

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

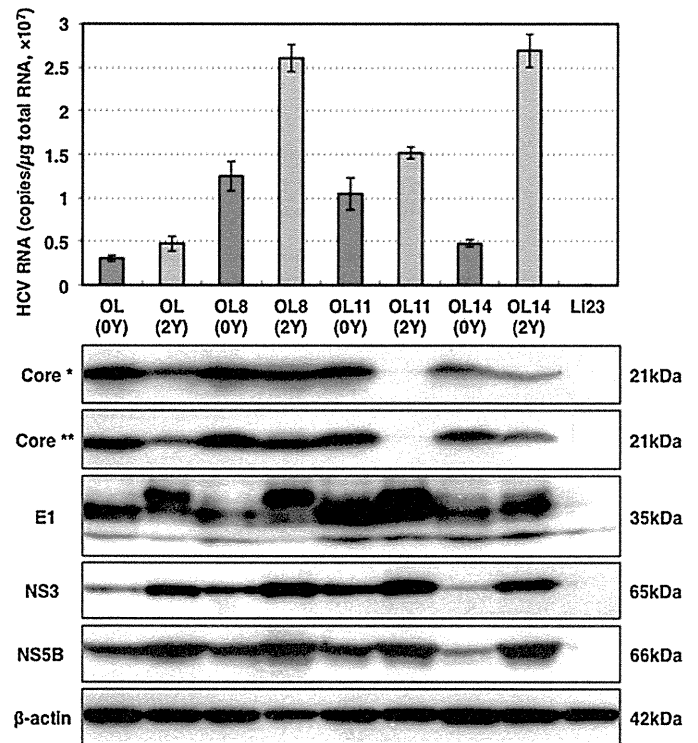
### 3.1. Efficient replication of genome-length HCV RNA is maintained in long-term cell culture

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).

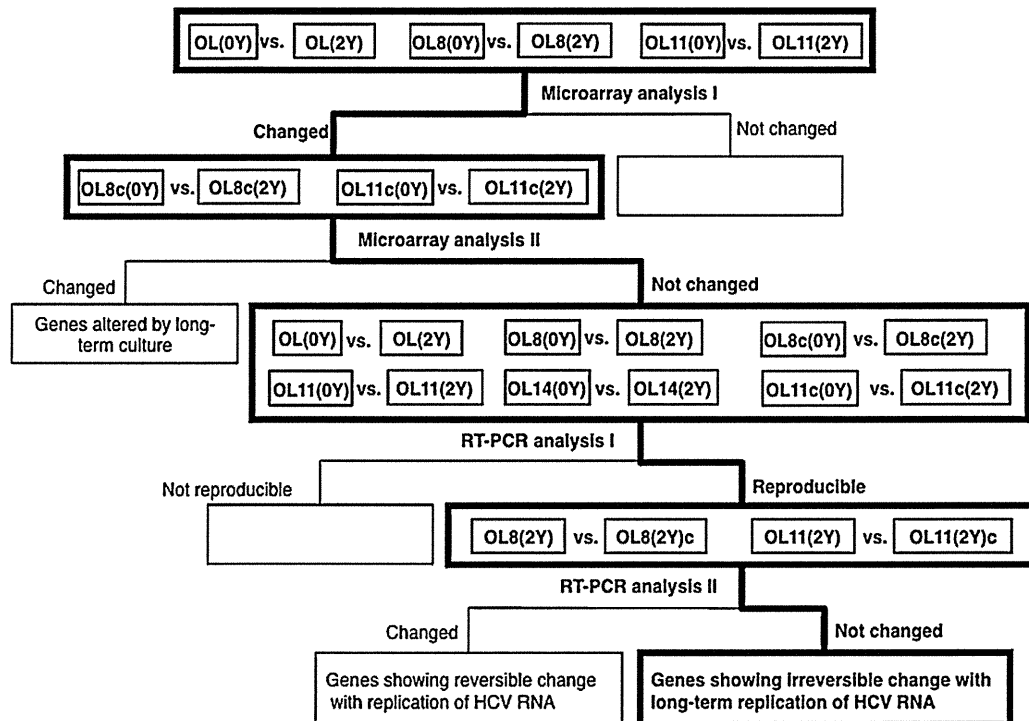
### 3.2. Selection of genes showing irreversible changes with long-term HCV RNA replication

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.  $\beta$ -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.

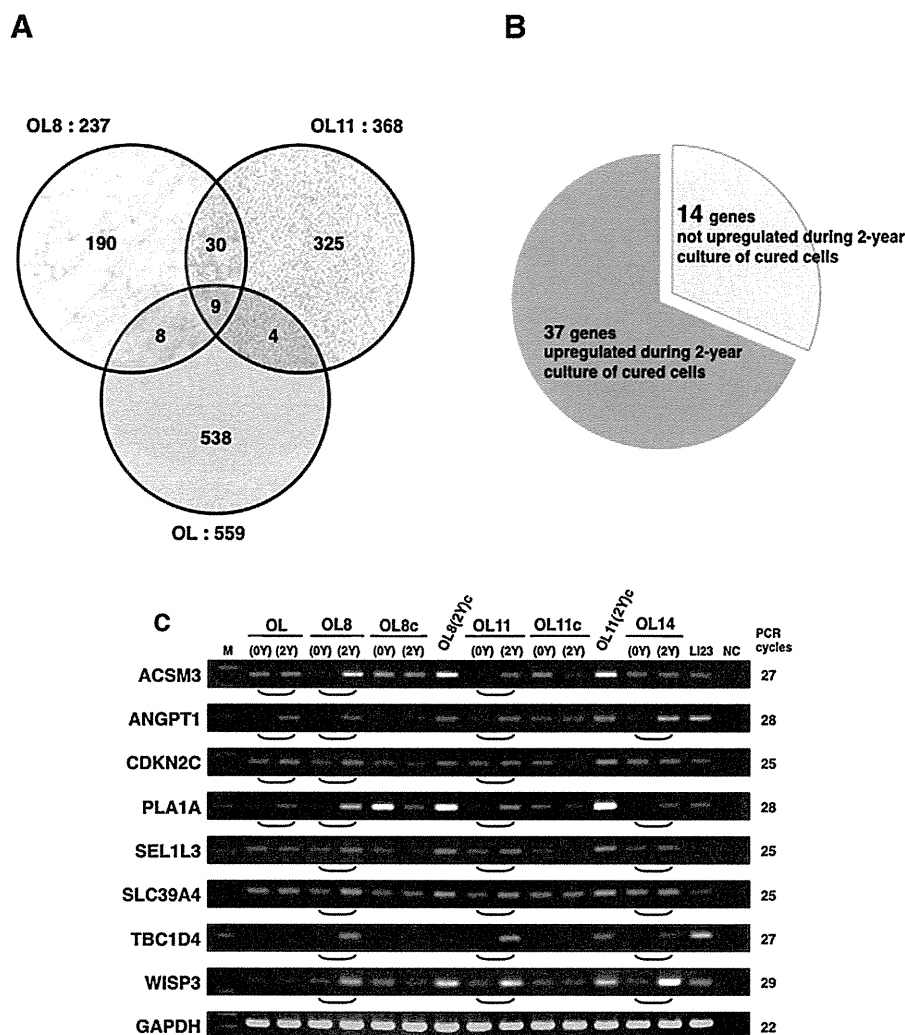
### 3.3. Selection and expression profiles of genes showing upregulated expression during long-term HCV RNA replication

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], angiotensinogen 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



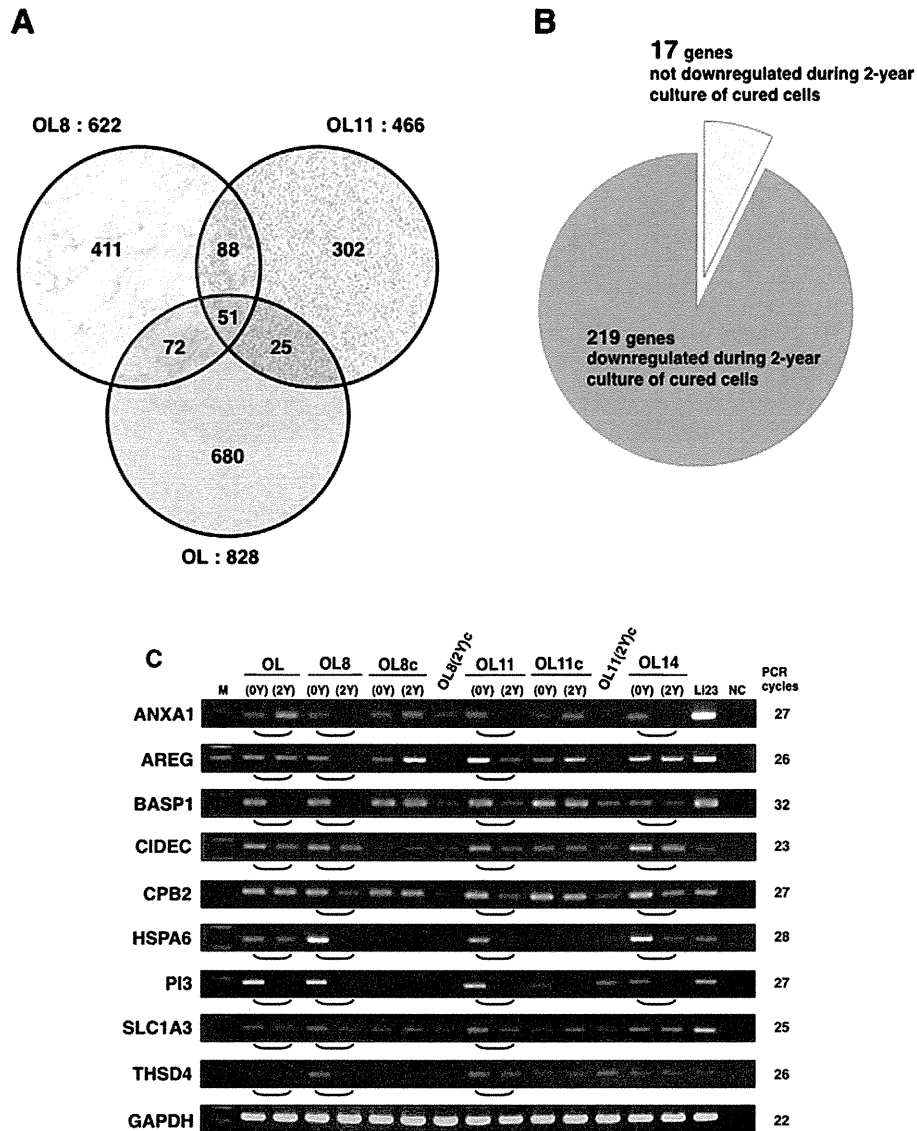
**Fig. 3.** Identification of genes irreversibly upregulated during 2-year replication of HCV RNA. (A) Upregulated genes obtained by microarray analysis I shown in Fig. 2. Genes whose expression levels were upregulated at ratios of more than 2 in the case of OL(0Y) versus OL(2Y) cells, OL8(0Y) versus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



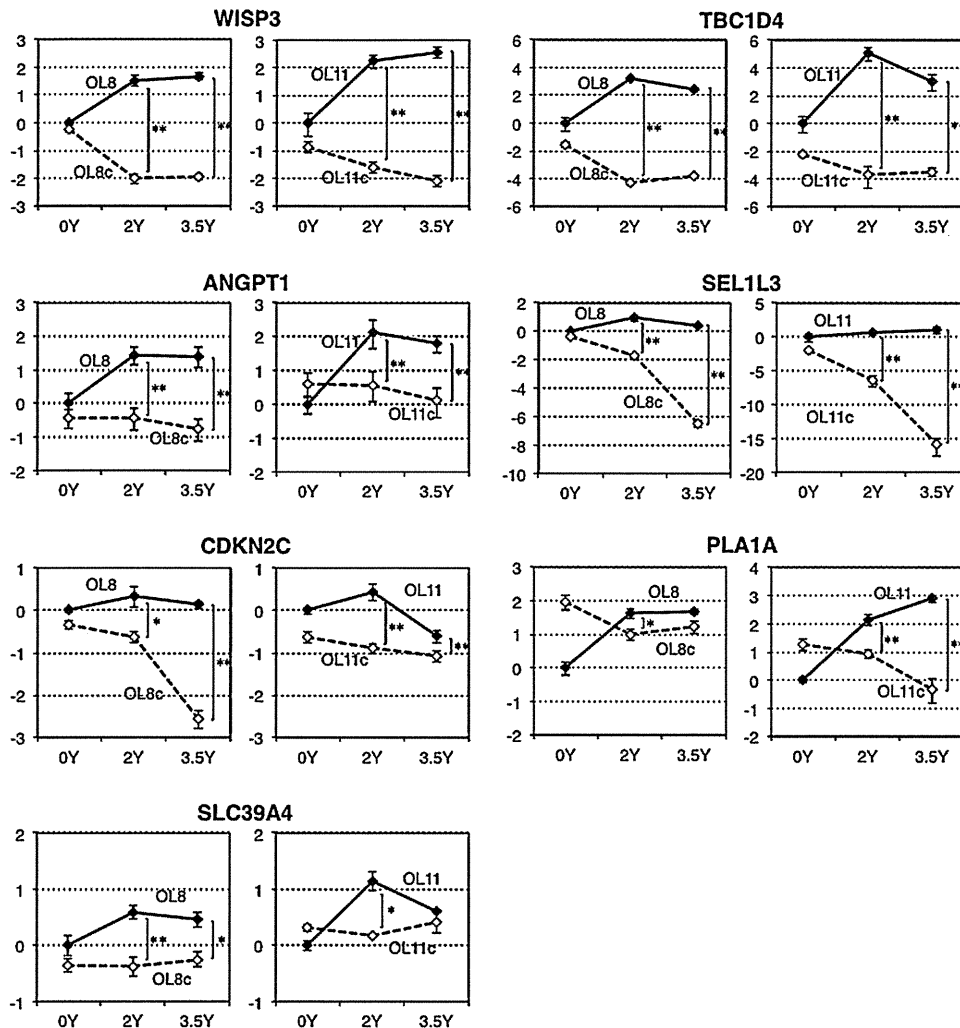
**Fig. 4.** Identification of genes irreversibly downregulated during 2-year replication of HCV RNA. (A) Downregulated genes obtained by microarray analysis I shown in Fig. 2. Genes were selected whose expression levels were downregulated at ratios of less than 0.5 in the case of OL(0Y) versus OL(2Y) 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*, *CIDECD*, and *THSD4*) (Fig. 6), although the expression levels (except for *AREG* in the OL11 series and *CIDECD* 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<sub>2</sub>, 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).

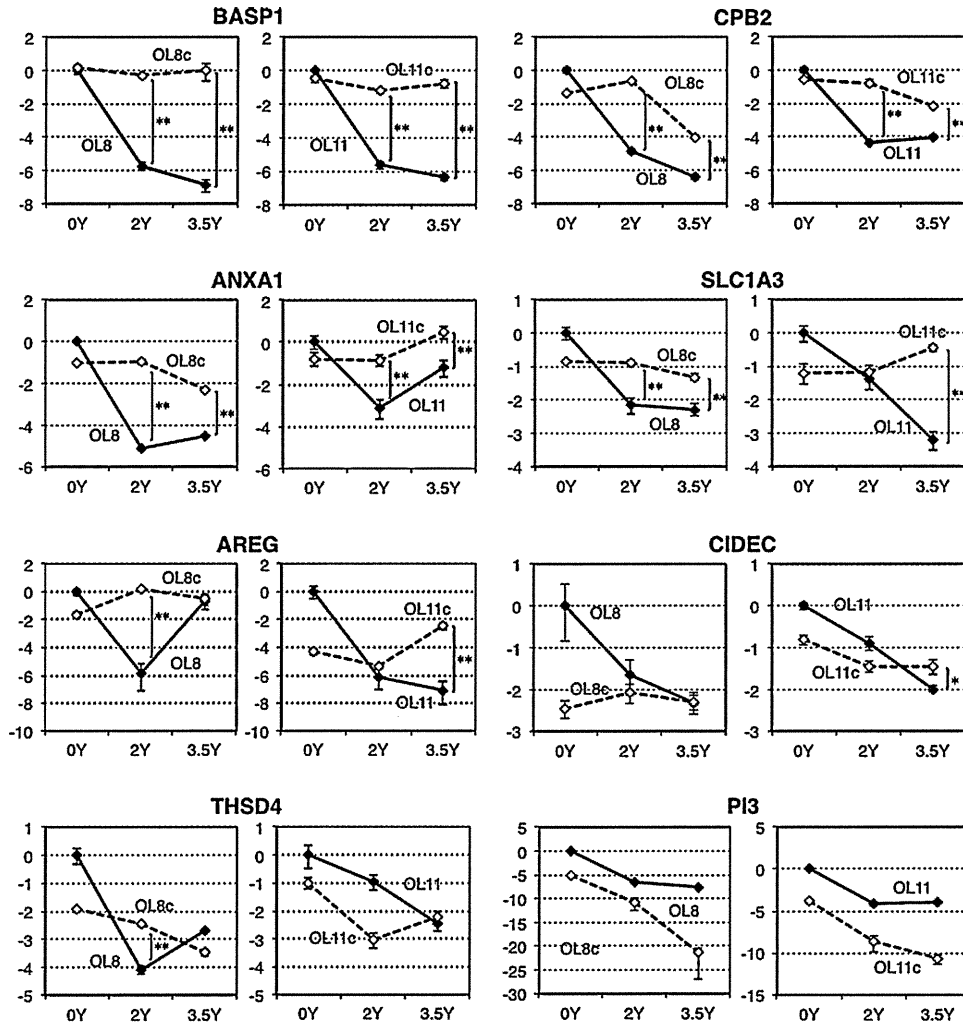
#### 4. Discussion

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 (Minea 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- $\kappa$ 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- $\kappa$ B activity by the binding of *ANXA1* to the p65 subunit of NF- $\kappa$ 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

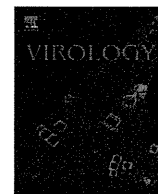
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## Trans-complemented hepatitis C virus particles as a versatile tool for study of virus assembly and infection

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### ABSTRACT

In this study, we compared the entry processes of *trans*-complemented hepatitis C virus particles (HCVtcp), cell culture-produced HCV (HCVcc) and HCV pseudoparticles (HCVpp). Anti-CD81 antibody reduced the entry of HCVtcp and HCVcc to almost background levels, and that of HCVpp by approximately 50%. Apolipoprotein E-dependent infection was observed with HCVtcp and HCVcc, but not with HCVpp, suggesting that the HCVtcp system is more relevant as a model of HCV infection than HCVpp. We improved the productivity of HCVtcp by introducing adapted mutations and by deleting sequences not required for replication from the subgenomic replicon construct. Furthermore, blind passage of the HCVtcp in packaging cells resulted in a novel mutation in the NS3 region, N1586D, which contributed to assembly of infectious virus. These results demonstrate that our plasmid-based system for efficient production of HCVtcp is beneficial for studying HCV life cycles, particularly in viral assembly and infection.

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### Introduction

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic liver diseases (Hoofnagle, 2002). HCV is an enveloped virus of the family *Flaviviridae*, and its genome is a positive-strand RNA consisting of the 5'-untranslated region (UTR), an open reading frame encoding viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) and the 3'-UTR (Suzuki et al., 2007).

Host–virus interactions are required during the initial steps of viral infection. It was previously reported that CD81 (Bartosch et al., 2003a, b; McKeating et al., 2004; Pileri et al., 1998), scavenger receptor class B type I (Bartosch et al., 2003a, b; Scarselli et al., 2002), claudin-1 (Evans et al., 2007; Liu et al., 2009) and occludin (Benedicto et al., 2009; Evans et al., 2007; Liu et al., 2009; Ploss et al., 2009) are critical molecules for HCV entry into cells. CD81 interacts with HCV E2 via a second extracellular loop (Bartosch et al., 2003a, b; Hsu et al., 2003) and its role in the internalization process was confirmed (Cormier et al., 2004; Flint et al., 2006). It has also been shown that infectious

HCV particles produced in cell cultures (HCVcc) exist as apolipoprotein E (ApoE)-enriched lipoprotein particles (Chang et al., 2007) and that ApoE is important for HCV infectivity (Owen et al., 2009).

Investigation of HCV had been hampered by difficulties in amplifying the virus *in vitro* before development of robust cell culture systems based on JFH-1 isolates (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Retrovirus-based HCV pseudoparticles (HCVpp), in which cell entry is dependent on HCV glycoproteins, have been used to study virus entry (Bartosch et al., 2003a; Hsu et al., 2003). Vesicular stomatitis virus (VSV)-based pseudotypic viruses bearing HCV E1 and E2 and replication-competent recombinant VSV encoding HCV envelopes have also been available as surrogate models for studies of HCV infection (Mazumdar et al., 2011; Tani et al., 2007).

It was recently shown that HCV subgenomic replicons can be packaged when structural proteins are supplied in *trans* (Adair et al., 2009; Ishii et al., 2008; Masaki et al., 2010; Steinmann et al., 2008). These *trans*-complemented HCV particles (HCVtcp) are infectious, but support only single-round infection and are unable to spread. Establishment of flexible systems to efficiently produce HCVtcp should contribute to studying HCV assembly, in particular encapsidation of the viral genome, and entry to cells with less stringent biosafety and biosecurity measures. Although single-round infection can be achieved by using the HCVcc system with receptor knock-out

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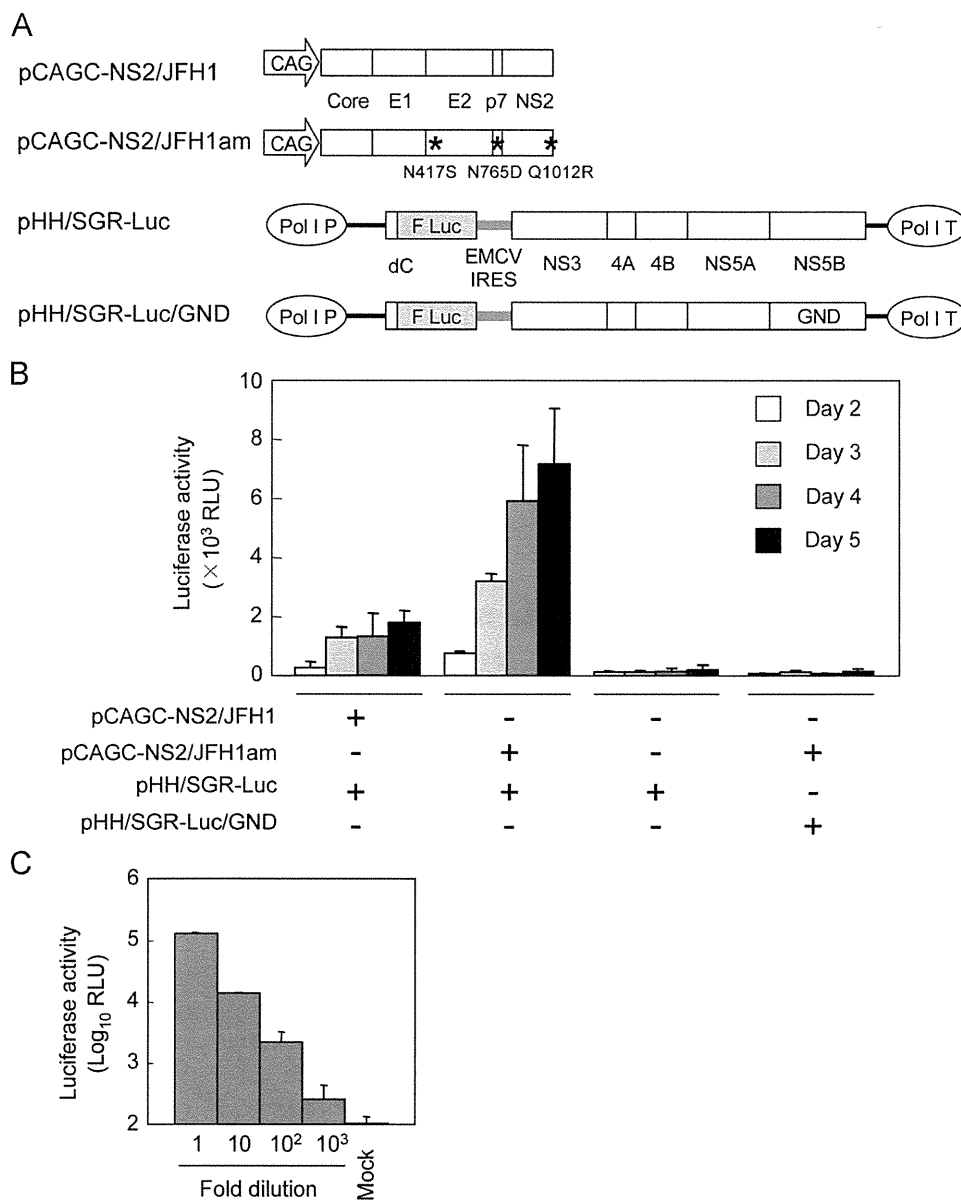
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cells, the single-round HCVcc system is not suitable for studying virus entry. We previously described plasmid-based production of HCVcc and HCVtcp (Masaki et al., 2010). Here, we demonstrated that HCVtcp production can be enhanced by introducing the previously reported cell-culture adaptive mutations and by deleting sequences not essential for replication in the subgenomic replicon construct. By providing genotype 1b-derived core-to-p7 in addition to intragenotypic viral proteins, chimeric HCVtcp were generated. Furthermore, blind passage of HCVtcp in the packaging cells resulted in the identification of a novel cell culture-adaptive mutation in NS3 that enables us to establish the efficient production of HCVtcp with structural proteins from various strains. Taken together, our system for producing single-cycle infectious HCV particles should be useful in the study of entry and assembly steps of the HCV life cycles. This technology may also have potential to be the basis for the safer vaccine development.

**Results**

*Enhancement of HCVtcp production by adaptive mutations in E2, p7 and NS2 and by deleting sequences not essential for replication from replicon construct*

In our HCVtcp system, the RNA polymerase I (Pol I)-driven replicon plasmid, which carries a dicistronic subgenomic luciferase reporter replicon of JFH-1 strain with a Pol I promoter and terminator (pHH/SGR-Luc), as well as a plasmid containing core-NS2 cDNA under the CAG promoter (pCAGC-NS2) were used (Masaki et al., 2010). In an effort to improve the yield of HCVtcp production, cell culture-adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) which were previously selected from serial passage of HCVcc (Russell et al., 2008) were introduced into the core-NS2 expression plasmid (Fig. 1A) (residues are numbered



**Fig. 1.** HCVtcp production by two-plasmid transfection. (A) Schematic representation of plasmids is shown. HCV polyproteins derived from JFH-1 are indicated by white boxes. HCV UTRs are indicated by bold lines. The internal ribosomal entry site from encephalomyocarditis virus (EMCV IRES) is denoted as gray lines. Adaptive mutations are indicated as asterisks. F Luc: firefly luciferase gene; CAG: CAG promoter; Pol I P: RNA polymerase I promoter; Pol I T: RNA polymerase I terminator; GND: replication-deficient GND mutation. (B) Luciferase activity in Huh7.5.1 cells inoculated with supernatant from cells transfected with indicated plasmids at the indicated time points. Data are averages of triplicate values with error bars showing standard deviations. (C) Luciferase activity in cells inoculated with serially diluted HCVtcp.

according to positions within the JFH-1 polyprotein). Supernatants of cells transfected with plasmids (Fig. 1A) were collected and were used to infect Huh7.5.1 cells, which were analyzed by luciferase assay. Introduction of adaptive mutations (pCAGC-NS2/JFH1am) resulted in more than 4-fold higher production of HCVtcp at 5 day post-transfection, as compared to wild-type (WT) (pCAGC-NS2/JFH1) (Fig. 1B), indicating that the adaptive mutations contribute to enhancing HCVtcp production. To confirm that luciferase activity levels in HCVtcp-infected cells are correlated with the number of infectious particles, Huh7.5.1 cells were inoculated with serial dilutions of HCVtcp. Luciferase activity was well correlated with viral load (Fig. 1C), indicating that luciferase assay in HCVtcp-infected cells can be used to quantify HCV infection.

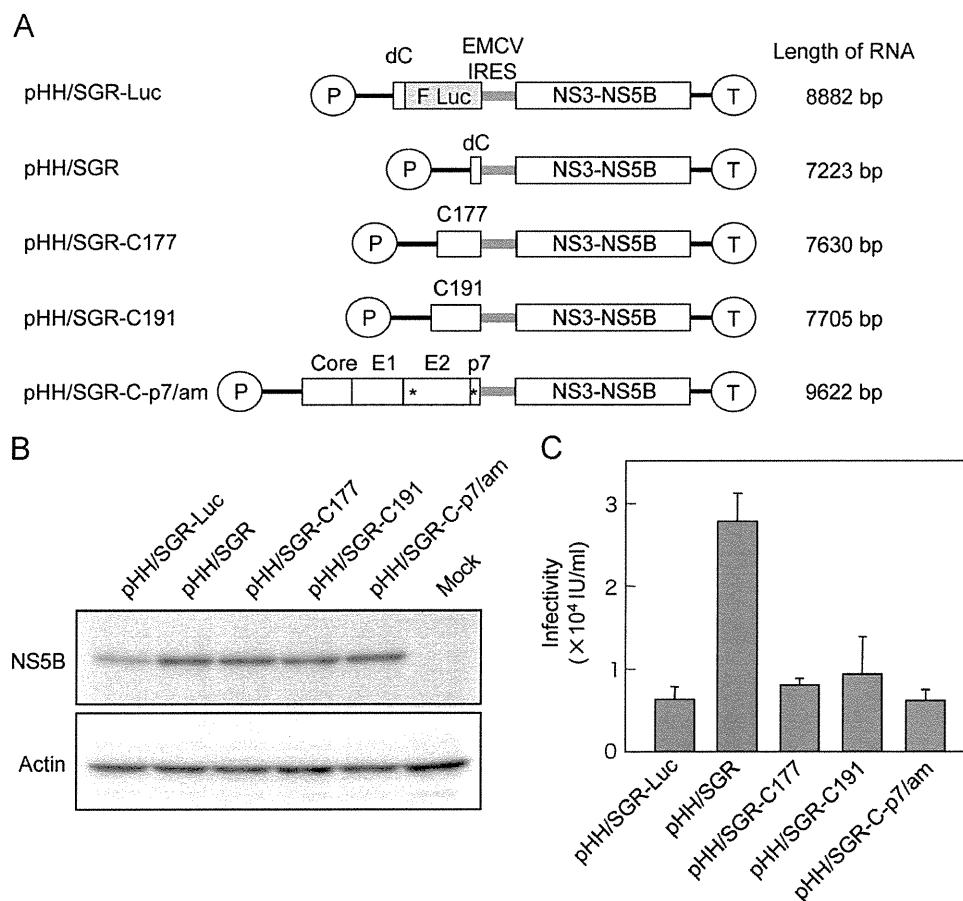
In order to further explore the efficient production of HCVtcp, we generated replicon constructs that lack the luciferase gene or include the partial coding sequences for structural proteins instead of reporter (Fig. 2A). Replication of each replicon in plasmid-transfected cells was then assessed by Western blotting (Fig. 2B). Among the constructs tested, NS5B levels were lowest in cells expressing pHH/SGR-Luc. NS5B levels in cells replicating other replicons appeared to be comparable. Cells were infected with supernatants of cells transfected with each replicon plasmid, along with pCAGC-NS2/JFH1am, followed by infectious unit assay (Fig. 2C). The highest production of HCVtcp was obtained from cells transfected with pHH/SGR, where the luciferase sequence was deleted from pHH/SGR-Luc, thus suggesting that deletion of the sequence not essential for RNA replication in the replicon may contribute to enhancing HCVtcp production.

#### Production of chimeric HCVtcp by providing heterologous core-p7

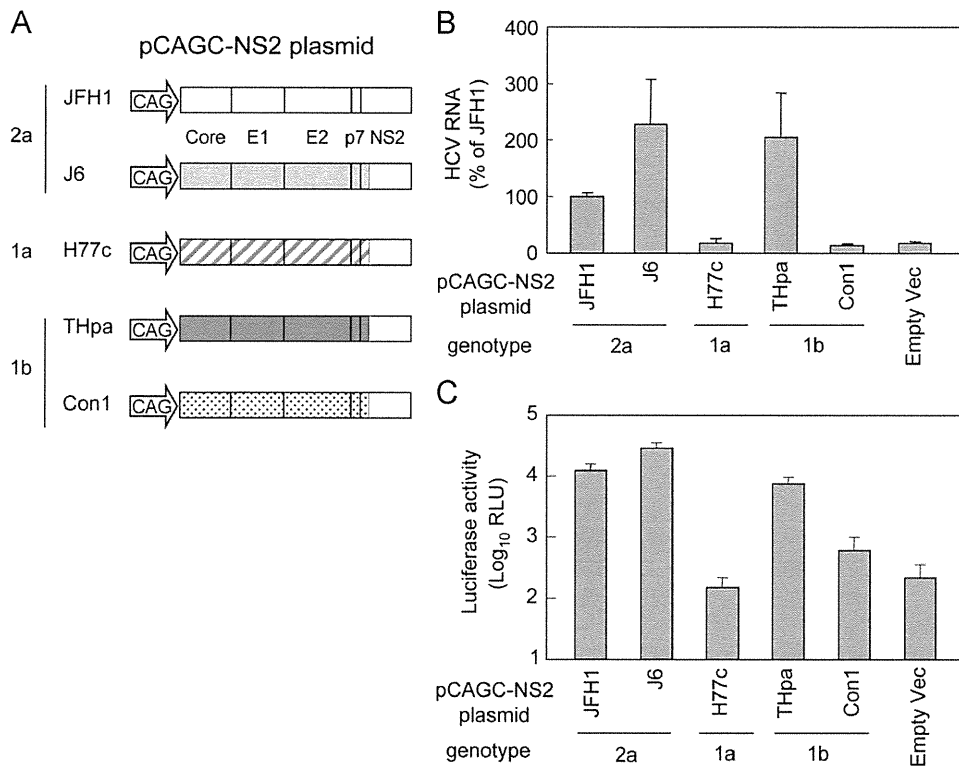
In order to elucidate whether *trans*-encapsidation of JFH-1 replicon can be achieved by providing core-p7 from other HCV strains, core-NS2 plasmids were constructed (Fig. 3A). In these plasmids, core through the N-terminal 33 aa of NS2, which contains transmembrane domain 1 of NS2, was derived from either H77c (genotype 1a), THpa (genotype 1b), Con1 (genotype 1b) or J6 (genotype 2a) strain. Residual NS2 was derived from JFH-1, as described previously (Pietschmann et al., 2006). HCVtcp was efficiently produced by core-p7 of J6 and THpa strains, but its production was less efficient in the case of Con1 strain. *Trans*-packaging was not detectable when core-p7 of H77c strain was used (Fig. 3C). Among HCV strains tested, difference in luciferase activity levels in HCVtcp-infected cells (Fig. 3C) were in agreement with that in the viral RNA levels in the culture supernatants of the transfected cells (Fig. 3B). Although the efficacy of *trans*-complementation was variable among strains, chimeric HCVtcp can be generated by providing genotype 1b-derived core-p7 in addition to intragenotypic viral proteins, and was used in subsequent studies.

#### ApoE- and CD81-dependent infection by HCVtcp

There is accumulating evidence that apolipoproteins, particularly ApoE, contribute to HCV production and infectivity (Chang et al., 2007; Owen et al., 2009). To determine whether ApoE is involved in infection of target cells by HCVtcp, we infected cells in the presence of increasing concentrations of anti-ApoE antibody.



**Fig. 2.** Production of HCVtcp with different replicon constructs. (A) Schematic representation of plasmids used for production of HCVtcp. Deduced length of transcribed RNA from each construct is shown on the right. HCV polyproteins from JFH-1 strain are indicated by open boxes. HCV UTRs are indicated by bold lines. The EMCV IRES is denoted by gray bars. Adaptive mutations are indicated by asterisks. F Luc: firefly luciferase gene; P: RNA polymerase I promoter; T: RNA polymerase I terminator. (B) Detection of NS5B and actin in Huh7.5.1 cells transfected with indicated plasmids at 4 day post-transfection. (C) Infectivity of culture supernatants from cells transfected with indicated replicon plasmids along with pCAGC-NS2/JFH1am at 4 day post-transfection.



**Fig. 3.** HCVtcp production with structural proteins from various strains. (A) Schematic representation of plasmids used. HCV polyproteins of JFH-1, J6, H77c, THpa and Con1 strain are shown in the open box, bright gray box, box with diagonal lines, dark gray box and dotted box, respectively. (B) Relative levels of HCV RNA in the supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc. (C) Luciferase activity in cells inoculated with supernatant from cells transfected with indicated plasmids along with pHH/SGR-Luc at 4 day post-transfection.

pCAGC-NS2/THpa and pCAGC-NS2/JFH1am were used as core-NS2 plasmids for HCVtcp production carrying core-p7 derived from genotypes 1b and 2a (HCVtcp-1b and HCVtcp-2a, respectively). HCVpp derived from JFH-1 and VSVpp were generated and used for comparison. Infection with HCVtcp-1b or HCVtcp-2a was blocked by anti-ApoE antibody in a dose-dependent manner. In contrast, anti-ApoE antibody did not affect infection with HCVpp and VSVpp (Fig. 4A).

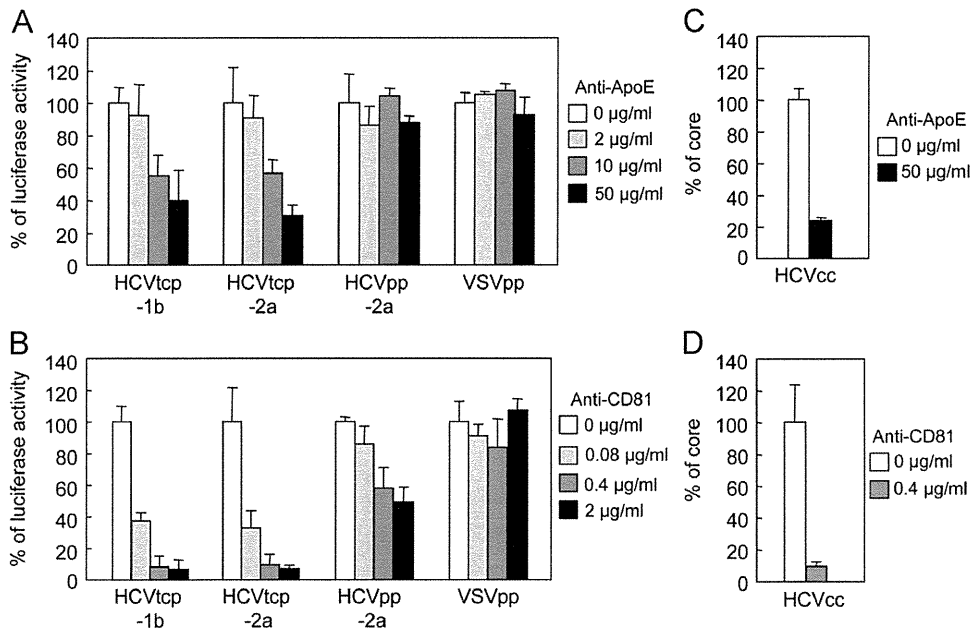
The CD81 dependence of infection was also compared between HCVtcp and HCVpp (Fig. 4B). Anti-CD81 antibody inhibited the entry of HCVtcp-1b, HCVtcp-2a, and HCVpp in a dose-dependent manner. The antibody had no effect on VSVpp infection. HCVtcp infection appears to be more sensitive to anti-CD81 antibody when compared with HCVpp infection; more than 60% inhibition was observed at 0.08  $\mu\text{g}/\text{mL}$  anti-CD81 antibody for HCVtcp-1b and HCVtcp-2a, whereas approximately 50% inhibition was observed for HCVpp at 2  $\mu\text{g}/\text{mL}$  antibody. Neutralization of HCVcc by anti-ApoE and anti-CD81 antibodies was also determined. Antibodies blocked HCVcc infection (Fig. 4C and D), as observed with HCVtcp. These results suggest that ApoE, as well as CD81, play an important role in HCVtcp infection. Thus, HCVtcp may be more useful for evaluating the HCV entry process than HCVpp.

#### Identification of novel culture-adaptive mutation in NS3 by serial passage of HCVtcp in packaging cells

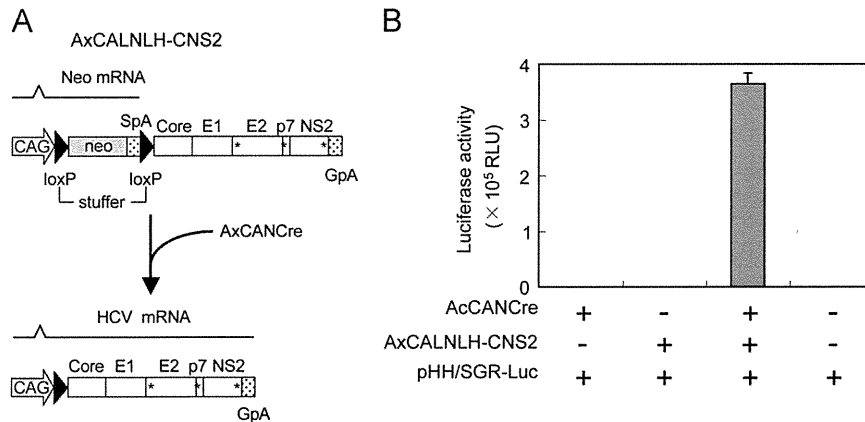
The HCVtcp system was further applied to analyses of genetic changes during serial passages in target cells. As an initial attempt, supernatants of cells co-transfected with pCAGC-NS2/JFH1am and pHH/SGR were inoculated into Huh7.5.1 cells transiently transfected with pCAGC-NS2/JFH1am. However, infectious titer was lost after repeated inoculation, likely due to low HCVtcp titers and

low efficiency of plasmid transduction (data not shown). To overcome this, we utilized recombinant adenovirus vectors (rAdVs) to provide core-NS2. As we were not able to obtain rAdV directly expressing core-NS2, conditional transgene expression based on a Cre-loxP strategy was employed (Kanegae et al., 1995). We constructed an rAdV containing core-NS2 gene downstream of a stuffer DNA flanked by a pair of loxP sites (AxCALNLH-CNS2). When cells were doubly infected with AxCALNLH-CNS2 and the Cre-expressing rAdV, AxCANCre (Kanegae et al., 1995), the Cre-mediated excisional deletion removed the stuffer DNA, resulting in core-NS2 expression under control of the CAG promoter (Fig. 5A). As expected, tightly regulated production of HCVtcp was observed. The cells infected with AxCANCre and AxCALNLH-CNS2 along with transduction of pHH/SGR-Luc produced HCVtcp at high levels. Production of HCVtcp was undetectable when either AxCANCre or AxCALNLH-CNS2 was not infected (Fig. 5B). The Cre-mediated rAdV expression system appears to have yielded considerably higher production of HCVtcp when compared with the settings for plasmid co-transfection.

Supernatants from cells in which core-NS2 was expressed using rAdVs and the subgenomic RNA derived from pHH/SGR replicated were inoculated into cells infected with AxCALNLH-CNS2 and AxCANCre (Fig. 6A). Blind passage was performed by sequentially transferring culture supernatants to cells infected with the above rAdVs. The two independent 10 blind passages (p10) showed virus titers of  $> 1 \times 10^6$  IU/mL, which were markedly higher than those of the passage 0 (p0) stock cultures ( $4 \times 10^4$  IU/mL). Side-by-side infection analysis revealed that the HCVtcp p10 #1 achieved a virus titer approximately 36 times higher than that of HCVtcp p0 on the packaging cells at 6 day post-infection (Fig. 6B). Sequencing of the entire replicon in the supernatants at p10 in two independent experiments revealed



**Fig. 4.** Effects of anti-ApoE and anti-CD81 antibodies on HCV entry. (A) Aliquots of virus sample were incubated with increasing concentrations of anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells. Luciferase activity was determined at 72 h post-infection and is expressed relative to activity without antibodies (white bar). (B) Huh7.5.1 cells were preincubated for 1 h with increasing concentrations of anti-CD81 antibodies, followed by inoculating virus samples. Luciferase activity was determined and expressed as shown in (A). (C) Aliquots of HCVcc were incubated with anti-ApoE antibodies for 1 h and were then added to Huh7.5.1 cells at an MOI of 0.05. Intracellular core levels were quantitated at 24 h post-infection and are expressed relative to levels without antibodies (white bar). (D) Huh7.5.1 cells were preincubated for 1 h with anti-CD81 antibodies. HCVcc infection and measurement of core proteins were performed as indicated in (C).



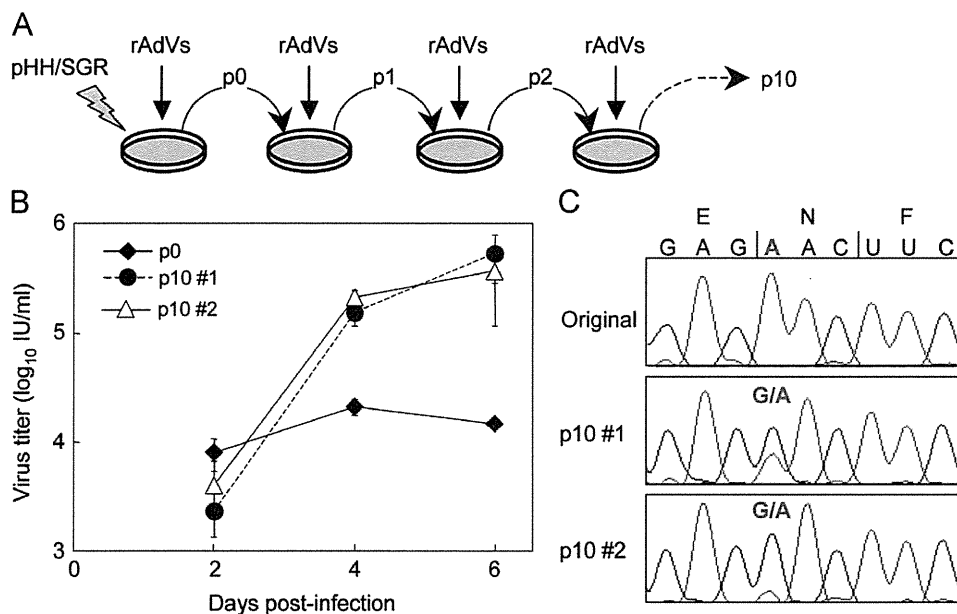
**Fig. 5.** Transgene activation mediated by rAdVs expressing Cre recombinase under control of CAG promoter. (A) Cre recombinase expressed by AxCANCre recognizes a pair of its target sequences loxP in AxCALNLH-CNS2, and removes the stuffer region resulting in expression of HCV core-NS2 polyprotein by CAG promoter. CAG: CAG promoter; SpA: SV40 early polyA signal; GpA: rabbit b-globin poly(A) signal. (B) Luciferase activity in Huh7.5.1 cells inoculated with 4-day post-transfection culture supernatant from cells transfected with pHH/SGR-Luc, and then infected with indicated rAdVs.

that both passaged HCVtcp had an identical nonsynonymous mutation in the NS3 region (N1586D) (Fig. 6C).

In order to examine the role of NS3 mutation identified on HCV RNA replication and on HCVtcp production, the N1586D mutation was introduced into pHH/SGR-Luc. Luciferase activities of the N1586D-mutated replicon were apparently lower than those of the WT-replicon, thus suggesting that the NS3 mutation reduced viral RNA replication (Fig. 7A). HCV RNA levels in the supernatants of cells transfected with WT- or mutant replicon plasmid along with pCAGC-NS2/JFH1am and luciferase activity in cells inoculated with supernatants from the transfected cells were then determined (Fig. 7B). The viral RNA level secreted from cells replicating the N1586D-mutated replicon was lower than that from cells replicating WT replicon (Fig. 7B, left). By contrast, a significantly higher infectivity of HCVtcp produced from the mutant replicon-cells was observed, as compared to WT replicon-cells (Fig. 7B, right),

suggesting that the adaptive mutation increased the specific infectivity (almost 9-fold) of the virus particles. To further determine whether the N1586D mutation affects infectious viral assembly and/or virus release, we used the CD81-negative Huh-7 subclone, Huh7-25 (Akazawa et al., 2007), which may produce infectious particles, but is not susceptible to HCV entry due to a lack of CD81 expression, therefore allowing us to examine viral assembly and release without the influence of reinfection by produced HCVtcp. Measurement of intracellular and extracellular HCVtcp indicated that Huh7-25 cells replicating the N1586D-mutated replicon produced more infectious virus than WT in both supernatants and cell lysates (Fig. 7C). Thus, it can be concluded that the N1586D mutation contributes to enhanced infectious viral assembly, not RNA replication. We could not exclude the possibility that N1586D mutation affects virus release, since the mutation enhanced extracellular virus titers more than did the intracellular titer.





**Fig. 6.** Genotypic changes in HCVtcp following blind passage. (A) Experimental procedure for blind passage of HCVtcp. Huh7.5.1 cells were transfected with pHH/SGR and were doubly infected with AxCANCre and AxCALNLH-CNS2. Culture fluids were collected and were inoculated into cells infected with AxCANCre and AxCALNLH-CNS2. These procedures were repeated 10 times with two independent samples (#1 and #2). (B) Growth curves of HCVtcp p0 and p10 on Huh7.5.1 cells expressing core-NS2. Cells were infected with HCVtcp at an MOI of 0.05, and medium was collected at the indicated time points and subjected to titration. (C) Nucleotide sequences of original and blind-passaged replicons from HCVtcp. Nucleotides of mutated position are shown in red and bold.

The impact of the N1586D mutation on production of intra- and intergenotypic HCVtcp chimeras was also investigated. The N1586D mutation in the replicon enhanced the production of chimeric HCVtcp by providing core-p7 from all strains examined, although not statistically significant in THpa, and Con1 strains (Fig. 7D). Finally, to determine whether the N1586D mutation was responsible for enhancing HCVcc production, this mutation was introduced into pHHJFH1, which carries the full-length wild-type JFH-1 cDNA (Masaki et al., 2010), yielding pHHJFH1N1586D. The virus titer obtained from cells transfected with the pHHJFH1N1586D was significantly higher than that of WT (Fig. 7E), thus demonstrating that the N1586D mutation enhances yields of HCVcc, in addition to HCVtcp.

## Discussion

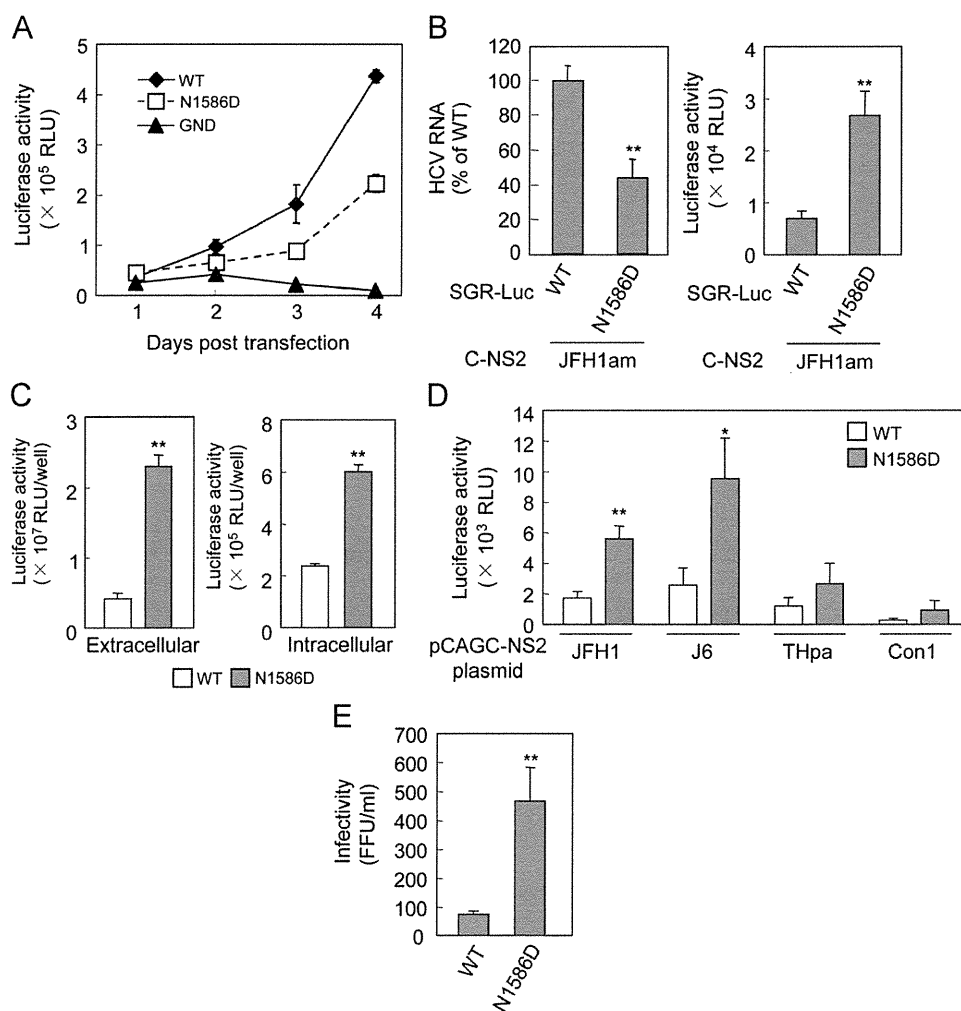
Single-round infectious viral particles generated by *trans*-packaging systems are considered to be valuable tools for studying virus life cycles, particularly the steps related to entry into target cells, assembly and release of infectious particles. However, limited HCV strains have been applied for the efficient production of HCVtcp to date. In this study, we improved the HCVtcp system in order to enhance the productivity of infectious particles. Production of chimeric HCVtcp by providing genotype 1b-derived core-p7, in addition to intragenotypic viral proteins, was also confirmed. Furthermore, we exploited the system to investigate genetic changes during serial passage of target cells and identified a novel cell culture-adaptive mutation in NS3, which also contributes to enhance the productivity of HCVtcp.

HCVpp (Bartosch et al., 2003a; Hsu et al., 2003) has proven to be a valuable surrogate system by which the study of viral and cellular determinants of the viral entry pathway is possible. Early steps of HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp (Lavie et al., 2007). However, as HCVpp is generated in non-hepatic cells such as the human embryo kidney cells 293T, it

is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc. Hepatocytes play a role in maintaining lipid homeostasis in the body by assembling and secreting lipoproteins, including VLDL. It is highly likely that HCV exploits lipid synthesis pathways, as there is a tight link between virion formation and VLDL synthesis. Down-regulation of ApoE considerably reduces HCV production (Benga et al., 2010; Chang et al., 2007; Hishiki et al., 2010; Jiang and Luo, 2009; Owen et al., 2009). Infectivity of HCVcc is also neutralized by anti-ApoE antibodies (Chang et al., 2007). These data suggest that ApoE is important for HCV infectivity. Furthermore, Niemann-Pick C1-like 1 (NPC1L1), involving cholesterol uptake receptor, was recently identified as a host factor for HCV entry (Sainz et al., 2012). Knockdown of NPC1L1 had no effect on the entry of HCVpp whereas HCVcc entry was impaired, possibly due to different cholesterol content of these particles. Here, we found that the anti-ApoE antibody neutralized infection by HCVtcp and HCVcc, but not by HCVpp (Fig. 4A and C), thus suggesting that biogenesis and/or secretion pathways of VLDL are involved in HCVtcp similarly to HCVcc, but not in HCVpp.

We also observed that infectivity of HCVtcp and HCVcc is more efficiently neutralized by the anti-CD81 antibody, as compared to that of HCVpp (Fig. 4B and D). It has recently been reported that E2 of HCVcc contained both high-mannose-type and complex-type glycans, whereas most of the glycans on HCVpp-associated E2 were complex-type, which is matured by Golgi enzymes (Vieyres et al., 2010). Mutational analysis of the N-linked glycosylation sites in E1/E2 demonstrated that several glycans on E2 may affect the sensitivity of HCVpp against antibody neutralization, as well as access of CD81 to its binding site on E2 (Helle et al., 2010). The differences in sensitivity between HCVtcp and HCVpp to neutralization by anti-CD81 antibody observed here may be due to differences in carbohydrate composition of HCV glycoproteins during expression and processing of E1/E2 in cells and morphogenesis of HCVtcp and HCVpp.

By analyzing the various replicons for *trans*-packaging, we observed the highest production of HCVtcp with replicons from pHH/SGR, which lacked sequences not essential for RNA



**Fig. 7.** Effects of N1586D mutation on RNA replication and production of HCVtcp or HCVcc. (A) RNA replication of replicons in cells transfected with pHH/SGR-Luc (WT) or N1586D mutant. Luciferase activities at 1 to 4 day post-transfection were determined. (B) Relative levels of HCV RNA in the supernatants from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am were shown in the left panel. Luciferase activities in cells inoculated with supernatants from cells transfected with indicated plasmids at 4 day post-transfection were shown in the right panel. (C) Luciferase activity in cells inoculated with supernatant and cell lysates from Huh7-25 cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with pCAGC-NS2/JFH1am at 5 day post-transfection. (D) Luciferase activity in cells inoculated with culture supernatant from cells transfected with pHH/SGR-Luc (WT) or N1586D mutant plasmid along with indicated core-NS2 plasmids at 4 day post-transfection. (E) Infectivity of supernatant from cells transfected with pHH/JFH1 (WT) or its derivative plasmid containing N1586D mutation at 6 day post-transfection. Statistical differences between WT and N1586D were evaluated using Student's *t*-test. \**p* < 0.05, \*\**p* < 0.005 vs. WT.

replication, while less efficient productivity was observed from pHH/SGR-Luc, pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am (Fig. 2C). Differences in the replication efficiency of the replicon do not appear to be a major determinant for HCVtcp productivity, at least in the present settings, as all replicon constructs except pHH/SGR-Luc replicated at similar levels, as confirmed by Western blotting (Fig. 2B). Although the shorter viral genome sequence may offer advantages over the longer sequence, further investigation is required in order to understand the molecular mechanisms underlying viral genome packaging. By comparing pHH/SGR vs. pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/am, it is likely that the expression of the structural protein *in cis* does not increase HCVtcp production when sufficient amounts of structural proteins are supplied *in trans*.

Blind passage of HCVtcp in packaging cells infected with rAdVs providing core-NS2 enabled us to identify a novel culture-adaptive mutation in NS3. The N-terminal third of NS3 forms a serine protease, together with NS4A, and its C-terminal two-thirds exhibits RNA helicase and RNA-stimulated NTPase activities. In addition, similarly to flaviviruses (Kummerer and Rice, 2002; Liu et al., 2002), it is now apparent that HCV NS3 is also involved in viral

morphogenesis (Han et al., 2009; Ma et al., 2008), although its precise role and underlying molecular mechanism(s) have not fully been elucidated. Two cell-culture adaptive NS3 mutations which are involved in HCV assembly have been identified. The Q1251L mutation in helicase subdomain 1 resulted in approximately 30-fold higher production of HCV without affecting NS3 enzymatic activities (Ma et al., 2008). The M1290K adaptive mutation was also located in subdomain 1 of the NS3 helicase (Han et al., 2009). The N1586D mutation identified here was located in subdomain 3 of helicase. Analogous to Q1251L and M1290K, the N1586D mutation enhanced the infectious viral assembly by increasing specific infectivity without affecting the efficiency of viral RNA replication. Considering the possibility that NS3 plays a role in linking between the viral replicase and assembly sites (Jones et al., 2011), it is likely that NS3 helicase is one of the determinants for interaction with the structural proteins. Our results, together with earlier studies, suggest that chimeric and defective mutations as well as supplying the viral components *in trans*, function as selective pressures in virion assembly.

In summary, we have established a plasmid-based reverse genetics for efficient production of HCVtcp with structural

proteins from various strains. Single-round infectious HCVtcp can complement the HCVcc and HCVpp systems as a valuable tool for the study of HCV life cycles.

## Materials and methods

### Cells

Huh7 derivative cell line Huh7.5.1 and Huh7-25 were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 10% fetal bovine serum at 37 °C in a 5% CO<sub>2</sub> incubator.

### Plasmids

Plasmids pHHJFH1, pHH/SGR-Luc, pHH/SGR-Luc/GND and pCAG/C-NS2 were as described previously (Masaki et al., 2010). In this study, plasmid pCAG/C-NS2 was designated as pCAGC-NS2/JFH. The plasmid pCAGC-NS2/JFH<sub>am</sub> having adaptive mutations in E2 (N417S), p7 (N765D), and NS2 (Q1012R) in pCAGC-NS2/JFH was constructed by oligonucleotide-directed mutagenesis. These mutations were also introduced in pHHJFH1, resulting in pHHJFH1<sub>am</sub>. To generate core-NS2 expression plasmids with different strains of HCV, the cDNA coding core to the first transmembrane region of NS2 (33 amino acids) in pCAGC-NS2/JFH was replaced with the corresponding sequence of the J6 (Lindenbach et al., 2005), H77c (Yanagi et al., 1997), THpa (Shirakura et al., personal communication) and Con1 (Koch and Bartenschlager, 1999) strains. The THpa sequence contained the P to A mutation at 328 aa at E1 in the original TH strain. To generate pHH/SGR, pHH/SGR-Luc was digested with MluI and PmeI, followed by Klenow enzyme treatment and self-ligation to delete the luciferase coding sequence. To generate pHH/SGR-C177, pHH/SGR-C191 and pHH/SGR-C-p7/<sub>am</sub>, cDNA coding the partial core and luciferase in pHH/SGR-Luc were replaced with coding sequences for mature core (177aa), full-length core (191aa) or core-p7 polyprotein containing adaptive mutations in E2 and p7, respectively. The selected NS3 mutation (N1586D) was introduced into pHH/SGR-Luc and pHHJFH1 by oligonucleotide-directed mutagenesis.

### Generation of viruses

HCVcc and HCVtcp were generated as described previously (Masaki et al., 2010). For the production of HCVpp-2a, plasmid pcDNAdeltaC-E1-E2(JFH1)<sub>am</sub> having adaptive mutations in E2 (N417S) in pcDNAdeltaC-E1-E2(JFH1) (Akazawa et al., 2007) was constructed by oligonucleotide-directed mutagenesis. Murine leukemia virus pseudotypes with VSV G glycoprotein expressing luciferase reporter (VSVpp) were generated in accordance with previously described methods (Akazawa et al., 2007; Bartosch et al., 2003a).

### Luciferase assay

Huh7.5.1 cells were seeded onto a 24-well plate at a density of  $3 \times 10^4$  cells/well 24 h prior to inoculation with reporter viruses. Cells were incubated for 72 h, followed by lysis with 100 µL of lysis buffer. Luciferase activity of the cells was determined using a luciferase assay system (Promega, Madison, WI). All luciferase assays were performed in triplicate.

### Quantification of HCV infectivity and HCV RNA

To determine the titers of HCVtcp and HCVcc, Huh7.5.1 cell monolayers prepared in multi-well plates were incubated with dilutions of samples and then replaced with media containing 10% FBS and 0.8% carboxymethyl cellulose. Following incubation for 72 h, monolayers were fixed and immunostained with rabbit polyclonal anti-NS5A antibody, followed by Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen), and stained foci or individual cells were counted and used to calculate a titer of focus-forming units (FFU)/mL for spreading infections or infectious units (IU)/mL for non-spreading infections. For intracellular infectivity, the cell pellet was resuspended in culture media, and cells were lysed by four freeze–thaw cycles. Cell debris was pelleted by centrifugation for 5 min at 4000 rpm. Supernatant was collected and used for titration. To determine the amount of HCV RNA in culture supernatants, RNA was extracted from 140 µL of culture medium by QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) and treated with DNase (TURBO DNase; Ambion, Austin, TX) at 37 °C for 1 h. Extracted RNA was further purified by using an RNeasy Mini Kit, which includes RNase-free DNase digestion (QIAGEN). Copy numbers of HCV RNA were determined by real-time quantitative reverse transcription-PCR as described previously (Wakita et al., 2005).

### Antibodies

Mouse monoclonal antibodies against actin (AC-15) and CD81 (JS-81) were obtained from Sigma (St. Louis, MO) and BD Biosciences (Franklin Lakes, NJ), respectively. Goat polyclonal antibody to ApoE (LV1479433) was obtained from Millipore (Tokyo, Japan). Anti-NS5A and anti-NS5B antibodies were rabbit polyclonal antibody against synthetic peptides.

### Neutralization assay

For neutralization experiments with anti-CD81 antibody, Huh7.5.1 cells were incubated with dilutions of anti-CD81 antibody for 1 h at 37 °C. Cells were then infected with viruses for 5 h at 37 °C. For neutralization experiments with anti-ApoE antibody, viruses were incubated with various concentrations of anti-ApoE antibody at room temperature for 1 h and cells were infected with viruses for 5 h at 37 °C. Following infection, supernatant was removed and cells were incubated with culture medium, and luciferase activity was determined at 3 day post-infection for HCVtcp and pseudotyped viruses. For neutralization experiments with HCVcc generated with pHHJFH1<sub>am</sub>, a multiplicity of infection (MOI) of 0.05 was used for inoculation, and intracellular core protein levels were monitored by ELISA (Ortho Clinical Diagnostics) at 24 h post-infection.

### Immunoblotting

Transfected cells were washed with PBS and incubated with lysis buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1% Triton X-100). Lysates were then sonicated for 5 min and were added to the same volume of SDS sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE, and transferred to PVDF membrane. After blocking, membranes were probed with first antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; PIERCE, Rockford, IL), in accordance with the manufacturer's protocols.

### Generation of recombinant adenoviruses

rAdV, AxCANCre, expressing Cre recombinase tagged with nuclear localization signal under CAG promoter was prepared as described previously (Baba et al., 2005). The target rAdV AxCALNLH-CNS2 expressing HCV core-NS2 polyprotein with adaptive mutations in E2, p7 and NS2 was generated as follows. Cosmid pAxCALNLwit2 is identical to pAxCALNLw (Sato et al., 1998), except that both the terminal sequences of the rAdV genome are derived from pAxCAwit2 (Fukuda et al., 2006). The core-NS2 fragment obtained from pCAGC-NS2/JFH1am by *StuI*-*EcoRI* digestion and subsequent Klenow treatment was inserted into the *SwaI* site of pAxCALNLwit2. The resultant cosmid pAx-CALNLH-CN2it2 was digested with *PacI* and transfected into 293 cells to generate rAdV AxCALNLH-CNS2.

### Preparation of packaging cells for HCVtcp

Huh7.5.1 cells were coinfecting with AxCANCre at an MOI of 1 and AxCALNLH-CNS2 at an MOI of 3 for expression of JFH-1 core-NS2 polyprotein containing the adaptive mutations in E2, p7 and NS2.

### RNA preparation, RT-PCR and sequencing

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription with random hexamer and Superscript III reverse transcriptase (Invitrogen). Three fragments of HCV cDNAs that cover the entire HCV subgenomic replicon genome, were amplified by nested PCR with TaKaRa Ex Taq polymerase (Takara, Shiga, Japan). Amplified products were separated by agarose gel electrophoresis, and were used for direct DNA sequencing.

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## Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1 $\alpha$ transduction

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**Background & Aims:** Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can be utilized as a tool for screening for hepatotoxicity in the early phase of pharmaceutical development. We have recently reported that hepatic differentiation is promoted by sequential transduction of SOX17, HEX, and HNF4 $\alpha$  into hESC- or hiPSC-derived cells, but further maturation of hepatocyte-like cells is required for widespread use of drug screening.

**Methods:** To screen for hepatic differentiation-promoting factors, we tested the seven candidate genes related to liver development.

**Results:** The combination of two transcription factors, FOXA2 and HNF1 $\alpha$ , promoted efficient hepatic differentiation from hESCs and hiPSCs. The expression profile of hepatocyte-related genes (such as genes encoding cytochrome P450 enzymes, conjugating enzymes, hepatic transporters, and hepatic nuclear receptors) achieved with FOXA2 and HNF1 $\alpha$  transduction was comparable to that obtained in primary human hepatocytes. The hepatocyte-like cells generated by FOXA2 and HNF1 $\alpha$  transduction exerted various hepatocyte functions including albumin and urea secretion, and the uptake of indocyanine green and low density lipoprotein. Moreover, these cells had the capacity to metabolize all nine tested drugs and were successfully employed to evaluate drug-induced cytotoxicity.

**Conclusions:** Our method employing the transduction of FOXA2 and HNF1 $\alpha$  represents a useful tool for the efficient generation of metabolically functional hepatocytes from hESCs and hiPSCs, and the screening of drug-induced cytotoxicity.

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### Introduction

Hepatocyte-like cells differentiated from human embryonic stem cells (hESCs) [1] or human induced pluripotent stem cells (hiPSCs) [2] have more advantages than primary human hepatocytes (PHs) for drug screening. While application of PHs in drug screening has been hindered by lack of cellular growth, loss of function, and de-differentiation *in vitro* [3], hESC- or hiPSC-derived hepatocyte-like cells (hESC-hepa or hiPSC-hepa, respectively) have potential to solve these problems.

Hepatic differentiation from hESCs and hiPSCs can be divided into four stages: definitive endoderm (DE) differentiation, hepatic commitment, hepatic expansion, and hepatic maturation. Various growth factors are required to mimic liver development [4] and to promote hepatic differentiation. Previously, we showed that transduction of transcription factors in addition to treatment with optimal growth factors was effective to enhance hepatic differentiation [5–7]. An almost homogeneous hepatocyte population was obtained by sequential transduction of SOX17, HEX, and HNF4 $\alpha$  into hESC- or hiPSCs-derived cells [7]. However, further maturation of the hESC-hepa and hiPSC-hepa is required for widespread use of drug screening because the drug metabolism capacity of these cells was not sufficient.

In some previous reports, hESC-hepa and hiPSC-hepa have been characterized for their hepatocyte functions in numerous ways, including functional assessment such as glycogen storage and low density lipoprotein (LDL) uptake [7]. To make a more precise judgment as to whether hESC-hepa and hiPSC-hepa can be applied to drug screening, it is more important to assess cytochrome P450 (CYP) induction potency and drug metabolism capacity rather than general hepatocyte function. Although Duan *et al.* have examined the drug metabolism capacity of hESC-hepa, drug metabolites were measured at 24 or 48 h [8]. To precisely

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