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 μM (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 μg) was reverse-transcribed with M-MLV reverse trascriptase (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	GCATTCAAGTTCTACCCAACCGAC	258	Brain abundant, membrane attached signal protein 1 (BASP1; NM_006317)	Forward	GGATGAATGCCAGCTTTCAGACAG	247
	Reverse	GGCTGCTGACAACAGCTGACTC			Reverse	ACTGGAACTGCAATGAACGCAGAC	
Angiopoietin 1 (ANGPT1; NM_001146)	Forward	ATACAACATCGTGAAGATGGAAGTC	287	Cell death activator CIDE-3 (CIDEC; 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	GGAACTGTCTCTAGTAGCCAGTG	242
	Reverse	CATAGAGCCTGGCCAAATCACAG			Reverse	CAGCGGCAAAAGCTTCTCTACAG	
Phospholipase A1 member A (PLA1A; NM_015900)	Forward	GGAGTTTCACTTGAAGGAACTGAG	292	Heat shock 70 kDa protein B' (HSPA6; NM_002155)	Forward	TGAAGCCGAGCAGTACAAGGCTG	235
	Reverse	GTTCACTGGTTCAGGTAAGCAGAC			Reverse	CTCCCTCTTCTGATGCTCATACTC	
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	GCCTGTTCCTCTACGTAGCACTC	158	Peptidase inhibitor 3 (PI3; NM_002638)	Forward	GGTTCTAGAGGCAGCTGTCACG	241[b]
	Reverse	GAAGGTGATGTCATCCTCGTACAG			Reverse	GCAGTCAGTATCTTTCAAGCAGC	
TBC1 domain family, member 4 (TBC1D4; NM_014832)	Forward	GGAGAGGGCCAATAGCCAACTG	198	Solute carrier family member 3 (SLC1A3; NM_004172)	Forward	CAATGGCGTGGACAAGCGCGTC	240
	Reverse	AGCTTCCGGAGTTGCTCCACTG			Reverse	CCGACAGATGTCAGCACAATGAC	
WNT1 inducible signaling pathway protein 3 (WISP3; NM_003880)	Forward	AGAGATGCTGTATCCCTAATAAGTC	129	Thrombospondin type-1 domain-containing protein 4 (THSD4; NM_024817)	Forward	TGGAGTCAGTGTTCCATCGAGTG	275
	Reverse	CAGGTTCTCTGCAGTTTCTCTGAC			Reverse	GGGTCACAGAGGTTACTTAGAGTC	
Annexin A1 (ANXA1; NM_000700)	Forward	GACTTGGCTGATTCAGATGCCAG	192	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046)	Forward	GACTCATGACCACAGTCCATGC	334
	Reverse	AATGTCACCTTTCAACTCCAGGTC			Reverse	GAGGAGACCACCTGGTGCTCAG	
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).

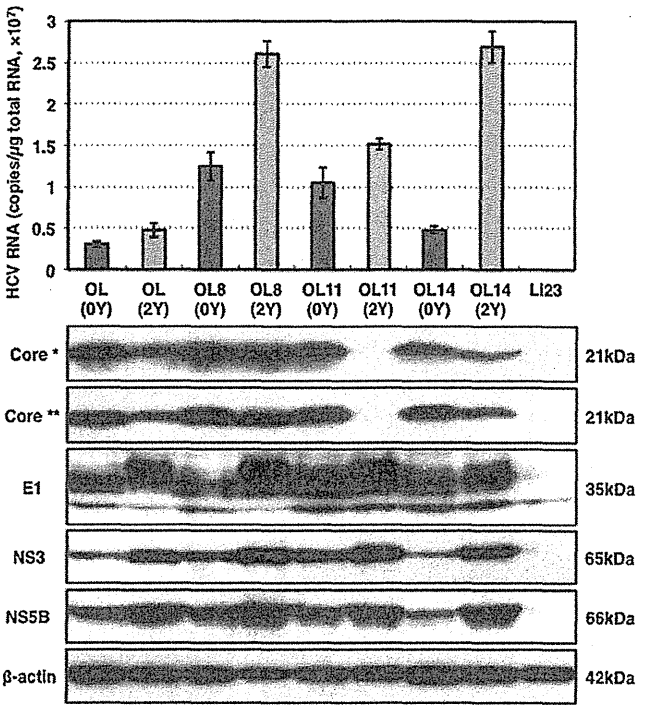

Fig. 1. Characterization of genome-length HCV RNA-replicating cells in long-term cell culture. The upper panel shows the results of a quantitative RT-PCR analysis of intracellular genome-length HCV RNA. Total RNAs from OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells after 2 years [OL(2Y), OL8(2Y), OL11(2Y), 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.

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 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.

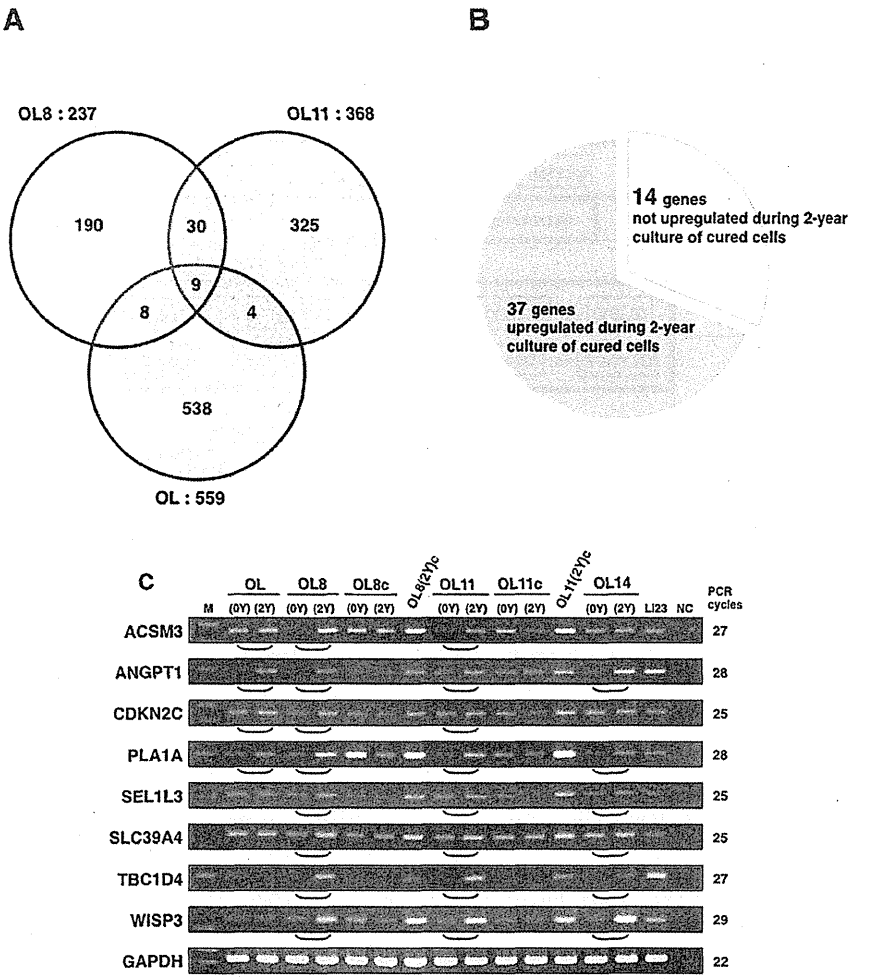

Fig. 3. Identification of genes irreversibly upregulated during 2-year replication of HCV RNA. (A) Upregulated genes obtained by microarray analysis I shown in Fig. 2. Genes whose expression levels were upregulated at ratios of more than 2 in the case of OL(0Y) versus OL(2Y) cells, OL8(0Y) versus OL8(2Y) 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 [*CIDEC*], 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*, *CIDEC*, *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),

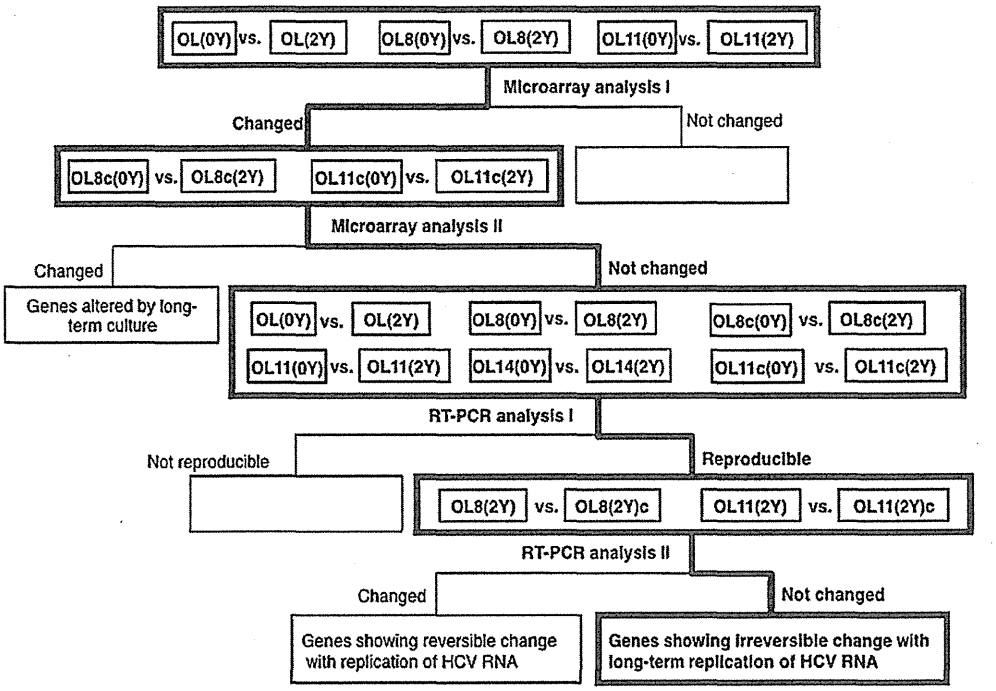

Fig. 2. Outline of selection process performed in this study. To obtain the objective genes, cDNA microarray analyses I and II were performed, and then RT-PCR analyses I and II were also performed.

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

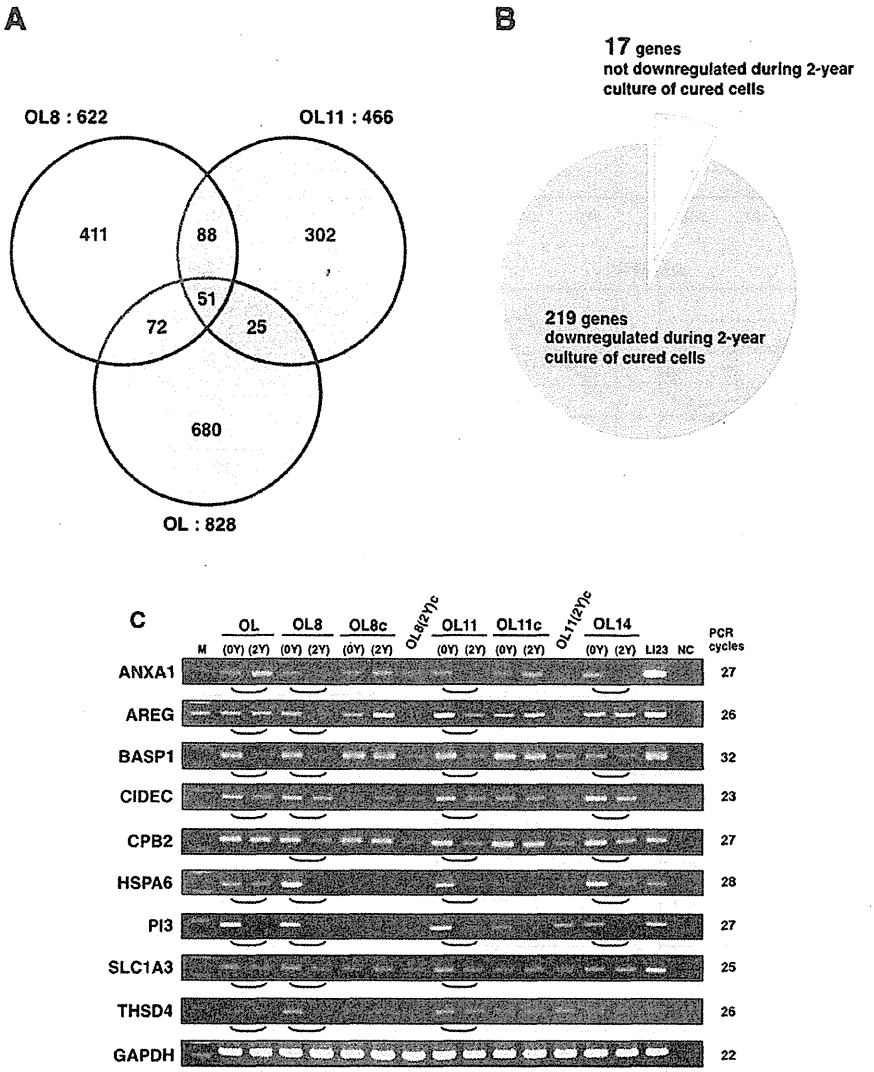

Fig. 4. Identification of genes irreversibly downregulated during 2-year replication of HCV RNA. (A) Downregulated genes obtained by microarray analysis I shown in Fig. 2. Genes were selected whose expression levels were downregulated at ratios of less than 0.5 in the case of OL(0Y) versus OL(2Y) cells, 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

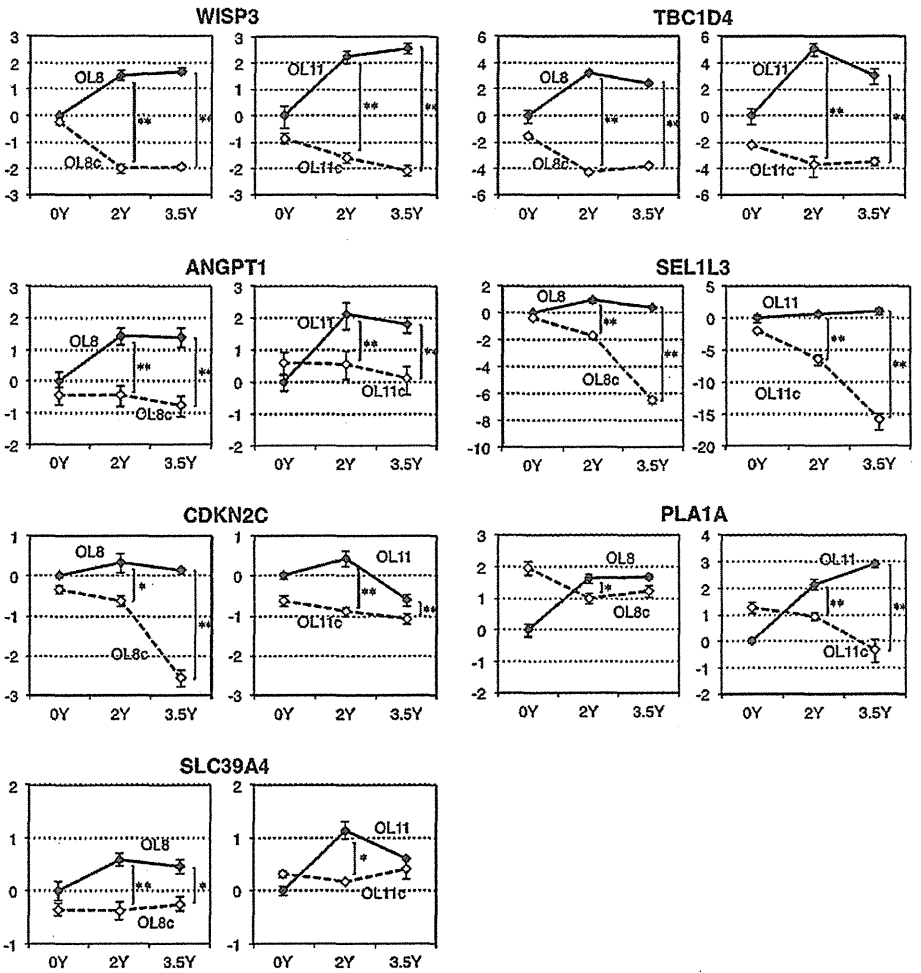

Fig. 5. Expression levels of genes selected as upregulated genes in 3.5-year cell culture. Quantitative RT-PCR analysis using the total RNAs derived from OL8(0Y), OL8(2Y), OL8(3.5Y), OL8c(0Y), OL8c(2Y), OL8c(3.5Y), OL11(0Y), OL11(2Y), OL11(3.5Y), OL11c(0Y), OL11c(2Y), and OL11c(3.5Y) cells was performed 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*, *CIDEC*, *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

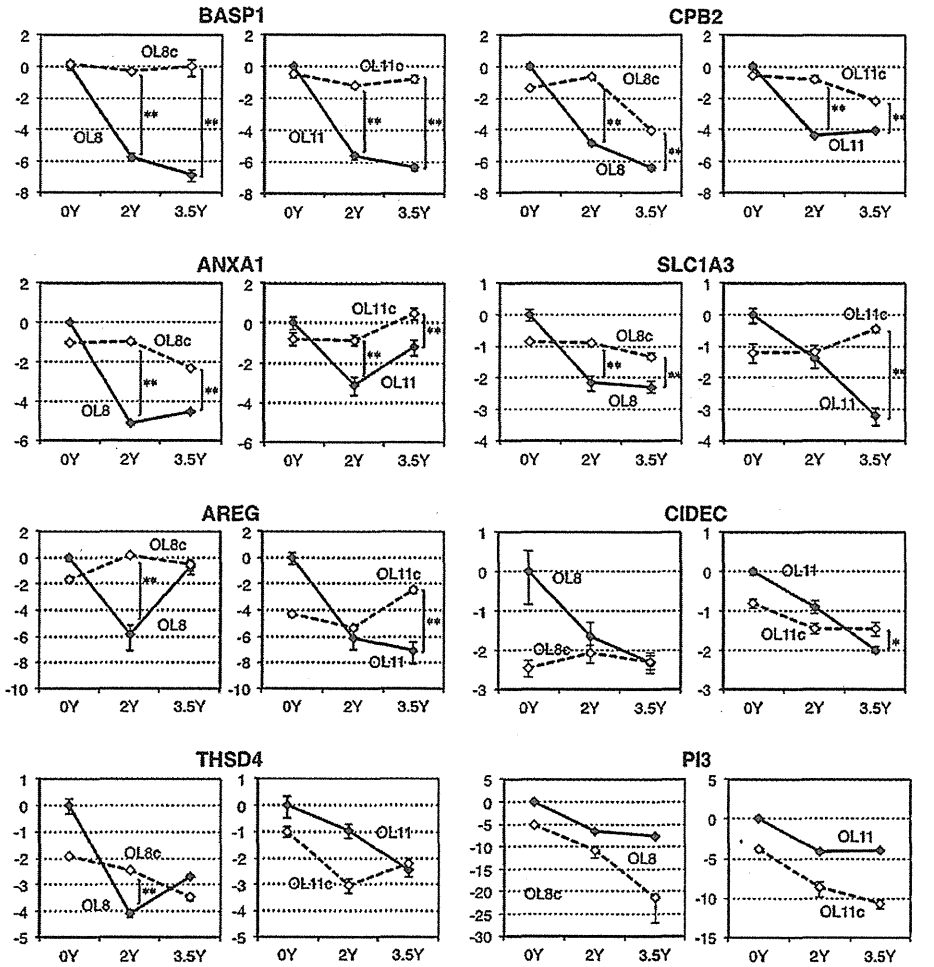

Fig. 6. Expression levels of genes selected as downregulated genes in 3.5-year cell culture. Quantitative RT-PCR analysis was performed as shown in Fig. 5, and the obtained results are also presented as shown in Fig. 5.

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 potently 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-κB 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-κB activity by the binding of ANXA1 to the p65 subunit of NF-κB 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.

Acknowledgements

We thank N. Kawahara, T. Nakamura, and K. Takeshita for their technical assistances. This work was supported by grants-in-aid for research on hepatitis from the Ministry of Health, Labor, and

Welfare of Japan. K.M. was supported by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science.

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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Raloxifene inhibits hepatitis C virus infection and replication

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ARTICLE INFO

Article history:
Received 21 May 2012
Received in revised form 27 July 2012
Accepted 8 August 2012

Keywords:
Hepatitis C virus
Raloxifene
Estrogen
Osteoporosis
Statin

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-α. These results suggest a link between the molecular biology of osteoporosis and the HCV life cycle.

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-O 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 17β-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 ERα or ERβ. 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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2211-5463/$36.00
http://dx.doi.org/10.1016/j.fob.2012.08.003

2. Materials and methods

2.1. Reagents and antibodies

Raloxifene was purchased from LKT Laboratories, Inc. (St. Paul, MN). IFN-α and tamoxifen were purchased from Sigma–Aldrich (St. Louis, MO). Pitavastatin (PTV) was purchased from Kowa Company (Nagoya, Japan). The antibodies used in this study were those specific to HCV Core (CP11, Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, Newcastle, UK), and β-actin (Sigma).

2.2. Cell culture and HCV RNAs

HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. HuH-7-derived OR6 and sOR cells were genome-length and subgenome HCV (O strain of genotype 1b) RNA harboring cells, respectively and cultured in the above medium supplemented with G418 (0.3 mg/ml; Geneticin, Invitrogen) [4]. HCVs replicating in OR6 and sOR cells contain *RL* and neomycin phosphotransferase (*NPT*) genes after 5′-untranslated region (UTR). HuH-7-derived RSc cells are cured cells, in which HCV RNA was eliminated by IFN-α; they are used for HCV JFH-1 infection [9]. RSc cells are also used for subgenomic JFH-1 RNA (JRN/35B) replication. JRN/35B contains *RL* and *NPT* genes after 5′-UTR.

2.3. RL assay

For the RL assay, 1.5×10^4 OR6 were plated onto 24-well plates in triplicate and cultured for 24 h. The cells were treated with each reagent for 72 h. Then the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol.

2.4. WST-1 cell proliferation assay

The cells (2×10^3 cells) were plated onto a 96-well plate in triplicate at 24 h before treatment with each reagent. At 72 h after treatment, the cells were subjected to a WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol.

2.5. Western blot analysis

For Western blot analysis, 4×10^4 cells were plated onto 6-well plates, cultured for 24 h, and then treated with reagent(s) for 72 h and 120 h. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were then performed as previously described [24]. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

2.6. HCV infection

RSc cells (1.5×10^4 cells) were plated onto a 24-well plate 24 h before infection. To evaluate the effect of the treatment prior to infection, the cells were first treated with raloxifene for 24 h, then inoculated with reporter JFH-1 (JR/C5B/BX-2) supernatant at a multiplicity of infection (MOI) of 0.2, cultured for 48 h, and subjected to RL assay as described previously [9]. The JR/C5B/BX-2 contains the *RL* gene in the first cistron following the encephalomyocarditis virus-internal ribosomal entry site (*EMCV-IRES*) gene and the open reading frame (ORF) of JFH-1 in the second cistron. To evaluate the effect of the treatment after infection, the cells were inoculated with reporter JFH-1 supernatant at MOI of 0.2, cultured for 72 h, and subjected to RL assay.

3. Results

3.1. Raloxifene inhibited HCV RNA replication

The HCV RNA that replicated in HuH-7-derived OR6 cells was a genome-length HCV with *RL*, *NPT*, and *EMCV-IRES* in the first cistron and the ORF of HCV (O strain of genotype 1b) in the second cistron [4]. OR6 cells could not produce infectious HCV. Therefore, we can monitor the replication step in the HCV life cycle using OR6 cells. Raloxifene inhibited HCV RNA replication in a dose-dependent manner, and its 50% effective concentration (EC_{50}) was 1 μM (Fig. 1A). Raloxifene did not exhibit cytotoxicity to OR6 cells until 2.5 μM (Fig. 1B). Raloxifene also inhibited intracellular Core and NS3 production in a dose- and time-dependent manner (Fig. 1C). The intensities of Core and NS3 in OR6 cells treated with 2.5 μM of raloxifene decreased to almost the level of cells treated with 10 IU/ml of IFN-α at 120 h after treatment. We also examined anti-HCV activity of raloxifene using subgenomic HCV replicon harboring sOR cells. Raloxifene exhibited weak anti HCV activity to sOR cells as compared with OR6 cells (Supplementary Figs. 1A and 1B). These results suggest that raloxifene exhibits anti-HCV activity and decreased the expression levels of HCV proteins more slowly compared to IFN-α.

3.2. Raloxifene enhanced anti-HCV activity of IFN-α

We investigated the anti-HCV activity of raloxifene in combination with a representative anti-HCV reagent, IFN-α. HCV RNA replication decreased in a dose-dependent manner after co-treatment with IFN-α and raloxifene (Fig. 2A). The results were almost similar to the expected effect of raloxifene in combination with IFN-α calculated from the anti-HCV activity of each reagent (Fig. 2B). These results indicate that the anti-HCV activity of raloxifene and IFN-α exhibited additive effect. We also examined the anti-HCV activity of previously reported SERM, tamoxifen. Tamoxifen also exhibited additive anti-HCV activity on HCV RNA replication in combination with IFN-α (Supplementary Figs. 2A–C). These results indicate that raloxifene as well as tamoxifen enhanced the anti-HCV activity of IFN-α. As both raloxifene and IFN-α are clinically used reagents, raloxifene seemed to be a candidate reagent as an add-on treatment to IFN-α in patients with CH–C.

3.3. Raloxifene antagonized anti-HCV activity of statin

We previously reported that statins exhibited anti-HCV activity using the OR6 assay system [14]. Statin is the first-choice reagent for dyslipidemia. As dyslipidemia and osteoporosis are major complications in postmenopausal women, we invested the effect of raloxifene on the anti-HCV activity of PTV. Raloxifene did not enhance the anti-HCV activity of PTV (Fig. 3A). Fig. 3B exhibits the expected anti-HCV activity of co-treatment with raloxifene and PTV calculated from the anti-HCV effect of either raloxifen or PTV alone. Raloxifene exhibited an antagonistic effect on PTV's anti-HCV activity. Raloxifene's antagonistic effect on PTV increased dose-dependently. The co-treatment with raloxifene (2.5 μM) and PTV (0.25, 0.5, and 1 μM) resulted in lower anti-HCV activity than did treatment with raloxifene alone (2.5 μM). These results suggest that we should be careful in the administration of statins with raloxifene to postmenopausal woman with CH–C.

3.4. Raloxifene inhibited infection of genotype 2a HCV

To further investigate the anti-HCV activity of raloxifene, we examined whether or not raloxifene could inhibit HCV infection. For this purpose, we used our recently developed JFH-1 reporter infection assay system [9]. HuH-7-derived RSc's are highly HCV-permissive cell lines. Raloxifene was pretreated at 24 h before HCV infection. The cells were inoculated with HCV JFH-1 virion with *RL* (JR/C5B/BX-2), and

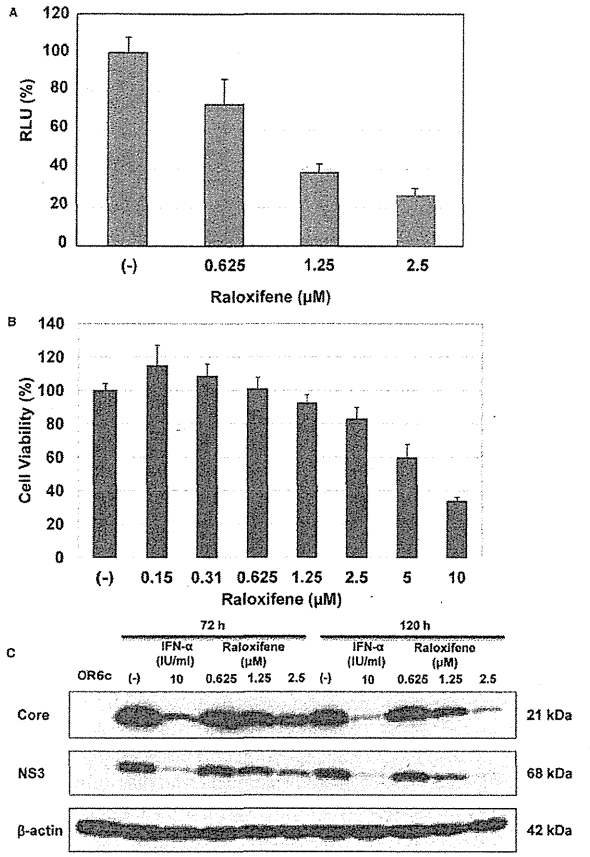

Fig. 1. Raloxifene inhibited HCV RNA replication. (A) Anti-HCV activity of raloxifene in OR6 cells. OR6 cells were treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) for 72 h. Relative RL activity (relative light unit: RLU) for HCV RNA replication is expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Effect of raloxifene on OR6 cell viability. Cell viability at 72 h after raloxifene treatment (0.15, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) was determined using WST-1 cell proliferation assay and is expressed as a percentage of control. (C) Raloxifene inhibited HCV proteins. OR6 cells were treated with IFN-α (10 IU/ml) or raloxifene (0, 0.625, 1.25, and 2.5 μM). After 72 or 120 h treatment, the production of Core and that of NS3 were analyzed by immunoblotting using anti-Core and anti-NS3 antibodies, respectively. OR6c cells were cured cells in which HCV RNA was eliminated using IFN-α, and were used as a negative control. β-actin was used as a control for the amount of protein loaded per lane.

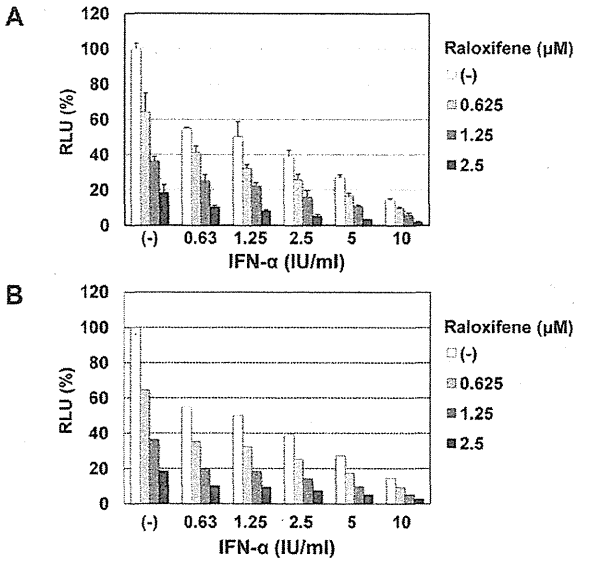

Fig. 2. Raloxifene enhanced the anti-HCV activity of IFN-α. (A) Anti-HCV activity of raloxifene in combination with IFN-α. OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and IFN-α (0, 0.63, 1.25, 2.5, 5, 10 IU/ml). Relative RL activity is shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Expected anti-HCV activity was calculated based on the results when the cells were treated with only raloxifene or IFN-α.

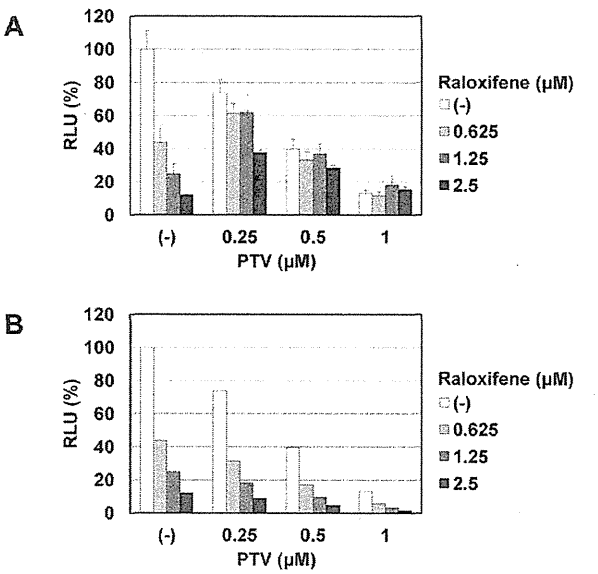

Fig. 3. Statin antagonized the anti-HCV activity of raloxifene. (A) OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and PTV (0, 0.25, 0.5, and 1 μM). Relative RL activity was shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (B) Expected anti-HCV activity was calculated based on the results when the cells were treated with only raloxifene or PTV.

the infection was monitored with RL activity at 48 h after infection. As shown in Fig. 4A, raloxifene inhibited HCV infection in RSc cells in a dose-dependent manner. Next we examined the effect of raloxifene after HCV infection. RSc cells were inoculated with HCV JFH-1 virion with *RL*. After HCV infection, the cells were treated with raloxifene for 72 h and raloxifene's inhibitory effect on post-infection was assessed using the RL assay. Raloxifene inhibited HCV proliferation in a dose-dependent manner when it was added to the cells after infection in RSc cells, although inhibitory effect of raloxifene on JFH-1 HCV RNA replication seemed to be weak compared to the genotype 1b HCV-O RNA replication (Fig. 4B). Raloxifene did not exhibit cytotoxicity to RSc cells until 2.5 μM (Fig. 4C). We found that raloxifene could not inhibit subgenomic JFH-1 HCV (JRN/35B) RNA replication (Fig. 4D). We further examined the inhibitory action of raloxifene around infection step. RSc cells were treated for short time with raloxifene around infection step: for 1, 4, and 4 h before, during, and after inoculation, respectively (Fig. 4E). Raloxifene inhibited JFH-1 infection, when it was treated during inoculation but not just before or after inoculation. In case of genotype 2a JFH-1, raloxifene's anti-HCV activity is mainly due to the inhibition of infection. These results indicate that raloxifene inhibits JFH-1 infection but not its RNA replication.

4. Discussion

In this study, we demonstrated that raloxifene, an osteoporotic reagent, inhibited the replication of genotypes 1b HCV RNA replication and inhibited genotype 2a HCV JFH-1 infection. Raloxifene additively enhanced the anti-HCV activity of IFN-α. On the other hand, raloxifene exhibited an antagonistic effect on statins.

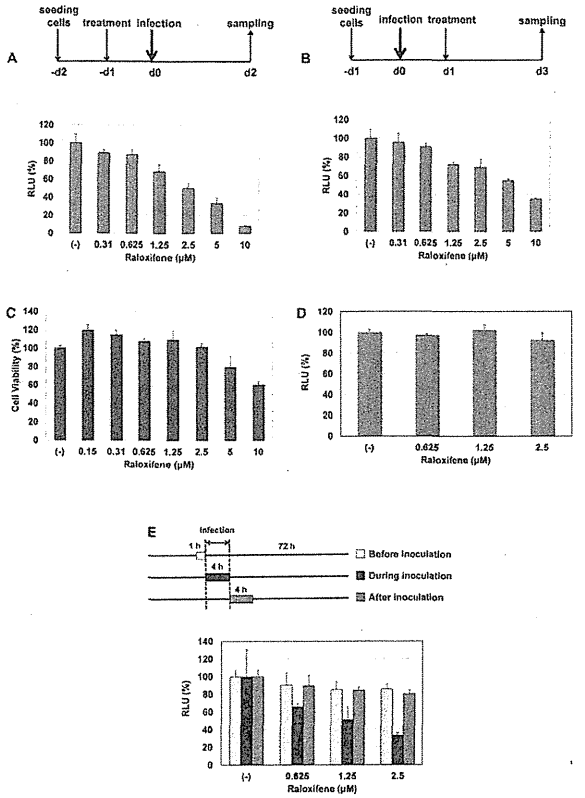

Fig. 4. Raloxifene inhibited genotype 2a HCV infection. (A) Raloxifene inhibited HCV JFH-1 infection. RSc cells were treated with raloxifene (0, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) 24 h before infection. HCV JFH-1 reporter virion was used as an inoculum after removal of raloxifene. The cells were then infected with reporter JFH-1 virion and cultured for 48 h. The inhibition of HCV infection was assessed by relative RL activity and expressed as a percentage of control. (B) Raloxifene inhibited HCV JFH-1 proliferation after infection. RSc cells were inoculated with HCV JFH-1 reporter virion and cultured for 24 h. Then the cells were treated with raloxifene (0, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) for 48 h. The inhibitory effect on HCV proliferation after infection was assessed by relative RL activity and expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (C) Effect of raloxifene on RSc cells viability. Cell viability at 72 h after raloxifene treatment (0.15, 0.31, 0.625, 1.25, 2.5, 5, and 10 μM) was determined using WST-1 cell proliferation assay and is expressed as a percentage of control. (D) Subgenomic JFH-1 RNA (JRN/35B) replicating RSc cells were treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) for 72 h. Relative RL activity for HCV RNA replication is expressed as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. (E) Raloxifene (0, 0.625, 1.25, and 2.5 μM) was treated for 1, 4, and 4 h before, during, and after JFH-1 inoculation to RSc cells at MOI of 0.2, respectively. The cells were then cultured for 72 h. The inhibition of HCV infection was assessed by relative RL activity and expressed as a percentage of control.

PEG-IFN/RBV therapy led to a 40–50% SVR rate among patients with CH–C. Telaprevir with PEG-IFN/RBV increases the effect of PEG-IFN/RBV therapy by 10–20%. However, the major complication of anemia in PEG-IFN/RBV therapy increased when telaprevir was added. Considering that PEG-IFN/RBV-based therapy is less effective on postmenopausal women, an alternative therapy with minimal side effects is needed. Add-on therapy for postmenopausal women may be a candidate for improving the SVR in these patients. We focused on the reagents, which compensate for the lack of estrogen function. Dyslipidemia and osteoporosis are the major complications in postmenopausal women, and these complications are attributable to the decrease in estrogen. Statins are clinically used reagents for dyslipidemia; they inhibit HCV RNA replication in vivo as well as in vitro [13–17]. Therefore, we investigated whether or not raloxifene exhibits anti-HCV activity using genotype 1b HCV RNA replication and genotype 2a infection systems. In the HCV life cycle, raloxifene inhibited genotype 2a HCV infection and genotypes 1b HCV RNA replication. Raloxifene may be a potential reagent with different anti-HCV mechanisms in the HCV life cycle. Further study is needed to clarify these underlying mechanisms.

Recently it was reported that vitamin D3, an osteoporotic reagent, inhibited HCV production in cell culture systems [20,21]. Furthermore, it was reported that vitamin D3 was associated with the effect of therapy for patients with CH–C [18,19]. Statins inhibited HCV RNA replication by suppressing geranylgeranyl pyrophosphate (GGPP) production [14]. Another osteoporotic reagent, bisphosphonate, may possess anti-HCV activity, because it also inhibited the biosynthesis of GGPP in the mevalonate pathway by inhibiting farnesyl pyrophosphate synthetase. Taken together, these findings indicate it is likely that the HCV life cycle is associated with osteoporosis.

Raloxifene and tamoxifen are SERMs for osteoporosis and breast cancer, respectively. Tamoxifen is used for estrogen receptor-positive breast cancer, and it inhibits HCV RNA replication in cell culture [23]. Tamoxifen's anti-HCV activity is associated with ERα. In our study, raloxifene inhibited HCV infection as well as replication. To clarify the multi-potential effects of raloxifene, further study is needed. The incidence of side effects including uterine cancer is lower in raloxifene therapy than in tamoxifen therapy [25]. This is another advantage of raloxifene in clinical use for patients with CH–C.

As for the precise role of ERα or ERβ on the HCV life cycle, we could not reach a clear conclusion because microarray analysis revealed an absence of expression for both ERα and ERβ in OR6 cells (data not shown). Hayashida et al. [22] reported that the most potent physiological estrogen, 17-β-estradiol, inhibited infectious HCV production using HuH-7.5 cells, and that ERα-selective agonist inhibited infectious HCV production whereas ERβ-selective agonist did not. Watashi et al. [23] reported that RNA interference-mediated knockdown of ERα reduced HCV RNA replication. In our study, the anti-HCV activity of raloxifene in infection and replication did not seem attributable to ERα or ERβ. It is not clear why our HuH-7-derived OR6 cells did not express ERα or ERβ. HuH-7 cells were developed in 1982 at Okayama University and distributed worldwide [26]. Recently, Bensadoun et al. [27] reported that the genetic background of the IL28B genotype of HuH-7 cells differed among different laboratories. This may be a consequence of the polyploidal nature of hepatoma cells. A similar mechanism might cause the different expression levels of ERα and ERβ. Another ER, GPR30 [28], was expressed in OR6 cells (data not shown; from microarray analysis). GPR30 may be the responsible host factor for anti-HCV activity in OR6 cells. Further study is needed to clarify this issue.

In conclusion, we found that raloxifene inhibited HCV RNA replication in genotype 1b and infection in genotype 2a. Raloxifene additively enhanced the anti-HCV activity of IFN-α. The antagonistic effects of statins and raloxifene will yield information on the clinical use of these reagents. Our results, as well as the reports of vitamin D3's anti-HCV activity, will open new fields of treatment for both osteoporosis and HCV infection.

Acknowledgments

The authors would like to thank Masayo Takemoto for her technical assistance. This work was supported by a Grant-In-Aid for Research on Hepatitis from the Ministry of Health, Labor and Welfare of Japan.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.08.003.

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Self-Enhancement of Hepatitis C Virus Replication by Promotion of Specific Sphingolipid Biosynthesis

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Abstract

Lipids are key components in the viral life cycle that affect host-pathogen interactions. In this study, we investigated the effect of HCV infection on sphingolipid metabolism, especially on endogenous SM levels, and the relationship between HCV replication and endogenous SM molecular species. We demonstrated that HCV induces the expression of the genes (*SGMS1* and *2*) encoding human SM synthases 1 and 2. We observed associated increases of both total and individual sphingolipid molecular species, as assessed in human hepatocytes and in the detergent-resistant membrane (DRM) fraction in which HCV replicates. SGMS1 expression had a correlation with HCV replication. Inhibition of sphingolipid biosynthesis with a hepatotropic serine palmitoyltransferase (SPT) inhibitor, NA808, suppressed HCV-RNA production while also interfering with sphingolipid metabolism. Further, we identified the SM molecular species that comprise the DRM fraction and demonstrated that these endogenous SM species interacted with HCV nonstructural 5B polymerase to enhance viral replication. Our results reveal that HCV alters sphingolipid metabolism to promote viral replication, providing new insights into the formation of the HCV replication complex and the involvement of host lipids in the HCV life cycle.

Citation: Hirata Y, Ikeda K, Sudoh M, Tokunaga Y, Suzuki A, et al. (2012) Self-Enhancement of Hepatitis C Virus Replication by Promotion of Specific Sphingolipid Biosynthesis. PLoS Pathog 8(8): e1002860. doi:10.1371/journal.ppat.1002860

Editor: Aleem Siddiqui, University of California, San Diego, United States of America

Received January 4, 2012; **Accepted** June 27, 2012; **Published** August 16, 2012

Funding: This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation of Japan; and the Ministry of Health, Labor, and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: M. Sudoh, A. Katsume, K. Okano, K. Ozeki, K. Kawasaki, T. Tsukuda, and Y. Aoki are employees of Chugai Pharmaceutical Co. Ltd. This does not alter our adherence to all PLoS Pathogens policies on sharing data and materials.

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Introduction

Lipids have long been known to play dual roles in biological systems, functioning in structural (in biological membranes) and energy storage (in cellular lipid droplets and plasma lipoproteins) capacities. Research over the past few decades has identified additional functions of lipids related to cellular signaling, microdomain organization, and membrane traffic. There are also strong indications of the important role of lipids in various stages of host-pathogen interactions [1].

Sphingomyelin (SM) is a sphingolipid that interacts with cholesterol and glycosphingolipid during formation of the raft domain, which can be extracted for study as a detergent-resistant membrane (DRM) fraction [2]. Recently, raft domains have drawn attention as potential platforms for signal transduction and pathogen infection processes [3,4]. For instance, raft domains may serve as sites for hepatitis C virus (HCV) replication [5,6]. Additionally, *in vitro* analysis indicates that synthetic SM binds to the nonstructural 5B polymerase (RdRp) of HCV [7]. This association allows RdRp to localize to the DRM fraction (known to be the site of HCV replication) and activates RdRp, although the degree of binding and activation differs among HCV genotypes [7,8]. Indeed, suppression of SM biosynthesis with a serine palmitoyltransferase (SPT) inhibitor disrupts the association between RdRp and SM in the DRM fraction, resulting in the suppression of HCV replication [7,9].

Multiple reports have indicated that HCV modulates lipid metabolism (e.g., cholesterol and fatty acid biosynthesis) to promote viral replication [10–12]. However, the effect of HCV infection on sphingolipid metabolism, especially on endogenous SM levels, and the relationship between HCV replication and endogenous SM molecular species remain to be elucidated as there are technical challenges in measuring SM levels (for both total and individual molecular species) in hepatocytes.

To address these questions, we first utilized mass spectrometry (MS)-based techniques and analyzed uninfected and HCV-

Author Summary

One of the key components for hepatitis C virus (HCV) propagation is lipids, some of which comprise membranous replication complexes for HCV replication. Research on cofactors that are involved in the formation of the membranous replication complex has advanced steadily; on the other hand, the lipids constituting the membranous replication complex remain to be elucidated. Here, we report that HCV modulates sphingolipid metabolism by promoting sphingolipid biosynthesis, to enhance viral replication. Specifically a specific molecular species of sphingomyelin (SM), a type of sphingolipid interacts with HCV nonstructural 5B polymerase, enhancing HCV replication. This work highlights the relationship between specific molecular species of SMs and HCV replication, giving new insight into the formation of the HCV replication complex and the involvement of host lipids in the HCV life cycle.

infected chimeric mice harboring human hepatocytes. Second, we developed a hepatotropic SPT inhibitor, NA808, and used this tool to elucidate the effects of inhibition of sphingolipid biosynthesis on hepatocyte SM levels. Third, we tested the inhibitor's anti-HCV activity in humanized chimeric mice, and demonstrated the relationship between HCV and endogenous SM in human hepatocytes. Finally, we identified the endogenous SM molecular species carried by the DRM fraction, defining the association between these molecular species and HCV replication.

Results

HCV upregulates SM and ceramide levels in hepatocytes of humanized chimeric mice

First, we examined the effects of HCV infection on SM biosynthesis in hepatocytes using humanized chimeric mice. The study employed a previously described mouse model (SCID/uPA) into which human hepatocytes were transplanted (see Materials and Methods). The average substitution rate of the chimeric mouse livers used in this study was over 80% [13], and HCV selectively infected human hepatocytes. This model supports long-term HCV infections at clinically relevant titers [13,14]. Indeed, the HCV-RNA levels reached (at 4 weeks post-infection) 10^8–10^9 copies/mL in the genotype 1a group (**Figure 1A**) and 10^6–10^7 copies/mL in the genotype 2a group (**Figure 1B**).

Once serum HCV-RNA levels had plateaued, we observed elevated expression of the genes (*SGMS1* and *2*) encoding human SM synthases 1 and 2; this pattern was HCV-specific, as demonstrated by the fact that the increase was not seen in hepatitis B virus-infected mice (**Figure 1C and Figure S1**). SM synthases convert ceramide to SM, so we next examined SM and ceramide levels in hepatocytes of both HCV-infected and uninfected chimeric mice. SM and ceramide levels were assessed using MS spectrometry, which allows analysis of samples at the single lipid species level as well as at the whole lipidome level. MS analysis showed that the level of ceramide, the precursor to SM, was increased in hepatocytes obtained from chimeric mice infected with HCV of either genotype (**Figure 1D**). Further, MS analysis showed that infection of chimeric mice with HCG9 (genotype 1a) was associated with increased SM levels in hepatocytes (**Figure 1E**). Similarly, SM levels were elevated in the hepatocytes of HCR24 (genotype 2a)-infected chimeric mice. These results indicate that infection with HCV increases total SM and ceramide levels in human hepatocytes.

MS analysis was conducted to determine which of several molecular species of SM [15] are present in HCV-infected hepatocytes. SM molecular species were analyzed in extracts obtained from a human hepatocyte cell line (HuH-7 K4) and from hepatocytes derived from the humanized chimeric mice. We identified four major peaks as SM molecular species (*d*18:1-16:0, *d*18:1-22:0, *d*18:1-24:0, and *d*18:1-24:1), and other peaks as phosphatidylcholine (**Figure 1F**). Infection-associated increases were seen for all ceramide molecular species, with significant changes in three of four species (excepting *d*18:1-16:0; $p<0.05$) with genotype 1a, and in all four species with genotype 2a ($p<0.05$) (**Figure 1G**). Upon infection with HCV of either genotype, hepatocytes tended to show increased levels of all four identified SM molecular species, but the changes were significant only for one species (*d*18:1-24:1; $p<0.05$) in genotype 1a and for two species (*d*18:1-16:0 and *d*18:1-24:1; $p<0.01$) in genotype 2a (**Figure 1H**). In cell culture, negligible amount of SM was likely increased by HCV infection. With respect to each molecular species, d18:1-16:0 SM was likely increased by HCV infection (**Figure S2**). These results indicate that HCV infection increases the abundance of several SM and ceramide molecular species.

Relationship between the SGMS genes and HCV infection

To clarify the relationship between SGMS1/2 and HCV, we investigated the correlation between SGMS1/2 expression and liver HCV-RNA in humanized chimeric mice. We found that SGMS1, but not SGMS2, had a correlation with liver HCV-RNA in HCV-infected humanized chimeric mice (**Figures 2A and 2B**).

Next, to clarify whether HCV infection of human hepatocytes increases the expression of the genes (*SGMS1* and *SGMS2*), we examined the effect of silencing HCV genome RNA on the expression of these genes in HCV-infected cells (**Figures 2C and 2D**). We found that silencing the HCV genome RNA decreases the expression of *SGMS1* and *SGMS2*.

The above results motivated us to examine the relationship between SGMS1/2 and HCV replication. Therefore, we examined the effect of SGMS1/2 mRNA silencing on HCV replication using subgenomic replicon cells [7,16]. We observed that silencing SGMS1 mRNA suppressed HCV replication, whereas silencing SGMS2 mRNA had no such effect (**Figures 2E and 2F**). These results indicate that SGMS1 expression has a correlation with HCV replication.

Characterization of the hepatotropic SPT inhibitor NA808

Based on our data, we hypothesized that HCV might alter the metabolism of sphingolipids, providing a more conducive environment for progression of the viral life cycle. To explore the relationship between HCV and sphingolipids, we investigated the effect of sphingolipid biosynthesis inhibition on HCV and the lipid profiles of SM and ceramide using HCV-infected chimeric mice harboring human hepatocytes. To inhibit the biosynthesis of sphingolipids, we used NA808, a chemical derivative of NA255, which is an SPT inhibitor derived from natural compounds [7]. We found that NA808 (**Figure 3A**) suppressed both the activity of SPT (**Figure 3B**) and biosynthesis of sphingolipids (**Figure 3C**) in a dose-dependent manner.

The conventional SPT inhibitor myriocin is not clinically beneficial due to immunosuppression through restriction of T-cell proliferation [17,18]. However, NA808 showed little immunosuppressive effect at the concentration at which NA808 suppressed HCV replication (**Figures 3D and 3E**). Moreover, pharmacokinetic analysis using [^{14}C]-labeled NA808 in rat models showed

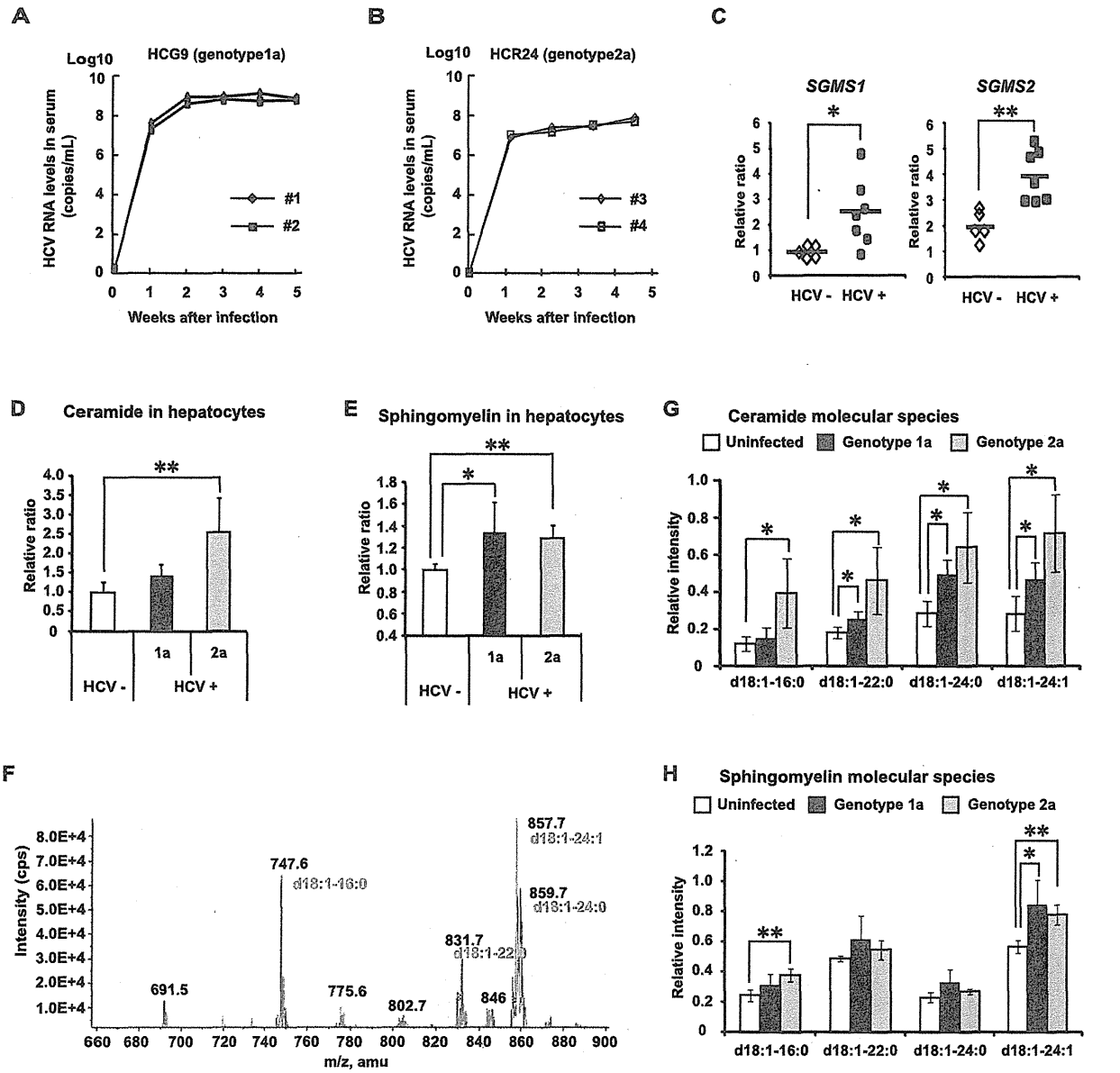

Figure 1. HCV alters sphingolipid metabolism. (**A, B**) Time-course studies of humanized chimeric mice inoculated with human serum samples positive for HCV genotype 1a (**A**) or 2a (**B**). (**C**) mRNA expression of *SGMS1* and *SGMS2* in uninfected (white, n = 5) and HCV genotype 1a-infected (black, n = 7) chimeric mice. (**D, E**) Effects of HCV infection on hepatocyte SM and ceramide levels in humanized chimeric mice. Relative intensity of total ceramide (**D**) and total shingomyelin (SM) (**E**) in uninfected mouse hepatocytes (white bar, n = 4), HCV genotype 1a-infected mouse hepatocytes (black bar, n = 5), and HCV genotype 2a-infected mouse hepatocytes (dark gray bar, n = 3). (**F**) Mass spectrum of SM in Bligh & Dyer extracts of a human hepatocyte cell line (HuH-7 K4). (**G, H**) Effects of HCV infection on hepatocyte SM and ceramide levels in humanized chimeric mice. Relative intensity of individual ceramide molecular species (**G**) and individual SM molecular species (**H**) in uninfected mouse hepatocytes (white bar, n = 3), HCV genotype 1a-infected mouse hepatocytes (black bar, n = 3), and HCV genotype 2a-infected mouse hepatocytes (dark gray bar, n = 3). In all cases, error bars indicate SDs. *$p<0.05$ and **$p<0.01$ compared with uninfected hepatocytes.
doi:10.1371/journal.ppat.1002860.g001

that NA808 mainly accumulated in the liver and small intestine (**Table S1**). These results indicate that NA808 suppressed SPT activity, with hepatotropic and low immunosuppressive properties.

Based on these results, we then examined the effects of inhibition of sphingolipid biosynthesis with NA808 on HCV replication using subgenomic replicon cells [7,16]. The luciferase activity of FLR3-1 showed that replication was suppressed by NA808 in a dose-dependent manner with no effect on cell viability, as measured by the WST-8 assay (**Figure 3E**). Similarly, western blot and immunofluorescence analysis showed that NA808 effectively suppressed HCV replication (**Figures 3F and 3G**).

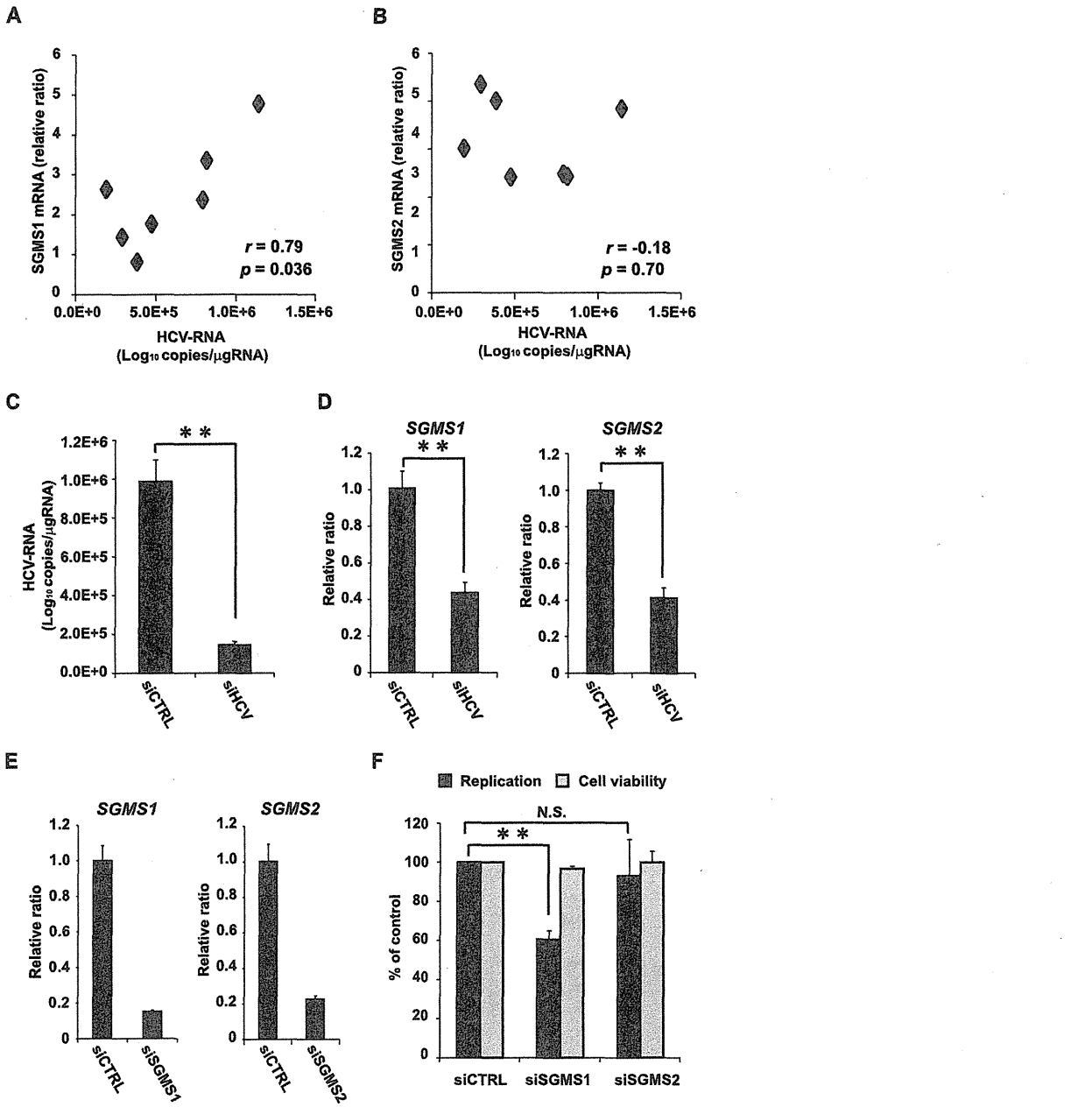

Figure 2. Relationship between the SGMS genes and HCV infection. (**A, B**) The correlation between SGMS1/2 and liver HCV-RNA of HCV infected humanized chimeric mice (n = 7). (**C**) The effect of silencing HCV genome RNA with siRNA (siE$_2$R7: 1 nM) on HCV in HCV-infected cells. (**D**) The effect of silencing HCV genome RNA with siRNA (siE-R7: 1 nM) on the expression of SGMS1/2 mRNA measured by RTD-PCR. (**E**) The effect of silencing SGMS1/2 mRNA with siRNA (3 nM each) measured by RTD-PCR. (**F**) The effect of silencing SGMS1/2 mRNA with siRNA (3 nM) on HCV replication in FLR 3-1. In all cases, error bars indicate SDs. *$p<0.05$ and **$p<0.01$.
doi:10.1371/journal.ppat.1002860.g002

Inhibition of sphingolipid biosynthesis impedes HCV infection of chimeric mice

To evaluate the effects of inhibition of sphingolipid biosynthesis in an animal model, we administered NA808 or pegylated interferon-α (PegIFN-α) via intravenous or subcutaneous injection to HCV-infected chimeric mice harboring human hepatocytes (**Table S2**). In chimeric mice infected with HCV genotype 1a, NA808 treatment led to a rapid decline in serum HCV-RNA (approximately 2–3 log units within 14 days). On the other hand, PegIFN-α produced less than a 1 log unit reduction, despite being delivered at 20 times the typical clinical dose (**Figure 4A**). Furthermore, results of 21-day NA808 treatment (5 mg/kg) in individual mice indicated that serum HCV RNA continued to decrease in all chimeric mice without viral breakthrough