

## Hepatitis C Virus Infection in Patients is Associated With C/EBP $\beta$ -Thr266 Phosphorylation and Hepatocyte Proliferation

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### ABSTRACT

**AIM:** Hepatitis C virus (HCV) infection in patients induces hepatocyte proliferation and also hepatocellular carcinoma. The mechanisms and the liver acinar distribution of the HCV infection remain unclear. The aim of this study was to determine the liver acinar localization of the infectious HCV and whether HCV infection is associated with the expression of proteins known to modulate hepatocyte proliferation.

**METHODS:** We analyzed normal ( $n = 6$ ), HCV genotype 1-infected non cirrhotic ( $n = 6$ ) and HCV genotype 1-infected cirrhotic liver samples ( $n = 6$ ). We performed immunofluorescent studies using antibodies against HCV Core, Glutamine synthetase (as a marker of hepatic acinar zone-3 in normal livers); phosphorylated C/EBP $\beta$ -Thr266 (since it is required for Transforming Growth Factor $\alpha$ - and Hepatic Growth Factor - induced hepatocyte proliferation) and ki-67 (as an indicator of hepatocyte proliferation). Also, we analyzed by QRT-PCR a proliferation microarray to compare the expression of genes associated with cell proliferation in HCV-infected patients.

**RESULTS:** In patients with HCV infection, the HCV Core protein was preferentially co-localized with hepatocytes expressing Glutamine synthetase, phosphorylated C/EBP $\beta$ -Thr266, HIF-1 $\alpha$  and  $\beta$ -Catenin. As expected, phosphorylated C/EBP $\beta$ -Thr266 was associated with hepatocyte proliferation in these patients. HCV infection markedly increased hepatocyte proliferation.

**CONCLUSION:** This study demonstrates that HCV infection is preferentially localized to an expanded acinar zone expressing GS, where enhanced hepatocyte proliferation occurs in association with phosphorylated C/EBP $\beta$ -Thr266. A better understanding of the mechanisms of HCV infection may facilitate additional studies and potential therapeutic interventions.

**Key words:** C/EBP $\beta$ Phosphorylation; Hepatitis C Virus Infection; Hepatocyte Proliferation; Liver Zonation; HIF-1 $\alpha$ ;  $\beta$ -Catenin

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### Abbreviations

HCV: Hepatitis C Virus

GS: Glutamine Synthetase

C/EBP: CCAAT/Enhancer Binding Protein

HIF: Hypoxia Inducible Factor

APC: Adenoma Polyposis Coli

TGF: Transforming Growth Factor

HGF: Hepatic Growth Factor

MAPK: Mitogen Activated Protein Kinase

PRKCA: Protein kinase C, Alpha

RBL2: Retinoblastoma-Like 2 (p130)

AKT2: V-akt murine thymoma viral oncogene homolog 2

MYD88: Myeloid differentiation primary response gene-88  
 Thr: Threonine  
 QRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction  
 IHC: Immunohistochemistry  
 HCC: Hepatocellular Carcinoma

## INTRODUCTION

An estimated 170 million individuals have chronic hepatitis C virus (HCV) infection worldwide<sup>[1]</sup>. About 70% of infected individuals develop a chronic infection; for some, this includes fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)<sup>[2,3]</sup>. Transgenic mouse lines in which HCV core protein is expressed constitutively in the liver at cellular levels similar to those found in chronic HCV-infected patients develop HCC, indicating that HCV core protein participates in HCV-related hepato-carcinogenesis<sup>[4]</sup>. The HCV core protein has been implicated in hepatocyte proliferation in the HCV core protein transgenic mice<sup>[4]</sup>.

Although it has been suggested that hepatocyte proliferation correlates with HCV-induced liver inflammation and requires PPAR $\alpha$  expression<sup>[4]</sup>, the precise mechanisms remain unknown. Further, hepatocyte proliferation rate is a good predictor of HCC development in patients with liver cirrhosis including those infected with HCV<sup>[5]</sup>. In this context, we have shown that TGF $\alpha$  induces phosphorylation of mouse C/EBP $\beta$  on Thr217 as well as hepatocyte proliferation<sup>[6]</sup>. The C/EBP $\beta$ -Thr217 phosphoacceptor is highly conserved through evolution<sup>[6,7]</sup>. We reported that expression of a catalytically inactive mutant RSK, which behaves as a dominant negative, blocks hepatocyte proliferation induced by TGF $\alpha$ , indicating that RSK activity is important for this effect<sup>[6]</sup>.

Phosphorylation of C/EBP $\beta$  on Thr217 is required for the stimulation of hepatocyte proliferation by TGF $\alpha$  since hepatocytes expressing a dominant negative, nonphosphorylatable C/EBP $\beta$ -Ala217 mutant, lacking the critical phosphoacceptor, are refractory to the stimulation of hepatocyte proliferation by TGF $\alpha$ <sup>[6]</sup>. Also, expression of the dominant positive, phosphorylation-mimic C/EBP $\beta$  Glu217 transgene was sufficient to induce hepatocyte proliferation in the absence of TGF $\alpha$ <sup>[6]</sup>. Thus, C/EBP $\beta$  PhosphoThr217 plays an active role in inducing hepatocyte proliferation after treatment with TGF $\alpha$ .

Liver zonation modulates many metabolic functions<sup>[8]</sup>. Zone-3 hepatocytes are characterized by the expression of Glutamine Synthetase (GS),  $\beta$ -Catenin and Hypoxia-Inducible Factor (HIF)-1<sup>[9-12]</sup>. Expression of the GS gene in liver acinar zone-3 hepatocytes is stimulated by C/EBP $\beta$ <sup>[13]</sup>. In this study we asked whether HCV infection in patients is associated with increased C/EBP $\beta$ -Thr266 phosphorylation (the human homologue phosphoacceptor) and whether it is also associated with hepatocyte proliferation and a zone-3 phenotype, as we reported in cultured human hepatocytes<sup>[14]</sup> using the HCV-infected primary human hepatocyte culture system that we developed<sup>[15]</sup>.

The primary aim was to assess whether the HCV infection in humans induces proliferation of infected hepatocytes and whether this is associated with the expression of phosphorylated-C/EBP $\beta$ -Thr266. A secondary aim was to analyze whether HCV infection also modulates liver zonation. An exploratory aim was to investigate whether a genetic proliferative profile is induced by the HCV infection.

## METHODS

### Ethical approval

Research Ethics Committee approval was obtained to utilize archival de-identified liver biopsies. We obtained anonymous, de-identified

liver samples from the University of California, San Diego and control subjects without liver disease from the NDRI. The protocol was approved by the University of San Diego, San Diego Human Protection Program. Because all these samples were excess, standard of care and archival samples it was an exempted, non-consented IRB approved protocol. The study was performed according to the World Medical Association Declaration of Helsinki <http://www.wma.net/e/policy/b3.htm>.

### Liver Samples

A total of 12 archival de-identified liver biopsies from HCV genotype 1-infected patients were included. Also, a total of 6 archival de-identified liver biopsies from healthy subjects (age 45 to 71) without any significant medical history or liver disease were included as controls. The demographics and clinical characteristics of the subjects with chronic HCV infection are shown in Table 1.

Research Ethics Committee approval was obtained for use of all clinical material. Six uninfected healthy control liver samples were obtained from the National Disease Research Interchange Repository. Exclusion criteria were other liver diseases and alcohol abuse.

### Microscopy Analysis

Fluorescent labels were observed using a quadruple channel fluorescent microscope<sup>[6,7,14,15]</sup>. Fluorochromes utilized were Alexa 488, 750, 350, 647, and 594. The expression of GS, phosphorylated-C/EBP $\beta$ -Thr266 and hepatocyte proliferation (ki-67) markers were determined and quantified in liver specimens. The number of positive cells was determined among those expressing the indicator protein. These values are reported as fold-increase from control samples. At least 100 cells were analyzed per experimental point<sup>[11,12]</sup>. These counts were performed with the tissue counting software from Keyence BZ-9000.

### Microarray Analysis

We utilized the PI3Kinase-AKT Signaling PCR Proliferation Array (Qiagen) to analyze hepatocyte proliferation, which consist of 90 genes, as we reported for q-PCR<sup>[7,15]</sup>. RNA was isolated from paraffin sections of liver samples of similar tissue age (procured within 6 months) and preserved in an identical manner.

This array contains a panel of proprietary controls to monitor genomic DNA contamination (GDC) as well as the first strand synthesis (RTC) and real-time PCR efficiency (PPC). The specificity of each gene amplification is guaranteed by the RT<sup>2</sup> SYBR Green PCR Array System (Qiagen).

**Table 1** Demographic and clinical characteristics of the HCV-infected subjects.

	Non-cirrhotic (mean +/- SD)	Cirrhotic (mean +/- SD)	P value
Number	6	6	NS
Gender (male)	6	5	NS
Age	56.2 (6.4)	58.6 (3.6)	0.2215
AST	85.0 (73.9)	114.7 (83.4)	0.2628
ALT	105.2 (82.9)	151.5 (106.7)	0.2037
Albumin	4.1 (0.2)	3.9 (0.4)	0.2862
Total bilirubin	0.5 (0.1)	0.7 (0.3)	0.1752
INR	1.0 (0.1)	1.1 (0.2)	0.2862
Platelets ( $\times 10^6$ )	188.2 (54.5)	133.5 (51.5)	0.1038
HCV GT-1	100%	100%	NS
HCV VL ( $\times 10^6$ )	4.1 (4.3)	3.5 (3.9)	0.8044
Liver Fibrosis (F = 0-6)	1.9 (0.8)	5.8 (0.4)	0.0051
Liver Necro-inflammation (A = 0-18)	3.7 (1.4)	9.0 (3.8)	0.0105

The differences between the non-cirrhotic and cirrhotic subjects were analyzed by the t-test for parametric data and with the U-test for non-parametric data.

### Statistical analyses

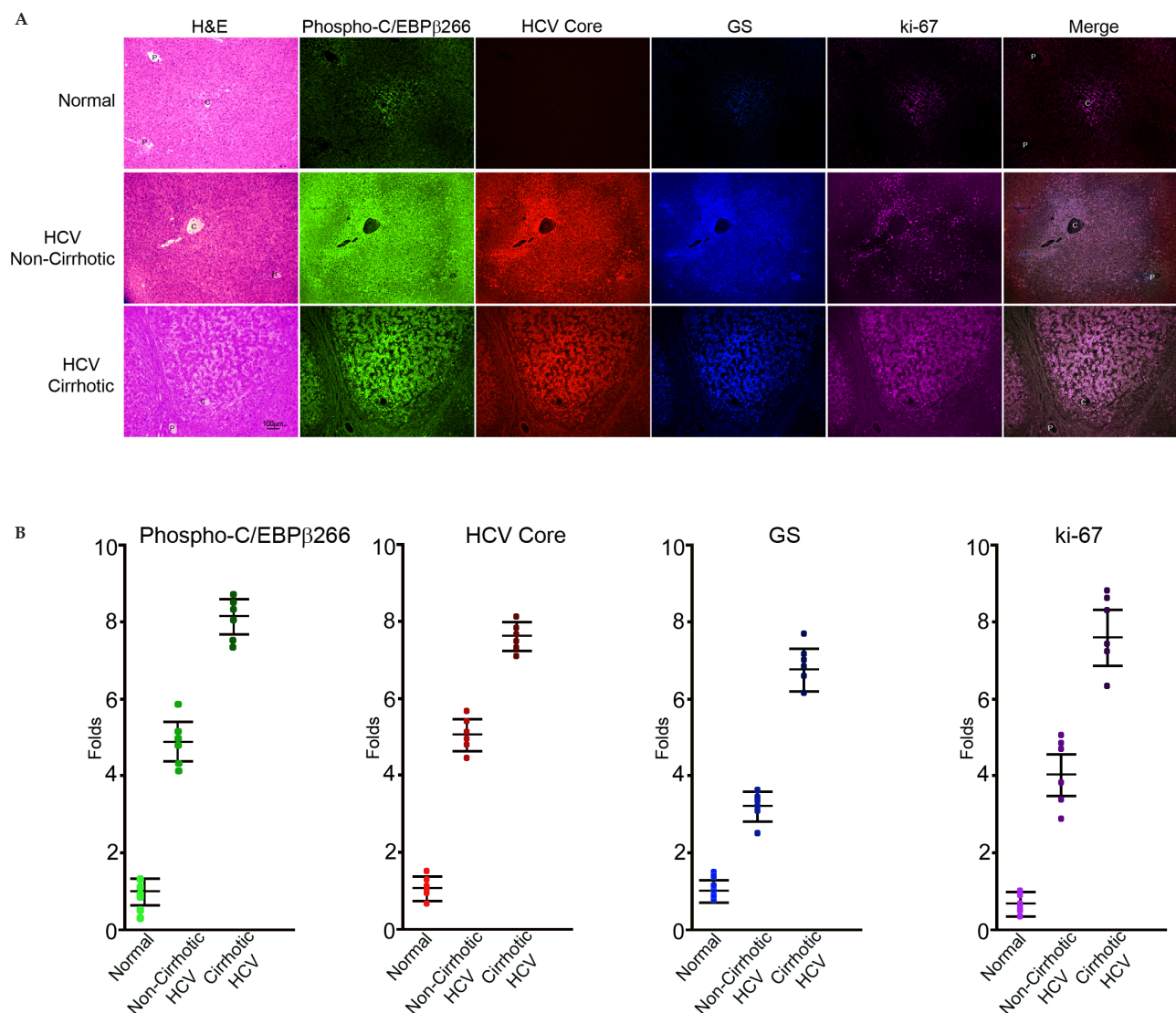
The endpoints were assessed with an unpaired-sample t-test (two-tails) for parametric data and the U-test (two-tails) for non-parametric data. The significance level was fixed at  $\alpha = 5\%$  for all tests. All analyses were carried using R version 2.12.2 (2011 Vienna, Austria; <http://www.R-project.org>).

## RESULTS

We studied liver specimens from 12 patients with chronic HCV infection (6 without cirrhosis and 6 with cirrhosis) and from 6 healthy control individuals without liver disease. The demographic and clinical parameters were not statistically significant between non-cirrhotic and cirrhotic subjects (Table 1). As expected, there was a statistically sig-

nificant difference between non-cirrhotic and cirrhotic subjects in the liver biopsy stage (F;  $1.9 \pm 0.8$  vs  $5.8 \pm 0.4$ ;  $p < 0.0051$ ) and the necro-inflammation score (A;  $3.7 \pm 1.4$  vs  $9.0 \pm 3.8$ ;  $p < 0.0105$ ) (Table 1).

As expected, the normal liver showed a classical zone-3 limited to the immediate hepatocytes surrounding the terminal hepatic venule with a similar restricted area of hepatocyte proliferation and a minimal expression of phosphorylated-C/EBP $\beta$ -Thr266 (Figure 1A). In contrast, we have found that patients with chronic HCV infection have a dramatically enlarged liver acinar expression of GS around the terminal hepatic venules<sup>[9]</sup> (Figure 1A). The HCV core protein was identified preferentially in hepatocytes also expressing GS in HCV non-cirrhotic patients (Figure 1A). Similarly, expression of phosphorylated-C/EBP $\beta$ -Thr266 (the human homologous phosphoacceptor of mouse Thr217) was identified in hepatocytes expressing both HCV



**Figure 1** Chronic HCV infection is associated with an enlarged liver acinar expression of phospho-C/EBP $\beta$ -Thr266 and hepatocyte proliferation. A: Low magnification ( $10 \times$ ) H&E histochemistry shows the structure of representative liver samples (P = portal triad; C = central hepatic venule). The scale bar represents 100  $\mu$ m. The HCV core protein was expressed preferentially in zone-3 hepatocytes (indicated by the expression of GS) in livers of HCV non-cirrhotic patients. Similarly, expression of phosphorylated-C/EBP $\beta$ -Thr266 was identified in zone-3 hepatocytes. Hepatocyte proliferation (as determined by the expression of ki-67) was mainly circumscribed to peri-central hepatic venule zone hepatocytes in patients with chronic HCV infection. These effects of chronic HCV infection on peri-central hepatic venule zone expansion and hepatocyte proliferation were more overt among cirrhotic patients compared to chronic HCV infected non-cirrhotic patients. In contrast, the normal liver showed a classical zone-3 proliferation and a minimal expression of phosphorylated-C/EBP $\beta$ -Thr266. B: Quantitative confocal microscopy was performed analyzing liver specimens containing at least 10 portal triads as described previously<sup>[16]</sup> for phospho-C/EBP $\beta$ -Thr266, HCV Core, GS and ki-67 ( $p < 0.001$  for non-cirrhotic and  $p < 0.0001$  for cirrhotic *vs* controls). Individual measurements, mean and SD are shown for phospho-C/EBP $\beta$ -Thr266, HCV Core, GS and ki-67.

core protein and GS (Figure 1A). As expected from the expression pattern of phosphorylated-C/EBP $\beta$ -Thr266, hepatocyte proliferation (as determined by the expression of ki-67) was mainly circumscribed to hepatocytes expressing phosphorylated-C/EBP $\beta$ -Thr266 in patients with chronic HCV infection (Figure 1A). These novel effects of chronic HCV infection on the expansion of GS expression and of hepatocyte proliferation were more overt among cirrhotic patients compared to chronic HCV infected non-cirrhotic patients. Omission of the primary antibodies resulted in undetectable fluorescence (data not shown).

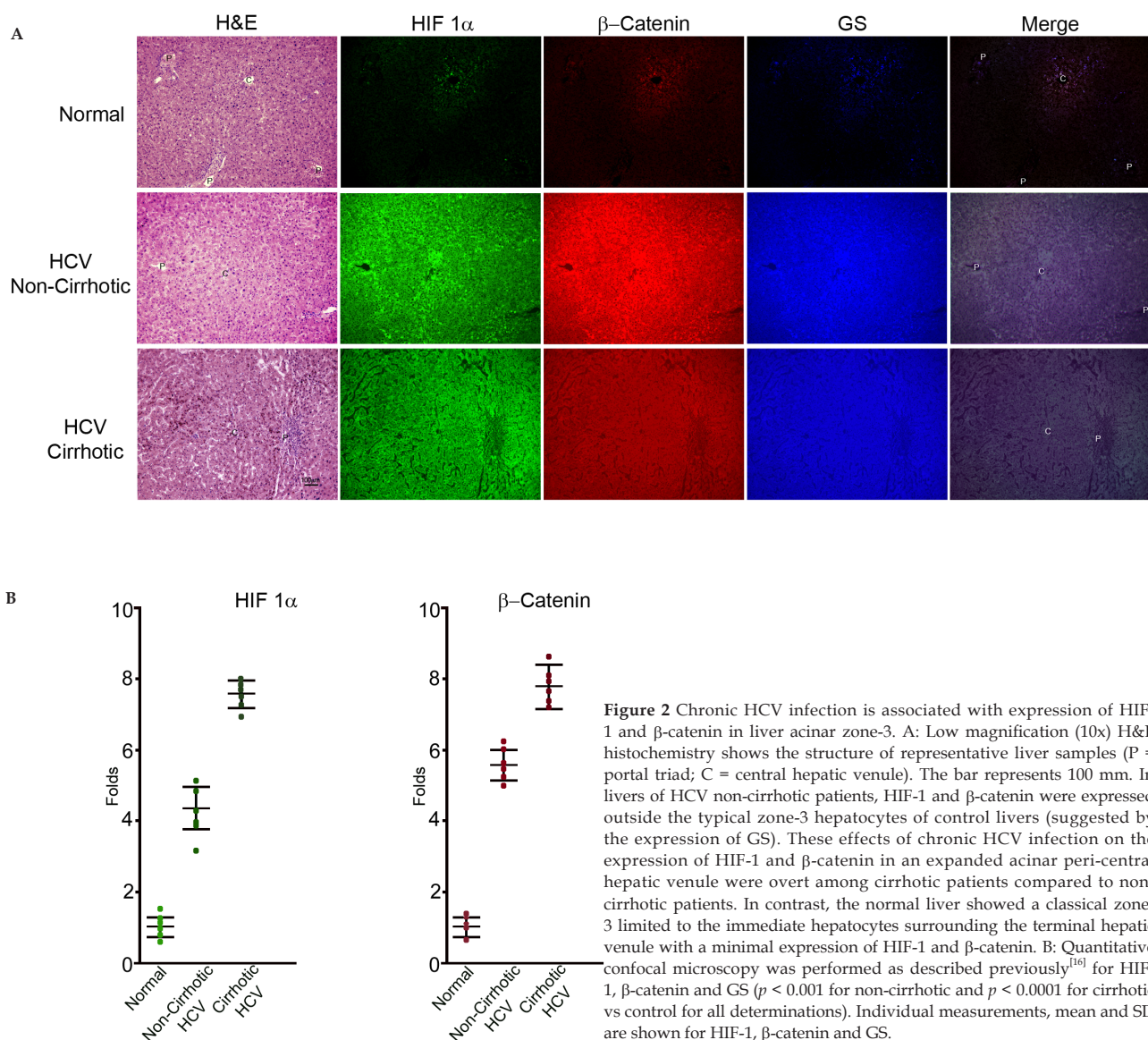
The expression of phospho-C/EBP $\beta$ -Thr266, HCV core protein, GS and ki-67 was increased 3-fold to 8-fold in HCV-infected patients ( $p < 0.001$  for non-cirrhotic and  $p < 0.0001$  for cirrhotic vs normal for all determinations) (Figure 1B), as measured by quantitative confocal microscopy performed as described previously<sup>[16]</sup>.

APC and  $\beta$ -Catenin modulate the liver acinar zonation and HIF-1 $\alpha$  is induced by hypoxia<sup>[10-12]</sup>. Therefore, we analyzed whether HCV infection affects the expression of  $\beta$ -Catenin in zone-3 and whether the HCV infection affects the predictable expression of HIF-1 $\alpha$  in the hypoxic acinar zone-3<sup>[10-12]</sup>. We found that both  $\beta$ -Catenin and HIF-1 $\alpha$  were expressed in a small acinar zone-3 in the liver from healthy

controls (Figure 2A). HCV infections expand the expression of  $\beta$ -Catenin and HIF-1 $\alpha$  in non-cirrhotic livers and to a larger extent in cirrhotic livers (Figure 2A and 2B). The expression of  $\beta$ -Catenin and HIF-1 $\alpha$  was increased 4-fold to 8-fold in HCV-infected patients ( $p < 0.001$  for non-cirrhotic and  $p < 0.0001$  for cirrhotic vs normal for all determinations).

We used the PI3Kinase-AKT Signaling PCR Proliferation Array, which consists of 90 genes, to analyze hepatocyte proliferation. The cell proliferation microarray assay showed a substantial increase in the expression of selective genes {PRKCA (Protein kinase C, alpha); FOS; RBL2 [Retinoblastoma-like 2 (p130)]; AKT2 (V-akt murine thymoma viral oncogene homolog 2); and MYD88 (Myeloid differentiation primary response gene-88)} among chronic HCV infected non-cirrhotic patients compared to control subjects (Figure 3A).

In addition, there was a substantial increase in the expression of some genes {PRKCA [Protein kinase C, alpha]; PDGFRA [Platelet-derived growth factor receptor, alpha polypeptide]; ILK [Integrin-linked kinase]; BAD [BCL2-associated agonist of cell death]; and RPLPO [Ribosomal protein, large, PO]} among chronic HCV infected cirrhotic patients compared to HCV-infected non-cirrhotic patients (Figure 3B).



In agreement with the immuno-histochemical studies for ki-67 (Figure 1) and  $\beta$ -Catenin (Figure 2) expression, these microarray findings provide a plausible explanation for the greater hepatocyte proliferation among HCV-infected cirrhotic patients when compared to HCV-infected non-cirrhotic patients, and perhaps, for their proclivity for HCC<sup>[2-4]</sup>.

## DISCUSSION

In this study, we found that HCV infection (as determined by HCV Core protein expression) is preferentially localized to liver acinar hepatocytes also expressing GS (a marker of zone-3 in normal livers) by immunofluorescent confocal microscopic analysis of liver biopsies (Figure 1A). The HCV infection expanded several-fold the liver acinar expression of GS compared to healthy, control liver biopsies. It remains to be determined whether zone-3 hepatocyte metabolic profile is critical for HCV survival and eventually, what are the mechanisms involved.

Further, HCV infection induced hepatocyte proliferation within the acinar hepatocytes expressing GS, as detected with ki67 immunofluorescent confocal microscopy analysis. The hepatocyte proliferation in this acinar zone increased by ~4-fold in HCV-infected non-cirrhotic patients and by ~8-folds among HCV-infected cirrhotic patients (Figure 1B).

Hepatocyte proliferation induced by the liver growth factors TGF $\alpha$  and HGF is mediated by C/EBP $\beta$ <sup>[6,17,18]</sup>. We have shown that TGF $\alpha$  induces phosphorylation of mouse C/EBP $\beta$  on Thr217 (the exact human homologue phosphoacceptor is C/EBP $\beta$ -Thr266) as well as proliferation of mouse primary hepatocyte cultures<sup>[6]</sup>. Also, expression of the phosphorylation-mimic C/EBP $\beta$  Glu217 transgene in mouse primary hepatocyte cultures was sufficient to induce their proliferation in the absence of a hepatocyte growth factor<sup>[6]</sup>.

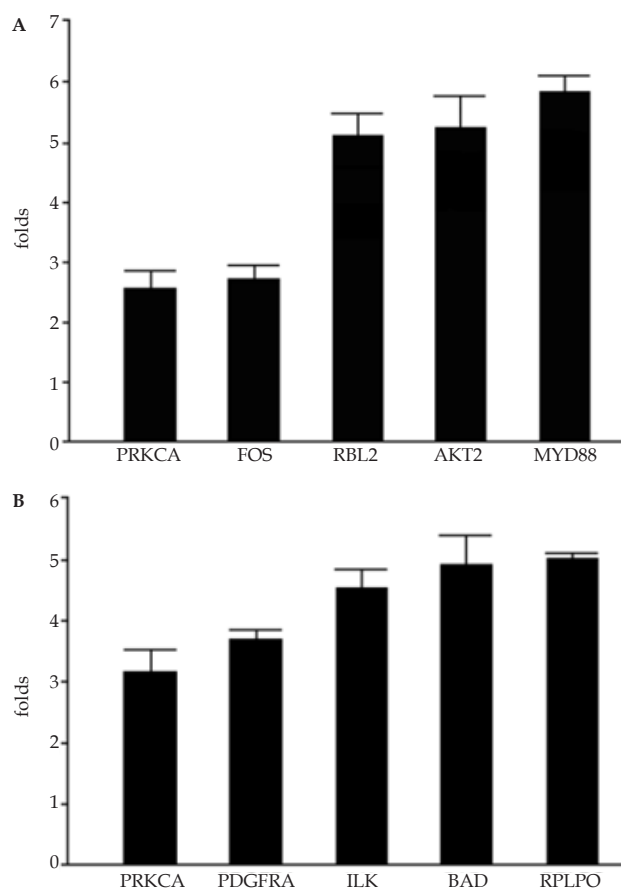
Similarly, HGF induces ~6-fold higher hepatocyte proliferation in C/EBP $\beta$ -wt compared to C/EBP $\beta$ -ko mice, and stimulates ERK1/2 and RSK activation<sup>[18]</sup>, a MAPK signaling pathway that results in the phosphorylation of mouse C/EBP $\beta$ -Thr217 (human Thr266)<sup>[6]</sup>. These data strongly support the hypothesis that phosphorylation of C/EBP $\beta$  on Thr217 (and of human phosphoacceptor Thr266) is critical for the excessive hepatocyte proliferation induced by liver growth factors in cellular and animal models, and probably by chronic HCV infection in patients.

The causality of the HCV-associated expansion of acinar zone expressing GS, phosphorylation of C/EBP $\beta$  Thr266,  $\beta$ -Catenin and HIF-1 $\alpha$ , as well as hepatocyte proliferation cannot be currently established in a human study. However, using a human primary hepatocyte culture system that allows efficient infection with intact HCV virions<sup>[15]</sup>, and by blocking C/EBP $\beta$  Thr266 phosphorylation with a designed inhibitory dominant negative peptide<sup>[7]</sup>, we were able to demonstrate a causal effect of C/EBP $\beta$  Thr266 phosphorylation on hepatocyte proliferation and the zone-3 phenotype<sup>[14]</sup>.

As previously reported<sup>[10-12]</sup>, we found that both  $\beta$ -Catenin and HIF-1 $\alpha$  were expressed in a small acinar zone-3 in the liver from healthy controls (Figure 2A). We have determined that HCV infections expand the expression of  $\beta$ -Catenin and HIF-1 $\alpha$  in non-cirrhotic livers and to a larger degree in cirrhotic livers (Figures 2A and 2B).

Although  $\beta$ -Catenin was increased in HCV-infected patients in acinar hepatocytes expressing GS, as expected the  $\beta$ -Catenin mRNA was not increased since  $\beta$ -Catenin expression is regulated post-translationally by  $\beta$ -Catenin protein ubiquitination<sup>[10]</sup>.

Several genes which are critical for cell proliferation were induced in HCV-infected livers (Figure 3A and 3B). Wnt/ $\beta$ -Catenin signaling



**Figure 3** Cell proliferation array in HCV-infected patients. A: Increase in the expression of PRKCA; FOS; RBL2; AKT2; and MYD88 among chronic HCV infected non-cirrhotic patients compared to control subjects. B: Increase in the expression of PRKCA; PDGFRA; ILK; BAD; and RPLPO among chronic HCV infected cirrhotic patients compared to HCV-infected non-cirrhotic patients. ( $p < 0.01$  for non-cirrhotic patients *vs* normal individuals, and  $p < 0.01$  for cirrhotic *vs.* non-cirrhotic patients for all determinations).

pathways stimulate the expression of Protein kinase C,  $\alpha$ <sup>[19]</sup> and in turn Retinoblastoma-like 2 (p130) modulates, together with APC, the nuclear localization of  $\beta$ -Catenin<sup>[20]</sup>. Further, Integrin-linked kinase enhances  $\beta$ -Catenin activity<sup>[21]</sup>. In addition, Rsk-2 kinase induces both FOS expression<sup>[22]</sup> and the phosphorylation of C/EBP $\beta$ -Thr217 (human Thr266)<sup>[6]</sup>, while phospho-C/EBP $\beta$ -Thr217 increases the expression of MYD-88<sup>[23]</sup>.

This coordinated regulation of proliferation genes by  $\beta$ -Catenin and phospho-C/EBP $\beta$ -Thr266 indicates that HCV-infection probably stimulates hepatocyte proliferation through these molecular mechanisms.

The Wnt/ $\beta$ -catenin signaling pathway modulates cell proliferation and when over-activated it can stimulate carcinogenesis<sup>[24]</sup>. Of great interest in liver physiology, hypoxia (as found in zone 3) is intimately related to oxidative stress (a condition that we have shown to induce cell proliferation and phosphorylation of mouse C/EBP $\beta$ -Thr217 (exact homologue of human Thr266)<sup>[6,25]</sup>. Indeed, the genetic disruption of the HIF-Prolyl Hydroxylase gene in hypoxic mice (by allowing HIF-1 $\alpha$  activation) lowers oxygen consumption in the mitochondria, reduces oxidative stress, and eventually enhances cellular survival<sup>[26]</sup>.

More conclusive evidence of an acinar zone expressing GS as a 'hot spot' for hepatocyte proliferation and tumorigenesis will require animal models with dominant negative and dominant

positive transgenes for the C/EBP $\beta$  Thr266 phosphorylation site<sup>[6]</sup>. It remains to be established whether  $\beta$ -Catenin and C/EBP $\beta$  Thr266 phosphorylation act synergistically in inducing hepatocyte proliferation in hepatocytes expressing GS, as well as tumorigenesis in chronic HCV infection.

It remains to be investigated whether other inducers of hepatocyte proliferation and tumorigenesis (e.g., chronic Hepatitis B viral infection) also activate C/EBP $\beta$  Thr266 phosphorylation and hepatocyte proliferation in hepatocytes expressing GS.

The mechanisms regulating the expression of C/EBP $\beta$  Thr266 phosphorylation almost exclusively in acinar zone 3 in normal livers are unknown but may involve the selective activation of signaling pathways in zone 3 or the presence of a phosphatase activity that unphosphorylates C/EBP $\beta$  Thr266 in acinar zones 1 and 2.

Collectively, these data suggest that HCV infection may induce hepatocyte proliferation and an expanded acinar zone expressing GS, which may provide an environmental advantage for HCV replication.

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### Competing interests

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### Author's Contributions

Martina Buck and Mario Chojkier participated in the concept and design of the study, and writing the manuscript. Martina Buck supervised the execution of the experimental data. Daniela Traykova and Martina Buck performed all the experiments. Mario Chojkier obtained the clinical specimens. Martina Buck and Mario Chojkier performed the statistical analysis, and interpreted all the data.

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