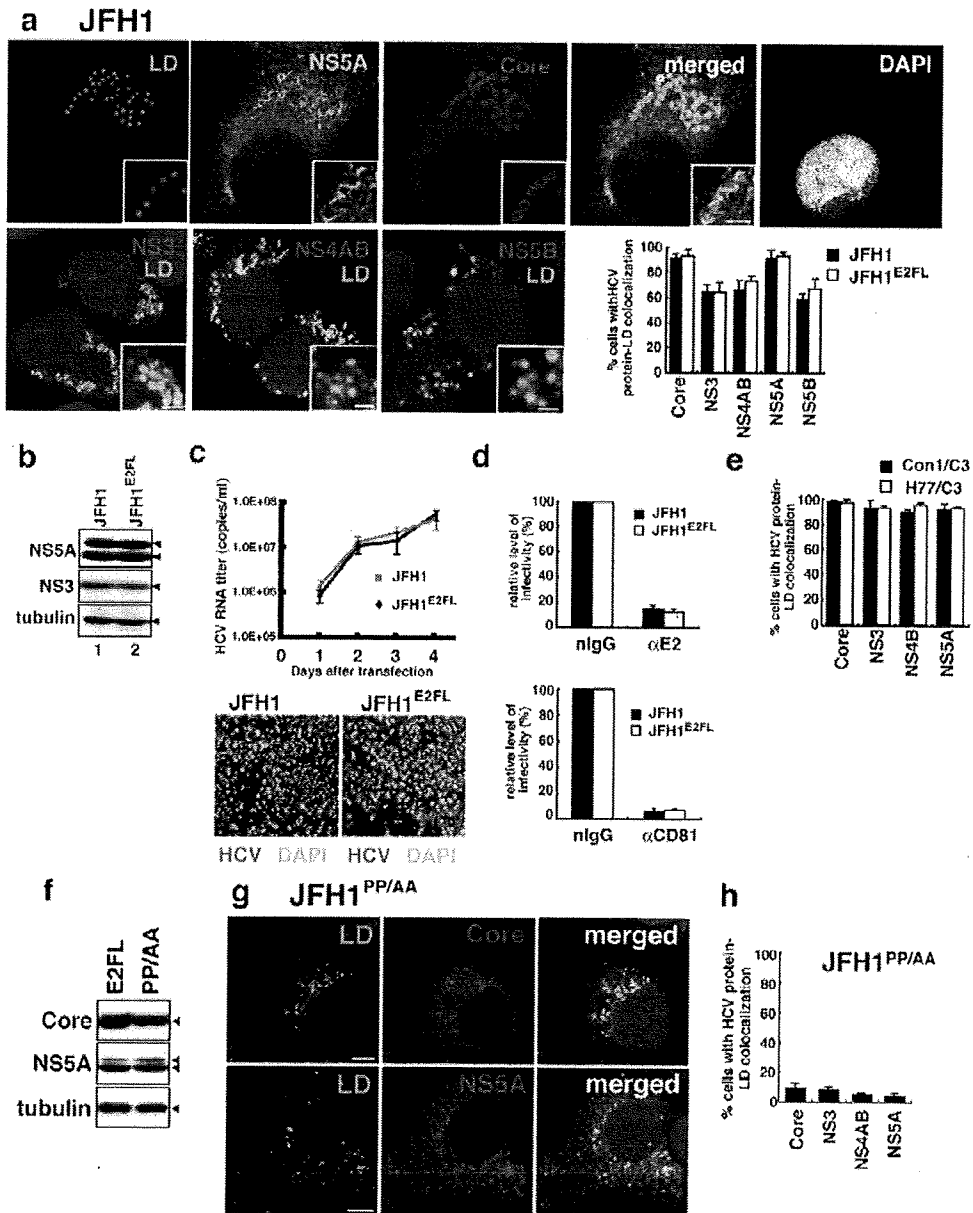


SUPPLEMENTARY INFORMATION



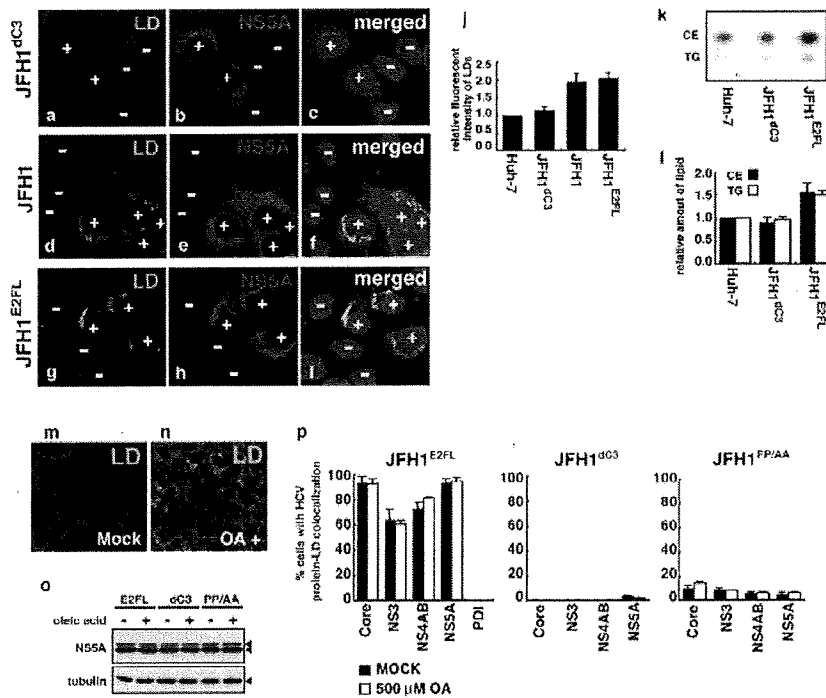
Supplementary Fig. S2 Kunitata Shimotohno
NCB-S11732B

Supplementary Fig. 2 Characterization of mutant JFH1s

(a) JFH1-replicating cells were stained with indicated antibodies, BODYPI 493/503 and

DAPI. Scale bars = 2 μm . Percentages of JFH1- and JFH1^{E2FL}-replicating cells with overlapping signals for LDs and HCV proteins ($n > 200$). (b) HCV proteins in JFH1 and JFH1^{E2FL} replicating cells were analyzed by western blotting with indicated antibodies. (c) Efficiency of virus production and infectivity of JFH1 and JFH1^{E2FL} viruses were analyzed as described in Fig. 4. ($n = 3$) (d) Inhibition of HCV infection by anti-E2 antibody and anti-CD81 antibody. JFH1 and JFH1^{E2FL} virus were pre-incubated with normal IgG (nIgG) or anti-E2 antibody for 1 hr at 4°C and were subsequently used to inoculate Huh-7.5 cells (upper panel). Huh-7.5 cells were pre-incubated with normal IgG (nIgG) or anti-CD81 antibody for 1 hr at 37°C before inoculation with JFH1 or JFH1^{E2FL} viruses (lower panel) ($n = 3$). No obvious difference with respect to the subcellular localization of viral proteins (a), efficiency of viral replication (b), virus production (c), and pathway of virus entry (d) were observed between JFH1^{E2FL} and JFH1 (e) Con1/C3 or H77/C3 replicating cells were stained for HCV antigens (Core or NS3 or NS4B or NS5A) and LDs. The graphs show the percentage of Con1/C3 (black) or H77/C3 (white) positive cells in which HCV protein signals overlapped with LD signals. The overlapping signals were detected by using the ImageJ RG2B software package ($n > 200$). (f) Whole-cell extracts of JFH1^{E2FL} (E2FL) and JFH1^{PP/AA} (PP/AA) replicon-bearing cells were analyzed by Western blot with indicated antibodies. Both Core^{Wt} and Core^{PP/AA} had an apparent molecular weight of about 21 kDa, indicating that Core^{PP/AA} was efficiently processed. The expression level of Core^{PP/AA}, however, was slightly lower than that of Core^{Wt}. (g) Analysis of the subcellular localization of Core and NS5A in cells bearing the JFH1^{PP/AA} mutant. Cells were labeled to detect LDs (green), Core (red), NS5A (red) and nuclei DAPI (blue). Scale bars = 10 μm . (h) The percentages of JFH1^{PP/AA} replicon-bearing cells positive for overlapping signals of LDs and HCV proteins are indicated ($n > 200$).

SUPPLEMENTARY INFORMATION

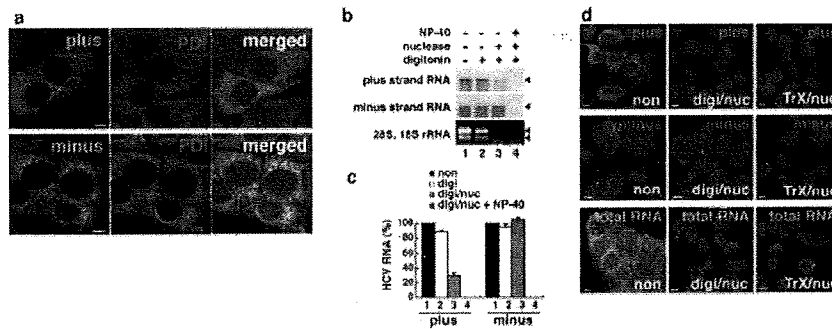


Supplementary Fig. S3 Kunitata Shimotohno
NCB-S11732B

Supplementary Fig. 3

Enhanced formation of LDs in JFH1- and JFH1^{E2FL}-replicating cells

Cells transfected with JFH1^{dc3} (a-c), JFH1 (d-f), and JFH1^{E2FL} RNA (g-i) were stained with BODIPY493/503 (green), DAPI (blue), and anti-NS5A antibody (red). + and - indicate HCV-positive and HCV-negative cells, respectively. Fluorescence intensities of LDs in Huh-7 cells, JFH1^{dc3}-, JFH1-, and JFH1^{E2FL}-replicating cells were measured by confocal microscopy. The results are represented as relative fluorescence intensity of LDs (j). (k and l) Lipid fraction extracted from Huh-7 cells, JFH1^{dc3}-, and JFH1^{E2FL}-replicating cells was analyzed by thin-layer chromatography. Each lane was loaded with lipid corresponding to an equal amount of protein. Cholesterol ester (CE) and triglyceride (TG) are indicated (k). The relative intensity of CE and TG in panel k is shown in l (n = 3). (m-p) JFH1^{E2FL} replicon-bearing cells were treated with 500 μM oleic acid for 24 hrs and were labeled with BODIPY493/503 (m and n). Cells transfected with JFH1^{E2FL} (E2FL), JFH1^{dc3} (dc3), or JFH1^{PP/AA} (PP/AA) RNA were treated with or without oleic acid. (o) The HCV protein level as represented by the level of NS5A was analyzed by western blot. (p) The percentages of cells positive for overlapping signals for LDs and the HCV proteins or PDI are indicated. The data was obtained in the presence (500 μM) or absence (MOCK) of oleic acid in the culture medium (n > 200).



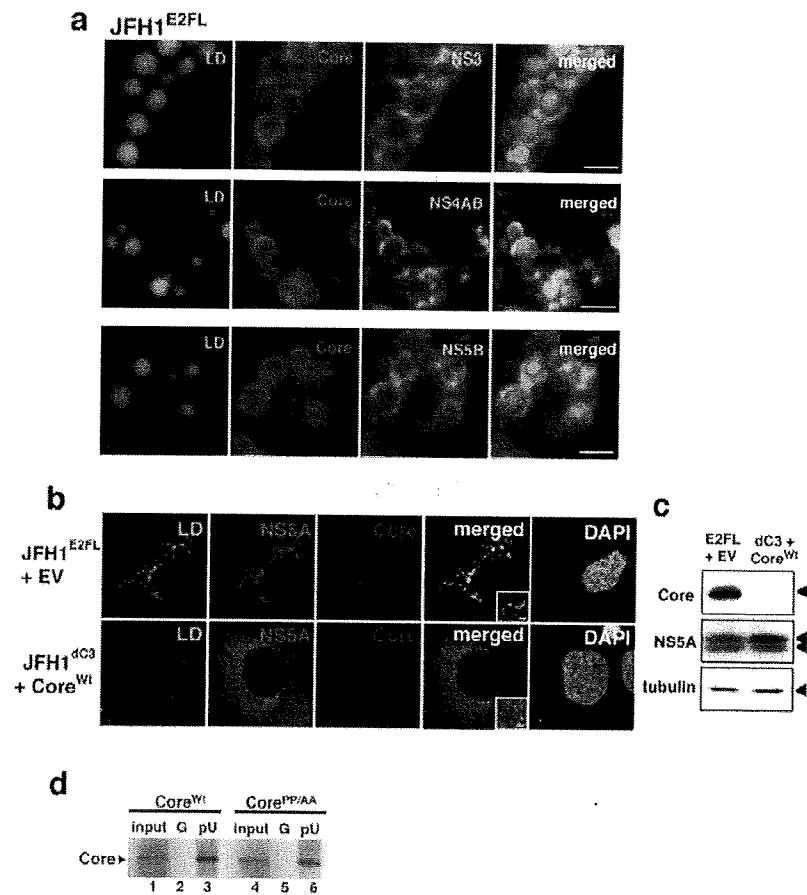
Supplementary Fig. S4 Kunitata Shimotohno
NCB-S11732B

Supplementary Fig. 4

Subcellular localizations of plus- and minus-strand HCV RNA

(a) JFH1^{E2FL} replicating cells were analyzed by *in situ* hybridization to detect plus- and minus-strand HCV RNA (green). The cells were also labeled with anti-PDI antibodies (red) and DAPI (blue). Scale bars = 10 μ m. (b, c) Relative amounts of plus- and minus-strand HCV RNAs. JFH1^{E2FL}-expressing cells were permeabilized with digitonin. The cells were treated with nuclease in the presence or absence of NP-40. Then, total RNA was analyzed by Northern blots with strand-specific HCV RNA probes. 28S and 18S ribosomal RNA was stained with ethidium bromide (b). The signals were quantified and plotted in c (n = 3). The amounts of plus- and minus-strand RNA were similar before and after digitonin treatment (lanes 1 and 2). The level of plus-strand RNA, however, was reduced by approximately 70% after nuclease treatment, whereas the level of minus-strand RNA remained constant (lanes 2 and 3). Nuclease treatment in the presence of NP-40 used to lyse the membranes caused both plus- and minus-strand HCV RNA to disappear (lane 4). This result suggests that ~30% and ~100% of plus- and minus-strand HCV RNA, respectively, are located in the replication complexes. (d) Localization of nuclease-resistant JFH1^{E2FL} RNA was analyzed by *in situ* hybridization. Digitonin-permeabilized cells were treated with nuclease in the presence (TrX/nuc) or absence (digi/nuc) of Triton X-100. Total RNA was visualized with SYTO RNaselect. "non" indicates cells without digitonin and nuclease treatment. Using the nuclease-resistant HCV RNA as a marker of replication complexes, we examined the localization of the replication complexes. Both plus- and minus-strand HCV RNA were detected in the perinuclear region even after the nuclease treatment. As expected, these RNAs were no longer detectable after nuclease treatment in the presence of Triton X-100. The intensity of the plus-strand RNA signal decreased after nuclease treatment (compare upper left and middle panels). However, the intensity of the minus-strand RNA signal remained unchanged after the treatment. These results correlated with the data obtained by Northern blotting analysis. For this reason, the percentages of cells with overlapping signals for LDs and plus- or minus-strand HCV RNA (Fig. 2c) were measured after lysis of cells with digitonin and nuclease treatment. Scale bars = 10 μ m.

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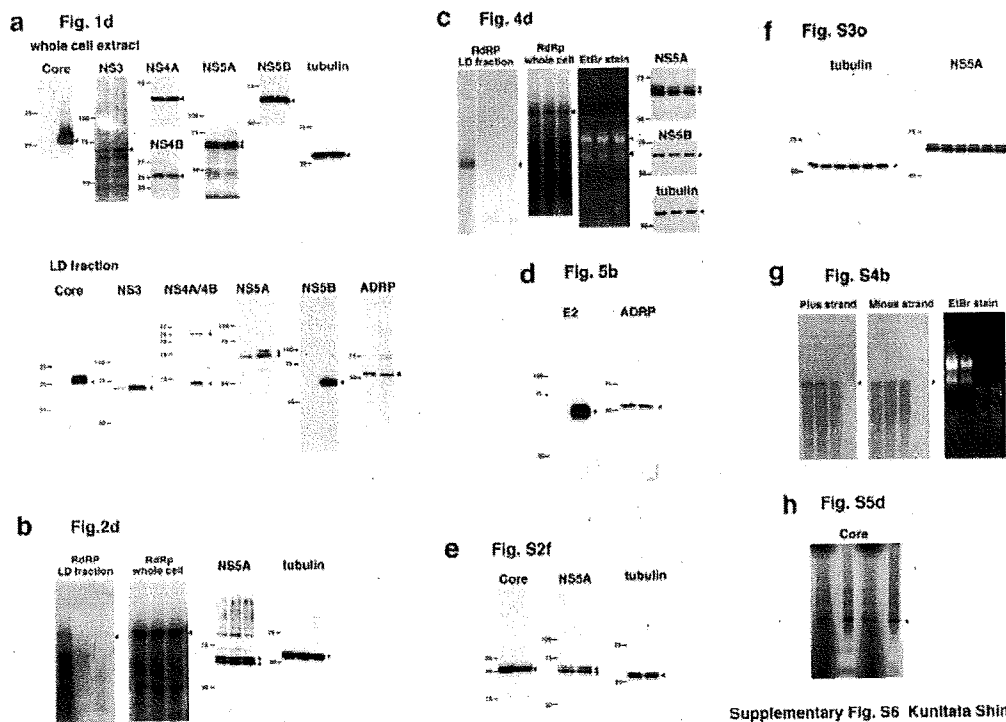


Supplementary Fig. S5 Kunitata Shimotohno
NCB-S11732B

Supplementary Fig. 5

Subcellular localization of HCV proteins in JFH1^{E2FL}-replicating cells and cells inoculated with rescued viruses, and RNA binding nature of Core.

(a) JFH1^{E2FL}-replicating cells were labeled to detect LDs (green), Core (red), NS3 (cyan), NS4AB (cyan), and NS5B (cyan). Scale bars = 2 μ m (b) The subcellular localizations of NS5A and Core in Huh-7.5 cells infected with viruses released from JFH1^{E2FL} replicon-bearing cells co-transfected with pcDNA3 (upper panels) and from JFH1^{dC3} replicon-bearing cells co-transfected with pcDNA3-Core^{WT} (lower panels). Cells were labeled with DAPI (white), BODIPY 493/503 (green), anti-Core (blue), and anti-NS5A (red) antibodies. The insets are high magnifications of the corresponding panel. (c) HCV proteins from these infected cells were analyzed by western blotting. (d) An RNA-protein binding precipitation assay was performed with *in vitro*-translated and ³⁵S-radiolabeled Core^{WT} (lane 1-3) and Core^{PP/AA} (lane 4-6). The resulting precipitates were analyzed by SDS-PAGE and detected by autoradiography. "G" and "pU" mark samples obtained using protein G Sepharose and poly-U Sepharose as the resin, respectively. "input" indicates 1/20 of the amount of translated product used in this assay.



Supplementary Fig. S6 Kunitata Shimotohno
NCB-S11732B

Supplementary Fig. 6

Full scan of key gel images depicted in the individual figures.

Full scans of (a) immunoblot detection of whole cell extract and LD fraction from JFH1^{E2FL}- and JFH1^{dC3}-replicating cells shown in Fig. 1d, (b) RNA synthesis assay and immunoblot detection of JFH1^{E2FL}-, JFH1^{dC3}-, and JFH1^{PP/AA}-replicating cells shown in Fig. 2d, (c) RNA synthesis assay and immunoblot detection of JFH1^{E2FL}-, JFH1^{AAA99}-, and JFH1^{AAA102}-replicating cells shown in Fig. 4d, (d) immunoblot detection of LD fraction from JFH1^{E2FL}- and JFH1^{dC3}-replicating cells shown in Fig. 5b, (e) immunoblot detection of JFH1^{E2FL}- and JFH1^{PP/AA}-replicating cells shown in Fig. S2f, (f) immunoblot detection of JFH1^{E2FL}-replicating cells shown in Fig. S3o, (g) Northern Blotting analysis of JFH1^{E2FL}-replicating cells shown in Fig. S4b, and (h) RNA-protein binding precipitation assay shown in Fig. S5d. Size of molecular weight markers (kDa) is indicated at the left side of western blot gel images. *In vitro* transcribed HCV RNA was used as a size marker for RNA gel electrophoresis (b, c, and g).

SUPPLEMENTARY INFORMATION

plasmid name	primer sequences (5' to 3')	template for PCR	restriction enzyme	original plasmid
pcDNA3-Core ²⁹¹	AGACCCAAGCTTCACCATGAGCACAAATCCTAAACC	pJFH1	HindIII	pcDNA3
	AATGGAATTCGAAGCAGAGACCGGAACGGTGATGC		EcoRI	
pcDNA3-TME2	AGACCCAAGCTTCACCATGAGCACAAATCCTAAACC	pJFH1	HindIII	pcDNA3
	AATGGAATTCGAAGCAGAGACCGGAACGGTGATGC		EcoRI	
ppDNA3-NS3	AGACCCAAGCTTCACCATGAGCACAAATCCTAAACC	pJFH1	HindIII	pcDNA3
	AATGGAATTCGAAGCAGAGACCGGAACGGTGATGC		EcoRI	
pcDNA3-NS4B	AGACCCAAGCTTCACCATGAGCACAAATCCTAAACC	pJFH1	HindIII	pcDNA3
	AATGGAATTCGAAGCAGAGACCGGAACGGTGATGC		EcoRI	
pcDNA3-NS5B	CTCGGATCCAGCATGTCATGTCATGTCGCTGGACC	pJFH1	BamHI	pcDNA3
	AATGGAATTCGAAGCAGAGACCGGAACGGTGATGC		EcoRI	
pcDNA3-Core ^{291,244}	CAATGGGTACATGCCCTGRTAGGGCGCGCGCTTARTGGGG	pJFH1 ^{291,244}	HindIII	pcDNA3
	CGCCACTAAGCGGGGGCCCTACGACGGCGATGTACCCGATG		EcoRI	
pJFH1 ²⁹¹	GATTACAAGGATGAGCAGBATAAGGGCGTGTTCAGCCATGGCCG	pJFH1	BstXI	pJFH1
	GTTATCGTCTGATCGTGTGTAATCAAGAGCCGCTCCAACGGTGG		NciI	
	GGGACATGATGATGAAGTGG			
	GTAATGTGAACACCGACCC			
pJFH1 ^{291,244}	GAAAAACCAAAGAAACACCAACTATGCAACAGGGAACTACG	pJFH1 ^{291,244}	EcoRI	pJFH1 ^{291,244}
	GTTGGTGTTCCTTTGGTTTTTG		BstXI	
	GGCACATGATGATGAAGTGG			
	GTAATGTGAACACCGACCC			
pJFH1 ^{291,244}	PATGGGTACATGCCCTGRTAGGGCGCGCGCTTARTGGGG	pJFH1 ^{291,244}	EcoRI	pJFH1 ^{291,244}
	CGCCACTAAGCGGGGGCCCTACGACGGCGATGTACCCGATG		NciI	
	GGCACATGATGATGAAGTGG			
	GTAATGTGAACACCGACCC			
pJFH1 ^{291,244} dBamHI	TAGTGGGTGGCATCCTGTGTGG	pJFH1 ^{291,244}	NsiI	pJFH1 ^{291,244}
	GGAGACAGGATGCCAGCCAGTA		RsrII	
	GTATTACGAATGAGGTGACC			
	GAACAATTTAGAGGTCAAGCC			
pJFH1 ^{291,244}	GGCCAGTCCGGCGGGCACCCCGCACG	pJFH1 ^{291,244}	BamHI	pJFH1 ^{291,244} dBamHI
	CGTCCCGGGTCCCGCCCGCACTGGCC		RsrII	
	TGGATGAACAGGCTTATTGC			
	GGTTGAAGCTCTACCTGATC			
pJFH1 ^{291,244}	CGCCGAAAAGCCGCGCGGAACCTACAAG	pJFH1 ^{291,244}	BamHI	pJFH1 ^{291,244} dBamHI
	GTTGTAGTTGGCGCGGGTTTCGGCCG		RsrII	
	TGUAIGAAACAGGCTTAAIGGC			
	GGTTGAAGCTCTACCTGATC			

Supplementary Table

A list of the plasmids used in this work. The sets of primers used to amplify the target genes, the template plasmids used in the PCRs, the restriction sites, and plasmids into which the amplified DNA fragments were inserted are shown.

Supplementary Materials and Methods

Cell culture

The human hepatoma cell lines Huh-7 and Huh-7.5¹ were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml nonessential amino acids (Invitrogen), and 100 µg/ml penicillin and streptomycin sulfate (Invitrogen). Huh-7.5 cells were obtained from Dr. C. Rice. (Rockefeller University, USA)

Plasmid construction

All plasmids were generated by insertion of PCR-amplified fragments into expression plasmids. The plasmids, the primer sequences, templates for the PCRs, and the restriction enzyme sites used to construct the plasmids are listed in Supplementary Table 1.

DNA and RNA transfection

Transfection of HCV RNA was performed as previously described². DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Northern blotting

Northern blot analysis was performed as described previously³ with strand specific RNA probes.

RT-PCR analysis

Quantitative real-time RT-PCR analysis of the HCV RNA titer was performed as described previously⁴.

ELISA for the detection of Core

Core in the culture medium was quantified with an ELISA according to the manufacturer's protocol (HCV antigen ELISA test, Ortho-Clinical Diagnostics).

Thin-layer chromatography

Lipid samples extracted from cells were dissolved in chloroform methanol and were

SUPPLEMENTARY INFORMATION

subjected to thin-layer chromatography with a high-performance TLC plate (Merck) by the two-step method⁵. The plate was charred by a copper acetate phosphoric acid solution at 180°C.

***In vitro* transcription**

RNA for transfection was synthesized using MEGAscript T7 (Ambion, TX, USA). Plasmids carrying the JFH1 RNA sequence were linearized with *Xba*I and used as templates for transcription. Probes for *in situ* hybridization were synthesized using MAXIscript Sp6 or T7 (Ambion) in the presence of the DIG RNA labeling mix (Roche). Probes for Northern blots were synthesized with MAXIscript Sp6 or T7 in the presence of 1.85 MBq of [α -³²P] UTP (Amersham Biosciences). For detection of plus-strand HCV RNA, minus-strand RNA probes were generated using pcDNA3-TME2 (*Hind*III for linearization), pcDNA3-NS3 (*Hind*III), and pcDNA3-NS5B (*Bam*HI) as templates for *in vitro* transcription. For detection of minus-strand HCV RNA, plus-strand RNA probes were generated using pcDNA3-TME2 (*Eco*RI), pcDNA3-NS3 (*Xba*I), and pcDNA3-NS5B (*Bam*HI) as templates. The RNA probes used for *in situ* hybridization were subjected to alkaline hydrolysis to generate fragments of ~170 nucleotides in length. Synthesized RNA probes were treated with DNase I (Ambion) and size fractionated using MicroSpin G-50 columns (Amersham Biosciences).

Sucrose density gradient centrifugation of culture medium

The 100-time concentrated medium from JFH1-bearing cells was loaded onto 20-50% [w/v] sucrose gradient containing 50 mM Hepes-KOH (pH 7.4), 100 mM NaCl and 1mM EDTA followed by centrifugation at 100,000 x g for 16 hrs using RPS40T rotor of HITACHI ultracentrifuge. The gradient was fractionated into 31 fractions. Buoyant density of each fraction was analyzed by Abe refractometer (ATAGO Inc., Japan). Each fraction was dialyzed against serum free DMEM and was used for the infection experiment as well as quantification of Core and HCV RNA titer as described above.

Infection experiments

Cells were cultured in DMEM containing 5% FBS. The medium was collected and mixed with a 0.01 volume of 1 M HEPES (pH 7.4). After filtering the sample through a 0.22- μ m filter (Millipore), the filtrate was concentrated by reducing the volume to

between 1/50 and 1/100 of the original volume with an Amicon Ultra-15 centrifugal filter with Ultracel-100 membrane (Millipore). Huh-7.5 cells seeded on a collagen-coated Labtech II 8-well chamber were incubated with 100 μ l of the concentrated medium for 120 min. Then, the cells were washed three times with DMEM. Twenty-four hours after the inoculation, the cells were labeled with serum from HCV-infected patients to determine the infectivity level.

***In situ* hybridization analysis**

Huh-7 cells transfected with JFH1 RNA were seeded on a collagen-coated Labtech II 8-well chamber (Nunc). Three days after seeding, the cells were washed twice with PBS and fixed with fixation solution for 15 min at room temperature. Then, the cells were permeabilized with 0.05% Triton X-100 in fixation solution for 15 min at room temperature. After washing the cells twice with cold DEPC-treated PBS, the cells were incubated in 95% formamide and 0.1x SSC (1x SSC: 150 mM NaCl and 15 mM sodium citrate) for 15 min at 65°C. After chilling the chamber on ice, the cells were incubated in 100 μ l of pre-hybridization solution for 60 min at room temperature. Pre-hybridization solution was composed of 50% formamide, 2x SSC, 1 μ g/ml of salmon sperm DNA (sonicated to 1-2 kb pieces, Roche), 1 μ g/ml of yeast tRNA (Roche), and 2 mM vanadyl ribonucleoside complex (NEB). Then, the cells were incubated in 100 μ l of hybridization solution (pre-hybridization solution containing 10% dextran sulfate and 100 to 500 ng/ml of the RNA probes) for 40 hrs at 42°C. After the hybridization, the slide glass in the chamber was transferred to a bucket filled with wash solution 1 (50% formamide and 2x SSC at pH 7.4) and washed three times for 20 min at 50°C with gentle agitation. Then, the slide was washed three more times in wash solution 2 (0.1x SSC at pH 7.4) for 20 min at 50°C with gentle agitation. The slide was incubated in blocking solution for 30 min at room temperature. To detect DIG-labeled probes, sheep anti-DIG antibodies (Roche) and Alexa 488 or Alexa 568 anti-sheep IgG antibodies (Invitrogen) were used as primary and secondary antibodies, respectively. When HCV RNA, Core, and NS5A were simultaneously labeled in the same sample, anti-DIG antibodies and the Alexa-conjugated antibodies were incubated with the samples separately to avoid cross-reaction of the Alexa 488 or Alexa 568 anti-sheep IgG antibodies with mouse and rabbit IgG. Briefly, the incubation with the anti-DIG antibodies and the Alexa 488 anti-sheep IgG antibodies was performed first. After

SUPPLEMENTARY INFORMATION

washing with PBS followed by a second fixation procedure, the cells were incubated with anti-Core and anti-NS5A antibodies followed by Alexa 568 anti-rabbit IgG and Alexa 647 anti-mouse IgG antibodies. For treatment with nuclease, digitonin-permeabilized cells were treated with 1 µg/ml of RNase A in the presence or absence of 0.05% Triton X-100 for 15 min at 37°C. After the treatment, RNase A was inactivated by incubation with 4% formaldehyde. Then, the cells were completely permeabilized with 0.05% Triton X-100 for 5 min at room temperature.

Statistical analysis of the recruitment of viral components to the LD

Only the cells that have any LDs surrounded by HCV proteins, PDI, or HCV RNA were counted as positive under immunofluorescence microscopy, and those adjacent to HCV signal were not included. The obtained cell number was divided by the total number of HCV replicating cells and is shown as “% cells with HCV protein-LD colocalization”. In case of the chimeras Con1/C3 and H77/C3 LD colocalization with HCV proteins was additionally analyzed by using the ImageJ RG2B software package (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2006.). Approximately 200 cells were examined for each antigen.

RNA-protein binding precipitation assay

In vitro translated [³⁵S]-labeled products (Core^{WT} and Core^{PP/AA}) were incubated with poly-U or protein G Sepharose resin in 50 mM HEPES (pH7.4), 100 mM NaCl, 0.1 % NP-40, and RNase inhibitor at 4°C for 2 hrs. After five washes, resin-bound radiolabeled proteins were analyzed by gel electrophoresis followed by autoradiography.

Supplementary References

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ERRATUM

In the letter by Miyanari *et al.* (*Nature Cell Biol.* 9, 1089–1097), Kunitada Shimotohno's affiliations were incorrectly listed. His affiliations should have been listed as 1, 2 and 6.

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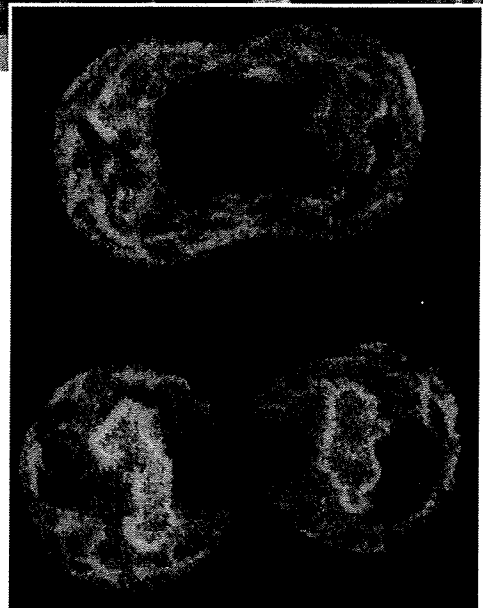
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Anti-hepatitis C Virus Activity of Tamoxifen Reveals the Functional Association of Estrogen Receptor with Viral RNA Polymerase NS5B*

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Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. HCV genome replication occurs in the replication complex (RC) around the endoplasmic reticulum membrane. However, the mechanisms regulating the HCV RC remain widely unknown. Here, we used a chemical biology approach to show that estrogen receptor (ESR) is functionally associated with HCV replication. We found that tamoxifen suppressed HCV genome replication. Part of ESR α resided on the endoplasmic reticulum membranes and interacted with HCV RNA polymerase NS5B. RNA interference-mediated knockdown of endogenous ESR α reduced HCV replication. Mechanistic analysis suggested that ESR α promoted NS5B association with the RC and that tamoxifen abrogated NS5B-RC association. Thus, ESR α regulated the presence of NS5B in the RC and stimulated HCV replication. Moreover, the ability of ESR α to regulate NS5B was suggested to serve as a potential novel target for anti-HCV therapeutics.

Estrogen receptor (ESR)² belongs to the steroid hormone receptor family of the nuclear receptor superfamily (1). ESR consists of two subtypes, ESR α and ESR β . As a primary physiological function, ESR is involved in the transcription for downstream genes in response to stimulation by the ligand, estradiol. In the normal state, ESR is mainly located in the cytoplasm and nucleus. Upon binding of the ligand, ESR dimerizes and translocates into the nucleus, where it binds to the ESR-responsive

elements (ERE) in the DNA promoter of downstream genes and drives transcription. In addition to this classical genomic action, a portion of ESR is located on the membrane, such as the plasma membrane, and involved in the nongenomic function of triggering signal transduction pathways, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C (2–4). Although the molecular basis of ESR membrane retention is not fully understood, one mechanism involves a membrane protein, caveolin (CAV); ESR α interacted with CAV, and this interaction facilitated ESR α localization to the membrane (5, 6). It was also reported that ESR α localizes to the lipid rafts on the plasma membrane (7). The lipid rafts are microdomains of the membrane that form platforms enriched in cholesterol and glycosphingolipids. However, the characteristics and relevance of membrane-associated ESR have not been fully disclosed. Here, we report the novel role of ESR α in the regulation of viral replication.

Hepatitis C virus (HCV), a causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, constitutes a serious health problem worldwide (8). HCV has a positive strand RNA genome that produces at least 10 functional viral proteins: core, envelope 1, envelope 2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS5B is an RNA-dependent RNA polymerase, which plays a central role in viral genome replication (11, 12). HCV genome replication can be evaluated using a HCV subgenomic replicon system, which Lohmann *et al.* (13) first established. In this system, cells carry an HCV subgenome RNA encoding NS3 to NS5B. Using this system, it has been proposed that HCV genome replication occurs in the replication complex (RC), which contains the viral genome RNA and HCV NS proteins. The RC forms on the surface of the intracellular membranes, including the endoplasmic reticulum (ER) membrane, and is surrounded by a membrane structure (14–17). It also has been reported that HCV genome replication associates with the lipid rafts on these intracellular membranes, such as the ER membrane (14, 18). These lipid rafts accumulate CAV2, and HCV proteins involved in viral genome replication cofractionate with CAV2 (18). However, it is largely unknown how the RC is formed and under what mechanism the HCV proteins participate in the RC.

A chemical biology approach is a useful method to analyze the molecular mechanism of viral life cycles as well as cellular physiological processes (19). We employed forward chemical genetics in which we analyzed HCV replication activity as a phenotypic indicator of a cell-based assay to screen chemical

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² The abbreviations used are: ESR, estrogen receptor; HCV, hepatitis C virus; RC, replication complex; ER, endoplasmic reticulum; TAM, tamoxifen; ERE, ESR-responsive element(s); CAV, caveolin; NS, nonstructural protein; MM, microsomal membrane; siRNA, small interfering RNA; si-ESR, small interfering ESR; GST, glutathione S-transferase; aa, amino acid(s); RT, reverse transcription; NS3, NS4A, NS4B, NS5A, and NS5B, nonstructural protein 3, 4A, 4B, 5A, and 5B, respectively.

Tamoxifen Suppresses HCV NS5B-Estrogen Receptor Association

compounds that inhibited HCV replication. Using this system, we previously identified an immunosuppressant, cyclosporin A, as an anti-HCV compound (20). We also reported that cyclophilin B regulated the RNA binding activity of NS5B (21). In the current study, this chemical screening approach linked ESR α to HCV replication. We showed that tamoxifen (TAM) suppressed HCV genome replication. Using TAM as a bioprobe, we found that ESR α interacted with NS5B and regulated the participation of NS5B in the RC.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Huh-7 and cured MH-14 cells (21) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acid (Invitrogen), and kanamycin (Meiji). MH-14 cells, carrying HCV subgenomic replicon (16), and LucNeo#2 cells, carrying luciferase-containing subgenomic replicon (22), were cultured in the same medium supplemented with 300 μ g/ml G418 (Invitrogen). Hus-E7/DN24 cells, a human hepatocyte cell line established by immortalization with HPV E6E7 and hTERT from human primary hepatocytes and introduction with a dominant negative form of interferon regulatory factor-7 (23), were cultured with Dulbecco's modified Eagle's medium with 20 mM Hepes (Invitrogen), 15 g/ml L-proline, 0.25 g/ml insulin (Sigma), 50 nM dexamethasone (Sigma), 44 mM NaHCO₃, 10 mM nicotinamide, 5 ng/ml epidermal growth factor, 0.1 mM Asc-2P, 100 IU/ml penicillin G (Invitrogen), 100 μ g/ml streptomycin (Invitrogen), 5% fetal bovine serum, 1% Dulbecco's modified Eagle's medium, and 2 UG/ml Fungizone (Invitrogen) (24). Plasmid transfection was performed with FuGENE 6 transfection reagent (Roche Applied Science), as described previously (25). RNA transfection was achieved using DMrie-C transfection reagent (Invitrogen), as described previously (21). siRNA was transfected by using siLentFect (Bio-Rad) (21).

Plasmid Construction—pCMV-FL-ESR α , encoding the whole open reading frame of ESR α fused with a FLAG tag, was generated by inserting the PCR product using 5'-GTTGAATTCATGACCATGACCCTCCAC-3' and 5'-GTTGATCTCGAGTCAGACTGTGGCAGGGAAAC-3' as primer set and human lymphocyte cDNA library (Clontech) as a template into the EcoRI-XhoI site of pCMV-FLAG vector (21). pCAG-HA-NS5B, encoding the NS5B protein fused with a hemagglutinin tag, was made by subcloning the PCR product with 5'-GTTGCGGCCGCTATGTCAATGTCCTACTCA-3' and 5'-GTTCTCGAGTCAACGGTTGGGGAGCAGGTA-3' as primers and pMH14 as a template into NotI-XhoI digestion of PCAG-HA vector (21). Expression plasmids for HCV NS3, NS4B, NS5A, and NS5B (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively) were described in Ref. 21. pGEX-ESR α A/B, C, D, and E/F, expressing the fusion protein of the domain A/B, C, D, and E/F of ESR α with GST, were prepared by the insertion of the PCR product with pCMV-FL-ESR α as a template and appropriate primers into the EcoRI-XhoI site of pGEX-6P1 vector (Clontech). The expression plasmids for the point mutants of ESR α , ESR α (L540Q), ESR α (255M), and ESR α (258M), of which Leu at aa 540, IRK at aa 255–257, and DRR at aa 258–260 were replaced by Gln, TGT, and ANT, respec-

tively, was generated by oligonucleotide-directed mutagenesis. pCMV-FL-CAV2, encoding FLAG-tagged CAV2, was prepared by inserting the PCR product amplified with 5'-GTTGTGCGACTATGGGGCTGGAGAC-3' and 5'-GTTAAGCTTTCAATCTGGCTC-3' as primers and human liver cDNA library (Clontech) as a template into the Sall-HindIII site of pCMV-FLAG vector (21). The mammalian expression vector for the C domain of ESR α was generated by replacing the EcoRI-XhoI digestion of pCMV-FLAG vector (21) by that of pGEX-ESR α C. pLMH14 was described previously (26). pGL3-EREX3-TATA-Luc, pcDNA3-ER α , pcDNA3-hER β were kindly provided by Dr. Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo). JFH1 expression plasmid was provided by Dr. Wakita (National Institute of Infectious Diseases).

Luciferase Assay—A luciferase assay monitoring HCV replication activity was performed as described previously (22, 26). In Fig. 1, A and F, we used LucNeo#2 cells, stably carrying luciferase-containing subgenomic replicon (22). In Figs. 2 (D and E), 4C, and 6A, we transiently transduced luciferase-containing replicon LMH14 RNA together with each expression plasmid into cured MH-14 cells (26). A luciferase assay detecting the transcriptional activity driven from the ERE was performed as described previously (25).

Real Time RT-PCR Analysis—Real time RT-PCR analysis was performed as previously described (20).

Immunoblot Analysis—Immunoblot analysis was performed as previously described (25). The antibodies used in this study are anti-NS5A (kindly provided by Dr. Takamizawa (Osaka University)), anti-NS5B (anti-NS5B#14; a generous gift from Dr. Kohara (Tokyo Metropolitan Institute of Medical Science)), anti-NS5B (NS5B#6; a kind gift from Dr. Fukuya (Osaka University)), anti-tubulin (Oncogene), anti-FLAG (Sigma), anti- κ B α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-calnexin (StressGen), and anti-caveolin-2 antibodies (BD Biosciences Pharmingen).

Indirect Immunofluorescence Analysis—Indirect immunofluorescence analysis was performed as described previously (25). The antibodies used were anti-NS5A and anti-protein-disulfide isomerase antibodies (StressGen).

siRNA—siRNA duplexes (5'-GUGUGCAAUGACUAUGCUUCA-3' for si-ESR α and 5'-CGCAUCGGGAUAUCACUAUGG-3' for si-ESR β) were synthesized (Proligo). A randomized siRNA, si-control, was purchased from Dharmacon (nonspecific control duplex IX).

Enzyme-linked Immunosorbent Assay—HCV core was quantified in the culture medium of the cells transfected with JFH1 RNA (29) with an enzyme-linked immunosorbent assay according to the manufacturer's protocol (HCV antigen enzyme-linked immunosorbent assay test; Ortho-Clinical Diagnostics).

RT-PCR Analysis—RT-PCR analysis was performed as described (20) by using the following primer sets: 5'-CCTACTA-CCTGGAGAACG-3' and 5'-GCTGGACACATATAGTCG-3' for the detection of ESR α and 5'-AGCCATGACATTCTAT-AGC-3' and 5'-CCACTTCGTAACACTTCC-3' for ESR β .

GST Pull-down Assay—The GST pull-down assay was conducted as described previously (25).

Immunoprecipitation Analysis—Immunoprecipitation analysis was performed as described previously (25). The antibodies

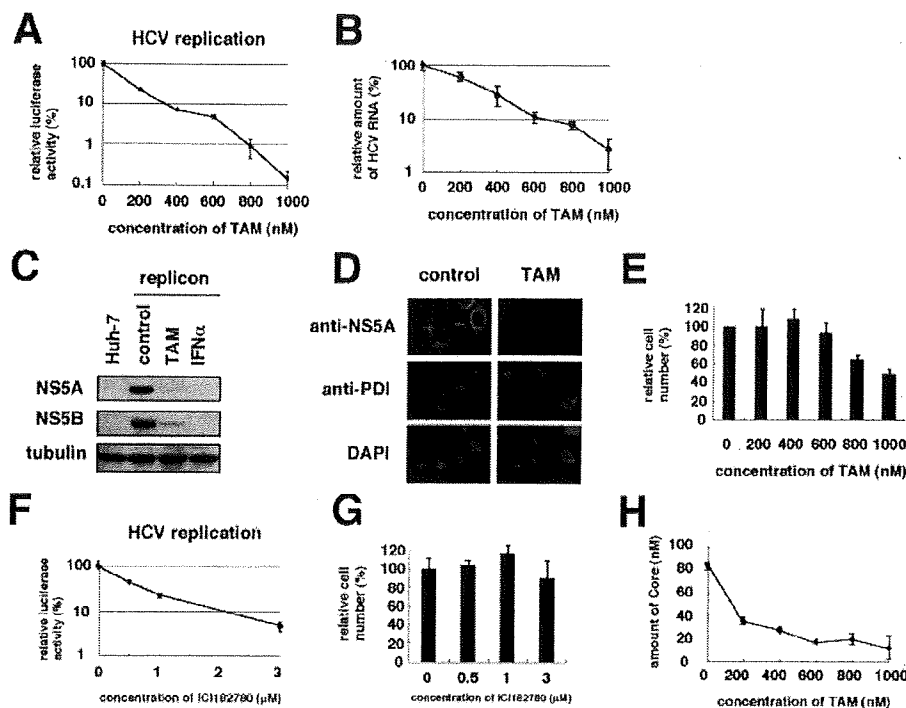


FIGURE 1. TAM suppressed the replication of the HCV genome. *A*, Luciferase activities were measured using the LucNeo#2 cells, which carried a luciferase-containing replicon RNA, upon treatment with TAM at the indicated doses for 7 days. Relative luciferase activities are plotted against the concentrations 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 ESR α 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 μ M PD98059 to stimulate HCV translation (27) (scheme in Fig. 6B). 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. 1C) and indirect immunofluorescence analyses (Fig. 1D), also drastically decreased by treatment with TAM. A high concentration of TAM decreased cell proliferation (Fig. 1E). 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. 1H) (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 ESR α and ESR β (Fig. 2A) reduced HCV RNA in replicon-containing cells to ~ 20 –40% and 60–70%, respectively (Fig. 2B). Transient transfection with ESR α and ESR β expression plasmids, which activated ERE-driven transcription 4–5-fold (Fig. 2C), showed that ectopically expressed ESR α augmented HCV replication activity in a dose-dependent manner, whereas ESR β did not (Fig. 2D). ESR α -induced augmentation of the replication was reversed upon TAM treatment (Fig. 2D). These results suggested a significant role of ESR, especially ESR α , in HCV

Tamoxifen Suppresses HCV NS5B-Estrogen Receptor Association

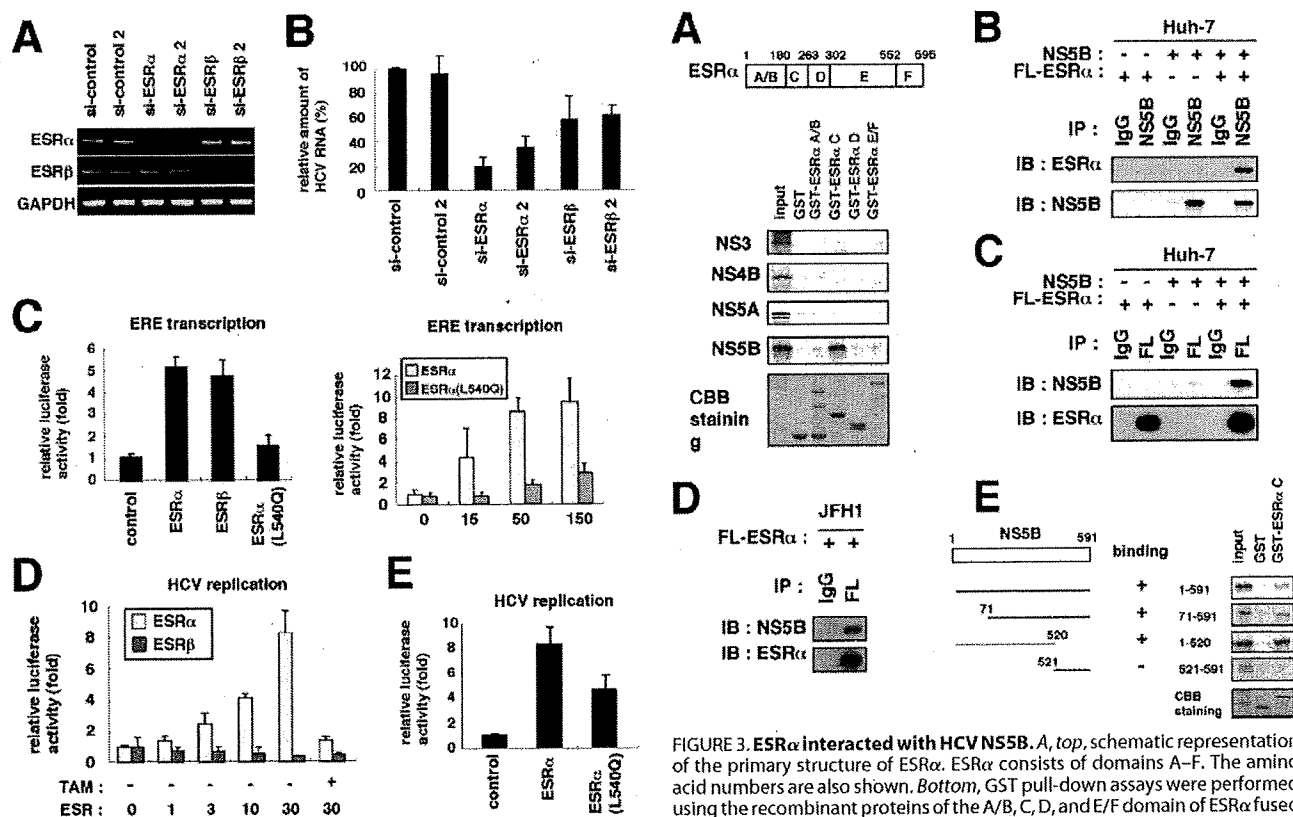


FIGURE 2. ESR was involved in HCV genome replication. *A*, specific knockdown of endogenous ESR α and ESR β . RT-PCR analysis was performed to detect the expression of ESR α , ESR β , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control in the cells transfected with siRNA recognizing ESR α (si-ESR α , si-ESR α 2), ESR β (si-ESR β , si-ESR β 2), 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-ESR α , si-ESR α 2, si-ESR β , and si-ESR β 2 for 5 days. *C*, the ERE-mediated transcriptional activities were measured by a luciferase assay using the lysates from the cells transfected with pGL3-ERE3-TATA-Luc reporter plasmid together with pcDNA3-ER α (ESR α), pcDNA3-hER β (ESR β), pcDNA-ESR α (L540Q), or the empty vector (control) (*left*) or varying amounts (ng) of pcDNA3-ER α (ESR α) or pcDNA-ESR α (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 ESR α or ESR β (*D*) or 30 ng of ESR α , ER α (L540Q), or the empty vector (control) (*E*) together with 0.125 μ g of LMH14 RNA without or with 1 μ M TAM for 4 days.

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

ESR α 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 ESR α and HCV proteins expressed in the HCV subgenomic replicon showed that the C domain of ESR α coprecipitated with NS5B but not NS3, NS4B, and NS5A (Fig. 3*A*). Other ESR α domains, A/B, D, and E/F, did not bind to any HCV proteins. A coimmunoprecipitation assay also indicated the presence of ESR α in the

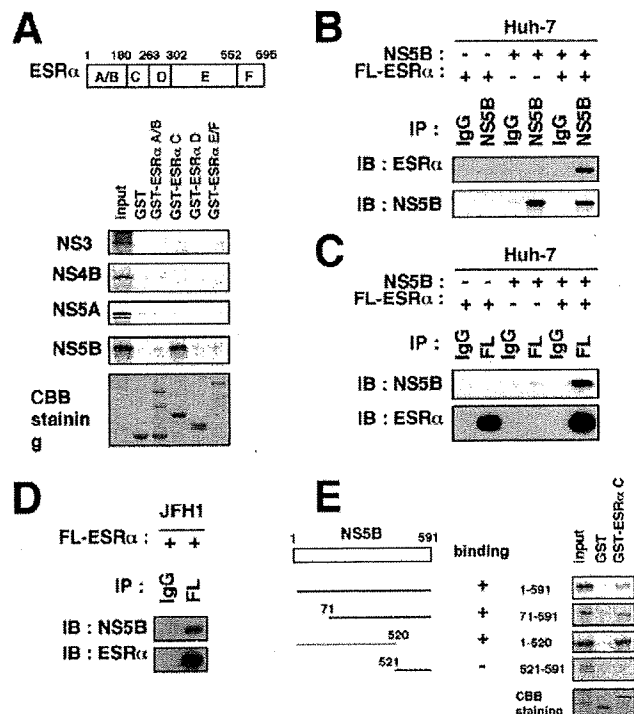


FIGURE 3. ESR α interacted with HCV NS5B. *A*, top, schematic representation of the primary structure of ESR α . ESR α consists of domains A–F. The amino acid numbers are also shown. *Bottom*, GST pull-down assays were performed using the recombinant proteins of the A/B, C, D, and E/F domain of ESR α 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 ESR α 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 ESR α 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 ESR α 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.

immunoprecipitate by anti-NS5B antibody (Fig. 3, *B* and *D*), and *vice versa* (Fig. 3*C*). Thus, ESR α 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 ESR α (Fig. 3*E*). This binding profile is different from that between cyclophilin B and NS5B, which we previously reported (21).

The ESR α -NS5B Interaction Was Important for the Regulation of HCV Genome Replication—To examine whether the interaction between ESR α and NS5B was essential for the ESR α -mediated regulation of HCV replication or not, we searched for a point mutant of ESR α that could not bind to NS5B by alanine-scanning mutation analysis. ESR α mutants, ESR α (255M) and ESR α (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 ESR α (255M) and ESR α (258M) caused only weak activations of HCV replication, compared with wild type ESR α (Fig. 4*C*). The data suggest that the interaction of ESR α with NS5B is

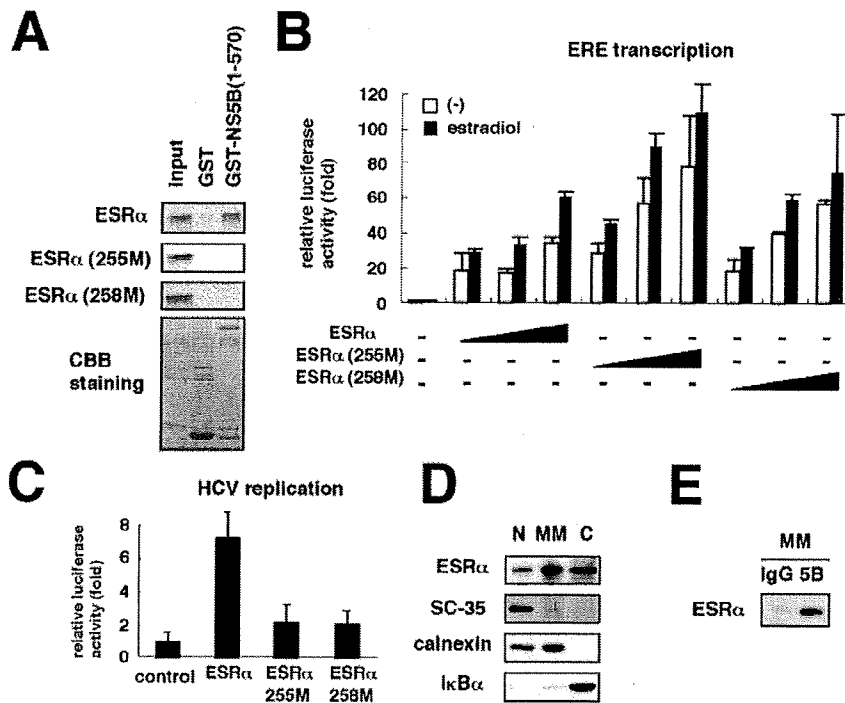


FIGURE 4. The interaction of NS5B mediated the regulation of HCV genome replication by ESR α . A, GST pull-down assays were performed as described in Fig. 3A using the wild type ESR α or point mutant of ESR α , ESR α (255M), and ESR α (258M). B, the mutation within ESR α (255M) and ESR α (258M) did not reduce the activation capacity of ERE-mediated transcription. Huh-7 cells were transfected with the expression plasmids for ESR α , ESR α (255M), or ESR α (258M) at doses of 10, 30, and 100 ng each together with pGL3-ERE3-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. 2D in the cells upon transfection with the expression plasmids for wild type ESR α , ESR α (255M), or ESR α (258M). D, the cells were fractionated into the nucleus (N), MM, and cytoplasm (C). Each fraction was detected for FLAG-tagged ESR α , SC-35, calnexin, and I κ B α , 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 ESR α .

critical for ESR α -mediated regulation of HCV genome replication.

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

ESR α 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. 5A).

In the experiment investigating the role of ESR α in NS5B-CAV2 association, the coprecipitation of NS5B with CAV2 was decreased upon the knocking down of ESR α (Fig. 5B). Treatment with TAM abrogated the association of NS5B with CAV2 (Fig. 5C), 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, ESR α was suggested to promote the association between NS5B and CAV2. Since a part of CAV2 resided on the lipid raft on the ER (18), ESR α -mediated binding between NS5B and CAV2 was possible to affect the localization of NS5B to the HCV RC. To see the consequential relevance of ESR α 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 I κ B α and calnexin; a cytosolic protein I κ B α was washed out following the treatment with digitonin (Fig. 5D, lanes 1 and 2), and ER protein calnexin, which did not accumulate in the RC, was digested by treatment with digitonin/protease (Fig. 5D, lanes 2–4). An ER lipid raft component, CAV2, was still detected under the digitonin/protease treatment (the RC-containing fraction) (Fig. 5D, lanes 3 and 4). Under this condition, a part of NS5B was detected in the digitonin/protease-resistant fraction, as described previously (16) (Fig. 5D, lanes 3 and 4). However, NS5B in this fraction was decreased upon treatment with TAM (Fig. 5D, lanes 3, 4, 7, and 8). On the other hand, the amount of NS5A was not significantly changed by TAM treatment. Knocking down of ESR α also disrupted the association of NS5B with the RC-containing fraction (Fig. 5E). From the above results, it was suggested that ESR α promoted the participation of NS5B in the RC (also see “Discussion”).

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

likely to function as a bridging factor that connects NS5B to CAV2, although we cannot fully neglect the possibility that ESR α 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 ESR α may recruit NS5B to the lipid raft and the HCV RC. In fact, ESR α promoted the participation of NS5B in the HCV RC. Thus, ESR α is suggested to escort NS5B to the HCV RC, although it is also possible that ESR α augments the number of the RC itself. However, ESR α at least augments the amount of NS5B involved in HCV replication machinery to stimulate the replication. It was reported that the membrane-associated ESR α 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 ESR α to form the RC for their efficient replication. Although the mechanisms of the nuclear receptor function of ESR α have been extensively elucidated, the functions of membrane-associated ESR α have not been widely characterized so far. This study suggested a novel physiological relevance of membrane-associated ESR α 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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