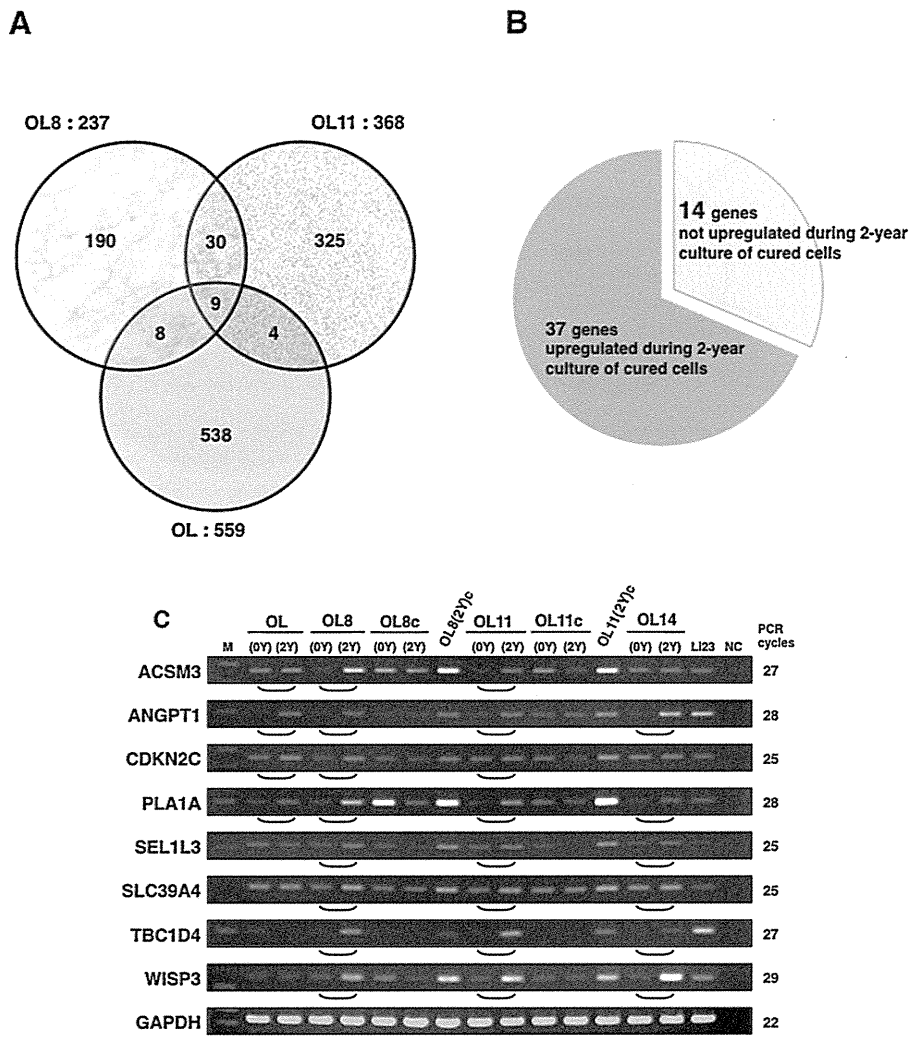

Fig. 3. Identification of genes irreversibly upregulated during 2-year replication of HCV RNA. (A) Upregulated genes obtained by microarray analysis I shown in Fig. 2. Genes whose expression levels were upregulated at ratios of more than 2 in the case of OL(0Y) versus OL(2Y) cells, OL8(0Y) versus OL8(2Y)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

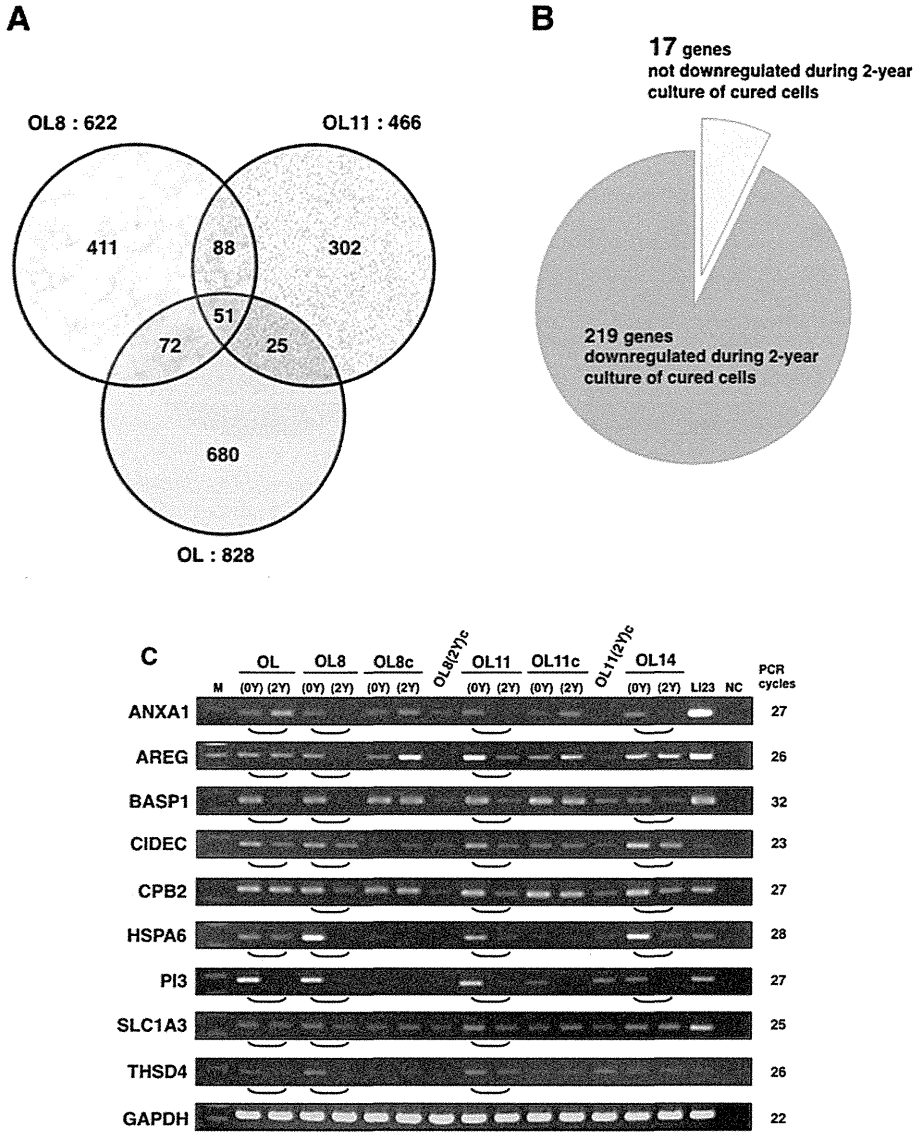

Fig. 4. Identification of genes irreversibly downregulated during 2-year replication of HCV RNA. (A) Downregulated genes obtained by microarray analysis I shown in Fig. 2. Genes were selected whose expression levels were downregulated at ratios of less than 0.5 in the case of OL(0Y) versus OL(2Y) cells,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

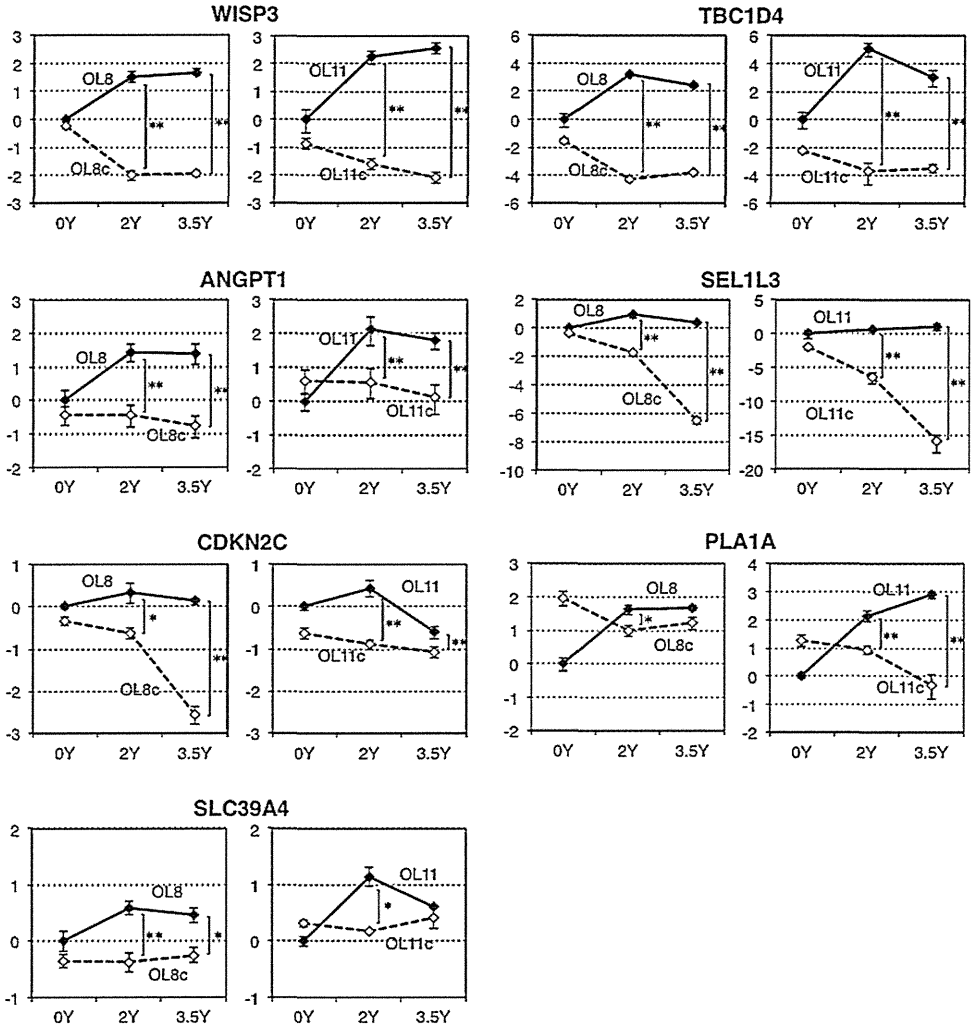

Fig. 5. Expression levels of genes selected as upregulated genes in 3.5-year cell culture. Quantitative RT-PCR analysis using the total RNAs derived from OL8(0Y), OL8(2Y), OL8(3.5Y), OL8c(0Y), OL8c(2Y), OL8c(3.5Y), OL11(0Y), OL11(2Y), OL11(3.5Y), OL11c(0Y), OL11c(2Y), and OL11c(3.5Y) cells was performed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

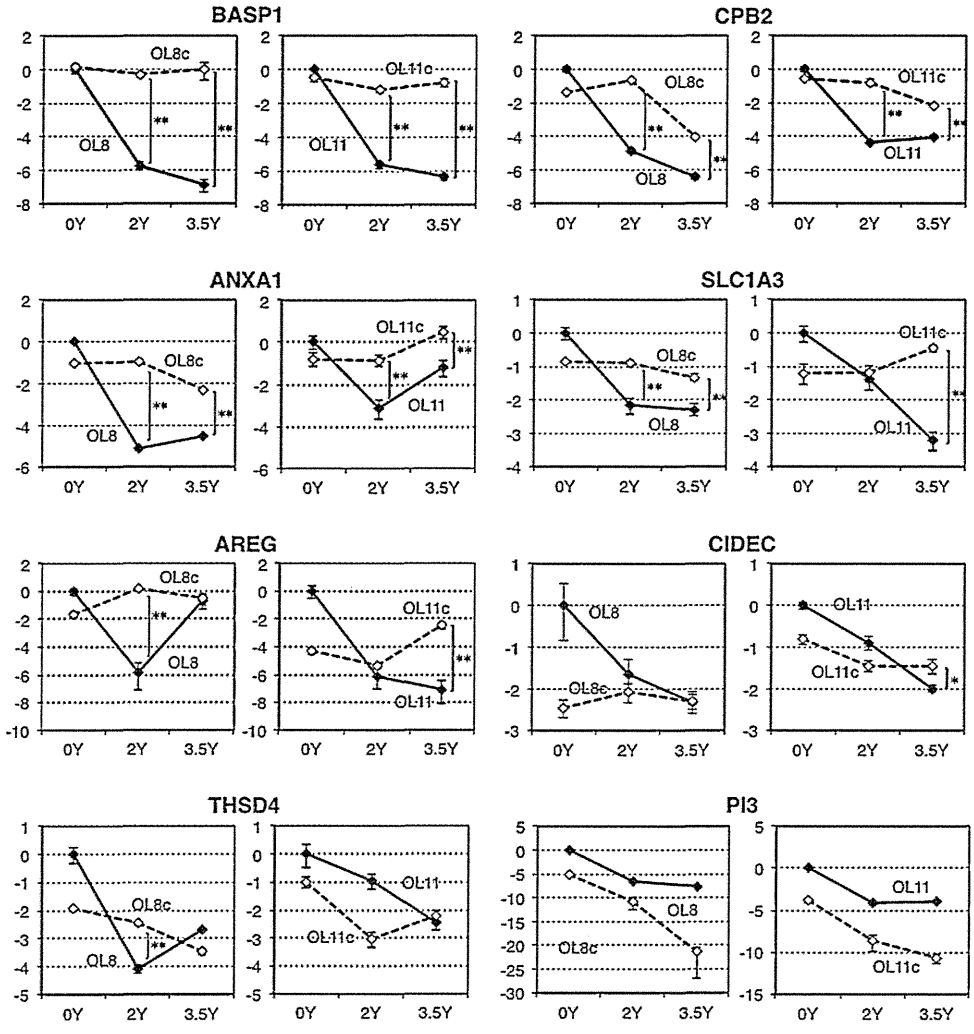

Fig. 6. Expression levels of genes selected as downregulated genes in 3.5-year cell culture. Quantitative RT-PCR analysis was performed as shown in Fig. 5, and the obtained results are also presented as shown in Fig. 5.

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.

References

Abe, K., Ikeda, M., Dansako, H., Naka, K., Kato, N., 2007. Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA. Virus Research 125, 88–97.

Ang, E.Z.-F., Nguyen, H.T., Sim, H.-L., Putti, T.C., Lim, L.H., 2009. Annexin-1 regulates growth arrest induced by high levels of estrogen in MCF-7 breast cancer cells. Molecular Cancer Research 7, 266–274.

Bartenschlager, R., 2005. The hepatitis C virus replicon system: from basic research to clinical application. Journal of Hepatology 43, 210–216.

Bartenschlager, R., Sparacio, S., 2007. Hepatitis C virus molecular clones and their replication capacity in vivo and in cell culture. Virus Research 127, 195–207.

Carpenter, B., Hill, K.J., Charalambous, M., Wagner, K.J., Lahiri, D., James, D.I., Andersen, J.S., Schumacher, V., Royer-Pokora, B., Mann, M., Ward, A., Roberts, S.G.E., 2004. BASP1 is a transcriptional cosuppressor for the Wilms' tumor suppressor protein WT1. Molecular and Cell Biology 24, 537–549.

Choo, Q., Kuo, G., Weiner, A., Overby, L., Bradley, D., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359–362.

Dansako, H., Naganuma, A., Nakamura, T., Ikeda, F., Nozaki, A., Kato, N., 2003. Differential activation of interferon-inducible genes by hepatitis C virus core protein mediated by the interferon stimulated response element. Virus Research 97, 17–30.

Fong, Y.-C., Lin, C.-Y., Su, Y.-C., Chen, W.-C., Tsai, F.-J., Tsai, C.-H., Huang, C.-Y., Tang, C.-H., 2012. CCN6 enhances ICAM-1 expression and cell motility in human chondrosarcoma cells. Journal of Cellular Physiology 227, 223–232.

Garcia Pedrero, J.M., Fernandez, M.P., Morgan, R.O., Herrero Zapatero, A., Gonzalez, M.V., Suarez Nieto, C., Rodrigo, J.P., 2004. Annexin A1 down-regulation in head and neck cancer is associated with epithelial differentiation status. American Journal of Pathology 164, 73–79.

Hartl, M., Nist, A., Khan, M.I., Valovka, T., Bister, K., 2009. Inhibition of Myc-induced cell transformation by brain acid-soluble protein 1 (BASP1). Proceedings of the National Academy of Sciences of the United States of America 106, 5604–5609.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., Shimotohno, K., 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proceedings of the National Academy of Sciences of the United States of America 88, 5547–5551.

Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Kimura, K., Shimotohno, K., 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proceedings of the National Academy of Sciences of the United States of America 90, 10773–10777.

Hillebrandt, S., Wasmuth, H.E., Weiskirchen, R., Hellerbrand, C., Keppeler, H., Werth, A., Schirin-Sokhan, R., Wilkens, G., Geier, A., Lorenzen, J., Koehl, J., Gressner, A.M., Matern, S., Lammert, F., 2005. Complement factor 5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans. Nature Genetics 37, 835–843.

Hu, N., Flaig, M.J., Su, H., Shou, J.-Z., Roth, M.J., Li, W.-J., Wang, C., Goldstein, A.M., Li, G., Emmert-Buck, M.R., Taylor, P.R., 2004. Comprehensive characterization of annexin I alterations in esophageal squamous cell carcinoma. Clinical Cancer Research 10, 6013–6022.

Huang, W., Gonzalez, M.E., Toy, K.A., Banerjee, M., Kleer, C.G., 2010. Blockade of ccn6 (WISP3) activates growth factor–independent survival and resistance to anoikis in human mammary epithelial cells. Cancer Research 70 (8), 3340–3350.

Huang, W., Zhang, Y., Varambally, S., Chinnaiyan, A.M., Banerjee, M., Merajver, S.D., Kleer, C.G., 2008. Inhibition of CCN6 (Wnt-1-induced signaling protein 3) down-regulates E-cadherin in the breast epithelium through induction of snail and ZEB1. American Journal of Pathology 172 (4), 893–904.

Huang, W.-C., Chan, S.-T., Yang, T.-L., Tzeng, C.-C., Chen, C.-C., 2004. Inhibition of ICAM-1 gene expression, monocyte adhesion and cancer cell invasion by targeting IKK complex: molecular and functional study of novel alpha-methylene-butyrolactone derivatives. Carcinogenesis 25, 1925–1934.

Ikeda, M., Abe, K., Dansako, H., Nakamura, T., Naka, K., Kato, N., 2005. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochemical and Biophysical Research Communications 329, 1350–1359.

Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., Kato, N., 1998. Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. Virus Research 56, 157–167.

Ikeda, M., Yi, M., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. Journal of Virology 76, 2997–3006.

Kane, S., Sano, H., Liu, S.C.H., Asara, J.M., Lane, W.S., Garner, C.C., Lienhard, G.E., 2002. A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. Journal of Biological Chemistry 277, 22115–22118.

Kato, N., Abe, K., Mori, K., Ariumi, Y., Dansako, H., Ikeda, M., 2009a. Genetic variability and diversity of intracellular genome-length hepatitis C virus RNA in long-term cell culture. Archives of Virology 154, 77–85.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., Shimotohno, K., 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proceedings of the National Academy of Sciences of the United States of America 87, 9524–9528.

Kato, N., Mori, K., Abe, K., Dansako, H., Kuroki, M., Ariumi, Y., Wakita, T., Ikeda, M., 2009b. Efficient replication systems for hepatitis C virus using a new human hepatoma cell line. Virus Research 146, 41–50.

Kato, N., Sugiyama, K., Namba, K., Dansako, H., Nakamura, T., Takami, M., Naka, K., Nozaki, A., Shimotohno, K., 2003. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochemical and Biophysical Research Communications 306, 756–766.

Krieger, N., Lohmann, V., Bartenschlager, R., 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. Journal of Virology 75, 4614–4624.

Lindenbach, B.D., Rice, C.M., 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436, 933–938.

Lohmann, V., Korner, F., Koch, J.-O., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Mîinea, C.P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peränen, J., Lane, W.S., Lienhard, G.E., 2005. As160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochemical Journal 391, 87–93.

Mori, K., Abe, K., Dansako, H., Ariumi, Y., Ikeda, M., Kato, N., 2008. New efficient replication system with hepatitis C virus genome derived from a patient with acute hepatitis c. Biochemical and Biophysical Research Communications 371, 104–109.

Mori, K., Ikeda, M., Ariumi, Y., Dansako, H., Wakita, T., Kato, N., 2011. Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system. Virus Research 157, 61–70.

Mori, K., Ikeda, M., Ariumi, Y., Kato, N., 2010. Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: comparison with HuH-7 and other hepatic cell lines. Hepatology Research 40, 1248–1253.

Moribe, T., Iizuka, N., Miura, T., Stark, M., Tamatsukuri, S., Ishitsuka, H., Hamamoto, Y., Sakamoto, K., Tamesa, T., Oka, M., 2008. Identification of novel aberrant methylation of BASP1 and SRD5A2 for early diagnosis of hepatocellular carcinoma by genome-wide search. International Journal of Oncology 33, 949–958.

Mosevitsky, M.I., Capony, J.P., Skladchikova, G.Y., Novitskaya, V.A., Plekhanov, A.Y., Zakharov, V.V., 1997. The BASP1 family of myristoylated proteins abundant in axonal termini. Primary structure analysis and physico-chemical properties. Biochimie 79, 373–384.

Myles, T., Nishimura, T., Yun, T.H., Nagashima, M., Morser, J., Patterson, A.J., Pearl, R.G., Leung, L.L.K., 2003. Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. Journal of Biological Chemistry 278, 51059–51067.

Naka, K., Dansako, H., Kobayashi, N., Ikeda, M., Kato, N., 2006. Hepatitis C virus NS5B delays cell cycle progression by inducing interferon- via toll-like receptor 3 signaling pathway without replicating viral genomes. Virology 346, 348–362.

Pennica, D., Swanson, T.A., Welsh, J.W., Roy, M.A., Lawrence, D.A., Lee, J., Brush, J., Taneyhill, L.A., Deuel, B., Lew, M., Watanabe, C., Cohen, R.L., Melhem, M.F., Finley, G.G., Quirke, P., Goddard, A.D., Hillan, K.J., Gurney, A.L., Botstein, D., Levine, A.J., 1998. WISP genes are members of the connective tissue growth factor family that are up-regulated in Wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proceedings of the National Academy of Sciences of the United States of America 95, 14717–14722.

Perretti, M., D'Acquisto, F., 2009. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nature Reviews Immunology 9, 62–70.

Redlitz, A., Tan, A.K., Eaton, D.L., Plow, E.F., 1995. Plasma carboxypeptidases as regulators of the plasminogen system. Journal of Clinical Investigation 96, 2534–2538.

Rowland, A.F., Fazakerley, D.J., James, D.E., 2011. Mapping insulin/GLUT4 circuitry. Traffic 12, 672–681.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q., Houghton, M., Kuo, G., 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proceedings of the National Academy of Sciences of the United States of America 87, 6547–6549.

Sharif, S.A., Du, X., Myles, T., Song, J.J., Price, E., Lee, D.M., Goodman, S.B., Nagashima, M., Morser, J., Robinson, W.H., Leung, L.L.K., 2009. Thrombin-activatable carboxypeptidase B cleavage of osteopontin regulates neutrophil survival and synoviocyte binding in rheumatoid arthritis. Arthritis and Rheumatism 60, 2902–2912.

Song, J.J., Hwang, I., Cho, K.H., Garcia, M.A., Kim, A.J., Wang, T.H., Lindstrom, T.M., Lee, A.T., Nishimura, T., Zhao, L., Morser, J., Nesheim, M., Goodman, S.B., Lee, D.M., Bridges Jr., S.L., Gregersen, P.K., Leung, L.L., Robinson, W.H., 2011. Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis. Journal of Clinical Investigation 121, 3517–3527.

Thomas, D., 2000. Hepatitis C epidemiology. Current Topics in Microbiology and Immunology 242, 25–41.
Thorstensen, L., Diep, C.B., Meling, G.I., Aagesen, T.H., Ahrens, C.H., Rognum, T.O., Lothe, R.A., 2001. Wnt1 inducible signaling pathway protein 3, WISP-3, a novel target gene in colorectal carcinomas with microsatellite instability. Gastroenterology 121, 1275–1280.
Tsunedomi, R., Ogawa, Y., Iizuka, N., Sakamoto, K., Tamesa, T., Moribe, T., Oka, M., 2010. The assessment of methylated BAPS1 and SRD5A2 levels in the detection of early hepatocellular carcinoma. International Journal of Oncology 36, 205–212.
Ueda, Y., Mori, K., Ariumi, Y., Ikeda, M., Kato, N., 2011. Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents. Biochemical and Biophysical Research Communications 409, 663–668.
Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.-G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nature Medicine 11, 791–796.
Xin, W., Rhodes, D.R., Ingold, C., Chinnaiyan, A.M., Rubin, M.A., 2003. Dysregulation of the annexin family protein family is associated with prostate cancer progression. American Journal of Pathology 162, 255–261.
Yazid, S., Ayoub, S.S., Solito, E., McArthur, S., Vo, P., Dufton, N., Flower, R.J., 2010. Anti-allergic drugs and the annexin-A1 system. Pharmacological Report 62, 511–517.
Zhang, Z., Huang, L., Zhao, W., Rigas, B., 2010. Annexin 1 induced by anti-inflammatory drugs binds to NF-kappaB and inhibits its activation: anticancer effects in vitro and in vivo. Cancer Research 70, 2379–2388.

Journal of General Virology (2012), 93, 1422–1431 DOI 10.1099/vir.0.040725-0

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

Midori Takeda,[1] Masanori Ikeda,[1] Yasuo Ariumi,[1]† Takaji Wakita[2] and Nobuyuki Kato[1]

Correspondence
Masanori Ikeda
maikeda@md.okayama-u.ac.jp

[1]Department of Tumor Virology, Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8544, Japan

[2]Department of Virology II, National Institute of Infectious Disease, Tokyo 162-8640, Japan

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

Received 21 December 2011
Accepted 26 March 2012

INTRODUCTION

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

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

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

†Present address: Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan.

Three supplementary figures are available with the online version of this paper.

revealed that HuH-7 and Li23 cells exhibited distinct gene-expression profiles (Mori *et al.*, 2010). For example, we identified three genes (New York oesophageal squamous cell carcinoma 1, β-defensin-1 and galectin-3) showing Li23-specific expression. Using HuH-7 and Li23 cells in combination with HCV strain O (genotype 1b), we developed drug-assay systems (OR6 and ORL8, respectively) by introducing the *Renilla* luciferase (RL) gene (Ikeda *et al.*, 2005; Kato *et al.*, 2009). We found and reported that the sensitivities to anti-HCV reagents were different between the HuH-7 and Li23 assay systems; for example, the Li23 assay system was 10 times more sensitive to ribavirin than the HuH-7 assay system (Mori *et al.*, 2011). Methotrexate showed very strong anti-HCV activity in the Li23 assay system, although it showed very weak anti-HCV activity in the HuH-7 assay system (Ueda *et al.*, 2011). These results encouraged us to develop a JFH-1 reporter-assay system using HuH-7 and Li23 cells. This JFH-1 reporter-assay system not only facilitated monitoring of virus infection and release steps, but also provided us with new information that could be missed in these steps when using only a HuH-7 assay system. However, increasing the size of the viral genome by introducing exogenous genes [RL and the encephalomyocarditis virus internal ribosomal site (EMCV-IRES)] reduced the efficiency of HCV RNA replication. To overcome this issue, we tried to improve the efficiency of HCV RNA replication by introducing adaptive mutations and by subcloning the parental cells.

Here, we developed JFH-1 HCV production reporter-assay systems in HuH-7- and Li23-derived cells using adaptive mutations and subcloned cells, which monitor the life cycle of HCV with luciferase activity. We also tested the effect of the mutations in NS5B from the JFH-1 strain on RNA replication of the specific genotype 1b O strain.

RESULTS

HCV mutations caused by long-term cell culture

The efficiency of HCV RNA replication depends on viral genetic mutations, host cells and viral genome size. For development of the HCV reporter-assay system, use of a longer viral genome reduced the efficiency of virus replication. To compensate for this issue, we tried to introduce adaptive mutations into the JFH-1 genome. We examined the viral sequences of JFH-1 130 days after infection of HuH-7-derived RSc cells. We performed RT-PCR for three parts of the viral genome: Core to NS2, NS3 to NS5A, and NS5B to 3′X. These three parts were separated by the *Age*I, *Spe*I, *Bsr*GI and *Xba*I sites on the viral genome. We introduced PCR products into the cloning vector and three independent clones were subjected to sequencing analysis.

In the Core to NS2 region between the *Age*I and *Spe*I sites (designated AS), there were eight common mutations with amino acid substitutions: lysine to glutamate at aa 78 (K78E) in Core, P251L and A351D in E1, V402A, I414T and K715N in E2, Y771C in p7, and D962G in NS2 (Fig. 1a). In the NS3 to NS5A region between *Spe*I and *Bsr*GI sites (designated SB), there were eight common mutations with amino acid substitutions: V1460I and M1611T in NS3, and I2270T, Q2307R, S2363L, M2392T, S2426A and C2441S in NS5A (Fig. 1b). In the NS5B to 3′X region between the *Bsr*GI and *Xba*I sites (designated BX), there was only one common mutation with an amino acid substitution, V2995L in NS5B (Fig. 1c). The determined sequences were studied further to enhance HCV RNA replication in the JFH-1 reporter assay.

Effect of genetic mutations on HCV RNA replication

To monitor the virus life cycle more easily, we constructed dicistronic JFH-1 with a reporter gene, pJR/C-5B. The first cistron contained the RL gene and was translated by the HCV-IRES. The second cistron contained the JFH-1 ORF and was translated by the EMCV-IRES. This construct facilitated monitoring of all steps of the virus life cycle by quantification of RL activity. However, the use of a longer viral genome resulted in lower replication efficiency. We tested the effect on HCV RNA replication of amino acid substitution caused during long-term cell culture.

The amino acid substitution clusters from three independent clones in Core to NS2 (AS-1, AS-2, AS-3) were introduced into pJR/C-5B. *In vitro*-transcribed HCV RNA was introduced into HuH-7-derived RSc cells, and RL activities were monitored 24, 48 and 72 h after electroporation (Fig. 2a). AS-3 exhibited higher replication efficiency than the wild type (WT). However, the replication efficiency of AS-2 was almost equal to that of the WT, and AS-1 exhibited lower replication efficiency than the WT. AS-3 possessed the highest replication efficiency among the tested JFH-1 mutants: at 72 h, the luciferase value of this clone was approximately 100 times that at 24 h.

The three pJR/C-5B constructs with mutations in NS3 to NS5A (SB-2, SB-3 and SB-4) were transcribed and introduced into RSc cells to compare the efficiency of HCV RNA replication (Fig. 2b). Unexpectedly, RL activity was not increased over 72 h after electroporation and exhibited a pattern similar to that of JFH-1 without the GDD motif. This result indicates that the mutation in NS3 to NS5A exhibited a negative effect on HCV RNA replication.

Finally, we tested the effect of the mutations in the NS5B region on HCV RNA replication. BX-2 contains two mutations with amino acid substitution (H2505Q and V2995L) and BX-7 contains only V2995L (Fig. 2c). JFH-1 with mutation(s) of BX-2 or BX-7 exhibited strong enhancement of HCV RNA replication. These results indicate that V2995L works as a strong replication-enhancing mutation (REM) in JFH-1 HCV RNA replication.

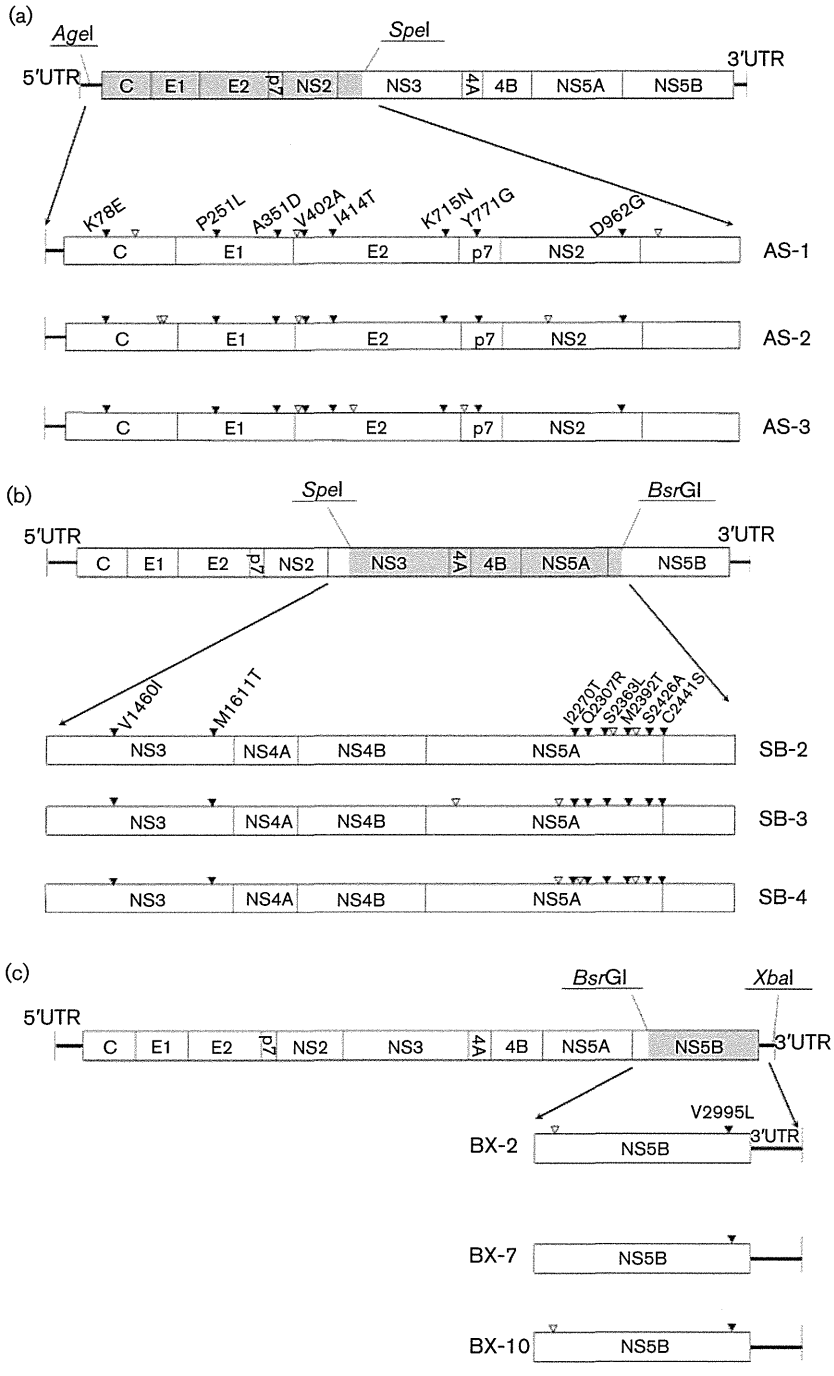

Fig. 1. Genetic mutations causing amino acid substitutions during long-term HCV infection. RT-PCR was performed for HCV RNAs from HuH-7 cells 130 days after JFH-1 infection. PCR products were subcloned into the pBluescript II plasmid. Three clones of (a) the Core to NS2 region between the *Age*I and *Spe*I sites (AS), (b) the NS3 to NS5A region between the *Spe*I and *Bsr*GI sites (SB) and (c) the NS5B to 3′X region between the *Bsr*GI and *Xba*I sites (BX) were subjected to sequencing analysis. ▼ and ▽ represent conservative and non-conservative amino acid substitutions, respectively.

Mutations in NS5B enhanced HCV RNA replication differently in genotypes 1b and 2a

V2995L in NS5B is a common substitution, occurring in three clones, and H2505Q is conserved in two clones (BX-2 and BX-10). We examined the corresponding amino acids at positions 2995 and 2505 in genotype 1b replication-competent HCV strains O, 1B-4 and KAH5 (Fig. 3a) (Nishimura *et al.*, 2009). The histidine at aa 2505 in JFH-1 is conserved in O, 1B-4 and KAH5 at the corresponding position, aa 2482. The valine at aa 2995 in JFH-1 is an alanine in O, 1B-4 and KAH5 at the corresponding position, aa 2972 (Fig. 3a). It is not clear whether the adaptive mutation found in genotype 2a is effective in genotype 1b HCV. Therefore, we investigated the effect of V2995L and/or H2505Q substitution on genotype 1b HCV RNA replication. We introduced substitutions V2995L and/or H2505Q into the subgenomic replicon, pOR/3-5B (HCV-O). In contrast to the case of JFH-1, H2505Q but not V2995L enhanced HCV-O RNA replication (Fig. 3b). These results indicate that the mutations in NS5B derived from JFH-1 functioned differently in genotype 1b HCV-O RNA replication.

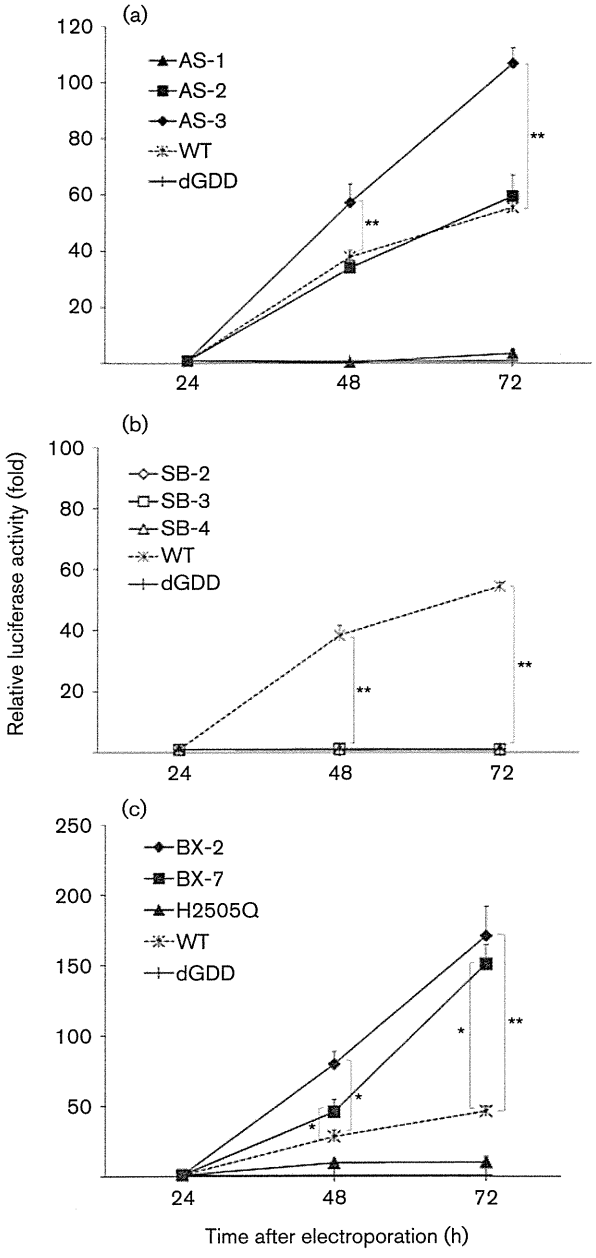

Fig. 2. Effect of amino acid substitutions on HCV RNA replication. (a) The Core to NS2 region; (b) the NS3 to NS5A region; (c) the NS5B to 3′X region. Amino acid substitutions were introduced into pJR/C5B and *in vitro*-synthesized RNAs were electroporated into HuH-7-derived RSc cells. RL activity was determined 24, 48 and 72 h after electroporation. dGDD, Negative control without the GDD motif; WT, wild type. *$P<0.05$; **$P<0.01$.

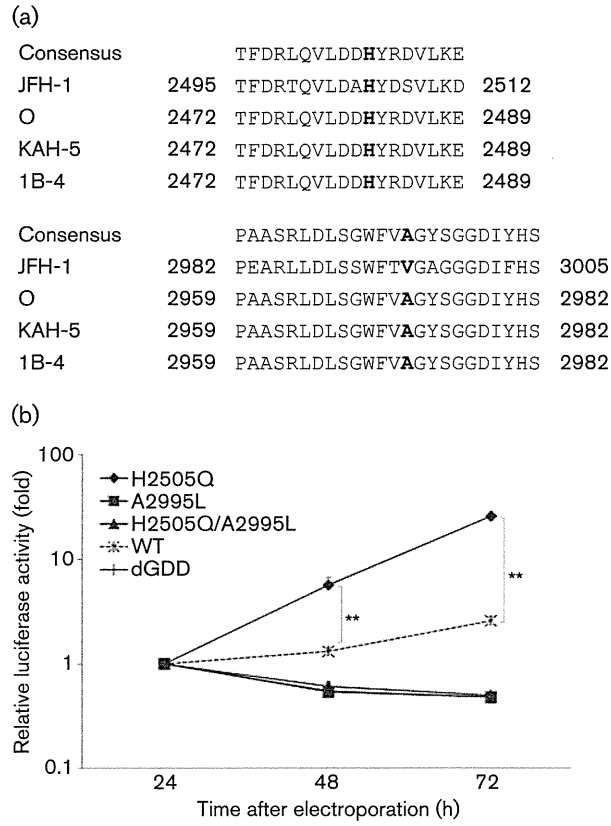

Fig. 3. Effect of amino acid substitutions in NS5B on genotype 1b and 2a HCV RNA replication. (a) Alignment of amino acids at positions 2505 (JFH-1) and 2482 (genotype 1b) and around the adjacent region (upper panel). Alignment of amino acids at positions 2995 (JFH-1) and 2972 (genotype 1b) and around the adjacent region (lower panel). The HCV strains O, KAH5 and 1B-4 belong to genotype 1b. (b) H2505Q and/or V2995L were introduced into the HCV-O subgenomic replicon (pOR/3-5B), and transcribed RNAs were electroporated into RSc cells. RL activities were tested 24, 48 and 72 h after infection. dGDD, Negative control without the GDD motif; WT, wild type. **$P<0.01$.

HCV infection in HuH-7- and Li23-derived cell lines

As well as viral genetic mutations, the choice of host cells is important for the efficiency of HCV RNA replication. Cured cells in which HCV RNAs were eliminated by IFN-α, such as HuH-7.5, HuH-7.5.1 and our RSc cells, exhibit higher replication efficiency than their parental HuH-7 cells (Ariumi *et al.*, 2007; Blight *et al.*, 2002; Zhong *et al.*, 2005). Therefore, we examined whether subcloned Li23 cells might enhance HCV RNA replication. We performed serial subcloning of Li23 cells from Li23-derived ORL8c cells by the limiting-dilution method (Fig. 4a). ORL8c cells are a cured cell line in which genome-length HCV RNAs were eliminated by interferon (IFN) treatment (Kato *et al.*, 2009). The subclonal Li23-derived cell lines were selected from among 50–100 independent single cells in 96-well plates by three-round limiting dilution from ORL8c cells (Fig. S1a, available in JGV Online). First, L8c15 cells were selected from their parental ORL8c cells by limiting dilution. Then, C22 cells were selected from their parental L8c15 cells by limiting dilution. Finally, D7 cells were selected from C22 cells by limiting dilution (Fig. S1b). Together, these steps resulted in the

selection of three subclonal cell lines that respectively exhibited the strongest replication efficiency in each round of selection. The lineages of the selected cell lines after three rounds of subcloning were designated L8c15, C22 and D7 cells, respectively.

We tested the subcloned cells for their HCV infectivities in comparison with those of HuH-7 and HuH-7-derived RSc cells. We reported previously that RSc cells could strongly support HCV replication and production (Kato *et al.*, 2009). Li23 and its derived ORL8c, L8c15, C22 and D7 cell lines were infected using the supernatant from RSc cells replicating JR/C-5B with BX-2 mutations at an m.o.i. of 0.2 (Fig. 4b, c). RL activities were determined 24, 48, 72 and 96 h after infection and f.f.u. ml^{-1} were determined 48 h after infection. The efficiency of HCV infectivity was highest in D7 cells, followed in order by C22, L8c15 and Li23 cells. HCV RNA replication in D7 cells was almost equal to that in RSc cells. These results suggest that the subcloned cell lines exhibit higher susceptibility to HCV infection than their parental cells.

Next, we further characterized the susceptibility of D7 cells to HCV infection in comparison with RSc cells, because D7 cells exhibited the highest susceptibility to HCV infection among the Li23-derived cell lines. D7 cells also exhibited the highest production and release of Core into the supernatant among the parental C22-derived subclonal

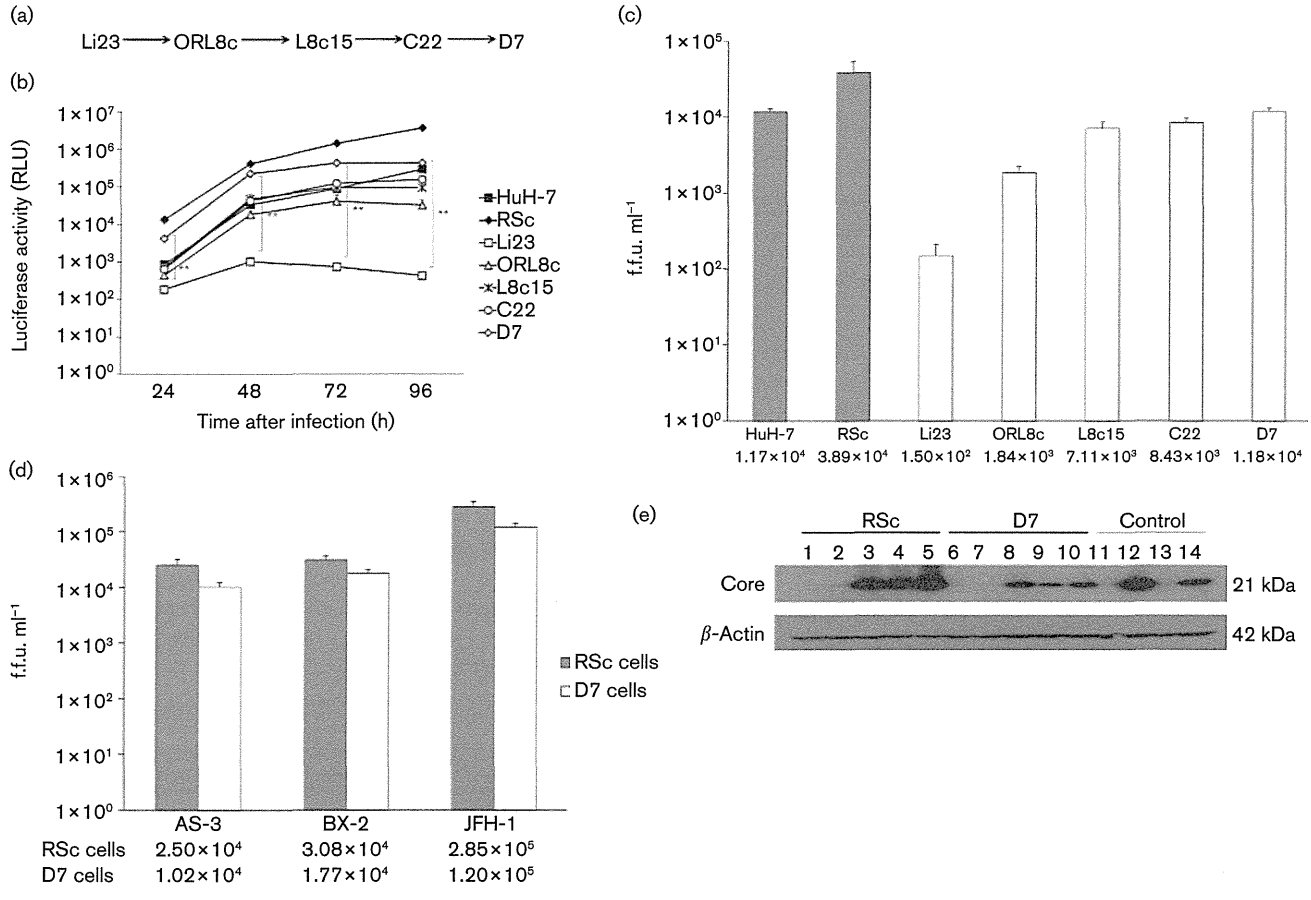

Fig. 4. HCV infection in HuH-7- and Li23-derived cell lines. (a) History of the selection of subclonal Li23-derived cell lines. (b) HuH-7, HuH-7-derived RSc, and Li23-derived ORL8c, L8c15, C22 and D7 cells were inoculated with supernatant from RSc cells replicating JR/C5B/BX-2. $^{**}P<0.01$. (c) f.f.u. ml^{-1} values were determined 48 h after infection of HuH-7- and Li23-derived cells with HCV using the supernatant from RSc cells replicating JR/C5B/BX-2 . (d) f.f.u. ml^{-1} values were determined 48 h after infection of RSc or D7 cells with HCV using the supernatant from RSc cells replicating JR/C5B/AS-3 or JR/C5B/BX-2. Supernatant from authentic JFH-1-replicating RSc cells was used as a positive control. (e) Core expression levels in RSc or D7 cells were determined 1, 2, 3 and 4 days after infection with JFH-1 with BX-2 mutations. Lanes: 1 and 6, mock-infected cells; 2 and 7, cells 1 day after infection; 3 and 8, cells 2 days after infection; 4 and 9, cells 3 days after infection; 5 and 10, cells 4 days after infection; 11 and 12, OR6c and OR6 cells, respectively; 13 and 14, ORL8c and ORL8 cells, respectively. OR6 and ORL8 were used as positive controls; OR6c and OR8c were used as negative controls. β-Actin was used as a control for the amount of protein loaded per lane.

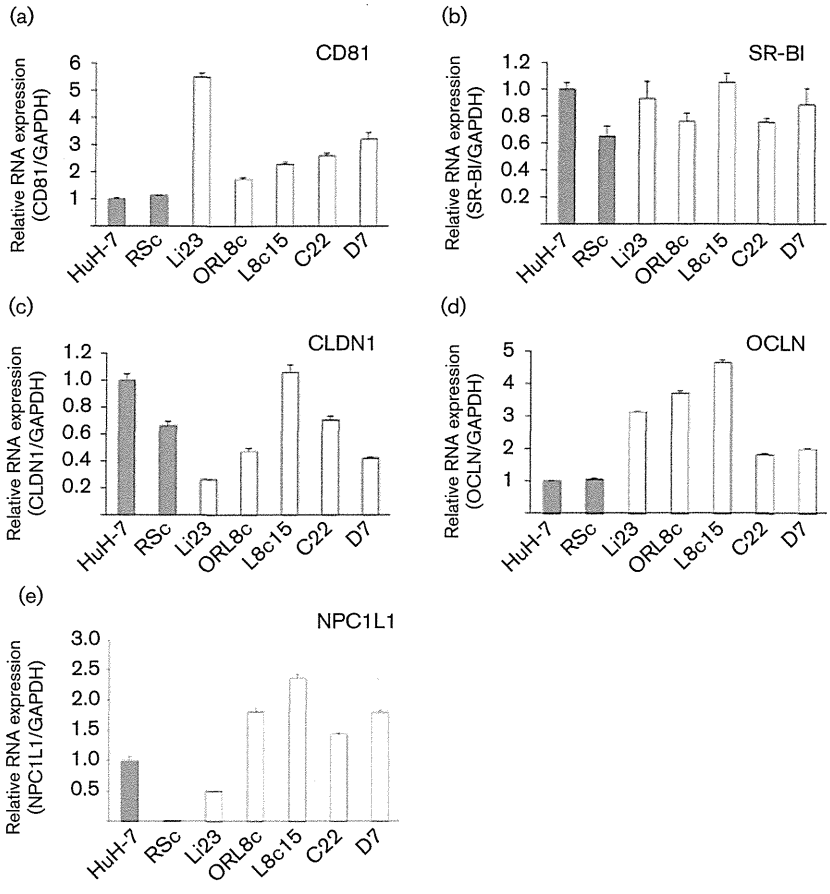

Fig. 5. Expression levels of HCV receptors in HuH-7- and Li23-derived cells. Quantitative RT-PCR was performed for CD81, SR-BI, CLDN1, OCLN and NPC1L1 as described in Methods. Relative expression levels of mRNA are shown, when the expression level of each receptor in HuH-7 was assigned to be 1. GAPDH was used as an internal control. Experiments were done in triplicate.

cells (Fig. S1b). The susceptibility of the HCV reporter-assay system to HCV infection was examined using HuH-7- and Li23-derived cells. Supernatants from RSc cells replicating JR/C-5B with AS-3 or BX-2 mutations were used as inocula. The supernatant from authentic JFH-1-replicating RSc cells was used as a positive control. RSc and D7 cells were inoculated with each HCV-containing supernatant and f.f.u. ml^{-1} were determined 48 h after infection. As shown in Fig. 4(d), the values of f.f.u. ml^{-1} for AS-3 were 2.5×10^4 and 1.0×10^4 in RSc and D7 cells, respectively; those for BX-2 were 3.1×10^4 and 1.8×10^4 in RSc and D7 cells, respectively; and those for authentic JFH-1 were 2.9×10^5 and 1.2×10^5 in RSc and D7 cells, respectively. These results indicate that the infectivities of these three inocula were almost equal in RSc and D7 cells.

Next we examined Core expression after infection of RSc and D7 cells with HCV, as D7 cells exhibited the highest infectivity among the Li23-derived cell lines (Fig. 4e). Core was detected 2, 3 and 4 days after infection of the supernatant from RSc cells infected by JR/C-5B with BX-2. Although Core expression in D7 cells was slightly weaker than that in RSc cells, the signal of Core in HCV-infected D7 cells was equal to that in stable ORL8 cells. These results suggest that the JFH-1 reporter-assay system in Li23 cells is useful not only for the RL assay, but also for Core expression.

Expression of HCV receptors in parental and subclonal hepatoma cell lines

We tested expression of the HCV receptors CD81, scavenger receptor class B member I (SR-BI), claudin-1 (CLDN1) and occludin (OCLN). We also examined the expression of the recently reported HCV entry factor Niemann–Pick C1-like 1 (NPC1L1) (Sainz *et al.*, 2012). Expression levels of CD81 in Li23 and its subclonal cells were higher than those in HuH-7 and RSc cells (Fig. 5a). Although expression of CD81 in Li23-derived cell lines was lower than that in parental Li23 cells, interestingly the expression levels of CD81 increased during the rounds of selection. There is no difference in the expression of SR-BI among the cell lines tested (Fig. 5b). The expression of CLDN1 in Li23-derived cells was higher than that in parental Li23 cells (Fig. 5c). Expression levels of OCLN in Li23 and its subclonal cells were higher than those in HuH-7 and RSc cells (Fig. 5d). Finally, the expression of NPC1L1 in Li23-derived cell lines was higher than that in parental Li23 cells (Fig. 5e). It is noteworthy that the expression level of NPC1L1 in RSc cells was approximately 2 $\log_{10}$ lower than that in parental HuH-7 cells. Taken together, these results indicate that the expression levels of CLDN1 and NPC1L1 in Li23-derived cells were higher than those in parental Li23 cells.

Life cycle of the HCV reporter-assay system in Li23-derived cells

We investigated whether D7 cells produce infectious HCV. First, D7 cells were inoculated using the supernatant from RSc cells replicating JR/C5B with BX-2, and the supernatant was stored at 17 days after infection. Then, the supernatant derived from the D7 cells was used as an inoculum for reinfection of naïve D7 cells. RL activities were determined 2, 6, 10 and 14 days after reinfection (Fig. 6). RL activity was increased after reinfection in D7 cells and reached a plateau 10 days after reinfection. These data indicate that the JFH-1 reporter-assay system is also useful for monitoring the HCV life cycle in Li23-derived cell lines.

DISCUSSION

In this study, we developed an HCV production reporter-assay system using two distinct hepatoma cell lines, HuH-7 and Li23. Robust HCV RNA replication and virus production were achieved by the introduction of REMs into the structural region or the NS5B region. These REMs were obtained from JFH-1-infected long-term-cultured cells. The two REMs in NS5B (H2505Q and V2995L substitutions) derived from JFH-1 had different effects on replication of genotype 1b HCV-O RNA and genotype 2a JFH-1 RNA. Furthermore, the subcloned Li23-derived D7 cells produced by serial limiting dilution supported this HCV production reporter-assay system.

Several groups have reported JFH-1 reporter virus systems (Koutsoudakis *et al.*, 2006; Marcello *et al.*, 2006; Pietschmann *et al.*, 2002; Wakita *et al.*, 2005). However, robust reporter virus production was limited within the study using HuH-7-derived cells. Therefore, we attempted to developed a JFH-1 reporter virus assay system using our previously reported line of Li23 cells (Kato *et al.*, 2009).

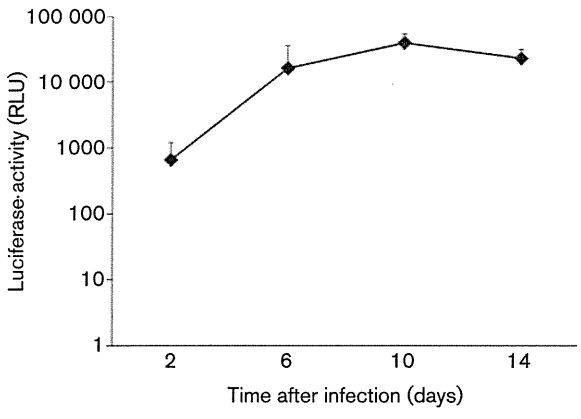

Fig. 6. HCV life cycle in Li23-derived D7 cells. D7 cells were inoculated with the supernatant from D7 cells after infection with JFH-1 with BX-2 mutants. RL activities were tested 2, 6, 10 and 14 days after infection.

The introduction of RL and EMCV-IRES genes into the HCV gene lengthened the genome of HCV by approximately 1.9 kb and led to a reduction in the efficiency of HCV RNA replication. To overcome this disadvantage, we adopted the following strategies: (i) introduce the REMs; (ii) select cloned Li23-derived cells with a highly permissive host condition by the serial limiting-dilution method. For the first purpose, we performed sequence analyses for HCV RNA from JFH-1-infected RSc cells. Mutations in the region from Core to NS2 or NS5B enhanced HCV RNA replication. However, combination of mutations from two different regions reduced HCV RNA replication (Fig. S2). The reason for this may be that these two mutation clusters were obtained from distinct RT-PCR-amplified clones and they were not necessarily located on the same viral genome. It has been reported that the combination of REMs exhibited an antagonistic effect on HCV RNA replication (Lohmann *et al.*, 2001). For the second purpose, we selected highly permissive Li23-derived clonal cells by the limiting-dilution method. We obtained three Li23-derived subclonal cell lines, L8c15, C22 and D7, in order from parental Li23-derived ORL8c cells. The efficiency of infectivity was highest in D7 cells, followed in order by C22, L8c15 and Li23 cells. D7 cells were highly permissive for infection of HCV with NS5B mutations.

As shown in Fig. 3(a), the histidine at aa 2505 in JFH-1 was conserved in the replication-competent O, 1B-4 and KAH5 strains at the corresponding position, aa 2482. The valine at aa 2995 in JFH-1 was alanine in strains O, 1B-4 and KAH5 at the corresponding position, aa 2972. The REMs in genotype 1b HCV were usually obtained by selection with neomycin after HCV RNA electroporation. Pietschmann *et al.* (2009) reported that REMs impaired infectious virus production. Most REMs are located in the NS3 and NS5A regions (Abe *et al.*, 2007; Blight *et al.*, 2002; Lohmann *et al.*, 2001; Pietschmann *et al.*, 2002). NS5A is a key molecule for virus production, and REMs affect the phosphorylation status of NS5A and the interaction with Core (Kato *et al.*, 2008; Masaki *et al.*, 2008; Tellinghuisen *et al.*, 2008). In contrast, our REMs in NS5B were obtained in JFH-1-infected long-term cell culture without drug selection. Taking this information into account, we considered that H2505Q in NS5B might not interfere with genotype 1b virus production. We attempted to apply this REM from genotype 2a to genotype 1b and found that H2505Q enhanced replication of the genotype 1b HCV-O replicon. We are currently investigating whether our NS5B REM could enhance genotype 1b HCV production. As for the substitution at aa 2995 in JFH-1 (aa 2972 in genotype 1b), we should be careful in interpretation, because the backgrounds at this position are different between genotypes 2 and 1. Analysis of an HCV database (http://s2as02.genes.nig.ac.jp/) revealed that the consensus amino acids at position 2995 in genotype 2 and at 2972 in genotype 1 were valine and alanine, respectively. Furthermore, alanine and valine are not found at position 2995 in genotype 2 or at 2972 in genotype 1, respectively. These observations

indicate that amino acid substitution between alanine and valine at these positions may be lethal for HCV of both genotypes. The amino acid at position 2995 in genotype 2 (2972 in genotype 1) is just upstream of a *cis*-acting replication element in NS5B. Therefore, the nucleotide at this position may affect the HCV RNA replication. To clarify this issue, further study will be needed.

A comparative study using HuH-7- and Li23-based JFH-1 reporter-assay systems would be expected to reveal new information on virus entry and release steps, because the backgrounds of these cells are different. Our recent study of these cells revealed the difference in sensitivities to anti-HCV reagents including ribavirin and methotrexate (Mori *et al.*, 2011; Ueda *et al.*, 2011). Furthermore, the IL28B genotype was different between HuH-7 and Li23 cells. The IL28B genotype (rs8099917) of HuH-7 cells renders them resistant to pegylated IFN and ribavirin, and Li23 cells are sensitive to pegylated IFN and ribavirin (M. Ikeda and N. Kato, unpublished data).

Recently, it was reported that stable expression of miR122 enhanced JFH-1 HCV production in Hep3B and HepG2 (Kambara et al., 2012; Narbus et al., 2011). It is noteworthy that the expression of miR122 in Li23-derived cells was almost the same as that in HuH-7 cells (Fig. S3). High-level expression of miR122 in Li23 cells may be one of the reasons that Li23 cells can support HCV production as robust as that in HuH-7 cells among the hepatocyte-derived cell lines. Interestingly, the expression levels of miR122 are higher in ORL8c, L8c15 and D7 cells, but not in C22 cells, than those in parental Li23 cells (Fig. S3). This result suggests that the expression level of miR122 may partly contribute to the fitness of HCV replication and production.

So far, we have only little information regarding the mechanism by which subclonal cells support HCV replication and production more efficiently than the parental cells. In this study, we found that the expression levels of CLDN1 and NPC1L1 in Li23-derived subclonal cells were higher than those in the parental Li23 cells. These results suggest that a high expression level of these entry factors in Li23-derived subclonal cells may contribute to enhanced virus entry. In the course of the experiment to determine the expression levels of NPC1L1 in HuH-7- and Li23-derived cell lines, we found that RSc cells expressed a very low level of NPC1L1 compared with the parental HuH-7 cells. Possible mechanisms for this are: (i) very low-level expression of NPC1L1 is sufficient for HCV entry; (ii) an unknown entry factor compensates for NPC1L1 in the entry step in RSc cells. Further study will be needed to clarify this issue.

In summary, we have developed JHF-1 reporter-assay systems using HuH-7-derived RSc and Li23-derived D7 cells. Expression levels of CLDN1 and NP1C1L were higher than those in the parental Li23 cells. We found different effects of REMs (V2995L and H2505Q) in NS5B on virus RNA replication in genotype 2a and 1b HCV strains. These findings will become useful tools for the study of the life cycle of HCV.

METHODS

Cell cultures. RSc and ORL8c cells were derived from the cell lines HuH-7 and Li23, respectively, as described previously (Kato *et al.*, 2009). L8c15, D7 and C22 cells were selected from ORL8c, L8c15 and C22 cells, respectively, by limiting dilution. HuH-7 and RSc cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies). Li23-derived cell lines were maintained in F12 medium (Life Technologies) and DMEM (1:1 in volume) supplemented with 1% FBS and epidermal growth factor (50 ng ml^{-1}; PeproTech, Inc.) as described previously (Kato *et al.*, 2009).

RT-PCR and sequencing analysis. RSc cells were infected with cell-culture-grown HCV (HCVcc) and cultured for 130 days. Total RNAs from these cells were prepared using an RNeasy extraction kit (Qiagen). These RNA samples were used for RT-PCR in order to amplify the Core to NS2 (4.0 kb), NS3 to NS5A (3.6 kb) and NS5B to 3′X (1.9 kb) regions. Reverse transcription was performed with an oligo$(dA)_{23}$ primer. The following primer pairs were employed: to amplify the Core to NS2 region, JFH-1/*Age*I (5′-CCCAAGCTTAC-CGGTGAGTACACCGGAATTGC-3′) and JFH-1/*Spe*IR (5′-TGCCA-TGTGCCTTGGATAGGTACG-3′); for the NS3 to NS5A region, JFH-1/*Spe*I (5′-CCCAGGGGTACAAAGTACTAGTGC-3′) and JFH-1/*Bsr*GIR (5′-CCCAAGCTTTACCTTTTTAGCCCTCTGTGAGGC-3′); for the NS5B to 3′X region, JFH-1/*Bsr*GI (5′-CCGCTCGAGACCC-TTTGAGTAACTCGCTGTTGC-3′) and JFH-1/*Xba*IR (5′-GCTCTA-GACATGATCTGCAGAGAGACCAGTTAC-3′). SuperScript III reverse transcriptase (Invitrogen) and KOD-plus DNA polymerase (TOYOBO) were used for reverse transcription and PCR, respectively. PCR products were ligated into pBluescript II (Fermentas) and three independent clones were subjected to sequencing analysis.

Plasmid construction. pJR/C-5B plasmid is a dicistronic HCV JFH-1 construct. The RL gene and HCV ORF were introduced into the first and second cistrons, respectively. To construct this plasmid, we fused the JFH-1 5′UTR with the RL gene by overlap PCR, and the PCR products were ligated into pFGR-JFH-1 (GenBank accession no. AB237837) at the *Age*I and *Pme*I sites. For the first PCR, the primer pair 5′-GCGCCTAGCCATGGCGTTAGTATG-3′ (J5dC) and 5′-AAGCCATGGCCGGCCCTGGGCGACGGTTGGTGTTTCTTTTGG-3′ (J5dCR) was employed to amplify the 5′UTR, and the primer pair 5′-AACCGTCGCCCAGGGCCGGCCATGGCTTCCAAGGTGTACG-ACCCC-3′ (JRL) and 5′-TCGAAATCTCGTGATGGCAGGTTGG-3′ (JRLR) was employed to amplify the RL region. These first PCR products were used in the second PCR as the templates. For the second PCR, the primer pair J5dC and JRLR was employed to amplify the 5′UTR and RL. KOD-plus DNA polymerase was used for PCR.

The H2505H and/or A2995L mutations were introduced into the HCV-O replicon by QuikChange mutagenesis (Stratagene) as described previously (Ikeda *et al.*, 2002).

Luciferase reporter assay. For the luciferase assay, approximately $1.0–1.5 \times 10^4$ HCV-harbouring cells were plated onto 24-well plates in triplicate and were cultured for 24–96 h after electroporation or infection, as described previously (Ikeda *et al.*, 2005). The cells were harvested with *Renilla* lysis reagent (Promega) and subjected to RL assay according to the manufacturer's protocol.

Western blot analysis. Preparation of cell lysates, SDS-PAGE and immunoblotting were performed as described previously (Kato *et al.*, 2003). The antibodies used in this study were Core (CP11; Institute of Immunology, Tokyo, Japan) and β-actin (AC-15; Sigma) antibodies. Immunocomplexes were detected with a Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science).

HCV infection and determination of f.f.u. To determine f.f.u. ml^{-1}, 6×10^3 cells were plated onto a 96-well plate 24 h before infection. The supernatant of HCV RNA-replicating cells was diluted serially and was used as an inoculum. Forty-eight hours after infection, the cells were fixed and Core was stained with anti-Core antibody and HRP-conjugated mouse anti-IgG antibody. Then, the expression of Core was visualized with a DAB substrate kit (DAKO). Culture supernatants and cells were collected for quantification of Core by ELISA (Mitsubishi Kagaku Bio-Clinical Laboratories).

Quantitative RT-PCR analysis. Quantitative RT-PCR analysis for HCV receptors was performed using real-time LightCycle PCR (Roche Diagnostics) as described previously (Ikeda *et al.*, 2005). The primer pairs for CD81, SR-BI, CLDN1 and OCLN were reported previously (Nakamuta *et al.*, 2011). The primer pair NPC1L1 (5′-AGATCTTCTTCTTCCGCCTCCA-3′) and NPC1L1R (5′-TGCCAG-AGCCGGGTTAAC-3′) was used for NPC1L1.

Statistical analysis. Luciferase activities were compared statistically between the various treatment groups using Student's *t*-test. *P*-values of <0.05 were considered statistically significant. The mean $\pm$ SD was determined from at least three independent experiments.

ACKNOWLEDGEMENTS

The authors would like to thank Masayo Takemoto, Takashi Nakamura and Keiko Takeshita for their technical assistance. This work was supported by Grants-in-Aid for Research on Hepatitis from the Ministry of Health, Labour, and Welfare of Japan.

REFERENCES

Abe, K., Ikeda, M., Dansako, H., Naka, K. & Kato, N. (2007). Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA. *Virus Res* **125**, 88–97.

Ariumi, Y., Kuroki, M., Abe, K., Dansako, H., Ikeda, M., Wakita, T. & Kato, N. (2007). DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. *J Virol* **81**, 13922–13926.

Blight, K. J., McKeating, J. A. & Rice, C. M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* **76**, 13001–13014.

Ikeda, M., Yi, M., Li, K. & Lemon, S. M. (2002). Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol* **76**, 2997–3006.

Ikeda, M., Abe, K., Dansako, H., Nakamura, T., Naka, K. & Kato, N. (2005). Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem Biophys Res Commun* **329**, 1350–1359.

Kambara, H., Fukuhara, T., Shiokawa, M., Ono, C., Ohara, Y., Kamitani, W. & Matsuura, Y. (2012). Establishment of a novel permissive cell line for the propagation of hepatitis C virus by expression of microRNA miR122. *J Virol* **86**, 1382–1393.

Kato, N. (2001). Molecular virology of hepatitis C virus. *Acta Med Okayama* **55**, 133–159.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. & Shimotohno, K. (1990). Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* **87**, 9524–9528.

Kato, N., Sugiyama, K., Namba, K., Dansako, H., Nakamura, T., Takami, M., Naka, K., Nozaki, A. & Shimotohno, K. (2003). Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. *Biochem Biophys Res Commun* **306**, 756–766.

Kato, T., Choi, Y., Elmowalid, G., Sapp, R. K., Barth, H., Furusaka, A., Mishiro, S., Wakita, T., Krawczynski, K. & Liang, T. J. (2008). Hepatitis C virus JFH-1 strain infection in chimpanzees is associated with low pathogenicity and emergence of an adaptive mutation. *Hepatology* **48**, 732–740.

Kato, N., Mori, K., Abe, K., Dansako, H., Kuroki, M., Ariumi, Y., Wakita, T. & Ikeda, M. (2009). Efficient replication systems for hepatitis C virus using a new human hepatoma cell line. *Virus Res* **146**, 41–50.

Koutsoudakis, G., Kaul, A., Steinmann, E., Kallis, S., Lohmann, V., Pietschmann, T. & Bartenschlager, R. (2006). Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* **80**, 5308–5320.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wölk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R. & other authors (2005). Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626.

Lohmann, V., Körner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Lohmann, V., Körner, F., Dobierzewska, A. & Bartenschlager, R. (2001). Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* **75**, 1437–1449.

Marcello, T., Grakoui, A., Barba-Spaeth, G., Machlin, E. S., Kotenko, S. V., MacDonald, M. R. & Rice, C. M. (2006). Interferons α and λ inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* **131**, 1887–1898.

Masaki, T., Suzuki, R., Murakami, K., Aizaki, H., Ishii, K., Murayama, A., Date, T., Matsuura, Y., Miyamura, T. & other authors (2008). Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. *J Virol* **82**, 7964–7976.

Mori, K., Ikeda, M., Ariumi, Y. & Kato, N. (2010). Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: comparison with HuH-7 and other hepatic cell lines. *Hepatol Res* **40**, 1248–1253.

Mori, K., Ikeda, M., Ariumi, Y., Dansako, H., Wakita, T. & Kato, N. (2011). Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system. *Virus Res* **157**, 61–70.

Narbus, C. M., Israelow, B., Sourisseau, M., Michta, M. L., Hopcraft, S. E., Zeiner, G. M. & Evans, M. J. (2011). HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *J Virol* **85**, 12087–12092.

Nakamuta, M., Fujino, T., Yada, R., Aoyagi, Y., Yasutake, K., Kohjima, M., Fukuizumi, K., Yoshimoto, T., Harada, N. & other authors (2011). Expression profiles of genes associated with viral entry in HCV-infected human liver. *J Med Virol* **83**, 921–927.

Nishimura, G., Ikeda, M., Mori, K., Nakazawa, T., Ariumi, Y., Dansako, H. & Kato, N. (2009). Replicons from genotype 1b HCV-positive sera exhibit diverse sensitivities to anti-HCV reagents. *Antiviral Res* **82**, 42–50.

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., Strand, D. & Bartenschlager, R. (2002). Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol* **76**, 4008–4021.

Pietschmann, T., Zayas, M., Meuleman, P., Long, G., Appel, N., Koutsoudakis, G., Kallis, S., Leroux-Roels, G., Lohmann, V. & Bartenschlager, R. (2009). Production of infectious genotype 1b virus particles in cell culture and impairment by replication enhancing mutations. *PLoS Pathog* **5**, e1000475.

Sainz, B., Jr, Barretto, N., Martin, D. N., Hiraga, N., Imamura, M., Hussain, S., Marsh, K. A., Yu, X., Chayama, K. & other authors (2012).

Identification of the Niemann–Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat Med* **18**, 281–285.

Tanaka, T., Kato, N., Cho, M. J., Sugiyama, K. & Shimotohno, K. (1996). Structure of the 3′ terminus of the hepatitis C virus genome. *J Virol* **70**, 3307–3312.

Tellinghuisen, T. L., Foss, K. L. & Treadaway, J. (2008). Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog* **4**, e1000032.

Ueda, Y., Mori, K., Ariumi, Y., Ikeda, M. & Kato, N. (2011). Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents. *Biochem Biophys Res Commun* **409**, 663–668.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Kräusslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* **102**, 9294–9299.

FEBS Open Bio 2 (2012) 279–283

FEBS openbio

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

Raloxifene inhibits hepatitis C virus infection and replication

Midori Takeda[a,1], Masanori Ikeda[a,*,1], Kyoko Mori[a], Masahiko Yano[a], Yasuo Ariumi[a,2], Hiromichi Dansako[a], Takaji Wakita[b], Nobuyuki Kato[a]

[a] Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama 700-8558, Japan
[b] Department of Virology II, National Institute of Infectious Disease, Tokyo 162-8640, Japan

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.

[1] These authors contributed equally to this work.
[2] Current address: Center for AIDS Research, Kumamoto University, Kumamoto 860–0811, Japan.
* Corresponding author. Fax: +81 86 235 7392.
E-mail address: maikeda@md.okayama-u.ac.jp (M. Ikeda).

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

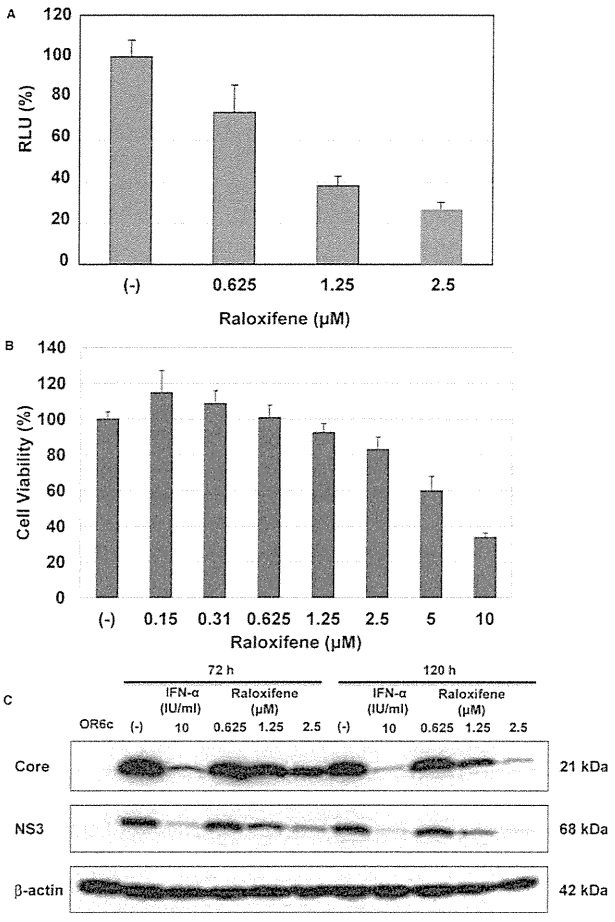

Fig. 1. Raloxifene inhibited HCV RNA replication. (A) Anti-HCV activity of raloxifene in OR6 cells. OR6 cells were treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) for 72 h. Relative RL activity (relative light unit: RLU) for HCV RNA replication is expressed as a percentage of control. Each bar represents 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.

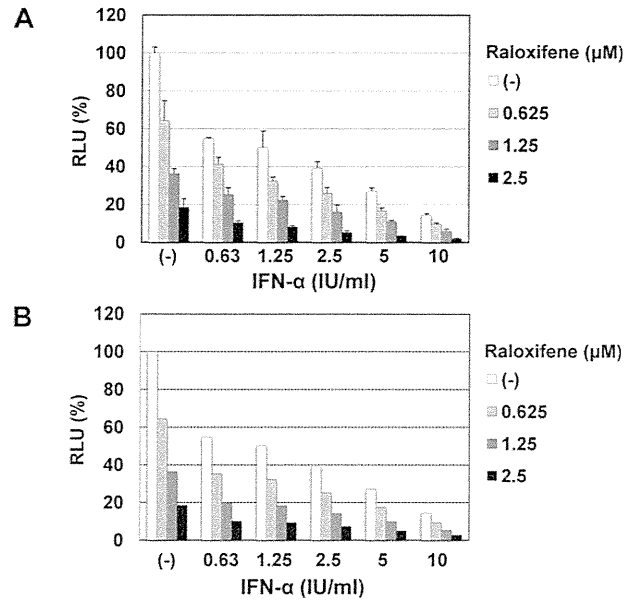

Fig. 2. Raloxifene enhanced the anti-HCV activity of IFN-α. (A) Anti-HCV activity of raloxifene in combination with IFN-α. OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and IFN-α (0, 0.63, 1.25, 2.5, 5, 10 IU/ml). Relative RL activity is shown as a percentage of control. Each 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.

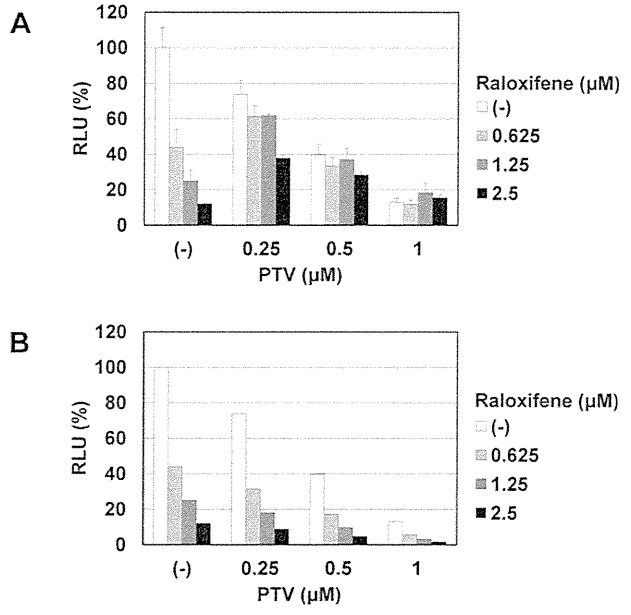

Fig. 3. Statin antagonized the anti-HCV activity of raloxifene. (A) OR6 cells were co-treated with raloxifene (0, 0.625, 1.25, and 2.5 μM) and PTV (0, 0.25, 0.5, and 1 μM). Relative RL activity was shown as a percentage of control. Each bar represents the average with standard deviations of triplicate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