Huh7 cells expressing hepatitis C virus Core or NS3/4A protein activate human LX-2 hepatic stellate cells through paracrine signaling: preliminary results

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Abstract

Chronic hepatitis C virus (HCV) infection is associated with development of hepatic fibrosis. HCV predominantly infects hepatocytes and the expression of HCV proteins in these cells induces oxidative stress. During liver injury hepatic stellate cells (HSCs) undergo a well-characterized activation process and are the major fibrogenic cells contributing to the excessive deposition of extracellular components leading to fibrosis. Although the activation of HSCs has been extensively studied, knowledge on the role of HCV protein expressing hepatocytes on HSC activation is scarce. The aim of this study is to establish an in vitro cell-cell interaction model to mimic conditions observed during HCV infection. LX-2 cells were used to represent human HSCs. LX-2 cells were either co-cultured with Huh7 cells that express HCV Core or NS3/4A protein or exposed to conditioned medium of these HCV protein-expressing Huh7 cells. Moreover, Huh7 cells were exposed to menadione to test whether exogenous oxidative stress to hepatocytes affects activation and/or stress response of HSCs.

mRNA levels of HSC activation markers COL1A1 and ACTA2 were increased in LX-2 cells when co-cultured with either Core- or NS3/4A-expressing Huh7 cells. In contrast, conditioned media of Core-expressing Huh7 cells increased TGF-1 mRNA levels in LX-2 cells, but not COL1A1 and ACTA2. HCV-protein expressing Huh7 cells did not cause oxidative stress or endoplasmic stress in LX-2 cells in co-culture conditions. Oxidative stress induction did not affect HSC activation or expression of enzymatic antioxidant markers as HMOX-1, SOD1, SOD2, and CAT neither cellular stress markers as HSPA5, ATF4, DDIT3 and sXBP1.

Our preliminary results demonstrate that transient expression of HCV Core or NS3/4A leads to the secretion of pro-fibrogenic factors by Huh7 cells expressing viral proteins, potentially revealing a pro-fibrotic paracrine interaction between HCV-infected hepatocytes and stellate cells in HCV patients.
Introduction

An estimated 80 million individuals worldwide are chronically infected with HCV and at risk to develop liver fibrosis, cirrhosis and, eventually, hepatocellular carcinoma (HCC) (1). It has been suggested that progression to chronic hepatitis C occurs in 55 to 85% of infected individuals while less that 20% resolve the infection (2,3).

Hepatic fibrosis is characterized by excessive deposition of extracellular matrix (ECM) components, like collagen type I and fibronectin in conjunction with decreased degradation of ECM through matrix metalloproteinases (MMP). Type I collagen is composed of two collagen type I alpha-1 chains (hereafter referred to as COL1A1) and one collagen type I alpha 2 chain (COL1A2), encoded by the COL1A1 and COL1A2 genes, respectively. The hepatic stellate cells (HSCs) are considered the most prominent fibrogenic cell type in the liver (4,5). HSCs undergo a well-characterized transdifferentiation and activation process from quiescent, non-proliferating vitamin A-storing cells in the healthy liver to highly proliferative, contractile, matrix-producing myofibroblasts in response to chronic liver inflammation that causes fibrosis (5).

HCV primarily infects hepatocytes, although evidence of extra-hepatocytic replication in several types of immune cells has also been reported (6–9). Human HSCs are not infected by HCV, despite the expression of the Cluster of Differentiation 81 (CD81), one of the surface molecules involved in HCV entry into hepatocytes (10). Thus, transdifferentiation and activation of HSCs in chronic HCV infection is the result of indirect effects and inflammatory cells are likely to mediate these indirect effects on HSCs via the secretion of pro-inflammatory and pro-fibrogenic factors (11).

However, HCV-infected hepatocytes, producing viral proteins, may also release signals that activate HSCs. HCV replication and expression of viral proteins, such as Core, NS3/4A and NS5A, in hepatocytes induce oxidative stress and endoplasmic reticulum (ER) stress (12). In addition, HCV can modulate lipid metabolism and signal transduction pathways (13–16). Thus, HCV-infected hepatocytes undergo phenotypic changes that may change their interaction with other hepatic cell types like HSCs. Schuzel-Krebs et al. determined the profibrogenic potential of conditioned media of Huh7 cells, stably expressing non-structural viral proteins (NS3-NS5B) on the activation of HSCs. They found that these cells release factors that modulate the...
expression of fibrogenic genes in HSCs, including increased expression of transforming growth factor beta 1 (TGF-β1), COL1A1 and Collagen Type III Alpha 1 Chain (COL3A1), in conjunction with decreased expression of fibrolytic matrix metalloproteinases (17). Recently, we developed an in vitro model using several sources of damage (Chapter 3 and 4), in which HCV protein-expressing (first injury) cells are additionally exposed to external oxidative stress (second injury) to mimic chronic HCV infection in vivo, in which HCV-infected hepatocytes are also exposed to oxidative stress elicited by inflammatory cells.

The aim of this pilot study is to establish an in vitro cell-cell interaction culture model to investigate the interaction between Huh7 cells expressing HCV proteins Core or NS3/4A and HSCs. Using this cell-cell interaction model, we investigated paracrine signaling between HCV protein-expressing Huh7 cells and HSCs. Moreover, we also investigated the effect of additional oxidative stress in Huh7 cells on HSC activation.

**Material and methods**

**Cell culture and treatments**

The human hepatoma cell line Huh7 (National Institutes of Biomedical Innovation, JCRB Cell Bank, Japan) and human LX-2 cells (kind gift from dr. S. Friedman) were maintained in Dulbecco’s modified Eagle medium (1X) + GlutaMAX™-I (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (100 IU/mL penicillin and 100 μg/mL streptomycin) (Gibco) in the presence of 5% CO₂ at 37°C. Recombinant human TGF-β1 (2.5 ng/mL) (Sigma-Aldrich, T-5050) was used in some experiments to induce activation of LX-2 cells. Two independent experiments in duplicate were conducted. The results are expressed as the mean of two independent experiments.

**Transfection of Huh7 cells**

Huh7 cells (1.8x10⁵) were seeded in 6-well plates and transfected after 24 hours (h) at a confluency of 70%. Lipofectamine™ 3000 (Invitrogen) and the expression vectors pTracer™-EF/V5-His (Invitrogen) (Huh7-Empty), pTracerCore (Huh7-Core) and pTracerNS3/4A (Huh7-NS3/4A) (Chapter 3) were used separately at a ratio of 4
µL:1 µg (Lipofectamine 3000: plasmid vector). Lipofectamine 3000 and the plasmids were prepared in OPTI-MEM™ I (1X) reduced serum medium (Gibco) following the manufacturer’s instructions. Six hours post-transfection (hpt), the plasmid DNA-Lipofectamine complexes were removed and medium was refreshed. The expression of green fluorescent protein (GFP) under the control of the human cytomegalovirus immediate-early promoter was used to determine the transfection efficiency 24 hpt. The expression of recombinant HCV Core and NS3/4A was under the control of the human Elongation Factor 1α (hEF-1α) promoter.

Transwell co-culture of transfected Huh7 cells with LX-2 cells

LX-2 cells (1x10^3) were seeded in 6-well plates in DMEM without FBS and antibiotics, 24 h prior to co-culture with transfected Huh7 cells. 24 hpt, Huh7-Empty, Huh7-Core, Huh7-NS3/4A and Huh7-NT cells were harvested using trypsin 1X (Gibco) and the viability of the cells was determined by trypan blue staining. Huh7 cells (1.5x10^5) were placed into the 6-well format cell culture insert with 0.4 µm pore PET track-etched membrane (Falcon). Huh7 cells and LX-2 cells were co-cultured for 24 h and 48 h, after which LX-2 cells were harvested for analysis. In some co-culture experiments, Huh7 cells seeded in the upper insert were treated with the superoxide anion donor menadione (50 µmol/L) to induce oxidative stress 6 h prior to harvesting HSCs. In some experiments, the anti-oxidant N-Acetyl-L-cysteine (NAC, 5 mmol/L, Sigma) was added to Huh7 cells 30 min before menadione treatment. The experiments were conducted twice with two biological repetitions. The results are expressed as the mean of two independent experiments.

Preparation of conditioned media and LX-2 treatment

Twenty-four hpt, Huh7-Empty, Huh7-Core, Huh7-NS3/4A and Huh7-NT (non-transfected) cells were washed 3 times with 1X Hank’s Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+}, followed by culture in DMEM without FCS or antibiotics for an additional 24 h. Then, medium was harvested, centrifuged at 1,000 rpm for 10 minutes to remove cell debris and used immediately for the treatment of LX-2 cells. Two independent experiments in duplicate were performed and the results are expressed as a mean.
RNA isolation and RT-qPCR

LX-2 cells were harvested on ice and washed three times with ice-cold 1X HBSS. Total RNA was isolated with TRI-reagent according to the manufacturer’s instructions (Sigma). Reverse transcription (RT) was performed using 2.5 µg of total RNA, 1X RT buffer (500 mmol/L Tris-HCl, pH 8.3; 500 mmol/L KCl; 30 mmol/L MgCl₂; 50 mmol/L DTT), 1 mmol/L deoxynucleotides triphosphate (dNTPs, Sigma), 10 ng/µL random nanomers (Sigma), 0.6 U/µL RNaseOUT™ (Invitrogen) and 4 U/µL M-MLV reverse transcriptase (Invitrogen) in a final volume of 50 µL. Reverse transcription program was 25°C/10 min, 37°C/60 min and 95°C/5 min. Complementary DNA (cDNA) was diluted 20X in nuclease-free water. Real-Time qPCR was carried out in a StepOnePlus™ (96-well) PCR System (Applied Biosystems, ThermoFisher) using TaqMan probes. The sequences of the probes and primers are described in Supplementary Table 1. For qPCR, 2X reaction buffer (dNTPs, HotGoldStar DNA polymerase, 5 mmol/L MgCl₂) (Eurogentech, the Netherlands), 5 µmol/L fluorogenic probe and 50 µmol/L of sense and antisense primers (Invitrogen) were used. mRNA levels were normalized to 18S and further normalized to the mean expression level of the control group.

Mitochondrial superoxide production determination

The fluorogenic probe MitoSOX™ Red reagent (Invitrogen) was used to quantify mitochondrial production of superoxide anions in Huh7 cells according to the manufacturer’s instructions. MitoSOX™ reagent working solution was added to the cells at final concentration of 5 mmol/L. After 10 min, media was removed and cells were washed three times with 1X HBSS Ca²⁺ Mg²⁺ (Gibco) and harvested for flow cytometry analysis using a 488 nm laser. Three independent experiments were carried out and the results are expressed as a mean.

Immunofluorescence microscopy

Huh7 cells (9.0x10⁴) were grown on glass cover slips placed in 12-well plates. After 24 h, attached cells were transfected according to the protocol described above. 24 hpt media were removed and cover slips were carefully washed three times with 1X HBSS Ca²⁺ Mg²⁺ (Gibco). Then, cells were fixed using a 4% paraformaldehyde solution in 1X HBSS Ca²⁺ Mg²⁺ (Gibco) for 10 min at room temperature and washed 3 times...
with 1X HBSS-10% FBS solution. Permeabilization was performed by incubation of the samples for 10 min in 1X HBSS containing 0.1% Triton X-100 (Sigma). 1% Bovine serum albumin (BSA, Sigma) in 1X HBSS + 0.1% Tween 20 (Sigma) solution was used to block non-specific binding of the antibodies for 30 min. Monoclonal antibodies against HCV Core (Clone B12-F8, kindly provided by prof. Dr. Mondelli (18)) and HCV NS3/4A (Clone 8 G2, (Abcam)) were used at a dilution of 1:1,000 in 1% BSA/1X HBSS in a humified chamber for 1 h at room temperature. Then, samples were washed three times with 1% BSA in 1X HBSS solution. Finally, cells were incubated with goat anti-mouse Alexa Fluor® 568 in 1% BSA/1X HBSS for 1 h at room temperature in the dark. Slides were evaluated using fluorescence microscopy and analyzed by Leica ALS AF Software (Leica).

**Western blotting**

Cells were scraped on ice and lysed by three cycles of freezing in liquid nitrogen and thawing at 37°C in lysis buffer (25 mmol/L HEPES, 150 mmol/L KAc, 2 mmol/L EDTA, 0.1% NP-40) supplemented with protease and phosphatase inhibitors (10 mmol/L NaF, 50 mmol/L PMSF, 1 µg/µL of α-protenin/pepstatin/leupeptin and 1 mmol/L DTT) followed by centrifugation for 10 minutes at 12,000 rpm. Cell lysates were resolved on Mini-PROTEAN® TGX Stain-Free™ Precast Gels (BioRad). Semi-dry blotting was performed using Trans-Blot Turbo Midi Nitrocellulose Membrane with Trans-Blot Turbo System Transfer (BioRad). Ponceau S 0.1% w/v (Sigma) staining was used to confirm protein transfer. The monoclonal antibodies against HCV Core and NS3/4A were used at a dilution of 1:1,000 and mouse anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Calbiochem) at a dilution of 1:10,000.

Monoclonal antibodies against Alpha-Smooth Muscle Actin (α-SMA) (Sigma-Aldrich) and Col1A1 (SouthernBiotech, USA) were used at 1:1,000 dilution. The primary antibodies were incubated overnight (16 h) at 4°C. Secondary anti-mouse-, anti-human- and anti-goat-horseradish peroxidase (HRP)-conjugated IgGs were used at 1:5,000 dilution and incubated for 1 h at room temperature. The blots were analyzed in a ChemiDoc XRS system (Bio-Rad). Protein band intensities were quantified by ImageLab software (BioRad).
Statistical analysis

The experiments described in this chapter have to be considered as pilot experiments. Two independent experiments were performed in duplicate and the mean ± standard deviation (s.d.) is depicted. To perform a thorough statistical analysis, part of the experiments need to be repeated. These experiments are currently in progress. The Graphpad Prism 5 software (GraphPad Software) was used and comparisons were evaluated by unpaired, two-tailed t-test. A p value of <0.05 was considered statistically significant.

Results

LX-2 hepatic stellate cells become activated when co-cultured with Huh7 cells that express HCV Core or NS3/4A.

Huh7 cells were transiently transfected with HCV Core or NS3/4A expression vectors. Transfection efficiency was 70% ± 5.0 for empty vector, 69.8 ± 6.5 for HCV Core vector and 68.7 ± 3.6 for HCV NS3/4A vector according to GFP detection by FACS (Supplementary Figure 1A). The expression of HCV Core and NS3/4A proteins was demonstrated by immunofluorescence 24 h post-transfection (hpt). Both proteins were detected at the perinuclear area and NS3/4A showed a stronger signal intensity than Core. No staining for Core or NS3/4A was detected in Huh7 cells transfected with the empty vector (Supplementary Figure 1B).

To mimic the in vivo situation of HCV infection, we employed trans-well co-cultures to study the effect of HCV-infected cells on LX-2 cells. LX-2 cells were co-cultured with non-transfected and transfected Huh7 cells. 24 hpt, Huh7 were harvested. Viability of cells was 96% ± 1.4, 94.5% ± 2.1, 94% ± 1.4 and 93.5% ± 3.5, for non-transfected Huh7, Huh7-Empty, Huh7-Core and Huh7-NS3/4A, respectively after harvesting (Supplementary Figure 2A). Transfected Huh7 cells were placed in the upper insert of the transwell system and their attachment to the insert membrane was confirmed by fluorescence microscopy at 24 h post-seeding (Supplementary Figure 2B). Co-culture of LX-2 cells for 24 h with Huh7 NS3/4A-transfected cells increased the expression of activation markers: a-SMA encoded by Actin Alpha 2, Smooth Muscle gene (ACTA2) and COL1A1, whereas expression of TGF-b1 was not changed. Co-culture of LX-2 cells
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with Huh7 cells expressing HCV Core did not induce the expression of HSC activation markers after 24 h (Figure 1A). However, when the co-culture was prolonged to 48 h, both Huh7 Core and NS3/4A induced expression of ACTA2, and Huh7

Figure 1. Transwell co-culture of LX-2 cells with Huh7 cells expressing HCV Core or NS3/4A increase the expression of activation markers. The mRNA levels of activation markers α-SMA, Col1A1 and pro-fibrogenic cytokine TGF-β1 were quantified in LX-2 cells after 24 h (A) and 48 h (B) of indirect co-culture with Huh7 cells expressing the empty vector, HCV Core or NS3/4A using a transwell system. The relative mRNA expression was normalized to 18S gene expression. The graphs show means ± s.d. of two independent experiments performed in duplicate. t test was performed to compare the means and the asterisks represent the p value as follow *<0.01 and **<0.006, (p values > 0.05 are considered not statistically significant). NT=Non-Transfected.

Core also induced expression of COL1A1 expression in LX-2 cells (Figure 1B). Again, no effect on TGF-β1 levels was observed. Next, the expression of HCV Core and NS3/4A proteins in Huh7 cells was evaluated by Western blotting after 24 and 48 hpt (Figure 2). Densitometry analysis revealed that HCV Core expression was significantly higher at 48 h co-culture compared to 24 h co-culture, whereas NS3/4A protein levels were similar at 24 h and 48 h of co-culture (Figure 2A, and 2B). Thus, the effects of Huh7 Core and NS3/4A on LX-2 activation correlated with the protein levels of Core and NS3/4A.
Figure 2. Expression of HCV Core and NS3/4A in transfected Huh7 cells. Protein levels of HCV Core (A) and NS3/4A (B) were determined and quantified in transfected Huh7 cells 24 and 48 h post-transfection (hpt). The relative HCV Core and NS3/4A protein expression was determined by densitometry in a representative experiment.

Next, we investigated the effects of Huh7 Core and NS3/4A on LX-2 activation markers at the protein level. Huh7 and LX-2 cells were co-cultured for 48 h and a-SMA and COL1A1 protein levels were evaluated by Western blotting and compared to treatment of LX-2 cells with recombinant TGF-β1. A significant increase in a-SMA and COL1A1 protein levels was observed after recombinant TGF-β1 treatment of LX-2 cells (Figure 3A-3C), indicating that the effect of TGF-1 treatment is mainly at the post-transcriptional level since ACTA2 and COL1A1 mRNA levels were not affected by TGF-β1 treatment (Figure 1A and 1B). Although a-SMA and COL1A1 protein levels were increased by co-culture of LX-2 cells with Huh7 cells expressing HCV Core protein, this increase was not statistically significant (Figure 3A-3C). Similarly, a slight but not significant increase of a-SMA in LX-2 cells co-cultured with Huh7 cells expressing NS3/4A was observed (Figure 3B).
Figure 3. HCV Core and NS3/4A transfected Huh7 cells increase protein levels of activation markers in LX-2 cells during transwell co-culture. Protein levels of activation markers α-SMA and Col1A1 were determined in LX-2 cells after treatment with 2.5 ng/ml human recombinant TGF-β1 and co-culture with Huh7 cells expressing empty vector, HCV Core or NS3/4A (A). The relative levels of α-SMA (B) and Col1A1 (C) protein expression were determined by densitometry in two independent experiments performed in duplicate and t test was performed to compare the means and the asterisks represent the p value as follow *<0.03, (p values > 0.05 are considered not statistically significant).
Conditioned media from hepatocytes expressing HCV Core and NS3/4A did not affect activation of hepatic stellate cells

Conditioned media from non-transfected and transfected Huh7 cells were harvested 48 hpt (which means 24 h-conditioned medium, see material and methods) and added to LX-2 cells. The expression of activation markers ACTA2, COL1A1 and TGF-β1 in LX-2 cells was measured and compared to the expression levels in LX-2 cells treated with recombinant TGF-β1. Treatment with recombinant TGF-β1 did not affect ACTA2 (Figure 4A) and COL1A1 (Figure 4B) expression at the transcriptional level. However, TGF-β1 did increase the expression of TGF-β1 in LX-2 cells, indicating a positive feedback effect after recombinant TGF-β1 treatment (Figure 4C). Conditioned media from Huh7 cells expressing HCV proteins did not induce activation of LX-2 cells (Figures 4A and 4B). TGF-β1 expression in LX-2 cells was significantly increased after treatment with conditioned medium from HCV Core expressing–Huh7 cells, mimicking the effect of recombinant TGF-β1 (Figure 4C).

Figure 4. Conditioned media from hepatocytes expressing HCV Core or NS3/4A does not increase the expression of activation markers in LX-2 cells. The mRNA levels of activation markers ACTA2 (A), COL1A1 (B) and pro-fibrogenic cytokine TGF-β1 (C) were quantified in LX-2 cells treated with conditioned media from Huh7 cells expressing the empty vector, HCV Core or NS3/4A and in LX-2 cells treated with 2.5 ng/mL recombinant TGF-β1. The relative mRNA expression was normalized to 18S gene expression. The graphs show means ± s.d. of two independent experiments. t test was performed to compare the means and the asterisks represent the p value: *<0.04, (p values > 0.05 are considered not statistically significant).
Menadione treatment of Huh7 cells expressing HCV Core and NS3/4A proteins has no additional effect on LX-2 activation

To mimic oxidative stress conditions observed during HCV infection, we induced external oxidative stress described earlier (Chapters 3 and 4) to the co-culture model, using the superoxide donor menadione. Menadione (50 mmol/L) increased mitochondrial superoxide anion production in both non-transfected and transfected Huh7 cells and this effect was abolished by the antioxidant NAC (Figure 5). Next, we evaluated the effect of external oxidative stress induced by menadione in transfected and non-transfected Huh7 cells on the expression of activation markers in LX-2 cells.

As shown in Figure 6, menadione treatment of Huh7 cells did not change the expression of the activation markers ACTA2, COL1A1 or TGF-b1 in LX-2 cells. In addition, menadione treatment of Huh7 cells did not change the expression of the antioxidant genes Heme Oxygenase 1 encoded by HMOX-1, Superoxide Dismutase 1 and 2 encoded by SOD1 and SOD2, respectively and Catalase (CAT) in LX-2 cells (Figure 7A-7D). Finally, we also determined the transcriptional expression of proteins involved in the ER stress response. For that, we quantified mRNA encoding Glucose-Regulated Protein 78 (GRP78, also known as immunoglobulin heavy chain-binding protein (BiP) encoded by the HSPA5 gene), which is an ER stress-inducible chaperone (Figure 8A). In addition, we determined the levels of the stress response inducible transcription factors ATF4 (Activation factor 4) (Figure 8B) and CCAAT/Enhancer-Binding Protein Homologous Protein (CHOP) encoded by the DDIT3 gene (Figure 8C) and spliced
version of the X-box binding protein 1 (XBP1) (Figure 8D). mRNA levels of none of these inducible stress response (ISR) markers were affected in LX-2 cells co-cultured with Huh7 cells expressing HCV proteins and additionally treated with menadione.

Figure 6. Oxidative stress induction in Huh7 cells expressing HCV Core and NS3/4A proteins has no additional effect on LX-2 activation markers. The mRNA levels of activation markers ACTA2 (A), COL1A1 (B) and pro-fibrogenic cytokine TGF-β1 (C) were quantified in LX-2 cells after 48 h of transwell co-culture with Huh7 cells expressing the empty vector, HCV Core or NS3/4A and treated with menadione 50 mmol/L. 5 mmol/L NAC was used to inhibit the menadione effect. The relative mRNA expression was normalized to 18S gene expression. Two independent experiments were performed in duplicate. The graphs show means ± s.d. of two independent experiments. t test was performed to compare the means (p values > 0.05 are considered not statistically significant).
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Figure 7. Oxidative stress induction in Huh7 cells expressing HCV Core and NS3/4A proteins has no effect on LX-2 expression of anti-oxidant genes. The mRNA levels of antioxidant enzymes HMOX-1 (A), SOD1 (B), SOD2 (C) and CAT (D) were quantified in LX-2 cells after 48 h of transwell co-culture with Huh7 cells expressing the empty vector, HCV Core or NS3/4A and menadione treatment (50 mmol/L) using a trans-well system. 5 mmol/L NAC was used to inhibit the menadione effect. The relative mRNA expression was normalized relative to 18S gene expression. The graphs show means ± s.d. of one experiment.

Discussion

Oxidative stress has been implicated in the development of chronic inflammatory diseases, including viral hepatitis (19). The goal of the present study was to mimic the interaction between hepatocytes expressing HCV viral proteins and HSCs in vitro, with a special emphasis on the significance of oxidative stress. First, we employed a transwell system for the co-culture of Huh7 cells expressing HCV Core or NS3/4A with LX-2 cells. This model more closely mimics the in vivo situation of HCV infection than the conditioned medium used in other studies (17), since the interacting cells are at close proximity of each other allowing the continuous exposure to any secreted
factors. After 24 h co-culture we observed a pro-fibrogenic effect of HCV NS3/4A-transfected Huh7 cells, whereas after 48 h of co-culture, we observed a pro-fibrogenic effect of both HCV NS3/4A and HCV Core-transfected Huh7 cells (Figure 1). The delayed pro-fibrogenic effect of HCV Core transfected cells compared to NS3/4A transfected Huh7 cells correlated with the protein levels of Core and NS3/4A in the transfected cells (Figure 2). The pro-fibrogenic effect of Core-transfected Huh7 cells appeared to be stronger than that of NS3/4A-transfected cells. This correlates with the relative pro-oxidant effect of these proteins in transfected Huh7 cells (Chapter 3 and 4).

Figure 8. Oxidative stress induction in Huh7 cells expressing HCV Core and NS3/4A proteins has no effect on LX-2 markers of ER stress. The mRNA levels of ER stress markers HSPA5 (A), DDIT3 (B) ATF4 (C) and XBP1 (D) were quantified in LX-2 cells after 48 h of indirect co-culture with Huh7 cells expressing the empty vector, HCV Core or NS3/4A and menadione treatment (50 mmol/L)) using a transwell system. 5 mmol/L NAC was used to inhibit the menadione effect. The relative mRNA expression was normalized to 18S gene expression. The graphs show means ± s.d. of two independent experiments. t test was performed to compare the means. (p values > 0.05 are considered not statistically significant).
We demonstrate that conditioned medium from Huh7 cells expressing HCV Core or NS3/4A proteins did not or very weakly activate LX-2 cells based on the expression of the activation and fibrogenic markers ACTA2, COL1A1 and TGF-β (Figure 4A and 4B). A significant effect of conditioned medium was only observed for conditioned medium from Core-transfected Huh7 cells and only for the expression of TGF-β1. HCV Core and NS3/4A proteins were chosen, because both proteins have been shown to induce oxidative stress in infected hepatocytes with HCV Core protein being a stronger inducer than NS3/4A (14,20–22). The lack of -or only weak- effect of conditioned medium may have several reasons, including the stability of secreted factors, the timing of the collection of the conditioned medium and/or the concentration of any secreted factors in the conditioned medium. On the other hand, recombinant TGF-β1 also induced only TGF-β1 expression, thus mimicking the effect of Core-transfected Huh7-conditioned medium.

Recently, Bataller et al. suggested that the direct interaction between HCV proteins and HSCs could play a role in HCV-induced liver fibrosis. They used two different approaches to test their hypothesis: human HSCs were incubated with recombinant HCV Core or NS3/4A proteins and additionally, HCV Core or NS3-NS5 proteins were expressed in HSCs using a recombinant adenovirus. Both HCV Core and NS3/4A induced oxidative stress in human HSCs, an effect that was attenuated by treatment with diphenylene iodonium, an NADPH oxidase inhibitor. Additionally, both viral proteins induced the activation and proliferation of HSCs. Thus, HCV proteins regulated key biological functions involved in liver fibrogenesis in human HSCs (23).

In another study, conditioned medium from HCV replicon transfected Huh7-5-15 cells had pro-fibrogenic effects on both rat and human hepatic stellate cells (30). Our results are in line with these results, however, our model more closely mimics the in vivo situation in which viral proteins are produced and secreted mainly by infected hepatocytes and not by hepatic stellate cells, since it has been shown that HSCs are not susceptible for HCV infection (11).

Although our results and the study of Bataller et al. suggest a direct pro-fibrogenic effect of HCV Core and NS3/4A proteins on hepatic stellate cells, it cannot be excluded that HCV-infected hepatocytes produce and secrete additional pro-fibrogenic cytokines. The most likely candidate is TGF-β1, as is also supported by our experiments with conditioned medium. Additional experiments, using neutralizing
antibodies to TGF-β1 in the co-culture system and/or the evaluation of pro-fibrogenic cytokines in the transfected Huh7 cells are necessary to identify these pro-fibrogenic factors. TGF-b1 is a cytokine involved in several important processes like cell growth, adhesion, migration, apoptosis and cell differentiation and is the most important cytokine involved in fibrogenesis (24). It has been suggested that Kupffer cells are the main source of TGF-b1. However it has also been reported that hepatocytes infected by HCV or expressing HCV viral proteins as Core can secrete TGF-b1 (25). Additionally, increased reactive oxygen species in HCV-infected hepatocytes induce TGF-b1 expression (26). Jee et al. reported that TGF-b1 production is increased in HCV-infected hepatocytes and in liver tissue of HCV-infected patients and this production was sufficient to activate HSC. The TGF-b1 production was triggered by HCV glycoprotein E2 (27).

The second goal of our study was to investigate whether HCV protein-expressing cells, subjected to an additional stress (described in Chapters 3 and 4) modulate the activation and/or stress response in LX-2 stellate cells. Huh7 cells, subjected to external oxidative stress did not increase any markers of oxidative stress or ER stress in LX-2 cells. These results suggest that Huh7 cells expressing HCV proteins and exposed to oxidative stress do not induce any stress response in stellate cells, indicating that the pro-fibrogenic signals from HCV protein expressing Huh7 cells is indeed the result of soluble, secreted factors and not the result of a stress response in LX-2 cells. This result is relevant, since it has been proposed that ER stress, autophagy and/or oxidative stress directly activate stellate cells. We did not observe any indications for ER stress or anti-oxidant response in LX-2 cells co-cultured with Huh7 cells subjected to external oxidative stress. However, we cannot completely rule out a role of oxidative stress in the pro-fibrogenic response of LX-2 cells, since we only measured expression levels of anti-oxidant genes and did not directly quantify oxidative stress, like ROS production or intracellular redox status.

The experiments described in this chapter have to be considered as pilot experiments. All experiments were performed at least two times. However, to perform a thorough statistical analysis, (part of) the experiments need to be repeated. These experiments are currently in progress.
In summary, in these pilot studies we established an *in vitro* model for cell-cell interaction between hepatocytes and HSCs to study the activation of HSCs and the cellular stress response in the context of HCV infection. In this model, we demonstrate a pro-fibrogenic effect of Huh7 cells expressing HCV Core or NS3/4A on LX-2 hepatic stellate cells. The identity of the pro-fibrogenic factors released by HCV-infected cells still needs to be elucidated, but TGF-b1 appears to be a likely candidate. The existence of a pro-fibrogenic action of HCV-infected cells on HSC, independent of inflammatory cells, provides novel insights in the pathogenesis of HCV-related liver fibrosis and may lead to novel therapeutic targets.

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Supplementary figures and tables

Supplementary Figure S1. Transfection efficiency and expression of HCV Core and NS3/4A in Huh7 cells. Transfection of Huh7 cells was determined by flow cytometry based on GFP expression of transfected cells. A representative experiment (dot plot cytogram) and the average of three independent experiments are shown (A). The expression of HCV Core and NS3/4A was demonstrated by immunofluorescence in Huh7 cells 24 hpt using anti-HCV Core and anti-NS3/4A antibodies. The red signal detecting the viral proteins is indicated by white arrows. Huh7 cells transfected with the empty vector were used as a control. The graphs show means ± s.d. of three independent experiments.
Huh7 cells expressing hepatitis C virus Core or NS3/4A protein activate human LX-2 hepatic stellate cells through paracrine signaling.

Supplementary Figure S2. Indirect co-culture of Huh7 cells expressing HCV Core and NS3/4A with LX-2 cells. Huh7 cells were harvested 48 hpt using 0.5 mg/mL trypsin. Then, cell viability was determined by trypan blue exclusion staining. The graphs show means ± s.d. of three independent experiments (A). Attachment of Huh7 cells to the membrane in the upper insert of indirect co-culture was confirm by fluorescent microscopy (B).
**Supplementary Table 1. Primer sets and probes for qPCR.**

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References


21. Ivanov A V, Smirnova OA, Ivanova ON, Masalova O V, Kochetkov SN, Isagulians MG. Hepatitis C virus proteins activate NRF2/ARE pathway by distinct ROS-


