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Cre-Estrogen Receptor-Mediated Hepatitis C Virus Structural Protein Expression in Mice:

HCV protein expression in mice

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Abstract

Hepatocyte apoptosis is an important feature of liver injury in hepatitis C virus (HCV) infection. However, the mechanism of apoptosis and consequences on disease progression *in vivo* have not been investigated fully in part due to the lack of adequate small animal models. In this study, transgenic (tg) mice were produced that express conditionally HCV structural proteins (core, E1, E2 and p7) in the liver following Cre-mediated DNA recombination. Using a novel Cre-estrogen receptor fusion protein (Cre-ER) induction strategy, tamoxifen was injected intraperitoneally (i.p.), which induced Cre nuclear translocation, transgene recombination and HCV protein expression in the liver. Hepatic expression of HCV core and envelope proteins resulted in increased hepatocyte apoptosis, detected by the TUNEL assay, between 7 and 33 days after induction. These results were confirmed by the presence of increased levels of apoptosis-associated cytokeratin 18 (CK18) in the sera of the same animals. The presence of cleaved caspase-3 and elevated levels of CHOP/GADD153 in the liver suggests an endoplasmic reticulum (ER) stress-associated apoptosis mechanism. This study suggests an *in vivo* correlation between HCV structural protein expression, ER stress and hepatocyte apoptosis, implicating a potentially important mechanism of HCV pathogenesis.

Keywords

viral hepatitis; transgenic mouse; animal model and liver injury

1. Introduction

The hepatitis C virus (HCV) is an emerging public health problem (Shepard et al., 2005). The mechanisms of HCV pathogenesis are complex and not understood fully. While T, NK and other immune cells in hepatitis C patients can cause liver injury (Bowen and Walker, 2005; Sun et al., 2004), HCV infection also alters hepatic gene expression, signaling pathways, lipid metabolism, cell proliferation, and oxidant production. Due to a lack of suitable small animal models that mimic human hepatitis C, however, a direct pathogenic role of HCV proteins has been difficult to establish. Several groups have generated conditional HCV transgenic (tg) mice, and induced these animals to express HCV proteins in the liver (Sun et al., 2005; Wakita et al., 1998). While a non-viral induction approach generated high levels of protein expression

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initially, expression declined gradually over the next few weeks (Sun et al., 2005), hampering studies on the chronic effects of HCV proteins.

Previously, apoptosis was considered to be a natural mechanism of cell removal without pathogenic consequences to the tissue; however, insufficient or excessive apoptosis is now known to cause tissue injury (Los et al., 1999; Takehara et al., 2004). In HCV infection, hepatocyte apoptosis is emerging as an important feature of liver injury (Bantel and Schulze-Osthoff, 2003; Calabrese et al., 2000; Patel et al., 1998; Rodrigues et al., 2000; Walsh et al., 2004). The ensuing responses of cell repair, regeneration and fibrosis may be triggered by this process (Canbay et al., 2004; Takehara et al., 2004). Many studies with a variety of cell culture systems have suggested that HCV core protein can either protect transfected cells from or sensitize them to apoptotic cell death (Balasubramanian et al., 2005). An array of agents has been used to induce apoptosis, including anti-Fas antibody (Ab) (Hahn et al., 2000; Marusawa et al., 1999), tumor necrosis factor- α (TNF- α), TRAIL (Chou et al., 2005), exogenous peroxides (Wen et al., 2004), or cisplatin and c-myc. In animals, some tg mice expressing HCV core displayed an increased apoptosis in the liver (Benali-Furet et al., 2005; Iken et al., 2006; Yang et al., 2002), while in others, the presence of the HCV proteins suppresses hepatocellular apoptosis (Kamegaya et al., 2005; Keasler et al., 2006; Machida et al., 2001). It has been speculated that various conditions being used in these experiments may have played a part in and/or contributed to those discrepancies. They include the tumorigenic origin of the cell lines for *in vitro* assays, beyond-physiologic conditions associated with apoptotic stimuli usage, and/or high levels of HCV protein expression in mice beginning *in utero*.

While the clinical implication of these studies remains unclear, a scenario has emerged in the past few years, suggesting that the HCV core can inhibit mitochondrial electron transport and increase reactive oxygen species (ROS) production (Korenaga et al., 2005; Okuda et al., 2002; Otani et al., 2005). Since HCV replicates from a ribonucleoprotein complex associated closely with the endoplasmic reticulum (ER) membrane (Lindenbach and Rice, 2005), HCV proteins and viral replication also have been suggested to induce ER stress and the unfolded protein response (UPR), resulting in cell injury and apoptosis (Benali-Furet et al., 2005; Liberman et al., 1999; Pavio et al., 2003; Tardif et al., 2002). In this study, a novel HCV tg mouse model was developed, which induced the persistent expression of HCV structural proteins in the liver. Under conditions mimicking more closely those in patients, HCV structural proteins were evaluated and shown to cause ER stress, hepatocyte injury and apoptosis *in vivo*.

2. Materials and Methods

2.1. Tg mice

HCV tg mice expressing conditionally the HCV structural genes encoding core, E1, and E2 of genotype 1b under the control of the murine albumin promoter-enhancer (SL24) have been described previously (Sun et al., 2005). The Cre-ER tg mice (Jackson Laboratories, Bar Harbor, ME) were constructed to express Cre DNA recombinase as a fusion protein between Cre and a mutated form of the ligand binding domain of the murine estrogen receptor (Hayashi and McMahon, 2002), which can be activated upon binding to an estrogen analogue, tamoxifen, or its metabolic derivative, 4-hydroxytamoxifen. The activated Cre-ER translocates to the nucleus to mediate DNA recombination, between the *loxP* sequences. To generate double tg mice, SL24 and Cre-ER mice were crossed. Offspring were genotyped by PCR with the tail DNA for HCV structural genes by using the primer pair, P1 and P3 (Sun et al., 2005) and for the Cre gene by using the primer pair (5'-ACGTTACACGGCATCAA CGT-3' and 5'-CTGCATTACCGGTCGATGCA-3'). Animals carrying a double or single (control) transgene were used in all the experiments reported in this study. All mice were housed in a specific pathogen-free facility with approval of the Institutional Animal Care and Use Committee.

2.2. Animal experiments

Tamoxifen (Sigma) was dissolved in warm sterilized corn oil (Sigma) at a concentration of 10 mg/ml, and placed in a water bath with occasional shaking. 0.1 mg of tamoxifen/g of body weight was injected intraperitoneally for three consecutive days. The animals were killed by CO₂ asphyxiation. The liver, spleen, heart, lungs and kidneys were harvested for analyses of HCV transgene recombination and protein expression at days 7, 14, 21, 33, and 45 from the first injection. The blood was collected for measurement of serum alanine aminotransferase (ALT), and the tissues were snap-frozen in liquid nitrogen for DNA and protein extraction or were embedded in the OCT compound (Tissue-Tek, Torrance, CA) for cryosection. Additional tissues were fixed in 10% buffered-formalin and embedded in paraffin for routine histological examination.

2.3. Determination of Cre-mediated DNA recombination

Total genomic DNA from the mouse organs was extracted using a GenElute Mammalian Genomic DNA Miniprep kit (Sigma, St. Louis, MO). Conventional PCR was undertaken to detect Cre-mediated recombination of HCV transgene as described previously (Sun et al., 2005). Copy numbers of the recombined transgene in the liver were assessed by real-time quantitative PCR on a LightCycler using a QuantiTect SYBR Green PCR kit (Qiagen, Inc., Valencia, CA). A standard curve was generated for the recombined transgene with pALB.HCV-S (Lerat et al., 2002), which was quantified with a Quant-iT PicoGreen dsDNA kit (Qiagen). The amplification reactions were carried out with 5- μ l sample aliquots in a 20- μ l final volume. Reaction cycles included denaturation at 95°C for 15 min, followed by cycling of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The standard curve of threshold cycles versus the template abundance was linear over 6 orders of magnitude, with a regression coefficient typically greater than 0.98. All data were combined from three independent experiments.

2.4. Western blotting and immunohistochemistry

Proteins were extracted from frozen liver tissues by homogenization, and soluble proteins (100 μ g) underwent electrophoresis in a precast, 4- to 12% gradient polyacrylamide gel (Invitrogen, San Diego, CA). Proteins were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and probed with a mouse monoclonal antibody (mAb) to HCV core (Anogen, Mississauga, Canada); E2 (clone Ap33) (Owsianka et al., 2005); or rabbit polyclonal antibodies to caspase-3 (Cell Signaling Technology, Beverly, MA), GRP78 or CHOP/GADD153 (Santa Cruz Biotechnology, San Diego, CA), followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL), and ECL detection reagents (Amersham Pharmacia, Buckinghamshire, England). Expression levels were evaluated by quantification of the relative density of each band normalized to β -actin. Liver tissue sections were prepared from OCT- or paraffin-embedded samples for immunohistochemistry. Cryosections were incubated in 5% normal goat serum in PBS/0.01% Triton X-100 (PBST), and then in anti-Cre Ab (BabCO, Richmond, CA) diluted 1:1000 in PBST containing 1% normal goat serum at room temperature for 1.5 hr. After four washes in PBST and once in PBS, slides were incubated for 1 hr in a 1:400 dilution of goat anti-rabbit IgG-FITC at room temperature. After 3 washes in PBST, sections were mounted in Vectashield mounting medium with DAPI (Vector, Burlingame, CA). Cells were imaged using a Zeiss LSM 510 confocal microscope and LSM Image 3.5 software.

2.5. TUNEL assay for detection of apoptosis *in situ*

The terminal deoxynucleotidyl transferase-mediated, dUTP-nicked end labeling (TUNEL) assay was carried out on cryosections after fixation in 1% paraformaldehyde and treatment

with proteinase K (20 mg/ml). After blocking endogenous peroxidase by immersion in 3% H₂O₂ for 5 min, we incubated the slides with a buffer solution containing terminal deoxynucleotide transferase and digoxigen-labeled deoxynucleotide triphosphates (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Intergen Company, Purchase, N.Y) in a humidified chamber at 37°C for 1 hr. An anti-digoxigenin peroxidase conjugate was added for 30 min, followed by 3,3'-diaminobenzidine tetrahydrochloride as a chromogen and counterstaining with methyl green. The total number of apoptotic cells in 20 randomly selected microscopic fields (×40) was counted blindly with respect to the tg status of the animal. Sections from three animals per group were examined by three individuals in a double-blinded fashion.

2.6. Serological measurement of caspase activity

Hepatic caspase activity was determined in serum by the quantitative measurement of the apoptosis-associated neopeptide in the C-terminal domain of cytokeratin-18 (CK-18) using the M30-Apoptosense ELISA kit (Peviva; Alexis, Grünwald, Germany) (Bantel et al., 2004), according to the manufacturer's instructions.

2.7. Statistical analysis

Statistical analyses were performed using a two-tailed, unpaired Student *t* test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Tamoxifen induction of HCV structural proteins in tg mice

This study employed a novel tamoxifen induction method in double tg mice carrying the conditional HCV-structural (SL24) (Sun et al., 2005) and the Cre-estrogen receptor (Cre-ER) transgenes (Hayashi and McMahon, 2002) (Fig. 1). In a non-induced state, the fusion protein Cre-ER is sequestered in the cytoplasm and, due to a mutation, unresponsive to endogenous estrogen. Binding of tamoxifen, an estrogen analog, to Cre-ER releases a molecular chaperone, resulting in nuclear translocation of Cre and HCV transgene recombination. SL24 and Cre-ER double tg mice, and control single or non-tg animals were i.p. injected with 0.2 mg/g tamoxifen, and genomic DNA from the spleen, kidneys, liver, lungs, and heart were analyzed by PCR. A 1.7-kb PCR fragment indicated the presence of the unrecombined transgene, and a 0.4-kb fragment suggested its recombination (Fig. 2A) (Sun et al., 2005). A single injection of tamoxifen was sufficient to induce a widespread recombination of HCV transgene in double tg mice, but not in the single HCV tg animals.

To induce sustained, high-level HCV expression, 10 groups of mice (3–5 animals/each group) were treated with three consecutive daily injections of tamoxifen (0.1 mg/g). As detected by PCR, multiple tamoxifen injections resulted in more efficient transgene recombination, as compared to a single tamoxifen injection (Fig. 2B) or hydrodynamic injection of naked Cre plasmid (Sun et al., 2005). Using highly sensitive, real-time quantitative PCR, we found that the recombined transgene copy number reached an average of 7.5×10^5 copies/ 10^5 hepatocytes at day 7 post-induction, and was sustained at this level through day 45 post-induction (Fig. 2C). Consistent with transgene recombination, HCV core protein expression was induced in the liver, but not in the spleen, kidney, lungs, and heart (Fig. 3A). Expression of core and E2 proteins persisted at 7, 21, 33 and 45 days post-transgene induction (Fig. 3B), which was much longer than that achieved previously through adenoviral and hydrodynamic inductions (approximately 14 days) (Sun et al., 2005; Wakita et al., 1998). Following a schedule of bimonthly tamoxifen injections, two Cre-ER single and seven SL24×Cre-ER double tg mice were injected i.p. with tamoxifen (0.1mg/g×2) at months 0, 2 and 4. When the animals were killed at month 5 (day 156), moderate-to-high levels of HCV core were detected in the livers

of the double tg mice (Fig. 3C). Consistent with previous finding (Sun et al., 2005), the extremely persistent expression of HCV structural proteins in the liver was not accompanied by spontaneous inflammation, steatosis and other histopathological abnormalities or serum ALT elevation (data not shown).

Immunohistochemical staining confirmed that, in double tg mice, Cre began to translocate from the cytoplasm to the nuclei at 3 days post-tamoxifen injection (Fig. 4). Significantly more nuclei were stained positively for Cre at days 5 and 7 post-induction, and it later disappeared from the nuclei via an as-yet-unidentified mechanism, at days 14 and 21 post-induction. In a previous study, constitutive version of the SL24 transgene resulted in cytoplasmic staining for core and E2 predominantly in pericentral hepatocytes (Lerat et al., 2002). Given the limitations associated commonly with the current constitutive and inducible tg animals, for example, immunotolerance due to premature HCV expression and inflammatory responses against adenoviral vector (Lerat et al., 2002; Sun et al., 2005; Wakita et al., 1998), this novel HCV mouse model offers several obvious advantages, including controlling temporally HCV expression without viral vector- or hydrodynamics-related complications in animals. It also provides a safer and more reliable induction technique and, more importantly, multiple deliveries of tamoxifen to ensure sustained, higher level protein expression *in vivo*.

3.2. HCV structural proteins induce apoptosis in the liver

Hydrodynamic injection was followed invariably by a mild and transient ALT elevation in animals (Liu et al., 1999; Sun et al., 2005). In this study, the effect of the hydrodynamic injection of pNLS-Cre and tamoxifen induction on hepatic apoptosis was compared. The use of the TUNEL assay revealed that the hydrodynamic injection of 50 µg pNLS-Cre triggered a burst of apoptosis in the liver at day 3, followed by a sharp decline and return to the baseline by 14 and 45 days post-injection (Fig. 5A). This result is consistent with earlier observations (Liu et al., 1999; Sun et al., 2005) and raises the possibility that this wave of apoptosis in mouse liver contributes to the progressive decline in transgene expression following hydrodynamic injection.

HCV core and envelope proteins have been shown to have various effects upon cells, depending on cell type and tissue culture conditions used. To test directly the effect of HCV structural proteins without having to use an apoptotic agent, we injected tamoxifen i.p. to double tg (SL24×Cre-ER) mice and control single tg animals (SL24 and Cre-ER), as described in Fig. 3, and assessed apoptosis in the liver with the TUNEL assay (Fig. 5B). Positively stained cells were easily detectable in both groups of mice, and the excellent contrast allowed accurate enumeration of apoptotic cells. In double tg mice expressing HCV core and envelope proteins, increased apoptosis was seen as early as 7 days post-induction, and it was significantly higher than that in single SL24 and Cre-ER tg ($P<0.05$; Fig. 5B and data not shown). The number of apoptotic cells from double tg animals continued to climb at day 14, peaked at day 21, and began to decline steadily thereafter. The increased apoptosis in double tg mice over the control single transgenic animals was statistically significant for days 14 ($P<0.05$) and 21 ($P<0.01$), but not for days 33 and 45 (both $P>0.05$) (Fig. 5B). It was considered that the hepatic apoptosis following transgene induction was neither due to nuclear translocation of Cre (Fig. 5B) nor attributable to injection of tamoxifen or its vehicle corn oil, since the control SL24 or Cre-ER single tg animals did not exhibit a comparable level of apoptosis. In additional experiments, normal mice of both sexes were injected i.p. with corn oil alone, or tamoxifen at 0.1 or 0.2 mg/g for 3 consecutive days, and sacrificed 10, 20 and 40 days later. Consistent with previous results with SL24 mice (Sun et al., 2005), these animals displayed no serum ALT elevation or histopathological abnormalities in the liver (data not shown).

To provide an independent measure of apoptosis in the liver, the serum cytokeratin-18 (CK-18) levels were determined in these animals. CK-18 is the major intermediate filament protein of

liver cells and is important for hepatocyte integrity. During apoptosis, CK-18 is cleaved by caspases, which expose a new epitope that can be recognized by a mAb, and is a biomarker for hepatitis C activity in clinical practice (Bantel et al., 2004; Feldstein and Gores, 2004; Seidel et al., 2005). Using a commercial ELISA kit, it was found that serum CK-18 values in tamoxifen-treated double tg mice were slightly higher than those in control animals (Fig. 5C), though not statistically significant ($P>0.05$).

Apoptosis is controlled by a family of intracellular cysteine proteases, called caspases, which cleave several cellular substrates (Los et al., 1999). Caspases are synthesized as inactive proenzymes and are processed proteolytically to active forms. Using Western blotting with a mAb that recognized both pro- and active forms, the activation of caspase-3, a master executioner, were examined in mouse liver extracts. An elevated level of cleaved caspase-3 (19 kDa) was detected in the liver samples of tamoxifen-induced double tg mice, but not in those of SL24 or Cre-ER single tg controls (Fig. 6A), which were incapable of expressing HCV proteins upon induction. These results demonstrate that hepatic expression of HCV core and envelope proteins contributes to apoptosis in the liver.

HCV structural proteins are processed and mature in the ER (Lindenbach and Rice, 2005). When the ER is stressed by, for example, virus, calcium depletion or accumulation of misfolded proteins, it activates the unfolded protein response and triggers a cascade of events (Lieberman et al., 1999; Tardif et al., 2002). Taking advantage of this HCV animal model, CHOP/GADD153, an ER stress marker and pro-apoptotic modulator was examined (Endo et al., 2005; Ji et al., 2005; Oyadomari and Mori, 2004). Mice expressing HCV core and envelope proteins displayed a 1.3- to 2.4-fold increase in the CHOP expression level in the liver, as compared with that in the SL24 controls (Fig. 6B). The levels of ER-associated proteins GRP78 and GRP94 were also elevated, though less so, in these animals 21 days post-tamoxifen induction (data not shown). Collectively, these results suggest that HCV structural proteins induce ER stress, leading to hepatocyte apoptosis *in vivo*.

4. Discussion

Understanding the involvement of HCV proteins and the mechanisms of host responses in liver injury and disease progression is an important goal for HCV research. Due to a lack of appropriate animal models, however, a direct pathogenic role of HCV proteins *in vivo* has been difficult to establish. In the present study, SL24 and Cre-ER double tg mice were established, and the expression of HCV structural proteins was induced in a fashion that was comparable to that in hepatitis C patients (Figs. 2 and 3)(Korenaga et al., 2005). The unique advantages of this murine system allowed us to assess the apoptotic effect of HCV core and envelope proteins *in vivo* without the influence of noticeable adverse effects frequently associated with viral and hydrodynamic induction methods (Liu et al., 1999; Wakita et al., 1998) or confounding factors such as pro-apoptotic agents and inflammatory cells (Kamegaya et al., 2005; Machida et al., 2001; Yang et al., 2002). Our data with the TUNEL, caspase-3 and CK-18 assays demonstrated that HCV structural proteins can induce low-grade, but not colossal, apoptosis in the livers of normal, non-tumorigenic animals (Figs. 5 and 6). These results were consistent with a clinical observation, in which up to 0.54% hepatocytes in chronic hepatitis C liver biopsies were found to be TUNEL-positive (Calabrese et al., 2000). Furthermore, the cell deaths observed in these animals were not readily detectable by the routine serum ALT test, a finding reminiscent of that seen in a subset of patients who have active HCV disease, as assessed by HCV RNA levels and liver histopathology, but who also exhibit persistently normal serum ALT values (Calabrese et al., 2000; Rodrigues et al., 2000; Shiffman et al., 2006).

Several mechanisms have been reported to be responsible for HCV-associated apoptosis, including immune-mediated cell destruction by activated NK, NKT, cytotoxic T cells and their

roinflammatory cytokines (Balasubramanian et al., 2005; Gremion et al., 2004). HCV protein expression in the absence of inflammation can also increase mitochondrial ROS production and lipid peroxidation, deplete mitochondrial glutathione, and sensitize cells to oxidant-induced cell injury (Choi and Ou, 2006; Otani et al., 2005; Wen et al., 2004); (Korenaga et al., 2005; Moriya et al., 1998; Okuda et al., 2002).

HCV structural proteins are processed and mature in close association with the ER (Lindenbach and Rice, 2005; Xu et al., 2003). When the ER is stressed by virus, calcium depletion or accumulation of misfolded proteins, it activates the unfolded protein response and triggers alterations in calcium homeostasis and secondary mitochondrial effects (Benali-Furet et al., 2005; Liberman et al., 1999; Pavio et al., 2003; Tardif et al., 2002; Tardif et al., 2005); (Gong et al., 2001). Severe prolonged ER stress can lead to apoptosis through over-expression of CHOP/GADD153 pro-apoptotic modulator and activation of the c-JUN kinase (Ji et al., 2005; Oyadomari and Mori, 2004). Since the HCV tg mice developed apoptosis shortly after induction without overt inflammatory responses in the liver, these results raised the possibility that HCV-induced ER stress contributes to hepatocyte apoptosis (Fig. 6). In summary, tg mice were established that conditionally express HCV structural proteins in the liver through a novel Cre-ER induction strategy. The transgene expression controlled temporally in the liver was robust and persistent, and affords a detailed investigation of the effects of HCV core and envelope proteins and host cell interaction under physiological conditions. This study demonstrates a correlation between HCV structural protein expression, and ER stress and hepatocyte apoptosis *in vivo*, suggesting a potentially important mechanism of HCV pathogenesis.

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Abbreviations

ALT	alanine aminotransferase
CK-18	cytokeratin 18
Cre-ER	Cre-estrogen receptor fusion protein
ER	endoplasmic reticulum
HCV	hepatitis C virus
mAb	monoclonal antibody
NS	HCV non-structural
ROS	reactive oxygen species
S	structural
tg	transgenic

Figure 1. Schematic representation of the tamoxifen-inducible HCV tg mouse

The conditional HCV-S tg mice (SL24) were bred to tg mice expressing the Cre DNA recombinase fused to a mutated murine estrogen receptor (Cre-ER) (Hayashi and McMahon, 2002; Sun et al., 2005). Upon binding to inducing agent tamoxifen, Cre-ER translocated to the nucleus and mediated DNA recombination between the two loxP sequences in SL24×Cre-ER double tg mice. The recombination event between the albumin promoter and HCV-S ORF was detectable by the presence of a 400-bp band amplified by PCR with primers P1 and P3 (Sun et al., 2005).

Figure 2. Tamoxifen injection induces persistent transgene recombination in SL24×Cre-ER mouse
 SL24 single and SL24×Cre-ER double tg mice were injected with tamoxifen (0.2 mg/g body weight) and killed at day 7 post-injection. DNA was extracted from organs and analyzed by PCR with primers P1 and P3 (Fig. 1) for detection of transgene recombination as indicated by the presence of 400-bp product. (A) Widespread tg recombination following a single injection of tamoxifen in double tg mice. (B) Efficiency of recombination induced by single (0.2 mg/g) and triple (0.1 mg/g×3) injections of tamoxifen in comparison with hydrodynamic delivery of plasmid DNA encoding Cre (Sun et al., 2005). Shown in A and B are results for representative tg animals 7 days after hydrodynamic and tamoxifen injection. (C) Recombination efficiency of the transgene was confirmed by highly sensitive, real-time quantitative PCR (see Materials

and Methods). Copy numbers of recombined transgenes were measured by using a genomic DNA isolated from the liver at indicated times after triple injection with 0.1 mg/g of tamoxifen. The purified transgene construct was used as the copy number control.

Figure 3. Liver-specific and persistent expression of HCV proteins

(A) Organs were harvested at day 7 after triple injection with tamoxifen (0.1 mg/g×3). Protein (100 µg) was extracted and loaded in each lane. Proteins resolved electrophoretically were blotted on a polyvinylidene difluoride membrane and probed with HCV core-specific mAb. (B) Expression of HCV core and E2 protein was sustained for at least 45 days post-induction, the endpoint of the experiments. A constitutive HCV tg mouse (lineage 139) and conditional SL24 single tg mouse were used as positive and negative controls, respectively. β-actin was used as a loading control. (C) Long-term HCV core expression following repeated tamoxifen injection. Cre-ER single and SL24×Cre-ER double tg mice were injected with tamoxifen

(0.1mg/g×2) i.p at months 0, 2 and 4, and the animals were sacrificed at month 5 (day 156). Shown are results for representative tg animals in each group.

Figure 4. Nuclear translocation of Cre DNA recombinase following tamoxifen injection
Shown are representative confocal micrographs of liver sections stained for Cre DNA recombinase. Tg- and SL24xCre-ER double tg mice were injected i.p with tamoxifen, and sacrificed on days 3, 5, 7, 14 and 21 as indicated. Cryosections of the liver were stained with a rabbit anti-Cre Ab, followed by FITC-conjugated anti-rabbit IgG. Nuclei were counterstained with DAPI (40 $\times$ objectives).

Figure 5. Hepatocyte apoptosis following HCV transgene induction

(A) Apoptosis was detected with the TUNEL assay in the livers of HCV tg mice (SL24) that received 50 mg pNLS-Cre hydrodynamically (Sun et al., 2005). (B) Apoptotic cells were found on liver cryosections of SL24×Cre-ER double tg mice and their single tg controls following an i.p. tamoxifen injection. Shown on the left panel are the results for representative animals in the control (SL24) and double tg groups. The right panel illustrates the average numbers of apoptotic cells per field (40× objectives) counted in 20 randomly selected fields. Both SL24 (○) and Cre-ER (□) single tg mice, depicted separately, were used as controls due to their lack of HCV protein expression upon tamoxifen induction. Three animals per group and time point were analyzed by three individuals in a double-blinded fashion. Error bars indicate ±SEM. (C)

Serum CK 18 from the control (SL24) and double tg animals was assessed with a commercial ELISA kit per the manufacturer's instructions (see Materials and Methods).

Figure 6. Hepatic expression of HCV proteins induces ER stress

Western blot analysis was performed on liver extracts from SL24×Cre-ER double tg mice in comparison with single SL24 and Cre-ER tg mice following i.p. tamoxifen injection. (A) Activation of caspase-3. (B) Increased expression of pro-apoptotic ER modulator CHOP/GADD153. Expression levels of CHOP/GADD153 were expressed as relative intensity and normalized to their respective β-actin band.