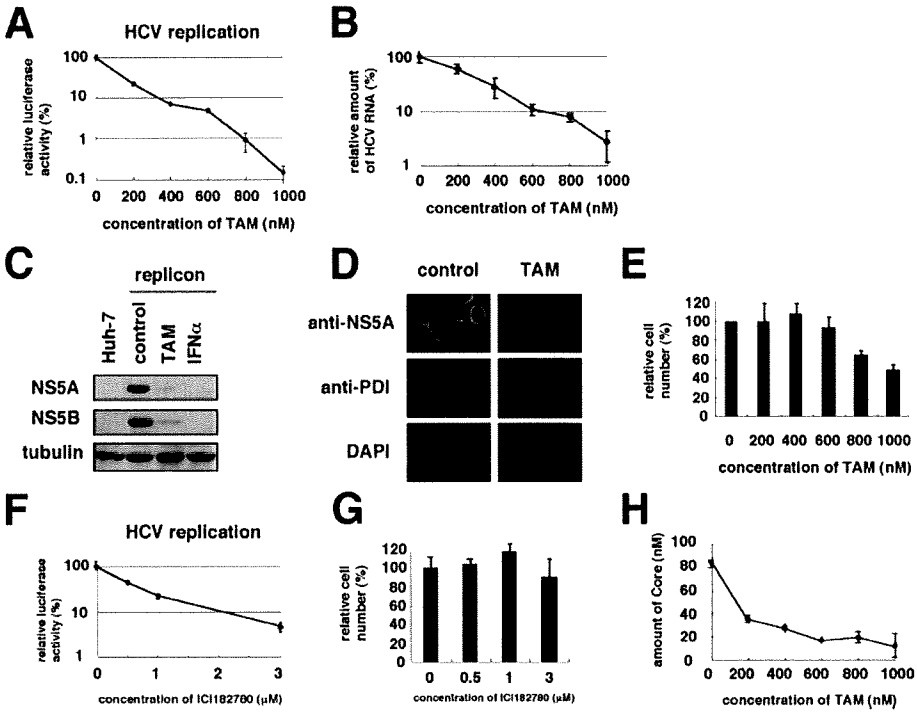

FIGURE 1. **TAM suppressed the replication of the HCV genome.** *A*, luciferase activities were measured using the LucNeo#2 cells, which carried a luciferase-containing replicon RNA, upon treatment with TAM at the indicated doses for 7 days. Relative luciferase activities are plotted against the concentrations of TAM. The data show the means of three independent experiments. The *error bars* are indicated. *B*, HCV RNA was quantified by real time RT-PCR analysis using the lysates from MH-14 cells, harboring the HCV subgenomic replicon, treated with the indicated doses of TAM for 7 days. Relative amounts of HCV RNA are shown. *C*, HCV NS5A and NS5B proteins as well as tubulin as an internal control were detected by immunoblot analysis in the lysates from MH-14 cells (replicon) treated without (control) or with 500 nM TAM or 100 IU/ml interferon-α as a positive control for 7 days and Huh-7 cells. *D*, HCV NS5A and protein-disulfide isomerase (*PDI*) as an internal control were detected by indirect immunofluorescence analysis in the cells treated without (control) or with 500 nM TAM for 7 days. 4′,6-Diamidino-2-phenylindole (*DAPI*) shows a nuclear staining. *E*, cell number was counted after 5 days upon treatment with various concentrations of TAM. Relative cell numbers are shown. *F*, luciferase activities with LucNeo#2 cells treated with various concentrations of ICI182780 were measured as described in *A*. *G*, cell number was counted under treatment with ICI182780 at the indicated concentrations. *H*, core in the culture medium of JFH1 RNA-transfected cells upon treatment with TAM was quantified as described under "Experimental Procedures."

used in this study were mouse normal IgG as a negative control (Zymed Laboratories), anti-NS5B (anti-NS5B#10; a generous gift from Dr. Kohara at the Tokyo Metropolitan Institute of Medical Science), anti-FLAG, and anti-caveolin-2 antibodies.

Fractionation of Cell Extracts—MH-14 cells transfected with the expression plasmid for FLAG-tagged ESRα were fractionated essentially as described previously (25).

HCV Replication Complex Assay—Isolation of HCV RC was done as described previously (16, 21).

In Vitro HCV Infection Experiment—*In vitro* HCV infection was conducted essentially as described (23). Briefly, HCV-infected serum ($\sim 2 \times 10^5$ copies) was inoculated into HuS-E7/DN24 cells (5×10^4 cells) for 24 h. After washes, cells were cultured in the medium supplemented with 10 μM PD98059 to stimulate HCV translation (27) (*scheme* in Fig. 6*B*). To observe HCV amplification, HCV RNA in the cells was quantified, since HCV RNA was hardly detected significantly in the culture medium (23).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay—The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to examine the cell viability using Cell Proliferation kit II, XTT (Roche Applied Science) according to the manufacturer's protocol.

RESULTS

Tamoxifen Suppressed HCV Genome Replication—We screened for agents that suppressed HCV genome replication using a HCV subgenomic replicon system (13, 16). Among the compounds tested, we observed that TAM inhibited HCV genome replication. HCV replication activity, monitored by luciferase activity (22), and the amount of HCV RNA were decreased with TAM treatment in a dose-dependent manner (Fig. 1, *A* and *B*). The expression of HCV proteins, NS5A and NS5B, detected by immunoblot (Fig. 1*C*) and indirect immuofluorescence analyses (Fig. 1*D*), also drastically decreased by treatment with TAM. A high concentration of TAM decreased cell proliferation (Fig. 1*E*). However, TAM suppressed HCV replication without any cytotoxicity in another cell line, HuS-E7/DN24 cells (Fig. 6, *C* and *D*). In addition, a pure anti-estrogen compound ICI182780, which had little cytotoxic effect, reduced HCV RNA (Fig. 1, *F* and *G*). Moreover, TAM inhibited the production of core in the culture medium of HCV JFH1-transfected cells, in a recently developed system of the production of infectious HCV particles (Fig. 1*H*) (28–30). The above data indicate that TAM suppresses HCV genome replication.

ESR Was Involved in HCV Genome Replication—Next, we investigated which cellular protein TAM targets to suppress HCV replication. It has been reported that TAM targets 1) ESR (31), 2) P-glycoprotein (32, 33), 3) calmodulin (34), 4) protein kinase C (35, 36), etc. Although other compounds targeting P-glycoprotein, calmodulin, and protein kinase C did not affect HCV replication in our screening (data not shown), ESR was suggested to play a role in HCV replication as shown below.

RNAi-mediated specific knockdown of endogenous ESRα and ESRβ (Fig. 2*A*) reduced HCV RNA in replicon-containing cells to ~20–40% and 60–70%, respectively (Fig. 2*B*). Transient transfection with ESRα and ESRβ expression plasmids, which activated ERE-driven transcription 4–5-fold (Fig. 2*C*), showed that ectopically expressed ESRα augmented HCV replication activity in a dose-dependent manner, whereas ESRβ did not (Fig. 2*D*). ESRα-induced augmentation of the replication was reversed upon TAM treatment (Fig. 2*D*). These results suggested a significant role of ESR, especially ESRα, in HCV

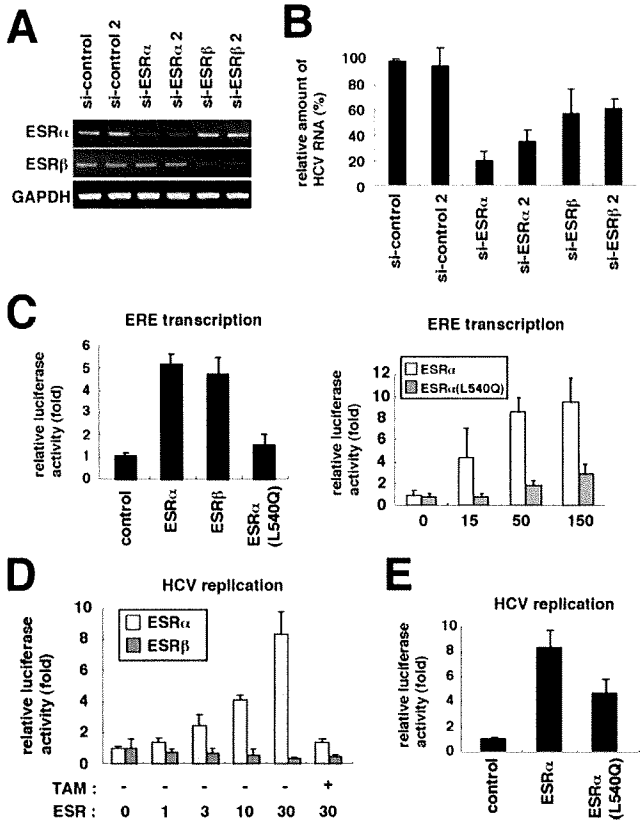

FIGURE 2. **ESR was involved in HCV genome replication.** *A*, specific knockdown of endogenous ESRα and ESRβ. RT-PCR analysis was performed to detect the expression of ESRα, ESRβ, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control in the cells transfected with siRNA recognizing ESRα (si-ESRα, si-ESRα2), ESRβ (si-ESRβ, si-ESRβ2), or randomized siRNA (si-control, si-control2). *B*, HCV RNA was quantified as shown in Fig. 1*B*, using the cells transfected with si-control, si-control2, si-ESRα, si-ESRα2, si-ESRβ, and si-ESRβ2 for 5 days. *C*, the ERE-mediated transcriptional activities were measured by a luciferase assay using the lysates from the cells transfected with pGL3-EREX3-TATA-Luc reporter plasmid together with pcDNA3-ERα (ESRα), pcDNA3-hERβ (ESRβ), pcDNA-ESRα(L540Q), or the empty vector (control) (*left*) or varying amounts (ng) of pcDNA3-ERα (ESRα) or pcDNA-ESRα(L540Q) (*right*) and treated with 100 nM estradiol for 36 h. *D* and *E*, HCV replication activities were examined by quantifying the luciferase activities using cured MH-14 cells transfected with the indicated doses (ng) of ESRα or ESRβ (*D*) or 30 ng of ESRα, ERα(L540Q), or the empty vector (control) (*E*) together with 0.125 μg of LMH14 RNA without or with 1 μM TAM for 4 days.

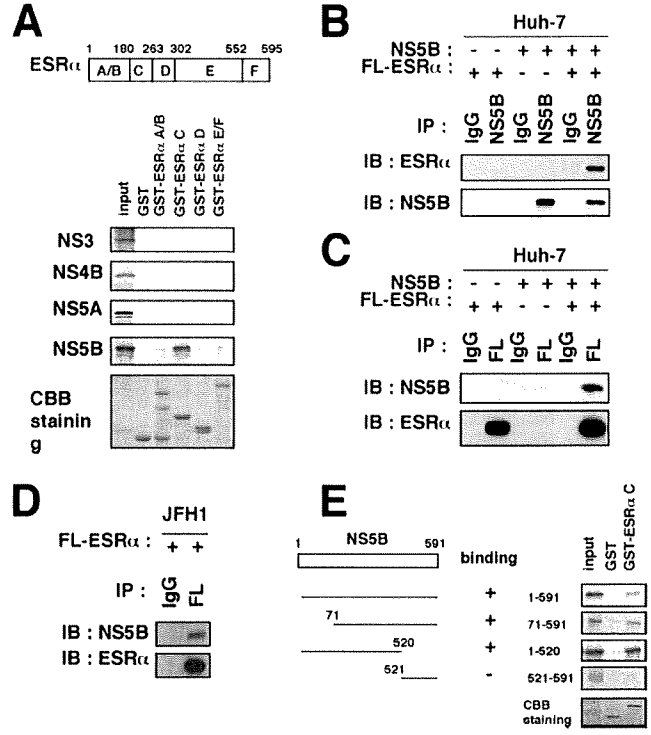

FIGURE 3. **ESRα interacted with HCV NS5B.** *A*, *top*, schematic representation of the primary structure of ESRα. ESRα consists of domains A–F. The amino acid numbers are also shown. *Bottom*, GST pull-down assays were performed using the recombinant proteins of the A/B, C, D, and E/F domain of ESRα fused with GST and *in vitro* translated HCV NS3, NS4B, NS5A, and NS5B protein. *Input*, the one-fifth amount of protein used for the pull-down assay. The Coomassie Brilliant Blue staining pattern of the precipitated fraction is also shown in the *bottom panel*. *B–D*, the lysates from the cells ectopically expressing NS5B (*B* and *C*) or the whole open reading frame of the HCV JFH1 strain (*D*) and/or FLAG-tagged ESRα were immunoprecipitated (*IP*) with anti-NS5B (*B*; NS5B), anti-FLAG antibody (*C* and *D*; FL), or mouse normal IgG as a negative control followed by the detection of ESRα and NS5B by immunoblot analysis (*IB*). *E*, deletion mutants of NS5B were subjected to a GST pull-down assay with GST-fused C domain of ESRα as described in *A*. The *left panel* shows a schematic representation of the full-length and truncated mutants of NS5B. The *numbers* indicate the amino acid numbers in NS5B.

genome replication. ESRα(L540Q), carrying a leucine to glutamine point mutation at aa 540 within the LXXLL motif (aa 536–540) of ESRα (37), had much lower transactivation activity driven from ERE (Fig. 2*C*). However, ESRα(L540Q) stimulated HCV replication activity ~5-fold, although the stimulation was less than that by wild-type ESRα (Fig. 2*E*). Thus, ESRα having lower transactivating capacity could still facilitate HCV replication.

ESRα Interacted with HCV NS5B—Thus, the chemical biology approach revealed the involvement of ESR in HCV genome replication. Then we investigated the molecular mechanism of ESR-induced HCV replication. A binding assay between ESRα and HCV proteins expressed in the HCV subgenomic replicon showed that the C domain of ESRα coprecipitated with NS5B but not NS3, NS4B, and NS5A (Fig. 3*A*). Other ESRα domains, A/B, D, and E/F, did not bind to any HCV proteins. A coimmunoprecipitation assay also indicated the presence of ESRα in the immunoprecipitate by anti-NS5B antibody (Fig. 3, *B* and *D*), and *vice versa* (Fig. 3*C*). Thus, ESRα specifically interacted with NS5B. Deletion analysis indicated that the region of 71–591 and 1–520 but not 521–591 of NS5B coprecipitated with the recombinant C domain of ESRα (Fig. 3*E*). This binding profile is different from that between cyclophilin B and NS5B, which we previously reported (21).

The ESRα-NS5B Interaction Was Important for the Regulation of HCV Genome Replication—To examine whether the interaction between ESRα and NS5B was essential for the ESRα-mediated regulation of HCV replication or not, we searched for a point mutant of ESRα that could not bind to NS5B by alanine-scanning mutation analysis. ESRα mutants, ESRα(255M) and ESRα(258M), in which IRK at aa 255–257 and DRR at aa 258–260 was replaced by TGT and AQT, respectively, had little affinity with NS5B (Fig. 4*A*) but still possessed the ERE-mediated transactivation capacity (Fig. 4*B*). However, both ESRα(255M) and ESRα(258M) caused only weak activations of HCV replication, compared with wild type ESRα (Fig. 4*C*). The data suggest that the interaction of ESRα with NS5B is

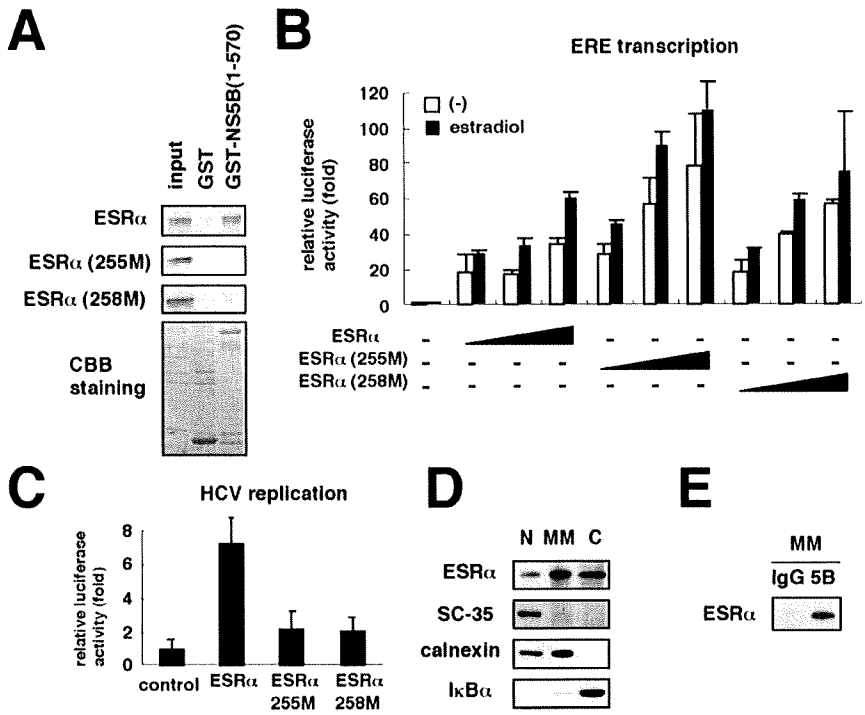

FIGURE 4. **The interaction of NS5B mediated the regulation of HCV genome replication by ESRα.** *A*, GST pull-down assays were performed as described in Fig. 3*A* using the wild type ESRα or point mutant of ESRα, ESRα(255M), and ESRα(258M). *B*, the mutation within ESRα(255M) and ESRα(258M) did not reduce the activation capacity of ERE-mediated transcription. Huh-7 cells were transfected with the expression plasmids for ESRα, ESRα(255M), or ESRα(258M) at doses of 10, 30, and 100 ng each together with pGL3-EREX3-TATA-Luc reporter plasmid and treated without (*white bar*) or with 100 nM estradiol (*black bar*) to quantify the luciferase activity. *C*, HCV replication activities were examined by quantifying the luciferase activities as described in the legend to Fig. 2*D* in the cells upon transfection with the expression plasmids for wild type ESRα, ESRα(255M), or ESRα(258M). *D*, the cells were fractionated into the nucleus (*N*), MM, and cytoplasm (*C*). Each fraction was detected for FLAG-tagged ESRα, SC-35, calnexin, and IκBα, respectively, by immunoblot analysis. Calnexin, an ER marker protein, was detected in the nucleus as well as MM, probably because of the existence of the nuclear membrane in the nuclear fraction. *E*, the MM fraction obtained in *D* was subjected to a coimmunoprecipitation assay using anti-NS5B or IgG followed by immunoblot analysis for the detection for ESRα.

critical for ESRα-mediated regulation of HCV genome replication.

Thus, ESRα interaction with NS5B regulates HCV replication. NS5B is mainly located on the cytoplasmic surface of the ER membrane (21, 38). On the other hand, ESRα as a nuclear hormone receptor is normally distributed in the cytoplasm and translocates into the nucleus upon ligand stimulation. In addition, a part of ESRα localizes on the membrane fraction. In our experiment, NS5B was mainly located around the ER, colocalized with the ER marker, protein-disulfide isomerase (data not shown) (21). Ectopically expressed ESRα showed diffuse distribution in the cells (data not shown). We fractionated cell homogenates and observed that a part of the ESRα resided in the microsomal membrane (MM) fraction (Fig. 4*D*). Moreover, ESRα in the MM fraction was coprecipitated with NS5B (Fig. 4*E*). It suggests the possibility that the interaction between NS5B and ESRα, at least in part of them, occurs on the ER membrane.

ESRα Promoted the Participation of NS5B in the HCV Replication Complex—It was reported that HCV proteins involved in the replication machinery was associated with the lipid raft on the ER and cofractionated with CAV2. A coimmunoprecipitation assay showed that NS5B associated with CAV2 (Fig. 5*A*). In the experiment investigating the role of ESRα in NS5B-CAV2 association, the coprecipitation of NS5B with CAV2 was decreased upon the knocking down of ESRα (Fig. 5*B*). Treatment with TAM abrogated the association of NS5B with CAV2 (Fig. 5*C*), although the total amount of NS5B in the cells is similar in the presence and absence of TAM for 24 h in this experiment (data not shown). Thus, ESRα was suggested to promote the association between NS5B and CAV2. Since a part of CAV2 resided on the lipid raft on the ER (18), ESRα-mediated binding between NS5B and CAV2 was possible to affect the localization of NS5B to the HCV RC. To see the consequential relevance of ESRα on NS5B function, we analyzed the HCV RC by treatment with digitonin/protease as described previously (16). HCV proteins involved in the RC and surrounded by the membrane structure are resistant to the treatment with digitonin followed by protease, whereas those unrelated to the replication outside the RC are digested by the treatment. By using this technique measuring the sensitivity to protease, HCV RC can be distinguished from the ER that is not related to the replication, although the RC and the nucleus cannot be separated. The experimental condition for fractionation was confirmed with the detection with IκBα and calnexin; a cytosolic protein IκBα was washed out following the treatment with digotinin (Fig. 5*D*, *lanes 1* and *2*), and ER protein calnexin, which did not accumulate in the RC, was digested by treatment with digotinin/protease (Fig. 5*D*, *lanes 2–4*). An ER lipid raft component, CAV2, was still detected under the digitonin/protease treatment (the RC-containing fraction) (Fig. 5*D*, *lanes 3* and *4*). Under this condition, a part of NS5B was detected in the digitonin/protease-resistant fraction, as described previously (16) (Fig. 5*D*, *lanes 3* and *4*). However, NS5B in this fraction was decreased upon treatment with TAM (Fig. 5*D*, *lanes 3*, *4*, *7*, and *8*). On the other hand, the amount of NS5A was not significantly changed by TAM treatment. Knocking down of ESRα also disrupted the association of NS5B with the RC-containing fraction (Fig. 5*E*). From the above results, it was suggested that ESRα promoted the participation of NS5B in the RC (also see "Discussion").

ESRα Could Serve as a Molecular Target of Anti-HCV Agents—Finally, we assessed the possibility that the association of ESRα with NS5B could serve as a target of anti-HCV agents. By introducing a decoy peptide against ESRα-NS5B interaction, consisting of the C domain of ESRα into replicon-bearing cells,

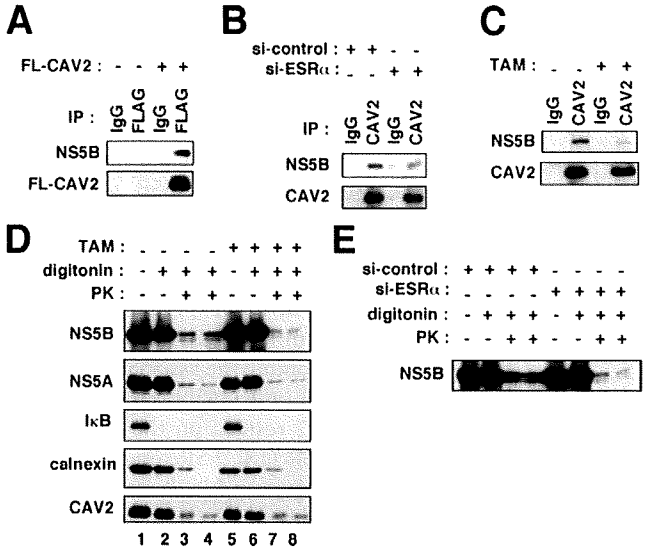

FIGURE 5. **ESRα promoted the participation of NS5B in HCV RC.** *A–C*, a coimmunoprecipitation assay (*IP*) was performed with anti-FLAG (*A*), anti-CAV2 (*B* and *C*) antibody, or mouse normal IgG from the lysates of the cells transfected without or with FLAG-tagged CAV2 (*A*), transfected with si-control or si-ESRα (*B*), or treated without or with 1 μM TAM (*C*). NS5B (*top*) and CAV2 (*bottom*) were detected by immunoblot analysis. *D*, detection of the amount of NS5B in the digitonin/protease-resistant fraction. MH-14 cells were treated without (*lanes 1–4*) or with 1 μM TAM (*lanes 5–8*) for 24 h. Cells were then treated without (*lanes 1* and *5*) or with digitonin (*lanes 2–4* and *6–8*), followed by digestion with proteinase K (0 μg/ml for *lanes 2* and *6*, 0.3 μg/ml for *lanes 3* and *7*, and 1 μg/ml for *lanes 4* and *8*). NS5B, NS5A, IκBα, calnexin, and CAV2 were detected by immunoblot analysis. *E*, HCV RC was isolated as described in *D* using the cells transfected with si-control or si-ESRα, and NS5B was detected. A similar result was obtained by using si-ESRα2.

HCV replication activity was reduced in a dose-dependent manner (Fig. 6*A*). To further observe the significance of ESRα in a physiological condition, we performed an *in vitro* infection experiment using serum from an HCV-infected patient as a nascent virus inoculum and nonneoplastic human hepatocytes as highly infection-permissive cells (Fig. 6*B*). Treatment with 1 μM TAM did not show a cytotoxic effect on these cells in any time course examined (Fig. 6*C*). However, treatment with TAM as well as cyclosporin A as a positive control inhibited the multiplication of viral genome RNA in the cells along with the time course (Fig. 6*D*). Thus, ESRα could serve as a potent molecular target of anti-HCV agents.

DISCUSSION

In general, viruses take advantage of host cell factors for their replication. So far, some factors have been shown to relevantly regulate HCV replication, including hVAP33 (39, 40), FBL2 (41), and cyclophilin B (21). Among these, FBL2 and cyclophilin B were identified by a chemical biological approach; FBL2 from the observation of an anti-HCV activity of lovastatin and an inhibitor of geranylgeranyl transferase (41–43); cyclophilin B from the inhibitory effect of cyclosporin A on HCV replication (20, 21). In this study, we found a suppressive capacity of TAM to HCV genome replication. Through further examination using TAM, we revealed ESRα as a host cell factor regulating HCV replication and suggested its regulation mechanism.

Currently, it is proposed that HCV RC that replicates the HCV genome is formed on the intracellular membrane, including the ER membrane (14–17). It was also reported that HCV genome replication was associated with the lipid raft on the intracellular membrane (18). Most HCV proteins are not related to the RC, whereas only a minor portion of HCV proteins take part in the RC to drive the viral replication (16). It has remained widely unknown, however, how HCV proteins are regulated to participate in the RC. It was reported that hVAP-33 binds to NS5A and NS5B, and this protein is related to the amount of NS5B in the lipid raft (40). hVAP-33 was speculated to recruit NS5B to the lipid raft, although its molecular mechanism has not been analyzed. This study suggested the interaction between ESRα and NS5B in the ER fraction, although we did not show the existence of ESRα in the RC, since the RC and the nucleus cannot be separated in the digitonin/protease treatment experiment. ESRα promoted the interaction of NS5B with CAV2. Previous papers reported that ESRα bound to CAV1 and CAV2 (6). From these observations, ESRα is

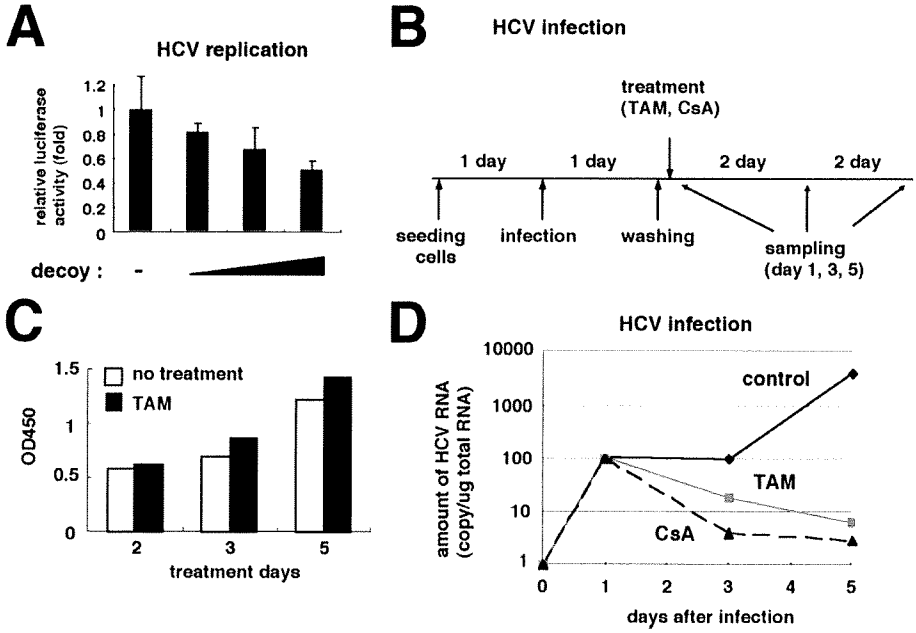

FIGURE 6. **ESRα could serve as a molecular target for anti-HCV agents.** *A*, HCV replication activity was measured by quantifying the luciferase activity as described in the legend to Fig. 2*D* in the cells overexpressing a decoy peptide consisting of the C domain of ESRα. *B*, experimental scheme of *in vitro* HCV infection experiment. After seeding the HuS-E7/DN24 cells, HCV-positive serum was inoculated for 24 h. After extensive washes, the cells were cultured with the medium supplemented without (control) or with 1 μM TAM or 3 μg/ml cyclosporin A. HCV genome RNA was quantified along with the time course (days 1, 3, and 5 postinoculation) by real time RT-PCR analysis. *C*, the treatment with 1 μM TAM did not show any cytotoxic effect on HuS-E7/DN24 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed as described under "Experimental Procedures" to examine the viability of the cells at days 2, 3, and 5 postinoculation. *D*, HCV genome RNA was quantified as described in *B* and plotted against the time course.

likely to function as a bridging factor that connects NS5B to CAV2, although we cannot fully neglect the possibility that ESRα augments NS5B-CAV2 binding via another function, such as transcriptional activity. Because CAV2 resided on the lipid raft of the intracellular membrane (18), this action of ESRα may recruit NS5B to the lipid raft and the HCV RC. In fact, ESRα promoted the participation of NS5B in the HCV RC. Thus, ESRα is suggested to escort NS5B to the HCV RC, although it is also possible that ESRα augments the number of the RC itself. However, ESRα at least augments the amount of NS5B involved in HCV replication machinery to stimulate the replication. It was reported that the membrane-associated ESRα served as a platform where signalsomes, including receptor tyrosine kinase, nonreceptor tyrosine kinase Src, and G proteins, assembled and activated downstream signaling pathways (44–46). HCV may also take advantage of such platform characteristics of ESRα to form the RC for their efficient replication. Although the mechanisms of the nuclear receptor function of ESRα have been extensively elucidated, the functions of membrane-associated ESRα have not been widely characterized so far. This study suggested a novel physiological relevance of membrane-associated ESRα as a regulator of the viral replication.

Until now, there are no clinical studies that report a direct interaction of TAM treatment with HCV replication in patients infected with HCV. Given our results, examinations on the effect of TAM or other anti-estrogen drugs may be one of the useful approaches to develop a new anti-HCV strategy. On the other hand, we disclosed the mechanism of ESR-mediated regulation of HCV genome replication. Screening for compounds that inhibit this mechanism expectedly led to novel types of anti-HCV agents. Further analyses on ESR are needed to develop anti-HCV therapeutics as well as reveal the regulation mechanism of HCV replication.

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Strain-Dependent Viral Dynamics and Virus-Cell Interactions in a Novel *In Vitro* System Supporting the Life Cycle of Blood-Borne Hepatitis C Virus

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We developed an *in vitro* system that can be used for the study of the life cycle of a wide variety blood-borne hepatitis C virus (HCV) from various patients using a three-dimensional hollow fiber culture system and an immortalized primary human hepatocyte (HuS-E/2) cell line. Unlike the conventional two-dimensional culture, this system not only enhanced the infectivity of blood-borne HCV but also supported its long-term proliferation and the production of infectious virus particles. Both sucrose gradient fractionation and electron microscopy examination showed that the produced virus-like particles are within a similar fraction and size range to those previously reported. Infection with different HCV strains showed strain-dependent different patterns of HCV proliferation and particle production. Fluctuation of virus proliferation and particle production was found during prolonged culture and was found to be associated with change in the major replicating virus strain. Induction of cellular apoptosis was only found when strains of HCV-2a genotype were used for infection. Interferon-alpha stimulation also varied among different strains of HCV-1b genotypes tested in this study. *Conclusion:* These results suggest that this *in vitro* infection system can reproduce strain-dependent events reflecting viral dynamics and virus-cell interactions at the early phase of blood-borne HCV infection, and that this system can allow the development of new anti-HCV strategies specific to various HCV strains. (HEPATOLOGY 2009;50:000-000.)

Hepatitis C virus (HCV) is a serious problem worldwide, with 3% of the world's population chronically infected.[1] Chronic infection with HCV may lead to high rates of liver cirrhosis and hepatocellular carcinoma.[2] Because the HCV standard therapy is still insufficient for treating many patients,[3] the development of more effective and less toxic anti-HCV agents is desired. The virological studies required to reach this goal need a reproducible and efficient HCV proliferation in cell culture. An *in vitro* infection system using recombinant HCV-JFH1 was developed. In this system, HuH7 cells transfected with *in vitro*-synthesized JFH1-RNA

Abbreviations: 2D, two-dimensional; 2D-HuS-E/2, HuS-E/2 cells cultured in two-dimensional condition; 3D, three-dimensional; 3D/HF, 3D hollow fibers; 3D-HuS-E/2, HuS-E/2 cells cultured in three-dimensional condition in the hollow fibers; HCV, hepatitis C virus; IFN-α, interferon alpha; LDH, lactate dehydrogenase; p.i., postinfection; RFB, radial-flow bioreactor; RT-PCR, reverse transcription polymerase chain reaction.

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were shown to secrete infectious viral particles.[4] This system, however, requires the combination of HuH-7-derived cell lines and JFH1-based constructs, limiting its usefulness for studying other HCV strains. Because HuH-7 cells cannot support the complete life cycle of blood-borne HCV (bbHCV) derived from clinical samples,[5] this system is insufficient for studying all the events related to bbHCV infection.

Many researchers have attempted to develop an *in vitro* system for bbHCV.[6-8] These current systems, however, are still insufficient due to their low efficiency for infectivity and replication of bbHCV. Working toward this same goal, we recently established immortalized primary human hepatocyte cell lines by transducing them with E6 and E7 genes from the human papilloma virus 18.[5,9] As expected, we observed improved infection and replication of bbHCV especially in one of these cell lines (HuS-E/2 cells) that showed a similar expression profile to that of human primary hepatocytes, but this strategy did not improve production of infectious particles.

Recently, a hybrid artificial liver support system was developed using animal hepatocytes cultured in a three-dimensional hollow fiber (3D/HF) system. This bioartificial liver showed several characteristic features of liver tissue for more than 4 months.[10-12] By growing our HuS-E/2 cells in a similar 3D culture[5] the gene expression profile was improved to more closely match that of human primary hepatocytes. Because the 3D cell culture condition more closely reproduces the *in vivo* environment of hepatocytes,[13] culturing these cells in this manner may support the entire HCV life cycle.

In this study we utilized this small 3D culture system and showed it to be ideal for culturing HuS-E/2 cells for the study of bbHCV infection. Using this system we are now able to study the variable patterns of the life cycle of different bbHCV strains as well as HCV-related cellular events.

Materials and Methods

Cell Culture. HuS-E/2 cells were cultured as previously described.[5] For the 3D/HF system, HuS-E/2 suspension was injected into the lumen of HF (Toyobo, Osaka, Japan) made from cellulose acetate and containing pores for nutrients and waste exchange (Supporting Fig. 1). The bundles were centrifuged to induce organoid formation. The cells in the fibers were cultured in 12-well plates (two capillary bundles per well) with gentle rotation using serum-free medium (Toyobo) in a CO_2 incubator at 37°C. The number of cells was adjusted to 3×105 cells per two-capillary bundle at the start of each experiment.

RNA Experiments. Total RNA was extracted from two-dimensional (2D) cultured cells, patient sera, or from 100 times concentrated culture medium as previously described.[4,5] For cells cultured in the 3D/HF, sterile scissors were used to cut each fiber into small pieces (1 mm^2 each), which were then solubilized in Sepasol RNA-1 (Nacalai Tesque, Kyoto, Japan). RNA was then extracted according to the manufacturer's protocol. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as described.[5]

HCV Infection. HCV infection experiments were carried out using sera from HCV patients. The amount of each inoculum was adjusted so as to add similar amount of HCV-RNA to the medium of the cells. After 24 hours, the cells were washed three times with phosphate-buffered saline (PBS) and cultured for the designated times. To assess the passage of infectivity, 12 mL of culture medium from the primary infected cells was collected, concentrated 100 times by filtration through Amicon Ultra-15, Ultracel-10K filters (Millipore, Carrigtwohill, Cork, Ireland), and 40 μL concentrated medium/well was used to infect naïve HuS-E/2 cells. All experiments were done with approval of the Ethical Committee of Kyoto University. Informed consent from patients was required for this approval.

Cloning and Sequencing. To amplify the complementary DNA (cDNA) fragment corresponding to hypervariable region 1 (HVR-1),[14] a nested RT-PCR was performed using Superscript III (Invitrogen, Carlsbad, CA) and PrimeSTAR HS DNA Polymerase (Takara, Tokyo, Japan). Reaction conditions were adjusted according to the manufacturer's protocol. Primers used were previously described[15] and are shown in Supporting Table 1. PCR products were then purified and cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Ten recombinant clones were randomly isolated for each PCR product and sequenced as described.[16]

Quantitative Detection of HCV Core and Interferon alpha (IFN-α) Protein by Enzyme-Linked Immunosorbent Assay (ELISA). The culture medium of infected cells was collected and concentrated 100 times as previously mentioned for the detection of HCV-core, or used directly for detection of IFN-α. HCV core protein was quantified using the Trak-C Core ELISA (Ortho Clinical Diagnostics, Neckargemünd, Germany). IFN-α was quantified using the Human IFN-A ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ). Light absorbance was then measured using a Wallac 1420 multilabel counter (PerkinElmer Life Science, Waltham, MA).

Cytotoxicity Assay. Culture medium was collected from HCV-infected cells and used for measuring lactate dehydrogenase (LDH) levels using an LDH cytotoxicity

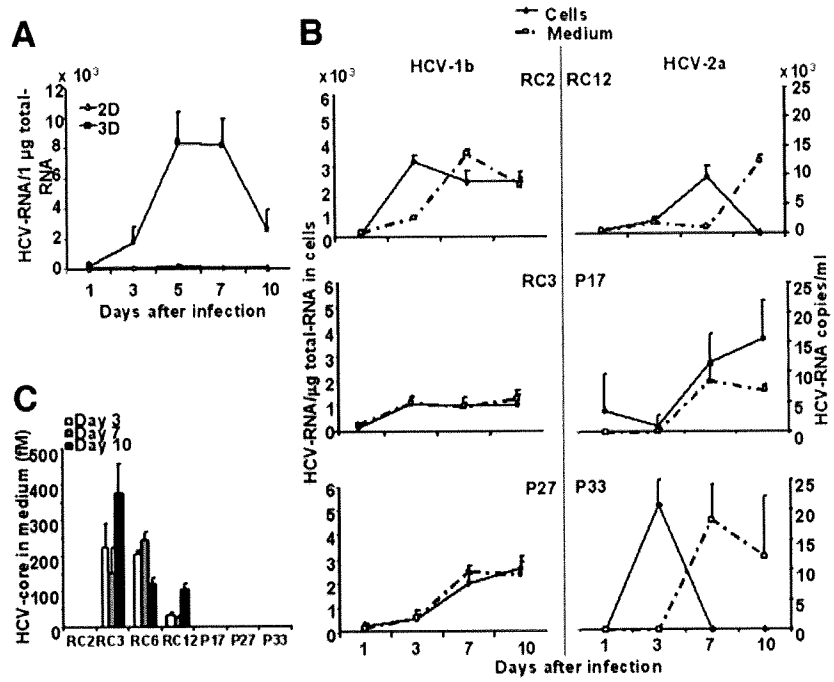

Fig. 1. Infection and proliferation of bbHCV in 3D-HuS-E/2 cells. (A) The quantity of HCV genomic RNA in 1 μg total RNA of 2D- or 3D-HuS-E/2 cells infected with HCV-RC6 was determined at each timepoint after infection by real-time RT-PCR analysis. (B) 3D-HuS-E/2 cells were infected with HCV-1b-containing sera: RC2, RC3, and P27; or HCV-2a-containing sera: 4: RC12, P17, P33. The quantity of HCV genomic RNA in the infected cells was determined as in (A). The culture medium from the last 2 days at each timepoint was collected, concentrated, and the amount of HCV-RNA (B) or HCV-core (C) was measured. Data represent the mean ± standard deviation (SD) of three independent experiments.

detection kit (Takara Biomedicals). Light absorbance was then measured as described above.

Sucrose Density Gradient. The culture medium of the infected cells was collected, concentrated 500 times, and loaded onto a 20%-50% (wt/vol) sucrose gradient containing 50 mM PBS, 100 mM NaCl, and 1 mM EDTA, followed by centrifugation at 100,000*g* for 16 hours at 4°C in a SW41Ti rotor (Beckman, Fullerton, CA). The gradient was fractionated into 31 fractions that were used for HCV-RNA and core detection and HCV infection into naïve cells as described above.

Electron Microscopy. The 1.12 g/mL fraction obtained by the sucrose density gradient showed the secondary infection activity as analyzed by transmission electron microscopy. The fraction was ultracentrifuged and the almost all supernatant was removed. The residual 10 μL of the solution was directly applied to a formvar-carbon grid for negative staining with 1% uranyl acetate solution and observed with an electron microscope (JEOL1010, JEOL, Tokyo, Japan).

Results

HuS-E/2 Cells Cultured in 3D/HF System Are Highly Permissive for Infection and Proliferation of bbHCV. We compared the ability of HuS-E/2 cells cultured in the 3D/HF system (3D-HuS-E/2 cells) to those cultured as a monolayer (2D-HuS/E2 cells) to reproduce infection by HCV genotype 1b (HCV-RC6), derived from patient serum (RC6). The HCV-RC6 RNA levels in the 3D-HuS/E2 cells were significantly higher at all timepoints (Fig. 1A), showing that the 3D/HF system greatly improves the proliferation of bbHCV in HuS-E/2 cells. We observed that both the early stages of infection and the continuous replication of HCV-RC6 in HuS-E/2 cells was improved by 3D/HF culture when the culture conditions were changed after the infection from 3D/HF to 2D and vice versa (Supporting Fig. 2).

As reported,[17] blocking CD81, an HCV-supposed entry receptor, during infection significantly impaired HCV proliferation into 3D-HuS-E/2 cells (Supporting Fig. 3), suggesting that CD81 is essential for HCV infectivity in 3D-HuS-E/2 cells. Although the expression level of CD81 mRNA in 3D-HuS-E/2 cells was observed, no significant change from 2D-HuS/E2 cells was found (data not shown), indicating that the quantity of CD81, at least, is not responsible for the improvement.

We then examined whether this system can be used for proliferation of six different bbHCV samples, three of which are HCV-1b (HCV-RC2, HCV-RC3, and HCV-P27) and three HCV-2a genotypes (HCV-RC12, HCV-P17, and HCV-P33) (Fig. 1B). Proliferation of HCV-RNA in the cells was seen in all six cases, suggesting that this system can be widely used for analysis of infection and proliferation of bbHCV strains. HCV-RNA and HCV-core were also detected in the culture medium (Fig. 1B). Different HCV strains showed variable patterns of proliferation and HCV-core secretion into the medium. Although HCV-core was detected from day 3 onward when

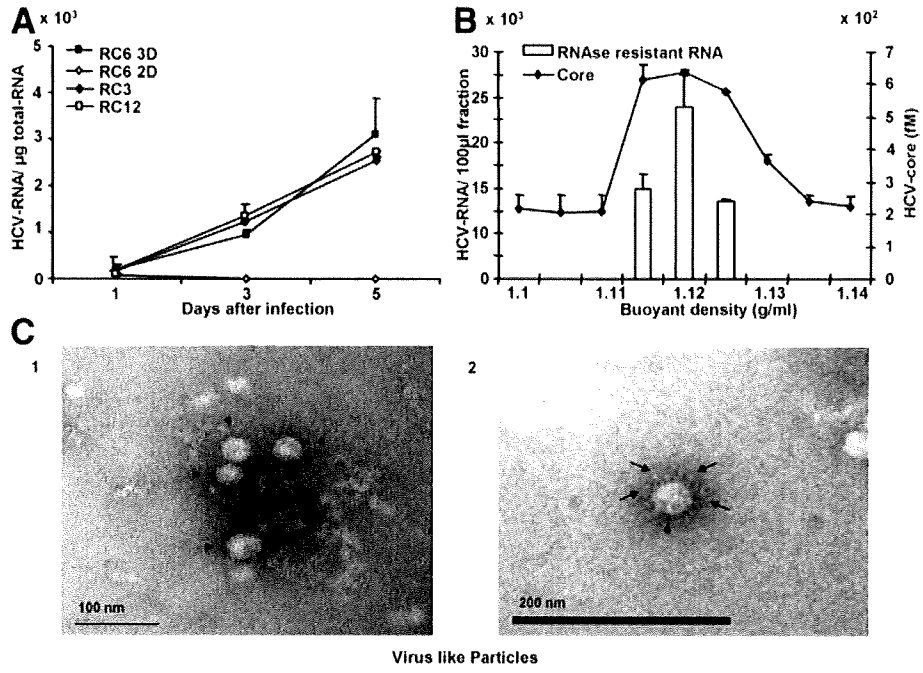

Fig. 2. Production of infectious virus-like particles from 3D-HuS-E/2 cells infected with different HCV strains. (A) The culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 or HCV-RC6 was collected from days 5 to 7 p.i. and for HCV-RC12 from days 23 to 25 p.i. The culture medium of 2D-HuS-E/2 cells infected with HCV-RC6 was also collected from days 5 to 7 p.i., and used to treat naïve 3D-HuS-E/2 cells. The quantity of HCV genomic RNA in 1 μg of total cellular RNA was determined as in Fig. 1. (B) The concentrated culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 was collected from days 5 to 7 p.i., and fractionated by ultracentrifugation with a 20%-50% sucrose density gradient. HCV-core protein and the RNase A-resistant HCV-RNA in the different fractions were quantitatively analyzed using an HCV-core ELISA kit and real-time RT-PCR, respectively. Data represent the mean ± SD of three independent experiments. (C) Photomicrograph showing negatively stained virus-like particles from the culture medium of HCV-RC3-infected 3D-HuS-E/2 cells (arrowheads, panels 1 and 2). The arrows indicate the spike-like structures found on the surface of the virus-like particles (panel 2).

RC3, RC6, and RC12 were used for infection, it was undetectable when RC2, P17, P27, and P33 sera were used, similar to 2D-HuS-E/2 cells infected with HCV-RC6 (Fig. 1C).

Production of Infectious Particles from 3D-HuS-E/2 Cells Infected with bbHCV. The culture media from 2D or 3D-HuS/E2 cells infected with RC6 serum (Fig. 1A) were collected from days 5 to 7 postinfection (p.i.), concentrated, and inoculated into naïve 3D-HuS-E/2 cell culture media. HCV-RNA's proliferation in the infected cells was only detected when using the culture medium from 3D-HuS-E/2 cells and not 2D-HuS-E/2 cells (Fig. 2A). Media collected from HCV-RC3 at days 5 to 7 and from HCV-RC12 from days 23 to 25 p.i. were also able to infect naïve cells (Fig. 2A). These data suggested the production and secretion of infectious virus-like particles. To investigate this further, biophysical analysis was performed. The culture medium of HCV-RC3 infected 3D-HuS-E/2 cells at day 7 p.i. was fractionated using a sucrose density gradient after RNase A treatment. HCV core was detected in the 1.11 to 1.14 g/mL fractions; similarly, the nuclease-resistant HCV RNA peaked in the 1.12 g/mL fraction (Fig. 2B). Furthermore, only the 1.12 g/mL fraction was able to infect naïve cells as examined above (data not shown). This fraction was pelleted by ultracentrifugation and examined by electron microscopy with negative staining. We observed 33-nm to 45-nm diameter spherical particles (Fig. 2C, panel 1) with spike-like structures from 7-9 nm in length on the surface (Fig. 2C, panel 2), consistent with HCV morphology reported previously in HCV patients.[18] These were detected in the sample collected from HCV-RC3-treated but not mock-treated 3D-HuS-E/2 cells. These data suggest that production of infectious virus-like particles occurs in 3D-HuS-E/2 cells infected with some bbHCV strains. It is therefore likely that 3D-HuS-E/2 cells can be used to reproduce nearly all steps in the HCV life cycle.

Prolonged Culture of HCV-Infected Cells in the 3D Hollow Fiber System. For HCV-RC6-infected cells (Fig. 3A), the amount of HCV-RNA in the cells fluctuated during the 30-day culture period. The levels of both HCV-RNA and HCV-core in the medium showed a similar pattern of fluctuations that peaked on days 5 and 20 p.i. Unlike RC6, the pattern of HCV-RNA levels in the medium of RC12-infected cells showed a negative

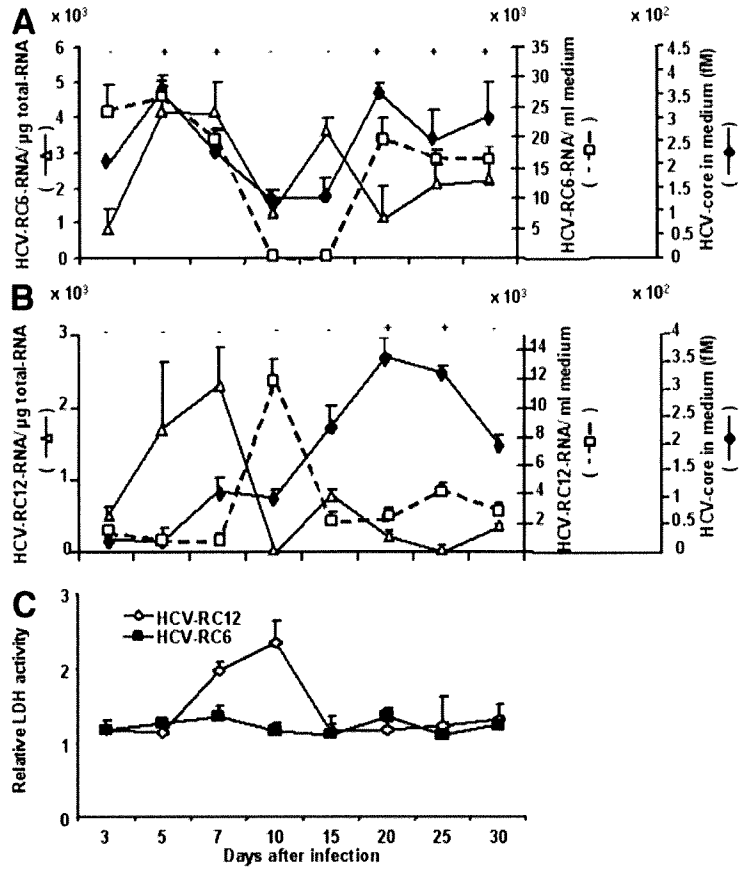

Fig. 3. Prolonged culture of HCV-infected cells in the 3D/HF system. After infection with HCV-RC6 (A) and HCV-RC12 (B), 3D-HuS-E/2 cells were cultured for 30 days with a medium change every 2 days. The HCV-RNA in the cells and medium as well as the HCV-core in the medium were quantitatively analyzed at the designated timepoints as in Fig. 1. Culture media were also used to treat naïve 3D-HuS-E/2 cells to examine the secondary infection as in Fig. 2. (+) and (−) indicate detection or no detection of secondary infection. (C) Culture media of HCV-RC6 and HCV-RC12 infected cells collected at each timepoint were used for the detection of LDH levels released from dead cells. LDH levels were normalized to uninfected cells cultured for the same time. Data represent the mean ± SD of three independent experiments.

correlation with that detected in the cells. This was clearly seen on day 10 p.i., when a sharp increase and decrease of HCV-RNA in the medium and the cells, respectively, was observed (Fig. 3B). Similarly, the amount of HCV-core detected in the medium throughout the culture was not correlated with RNA levels in the medium. Instead, core levels were very low in the first 10 days, at which time levels increased, reaching a peak on day 20 p.i. (Fig. 3B). Culture media from cells infected with HCV-RC6 from days 5 to 7 and 20 to 30 p.i. (Fig. 3A) and that from HCV-RC12 from days 20 to 25 p.i. showed passage of infectivity (Fig. 3B). All culture media showing infectivity appeared to have a high amount of HCV-core protein.

Clonal Changes in HCV During Prolonged Culture. In order to perform a populational analysis to understand the fluctuating pattern seen during HCV proliferation, two sera with limited HCV variants, HCV-RC6 (two major strains) and -RC12 (single major strain) from immunosuppressed liver transplantation patients with recurrent HCV were used in the previous prolonged infection experiment. The variants' composition was analyzed by single-strand confirmation polymorphism analysis for HCV-HVR1 (Supporting Fig. 4). RC6 serum (Fig. 4A) showed two different major sequences, HCV-RC6-1 and -2 strains, which constituted 60% and 40%, respectively, and shared 85% homology. In cells infected with HCV-RC6 the nucleotide sequence of HVR1 on day 5 showed 97% homology to HCV-RC6-1, and on day 20 p.i. it showed 97% homology to HCV-RC6-2. These data suggest selection of the dominant HCV strain in the cells over time. For RC12 (Fig. 4B), the nucleotide sequence on day 5 p.i. had only one nucleotide difference from that of the HCV from the original serum. The sequence from day 20 p.i. was four nucleotides different from that from the serum, and five different from the cells on day 5 p.i. These data indicated that each peak of HCV-RNA that appeared in the cells infected with RC12 serum included primarily a single HCV strain with a slightly different genomic sequence. This suggests that the periodic appearance of HCV-RNA peaks in the cells infected with a particular HCV strain is a result of selection and/or mutation of HCV strains during the prolonged culture period.

Cellular Response Induced by bbHCV Infection. At day 10 p.i., HCV-RNA levels in the culture medium rose and RNA levels in 3D-HuS-E/2 cells infected with HCV-RC12 dropped (Figs. 1B, 3B). To determine if this was caused by a cytotoxic effect of HCV infection, LDH levels were measured in the culture medium of HCV-RC6- and

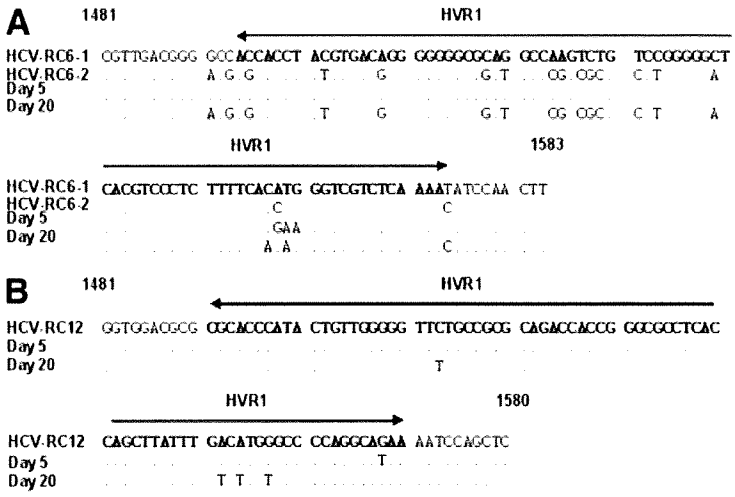

Fig. 4. Comparison of HCV-HVR1 sequences in the serum used for infection and the HCV replicating in the cells on days 5 and 20 after infection of HCV-RC6 (A) or HCV-RC12 (B). Nucleotide numbering was based on HCV-J1 sequence (GenBank Access. No. D10749). Three additional nucleotides were found at the 5'-terminal end of the E2 regions of all RC6 sequences. The major sequence present in the serum used for infection is shown in the upper row in each panel. Dots represent the identical nucleotides.

HCV-RC12-infected 3D-HuS-E/2 cells. LDH activity showed a strong correlation with HCV-RNA levels in the medium on day 10 p.i. in HCV-R12-infected cells (Fig. 3B), suggesting a cytotoxic effect of HCV-RC12 that was not observed in the case of HCV-RC6 (Fig. 3A,C). To determine if this HCV infection-mediated cytotoxicity is due to apoptosis, as with other viruses belonging to the Flaviviridae family,[19] the involvement of caspase was examined using the caspase inhibitor z-VAD-fmk. A significant dose-dependent reduction in HCV-RNA levels in the medium and LDH activity (Fig. 5A,B) was found, whereas no significant effect was observed on the viability of noninfected cells (Fig. 5B) or intracellular HCV-RNA levels (Fig. 5A). This suggested that the cytotoxic effect of HCV infection is mediated by apoptosis. It is noteworthy that HCV-induced cytopathicity was also found when HCV-P17 and HCV-P33 samples were used for infection (both are HCV-2a genotype) and was not reproduced in any of the HCV-1b genotype samples used in this work (Fig. 5C).

After infection with HCV-RC6, no cytotoxicity was detected that might have inhibited HCV-RC6-1 proliferation in the cells. However, HCV-RC6-2 RNA replaced HCV-RC6-1 RNA during prolonged culture. To assess a

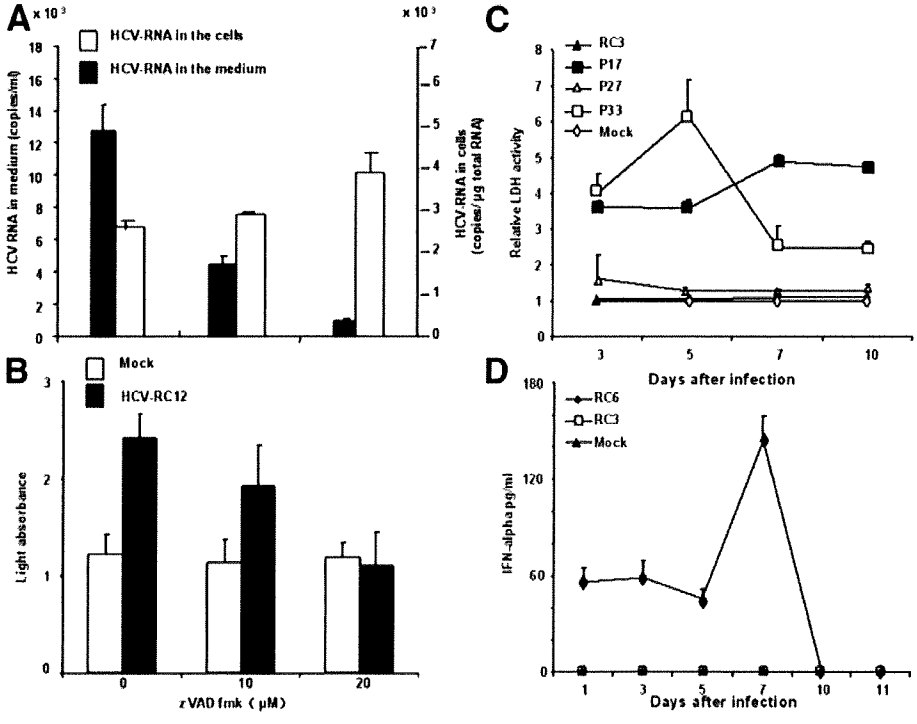

Fig. 5. Cellular response of 3D-HuS-E/2 cells infected with bbHCV. 3D-HuS-E/2 cells infected with HCV-RC12 and mock-treated cells were cultured for 10 days in the presence of z-VAD-fmk (0, 10, and 20 μM). (A) HCV-RNA in the cells and medium on day 10 was measured as in Fig. 1. (B) LDH levels in the medium on day 10 after infection with HCV-RC12 was measured as in Fig. 3. (C) Culture media of HCV-RC3, HCV-P17, HCV-P27, HCV-P33, and mock-infected cells collected at designated points were used for the detection of LDH levels. (D) IFN-α levels in the culture media of HCV-RC6, HCV-RC3, and mock-infected cells collected at each designated timepoint were measured by ELISA. Data represent the mean $\pm$ SD of three independent experiments.

possible role of the innate-immune response in this phenomenon, the production of IFN-α in the medium was measured during the first 11 days p.i. IFN-α production was detected as early as day 1 p.i., reached a peak at day 7 p.i., and was then rapidly lost (Fig. 5D). These data suggest that HCV-RC6-1 infection induced the innate-immune response of the cells, possibly leading to suppression of its proliferation. In contrast to HCV-RC6-1, HCV-RC3 did not show any stimulation of IFN-α production upon infection in the first 10 days, showing a possible strain-dependent evasion from the host defense within the same genotype.

Discussion

In this study we report the development of a novel system that reproduces bbHCV infection, proliferation, and production of infectious virus. The most recent models used in the study of the life cycle of HCV infection are based on subclones of HuH-7 cells infected with JFH1 recombinant virus or its derivatives.[4] HuH-7 cells and its subclones, however, do not support the entire life cycle of the bbHCVs present in patients' blood.[5] Moreover, HCV has considerable diversity and variability. It is generally classified into six major genotypes and more than 100 subtypes.[20] This huge pool of natural HCV variants causes a wide variety of diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[21] JFH1, however, is a single isolate of HCV genotype 2a that was originally derived from a patient with rare fulminant hepatitis.[4] We suggest that our newly established system has an important advantage because it supports the entire life cycle of a variety of HCV strains and genotypes.

Due to the lack of some *in vivo* factors, including host immune response, *in vitro* systems may not completely reproduce the *in vivo* situation. However, *in vitro* experimental systems seem to be important to simplify particular events from the complex situation *in vivo*. From that standpoint, our cell culture system is likely reproducing the early event of HCV infection in the absence of host-immune responses and supporting whole life cycle of the blood-borne HCV. Several *in vitro* hepatocyte culture systems have been reported to be useful for studying the infection and replication of bbHCV.[5-8,22] Only the radial-flow bioreactor (RFB) 3D culture system demonstrated production of infectious viruses.[22] In our studies we observed not only the enhancement of HCV replication, but also the production of infectious HCV particles in the medium using the 3D/HF system. These data suggest that some structure of the cell mass formed by the 3D culture system, most likely the polar character, is essential for the life cycle of bbHCV. The RFB system is composed of a dedicated device containing 1×10^9 FLC4 cells with a culture area of 2.7 m^2.[22] It can only be used to study HCV particle production in the medium and not the cellular events that accompany the HCV life cycle. In contrast, because cells grown in our 3D/HF system are cultured in 12-well plates at a density of 3×10^5/fiber, it is much simpler to study both viral and cellular events.

The production of infectious particles was not detected with infection by different HCV strains, despite detecting equivalent levels of HCV-RNA in the cells (Fig. 1B,C). Delayed production of infectious particles was also observed in cells infected with HCV-RC12 after prolonged culture. A similar delay was also observed in the RFB system.[22] Considering the relative stability of HuS-E/2 cells[5] and the relatively high frequency of the change in HCV population in the cells,[16] it is likely that mutation of the HCV genome and/or selection of clones during prolonged culture improved the productivity of infectious particles. A marked improvement of infectious particle production by substitution of the structural proteins of the genome was also reported in the recombinant HCV production system.[23] The lack of production of infectious particles soon after infection may serve to avoid an early strong response from the host immune system, and demonstrates a novel mechanism of latent infection by HCV. Although they may not be associated with plasma components as those present *in vivo*, HCV virus-like particles produced by this system showed a close resemblance to those isolated from infected HCV patients because they showed the same size[18] and were within the fraction range.[24] They may help in the study of viral and cellular factors required for particle production and the possible receptors utilized for infection with different HCV strains.

Fluctuation in HCV proliferation was observed during the prolonged culture of 3D-HuS-E/2 cells infected with bbHCV (Fig. 3A,B), consistent with previous reports in other culture systems.[6,22] This fluctuation was associated with a change in viral quasispecies, suggesting that an HCV strain having a growth advantage proliferates selectively and dominantly in these culture conditions. Because the progressive emergence of each dominant strain was only temporary, it is highly likely that the infection and proliferation of such an HCV strain is suppressed by cellular mechanism(s). Our results suggest that there are actually two cellular mechanisms functioning to do this. The first is the involvement of the innate immune system, as evidenced by the secretion of IFN-α during the first week of infection (Fig. 5D). This is the first report of secretion of IFN-α from cultured cells infected with bbHCV. Although recent reports suggest that stimulation of the IFN pathway by HCV infection could be impaired by HCV NS3-4a proteinase-mediated cleavage of IPS-

1,[25] our results suggest that not all bbHCVs possess a host cell suppressive function. The second mechanism is HCV-induced cell death (Fig. 3C). Almost all the studies reporting HCV-induced apoptosis used hepatocellular carcinoma cell lines.[26,27] Because it has been established that the inability to undergo apoptosis is essential for the development of cancer,[28-30] our use of immortalized, noncancerous HuS-E/2 hepatocytes may make it possible to reproduce the physiological response of the cells to bbHCV infection more closely. Although HCV-induced apoptosis was not found when HCV-1b was used for infection, it was found in all cases where HCV-2a was used, suggesting a higher cytopathic tendency of the HCV-2a genotype. HCV proliferation was continuously found even after the suppression of the first peak of RNA production during prolonged culture. How HCV survives under those conditions is still unknown. Further studies to clarify the molecular mechanisms involving the HCV-cell interaction can be done using this novel 3D culture system that reproduces the infection of a variety of bbHCVs.

In conclusion, we have established a new *in vitro* culture system that can support the entire life cycle of a variety of HCV isolates and genotypes. Although this *in vitro* model system may not completely reproduce the *in vivo* situation, we believe it is the first *in vitro* system showing HCV strain-dependent virus/cell interaction including induction of cellular apoptosis and/or evasion from cellular innate immune response, which may make it a good tool for analysis of virus/host interaction together with the development of new anti-HCV strategies for the different bbHCV strains.

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Suppression of hepatitis C virus replication by baculovirus vector-mediated short-hairpin RNA expression

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Abstract Short-hairpin RNAs (shRNAs) inhibit gene expression by RNA interference. Here, we report on the inhibition, by baculovirus-based vector-derived shRNAs, of core-protein expression in full-length hepatitis C virus (HCV) replicon cells. shRNAs were designed to target the highly conserved core region of the HCV genome. In particular, the core-shRNA452 containing nucleotides 452–472, as the target in the HCV core gene, dramatically inhibited the expression of the HCV core protein in replicon cells. Furthermore, HCV core-protein expression was inhibited more strongly by the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped baculovirus vector than by the wild-type baculovirus vector.

Keywords: Baculovirus vector; Hepatitis C virus; RNA interference; Short-hairpin RNA; Vesicular stomatitis virus glycoprotein

1. Introduction

The hepatitis C virus (HCV) is a major cause of liver-related morbidity and mortality [1]. The virus establishes a persistent liver infection, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas [1,2]. However, a highly effective anti-HCV drug has yet to be developed, partly due to the lack of detailed information about the life cycle of the virus. In an effort to develop an alternative to combined interferon–ribavirin treatment [3,4], we used RNA interference (RNAi) based on short-hairpin RNA (shRNA), which is a powerful tool for suppressing gene function [5]. RNAi is triggered by small-interfering RNAs (siRNAs) that are processed from long double-stranded or hairpin precursors, and become part of the ribonucleoprotein complex, the RNA-induced silencing complex (RISC) [6,7]. siRNAs expressed from DNA templates through the action of the Dicer enzyme silence gene expression as effectively as exogenously introduced synthetic siRNAs [5,8,9]. The use of RNAi has recently been extended to differentiated cultured mammalian cells [10]. It has also been used to inhibit viral replication in the HCV subgenomic replicon [11–13].

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has long been used as a biopesticide, and as a tool for efficient recombinant protein production in insect cells [14]. Its host specificity was originally thought to be restricted to cells derived from arthropods; however, with an appropriate eukaryotic promoter, it can express foreign genes in several types of mammalian cells [15–17] and animal models [18,19]. Its advantages for use in gene-therapy applications are its inherent inability to replicate, its lack of cytopathic effect (CPE) in mammalian cells even at a high multiplicity of infection (MOI), and the absence of preexisting antibodies against baculoviruses in animals.

Here we designed baculovirus vector-mediated shRNAs against the highly conserved core-protein region of the HCV [20,21]. The shRNA452 construct mediated more effective inhibition of HCV replication than the other core-shRNAs (Ac-shRNA479 and Ac-shRNA523). We also found that HCV core-protein expression was more significantly inhibited by the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped baculovirus vector than by the wild-type baculovirus vector.

2. Materials and methods

2.1. Baculovirus transfer vector constructs

We designed baculovirus transfer vectors expressing shRNAs against the following regions of the HCV core-protein sequence: nucleotides 452–472, containing the nuclear localization signal site (pU6-core-shRNA452); nucleotides 479–499 (pU6-core-shRNA479); and nucleotides 523–543 (pU6-core-shRNA523) [22]. Sense and antisense strands of shRNA oligonucleotides were synthesized, annealed at 95 °C for 3 min, and then slowly cooled in phosphate-buffered saline (PBS; pH 7.4, containing 50 mM NaCl). The oligonucleotides contained the loop CCACACC sequence, and Kpn I and BamHI ends, which were inserted into a pU6 vector, based on pSV2-neo. A Pol III-type U6 promoter allowed the constant expression of shRNAs. The following three sites in the core region of the common sequences of the HCV strain M1LE (GenBank accession number AB080299) were chosen as the targets for shRNAs: 5′-GCCGCGCAGGGGCCC-CAGGUU-3′ (shRNA452); 5′-GCGCGCGACUAGGAAGACUUC-3′ (shRNA479); and 5′-GCGACAACCUAUCCCCAAGGC-3′ (shRNA523). Fragments of U6-core-shRNAs, ranging from the Eco-RI site upstream of the U6 promoter to the BamHI site downstream of the terminating sequences, were sequenced and then inserted into the cloning site of the baculovirus transfer vector, pVL1393 (BD Biosciences, San Jose, CA, USA) in an opposite orientation to the PH promoter (Fig. 1C), in order to create pVL1393-core-shRNA452, shRNA479, shRNA523, and pVL1393-U6-terminator. A spacer was inserted between the inverted sequences to form a hairpin structure,

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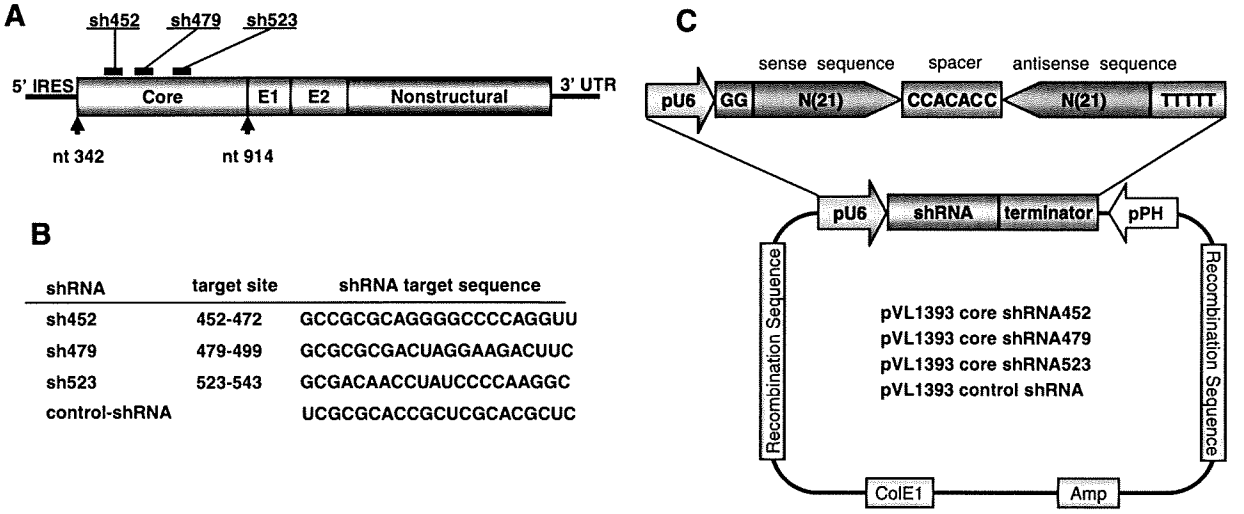

shRNA	target site	shRNA target sequence
sh452	452-472	GCCGCGCAGGGGCCCCAGGUU
sh479	479-499	GCGCGCGACUAGGAAGACUUC
sh523	523-543	GCGACAACCUAUCCCCAAGGC
control-shRNA		UCGCGCACCGCUCGCACGCUC

Fig. 1. (A) Genomic profile of HCV showing both coding and non-coding genes. (B) HCV core-region target sites and sequences used for the design of shRNAs. (C) Construction and schematic representation of baculovirus transfer vector expressing HCV core-shRNA.

and to enhance its stability (Fig. 1C). Scrambled shRNA (control-shRNA) cloned into the same vector was used as a negative control (pVL1393-control-shRNA) in all experiments. The VSV-G-pseudotyped baculovirus vector-transduced shRNA452 was constructed following previously published procedures [23].

2.2. Preparation of baculoviruses

Recombinant baculovirus containing the shRNA genome (Ac-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) following previously published procedures [23].

2.3. Cell culture

NNC#2 (NN/1b/FL) cells [24] carrying a full genome replicon were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino-acids, L-glutamine, and 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA).

2.4. Measurement of HCV core protein

AcU6-HCV-core-shRNAs or AcU6-VSV-HCV-core-shRNAs were used to infect HCV replicon cells. After 48 h, intracellular HCV core-protein levels were measured using a fully automated HCV core-protein antigen chemiluminescent enzyme immunoassay (CLEIA) according to the manufacturer's instructions [25]. The relative chemiluminescence unit was measured, and was used to determine the concentration of HCV core antigen according to a standard curve generated using recombinant HCV core antigen. The concentration was expressed in units of femto-mol/L (fmol/L). Each CLEIA assay was performed in triplicate.

3. Results

3.1. Construction of baculovirus transfer vectors carrying shRNA-synthesizing cassettes

The core-protein forms the nucleocapsid, and modulates gene transcription, cell proliferation, and apoptosis [21]. HCV functions as a messenger RNA (mRNA) with a single-stranded RNA genome; thus, we hypothesized that cleavage of the core-protein mRNA would inhibit nuclear transport and virus duplication.

To characterize the efficiency of baculoviruses as vehicles for gene therapy, we selected the HCV core region as a target site (Fig. 1A and B), and constructed a recombinant baculovirus vector expressing the shRNA. Fig. 1C shows the baculovirus-transfer vectors used in this study. The baculovirus-transfer vector pVL1393-core-shRNA carries core-shRNA under the control of the PolIII, U6 promoter. Recombinant baculovirus containing the shRNA genome (Ac-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) in Sf9 cells. Viruses were produced at high titers, ranging from 1.5×10^8 to 1.2×10^9 pfu/ml. shRNA452 expression was confirmed by Northern blot analysis in Ac-shRNA452-infected Huh-7 cells (Supplementary Fig. S1 and Supplementary methods).

3.2. Inhibition of HCV RNA replication of baculovirus-mediated shRNA-expression vectors in the HCV replicon

We investigated whether intracellular expression of shRNA inhibited viral replication and affected HCV RNA levels in NNC#2 cells. The baculovirus-infection efficiency of NNC#2 cells ranged from 80 to 90% (Fig. 2A and Supplementary methods). The real-time reverse-transcription (RT) polymerase chain reaction (PCR) was used to examine the presence of HCV RNA, and the ability to induce RNA silencing in NNC#2 cells 48 h post-infection. When NNC#2 cells were infected with Ac-shRNAs at a MOI of 100, a significant reduction in HCV RNA levels was observed compared with a non-related shRNA control (Fig. 2B and Supplementary methods). Although inhibition of HCV RNA levels occurred with all three constructs, the greatest effect occurred with sh452 (68%), while sh479 decreased the levels by approximately 55% and sh523 by 25%. By contrast, the control baculovirus vectors (Ac-U6-terminator and Ac-control-shRNA [random sequence]) had no inhibitory effect on HCV replication (Fig. 2B). These results point to a sequence-specific inhibitory effect of shRNA on HCV replication.

3.3. Silencing of HCV core-protein expression through baculovirus-mediated shRNA in the HCV replicon

We confirmed the inhibitory effect of shRNAs using fluorescence microscopy to assess the localization and expression of the HCV core protein 48 h post-infection. The core proteins

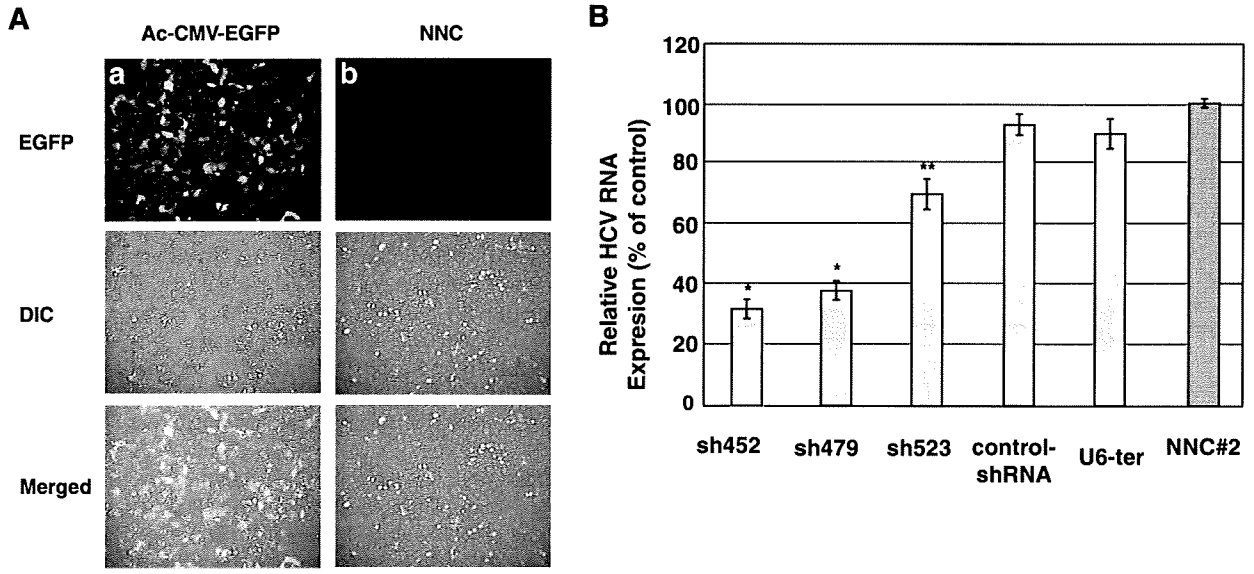

Fig. 2. Inhibition of HCV RNA by baculovirus-mediated shRNA expression vector. (A) Fluorescence-microscopic analyses of HCV full replicon cells (NNC#2, 4×10^4 cells/well) infected with enhanced green fluorescent protein (EGFP) baculovirus (Ac-CMV-EGFP) at a MOI of 100. Images were taken at a ×10 magnification 2 d post-infection: (a) Ac-CMV-EGFP; (b) NNC cells alone. (B) Real-time PCR analysis of HCV RNA expression after transduction of HCV full replicon cells (NNC#2, 4×10^4 cells/well) with a MOI of 100 for baculovirus-mediated shRNA. Relative HCV RNA values are shown. Error bars represent S.E. of the mean from three different experiments. $^*P < 0.01$; $^{**}P < 0.05$.

were localized in the cytoplasm of core protein-shRNA (sh452, sh479, and sh523)-expressing NNC#2 cells (Fig. 3A and Supplementary methods), as well as control cells (control-shRNA). Inhibition of the HCV core protein was observed following infection by all three constructs, although the greatest effect occurred with sh452.

We next examined whether shRNA against the HCV core protein inhibited viral replication, using a CLEIA assay. When NNC#2 cells were infected with Ac-shRNAs at MOIs of 100 and 200, a significant reduction in core-protein expression was observed compared with a non-related shRNA control (Fig. 3B). The sh452 construct mediated more effective inhibition of HCV replication than the other core-shRNAs (Ac-shRNA479 and Ac-shRNA523). The control-Ac-U6-terminator and Ac-control-shRNA baculovirus vectors had no inhibitory effect on HCV replication.

Furthermore, enhanced gene-transfer efficiency was observed in a range of cell lines with recombinant baculovirus vectors expressing surface glycoprotein G of VSV-G (Fig. 4A). VSV-G enhances the escape of baculovirus vectors from intracellular endosomes, thereby increasing the transduction efficiency of the virus [17]. The VSV-G-modified baculovirus

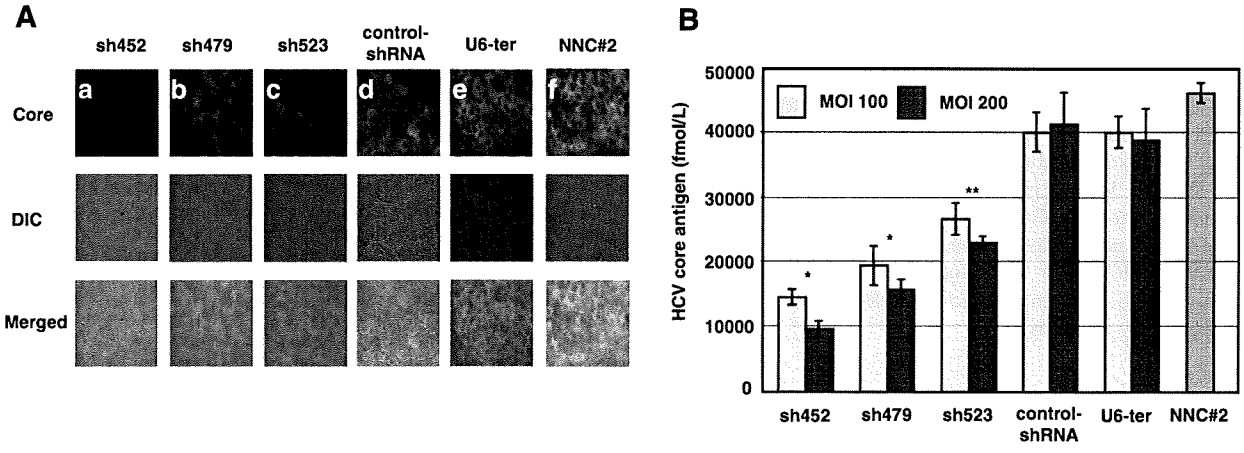

Fig. 3. Suppression of HCV core protein by baculovirus-mediated core-shRNA expression vectors. (A) Immunofluorescence microscopy of anti-HCV core-protein antibody (green) following infection of NCC#2 cells with the following: (a) sh452 vector; (b) sh479 vector; (c) sh523 vector; (d) control-shRNA vector; (e) U6-terminator vector; (f) NNC cells alone. (B) Ac-shRNAs were used to infect NCC#2 cells and intracellular HCV core-protein levels were measured after 48 h by an HCV protein antigen CLEIA assay. Error bars represent S.E. of the mean from three different experiments. $^*P < 0.01$; $^{**}P < 0.05$.

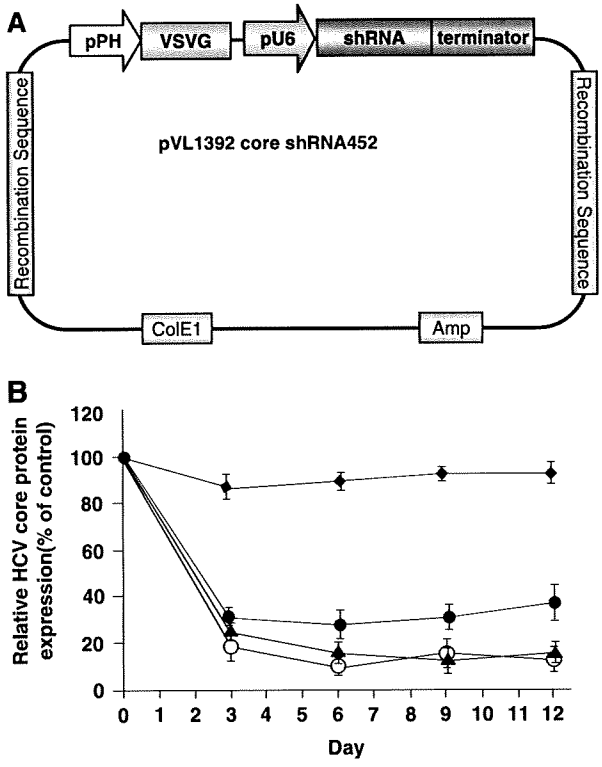

Fig. 4. Inhibition of HCV core protein by VSV-G-pseudotyped and wild-type baculovirus-mediated shRNAs. (A) Construction and schematic representation of VSV-G-pseudotyped baculovirus transfer vector expressing HCV core-shRNA. (B) CLEIA analysis of HCV core-protein expression after transduction of HCV full replicon cells (NNC#2, 4×10^4 cells/well) with Ac-shRNA452 (MOI = 100 [solid circle] or 200 [solid triangle]), Ac-VSV-G-shRNA452 (MOI = 100 [open circle]) or control-shRNA expression vector (MOI = 100 [solid diamond]).

(Ac-VSV-G-shRNA452) was propagated following previously published procedures [23]. Based on the shRNA452-expression data, we evaluated the effectiveness of shRNA452-mediated inhibition of HCV core expression by VSV-G-pseudotyped or wild-type baculovirus vector-transduced shRNA in NNC#2 cells. When Ac-VSV-G-shRNA452 (MOI = 100) or Ac-shRNA452 (MOI = 00 or 200) was added to the cells at 3 d intervals for 12 d, a significant reduction in HCV RNA (70–90%) was sustained from day 3 to day 12. The results indicated that HCV core protein expression was more strongly inhibited by the VSV-G-pseudotyped baculovirus vector than by the wild-type baculovirus vector (Fig. 4B). The ability of Ac-VSV-G-shRNA452 to reduce HCV core-protein levels was not due to a cytotoxic effect (Supplementary Fig. S2 and Supplementary methods).

4. Discussion

There is a need for a highly effective anti-HCV drug to be developed. The current study aimed to establish a universal baculovirus vector for intracellular shRNA generation and inhibition of HCV gene expression in NNC#2 cells. shRNAs that were designed to target core regions of HCV replicon RNA varied in their ability to inhibit HCV replication. It is important to note that the core protein is synthesized as the initial polypeptide from the HCV polyprotein precursor. However, the inhibition of the HCV core protein upon the introduction of siRNAs was not determined in these studies [11,12]. Our current work revealed that baculovirus-mediated shRNA452 expression significantly inhibits HCV replication.

The delivery of siRNA to appropriate cells or tissues is a major challenge. Several approaches have been described for generating loss-of function phenotypes in mammalian systems using siRNA; however, these techniques are limited and are not suitable for generating a long-term silencing effect in vivo [26,27]. Efficient and safe delivery systems have not yet been established for the suppression of HCV replication. Baculoviruses appear to be useful viral vectors, not only for the abundant expression of foreign genes in insect cells, but also for efficient gene delivery to the hepatoma lines HepG2 and Huh7 [28], and the osteogenic sarcoma line Saos2 [29].

In this study, the introduction of three different baculovirus-mediated shRNAs into target cells containing HCV replicon RNA caused a dose-dependent reduction in the level of viral proteins and RNA. In particular, protein-expression analyses revealed that shRNA452 at a MOI of 200 suppressed HCV core-protein levels by 75%. By contrast, the control baculovirus vectors (Ac-U6-terminator and Ac-control-shRNA) had no inhibitory effect on HCV replication. We also examined the suppression of HCV core protein by VSV-G-pseudotyped baculovirus-mediated shRNA452. HCV core-protein expression was more strongly inhibited by the VSV-G-pseudotyped baculovirus vector than by the wild-type baculovirus vector (Fig. 4B). Baculovirus vectors carrying shRNA thus appear to be able to inhibit HCV replication more effectively than simple siRNA.

In conclusion, we have demonstrated efficient inhibition of intracellular HCV replication by baculovirus-based shRNA-expressing vectors. The reduction in the amount of HCV mRNA is consistent with an shRNA-mediated degradation effect at the post-transcriptional level; this suggests that our RNAi approach might provide effective gene therapy for HCV infection, especially at the nuclear localization signal site (the shRNA452 target site) within the conserved core region. Furthermore, the VSV-G-modified baculovirus (Ac/VSV-G) transduced genes into NNC#2 cells more efficiently than wild-type baculovirus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.056.

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Heat-shock Protein 90 Is Essential for Stabilization of the Hepatitis C Virus Nonstructural Protein NS3*

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The hepatitis C virus (HCV) is a major cause of chronic liver disease. Here, we report a new and effective strategy for inhibiting HCV replication using 17-allylaminogeldanamycin (17-AAG), an inhibitor of heat-shock protein 90 (Hsp90). Hsp90 is a molecular chaperone with a key role in stabilizing the conformation of many oncogenic signaling proteins. We examined the inhibitory effects of 17-AAG on HCV replication in an HCV replicon cell culture system. In HCV replicon cells treated with 17-AAG, we found that HCV RNA replication was suppressed in a dose-dependent manner, and interestingly, the only HCV protein degraded in these cells was NS3 (nonstructural protein 3). Immunoprecipitation experiments showed that NS3 directly interacted with Hsp90, as did proteins expressed from ΔNS3 protease expression vectors. These results suggest that the suppression of HCV RNA replication is due to the destabilization of NS3 in disruption of the Hsp90 chaperone complex by 17-AAG.

Infection by the hepatitis C virus (HCV)[2] is a major public health problem, with 170 million chronically infected people worldwide (1, 2). The current treatment by combined interferon-ribavirin therapy fails to cure the infection in 30–50% of cases (3, 4), particularly those with HCV genotypes 1 and 2. Chronic infection with HCV results in liver cirrhosis and can lead to hepatocellular carcinoma (5, 6). Although an effective combined interferon-α-ribavirin therapy is available for about 50% of the patients with HCV, better therapies are needed, and preventative vaccines have not yet been developed.

HCV is a member of the *Flaviviridae* family and has a positive strand RNA genome (7, 8) that encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, E1 (envelope 1), E2, p7, NS2 (nonstructural protein 2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS2 and the amino terminus of NS3 comprise the NS2-3 protease responsible for cleavage between NS2 and NS3 (9, 11), whereas NS3 is a multifunctional protein consisting of an amino-terminal protease domain required for processing NS3 to NS5B (12, 13). NS4A is a cofactor that activates the NS3 protease function by forming a heterodimer (14–17), and the hydrophobic protein NS4B induces the formation of a cytoplasmic vesicular structure, designated the membranous web, which is likely to contain the replication complex of HCV (18, 19). NS5A is a phosphoprotein that appears to play an important role in viral replication (20–23), and NS5B is the RNA-dependent RNA polymerase of HCV (24, 25). The 3′-untranslated region consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region and is critical for HCV RNA replication and HCV infection (26–29).

Hsp90 (heat-shock protein 90) is a molecular chaperone that plays a key role in the conformational maturation of many cellular proteins. Hsp90 normally functions in association with other co-chaperone proteins, which together play an important role in folding newly synthesized proteins and stabilizing and refolding denatured proteins in cells subjected to stress (30–34). Its expression is induced by cellular stress and is also associated with many types of tumor. Hsp90 inhibitors are currently showing great promise as novel pharmacological agents for anticancer therapy.

Hsp90 inhibitors have two major modes of action as preferential clients for protein degradation or as Hsp70 inducers. The benzoquinone ansamycin antibiotic geldanamycin and its less toxic analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) directly bind to the ATP/ADP binding pocket of Hsp90 (34–36) and thus prevent ATP binding and the completion of client protein refolding. Recently, Waxman *et al.* (37) demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protease, using NS2/3 translated by rabbit reticulocyte lysate. Nakagawa *et al.* (38) also reported that inhibition of Hsp90 is highly effective in suppressing HCV genome replication. Hsp90 may directly or indirectly interact with any of the proteins NS3 through NS5B to regulate replication of the HCV replicon. More recently, Okamoto *et al.* (39) reported that Hsp90 could bind to FKBP8 (FK506-binding protein 8) and form a complex with NS5A. The interaction with FKBP8 has also been shown to be the mechanism by which Hsp90 regulates HCV RNA replication, a process in which Hsp90 clearly plays an important role.

In this study, we have demonstrated that NS3 also forms a complex with Hsp90, which is critical for HCV replication. On the basis of the findings that treating HCV replicon cells with

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[2] The abbreviations used are: HCV, hepatitis C virus; 17-AAG, 17-allylamino-17-demethoxygeldanamycin.