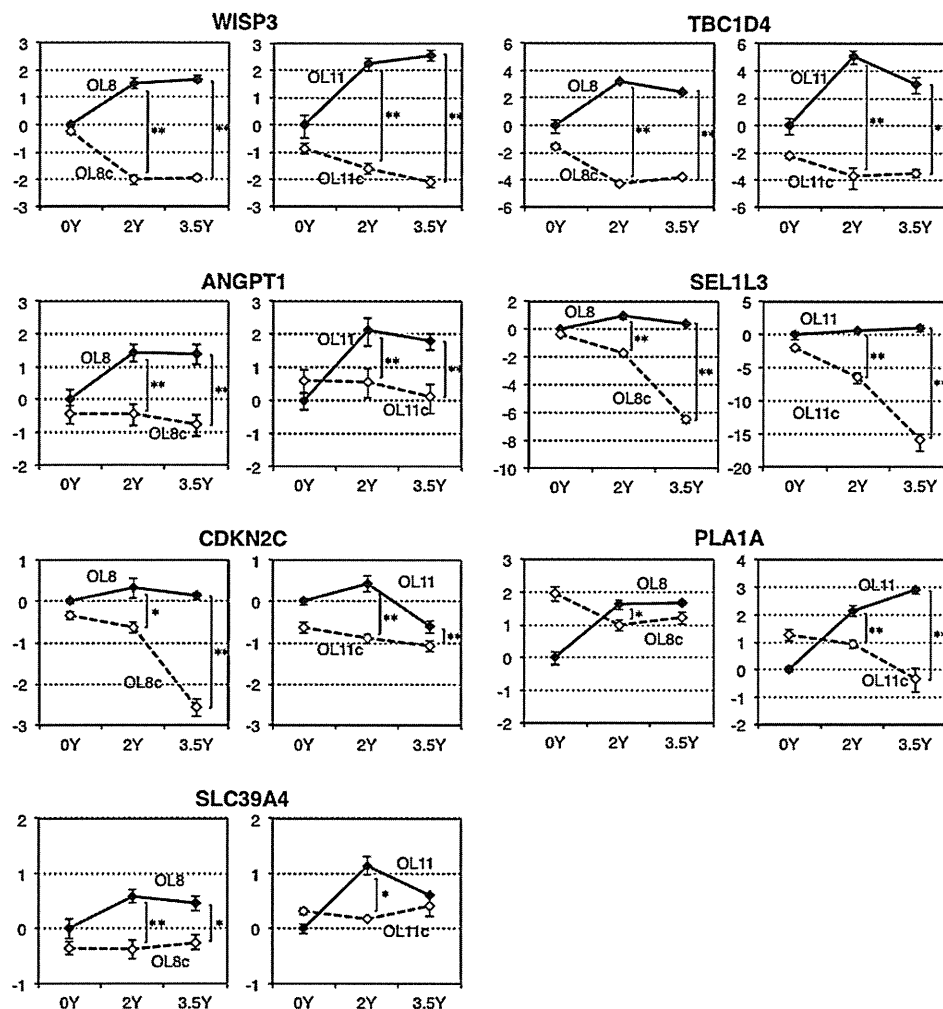


Fig. 5. Expression levels of genes selected as upregulated genes in 3.5-year cell culture. Quantitative RT-PCR analysis using the total RNAs derived from OL8(0Y), OL8(2Y), OL8(3.5Y), OL8c(0Y), OL8c(2Y), OL8c(3.5Y), OL11(0Y), OL11(2Y), OL11(3.5Y), OL11c(0Y), OL11c(2Y), and OL11c(3.5Y) cells was performed as described in Section 2. Experiments were done in triplicate. The vertical lines indicate the expression levels, with the fold in the scale of log 2, when the level in OL8(0Y) or OL11(0Y) cells was assigned to be 1. Asterisks indicate significant differences between mRNA levels of HCV RNA-replicating cells and their cured counterparts. * $P < 0.05$; ** $P < 0.01$.

differences, as depicted in Fig. 4C. Quantitative RT-PCR analysis revealed that the expression levels of *PI3* gene drastically decreased during 3.5-year culture of cured cells, although *PI3* gene expression was very low level in cured cells (Fig. 6). These results suggest that the downregulated expression of *AREG*, *CIDEA*, *THSD4*, or *PI3* gene is not irreversible change by long-term replication of HCV RNA. The most drastic difference between mRNA levels of HCV RNA-replicating cells and their cured counterparts was observed in the case of the *BASP1* gene (Fig. 6).

4. Discussion

In this study, we performed cDNA microarray and RT-PCR analyses using genome-length HCV RNA-replicating Li23-derived cells cultured for 2 years after the cells had been established as cell lines, and we performed quantitative RT-PCR analyses using these cells and additional cells cultured for a period of up to 3.5 years. Consequently, we identified 5 genes (*WISP3*, *TBC1D4*, *ANGPT1*, *SEL1L3*, and *CDKN2C*) showing irreversible upregulated expression, and 4 genes (*BASP1*, *CPB2*, *ANXA1*, and *SLC1A3*) showing irreversible downregulated expression with the persistent 3.5-year replication of HCV RNA.

Two possibilities can be considered as plausible biological explanations for the irreversible changes in expression levels of these identified genes. First, it is possible that these genes play roles in the optimization of the environment in HCV RNA replication. Indeed, in the present study, we observed that the levels of HCV RNAs increased in all cases after constitutive HCV RNA replication of 2 years (Fig. 1). However, the expression levels of these genes did not differ between HCV RNA-replicating cells and the corresponding cured cells at the time at which the cells were first established (Figs. 5 and 6). Since, to date, no studies reported in the literature have demonstrated that these genes are required for HCV RNA replication or that the level of HCV RNA replication is regulated by these genes, further comparative analysis such as the quantification of HCV RNA levels in the cells forced to express these genes will be needed to clarify these points.

A second possible explanation for the observed irreversible changes would be that these genes play roles in the progression of HCV-associated hepatic diseases. We focused on this possibility, due to the number of reports in the literature regarding these genes.

Among the upregulated genes identified in this study, *WISP3* is most interesting. *WISP3* is a Wnt1-inducible cysteine-rich protein (CCN6) that belongs to the CCN family. Previous studies have

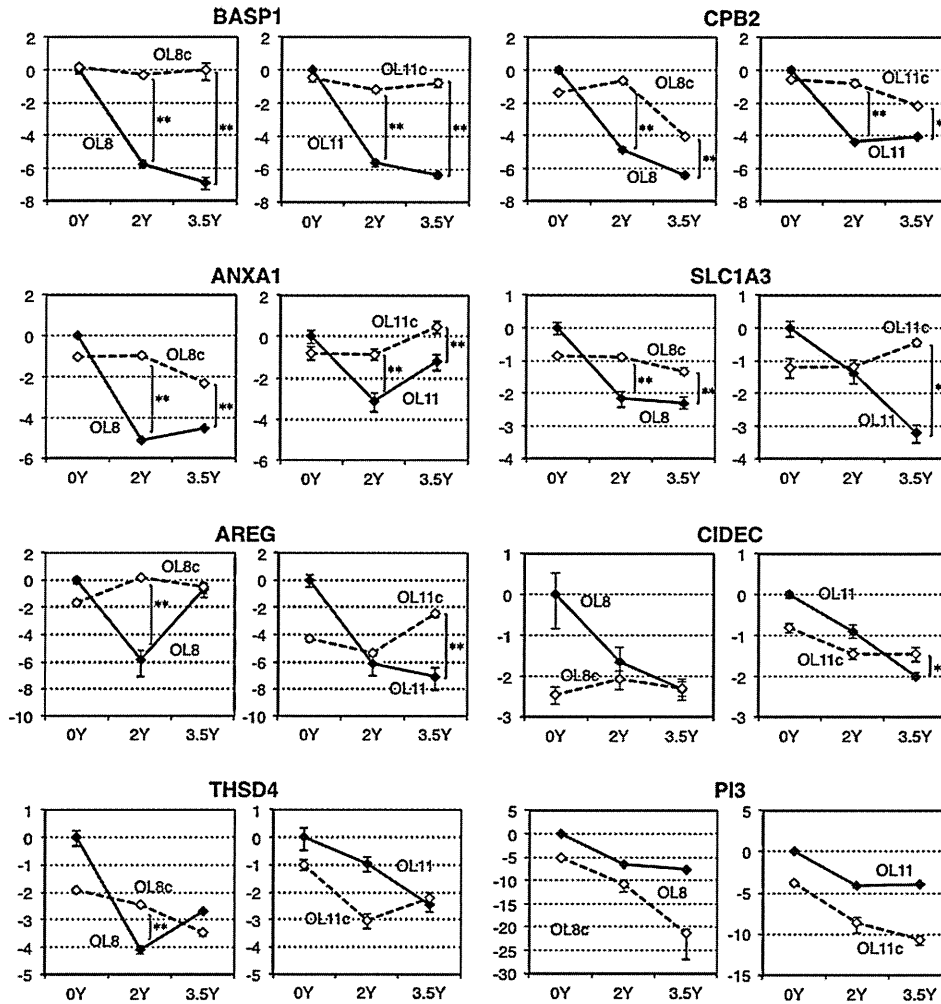


Fig. 6. Expression levels of genes selected as downregulated genes in 3.5-year cell culture. Quantitative RT-PCR analysis was performed as shown in Fig. 5, and the obtained results are also presented as shown in Fig. 5.

linked the overexpression of WISP3/CCN6 to colon cancer (Pennica et al., 1998; Thorstensen et al., 2001), suggesting that overexpression of this protein is associated with the development of this type of cancer. However, recent studies revealed that WISP3 exerts both tumor-growth and invasion-inhibitory functions in inflammatory breast cancer and aggressive non-inflammatory breast cancer (Huang et al., 2008, 2010). Although the role of WISP3 in the development of symptomatic cancer is controversial and unproven, enhancement of WISP3 expression in liver tissue may be involved in the progression of hepatic cancer. On the other hand, it was recently reported that WISP3 increased the migration and the expression of intercellular adhesion molecule-1 (ICAM-1) in human chondrosarcoma cells (Fong et al., 2012). Since ICAM-1 may facilitate the movement of cells through the extracellular matrix, ICAM-1 is expected to play an important role in cancer cell invasion and metastasis (Huang et al., 2004). Therefore, irreversible enhancement of WISP3 by long-term HCV RNA replication, as shown in this study, may be involved in tumor invasion or metastasis, i.e., the transition to the aggressive phenotype of human cancers. However, we could not confirm an enhancement of ICAM-1 expression in our microarray analysis. Therefore, further experiments will be necessary to clarify the biological significance of enhanced WISP3 expression by HCV.

TBC1D4 is also of interest as an enhanced gene during the long-term replication of HCV RNA. *TBC1D4* was discovered as a substrate

phosphorylated by insulin-activated serine–threonine kinase Akt (Kane et al., 2002). This protein, which was initially designated as AS160 (Akt substrate of 160 kDa), has a GTPase-activating protein (GAP) and shows GAP activity with Rab 2A, 8A, 10, and 14, which participate in the translocation of the GLUT4 glucose transporter from intracellular storage vesicles to the plasma membrane (Mîinea et al., 2005). Therefore, *TBC1D4* functions as a Rab inhibitor in insulin-regulated GLUT4 trafficking (Rowland et al., 2011). Since we observed the enhancement of *TBC1D4* expression in this study, we simply inferred that insulin-dependent glucose uptake might be suppressed in long-term cultured cells replicating HCV RNA. However, we found very low levels of expression of GLUT4 in the Li23-derived cells used in this study, suggesting that an enhancement of *TBC1D4* may be involved in the trafficking of molecule(s) other than the GLUT4 transporter.

Among the downregulated genes identified in this study, three genes of interest showing altered expression levels were clearly identified by quantitative RT-PCR. The first of the three is *BASP1*, which was originally isolated as a membrane-bound phosphoprotein abundant in nerve terminals (Mosevitsky et al., 1997). Although the function of *BASP1* in the nervous system is still unclear, it has been reported to be a transcriptional co-suppressor for Wilms' tumor suppressor protein WT1 (Carpenter et al., 2004). In addition, it has also been found that *BASP1* can inhibit cellular transformation by the *v-Myc* oncogene, and can block the

regulation of Myc target genes (Hartl et al., 2009). These studies suggest that *BASP1* probably acts as a tumor suppressor. Furthermore, it has been reported that *BASP1* is suppressed by the methylation of the *BASP1* gene in a significant proportion of HCCs, and the suppression of this gene has been identified as a useful biomarker for the early diagnosis of HCC (Moribe et al., 2008; Tsunedomi et al., 2010). In this context, the suppression of *BASP1* expression observed in this study may be due to the methylation of the *BASP1* gene. If so, this type of methylation would likely be induced during the long-term replication of HCV RNA, as the long-term culture of cured cells did not induce a suppression of *BASP1* expression. To obtain additional information, we compared the mRNA levels of *BASP1* among HuH-7-derived HCV RNA-replicating O cells, those cells cultured for 2 years, and the corresponding cured cells (Ikeda et al., 2005; Kato et al., 2009a). The preliminary results revealed that the mRNA levels of *BASP1* in these cells were remarkably lower than those in the Li23-derived cells, and no significant differences were observed among the HuH-7-derived cells (data not shown). These results are consistent with the results in a previous report (Tsunedomi et al., 2010) describing hypermethylation of the *BASP1* gene in HuH-7 cells. However, we observed that the mRNA levels of *BASP1* in Li23-derived cells (e.g., OL8, OL11) were similar to those in the immortalized hepatocyte PH5CH8 and NKNT3 cell lines (Ikeda et al., 1998; Naka et al., 2006), suggesting that the methylation status of the *BASP1* gene in these cell lines is lower than that of HuH-7 cells. The results, taken together, led us to speculate that persistent HCV replication may induce the methylation of the *BASP1* gene, although no association of *BASP1* suppression with the aggressive phenotype of HCC has been reported to date. To clarify this point, further analysis will be needed.

A second intriguing gene is *CPB2*, which is produced mainly by the liver and circulates in plasma as a plasminogen-bound zymogen. Thus far, it is known that *CPB2* potentially attenuates fibrinolysis by removing the fibrin C-terminal residues that are needed for the binding and activation of plasminogen (Redlitz et al., 1995). On the other hand, several proinflammatory mediators (e.g., C5a, osteopontin, and bradykinin) have been identified as substrates of *CPB2* in vitro (Myles et al., 2003; Sharif et al., 2009). Therefore, it has been considered that *CPB2* may serve an anti-inflammatory function. Indeed, a recent study demonstrated that *CPB2* plays a central role in down-regulating C5a-mediated inflammatory responses in autoimmune arthritis in mice and humans (Song et al., 2011). These findings led to the hypothesis that the suppression of *CPB2* in HCV-infected hepatocytes leads to the proinflammatory status in vivo. The specific suppression of *CPB2* obtained as an HCV-induced irreversible change in host cells supports the above hypothesis. Furthermore, since it has been reported that C5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans, and that it plays a causative role in human liver fibrosis (Hillebrandt et al., 2005), the suppression of *CPB2* during the long-term replication of HCV RNA may be involved in liver fibrogenesis.

The third gene of interest in this context is *ANXA1*, a member of the superfamily of annexin proteins that bind acidic phospholipids with high affinity in the presence of Ca^{2+} . *ANXA1* is found in many differentiated cells, particularly those of the myeloid lineage, and is known to be a downstream mediator of glucocorticoids (Yazid et al., 2010). Recent reports have shown that glucocorticoids can differentially affect the *ANXA1* pathway in cells of the innate and adaptive immune system, and that *ANXA1* is an important mediator of the anti-inflammatory effects of glucocorticoids (Perretti and D'Acquisto, 2009). Furthermore, it was reported recently that *ANXA1* is an endogenous inhibitor of NF- κ B which can be induced in human cancer cells and mice by anti-inflammatory glucocorticoids and modified nonsteroidal anti-inflammatory drugs (Zhang et al., 2010). The suppression of NF- κ B activity by the binding of *ANXA1* to the p65 subunit of NF- κ B was accompanied by enhanced

apoptosis and inhibition of cell growth. In this context, the irreversible suppression of *ANXA1* observed in the present study may weaken the anti-inflammatory effects of glucocorticoids. However, in our microarray analysis, no expression of the *ANXA1* receptor (ALXR; formyl peptide receptor 2 known as ALXR in humans) was observed. Therefore, it is unlikely that Li23-derived cells respond to glucocorticoids in an autocrine manner leading to the anti-inflammatory state, although secreted *ANXA1* may interact with its target cells in a paracrine manner. On the other hand, *ANXA1* has been shown to be strongly suppressed in prostate cancer (Xin et al., 2003), head and neck cancer (Garcia Pedrero et al., 2004), and esophageal cancer (Hu et al., 2004). Moreover, a recent study showed that *ANXA1* regulates the proliferative functions of estrogens in MCF-7 breast cancer cells (Ang et al., 2009). In that study, it was revealed that high physiologic pregnancy levels (up to 100 nM) of estrogen enhanced *ANXA1* expression and induced a growth arrest of MCF-7 cells, whereas physiologic levels of estrogen (1 nM) induced the proliferation of these cells. Furthermore, silencing of *ANXA1* expression using *ANXA1* siRNA reversed this estrogen-dependent proliferation as well as growth arrest [51]. These results suggest that *ANXA1* may act as a tumor suppressor gene and modulate the proliferation function of estrogens. In this context, suppression of *ANXA1* expression by long-term HCV RNA replication may modulate cell proliferation. Therefore, it is of interest whether *ANXA1* acts as an anti-proliferative mediator on the Li23-derived hepatoma cell lines used in this study. To clarify this point, further experiments involving *ANXA1* overexpression or silencing will be needed.

This study revealed irreversible changes in host gene expression due to the long-term replication of HCV RNA in cell culture, but not with simple long-term cell culture in the absence of HCV. However, we can not exclude completely the possibility that G418, but not HCV, cause the irreversible changes in the gene expression profiles of Li23-derived cells, since HCV RNA replicating cells were cultured under selective pressure of G418, while the control cured cells were cultured in the absence of G418, except for a few passages before mRNA profiling. To resolve this issue, a long-term culture of G418-resistant cured cells may be the best way, however, it would take a long time to obtain the conclusion. Alternatively, to examine this point, regarding the genes selected in this study, we fortunately could compare the mRNA levels by RT-PCR analysis among HuH-7-derived HCV RNA-replicating O cells, those cells cultured for 2 years, and the corresponding cured cells obtained in previous studies (Ikeda et al., 2005; Kato et al., 2009a). The results revealed that eight genes except for *BASP1*, which was very low expression level in HuH-7-derived cells, showed no such upregulated or downregulated expression profiles obtained in this study (data not shown). Therefore, it is unlikely that the genes identified in this study have been selected by the long-term treatment with G418.

Although we have not yet clarified how these irreversible changes in the expression of identified genes modify cellular function, we may speculate about the nature of the functional changes in several of these genes, as described above. Additional studies using primary hepatocytes or immortalized noncancerous hepatocytes will be needed to clarify the biological significance of expressional changes of the identified genes. Such studies would lead to a better understanding of the mechanisms underlying the long-term persistent replication of HCV RNA that account for how such long-term replication modifies gene function in host cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2012.04.008>.

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PML tumor suppressor protein is required for HCV production

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ABSTRACT

PML tumor suppressor protein, which forms discrete nuclear structures termed PML-nuclear bodies, has been associated with several cellular functions, including cell proliferation, apoptosis and antiviral defense. Recently, it was reported that the HCV core protein colocalizes with PML in PML-NBs and abrogates the PML function through interaction with PML. However, role(s) of PML in HCV life cycle is unknown. To test whether or not PML affects HCV life cycle, we examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, stably expressing short hairpin RNA targeted to PML. In this context, the level of secreted HCV core and the infectivity in the supernatants from PML knockdown cells was remarkably reduced, whereas the level of HCV RNA in the PML knockdown cells was not significantly affected in spite of very effective knockdown of PML. In fact, we showed that PML is unrelated to HCV RNA replication using the subgenomic HCV-JFH1 replicon RNA, JRN/3-5B. Furthermore, the infectivity of HCV-like particle in the culture supernatants was significantly reduced in PML knockdown JRN/3-5B cells expressing core to NS2 coding region of HCV-JFH1 genome using the *trans*-packaging system. Finally, we also demonstrated that INI1 and DDX5, the PML-related proteins, are involved in HCV production. Taken together, these findings suggest that PML is required for HCV production.

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1. Introduction

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of the 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 [1,2]. HCV core protein forms a viral capsid and is essential for infectious virion production. The core protein is targeted to lipid droplets. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [3].

In addition, HCV core has been reported to facilitate cellular transformation as well as development of hepatocellular

carcinoma in HCV core-transgenic mice [4]. Interactions of core with tumor suppressor proteins such as p53 and DDX3 may lead to enhanced cellular proliferation [4]. Indeed, HCV core interacts with promyelocytic leukemia (PML) protein and inhibits the PML tumor suppressor pathway through interfering with the PML-mediated apoptosis-inducing function [5]. PML forms discrete nuclear structures termed PML-nuclear bodies (PML-NBs) and associates with several cellular functions, including cell proliferation, apoptosis and antiviral defense [6,7]. In acute promyelocytic leukemia (APL) patient, the PML gene is fused with the retinoic acid receptor- α (RAR α) gene, thus resulting in expression of an oncogenic PML-RAR α fusion protein [6,7]. Conversely, treatment of APL patient with arsenic trioxide leads to reformation of PML-NBs and results in disease remission [6,7], indicating that PML is a target of arsenic trioxide. Interestingly, we have recently demonstrated that arsenic trioxide strongly inhibited HCV infection and HCV RNA replication without cell toxicity [8]. However, the role of PML in HCV life cycle yet remains unclear. To investigate the possible involvement of PML in HCV life cycle, we examined the accumulation of HCV RNA as well as the release of HCV core into culture

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supernatants from cells rendered defective for PML by RNA interference. The results provide evidence that PML is required for HCV production.

2. Materials and methods

2.1. Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). The three HuH-7-derived cell lines: RSc cured cells that cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) [9] could infect and effectively replicate [10–13], OR6c cells is cured cells of OR6 cells harboring the genome-length HCV-O RNA with luciferase as a reporter [14] or OR6c JRN/3-5B cells harboring the subgenome HCV-JFH1 RNA with luciferase as a reporter were cultured in DMEM with 10% FBS as described previously [13].

2.2. RNA interference

Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences targeted to DDX5 in a lentiviral vector: 5'-GATCCCCCTCTAATGTGGAGTGCAGCTTCAAGAGAGTGCAGCTCCACA TTAGAGTTTTGGAAA-3' (sense), 5'-AGCTTTTCCAAAACCTCTAATGT GGAGTGCAGCTCTTGAAGTGCAGCTCCACATTAGAGGGG-3' (antisense). The oligonucleotides above were annealed and subcloned into the *Bgl*III-*Hind*III site, downstream from an RNA polymerase III promoter of pSUPER [15], to generate pSUPER-DDX5i. To construct pLV-DDX5i, the *Bam*HI-*Sall* fragments of the pSUPER-DDX5i were subcloned into the *Bam*HI-*Sall* site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transduced cells [16]. We previously described pLV-PMLi [8] and pLV-INI1i [17], respectively.

2.3. Lentiviral vector production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [18,19]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV- Δ R8.91 [18,19] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pLV-PMLi into 293FT cells with FuGene6 (Roche Diagnostics, Mannheim, Germany).

2.4. HCV infection experiments

The supernatants was collected from cell culture-generated HCV-JFH1-infected RSc cells at 5 days post-infection and stored at -80°C after filtering through a $0.45\ \mu\text{m}$ filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1 virus or J6/JFH1 [20], RSc cells (5×10^4 cells/well) were plated onto 6-well plates and cultured for 24 h (hrs). We then infected the cells at a multiplicity of infection (MOI) of 0.05. The culture supernatants were collected at the indicated time post-infection and the levels of the core protein were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using RNeasy mini kit (Qiagen, Hilden, Germany) for quantitative RT-PCR analysis of intracellular HCV RNA. The infectivity of HCV-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h post-infection.

2.5. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously [14]. We used the following forward and reverse primer sets for the real-time LightCycler PCR: PML, 5'-GAGGAGTTCCAGTTTCT GCG-3' (forward), 5'-GCGCCTGGCAGATGGGGCAC-3' (reverse); DDX5, 5'-ATGTCCGGTTATTCGAGTGA-3' (forward), 5'-TTTCTCC CCAGGGTTTCCAA-3' (reverse); INI1, 5'-ATGATGATGATGGCGCTG AG-3' (forward), 5'-TCGGAACATACGGAGGTAGT-3' (reverse); β -actin, 5'-TGACGGGGTCACCCACTG-3' (forward), 5'-AAGCTGTAG CCGCGCTCGGT-3' (reverse); and HCV-JFH1, 5'-AGAGCCATAGTGGT CTGCGG-3' (forward), 5'-CTTTCGCAACCCAACGCTAC-3' (reverse).

2.6. Western blot analysis

Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-HCV core (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan) or anti- β -actin antibody (Sigma).

2.7. WST-1 assay

RSc or OR6c JRN/3-5B cells (1×10^3 cells/well) were plated onto 96-well plates and cultured. The cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. The absorbance was read using a microplate reader at 440 nm with a reference wavelength of 690 nm.

2.8. Renilla luciferase (RL) assay

OR6c JRN/3-5B cells (1.5×10^4 cells/well) were plated onto 24-well plates and cultured for 72 h, then, subjected to the RL assay according to the manufacturer's instructions (Promega, Madison, WI, USA). A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

2.9. RNA synthesis and transfection

Plasmid pJRN/3-5B was linearized by digestion with *Xba*I and was used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [13]. *In vitro* transcribed RNA was transfected into OR6c cells by electroporation as described previously [14].

2.10. Immunofluorescence and confocal microscopic analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% Nonidet P-40 in PBS at room temperature, and incubated with anti-PML antibody (PM001, MBL) and anti-HCV core at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37°C for 30 min. They were then stained with anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 647-conjugated anti-rabbit antibody (Molecular Probes, Invitrogen) at a 1:300 dilution in PBS containing BSA at 37°C for 30 min. Lipid droplets and nuclei were stained with BODIPY 493/503 (Molecular Probes, Invitrogen) and DAPI (4',6'-diamidino-2-phenylindole), respectively. Following extensive washing in PBS, the cells were mounted on slides using a mounting media of SlowFade Gold antifade reagent (Invitrogen) added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan).

3. Results

3.1. PML is involved in the propagation of HCV

To investigate the potential role(s) of PML in HCV life cycle, we first used lentiviral vector-mediated RNA interference to stably knockdown PML in HuH-7-derived RSc cells that HCV-JFH1 [9] could infect and effectively replicate [10–13]. Real-time RT-PCR analysis for PML demonstrated a very effective knockdown of PML in RSc cells transduced with lentiviral vector expressing shRNA targeted to PML (Fig. 1A). To test the cell toxicity of shRNA, we examined WST-1 assay. In spite of very effective knockdown of PML, we demonstrated that the shRNA targeted to PML did not affect the cell viabilities (Fig. 1B). We next examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in PML knockdown RSc cells 24, 48, or 72 h after HCV-JFH1 infection at an MOI of 0.05. The results showed that the level of HCV RNA in PML knockdown cells was not affected until 72 h post-infection (Fig. 1C), while the release of HCV core protein into the culture supernatants

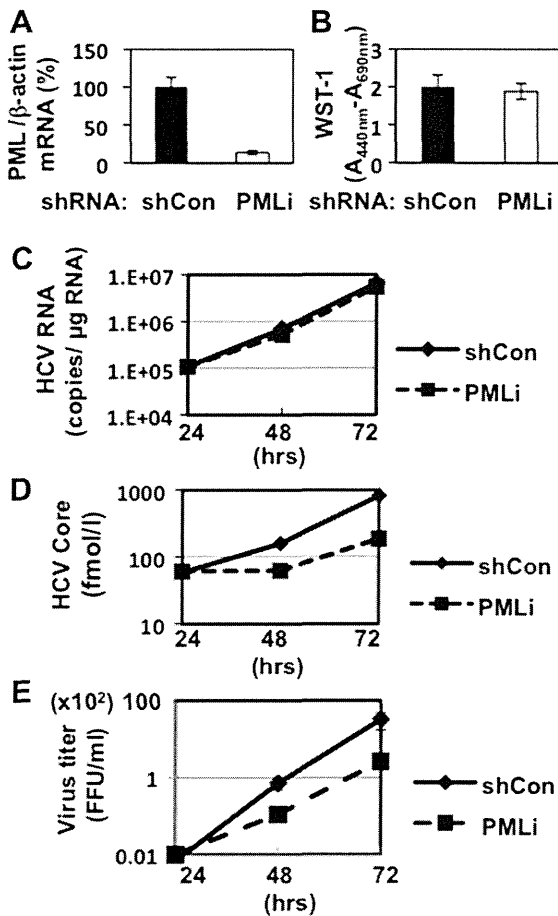


Fig. 1. PML is required for infectious HCV production. (A) Inhibition of PML mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for PML was performed as well as for β -actin mRNA. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon) which was assigned as 100%. (B) WST-1 assay of the PML knockdown (PMLi) or the control (shCon) RSc cells. (C) The levels of intracellular genome-length HCV-JFH1 RNA in the PML knockdown or the control cells at 24, 48 or 72 h post-infection at an MOI of 0.05 were monitored by real-time LightCycler RT-PCR. (D) The levels of HCV core in the culture supernatants from the PML knockdown or the control RSc cells 24, 48 or 72 h after inoculation of HCV-JFH1 were determined by ELISA. (E) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. All experiments were done in triplicate.

was significantly suppressed in PML knockdown cells at 48 or 72 h post-infection (Fig. 1D). Consistent with this finding, the infectivity of HCV in the culture supernatants was also significantly suppressed in the PML knockdown cells at 48 or 72 h post-infection (Fig. 1E). We also obtained similar results using siRNA specific for human PML (siGENOME SMRT pool M-006547-01-0005, Dharmacon, Thermo Fisher Scientific, Waltham, MA) (data not shown). These results suggested that PML is associated with propagation of HCV.

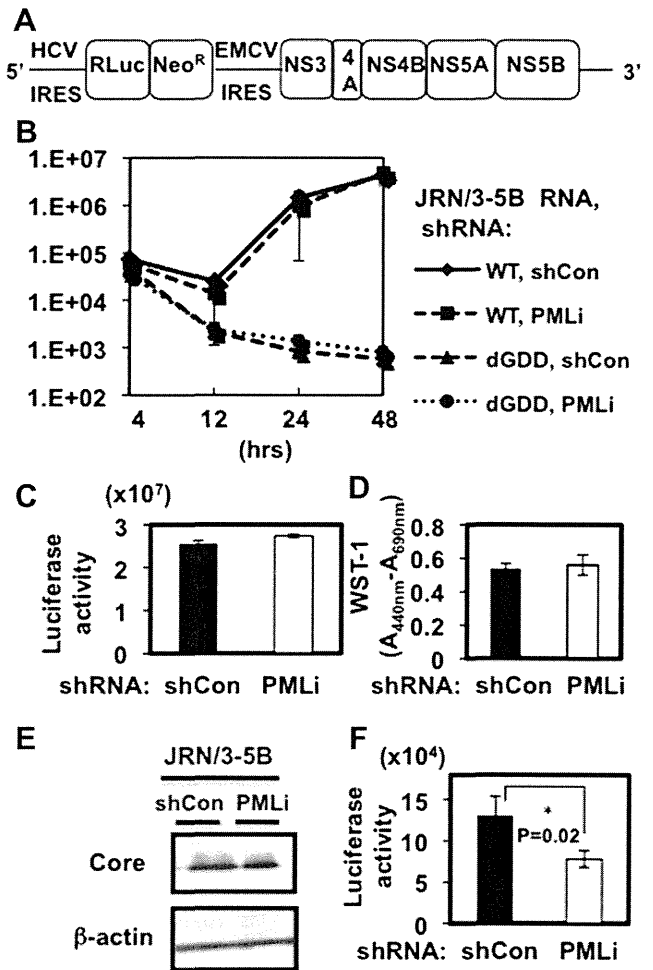


Fig. 2. PML is unrelated to the HCV RNA replication. Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding *Renilla* luciferase (RL) gene. *Renilla* luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with Neo. (B) The transient replication of subgenomic HCV-JFH1 replicon in the PML knockdown (PMLi) or the control OR6c cells (shCon) after electroporation of *in vitro* transcribed JRN/3-5B RNA (10 μ g) was monitored by RL assay at the indicated time. The results of *Renilla* luciferase activity are shown. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the subgenomic HCV replicon with the deletion of GDD was used as a negative control. (C) The level of HCV RNA replication in PML knockdown (PMLi) or the control (shCon) OR6c JRN/3-5B cells was monitored by RL assay. The results shown are means from three independent experiments. (D) WST-1 assay of the PML knockdown or the control JRN/3-5B cells. (E) The level of HCV core protein in OR6c JRN/3-5B cells by expression of HCV core to NS2 coding region of HCV-JFH1 using mouse retroviral vector. pCX4bsr-JFH1-myc-C-NS2 and pMDG2 were cotransfected into Plat-E cells, mouse retroviral packaging cells. Mouse retroviral vector was obtained from their culture supernatants and transduced into OR6c JRN/3-5B PML knockdown or the control cells. The results of Western blot analysis of cellular lysates with anti-HCV core or an anti β -actin antibody are shown. (F) The level of HCV RNA replication in RSc cells 72 h after inoculation of HCV-like particles produced using *trans*-packaging system was monitored by RL assay. Asterisk indicates significant difference compared to the control. *P = 0.02.