Nuclear receptor 4 group A member 1 determines hepatitis C virus entry efficiency through the regulation of cellular receptor and apolipoprotein E expression

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, affecting over 185 million people worldwide (Mohd Hanafiah et al., 2013). The treatment for chronic hepatitis C still depends mainly on IFN (Schinazi et al., 2014), despite significant progress in the development of direct-acting antiviral agents due to increased understanding of the HCV life cycle in recent years. Novel targets for antiviral therapy based on host factors will help to provide broad and optimal therapeutic options and reduce the costs of HCV treatment.

It is generally accepted that the life cycle of HCV from entry to release is closely tied to lipid metabolism, e.g. HCV particles purified from serum are associated with lipoproteins and exist as lipo-viroparticles (André et al., 2002; Gastaminza et al., 2006; Merz et al., 2011). The decoration of HCV by apolipoprotein E (ApoE) is important for virus infection because the interaction of ApoE with cell-surface heparin sulfate mediates the attachment of HCV (Jiang et al., 2012). Several enzymes involved in lipid metabolism, such as phosphatidylinositol 4-kinase (PI4KA), cytosolic phospholipase A2 (PLA2) and the triglyceride-synthesizing enzyme diacylglycerol acyltransferase-1 (DGAT1) are required for HCV replication (Borawska et al., 2009; Camus et al., 2013; Herker et al., 2010; Menzel et al., 2012; Tai et al., 2009; Xu et al., 2012). HCV assembly is believed to occur near lipid droplets (Miyanari et al., 2007), and its release is thought to be co-opted with the secretion of very-low-density lipoprotein (VLDL) (Coller et al., 2012; Gastaminza et al., 2008; Huang et al., 2007). On the other side, about half of chronic HCV-infected individuals develop fatty liver disease (steatosis), probably as a consequence of disturbed lipid metabolism in HCV infection.

Nuclear receptors are a class of proteins that have the activity of transcription factors and exert pleiotropic effects on lipid and glucose metabolism, inflammation, differentiation and proliferation (Francis et al., 2003; Wagner et al., 2011). The expression of many host nuclear factors is modulated by HCV infection. For example, HCV Core protein interacts with retinoid X receptor alpha (RXRα) and modulates its transcriptional activity, thus contributing to the pathogenesis of HCV infection (Tsutsu et al., 2002). HCV Core protein also stimulates lipogenesis.
through the induction of liver X receptor (LXR), sterol regulatory element-binding protein C (SREBP1c) and peroxisome proliferator-activated receptor γ (PPARγ) activity (García-Mediavilla et al., 2012; Kim et al., 2007). Nuclear receptors might be potential anti-HCV targets, as agonists to PPARx, PPARγ and LXR suppress HCV replication (García-Mediavilla et al., 2012; Goldwasser et al., 2011; Rakic et al., 2006; Zeng et al., 2012).

The nuclear receptor subfamily 4 group A member 1 (NR4A1, also known as TR3, Nur77 and nerve growth factor-induced gene-B), is an inducible transcription factor that is widely expressed in various tissues. NR4A1 consists of an N-terminal activation function 1 (AF-1) domain, a DNA-binding domain, a ligand-binding domain and a C-terminal ligand-dependent activation AF-2 domain (Chang et al., 1989). NR4A1 modulates hepatic metabolic processes such as lipogenesis, glucose homeostasis and apoptosis in hepatic cells (Pei et al., 2006; Pols et al., 2008; Yeo et al., 2005). The expression of NR4A1 may be induced via transactivation by several viruses, such as hepatitis B virus, human T-lymphotropic virus type 1 and adenovirus (Chen et al., 1998; Granberg et al., 2006; Lee et al., 2001; Liu et al., 1999). Furthermore, the interaction of Epstein–Barr virus nuclear antigen 2 with NR4A1 retains NR4A1 in the nucleus and is thought to be a viral strategy to block apoptosis, whilst the interaction of NR4A1 with human papillomavirus 16 E2 protein has been proposed to modulate viral genome transcription (Lee et al., 2002, 2004; Olejnik-Schmidt et al., 2006). In the present study, we analysed the expression of NR4A1 during HCV infection and explored its role in the HCV life cycle.

RESULTS

HCV infection upregulates NR4A1 expression

We previously performed a gene array analysis to identify host factors relevant to HCV replication and confirmed the participation of two host factors, AREG and PLA2G4C, in the HCV life cycle (Pei et al., 2011; Xu et al., 2012). Among the host genes upregulated during HCV infection in Huh7.5.1 cells, NR4A1, a nuclear receptor that modulates hepatic lipid and glucose metabolism, was of particular interest. After HCV infection, the level of NR4A1 mRNA increased in parallel with that of HCV RNA (Fig. 1a, b). At 48 h post-infection (p.i.), the NR4A1 mRNA level was upregulated fivefold in cells infected with HCV J399EM strain at an m.o.i. of 1. A 6.6-, 8.6- and 16.2-fold increase in NR4A1 mRNA expression was measured at 72 h p.i. in cells infected with J399EM at an m.o.i. of 0.01, 0.1 and 1, respectively, compared with uninfected cells (Fig. 1b). Consistent with the changes in mRNA level, NR4A1 protein expression level was increased significantly (Fig. 1c).

NR4A1 protein expression and its subcellular localization were analysed further by indirect immunofluorescence. The levels of endogenous NR4A1 expression in Huh7.5.1 cells were increased by HCV infection, as indicated by the stronger intensity of fluorescence (Fig. 1d). Surprisingly, a cytoplasmic distribution of NR4A1 was observed both in uninfected and HCV-infected Huh7.5.1 cells, which was different from the nuclear localization of NR4A1 protein in other cell lines, such as HeLa cells, as reported previously (Humphries et al., 2004) (Fig. S1, available in the online Supplementary Material).

Knockdown of NR4A1 expression impairs HCV infection

To determine the role of NR4A1 in HCV replication, Huh7.5.1 cells were transfected with small interfering (si)RNAs targeted to NR4A1 (siNR4A1-1, -2 and -6) and subsequently infected with J399EM. siHCV and siNC were included as positive and negative controls, respectively. Cell viability was not impacted by transfection with siRNAs as shown by a WST1 assay (Fig. S2). Transfection of siNR4A1 efficiently downregulated NR4A1 expression and blocked the upregulation of NR4A1 by HCV infection (Fig. 2a), whilst both the intracellular HCV RNA level (Fig. 2b) and HCV NS3 and Core protein expression (Fig. 2c) were decreased. The siNR4A1-6 most efficiently inhibited NR4A1 expression and HCV replication at 72 h p.i. (Fig. 2b, c and Fig. S3). The samples at this time point (72 h p.i.) were analysed further to determine the supernatant and intracellular HCV RNA copy numbers and viral titres. The assembly and budding efficiency of HCV were calculated, indicated by the ratio of supernatant HCV RNA copies to intracellular HCV RNA copies and supernatant HCV titre to intracellular HCV titre, respectively. Whilst lower levels of HCV RNA copy number in the supernatant (about a 2.5-fold decrease) and viral titres (four- to fivefold decrease) were observed, the maturation process of HCV, including assembly (Fig. 2d) and budding (Fig. 2e), was not affected by NR4A1 knockdown. Interestingly, the specific infectivity of HCV particles, defined as the ratio of the infective titre to HCV RNA copy number, decreased by 42 % after NR4A1 knockdown (Fig. 2f).

To further confirm the role of NR4A1 in the HCV life cycle, two NR4A1 stable knockdown cell lines (Huh7.5.1-shNR4A1-UTR and Huh7.5.1-shNR4A1-CDS2) were generated based on the Huh7.5.1 cell line using the pSUPER RNAi system with small hairpin (sh)RNAs targeted to NR4A1 (siNR4A1-1, -2 and -6) and subsequently infected with J399EM. shHCV and shNC were included as positive and negative controls, respectively. Cell viability was not impacted by transfection with shRNAs as shown by a WST1 assay (Fig. S2). Transfection of siNR4A1 efficiently downregulated NR4A1 expression and blocked the upregulation of NR4A1 by HCV infection (Fig. 2a), whilst both the intracellular HCV RNA level (Fig. 2b) and HCV NS3 and Core protein expression (Fig. 2c) were decreased. The siNR4A1-6 most efficiently inhibited NR4A1 expression and HCV replication at 72 h p.i. (Fig. 2b, c and Fig. S3). The samples at this time point (72 h p.i.) were analysed further to determine the supernatant and intracellular HCV RNA copy numbers and viral titres. The assembly and budding efficiency of HCV were calculated, indicated by the ratio of supernatant HCV RNA copies to intracellular HCV RNA copies and supernatant HCV titre to intracellular HCV titre, respectively. Whilst lower levels of HCV RNA copy number in the supernatant (about a 2.5-fold decrease) and viral titres (four- to fivefold decrease) were observed, the maturation process of HCV, including assembly (Fig. 2d) and budding (Fig. 2e), was not affected by NR4A1 knockdown. Interestingly, the specific infectivity of HCV particles, defined as the ratio of the infective titre to HCV RNA copy number, decreased by 42 % after NR4A1 knockdown (Fig. 2f).
of HCV were comparable in the Huh7.5.1-shNR4A1-UTR and Huh7.5.1-shNR4A1-CDS2 cell lines to that in the Huh7.5.1-shNC cells. Taken together, these data clearly demonstrated that NR4A1 plays a role in HCV infection.

**Inhibition of NR4A1 impairs HCV entry**

NR4A1 may also be involved in HCV entry, RNA replication or translation. To dissect these steps, we first explored transduction with HCV pseudoparticles (HCVpps) to examine the role of NR4A1 at HCV entry. Huh7.5.1 cells were transfected with individual siRNAs and then transduced with HCVpps. Luciferase activities in transduced cells were measured 48 h after transduction and used as the indicator of HCV entry efficiency. Silencing of NR4A1 inhibited HCV entry efficiency to 44 % of that of the siNC controls (Fig. 3a). The positive control siCD81 significantly blocked HCV entry into Huh7.5.1 cells. Consistent with these observations, HCV entry efficiency was reduced to 24 and 33 %, respectively, in Huh7.5.1 shNR4A1-UTR and shNR4A1-CDS2 cell lines compared with the shNC cell line (Fig. 3b), indicating an important role of NR4A1 in the entry step of HCV infection.

To examine whether NR4A1 is required for HCV translation, pHCV-IRES was transfected into NR4A1 stable knockdown cell lines and HCV internal ribosome entry site (IRES)-dependent luciferase expression was measured. No significant difference in HCV IRES-dependent luciferase expression was observed in NR4A1 stable knockdown cell lines and the control cell line (Fig. 3c), suggesting that NR4A1 did not affect HCV translation.

To further determine the influence of NR4A1 on HCV RNA replication, specific and control siRNAs were transfected into Con1 cells harbouring a subgenomic HCV replicon. The NR4A1 mRNA level was efficiently reduced by siNR4A1-6 (Fig. 3d). Although a slight decrease in HCV RNA was observed at 48 h post-transfection (p.t.), there was no significant difference in HCV RNA levels at 72 h p.t. between NR4A1 knockdown and control cells (Fig. 3e). Moreover, the level of HCV NS3 protein did not change significantly (Fig. 3f). In contrast, the positive control with siHCV decreased both HCV RNA and protein levels. Similar results were obtained in a Huh7.5.1 cell line with a JFH1-SGR (genotype 2a) subgenomic replicon (Fig. S5). These data suggested that knockdown of NR4A1 specifically inhibited HCV entry.

**NR4A1 regulates HCV receptor expression**

NR4A1, a transcription factor, is involved in HCV entry and might regulate the expression of HCV receptors and co-receptors such as CD81, scavenger receptor BI (SR-BI),...
NR4A1 determines HCV entry efficiency

Fig. 2. Role of NR4A1 in the HCV life cycle. (a–f) Huh7.5.1 cells were transfected with different siRNAs before J399EM infection (m.o.i.=0.1). The relative intracellular NR4A1 mRNA levels (a), HCV RNA levels in cells (b) and HCV NS3 and Core expression (c) were measured at 72 h p.i. The numbers below the blot indicate the arbitrary units of the densitometry analysis normalized against actin. The assembly efficiency (Sup HCV copies/intra HCV copies) (d), the intracellular (Intra) and supernatant (Sup) HCV titres and the budding efficiency (e), and the specific infectivity of the virus in the supernatant (Sup HCV titre/sup HCV copies) (f) were calculated. (g–i) Huh7.5.1 shNR4A1 and shNC cells were infected with J399EM at an m.o.i. of 0.1 and the samples were collected at 72 h p.i. The intracellular HCV RNA level in cells (g), the assembly efficiency (h), the intracellular (Intra) and supernatant (Sup) HCV titres and the budding efficiency (i) were calculated. The results shown are representative of three independent experiments (means ± SD). *P<0.05; **P<0.01; #P<0.001; n.s., no significant difference.
tight-junction proteins claudin-1 (CLDN1) and occludin (OCLN), and epidermal growth factor receptor (EGFR) (Evans et al., 2007; Lupberger et al., 2011; Pileri et al., 1998; Ploss et al., 2009; Scarselli et al., 2002). Quantitative reverse transcription-PCR and Western blot analysis demonstrated that NR4A1 silencing significantly reduced both the mRNA (Fig. 4a) and protein (Fig. 4b) levels of SR-BI, CLDN1, OCLN and EGFR but not those of CD81. Consistent with these observations, the expression of SR-BI, CLDN1, OCLN and EGFR in the NR4A1 stable knockdown cell lines Huh7.5.1 shNR4A1-UTR and shNR4A1-CDS2 were lower than in control cells (Fig. 4c). Thus, siNR4A1 inhibited HCV entry by downregulation of the expression of multiple HCV receptors.

**NR4A1 modulates the expression of ApoE protein**

It has been reported that NR4A1 modulates hepatic lipogenesis (Pols et al., 2008). Because the specific infectivity of HCV in NR4A1-silenced cells was reduced, we suspected that NR4A1 might also regulate the expression of apolipoproteins such as ApoE, which associate with mature HCV virions and play an important role in virion infectivity (Hueging et al., 2014; Jiang et al., 2012). As shown in Fig. 5(a), siNR4A1 reduced ApoE mRNA to 50% in Huh7.5.1 cells either infected or uninfected by HCV. Correspondingly, the intracellular expression of ApoE protein was significantly reduced by siNR4A1 (Fig. 5b). Interestingly, the level of HCV virions associated with ApoE was significantly reduced in siNR4A1-transfected cells, as indicated by a co-immunoprecipitation assay with antibody to ApoE (Fig. 5c). The buoyant density distribution of the HCV particles released in the culture medium of siNC- and siNR4A1-transfected cells was then analysed using iodixanol density gradients (Fig. 5d, e).

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**Fig. 3.** Inhibition of NR4A1 impairs HCV entry. (a) Huh7.5.1 cells were transfected with siNR4A1-6 for 48 h and then transduced with HCVpp. Luciferase activities were measured 48 h later. Naïve Huh7.5.1 cells without HCVpp transduction served as a negative control of the luciferase assay. (b) Huh7.5.1 shNC, shNR4A1-CDS2 and shNR4A1-UTR cells were transduced with HCVpp and luciferase activities measured 48 h later. (c) Huh7.5.1 shNC, shNR4A1-CDS2 and shNR4A1-UTR cells were transfected with pHCV-IRES and luciferase activities were measured 48 h later. Translation efficiency was determined by the ratio of firefly luciferase (F-Luc) activity to Renilla luciferase (R-Luc) activity. Results are shown as means ± SD of experiments carried out in triplicate. (d–f). Con1 cells were transfected with the indicated siRNAs. NR4A1 mRNA (d), HCV RNA (e) and NS3 protein expression (f) levels were determined at 48 h and 72 h post-transfection (p.t.). The results shown are representative of three independent experiments (means ± SD). *P<0.05; **P<0.01; #P<0.001.
Fig. 4. NR4A1 regulates the expression of HCV receptors. (a, b) Huh7.5.1 cells were transfected with siNR4A1-6 and the mRNA levels (a) and protein expression (b) of the HCV receptors CD81, SR-BI, CLDN1, OCLN and EGFR were determined. (c) Expression of HCV receptors in NR4A1 stable knockdown cells. Whole-cell lysates of Huh7.5.1 shNC, shNR4A1-UTR and shNR4A1-CDS2 were collected to measure the protein expression of HCV receptors. The numbers below the blot indicate the arbitrary units of the densitometry analysis normalized against actin. The results shown are representative of three independent experiments (means ± SD). *P<0.05; **P<0.01; #P<0.001.

Fig. 5. NR4A1 modulates the expression of ApoE protein. Huh7.5.1 cells were transfected with siNC or siNR4A1-6 and then infected with J399EM at an m.o.i. 0.1 for 72 h. (a) Levels of NR4A1 mRNA, ApoE mRNA and HCV RNA were determined. (b) The expression of ApoE protein, HCV NS3 and Core were determined by Western blotting using primary antibodies against the respective proteins. (c) ApoE-associated virus particles were collected by immunoprecipitation as described in Methods. The HCV RNA levels of immunoprecipitated viral particles were measured by real-time RT-PCR. (d, e) The buoyant density distribution of the HCV particles recovered from the siNC (d)- or siNR4A1 (e)-transfected cells was measured. The results shown are representative of three independent experiments (means ± SD). *P<0.05; **P<0.01; #P<0.001.
from 1.01 to 1.30 g ml$^{-1}$, and the infectious virions covered densities ranging from 1.02 to 1.12 g ml$^{-1}$. There was no difference in the distribution patterns between the two groups, which is consistent with a previous report showing that reducing ApoE expression did not change the buoyant density distribution of HCV virions (Hishiki et al., 2010).

**Inhibition of NR4A1 delays HCV spread in cell culture**

The results described above indicated that NR4A1 influences HCV replication through the control of HCV receptor expression to affect virus entry and through the control of ApoE expression to affect viral infectivity. Therefore, virus spread should be modulated by NR4A1. To verify this hypothesis, HCV infection was monitored by EGFP expression in cells infected with HCV J399EM by flow cytometry analysis. The percentage of EGFP-positive cells was calculated at 24, 48, 72 and 96 h p.i. (Fig. 6a, b). When infected at a low m.o.i. (0.05), the percentage of EGFP-positive cells was only 0.8% at 24 h p.i. and increased to 2.7% at 48 h p.i. A sharp increase to 40 and 82% occurred at 72 and 96 h p.i. NR4A1 silencing reduced the level of EGFP-positive cells to only 16 and 35% at 72 and 96 h p.i., respectively (Fig. 6a). When a higher m.o.i. was used in the initial infection, 83% of cells of the control were positive for EGFP at 72 h p.i., whilst only 54% of siNR4A1-transfected cells were EGFP positive (Fig. 6b). However, the majority of the siNR4A1 transfected cells (73%) were EGFP positive at 96 h p.i. The fluorescence intensity was similar in siNC- and siNR4A1-transfected cells. These results showed that NR4A1 knockdown delayed HCV spread but did not totally block viral entry or replication.

**DISCUSSION**

Many nuclear receptors have been reported to participate in HCV replication through the modulation of cell processes. Upon HCV infection, NR4A1, a member of the nuclear receptor family, demonstrated both mRNA and protein upregulation. Knockdown of NR4A1 in Huh7.5.1 cells downregulated the expression of ApoE and HCV receptors SR-BI, OCLN, CLDN and EGFR and impaired HCV entry and spread.

Previous studies have demonstrated that members of the NR4A subgroup were tremendously induced in different tissue types and cultured cells upon stimulation with a variety of factors including inflammatory cytokines, growth factors, and stress and peptide hormones (Maxwell & Muscat, 2006; Pei et al., 2005; Tetradis et al., 2001; Williams & Lau, 1993). Expression of the HCV structural
and non-structural proteins alone was not able to induce NR4A1 expression (data not shown). However, we found that U0126, a specific inhibitor of MEK1/2, interfered with HCV-induced NR4A1 expression (Fig. S6). These results suggest that HCV infection might induce NR4A1 expression through the mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway, which is activated by HCV infection (Pei et al., 2011) and has been reported to be involved in regulating the transcription of NR4A1 induced by several stimuli (Darragh et al., 2005). The induction of NR4A1 by purified virus appeared at late stage of virus infection (48–72 h.p.i.); however, NR4A1 was induced as early as 6 h.p.i. when unpurified virus was inoculated (data not shown), implying the indirect induction of NR4A1 by the cytokines in the supernatant. The details of this possible mechanism are under investigation.

As a transcription factor, NR4A1 localizes to the nucleus of various cell types. After stimulation, NR4A1 is translocated from the nucleus to mitochondria and mediates apoptosis in different cell lines (Chen et al., 2013; Lin et al., 2004; Thompson & Winoto, 2008; Wilson et al., 2003). The NR4A1 function in transcription activity and in induction of apoptosis by mitochondrial localization is negatively regulated by post-translational modification. The phosphorylation of NR4A1 at Ser350 by Akt, which resides within its DNA-binding domain, has been shown to decrease the transcriptional activity of NR4A1 (Hirata et al., 1993; Pekarsky et al., 2001). In addition, the phosphorylation of NR4A1 at Ser105 by nerve growth factor promotes NR4A1 translocation from the nucleus to the cytoplasm together with RXR, thus reducing the transcriptional activity of RXR (Katagiri et al., 2000). In another report, phosphorylation of NR4A1 at the N terminus by Akt blocks its mitochondrial localization in the cytoplasm and protects the cells from apoptosis (Chen et al., 2008). In the present study, we found that NR4A1 was distributed predominantly in the cytoplasm of Huh7.5.1 cells, which maintained normal cell proliferation activity. Further research is needed to determine the type of post-translational modification of NR4A1 that results in its unique activity in Huh7.5.1 cells.

NR4A1 can both positively and negatively regulate cellular gene expression through transcriptional regulation activity in the nuclear (genomic action) and extra-nuclear effect independent of its transcriptional function (non-genomic actions) (To et al., 2012). For direct transcriptional regulation, NR4A1 functions as a transcription factor that can bind to DNA as monomers, homodimers and heterodimers to regulate gene expression in nucleus (Perlmann & Jansson, 1995; Philips et al., 1997; Wilson et al., 1991). Non-genomic regulation of gene expression by NR4A1 has also been reported but remains to be explored; for example, cytoplasmic NR4A1 degrades β-catenin and thus suppresses the expression of genes downstream of β-catenin signalling (Sun et al., 2012). According to our data, knockdown of NR4A1 in Huh7.5.1 cells in which NR4A1 localized mainly to the cytoplasm resulted in a decrease in SR-BI, CLDN1, OCLN, EGFR and ApoE expression levels but not CD81, indicating that cytoplasmic NR4A1 also specifically regulates cellular gene expression. It is not clear how NR4A1 regulates the expression of these genes. NR4A1 localized mainly to the cytoplasm in Huh7.5.1 cells, although the possibility exists that transient or low levels of NR4A1 appeared in the nucleus, which cannot be detected under the experimental conditions, it is unlikely that NR4A1 binds directly to the promoters of these genes, as neither the canonical NR4A1 binding sequence NGFI-B response element nor a similar sequence was found in the promoter region of these genes. One possibility is that the interaction or crosstalk of NR4A1 with other transcription factors such as RXR and NF-κB, which might be regulators of these genes, was affected and the cytoplasmic localization of NR4A1 restricted the function of these transcription factors. It is also possible that the modulation of these genes is related to the other functions of NR4A1 in the cytoplasm such as the degradation of β-catenin. Thus, NR4A1 expression induced by HCV infection might upregulate SR-BI, CLDN1, OCLN, EGFR and ApoE expression independently of its transcription activity and further enhance HCV entry and infectivity.

Infectious HCV particles are believed to associate with lipoproteins, especially VLDLs and low-density lipoproteins (LDLs) (Gastaminza et al., 2006; Merz et al., 2011), which might occur during HCV assembly and release (Coller et al., 2012; Gastaminza et al., 2008; Huang et al., 2007). VLDL is assembled by triglycerides, cholesterol and apolipoproteins, including ApoB100, ApoC1 and ApoE. Previously, NR4A1 was reported to modulate lipid metabolism in the liver (Pols et al., 2008), as both triglyceride and cholesterol content was reduced by NR4A1, most likely through reduced lipid uptake (Zhang et al., 2012). However, in the NR4A1 knockdown Huh7.5.1 cell line, we did not observe significant changes in quantity or size of lipid droplets, which are triglyceride and cholesterol storage organelles and the lipid source for VLDL formation (Gibbons et al., 2000). In contrast, the expression and secretion of ApoE were clearly impaired by the silencing of NR4A1, and levels of ApoE-associated HCV virions were significantly reduced. Nonetheless, the reduced expression of ApoE lowered the infectivity of HCV particles (Benga et al., 2010; Chang et al., 2007; Hishiki et al., 2010). Notably, in mice expressing NR4A1 in the liver, total plasma triglyceride and cholesterol were not changed; however, plasma lipid distribution profiles were modified, as indicated by reduced high-density lipoprotein-cholesterol, and increased LDL-cholesterol and LDL-triglyceride (Pols et al., 2008). It is possible that, in vivo, altered lipid profiles in the plasma influence the infectivity of HCV particles, but further evidence is needed to confirm this hypothesis.

In summary, the data presented in this study provide evidence to illustrate the important role of NR4A1 in the HCV life cycle. NR4A1 induced by HCV infection directly
or indirectly might provide an environment facilitating HCV infection and promote virus spread. As a transcription factor, NR4A1 might participate in the progression of chronic hepatitis C infection and hepatocellular carcinoma. A comprehensive understanding of the complex function of NR4A1 in HCV infection will be helpful in the development of effective treatment strategies against HCV infection and HCV-related liver diseases.

**METHODS**

**Cell lines and virus.** Huh7.5.1 cells (kindly provided by Professor F. Chisari, the Scripps Research Institute) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10 % FBS, 100 U penicillin ml⁻¹ and 100 μ streptomycin ml⁻¹ at 37 °C in a 5 % CO₂ incubator. The subgenomic HCV replicon cell lines (Con1 and Huh7.5 JFH1-SGR) (Pei et al., 2011; Xu et al., 2012) were grown in medium containing 500 μ G418 ml⁻¹. The HCV J399EM strain was derived from the JFH-1 virus (kindly provided by Professor T. Wakita, National Institute of Infectious Diseases, Japan) by introducing EGFP into the HCV NS5A locus (Han et al., 2009). To generate viral stocks, Huh7.5.1 cells were inoculated with J399EM at an m.o. of 0.01 and passaged once. The supernatant was collected at 7 days p.i., and virus was concentrated and purified by polyethylene glycol-8000 precipitation and sucrose-cushion centrifugation. Viral stocks were aliquotted and stored at −80 °C.

**Antibodies.** The antibodies used in this study are commercially available: antibodies against HCV Core, anti-HCV NS3 and anti-ApoE were from Abcam; anti-NR4A1 was from Cell Signalling Technology; anti-CDBI, anti-actin and anti-EGFR were from Santa Cruz Biotechnology; anti-SR-BI was from Novus Biologicals; anti-claudin-1 and anti-occludin were from Life Technologies Corp. HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch. Alexa Fluor 561-conjugated secondary antibodies used for indirect immunofluorescence were obtained from Invitrogen.

**Construction of plasmids.** The coding sequence of human NR4A1 was amplified from cDNA of 293T cells by using the primers NR4A1/F and NR4A1/R (Table S1) according to the published reference sequence (GenBank accession no. NM_002135) and inserted into the HindIII and KpnI restriction sites of the pXJ40-HA plasmid to generate the N-terminally haemagglutinin (HA)-tagged expression construct pHA-NR4A1. Expression plasmids for HCV proteins and the bicistronic reporter pHCV-IRES have been described previously (Xu et al., 2012).

**Transfection of plasmid DNA and siRNA.** The siRNAs targeting NR4A1 and negative-control siRNA were obtained from Qiagen and GenePharma, respectively. The target sequences of siRNAs were as follows: NR4A1-1, 5'-CTCCAGTGGCCTGCTGACTA-3' (Qiagen); NR4A1-1, 5'-CAGUCGCGCCGAGGCUGCUCCU-3'; NR4A1-2, 5'-GGAAGGGTGTCGGAACAGAC-3'. The siRNAs and plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. To achieve a prolonged gene-silencing effect, cells were split 24 h after the first transfection and transfected with the same siRNA again. HCV infection was performed at 6 h after the second transfection.

**Generation of NR4A1 knockdown cell lines.** The coding sequences for the siRNAs targeting the NR4A1 gene (shUTR, 5'-TCCCTTCACAGTACATAAAC-3'; shCDS2, 5'-CTCGAGTGGGTTCTGACTA-3') and a negative-control siRNA (5'-GTTGCA-GCAGATTGTGAAATCCCCTATGAA-3') were cloned into the shRNA expression vector pSUPER.retro.neo (OligoEngine) following the manufacturer’s instructions. To obtain the retrovirus, 1.5 μg shNR4A1-UTR, shNR4A1-CDS2 or shNC plasmids were transfected together with 1.5 μg pVPack-GP (Stratagene) and 1.5 μg pVPack-VSV-G (Stratagene) into 293T cells by Lipofectamine 2000 (Invitrogen). The supernatants of transfected cells were collected 60 h later and filtered through a 0.45 μm syringe filter (Millipore) and mixed with 8 μg polybrene ml⁻¹.

To generate stable knockdown cell lines, HuH7.5.1 cells were seeded in a six-well plate at a density of 3 × 10⁵ cells per well and incubated with the retrovirus-containing supernatants. Spinnoculation was performed at 1100 g at 32 °C for 30 min. Cells were transferred into 10 cm plates 2 days later and selected in medium containing 1000 μg G418 ml⁻¹ for 3 weeks. The cell clones were pooled and cultured for further analysis.

**Quantitative real-time RT-PCR.** Total cellular RNA was prepared using TRIzol reagent (Invitrogen) and digested with RNase-free DNase (Promega) according to the manufacturer’s protocols. HCV RNA in the supernatant was extracted using TRIzol LS reagent (Invitrogen). Specific mRNAs and HCV RNAs were quantified using a Quantitect SYBR Green RT-PCR kit (Applied Biosystems). The levels of mRNAs or HCV RNAs were normalized against β-actin mRNA. The primers used are listed in Table S1.

**Western blotting and indirect immunofluorescence staining.** Whole-cell lysates were prepared and quantified using a Bio-Rad protein assay following the manufacturer’s instructions (Xu et al., 2012). Equal amounts of protein samples (30 μg) were subjected to SDS-PAGE and transferred onto a nitrocellulose filter membrane (Millipore). After blocking with 5 % non-fat milk in TBS with 0.05 % Tween 20, membranes were incubated with specific primary antibodies and corresponding HRP-conjugated secondary antibodies. Specific protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech), and visualized and quantified on a PhosphorImager (Typhoon 8000; Amersham Pharmacia Biotechnology). Indirect immunofluorescence staining was performed as described previously (Xu et al., 2012).

**Transduction of HCV HCVpps and titration of cell-culture-derived infectious HCV (HCVccs).** HCVpps containing the luciferase transgene were generated by transfection of 293T cells with pNL4.3.lucR’E” and pCDNA3.1-E1E2 (a gift from Dr Jin Zhong, Institute Pasteur of Shanghai, China) plasmids (Tao et al., 2009) with Lipofectamine 2000 (Invitrogen), as described previously (Pei et al., 2011). HuH7.5.1 cells were transfected with siRNAs as indicated and then transduced with HCVpps. After 6 h incubation at 37 °C, HCVpp-containing medium was replaced with fresh medium. Forty-eight hours later, luciferase assays were performed using a Steady-Glo luciferase assay kit (Promega).

The HCVcc titres in culture supernatants and cell lysates were determined by a modified end-point dilution assay described by Lindenbach (2009). Briefly, serially diluted samples containing HCVcc particles were used to infect the Huh7.5.1 cells in a 96-well-plate (eight wells per dilution). Viral titres were calculated with the EGFP-positive wells counted under a fluorescence microscope using the method described by Lindenbach (2009).

**Determination of cell viability.** Cell viability after transfection of siRNAs was determined by a WST-1 assay (Roche). Briefly, HuH7.5.1 cells were transfected with siRNAs and reseeded in 96-well plates at a density of 5 × 10⁴ cells per well and cultured for the indicated periods. WST-1 reagent (10 μl) was added to the medium and incubated for 2 h at 37 °C. Absorbance at 450 nm was measured using an Epoch microplate spectrophotometer (Biotek).
Immunoprecipitation of ApoE-associated viral particles. Three micrograms of normal mouse IgG and ApoE-specific antibody (Abcam) were each mixed with 300 µl HCVcc supernatant and rotated for 16 h at 4 °C. After incubation, 50 µl washed protein G-conjugated agarose beads (Millipore) were added, and the samples were rotated for 6 h at 4 °C. Unbound antibodies were removed by at least three washes in PBS. HCV particle-bound beads (ApoE associated) were resuspended into 250 µl PBS, and HCV RNA was extracted with 750 µl TRIzol LS reagent (Invitrogen). HCV RNA copy numbers were quantified by one step real-time RT-PCR as described above.

Iodixanol density gradient analysis. HCV culture supernatants were collected at 72 h p.i. For iodixanol density gradient analysis, 10–40% iodixanol gradients were prepared as described previously (Lindenbach et al., 2005). HCV culture medium was precipitated with polyethylene glycol-8000, resuspended in Opti-MEM (Gibco) and then loaded on top of the 10% gradient. Gradients were centrifuged at 40,000 r.p.m. for 16 h at 4 °C in an SW-41 Ti rotor (Beckman). Fractions (0.5 ml) were collected from the top of tube. HCV RNA from each fraction was extracted using TRIzol LS reagent (Invitrogen) and quantified by quantitative RT-PCR. HCV infectivity titre was measured by the modified end-point dilution assay described above.

Determination of HCV infection by flow cytometry. Infection rates were determined by flow cytometric analysis of EGFP expression in HCV-infected cells. Cells were trypsinized, washed with PBS at different time points after J99EM infection and fixed with 2% paraformaldehyde. Uninfected HuH7.5.1 cells were used as a negative control. Prepared samples were subjected to flow cytometric analysis using a BD Accuri C6 flow cytometer (BD Biosciences).

Statistical analysis. A two-tailed unpaired t-test was applied to analyse the statistical significance of the data. A P value of less than 0.05 was considered as statistically significant. Data are presented as means ± SD.

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