transgenic mice than in non-transgenic mice, but was not significantly different. These results suggested that additive HCV-induced ROS production was unlikely to be the cause of the significantly increased ROS production after ovariectomy in the transgenic mice. The other possibility is HCV-associated attenuation of antioxidant potential against ovariectomy-induced oxidative stress. In this respect, OVX transgenic mice had a lower ratio of BAP to dROM than OVX non-transgenic mice and the expression of SOD2 and GPx1 in the liver was not increased. These results suggest that HCV protein attenuated antioxidant potential against ovariectomy-induced oxidative stress.

Proliferator-activated receptor-γ co-activator-1α is required for the induction of many ROS-detoxifying enzymes upon oxidative stress.[26] SIRT3 has been shown to function as a downstream target gene of PGC-1α and mediate the PGC-1α-dependent induction of ROS-detoxifying enzymes.[27] Additionally, AMPK, which is a crucial cellular energy sensor, regulates PGC-1α activity through both modulation of PGC-1α transcription and phosphorylation of the PGC-1α protein.[28,37] Thus, AMPK/PGC-1α signaling is one of the important pathways that protect cells from oxidative stress through the induction of several key ROS-detoxifying enzymes. Recent evidence indicating that HCV replication inhibits AMPK activity[29] prompted us to investigate whether the antioxidant potential against ovariectomy-induced oxidative stress in FL-N/35 transgenic mice was attenuated through inhibition of this signaling pathway. As expected, upon ovariectomy, AMPK was activated in non-transgenic mice, but not in transgenic mice. This, in turn, led to the lower expression of PGC-1α in the nuclear fraction of the liver in OVX transgenic mice than in OVX non-transgenic mice, resulting in the absence of significant induction of SIRT3 in the mitochondrial fraction of the liver in the OVX transgenic mice. Thus, ROS production in the liver in OVX transgenic mice was increased by attenuation of the antioxidant potential through inhibition of AMPK/PGC-1α signaling. However, it remains unknown why the expression of PGC-1α in the nuclear fraction was significantly increased in OVX transgenic mice regardless of the lack of activation of AMPK. Various kinases other than AMPK and post-translational modifications other than phosphorylation have been shown to regulate PGC-1α expression.[28] Therefore further investigations are required to clarify this issue.

Of particular concern is the relevance of the present results to HCC development in patients with HCV-associated chronic liver diseases. A recent study from Japan demonstrated a higher proportion of females, especially among elderly patients with HCV-related HCC, suggesting that the sex disparity in HCC development becomes less distinct as the patient's age at HCC diagnosis increases.[6] In general, ROS production creates a pro-carcinogenic environment under which chromosomal damage is likely to occur. The present findings that OVX transgenic mice have increased hepatic ROS production compared with that in OVX non-transgenic mice may indicate one of the mechanisms by which women with HCV infection are at high risk for HCC development when some period has passed after menopause, even though we need to clinically ascertain the increased hepatic oxidative stress in HCV-infected menopausal women with HCC.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported by a Grant-in-Aid for Scientific Research (B) (no. 23390201) and a Grant-in-aid for Exploratory Research (no. 25670374) from the Japan Society for the Promotion of Science, a Health and Labor Sciences Research Grant for Research on Hepatitis from the Ministry of Health, Labor and Welfare of Japan, and Research Project Grant P2 from Kawasaki Medical School.

REFERENCES

1 Chen DS, Locarnini S, Wait S *et al.* Report from a Viral Hepatitis Policy Forum on implementing the WHO framework for global action on viral hepatitis in North Asia. *J Hepatol* 2013. doi: 10.1016/j.jhep.2013.06.029.
2 Bruno S, Silini E, Crosignani A *et al.* Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. *Hepatology* 1997; 25: 754–8.
3 Degos F, Christidis C, Ganne-Carrie N *et al.* Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; 47: 131–6.
4 Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; 127: S5–S16.
5 Naugler WE, Sakurai T, Kim S *et al.* Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 2007; 317: 121–4.
6 Kumada T, Toyoda H, Kiriyama S *et al.* Characteristics of elderly hepatitis C virus-associated hepatocellular carcinoma patients. *J Gastroenterol Hepatol* 2013; 28: 357–64.
7 Farinati F, Cardin R, De Maria N *et al.* Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. *J Hepatol* 1995; 22: 449–56.
8 Barbaro G, Di Lorenzo G, Asti A *et al.* Hepatocellular mitochondrial alterations in patients with chronic hepatitis C:

ultrastructural and biochemical findings. *Am J Gastroenterol* 1999; 94: 2198–205.
9 Tanaka H, Fujita N, Sugimoto R *et al.* Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C. *Br J Cancer* 2008; 98: 580–6.
10 Moriya K, Nakagawa K, Santa T *et al.* Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001; 61: 4365–70.
11 Furutani T, Hino K, Okuda M *et al.* Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2006; 130: 2087–98.
12 Ji H, Zheng W, Menini S *et al.* Female protection in progressive renal disease is associated with estradiol attenuation of superoxide production. *Gend Med* 2007; 4: 56–71.
13 Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radic Biol Med* 2003; 34: 546–52.
14 Beard MR, Abell G, Honda M *et al.* An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* 1999; 30: 316–24.
15 Lerat H, Honda M, Beard MR *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; 122: 352–65.
16 Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; 37: 911–7.
17 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265–75.
18 Nishina S, Hino K, Korenaga M *et al.* Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. *Gastroenterology* 2008; 134: 226–38.
19 Harrison-Findik DD, Schafer D, Klein E *et al.* Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression. *J Biol Chem* 2006; 281: 22974–82.
20 Cesarone MR, Belcaro G, Carratelli M *et al.* A simple test to monitor oxidative stress. *Int Angiol* 1999; 18: 127–30.
21 Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 2001; 276: 7806–10.
22 Nemeth E, Tuttle MS, Powelson J *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004; 306: 2090–3.
23 Fenton HJH. Oxidation of tartaric acid in presence of iron. *J Chem Soc* 1894; 65: 899–910.
24 Pietrangelo A, Dierssen U, Valli L *et al.* STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. *Gastroenterology* 2007; 132: 294–300.
25 Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* 2004; 18: 357–68.
26 St-Pierre J, Drori S, Uldry M *et al.* Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 2006; 127: 397–408.
27 Kong X, Wang R, Xue Y *et al.* Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. *PLoS ONE* 2010; 5: e11707.
28 Fernandez-Marcos PJ, Auwerx J. Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. *Am J Clin Nutr* 2011; 93: 884S–90.
29 Mankouri J, Tedbury PR, Gretton S *et al.* Enhanced hepatitis C virus genome replication and lipid accumulation mediated by inhibition of AMP-activated protein kinase. *Proc Natl Acad Sci U S A* 2010; 107: 11549–54.
30 Hawley SA, Boudeau J, Reid JL *et al.* Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* 2003; 2: 28.
31 Rogers NH, Perfield JW 2nd, Strissel KJ, Obin MS, Greenberg AS. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology* 2009; 150: 2161–8.
32 Pighon A, Gutkowska J, Jankowski M, Rabasa-Lhoret R, Lavoie JM. Exercise training in ovariectomized rats stimulates estrogenic-like effects on expression of genes involved in lipid accumulation and subclinical inflammation in liver. *Metabolism* 2011; 60: 629–39.
33 Hong J, Stubbins RE, Smith RR, Harvey AE, Nunez NP. Differential susceptibility to obesity between male, female and ovariectomized female mice. *Nutr J* 2009; 8: 11.
34 Kamada Y, Kiso S, Yoshida Y *et al.* Pitavastatin ameliorated the progression of steatohepatitis in ovariectomized mice fed a high fat and high cholesterol diet. *Hepatol Res* 2013; 43: 401–12.
35 Korenaga M, Wang T, Li Y *et al.* Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005; 280: 37481–8.
36 Okuda M, Li K, Beard MR *et al.* Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; 122: 366–75.
37 Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* 2012; 13: 251–62.

Hepatology Research

Hepatology Research 2014; 44: 123–132 doi: 10.1111/hepr.12247

Review Article

Mitochondrial reactive oxygen species as a mystery voice in hepatitis C

Keisuke Hino, Yuichi Hara and Sohji Nishina

Department of Hepatology and Pancreatology, Kawasaki Medical School, Kurashiki, Japan

There are several lines of evidence suggesting that oxidative stress is present in hepatitis C to a greater degree than in other inflammatory liver diseases and is closely related to disease progression. The main production site of reactive oxygen species (ROS) is assumed to be mitochondria, which concept is supported by evidence that hepatitis C virus (HCV) core protein is directly associated with them. The detoxification of ROS also is an important function of the cellular redox homeostasis system. These results draw our attention to how HCV-induced mitochondrial ROS production is beyond redox regulation and affects the disease progression and development of hepatocellular carcinoma (HCC) in chronic hepatitis C. On the other hand, HCV-related chronic liver diseases are characterized by metabolic alterations such as insulin resistance, hepatic steatosis and/or iron accumulation in the liver. These metabolic disorders also are relevant to the development of HCC in HCV-related chronic liver diseases. Here, we review the mechanisms by which HCV increases mitochondrial ROS production and offer new insights as to how mitochondrial ROS are linked to metabolic disorders such as insulin resistance, hepatic steatosis and hepatic iron accumulation that are observed in HCV-related chronic liver diseases.

Key words: calcium signaling, mitochondrial electron transport, mitophagy, hepcidin, insulin resistance, iron metabolism

INTRODUCTION

APPROXIMATELY 170 MILLION people worldwide are infected with hepatitis C virus (HCV).[1] HCV infection often remains asymptomatic, but can lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).[2] Although the mechanisms of its pathogenesis are incompletely understood, there are several lines of evidence suggesting that oxidative stress is present in hepatitis C to a greater degree than in other inflammatory liver diseases and is closely related to disease progression.[3–6] Previous *in vitro* and *in vivo* studies have shown that HCV core protein induces the production of reactive oxygen species (ROS)[7–9] and that mitochondrial electron transport inhibition by HCV core protein is associated with ROS production.[10,11] These results draw our attention to how HCV-induced mitochondrial injury contributes to disease progression and hepatocarcinogenesis in hepatitis C.

On the other hand, HCV-related chronic liver diseases are characterized by metabolic alterations such as insulin resistance,[12–14] hepatic steatosis[15,16] and/or iron accumulation in the liver.[3,17] These metabolic disorders also are relevant to the development of HCC in HCV-related chronic liver diseases.[18–21] The present review highlights the mechanisms underlying the production of mitochondrial ROS by HCV and the metabolic disorders induced by mitochondrial dysfunction, and discuss how mitochondrial ROS contribute to the disease progression and hepatocarcinogenesis in hepatitis C.

MITOCHONDRIAL ROS PRODUCTION

Mitochondrial electron transport and ROS production

THE MITOCHONDRIAL ELECTRON transport system consists of several multi-polypeptide protein complexes (I–V) embedded in the inner mitochondrial membrane that receive electrons from reducing equivalents (i.e. nicotinamide adenine dinucleotide and

Correspondence: Professor Keisuke Hino, Department of Hepatology and Pancreatology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan. Email: khino@med.kawasaki-m.ac.jp

Received 25 May 2013; revision 5 September 2013; accepted 19 September 2013.

$FADH_2$) generated by dehydrogenases (e.g. pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, acyl-coenzyme A dehydrogenase). These electrons flow through complex I, the ubiquinone cycle (Q/QH_2), complex III, cytochrome c, complex IV, and to the final acceptor O_2 to form H_2O. Electron flow through complexes I, III and IV results in the pumping of protons to the outer surface of the inner membrane, establishing a membrane potential that is used by adenosine triphosphate synthetase to drive the re-phosphorylation of adenine dinucleotide phosphate. Several of the redox couples within the electron transport chain transfer single rather than two electrons and are therefore susceptible to leaking electrons directly to surrounding O_2 to form the free-radical superoxide ($O_2^{\bullet-}$). The detoxification of ROS is an important function of the cellular redox homeostasis system. Cells rapidly convert $O_2^{\bullet-}$ into the two-electron non-radical hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD). H_2O_2 in turn can be further reduced to H_2O in the mitochondrial matrix by glutathione (GSH) or the thioredoxin/peroxiredoxin systems, or can freely diffuse out of the mitochondria where it again is buffered by GSH.[22]

Interaction of HCV core protein with mitochondria and mitochondrial ROS production

Hepatitis C virus core protein has been shown to directly associate with mitochondria. While the initial reports showed that HCV core protein associated exclusively with the mitochondrial outer membrane via a C-terminal motif,[10,23] a recent study using electronic microscopy suggests that HCV core protein is also associated with the mitochondrial inner membrane.[24] Importantly, Schwer *et al.* have demonstrated that core protein associates with the mitochondria-associated membrane (MAM) fraction, a point of close contact between the endoplasmic reticulum (ER) and mitochondrion.[23] In addition, biochemical evidence suggests that the interaction also takes place in the context of productively replicative HCV in cell culture (HCVcc),[25] even though subcellular analysis using confocal microscopy did not confirm a direct interaction of the HCV core with mitochondria in HCVcc infected Huh7.5 cells.[26] Direct interaction of HCV core protein with mitochondria potentially modifies mitochondrial ROS production and scavenging, which subsequently induce oxidative stress. The effects of HCV on ROS production and scavenging are summarized in Table 1.[27] When mitochondrial electron transport activity is inhibited by HCV core protein,[10,28] electrons are likely to leak from the electron transport chain transfer, accelerating mitochondrial $O_2^{\bullet-}$ production and/or H_2O_2 emission. Induction of mitochondrial and/or cellular antioxidant enzymes concomitantly with ROS production may be explained by antioxidant defense mechanisms rather than direct induction of antioxidant enzymes by HCV, even though HCV core and non-structural proteins have been reported to lead to different effects on cellular

Table 1 Effects of HCV on ROS production or scavenging *in vitro* and *in vivo*

Effect on ROS production or scavenging	*In vitro* and *in vivo* HCV models	References
Inhibition of mitochondrial electron transport	Structural gene transgenic mice, core gene transgenic mice, full genomic replicon cells	10, 28
Oxidation of glutathione pool	Structural gene transgenic mice, core- and non-structural protein-expressing cell lines	10, 29
Induction of mitochondrial antioxidant enzymes	Non-structural protein-expressing cell lines	29
Inhibition of gastrointestinal-glutathione peroxidase	Subgenomic replicon cells	30
Induction of glutathione peroxidase	Core-expressing cell line, subgenomic replicon cells	8, 30
Oxidation of the thioredoxin pool	Core-expressing cell line	29
Increase in lipid peroxidation	Core-expressing cell line, core gene transgenic mice	8, 31
Induction of metallothionein	Core-expressing cell line	8, 32
Cytoplasmic ROS production by NADPH oxidase	Core-expressing cell line, subgenomic replicon cells, full genomic replicon cells, HCV-infected Huh7 cells (JFH)	33, 34

HCV, hepatitis C virus; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

antioxidant defenses.[29] Thus, one of the major sources for intracellular ROS production by core protein is the mitochondrion, even though the core is also involved in ROS production at the plasma membrane by activating nicotinamide adenine dinucleotide phosphate oxidase 4.[33,34]

Modulated calcium signaling by HCV and mitochondrial ROS production

The close physical association between the ER and mitochondria mediated by MAM results in Ca^{2+} microdomains at contact points that facilitate efficient Ca^{2+} transmission from the ER to mitochondria.[35] Although sufficient intra-organelle Ca^{2+} concentrations are required to stimulate metabolism by activating enzymes critical for maintenance of the tricarboxylic acid (TCA) cycle,[36] prolonged increases of Ca^{2+} can, in turn, interfere with the activity of these enzymes. The TCA cycle activity affects the electron transport chain activity, which in turn affects the mitochondrial membrane potential ($\Delta\Psi$). Thus, increased Ca^{2+} influx to mitochondria induces a substrate imbalance of the TCA cycle that leads to the generation of mitochondrial ROS, probably through the inhibition of electron transport chain activity. There are several lines of evidence indicating that HCV increases mitochondrial ROS production by modulating calcium signaling.[37–39] The HCV NS5A protein is reported to cause a disturbance of intracellular Ca^{2+} signaling, which triggers mitochondrial ROS production.[37] As shown in Figure 1, HCV core protein also enhances mitochondrial Ca^{2+} uptake in response to ER Ca^{2+} release through activation of the mitochondrial Ca^{2+} uniporter, which leads to increased mitochondrial ROS production.[38,39] Pharmacological inhibition of ER–mitochondrial Ca^{2+} fluxes, but not ROS scavengers, has been shown to normalize all aberrant effects induced by HCV: normalization of the electron transport chain complex I activity, restoration of mitochondrial $\Delta\Psi$ and normalization of ROS concentrations. More importantly, the time course and titration of HCV polyprotein expression suggest that mitochondrial Ca^{2+} uptake is the earliest of these above events induced by HCV.[39] Thus, mitochondrial Ca^{2+} uptake may be the initial event associated with mitochondrial dysfunction induced by HCV and may, in turn, trigger complex I inhibition, loss of mitochondrial $\Delta\Psi$ and ROS production. All these effects could be counteracted by intracellular Ca^{2+} chelation, suggesting that control of mitochondrial Ca^{2+} uptake may be useful as a new therapeutic intervention.

MITOCHONDRIAL QUALITY CONTROL

AS MENTIONED ABOVE, the detoxification of ROS is an important function of the cellular redox homeostasis system. Under resting cellular conditions, the intracellular redox environment is in a relatively reduced state.[40] Therefore, the next question is how HCV core-induced mitochondrial ROS production and the subsequent oxidative stress persist in spite of the presence of ROS-detoxifying agents such as MnSOD and/or GSH or the thioredoxin/peroxiredoxin systems. There are several lines of evidence indicating that mitochondrial injury is present in patients with chronic hepatitis C[4] and transgenic mice expressing the HCV core protein.[19] Although it remains unknown whether damaged mitochondria behave as an active ROS source, they are assumed to have less ROS-detoxifying activity than intact mitochondria. In mammalian cells, the autophagy-dependent degradation of mitochondria (mitophagy) is thought to maintain mitochondrial quality by eliminating damaged mitochondria.[41,42] Indeed, mitophagy plays an essential role in reducing mitochondrial ROS production and mitochondrial DNA mutations in yeast.[43] Mitochondrial membrane depolarization precedes the induction of mitophagy,[44] which is selectively controlled by a variety of proteins in mammalian cells, including phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin.[41,45] PINK1 facilitates Parkin targeting to the depolarized mitochondria[45] and, although Parkin ubiquitinates a broad range of mitochondrial outer membrane proteins,[45] it remains unclear how Parkin enables damaged mitochondria to be recognized by the autophagosome. We recently found that HCV core protein suppresses mitophagy by inhibiting the translocation of Parkin to the mitochondria via a direct interaction with it (Yuichi Hara, unpubl. data, 2013). Considering that oxidative stress and/or hepatocellular mitochondrial alterations are present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases[3–6] and that mitophagy is important for maintaining mitochondrial quality by eliminating damaged mitochondria, our finding that HCV core protein suppresses mitophagy may in part explain the pathophysiology of chronic hepatitis C. However, in contrast to our results, Siddiqui *et al.* have shown that HCV induces the mitochondrial translocation of Parkin and subsequent mitophagy.[46] In addition, their results indicated that the HCV-mediated decline of mitochondrial complex I enzyme activity was rescued by chemical inhibition of mitophagy or by Parkin silenc-

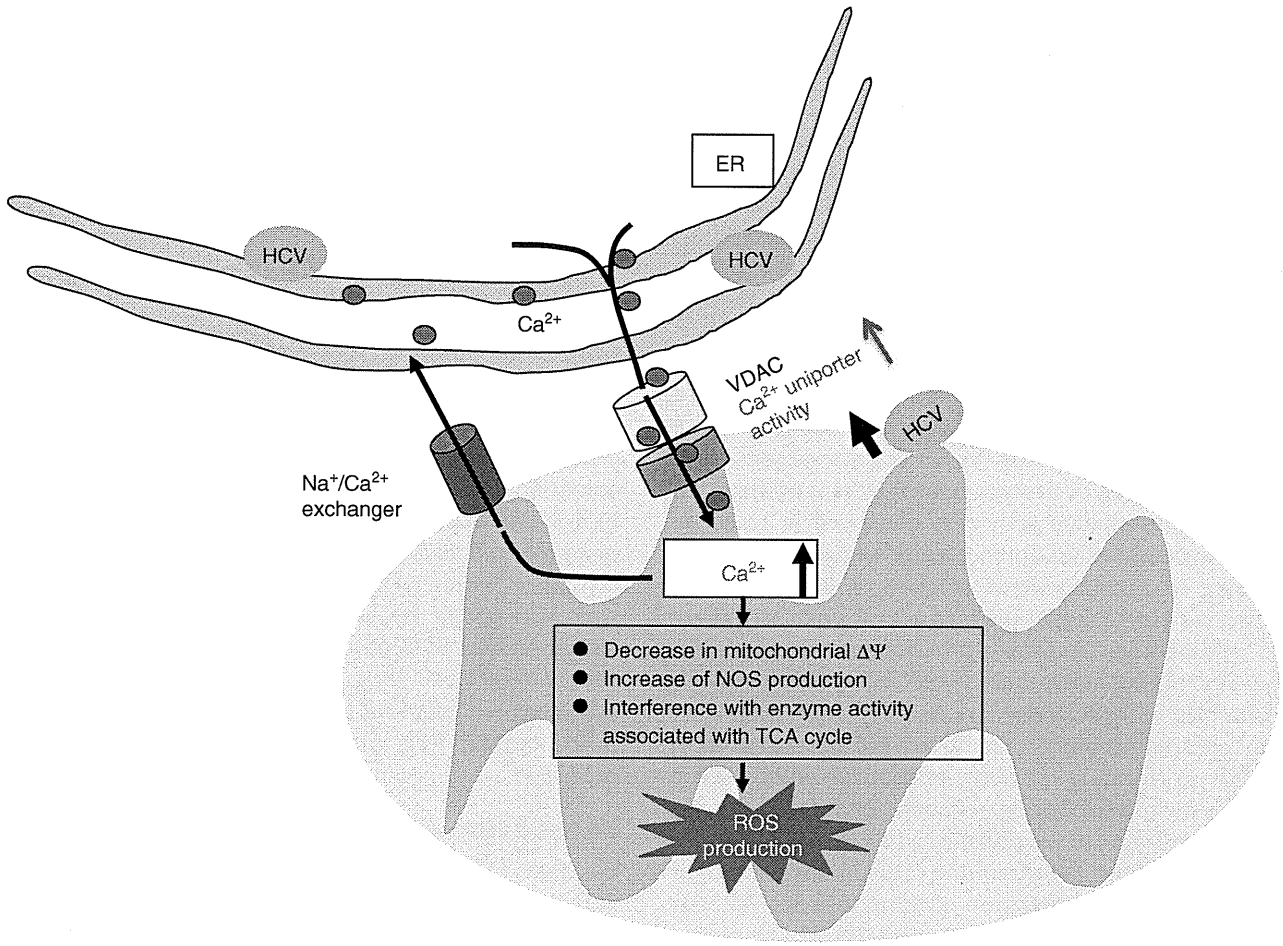

Figure 1 Schematic diagram depicting hepatitis C virus (HCV)-related calcium transfer from the endoplasmic reticulum (ER) to the mitochondria in the mitochondria-associated membrane (MAM) fraction. HCV core protein enhances mitochondrial Ca^{2+} uptake in response to ER Ca^{2+} release through activation of the mitochondrial Ca^{2+} uniporter, which leads to increased mitochondrial ROS production The voltage-dependent anion channel (VDAC) is the major component of the mitochondrial permeability transition (MPT) pore. It is assumed that there is spatial proximity of the complexes responsible for ER Ca^{2+} release, mitochondrial Ca^{2+} uptake and the MPT pore.

ing, and suggested that induction of mitophagy by HCV may significantly contribute to the mitochondrial injury associated with chronic hepatitis C. Thus, it still remains a matter of debate as to whether HCV induces or suppresses mitophagy and how oxidative stress persists in HCV infection.

METABOLIC ALTERATIONS BY MITOCHONDRIAL ROS

Insulin resistance

TYPE 2 DIABETES mellitus is one of the important extrahepatic manifestations associated with chronic HCV infection.[47,48] The final common pathway responsible for the development of type 2 diabetes mellitus is the failure of the pancreatic β-cells to compensate for insulin resistance. Although the molecular mechanisms by which HCV promotes insulin resistance have not been fully elucidated, there are several lines of evidence suggesting that HCV directly induces insulin resistance.[49–51] Insulin receptor substrate (IRS)1 and IRS2 are normally expressed in hepatocytes and central molecules of the hepatic insulin signal cascade. HCV core protein is reported to upregulate suppressor cytokine signal (SOCS)3 and cause ubiquitination of IRS1 and IRS2, leading to their proteosomal degradation.[50] SOCS3 also suppresses phosphorylation of tyrosine within IRS1.[52,53] Inhibition of tyrosine phos-

phorylation within IRS1 due to a high level of tumor necrosis (TNF)-α which leads to suppression of downstream insulin signals has been shown in HCV core transgenic mice.[49]

What roles do mitochondrial ROS play in HCV-induced insulin resistance? TNF-α phosphorylates Ser^{307} of IRS1 through serine (Ser) kinases such as c-Jun N-terminal kinase (JNK), which disrupts the interaction between the catalytic domains of the insulin receptors and the phosphotyrosine-binding domain of IRS1,[54,55] even though there is a contrary report that Ser^{307} promotes insulin sensitivity in mice.[56] The intracellular redox condition is a master regulator of phosphorylation/dephosphorylation events due to the presence of redox-sensing cystein (Cys) residues within nearly all classes of protein phosphatase enzymes.[57] In general, phosphatase activity is depressed in response to an oxidative shift in the redox environment, thus leading to a concomitant increase in kinase activity either via direct oxidant-induced activation or secondary to phosphatase inactivation.[57] Inactivation of protein tyrosine phosphatases is mediated via the oxidation of a conserved redox sensitive Cys residue within their catalytic sites, which must be in the reduced state as the thiol (-SH) to form a cysteinyl-phosphate intermediate during hydrolysis.[58] Thus, research conducted over the past several years has established a role for the activation of stress sensitive Ser/threonine (Thr) kinases and their subsequent phosphorylation of inhibitory Ser/Thr residues within the insulin receptor and IRS1/2 as a potential mechanism of insulin resistance.[59] In agreement with the evidence suggesting a central role for ROS in the development of insulin resistance,[60] HCV core-induced mitochondrial ROS production is presumed to induce insulin resistance through activation of Ser/Thr kinases such as JNK1 and subsequent inhibition of tyrosine phosphorylation within IRS1 (Fig. 2). Hepatic insulin resistance induces suppressed insulin clearance as well as increased insulin secretion from pancreatic β-cells, which leads to hyperinsulinemia and represses whole-body insulin sensitivity.[61]

Hepatic steatosis

Hepatic steatosis is also one of the pathophysiological features of HCV-associated chronic liver disease.[15,16] It is characterized by the cytoplasmic accumulation of lipid droplets, mainly composed of triglyceride and cholesterol ester. The composition of triglycerides in the liver is uniquely and significantly enriched in carbon monosaturated (C18:1) fatty acids in chronic hepatitis C,[62] which is distinct from what occurs in obese patients. The mechanisms underlying HCV-related steatosis are diverse: decreased lipoprotein secretion from hepatocytes, increased synthesis of fatty acids, decreased fatty acid oxidation and increased fatty acid uptake by hepatocytes. The HCV core protein has been demonstrated to inhibit microsomal transfer protein activity[63] and to upregulate transcriptional activity of sterol regulatory element-binding protein 1, a transcription factor involved in lipid synthesis.[64] These observations underscore the importance of the core as a direct and principal regulator of HCV-associated steatosis. On the other hand, decreased fatty acid oxidation and increased fatty acid uptake are related to mitochondrial dysfunction and hyperinsulinemia, respectively. Indeed, we previously demonstrated impaired mitochondrial fatty acid oxidation concomitant with increased ROS production in iron-overloaded transgenic mice expressing the HCV polyprotein.[65] Hyperinsulinemia derived from insulin resistance inhibits lipolysis in the liver and increases fatty acid uptake by hepatocytes. As described above, mitochondrial ROS production is presumed to induce insulin resistance. Thus, inhibited fatty acid oxidation and increased fatty acid uptake are potentially related to mitochondrial ROS production induced by the core protein.

Hepatic iron accumulation

Elevated iron-related serum markers and increased hepatic iron accumulation are relatively common and correlate with the severity of hepatic inflammation and fibrosis in patients with chronic hepatitis C. Excess divalent iron can be highly toxic, mainly via the Fenton reaction producing hydroxyl radicals.[66] This is particularly relevant for chronic hepatitis C, in which oxidative stress has been proposed as a major mechanism of liver injury. Oxidative stress and increased iron levels strongly favor DNA damage, genetic instability and tumorigenesis. Indeed, a significant correlation between 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidatively generated DNA damage,[67] and hepatic iron excess has been shown in patients with chronic hepatitis C.[68] We showed that transgenic mice expressing the HCV polyprotein fed an excess-iron diet developed HCC through hepatic accumulation of 8-OHdG.[65]

Here, we discuss the mechanisms by which hepatic iron accumulates in chronic hepatitis C, focusing on the relationship between HCV-induced ROS production and iron metabolic disorder. Systemic iron homeostasis is mainly regulated both by intestinal absorption and macrophage recycling of iron from hemoglobin because there is no efficient pathway for iron excretion.[69]

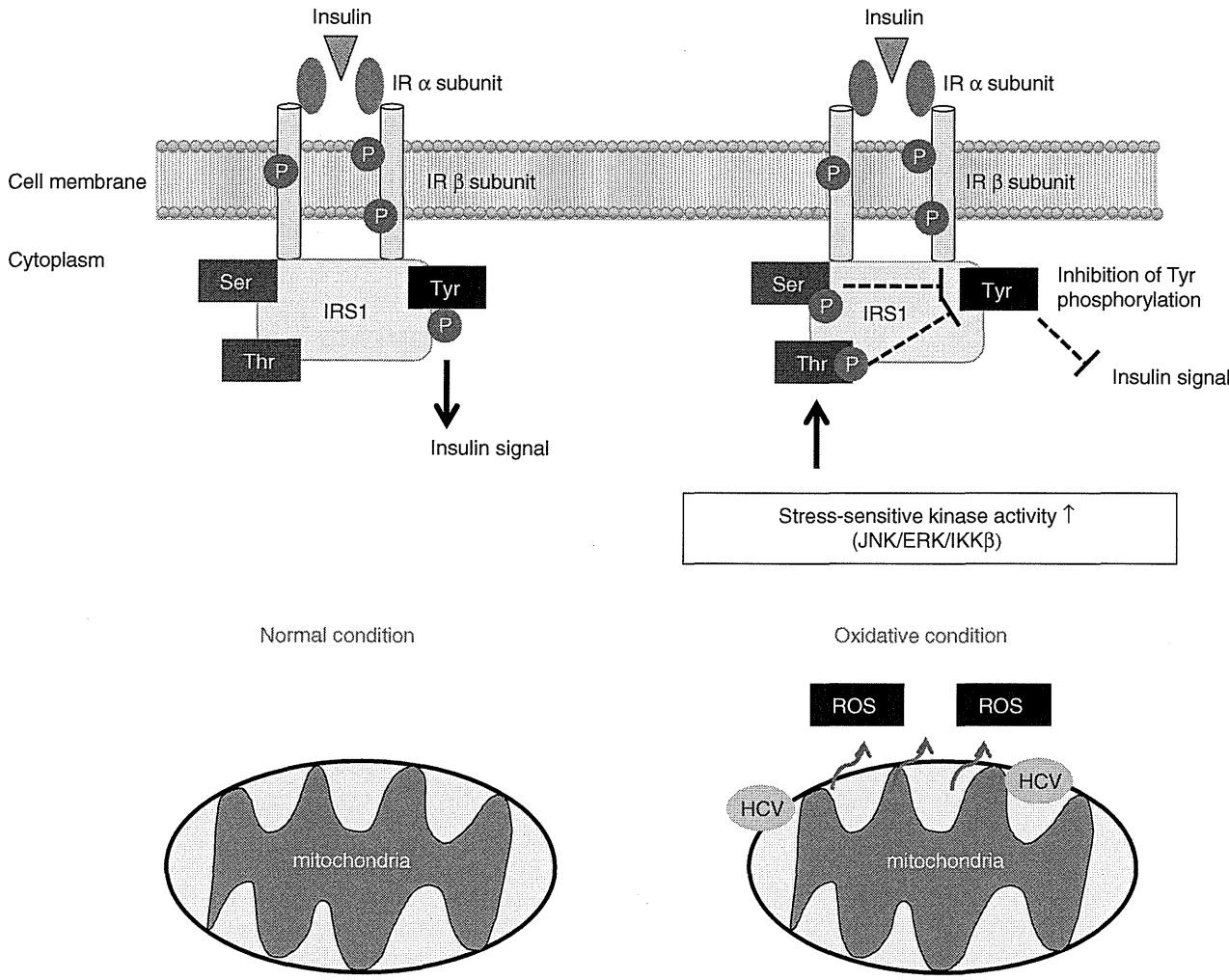

Figure 2 Schematic diagram depicting the presumed mechanism underlying the inhibitory effect on tyrosine phosphorylation within insulin receptor substrate 1 (IRS1). Hepatitis C virus (HCV) core-induced mitochondrial reactive oxygen species (ROS) production is presumed to induce insulin resistance through activation of Ser/Thr kinases such as c-Jun N-terminal kinase-1 (JNK1) and subsequent inhibition of tyrosine phosphorylation within IRS1. IR, insulin receptor; Ser, serine; Thr, threonine; Tyr, tyrosine; ERK, extracellular signal-regulated kinase; IKKβ, inhibitory-κβ kinase β.

Hepcidin, which was originally isolated from human serum and urine as a peptide with antimicrobial activity,[70] is a hormone exclusively synthesized in the liver and a soluble regulator that acts to attenuate both intestinal iron absorption and iron release from reticuloendothelial macrophages.[71] Hepatic mRNA levels[72] and the 25 amino acid bioactive hepcidin levels in serum[73] are lower in chronic hepatitis C than in chronic hepatitis B or controls, despite a significant correlation between hepcidin and serum ferritin or the histological iron score. Thus, the relatively decreased synthesis of hepcidin in chronic hepatitis C contrasts with the absolute deficit or lack of hepcidin synthesis observed in hereditary hemochromatosis. The detailed mechanisms underlying the transcriptional regulation of hepcidin are discussed elsewhere. Interestingly, alcohol metabolism-mediated ROS were shown to suppress hepcidin transcription via CCAAT/enhancer-binding protein α (C/EBPα).[74] In parallel with these results, we found that hepcidin promoter activity and the DNA binding activity of C/EBPα were downregulated concomitant with increased expression of C/EBP homology protein (CHOP), an inhibitor of C/EBP DNA binding activity, and with increased levels of mitochondrial ROS in

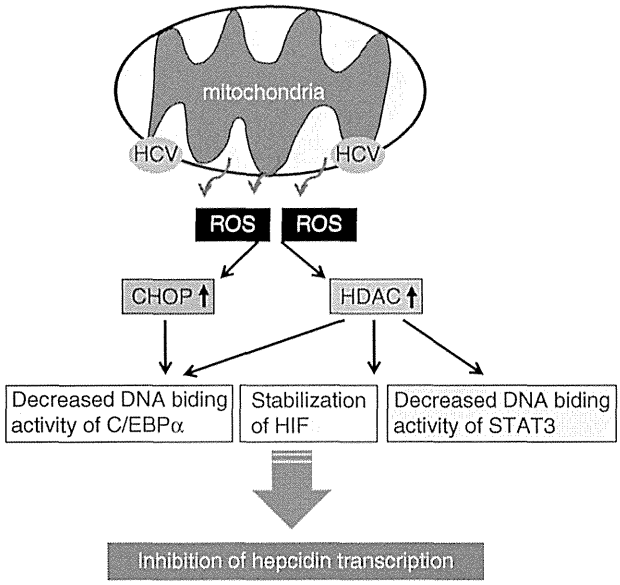

Figure 3 Schematic diagram depicting the mechanism by which mitochondrial reactive oxygen species (ROS) suppress hepcidin transcription in transgenic mice expressing hepatitis C virus (HCV) polyprotein or full-length HCV replicon cells. CHOP, CCAAT/enhancer-binding protein (C/EBP) homology protein; HDAC, histone deacetylase; HIF, hypoxia inducible factor; STAT3, signal transduction and activator of transcription 3.

transgenic mice expressing the HCV polyprotein.[75] There are several lines of evidence indicating that ROS upregulate the expression of CHOP.[76] In agreement with our observation, an *in vitro* study using hepatoma cells showed that HCV-induced ROS inhibited the binding activity of C/EBPα and signal transduction and activator of transcription 3 to the hepcidin promoter in addition to stabilization of hypoxia-inducible factor through increased histone deacetylase activity.[77] Thus, HCV core-induced mitochondrial ROS accumulate hepatic iron through the inhibition of hepcidin transcription (Fig. 3).

CONCLUSION

IN THE PRESENT review we discussed how HCV interacts with mitochondria and how subsequently occurring mitochondrial ROS production contributes to the pathophysiology of HCV-related chronic liver diseases. The mitochondrion is the key organelle that determines the cellular response to various kinds of biological stress. Therefore, it may not be surprising that HCV-induced alterations of mitochondrial functions have a critical impact on disease progression towards hepatocarcinogenesis by creating an oxidatively stressed liver microenvironment through mitochondrial ROS production. However, the molecular details underlying HCV-induced mitochondrial dysfunctions remain confusing and are still a matter of debate, which undoubtedly requires further investigation to shed light on the questions in this field.

ACKNOWLEDGMENTS

THIS RESEARCH WAS supported by a Grant-in-Aid for Scientific Research (B) (no. 23390201) from the Japan Society for the Promotion of Science, by a Health and Labor Sciences Research Grant for Research on Hepatitis from the Ministry of Health, Labor and Welfare of Japan and by a P2 Research Project Grant from Kawasaki Medical School.

REFERENCES

1 Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet* 2003; 362: 2095–100.
2 Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002; 36: S35–46.
3 Farinati F, Cardin R, De Maria N *et al.* Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. *J Hepatol* 1995; 22: 449–56.
4 Barbaro G, Di Lorenzo G, Asti AM *et al.* Hepatocellular mitochondrial alterations in patients with chronic hepatitis C: ultrastructural and biochemical findings. *Am J Gastroenterol* 1999; 942: 198–205.
5 Valgimigli M, Valgimigli L, Trere D *et al.* Oxidative stress EPR measurement in human liver by radical-probe technique. Correlation with etiology, histology and cell proliferation. *Free Radic Res* 2002; 36: 939–48.
6 Kitase A, Hino K, Furutani T *et al.* In situ detection of oxidized n-3 polyunsaturated fatty acids in chronic hepatitis C: correlation with hepatic steatosis. *J Gastroenterol* 2005; 40: 617–24.
7 Moriya K, Nakagawa K, Santa T *et al.* Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001; 61: 4365–70.
8 Okuda M, Li K, Beard MR *et al.* Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; 122: 366–75.
9 Otani K, Korenaga M, Beard MR *et al.* Hepatitis C virus core protein, cytochrome P450 2E1, and alcohol produce combined mitochondrial injury and cytotoxicity in hepatoma cells. *Gastroenterology* 2005; 128: 96–107.

10 Korenaga M, Wang T, Li Y *et al.* Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005; **280**: 37481–8.
11 Ando M, Korenaga M, Hino K *et al.* Mitochondrial electron transport inhibition in full genomic hepatitis C virus replicon cells is restored by reducing viral replication. *Liver Int* 2008; **28**: 1158–66.
12 Allison ME, Wreghitt T, Palmer CR, Alexander GJ. Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. *J Hepatol* 1994; **21**: 1135–9.
13 Caronia S, Taylor K, Pagliaro L *et al.* Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 1999; **30**: 1059–63.
14 Mason AL, Lau JY, Hoang N *et al.* Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 1999; **29**: 328–33.
15 Moriya K, Yotsuyanagi H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997; **78**: 1527–31.
16 Rubbia-Brandt L, Quadri R, Abid K *et al.* Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. *J Hepatol* 2000; **33**: 106–15.
17 Kageyama F, Kobayashi Y, Kawasaki T, Toyokuni S, Uchida K, Nakamura H. Successful interferon therapy reverses enhanced hepatic iron accumulation and lipid peroxidation in chronic hepatitis C. *Am J Gastroenterol* 2000; **95**: 1041–50.
18 Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut* 2005; **54**: 533–9.
19 Moriya K, Fujie H, Shintani Y *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065–7.
20 Lerat H, Honda M, Beard MR *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122**: 352–65.
21 Kato J, Miyanishi K, Kobune M *et al.* Long-term phlebotomy with low-iron diet therapy lowers risk of development of hepatocellular carcinoma from chronic hepatitis C. *J Gastroenterol* 2007; **42**: 830–6.
22 Fisher-Wellman KH, Neufer PD. Linking mitochondrial bioenergetics to insulin resistance via redox biology. *Trends Endocrinol Metab* 2012; **23**: 142–53.
23 Schwer B, Ren S, Pietschmann T *et al.* Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. *J Virol* 2004; **78**: 7958–68.
24 Chu VC, Bhattacharya S, Nomoto A *et al.* Persistent expression of hepatitis C virus non-structural proteins leads to increased autophagy and mitochondrial injury in human hepatoma cells. *PLoS ONE* 2011; **6**: e28551.
25 Wang T, Campbell RV, Yi MK, Lemon SM, Weinman SA. Role of Hepatitis C virus core protein in viral-induced mitochondrial dysfunction. *J Viral Hepat* 2010; **17**: 784–93.
26 Rouille Y, Helle F, Delgrange D *et al.* Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. *J Virol* 2006; **80**: 2832–41.
27 Brault C, Levy PL, Bartosch B. Hepatitis C virus-induced mitochondrial dysfunctions. *Viruses* 2013; **5**: 954–80.
28 Tsutsumi T, Matsuda M, Aizaki H *et al.* Proteomics analysis of mitochondrial proteins reveals overexpression of a mitochondrial protein chaperon, prohibitin, in cells expressing hepatitis C virus core protein. *Hepatology* 2009; **50**: 378–86.
29 Abdalla MY, Ahmad IM, Spitz DR, Schmidt WN, Britigan BE. Hepatitis C virus-core and nonstructural proteins lead to different effects on cellular antioxidant defenses. *J Med Virol* 2005; **76**: 489–97.
30 Morbitzer M, Herget T. Expression of gastrointestinal glutathione peroxidase is inversely correlated to the presence of hepatitis C virus subgenomic RNA in human liver cells. *J Biol Chem* 2005; **280**: 8831–41.
31 Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol* 2006; **80**: 7199–207.
32 Li K, Prow T, Lemon SM, Beard MR. Cellular response to conditional expression of hepatitis C virus core protein in Huh7 cultured human hepatoma cells. *Hepatology* 2002; **35**: 1237–46.
33 Boudreau HE, Emerson SU, Korzeniowska A, Jendrysik MA, Leto TL. Hepatitis C virus (HCV) proteins induce NADPH oxidase 4 expression in a transforming growth factor beta-dependent manner: a new contributor to HCV-induced oxidative stress. *J Virol* 2009; **83**: 12934–46.
34 deMochel NS, Seronello S, Wang SH *et al.* Hepatocyte NAD(P)H oxidases as an endogenous source of reactive oxygen species during hepatitis C virus infection. *Hepatology* 2010; **52**: 47–59.
35 Rieusset J, Fauconnier J, Paillard M *et al.* Disruption of cyclophilin d-mediated calcium transfer from the ER to mitochondria contributes to hepatic ER stress and insulin resistance. *Hepatology* 2013; **58**: 1195.
36 Balaban RS. Cardiac energy metabolism homeostasis: role of cytosolic calcium. *J Mol Cell Cardiol* 2002; **34**: 1259–71.
37 Gong G, Waris G, Tanveer R, Siddiqui A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc Natl Acad Sci U S A* 2001; **98**: 9599–604.
38 Li Y, Boehning DF, Qian T, Popov VL, Weinman SA. Hepatitis C virus core protein increases mitochondrial ROS production by stimulation of Ca2+ uniporter activity. *FASEB J* 2007; **21**: 2474–85.

39 Piccoli C, Scrima R, Quarato G *et al.* Hepatitis C virus protein expression causes calcium-mediated mitochondrial bioenergetic dysfunction and nitro-oxidative stress. *Hepatology* 2007; 46: 58–65.
40 Maher P. Redox control of neural function: background, mechanisms, and significance. *Antioxid Redox Signal* 2006; 8: 1941–70.
41 Narendra D, Tanaka A, Suen DF *et al.* Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* 2008; 183: 795–803.
42 Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol* 2011; 12: 9–14.
43 Kurihara Y, Kanki T, Aoki Y *et al.* Mitophagy plays an essential role in reducing mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast. *J Biol Chem* 2012; 287: 3265–72.
44 Elmore SP, Qian T, Grissom S *et al.* The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J* 2001; 15: 2286–7.
45 Geisler S, Holmstrom KM, Skujat D *et al.* PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 2010; 12: 119–31.
46 Kim SJ, Syed GH, Siddiqui A. Hepatitis C virus induces the mitochondrial translocation of Parkin and subsequent mitophagy. *PLoS Pathog* 2013; 9: e1003285.
47 Hui JM, Sud A, Farrell GC *et al.* Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression. *Gastroenterology* 2003; 125: 1695–704.
48 Aytug S, Reich D, Sapiro LE, Bernstein D, Begum N. Impaired IRS-1/PI3-kinase signaling in patients with HCV: a mechanism for increased prevalence of type 2 diabetes. *Hepatology* 2003; 38: 1384–92.
49 Shintani Y, Fujie H, Miyoshi H *et al.* Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; 126: 840–8.
50 Kawaguchi T, Yoshida T, Harada M *et al.* Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am J Pathol* 2004; 165: 1499–508.
51 Shlomai A, Rechtman MM, Burdelova EO *et al.* The metabolic regulator PGC-1alpha links hepatitis C virus infection to hepatic insulin resistance. *J Hepatol* 2012; 57: 867–73.
52 Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D, Van Obberghen E. SOCS-3 is an insulin-induced negative regulator of insulin signaling. *J Biol Chem* 2000; 275: 15985–91.
53 Ueki K, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol Cell Biol* 2004; 24: 5434–46.
54 Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996; 271: 665–8.
55 Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J Biol Chem* 2002; 277: 1531–7.
56 Copps KD, Hancer NJ, Opare-Ado L, Qiu W, Walsh C, White MF. Irs1 serine 307 promotes insulin sensitivity in mice. *Cell Metab* 2010; 11: 84–92.
57 Chiarugi P. PTPs versus PTKs: the redox side of the coin. *Free Radic Res* 2005; 39: 353–64.
58 Tonks NK. Redox redux: revisiting PTPs and the control of cell signaling. *Cell* 2005; 121: 667–70.
59 Boura-Halfon S, Zick Y. Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab* 2009; 296: E581–91.
60 Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 2006; 440: 944–8.
61 Michael MD, Kulkarni RN, Postic C *et al.* Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* 2000; 6: 87–97.
62 Moriya K, Todoroki T, Tsutsumi T *et al.* Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biochem Biophys Res Commun* 2001; 281: 1207–12.
63 Perlemuter G, Sabile A, Letteron P *et al.* Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* 2002; 16: 185–94.
64 Moriishi K, Mochizuki R, Moriya K *et al.* Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc Natl Acad Sci U S A* 2007; 104: 1661–6.
65 Furutani T, Hino K, Okuda M *et al.* Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2006; 130: 2087–98.
66 Fenton HJH. Oxidation of tartaric acid in presence of iron. *J Chem Soc* 1894; 65: 899–910.
67 Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* 1991; 349: 431–4.
68 Fujita N, Horiike S, Sugimoto R *et al.* Hepatic oxidative DNA damage correlates with iron overload in chronic hepatitis C patients. *Free Radic Biol Med* 2007; 42: 353–62.
69 Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. *Cell* 2004; 117: 285–97.
70 Krause A, Neitz S, Magert HJ *et al.* LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. *FEBS Lett* 2000; 480: 147–50.

71 Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 2001; **276**: 7806–10.
72 Fujita N, Sugimoto R, Takeo M *et al.* Hepcidin expression in the liver: relatively low level in patients with chronic hepatitis C. *Mol Med* 2007; **13**: 97–104.
73 Girelli D, Pasino M, Goodnough JB *et al.* Reduced serum hepcidin levels in patients with chronic hepatitis C. *J Hepatol* 2009; **51**: 845–52.
74 Harrison-Findik DD, Schafer D, Klein E *et al.* Alcohol metabolism-mediated oxidative stress down-regulates hepcidin transcription and leads to increased duodenal iron transporter expression. *J Biol Chem* 2006; **281**: 22974–82.
75 Nishina S, Hino K, Korenaga M *et al.* Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. *Gastroenterology* 2008; **134**: 226–38.
76 Bek MF, Bayer M, Muller B *et al.* Expression and function of C/EBP homology protein (GADD153) in podocytes. *Am J Pathol* 2006; **168**: 20–32.
77 Miura K, Taura K, Kodama Y, Schnabl B, Brenner DA. Hepatitis C virus-induced oxidative stress suppresses hepcidin expression through increased histone deacetylase activity. *Hepatology* 2008; **48**: 1420–9.

Journal of General Virology (2014), 95, 2658–2667 DOI 10.1099/vir.0.068528-0

Alternative endocytosis pathway for productive entry of hepatitis C virus

Mami Matsuda,[1] Ryosuke Suzuki,[1] Chikako Kataoka,[1] Koichi Watashi,[1] Hideki Aizaki,[1] Nobuyuki Kato,[2] Yoshiharu Matsuura,[3] Tetsuro Suzuki[4] and Takaji Wakita[1]

Correspondence
Ryosuke Suzuki
ryosuke@niid.go.jp

[1]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

[2]Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

[3]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

[4]Department of Infectious Diseases, Hamamatsu University School of Medicine, Shizuoka, Japan

Received 29 May 2014
Accepted 1 August 2014

Previous studies have shown that hepatitis C virus (HCV) enters human hepatic cells through interaction with a series of cellular receptors, followed by clathrin-mediated, pH-dependent endocytosis. Here, we investigated the mechanisms of HCV entry into multiple HCV-permissive human hepatocyte-derived cells using trans-complemented HCV particles (HCVtcp). Knockdown of CD81 and claudin-1, or treatment with bafilomycin A1, reduced infection in Huh-7 and Huh7.5.1 cells, suggesting that HCV entered both cell types via receptor-mediated, pH-dependent endocytosis. Interestingly, knockdown of the clathrin heavy chain or dynamin-2 (Dyn2), as well as expression of the dominant-negative form of Dyn2, reduced infection of Huh-7 cells with HCVtcp, whereas infectious entry of HCVtcp into Huh7.5.1 cells was not impaired. Infection of Huh7.5.1 cells with culture-derived HCV (HCVcc) via a clathrin-independent pathway was also observed. Knockdown of caveolin-1, ADP-ribosylation factor 6 (Arf6), flotillin, p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A had no inhibitory effects on HCVtcp infection into Huh7.5.1 cells, thus suggesting that the infectious entry pathway of HCV into Huh7.5.1 cells was not caveolae-mediated, or Arf6- and flotillin-mediated endocytosis and macropinocytosis, but rather may have occurred via an undefined endocytic pathway. Further analysis revealed that HCV entry was clathrin- and dynamin-dependent in ORL8c and HepCD81/miR122 cells, but productive entry of HCV was clathrin- and dynamin-independent in Hep3B/miR122 cells. Collectively, these data indicated that HCV entered different target cells through different entry routes.

INTRODUCTION

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). HCV is an enveloped virus belonging to the family *Flaviviridae*. Its genome is an uncapped 9.6 kb positive-stranded RNA consisting of the 5′-UTR, an ORF encoding viral proteins and the 3′-UTR (Suzuki *et al.*, 2007). A precursor polyprotein is further processed into structural proteins (core, E1, and E2), followed by p7 and non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), by cellular and viral proteases.

Two supplementary figures are available with the online version of this paper.

Host–virus interactions are required during the initial steps of viral infection. Viruses enter the cells by various pathways, such as receptor-mediated endocytosis followed by pH-dependent or -independent fusion from endocytic compartments, or pH-independent fusion at the plasma membrane coupled with receptor-mediated signalling and coordinated disassembly of the actin cortex (Grove & Marsh, 2011). It was reported previously that CD81 (Bartosch *et al.*, 2003; McKeating *et al.*, 2004; Pileri *et al.*, 1998), scavenger receptor class B type I (SR-BI) (Bartosch *et al.*, 2003; Scarselli *et al.*, 2002), claudin-1 (Evans *et al.*, 2007; Liu *et al.*, 2009) and occludin (Benedicto *et al.*, 2009; Liu *et al.*, 2009; Ploss *et al.*, 2009) are critical molecules for HCV entry into cells. Recently, epidermal growth factor receptor and ephrin receptor type A2 were also identified as host cofactors for HCV entry, possibly by modulating interactions between CD81 and claudin-1 (Lupberger *et al.*,

2011). In addition, Niemann–Pick C1-like 1 (NPC1L1) cholesterol absorption receptor has been shown to play a role in HCV entry, probably at the fusion step (Sainz *et al.*, 2012).

Following receptor binding, HCV has been reported to enter cultured cells via clathrin-mediated endocytosis, the most common and best-characterized mode of endocytosis, following membrane fusion in early endosomes (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009) using retrovirus-based HCV pseudoparticles (HCVpp) and cell culture-produced HCV (HCVcc). Early steps in HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp. However, as HCVpp are generated in non-hepatic cells such as human embryo kidney 293T cells, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc.

In the present study, we readdressed the HCV endocytosis pathway using trans-complemented HCV particles (HCVtcp) (Suzuki *et al.*, 2012), of which the packaged genome is a subgenomic replicon. HCVtcp, generated in Huh-7 or its derivative cell lines with two plasmids, are infectious, but support only single-round infection, thereby allowing us to examine infectious viral entry without the influence of reinfection. In addition, HCVtcp is useful for quantifying productive infection by measuring luciferase activity. Furthermore, it has been shown that the HCVtcp system is more relevant as a model of HCV infection than HCVpp (Suzuki *et al.*, 2012). Our results demonstrated conclusively that, in addition to the clathrin-mediated endocytosis pathway, HCV was capable of utilizing the clathrin- and dynamin-independent pathways for infectious entry of HCV into human liver-derived cells.

RESULTS

HCV entry depends on receptor-mediated, pH-dependent endocytosis

HCV has been shown to enter permissive cells through clathrin-mediated endocytosis and low pH-dependent fusion with endosomes mostly using HCVpp (Codran *et al.*, 2006; Meertens *et al.*, 2006; Trotard *et al.*, 2009), although some researchers have used HCVcc with limited cell lines (Blanchard *et al.*, 2006; Coller *et al.*, 2009). However, several distinct characteristics between HCVpp and HCVcc have recently been revealed with regard to morphogenesis and entry steps (Helle *et al.*, 2010; Sainz *et al.*, 2012; Suzuki *et al.*, 2012; Vieyres *et al.*, 2010). Therefore, in this study, we used HCVtcp, which exhibit similar characteristics to HCVcc when compared with HCVpp and support single-round infection (Suzuki *et al.*, 2012).

Initially, to determine whether receptor candidates such as CD81, claudin-1, occludin and SR-BI are essential for HCV entry into Huh-7 and Huh7.5.1 cells, we examined the knockdown effect of these molecules on HCVtcp infection. Knockdown of these receptors was confirmed by immunoblotting (Fig. 1a) and FACS analysis (Fig. 1b). It should be noted that the luciferase activity in Huh7.5.1 was approximately four times higher than that in Huh-7 cells when the same amount of inoculum was used for infection (Fig. S1, available in the online Supplementary Material), and knockdown did not affect cell viability (data not shown). Knockdown of CD81 and claudin-1 significantly reduced the infection of Huh-7 and Huh7.5.1 cells with HCVtcp derived from genotype 2a (Fig. 1c). Knockdown of occludin led to a moderate reduction in infection; however, only a marginal effect was observed in SR-BI knockdown in both Huh-7 and Huh7.5.1 cells (Fig. 1c), possibly due to the reduced requirement for SR-BI during virus entry by adaptive mutation in E2 (Grove *et al.*, 2008).

Next, to examine whether HCV entry was pH-dependent, Huh-7 and Huh7.5.1 cells were pretreated with bafilomycin A1, an inhibitor of vacuolar H^+-ATPases that impairs vesicle acidification, and then infected with HCVtcp. At 72 h post-infection, luciferase activity and cell viability were determined. Bafilomycin A1 inhibited HCVtcp infection in a dose-dependent manner without affecting cell viability in both Huh-7 and Huh7.5.1 cells (Fig. 2a, b). We also confirmed that treatment with bafilomycin A1 after HCVtcp infection had a minor effect on luciferase activity (Fig. 2c). These results indicated that the infectious route of HCVtcp into Huh-7 and Huh7.5.1 cells is receptor-mediated and involves pH-dependent endocytosis.

Knockdown of clathrin heavy chain (CHC) or dynamin-2 (Dyn2) reduces HCVtcp infection in Huh-7 cells, but not in Huh7.5.1 cells

Among the known pathways of pH-dependent viral endocytosis, clathrin-mediated dynamin-dependent endocytosis is a major endocytosis pathway. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis, has been commonly used to study clathrin-mediated endocytosis; however, it exerts multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Therefore, we examined the HCV endocytosis pathway by knockdown of specific molecules required for the endocytosis pathway. CHC, a major structural protein in clathrin-coated vesicles, and Dyn2, a GTPase essential for clathrin-coated-pit scission from the plasma membrane, play important roles in the clathrin-mediated pathway. Another well-studied model of viral entry is caveolin-mediated endocytosis. The role of dynamin in both clathrin-mediated endocytosis and caveolae-dependent endocytosis has been established (Marsh & Helenius, 2006; Miaczynska & Stenmark, 2008). To examine the endocytosis pathways of HCV, small interfering RNAs (siRNAs) for CHC, Dyn2 and caveolin-1 (Cav1), or scrambled control siRNA, were transfected into Huh-7 or

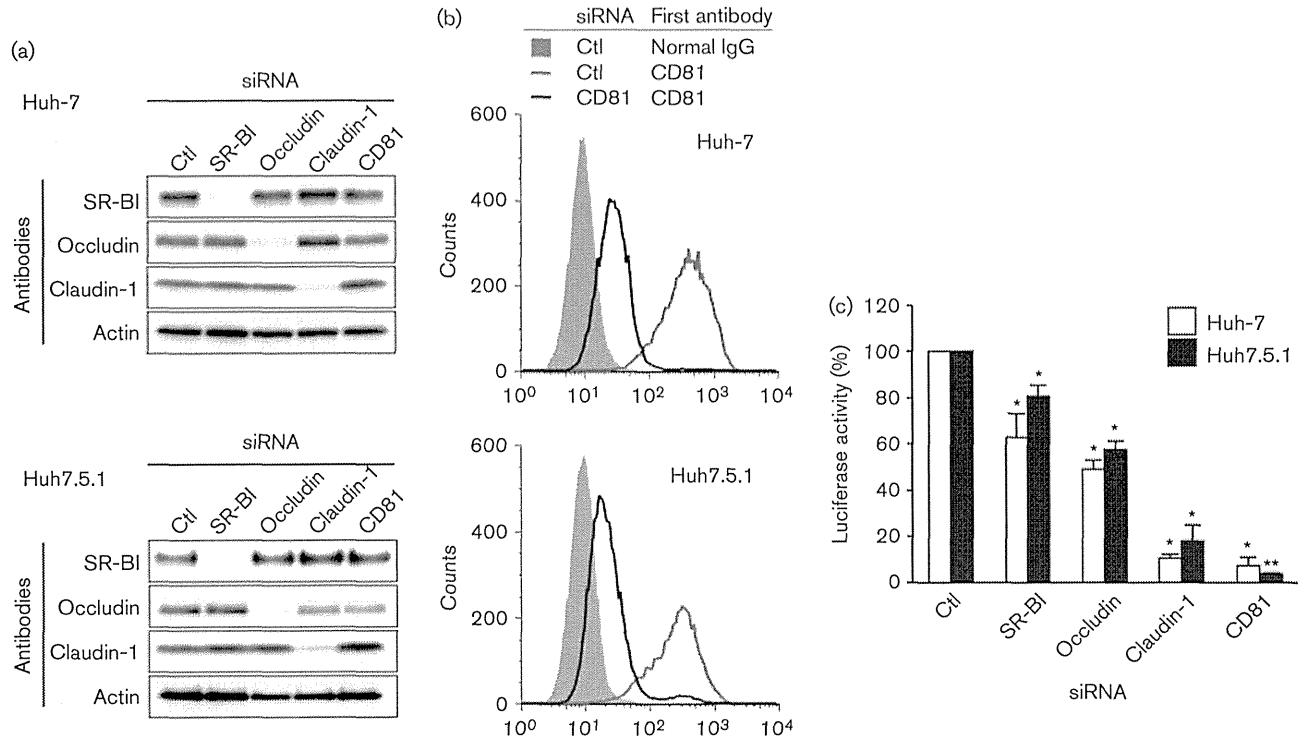

Fig. 1. Knockdown effect of receptor candidate molecules on HCV infection. (a) Huh-7 or Huh7.5.1 cells were transfected with the indicated small interfering RNAs (siRNA), harvested at 48 h post-transfection and the specific knockdown of each protein was verified by immunoblotting. (b) Huh-7 or Huh7.5.1 cells were transfected with CD81 or control siRNAs, harvested at 48 h post-transfection and the cell surface expression of CD81 was verified by FACS analysis. (c) Cells transfected with siRNA were infected with the same amount of HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was determined at 72 h post-infection and is expressed relative to the activity with control siRNA transfection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

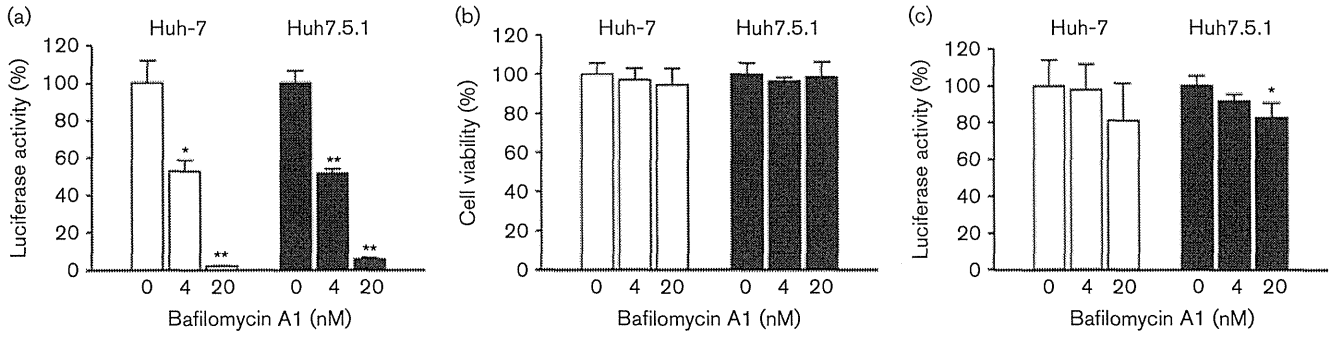

Fig. 2. Role of endosomal low pH in HCV infection. Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations and infected with HCVtcp. (a, b) Luciferase activity (a) and cell viability (b) were determined at 72 h post-infection, and expressed relative to amounts observed in controls. (c) Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations 48 h after HCVtcp infection. Luciferase activity was determined at 10 h post-treatment and expressed relative to amounts observed in controls. Data represent the mean ± SD. Statistical differences between controls and indicated concentrations were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

Huh7.5.1 cells, followed by infection with HCVtcp. Expression of CHC, Dyn2 and Cav1 was downregulated by transfection of specific siRNAs (Fig. 3a, b), whereas expression of SR-BI, occludin, claudin-1 and CD81 was not reduced (Figs 3a and S2). As indicated in Fig. 3(c), luciferase activity from HCVtcp was significantly reduced by knockdown of CHC and Dyn2 in Huh-7 cells, but not in Huh7.5.1 cells. Knockdown of Cav1 showed no inhibitory effects on HCVtcp entry into either cell line. Dynamin-independent entry in Huh7.5.1 cells was also observed using HCVtcp derived from genotype 1b (data not shown). Knockdown of CHC or Dyn2 also reduced entry of HCVcc in Huh-7 cells, but had no inhibitory effects in Huh7.5.1 (Fig. 3d). To rule out the possibility of effects on CHC and Dyn2 knockdown on viral RNA replication, HCVtcp were also inoculated before siRNA transfection. Luciferase activity was not affected by knockdown of CHC or Dyn2 in either cell line, whereas marked inhibition was observed for phosphatidylinositol 4-kinase (PI4K) (Fig. 3e). These data suggested that HCV entry was clathrin-mediated and dynamin-dependent in Huh-7 cells, but productive entry of HCV was clathrin- and dynamin-independent in Huh7.5.1 cells.

Expression of the dominant-negative form of Dyn2 reduces HCV infection in Huh-7 cells, but not in Huh7.5.1 cells

We also examined the role of dynamin in infectious entry of HCV into Huh-7 and Huh7.5.1 cells by overexpression of the dominant-negative form of Dyn2 (Dyn-K44A), which

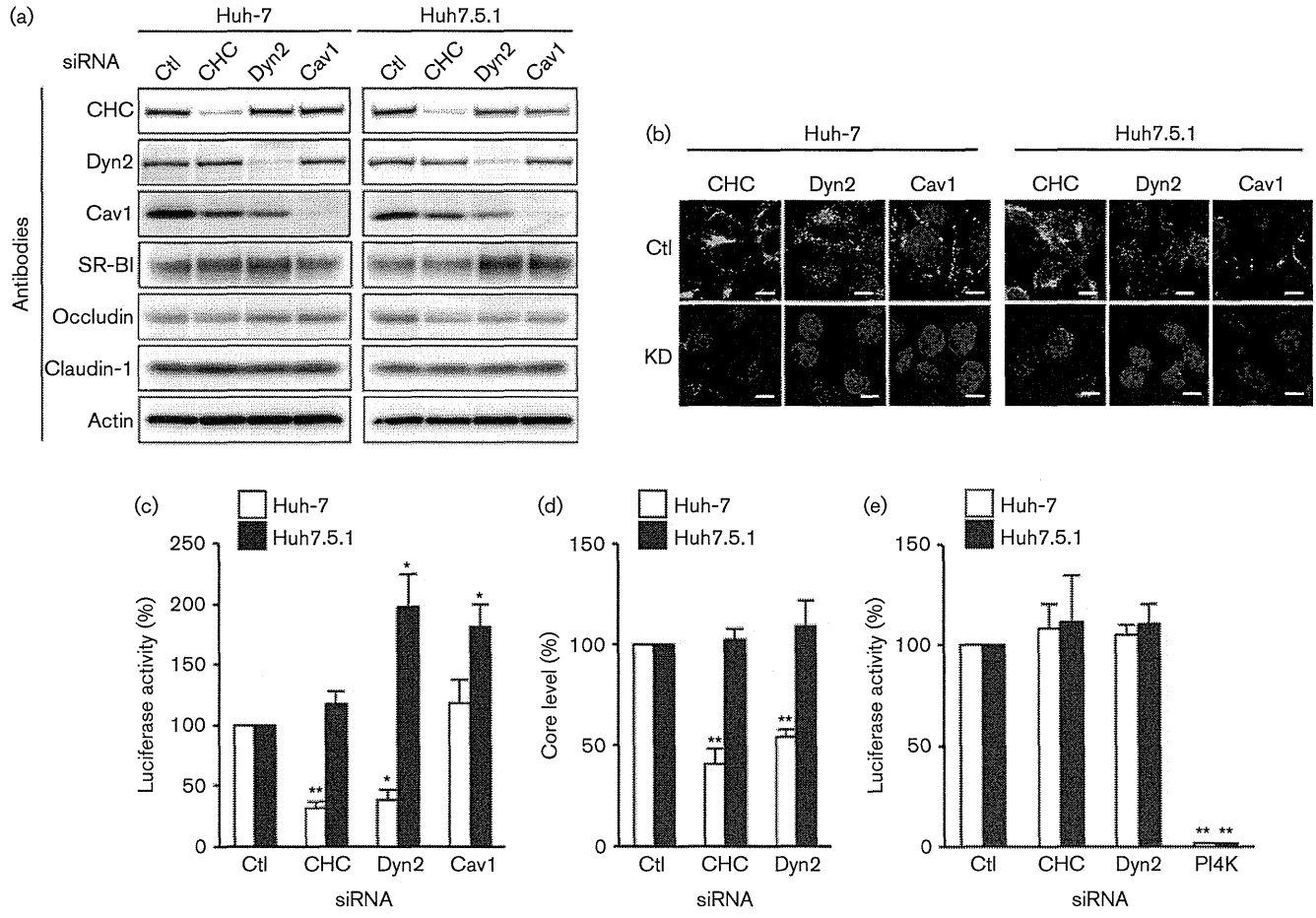

Fig. 3. Effects of CHC, Dyn2 and Cav1 knockdown on HCV infection. (a, b) Huh-7 cells or Huh7.5.1 cells were transfected with the indicated siRNAs and the specific knockdown (KD) of each protein was verified by immunoblotting (a) or immunostaining (b) at 48 h post-transfection. Bar, 50 μm. (c) Cells were transfected with the indicated siRNAs, followed by infection with HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was subsequently determined at 3 days post-infection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. (d) Cells were transfected with siRNA, followed by infection with HCVcc at 48 h post-transfection. Intracellular core levels were quantified at 24 h post-infection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. (e) Cells were infected with HCVtcp, followed by transfection with the indicated siRNAs. Luciferase activity in the cells was subsequently determined at 2 days post-transfection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

has been shown to effectively block clathrin-dependent and caveolar endocytosis (Damke *et al.*, 1995). Expression of haemagglutinin (HA)-tagged Dyn-K44A reduced the number of HCV-infected Huh-7 cells, but not Huh7.5.1 cells, as compared with WT HA-tagged Dyn2 (Dyn-WT), as shown in Fig. 4(a, b). Interestingly, internalization of transferrin, which is known to be mediated by clathrin-dependent endocytosis, was reduced in both Huh-7 and Huh7.5.1 cells expressing Dyn-K44A, whereas cells expressing Dyn-WT showed efficient endocytosis of transferrin (Fig. 4c, d). Collectively, these results suggested that dynamin participated in the internalization of HCV in Huh-7 cells, but was not absolutely required in Huh7.5.1 cells, although transferrin was taken up via dynamin-dependent endocytosis in both Huh-7 and Huh7.5.1 cells.

Flotillin-1 or the GTPase regulator associated with focal adhesion kinase 1 (GRAF1) play no major role during HCV infection of Huh7.5.1 cells

In order to dissect the major endocytosis pathways of HCVtcp in Huh7.5.1 cells, we investigated the role of alternative routes of HCV entry by siRNA knockdown. We silenced essential factors for the clathrin- or dynamin-independent pathways

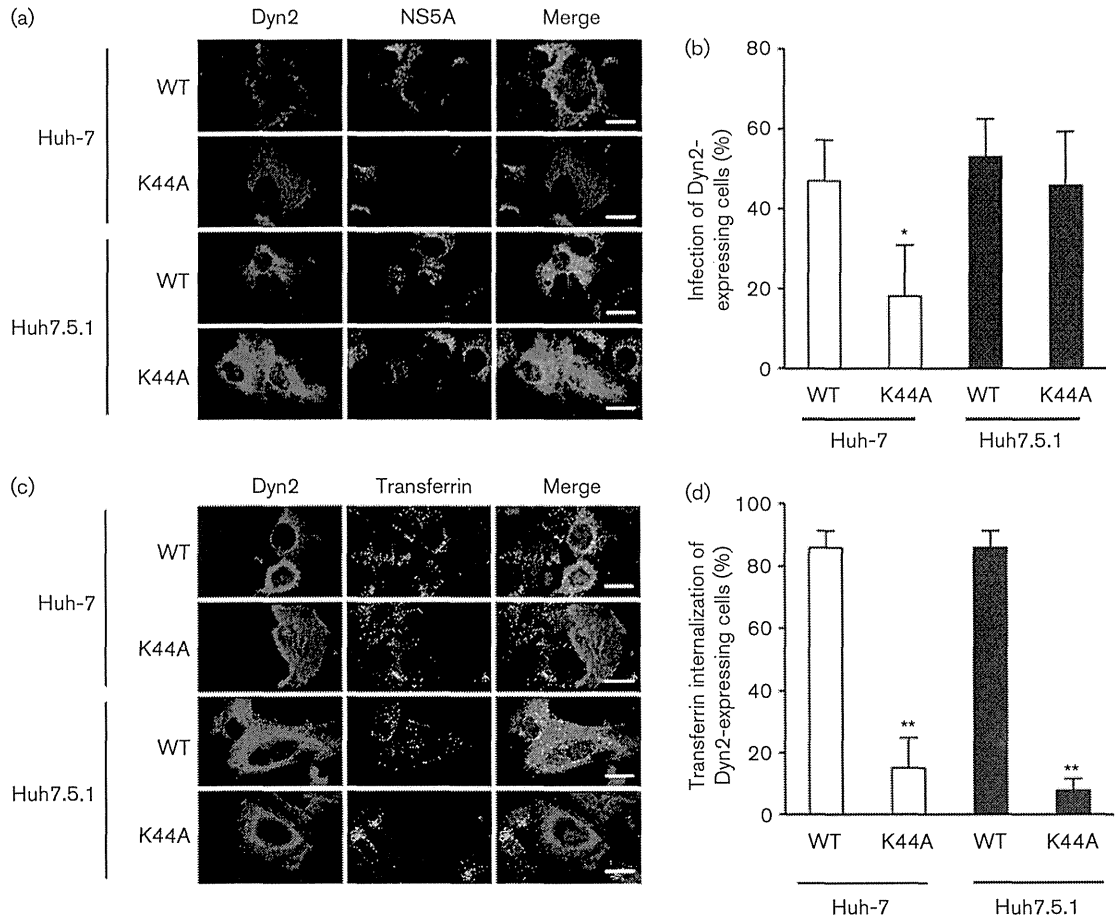

Fig. 4. Dynamin participates in the internalization of HCV in Huh-7 cells, but not in Huh7.5.1 cells. (a) Cells were transfected with HA-tagged WT Dyn2 (Dyn-WT) or dominant-negative Dyn2 (Dyn-K44A) expression plasmids. At 2 days post-transfection, cells were infected with HCVtcp, which possessed a subgenomic replicon without the luciferase gene. After 3 days, cells were fixed and HA-Dyn2 or HCV NS5A stained with anti-HA or anti-NS5A antibodies, respectively. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (b) Data were quantified as the population of HCVtcp-infected cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. (c) Cells were transfected with HA-tagged Dyn-WT or Dyn-K44A expression plasmids. At 2 days post-transfection, cells were incubated with Alexa Fluor-488 labelled transferrin at 37 °C in a 5 % CO_2 incubator. After 30 min of incubation, cells were washed, fixed and stained with anti-HA antibodies. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (d) Data were quantified as the population of transferrin-internalized cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. Statistical differences between Dyn-WT and Dyn-K44A were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus Dyn-WT.

including flotillin-dependent endocytosis, ADP-ribosylation factor 6 (Arf6)-dependent endocytosis, clathrin-independent carrier/glycosylphosphatidylinositol-enriched early endosomal compartment (CLIC/GEEC) endocytic pathway and macropinocytosis in Huh7.5.1 cells. Flotillin-1 and Arf6 are indispensable components of the flotillin and Arf6 pathways, respectively. Knockdown of flotillin-1 or Arf6 had no inhibitory effects on HCVtcp infection in Huh7.5.1 cells (Fig. 5a). The CLIC/GEEC endocytic pathway has recently become better defined and is regulated by the GTPase regulator associated with focal adhesion kinase-1 (GRAF1). However, GRAF1 was not detected in Huh-7 or Huh7.5.1 cells (Fig. 5b); thus, it is unlikely that the CLIC/GEEC pathway was involved in HCV entry in Huh7.5.1 cells. In addition, knockdown of p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A (CtBP1), which play important regulatory roles in the process of macropinocytosis, did not inhibit HCVtcp infection in Huh7.5.1 cells (Fig. 5c). Taken together, these results suggested that the entry of HCVtcp into Huh7.5.1 cells was not mediated mainly by flotillin-dependent endocytosis, Arf6-dependent endocytosis, the CLIC/GEEC endocytic pathway and macropinocytosis.

Clathrin-dependent and -independent pathways for HCV entry in other hepatic cells

We further examined the endocytosis pathways for HCV in non-Huh-7-related human liver-derived cell lines. Three HCVcc permissive hepatocellular carcinoma cell lines, Li23-derived ORL8c (Kato *et al.*, 2009), HepCD81/miR122 cells (HepG2/CD81 cells overexpressing miR122) and Hep3B/miR122 (Kambara *et al.*, 2012), were transfected with siRNA for CHC, Dyn2 or claudin-1, followed by infection with HCVtcp. Immunoblotting was performed in order to confirm knockdown of target proteins (Fig. 6a). Although knockdown of CHC or Dyn2 expression inhibited HCVtcp infection of ORL8c and HepCD81/miR122 cells, HCVtcp infection of Hep3B/miR122 cells was not affected (Fig. 6b), thus suggesting that productive entry of HCV is clathrin- and dynamin-independent in Hep3B/miR122 cells.

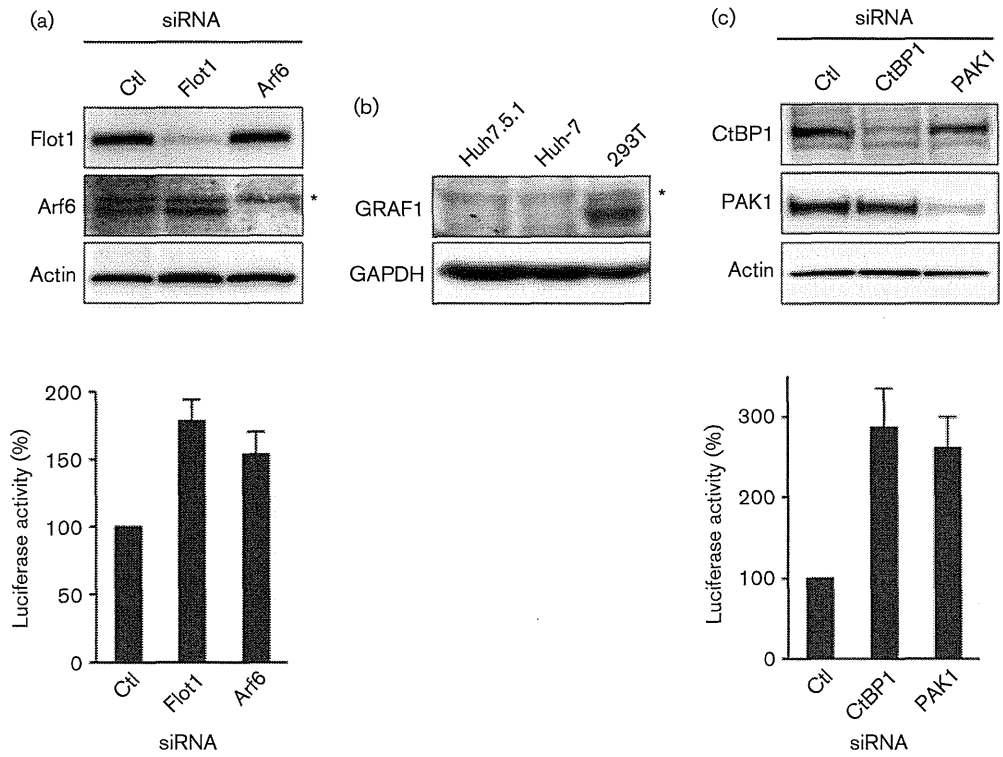

Fig. 5. Role of an alternative endocytosis pathway of HCV in Huh7.5.1 cells. (a) Huh7.5.1 cells were transfected with flotillin-1 (Flot1) or Arf6 siRNAs and specific knockdown of each protein was verified by immunoblotting (upper). Non-specific bands are marked with an asterisk. Cells transfected with siRNA were infected with HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection, Data represent the mean ± SD. (b) Expression of GRAF1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in Huh7.5.1, Huh-7 and 293T cells was analysed by immunoblotting. Non-specific bands are marked with an asterisk. (c) Huh7.5.1 cells were transfected with CtBP1 or PAK1 siRNA and specific knockdown of each protein was verified by immunoblotting (upper). Cells transfected with siRNA were infected with the HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection. Data represent the mean ± SD.

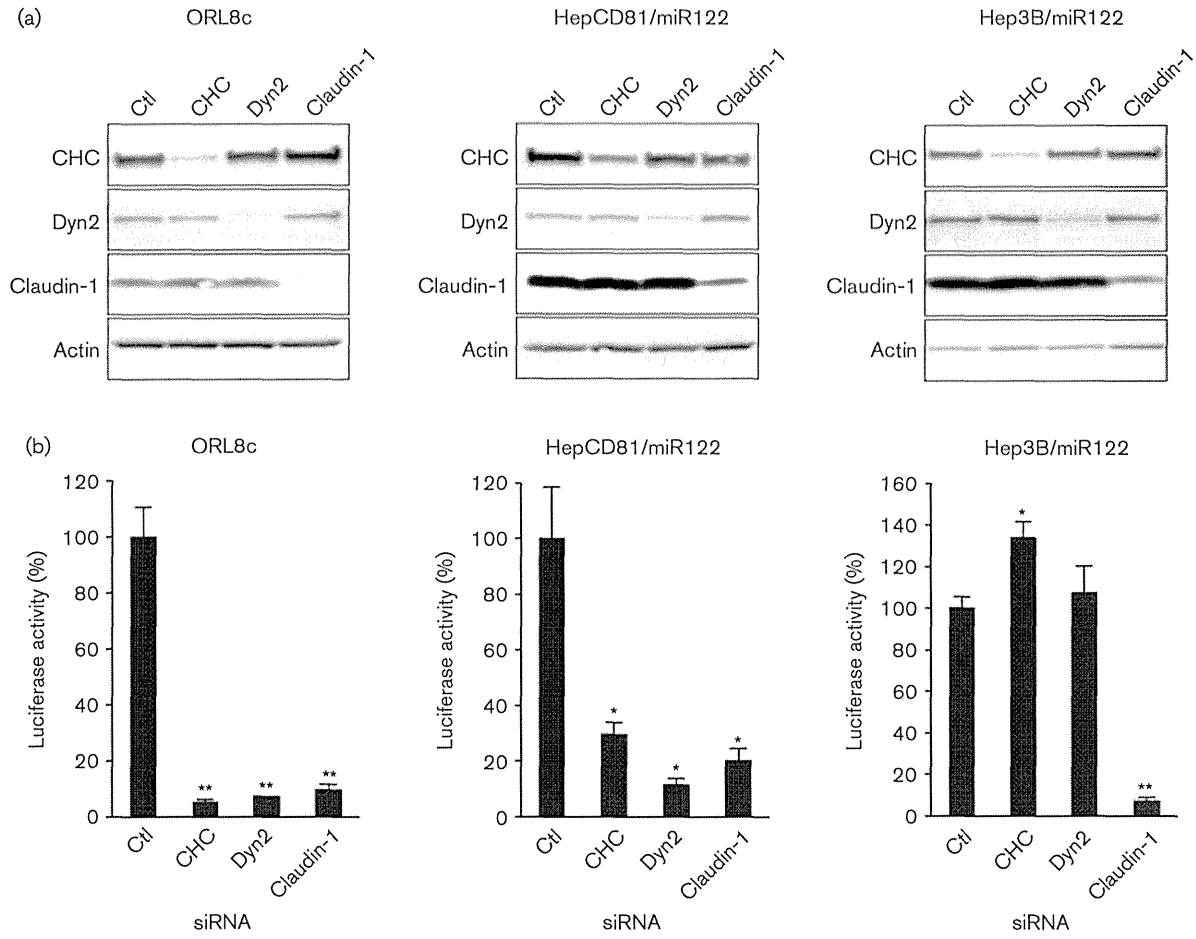

Fig. 6. Clathrin-dependent and -independent pathway of HCV entry in other HCV-permissive cells. The indicated cells were transfected with the indicated siRNAs and then infected with HCVtcp at 48 h post-transfection. (a) Specific knockdown of each protein was verified by immunoblotting. (b) Luciferase activity was determined at 72 h post-infection and expressed relative to the amount observed in the control (Ctl) siRNA transfection. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.001$ versus control.

In summary, we identified an alternative clathrin- and dynamin-independent entry pathway for HCV in at least two independent cell lines, Huh7.5.1 and Hep3B/miR122 cells, in addition to the previously reported clathrin- and dynamin-dependent pathway. These findings provided clues for understanding the molecular mechanisms of the endocytosis pathway for HCV infection.

DISCUSSION

Many viruses have been shown to utilize a number of different endocytic pathways to productively infect their hosts. Clathrin-dependent endocytosis would appear to be the most commonly used, but it is increasingly clear that a number of clathrin-independent endocytosis pathways are also used by several different viruses (Mercer *et al.*, 2010). In the case of HCV, it has been reported that viral entry is mediated by clathrin-dependent endocytosis (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009). In these papers, HCVpp was used at least in part for analysis of HCV entry pathway. However, recent reports have revealed several different characteristics between HCVpp and HCVcc.

Viral entry has been addressed primarily by pharmacologic inhibitor studies, immunofluorescence and electron microscopy, by transfection with dominant-negative constructs, and more recently by siRNA knockdown. Analysis of endocytosis pathways using pharmacological inhibitors has raised concerns about specificity. For example, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, has been shown to exert multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Methods for elucidating the viral endocytosis pathway by co-localization of virus particles with host factor also have limitations. Electron and

fluorescence microscopy, which require a high particle number, do not allow the differentiation of infectious and non-infectious particles. Infectious particles of HCV in the supernatant of infected cells appeared to represent only a small portion of secreted virus particles (Akazawa *et al.*, 2008) and it is unclear whether the viral particles observed by microscopy could lead to productive infection. Therefore, we utilized HCVtcp, which is useful for determining productive entry of the virus without reinfection, and a combination of siRNA knockdown and dominant-negative mutants for analysis of the productive route of infection. Although HCVcc is also utilized in analysis of productive entry, it cannot completely exclude the effects of reinfection by virus produced by infected cells. Reduction of HCVcc infection by knockdown of CHC and Dyn2 was moderate when compared with that of HCVtcp (Fig. 3c, d), thus suggesting slight effects due to reinfection in HCVcc.

The data presented here demonstrate for the first time to our knowledge that HCV is able to enter cells via dynamin-independent endocytosis in addition to the previously described classical clathrin- and dynamin-dependent pathway. First, knockdown of CHC and Dyn2 had no inhibitory effects on HCVtcp and HCVcc entry into Huh7.5.1 cells. Second, overexpression of dominant-negative Dyn2 had no inhibitory effects on HCVtcp in Huh7.5.1 cells. Finally, in addition to Huh7.5.1 cells, Hep3B/miR122 cells were also shown to be infected with HCV via clathrin- and dynamin-independent pathways. We further investigated the role of alternative minor routes of HCV entry into Huh7.5.1 cells; however, the productive endocytosis pathway could not be defined. It should be noted that inhibition of alternative endocytosis routes by siRNA led to an increase of luciferase activity (Figs 3c and 5a, c). This could be explained by the inhibition of a particular endocytosis pathway resulting in a compensatory increase in alternative endocytosis pathways (Damke *et al.*, 1995).

Although we confirmed an alternative endocytosis pathway for the productive entry of HCV, it is not clear why and how the two independent endocytosis pathways operate in different cell lines. SV40 can enter cells via caveolae-dependent (Norkin *et al.*, 2002; Pelkmans *et al.*, 2001) and -independent (Damm *et al.*, 2005) pathways. Influenza virus enters cells via clathrin-mediated endocytosis (Matlin *et al.*, 1981) in addition to non-clathrin-mediated, non-caveola-mediated internalization pathways (Sieczkarski & Whittaker, 2002b). Entry of dengue virus type 2 is clathrin-dependent in HeLa and C6/36 cells (Acosta *et al.*, 2008; Mosso *et al.*, 2008; van der Schaar *et al.*, 2008), and is clathrin-independent in Vero cells (Acosta *et al.*, 2009). Different receptor usage may determine the consequential route of entry. However, we did not observe any differences between Huh-7 and Huh7.5.1 cells in terms of knockdown effects of receptor candidate molecules on HCV infection, as shown in Fig. 1(c), although we cannot exclude the possibility that other undefined receptors are associated with viral entry. Huh7.5.1 cells were established by elimination of the HCV genome from replicon cells derived from Huh-7 cells (Blight *et al.*, 2002; Zhong *et al.*, 2005) and they exhibit more potent replication of HCV than the original Huh-7 cells. Further study showed that the increased permissiveness of cured cells results from a mutation in the retinoic acid-inducible gene I (Sumpter *et al.*, 2005), which impairs IFN signalling. In addition, it has been shown that cured cell lines express higher levels of miR122 than parental cells participating in the efficient propagation of HCVcc (Kambara *et al.*, 2012). As it is unclear whether these changes are the reason for a distinct endocytosis pathway, it will be of interest to explore these associations in further studies.

In conclusion, we confirmed an alternative clathrin-independent endocytosis pathway in HCV-permissive human hepatic-derived cells, in addition to the previously reported clathrin-dependent endocytosis pathway. This paper highlights the fact that clathrin- and dynamin-mediated endocytosis is the main route of HCV entry for Huh-7, HepCD81/miR122 and ORL8c cells, whilst clathrin and dynamin do not play a major role during the productive route of HCV infection in Huh7.5.1 and Hep3B/miR122 cells. Taken together, these studies suggest that different cell entry pathways for HCV infection may be utilized in different cell types, although further studies are necessary in order to understand this phenomenon.

METHODS

Cells. The human hepatocellular carcinoma cell lines Huh-7, Huh7.5.1, Hep3B/miR122 and HepG2/CD81, which overexpressed miR122 (Kambara *et al.*, 2012), were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) containing non-essential amino acids, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10 % FBS. Li23-derived ORL8c cells (Kato *et al.*, 2009) were maintained in F12 medium and DMEM (1 : 1, v/v) supplemented with 1 % FBS, epidermal growth factor (50 ng ml^{-1}), insulin (10 μg ml^{-1}), hydrocortisone (0.36 μg ml^{-1}), transferrin (5 μg ml^{-1}), linoleic acid (5 μg ml^{-1}), selenium (20 ng ml^{-1}), prolactin (10 ng ml^{-1}), gentamicin (10 μg ml^{-1}), kanamycin monosulfate (0.2 mg ml^{-1}) and fungizone (0.5 μg ml^{-1}). All cell lines were cultured at 37 °C in a 5 % CO_2 incubator.

Preparation of viruses. HCVtcp and HCVcc derived from JFH-1 with adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) were generated as described previously (Suzuki *et al.*, 2012). For HepCD81/miR122 and ORL8c cells, HCVtcp containing the *Gaussia* luciferase (GLuc) reporter gene were used. To do this, plasmid pHH/SGR-JFH1/GLuc/NS3m carrying the bicistronic subgenomic HCV replicon containing the GLuc reporter gene and the NS3 adaptive mutation was constructed by replacement of the firefly luciferase (FLuc) gene of pHH/SGR-Luc containing the NS3 mutation (N1586D) (Suzuki *et al.*, 2012) with the GLuc gene of pCMV-GLuc (NEB).

Plasmids. HA-tagged Dyn2, a dominant-negative Dyn2 (K44A) in which Lys44 was replaced with Ala, was cloned into pcDNA3.1 as described previously (Kataoka *et al.*, 2012).

Gene silencing by siRNA. siRNAs were purchased from Sigma-Aldrich and were introduced into the cells at a final concentration of