

**Fig. 4.** Effects of changes in viral parameters. (a) The higher the initial virus count, the greater is the increase in the rate of formation of chronic infection, but (b) there was no effect on the conditions in the equilibrium phase. (c) Extending the virus lifespan increased the virus count. (d) Increasing the speed of virus movement to 100 grids/tic increased the virus count, but increasing it to 500 grids/tic had the opposite effect, with a slight declining trend. (a) Black bars: number of infections produced; (b–d) black circles: virus count; line: virus count approximation curve; white bars: uninfected cell count; black bars: infected cell count.

established. When the simulation was performed multiple times, the features described above were maintained, and the average values for virus, infected cell, and uninfected cell counts during the equilibrium state were all consistent.

Fig. 1d shows the simulation screen of the RePast. When we attempted setting all the initial parameters to the same values as those in the StarLogo, the results were not consistent. When we recalculated the parameters from the simulation results, in RePast, the parameters were largely maintained at the levels of the settings, but in StarLogo, the lifespans of both cell types became shorter than the settings while the simulation was in progress. We made the results of both simulations consistent by using the same parameters during the actual simulation (Fig. 2a and b).

### 3.2. Comparison Between Agent-based Simulation Models and Mathematical Simulation Model

We investigated whether the results of a chronic viral infection disease model produced by RePast would be consistent with the results of a mathematical model. For the mathematical model, we carried out an approximate integration using a four-dimensional Runge–Kutta method to ensure that the uninfected cell count and infected cell count would be in the same class. Parameters were always fixed as constant between simulations. The simulation results were consistent for the equilibrium

phase, but transitions in virus count during the transient phase varied, with a shift to equilibrium state following two overshoots in the mathematical model, but a monotonic increase following a logistic curve in the agent-based model (Fig. 3). In the mathematical model, when the equilibrium condition was calculated with  $dT/dt = dI/dt = dV/dt = 0$ , the equilibrium-phase virus count, uninfected cell count, and infected cell count were very similar to those of the agent-based model (virus count: mathematical model 371.8/space, agent-based model  $371.1 \pm 32.4$ /space [average  $\pm$  SD]; uninfected cell count: mathematical model 1605/space, agent-based model  $1454 \pm 194$ /space; infected cell count: mathematical model 115.9/space, agent-based model  $108.3 \pm 14.2$ /space).

### 3.3. Usability of the Models; Effect of Changing Parameters

We investigated the changes in the equilibrium phase brought about by changing each parameter. All the investigations below were carried out by using RePast, and we used the average values from ten simulations.

### 3.4. Viral Parameters

The lower the virus counts at the beginning of the simulation, the lower the probability of a chronic infection (Fig. 4a). However, the initial virus count did not have any effect on the equilibrium

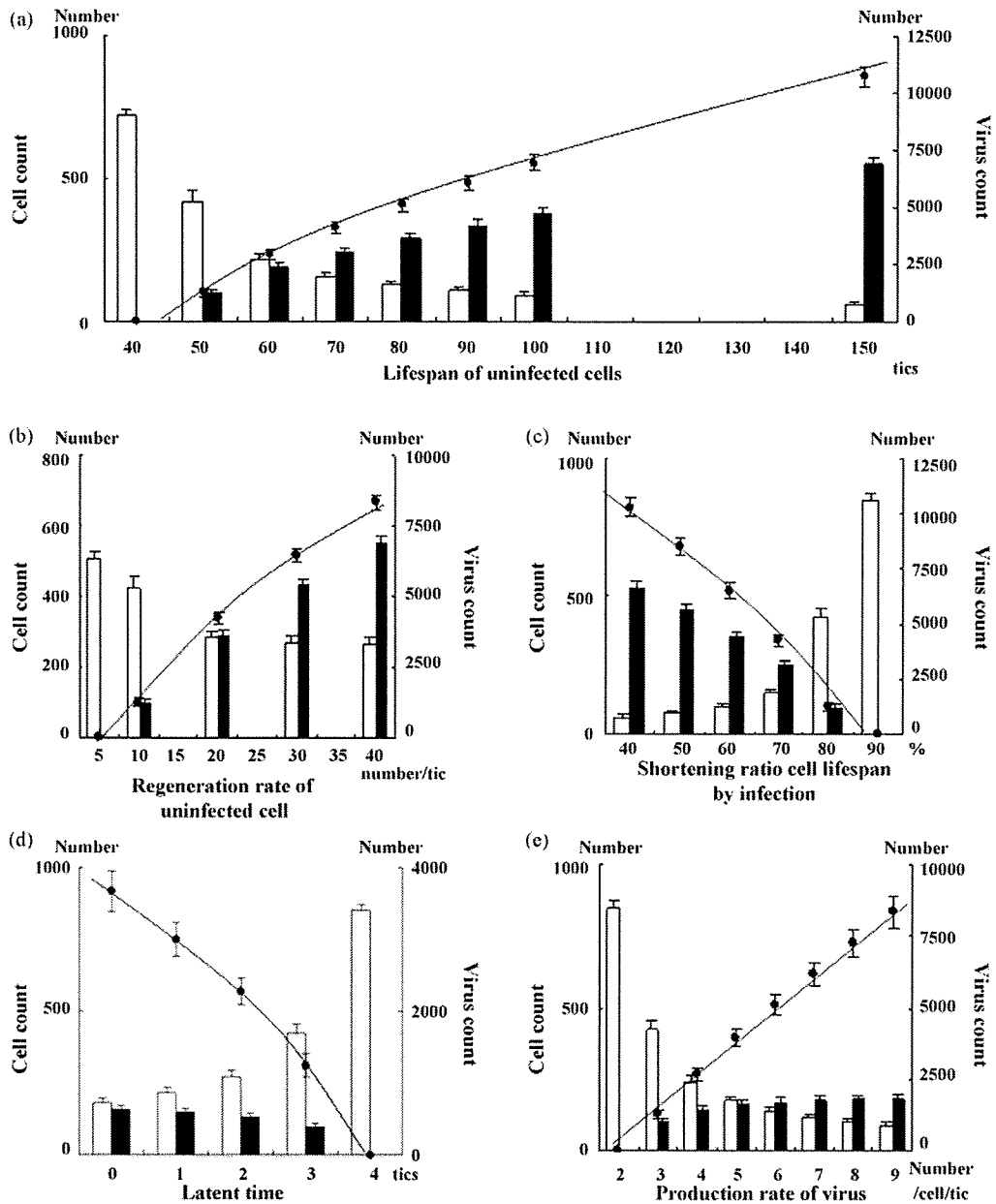


Fig. 5. Effects of changes in cell parameters. (a) Extending the uninfected cell lifespan and (b) increasing the uninfected cell regeneration rate increased the virus count. (c) Raising the lifespan-shortening ratio as a result of infection shortened the lifespan of infected cells, thereby decreasing the virus count. (d) Extending the latent period shortened the period of virus production from infected cells, thereby decreasing the virus count. (e) Increasing the virus production count resulted in a linear increase in equilibrium-phase virus count. Black circles: virus count; line: virus count approximation curve; white bars: uninfected cell count; black bars: infected cell count.

phase itself (Fig. 4b). Extending the lifespan of viruses resulted in a linear increase in equilibrium-phase virus count (Fig. 4c). Although the infected cell count increased, the rate of increase gradually declined. Changing the speed of viral movement resulted in the equilibrium-phase virus count to eventually decline after 100 grids/tic was reached, allowing movement over an area twice the size of the simulation space (Fig. 4d).

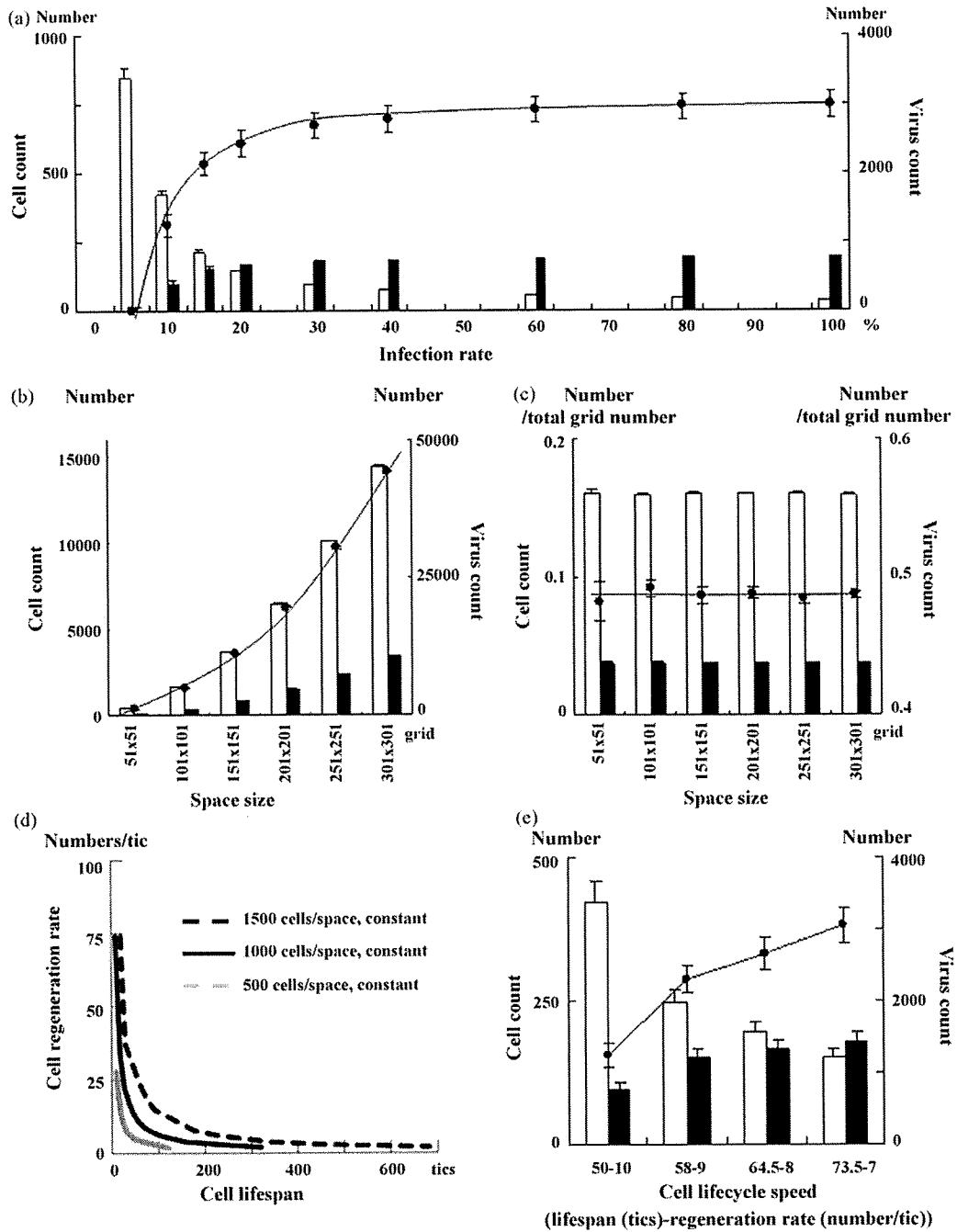
### 3.5. Uninfected Cell Parameters

Extending the lifespan of uninfected cells led to an increased virus count during the equilibrium phase (Fig. 5a). Increasing the uninfected cell regeneration rate also contributed to increased equilibrium-phase virus count (Fig. 5b). In both the cases, the

increases in virus count and infected cell count were not linear, but showed a tendency for the rate of increase to decline gradually.

### 3.6. Infected Cell Parameters

We carried out an investigation of the effects of variation in the lifespan-shortening ratio on the virus count on the assumption that cell lifespan is shortened by infection. When this ratio was increased, the virus count decreased (Fig. 5c). An extended latent period was also related to a decreased virus count (Fig. 5d). However, the virus production from infected cells led to a linear increase in the virus count (Fig. 5e).



**Fig. 6.** (a) Increasing the infection rate increased the virus count in equilibrium periods, but the virus count did not change at infection rates of 30% or more. (b) The size of the simulation space increased not only virus count but also the cell count; however, (c) when virus and cell counts were divided by the total number of grids in the space, they were constant for all space sizes. (d) Changing the lifespan and regeneration rate of uninfected cells in opposite directions at the same time makes it possible to change only the cell cycle speed without altering the uninfected cell count. (e) When the cell cycle speed was reduced, the virus count increased toward the right of the graph. This may be because the effect of extending the lifespan of cells exceeds that of reducing their regeneration rate. (a–c and e) Black circles: virus count; line: virus count approximation curve; white bars: uninfected cell count; black bars: infected cell count.

### 3.7. Infection Rate and Space Size

Increasing the infection rate caused an increase in the virus count, but the change was minimal at an infection rate of 30% or more. The same results were seen for infected cell count, but a decrease in uninfected cell count resulted in a tendency for the infection rate to decrease by up to 60% (Fig. 6a).

The larger the space, higher the increase in both virus and cell counts (Fig. 6b). This increase was proportional to space size, how-

ever, when virus and cell counts were divided by the total number of grids in the space they were all constant (Fig. 6c).

### 3.8. Cell Cycle Speeds

Running a simulation with the initial virus count set to zero enables only the equilibrium condition for uninfected cells to be simulated. Changing the lifespan and regeneration rate of uninfected cells in opposite directions at the same time makes it possible

to change the cell cycle speed without altering the uninfected cell count (Fig. 6d). We used this technique to investigate how changing the cell cycle speed affected the equilibrium phase. Fig. 6e shows the results. Cell lifespan increases while the cell cycle speed declines. The equilibrium virus count increased in accordance with slower cell cycle speeds.

#### 4. Discussion

In this study, we investigated the models using two agent-based simulation methods to program a simple virus–host chronic infection model. The same model written in two different programming language systems displayed the same results. The transient phase was unlike that seen in a mathematical simulation with no overshoot in virus count, but rather a smooth transition to the equilibrium phase. The virus count at the start of the simulation only had effect on the rate of infection development. Increases in virus lifespan, uninfected cell lifespan, uninfected cell regeneration rate, virus production count from infected cells, and infection rate all led to increased equilibrium-phase virus count. Rises in the infected cell lifespan-shortening ratio, latent period, and cell cycle speed decreased the equilibrium-phase virus count. The size of the space itself had no innate effect on the equilibrium phase, but a speed of movement of the virus that was twice the size of the space produced the maximum virus count.

Reproducibility is the basis for all scientific study, but there are many problems to prove it in computer simulations, such as programming bugs. As agent-based simulation deals with numerous agents individually, it requires vast amounts of calculations. Accumulation of very small change of values leads to large differences of results. In this study, we investigated two programs based on two programming languages to confirm the reproducibility of our simulation results in different programming languages. The results of two simulations were consistent, but in StarLogo, the lifespan parameters had a tendency to be lower than when they were set while simulations were actually in progress. This may be because the number of digits used in calculations was different between the two programs. RePast performs calculations to at least eight decimal places. In StarLogo, the library settings only enable settings to be made up to five decimal places. It is probable that these small differences accumulate during repeated calculations and are reflected in the simulation. Ultimately, we confirmed that the differences in results obtained by using different libraries and programming languages were not innate and by making the parameters consistent during simulation, consistent results were obtained.

Mathematical models using formulae for HIV therapy was published in 1994, the method has since been applied to HBV and HCV (Ho et al., 1995; Nowak et al., 1996; Neumann et al., 1998), and they were thought to be good reflections of the reality. In the mathematical model, viruses and cells are conceived as individuals in the concept itself, but both of them are perceived *en masse* when calculations are performed. However a feature of the agent-based simulation is that it deals with individual viruses and cells as separate agents. By moving each agent individually, it probes the factors influencing overall shifts from the micro viewpoint. When the space is viewed as a whole, it is possible to watch on the screen the collective movement of groups of agents. Recently, models that provide a visual representation of Epstein-Barr virus and HIV infection have been reported, both of which are useful for an instinctive and intuitive understanding (Duca et al., 2007; Shapiro et al., 2008; Castiglione et al., 2007).

In agent-based simulation model, virus count transit smoothly to the equilibrium phase. On the other hand, virus counts overshoot during transient phase in mathematical model. We think this difference is derived from technicality of different model-

ing. The difference in concepts between mathematical models and agent-based models is the space. The mathematical model has no space in concept, but agents move across the space in the agent-based model. In agent-based models, the densities of virus and cells change overtime especially in the transition phase because of the limited space. These changes of the densities of virus and cells lead to the dynamic change of the encounter rate of viruses and cells. The mathematical model does not make such concept of the density; the encounter rate is constant. This may be the reason for the difference between two models in the transition phase. Since no overshoot of virus counts in transient phase had been reported from *in vivo* studies of hepatitis C virus and simian immunodeficiency virus (Dahari et al., 2005; Nowak et al., 1997), agent-based model correlates with actual biology *in vivo* at least for these viruses. The increase of initial virus count at the start of simulation correlates with higher encounter rate of viruses and cells which make the linear increasing of infection forming rate. Mathematical model can only express the infection formation rate as “infected or not”.

The importance of viral passing speed in the agent-based model is also explained by the “space”. Although the virus actually moves through the blood stream in our body and virus could not decide their moving speeds by themselves, there is most appropriate speed for virus to meet the cells on the simulation space by the highest probability. The effect of cell cycle speed should be mentioned by another affection of the space. A fast cell cycle speed means that the lifespan of uninfected cells is short. Then fast cell cycle speed leads to the short lifespan of infected cells. A higher regeneration rate for uninfected cells results in a higher rate of infection among uninfected cells by viruses, but in situations where viruses and cells are dispersed around the space this is ineffective in increasing the infection rate, as the latter depends on the probability that they will encounter one another. As a result, the infected cell count decreases during the equilibrium phase, as does the virus count.

In this study, we confirmed the reproducibility and usability of agent-based models in expressing the interaction between viruses and cells. A feature of this simulation system is that it uses the concept of space as actual space, which means that the existence of the space becomes an additional controlling factor on the simulation results. This is a concept that is absent from mathematical models. The reality is that we have a spatial existence, and an advantage of the agent-based simulation system is the fact that it accounts for the space. Another feature of the simulation system is that it enables the condition to be perceived in visual terms, making it easy to understand. However it may be affected by computer performance and by the limitations of programming languages or the program itself, this system may offer a powerful tool for the future analysis of real virus–host interaction disease.

#### Conflict of interest

No conflicts of interest exist for all authors.

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## Original Article

Two flavonoids extracts from *Glycyrrhizae radix* inhibit *in vitro* hepatitis C virus replication

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**Aim:** Traditional herbal medicines have been used for several thousand years in China and other Asian countries. In this study we screened herbal drugs and their purified compounds, using the Feo replicon system, to determine their effects on *in vitro* HCV replication.

**Methods:** We screened herbal drugs and their purified extracts for the activities to suppress hepatitis C virus (HCV) replication using an HCV replicon system that expressed chimeric firefly luciferase reporter and neomycin phosphotransferase (Feo) genes. We tested extracts and 13 purified compounds from the following herbs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae capillari spica*; and *Rhei rhizoma*.

**Results:** The HCV replication was significantly and dose-dependently suppressed by two purified compounds, isoliquiritigenin and glycy coumarin, which were from *Glycyrrhizae*

*radix*. Dose-effect analyses showed that 50% effective concentrations were  $6.2 \pm 1.0 \mu\text{g/mL}$  and  $15.5 \pm 0.8 \mu\text{g/mL}$  for isoliquiritigenin and glycy coumarin, respectively. The MTS assay did not show any effect on cell growth and viability at these effective concentrations, indicating that the effects of the two compounds were specific to HCV replication. These two compounds did not affect the HCV IRES-dependent translation nor did they show synergistic action with interferon-alpha.

**Conclusion:** Two purified herbal extracts, isoliquiritigenin and glycy coumarin, specifically suppressed *in vitro* HCV replication. Further elucidation of their mechanisms of action and evaluation of *in vivo* effects and safety might constitute a new anti-HCV therapeutics.

**Key words:** hepatitis C virus, herbal drugs, replicon

## INTRODUCTION

HEPATITIS C VIRUS (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy.<sup>1,2</sup> The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently, combination therapy using pegylated interferon-alpha (IFN) and ribavirin has been used worldwide.<sup>3-5</sup> The success rates, however, are almost half of patients

treated. Furthermore, these therapies carry a significant risk of serious side effects. Thus, the development of alternative therapeutic agents against HCV is our high priority goal.

We have reported an HCV subgenomic replicon that expresses chimeric luciferase reporter "Feo" protein.<sup>6</sup> This Feo replicon supports stable and high levels of autonomous HCV RNA replication in transfected cells. Furthermore, the level of luciferase correlates well with levels of HCV RNA production, so that luciferase can be used as a reliable surrogate marker for HCV replication. This chimeric reporter replicon system has contributed the discovery of novel anti-HCV substances such as cyclosporins,<sup>7-9</sup> short interfering RNA,<sup>10,11</sup> interferon-gamma<sup>12</sup> and HMG-CoA reductase inhibitors.<sup>13,14</sup>

Traditional herbal drugs have been used for several thousand years in China and other Asian countries. Although these pharmacological activities are not fully

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Table 1 List of herbal drugs and their purified extracts

Herbal drug	Purified compound
<i>Glycyrrhizae radix</i>	Isoliquiritigenin Glycoumarin Isoliquiritin Licuroside
<i>Paeoniae radix</i>	Paeoniflorin 1,2,3,6-tetra-O-galloyl- $\beta$ -D-glucose
<i>Rhei Rhizoma</i>	Rhein 8-O- $\beta$ -glucoside
<i>Rehmanniae radix</i>	Acteoside Martynoside Isoacteoside
<i>Artemisiae capillari spica</i>	Demethoxycapillarisin 3,4-di-o-galloylquinic acid Acteosyringone

characterized, they also have been safely used for many clinical conditions in Japan. For example, Sho-saiko-to (TJ-9; Xiao-Chae-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix*, *Pinelliae tuber*, *Scutellariae radix*, *Ginseng radix*, *Glycyrrhizae radix*, and *Zingiberis rhizoma*),<sup>15</sup> has been clinically used for the treatment of chronic viral liver disease. It has been reported to regulate the cytokine production system in patients with hepatitis C<sup>16</sup> and to prevent the development of HCC in patients with non-B cirrhosis.<sup>17</sup> *Glycyrrhizin*, the major component of *Glycyrrhizae radix* (licorice), has also been used for the treatment of chronic hepatitis in Japan, known to have an alanine transaminase-lowering effect.<sup>18,19</sup> Despite the clinical effects of these herbal drugs, they did not suppress the HCV replication *in vitro*.<sup>15</sup>

In the present study, we applied the Feo replicon system to screen the herbal drugs and their purified compounds for their effects on *in vitro* HCV replication. Here, we show that two purified compounds from the herbal extracts specifically and substantially suppressed HCV replication.

## MATERIALS AND METHODS

### Purified compounds (Table 1)

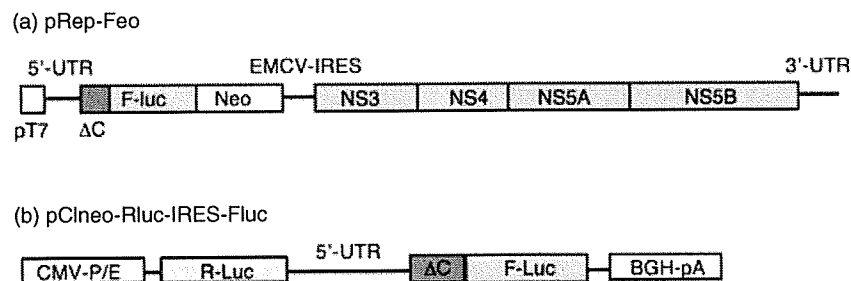
THIRTEEN COMPOUNDS WERE purified from five herbal drugs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae Capillari Spica*; and *Rhei Rhizoma* (Table 1; Tsumura, Tokyo, Japan). These extracts were prepared at concentrations of 5 mg/mL in dimethyl sulfoxide (DMSO), then stored at  $-20^{\circ}\text{C}$  until use. Recombinant human interferon (IFN) alpha-2b was obtained from Schering-Plough (NJ, USA).

### Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal calf serum at  $37^{\circ}\text{C}$  under 5%  $\text{CO}_2$ . Huh7 cells expressing the HCV replicon were cultured in a medium containing 200  $\mu\text{g}/\text{mL}$  G418 (Wako, Osaka, Japan).

### HCV subgenomic replicon construct

An HCV subgenomic replicon plasmid, pHCV1bneo-delS,<sup>20</sup> was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-



**Figure 1** HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo". NS, nonstructural region; pT7, T7 promoter; 3' UTR, 3' untranslated region. (b) A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV-IRES-mediated translation efficiency. The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-internal ribosome entry site (IRES)-mediated initiation, was stably transfected into Huh7 cells.

Feo and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.<sup>10,21</sup>

### HCV-IRES reporter construct

A plasmid, pCIneo-Rluc-IRES-Fluc, was used to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Fig. 1b).<sup>22</sup> The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-IRES-mediated initiation, was stably transfected into Huh7 cells. After culture in the presence of G418, Huh7/CRIF cells were established.<sup>9</sup> Activities of the HCV-IRES-mediated translation were measured by culture of Huh7/CRIF cells in the presence of drugs and by dual luciferase assays after 48 h.

### Luciferase assays and measurements of antiviral activity

Huh7/Rep-Feo cells were cultured with various concentrations of herbal extracts or compounds. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega, WI, USA) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means  $\pm$  SD as percentages of the controls. The 50% effective concentrations (EC<sub>50</sub>) were calculated using probit method. The determination of EC<sub>50</sub> was performed three times, and presented as mean  $\pm$  SD in each compound.

### Realtime RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Two  $\mu$ g of total cellular RNA was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen, CA, USA). The replicon RNA expression levels were measured using the Applied Biosystems 7500 Fast Realtime PCR System (Applied Biosystems, CA, USA) and QuantiTect SYBR Green PCR Kit (QIAGEN, CA, USA). Sequences of a pair of primers has been described elsewhere.<sup>23</sup>

### Northern blottings

Expression of HCV subgenomic RNA was detected as previously reported.<sup>24</sup> Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Fifteen micrograms of the total cellular RNA was electrophoresed on a 1.0% denaturing agarose-

formaldehyde gel and was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Sweden). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for beta-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Germany) and visualized using a Fluoro-Imager (Roche).

### Western blottings

Western blotting was done as reported previously.<sup>24</sup> Thirty micrograms of total cell lysate was separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, CA, USA) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche). The antibodies used were anti-NS5A (BioDesign, ME, USA), anti-core (provided by Dr. Wakita), and anti-beta-actin antibodies (Sigma).

### MTS assays

To evaluate cell viability, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

### HCV-JFH1 virus cell culture

An *in vitro* transcribed HCV-JFH1 RNA<sup>25</sup> was transfected into Huh7.5.1 cells.<sup>26</sup> Naïve Huh7.5.1 cells were subsequently infected by culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to culture in the presence of drugs. Culture medium was collected serially and HCV core antigen was measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics, Tokyo, Japan). Cellular virus expression was measured by the Western blotting using anti-core antibodies.<sup>27</sup>

### Statistical analyses

Statistical analyses were performed using Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.



**RESULTS**

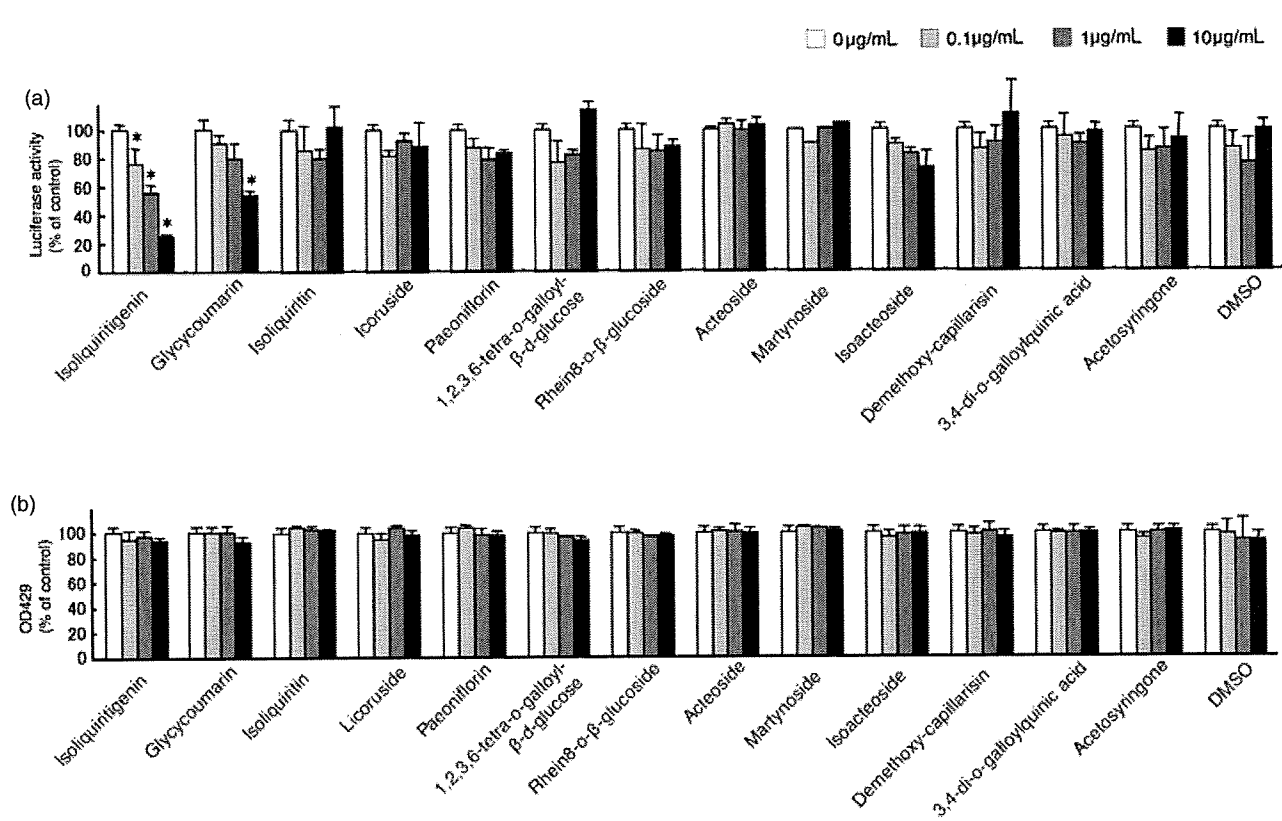
**Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycomarin**

**T**O SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix*, *Rhemanniae radix*, *Paeoniae radix*, *Artemisiae capillari spica*, and *Rhei rhizoma*, and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycomarin, which were purified from *Glycyrrhizae radix*, suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were  $6.2 \pm 1.0$  and

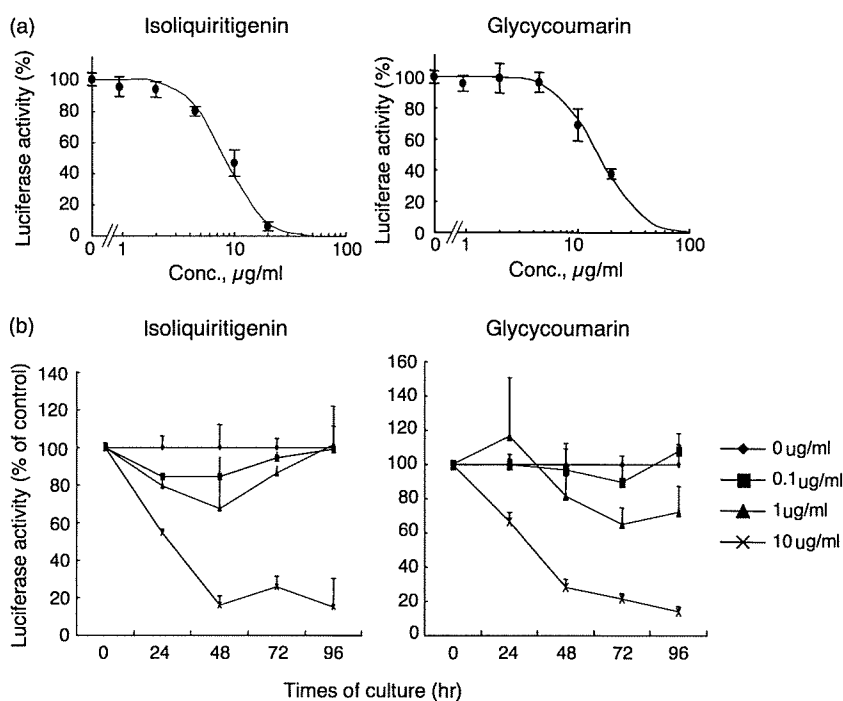
$15.5 \pm 0.8 \mu\text{g/mL}$  for isoliquiritigenin and glycycomarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycomarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

**Realtime-RT-PCR and Western blotting analyses**

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-



**Figure 2** Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10  $\mu\text{g/mL}$ . The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean  $\pm$  SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean  $\pm$  SD.



**Figure 3** Dose- and time-dependent suppression of HCV replication by isoliquiritigenin and glycycomarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycomarin. Error bars indicate mean  $\pm$  SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycomarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean  $\pm$  SD.

itigenin and glycycomarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycomarin (Fig. 4c). Densitometric analysis of NS5A protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

#### Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycomarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFN $\alpha$ -2b and isoliquiritigenin or glycycomarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycomarin of 0, 0.1, 1, 10  $\mu$ g/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycomarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or

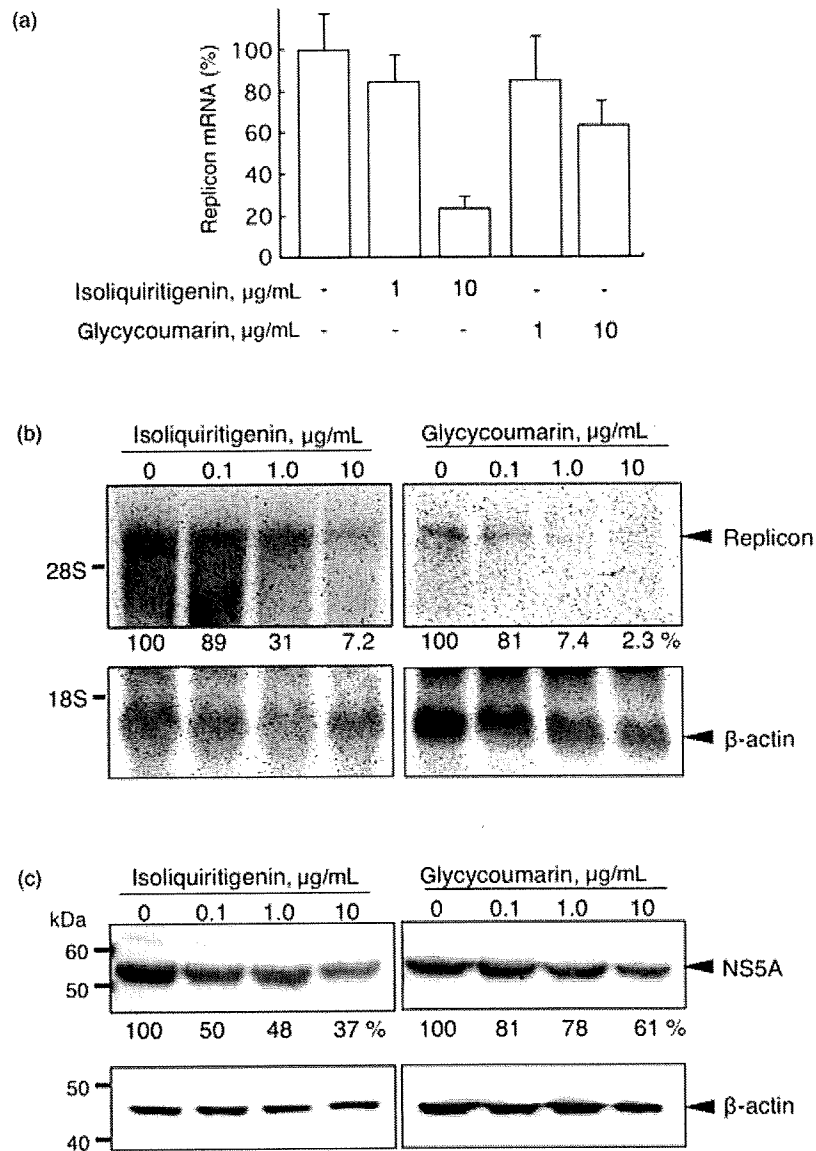
glycycomarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

#### Isoliquiritigenin and glycycomarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycomarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

#### Isoliquiritigenin and glycycomarin suppress HCV-JFH1 virus cell culture

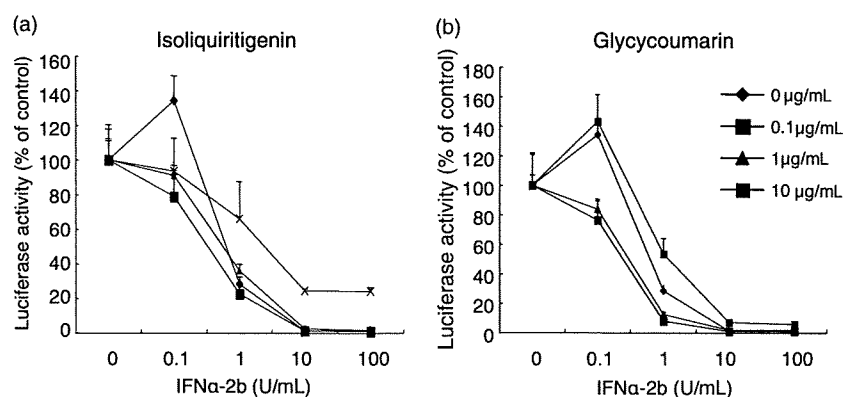
The demonstrated inhibitory effects isoliquiritigenin and glycycomarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.<sup>25</sup> As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent



**Figure 4** Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycy coumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycy coumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot hybridization. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the hepatitis C virus replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with beta-actin probe. Densitometry for replicon RNA was performed and indicated as percents of drug-negative control. (c) Western blotting. Thirty micrograms of total cellular protein was electrophoresed in each lane. Densitometry of NS5A protein was performed and indicated as percents of drug-negative control.

increase of HCV core antigen in the medium. In all time points, core antigen levels were significantly lower in culture that were treated with isoliquiritigenin and glycy coumarin than the untreated culture. The effect of glycy coumarin was partly reversed on day six probably

due to chemical instability of the compound. Consistently, the Western blot showed that the cellular HCV core protein expression was substantially suppressed by treatment with isoliquiritigenin and glycy coumarin (Fig. 7b).

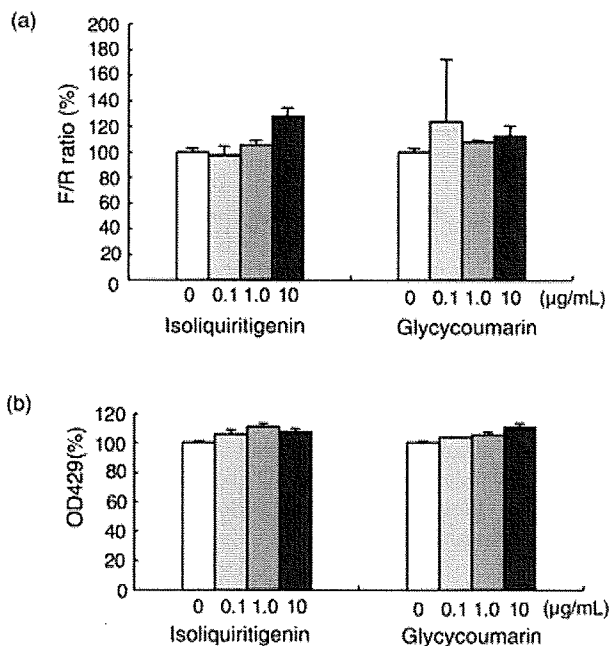


**Figure 5** Effects of (a) isoliquiritigenin and (b) glycycomarin used in combination with interferon(IFN)- $\alpha$  on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN- $\alpha$ -2b and isoliquiritigenin or glycycomarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean  $\pm$  SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycomarin and without IFN.

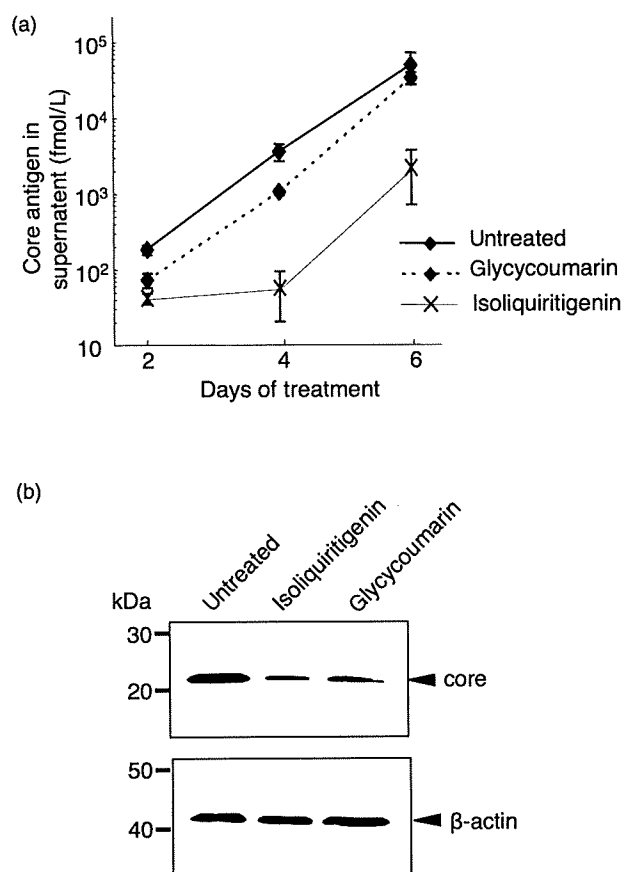
## DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycomarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,<sup>28</sup> antioxidative and anticarcinogenic activities.<sup>29</sup> Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,<sup>30</sup> an antiplatelet aggregation effect,<sup>31</sup> an inhibitory effect on aldose reductase activity,<sup>32</sup> estrogenic properties<sup>33</sup> and selective inhibition of H2 receptor-mediated signaling.<sup>34</sup>



**Figure 6** Isoliquiritigenin and glycycomarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably transfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycomarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean  $\pm$  SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycomarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean  $\pm$  SD.



**Figure 7** Suppression of HCV-JFH1 virus expression by *isoliquiritigenin* and *glycycomarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean  $\pm$  SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,<sup>35</sup> and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glycyrrhizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycomarin. Glycycomarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes*, *Haemophilus influenzae* and *Moraxella*

*catarrhalis*,<sup>36</sup> and methicillin-resistant *Staphylococcus aureus*,<sup>37</sup> but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycomarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glycyrrhizae radix*. However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycomarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

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## HEPATOLOGY

## Inhibition of hepatitis C virus infection and expression *in vitro* and *in vivo* by recombinant adenovirus expressing short hairpin RNA

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### Key words

adenovirus vector, hepatitis C virus, RNA interference.

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### Abstract

**Background and Aim:** We have reported previously that synthetic small interfering RNA (siRNA) and DNA-based siRNA expression vectors efficiently and specifically suppress hepatitis C virus (HCV) replication *in vitro*. In this study, we investigated the effects of the siRNA targeting HCV-RNA *in vivo*.

**Methods:** We constructed recombinant retrovirus and adenovirus expressing short hairpin RNA (shRNA), and transfected into replicon-expressing cells *in vitro* and transgenic mice *in vivo*.

**Results:** Retroviral transduction of Huh7 cells to express shRNA and subsequent transfection of an HCV replicon into the cells showed that the cells had acquired resistance to HCV replication. Infection of cells expressing the HCV replicon with an adenovirus expressing shRNA resulted in efficient vector delivery and expression of shRNA, leading to suppression of the replicon in the cells by  $\sim 10^{-3}$ . Intravenous delivery of the adenovirus expressing shRNA into transgenic mice that can be induced to express HCV structural proteins by the Cre/loxP switching system resulted in specific suppression of virus protein synthesis in the liver.

**Conclusion:** Taken together, our results support the feasibility of utilizing gene targeting therapy based on siRNA and/or shRNA expression to counteract HCV replication, which might prove valuable in the treatment of hepatitis C.

### Introduction

Hepatitis C virus (HCV), which affects 170 million people worldwide, is one of the most important pathogens causing liver-related morbidity and mortality.<sup>1</sup> The difficulty in eradicating HCV is attributable to limited treatment options against the virus and their unsatisfactory efficacies. Even with the most effective regimen with pegylated interferon (IFN) and ribavirin in combination, the efficacies are limited to less than half of the patients treated.<sup>2</sup> Given this situation, the development of safe and effective anti-HCV therapies is one of our high-priority goals.

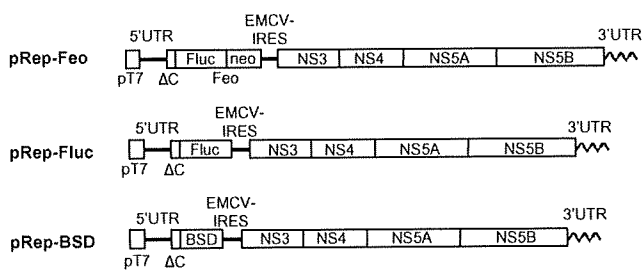
RNA interference (RNAi) is a process of sequence-specific, post-transcriptional gene silencing that is initiated by double-stranded RNA.<sup>3,4</sup> Because of its potency and specificity, RNAi rapidly has become a powerful tool for basic research to analyze gene functions and for potential therapeutic applications. Recently,

successful suppression of various human pathogens by RNAi have been reported, including human immunodeficiency viruses,<sup>5,6</sup> poliovirus,<sup>7</sup> influenza virus,<sup>8</sup> severe acute respiratory syndrome (SARS) virus<sup>9</sup> and hepatitis B virus (HBV).<sup>10-13</sup>

We and other researchers have reported that appropriately designed small interfering RNA (siRNA) targeting HCV genomic RNA can efficiently and specifically suppress HCV replication *in vitro*.<sup>14-19</sup> We have tested siRNA designed to target the well-conserved 5'-untranslated region (5'-UTR) of HCV-RNA, and identified the most effective target, just upstream of the translation initiation codon. Furthermore, transfection of DNA-based vectors expressing siRNA was as effective as that of synthetic siRNA in suppressing HCV replication.<sup>14</sup>

In this study, we explored the further possibility that efficient delivery and expression of siRNA may be effective in suppression and elimination of HCV replication and that delivery of such





**Figure 1** Structures of HCV replicon plasmids. The pRep-Feo expressed a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase (GenBank accession No. AB119282).<sup>14,20</sup> The pRep-Fluc expressed the Fluc protein. The pRep-BSD expressed the blasticidin S (BSD) resistance gene. pT7, T7 promoter; 5'UTR, HCV 5'-untranslated region; ΔC, truncated HCV core region (nt. 342–377); neo, neomycin phosphotransferase gene; EMCV, encephalomyocarditis virus; NS3, NS4, NS5A and NS5B, genes that encode HCV non-structural proteins; 3'UTR, HCV 3'-untranslated region.

HCV-directed siRNA *in vivo* may be effective in silencing viral protein expression in the liver. Here, we report that HCV replication was suppressed *in vitro* by recombinant retrovirus and adenovirus vectors expressing short hairpin RNA (shRNA) and that the delivery of the adenovirus vector to mice *in vivo* specifically inhibited viral protein synthesis in the liver.

## Methods

### Cells and cell culture

Huh7 and Retro Pack PT67 cells (Clontech, Palo Alto, CA, USA) were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO<sub>2</sub>. To maintain cell lines carrying the HCV replicon, G418 (Wako, Osaka, Japan) was added to the culture medium to a final concentration of 500 µg/mL.

### HCV replicon constructs and transfection

HCV replicon plasmids, pRep-Feo, pRep-Fluc and pRep-BSD were constructed from a virus, HCV-N strain, genotype 1b.<sup>21</sup> The pRep-Feo expressed a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase.<sup>14,20</sup> The pRep-Fluc and the pRep-BSD expressed the Fluc and blasticidin S (BSD) resistance genes, respectively (Fig. 1). The replicon RNA synthesis and the transfection protocol have been described previously.<sup>22</sup>

### Synthetic siRNA and siRNA-expression plasmid

The design and construction of HCV-directed siRNA vectors have been described.<sup>14</sup> Briefly, five siRNA targeting the 5'-UTR of HCV RNA were tested for their efficiency to inhibit HCV replication, and the most effective sequence, which targeted nucleotide position of 331 through 351, was used in the present study. To construct shRNA-expressing DNA cassettes, oligonucleotide inserts were synthesized that contained the loop sequence (5'-TTC AAG AGA-

3') flanked by sense and antisense siRNA sequences (Fig. 2a). These were inserted immediately downstream of the human U6 promoter. To avoid a problem in transcribing shRNA because of instability of the DNA strands arising from the tight palindrome structure, several C-to-T point mutations, which retained completely the silencing activity of the shRNA, were introduced into the sense strand of the shRNA sequences (referred to as 'm').<sup>23</sup> A control plasmid, pUC19-shRNA-Control, expressed shRNA directed towards the Machado–Joseph disease gene, which is a mutant of ataxin-3 gene and is not normally expressed. We have previously described the sequence specific activity of the shRNA-Control.<sup>24</sup>

Prior to construction of the virus vectors, we tested silencing efficiency of five shRNA constructs of different lengths that covered the target sequence (Fig. 2a). The shRNA-HCV-19, shRNA-HCV-21 and shRNA-HCV-27 had target sequences of 19, 21 and 27 nucleotides, respectively. Transfection of these shRNA constructs into Huh7/pRep-Feo showed that shRNA with longer target sequences had better suppressive effects (Fig. 2b). Therefore, we used shRNA-HCV-27m (abbreviated as shRNA-HCV) in the following study.

### Recombinant retrovirus vectors

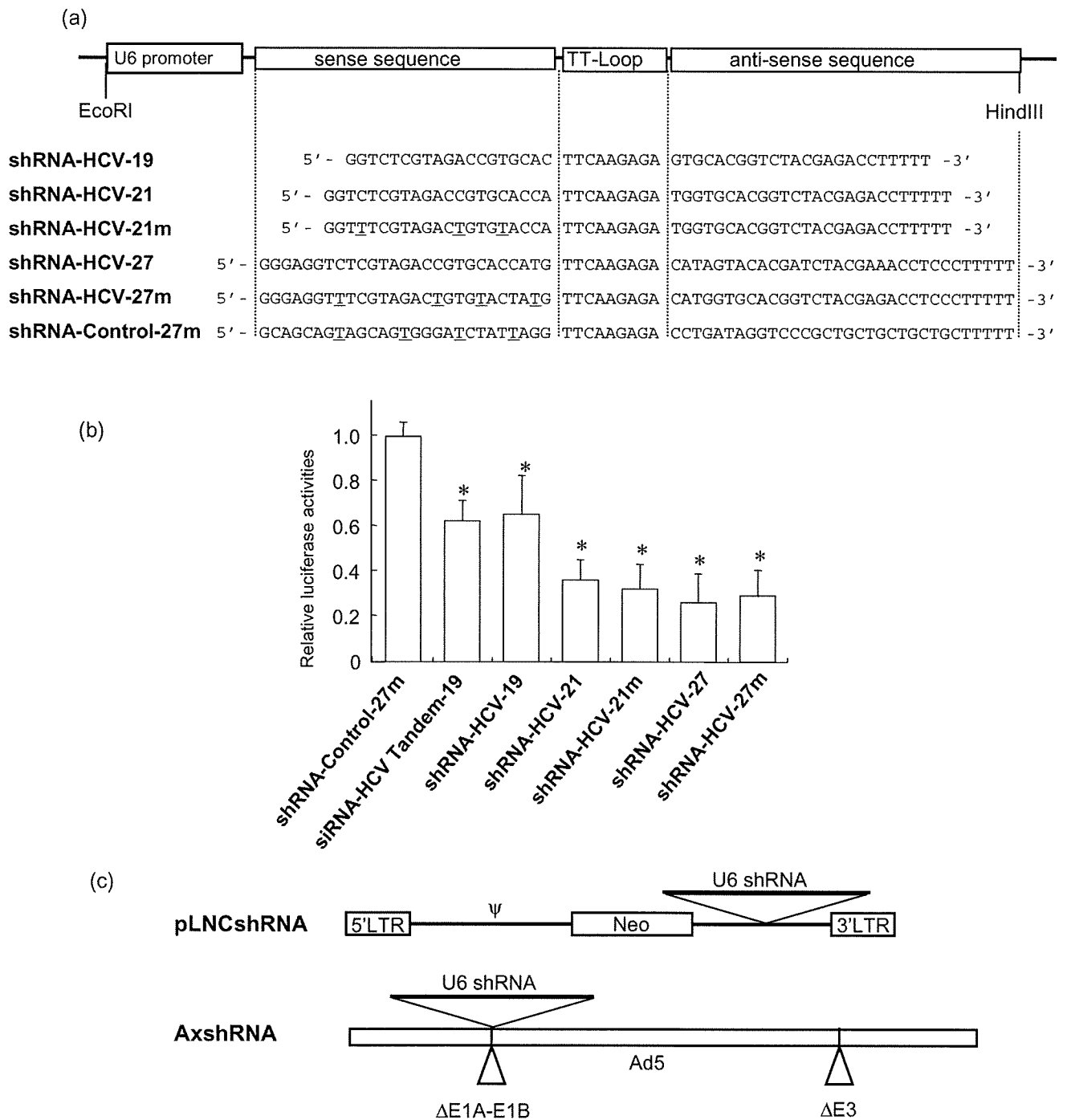
The U6-shRNA expression cassettes were inserted into the *StuI/HindIII* site of a retrovirus vector, pLNCX2 (Clontech) to construct pLNCshRNA-HCV and pLNCshRNA-Control (Fig. 2c). The plasmids were transfected into the packaging cells, Retro Pack PT67. The culture supernatant was filtered and added onto Huh7 cells with 4 µg/mL of polybrene. Huh7 cell lines stably expressing shRNA were established by culture in the presence of 500 µg/mL of G418.

### Recombinant adenovirus

Recombinant adenoviruses expressing shRNA were constructed using an Adenovirus Expression Vector Kit (Takara, Otsu, Japan). The U6-shRNA expression DNA cassette was inserted into the *SwaI* site of pAxcw to construct pAxshRNA-HCV and pAxshRNA-Control. The adenoviruses were propagated according to the manufacturer's protocol (AxshRNA-HCV and AxshRNA-Control; Fig. 2c). A 'multiplicity of infection' (MOI) was used to standardize infecting doses of adenovirus. The MOI stands for the ratio of infectious virus particles to the number of cells being infected. An MOI = 1 represents equivalent dose to introduce one infectious virus particle to every host cell that is present in the culture.

### Plasmids for assays of interferon responses

pISRE-TA-Luc (Invitrogen, Carlsbad, CA, USA) contained five copies of the consensus interferon stimulated response element (ISRE) motifs upstream of the Fluc gene. pTA-Luc (Invitrogen), which lacks the enhancer element, was used for background determination. The pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega, Madison, WI, USA), which expresses the *Renilla* luciferase protein, was used for normalization of transfection efficiency.<sup>25</sup> A plasmid, pEGFPneo (Invitrogen), was used to monitor percentages of transduced cells.



**Figure 2** Structure of shRNA-expression constructs and shRNA sequences. (a) Structure of shRNA-expression cassette and shRNA sequences. TT-Loop, the loop sequence. The shRNA-Control was directed toward an unrelated target, Machado–Joseph disease gene. Underlined letters indicate C-to-T point mutations in the sense strand. (b) The shRNA-expression plasmids were transfected into Huh7/pRep-Feo cells, and internal luciferase activities were measured at 48 h of transfection. Each assay was done in triplicate, and the values are displayed as mean + SD. \**P* < 0.05. (c) pLNCshRNA, structure of a recombinant retrovirus expressing shRNA.  $\Psi$ , the retroviral packaging signal sequence. AxshRNA, structure of a recombinant adenovirus expressing shRNA.

### Real-time RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Total cellular RNA (2 µg) was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen). The mRNA expression levels were measured using the Light Cycler PCR and detection system (Roche, Mannheim, Germany) and Light Cycler Fast Start DNA Master SYBR Green 1 mix (Roche).

### Luciferase assays

Luciferase activity was measured using a luminometer, Lumat LB9501 (Promega) and the Bright-Glo Luciferase Assay System (Promega) or the Dual-Luciferase Reporter Assay System (Promega).

### Northern and western hybridization

Total cellular RNA was separated by denaturing agarose-formaldehyde gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridized with a digoxigenin-labeled probe specific for the full-length replicon sequence, and subsequently with a probe specific for beta-actin. The signals were detected by chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche), and visualized by Fluoro-Imager (Roche). For the western blotting, 10 µg of total cell lysate was separated on NuPAGE 4.12% Bis-TrisGel (Invitrogen), and blotted onto an Immobilon PVDF Membrane (Roche). The membrane was incubated with monoclonal antibodies specific for HCV-NS5A (BioDesign, Saco, ME, USA), NS4A (Virogen, Watertown, MA, USA), or beta-actin (Sigma), and detected by a chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; POD, Roche).

### Transient-replication assays

A replicon, pRep-Fluc, was transfected into cells and the luciferase activities of the cell lysates were measured serially. To correct the transfection efficiency, each value was divided by the luciferase activity at 4 h after the transfection.

### Stable colony formation assays

Cells were transfected with a replicon, pRep-BSD, and were cultured in the presence of 150 µg/mL of BSD (Invitrogen). BSD-resistant cell colonies appeared after ~3 weeks of culture, and were counted.

### HCV-JFH1 virus cell culture

An *in-vitro* transcribed HCV-JFH1 RNA<sup>26</sup> was transfected into Huh7.5.1 cells.<sup>27</sup> Naive Huh7.5.1 cells were subsequently infected by the culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to siRNA or drug treatments. Replication levels of HCV-RNA were quantified by the realtime RT-PCR by using primers that targeted HCV-NS5B region, HCV-JFH1 sense: 5'-TCA GAC AGA GCC TGA GTC CA-3', and HCV-JFH1 anti-sense: 5'-AGT TGC TGG AGG GCT TCT GA-3'.

### Mice and adenovirus infection

Transgenic mice, CN2-29, inducibly express mRNA for the HCV structural proteins (genotype1b, nucleotides 294–3435) by the Cre/loxP switching system.<sup>28</sup> The transgene does not contain full-length HCV 5'-UTR, but shares the target sequence of the shRNA-HCV. Although the transgenic mouse CN2 has been previously reported as expressing higher levels of the viral proteins, the expression levels of the viral core protein in the CN2-29 mice are modest and similar to that in the liver of HCV patients. Thus, we chose CN2-29 mice in the present study.

The mice were infected with AxshRNA-HCV or controls (AxshRNA-Control or AxCAw1) in combination with AxCAN-Cre, which expressed Cre recombinase. Three days after the infection, the mice were killed and HCV core protein in the liver was measured as described below. The BALB/c mice were maintained in the Animal Care Facility of Tokyo Medial and Dental University, and transgenic mice were in the Tokyo Metropolitan Institute of Medical Science. Animal care was in accordance with institutional guidelines. The review board of the university approved our experimental animal studies and all experiments were approved by the institutional animal study committees.

### Measurement of HCV core protein in mouse liver

The amounts of HCV core protein in the liver tissue from the mice was measured by a fluorescence enzyme immunoassay (FEIA)<sup>29</sup> with a slight modification. Briefly, the 5F11 monoclonal anti-HCV-core antibody was used as the first antibody on the solid phase, and the 5E3 antibody conjugated with horseradish peroxidase was the second antibody. This FEIA can detect as little as 4 pg/mL of recombinant HCV-core protein. Contents of the HCV core protein in the liver samples were normalized by the total protein contents and expressed as pg/mg total protein.

### Immunohistochemical staining

Liver tissue was frozen with optimal cutting temperature (OTC) compound (Tissue Tek; Sakura Finetechnical, Tokyo, Japan). The sections (8 µm thick) were fixed with a 1:1 solution of acetone : methanol at -20°C for 10 min and then washed with phosphate-buffered saline (PBS). Subsequently, the sections were incubated with the IgG fraction of an anti-HCV core rabbit polyclonal antibody (RR8)<sup>28</sup> in blocking buffer or antialbumin rabbit polyclonal antibody (Dako Cytomation, Glostrup, Denmark) in PBS overnight at 4°C. The sections were incubated with secondary antibody, Alexa-antirabbit IgG (Invitrogen) or TRITIC-antirabbit IgG (Sigma), for 2 h at room temperature. Fluorescence was observed using a fluorescence microscope.

### Statistical analyses

Statistical analyses were performed using Student's *t*-test; *P*-values of less than 0.05 were considered to be statistically significant.

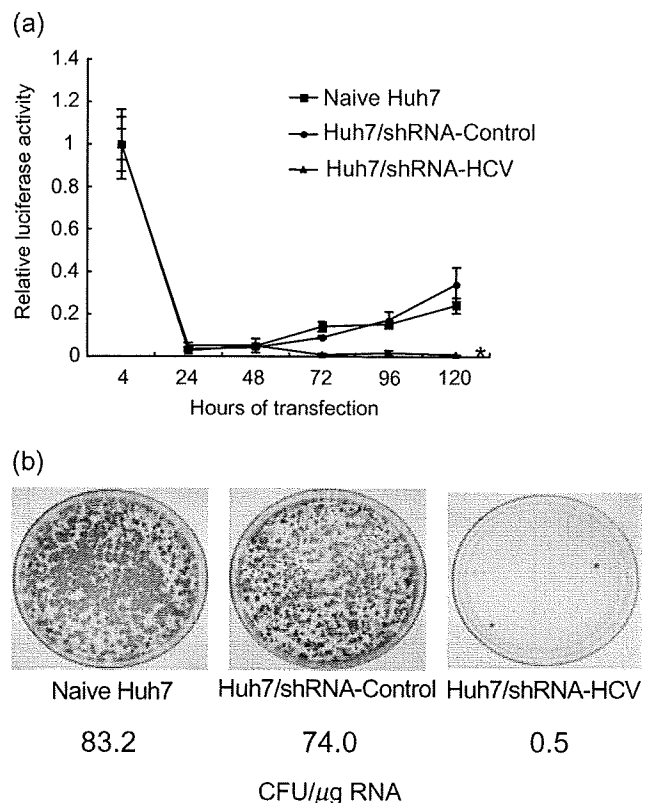
## Results

### Retrovirus transduction of shRNA can protect from HCV replication

Retrovirus vectors propagated from pLNCshRNA-HCV and pLNCshRNA-Control were used to infect Huh7 cells, and cell lines were established that constitutively express shRNA-HCV and shRNA-Control (Huh7/shRNA-HCV and Huh7/shRNA-Control, respectively). There were no differences in the cell morphology or growth rate between shRNA-transduced and non-transduced Huh7 cells (data not shown). The HCV replicon, pRep-Fluc, was transfected into Huh7/shRNA-HCV, Huh7/shRNA-Control and naive Huh7 cells by electroporation. In Huh7/shRNA-Control and naive Huh7 cells, the initial luciferase activity at 4 h decreased temporarily, which represents decay of the transfected replicon RNA, but increased again at 48 h and 72 h, which demonstrate *de novo* synthesis of the HCV replicon RNA. In contrast, transfection into Huh7/shRNA-HCV cells resulted in a decrease in the initial luciferase activity, reaching background by 72 h (Fig. 3a). Similarly, transfection of the replicon, pRep-BSD, into Huh7 cells and BSD selection yielded numerous BSD-resistant colonies in the naive Huh7 (832 colonies) and Huh7/shRNA-Control cell lines (740 colonies), while transfection of Huh7/shRNA-HCV, which expressed shRNA-HCV, yielded obviously fewer colonies (five colonies), indicating reduction of colony forming units by  $\sim 10^2$  (Fig. 3b). There was no difference in shape, growth or viability between cells expressing the shRNA or not. These results indicated that cells expressing HCV-directed shRNA following retrovirus transduction acquired resistance to HCV replication.

### Effect of recombinant adenoviruses expressing shRNA on *in vitro* HCV replication

We investigated subsequently the effects of recombinant adenovirus vectors expressing shRNA. AxshRNA-HCV and AxshRNA-Control were used separately to infect Huh7/pRep-Feo cells, and the internal luciferase activities were measured sequentially (Fig. 4a). AxshRNA-HCV caused continuous suppression of HCV RNA replication. Six days postinfection, the luciferase activities fell to background levels. In contrast, the luciferase activities of the Huh7/pRep-Feo cells infected with AxshRNA-Control did not show any significant changes compared with untreated Huh7/pRep-Feo cells (Fig. 4a). The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay showed no significant difference between cells that were infected by recombinant adenovirus and uninfected cells (Fig. 4b). In the northern blotting analysis, the cells were harvested 6 days after infection with the adenovirus at an MOI of 1. Feo-replicon RNA of 9.6 kb, which was detectable in the untreated Huh7/pRep-Feo cells and in the cells infected with AxshRNA-Control, diminished substantially following infection with the AxshRNA-HCV (Fig. 4c). Densitometries showed that the intracellular levels of the replicon RNA in the Huh7/pRep-Feo cells correlated well with the internal luciferase activities. Similarly in the western blotting, cells were harvested 6 days after infection with adenovirus. Levels of the HCV NS4A and NS5A proteins that were translated from the HCV replicon decreased following infection with the AxshRNA-HCV



**Figure 3** HCV replication can be inhibited by shRNA-HCV which was stably transfected into cells. Huh7/shRNA-HCV and Huh7/shRNA-Control stably express shRNA-HCV or shRNA-Control, respectively, following retroviral transduction. (a) Transient replication assay. An HCV replicon RNA, pRep-Fluc, was transfected into naive Huh7, Huh7/shRNA-HCV and Huh7/shRNA-Control cells. Luciferase activities of the cell lysates were measured serially at the times indicated, and the values were plotted as ratios relative to luciferase activities at 4 h. The luciferase activities at 4 h represent transfected replicon RNA. The data are mean  $\pm$  SD. An asterisk denotes a *P*-value of less than 0.001 compared with the corresponding value of the naive Huh7 cells. (b) Stable colony formation assay. The HCV replicon, pRep-BSD, was transfected into naive Huh7, Huh7/shRNA-HCV and Huh7/shRNA-Control cells. The cells were cultured in the presence of blasticidin S (BSD) in the medium for  $\sim 3$  weeks, and the BSD-resistant colonies were counted. These assays were repeated twice. The colony-forming units per microgram RNA (CFU/ $\mu$ g RNA) are shown at the bottom.

(Fig. 4d). These results indicated that the decrease in luciferase activities was due to specific suppressive effects of shRNA on expression of HCV genomic RNA and the viral proteins, and not due to non-specific effects caused by the delivery of shRNA or to toxicity of the adenovirus vectors.

### Absence of interferon-stimulated gene responses by siRNA delivery

It has been reported that double-stranded RNA may induce interferon-stimulated gene (ISG) responses which cause instability of mRNA, translational suppression of proteins and apoptotic cell