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Liver X receptor α-mediated regulation of lipogenesis by core and NS5A proteins contributes to HCV-induced liver steatosis and HCV replication

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Molecular mechanisms contributing to hepatitis C virus (HCV)-associated steatosis are not well established, although HCV gene expression has been shown to alter host cell cholesterol/lipid metabolism. As liver X receptors (LXRs) play a role as key modulators of metabolism signaling in the development of steatosis, we aimed to investigate in an HCV *in vitro* model the effect of HCV NS5A protein, core protein, and viral replication on the intracellular lipid accumulation and the LXRα-regulated expression of lipogenic genes. The effects of LXRα siRNA or agonist GW3965 treatment on lipogenesis and HCV replication capacity in our HCV replicon system were also examined. NS5A- and core-expressing cells and replicon-containing cells exhibited an increase of lipid accumulation by inducing the gene expression and the transcriptional activity of LXRα, and leading to an increased expression of its lipogenic target genes sterol regulatory element binding protein-1c, peroxisome proliferator-activated receptor-γ, and fatty acid synthase. Transcriptional induction by NS5A protein, core protein, and viral replication occurred via LXR response element activation in the lipogenic gene promoter. No physical association between HCV proteins and LXRα was observed, whereas NS5A and core proteins indirectly upregulated LXRα through the phosphatidylinositol 3-kinase pathway. Finally, it was found that LXRα knockdown or agonist-mediated LXRα induction directly regulated HCV-induced lipogenesis and HCV replication efficiency in replicon-containing cells. Combined, our data suggest that LXRα-mediated regulation of lipogenesis by core and NS5A proteins may contribute to HCV-induced liver steatosis and to the efficient replication of HCV.
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Hepatitis C virus (HCV) is a positive-sensed, single-stranded RNA virus of the *Flaviviridae* family.[1] The genome of the human HCV encodes a polyprotein posttranslationally cleaved by both viral/cellular proteases to produce four structural (core, E1, E2, and p7) and six nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins.[2] Over 170 million individuals worldwide are infected with HCV, which is a major etiological agent of liver diseases, including hepatic steatosis, cirrhosis, and hepatocellular carcinoma.[3]

Steatosis is present in almost 50% of patients infected by HCV,[4] and it has been reported to be associated with insuline resistance and hepatic fibrosis in some studies.[5–7] The mechanisms involved in HCV-induced steatosis seem to be mediated by HCV proteins, whose expression is associated with changes in host cell cholesterol/lipid metabolism.[8–10] Thereby, HCV core protein expression has been demonstrated to activate various pathways of lipid metabolism, contributing to the development of HCV-associated steatosis.[11–13] Other HCV proteins, such as NS2, NS4B, and NS5A, have also been shown to be able to modulate lipogenic gene expression.[14–16] However, the precise functions of HCV proteins in the development of fatty liver remain unknown.

Liver X receptors (LXRs) are members of the nuclear hormone receptor superfamily that work as fatty acid-activated transcription factors.[17] There are two isoforms termed LXRα and LXRβ, both expressed in the liver among other locations.[18] Peroxisome proliferator-activated receptor-γ (PPAR-γ) and sterol regulatory element-binding protein-1c

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(SREBP-1c) are two transcription factors activated by LXRα with an important role in regulating fatty acid synthesis.[19,20] Little is known, however, on the role of LXRα in the development of hepatic steatosis in HCV infection. We have recently shown that the LXRα gene and its lipogenic targets PPAR-γ, SREBP-1c, SREBP-2, fatty acid synthase (FAS), and fatty acid translocase CD36 are overexpressed in the liver of HCV genotype 1 patients.[21,22] Moreover, it has been described that HCV gene expression may activate the LXRα signaling pathway in *in vivo* and *in vitro* models.[15,23] Based on these data, it is conceivable that LXRα may be a key regulator of hepatic lipogenesis in HCV infection.

To gain some insight on this hypothesis, in this study we explored the effects of HCV NS5A protein, core protein, and virus replication on LXRα-mediated lipogenic gene expression in an *in vitro* model. Furthermore, several reports suggest that lipid biosynthesis affects HCV replication.[24–27] Therefore, we also examined the effects of LXRα knockdown or agonist-mediated LXRα induction on HCV RNA replication efficiency in HCV replicon-containing cells.

MATERIALS AND METHODS

Plasmid Constructs

The plasmid pcDNA3.1–NS5A was generated by subcloning into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) a PCR fragment encoding full-length NS5A protein from HCV genotype 1b (kindly provided by Dr Lai, University of Southern California, Los Angeles, CA, USA). The expression vector pEF–core was obtained by subcloning into pEF1α, previously described as pcDEF,[28] a PCR fragment encoding full-length core protein from HCV genotype 1b.

Cells, Cell Culture, and Treatment Protocols

Chang liver cells (CCL13; American Type Culture Collection, Manassas, VA, USA) and their derivative CHL–NS5A and CHL–core have been used.[29] CHL–NS5A and CHL–core were generated by stable transfection of CHL cells with pcDNA3.1–NS5A and pEF–core expression vectors, respectively. Western blot analysis confirms NS5A and core gene expression in transfectants. Empty vector (pcDNA3.1 or pEF, respectively) transfected cells were used as control (CHL). Huh7 cells expressing full-length genotype 1b HCV replicons (HCV-G1) were established as previously described.[30] HCV-G1 cells were treated with human interferon α-2b (IFNα-2b) to eliminate replicons,[30] and were used as control (Huh7). Huh7 and Chang liver cells and their derivatives were grown at 37 °C with a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamycin. CHL–NS5A, CHL–core, and HCV-G1 were selected by growth in culture medium containing G418. In order to prevent phenotypic drift, the cultures were used for only 8–10 weeks before reverting to frozen stocks from an early passage.

Table 1 Primers and probes used for the RT-qPCR

Gene	Genbank	Assay ID	Amplicon size	Dye
LXRα	NM_005693.2	Hs00172885_m1	78	FAM™
PPAR-γ	NM_015869.4	Hs01115510_m1	92	FAM
SREBP-1c	NM_004176.3	Hs00231674_m1	84	FAM
FAS	NM_004104	Hs01005611_m1	103	FAM
GAPDH	NM_002046.3	4326317E	122	VIC™

LXRα, liver X receptor α; PPAR-γ, peroxisome proliferator-activated receptor-γ; SREBP, sterol regulatory element-binding protein; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Triglyceride Assay

Intracellular triglyceride (TG) accumulation was evaluated after the lysis of cells. We collected the supernatants of each group to determine the TG content in the cell lysates. TG levels were determined with a kit from Biovision Research Products (Mountain View, CA, USA) following the guide provided by the company.

Flow Cytometry and Fluorescence Microscopy

The lipid content in cultured cells was determined by flow cytometry using Nile Red, a vital lipophilic dye used to label fat accumulation in the cytosol. Briefly, cell monolayers were washed twice with PBS and incubated for 15 min with Nile Red solution at a final concentration of 1 mg/ml in PBS at 37 °C, then washed twice, resuspended in PBS, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Nile Red fluorescence of 10 000 cells was analyzed using Cell Quest software (Becton Dickinson Biosciences). Indeed, intracellular lipid accumulation was corroborated by fluorescence microscopy using a Nikon Eclipse Ti inverted microscope (Nikon, Amstelveen, The Netherlands).

Quantitative Real-Time PCR

Total RNA was obtained by using a Trizol reagent (Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). For gene expression assays, cDNA was amplified using multiplex real-time PCR reactions on a StepOne Plus (Applied Biosystems).[21] TaqMan primers and probes were derived from the commercially available TaqMan® Gene Expression Assays (Applied Biosystems) (Table 1). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of GAPDH detection, referred to as ΔCT. PCR efficiency was determined by TaqMan analysis on a standard curve for targets and endogenous control amplifications that were highly similar. Replication studies were carried out using a SYBR Green kit (Roche Diagnostics GmbH) and two specific primer sets (5′-CCTGTGAGGAAC TACTGTCT-3′ and 5′-CTATCAGGCAGTACCACAAG-3′ for

HCV, spanning 255 nucleotides of the 5′ nontranslated region; 5′-AAAGCCGCTCGCAAGAGTGCG-3′ and 5′-ACTTGCCT CCTGCAAAGCAC-3′ for histone H3).[31] The number of HCV RNA copies was determined by crossing point interpolation into standard curves, which were generated by reverse transcription of serially diluted, *in vitro* synthesized viral RNA (genomic) followed by quantitative PCR. The total amount of RNA per reaction was kept constant (1 μg) by the addition of Huh7 RNA.

Western Blot

Protein extraction and western blotting were performed as described,[32] using rabbit polyclonal antibodies against LXRα (Abcam, Cambridge, UK), SREBP-1c (Abcam), PPAR-γ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-AKT (Ser 473; Santa Cruz Biotechnology), and AKT (Santa Cruz Biotechnology) or mouse monoclonal antibodies against FAS (Abcam), HCV NS5A protein (ViroStat, Portland, ME, USA), and HCV core protein (Thermo Scientific Pierce, Rockford, IL, USA). Bound primary antibody was detected with HRP (horseradish peroxidase)-conjugated anti-rabbit or anti-mouse antibodies (DAKO, Glostrup, Denmark), and blots were developed using an enhanced chemiluminescence detection system (ECL kit, Amersham Pharmacia, Uppsala, Sweden). The density of the specific bands was quantified with an imaging densitometer (Scion Image, Frederick, MD, USA).

Electrophoretic Mobility Shift Assays

Binding activity of LXRα was determined in nuclear cell extracts by means of electrophoretic mobility shift assay (EMSA). Nuclear cells extracts were prepared as previously described.[33] To assess the purity of the nuclear extracts, western blots for several enzyme-specific subcellular fractions were carried out. No contamination with the membrane and the cytosolic or mitochondrial fractions occurred. Oligonucleotides were end-labeled with [γ-32P]ATP to a specific activity $>5\times10^7$ c.p.m./μg DNA-LXR response element (LXRE) consensus: 5′-AGCTTGAATGACCAGCAGTAACCT CAGC-3′ (Panomics, Fremont, CA, USA). Nuclear extract (40 μg) was incubated for 20 min at room temperature in binding buffer in the presence of $\approx$1 ng labeled oligonucleotide ($\approx$250 μCi (Amersham Redivue)). To verify that the results from EMSA analysis did not arise from nonspecific binding, competition experiments were also carried out using a negative control (Cold probe) and cell sample + nonspecific competitor (NC probe). The nonspecific competitor reaction used an oligonucleotide with a different sequence to the specific oligonucleotide listed above. In this instance, SP1 oligonucleotide (Promega, Madison, WI, USA) was used. For specific signal, addition of nonradiolabeled specific competitor would decrease the signal intensity. An additional aliquot was prepared and loaded onto the gel, which contained all reagents with the exception of sample (negative control or cold probe). Protein–DNA complexes were separated from the free DNA probe by electrophoresis through 6% native polyacrylamide gels containing 10% ammonium persulfate and 0.5× Tris-borate-EDTA buffer. Gels were dried under vacuum on Whatmann DE-81 paper and exposed for 48–72 h to Amersham Hyperfilms at −80 °C.

ChIP (Chromatin Immunoprecipitation) Assays

Chromatin of cultured cells was fixed and immunoprecipitated according to Borrás *et al*.[34] Crosslinking between transcription factors and chromatin was achieved via the addition of formaldehyde (1% final concentration) for 10 min at 37 °C, and then halted via the addition of 125 mM glycine for 5 min at room temperature (25 °C). Chromatin solutions were sonicated and incubated with anti-LXRα, anti-SREBP-1c (both from Abcam), and an antibody against RNA polymerase II (Santa Cruz Biotechnology),[35] and then rotated overnight at 4 °C. The immune complexes were collected with Protein A or G Sepharose slurry (Invitrogen) and salmon sperm DNA for 4 h with rotational washing and then incubated overnight at 65 °C for reverse crosslinking. The DNA from all the samples was purified with a PCR purification kit (Qiagen) and used for PCR analysis of the target genes.

PCR Analysis of the Immunoprecipitated Chromatin

Chromatin DNA was subjected to PCR analysis with appropriate primers pairs to amplify products of 200–300 bp in length, corresponding to the flanking region of the LXRE and sterol response element (SRE) binding sites on the human SREBP-1c and FAS promoter or the coding regions of the target genes (Table 2). PCR fragments were size-fractionated by 2% (w/v) agarose gel electrophoresis and stained with ethidium bromide.

Table 2 Primer sequences for ChIP assays

Gene	Promoter regions
SREBP-1c	5′-GCAGGAAGATGAGACAAATAAAGACA-3′
	5′-AATGTGGTGAGACCAACAAGCA-3′
FAS	5′-CTTGCTCCATGGGAATCCA-3′
	5′-GGGCGACAGAGCAAGACTCT-3′
HPRT	5′-ATTCGTTCAAATGCATCCTGAAAA-3′
	5′-GGTCGTAGTCGTAGTGTCAAAAGT-3′
Gene	Coding regions
SREBP-1c	5′-CCCATTCCCCAACCTAAAGC-3′
	5′-TCCTTAAGAGTAAAAAACAGTCATTGCA-3′
FAS	5′-GAACTCCTTGGCGGAAGAGA-3′
	5′-CTCGTTGAAGAACGCATCCA-3′
HPRT	5′-CATTATTTAAACAGGGTAGCAGTAC-3′
	5′-CAATGTGTGCAGTAGTCAGTAAATC-3′

SREBP, sterol regulatory element binding protein; FAS, fatty acid synthase; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

Immunofluorescence Analysis

To study the localization of LXRα and HCV proteins, double staining of LXRα and core or NS5A was performed on HCV-G1 cells. Cells were grown on coverslips for 48 h. Cells were washed with PBS and fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and pretreated with blocking solution. After fixation and after blocking the nonspecific binding, the coverslips were incubated with rabbit anti-LXRα (Abcam) and mouse anti-core (Thermo Scientific Pierce) or anti-NS5A (Virostat) antibodies at 4 °C overnight. Thereafter, the secondary antibodies donkey anti-rabbit conjugated with FITC (Jackson ImmunoResearch, Baltimore, PA, USA) or donkey anti-mouse conjugated with Texas Red (Jackson ImmunoResearch) were applied. After washing, the coverslips were mounted on DakoCytomation Fluorescent Mounting Medium (DAKO). The preparations were analyzed with an inverted fluorescence microscope (Nikon Eclipse Ti).

Co-Immunoprecipitation

Huh7 and HCV-G1 cells were washed with ice-cold PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% NP-40, 1 mM NaF, 1 mM Na_3VO_4, and EDTA-Free Halt Protease Inhibitor Cocktail (Thermo Scientific Pierce) for 30 min on ice. Total cell lysates (1 mg of protein) were subjected to immunoprecipitation with 2 μg of anti-LXRα (Santa Cruz Biotechnology), anti-core (Thermo Scientific Pierce), or anti-NS5A (Virostat) overnight at 4 °C. Protein G Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was added and incubation continued for 1 h at 4 °C. Precipitates were washed three times with ice-cold lysis buffer, resuspended in Laemmli buffer, and boiled for 10 min. Bound proteins were separated on a SDS-polyacrylamide gel and analyzed by western blotting using the indicated antibodies.

LXRα Small Interfering RNA (siRNA) and siRNA Transfection

For the siRNA-mediated downregulation of LXRα, LXRα-specific siRNA and negative control siRNA were purchased from Ambion (Austin, TX, USA). Delivery of siRNAs into HCV-G1 cells was performed by reverse transfection methods as per the manufacturer's protocol. Nontargeting siRNA (17 μl) or LXRα siRNA (2 μM) and 283 μl of OPTI-MEM® I Reduced Serum Medium (GIBCO, Grand Island, NY, USA) were mixed in six-well plastic plates and incubated at room temperature for 10 min after addition of 5 μl of siPORT (Ambion). Then, 2.4 ml of suspended HCV-G1 cells (2.0×10^5 cells/ml) in growth medium without antibiotics was added. Following siRNA transfection (30 h), the medium was replaced with fresh normal growth medium and then the cells were used for analysis and experimentation at 48 h.

LXRα Agonist Treatment

LXR agonist GW3965 was kindly provided by GlaxoSmith Kline (Cambridge, UK). HCV replicon-containing cells were serum-deprived and treated with the LXR ligand GW3965 (5 μM) for 24 h. For all data shown, individual experiments were repeated at least three times with different preparation of cells.

Statistical Analysis

Results are expressed as the mean ± s.d. Significant differences were evaluated by one-way analysis of variance (ANOVA) and Newman–Keuls test. $P < 0.05$ was considered to be significant for a difference.

RESULTS

HCV NS5A Expression, Core Expression, and Viral Replication Induce Intracellular Lipid Accumulation

Results of flow cytometric analysis of Nile Red-stained CHL–NS5A, CHL–core, and HCV-G1 cells are presented in Figure 1a. Lipid accumulation increased significantly in cells expressing HCV proteins compared with control cells (CHL–NS5A: + 245%, CHL–core: + 107%). Representative fluorescence images of Nile Red-stained cells, corroborating the results obtained by flow cytometry, are exhibited in Figure 1b. Similarly, intracellular triglyceride content was significantly enhanced when HCV proteins were expressed (CHL–NS5A: + 144%, CHL–core: + 56%; Figure 1c). Noteworthy, total lipid and TG accumulation in CHL–NS5A cells were significantly higher than in CHL–core cells. We next analyzed the effect of HCV replication on intracellular total lipid and triglyceride accumulation (Figure 1a–c) and, as expected, a significant increase in Nile Red fluorescence and TG content was observed in HCV replicon-containing cells compared with Huh7 cells (+ 223% and + 169%, respectively).

HCV NS5A Protein, Core Protein, and Viral Replication Increase LXRα and Its Related Lipogenic Gene Expression

The expression of HCV core and NS5A proteins in CHL–core and CHL–NS5A cells, respectively, or in HCV-G1 cells were confirmed by immunoblotting (Figure 2c). We observed a significant induction of LXRα gene expression in CHL–NS5A (mRNA, + 190%, protein, + 65.9%) and CHL–core cells (mRNA, + 40%, protein, + 31.3%) compared with control cells (Figure 2a–c). Regarding gene expression of LXRα-related lipogenic genes, we also found a significant overexpression of SREBP-1c, PPAR-γ, and FAS in NS5A-expressing cells (mRNA, + 330%, + 307%, and + 90%, respectively; protein, + 81.8%, + 56%, and + 53.1%, respectively) and in CHL–core cells (mRNA, + 60%, + 205%, and + 50%, respectively; protein, + 38.1%, + 28.5%, and + 40.2%, respectively) compared with CHL cells (Figure 2a–c). Interestingly, LXRα, SREBP-1c, and PPAR-γ upregulation was significantly higher when NS5A protein was expressed. Finally, LXRα and its

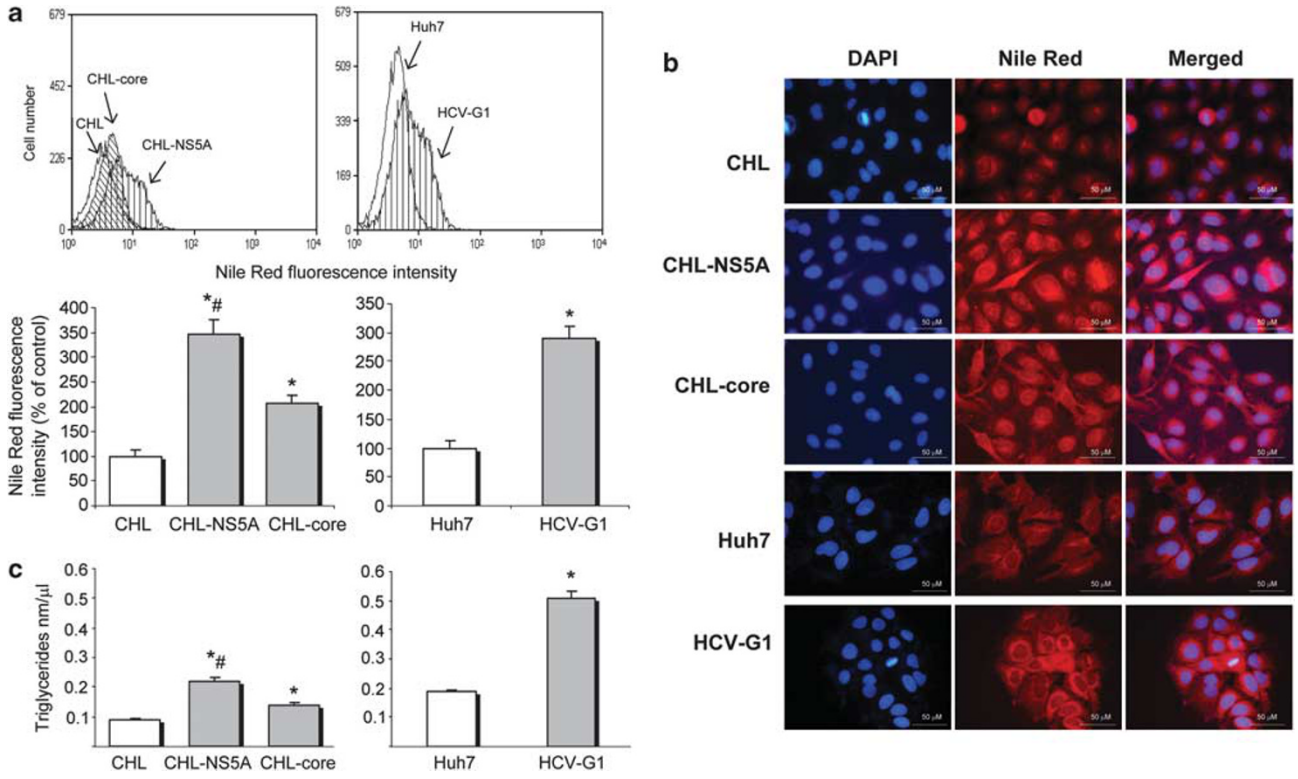

Figure 1 Effect of HCV proteins and viral replication on intracytoplasmic lipid accumulation. (**a**) Representative histograms of Nile Red fluorescence obtained by flow cytometry in CHL, CHL–NS5A and CHL–core cells or Huh7 and HCV-G1 cells (see Materials and Methods section for experimental details). Results are expressed as Nile Red relative fluorescence intensity and normalized to control cells. (**b**) Representative fluorescent images of Nile Red-treated cells. Nuclei were stained with DAPI (blue). Photographs shown are typical results of six independent experiments. (**c**) Triglyceride assay was performed on cell lysates as indicated in the Materials and Methods. Data are described as the mean values ± s.d. of six experiments (*$P<0.05$ *vs* CHL or Huh7, #$P<0.05$ *vs* CHL–core).

related lipogenic genes were significantly overexpressed in HCV-replicating cells (mRNA, LXRα: +140%; SREBP-1c: +123%; PPAR-γ: +221%; FAS: +70%; protein, LXRα: +161.8%; SREBP-1c: +165.9%; PPAR-γ: +118.3%; FAS: +64.5%; Figure 2a–c). Overall, these results indicate that HCV NS5A and core proteins may contribute to a different extent to the hepatic lipid accumulation observed in replicating cells through the overexpression of LXRα and its related target genes.

LXRα Mediates HCV NS5A, Core, and Viral Replication-Induced Lipogenic Gene Overexpression

To establish the contribution of LXRα activation to HCV-mediated induction of lipogenic genes described above, EMSA and ChIP assays were carried out. Figure 3a and b depicts EMSA results for LXRE binding in CHL and Huh7 and their derivative cells. HCV proteins and viral replication caused a significant activation of LXRα compared with their controls (CHL–NS5A: +101%, CHL–core: +98%, *vs* CHL; HCV-G1: +58%, *vs* Huh7). To determine the *in vivo* nuclear binding of LXRα to the SREBP-1c and FAS LXRE promoter region, ChIP assays were performed with an affinity-purified antibody directed against LXRα (Figure 3c and e). Our data clearly show the binding of LXRα to the SREBP-1c and FAS promoters in CHL–NS5A and CHL–core cells, whereas it was undetectable in CHL cells (Figure 3c). However, binding to LXRE promoter region of SREBP-1c and FAS was also evident in HCV-G1 cells (Figure 3e). Binding to the HPRT promoter (used as a negative control) was not observed, indicating specific binding of LXRα to LXRE in SREBP-1c and FAS promoters. When the same crosslinked chromatin samples used for the ChIP assay with anti-LXRα antibody were also used for ChIP assay with an antibody against RNA polymerase II, no signal was observed in CHL or Huh7 cells (Figure 3d and f). However, the RNA polymerase was bound to the coding region of SREBP-1c and FAS in CHL-NS5A, CHL-core, and HCV-G1 cells, providing an excellent internal control of real-time transcription (Figure 3d and f). To examine the contribution of SREBP-1c activation to LXRα-mediated lipogenic induction, we also conducted ChIP assays for the analysis of the binding of SREBP-1c to SRE within the promoter region of SREBP-1c and FAS. As shown in Figure 4a, SRE was activated in the SREBP-1c and FAS promoter regions of CHL–NS5A and CHL–core cells. No PCR products were detected in control group. ChIP assay also demonstrated that SRE was clearly activated in the

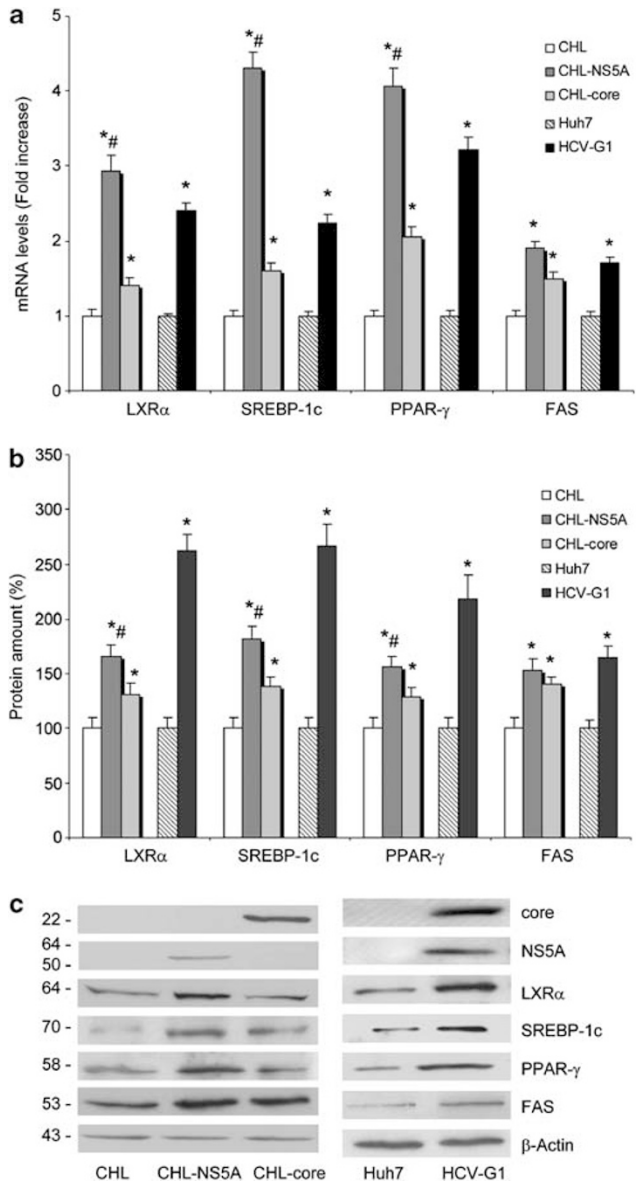

Figure 2 Overexpression of LXRα and its related lipogenic genes in CHL–NS5A, CHL–core, and HCV-G1 cells. (**a**) Bar graph shows LXRα, SREBP-1c, PPAR-γ, and FAS mRNA levels determined by real-time qRT-PCR as described. (**b**, **c**) LXRα, SREBP-1c, PPAR-γ, and FAS protein levels in CHL, CHL–NS5A, and CHL–core cells or Huh7 and HCV-G1 cells were analyzed by western blot. Densitometry analysis of specific bands expressed as percentage relative to CHL cells (100%). β-Actin levels were used as loading control. Molecular weight markers (kDa) are indicated on the left. Photographs are representative of six independent experiments. Data are described as the mean values ± s.d. of six experiments (*$P<0.05$ *vs* CHL or Huh7, #$P<0.05$ *vs* CHL–core).

SREBP-1c and FAS promoter of replicon-containing cells, postulating to be involved in the modulation of the expression of both genes (Figure 4c). The presence of RNA polymerase II at the coding region of SREBP-1c and FAS indicates the current transcription of the genes (Figure 4b and d). Together, these results indicate that HCV core and NS5A proteins also induced SREBP-1c transcriptional activity, at least in part in a LXRα-mediated manner, contributing to increased lipogenesis associated to HCV.

LXRα Does Not Physically Interact with HCV NS5A or Core Proteins in HCV-G1 Cells

To characterize crosstalk between LXRα and HCV proteins, we examined whether these proteins were physically associated. First, we performed double immunofluorescence staining of LXRα and HCV NS5A or core proteins in cells (Figure 5a). Fluorescence microscopy analysis showed that NS5A and core are mostly localized in the cytoplasm, with a minimal presence in the nucleus. Nevertheless, LXRα is mainly localized in the nucleus of HCV-G1 cells, and although it is also present in the cytoplasm in a lesser extent, no apparent association with HCV proteins was found. Second, reciprocal co-immunoprecipitation experiments were carried out. The results obtained showed that LXRα and HCV proteins do not co-immunoprecipitate, and this indicated that these proteins are not physically associated in HCV-G1 cells (Figure 5b).

Inhibition of the PI3K/AKT Pathway Attenuates HCV NS5A Protein, Core Protein, and Viral Replication-Mediated LXRα Upregulation

We investigated the effect of phosphatidylinositol 3-kinase (PI3K) chemical inhibition with LY294002 (Tocris Bioscience, Bristol, UK)[15] on LXRα upregulation associated to HCV protein expression and viral replication. AKT activation was evaluated by western blot using antibodies against phospho-AKT and AKT. We observed a significant induction of AKT phosphorylation in CHL–NS5A (+132%), CHL–core (+52%), and HCV-G1 cells (+140%) compared with their controls (Figure 6a and b). LY294002-treated cells exhibited a reduced AKT activity compared with nontreated cells (CHL–NS5A: −91%, CHL–core: −72%, and HCV-G1: −79%; Figure 6a and b), indicating that AKT phosphorylation induced by HCV proteins and viral replication was regulated by PI3K. Additionally, AKT activity inhibition was accompanied by a reduction of HCV NS5A and core proteins and viral replication-mediated LXRα overexpression (mRNA, CHL–NS5A: −45%, CHL–core: −58%, HCV-G1: −46%; protein, CHL–NS5A: −81%, CHL–core: −68%, HCV-G1: −84% *vs* non-treated cells; Figure 6 a–c). These results suggested a role for PI3K/AKT signaling pathway activity in HCV NS5A and core proteins and viral replication-mediated LXRα modulation.

Effect of LXRα Knockdown and LXRα Activation on Lipogenic Gene Expression and HCV RNA Replication in HCV-G1 Cells

To confirm that HCV-induced lipogenesis is associated with the LXRα-dependent pathway, we examined the effect of LXRα knockdown or GW3965 LXR agonist treatment on lipid accumulation and lipogenic gene expression in HCV-G1

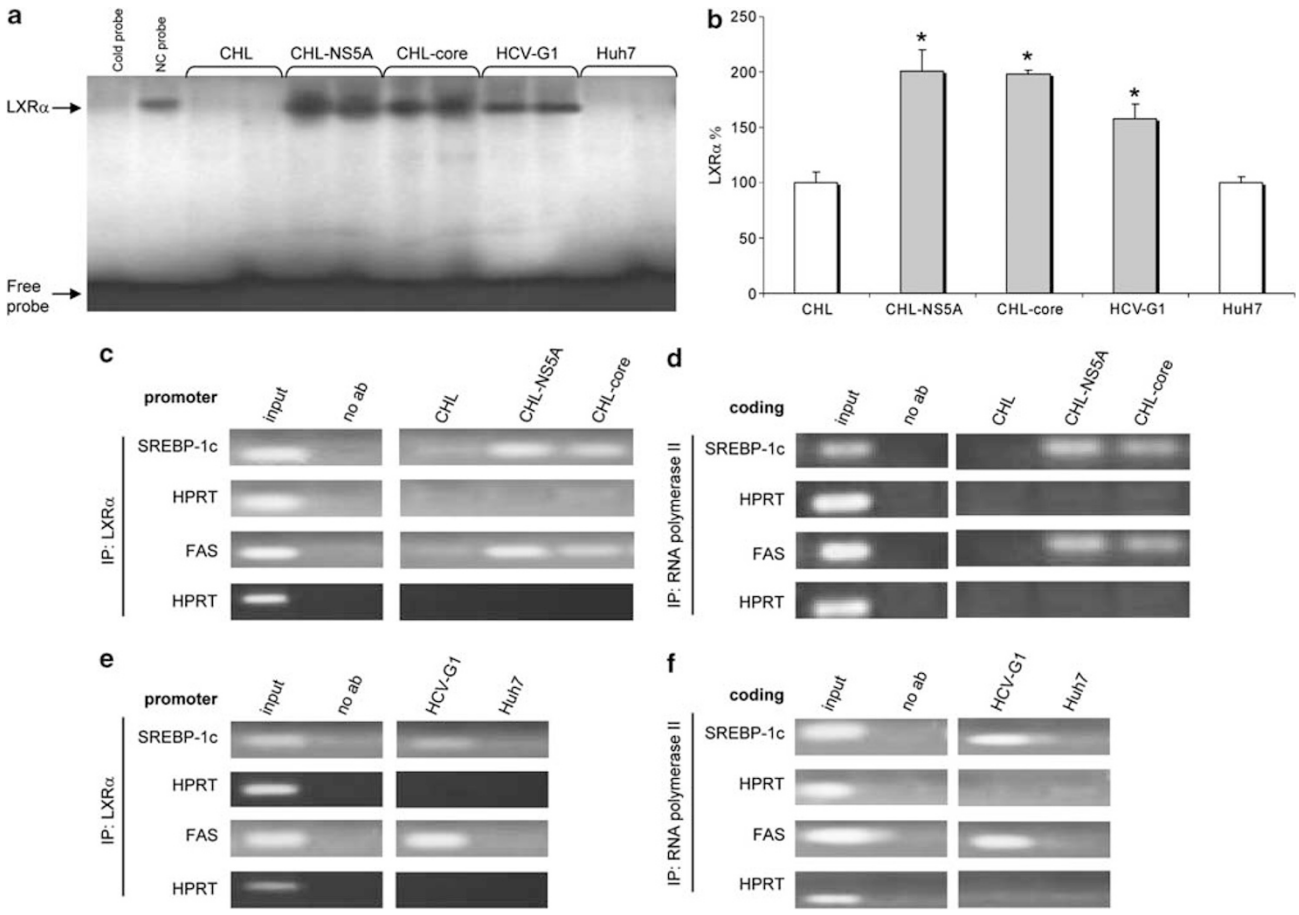

Figure 3 LXRα mediates HCV core and NS5A proteins and viral replication-induced lipogenic gene overexpression. (**a**, **b**) LXRα activation is determined by electrophoretic mobility shift assay (EMSA) in cell nuclear extracts. Left: representative EMSA results. Specific binding was verified by the addition of unlabelled oligonucleotide (Cold probe) or labelled oligonucleotide mutate (non-competitor, NC probe) (**a**). Right: densitometric analysis of EMSA. Data are described as the mean values ± s.d. of four separate experiments (*$P<0.05$ *vs* CHL or Huh7) (**b**). (**c**–**f**) Schematic presentation of the chromatin immunoprecipitation assay (ChIP assay). Immunoprecipitation of formaldehyde-crosslinked chromatin with specific antibodies against the LXRα (**c**, **e**) or RNA polymerase II (**d**, **f**) immunoprecipitates were aliquoted and subsequently analyzed by PCR with specific primers either for the SREBP-1c and FAS promoters (**c**, **e**) or the SREBP-1c and FAS coding regions to study the current transcription of the gene (RNA polymerase II ChIP assay) (**d**, **f**). Total chromatin (Input) and samples containing no antibodies (no ab) were included in the PCR reactions. The PCR products obtained with specific oligonucleotides for the HPRT promoter and coding region were included as negative controls. Results are representative of three independent experiments.

cells. Negative control siRNA did not affect either LXRα gene expression or HCV RNA replication and HCV protein expression, constituting a suitable control in this study. As shown in Figure 7a, siRNA for LXRα significantly decreased mRNA levels of LXRα (−92%) and its lipogenic target genes (SREBP-1c: −74%; PPAR-γ: −72%; and FAS: −77%) compared with negative control siRNA-treated HCV-G1 cells. In the same manner, intracytoplasmic lipid accumulation was significantly reduced in the LXRα knockdown HCV-G1 cells (−60% *vs* control siRNA-treated HCV-G1 cells). As shown in Figure 7b and c, siRNA LXRα-mediated lipogenic inhibition was accompanied by a significant reduction in number of copies of HCV RNA (−35%) and HCV NS5A and core protein levels. On the other hand, mRNA levels of LXRα, SREBP-1c, PPAR-γ, and FAS were significantly increased by LXR agonist treatment (+361%, +337%, +273%, and +154%, respectively) compared with negative control siRNA-treated HCV-G1 cells (Figure 7a). Furthermore, lipogenic gene upregulation was accompanied with lipid overaccumulation in GW3965-treated cells (+143% *vs* control siRNA-treated HCV-G1 cells). Additionally, LXR agonist treatment also induced HCV RNA replication (+160%) and NS5A and core expression (Figure 7b and c). To examine whether the effect of agonist on HCV replication is indeed due to LXRα activation, LXRα knockdown HCV-G1 cells were treated with GW3965. Interestingly, LXR agonist treatment did not affect HCV RNA replication and NS5A and core expression in siRNA LXRα-treated HCV-G1 cells (Figure 7b and c). In view of these findings, we suggest a potential role of LXRα in the regulation of HCV RNA replication.

DISCUSSION

Despite the fact that HCV infection is often associated with hepatic steatosis, the molecular mechanisms of HCV-medi-

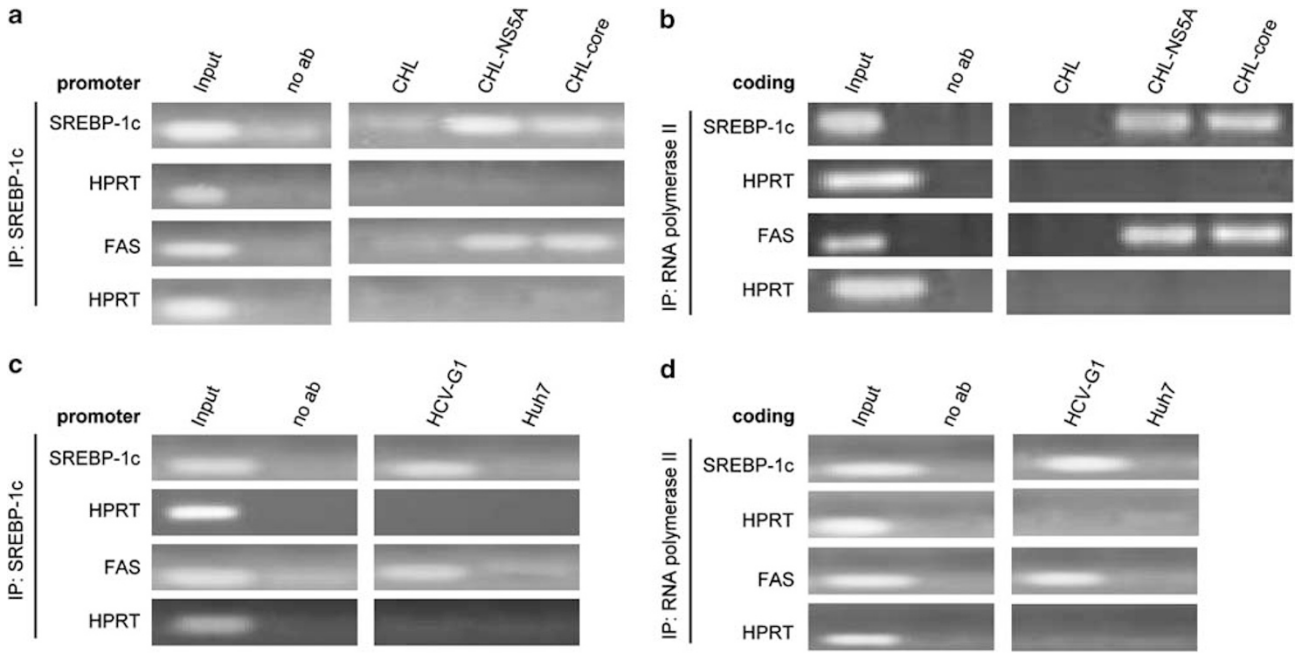

Figure 4 HCV core and NS5A proteins and viral replication enhance the binding to sterol regulatory element (SRE) within SREBP-1c and FAS promoters. Schematic presentation of the chromatin immunoprecipitation assay (ChIP assay). Immunoprecipitation of formaldehyde-crosslinked chromatin with specific antibodies against the SREBP-1c (**a**, **c**) or RNA polymerase II (**b**, **d**) immunoprecipitates were aliquoted and subsequently analyzed by PCR with specific primers either for the SREBP-1c and FAS promoters (**a**, **c**) or the SREBP-1c and FAS coding regions to study the current transcription of the gene (RNA polymerase II ChIP assay) (**b**, **d**). Total chromatin (Input) and samples containing no antibodies (no ab) were included in the PCR reactions. The PCR products obtained with oligonucleotides specific for the HPRT promoter and coding region were included as negative controls. Results are representative of three independent experiments.

ated steatosis are not completely understood.[8,9,36] Here, we show the implication of the LXRα regulatory pathway on HCV-mediated lipogenesis. The most interesting finding of this study is that HCV replication (genotype 1b) induced a LXRα-mediated intracellular lipid accumulation and lipogenic gene upregulation, supporting the results previously obtained in HCV patients.[21] In turn, we demonstrate that both HCV core and NS5A proteins contribute to the LXRα-mediated lipogenesis associated to HCV expression by indirect upregulation of LXRα through the PI3K pathway. Finally, we show that LXRα expression blockade or agonist-mediated LXRα induction directly regulate HCV-induced lipogenesis and viral replication capacity.

There is experimental evidence that LXRα induces the expression of lipogenic genes involved in fatty acid synthesis.[19,20] In our study, HCV NS5A and core proteins induced intracellular total lipid and triglyceride accumulation by upregulating gene expression and the transcriptional activity of LXRα, and leading to an increased expression of its lipogenic target genes SREBP-1c, PPAR-γ, and FAS. Interestingly, lipogenic capacity was significantly higher when NS5A protein was expressed. LXRα forms heterodimers with the retinoid X receptor (RXR)α and activates SREBP-1c, PPAR-γ, and FAS transcription by binding to the LXRE sequences in their promoters.[20,37,38] In turn, SREBP-1c activates genes involved in lipid and cholesterol metabolism, such as FAS, through binding to the SRE in the gene promoters.[38,39] In the current study, transcriptional regulation occurred via activation of the LXRE present in the promoter of lipogenic genes, thereby suggesting that the core and NS5A-mediated regulation of lipogenesis involves direct targeting of LXRα. We also showed that SRE in the SREBP-1c promoter was involved in SREBP-1c activation by HCV core and NS5A proteins. Furthermore, we observed that FAS transcription was upregulated by both HCV proteins, as a result of LXRα and SREBP-1c activation.

It has been previously described that HCV core protein can induce transcriptional activation of the SREBP-1c promoter by increasing the binding of LXRα/RXRα to LXRE in an *in vivo* model.[40] However, HCV core protein was not included in the LXRα/RXRα-LXRE complex, suggesting that HCV core protein indirectly activates the SREBP-1c promoter.[40] Moreover, it has been shown that NS5A protein augments hepatic lipid accumulation by inducing the activation of PPAR-γ and SREBP-1c.[14,41] However, to our knowledge, this is the first line of evidence that HCV NS5A induces LXRα-mediated lipogenic pathways. Nevertheless, the exact mechanisms responsible for LXRα activation by HCV NS5A and core proteins are not clear. It has been shown that hepatitis B virus X protein induces lipogenic genes through the activation of LXRα.[42] Thus, hepatitis B virus X protein physically interacts with LXRα in the nucleus and

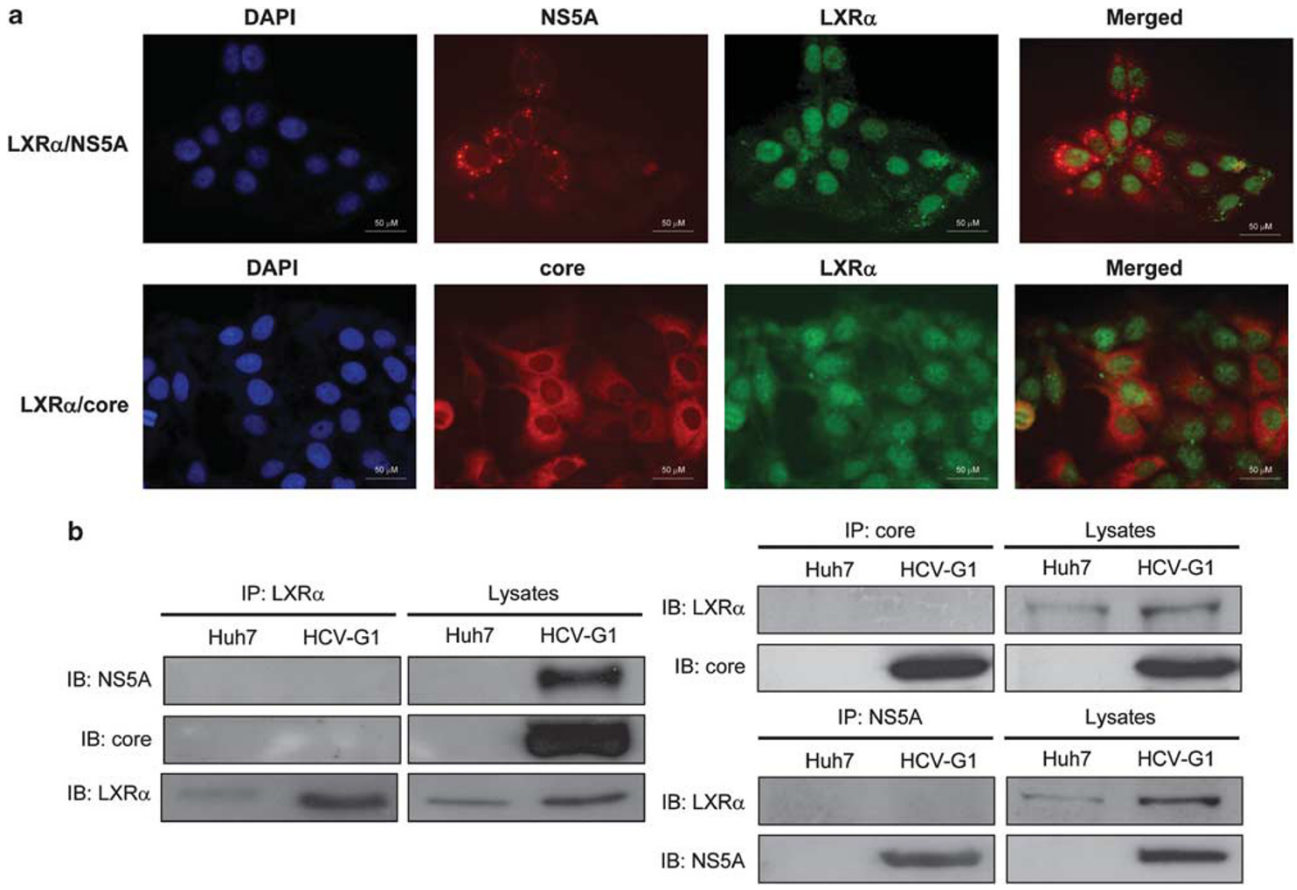

Figure 5 LXRα does not physically interact with HCV NS5A or core proteins in genomic replicon-containing cells. (**a**) HCV-G1 cells were processed for double immunostaining using anti-LXRα (green) and anti-core (red) or anti-NS5A protein (red)-specific antibodies. Nuclei were stained with DAPI (blue). The merged image with green and red fluorescence is shown (right). Photographs shown are typical results of six independent experiments. (**b**) Co-immunoprecipitation of LXRα and HCV proteins. Total lysates from Huh7 and HCV-G1 cells were subjected to immunoprecipitation (IP) using antibodies specific for LXRα, NS5A, or core proteins. Both lysates and precipitates were analyzed via immunoblotting (IB) with the indicated antibodies.

enhances the binding of LXRα to LXRE (LXR-response element).[43] In this study, core and NS5A are mainly detected in the cytoplasm of replicating cells, as previously described.[44,45] Meanwhile, LXRα localizes mainly in the nucleus of genomic replicon-containing cells where the presence of proteins of the virus are minimal. Moreover, reciprocal co-immunoprecipitation experiments indicate that LXRα does not crossreact with HCV proteins. Therefore, no physical association between viral proteins and nuclear receptor has been observed, supporting that both NS5A and core proteins indirectly increased the transcriptional activity of LXRα.

In the current research, both HCV core and NS5A proteins activated AKT, an important kinase downstream target for PI3K. These results are consistent with previous reports showing that HCV proteins activate the PI3K/AKT pathway.[46–48] It has been suggested that HCV gene expression induces activation of PI3K/AKT via oxidative stress and calcium signaling.[15] We have previously described that NS5A and core proteins induce oxidative stress-mediated Ca^{2+} homeostasis alterations in CHL, which might underlie the effects of both proteins on the PI3K/AKT pathway.[31] It has been proposed that the PI3K/AKT pathway activation is required for HCV core-mediated SREBP-1 induction,[46] process synergistically supported by HCV NS4B protein.[15,49] Thus, AKT has been shown to be able to increase endoplasmic reticulum to Golgi transport of SREBP and mature SREBP levels and SREBP phosphorylation, whereas the effects on SREBP transcription has not been observed.[15,46] Moreover, it has been recently described that the induction of gene expression by LXR agonists is attenuated by inhibitors of PI3K pathway in macrophages.[50] In our study, LY294002-mediated PI3K pathway inhibition attenuates LXRα upregulation induced by HCV core and NS5A proteins. These results indicate for the first time a role for PI3K signaling pathway in the HCV core and NS5A-mediated LXRα activation, suggesting a specific mechanism in which HCV infection alters the cellular lipid profile and causes steatosis. However, the exact mechanism by which HCV proteins induce LXRα through the PI3K/AKT pathway remains to be determined.

As described in HCV–NS5A and HCV–core cells, in our replicon-containing cells a parallel upregulation of the LXRα-mediated transcription of SREBP-1c and FAS through LXRE

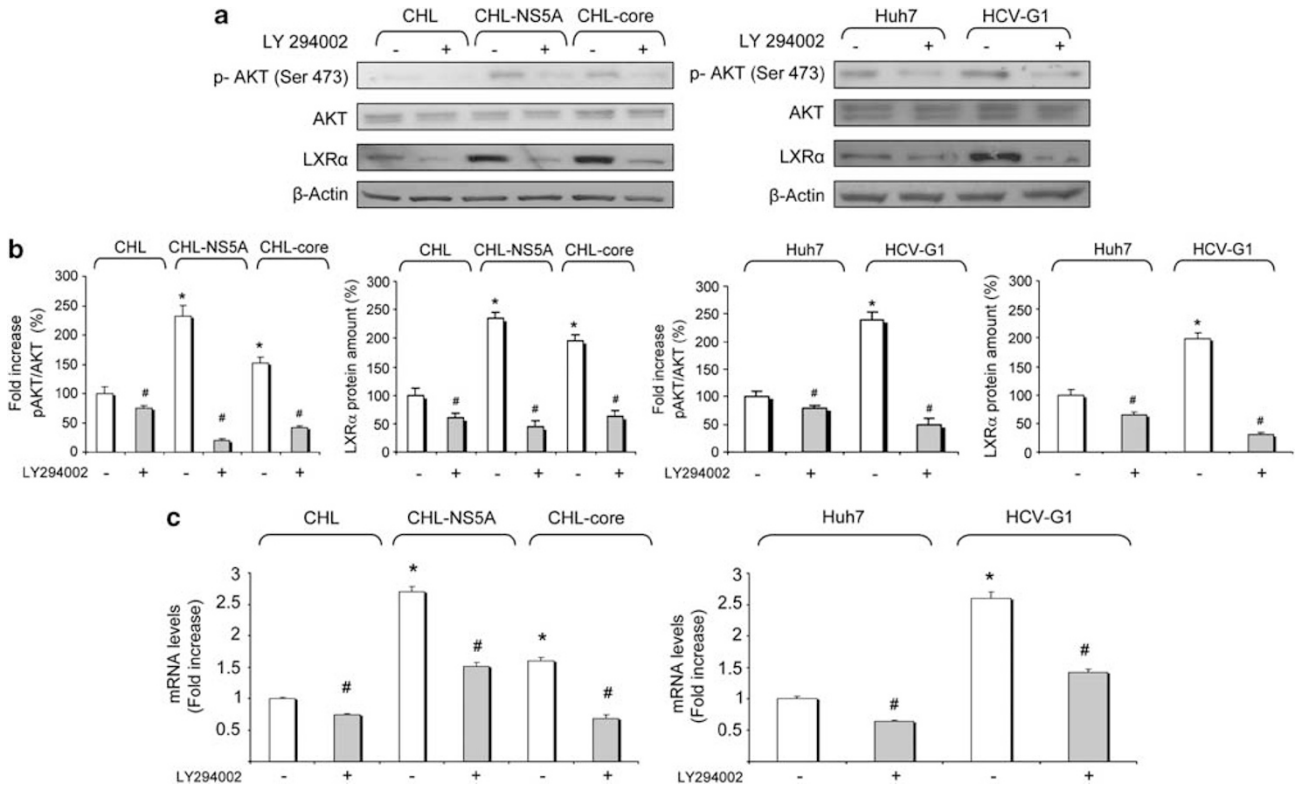

Figure 6 Effect of PI3K/AKT pathway inhibition on HCV core and NS5A proteins and viral replication-mediated LXRα upregulation. Cultured cells were treated with the PI3K-inhibitor LY294002 (50 μM) for 24 h. (**a**, **b**) p-AKT (Ser 473), AKT, and LXRα protein levels in CHL, CHL–NS5A, and CHL–core cells or Huh7 and HCV-G1 cells were analyzed by western blot. Densitometry analysis of specific bands expressed as percentage relative to their controls (100%). β-Actin levels were used as loading control. Molecular weight markers (kDa) are indicated on the left. Photographs are representative of six independent experiments. (**c**) Bar graphs show LXRα mRNA levels determined by real-time qRT-PCR as indicated. Data are described as the mean values ± s.d. of six experiments ($^{*}P<0.05$ *vs* CHL or Huh7, $^{\#}P<0.05$ *vs* nontreated cells).

and SRE motifs in their promoters was observed. These results are consistent with previous studies in which genotype 1b or genotype 2a HCV replication systems induced lipogenic genes,[15,25] but differ from data obtained in patients infected with HCV genotype 3, in which steatosis was not associated with induction of genes involved in lipogenesis.[51] Similarly, overexpression of LXRα in HCV-replicating cells seems to be mediated by PI3K/AKT pathway activation. Although the LXRα-mediated lipogenesis observed in replicon-containing cells could be partially induced by the expression of HCV NS5A and core proteins, other HCV proteins, such as NS2 and NS4B, may contribute to higher LXRα levels, and increased SREBP-1c and FAS expression, as previously indicated.[15,16,49] Future studies should determine whether different HCV proteins regulate lipid metabolism through different mechanisms.

It has been described that saturated and monounsaturated fatty acids are required for efficient HCV replication, most likely, by maintaining optimal membrane structure.[10,24,25] Thus, it has been shown that reduction of FAS by RNA interference suppressed HCV replication in both replicon and infection systems, consistent with the requirement of the fatty acid biosynthetic pathway in HCV replication.[24,26] Similarly, SREBP-1c suppression by curcumin also inhibits hepatitis C virus replication in an *in vitro* model.[52] In our replication model, LXRα knockdown decreases lipid accumulation as well as the expression of the lipogenic genes. siRNA LXRα-mediated lipogenic inhibition is accompanied by a partial blockage of HCV RNA replication and NS5A and core expression. These results differ from those obtained by Kapadia and Chisari,[25] which showed that polyunsaturated fatty acids inhibited HCV RNA replication by a mechanism independent of their ability to antagonize LXRα, and that LXR agonist T0901317 had no effect on baseline or polyunsaturated fatty acid-inhibited HCV RNA replication. However, in our study, treatment of replicon-containing cells with GW3965, a more specific LXR agonist,[53] increased lipogenic gene expression, HCV RNA replication, and NS5A and core expression. Moreover, the modulation of HCV replication capacity by GW3965 seems to be mediated by LXRα activation as indicated by the lack of effect observed in LXRα knockdown HCV-G1 cells treated with the LXR agonist. These data suggest that LXRα-mediated regulation of lipid metabolism may contribute to the efficient replication of HCV.

Overall, these results place LXRα in a key position within the HCV-induced lipogenic pathways, and suggest a

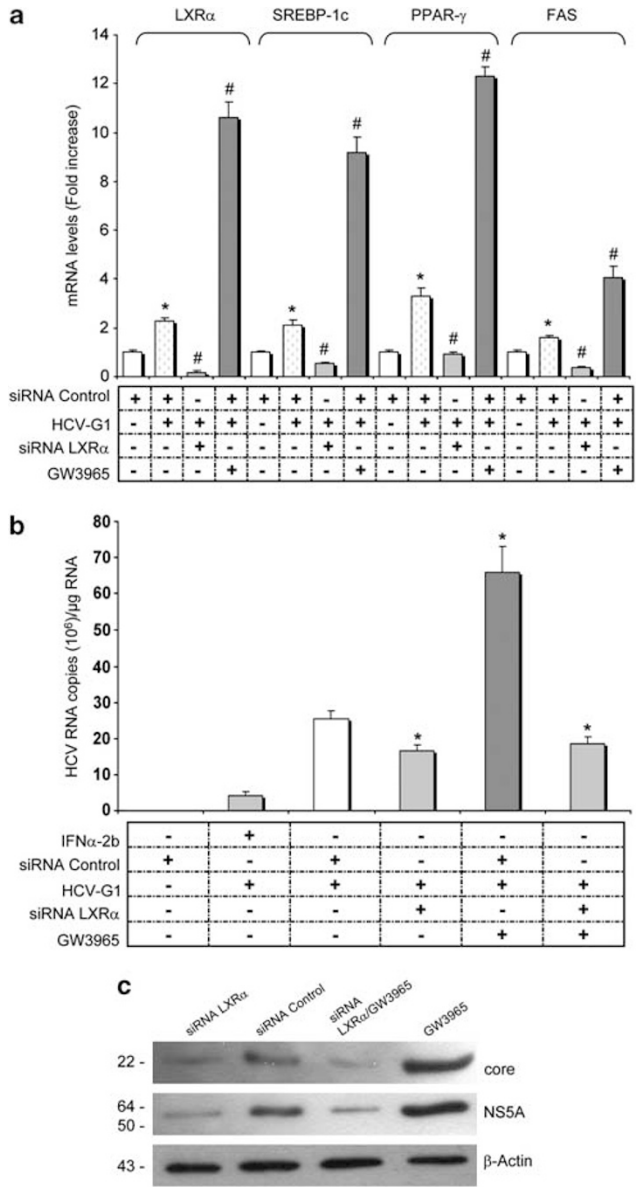

Figure 7 Effect of LXRα siRNA and agonist treatment on lipogenic gene expression, HCV RNA replication, and HCV protein expression in replicon-containing cells. HCV-G1 cells were transfected with negative control or LXRα siRNA (10 nM) for 48 h and treated with LXR agonist GW3965 (5 μM) for 24 h. (**a**) Bar graph shows LXRα, SREBP-1c, PPAR-γ, and FAS mRNA levels determined by real-time qRT-PCR as described (*$P<0.05$ *vs* control siRNA-treated Huh7 cells, $^{\#}P<0.05$ *vs* control siRNA-treated HCV-G1 cells). (**b**) Samples of 1 μg RNA were analyzed by real-time RT-PCR using specific primers to determine HCV RNA levels. Histone H3 mRNA levels were used for sample normalization. HCV-G1 cells were treated with IFNα-2b (0.5 U/ml) as control of replication inhibition. Results are expressed as HCV RNA copies/μg total RNA. Data are described as the mean values ± s.d. of six experiments (*$P<0.05$ *vs* control siRNA-treated HCV-G1 cells). (**c**) HCV core and NS5A protein levels were analyzed by western blot. β-Actin levels were used as loading control. Molecular weight markers (kDa) are indicated on the left. Photographs are representative of six independent experiments.

molecular mechanism through which HCV gene expression can stimulate hepatic lipid accumulation and, in turn, regulate viral replication efficiency.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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