INTRODUCTION

Hepatitis C is a blood-borne infectious disease that is caused by the hepatitis C virus (HCV) (Choo et al., 1989). The estimated prevalence of HCV infection is 2.2% worldwide (Alter, 2007). In most cases, the infection causes hepatic inflammation (hepatitis) that is often asymptomatic, but about 80% of infected individuals develop chronic hepatitis that may ultimately lead to cirrhosis and hepatocellular carcinoma (HCC) (Lauer & Walker, 2001). HCV is able to establish a persistent infection also because of its efficiency in evading the immune response of the host (Sklan et al., 2009). HCV is a serious health care problem that requires careful understanding of the molecular mechanisms that regulate its replication in the host cells in order to develop efficient antiviral strategies. The outcome of an HCV infection depends both on the host genetic background and on the viral subtype as well as from external factors. Among these, iron (Fe) overload has been shown to be common among patients with chronic hepatitis C (Bonkovsky, 2002; Boucher et al., 1997; Di Bisceglie et al., 1992) and to correlate with a poor response to antiviral therapy (Franchini et al., 2008; Sherrington & Olynyk, 2002).

Fe is subject to a tightly coordinated regulation by cellular factors involved in Fe absorption, transport, uptake and storage (Hentze et al., 2004). Circulating transferrin (Tf) bound to Fe$^{3+}$ is endocytosed into cells by the transferrin receptor 1 (TfR1) through clathrin-coated pits into endosomes. The divalent metal transporter 1 (DMT-1, also known as DCT1 or Nramp2) transfers Fe$^{2+}$ across the endosomal membrane into the cytoplasm, but is also able to mediate direct absorption of non-Tf-bound Fe from the plasma in the intestine (Fleming et al., 1997). Fe is stored in the cells in a complex with ferritin (Torti & Torti, 2002) and is exported from cells by the Fe exporter ferroportin 1 (FPN1, also known as Ireg1) (Abboud & Haile, 2000; Donovan et al., 2000; McKie et al., 2000). Regulation of Fe homeostasis is orchestrated by the antimicrobial peptide hormone hepcidin (encoded by the Hamp gene) (Ganz, 2006). Hepcidin is mainly secreted by the liver and induces internalization and degradation of the Fe exporter ferroportin thereby inhibiting Fe absorption from the intestine and Fe release from reticuloendothelial cell stores (Nemeth et al., 2003). Defects in the Hamp gene result in a form of inherited Fe overload disorder (haemochromatosis) that induces increased intestinal Fe absorption and deposition of Fe in parenchymal organs. The classical hereditary haemochromatosis (HH), an autosomal recessive disorder, is most often caused by mutations in a gene encoding a MHC class I-like protein with unknown function (HFE) (Feder et al., 1996). However, juvenile haemochromatosis or haemochromatosis type 2 A (HFE2A) is caused by mutations in the gene encoding haemjuvelin (HJV) (Papanikolaou et al., 2004). HJV is a coreceptor for bone morphogenetic proteins (BMPs), and inhibition of endogenous BMP signalling reduces hepcidin expression and increases serum Fe in mice.

Several clinical observations point to an intricate crosstalk between iron (Fe) metabolism and chronic hepatitis C virus (HCV) infection. In this study, we wanted to investigate the molecular control that Fe levels exert on HCV replication at the hepatocyte level. In keeping with previous observations we confirmed that supra-physiological intracellular Fe induced by haemin treatment down-modulated HCV replication from subgenomic replicons. We also found that RNAi-mediated knockdown of the key Fe modulator hepcidin increased intracellular ferritin and inhibited HCV replication. Conversely, HCV replication did not modulate ferritin content in hepatocytes. Finally, we demonstrated that hepcidin is modulated at the mRNA level by alpha interferon through STAT3. We propose that in Huh7 cells hepcidin modulation leads to an unfavourable intracellular environment for HCV replication. These data may therefore contribute to a better understanding of the complex interplay between HCV and cellular physiology during infection.
(Andriopoulos et al., 2009; Babitt et al., 2006). Genetic conditions that cause increased intestinal Fe absorption and Fe deposition in organs aggravate the clinical picture of chronic hepatitis C infection (Pietrangelo, 2003).

Many studies have confirmed the existence of a tight correlation between Fe metabolism and the immune system (Ganz, 2006). Importantly, during infectious/inflammatory conditions Fe levels are modulated in order to reduce Fe availability for bacterial growth (Walker & Walker, 2000). There is clinical evidence that HCV infection influences the levels of Fe body stores. Elevated Fe serum markers and Fe accumulation in the liver is frequent in subjects with chronic hepatitis C (Boucher et al., 1997; Di Bisceglie et al., 1992). Liver Fe accumulation is hepatotoxic, may cause worsening of liver injury, and makes patients with chronic hepatitis C resistant to interferon-based therapy because the oxidative stress induced by Fe impairs the alpha interferon (IFN-α) signal by blocking the janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway (Bassett et al., 1999; Di Bona et al., 2006; Distante et al., 2002; Fujita et al., 2007; Nishina et al., 2008; Olynnyk et al., 1995; Souza et al., 2006; Van Thiel et al., 1994). Phlebotomy improves the therapeutic response to interferon in chronic HCV (Desai et al., 2008). Serum hepcidin-to-ferritin ratios were significantly lower in HCV-positive patients than in HCV-negative controls. After successful HCV eradication by antiviral therapy, hepcidin levels returned to normal concomitantly with the improvement of the Fe-overload condition (Fujita et al., 2008). Interestingly, HFE mutations that are associated with increased Fe loading are also associated with a significantly higher likelihood of sustained virological response to treatment in chronic HCV (Bonkovsky et al., 2006). A long-term survey of patients with advanced chronic hepatitis C recently confirmed that the presence and degree of stainable Fe in hepatocytes and portal stromal cells was predictive of adverse clinical outcomes, further reinforcing the notion that Fe is an important comorbid factor in chronic hepatitis C infection (Lambrecht et al., 2011).

From the clinical point of view it seems clear that Fe is an unfavourable risk factor in chronic hepatitis C and HCC prognosis. On the other hand, in vitro studies on HCV replication gave controversial results: Fe helps HCV translation by the internal ribosome entry site (IRES) of HCV and by increasing the expression of the translation initiation factor eIF3 (Cho et al., 2008; Theurl et al., 2004), while Fe represses HCV replication by inhibiting the enzymic activity of the RNA-dependent RNA polymerase of HCV (NS5B) (Fillebeen et al., 2005). It has been shown that Huh7 human hepatoma cells, carrying a replicating HCV replicon, show differences in the expression of genes related directly or indirectly to Fe metabolism, when compared to their parent counterparts (Fillebeen et al., 2007). The interpretation of this differential expression pattern suggests that cells that are in a condition that mimics chronic HCV infection may be Fe-deficient. Recent findings from the same group confirmed the Fe-mediated inhibition of HCV replication in vitro (Fillebeen & Pantopoulos, 2010).

In this study, we confirm that, under our experimental conditions, excess intracellular Fe induced by haemin treatment is detrimental for HCV replication, but HCV replication per se does not affect Fe levels measured by ferritin content in hepatocytes. Knockdown of the Fe metabolism-related protein hepcidin results in higher levels of intracellular Fe content and in the inhibition of HCV replication. We also show that hepcidin is the only Fe-related gene modulated by IFN-α signaling of HCV replication of Huh7 human hepatoma cells, carrying a replicating HCV replicon, show differences in the expression of genes related directly or indirectly to Fe metabolism, when compared to their parent counterparts (Fillebeen et al., 2007). The interpretation of this differential expression pattern suggests that cells that are in a condition that mimics chronic HCV infection may be Fe-deficient. Recent findings from the same group confirmed the Fe-mediated inhibition of HCV replication in vitro (Fillebeen & Pantopoulos, 2010).

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**RESULTS**

**Fe accumulation into the host cell affects HCV replication**

Clinical data are consistent in correlating high cellular and systemic Fe levels in HCV patients with poor prognosis and dramatic deregulation of liver physiology (Bonkovsky, 2002; Boucher et al., 1997; Di Bisceglie et al., 1992). Conversely, in vitro studies do not provide a clear role for cellular Fe in HCV replication and translation (Fillebeen & Pantopoulos, 2011; Sikorska et al., 2011). Fe helps HCV translation by the IRES of HCV and by increasing the expression of the translation initiation factor eIF3 (Cho et al., 2008; Theurl et al., 2004), while Fe represses HCV replication by inhibiting the enzymic activity of NS5B (Fillebeen et al., 2005).

To resolve this issue in cells naïve for HCV replication we used the human hepatocellular carcinoma cell line Huh7 and subgenomic replicons derived from the HCV genotype 2a JFH1 strain cloned from an individual with fulminant hepatitis (Kato et al., 2005). These replicons carry the luciferase reporter gene for the quantification of HCV replication. We incubated Huh7 cells transfected with HCV-RNA in the presence of haemin for 6 or 24 h in order to monitor the effect of cellular Fe loading on HCV replication (Fig. 1a). Cells were harvested at various time points post-HCV-RNA transfection and luciferase activity assessed. By comparison with the untreated control, luciferase activity was significantly reduced after haemin treatment. To confirm that the effect was specific on HCV replication, cell lysates were immunoblotted with an antibody against viral NS5A. As shown in Fig. 1(b), haemin treatment for 6 and 24 h reduced the level of HCV proteins at 72 h post-transfection.

To confirm the effect of haemin on HCV replication we repeated the experiment using the HCV replicon Luc-JFH1/ΔE1-E2 that carries only a deletion of the E1/E2 region, but maintains all the rest of the HCV genome (Koutsoudakis et al., 2009). Genetic conditions that cause increased intestinal Fe absorption and Fe deposition in organs aggravate the clinical picture of chronic hepatitis C infection (Pietrangelo, 2003).
et al., 2006; Wakita et al., 2005). When we used the same protocol as above, where incubation with haemin was conducted between 24 and 48 h post-electroporation of the replicon, inhibition of HCV replication measured as luciferase activity was not observed with 6 or 24 h of haemin incubation (data not shown). However, if haemin was added at the time of electroporation for 24 h, luciferase activity was significantly reduced at 48 and 72 h (Fig. 1c). This treatment also reduced the level of the NS5A viral protein (Fig. 1d).

To verify that haemin increased the intracellular Fe, we measured the ferritin content of Huh7 cells. As shown in Fig. 2(a), a 6 h treatment of Huh7 cells with 50 μM haemin was able to induce an eightfold increase of intracellular ferritin (Fig. 2a).

Finally, we explored the effect of haemin treatment at the highest concentration used (100 μM for 24 h) on the proliferation of Huh7 cells. As shown in Fig. 2(b), cell viability was not affected. Furthermore, the cell cycle profile also remained unaffected by the treatment (Fig. 2c). We conclude that treatment of haemin did not affect cell proliferation in the time frame of the experiments, in keeping with the work of Fillebeen et al. (2005) that reported concentrations of haemin up to 200 μM and the work of Paeshuysse et al. (2006) that measured a 50% cytostatic concentration of haemin above 50 μM, both in Huh7 cells. These observations confirm the inhibitory role of intracellular Fe on HCV replication.

**Hepcidin down-modulation affects HCV replication and correlates with increase ferritin**

The intracellular Fe balance is maintained by several regulatory factors (Hentze et al., 2004). Among them, hepcidin is the key regulator of Fe body levels. To
determine whether hepcidin plays a role in the regulation of HCV replication we knocked down hepcidin in Huh7 cells transfected with HCV replicons. siRNA-mediated knockdown of hepcidin showed a reduction of about 70% at the mRNA level after 24 h post-transfection (Fig. 3a). HCV replication was also significantly reduced after siRNA transfection (Fig. 3b). The down modulation of HCV replication is related to Fe increase (ferritin) due to hepcidin decrease as demonstrated in Fig. 3(c) where knockdown of hepcidin induced intracellular ferritin. This effect is similar to what was obtained by inducing ferritin by haemin (Fig. 2a) and HCV replication inhibition (Fig. 1a).

Next, we wished to explore the effect of HCV replication on the intracellular Fe homeostasis. To this end, we transfected Huh7 cells with the HCV replicon encoding luciferase or with the non-replicating replicon carrying the GND mutation in NS5B (Fig. 3d). HCV replication measured as luciferase activity peaked at 48 h post-transfection. As shown in Fig. 3(e), HCV replication did not induce intracellular Fe accumulation measured as ferritin content in Huh7 hepatocytes. This measurement was taken at 48 h post-replicon transfection, at the peak of viral replication, when transcription from the input RNA is negligible (GND mutant). As a control, we transfected a synthetic dsRNA that is used to mimic replication intermediates of RNA viruses.

We conclude that hepcidin knockdown induces both intracellular ferritin increase and a decrease of HCV replication, but HCV replication per se is unable to modulate intracellular ferritin content.

Hepcidin is induced by IFN-α

We decided to measure the response to IFN on a panel of Fe-related genes to explore the possibility of interplay between infection, the antiviral response and Fe levels. The genes included in the analysis were the following: DMT-1, the hypoxia inducible factor 1 alpha-subunit (HIF1α); FPN1; the HH genes HFE, HJV and Hamp. mRNA levels were monitored for 48 h after exposure of the cells to 200 U IFN-α ml⁻¹ and compared to mock-treated cells (Supplementary Fig. S1, available in JGV Online). The IFN inducible gene 29,5'-oligoadenylate synthetase 1 (OAS1) was used as positive control. Among the genes tested only the mRNA expression of endogenous hepcidin was modulated by IFN-α administration. A slight but significant increase of Hamp mRNA was observed 2 h after induction with IFN-α (Fig. 4a).

Expression of the chemokine gene CXCL11 is highly inducible by IFN (Indraccolo et al., 2007; Yang et al., 2007). As shown in Fig. 4(b), the expression of both Hamp and CXCL11 where significantly increased 2 h after exposure of cells to IFN-α. Higher IFN-α concentrations (500–1000 U ml⁻¹) were used to match those previously shown to be able to induce CXCL11 (Indraccolo et al., 2007; Yang et al., 2007). CXCL11 mRNA levels continued to increase thereafter, while Hamp mRNA returned to baseline even at higher doses of IFN. CXCL11 is induced by IFN-α through a STAT3-dependent pathway (Yang et al., 2007). Hamp carries a STAT3-binding motif in its promoter and STAT3 has been shown to be critical for the control of baseline hepcidin mRNA expression and under
inflammatory conditions (Verga Falzacappa et al., 2007). Since it has been already shown that IFN-α treatment of Huh7 hepatocytes efficiently induced STAT3 phosphorylation (Zhu et al., 2003, 2004) we wondered if the STAT3-binding site in the Hamp promoter was necessary for IFN induction. To this end, we transfected Huh7 cells with reporter plasmids that express luciferase either under the control of the wild-type hepcidin promoter, or under the control of a deletion mutant in the STAT3-binding motif located at position –64/–72 of the promoter (Verga Falzacappa et al., 2007). The deletion of this STAT3-binding motif completely abolished the IFN-α-dependent increase in luciferase activity (Fig. 4c). These data show that a functional STAT3-binding motif within the hepcidin promoter mediates the hepcidin response to IFN-α.

**DISCUSSION**

Clinical evidence indicates that liver Fe overload and HCV infection are two independent but synergistic risk factors for the progression of liver disease. In addition, response to IFN, the only treatment for HCV infection available to date, is poor in conditions of Fe overload resulting in progression of liver damage and increasing the probability of developing HCC (Franchini et al., 2008; Sherrington & Olynyk, 2002). However, despite a wealth of convincing
clinical data, the molecular basis that supports a crosstalk between Fe metabolism and HCV infection is still poorly investigated.

Fe is a modulator of HCV replication (Fillebeen & Pantopoulos, 2010; Fillebeen et al., 2005; Hou et al., 2009). In this work, we confirm that haemin treatment of the hepatocyte inhibits HCV replication (Fig. 1) consistent with other reports (Fillebeen et al., 2005), but contrary to reports that showed increased translation initiation mediated by the IRES of HCV (Cho et al., 2008; Theurl et al., 2004). However, the latter studies focussed on the IRES response and did not consider the replication of the full genome. We also demonstrate that haemin down modulation of HCV replication is independent from the requirement of cell lines selected for HCV replicon maintenance as previously used (Fillebeen et al., 2005). We confirmed our results also using an alternative HCV replicon that maintains most of the HCV genome including the core that is secreted by the construct, albeit with lower efficiency with respect to wild-type (Fig. 1c, d) (Koutsoudakis et al., 2006; Wakita et al., 2005). Therefore, any core-mediated effect on the HCV response to haemin-mediated Fe overload could be ruled out (Miura et al., 2008).

Haemin is as a convenient Fe donor that has been used to explore the effect of Fe overload on HCV replication (Fillebeen et al., 2005). We confirm that ferritin is increased upon haemin treatment (Fig. 2a) at concentrations that do not affect cell proliferation (Fig. 2b, c). It should be noted that haemin and other metalloporphyrins may have additional effects on HCV replication, other than Fe overload (Hou et al., 2009, 2010; Shan et al., 2006, 2007). Therefore, haemin treatment may lead to additional/alternative pathways of HCV inhibition. However, Fe delivery through other cell permeable Fe donors, like Fe-SIH for example, showed similar effects on HCV replication.
replication pointing to a role of intracellular Fe (Fillebeen & Pantopoulos, 2010).

Next, we observed that the knockdown of hepcidin by RNAi significantly reduced HCV replication (Fig. 3). This finding is consistent with a similar observation obtained from a functional genomic screen of cellular cofactors for HCV replication (Tai et al., 2009). Knockdown of hepcidin upregulates intracellular ferritin (Fig. 3c) contributing to an intracellular environment that is not favourable to HCV replication as demonstrated by inducing Fe uptake by haemin treatment (Fig. 1). The mechanism of ferritin increase in Huh7 cells where hepcidin is down-modulated by siRNA was not investigated. Hepcidin and ferroportin control Fe availability at the systemic level (Hentze et al., 2004). Although ferroportin is expressed in Huh7 cells (Fillebeen et al., 2007) (Supplementary Fig. S1c), loss of hepcidin control would lead to ferroportin stabilization and increase Fe efflux from the hepatocyte, leading to intracellular Fe deficiency instead of overload. However, upregulation of ferroportin did not affect ferritin levels in Huh7 cells (Fillebeen et al., 2007) reinforcing the notion that ferroportin may not be involved in the mechanism of ferritin increase when hepcidin is down-regulated in Huh7 cells.

We also verified that HCV replication is unable to modulate the intracellular Fe content (Fig. 3e). This finding appears to contradict a recent work that proposes a reduction of intracellular Fe levels in Huh7 cell lines stably expressing a subgenomic HCV replicon (Fillebeen et al., 2007). However, also in their case ferritin levels remained unaltered. Increased cellular Fe levels appear initially in the cytosolic labile Fe pool only loosely associated to macromolecular complexes and then eventually sequestered into ferritin molecules. Cells chronically harbouring the HCV replicon show a reduction of this labile Fe pool (Fillebeen et al., 2007). Therefore, it remains to be addressed if cells transiently treated with an HCV replicon like in our case, or infected by HCV (Fillebeen & Pantopoulos, 2010), may be also able to modulate the labile intracellular Fe pool.

We also wanted to assess whether IFN-α triggers Fe-related proteins. IFN treatment is able to potently suppress HCV-JFH1 replication in Huh7 cells (Kato et al., 2005). Among the several factors analysed, we found that the Fe hormone hepcidin is the only one induced upon IFN-α induction (Fig. 4). *Hamp* is upregulated very early after induction, similarly to what has been observed after inflammatory stimulation (Verga Falzacappa et al., 2007). Compared to the IFN-α-mediated induction of the CXCL11 chemokine, *Hamp* is induced with the same initial kinetic, but then tails off, while CXCL11 mRNA continues to increase (Fig. 4b). Like CXCL11, *Hamp* requires a STAT3-binding site in its promoter to be able to respond to IFN-α (Fig. 4c). Further studies will be required to understand the molecular pathways leading to IFN-α-mediated modulation of hepcidin mRNA. Clearly, induction of hepcidin does not participate in the several mechanisms that contribute to the intracellular antiviral activity of IFN-α, given our previous results. We may argue that one of the beneficial effects of IFN-α treatment for chronic HCV infection is related to its ability to increase ferricidin levels, thus reducing systemic Fe absorption. In favour of this hypothesis is the clinical observation that anti-HCV therapy based on pegylated IFN and ribavirin caused patients to exhibit anaemia (Lin & Yin, 2008).

It is obviously difficult to extend these findings, obtained on isolated cells in the test tube, to the complex pathophysiological conditions found in the infected patient. However, this contribution may furnish a rationale on the many, sometimes contradictory clinical observations and direct subsequent studies towards a better definition of the phenomenon.

**METHODS**

**Cell culture.** The human hepatocarcinoma Huh7 cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM; high glucose; Invitrogen) supplemented with 10% heat-inactivated low endotoxin FBS (Invitrogen), 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Cell cultures were maintained at 37 °C under 5% CO₂.

IFN-α was obtained from the Biotechnology Development Group of the ICGEB and was used at a final concentration of 200, 500 or 1000 U ml⁻¹.

A stable aqueous solution of haemin-arginate, containing 25 g haemin (Sigma-Aldrich) 1⁻¹, 26.7 g l-arginine (Fluka) 1⁻¹, 100 g ethanol (99.8 %) 1⁻¹ and 400 g propylene glycol 1⁻¹ was prepared according to Tenhunen et al. (1987). The solution was diluted to obtain a 10 mM haemin stock solution, stabilized by filtration through a 0.22 μm filter, distributed into aliquots and kept frozen until used only once at a final concentration of 50–100 μM.

Cell viability was assessed by the 0.4% trypan blue dye exclusion test. Flow cytometry of propidium iodide-treated Huh7 cells was performed by standard procedure on a FACSCalibur cell analyzer from Becton Dickinson.

**RNA isolation, reverse transcription and quantitative real-time PCR analysis.** Total RNAs were extracted by TRIzol (Invitrogen) from cells according to the manufacturer’s instructions, treated with DNase I (Invitrogen) and then quantified. Reverse transcription and real-time PCR were carried out using M-MLV reverse transcriptase (Invitrogen) and iQ SYBR Green Supermix (Bio-Rad), respectively. Real-time PCR was performed using the CFX96TM Real-Time System (Bio-Rad). Amplification reactions were carried out in 20 μl volume using SYBR Green 1 dye and the following amplification conditions: 50 °C 2 min, 95 °C 10 min (95 °C 15 s and 60 °C 15 s) x 45 cycles. Primers were designed to specifically amplify 123 bp of hepcidin cDNA (forward 5’-CTCTGTTTTTTCCCACAACAGC-3’, reverse 5’-ATGGGAAATGGGTTGCTC-3’), 113 bp of GAPDH cDNA (forward 5’-CATGAGAATGTAGACACCC-3’, reverse 5’-AGTCCCTTCCACAGTACAAAGG-3’); 126 bp of the HPV DNA (forward 5’-CACCGGGAAAGTCTACC-3’, reverse 5’-GGTCGGTCACCTCGCATTGAT-3’); 75 bp of the HIF-1α cDNA (forward 5’-CTCTCAGTACC-3’, reverse 5’-TCCTAGAGAT-3’); 55 bp of the ICGE-α cDNA (forward 5’-TACCCCAAGTTCTAGCTAC-3’, reverse 5’-GGACTATTAGGCTCAGGT-3’); 178 bp of the FPN1 cDNA (forward 5’-AGTGTCGTCTGGTGGCAGG-3’); 187 bp of the NR2A cDNA (forward 5’-GCTGACCTGGTCTTTATGGCAGG-3’); 108 bp of the HFE cDNA (forward 5’-GTTCTGCTGCTAATCTACTGAC-3’. In all cases the fold induction was calculated with the ΔΔ Ct method.
and 73 bp of the DMT-1 cDNA (forward 5′-AATGAGTGCATT-TGCCATAAGGA-3′, reverse 5′-GATGGAACAGTATAGGAACCAA-5′); 160 bp of the OAS cDNA (forward 5′-AGAAATACCC-CAGCACAATCTCT-3′, reverse 5′-TGAGAGCCACCCCTTACCA-3′). The mRNA/cDNA abundance of each gene was calculated relative to the expression of a housekeeping gene, GAPDH (glyceraldehyde-3-phosphate-dehydrogenase).

Primers for RT-PCR of CXCL11 were described in Indraccolo et al. (2007). These primers amplify a 132 bp fragment (forward: 5′-CTTGGGCTGTATATTGTCG-3′, reverse: 5′-GGGTACATTATG-GAGGCTTTC-3′).

**In vitro transcription and electroporation of RNA.** Plasmid pSGR-JFH1/Luc and the non-replicating control pSGR-JFH1/Luc-GND were kindly provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) (Kato et al., 2005). Plasmid pFK-Luc-JFH/AE1-E2 was kindly provided by R. Bartenschlager (University of Heidelberg, Germany). The construct containing the subgenomic (JFH1) sequence was linearized with XbaI and treated with mung bean nuclease as described previously (Kato et al., 2006; Miorin et al., 2008). RNA was transcribed in vitro from linearized constructs using the MEGAscript T7 kit (Ambion). Synthesized RNA was treated with DNase I and used for electroporation.

The RNA transcribed from pSGR-JFH1/Luc was introduced into HuH7 cells by electroporation as follows. HuH7 cells were washed with PBS supplemented with salts and resuspended in 800 μl of PBS supplemented with salts. Synthesized HCV-RNA (10 μg) along with a control plasmid containing the Renilla gene under the control of the CMV promoter (details of the control plasmid are available upon request) were mixed within the cell suspension. These cells were transferred to an electroporation cuvette (0.4 cm Gene Pulser Cuvette; Bio-Rad) and pulsed at 250 mV and 960 μF with the Gene Pulser apparatus (Bio-Rad). Transfected cells were immediately transferred to 1.2 ml of culture medium and seeded into six-well culture plates.

Poly I:C (polynosinic:polycytidylic acid sodium salt, 1 μg; Invivogen) was also electroporated as described above.

**siRNA-mediated knockdown of hepcidin.** These experiments were conducted essentially as described previously (Pegoraro et al., 2006; Vardabasso et al., 2008). Briefly, HuH7 cells were seeded at 30% confluency in DMEM supplemented with 10% low endotoxin FBS without antibiotics. After 24 h, cells were transfected using Oligofectamine Reagent (Invitrogen) and 100 nM of siRNA directed against hepcidin (Hs_HAMP_3_HP siRNA; Qiagen), or control siRNA (non-silencing control, siRNA; Qiagen). Cells were harvested 24 h post-transfection. The efficiency of the knockdown was analysed at the mRNA level by quantitative real-time PCR.

**HCV-RNA electroporation with subsequent siRNA-mediated knockdown of hepcidin and luciferase assay.** HuH7 cells were electroporated (see in vitro transcription and electroporation) with pSGR-JFH1/Luc-RNA together with a control plasmid containing the Renilla gene under the control of the CMV promoter (details of the control plasmid are available upon request) (Hoenninger et al., 2008; Miorin et al., 2008). After 24 h, cells were transfected using the Oligofectamine Reagent (Invitrogen) and 100 nM of siRNA directed against hepcidin (Hs_HAMP_3_HP siRNA; Qiagen), or non-silencing control siRNA. After 24 and 48 h, the cells were lysed in passive lysis buffer (Promega Corporation) and cellular extracts were analysed for luciferase activity using the Dual-Luciferase-Reporter assay system (Promega) and a Multilabel Reader EnVision luminometer (PerkinElmer). Protein content was measured by the Bradford assay.

**Plasmid transfection.** Luciferase reporter vectors either containing the wild-type sequence (942 bp) upstream of the transcription start site of the human hepcidin promoter or the deletion mutant of the STAT-binding motif at position −64−72 were previously described in Verga Falzacappa et al. (2007). HuH7 cells were plated overnight and then transfected with the corresponding plasmids (200 ng) and the control plasmid encoding Renilla (50 ng) using lipofectamine (Invitrogen). After 4 h, IFN-α (1000 U ml−1) was added to the cells that were subsequently harvested in passive lysis buffer. The luciferase assay was conducted as described above.

**Immunoblots.** Immunoblots were performed as described previously (De Marco et al., 2010). To assess intracellular viral protein content we used a sheep polyclonal against viral NS5A generously provided by Dr Mark Harris (University of Leeds, UK). Total protein content was assessed with an antibody against β-tubulin (T6557; Sigma).

**Ferritin ELISA-test.** HuH7 cells after the indicated treatments were harvested with 100 ml lysis buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 1% Triton X-100) and the protein concentration has been measured by Bradford assay. A quantitative determination of ferritin in the protein lysate has been obtained performing a ferritin-ELISA (IBL International) according to the manufacturer’s instructions.

**Statistics.** Results are expressed as mean ± SD. Student’s t-test was used for estimation of statistical significance.

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**REFERENCES**


