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Hepatitis C Virus Proteins Induce Cirrhosis Antigen Expression on Human Hepatoma Cells *In Vitro*: Implications for Viral Mechanisms in Hepatitis C Fibrogenesis

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1. Introduction

Hepatitis C virus (HCV), an RNA virus classified within the *Flaviviridae* (12), has a remarkable propensity for persistence in the human host following acute infection. Infections spontaneously resolve in 20-30% of infected individuals, while 70-80% of infections result in long-term persistent viremia. Chronically infected individuals are at an increased risk of developing hepatocellular injury compared to subjects with acute resolved infections (25), with manifestations progressing from mild to severe (bridging) fibrosis, and, ultimately cirrhosis, in 10-30% of chronic infections. Cirrhosis underlies life-threatening complications of end stage liver disease and/or hepatocellular carcinoma, after a long and extremely variable disease incubation period (25, 38). It is presently impossible to predict which persons with chronic hepatitis C are at greatest risk for disease progression, and, likewise, host-virus relationships are most important for driving chronic hepatitis C disease progression have not been defined. *In vitro* evidence supporting the concept of virus-mediated liver injury has recently been reported, including isolation of cytopathic derivatives of HCV infectious clone JFH1 (27), and also in the chimeric mouse (immune deficient mouse with humanized liver) hepatitis C model (18).

Based on experimental data from acute liver injury rodent model systems, where massive hepatic necrosis is experimentally induced by toxins such as carbon tetrachloride (29), the master mediators of hepatic fibrogenic processes are Transforming Growth Factor beta (TGFbeta) and Platelet Derived Growth factor (PDGF) (17). Hepatic Stellate Cells (HSCs) and macrophages appear to be the major cell types regulating hepatic fibrosis (17, 46). HSCs, normally quiescent in the liver, and potentially derived from hepatic oval cells (45), respond to injury by proliferation and secretion of large amounts of extracellular matrix proteins, in addition to pro-fibrogenic cytokines including TGFbeta. During the process, HSCs are transformed into fibrogenic myofibroblasts. A well characterized liver cirrhosis-

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associated antigen, Glial Fibrillary Acidic Protein (GFAP), is induced on pro-fibrotic cells such as HSCs, and is a definitive marker of fibrogenic pathway activation in this latter cell type (29). Studies of fibrosis mechanisms in human liver are limited. One longitudinal study, after liver transplantation, reported that increased density of GFAP in liver biopsy specimens predicted subsequent advanced fibrosis or cirrhosis (9). Although cells harboring GFAP were only presumed to be activated HSCs, the study concluded that 30% of cells in cirrhotic livers may be activated HSCs. However, the possibility of a direct effect of HCV on GFAP expression in hepatocytes was not investigated.

The present study therefore examined the effect of HCV on hepatic fibrosis marker expression, using two human hepatoma cell line model systems, capable of supporting either non-productive HCV replication (HCV replicon, (22)), or productive HCV infection (genotype 2a infectious clone JFH1; (44)). The study also examined liver biopsy samples from HCV infected patients for the simultaneous presence of GFAP and HCV replicative intermediate RNA. Finally, microarrays were used to analyze expression of multiple cellular genes linked with liver fibrosis, in human hepatoma cell lines plus or minus HCV. The effect of HCV on differential expression of 153 genes (1, 3, 17, 28, 37) either involved in, or associated with, with the process of liver fibrosis, is reported.

2. Methods

2.1 Human liver biopsy specimens

Thirty-two liver biopsy specimens, obtained under informed consent and per IRB-approved protocol, were available for study. All 32 subjects had chronic, active (viremic) HCV genotype 1 infections. During procurement, the specimens were immediately preserved in OCT buffer and snap frozen at the bedside. Parallel sections of the liver biopsies were reviewed by a single pathologist who was blinded to HCV status and all other data. Liver fibrosis severity, staged as 0 (no fibrosis) through 4 (cirrhosis), was assigned according to the system described by Batts and Ludwig (5). For the present study, the liver specimens were de-identified for all information except HCV replication status and fibrosis severity. Fresh thin sections were obtained for the GFAP immunostaining experiments described below.

Parallel sections of all 32 liver biopsy specimens were assayed for GFAP expression by immunocytochemistry. 29 of the specimens had been previously analyzed for both HCV genomic (G) and replicative intermediate (RI) RNAs by strand-specific *in situ* hybridization (ISH). Details of the ISH assay, and assay results for a larger sample of hepatitis C cases, were previously reported (31). Of 29 specimens with both GFAP and HCV replication data, HCV RNA was determined as either positive (G+RI+; 20 specimens), or negative (G-RI-; 9 specimens), and GFAP staining level (% of cells per biopsy staining positive for GFAP, or %GFAP) was then analyzed as a function of HCV infection/replication status, and fibrosis stage.

2.2 Hepatoma cell infection by JFH1 HCV

Huh7.5.1 cells (48) were generously provided by Francis Chisari (Scripps Institute, La Jolla, CA). Infection of Huh7.5.1 cells with the HCV JFH1 genotype 2a clone was performed as previously described (43), including the preparation of the JFH1 viral stock, cell infection, and titration. Briefly, we inoculated naïve Huh7.5.1 cells with supernatant harvested from JFH1 RNA transfected cells. Naïve Huh7.5.1 cells were seeded 24 h before infection at a density of 1×10^6 per 10 cm dish. The cells were incubated with 2.5 ml of the JFH1 inoculum at an multiplicity of infection of 0.01 for 3 h, washed three times with PBS, and

supplemented with fresh complete Dulbecco's modified Eagle's medium. Cells were collected 72 hours post infection and assayed for HCV infection and replication by western blot analysis, immunocytochemistry and/or real time PCR.

2.3 Huh7-HCV replicon cells

Huh7 cells containing either full-length genotype 1b HCV replicon, or a Huh 7.5 hepatoma subline with genotype 1a strain H77 replicon (FL-Neo replicon) (7), were obtained from C. Rice, Rockefeller Institute, and maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 400 μg/ml of G418 (Calbiochem; San Diego, CA) supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin/streptomycin at 37 °C in a 5 % CO_2 atmosphere.

2.4 Transfection of HCV genes

Conditions for transient transfection of genotype 1a HCV core, NS3/4A, and NS5A genes cloned into expression vector pcDNA3.1 were previously described (30). The day prior to transfection, $0.5x10^6$ Huh7.5.1 cells were plated overnight onto chamber slides. Endotoxin-free plasmid DNA (0.5 μg) was purified (Endofree kit; QIAGEN, CA) and transfected with Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen, CA).

2.5 Transfection of TGF beta siRNA

100pmol of TGFbeta-specific siRNA was transfected in $0.5x10^6$ cells 24 h after HCV gene transfection. Transfection of siRNA was carried out using Ambion si RNA transfection reagent kit (Ambion, TX) according to the manufacturer's protocol. Mock, non-targeting control siRNA, with limited sequence similarity to known genes (Silencer® Negative Control), was used as negative control (Ambion). At 48 h post-transfection, total RNA and protein were harvested for immunoblot assay and real time RT-PCR, respectively. For immunofluorescence assays, cells were grown in chamber slides for transfection, and fixed in 10% neutral buffered formalin at various times post-transfection, as indicated in the Results section.

2.6 Antibodies and immunoblot analysis

Protein concentrations in cell lysates were quantified (BCA Protein Assay; Pierce), and equal amounts of total protein (10-20 μg) were separated on 4 to 20% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels. Immunoblot analysis was performed using a GFAP-specific monoclonal antibody (Dako, CA) followed by secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch). The relative levels of GFAP protein were quantified in immunoblots using ImageQuant (version 5.1). The signals from the immunoblot were normalized against the signal from a common cellular housekeeping gene (GADPH), and the ratio of GFAP-specific signal to control GADPH signal was determined.

2.7 Immunocytochemistry

Methods for immunocytochemistry (ICC) were as previously described (31, 32). Briefly, snap frozen liver sections, or hepatoma cells from culture, were fixed in 10% neutral buffered formalin and subjected to immunohistochemistry. Mouse monoclonal antibodies against GFAP were used at 1:100 dilution for 60 minutes (Affinity BioReagents, Co), followed by biotinylated goat antimouse immunoglobulins (dilution 1:200) for 30 minutes at room temperature. Samples were incubated with the Vectastain ABC alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature to develop the vector red substrate. For double immmunostaining, anti-HCV core (Affinity

BioReagents, Co) anti-HCV NS3 (Vision Biosystem, MA) or anti-NS5A antibodies were used in combination staining, at 1:100 dilution for 60 minutes, followed by FIT-C conjugated goat antimouse immunoglobulins (dilution 1:200) for 30 minutes at room temperature. Mounting media containing DAPI (Vector laboratories, CA) was used to counter stain.

2.8 RNA extraction

Total cellular RNA was isolated from either HCV replicon, or negative control Huh7.5 cells, using 10^6 cells and an RNeasy miniprep Kit with an on column DNAse treatment following the manufacturer's protocol (Qiagen). The RNA was quantified and quality checked using an Agilent Bioanalyzer platform (Agilent Technologies); using this standard, all RNA preparations were of highest quality and integrity. The RNA Integrity Numbers (RINs) of all the RNA samples were between 9.7 and 10.0. RINs greater than 6 represent RNA of sufficient quality for quantification experiments (13).

2.9 Real-time RT-PCR

Total RNA was extracted from uninfected or infected hepatoma cells, and reverse transcribed into cDNA using the superscript II first strand synthesis system according to the manufacturer's protocol (Invitrogen, CA). Real-time quantitative PCR was carried out with an ABI 7900 Real-time PCR System, using the GAPDH gene as a reference (30). Three independent experiments were performed, and standard deviations calculated.

2.10 Microarray Hybridization

0.5µg of total RNA was used for a linear T7-based amplification step. To produce Cy3 labeled cRNA, the RNA samples were amplified and labeled using the Agilent qucik Amp Labeling kit (Agilent Technologies). Yields of cRNA and dye incorporation rate were measured with a ND-1000 spectrophotometer (Nano-Drop Technologies). Agilent whole Human genome Oligo Microarrays 4X44K (Miltenyi Biotech, Germany) were used to compare RNA samples from genotype 1a HCV replicon and Huh7.5 cells. The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression hybridization kit (Agilent Technologies). Briefly, 1.65µg Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight at 65°C. The microarrays were washed once with the Agilent Gene Expression Wash Buffer 1 for 1 min at room temperature followed by a second wash with preheated Agilent Gene Expression Wash Buffer 2 (37°C) for 1 min. The last washing step was performed with acetonitrile. Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies).

2.11 Image and data analysis

The Agilent Feature Extraction Software (FES) was used to process the microarray image files to determine feature intensities (including background subtraction) and reject outliers. All samples were labeled with Cy3. Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware) was used to build pair-wise ratios and for data normalization.

2.12 Statistical analyses

In all experiments, including pixel count for GFAP fluorescence, densitometric scans of Western blots, RT-PCR analysis and GFAP antigen expression in liver biopsies by ICC,

results were calculated as the means (±S.D.) of three independent experiments. For liver biopsy specimens, 3 different microscope fields were each read by 3 different trained study investigators, and results were averaged and compared using one-way analysis of variance. P values of 0.05 or less were considered statistically significant.

3. Results

3.1 Detection of GFAP and HCV RNA in parallel sections from HCV-infected human liver biopsies

A previous study correlating intrahepatic HCV replication with liver fibrosis stage suggested a potential involvement of HCV in liver fibrogenesis [31]. Glial fibrillary acidic protein (GFAP) is a well-known marker of hepatic stellate cell (HSC) activation and liver cirrhosis. Therefore, for the present study, GFAP expression was analyzed in parallel sections from *ex vivo* liver biopsy specimens obtained from 32 HCV genotype 1-infected research subjects. All specimens had been scored previously for both liver fibrosis stage, while 29 specimens had been scored for HCV infection and replication status using viral RNA strand-specific *in situ hybridization* (ISH), as described previously (18). GFAP was determined by ICC and counting percentage of positive cells per biopsy. As expected, there was a significant correlation between percentage of liver cells staining positive for GFAP, and liver fibrosis stage ($p<0.005$) (Figure 1A). 20 specimens were positive for both HCV genomes and replicative intermediate RNAs (G+RI+), while 9 specimens were negative for both markers (G-RI-) despite chronic HCV infection confirmed by both enzyme immunoassay for HCV antibodies, and serum PCR for viral RNA. Figure 1B demonstrates that GFAP staining was significantly increased in parallel sections from the HCV replication positive (G+RI+) liver biopsies, compared to the biopsies lacking HCV RNA signal (G-RI-) (mean 55% GFAP positive cells versus 16% GFAP positive, respectively; $p<0.01$).

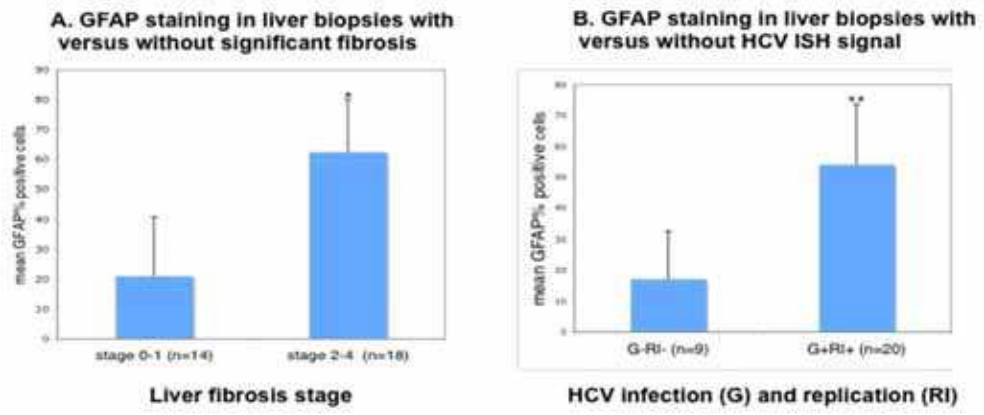


Fig. 1. Relationships between degree of liver fibrosis, GFAP expression, and genotype 1 HCV infection and replication status, in parallel sections of 32 liver biopsy specimens from research subjects. Panel A: GFAP staining according to degree of liver fibrosis in parallel sections from 32 biopsies. Panel B: GFAP staining according to HCV replication status in parallel liver biopsy sections from 29 of the 32 subjects with chronic hepatitis C. * $p<0.005$; ** $p<0.01$.

3.2 HCV induces GFAP expression in cultured human hepatoma cells

To follow up these observations, a series of experiments examined the effect of HCV on cirrhosis antigen (GFAP) expression in well-characterized human hepatoma cell lines. The HCV replicon efficiently replicates, but does not produce progeny virus, in hepatoma Huh 7.5 cells (7, 22). For this experiment, a gentotype 1b replicon was used. Dual immunostaining of negative control Huh7.5 cells showed absence of the HCV core protein, and low or undetectable expression of the liver cirrhosis antigen, Glial Fibrillary Acidic protein (GFAP) (Figures 2A and B, respectively). The presence of the HCV genotype 1b replicon, lead to significant induction of GFAP expression in Huh7.5 cells (Figures 2C and D, respectively). These data were the first suggestion that GFAP expression was up regulated in human liver cells by HCV. To confirm this result, and investigate if the results extend to more than one HCV genotype, GFAP expression was assayed in the human hepatoma cell line Huh7.5.1, in the presence, or absence, of the HCV genotype 2a infectious clone, JFH1 (44, 48). Figures 2E and 1F show negative dual immunostaining of uninfected Huh7.5.1 cells for HCV core and GFAP, respectively, while increased staining of HCV core protein (Figure 2G) and GFAP (Figure 2H) were observed in the presence of JFH1 infection. Quantification of immunofluorescence signals (right hand panel of Figure 2) confirmed highly significant increases in GFAP antigen signal in hepatoma cells in the presence of either HCV replicon, or infectious clone JFH1, respectively ($p<0.001$ in both cases).

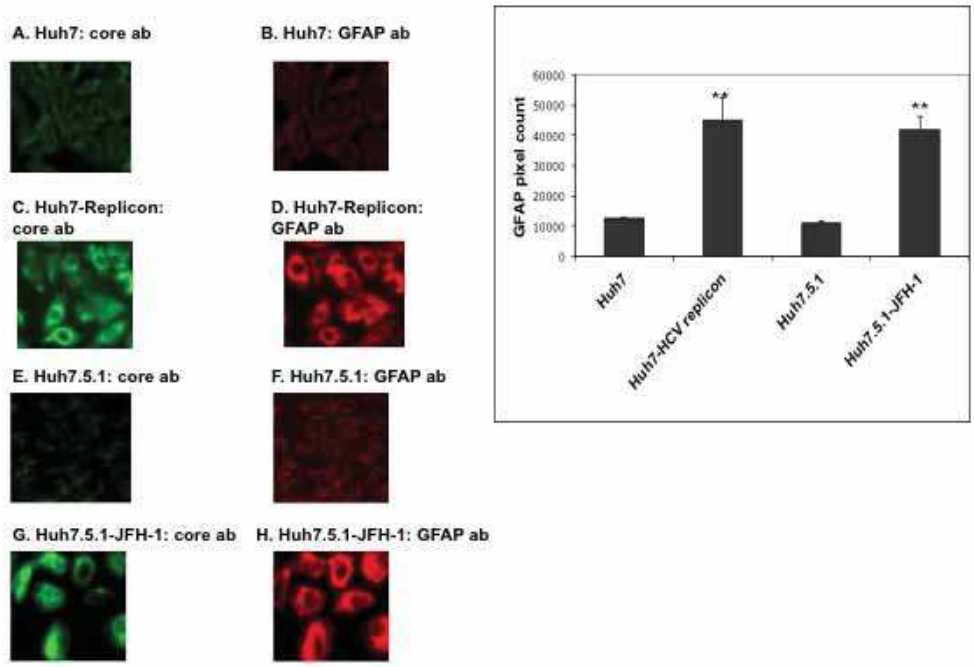


Fig. 2. Induction of GFAP in cultured human hepatoma cells. HCV core and GFAP signals were detected by dual label immunocytochemistry (ICC) using FITC and Texas Red channels, respectively. 40X magnification. In the bar graph, mean GFAP antibody fluorescence from triplicate experiments was quantified by metamorph and expressed as pixel count. ** indicates a significant increase in GFAP fluorescence in the presence of HCV, $p < 0.001$.

3.3 HCV core and NS5A genes both induce GFAP expression in hepatoma cells

The HCV core gene encodes a virion structural component that is known to influence multiple cellular processes (25). Both NS3/4A (helicase and protease) and NS5A (kinase) genetic cassettes also encode cell regulatory functions (14, 23). Thus, these three HCV genes (all derived from genotype 1b HCV) were each tested individually for induction of GFAP protein and RNA. Huh7.5.1 cells were transfected with either empty, or HCV-protein-containing expression vectors, and dual-immunofluorescence was performed to detect GFAP, and the corresponding HCV antigens, 48 hrs after transfection (Figure 3). Figures 3A, B and C illustrate negative immunostaining for HCV core, GFAP and HCV NS5A antigens, respectively, following control vector transfection. Transfection of the NS3/4A cassette also gave negative GFAP signal (data not shown). However, GFAP signal was greatly enhanced in Huh7.5.1 cells transfected with either the HCV core gene (Figure 3E), or the NS5A gene (Figure 3F). Figures 3D and F confirm expression of the HCV core and NS5A proteins in transfected Huh7.5.1 cells, respectively. Interestingly, although HCV core expression was limited to a relatively small subset of transfected cells (Figure 3D), GFAP expression was widespread, and signal was strong even in cells that stained negative for HCV core antigen (compare green and red signals in Figures 3D and E). This result suggested that a diffusible intermediate might be responsible for core-mediated GFAP induction in hepatoma cells. In contrast, NS5A expression was more widespread throughout the transfected cells, the pattern of GFAP expression in response to the NS5A protein was more punctate and discrete than that observed following core transfection, and NS5A staining was observed in the same cells that showed increased GFAP expression (Figure 3F). Quantitative assessment of GFAP immunofluorescence signals, summarized by bar graph in Figure 3, confirmed that the HCV core and NS5A proteins both significantly increased expression of GFAP in cultured hepatoma cells ($p<0.001$ in both cases).

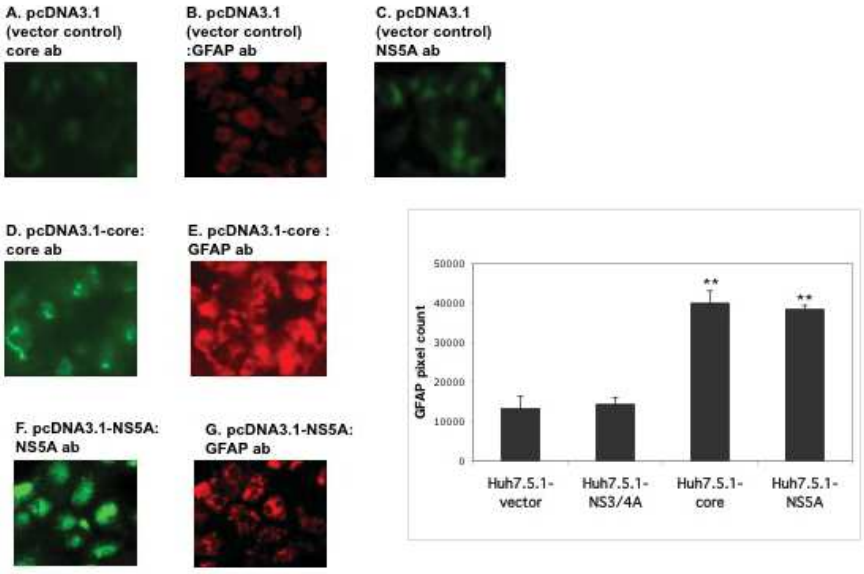


Fig. 3. HCV core and NS5A genes induce GFAP in hepatoma cell lines.HCV antigens and GFAP signals were detected using FITC and Texas Red channels, respectively. 40X magnification. The bar graph compares GFAP signal intensity in the presence of vector or HCV gene products core, NS3/4A, or NS5A.

Experiments using cell extracts, summarized in Figure 4, were performed to confirm the results of cell surface staining. To examine effect of HCV on GFAP protein expression within cells, total cell protein was harvested from Huh7.5 cells, HCV replicon-containing Huh7.5 cells, Huh7.5.1 cells infected with JFH1 virus for 72 hours, and Huh7.5.1 cells following transient transfection with individual HCV core, NS3/4A, or NS5A constructs. Protein extracts were subjected to Western Blot using the same panel of antibodies as used for Figures 2 and 3. GFAP expression was determined by quantification of GFAP pixel intensity in blot images, using GAPDH as control (Figure 4A).

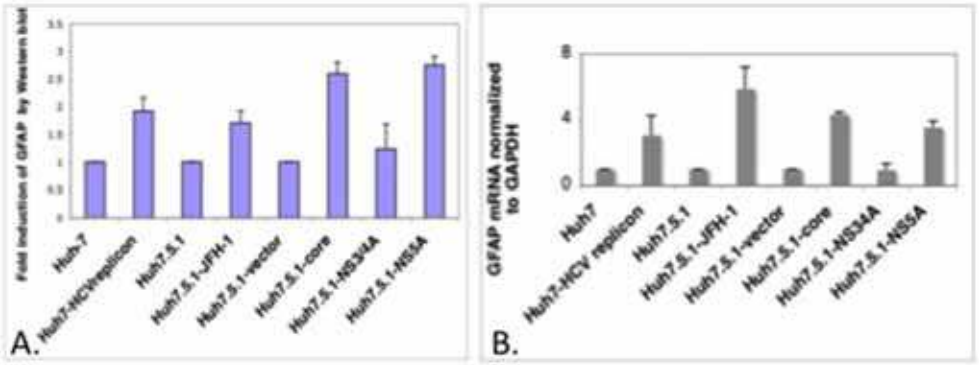


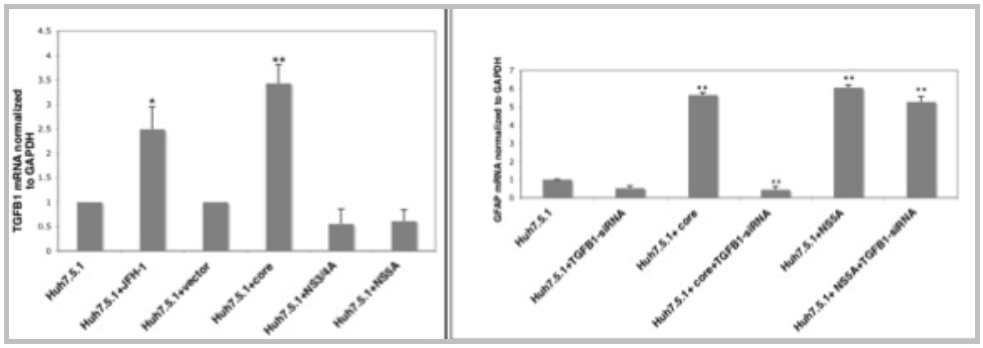
Fig. 4. Quantification of HCV effect on GFAP protein and RNA expression levels, by Western Blot (Panel A) and real time PCR (Panel B), respectively. Experiments were as summarized for Figures 2 and 3. Quantities of GFAP protein or RNA expression were averaged from three experiments. All data were normalized to GAPDH protein (Panel A) or RNA (Panel B) expression levels. * $p < 0.05$; **$p<0.001$.

GFAP protein levels harvested from culture lysate were increased 1.8-fold in cells containing the HCV replicon, and approximately 1.7-fold in JFH1-infected cells relative to control cells. In cell transfection experiments using Huh7.5.1 cells as control, quantities of GFAP protein expression were increased by either the HCV NS5A gene (approximately 2.8-fold), or the HCV core gene (approximately 2.6-fold), while no significant effect on GFAP expression was observed following NS3/4A transfection. The Western blot experiment confirmed that the genotype 1b HCV replicon, the genotype 2a HCV infectious clone JFH1, the HCV core gene product, and the NS5 gene product were all able to up regulate expression of GFAP protein in cultured hepatoma cells. The effect of HCV on GFAP RNA expression in cultured hepatoma cells is presented in Figure 4B, under identical conditions to that described in Figure 4A. GFAP RNA expression was increased in both HCV replicon cells (approximately 3-fold), and JFH1-infected cells (approximately 6-fold), compared to cultured hepatoma cell line controls. Furthermore, Huh7.5.1 cells transfected with either the core construct, or the NS5A construct, showed approximately 3-fold increases in GFAP RNA expression, while no change in GFAP RNA expression was found following NS3/4A transfection (Figure 4B).

3.4 TGFbeta mediates core induction of GFAP, but not NS5A induction of GFAP

TGF-β is a known modulator of GFAP expression in human astrocytes (35), and the HCV core product is known to induce TGF-β expression *in vitro* (42). Thus, we anticipated that HCV core induction of GFAP in hepatoma cells was TGF-β dependent. As indicated in

Figure 5A, TGF-β RNA expression was increased approximately 3-fold in the presence of either JFH1 infection, or HCV core gene transfection, but not in the presence of either NS3/4A, or NS5A gene transfection. RNA knockdown of TGF-β RNA, using TGF-β - specific siRNA, blocked GFAP induction by the HCV core protein, but had no effect on GFAP induction by the NS5A protein (Figure 5B). The data implicates a TGF-β dependent mechanism for induction of GFAP by the HCV core protein. However, the data argue that the HCV NS5A gene product most likely induces GFAP expression by a TGF-β - independent mechanism.



(A) (B)

Fig. 5. Panel A: HCV induction of TGFbeta expression by real-time RT-PCR using cells transfected with either core, NS3/4A, or NS5A in pcDNA3.1 plasmids. Data were normalized to GAPDH RNA. *, $p < 0.05$; **, $p < 0.001$. Panel B: Differential effect of TGFbeta knockdown by siRNA on GFAP induction by HCV core and NS5A. (** Indicates $p < 0.001$).

3.5 Extracellular matrix gene transcriptional responses induced in hepatoma cells by the HCV replicon

To determine the breadth of effect of HCV on the extracellular matrix, microarray analysis was used to analyze, within hepatoma cells, the expression of 153 cellular genes associated with liver fibrosis (1, 3, 17, 28, 37), and to determine the effect of genotype 1a hepatitis C virus replication on matrix gene expression. Genes associated with cell-cell and cell-matrix interactions, along with those implicated in dysregulated tissue remodeling during repair and wound healing, were assessed in Huh7.5 cells in the presence or absence of the HCV replicon. A change in gene expression was considered significant based on two criteria, a greater than 99% probability of being expressed differentially ($P \leq 0.01$), and a fold change of 1.5 or greater, which is conservative. Figure 6 shows the log scatter plot of signal intensities of all spots representing expression levels of individual cellular genes in the presence (Y axis), or absence (X-axis), of the HCV replicon.

The gene panel we analyzed included 20 collagen genes, and 133 non-collagen genes associated with the extracellular matrix (ECM) and cellular adhesion, including remodeling enzymes, cytokines, chemokines, growth factors, and genes involved in signal transduction. From the total of 133 non-collagen genes analyzed, 34 genes were induced by HCV, with induction values ranging from 1.5 fold to 100 fold (Figure 7 and Table I). Another 30 genes were down regulated in the presence of HCV; again with a range of 1.5 fold to 100 fold (Figure 7 and Table I). Finally, 69 non-collagen genes showed no significant change in gene expression in the presence of HCV, relative to control cells (Table II).

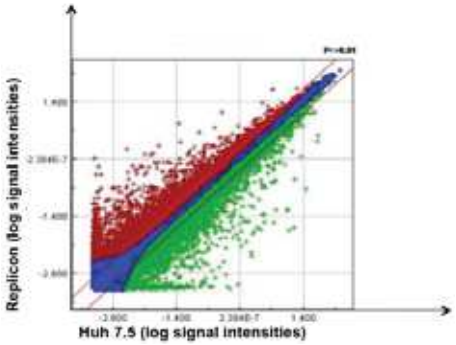


Fig. 6. Pair-wise comparison of signal intensity on DNA microarrays hybridized with RNA from Replicon 1a (Fl-Neo) and Huh 7.5 -control cell lines. The red lines depict the p value cut off (p=0.01), while red and green crosses indicate up and down regulated genes in the Replicon 1a cell line relative to the control cell line.

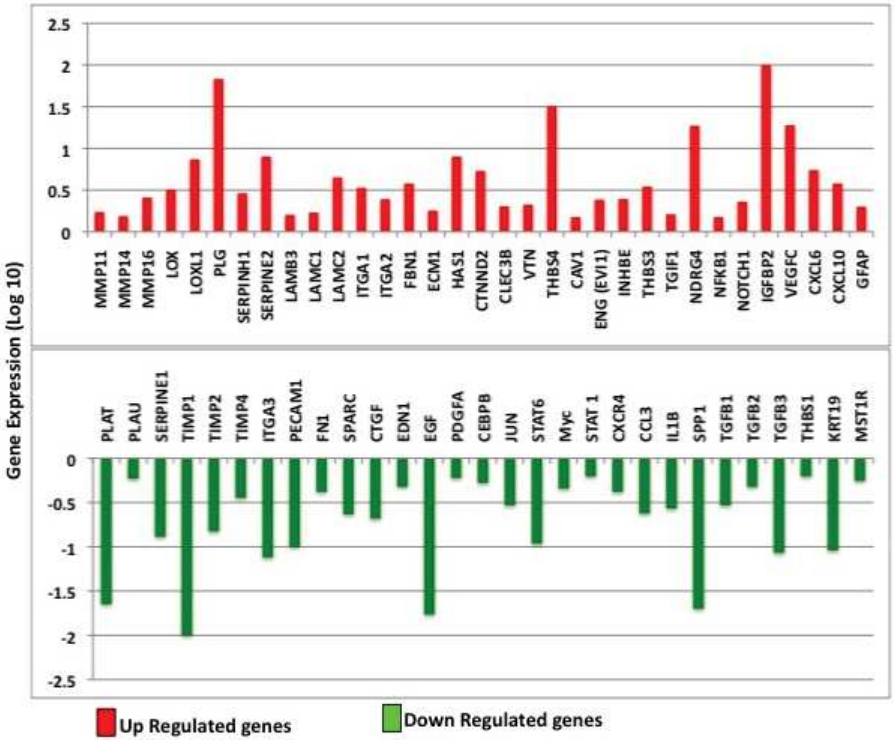


Fig. 7. Regulation of genes linked with hepatic fibrosis, by HCV. The red and green bar graph shows increased or decreased expression (relative to the control Huh 7.5 cells) of various cellular genes linked to liver fibrosis. Expression level, on the vertical axis, refers to the Log-fold change in the transcript abundance of individual genes arranged on the horizontal axis.

Gene Characterization	Gene Symbol	Fold change	P value
Significantly up regulated in Replicon cells			
ECM and Cell adhesion			
Remodeling Enzymes	MMP11	1.73	0.00025
	MMP14	1.54	0.00321
	MMP16	2.57	5 x10-10
	LOX	3.2	0.0
	LOXL1	7.4	0.00005
	PLG	68.079	8x10-6
	SERPINH1	2.9	5x10-10
	SERPINE2	8.0	4x10-18
ECM proteins	LAMB3	1.6	0.003
	LAMC1	1.7	0.0004
	LAMC2	4.5	3x10-14
Cell Adhesion	ITGA1	3.37	1x10-11
	ITGA2	2.46	9x10-7
	FBN1	3.8	1x10-12
	ECM1	1.8	0.007
	HAS1	8.0	0.00004
	CTNND2	5.37	3x10-16
	CLEC3B	2.022	0.00005
	VTN	2.12	1x10-6
	THBS4	32.0	3x10-22
TGFb super family	CAV1	1.5	2x10-21
	ENG (EVI1)	2.43	8x10-6
	INHBE	2.47	3x10-8
	THBS3	3.49	6x10-12
	TGIF1	1.62	0.001
	NDRG4	18.7	3x10-21
Transcription Factors	NFKB1	1.5	5x10-18
	NOTCH1	2.3	0.0
Growth Factors	IGFBP2	100	2x10-23
	VEGFC	19.0	2x10-14
Cytokines	CXCL6	5.5	1x10-9
	CXCL10	3.8	0.0004
	GFAP	2.0	8x10-7
Significantly down regulated in Replicon cells			
ECM and Cell adhesion			
Remodeling Enzymes	PLAT	44.6	0.0
	PLAU	1.7	3x10-29
	SERPINE1	7.7	0.0
	TIMP1	100	3x10-23
	TIMP2	6.7	9x10-19
	TIMP4	2.8	1x10-9

Table 1. Significantly dysregulated transcripts in Replicon Fl-Neo

Gene Characterization	Gene Symbol	Fold change	P value
Significantly down regulated in Replicon cells			
ECM and Cell adhesion			
Cell Adhesion Molecules	ITGA3	13.2	1x10-10
	PECAM1	10.0	3x10-19
	FN1	2.4	2x10-8
Extracellular matrix protein	SPARC	4.3	8x10-14
Growth Factors	CTGF	4.8	8x10-15
	EDN1	2.1	0.0
	EGF	58.3	1x10-22
	PDGFA	1.67	0.0005
Transcription Factors	CEBPB	1.9	0.0006
	JUN	3.4	0.0
	STAT6	9.2	7x10-19
	Myc	2.2	0.0
	STAT 1	1.6	8x10-26
Inflammatory cytokines and chemokines	CXCR4	2.4	3x10-5
	CCL3	4.2	0.0
	IL1B	3.7	1x10-45
	SPP1	50.1	0.0
TGFb super family	TGFB1	3.4	0.0
	TGFB2	2.1	0.001
	TGFB3	11.7	2x10-19
	THBS1	1.6	0.0
Cytoskeletal	KRT19	11.0	1x10-19
Kinase	MST1R	1.8	0.0001

Table 1. Significantly dysregulated transcripts in Replicon Fl-Neo (continuation)

Pro-Fibrotic: ACTA2; SNAI1
Basement Membrane: COL10A1
FACITs : COL19A1, COL20A1
Transmembrane: COL17A1
Multiplexin Collagens: COL13A1, COL15A1, COL18A1
ECM: LAMA1, LAMA2, LAMA3, LAMB1
Remodelling Enzymes: MMP1, MMP2,MMP3, MMP9, MMP13, MMP14, ADAMTS1, ADAMTS8, ADAMTS13, SERPINA1
Cellular Adhesion: ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, ITGB6, ITGB8, ITGAV, ITGA4, ITGA5, ITGA6, ITGA7, ITGAL. ITGAM, CDH1
Inflammatory cytokines and chemokines: CCR2, CXCR4, CCL11, CCL2(MCP-1), IL-13, IL13RA2, IL4, IL5, IFNG, IL13RA2, IL1A, ILK, IL1RN
Growth factors: AGT, PDGFB, VEGFA
TGF beta super family: BMP7, DCN, GREM1, LTBP1, SMAD2, SMAD3, SMAD4, SMAD6, SMAD7, TGFBR1, TGFBR2, THSP2
Transcription Factors: SP1, MITF

Table 2. Transcripts with no significant change in gene expression

Gene Characterization	Gene Symbol	Fold Change	P values
Significantly up-regulated in Replicon cells			
Fibril forming	COL2A1	100	2x10-23
	COL5A2	2.9	8x10-9
	COL11A1	2.8	1x10-9
Non-Fibrillar			
Basement Membrane Collagen	COL4A2	2.2	4x10-7
	COL4A5	1.6	0.008
	COL4A6	1.9	0.00002
	COL8A2	2.0	0.0006
FACITs	COL9A2	2.4	4x10-8
	COL9A3	10.0	3x10-19
	COL12A1	3.2	2x10-8
	COL14A1	53	6x10-18
	COL16A1	15.58	9x10-21
Significantly down regulated in Replicon cells			
Fibril Forming	COL1A1	2.5	3x10-19
	COL1A2	4.6	0.0
	Col3A1	3.8	1.3x10-9
Non-Fibrillar			
Anchoring Filaments Collagen	COL7A1	5.7	4x10-16
Collagen of beaded microfilaments	Col6A1	5.9	3x10-16
	Col6A2	8.4	8.7x10-13

Table 3. Significantly dysregulated Collagen transcripts in Replicon Fl-Neo

The effect of HCV on expression of twenty known collagen genes is presented in Table III. These were grouped into two main molecular classes: the fibril forming species (collagens 1,2,3,5), and non-fibrillar collagens (37). The non-fibrillar collagens were further subdivided. Nine collagen genes had increased transcript abundance in the HCV replicon positive cell line, while four collagen genes showed decreased transcript abundance in the presence of HCV. There was no significant change in the expression of remaining 7 collagen genes when compared to the control Huh 7.5 cells. We observed an increase in fibril forming collagen type 2 and 5. Col2A1 showed an increase of 100-fold. We also found an increase in expression of fibril forming, basement membrane and FACIT collagen molecules in the replicon cell line when compared to the huh7.0 cell line (Table III). However, of interest is the note that the expression of Col1A1 and Col1A2 was down regulated in the presence of HCV (Table I). Col3A1 was also down regulated in the presence of HCV.

There was a 8-fold increase in hyaluronan synthase 1 (Has1) mRNA, along with an increase in transcript abundance of Integrins (ITGA1, ITGA2) and Laminin (LAMC1, LAMC2, LAMB3). Laminin is the main type of adhesive ECM component, and is associated with the basement membrane formation in liver during cirrhosis (Table I).

The expression of fibrosis associated antigen, GFAP, was increased 2-fold in the presence of the genotype 1a HCV replicon, which is very similar to the value observed using real time

PCR analysis of GFAP RNA in the presence of the genotype 1b replicon (Figure 4B). The GFAP data, across multiple HCV genotypes, and different methods of analysis, argue for consistency of the observations of effect of HCV on cell matrix. TGFβ is a known modulator of GFAP in mature glial cells of the CNS, and the major mediator of fibrogenesis in Hepatic Stellate Cells. Surprisingly, TGFβ1, TGFβ2 and TGFβ3 were all down regulated in the presence of HCV replicon, compared to Huh7.5 controls. However, several of the other members of TGFβ super family (CAV1, ENG, INHBE, TGIF1, THBS3 and NDRG4) showed a significant increase in expression in the presence of HCV.

4. Discussion

Why fibrosis and cirrhosis are variable in chronic hepatitis C is unknown. The present study 1) describes a significant relationship between HCV replication and cirrhosis antigen expression *in vivo*, 2) focused on the effect of 3 different HCV genotypes (1a, 1b and 2a) on expression of the liver cirrhosis-associated antigen Glial Fibrillary Acidic Protein (GFAP), and 3) also examined direct effects of hepatitis C virus on 153 cellular genes that contribute to the extracellular matrix (ECM). The data indicate that HCV induces dramatic change in collagens, as well as some known mediators of fibrogenesis, including the cirrhosis antigens GFAP and Smooth Muscle Actin (data not shown). Focus on GFAP revealed two distinct pathways: one TGFbeta dependent, and the other, TGFbeta independent, mediated by two different HCV proteins, core and NS5A. Independent modulation of GFAP expression by two different HCV proteins, via different mechanisms, implies an important function. Two possible implications of the results are: 1) That specific cell surface components play an important role the HCV life cycle; and 2) That HCV may directly accelerate fibrogenesis from within hepatocytes, since many of the molecules induced are profibrogenic in experimental models.

The ECM is a complex molecular network that helps determine the specific architecture of a tissue. During hepatic fibrogenesis, major changes occur in both the quantity and quality of hepatic ECM (*for review*, *see ref* (37)). Increase in abundance of collagen molecules is the major hallmark of liver fibrosis (6). In the normal liver, the sub-endothelial space of Disse contains both an interstitial and a basement membrane-like ECM of low density. The perisinusoidal matrix is composed of fibrillar collagen types I, III, and V, microfibrillar collagen VI, basement membrane collagens IV and XVIII, traces of FACIT collagens XIV, and small proteoglycans decorin, fibronectin, tensacin-c, laminin and others (36). As the liver becomes fibrotic, significant qualitative and quantitative changes of the ECM occur, predominantly in the periportal and perisinusoidal space, while the total content of collagens and noncollagenous components increases up to tenfold (34). Thus, the perisinusoidal low-density ECM is transformed to a high-density matrix characterized by accumulation of bundles of collagen fibrils and an electron-dense basement membrane.

In the advanced stages of liver fibrosis, Collagens I and II are concentrated in the ECM, to levels elevated 6-fold compared to normal states (6). In our present study, Collagen II A1 mRNA was upregulated 100-fold, while Collagens XII A1 and XIV A1 mRNAs, implicated in stabilizing collagen fibril structure during development and remodeling (24, 47), were up-regulated 3.2 and 53 fold, respectively (Table III). The mRNA for lysyl oxidase (LOX), required for crosslinking of collagens (2, 41), was also upregulated, by 3.2 fold (Table I).

Thus, the effect of HCV on hepatoma cell ECM resembled that observed during liver fibrogenesis. However, the results need to be confirmed in normal liver.

Thrombospondins (THBS3 and THBS4) and chemokines (CXCL10 and CXCL6) were upregulated in Replicon cell line. IGFBP2 gene expression was also significantly upregulated (Table I). IGFBP2 expression is increased in HBV associated HCC (19). In this study, THBS1, TIMP1, TIMP2, TIMP4 were down regulated. Laminin is the main type of adhesive ECM and is associated with the formation of Basement membrane in liver during cirrhosis. Our study shows that LAMB3, LAMC1 and LAMC2 were increased in Replicon cell line (Table I).

The topic of HCV and the cell surface is of high interest, from a receptor standpoint, since the mechanism of HCV infection is rather unique (26). Recent studies have indicated an alternative, claudin-mediated pathway of direct spread of HCV from cell to cell, without an extracellular viral stage (8). Of interest, claudin 6 was significantly upregulated (8.4 fold) (data not shown) in our study, but the significance towards HCV infection dynamics was not assessed.

HCV involvement in hepatic fibrogenesis, through a direct effect on Hepatic Stellate Cells (HSCs), has previously been suggested (39). In separate studies, HCV core isolated from hepatocellular carcinoma cells *ex vivo* was capable of shifting TGF-β responses from cytostatic effects, toward Epidermal Mesenchymal Transition (EMT), in primary mouse or human hepatocytes (4, 33). The present study is the first to describe a potential fibrogenic effect of HCV on hepatocyte-derived hepatoma cells, and potential role of TGFbeta in this effect. Natural variation in HCV core interaction with the TGFbeta pathway has been implicated in liver oncogenesis (4, 33). In the present study, although TGFβ1 was down regulated in Replicon 1a cells, several other genes which interact with TGFβ were upregulated, such as FBN1 (Fibrillin 1) LOXL1, NDRG4, Collagen type XVI type, and several other transcription factors including HOXD1, HOXC10 and CAND2. Since TGFbeta is a pluripotent master regulator of many processes, including injury repair, the overall effect of HCV on TGFbeta regulated mechanisms, and visa versa, needs to be determined.

Induction of GFAP by NS5A, via a TGFbeta-independent mechanism, is a novel finding of the present study. Modulation of cellular gene expression is a well-known function of NS5A (26), and NS5A has been reported to be a negative modulator of the TGF-beta1 signal transduction pathway (11); reduced phosphorylation and nuclear translocation of Smad2, as well as reduced heterodimerization of Smad3 with Smad4, were both observed in the presence of NS5A, and were apparently mediated via direct binding of NS5A to the TGF-beta1 receptor, TbetaR1. Whether or not the NS5A effect was mediated by a soluble factor was not assessed for the present study, and requires further investigation. In the CNS, GFAP is induced by both TGFbeta dependent and independent mechanisms (27), and mediators of TGFbeta independent induction of GFAP have yet to be defined. Determining the mechanism of GFAP induction by NS5A, and determining the overall significance to liver infection by HCV, are important goals of future research.

The present results also raise a diagnostic question: the possibility that increases in GFAP positivity with advanced hepatitis C liver disease may in fact be due to HCV infection of hepatocytes. Evidence for this in the present study included 1) co-localization of GFAP signal with either core or NS5A protein in hepatoma cell culture following gene

transfection (in addition to diffuse GFAP signal in cells neighboring core-expressing cells); 2) high correlation between percentage of cells harboring GFAP and HCV replicative intermediate (RI) RNA in serial sections from liver biopsies of HCV-infected study subjects; and 3) high correlation between both markers and degree of hepatic fibrosis in the same liver biopsies. Other lines of human investigation have reveled highly significant associations between HCV genotype 1 infection virology in humans, and hepatitis C disease severity. Both iatrogenic and viral induced immune suppression dramatically accelerates rates and incidence of hepatitis C progression (15, 40). Longitudinal studies have identified genetic evidence of selective outgrowth of high fitness viral variants during disease progression (20, 21). Finally, as alluded to above, intrahepatic HCV replication, as well as nonstructural antigen expression, have been highly correlated with hepatitis C liver disease severity, in the settings of liver transplantation (10, 16), natural infection, and HIV coinfection (31).

These findings collectively support an intriguing hypothesis, that fibrogenesis in hepatitis C may in part be mediated by fibrogenic-like transitions of infected hepatocytes.

In particular, the study draws attention to major cytoskeletal effects of HCV on hepatoma cells, and suggests that such effect may potentially represent a pro-fibrogenic response. The study implicates two HCV proteins as potential mediators of the cytoskeletal changes: HCV core and NS5A. Questions as to the relevance and consequences of HCV modulation of the extracellular matrix remain to be addressed. The potential significance of the observed interaction to HCV life cycle, also needs further investigation.

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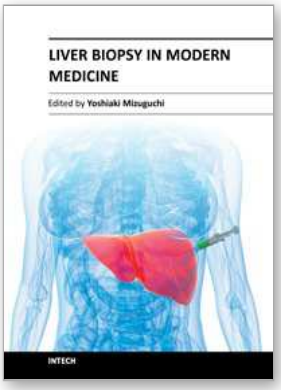

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Liver biopsy, first performed by Paul Ehrlich in 1883, remains an important diagnostic procedure for the management of hepatobiliary disorders and the candidate/donated organ for transplantation. The book "Liver biopsy in Modern Medicine" comprises 21 chapters covering the various aspects of the biopsy procedure in detail and provides an up-to-date insightful coverage to the recent advances in the management of the various disorders with liver biospy. This book will keep up with cutting edge understanding of liver biopsy to many clinicians, physicians, scientists, pharmaceutics, engineers and other experts in a wide variety of different disciplines.

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