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

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

Abstract

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

a 2-year culture period in cured cells. We further examined the expression levels of the selected candidate genes by RT-PCR analysis using additional specimens from the cells cultured for 3.5 years. Reproducibility of the RT-PCR analysis using specimens from recultured cells was also confirmed. Finally, we identified 5 upregulated genes and 4 downregulated genes, the expression levels of which were irreversibly altered during 3.5-year replication of HCV RNA. These genes may play key roles in the progression of HCV-associated hepatic diseases.

Keywords: HCV, HCV RNA replication system, Li23 cells, long-term RNA replication, upregulated host genes, downregulated host genes.

1. Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma (HCC) (Choo et al., 1989; Saito et al., 1990; Thomas, 2000). However, the mechanisms of HCV-associated hepatocarcinogenesis and disease progression are still unclear. HCV is an enveloped virus with a positive single-stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993; Kato et al., 1990).

The initial development of a cell culture-based subgenomic replicon system (Lohmann et al., 1999) and a genome-length HCV RNA-replication system (Ikeda et al., 2002) using genotype 1b strains enabled the rapid progression of investigations into the mechanisms underlying HCV replication (Bartenschlager, 2005; Lindenbach and Rice, 2005). Furthermore, these RNA replication systems have been improved such that they have become suitable for the screening of anti-HCV reagents by the introduction of

reporter genes such as luciferase (Ikeda et al., 2005; Krieger et al., 2001). Moreover, in 2005, an efficient virus production system using the JFH1 genotype 2a strain was developed using human hepatoma cell line HuH-7-derived cells (Wakita et al., 2005). However, to date, HuH-7-derived cells are used as the only cell culture system for robust HCV replication (Bartenschlager and Sparacio, 2007; Lindenbach and Rice, 2005). Most studies of HCV replication or anti-HCV reagents are currently carried out using a HuH-7-derived cell culture system. Therefore, it remains unclear whether or not recent advances obtained from the HuH-7-derived cell culture system reflect the general features of HCV replication or anti-HCV targets. To resolve this issue, we aimed to find a cell line other than HuH-7 that enables robust HCV replication. We recently found a new human hepatoma cell line, Li23, that enables efficient HCV RNA replication and persistent HCV production (Kato et al., 2009b). We further developed Li23-derived drug assay systems (ORL8 and ORL11) (Kato et al., 2009b), which are relevant to the HuH-7-derived OR6 assay system (Ikeda et al., 2005). Since we demonstrated that the gene expression profile of Li23 cells was distinct from that of HuH-7 cells (Mori et al., 2010), we expected to find that the host factors required for HCV replication or

anti-HCV targets in Li23-derived cells would also be distinct from those in HuH-7-derived cells. Indeed, we found that treatment of the cells with approximately 10 μM (a clinically achievable concentration) of ribavirin, an anti-HCV drug, efficiently inhibited HCV RNA replication in both the Li23-derived ORL8 and ORL11 assay systems, but not in the HuH-7-derived OR6 assay system (Mori et al., 2011). We further demonstrated that more than half of the 26 anti-HCV reagents that have been reported by other groups as anti-HCV candidates using HuH-7-derived assay systems other than OR6 assay system exhibited different anti-HCV activities from those of the previous studies (Ueda et al., 2011). In addition, we observed that the anti-HCV activities evaluated by the OR6 and ORL8 assay systems were also frequently different (Ueda et al., 2011). Furthermore, Li23-derived cells showed epidermal growth factor (EGF)-dependent growth (Kato et al., 2009b)-like immortalized or primary hepatocyte cells (e.g., PH5CH8 (Ikeda et al., 1998)), whereas HuH-7-derived cells can grow in an EGF-independent manner. Our findings, when taken together, suggested that a study using Li23-derived cells might yield unexpected results, since only HuH-7-derived cells are commonly used in a wide range of HCV studies.

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

2. Materials and methods

2.1. Cell culture

The Li23 cell line consists of human hepatoma cells from a Japanese male (age 56) was established and characterized in 2009 (Kato et al., 2009b). Li23 cells were maintained in modified culture medium for the PH5CH8 human immortalized hepatocyte cell line (Ikeda et al., 1998), as described previously (Kato et al., 2009b). Genome-length HCV RNA-replicating cells (Li23-derived OL, OL8, OL11, and OL14 cells) were also maintained in the medium for the Li23 cells in the presence of 0.3

mg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA). Cured cells (OL8c and OL11c cells), from which the HCV RNA had been eliminated by interferon (IFN)-γ treatment (Abe et al., 2007), were cultured in the medium for the Li23 cells. These cells were passaged every 7 days for 3.5 years. In this study, OL, OL8, OL11, OL14, OL8c, and OL11c cells were renamed as OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells, respectively, to specify the time at which the cells were established. Two-year cultures of OL(0Y), OL8(0Y), OL11(0Y), OL14(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL(2Y), OL8(2Y), OL11(2Y), OL14(2Y), OL8c(2Y), and OL11c(2Y) cells, respectively. The 3.5-year cultures of OL8(0Y), OL11(0Y), OL8c(0Y), and OL11c(0Y) cells were designated as OL8(3.5Y), OL11(3.5Y), OL8c(3.5Y), and OL11c(3.5Y) cells, respectively. The cured cells obtained from OL8(2Y) and OL11(2Y) cells by IFN-γ treatment (Abe et al., 2007) were designated as OL8(2Y)c and OL11(2Y)c cells, respectively, and were maintained in the medium for the Li23 cells.

2.2. cDNA microarray analysis

OL(0Y), OL(2Y), OL8(0Y), OL8(2Y), OL11(0Y), OL11(2Y), OL8c(0Y), OL8c(2Y), OL11c(0Y), and OL11c(2Y) cells were cultured in the medium without G418 during a few passages, and then these cells (1 x 10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 or 3 days. Total RNAs from these cells (approximately 70-80% confluency) were prepared using the RNeasy extraction kit (QIAGEN, Hilden, Germany). The cDNA microarray analysis was performed according to previously described methods (Kato et al., 2009b; Mori et al., 2010). Differentially expressed genes were selected by comparing the arrays from the genome-length HCV RNA-replicating cells, and the selected genes were further compared with the arrays from the cured cells (see Fig. 2 for details).

2.3. RT-PCR

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

primers arranged for this study are listed in Table 1.

2.4. Quantitative RT-PCR analysis

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

2.5. Western blot analysis

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

gift from Dr. M. Kohara), and NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK). β-actin antibody (Sigma, St. Louis, MO) was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected by the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Sciences, Boston, MA).

2.6. Statistical analysis

Statistical comparison of the luciferase activities between the treatment groups and controls was performed using Student's t-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

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

To prepare specimens for the cDNA microarray analysis, genome-length HCV RNA-replicating OL(0Y), OL8(0Y), OL11(0Y), and OL14(0Y) cells were cultured for 2 years, and were designated as OL(2Y), OL8(2Y), OL11(2Y), and OL14(2Y) cells,

1 respectively. Using the total RNA specimens obtained from these cells, the levels of
2 genome-length HCV RNAs were examined by quantitative RT-PCR analysis. The
3 results revealed that the levels of the genome-length HCV RNAs had increased in all
4 cases after a 2-year period of HCV RNA replication (Fig. 1). The levels of HCV
5 proteins (Core, E1, NS3, and NS5B) were also examined by Western blot analysis. The
6 E1, NS3, and NS5B were detected in all specimens, except for the Li23 cells, although a
7 little larger size of E1 was additionally detected in the specimens from 2-year culture
8 (Fig. 1). This phenomenon may indicate the appearance of additional N-glycosylation
9 sites by mutations caused during the 2-year replication of the HCV RNA, as observed in
10 a previous report (Mori et al., 2008). In addition, Core was not detected in the cultures
11 of OL11(2Y) cells, even when polyclonal anti-Core antibody was used (Fig. 1). A
12 similar phenomenon was observed in a previous study using HuH-7-derived
13 genome-length HCV RNA-replicating cells (Kato et al., 2009a). Since we detected the
14 full-sized HCV RNA by RT-PCR analysis (data not shown), this result suggests that
15 marked sequence variations within the epitopes of the anti-Core antibody occurred
16 during long-term cell culture, as was also observed in a previous study using

HuH-7-derived cells (Kato et al., 2009a).

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

To identify those genes whose expression levels were irreversibly altered by the long-term replication of HCV RNA, we performed a combination of cDNA microarray and RT-PCR analyses using several Li23-derived cell lines. An outline of the selection process performed in this study is provided in Figure 2. The first microarray analysis I was carried out by the comparison of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells. In this step, we selected those genes whose expression levels commonly showed changes in at least two of three comparative analyses. As regards the selected genes, a microarray analysis II was performed in which OL8c(0Y) cells were compared to OL8c(2Y) cells, and OL11c(0Y) cells were compared to OL11c(2Y) cells. In this step, the genes were excluded from those selected by the microarray analysis I if their expression levels had changed during the 2-year culture of cured cells. As regards the selected genes, we next performed a RT-PCR analysis I to examine the reproducibility of changes in gene

expression levels. In this step, we added the results of a new comparative series, OL14(0Y) versus OL14(2Y), to arrive at the judgment to advance to the next step of analysis. We selected genes for which expression levels had changed in more than five of six comparative series (Fig. 2). At the last step, we confirmed by RT-PCR analysis II whether or not the expression levels of the selected genes in OL8(2Y) or OL11(2Y) cells had changed by HCV RNA replication. When the gene expression levels had not changed in two comparative series (OL8(2Y) versus OL8(2Y)c and OL11(2Y) versus OL11(2Y)c), the genes were selected as the candidates exhibiting irreversible changes after 2-year HCV RNA replication.

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

The process outlined in Figure 2 was used to identify those genes that exhibited irreversibly upregulated expression during the 2-year replication of HCV RNA. Microarray analysis I revealed 1912, 1148, and 1633 probes, the expression levels of which were upregulated at a ratio of more than 2 in the case of OL(0Y) cells versus

OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. From among these probes, we selected those showing ratios of more than 4 with an expression level of more than 100 (actual value of measurement), or those showing ratios of more than 3 with an expression level of more than 200, or those showing an expression level of 1000. By this selection process, 559, 237, and 368 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 3A). At this step, we obtained 51 genes as candidates exhibiting upregulation in more than two of three comparisons. Based on the results of the subsequent microarray analysis II, we further selected 14 genes from a total of 51 genes, because the expression levels of the remaining 37 genes increased during the 2-year culture of cured cells (Fig. 3B). As regards the 14 selected genes, we performed an RT-PCR analysis I to confirm the results obtained by the cDNA microarray analysis and to examine the status of gene expression in an additional comparison of OL14(0Y) cells versus OL14(2Y) cells. This analysis revealed that the mRNA levels of 6 of 14 genes showed no enhancement in two of four comparative

series (data not shown). Therefore, in this step, these 6 genes were excluded from the candidate genes. However, the mRNA levels of the remaining 8 genes (acyl-CoA synthetase medium-chain family member 3 [*ACSM3*], angiopoietin 1 [*ANGPT1*], cyclin-dependent kinase inhibitor 2C [*CDKN2C*], phospholipase A1 member A [*PLA1A*], Sel-1 suppressor of lin-12-like 3 [*SEL1L3*], solute carrier family 39 member 4 [*SLC39A4*], TBC1 domain family member 4 [*TBC1D4*], and WNT1 inducible signaling pathway protein 3 [*WISP3*]) were enhanced in more than three of four comparative series (Fig. 3C). Furthermore, we demonstrated by RT-PCR analysis II that the expression levels of these 8 genes did not return to initial levels, even after elimination of HCV RNA from OL8(2Y) or OL11(2Y) cells (Fig. 3C). It was noteworthy that the mRNA levels of the *ANGPT1* and *PLA1A* genes were enhanced in all comparative series (Fig. 3C).

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

To obtain genes showing irreversibly downregulated expression during the 2-year

HCV RNA replication period, we performed a selection of genes according to the methods described for the selection of upregulated genes. The first microarray analysis I in this series revealed 1901, 2128, and 1579 probes whose expression levels were downregulated at a ratio of less than 0.5 in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively. From among these probes, we selected those showing ratios of less than 0.25 with an initial expression level of more than 1000 (actual value of measurement), or those showing ratios of less than 0.33 with an initial expression level of more than 200, or those showing an initial expression level of 100. By this selection process, 828, 622, and 466 genes (redundant probes excluded) were assigned in the case of OL(0Y) cells versus OL(2Y) cells, OL8(0Y) cells versus OL8(2Y) cells, and OL11(0Y) cells versus OL11(2Y) cells, respectively (Fig. 4A). At this step, we obtained 236 genes as candidates showing downregulation in more than two of three comparisons. Based on the results 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). 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), 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 Figures 3C and 4C. The results revealed that most of the genes examined showed reproducible results, as shown in Figures 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 differences, as depicted in Figure 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. However, in the present study, we did not observe any drastic enhancement of HCV RNA replication levels during a period of at least two years of culture (Fig. 1). Furthermore, 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), suggesting that these genes are not host factors determinative of HCV RNA levels. Therefore, it is unlikely that these genes are related to environmental factors required for HCV RNA replication. Indeed, 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. 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 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. 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

1 is an important mediator of the anti-inflammatory effects of glucocorticoids (Perretti
2 and D'Acquisto, 2009). Furthermore, it was reported recently that ANXA1 is an
3 endogenous inhibitor of NF-κB which can be induced in human cancer cells and mice
4 by anti-inflammatory glucocorticoids and modified nonsteroidal anti-inflammatory
5 drugs (Zhang et al., 2010). The suppression of NF-κB activity by the binding of
6 ANXA1 to the p65 subunit of NF-κB was accompanied by enhanced apoptosis and
7 inhibition of cell growth. In this context, the irreversible suppression of ANXA1
8 observed in the present study may weaken the anti-inflammatory effects of
9 glucocorticoids. However, in our microarray analysis, no expression of the ANXA1
10 receptor (ALXR; formyl peptide receptor 2 known as ALXR in humans) was observed.
11 Therefore, it is unlikely that Li23-derived cells respond to glucocorticoids in an
12 autocrine manner leading to the anti-inflammatory state, although secreted ANXA1 may
13 interact with its target cells in a paracrine manner. On the other hand, ANXA1 has been
14 shown to be strongly suppressed in prostate cancer (Xin et al., 2003), head and neck
15 cancer (Garcia Pedrero et al., 2004), and esophageal cancer (Huet al., 2004). Moreover,
16 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. Although we have not yet clarified how these irreversible changes in the expression of identified genes modify cellular function, we may speculate about the nature of the functional changes in several of these genes, as described above.

Additional studies using primary hepatocytes or immortalized noncancerous hepatocytes will be needed to clarify the biological significance of expressional changes of the identified genes. Such studies would lead to a better understanding of the mechanisms underlying the long-term persistent replication of HCV RNA that account for how such long-term replication modifies gene function in host cells.

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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.

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. Mol. Cancer Res. 7, 266–274.

Bartenschlager, R., 2005. The hepatitis C virus replicon system: From basic research to clinical application. J. Hepatol. 43, 210–216.

Bartenschlager, R., Sparacio, S., 2007. Hepatitis C virus molecular clones and their replication capacity in vivo and in cell culture. Virus Res. 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. Mol. Cell Biol. 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 Res. 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. J. Cell Physiol. 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. Am. J. Pathol. 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). Proc. Natl. Acad. Sci. U. S. A. 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. Proc. Natl. Acad. Sci. U. S. A. 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. Proc. Natl. Acad. Sci. U. S. A. 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. Nat. Genet. 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. Clin. Cancer Res. 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 Res. 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. The Am. J. of Pathol. 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. Biochem. Biophys. Res. Commun. 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 Res. 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. J. Virol. 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. J. Biol. Chem. 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. Arch. Virol. 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. Proc. Natl. Acad. Sci. U.S.A. 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 Res. 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. Biochem. Biophys. Res. Commun. 306, 756–766.

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

Lindenbach, B. D., Rice, C. M., 08 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. Biochem. J. 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. Biochem. and Biophys. Res. Commun. 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 Res. 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. Hepatol. Res. 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. Int. J. Oncol. 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. J. Biol. Chem. 278, 51059–51067.

Naka, K., Dansako, H., Kobayashi, N., Ikeda, M., Kato, N., 3 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. Proc. Natl. Acad. Sci. U. S. A. 95, 14717–14722.

Perretti, M., D'Acquisto, F., 2009. Annexin A1 and glucocorticoids as effectors of the

resolution of inflammation. Nat. Rev. Immunol. 9, 62–70.

Redlitz, A., Tan, A. K., Eaton, D. L., Plow, E. F., 1995. Plasma carboxypeptidases as regulators of the plasminogen system. J. Clin. Invest. 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. Proc. Natl. Acad. Sci. U. S. A. 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., and Leung, L. L. K., 2009. Thrombin-activatable carboxypeptidase B cleavage of osteopontin regulates neutrophil survival and synoviocyte binding in rheumatoid arthritis, Arthritis. Rheum. 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, S. L. Jr., Gregersen, P. K., Leung, L. L., Robinson, W. H., 2011. Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis. J. Clin. Invest. 121, 3517–3527.

Thomas, D., 2000. Hepatitis C epidemiology. Curr. Top. Microbiol. Immunol. 242, 25–41.

Thorstensen, L., Diep, C. B., Meling, G. I., Aagesen, T. H., Ahrens, C. H., Rognum, T. O., Lothe, R. A., 12 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. Int. J. Oncol. 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. Biochem. Biophys. Res. Commun. 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. Nat. Med. 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. Am. J. Pathol. 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. Pharmacol. Rep. 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 Res. 70, 2379–2388.

Figure Legends

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

Fig. 2. Outline of selection process performed in this study. To obtain the objective

genes, cDNA microarray analyses I and II were performed, and then RT-PCR analyses I and II were also performed.

Fig. 3. Identification of genes irreversibly upregulated during 2-year replication of HCV RNA. (A) Upregulated genes obtained by microarray analysis I shown in Figure 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 Figure 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 Figure 2 were performed as described in Materials and Methods. PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels.

Fig. 4. Identification of genes irreversibly downregulated during 2-year replication of

HCV RNA. (A) Downregulated genes obtained by microarray analysis I shown in Figure 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 Figure 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 Figure 2, were performed as described in Figure 3C.

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 Materials and Methods. Experiments were done in triplicate. The vertical lines indicate

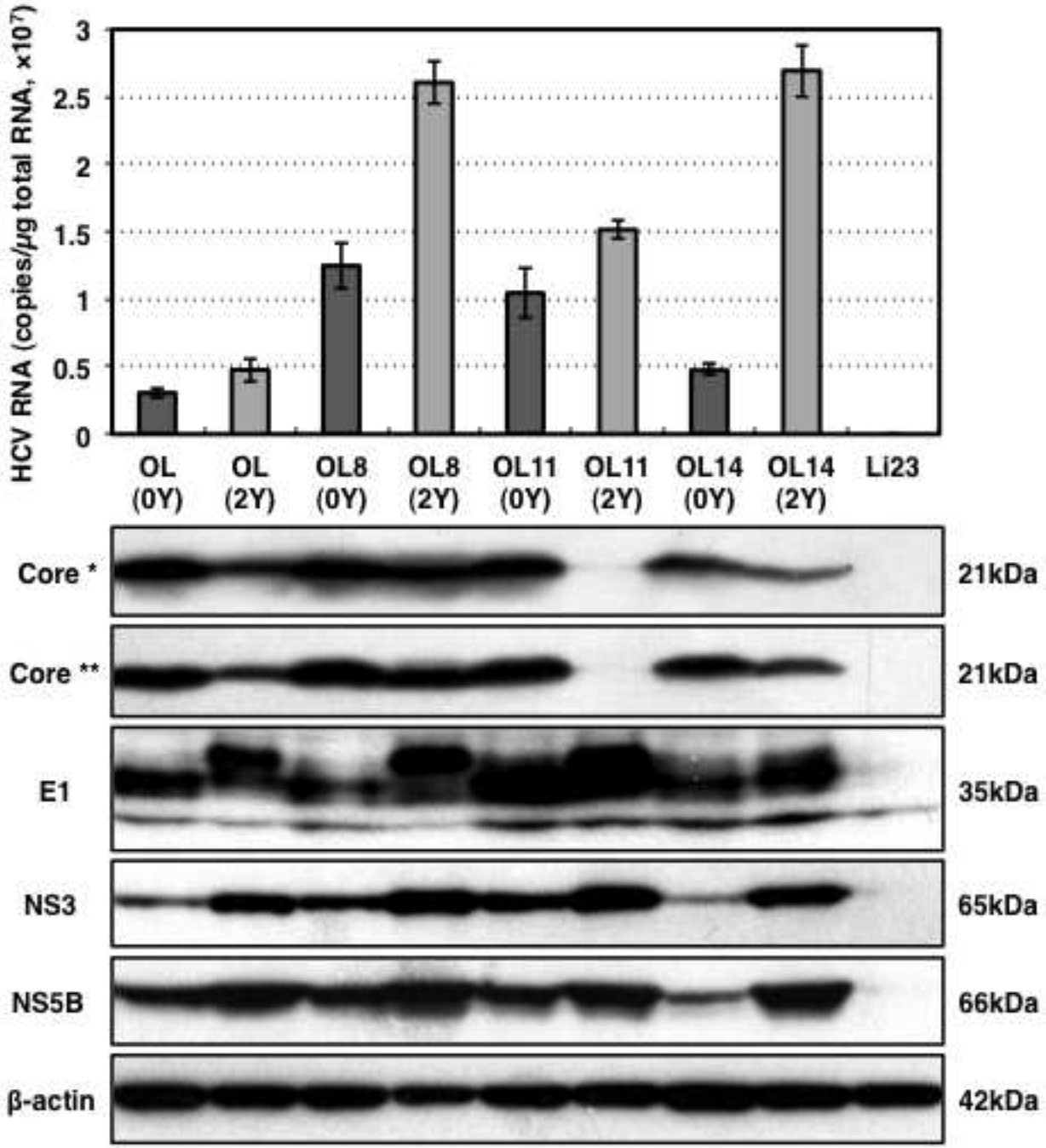
1 the expression levels, with the fold in the scale of log2, when the level in OL8(0Y) or
2 OL11(0Y) cells was assigned to be 1. Asterisks indicate significant differences between
3 mRNA levels of HCV RNA-replicating cells and their cured counterparts. * $P < 0.05$;
4 ** $P < 0.01$.
5
6 **Fig. 6.** Expression levels of genes selected as downregulated genes in 3.5-year cell
7 culture. Quantitative RT-PCR analysis was performed as shown in Figure 5, and the
8 obtained results are also presented as shown in Figure 5.
9
10

Table 1 Primers used for RT-PCR analysis

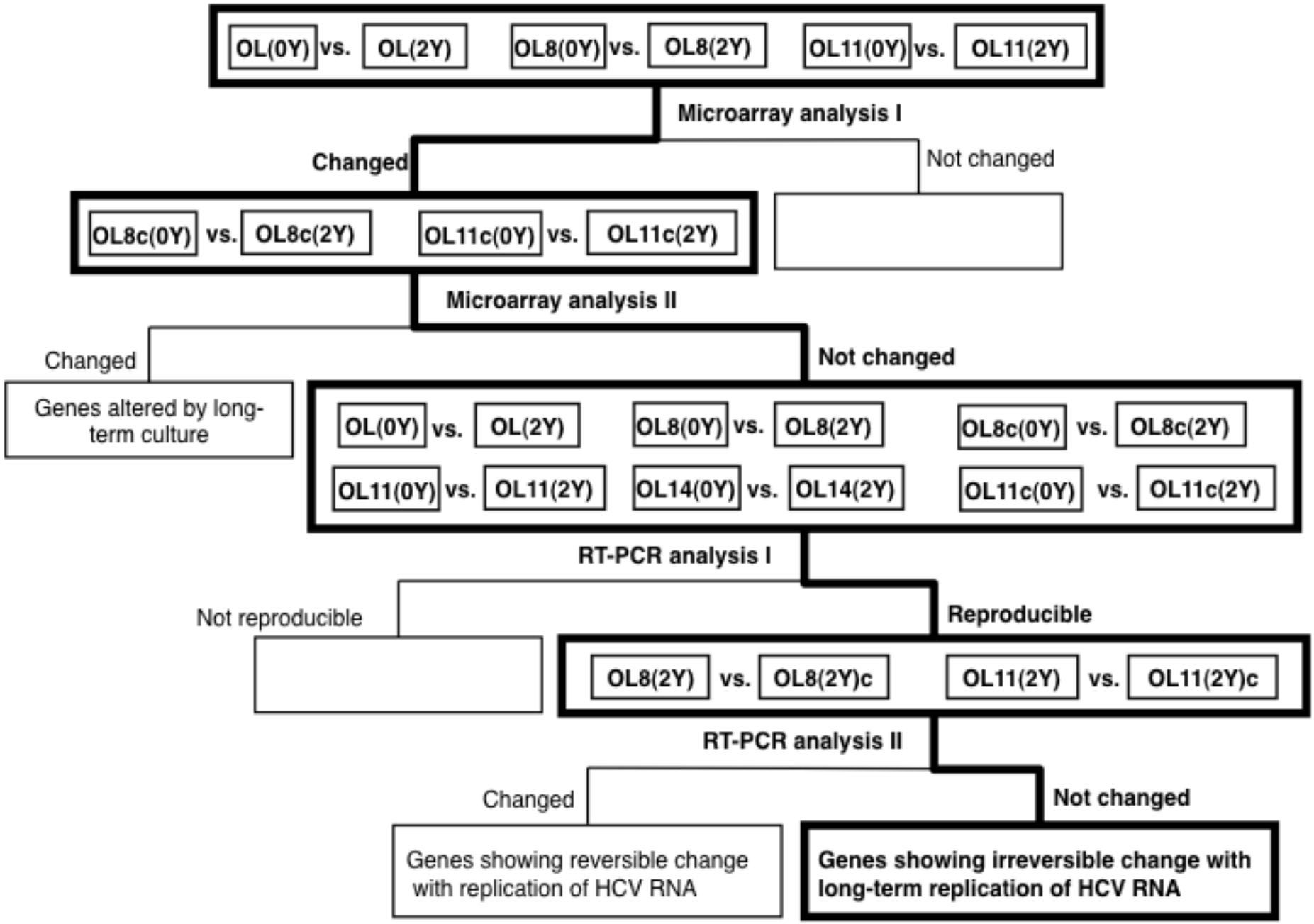
Gene (Accession No.)	Direction	Nucleotide sequence (5' – 3')	Products (bp)	Gene (Accession No.)	Direction	Nucleotide sequence (5' – 3')	Products (bp)
Acyl-CoA synthetase medium-chain family member 3 (ACSM3; NM_005622)	Forward Reverse	GCATTCAAGTTCTACCCAACCGAC GGCTGCTGACAACAGCTGACTC	258	Brain abundant, membrane attached signal protein 1 (BASP1; NM_006317)	Forward Reverse	GGATGAATGCCAGCTTTCAGACAG ACTGGAACTGCAATGAACGCAGAC	247
Angiopoietin 1 (ANGPT1; NM_001146)	Forward Reverse	ATACAACATCGTGAAGATGGAAGTC CCGTGTAAGATCAGGCTGCTCTG	287	Cell death activator CIDE-3 (CIDEC; NM_022094)	Forward Reverse	GATCTGTACAAGCTGAACCCACAG GACAGGTCGGGATAAGGGATGAG	265
Cyclin-dependent kinase inhibitor 2C (CDKN2C; NM_001262)	Forward Reverse	AAGACCGAACTGGTTTCGCTGTC CATAGAGCCTGGCCAAATCACAG	246	Carboxypeptidase B2 (CPB2; NM_001872)	Forward Reverse	GGAACTGTCTCTAGTAGCCAGTG CAGCGGCAAAAGCTTCTCTACAG	242
Phospholipase A1 member A (PLA1A; NM_015900)	Forward Reverse	GGAGTTTCACTTGAAGGAACTGAG GTTCACTGGTTCAGGTAAGCAGAC	292	Heat shock 70 kDa protein B' (HSPA6; NM_002155)	Forward Reverse	TGAAGCCGAGCAGTACAAGGCTG CTCCCTCTTCTGATGCTCATACTC	235
Sel-1 suppressor of lin-12-like 3 (SEL1L3; NM_015187)	Forward Reverse	ACCTGCACTTGCGGCTTCTCTG AGAGGCATCTGCAGCTGGAGTC	212	Peptidase inhibitor 3 (PI3; NM_002638)	Forward Reverse	GGTTCTAGAGGCAGCTGTCACG CCGCAAGAGCCTTCACAGCAC	276[a]
Solute carrier family 39 member 4 (SLC39A4; NM_017767)	Forward Reverse	GCCTGTTCCTCTACGTAGCACTC GAAGGTGATGTCATCCTCGTACAG	158	Peptidase inhibitor 3 (PI3; NM_002638)	Forward Reverse	GGTTCTAGAGGCAGCTGTCACG GCAGTCAGTATCTTTCAAGCAGC	241[b]
TBC1 domain family, member 4 (TBC1D4; NM_014832)	Forward Reverse	GGAGAGGGCCAATAGCCAACTG AGCTTCCGGAGTTGCTCCACTG	198	Solute carrier family member 3 (SLC1A3; NM_004172)	Forward Reverse	CAATGGCGTGGACAAGCGCGTC CCGACAGATGTCAGCACAATGAC	240
WNT1 inducible signaling pathway protein 3 (WISP3; NM_003880)	Forward Reverse	AGAGATGCTGTATCCCTAATAAGTC CAGGTTCTCTGCAGTTTCTCTGAC	129	Thrombospondin type-1 domain-containing protein 4 (THSD4; NM_024817)	Forward Reverse	TGGAGTCAGTGTTCCATCGAGTG GGGTCACAGAGGTTACTTAGAGTC	275
Annexin A1 (ANXA1; NM_000700)	Forward Reverse	GACTTGGCTGATTCAGATGCCAG AATGTCACCTTTCAACTCCAGGTC	192	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046)	Forward Reverse	GACTCATGACCACAGTCCATGC GAGGAGACCACCTGGTGCTCAG	334
Amphiregulin (AREG; NM_001657)	Forward Reverse	CGGGAGCCGACTATGACTACTC AAGGCAGCTATGGCTGCTAATGC	391				

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

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

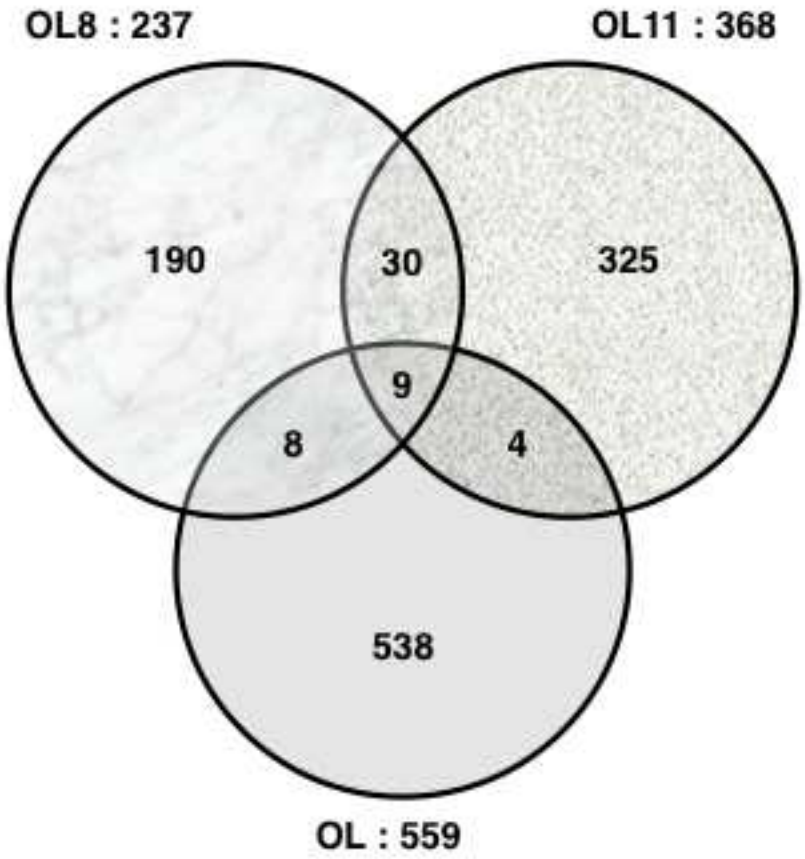


Sejima et al., Fig. 1

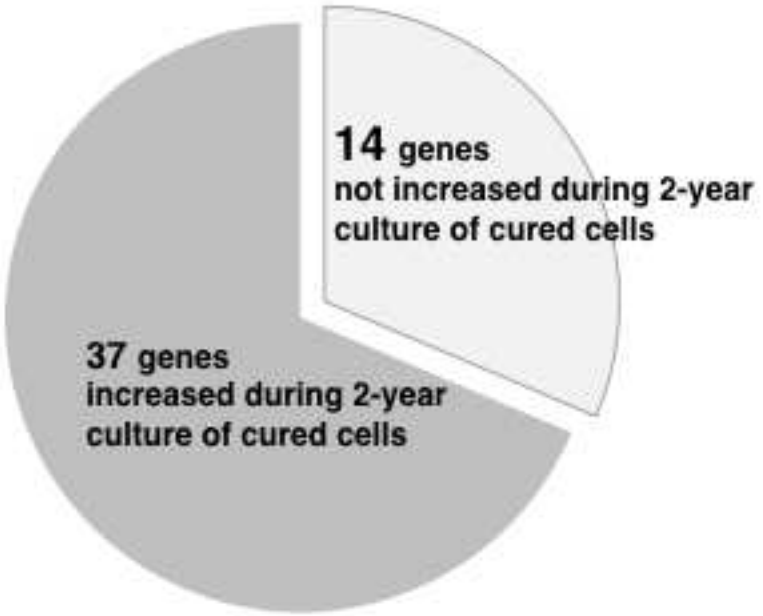


Sejima et al., Fig. 2

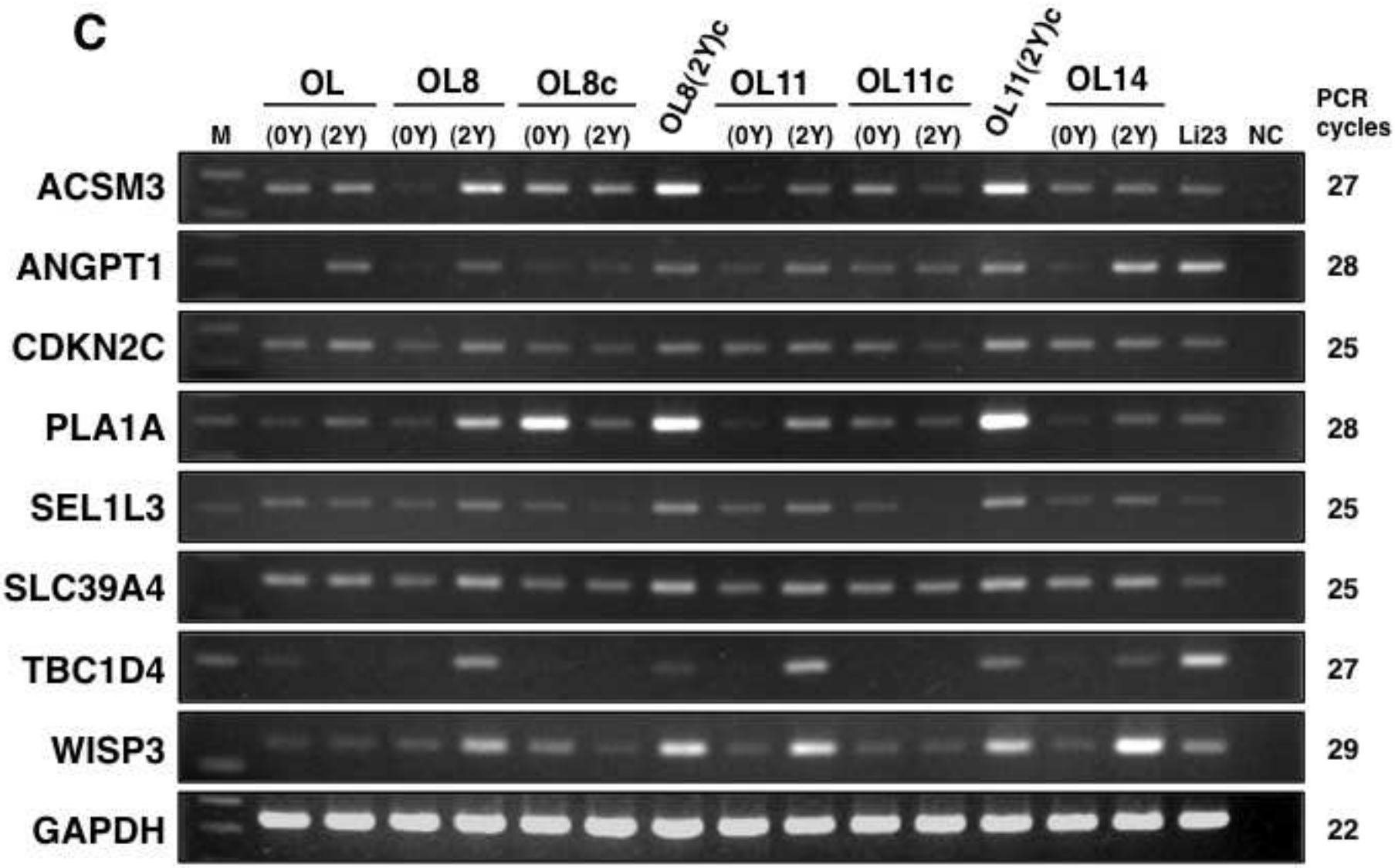
A



B

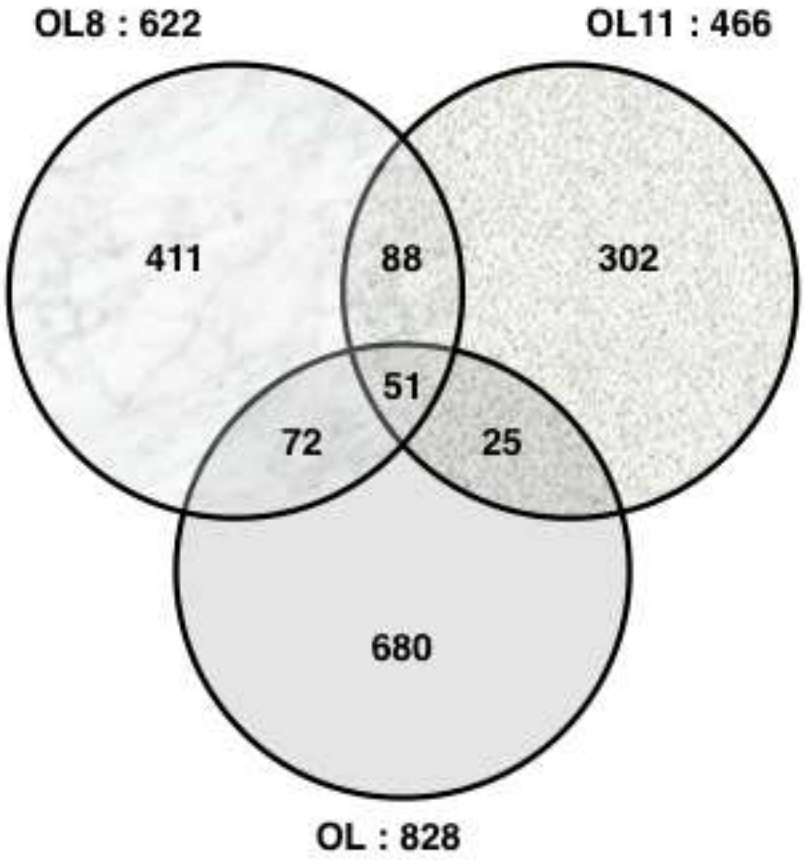


Sejima et al., Figs. 3A & 3B

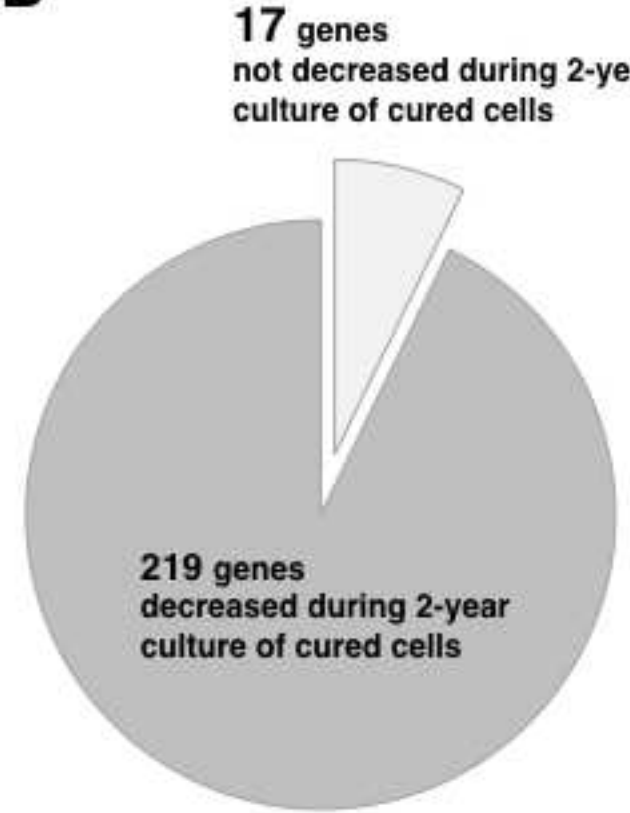


Sejima et al., Fig. 3C

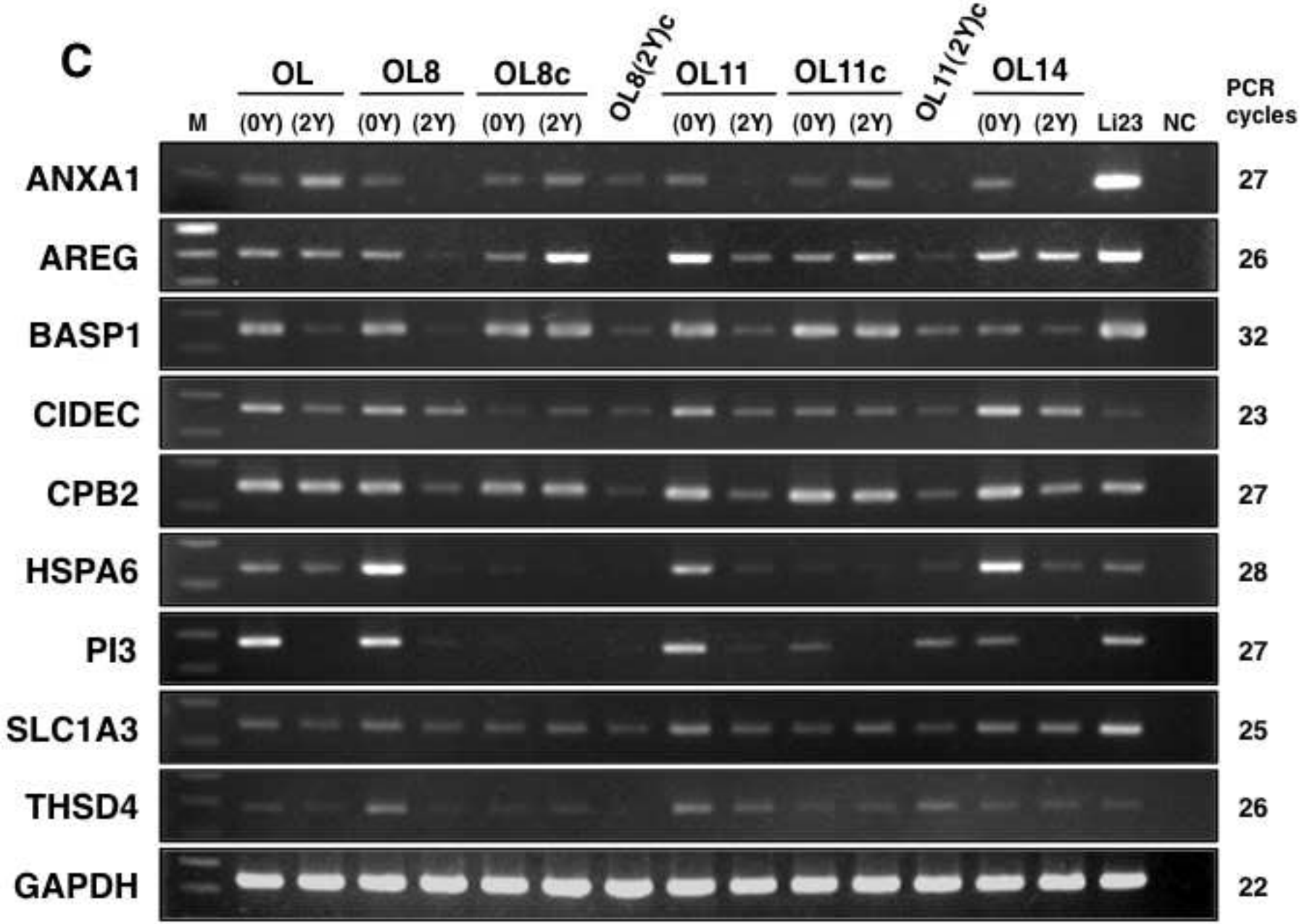
A



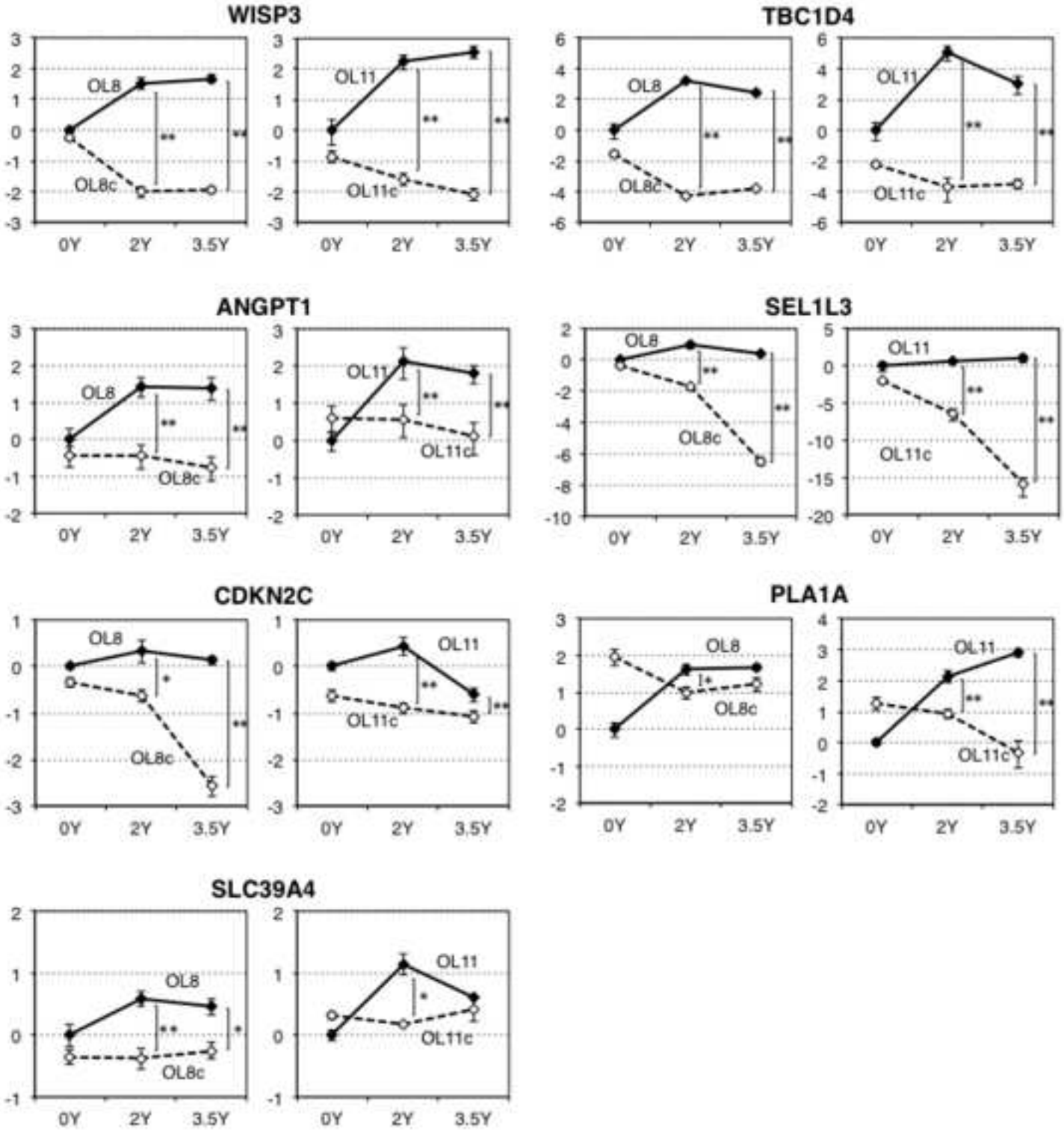
B



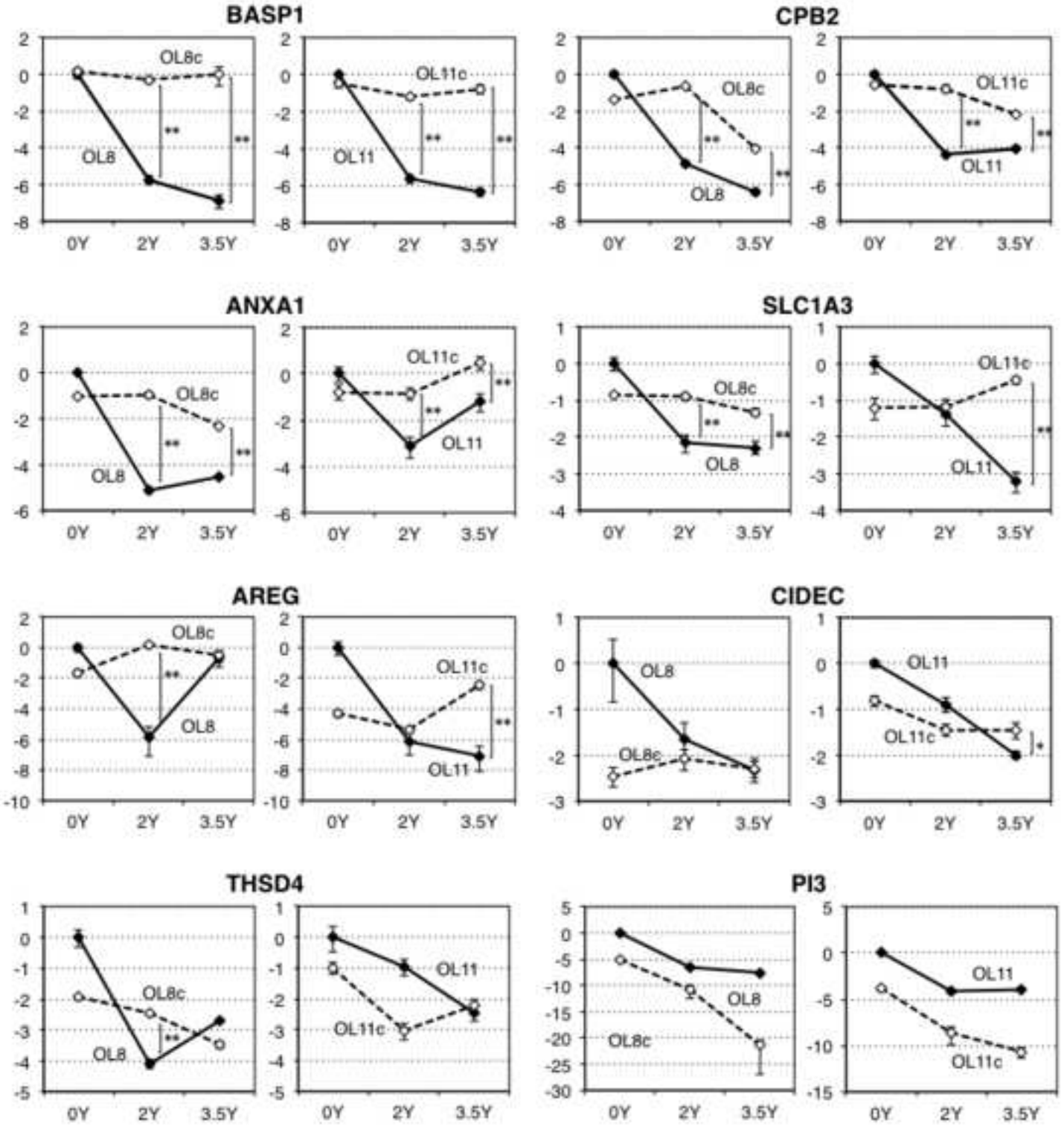
Sejima et al., Figs. 4A & 4B



Sejima et al., Fig. 4C



Sejima et al., Fig. 5



Sejima et al., Fig. 6