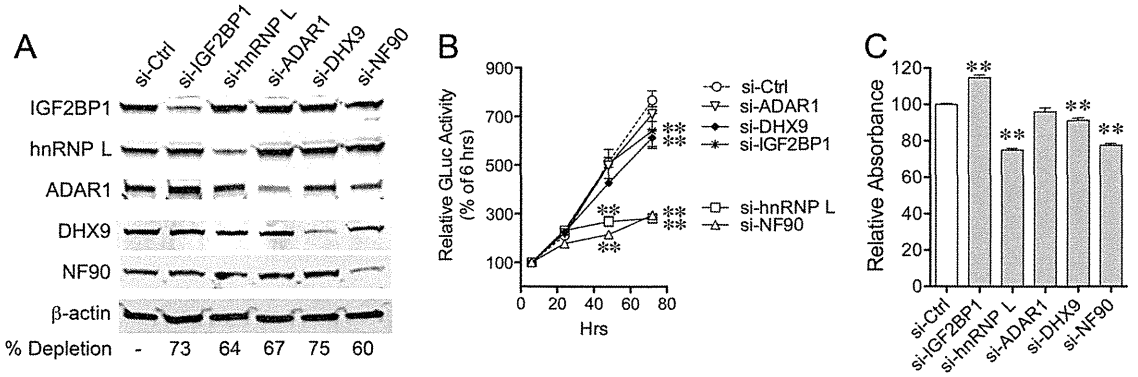

FIG 2 Depletion of hnRNP L or NF90 impairs HCV replication. Huh-7.5 cells were transfected with siRNAs targeting the indicated proteins or scrambled si-Ctrl and then 48 h later retransfected with H77S.3/GLuc2A RNA. (A) Immunoblots of IGF2BP1, hnRNP L, ADAR1, DHX9, and NF90 72 h after siRNA transfection. β-Actin was included as a loading control. The percent depletion of each protein is shown. (B) Relative GLuc activity in supernatant fluids from Huh-7.5 cells transfected with HCV RNA and the indicated siRNAs. The results shown represent the means of three replicate experiments ± the standard deviations. Values at 6 h were arbitrarily set to 100. **, $P < 0.01$ (compared to si-Ctrl by two-way analysis of variance [ANOVA]). (C) WST-1 assay measurement of proliferation of cells transfected with indicated siRNAs at 72 h after HCV RNA transfection. Values for control siRNA were arbitrarily set to 100. **, $P < 0.01$ (compared to si-Ctrl by two-way ANOVA with Bonferroni's multiple-comparison test.)

in cell proliferation (Fig. 3C). We confirmed these results by infecting Huh-7.5 cells with HJ3-5 virus, an HCV genotype 1a/2a chimera that efficiently replicates and produces infectious particles in cell culture. Knockdown of hnRNP L dramatically diminished HJ3-5 replication, as measured by expression of HCV core and NS5A proteins; knockdown of NF90 also modestly reduced the abundance of core and NS5A proteins (Fig. 3D). Consistently, hnRNP L and NF90 knockdown resulted in 3- and 1.5-fold reductions in infectious virus yield after 72 h compared to cells transfected with a nontargeting control siRNA (Fig. 3E). The above-described experiments used siRNA pools targeting hnRNP L and NF90. To assess the possibility that inhibition of replication resulted from off-target effects of these siRNAs, we compared the knockdown efficiency of individual siRNAs with their impact on viral replication. We observed substantial variation in the capacity of individual siRNAs to deplete hnRNP L or NF90 (Fig. 3F), but with both proteins the degree of depletion strongly correlated with the magnitude of the reduction in HCV replication, as measured by GLuc assay (Fig. 3G). Thus, the reduction of HCV replication was likely caused by specific depletion of hnRNP L and NF90 and not spurious off-target effects of the siRNA. Collectively, these data strongly suggest a role for hnRNP L and NF90 in efficient HCV genome amplification.

To examine whether hnRNP L and NF90 are required for HCV IRES-mediated translation, we transfected cells with an HCV minigenome RNA comprised of the HCV 5′UTR and 3′UTR flanking the GLuc sequence as reporter (Fig. 4A). This RNA was transfected into Huh-7.5 cells depleted of hnRNP L and NF90, and secreted GLuc activity was measured over the ensuring 24 h. Depletion of hnRNP L and NF90 resulted in little difference in GLuc activity (Fig. 4B) or GLuc mRNA abundance at 24 h (Fig. 4C). These data suggest that hnRNP L and NF90 are not essential for HCV IRES-mediated translation and that they do not significantly influence the stability of RNAs containing the HCV 5′UTR. Since hnRNP L binds to the 5′ end of HCV RNA in competition with miR-122 (Fig. 1F), while NF90 binds nearby, we also tested whether hnRNP L and NF90 depletion would affect the capacity of miR-122 to promote HCV replication. We transfected H77S.3/GLuc2A RNA into control, hnRNP L- or NF90-depleted cells, together with miR-122 or miR-124. Consistent with previous reports (9, 10), miR-122 supplementation resulted in an ~2-fold increase in GLuc activity compared to a control miRNA, miR-124 (Fig. 4D). The fold increase in GLuc activity caused by miR-122 supplementation (2.06) was unchanged in hnRNP L- and NF90-depleted cells (1.95 to 2.06, Fig. 4D), indicating that significant reductions in hnRNP L and NF90 abundance do not influence miR-122 enhancement of HCV replication.

hnRNP L and NF90 have been reported to be nuclear proteins (29–31). We confirmed this by laser-scanning confocal fluorescence microscopy (data not shown). However, since the HCV life cycle is restricted to the cytoplasm, we further analyzed the distribution of hnRNP L and NF90 in Huh-7.5 cells by cytoplasmic-nuclear fractionation. As shown in Fig. 5A, hnRNP L was largely localized to the nuclear fraction, and yet a small proportion of it was present in cytoplasm. Approximately half of the NF90 abundance localized to the cytoplasm, while NF110, an isoform of NF90, was predominantly detected in the nuclear fraction. NF90 and NF110 share antigenic specificity, and this likely explains the discrepant microscopy findings. HCV infection did not alter the intracellular distribution of hnRNP L or NF90 (Fig. 5A, right).

To determine whether hnRNP L and NF90 bind to HCV RNA in infected cells, as they do *in vitro* (Fig. 1), we immunoprecipitated hnRNP L and NF90 from HJ3-5 virus-infected Huh-7.5 cells and examined the precipitate for the presence of viral RNA. hnRNP L and NF90 were efficiently pulled down by specific antibodies compared to an isotype control IgG (Fig. 5B). RNA coprecipitating with hnRNP L and NF90 was purified and HCV RNA amplified and detected by RT-PCR. As shown in Fig. 5B, HCV RNA coprecipitated with both hnRNP L and NF90, indicating that hnRNP L and NF90 associate with HCV RNA within infected cells. In contrast, antibody to Dcp1a, an mRNA-decapping protein that does not colocalize with HCV RNA in cells (10), did not pull down HCV RNA. hnRNP L and NF90 were also found to coprecipitate with NS5A in HCV-infected cells by immunoblot assay (Fig. 5C). This association was RNA dependent since RNase treatment completely abolished the interaction (Fig. 5C). To determine whether the RNA mediating the interaction between NS5A and hnRNP L or NF90 is HCV specific, we repeated this

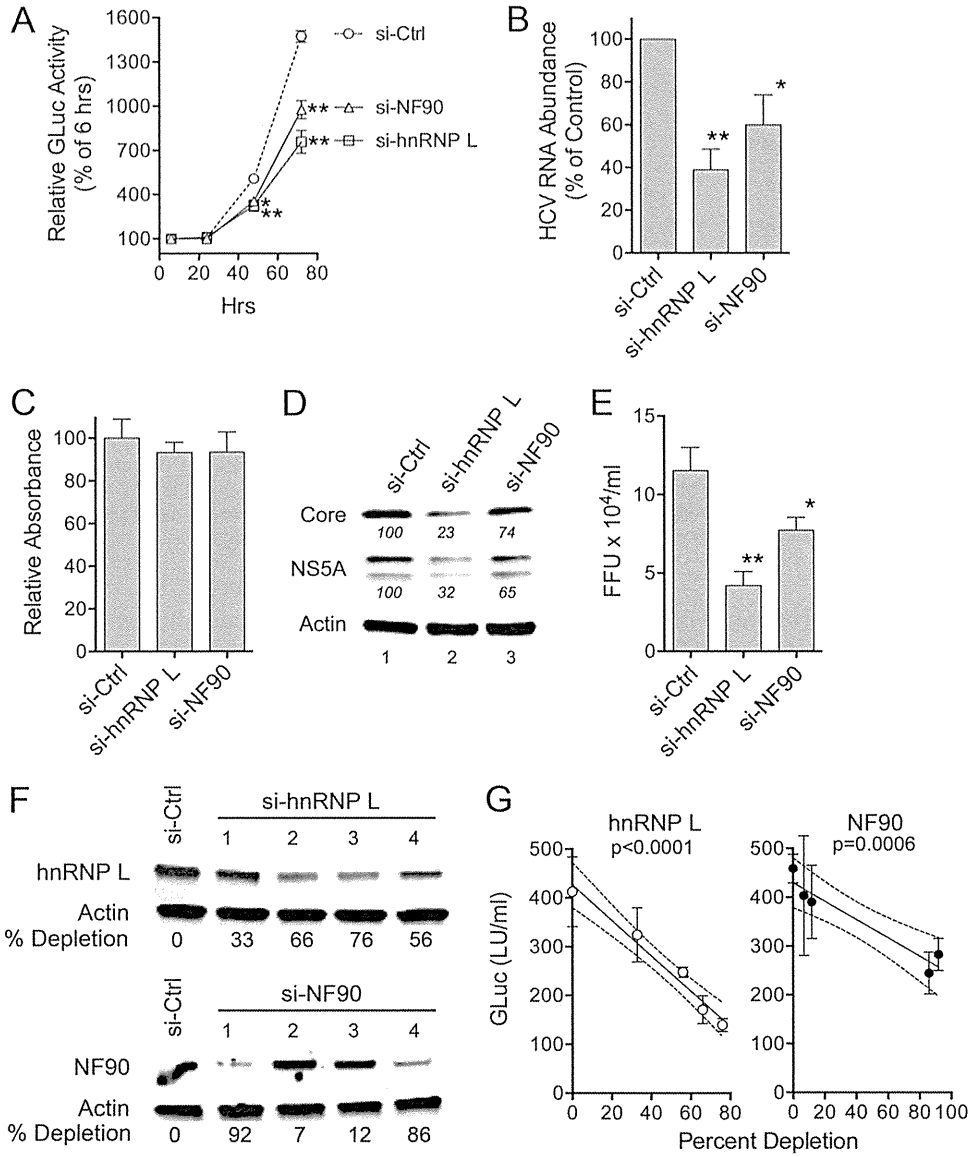

FIG 3 hnRNP L and NF90 are required for efficient HCV replication. (A) Huh-7.5 cells were maintained in growth media containing 2% serum, and transfected with siRNAs for hnRNP L, NF90, or scrambled si-Ctrl and then 48 h later retransfected with H77S.3/GLuc2A RNA. Supernatant fluids were removed at intervals and assayed for GLuc activity. Values at 6 h were arbitrarily set to 100. The results shown represent the means of three replicate experiments ± the standard errors of the mean. *, $P < 0.05$; **, $P < 0.01$ (compared to si-Ctrl by two-way ANOVA). (B) HCV RNA was quantified relative to β-actin mRNA by real-time RT-PCR 72 h after HCV RNA transfection. *, $P < 0.05$; **, $P < 0.01$ (compared to si-Ctrl by one-way ANOVA with Bonferroni's correction for multiple comparisons). (C) WST-1 assay for measurement of proliferation of cells at 72 h after HCV RNA transfection. Values for control siRNA were arbitrarily set to 100. Values do not differ significantly from si-CTRL (one-way ANOVA with Bonferroni's post test). (D) Immunoblots of HCV core and NS5A proteins at 72 h postinfection, with β-actin as a loading control. Huh-7.5 cells were maintained in growth medium containing 2% serum and transfected with siRNAs for hnRNP L, NF90, or scrambled si-Ctrl and then 48 h later infected with HJ3-5 virus. Italicized labels show quantification of core and NS5A proteins relative to β-actin, with values for si-Ctrl arbitrarily set to 100. (E) The infectious virus titers of supernatant fluids from panel D were determined by a fluorescent focus formation assay. The results shown represent the means of three replicate experiments ± the standard deviations. *, $P < 0.05$; **, $P < 0.01$ (compared to si-Ctrl by one-way ANOVA with Bonferroni's post test). (F) Individual siRNAs targeting hnRNP L or NF90 (four each) were transfected into cells, and hnRNP L and NF90 protein abundance was determined by immunoblotting 96 h later. β-Actin was included as a loading control. hnRNP L and NF90 levels were normalized to β-actin, and the percent depletion compared to si-Ctrl was calculated for each siRNA. (G) H77S.3/GLuc2A RNA was transfected into the hnRNP L- and NF90-depleted cells shown in panel F, and GLuc activity secreted from the cells between 48 and 72 h was assessed. The results shown represent mean GLuc ± the standard deviations in three replicate cultures, plotted against the percent depletion of target protein. Dashed lines represent the outer limits of the 95% confidence-interval band for a best-fit plot. The *P* values indicate the likelihood that the slope of this line diverges from "0". Inhibition of HCV replication was highly correlated with the degree of depletion of hnRNP L and NF90.

assay using U2OS cells that conditionally express only the NS5A protein (Fig. 5D). Little interaction was evident between NS5A and hnRNP L or NF90 under these conditions (Fig. 5E), suggesting that the coprecipitation of hnRNP L and NF90 with NS5A in infected cells may be largely mediated by HCV RNA. We found no evidence that either protein directly interacts with NS5A.

Previous studies indicate that HCV RNA replication complexes are localized to detergent-resistant intracellular mem-

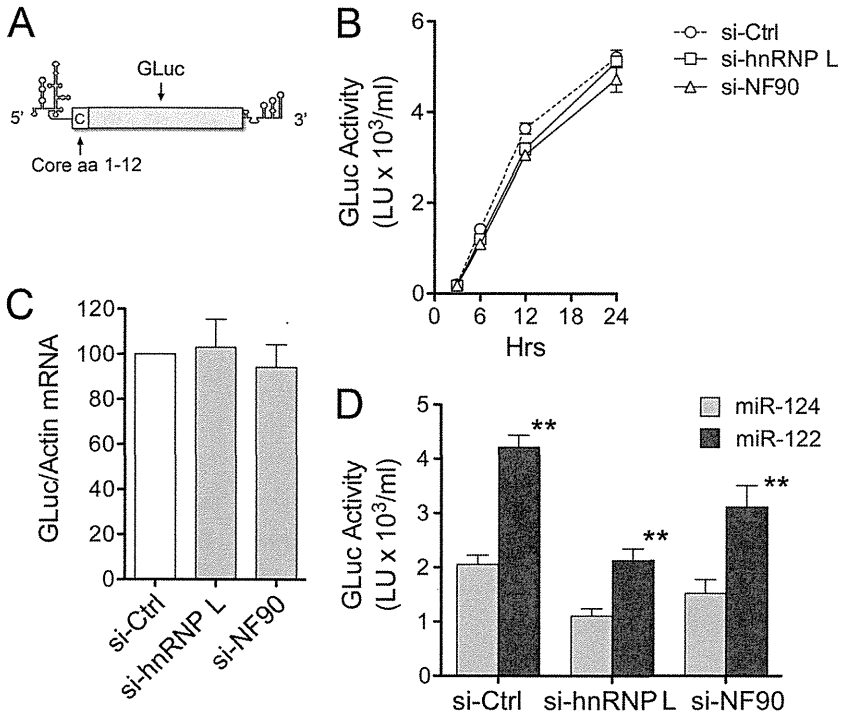

FIG 4 hnRNP L and NF90 depletion do not affect HCV IRES translation or miR-122 enhancement of HCV replication. (A) Diagram of the HCV minigenome RNA comprising the HCV 5′UTR followed by N-terminal core protein sequence fused to GLuc and the HCV 3′ UTR. (B) Huh-7.5 cells were transfected with siRNAs for hnRNP L, NF90 or scrambled si-Ctrl, and then 48 h later retransfected with HCV minigenome RNA. GLuc activity in supernatant fluids was measured at indicated times. The results shown represent the means of three replicate experiments ± the standard deviations. Values at 3 h were arbitrarily set to 100. (C) GLuc RNA levels were determined by real-time RT-PCR 24 h after HCV minigenome RNA transfection relative to β-actin mRNA. The differences in GLuc activity between hnRNP L- and NF90-depleted cells and cells transfected with si-Ctrl at 24 h (see panel B) were not significant when normalized to mRNA abundance ($P > 0.05$ as determined by two-sided t test). (D) Huh-7.5 cells were transfected with siRNAs for hnRNP L, NF90, or scrambled si-Ctrl and then 48 h later retransfected with H77s-GLuc RNA, together with 100 nM duplex miR-122 or miR-124. The GLuc activities were measured 72 h later. The results shown represent the means of three replicate experiments ± the standard deviations. **, $P < 0.01$ (compared to miR-124 by two-sided t test).

branes (DRMs) (32). Thus, to examine the possibility that hnRNP L and NF90 might be associated with replication complexes, we investigated their distribution within membrane fractions recovered from a flotation gradient. Cytoplasmic membranes from uninfected or HJ3-5 virus-infected Huh-7.5 cells were treated with Triton X-100, overlaid with a density gradient, and subjected to centrifugation (see Methods). Partitioning of DRMs and detergent soluble membranes (DSM) into separate fractions was confirmed by distinct distributions of caveolin-2 and calrecticulin among these fractions (Fig. 6A). HCV replication complexes were largely associated with the DRM, as shown by the distribution of NS5A. Although hnRNP L and NF90 were readily detected in DRM fractions from uninfected cells (3.0 to 6.85% and 2.2 to 3.2% of the total of hnRNP L and NF90, respectively, in the gradient were present in fractions 1 to 4), HCV infection increased the proportion of the proteins associating with the DRM (10.0 to 21.9% hnRNP L and 12.7 to 27.6% NF90 in replicate experiments) (Fig. 6A and B). In contrast, IGF2BP1, which binds nonspecifically to our RNA bait (Fig. 1D) and the depletion of which had relatively little effect on HCV replication (Fig. 2B), was barely detectable in DRM fractions, and its distribution was not altered by HCV infection (Fig. 6A). These data indicate that HCV infection results in an increased association of hnRNP L and NF90 with DRMs, providing additional evidence that these proteins are likely to be associated with the replication complex.

To further confirm this association, we treated the DRM fractions with proteinase K. Proteins present within membranous vesicles within the DRM fraction should be resistant to proteases in the absence of strong detergents. hnRNP L and NF90 in DRM fractions from uninfected cells were completely digested by proteinase K (Fig. 6C), indicating that they are not protected by membranes. In contrast, a significant fraction of hnRNP L and NF90 in DRM fractions from HCV-infected cells was resistant to protease treatment (Fig. 6C). Pretreatment of these fractions with 1% NP-40, which should disrupt all membrane structures, rendered these proteins sensitive to proteinase K. Taken together with the increased distribution of hnRNP L and NF90 into the DRM fractions (Fig. 6A and B), these data suggest that hnRNP L and NF90 are recruited into replication complexes to facilitate replication.

DISCUSSION

The 5′-terminal sequence of the HCV genome contains two miR-122 binding sites and is known to have an important regulatory role in viral RNA replication (3, 4, 19). The predicted secondary structure of the 47-nucleotide terminal sequence contains a conserved stem-loop (SLI), followed by an unstructured region within which the miR-122 seed sequence-binding sites are located (Fig. 1A, left). We hypothesized that these conserved regions may interact with host proteins that regulate viral RNA translation and/or replication. To assess this hypothesis, we used a biotin-RNA pulldown strategy to identify proteins that bind to this 5′-terminal sequence. In addition to Ago2 and PCBP2, which have

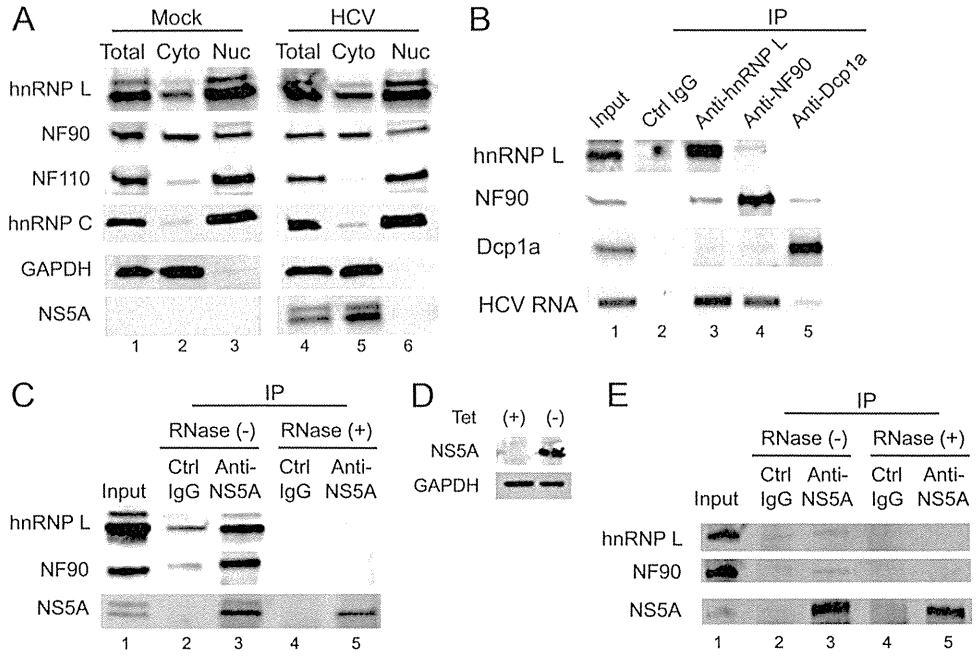

FIG 5 Cellular localization and association of hnRNP L and NF90 with HCV RNA and NS5A. (A) Distribution of hnRNP L and NF90 in cytoplasmic and nuclear fractions. Total lysate and cytoplasmic and nuclear fractions of Huh-7.5 cells with or without HCV infection were prepared, and the distribution of the indicated proteins was assessed using immunoblotting. hnRNP L was largely localized to the nuclear fraction, with a small amount in the cytoplasm. NF90 protein was equally distributed between cytoplasmic and nuclear fractions, while NF110, an isoform of NF90, predominantly localized to the nucleus. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as cytoplasmic marker and hnRNP C as a nuclear marker. HCV NS5A protein was detected only in the cytoplasmic fraction from infected cells. (B) hnRNP L and NF90 associate with HCV RNA in infected cells. hnRNP L or NF90 proteins were immunoprecipitated by specific antibodies from a cytoplasmic lysate of Huh-7.5 cells infected with HJ3-5 virus. Precipitation of hnRNP L and NF90 proteins was confirmed by immunoblotting. RNAs were extracted from the precipitates, and HCV RNA was detected by RT-PCR. Isotype control IgG was used as negative control. 5% input lysate was loaded as a reference. (C) RNA-dependent association of NS5A with hnRNP L and NF90. NS5A protein was immunoprecipitated by specific antibody or control IgG from HJ3-5 virus-infected Huh-7.5 cytoplasmic lysate with or without RNase treatment. NS5A, hnRNP L, and NF90 proteins in the precipitates were detected by immunoblotting. 5% input lysate was loaded as reference. hnRNP L and NF90 coimmunoprecipitate with NS5A only in non-RNase-treated samples. (D) U2OS cells with tet-regulated expression of NS5A protein were treated with or without 2 μg of tetracycline/ml for 48 h, and the expression of NS5A was detected by immunoblotting with GAPDH as a loading control. (F) NS5A immunoprecipitation was carried out as in panel C for U2OS cells expressing NS5A protein. Little coprecipitation of hnRNP L and NF90 with NS5A was observed.

been shown previously to bind this region of the genome (9, 14, 20), we documented the binding of several novel proteins, including hnRNP L, DHX9, ADAR1, and NF90 (Fig. 1B and C). hnRNP L binds ssRNA, whereas DHX9, ADAR1, and NF90 bind the double-strand complement of this RNA segment. Among these proteins, siRNA-mediated knockdowns of either hnRNP L or NF90 reduced HCV RNA and protein levels within infected cells (Fig. 3), suggesting that both are required for efficient HCV replication. hnRNP L and NF90 function in the HCV life cycle independently of miR-122, because depletion of either did not affect the capacity of miR-122 to stimulate HCV replication (Fig. 4D). hnRNP L and NF90 depletion had no impact on GLuc activity expressed by an HCV minigenome (Fig. 4B), suggesting they are not required for HCV IRES-mediated translation.

Previous studies have demonstrated that HCV RNA replication complexes consist of viral and host proteins assembled on DRMs (32). The localization of hnRNP L and NF90 to the DRM fractions of HCV-infected cells (Fig. 6A and B) thus suggests that they may be associated with the RNA replication complex. The fact that both proteins are protected by membranes from protease digestion in these DRM fractions provides further support for this (Fig. 6C). Immunoprecipitation of hnRNP L and NF90 in HCV-infected cells confirmed their association with HCV RNA (Fig. 5B). It is particularly interesting that hnRNP L and NF90 coimmunoprecipitated with NS5A (Fig. 5C), an essential component of the replication complex. The interaction was dependent on RNA, suggesting that while hnRNP L and NF90 do not physically interact with NS5A, they may form an RNA-protein complex together, possibly in association with HCV RNA in the replication complex.

hnRNPs are proteins that interact with heterogeneous nuclear RNAs (hnRNAs) (33). Several functions have been suggested for hnRNPs, including pre-mRNA processing, mRNA translocation from the nucleus to the cytoplasm, and translation (33). This group of RNA-binding proteins has been shown to participate in both viral IRES mediated-translation and viral replication. For example, PTB (hnRNP I) has been shown to enhance IRES-dependent translation of encephalomyocarditis virus and foot-and-mouth disease virus, both picornaviruses, as well as HCV (16, 34, 35). PCBP2 (hnRNP E2) is required for efficient translation and replication of both poliovirus and HCV RNA (14, 36). hnRNP L has been reported to be localized mainly in the nucleus (29). However, we detected hnRNP L in the cytoplasm both in the presence and in the absence of HCV infection (Fig. 5), suggesting that it may shuttle between the nucleus and cytoplasm. hnRNP L was previously reported to be required for HCV IRES-dependent translation because an RNA aptamer specific for hnRNP L blocked reporter protein translation directed by the HCV IRES

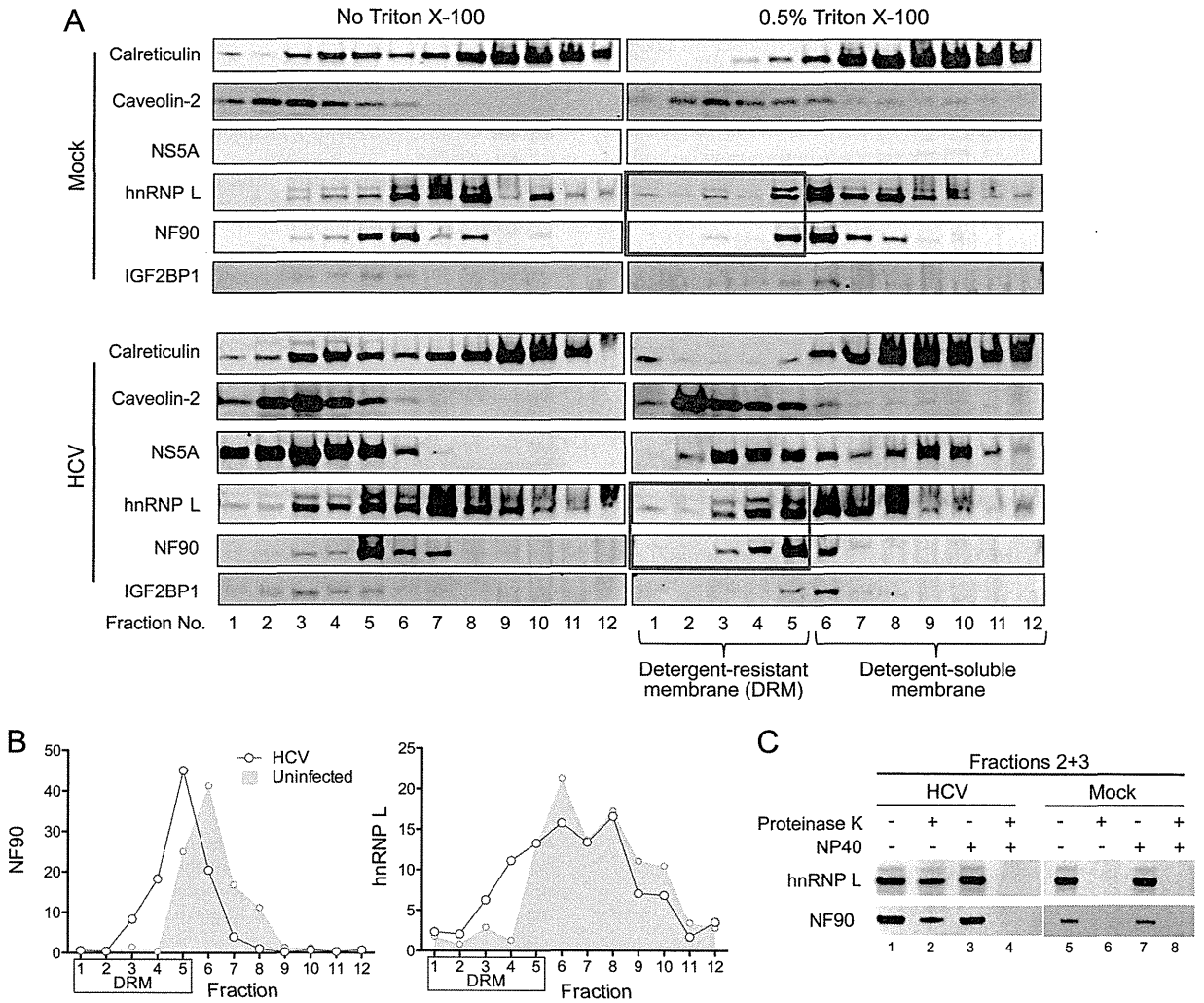

FIG 6 Membrane flotation assay. (A) Analysis of the distribution of hnRNP L and NF90 in fractions recovered from a membrane flotation assay. Cytoplasmic membranes extracted from FT3-7 cells with or without HCV infection were treated or not treated with Triton X-100, overlaid with a density gradient, and subjected to centrifugation. Fractions were subsequently recovered from the top of the gradient, and proteins in each were precipitated, resolved by SDS-PAGE, and analyzed by immunoblotting. Calrecticulin and caveolin-2 were detected as markers for detergent-soluble membrane (DSM) and detergent-resistant membrane (DRM), respectively. A substantial proportion of NS5A was located in DRM fractions, consistent with its presence in the HCV replication complex. hnRNP L and NF90 were readily detected in DRM, while little IGF2BP1 protein was found present in DRM. The image shown is representative of replicate experiments. (B) Quantification of the distribution of hnRNP L and NF90 proteins in fractions from the flotation gradients shown in panel A. Protein bands in immunoblots of each fraction were quantified with an Odyssey infrared imaging system. (C) hnRNP L and NF90 distributed to the DRM are protected from protease digestion in gradients loaded with material from HCV-infected cells. Fractions 2 and 3 (no Triton X-100) from panel A were treated with proteinase K with or without prior treatment with 1% NP-40. hnRNP L and NF90 proteins were detected by immunoblotting. hnRNP L and NF90 were uniformly sensitive to protease K in uninfected cells, whereas hnRNP L and NF90 associating with the DRM were resistant to proteinase K in HCV-infected cells. All proteins in fractions 8 and 9 from either uninfected or infected cells were sensitive to protease treatment (data not shown).

(37). However, in our study hnRNP L had little effect on the translation of a minigenome reporter containing both HCV UTRs, indicating that it is not essential for HCV IRES-dependent translation initiation (Fig. 4). It is possible the aptamer used in the previous report caused a nonspecific inhibition of IRES activity.

It is intriguing that the hnRNP L protein binding site appears to overlap with miR-122 binding sites in HCV 5′UTR, since the p6 mutation in the S1 and S2 sites reduced hnRNP L binding to the RNA bait in the pulldown experiments (Fig. 1E). However, the binding of hnRNP L to the HCV 5′ terminus is independent of miR-122, since the anti-miR-122 antagomir did not block binding of hnRNP L (Fig. 1F). In contrast, preincubation of the RNA bait with miR-122 diminished hnRNP L binding, indicating that hnRNP L and miR-122 bind competitively to overlapping sites. It is puzzling why two proviral factors, miR-122 and hnRNP L, would compete with each other. One plausible explanation is that hnRNP L and miR-122 are involved in different stages of the HCV life cycle and that a change of binding factors at the 5′ RNA terminus is accompanied by a transition between these different stages. Further work will be required to investigate this hypothesis.

NF90 belongs to a family of proteins that contain a double-strand RNA binding motif and exert a variety of functions involving RNA recognition, RNA processing, and RNA stability (38). NF90 has previously been reported to form a complex with DHX9

(39). Although DHX9 and NF90 both bound to the dsRNA bait (Fig. 1), depletion of NF90 had a much greater impact on HCV replication (Fig. 2B). NF90 has been shown to positively regulate replication of several positive-strand RNA virus, including dengue virus and bovine viral diarrhea virus, a pestivirus closely related to HCV (40, 41). This suggests its host factor function may be conserved among the *Flaviviridae*. It binds to the 5′UTRs and/or 3′UTRs of these viruses and promotes their replication. It has also been reported to associate with both the 5′UTRs and 3′UTRs of HCV in a cross-linking assay and to promote interactions between the 5′ and 3′ ends of the genome (42). Although we found that NF90 bound to the dsRNA bait (Fig. 1) and was required for efficient HCV replication (Fig. 3), we do not know whether NF90 mediates HCV replication through its direct association with dsRNA (as we have demonstrated) or by binding to structured elements within the HCV 5′UTRs and 3′UTRs. Nevertheless, the increased distribution of NF90 into DRM following HCV infection (Fig. 6) suggests the association of NF90 with viral replicase complexes.

How hnRNP L and NF90 function in HCV replication remains to be determined. The binding of hnRNP L or NF90 could facilitate replication complex assembly by recruiting other viral and host factors onto HCV RNA or possibly induce conformational changes in the RNA that promote viral RNA synthesis. Further studies are needed to dissect the individual steps in the viral life cycle impacted by hnRNP L and NF90 and to identify other host and viral proteins involved. Further elucidation of the molecular basis of the function of hnRNP L and NF90 on HCV replication could yield important insight into unrecognized actions of RNA-binding proteins in the viral life cycle and possibly provide clues for development of broadly acting antiviral therapies.

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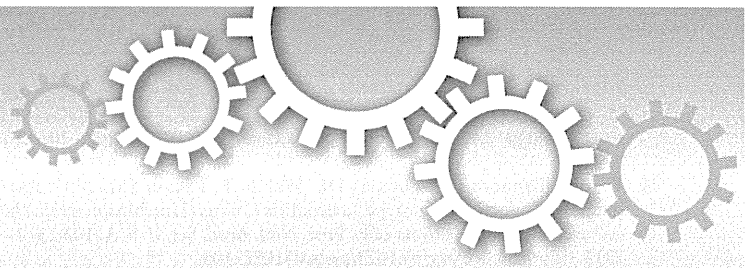

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The Acyclic Retinoid Peretinoin Inhibits Hepatitis C Virus Replication and Infectious Virus Release *in Vitro*

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Clinical studies suggest that the oral acyclic retinoid Peretinoin may reduce the recurrence of hepatocellular carcinoma (HCC) following surgical ablation of primary tumours. Since hepatitis C virus (HCV) infection is a major cause of HCC, we assessed whether Peretinoin and other retinoids have any effect on HCV infection. For this purpose, we measured the effects of several retinoids on the replication of genotype 1a, 1b, and 2a HCV *in vitro*. Peretinoin inhibited RNA replication for all genotypes and showed the strongest antiviral effect among the retinoids tested. Furthermore, it reduced infectious virus release by 80–90% without affecting virus assembly. These effects could be due to reduced signalling from lipid droplets, triglyceride abundance, and the expression of mature sterol regulatory element-binding protein 1c and fatty acid synthase. These negative effects of Peretinoin on HCV infection may be beneficial in addition to its potential for HCC chemoprevention in HCV-infected patients.

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC); therefore, the eradication of HCV from an infected liver could reduce death from HCV-related liver disease. Combination therapy of PEGylated-interferon (PEG-IFN) and ribavirin has long been the standard of care for patients with chronic hepatitis C (CH-C); however, a sustained viral response (SVR) is obtained in only ~50% of treated patients infected with genotype 1 HCV[1]. Recently, several classes of direct-acting antiviral agents (DAAs) have entered into clinical use. In the United States, two NS3/4A protease inhibitors, telaprevir and boceprevir, were approved for use in combination with PEG-IFN and ribavirin in 2011. Although the addition of these DAAs dramatically improves the SVR rate, 20–30% of patients still fail to eradicate HCV due to breakthrough by drug-resistant mutants or null response to therapy[2]. More potent DAAs are currently in late clinical development and promise much higher SVR rates even in the absence of PEG-IFN therapy; however, HCV-related HCC is likely to continue to be a significant clinical issue for many years because it will take time for potent DAAs to be distributed worldwide.

Peretinoin (generic name code: NIK-333) is an oral acyclic retinoid with a vitamin A-like structure that targets retinoid nuclear receptors, such as retinoid X receptor and retinoic acid receptor. The oral administration of Peretinoin significantly reduces the incidence of post-therapeutic HCC recurrence and improves the survival rate of patients in clinical trials[3,4]. In addition, Peretinoin prevents the development of hepatoma in several different hepatoma models[5,6]. Larger-scale clinical studies are currently ongoing in various countries to confirm its clinical efficiency. Depending on the results of these studies, Peretinoin may be used in CH-C patients to prevent HCC. Therefore, we sought to understand the effect of Peretinoin on HCV replication.

Peretinoin is categorised as a vitamin A or retinoid compound, and conflicting reports have described the effects of vitamin A compounds on HCV replication. One report showed that 3 retinoids, 9-cis retinoic acid (RA), 13-cis RA, and all-trans RA (ATRA), suppressed the replication of a sub-genomic HCV replicon[7]. However, vitamin A also reportedly enhances the replication of genome-length HCV in Huh-7 cells[8]. Here, we describe the impact of Peretinoin on different steps of the HCV life cycle, including translation, RNA amplification, virus assembly, and secretion, and its impact on host lipid metabolism *in vitro*. Our results clearly demonstrate that Peretinoin inhibits HCV RNA amplification and virus release by altering lipid metabolism.

Results

Inhibition of HCV RNA replication by retinoids. Several studies have tested the effects of vitamin A on HCV replication; these studies used a sub-genomic or full-genomic replicon, which contains 2 cistrons, one driven by HCV internal ribosome entry sites (IRES) and the other by encephalomyocarditis virus IRES[7,8]. We reported the usefulness of HCV genomes containing *Gaussia princeps* luciferase (GLuc) between p7 and NS2, followed by foot-and-mouth disease virus 2A, to monitor HCV RNA replication[9,10], and this system is closer to physiological HCV replication than the bicistronic replicon systems (Fig. 1A). In addition to GLuc-containing HCV genomes in the backbone of genotype 1a H77S.3, a chimeric clone of H77S and genotype 2a JFH1, HJ3-5[11], with structural proteins from H77S and non-structural proteins from JFH1, we also constructed GLuc-containing genomes in the backbone of genotype 1b N[12] and 2a JFH1[13] and confirmed their efficient replication in Huh-7.5 cells. Importantly, all of the strains used here are derived from cDNA clones that are infectious to chimpanzees.

We initially examined the effects of 4 different retinoids, namely ATRA, 9-cis RA, 13-cis RA, and Peretinoin, on HCV replication by using these 4 HCV genomes containing GLuc, according to the use of GLuc activity as an indicator of RNA replication, and the structures of each retinoid were shown in Supplementary Fig. S1 online. Peretinoin inhibited the replication of H77S.3/GLuc2A in a dose-dependent manner (Fig. 1B). As the other retinoids also suppressed HCV replication, we determined the antiviral half maximal effective concentrations (EC_{50}s) of these retinoids for each HCV genotype. Whilst Peretinoin showed the strongest antiviral effect on all genotypes tested, ATRA exerted a moderate effect, and 9-cis and 13-cis RA generated a weaker effect (Table 1). Especially, Peretinoin suppressed the RNA replication of H77S.3/GLuc2A most efficiently and its EC_{50} was 9 μM.

We also determined the half maximal cytotoxicity concentrations (CC_{50}s) of these retinoids in H77S.3/GLuc2A-replicating Huh-7.5 cells by using the WST-8 assay, which reflects cell number. The CC_{50}s of ATRA, 9-cis RA, and 13-cis RA were more than 100 μM; however, the CC_{50} of Peretinoin was 68 μM when the cells were treated for 72 h (Table 2). Although Peretinoin had a slightly negative impact on cell growth, as it showed the strongest antiviral effect and may be used for HCC chemoprevention in HCV-infected patients in the future, we focused upon the action of Peretinoin among these retinoids.

Inhibition of HCV RNA replication by Peretinoin. We examined the time dependence of the antiviral effect of Peretinoin. After HCV RNA transfection, we treated the transfected cells with Peretinoin at a range of concentrations (10–40 μM) and monitored RNA replication every 24 h until 72 h. Peretinoin started to show an antiviral effect from 24 h after treatment, which continued until 72 h. Peretinoin suppressed RNA replication in a time-dependent manner for all genotypes tested (Fig. 1C).

We also examined whether Peretinoin could also suppress RNA replication in a sub-genomic replicon system (Fig. 1D), in which infection should not occur due to the lack of structural proteins. Peretinoin was also able to suppress RNA replication in a dose-dependent manner in bicistronic sub-genomic RNA-transfected cells (Fig. 1E).

Importantly, when we treated HCV (H77S.3/GLuc2A)-replicating and HCV-non-replicating Huh-7.5 cells with Peretinoin at a range of concentrations (5–50 μM), the cell numbers were identical under the conditions tested (Fig. 1F).

As Peretinoin could suppress GLuc activity itself, we then examined directly its antiviral effect in the context of an HCV genome lacking the GLuc genome. For this purpose, Huh-7.5 cells infected with cell culture-derived HCV (HCVcc) of HJ3-5 were treated with different concentrations of Peretinoin. When we monitored HCV RNA replication by using quantitative real-time detection-polymerase chain reaction (RTD-PCR) (Fig. 2A) and protein expression by western blotting for the HCV core protein (Fig. 2B, see Supplementary Fig. S2 online), Peretinoin suppressed RNA replication and protein expression in a dose-dependent manner, which is consistent with the GLuc activity results. We also tested infectious virus production from Peretinoin-treated cells using a conventional focus forming unit (FFU) assay, and found that Peretinoin also reduced this in a dose-dependent manner (Fig. 2C).

Effect of Peretinoin on translation driven by HCV IRES. We also tested the effect of Peretinoin on translation directed by HCV IRES. For this purpose, we used a mini-genome RNA which has, sequentially, the HCV 5′-untranslated region (UTR), GLuc, and HCV 3′-UTR, and cap-*Cypridina* luciferase (CLuc)-polyA RNA as a control (see Supplementary Fig. S1 online). After we treated Huh-7.5 cells with different concentrations of Peretinoin for 24 h, we co-transfected the cells with these RNAs and measured GLuc and CLuc activity every 3 h from 3 to 12 h. When we normalised GLuc activity to CLuc activity at each time point, we did not observe a significant difference among the cells treated with the different concentrations of Peretinoin (see Supplementary Fig. S3 online), suggesting that Peretinoin does not have an effect on protein expression directed by HCV IRES.

Effect of Peretinoin on cellular interferon signalling. We hypothesised that the suppression of RNA replication by Peretinoin could be due to the activation or enhancement of cellular interferon (IFN) signalling. To examine this, we treated HCV (H77S.3/GLuc2A)-non-replicating and HCV-replicating Huh-7.5 cells with either IFNα-2b (10 IU/mL) or Peretinoin (10–40 μM) and monitored the expression of total and phosphorylated signal transducer and activator of transcription 1 (STAT1). Peretinoin did not alter the expression of either total or phosphorylated STAT1 in HCV-non-replicating Huh-7.5 cells or HCV-replicating cells (see Supplementary Fig. S4 online). In addition, Peretinoin did not further enhance the amount of phosphorylated STAT1 activated by IFNα-2b in HCV-non-replicating Huh-7.5 cells or HCV-replicating cells (see Supplementary Fig. S4 online). These data suggest that Peretinoin suppresses RNA replication without either activating or enhancing cellular IFN signalling.

Impact of Peretinoin on lipid metabolism. As lipid metabolism has an important role in various aspects of HCV infection[14–16], we examined the impact of Peretinoin on lipid metabolism. However, as it is sometimes difficult to detect small changes in lipid metabolism, we tested the effect of Peretinoin under oleic acid (OA) treatment, which amplifies changes in lipid metabolism. We treated H77S.3/GLuc2A-replicating Huh-7.5 cells with 40 μM Peretinoin and 250 μM OA, fixed and stained the cells with BODIPY 493/503 for lipid droplets (LDs) and 4′, 6-diamidino-2-phenylindole (DAPI) for nuclei, and used an anti-core protein antibody to detect HCV. When we stained LDs in the presence of 250 μM OA and the absence of Peretinoin, we observed intense signals (Fig. 3A); however, when it was accompanied with 40 μM Peretinoin, the signals from LDs were dramatically reduced, and at the same time, the expression of HCV core protein was also down-regulated (Fig. 3B). When we quantitated the signal strength from LDs and HCV core protein in 4 different fields, Peretinoin significantly reduced the signals from LDs and HCV core protein (two-tailed Student's t test, $p<0.0001$ for each) (Fig. 3C). This reduction was also confirmed by the quantitation of the 5 cells which were positive for both LDs and HCV core (see Supplementary Fig. S5 online). The reduced expression of HCV core protein was also observed by western blot analysis (Fig. 3D, see Supplementary Fig. S6 online). We next investigated the

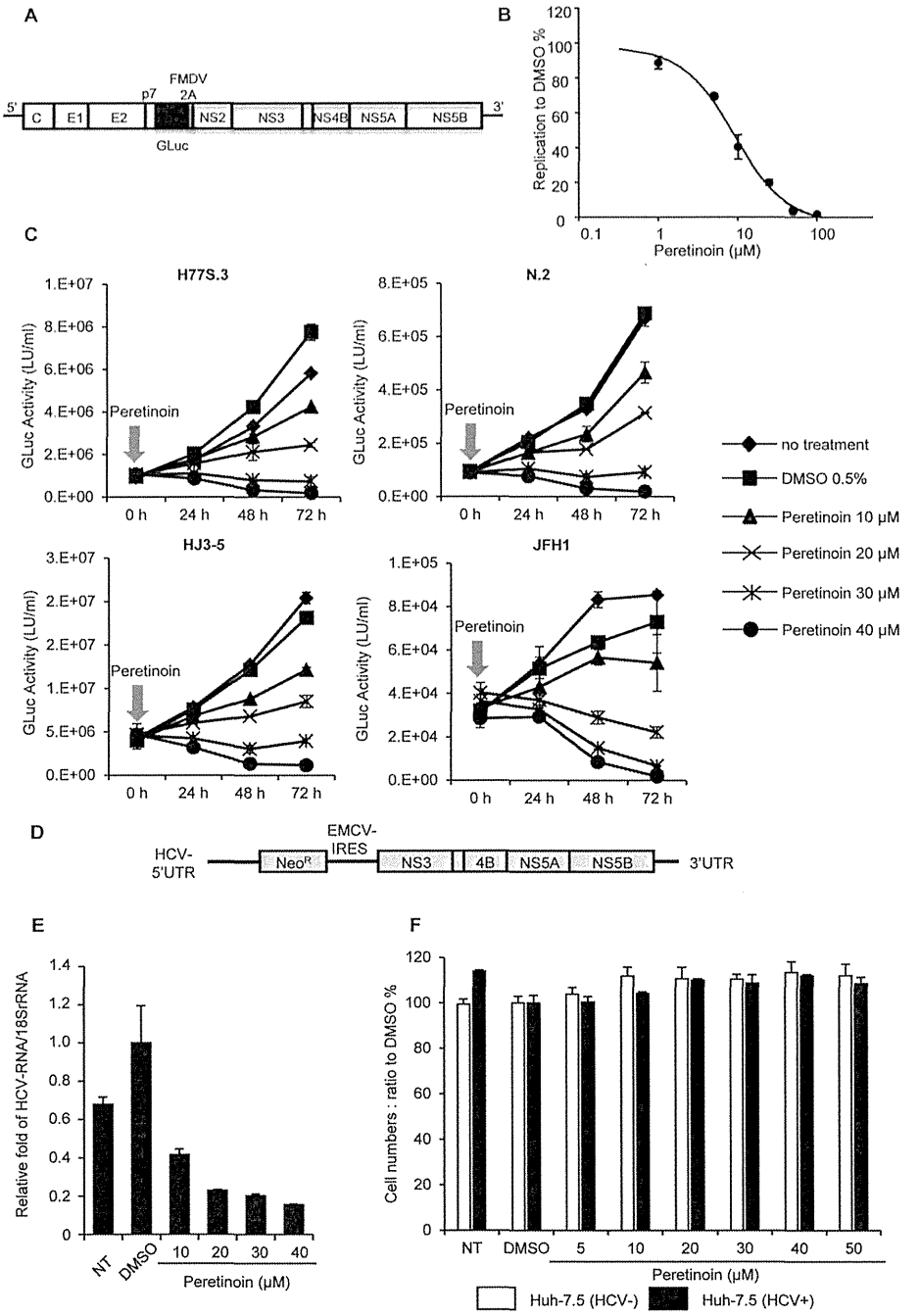

Figure 1 | Antiviral effects of several retinoids and their effects on cell growth. (A) Schematic representation of the GLuc-containing HCV genome. (B) Huh-7.5 cells were transfected with H77S.3/GLuc2A RNA, and 48 h later, 0.5% DMSO or Peretinoin was added at concentrations ranging from 1 to 100 μM. Fresh medium containing Peretinoin was added every 24 h, and 72 h after adding Peretinoin, secreted GLuc activity was measured. The GLuc activity from Peretinoin-treated cells was normalised to that with DMSO treatment. Data show the mean inhibition to DMSO treatment in each concentration of Peretinoin ± SD from 3 independent experiments. (C) Huh-7.5 cells were transfected with H77S.3/GLuc2A, N.2/GLuc2A, HJ3-5/GLuc2A, and JFH1/GLuc2A RNAs, and 48 h later, 0.5% DMSO or Peretinoin was added at the indicated concentrations. The medium was collected and replaced with fresh medium every 24 h until 72 h. GLuc activity was determined at each time point. The results shown represent the mean GLuc activity ± SD from 3 different plates. (D) Schematic representation of the bicistronic sub-genomic HCV RNA (E) Huh-7.5 cells were transfected with bicistronic sub-genomic RNA. At 48 h later, the transfected cells were treated with the indicated concentrations of Peretinoin for 72 h. Quantification of HCV RNA and 18S rRNA levels was performed and relative HCV RNA abundance normalised to the amount of 18S rRNA is presented as fold change ± SD compared to DMSO-treated cells from 3 independent experiments. (F) Huh-7.5 cells were transfected with H77S.3/GLuc2A RNA, and 7 days later, HCV (H77S.3/GLuc2A)-replicating Huh-7.5 cells, depicted as 'HCV+', were treated with the indicated concentrations of Peretinoin and HCV-non-replicating Huh-7.5 cells, depicted as 'HCV-', were also treated in a same way. At 72 h after Peretinoin treatment, cell numbers were determined by using a Cell Counting Kit-8. Data represent relative cell numbers ± SD from 3 independent experiments to DMSO-treated cells. EMCV, Encephalomyocarditis virus; Neo^R, Neomycin resistance gene; NT, no treatment.

Table 1 | EC_{50} of vitamin A compounds on HCV RNA replication

	Peretinoin		ATRA		9-cis RA		13-cis RA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
HCV	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)
H77S.3	9	1	32	3	29	7	41	4
N.2	19	1	53	5	75	8	83	17
HJ3-5	18	2	25	1	51	6	82	17
JFH1	20	1	25	1	61	8	78	11

impact of Peretinoin on lipid metabolism by measuring intracellular triglyceride (TG) levels, which should mainly reflect the amount of LDs, following treatment with 0–40 μM Peretinoin with or without HCV replication and OA treatment. Peretinoin reduced intracellular TG levels in a dose-dependent manner, regardless of OA treatment and HCV replication (Fig. 4A). These effects may be primarily due to its transcriptional modulation. To address this possibility, we examined the effect of Peretinoin on the transcription of fatty acid synthase (FASN) using RTD-PCR under 0–40 μM Peretinoin with or without HCV replication and OA treatment, because FASN is a key enzyme for the synthesis of fatty acids, which are an essential component of TGs. Peretinoin reduced the mRNA levels of FASN in a dose-dependent manner, regardless of OA treatment and HCV replication (Fig. 4B). We also examined FASN protein expression as well as the levels of precursor and mature sterol regulatory element-binding protein 1c (SREBP1c), which is a critical transcription factor for FASN. Peretinoin reduced the expression of FASN protein, which is consistent with the RTD-PCR results (Fig. 4C, see Supplementary Fig. S7 online). Although Peretinoin did not have an effect on precursor SREBP1c protein expression, it dramatically reduced the levels of mature SREBP1c (Fig. 4C, see Supplementary Fig. S7 online). We also observed a reduction of FASN mRNA levels by Peretinoin in an immortalised human hepatocyte cell line (Fig. 5A), and a similar reduction was also observed for ATRA, 9-cis RA, and 13-cis RA treatment of HCV-replicating Huh-7.5 cells (Fig. 5B). These results indicate that Peretinoin reduced intracellular lipid levels by reducing the amount of mature SREBP1c and, subsequently, FASN.

Specific inhibition of virus secretion by Peretinoin. Recently, lipids including LDs and TG have been reported to be important for efficient infectious virus production[14–16]. Due to its huge impact on lipid metabolism, Peretinoin could affect virus assembly or secretion as well as RNA amplification. To test the effect of Peretinoin on infectious virus production, we determined intra- and extracellular infectivity and the virus secretion ratio by measuring the amount of intra- and extra-cellular infectious virus from HJ3-5/GLuc2A-replicating FT3-7 cells treated with various concentrations of Peretinoin. We infected naïve Huh-7.5 cells with intra- and extra-cellular virus derived from HJ3-5/GLuc2A-replicating cells after Peretinoin treatment and used GLuc activity as an indicator of infectious virus production because FFUs and GLuc activity were well correlated (see Supplementary Fig. S8 online),

Table 2 | CC_{50} of vitamin A compounds on Huh-7.5 cells supporting HCV replication

Peretinoin		ATRA	9-cis RA	13-cis RA
Mean	SD	Mean	Mean	Mean
(μM)	(μM)	(μM)	(μM)	(μM)
68	5.2	>100	>100	>100

and a previous report also showed a good correlation between them[17]. Although Peretinoin did not show a significant impact on intracellular infectivity at 10–30 μM, it dramatically reduced extracellular infectivity and virus secretion from 10 μM when we normalised intra- and extra-cellular infectivity by the replication capacity of the virus producing the intra- and extra-cellular virus, as determined by GLuc activity (Fig. 6A). This result was also confirmed by using the extra-cellular virus which was prepared by centrifugation and subsequently re-suspended to fresh medium without containing Peretinoin, indicating that possible carryover of Peretinoin in the medium from extra-cellular cultures does not affect the result shown in Figure 6A (Supplementary Fig. S9 online). Interestingly, the expression of apolipoprotein E3 (ApoE3), which is essential for virus secretion, was also suppressed by Peretinoin (Fig. 4C, see Supplementary Fig. S7 online). Furthermore, we compared the buoyant density of HCVcc derived from HJ3-5/GLuc2A-replicating FT3-7 cells by equilibrium gradient ultracentrifugation. HCVcc from HJ3-5/GLuc2A-replicating cells treated with dimethyl sulfoxide (DMSO) or 30 μM Peretinoin showed exactly the same peak of infectivity at 1.107 g/cm^3 (Fig. 6B, 6C). Specific infectivity, as calculated from both peaks of HCV RNA and GLuc activity, was 0.0381 ± 0.0209 (standard deviation, SD) light units (LU)/copy for DMSO-treated cells, and 0.0799 ± 0.0457 LU/copy for Peretinoin-treated cells, which did not show a considerable difference. Furthermore, Peretinoin did not affect virus entry of HCVcc when we tested it by RT-PCR for HCV RNA at 5 h after infection and an FFU assay at 72 h after infection (see Supplementary Fig. S10 online). Collectively, Peretinoin seems to inhibit virus release in addition to viral RNA amplification.

Discussion

In the present study, we clearly showed that Peretinoin, as well as ATRA, 9-cis RA, and 13-cis RA, suppressed HCV RNA replication (Table 1). While previous reports used replicon systems to test the effects of retinoids, we used a genome-length HCV containing a GLuc-coding sequence between p7 and NS2, which is more physiological than replicons. The inhibitory effect of retinoids was universal among the HCV genotypes tested, and all retinoids tested showed an inhibitory effect on HCV replication (Table 1). In addition, we also observed the antiviral effect of Peretinoin in the replicon system (Fig. 1E). Therefore, our present data strongly support the notion that retinoids exert an antiviral effect *in vitro*. The antiviral effect of retinoids has also been confirmed in a clinical study. Even when CH-C patients were treated with ATRA, the viral load dropped by 1–2 log units in 50% of the patients enrolled. In addition, when CH-C patients who showed no response to prior IFN/PEG-IFNα and ribavirin therapy were treated with a combination of ATRA and PEG-IFNα-2a, 30% of patients showed a significant viral reduction[18]. Recently, combined vitamin A and D deficiency prior to IFN-based therapy was shown to be a strong independent predictor of non-response to antiviral therapy[19]. Collectively, our data and the clinical findings indicate that retinoids possess inhibitory effects on HCV replication.

Peretinoin showed the strongest antiviral effect among the retinoids tested (Table 1); thus, we focused on Peretinoin to clarify its antiviral mechanism. A previous report showed that 9-cis RA enhanced the antiviral effect of IFNα by increasing the expression of the IFNα receptor[20]; however, another study showed that ATRA did not induce the activation of dsRNA-activated protein kinase R, which is a key player in the IFN-induced antiviral response[7]. In the present study, Peretinoin did not increase the amount of the activated form of STAT-1, which is pSTAT1, contrary to IFNα-2b, both in HCV-replicating and HCV-non replicating Huh-7.5 cells, and dual treatment of Huh-7.5 cells with IFNα-2b and Peretinoin did not show a further increase of the pSTAT1 levels induced by only IFNα-2b (see Supplementary Fig. S4 online), indicating that

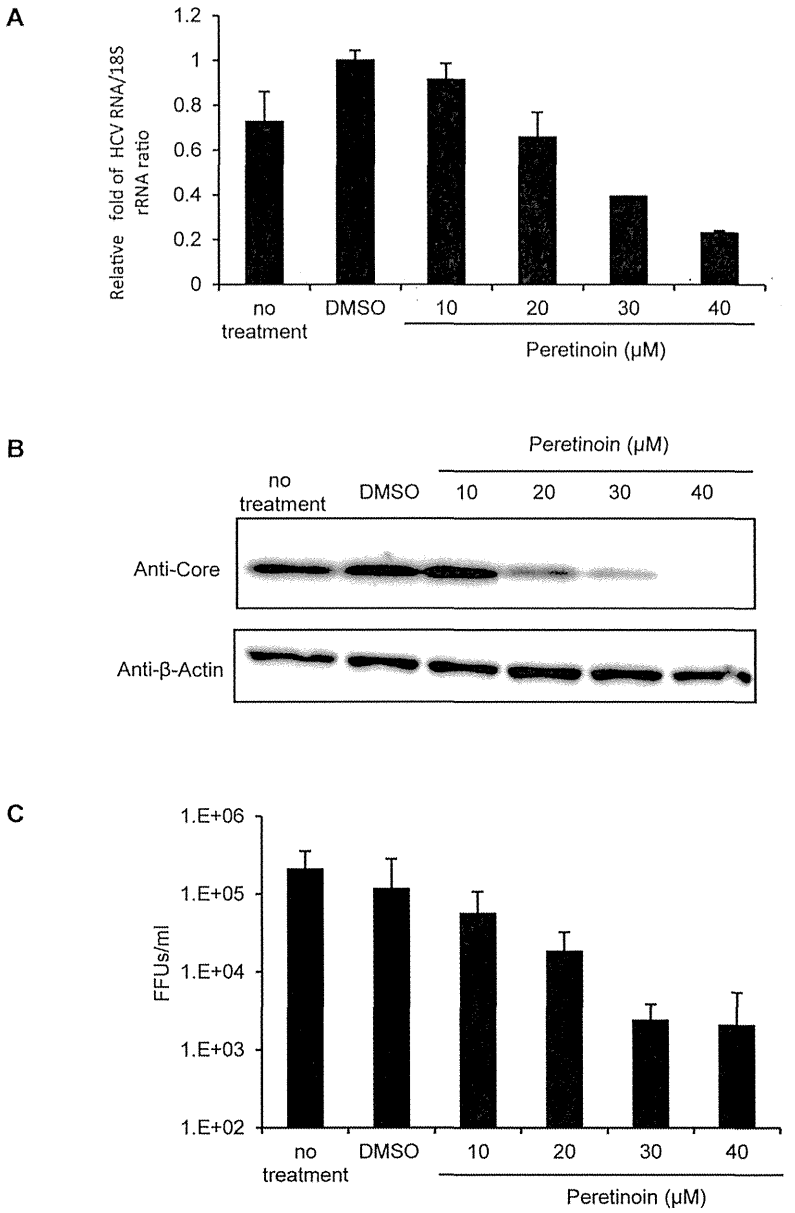

Figure 2 | Inhibition of HCV replication and infectious virus production. Huh-7.5 cells were infected with the HJ3-5 virus at a multiplicity of infection (MOI) of 1, and 72 h later, DMSO or Peretinoin was added at the indicated concentrations. The medium was replaced with fresh medium every 24 h until 72 h. (A) At 72 h after adding Peretinoin, total cellular RNA was extracted, and the amount of HCV RNA and 18S rRNA was quantitated by RTD-PCR. Relative HCV RNA abundance normalised to the amount of 18S rRNA is presented as fold change ± SD compared to DMSO-treated cells from 3 independent experiments. (B) At 72 h after Peretinoin treatment, the cell lysates were collected and subjected to western blot analysis using anti-core protein and anti-β-actin antibodies. Full-length blots/gels are presented in Supplementary Fig. S2 online. (C) The medium was collected at 72 h after Peretinoin treatment, and immediately, naïve Huh-7.5 cells were infected with serially diluted medium. At 72 h after infection, the infectious virus titre of HCVcc from Peretinoin-treated cells was determined by an FFU assay. Data shown here represent the mean FFUs/mL ± SD from 2 independent experiments.

Peretinoin did not activate or enhance cellular IFN signalling. Our results also indicate that the antiviral effect of Peretinoin is not due to the suppression of HCV translation directed by HCV IRES (see Supplementary Fig. S3 online). As Peretinoin suppressed the RNA replication of bicistronic sub-genomic replicons (Fig. 1E), it seems to suppress RNA amplification itself (see also the later description of FASN). A report showed that retinoids inhibited HCV RNA replication by enhancing the expression of gastrointestinal-glutathione peroxidase (GI-GPx) only in the presence of sodium selenite[7]; however, in the present study, we demonstrated that all retinoids tested inhibited HCV replication, even in the absence of sodium selenite. Thus, our results support the notion that the observed antiviral effects could be independent of GI-GPx, although supplementation with sodium selenite may further enhance the antiviral effects of retinoids.

To clarify the mechanism underlying the antiviral effect of Peretinoin further, we focused on the effect of Peretinoin on lipid metabolism because it has been shown to modify multiple aspects of HCV infection[14–16], and we detected a significant reduction of FASN

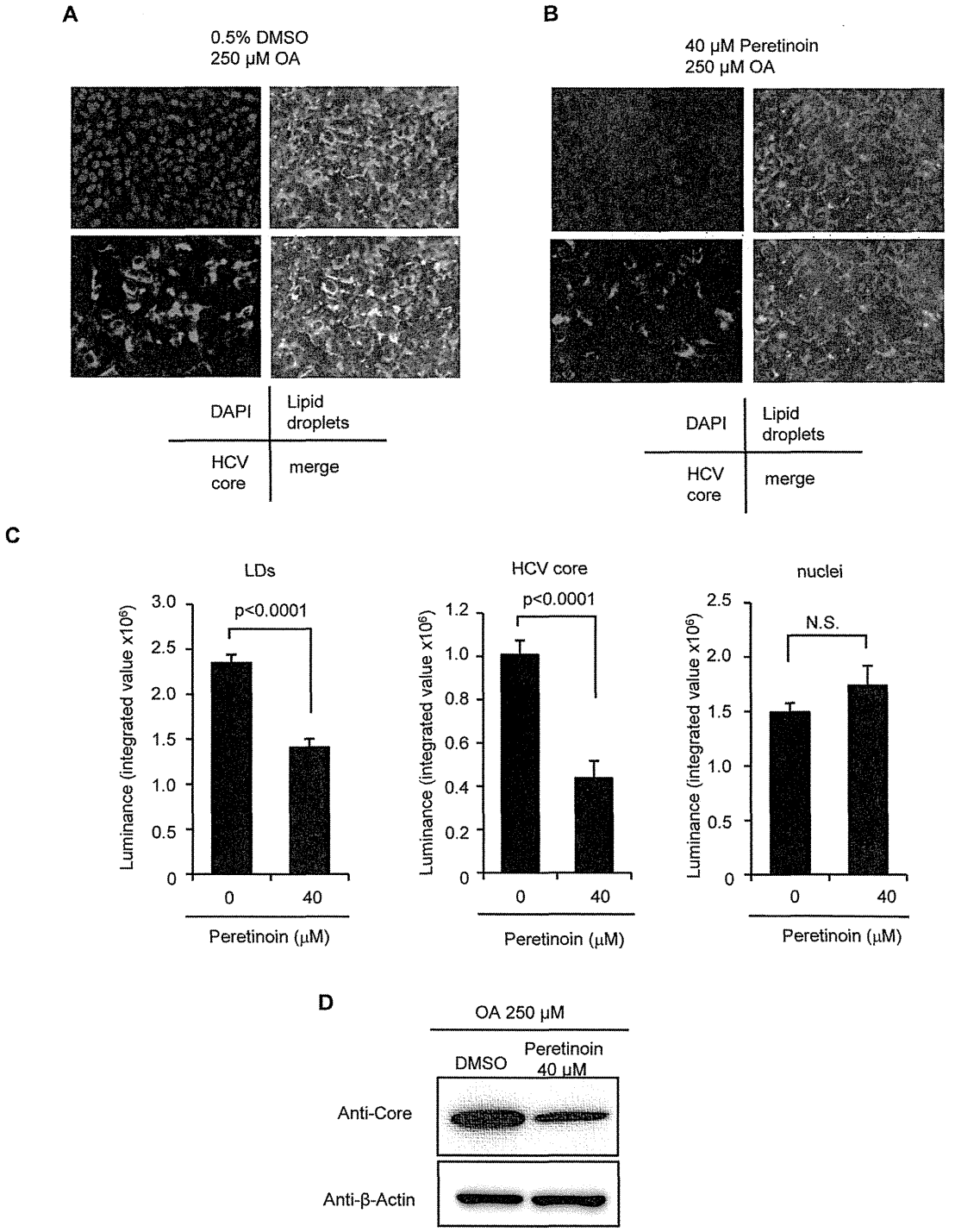

Figure 3 | Reduction of LD signals by Peretinoin. Huh-7.5 cells were infected with HJ3-5 virus at an MOI of 1, and 72 h later, 250 μM OA and DMSO or 250 μM OA and 40 μM Peretinoin were added, and the following assay was performed at 72 h later. (A, B) At 72 h later, the cells were fixed and stained for nuclei, LDs, and HCV core protein. (A) Shows 250 μM OA and DMSO-treated cells and (B) shows 250 μM OA and 40 μM Peretinoin-treated cells. The photos in (A) and (B) were taken under exactly the same conditions. (C) The signal intensity from LDs, HCV core protein, and nuclei was quantitated as described in the Methods. Data shown represent mean signal intensity ± SD from 4 different areas, and the difference was analysed statistically using Student's t-test. (D) Cell lysates were collected and subjected to western blot analysis using anti-core protein and anti-β-actin antibodies. Full-length blots/gels are presented in Supplementary Fig. S6 online. N.S., not significant.

mRNA levels by Peretinoin in a mouse hepatoma model, implying its possible effect on lipid metabolism[5]. Surprisingly, Peretinoin strongly reduced the signal from LDs in the presence of OA and intracellular TGs (Fig. 3A–C, 4A). LDs are known to have an essential role in the assembly of HCV virus particles by interacting with HCV core protein and NS5A[21,22]. Therefore, we examined the effect of Peretinoin on several steps of infectious virus production, such as assembly and secretion. Interestingly, Peretinoin specifically

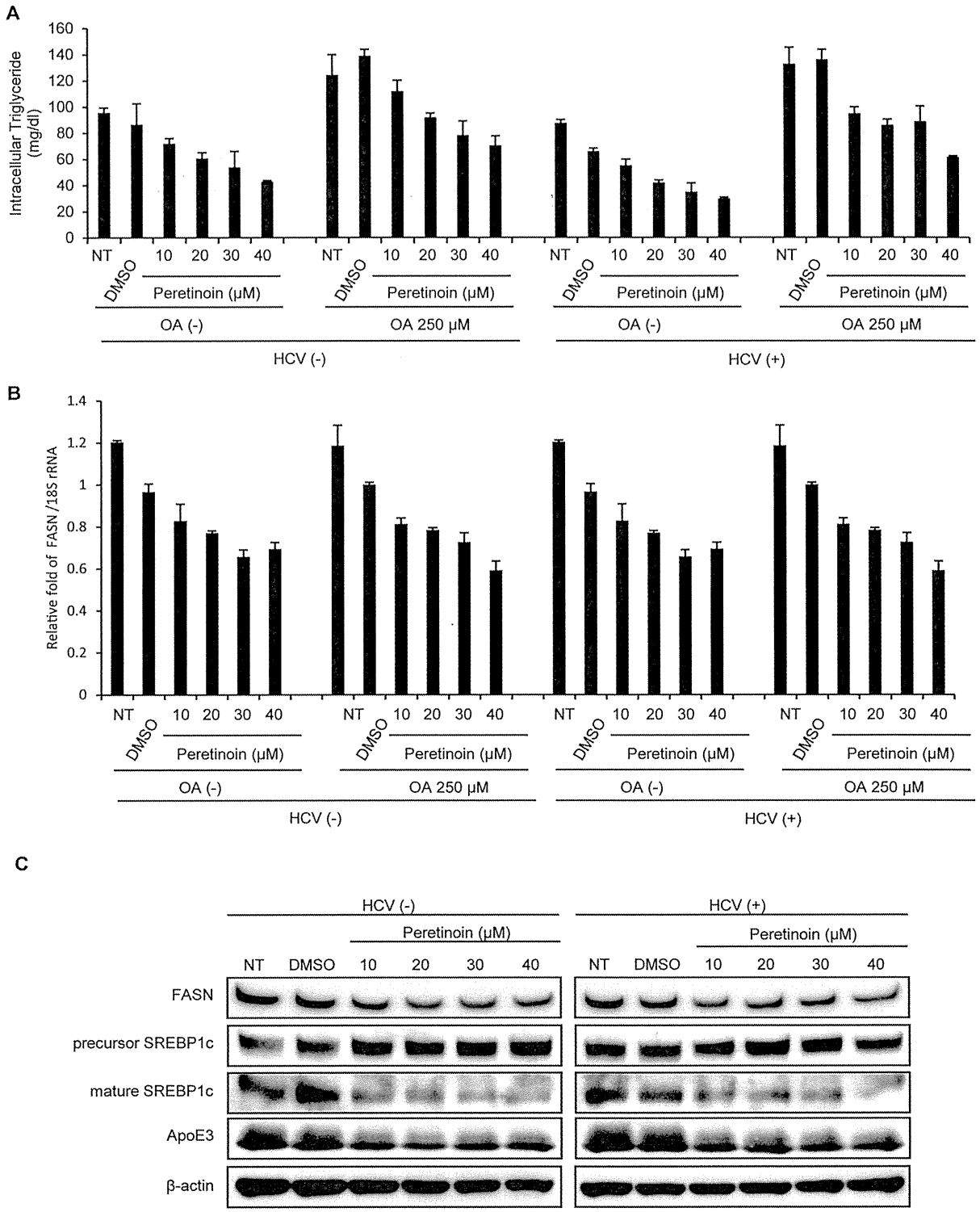

Figure 4 | Mechanism by which Peretinoin alters lipid metabolism. Huh-7.5 cells were transfected with H77S.3/GLuc2A RNA, and 72 h later, the transfected cells, depicted as 'HCV(+)', and non-transfected Huh-7.5 cells, depicted as 'HCV(-)', were treated with or without 250 μM OA in the presence of 2% fatty acid-free BSA with 0.5% DMSO or 10–40 μM Peretinoin, and the following assay was performed at 72 h later. (A) The concentration of intracellular TGs was measured. Data shown represent mean concentration ± SD from 3 independent experiments. (B) RNA was extracted and the levels of FASN mRNA and 18S rRNA were quantitated by RTD-PCR. FASN levels were normalised to those of 18S rRNA, and the ratio was furthermore normalised to that from DMSO-treated cells. The results presented here represent the relative fold of FASN/18S rRNA ± SD from 3 independent experiments at the indicated conditions. (C) Lysates from the cells without OA treatment were collected and subjected to western blot analysis using anti-FASN, anti-precursor SREBP1c, anti-mature SREBP1c, anti-ApoE3, and anti-β-actin antibodies. Full-length blots/gels are presented in Supplementary Fig. S7 online.

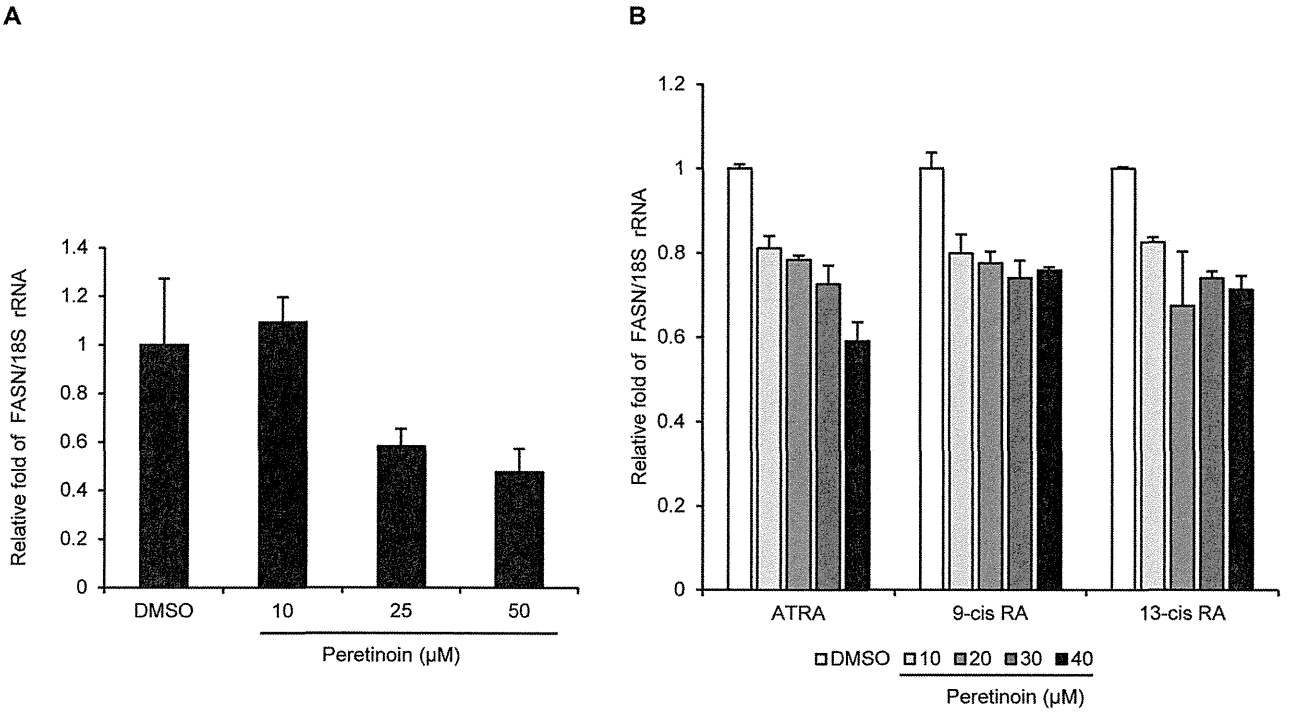

Figure 5 | Reduction of FASN mRNA levels by Peretinoin in a human hepatocyte cell line and the effects of ATRA, 9-cis RA, and 13-cis RA on the expression of FASN mRNA. (A) An immortalised human hepatocyte cell line, THLE-5b cells, was treated with the indicated concentrations of Peretinoin. At 72 h later, RNA was extracted and reverse transcribed, and the levels of FASN mRNA and 18S rRNA were quantified by RTD-PCR. The relative amount of FASN mRNA normalised to that of 18S rRNA is presented as fold change compared to DMSO-treated cells from 3 independent experiments at the indicated conditions. (B) Huh-7.5 cells were transfected with H77S.3/GLuc2A RNA. At 72 h later, the transfected cells were treated with DMSO or 10–40 μM ATRA, 9-cis RA, and 13-cis RA. At 72 h later, RNA was extracted and the levels of FASN mRNA and 18S rRNA were quantified by RTD-PCR. The relative amount of FASN mRNA was determined as described in Fig. 4 and presented as fold change compared to DMSO-treated cells from 3 independent experiments at the indicated conditions.

impaired virus secretion without affecting assembly at 10–30 μM, whilst 40 μM Peretinoin impaired virus secretion and assembly (Fig. 6A). The role of LDs in virus secretion has not been fully characterised, but virus should be secreted through the production and release of very low-density lipoproteins. In addition to microsomal triglyceride transfer protein and several apolipoproteins, such as ApoB and ApoE[23], small interfering RNA screening revealed that multiple components of the secretary pathway, including endoplasmic reticulum to Golgi trafficking and lipid and protein kinases, are involved in HCV secretion[24]. Thus, the mechanism underlying this specific inhibition of virus secretion by Peretinoin remains to be addressed. One possible explanation for its action is the reduction of ApoE3 expression (Fig. 4C), because ApoE3 was shown to have an important role in virus secretion with a minimal impact on assembly[25].

Several reports showed that LDs play an essential role in RNA amplification and virus assembly. The hypolipidemic agent nordihydroguaiaretic acid reduced the number of LDs, resulting in the suppression of RNA amplification and virus secretion, as Peretinoin did[26]. Furthermore, inhibition of tail-interacting protein 47, which coats LDs and is involved in their generation and turnover, suppressed HCV RNA replication and assembly[27,28]. Thus, the inhibition of RNA replication by Peretinoin could be explained by its direct effect on LDs. In addition, a recent report suggested that FASN may localise within HCV replication complexes through an interaction with NS5B, thereby increasing its RNA-dependent RNA polymerase activity[29]. Thus, Peretinoin may inhibit RNA replication not only by reducing the signalling of LDs but also inhibiting the expression of FASN.

We also demonstrated that Peretinoin reduced the levels of mature SREBP1c by inhibiting the proteolysis of its precursor, and subsequently the transcription and expression of FASN (Fig. 4C), which could be the main reason for the alteration of lipid metabolism by Peretinoin; however, the mechanism by which it inhibits proteolysis should be addressed in a future study. Several reports have shown that the expression of SREBP1c and/or FASN is increased in HCV-infected patients[30], Huh-7 cells[31], and a transgenic mouse expressing the full-length HCV polyprotein[32]. In addition, HCV infection was shown to enhance the proteolytic cleavage of precursor SREBP1c, resulting in an increase in its mature form[33]. Taken together, HCV induces lipogenesis to make infected cells more supportive for its propagation. In contrast to HCV, Peretinoin seems to suppress lipogenesis by inhibiting the SREBP1c-FASN axis, which is highly activated by HCV infection. It is also important to note that this effect did not depend on HCV infection, indicating that Peretinoin should exert a hypolipidemic effect, as we also observed a reduction of FASN mRNA levels following Peretinoin treatment of an immortalised human hepatocyte cell line (Fig. 5A). Interestingly, this effect was universal among retinoids because the other retinoids examined also reduced FASN mRNA levels (Fig. 5B). These findings suggest that Peretinoin could also be useful for the treatment of non-alcoholic fatty liver disease, whose hallmark is hepatic fat accumulation.

The antiviral EC_{50} of Peretinoin seems to be closer to its CC_{50} than that of the other retinoids in Huh-7.5 cells because several papers have shown that Peretinoin inhibits the growth of hepatoma cells *in vivo* and *in vitro*[34,35], induces apoptosis in human hepatoma cell lines[36], and causes an arrest of the cell cycle in G0-G1 in human hepatoma cell lines[35], indicating that Peretinoin should selectively

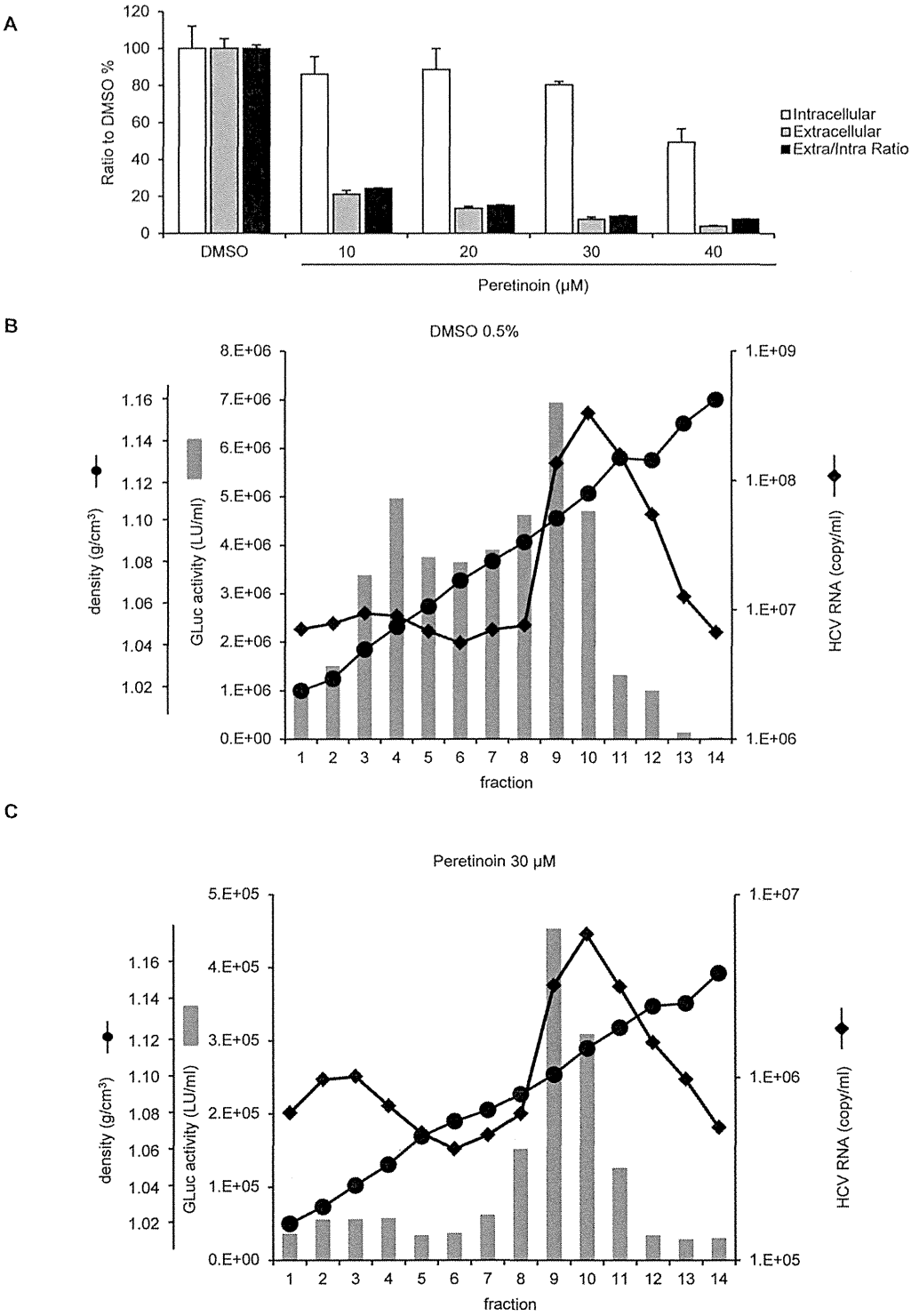

Figure 6 | Impact of Peretinoin on infectious virus production. (A) FT3-7 cells were transfected with HJ3-5/GLuc2A RNA, and 7 days later, 0.5% DMSO, or 10–40 μM Peretinoin, were added. At 72 h later, extra- and intra-cellular viruses were collected and used to infect naïve Huh-7.5 cells. Replication capacity was also determined by measuring secreted GLuc activity. At 48 h after infection, we determined the amount of infectious virus from extra- and intra-cellular media by using GLuc activity as an indicator of the amount of infectious virus. Intra- and extra-cellular infectivity was normalised to replication capacity at infection, and these were then normalised to those of DMSO-treated cells, which were set to 100%. The ratio of extracellular infectious virus to intracellular virus was calculated at the indicated conditions, and it was then normalised to DMSO-treated cells, which were set to 100%. Data show the mean ratio to that of DMSO-treated cells ± SD from 3 independent experiments. (B, C) FT3-7 cells were transfected with HJ3-5/GLuc2A RNA, and 7 days later, 0.5% DMSO or 30 μM Peretinoin were added, and then 72 h later, the medium was collected and subjected to equilibrium ultracentrifugation. Fourteen fractions were taken and analysed for density (circles), HCV RNA levels (diamonds), and infectious virus titres determined by GLuc activity (grey bars). (B) shows the results from DMSO-treated cells, whilst (C) shows those for Peretinoin-treated cells.

suppress the growth of hepatoma cells, although the mechanism has not been fully understood. However, pharmacokinetic data from humans showed that the mean plasma concentration of lipid-bounded Peretinoin is 7.3 μM when patients received 600 mg Peretinoin daily for 8 weeks[37]. This concentration is very close to the antiviral EC_{50} and could have an inhibitory effect on HCV replication, indicating that we could expect an antiviral effect at this dose in humans. Peretinoin showed an additive antiviral effect when combined with IFNα-2b (data not shown); furthermore, HCV did not acquire resistance to Peretinoin after 14 days treatment with 10–40 μM Peretinoin (see Supplementary Fig. S11 online). Although it could be difficult to eradicate HCV only by Peretinoin due to its low selective index (CC50/EC50), combination therapy with Peretinoin plus PEG-IFN, ribavirin, or DAAs may further improve the SVR rate, as vitamin D has been proved to do[38,39].

In summary, we have demonstrated that Peretinoin, which may in the future be administered to patients infected with HCV to prevent HCC, inhibits HCV RNA replication and infectious virus release by modifying several aspects of lipid metabolism.

Methods

Cell lines. Huh-7.5 (kindly provided by Professor C. M. Rice, Rockefeller University, New York, NY), and FT3-7 cells (both clonal derivatives of Huh-7 cells) were maintained as described previously[9]. We used an immortalised human hepatocyte cell line, THLE-5b cells[40], for the indicated experiments.

Reagents. Peretinoin and IFNα-2b were kindly provided by KOWA Company, Ltd. (Tokyo, Japan). ATRA, 9-cis RA, and 13-cis RA, were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Stock solutions were prepared in DMSO, and all final dilutions contained 0.5% DMSO.

Plasmids. The GLuc coding sequence, followed by the FMDV2A sequence, was inserted between p7 and NS2 in pJFH1 and pHCV-N.2, which encode cDNA of genotype 2a JFH1[13] and genotype 1b N[12], carrying several replication-enhancing mutations to be described elsewhere, respectively, by the same strategy adopted previously for H77S[10]. pH77S.3/GLuc2A[10], pHJ3-5/GLuc2A[9], and pHJ3-5[11] have been described previously.

Antiviral activity assay. The indicated HCV RNAs were transfected by electroporation. The medium was replaced with fresh medium containing serial dilutions of the antiviral compounds at 48 h, and at 24 h intervals thereafter. Secreted GLuc activity was determined at 72 h after adding the antiviral compounds. The concentration of each compound required to reduce the amount of secreted GLuc activity by 50% (EC_{50}) was determined using a 3-parameter Hill equation (Sigma Plot 10.0).

Cell number determination. Huh-7.5 cells were seeded in 96-well plates at a density of 5,000 cells/well, and at 24 h later, the indicated compounds were added. Cell numbers were determined by a WST-8 assay using Cell Counting Kit-8. The concentration of each compound required to reduce the amount of cell number by 50% (CC_{50}) was determined using a 3-parameter Hill equation (Sigma Plot 10.0).

RNA transcription. HCV RNAs were synthesised using a MEGAscript T7 Kit, and synthesised RNA was purified using an RNeasy Mini Kit.

Virus yield determination. Huh-7.5 cells were seeded in 48-well plates at a density of 4.0×10^4 cells/well at 24 h prior to inoculation with 100 μL of virus-containing medium. The cells were maintained at 37°C in a 5% CO_2 environment and fed with 300 μL medium at 24 h later. Following 48 h of additional incubation, the cells were fixed in methanol-acetone (1 : 1) at room temperature for 9 min and stained with a C7-50 monoclonal antibody to the HCV core protein (1 : 300). After extensive washing, the cells were stained with Alexa Fluor 568-conjugated anti-mouse IgG antibodies. A cluster of infected cells staining for core antigen was considered to constitute a single infectious FFU; virus titres are reported as FFUs/mL.

Western blotting and immunostaining. Western blotting and immunostaining were performed as described previously[41,42]. Briefly, the cells were washed in phosphate-buffered saline (PBS) and lysed in a radioimmunoprecipitation assay buffer containing complete Protease Inhibitor Cocktail and PhosSTOP. The membranes were blocked in Blocking One or Blocking One-P solution, and the expression of HCV core protein, FASN, precursor and mature SREBP1c, ApoE3, and β-actin was evaluated with mouse anti-core protein, rabbit anti-FASN, rabbit anti-SREBP1c, goat anti-ApoE3, and rabbit anti-β-actin antibodies, respectively.

For immunofluorescence staining, the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. After washing again with PBS, the cells were permeabilised with 0.05% Triton X-100 in PBS for 15 min at room temperature. They were incubated in a blocking solution (10% foetal bovine serum and 5% bovine serum albumin [BSA] in PBS) for 30 min, and then with the anti-core protein monoclonal antibodies. The fluorescent secondary antibodies were Alexa Fluor 568-conjugated anti-mouse IgG antibodies. Nuclei were labelled with DAPI, and LDs were visualised with BODIPY 493/503. Imaging was performed on a BIOREVO fluorescence microscope (Keyence Corporation, Osaka, Japan). The signal strength of LDs, core protein, and nuclei was quantitated by using Measurement Module BZ-H1M (Keyence Corporation).

Quantitative RTD-PCR. The primer pairs and probes for FASN and 18S rRNA were obtained from the TaqMan assay reagents library. HCV RNA was detected as described previously[43].

Secreted luciferase assay. Cell culture supernatant fluids were collected at intervals after RNA transfection and the cells were re-fed fresh medium. Secreted GLuc was measured as described previously[9].

Fatty acid treatment and measurement of TGs. The cells were treated with the indicated concentrations of OA in the presence of 2% fatty acid-free BSA. Intracellular TG content was measured using a TG Test according to the manufacturer's instructions.

Intra- and extra-cellular infectivity assay. To determine the amount of intra-cellular infectious virus, cell pellets of HJ3-5/GLuc2A-replicating FT3-7 cells harvested after trypsinization were resuspended in complete medium, washed twice with PBS, and lysed by 4 cycles of freezing and thawing. The lysates were clarified by centrifugation at 2,300 × *g* for 5 min prior to inoculation onto naïve Huh-7.5 cells. At the same time, extra-cellular medium was also collected. The medium derived from extra- and intra-cellular cultures was used to infect naïve Huh-7.5 cells, which were plated in 48-well plates at a density of 4.0×10^4 cells/well at 24 h prior to infection. After 6 h inoculation, medium containing virus and possible carryover of Peretinoin was removed by extensive wash, and medium was replaced with fresh one every 24 h until 48 h. At 48 h after infection, we determined GLuc activity and used it as an indicator of the infectious virus titre.

Equilibrium ultracentrifugation of HJ3-5/GLuc2A virus particles using an isopycnic iodixanol gradient. Filtered supernatant fluids collected from HJ3-5/GLuc2A virus-replicating FT3-7 cells treated with DMSO or 30 μM Peretinoin for 72 h were concentrated 30-fold using a Centricon PBHK Centrifugal Plus-20 Filter Unit with an Ultracel-PL membrane (100-kDa exclusion; Merck Millipore, Billerica, MA), then layered on top of a preformed continuous 10–40% iodixanol gradient in Hanks' balanced salt solution. The gradients were centrifuged in an SW41 rotor at 209,678 × *g* for 16 h at 4°C, and fractions (500 μL each) were collected from the top of the tube. The density of each fraction was determined using a digital refractometer. Virus RNA was isolated from each gradient fraction using a QIAamp Viral RNA Kit, and cDNA was synthesised using a High Capacity cDNA Reverse Transcription Kit. RTD-PCR to quantitate the amount of HCV RNA was performed using a 7500 Real Time PCR System. Each fraction was used to infect naïve Huh-7.5 cells for 6 h, followed by extensive washing to ensure GLuc activity was reduced to background. The infected cells were inoculated and the medium was replaced with fresh medium every 24 h. GLuc activity, which was used an alternative to the infectious virus titre, was determined at 72 h after infection.

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Author contributions

Study design and concept; T.S., T.S. and D.Y., Acquisition of data; T.S., T.S., F.L., K.M., T.S., R.T. and M.F., Drafting of the manuscript; T.S. and T.S., Critical revision of the manuscript for important intellectual content; M.H., D.Y., S.M., S.L. and S.K., Study supervision; M.H. and S.K. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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Hepatic Interferon-Stimulated Genes Are Differentially Regulated in the Liver of Chronic Hepatitis C Patients With Different Interleukin-28B Genotypes

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Pretreatment up-regulation of hepatic interferon (IFN)-stimulated genes (ISGs) has a stronger association with the treatment-resistant interleukin (IL)28B minor genotype (MI; TG/GG at rs8099917) than with the treatment-sensitive IL28B major genotype (MA; TT at rs8099917). We compared the expression of ISGs in the liver and blood of 146 patients with chronic hepatitis C who received pegylated IFN and ribavirin combination therapy. Gene expression profiles in the liver and blood of 85 patients were analyzed using an Affymetrix GeneChip (Affymetrix, Santa Clara, CA). ISG expression was correlated between the liver and blood of the MA patients, whereas no correlation was observed in the MI patients. This loss of correlation was the result of the impaired infiltration of immune cells into the liver lobules of MI patients, as demonstrated by regional gene expression analysis in liver lobules and portal areas using laser capture microdissection and immunohistochemical staining. Despite having lower levels of immune cells, hepatic ISGs were up-regulated in the liver of MI patients and they were found to be regulated by multiple factors, namely, IL28A/B, IFN-λ4, and wingless-related MMTV integration site 5A (WNT5A). Interestingly, WNT5A induced the expression of ISGs, but also increased hepatitis C virus replication by inducing the expression of the stress granule protein, GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1), in the Huh-7 cell line. In the liver, the expression of WNT5A and its receptor, frizzled family receptor 5, was significantly correlated with G3BP1. *Conclusions:* Immune cells were lost and induced the expression of other inflammatory mediators, such as WNT5A, in the liver of IL28B minor genotype patients. This might be related to the high level of hepatic ISG expression in these patients and the treatment-resistant phenotype of the IL28B minor genotype. (HEPATOLOGY 2014;59:828-838)

Interferon (IFN) and ribavirin (RBV) combination therapy has been a popular modality for treating patients with chronic hepatitis C (CHC); however, ~50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.[1] The recently developed direct-acting antiviral drug, telaprevir, combined with pegylated (Peg)-IFN plus RBV, significantly improved sustained virologic response (SVR) rates; however, the SVR rate was not satisfactory (29%-33%) in patients who had no

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL, CC chemokine ligand; CHC, chronic hepatitis C; CLLs, cells in liver lobules; CPAs, cells in portal areas; CXCL10/IP-10, chemokine (C-X-C motif) ligand 10/interferon-gamma-induced protein 10; CXCR3, chemokine (C-X-C motif) receptor 3; DCs, dendritic cells; DVL, disheveled; FZD5, frizzled family receptor 5; G3BP1, GTPase-activating protein (SH3 domain)-binding protein 1; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; IFI44, interferon-induced protein 44; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IFN, interferon; IHC, immunohistochemical; IL, interleukin; ISGs, interferon-stimulated genes; JFH-1, Japanese fulminant hepatitis type 1; LCM, laser capture microdissection; MA, major genotype; MAd, major genotype, down-regulated; MAu, major genotype, up-regulated; MI, minor genotype; Mx, myxovirus (influenza virus) resistance; NK, natural killer; OAS2, 2′-5′-oligoadenylate synthetase 2; PALT, portal-tract-associated lymphoid tissue; Peg_IFN, pegylated IFN; RBV, ribavirin; RTD-PCR, real-time detection polymerase chain reaction; SG, stress granule; siRNA, small interfering RNA; SVR, sustained virologic response; WNT5A, wingless-related MMTV integration site 5A.

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response to previous therapy.[2] Therefore, IFN responsiveness is still an essential clinical determinant for treatment response to triple (Peg-IFN+RBV+DAA) therapy.

A recent landmark genome-wide association study identified a polymorphism in the interleukin (IL)28B, IFN-λ3) gene that was associated with either a sensitive (major genotype; MA) or resistant (minor genotype; MI) treatment response to Peg-IFN and RBV combination therapy and was characterized by either up- (-u) or down-regulation (-d) of interferon-stimulated genes (ISGs).[3-5] However, the underlying mechanism for the association of this polymorphism and treatment response has not been clarified. Previously, we showed that up-regulation of the pretreatment expression of hepatic ISGs was associated with an unfavorable treatment outcome and was closely related to the treatment-resistant IL28B genotype (TG or GG at rs8099917).[6] It could be speculated that the pretreatment activation of ISGs would repress additional induction of ISGs after treatment with exogenous IFN. However, it is unknown how hepatic ISGs are up-regulated in treatment-resistant CHC patients and why patients with high levels of ISG expression cannot eliminate HCV. Therefore, other mechanisms should be involved in the unfavorable treatment outcome of patients with the treatment-resistant IL28B genotype.

In the present study, we performed gene expression profiling in the liver and blood and compared the expression of ISGs between them. Furthermore, ISG expression in liver lobules and portal areas was analyzed separately using a laser capture microdissection (LCM) method. Finally, we identified an immune factor that is up-regulated in patients with the treatment-resistant IL28B genotype and mediates favorable signaling for HCV replication.

Materials and Methods

Patients. We analyzed 168 patients with CHC who had received Peg-IFN-α2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks at the Graduate School of Medicine, Kanazawa University Hospital, Japan and its related hospitals, as reported previously (Table 1 and Supporting Table 1).[6]

Preparation of Liver Tissue and Blood Samples. A liver biopsy was performed on samples from 168 patients, and blood samples were obtained from 146 of these patients before starting therapy (Table 1 and Supporting Table 1). Detailed procedures are described in the Supporting Materials and Methods.

Affymetrix GeneChip Analysis. Liver tissue samples from 91 patients and blood samples from 85 patients were analyzed using an Affymetrix GeneChip (Affymetrix, Santa Clara, CA). LCM analysis was performed in 5 MAu, MAd, and MI patients. Affymetrix GeneChip analysis and LCM were performed, as described previously.[6,7] Detailed procedures are described in the Supporting Materials and Methods.

Hierarchical Clustering and Pathway Analysis of GeneChip Data. GeneChip data analysis was performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.htm), as described previously.[7] Pathway analysis was performed using MetaCore (Thomson Reuters, New York, NY). Detailed procedures are described in the Supporting Materials and Methods.

Quantitative Real-Time Detection Polymerase Chain Reaction, Cell Lines, Cell Migration Assay, Vector Preparation, HCV Replication Analysis, and Statistical Analysis. These procedures are described in detail in the Supplemental Material and Methods.

Results

Differential ISG Expression in Liver and Blood of Patients With Different IL28B Genotypes. Previously, we showed that pretreatment up-regulation of hepatic ISGs was associated with an unfavorable treatment outcome and was closely related to the treatment-resistant IL28B MI (TG or GG at rs8099917).[6] To examine whether expression of hepatic ISGs would reflect the expression of blood ISGs, we compared ISG expression between the liver and blood. We utilized three ISGs (interferon-induced protein 44 [IFI44], interferon-induced protein with

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