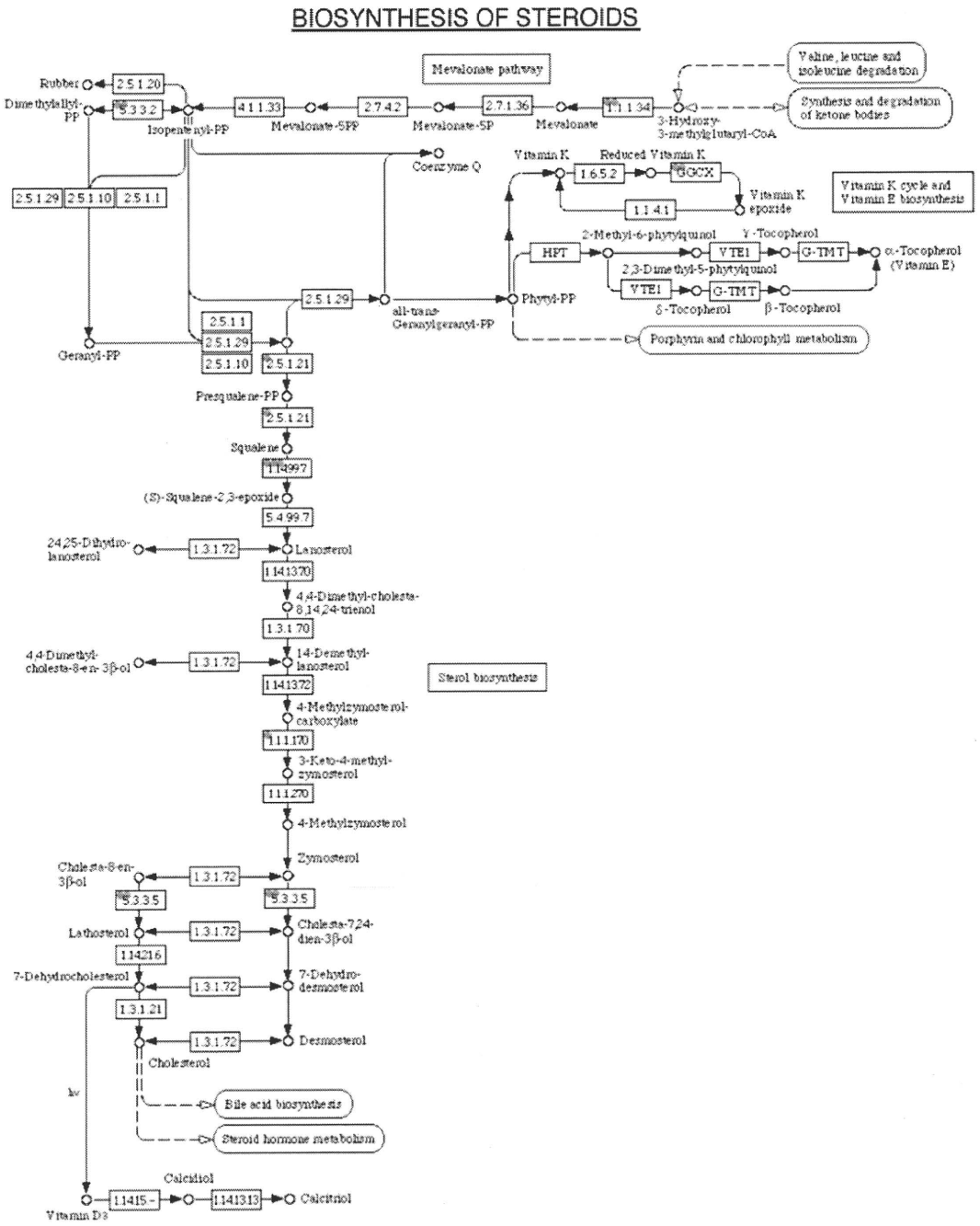

Fig. 5 KEGG Pathway map and array data (biosynthesis of steroids). Gene expression changes were mapped on the pathways. Each *circle* within a *box* represents the corresponding probe set on Human Genome U133 Plus 2.0 array because multiple probe sets are sometimes designed for a single gene. *Red circles* indicate overexpressed genes in *cured* cells compared to parental Huh7 cells. The *dotted numerical code* in each *box* represents the Enzyme Commission (EC) number based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB). Correspondence between the genes that were examined in the microarray analyses and enzymes that are presented in Fig. 5 is shown in Supplementary Table 4

Rep-Feo cells showed that the replication of the HCV replicon was suppressed by clofibrate and fenofibrate in a dose-dependent manner, whereas pioglitazone and troglitazone elevated expression levels of replicon. The MTS assay did not show any effect on cell viability or replication. These results suggest that the decrease or increase in HCV replication is due to specific effects of PPAR-alpha or gamma agonists on HCV replication.

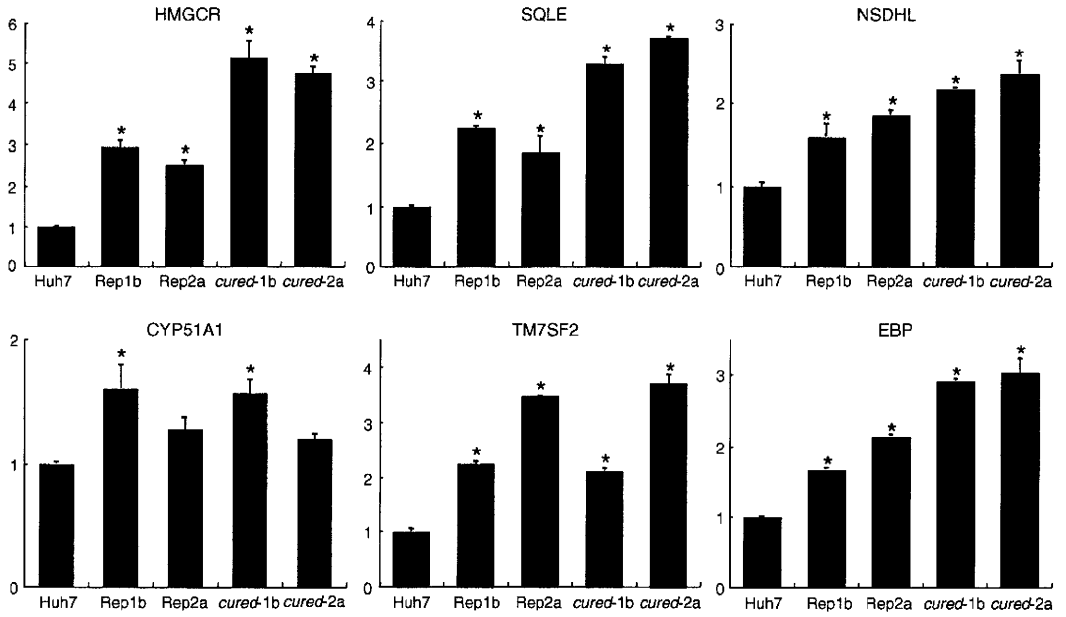

Fig. 6 Real-time detection RT-PCR. Real-time RT-PCR was performed to verify expression levels of genes that were listed in the cholesterol biosynthesis pathway in Fig. 4c and that showed differences in their expression levels by microarray analyses. Assays were done in triplicate, and *asterisks* indicate *P*-values of less than 0.05

Discussion

In our present analyses, we identified MAPK signaling, biosynthesis of steroid related and TGF-beta signaling pathways as significantly changed pathway processes by comparing replicon-expressing and *cured* cells (Supplementary Table 2). The results suggest that these pathways were primarily affected by HCV replication. Comparison of *cured* cells and naïve Huh7 cells identified cell cycle, TGF-beta, sphingolipid metabolism, and biosynthesis of steroids pathways as significantly changed pathways. Interestingly, cholesterol biosynthesis pathways were significantly changed in both comparisons (Supplementary Tables 2, 3). These data suggest that these pathways may positively regulate cellular HCV replication and that cholesterol biosynthesis pathways are primarily activated by HCV replication and may be essential for continuous virus replication.

There are several studies that report gene expression changes in replicon-expressing Huh7 cells as compared with the naïve cells [30–32]. In those studies, however, the changes in gene expression do not only reflect the effect of intracellular HCV replication, but also reflect alteration of host cell clonalities. Indeed, there are inconsistencies among studies. Use of the cured Huh7 cells can minimize the effect of cellular clonal changes because such Huh7 subclones have already been selected through HCV replicon transduction, drug-resistance selection and subsequent HCV elimination [33]. In our study, we have compared gene expression between genotype 1b and 2a replicon cells, respective cured cells and the naïve parental cells, and have identified molecular signaling or metabolic pathways that were differentially up- or down-regulated over different HCV genotypes.

Comprehensive microarray analyses and pathway analyses were very useful for the identification of molecular mechanisms of HCV infection and replication in the host cells. We used the KEGG Pathway database [28], a knowledge-based database of biological systems that integrates genomic, chemical and systemic functional information. KEGG provides a reference knowledge base for linking genome to life through the process of PATHWAY mapping, which is to map, for example, a genomic or transcriptomic content of genes to KEGG reference pathways to infer systemic behavior of the cells or the organism. These pathway databases are free on-line resources. Using these analyses, the close relation between cholesterol metabolism and HCV replication was demonstrated. Moreover, in relation to this, when we examined the pathways of other lipid metabolism, it was shown that fatty acid biosynthesis metabolism-related pathways were significantly changed in *cured* cells, and indeed we found a large number of lipid droplets in the cytosol of replicon cells and *cured* cells.

The HCV-JFH1 strain is the basis of a robustly replicating cell culture system reported recently [5]. We have performed comprehensive gene expression analyses using the HCV-JFH1 and the cured Huh7.5.1 cell line [6]. The

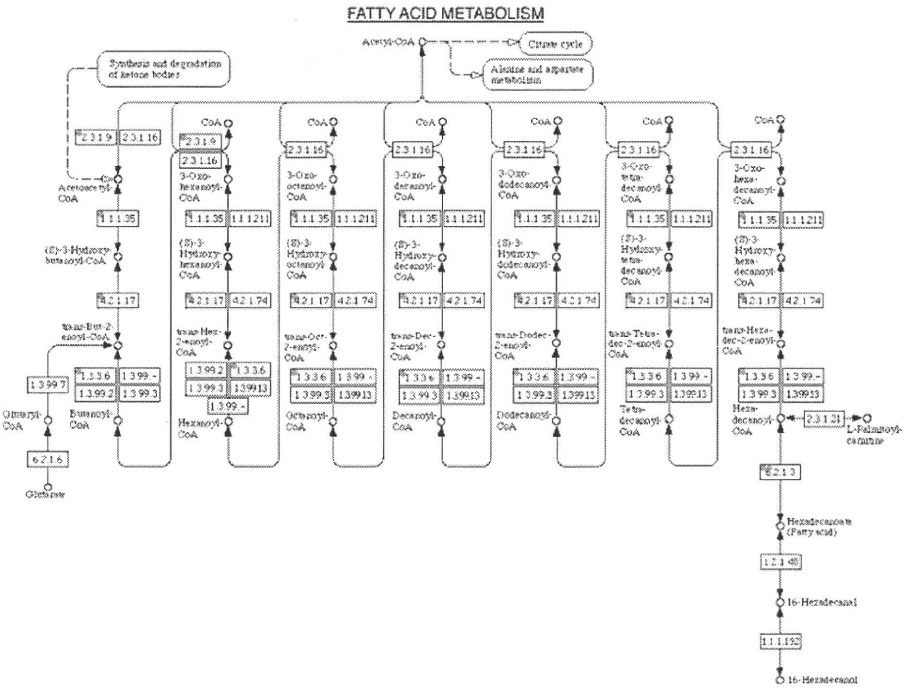

Fig. 7 KEGG Pathway map and array data (fatty acid metabolism). Gene expression changes were mapped on the pathways. Each *circle* within a *box* represents the corresponding probe set on Human Genome U133 Plus 2.0 array because multiple probe sets are sometimes designed for a single gene. *Red circles* indicate overexpressed genes in *cured* cells compared to parental Huh7 cells. The *dotted numerical code* in each *box* represents the Enzyme Commission (EC) number. Correspondence between the genes that were examined in the microarray analyses and enzymes that are presented in Fig. 7 are shown in Supplementary Table 4

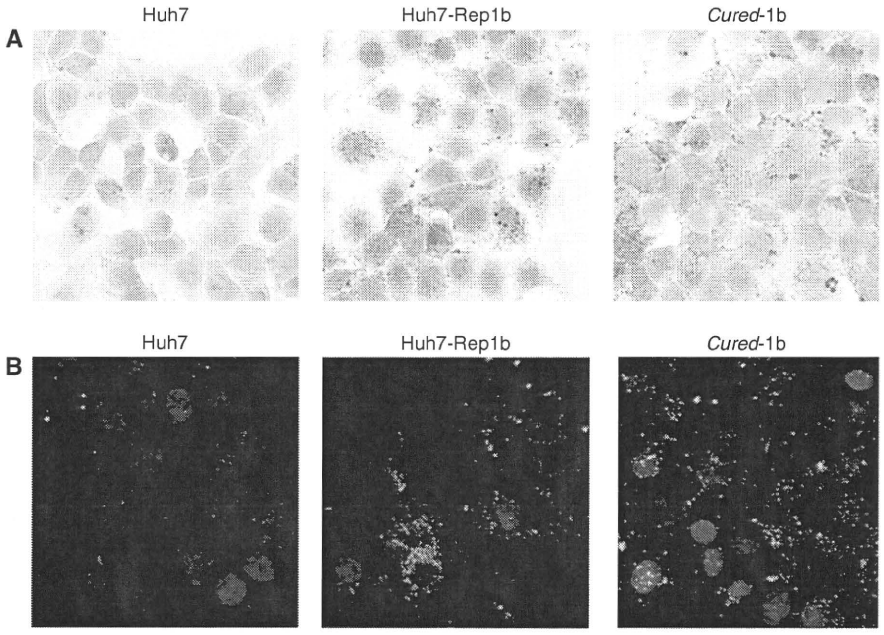

Fig. 8 Detection of intracellular lipid droplets and HCV NS protein. **a** Huh7 cells, replicon cells and *cured* cells were fixed and stained with Oil red O and Mayer's hematoxylin. Intracellular lipid droplets were detected as *red spheres* in the cells. Nuclei are stained in *blue*. **b** Rep1b/Huh7 cells were labeled with antibodies against NS5A (*red*). Lipid droplets and nuclei were stained with BODIPY493/503 (*green*) and DAPI (*blue*), respectively

KEGG Pathway analyses have identified several significantly affected pathways that are involved in the cell cycle, TGF-beta signaling, PPAR signaling and sterol biosynthesis. These findings are consistent with our present results using the HCV subgenomic replicon (see the Supplementary Table 5; Supplementary Figs. 4, 5).

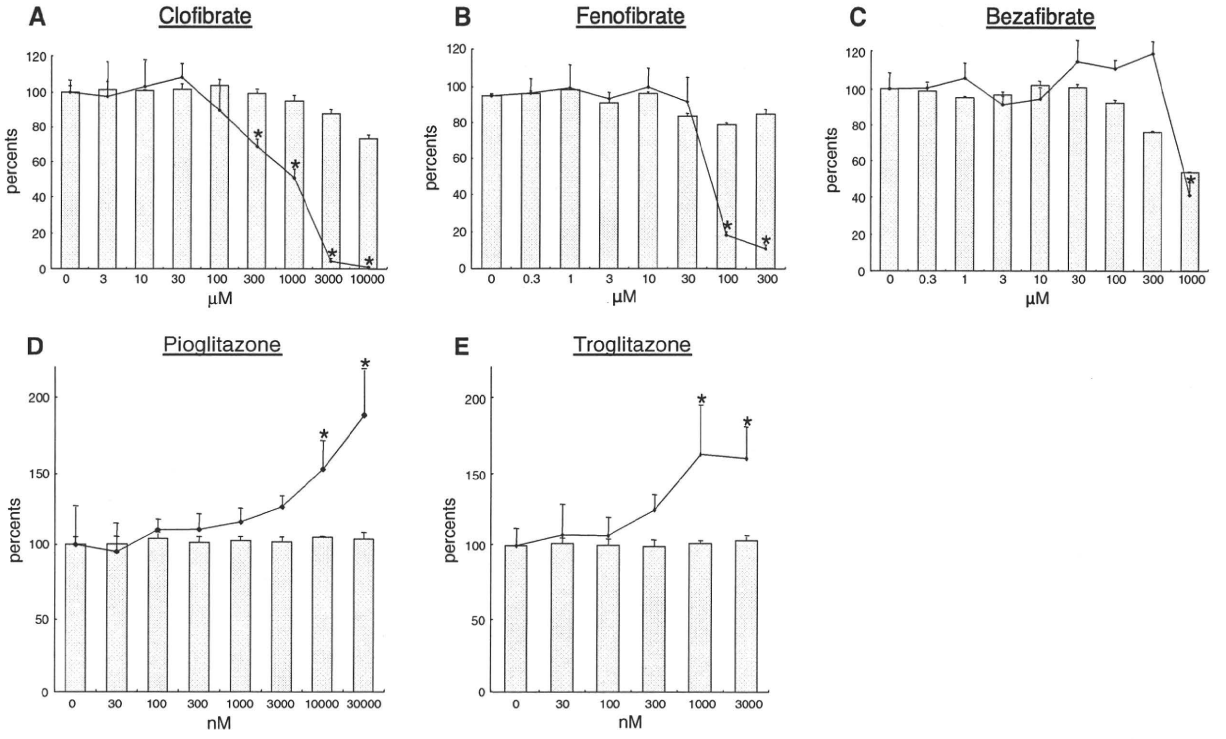

Fig. 9 Results of secondary screening with PPAR-alpha and -gamma agonists. Luciferase activity for HCV replication levels is shown as a percentage of the control. Cell viability is also shown as percentage of the control. Each *bar* represents the average of quadruplicate data points with standard deviation represented as the *error bar*. *Asterisks* denotes a significant difference from the control of at least $P < 0.05$

The JFH1 strain, however, showed substantial cytopathic effects on cultures of more than 5 days accompanied by overall induction of apoptosis-related genes and massive cell death [34]. Thus, it was difficult to conduct gene expression studies consistently.

Lipid metabolism is involved in the life cycle of many viruses. Recent studies have demonstrated the localization of HCV nonstructural proteins in the lipid raft in the endoplasmic reticulum (ER) forming intracellular replication complexes, called membranous webs [35, 36]. Because the lipid raft is enriched in cholesterols and sphingolipids, depletion of these lipids leads to inhibition of HCV genomic replication [19]. Amemiya et al. [37] reported that another serine palmitoyltransferase, myriocin, depleted cellular sphingomyelin contents and inhibited HCV replication.

It has been reported that statins efficiently suppress HCV replication in vitro and in vivo [38–40]. Statins are inhibitors of HMG-CoA reductase and shut down cholesterol biosynthesis by preventing the formation of mevalonate from 3-hydroxy-3-methyl-glutaryl CoA. As we have shown in the results, all enzymes in the cholesterol synthesis pathway were upregulated in the replicon-expressing and the cured Huh7 cells. In addition to lowering intracellular levels of sterols, statins also reduce levels of isoprenoids, which are derived from mevalonate. Isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate serve as lipid attachments for a variety of intracellular signaling molecules. In our results, the cholesterol biosynthesis pathway was also upregulated between cured versus naïve cell lines as well as replicon versus cured cell lines. These results suggest that HCV replication may promote synthesis of lipids including steroids that were essential for the viral efficient replication.

It has been recognized that HCV infection causes hepatic steatosis and subclinical insulin resistance and that they are independent of other risk factors such as obesity or the presence of diabetes mellitus. Similarly, in HCV cell cultures, Yang et al. [41] have reported that cellular fatty acid synthase is upregulated in HCV-infected Huh7 cells and specific inhibition of the enzymatic activity caused suppression of HCV replication. In the present study, although lipid metabolism-related genes were upregulated in cured cells, which supports efficient HCV replication, there was not significant change in lipid-related genes between replicon-expressing as compared with cured cells (Fig. 7). These results suggest that HCV subgenomic replication does not cause steatosis as it did in full-length HCV cell culture [41]. These discrepancies might be due to the absence of the presence of HCV structural genes including core and envelope proteins.

We have shown an increase in lipid droplets in HCV replicon-positive cells and their cured cell lines as a phenotype of the gene expression profiles (Fig. 8). On the other hand, ACOX1, a rate-limiting enzyme of peroxisomal beta-oxidation, was higher in cured cells than parental Huh7 cells (Fig. 7) [42]. We have shown preliminarily that cellular SREBP1 (sterol regulatory element-binding protein 1), which regulates a set of triglyceride synthesis enzymes en bloc, is upregulated in HCV replicon-positive cell lines. These discrepancies might be due to more proficient activation of SREBP1-induced fatty acid biosynthesis pathways. Collectively, our results suggest that the overall fatty acid synthesis pathway, not only fatty acid synthase, is activated by upregulation of a set of responsible enzymes.

We have investigated effects of PPAR agonists to HCV replication. PPAR-alpha agonists, clofibrate and fenofibrate suppressed HCV replication (Fig. 9). PPAR-alpha, not PPAR-gamma, is expressed in hepatocytes, recognizes cellular free fatty acids and leukotriene B4 as a specific ligands, and mediates oxidative degradation of triglyceride and depletion of intracellular fat droplets [43, 44]. These properties of PPAR-alpha agonists suggest that the level of HCV replication is affected by the increased production of fatty acids, but not by the overexpression of their related enzymes. PPAR-gamma agonists, in contrast, amplified HCV replication. Because PPAR-gamma is a regulator of fatty acid metabolism in peripheral tissue and is not expressed in the hepatocytes or in Huh7 cells (data not shown), it is possible that the effects of the PPAR-gamma agonists on HCV replication may be through its pleiotropic side effects such as p38 MAPK activation [45]. Very recently, it has been reported that HCV-NS5A proteins induce expression of PPARgamma [46].

In conclusion, comprehensive gene expression and pathway analyses were useful to study molecular pathways that were involved in HCV pathogeneses and to identify host factors for HCV replication that could constitute antiviral targets.

Acknowledgment This study was supported by grants from Ministry of Education, Culture, Sports, Science and Technology-Japan, the Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare-Japan, Japan Health Sciences Foundation, and National Institute of Biomedical Innovation.

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Curcumin inhibits hepatitis C virus replication via suppressing the Akt-SREBP-1 pathway

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ABSTRACT

A polyphenolic compound from the curry spice turmeric, curcumin, is known to show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus. However, it remains to be determined whether curcumin can inhibit the replication of hepatitis C virus (HCV). In this study, we showed that curcumin decreases HCV gene expression via suppression of the Akt-SREBP-1 activation, not by NF-κB pathway. The combination of curcumin and IFNα exerted profound inhibitory effects on HCV replication. Collectively, our results indicate that curcumin can suppress HCV replication in vitro and may be potentially useful as novel anti-HCV reagents.

1. Introduction

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped virus with a single-stranded 9.6 kb RNA genome. HCV infection is characterized by a high rate of progression to fibrosis and chronic hepatitis, resulting in cirrhosis, and ultimately in hepatocellular carcinoma [1]. The best anti-viral therapy presently known involves the combination of pegylated interferon (IFN) alpha and ribavirin, but almost half of all patients manifest no response to exogenous IFNα [2]. Therefore, the development of novel drugs for the safer and more efficient treatment of HCV is urgently required.

Many bioactive polyphenolic compounds have been shown to perform candidate agent functions in chemoprevention and in cancer chemotherapy [3]. Among this class, curcumin (diferuloylmethane) is one of the most widely studied compounds. Curcumin is the major component of the curry spice tumeric (*Curcuma longa* Linn) and can affect the metabolism of cells and organisms in a number of ways, including anti-inflammatory, anti-oxidant, and anti-proliferative properties via the modulation of multiple cellular mechanisms [4,5]. Furthermore, some recent reports have shown that these compounds show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus [6–9]. Also, curcumin has been shown to suppress transcription activation by the host protein AP-1, leading to diminished HTLV-1 and HPV-mediated cellular transformation [10]. However, it remains to be determined whether curcumin can inhibit the gene expression of HCV.

On the basis of our previous knowledge of the regulation of HCV replication and the biological properties of curcumin, we evaluated the effects of curcumin on the intracellular replication of the HCV genome in vitro, using an HCV replicon system. We showed that curcumin at concentrations that do not affect cell viability reduced HCV RNA replication in vitro to a significant degree. Curcumin inhibited a lipogenic transcription factor, sterol regulatory element binding protein-1 (SREBP-1)-induced HCV replication via the PI3K/Akt pathway. Finally, the combination of curcumin and IFNα showed cooperative inhibitory effects on HCV RNA replication. Our results indicate that curcumin may potentially prove useful as a treatment for HCV infection.

2. Materials and methods

2.1. Plasmid constructs

pEMCV/IRES-Rluc was utilized as a control for the analysis of translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (EMCV-IRES) which mediates the translation of the HCV non-structure gene of replicon constructs, Huh7/Rep-Feo [11]. pCIneo-Rluc-IRES-Fluc was constructed in or-

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der to evaluate HCV internal ribosome entry site (IRES)-mediated translation efficiency [12]. The plasmid expressed a bicistronic RNA, in which Rluc was translated in a cap-dependent manner and Fluc was translated via HCV IRES-mediated initiation.

2.2. Cell cultures and treatments

Huh7 cells expressing the HCV replicon (Huh7/Rep-Feo) were maintained in DMEM supplemented with 10% FBS containing 500 μg/ml of G418 (Calbiochem). Huh7/Rep-Feo and Huh7 cells were plated at 70–80% confluence and treated with various concentrations of curcumin or vehicle controls and incubated for 24 h at 37 °C. The concentration ranges of 5–15 mM curcumin (obtained from Sigma) were tested. Control vehicle treatment (DMSO) was equivalent to the highest concentrations in the dose range experiments for each of the tested drugs.

2.3. HCV replicons

An HCV subgenomic replicon plasmid, pRep-Feo (Fig. 1A), was derived from pRep-Neo (originally referred to as pHCVlbneo-delS) [13]. Replicon RNA was synthesized in vitro using T7-RNA polymerase (Promega, Madison, WI) and transfected into the Huh7 cells via electroporation. After culturing in the presence of G418, the cell lines stably expressing the replicons were established and designated Huh7/Rep-Feo.

2.4. Transient transfection and luciferase reporter assay

Plasmid transfection was conducted using PolyFect (QIAGEN) in accordance with the manufacturer's instructions. The pcDNA3.1 empty vector was added to the transfections in order to achieve the same total amount of plasmid DNA per transfection. The cells were lysed in cell culture lysis buffer (Promega). The luciferase activity was evaluated using an analytical luminescence luminometer in accordance with the manufacturer's instructions.

2.5. MTT assay

For cell viability assay, Huh7/Rep-Feo cells were seeded in a 24-well tissue culture plate and incubated for 24 h. Cells were treated with curcumin or Bay11-7082. After 24 h, MTT (3-(4,5-dimethylthiozol-2-ly)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/ml) was added to each well. After incubation for 2 h at 37 °C, formazan crystals in viable cells were soluble in 200 μl of DMSO. The soluble formazan product was spectrophotometrically quantified using an ELISA leader at 570 nm.

2.6. siRNA design and siRNA transfection

RNA oligonucleotides were synthesized by Bioneer (Daejeon, Republic of Korea). The sequences of siRNA targeting human p65 were sense, 5′-GAU UGA GGA GAA ACG UAA A-3′ and antisense, 5′-UUU ACG UUU CUC CUC AAU C-3′. The sequence of siRNA targeting human SREBP-1 were sense, 5′-UGA GUG GCG GAA CCA UCU U-3′ and antisense, 5′-AAG AUG GUU CCG CCA CUC A-3′. The scramble control siRNA sequences were sense, 5′-CCU ACG CCA CCA AUU UCG U-3′ and antisense, 5′-ACG AAA UUG GUG GCG UAG G-3′. The cells were transfected with siRNA using HiPerFect (QIAGEN) according to the instructions of the manufacturer.

2.7. RT-PCR analysis

Total RNA from the curcumin-treated Huh7/Feo cells was prepared using TRIzol reagent (Invitrogen) by following the manufacturer's instructions. cDNA was used as a template for real-time PCR using gene-specific primers: NS3, 5′-TCG TGG CAA CAG ACG CTC TAA TGA-3′ (forward) and 5′-AGA ACT CCA G AT GGT CCT GGC AAA-3′ (reverse); NS5A, 5′-TAG CAG TGC TCA CTT CCA TGC TCA-3′ (forward) and 5′-AGG ATC TCC GCC GCA ATG GAT ATT-3′ (reverse); β-actin, 5′-GAC TAC CTC ATG AAG ATC-3′ (forward), 5′-GAT CCA CAT CTG CTG GAA-3′ (reverse).

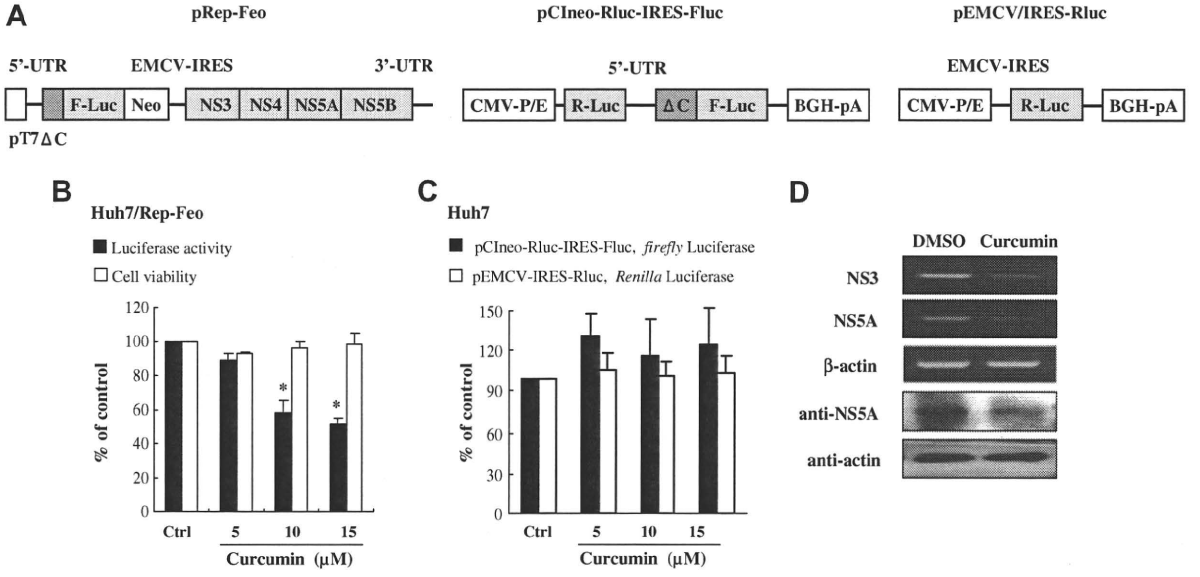

Fig. 1. Curcumin suppresses HCV RNA replication. (A) Structures of the hepatitis C virus (HCV) replicon, pEMCV/IRES-Rluc, and pCIneo-Rluc-IRES-Fluc plasmids. (B) The effects of curcumin on Huh7/Rep-Feo cells on luciferase activity and MTT assays. For the luciferase assay, the Huh7/Rep-Feo cells were cultured in the presence of the indicated curcumin concentrations, and the luciferase activity was determined at 24 h of treatment. The values are expressed as the means ± S.D. for at least three independent experiments. *$P < 0.05$ compared with control. (C) The luciferase activity effects on HCV IRES-mediated translation and EMCV/IRES-mediated translation by curcumin. The data are expressed as the means ± S.D. ($n = 3$). (D) The inhibitory effect of HCV RNA and protein level by curcumin. Huh7/Rep-Feo cells were cultured for 24 h in the presence of 15 μM curcumin. Total RNA was extracted from the cells, and the levels of NS3 and NS5A mRNA were determined by RT-PCR and β-actin expression is shown as an mRNA-loading control. Total protein extracts were blotted with anti-NS5A antibody and actin expression is shown as a protein-loading control. Three independent experiments were reproduced.

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2.8. Nuclear/cytosolic fractions analysis

Cells were lysed in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5% NP-40, 1 mM PMSF, protease inhibitors) and incubated for 10 min on ice. The supernatants (cytosolic lysates) were collected by centrifugation (3300×*g*) at 4 °C for 5 min. The nuclear pellets were then washed with ice-cold PBS to avoid contamination of cytosolic proteins and lysed in buffer B (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5% NP-40, 1 mM PMSF, protease inhibitors). After incubation on ice for 25 min, the supernatants (nuclear lysates) were collected by centrifugation (13 000×*g*) at 4 °C for 5 min.

2.9. Statistical analysis

Statistical analyses were carried out by unpaired or paired *t* test as appropriate. All data are reported as means ± S.D. *P* value of <0.05 was considered significant.

3. Results

3.1. Curcumin suppresses HCV RNA replication

In order to evaluate the effects of curcumin on the intracellular replication of the HCV genome, Huh7/Rep-Feo cells were treated with various concentrations of curcumin. The luciferase activity of the Huh7/Rep-Feo cells demonstrated that the replication of the HCV replicon was suppressed by curcumin. The MTT assay showed no effects on cell viability at various concentrations of these compounds (Fig. 1B). Moreover, the efficiency of EMCV-IRES-mediated translation was not affected by curcumin (Fig. 1C). These data indicate that the inhibitory effects on HCV replication exerted by curcumin are not attributable to cytotoxicity or to an artificial effect on the EMCV-IRES, which directly translates the HCV non-structure protein of the replicon. We then attempted to determine whether these effects of curcumin on the replication of the HCV replicon are involved in HCV IRES-dependent translation. We determined that curcumin gives no effects on the activity of *Firefly* luciferase in Huh7 cells that were transiently transfected with the pCIneo-Rluc-IRES-Fluc reporter (Fig. 1C). Taken together, these results indicate that curcumin suppresses HCV replication in vitro and that these effects are not involved in cell viability, EMCV-IRES-mediated translation, or HCV IRES-dependent translation.

In an effort to confirm these inhibitory effects on the HCV replicon by curcumin, we attempted to determine whether curcumin affects the HCV RNA and protein level. HCV replicon RNA level was detected by RT-PCR by using primers specific to NS3 and NS5A and protein level was assayed by Western blots with the anti-NS5A antibody. As shown in Fig. 1D, curcumin-treated cells expressed lower levels of the HCV replicon RNA as compared with the control vehicle-treated Huh7/Rep-Feo cells. Also, we showed that curcumin decreases the protein level of HCV NS5A, translated from the

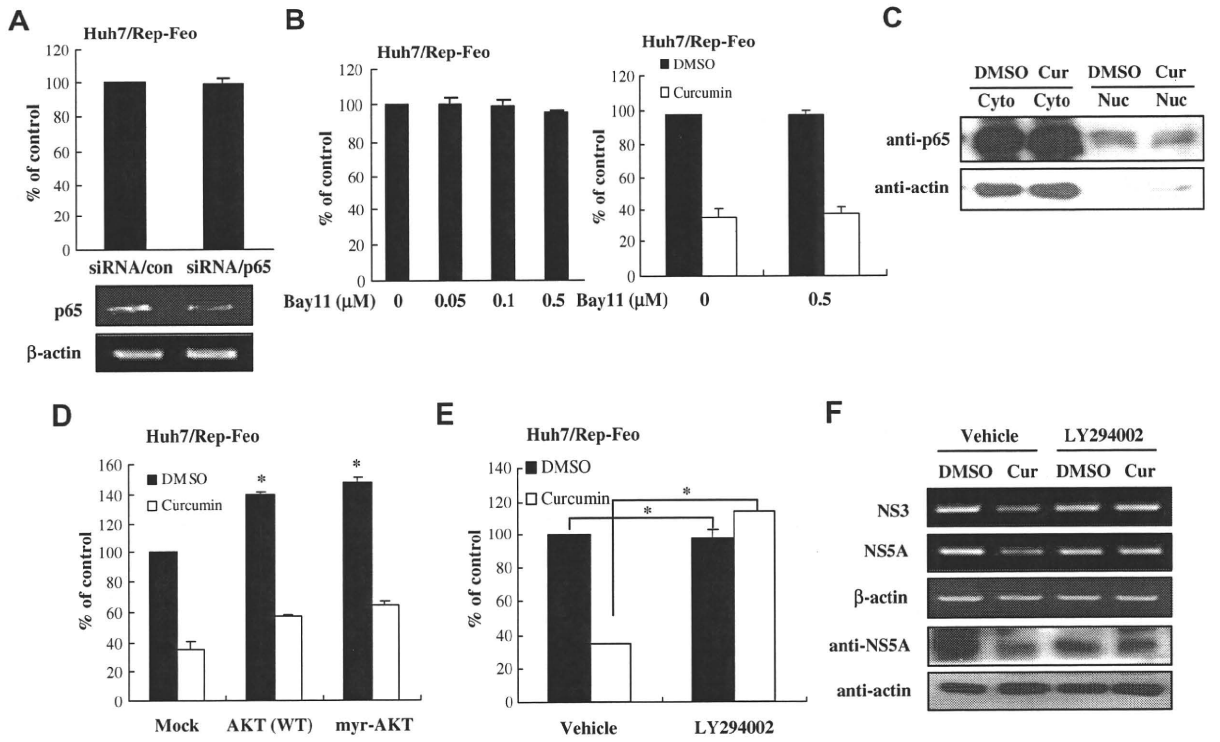

Fig. 2. Curcumin suppressed HCV replication via the PI3 K/Akt pathway. (A) The effects of p65 siRNA on HCV replication. Huh7/Rep-Feo cells were seeded in 6-well culture plates and transfected with scramble control siRNA and p65 siRNA and luciferase activities are measured ($n = 3$). (B) The effects of Bay11-7082 on HCV replication. Huh7/Rep-Feo cells were seeded in 24-well culture plates and treated with the Bay11-7082. The effects of curcumin on Huh7/Rep-Feo cells were identified by MTT assays (left) and luciferase activity (right) ($n = 3$). (C) The effects of curcumin on the nuclear localization of p65 protein. Huh7/Rep-Feo cells were seeded in 6-well culture plates and treated with curcumin (15 μM). The cyto or nuc indicates the cytosolic or nuclear extracts. (D) The effect of Akt on HCV RNA replication. Huh7/Rep-Feo cells were transfected with the plasmid for Akt (WT) or myr-Akt and cells were treated for 24 h with 15 μM curcumin or vehicle (DMSO). Luciferase activity was measured and the values are expressed as the means ± S.D. for at least three independent experiments. (E and F) The effect of LY249002 on curcumin-suppressed HCV RNA replication. Huh7/Rep-Feo cells were pretreated with 25 μM LY294002 for 2 h, and then incubated further in the absence or presence of 15 μM curcumin for 24 h. Luciferase activities were measured ($n = 3$) (E). NS3 or NS5A mRNA levels were detected using RT-PCR and protein levels of NS5A were detected by Western blotting ($n = 2$) (F).

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HCV replicon (Fig. 1D). These data correlate well with the luciferase activity of Huh7/Rep-Feo cells.

3.2. Curcumin inhibits HCV replication via the PI3K/Ak pathway, not NF-κB pathway

In an effort to gain insight into the molecular mechanism by which curcumin suppresses HCV replication, we then analyzed the signaling pathways involved in curcumin-inhibited HCV replication. Previously, it was suggested that the activation of the NF-κB pathway is involved in the increase of HCV replication [14,15]. To evaluate the involvement of NF-κB pathway on the curcumin-mediated inhibition of HCV replication, p65 (of NF-κB) siRNA and Bay11-7082 (a specific inhibitor of NF-κB pathway) were exploited. However, in contrast to our expectations, p65 siRNA and Bay11-7082 had no effect on HCV replication (Fig. 2A and B). Furthermore, curcumin also had no effect on the nuclear localization of p65 (Fig. 2C). These results show that PDTC and curcumin exert a synergic-inhibitory effect on HCV replication via NF-κB-independent pathway.

We next explored other potential pathways for HCV replication by curcumin. Several signaling pathways and transcription factors including AP-1, mitogen-activated protein kinases (MAPKs), and cell cycle machinery have been suggested as the targets of curcumin. We examined the effect of several pathways on HCV replication using treatments of specific kinase inhibitors or transfection of plasmid for specific kinases, including ERK, JNK, p38, Akt, and PKA. Among many signaling pathways, transfection of plasmid for wild type (WT)-Akt or constitutive active (myr)-Akt significantly augmented the luciferase activities of HCV replicon (Fig. 2D). Also, LY294002, a specific inhibitor of the PI3K/Akt pathway, inhibited curcumin-suppressed activity of HCV replicon (Fig. 2E). To confirm these effects on the HCV replicon induced by curcumin, we examined the HCV RNA and protein levels. As shown in Fig. 2F, LY294002-treated cells restrained the RNA and protein levels of HCV replicon inhibited by curcumin. Taken together, these results suggest that curcumin suppresses HCV replicon expression via the PI3K/Akt pathway.

3.3. SREBP-1 increases the HCV replicon expression via the PI3K/Akt pathway

To identify the downstream target of the PI3K/Akt pathway on HCV RNA replication, we tested several transcription factors regulated by the PI3K/Akt pathway. Among them, two SREBP-1 isoforms, SREBP-1a and SREBP-1c, induced the luciferase activity of the Huh7/Rep-Feo cells. Especially, SREBP-1a showed a profound effect on HCV replication (Fig. 3A). Previously, it has been suggested that the activation of Akt was able to increase the SREBP-1 gene expression [16]. Also, in an effort to determine whether SREBP-1 performs a function in HCV RNA replication, we attempted to knockdown SREBP-1 expression using siRNA for SREBP-1. As shown in Fig. 3B, siRNA/SREBP-1-transfected cells decreased the luciferase activity of the Huh7/Rep-Feo cells.

In an effort to confirm these effects on the HCV replicon exerted by SREBP-1, we attempted to determine whether SREBP-1 affects the HCV RNA and protein level. As shown in Fig. 3C, SREBP-1a-transfected cells enhanced the levels of the HCV replicon RNA as

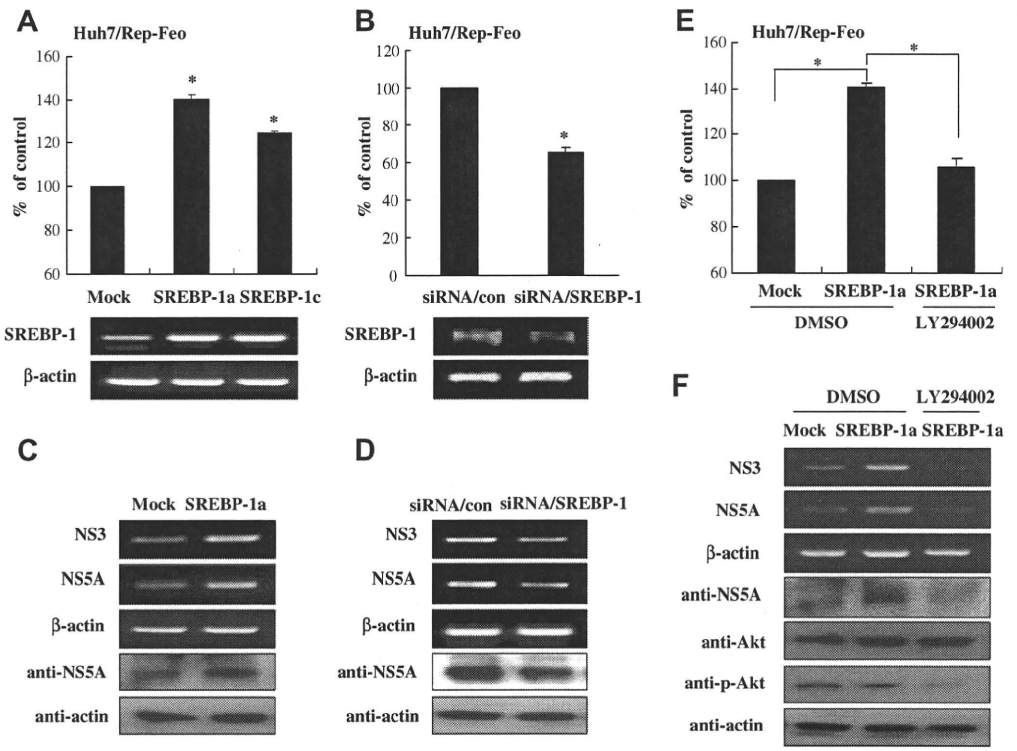

Fig. 3. SREBP-1 increases the HCV replicon expression via the PI3 K/Akt pathway. (A and B) The effects of SREBP-1 on HCV replication. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a or SREBP-1c and luciferase activities were measured ($n = 3$) (A). Huh7/Rep-Feo cells were transfected with scramble control siRNA and SREBP-1 siRNA and luciferase activities were measured ($n = 2$) (B). *$P < 0.05$ compared with control. (C and D) The effects of SREBP-1 on HCV RNA and protein levels. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a (C) or siRNA for SREBP-1 (D). NS3 or NS5A mRNA levels were detected using RT-PCR and protein levels of NS5A were detected by Western blotting ($n = 2$). (E and F) The effect of LY294002 on SREBP-1-induced HCV replication. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a and then incubated further in the absence or presence of 25 μM LY294002 for 24 h. Luciferase activities were measured ($n = 3$) (E) and NS3 or NS5A mRNA and NS5A protein levels were determined using RT-PCR or Western blotting ($n = 2$) (F).

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compared with the control transfected-Huh7/Rep-Feo cells. Also, we showed that the knockdown of SREBP-1 expression decreases the RNA and protein level of HCV replicon (Fig. 3D). Furthermore, SREBP-1a-transfected cells did not induce the HCV RNA replication in the presence of LY294002 (Fig. 3E and F). Taken together, SREBP-1 can increase the HCV replication via the PI3 K/Akt pathway.

3.4. Curcumin suppresses the SREBP-1-induced HCV replication

In order to determine whether SREBP-1-increased HCV RNA replication is inhibited by curcumin, Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a in the absence or presence of curcumin treatment. As shown in Fig. 4A, the luciferase activities of Huh7/Rep-Feo cells were inhibited by curcumin in the SREBP-1a transfection. Also, these effects were confirmed on the RNA and protein levels of HCV replicon (Fig. 4B).

3.5. Curcumin and IFNα have synergistic inhibitory effects on HCV replication

Finally, we examined whether curcumin can affect IFNα-based inhibition of HCV. IFNα has been shown to exert inhibitory effects on HCV replication. In order to determine whether curcumin could affect the IFNα-mediated inhibitory effect on HCV replicon, Huh7/Rep-Feo cells were treated with a combination of IFNα and curcumin. As shown in Fig. 5A and B, curcumin significantly augmented the IFNα-mediated inhibition of HCV replication. Thus, these findings demonstrate that cotreatment with curcumin was more effective than treatment with IFNα alone.

4. Discussion

There is abundant evidence indicating that dietary phytochemicals, including epigallocatechin gallate (EGCG), curcumin, resveratrol, and genistein, show anti-viral effects in a variety of virus types [6–9,17,18]. However, it remains to be determined whether dietary phytochemicals have anti-viral activity in cases of Hepatitis C. It was reported that curcumin exhibits anti-oxidant activity by reducing the generation of reactive oxygen species (ROS). Although it has been known that oxidative stress decreases HCV replication [19], a recent study showed that ROS is able to increase the replication of HCV [20]. According to this study, anti-oxidant pyrrolidine dithiocarbamate (PDTC) treatment decreased the expression of HCV RNA in Huh7 cells expressing HCV subgenomic replicons. In the present study, we observed that PDTC reduces the activity of HCV luciferase replicons in Huh7/Rep-Feo cells (data not shown). In addition, curcumin suppressed the HCV replication in cooperation with PDTC. Although curcumin had no effect on the

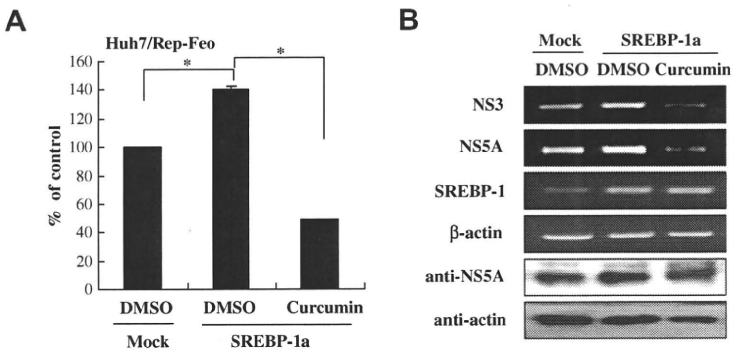

Fig. 4. Curcumin suppresses the SREBP-1-induced HCV replication. (A and B) The effects of SREBP-1 on curcumin-treated HCV replicon. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a and then incubated further with or without 15 μM curcumin for 24 h. Luciferase activities were measured ($n = 3$) (A) and NS3 or NS5A mRNA and NS5A protein levels were determined using RT-PCR or Western blotting ($n = 2$) (B).

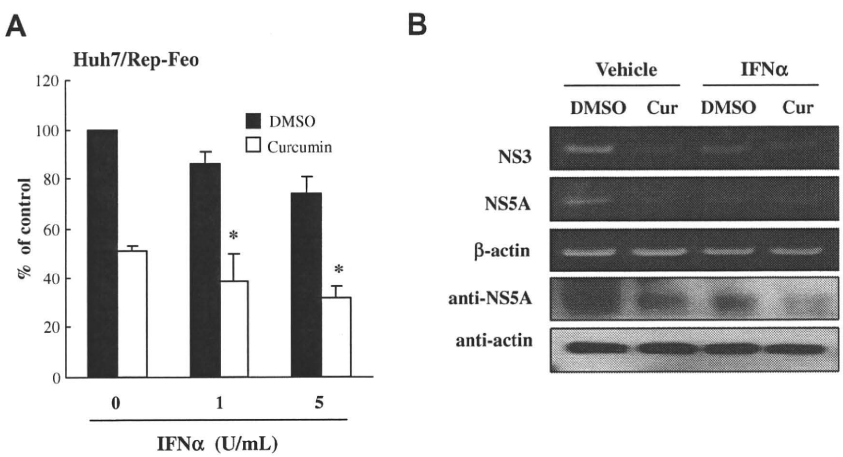

Fig. 5. curcumin and IFNα have synergistic inhibitory effects on HCV replication. (A) Huh7/Rep-Feo cells were cultured in the presence or absence of curcumin (15 μM) and IFNα, (0, 1, 5 U/ml). After 24 h of treatment, the cell lysates were obtained and luciferase activity was measured. The data shown are expressed as the means ± S.D. ($n = 3$). *$P < 0.05$ compared with curcumin alone or IFNα alone. (B) Huh7/Rep-Feo cells were incubated in the presence or absence of curcumin (15 μM) and IFNα (5 U/ml). After 24 h, NS3 or NS5A mRNA levels or NS5A protein levels were detected using RT-PCR or Western blotting ($n = 2$).

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gene expression change of anti-oxidant enzymes, such as, Cu/Zn-superoxide dismutase (Cu/Zn-SOD), Mn-superoxide dismutase (Mn-SOD) (data not shown), because curcumin exert the function as anti-oxidant through various pathways, we could not rule out the possibility that curcumin decreases HCV replication as anti-oxidant.

Some reports were demonstrated that despite its beneficial, direct anti-tumor actions, curcumin (and potentially other natural products) may adversely modulate the cellular response to clinically relevant cytokines or cytotoxic activities against a variety of tumor targets [21,22]. Therefore, curcumin experimentally evaluated has been found to be non-toxic or to have effective doses far below its toxic doses in the cancer therapy or blood lipid profile [23,24]. It is necessary to perform experiments to identify the adequate concentrations of curcumin in relation to inhibition of HCV replication in vivo.

Curcumin is considered to be a potentially important chemopreventive agent against a variety of cancers, including liver cancer [25]. Recently, it has been reported that curcumin inhibits the development of human hepatocellular carcinoma [26,27]. In this report, we demonstrated that curcumin inhibits HCV replicon expression via the PI3K/Akt-SREBP-1 pathway. Taken together with previous findings, although hepatocellular carcinoma is the outcome of complicated processes by various genetic factors and environmental factors, our current data suggests the possibility that curcumin may hinder the development of liver cancer via the inhibition of HCV replication in HCV-induced hepatocellular carcinoma.

Acknowledgments

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Inhibition of hepatitis C virus replication by chloroquine targeting virus-associated autophagy

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Abstract
Background Autophagy has been reported to play a pivotal role on the replication of various RNA viruses. In this study, we investigated the role of autophagy on hepatitis C virus (HCV) RNA replication and demonstrated anti-HCV effects of an autophagic proteolysis inhibitor, chloroquine.
Methods Induction of autophagy was evaluated following the transfection of HCV replicon to Huh-7 cells. Next, we investigated the replication of HCV subgenomic replicon in response to treatment with lysosomal protease inhibitors or pharmacological autophagy inhibitor. The effect on HCV replication was analyzed after transfection with siRNA of ATG5, ATG7 and light-chain (LC)-3 to replicon cells. The antiviral effect of chloroquine and/or interferon-α (IFNα) was evaluated.
Results The transfection of HCV replicon increased the number of autophagosomes to about twofold over untransfected cells. Pharmacological inhibition of autophagic proteolysis significantly suppressed expression level of HCV replicon. Silencing of autophagy-related genes by siRNA transfection significantly blunted the replication of HCV replicon. Treatment of replicon cells with chloroquine

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suppressed the replication of the HCV replicon in a dose-dependent manner. Furthermore, combination treatment of chloroquine to IFNα enhanced the antiviral effect of IFNα and prevented re-propagation of HCV replicon. Protein kinase R was activated in cells treated with IFNα but not with chloroquine. Incubation with chloroquine decreased degradation of long-lived protein leucine.

Conclusion The results of this study suggest that the replication of HCV replicon utilizes machinery involving cellular autophagic proteolysis. The therapy targeted to autophagic proteolysis by using chloroquine may provide a new therapeutic option against chronic hepatitis C.

Keywords Autophagy · Autophagosome · HCV replicon · Chloroquine

Introduction

The genome of HCV, a member of the family Flaviviridae, consists of a positive-sense single-stranded RNA. Peg-interferon/ribavirin combination therapy, which is the most effective therapy against HCV infection, is effective in around 50% for genotype 1 and 80% for genotypes 2 and 3 [1–3], however, many people cannot tolerate the serious side effects and are resistant to Peg-interferon/ribavirin combination therapy. Difficulties in eradicating HCV are attributable to the limited number of treatment options against HCV [4, 5]. Therefore, the search for novel therapeutic agents remains a strong aspiration.

Autophagy is an evolutionarily conserved cellular pathway in which the cytoplasm and organelles are engulfed within double-membraned vesicles, known as autophagosomes. While cellular autophagy is thought to be in preparation for the turnover and recycling of cellular constituents [6–8], this process has been proposed as a mechanism of virus replication complex formation in positive-stranded RNA viruses including poliovirus, equine arteritis virus and coronavirus [9–12]. In these viruses, the replication complexes consist of double membrane vesicles in the cytoplasm, suggestive of an autophagosome origin [9, 12]. Recently, it was reported that transfection of HCV replicon induced autophagy [11]. Additionally, Sir et al. [13] demonstrated that the suppression of autophagy inhibited the replication of HCV. These findings suggested that the autophagy plays a pivotal role in HCV replication.

Chloroquine, which is widely used for the treatment of malaria, is a well-established inhibitor of autophagic proteolysis which acts by inhibiting acidification of lysosomes and endosomes [14]. It has been reported that chloroquine exerts direct antiviral effects on several RNA viruses including coronaviruses, flaviviruses and human immunodeficiency virus (HIV) [8, 15–17]. Moreover, clinical studies have demonstrated the safety, tolerability, and efficacy of chloroquine in the antiviral treatment of HIV infection [18, 19]. Here, we have demonstrated that autophagic proteolysis plays a pivotal role on HCV replication, moreover, the inhibition of autophagic proteolytic pathways can constitute an effective new therapeutic target against HCV.

Materials and methods

Cell culture and treatment

Huh-7 cells were stably transfected with HCV replicon expressing chimeric protein of firefly luciferase and neomycin phosphotransferase [20, 21]. They were cultured in Dulbecco's modified essential medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% foetal bovine serum (FBS) at 37°C under 5% CO_2. To maintain cell lines carrying the HCV replicon, G418 (Wako, Osaka, Japan) was added to the medium at a final concentration 500 μg/ml.

Luciferase assay

Luciferase activities were quantified to evaluate the replication of HCV replicon by a luminometer (Lumat LB9507; Berthold, Germany) using a Bright-Glo Luciferase Assay System (Promega, Madison, WI). Assays were performed in triplicate, and the results were expressed as mean ± SD as percents of controls.

Cell viability assay

The viability of cells was assessed by WST-1 assay. Cells were cultured in 96-well plates at 5×10^3/well for 24 h, and then treated with 3-methyladenine [22] (10 mM), mixture of E64d (1 μg/ml) and pepstatin A [23] (1 μg/ml), and chloroquine (10^{-6}–10^{-3} M) for 18 h. Cell proliferation reagent WST-1 (Roche, Swiss) was added to each well, and the cells were incubated for another 1 h at 37°C. The absorbance was measured against a background control by microplates reader (SPECTRA max 340PC, Molecular Devices, Sunnyvale, CA) at 450 nm. The reference wavelength was 650 nm.

Inhibition of autophagy and replication of HCV replicon

Cells were treated with 3-methyladenine (10 mM) or mixture of E64d (1 μg/ml) and pepstatin A (1 μg/ml), chloroquine (10^{-7}–10^{-3} M), interferon (IFN)α (100 U/ml) for 18 h, the levels of replication of HCV replicon were assessed by luciferase assay. Moreover, cells were cultured

with chloroquine (10^{-5} M) and/or IFNα (100 U/ml) for 7 days, then continued to incubate without drugs for another 21 days. Replication levels of HCV replicon were determined by luciferase assay at 7th and 21st days from cessation of drugs.

Identification of autophagosomes

Naïve Huh7 cells, Huh7/Rep-Feo cells, and Huh7/Rep-Feo treated with IFNα for 14 days were seeded on 30 mm dishes and incubated for 48 h. In addition, Huh7/Rep-Feo cells were treated with chloroquine (10^{-5} M) for 18 h. Cells were prefixed with 2% glutaraldehyde, post-fixed with 1% osmic acid, dehydrated in graded ethanol, embedded in resin, and cut into sections on an ultramicrotome. The cells were analyzed by a transmission electron microscope (Hitachi H7100, Japan). The number of autolysosomes in 100 μm^2 of cytoplasm was counted by using transmission electron microscopy.

Small interfering RNA knockdown of ATG5, 7, LC-3

A combination of four chemically synthesized siRNA duplex molecules targeted to the human ATG5, 7, LC-3α, LC-3β mRNA sequence (Dharmacon, Lafayette, CO) was transiently transfected (final concentration 50 nM) into Huh7/Rep-Feo cells using a transfection reagent (Dharmacon, Lafayette, CO). siRNA targeted to enhanced green fluorescence protein was used as a control. Forty-eight hours after transfection, levels of HCV replication were analyzed by luciferase assay.

Western blot analysis

Twenty-five micrograms of total cell lysates were subjected to SDS/PAGE on a 10% gradient gel and electrophoretically transferred onto polyvinylidene fluoride membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline, membranes were incubated with primary rabbit monoclonal antibody against Phospho-protein kinase R (P-PKR) (Cell Signaling Technology, Danvers, MA) or light-chain 3 (LC3), followed by a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA). Subsequently, specific bands were visualized using ECL detection kit (Amersham Pharmacia Biotech, Midland, ON, Canada).

Protein degradation assay

Long lived protein is mainly degraded by autophagy [24]. Cells were incubated with Williams' E/10% FBS containing 0.5 μCi/ml [^{14}C]leucine for 24 h to label long-lived proteins. Cells were washed with Williams' E/10% FBS containing 10^{-5} M of unlabeled leucine and incubated with the medium for 2 h to allow degradation of short-lived proteins and minimize the incorporation of labeled leucine. The cells were then washed with phosphate-buffered saline (PBS) and incubated at 37°C with Williams' E/10% FBS in the presence or absence of chloroquine (10^{-5} M). After 4 h, aliquots of the medium were taken and a one-tenth volume of 100% trichloroacetic acid was added to each aliquot. The mixtures were centrifuged at 12,000*g* for 5 min, and the acid-soluble radioactivity was determined using a liquid scintillation counter. At the end of the experiment, the cultures were washed twice with PBS, and 1 ml of cold trichloroacetic acid was added to fix the cell proteins. The fixed cell monolayers were washed with trichloroacetic acid and dissolved in 1 ml of 1 N NaOH at 37°C. Radioactivity in an aliquot of 1 N NaOH was determined by liquid scintillation counting. The percentage of protein degradation was calculated according to published procedures [25].

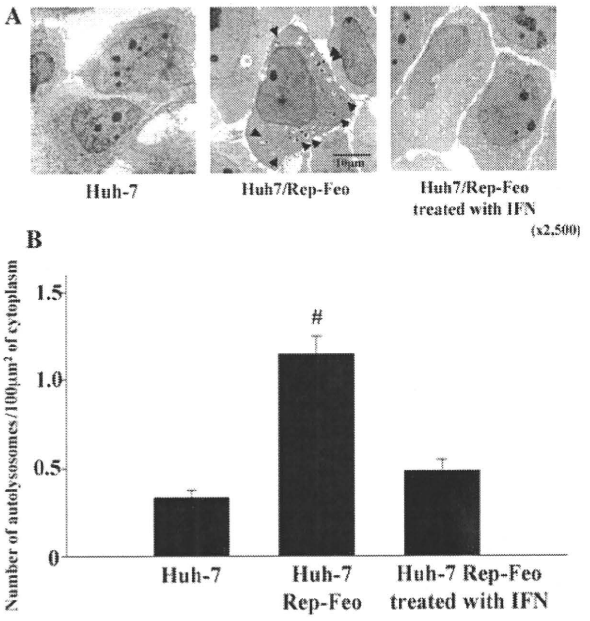

Fig. 1 Expression of autophagy is changed by presence or absence of HCV replicon. **a** Naïve Huh7 cells, Huh7/Rep-Feo cells, and Huh7/Rep-Feo treated with IFNα for 14 days were seeded on 30 mm dishes and incubated for 48 h. The cells were analyzed by a transmission electron microscopy. Autophagosomes (*arrow heads*) were detected by transmission electron microscopy. **b** The number of autolysosomes in 100 μm^2 of cytoplasm was counted by using transmission electron microscopy

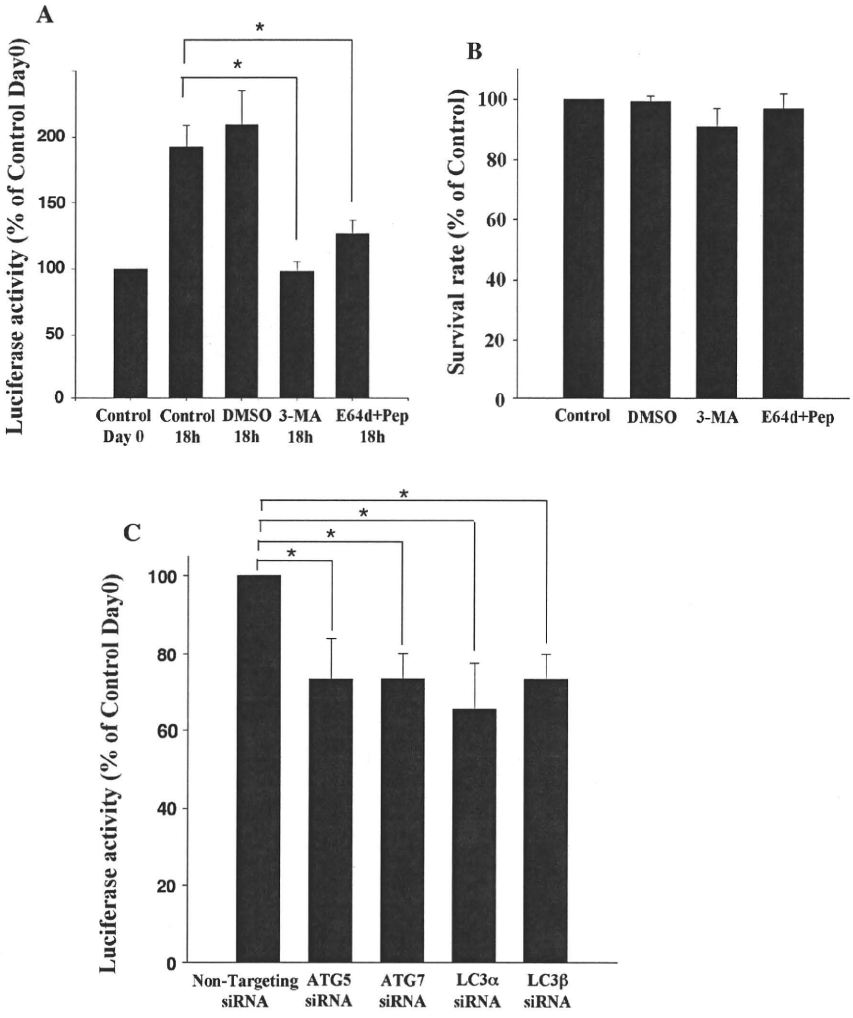

Fig. 2 Inhibition of autophagy suppressed replication of HCV replicon. **a** Cells were treated with 3-methyladenine (3-MA) (10 mM) or a mixture of E64d (1 μg/ml) and pepstatin A (Pep) (1 μg/ml) for 18 h, the levels of replication of HCV replicon were assessed by luciferase assay. **b** Cells were treated with 3-methyladenine (10 mM), mixture of E64d (1 μg/ml) and pepstatin A (1 μg/ml) for 18 h. Cell proliferation reagent WST-1 was added to each well, and the cells were incubated for 1 more hour at 37°C. The absorbance was measured against a background control by microplates reader at 450 nm. The reference wavelength was 650 nm. **c** A combination of four chemically synthesized siRNA duplex molecules targeted to the human ATG5, 7, LC-3α, LC-3β mRNA sequence was transiently transfected into Huh7/Rep-Feo cells using a transfection reagent. siRNA targeted to enhanced green fluorescence protein was used as the control. Forty-eight hours after transfection, levels of HCV replication were analyzed by luciferase assay

Statistical analysis

Differences were compared using ANOVA. Basically P values less than 0.05 were considered as statistically significant.

Results

The inhibition of autophagy suppressed replication of HCV replicon

We counted numbers of autophagosome and autolysosome in cells transduced with HCV replicon Rep-Feo by using electron microscopy. Double membrane vesicles with the morphology of autophagosomes were identified at 2.3 vacuoles/cells in naïve Huh-7 cells, while transfection of HCV replicon increased the number of vacuoles to about fourfold over untransfected Huh-7 cells (Fig. 1a, b). Subsequent treatment of the cells with IFNα (100 U/ml) for 14 days to eliminate HCV replicon substantially decreased the autophagolysosome in cytoplasm of Huh7/Rep-Feo cells (Fig. 1a, b). These observations suggested that HCV replicon induces formation of autophagosomes. To clarify the role of autophagy on the replication of HCV, Huh7/Rep-Feo cells were treated with 3-methyladenine (10 mM) or a mixture of E64d (10 μg/ml) and pepstatin A (10 μg/ml) which inhibited autophagic protein degradation. Replication level of HCV replicon in cells was increased to about twofold after 18 h in control media, however incubation with 3-methyladenine completely blunted increases in replication of HCV replicon. Treatment with 3-methyladenine decreased the number of autophagosomes to about 19% of Huh7/Rep-Feo cells. Furthermore co-incubation with E64d and pepstatin A decreased replication of HCV replicon to about 66% of control (Fig. 2a). Next, WST-1 assay was performed to check the cytotoxicity of these drugs. Treatment with 3-methyladenine or a mixture of

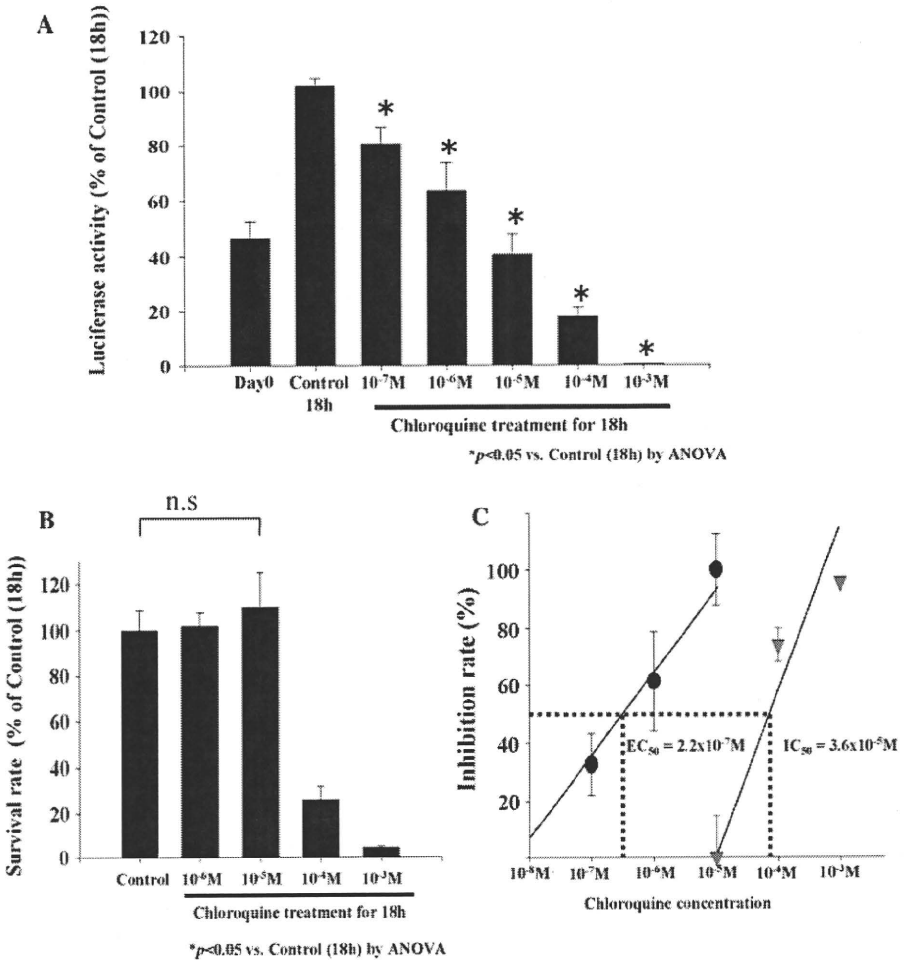

Fig. 3 Effect of chloroquine on inhibition of HCV replication and cell viability. **a** Effect of chloroquine on replication of HCV replicon. Huh-7 Rep/Feo cells were seeded in 48-well plate and incubated with chloroquine (10^{-7}–10^{-3} M) for 18 h. Replication levels of HCV replicon were determined by luciferase assay. Values are shown as percentages of the control cells. [$^*P < 0.05$ vs. control (18 h) by ANOVA]. **b** Effect of chloroquine on proliferation of Huh-7 Rep/Feo cell lines in vitro. Cells seeded in 96-well plates were treated with 10^{-6} to 10^{-3}M of chloroquine. After 18 h, effects on cell proliferation were determined by WST-1 assay. [$^*P < 0.05$ vs. control (18 h) by ANOVA]. **c** Calculation of EC_{50} and IC_{50}. Concentration of chloroquine inhibiting 50% of the replication of HCV replicon is showed as EC_{50}. IC_{50} is the concentration of chloroquine which inhibits 50% of the cell proliferation of Huh-7 Rep/Feo cells

E64d and pepstatin A did not affect cell viability (Fig. 2b). To clarify the role of autophagy induction in the replication of HCV, we suppressed the induction of autophagy by silencing autophagy-related genes (ATG5, ATG7, LC-3α and LC-3β) by siRNA transfection. Silencing of autophagy-related genes reduced the replication of HCV replicon to about 70% of control (Fig. 2c). Transfection with siRNA of autophagy related genes decreased the number of autophagosomes to about 30% of control. These results indicated that autophagy plays a pivotal role in replication of HCV.

Chloroquine inhibits the replication of HCV replicon

Next, we evaluated the anti-HCV effect of chloroquine, which is a lysosomotropic agent that raises intralysosomal pH and impairs autophagic protein degradation. To assess the effects of chloroquine on the intracellular replication of the HCV replicon, Huh7/Rep-Feo cells were cultured with various concentrations of chloroquine in the medium. The replication of the HCV replicon was increased to about twofold within 18 h in the control media, however, which was suppressed by chloroquine in a dose-dependent manner (Fig. 3a). Next, cytotoxicity of chloroquine was analysed by WST-1 assays. Huh7/Rep-Feo cells treated with chloroquine showed no significant effect on cell viability in doses of lower than 10^{-5} M (Fig. 3b). However, incubation with 10^{-4} M of chloroquine reduced the cell viability to 25% of control. On the basis of the toxicity curve, the IC_{50} of the drug was calculated to be 3.6×10^{-5} M (Fig. 3c). The average EC_{50} of chloroquine was calculated as 2.2×10^{-7} M (Fig. 3c). The replication of HCV replicon was suppressed to nearly 40% of control at 10^{-5} M of chloroquine, which did not affect cell viability. These data indicated that chloroquine efficiently inhibited the replication of HCV replicon in the absence of toxic effect to cells at the concentration of 10^{-5} M. Accordingly, we used 10^{-5} M of chloroquine for the following study.

Next, we conducted the following assay to determine the synergistic inhibitory effect of chloroquine to IFNα on HCV replication. Treatment with chloroquine for 18 h

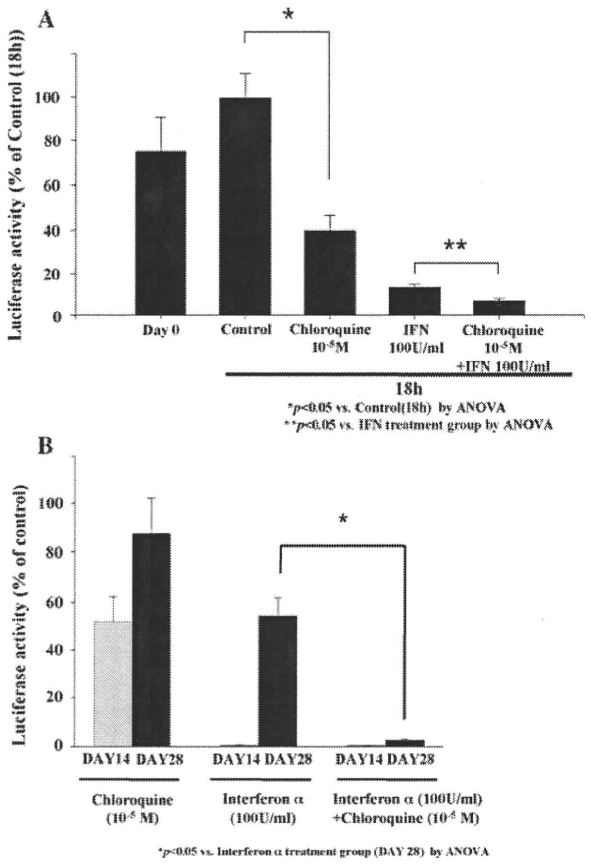

Fig. 4 Combination effect of chloroquine with IFNα on HCV replication. **a** Huh-7 Rep/Feo cells were treated with chloroquine (10^{-5} M) and/or IFNα (100 U/ml) for 18 h. Values are shown as percentages of the control cells [$*P < 0.05$ vs. control (18 h) by ANOVA, $**P < 0.05$ vs. IFNα treatment group by ANOVA]. **b** Assessment of re-propagation of HCV replicon after long term treatment of chloroquine and/or IFNα. Huh-7 Rep/Feo was incubated with chloroquine (10^{-5} M) and/or IFNα (100 U/ml) for 7 days, then drugs were removed from the medium and incubation continued for another 21 days. Luciferase assay was performed at the 7th and 21st days from cessation of drugs. Values are shown as percentages of the control cells [$*P < 0.05$ vs. IFNα treatment group (day 28) by ANOVA]

resulted in a significant decrease of HCV replicon to about 40% of control. On the other hand, incubation with IFNα for 18 h inhibited the replication of HCV replicon to the levels about 15% of controls as expected. However, co-incubation with 100U/ml of IFNα and 10^{-5} M of chloroquine further decreased HCV replication significantly (Fig. 4a).

To determine whether long-term chloroquine treatment inhibits post-treatment re-propagation of HCV replicon, we followed up luciferase activity of the cells at the 7th and 21st days after 7 days of treatment with chloroquine and/or IFNα (Fig. 4b). In HCV replicon cells treated by chloroquine, luciferase activities recovered to 53 and 88% on 7 and 21 days after cessation of treatment. In cells that were treated by IFNα, luciferase activity maintained background level for 7 days post-treatment. However, it reappeared in 21 days. In sharp contrast, co-incubation with IFNα and chloroquine for 7 days suppressed HCV replication for the extensive period up to 21 days, even in the absence of these drugs (Fig. 4b).

Anti-HCV effect of chloroquine independent of IFN signaling pathway

IFN-inducible double-stranded RNA-activated protein kinase R (PKR) plays a key antiviral role against hepatitis C virus [26, 27]. To elucidate the mechanisms of the inhibitory effect of chloroquine on HCV replication, phosphorylated PKR (P-PKR) was evaluated by western blotting analysis. P-PKR was detectable in cells treated with IFNα after 24 h; this increase in P-PKR expression peaked at 24 h after IFNα treatment and was reduced at 48 h (Fig. 5a). In contrast, P-PKR was not observed in cells treated with chloroquine at any time point.

Chloroquine blunts autophagic proteolysis in cells transfected with HCV replicon

It is reported that chloroquine disrupts lysosomal function, preventing effective autophagic protein degradation, leading to the accumulation of ineffective autophagosomes [28]. Therefore, we investigated if chloroquine led to the accumulation of autolysosomes as a result of suppression of proteolysis. We performed electron microscopic investigation to evaluate quantities of autophagosomes and autolysosomes. Ultrastructural analysis identified 0.94 ± 0.1 vacuoles/100 μm^2 of autolysosomes in control cells; however, treatment with chloroquine increased the number of autolysosomes dramatically to about 13-fold over control (Fig. 5b). Furthermore, the molecular form of LC3 protein of the cells, which is a component of autophagosomes, was examined by western blot analysis to ensure that chloroquine treatment leads to the accumulation of autophagosomes and autolysosomes. As shown in Fig. 5c, immunopositive protein bands for LC3-I and LC3-II forms were clearly evident in control cells. After chloroquine treatment, LC3-II expression increased at 4 h (Fig. 5c) to about threefold over control without enhancing LC3-I expression, and at 8 h (Fig. 5c) LC3-II expression was further enhanced. Finally, we evaluated turnover of the long-lived protein leucine, which was mainly degraded by autophagy. Huh7/Rep-Feo cells were labeled with [^{14}C]leucine for 24 h, and degradation of [^{14}C]leucine in cells treated with or without chloroquine was measured. Chloroquine treatment decreased degradation of leucin to 76% of control, indicating that chloroquine blunts degradation of proteins via an autophagic

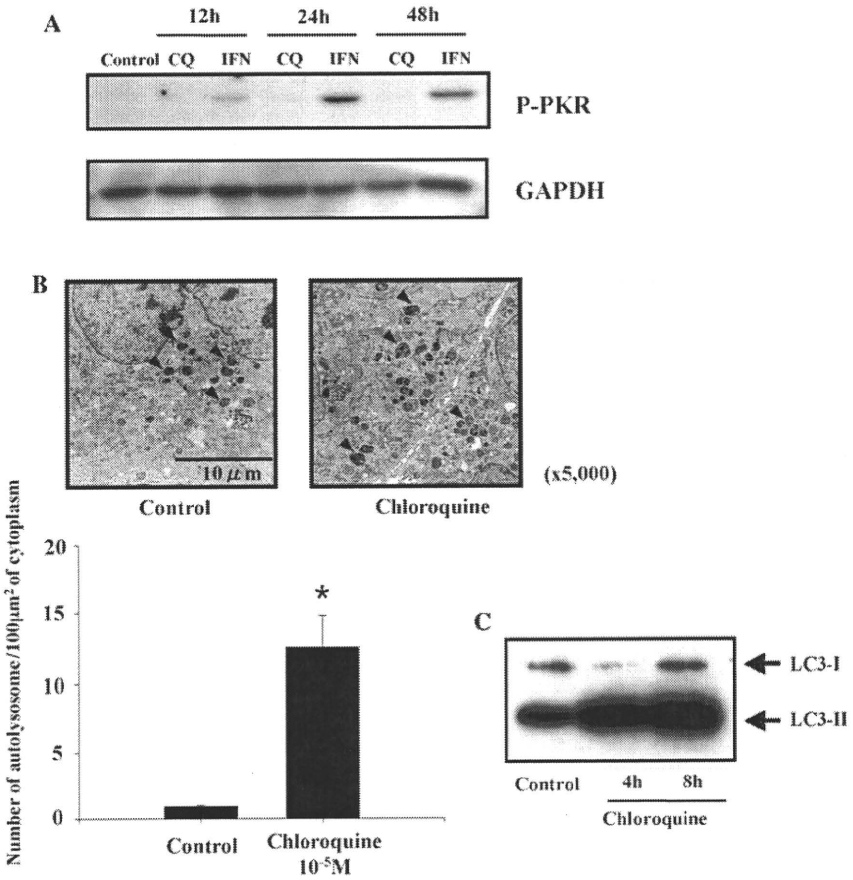

Fig. 5 Chloroquine suppresses autophagic protein degradation, not interferon pathways. **a** Cells were treated with 10^{-5} M of chloroquine (CQ) or 100 U/ml of IFNα for 24–48 h. Phosphorylation of PKR was assessed by western blot analysis. GAPDH was used as loading control. **b** Ultrastructural analysis showing the effect of chloroquine on the number of autolysosomes. Huh-7/Rep-Feo cells were incubated with chloroquine for 18 h. Autolysosomes were identified as the double membrane vesicles (arrow heads) of cytoplasm in Huh-7 Rep/Feo. The number of autolysosomes in 100 μm^2 of cytoplasm was counted by using transmission electron microscopy. Data represent mean ± SEM of individual preparations from pictures (*$P < 0.05$ vs. control by ANOVA). **c** Western blot analysis of LC3 in Huh7 Rep/Feo. The lysate of Huh7 Rep/Feo treated with chloroquine for 4–8 h were immunoblotted with LC3. GAPDH was used as loading control

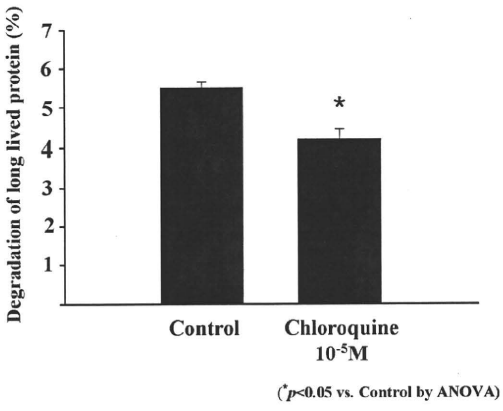

Fig. 6 Turnover of long lived protein. Huh-7 Rep/Feo cells were labeled with [^{14}C]leucin for 4 h, then degradation of long-lived protein in chloroquine treated cells was measured as described in Materials and Methods. The percentage of protein degradation was calculated by dividing the amount of acid-soluble radioactivity in the medium at that time by the amount of acid-precipitable radioactivity present in the cells at time zero. Data are mean ± SEM of value of triplicate in each group (*$P < 0.05$)

pathway (Fig. 6). These results demonstrate that chloroquine-induced the accumulation of autolysosomes was due to disruption of autophagic proteolysis.

Discussion

Previous reports have disclosed that autophagy plays a pivotal role on the replication of several RNA viruses [10–12]. Our present results demonstrate that autophagy is induced by transfection of HCV replicon and is reduced by deletion of replicon due to IFNα (Fig. 1a, b). These results suggest that autophagy is induced in the presence of HCV replication in its host cells. However, the role of autophagy in the pathogenesis of HCV is largely unclear. We found that the inhibition of autophagosome formation and autophagic proteolysis blunt the replication of genotype 1b subgenomic HCV replicon (Fig. 2a, c). Sir et al. [13] reported that inhibition of autophagy also reduced the replication of the JFH1-based full length genotype 2a genome. Therefore, the utilization of autophagy on viral replication is shown by HCV strains across different genotypes.

On the other hand, not only a silencing of autophagic gene but also pharmacological inhibition of autophagic proteolysis possesses anti-HCV effects (Fig. 2a, c). However, treatment with both chloroquine and the mixture of E64d and pepstatin induced the accumulation of

autophagosomes in cytoplasm. Therefore, it is likely that HCV does not utilize the double membrane structure as the localization of the viral replication formation. These results support the hypothesis that protein degradation due to autophagy is important for HCV replication.

Chloroquine is a well-known inhibitor of autophagic protein degradation and is often used as an anti-malarial agent. Moreover, the anti-viral effect of chloroquine on other RNA viruses has been already reported in clinical trials [15, 16]. In our results, chloroquine inhibits the intracellular replication of an HCV replicon in a dose-dependent manner (Fig. 3a). This antiviral effect of chloroquine was clearly not due to cytotoxic effects (Fig. 3b). Moreover, chloroquine possesses a synergistic effect with IFNα on HCV replication (Fig. 4a). Although IFNα possesses strong anti-HCV effects, re-propagation of HCV replicon was observed after 3 weeks following 7 days of treatment with IFNα. Interestingly, co-incubation with IFNα and chloroquine for 7 days prevented re-propagation of HCV replicon (Fig. 4b). Chloroquine is a lysosomal weak base that is known to affect acid vesicles leading to dysfunction of several proteins [29]. It was demonstrated that disruption of lysosomal function impairs maturation of viruses through inhibiting the low-pH dependent proteases in trans-Golgi vesicles in HIV and the SARS coronavirus infection in vitro [15, 29]. However, little is understood about the mechanism of its antiviral effect. In previous reports, various drugs which possess inhibitory effects on the replication of HCV and have a synergistic action with IFNα have been proposed as new therapeutic agents to treat HCV. Some of them have proved to exhibit their anti-HCV effects through augmentation of IFN-induced antiviral gene responses [30, 31]. However, the anti-HCV effect of chloroquine was not associated with activation of one of IFN receptors signaling molecule PKR (Fig. 5a). Our results showed chloroquine induced the accumulation of ineffective autophagosomes in cytoplasm of Huh7/Rep-Feo cells (Fig. 5b) and inhibited the degradation of long-lived protein leucine (Fig. 6). These findings imply that chloroquine effectively impairs the function of autophagy in our experiment. These results indicated that chloroquine is a new anti-HCV agent that targets the autophagic proteolysis.

Previous reports have shown that chloroquine possesses anti-viral effects on various RNA viruses. Its best-studied effects are those against HIV replication, which are being tested in clinical trials [17, 18]. HCV co-infection is common in HIV-positive patients in USA and Europe [32, 33]. Since HIV infection accelerates the progression of HCV-related liver disease, treatment of HCV is generally recommended. However, co-infected patients have a greater risk of antiretroviral therapy-associated hepatotoxicity than patients with HIV only [34]. Moreover, treatment with ribavirin is believed to increase the risk of anemia in patients taking the HIV drug zidovudine [35]. A clinical study designed for HIV patients showed the safety and efficacy of chloroquine used for long terms up to 48 weeks [36]. Therefore, the combination therapy of interferon and chloroquine is, possibly, a hopeful therapy for HCV–HIV co-infected patients. Since chloroquine is known as one of the inexpensive drugs, therefore, chloroquine might provide a new effective, safe and economical therapeutic option for patients with HCV. In conclusion, autophagic proteolysis might be a new therapeutic target on the replication of HCV.

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