Abstract
Liver acinar zonation allows for diverse functions to be performed on demand by specialized hepatocytes. Therefore, understanding how hepatitis C virus (HCV) infection may modulate the acinar zone phenotypes should provide insights into the pathophysiology of HCV infection. We assessed the effects HCV infection in the highly-differentiated primary human hepatocyte culture that we have developed for the efficient replication of intact HCV virions.

We found that HCV genotype 1 infection stimulated the zone-3 phenotype after 72 hr since the cultured hepatocytes expressed GS, β-Catenin and HIF-1α, markers of zone-3 hepatocytes. HCV infection also induced phosphorylated-C/EBPβ-Thr266 and proliferation in these cultured hepatocytes. A peptide designed to selectively inhibit phosphorylation of C/EBPβ-Thr266 blocked this phosphorylation, the zone-3 phenotype and hepatocyte proliferation induced by HCV infection in these cultured human hepatocytes. In addition, we found that in normal, uninfected human hepatocytes, unphosphorylated-C/EBPβ-Thr266 was associated with protein von Hippel Lindau (pVHL) and Axin (inhibitors of HIF-1α and β-catenin activation, respectively). These associations were blocked by the HCV infection since phosphorylated C/EBPβ-Thr266 was not associated with either pVHL or Axin. The inhibition of C/EBPβ-Thr266 phosphorylation normalized the association of unphosphorylatable C/EBPβ-Thr266 with pVHL and Axin, which is expected to inhibit the activation of HIF-1α and β-catenin. Collectively, these results strongly suggest that C/EBPβ-Thr266 phosphorylation is indispensable for the activation of GS, HIF-1α and β-catenin, and the induction of zone-3 phenotype in HCV-infected human hepatocytes.

Keywords
Human hepatocytes; Hepatitis C; Liver zone-3; C/EBPβ; Glutamine synthetase; HIF-1; β-catenin

Abbreviations
C/EBP: CCAAT/Enhancer Binding Protein; HIF: Hypoxia Inducible Factor; GS: Glutamine Synthetase; HCC: Hepatocellular Carcinoma; pVHL: protein von Hippel Lindau; HCV: Hepatitis C Virus

Introduction
The hepatocytes in different liver acinar zones are under different O2 tension and perform different metabolic activities [1]. Hepatocytes in acinar zone-1 are under normoxic conditions, which stimulate aerobic (mitochondrial oxidative) metabolism. By contrast hepatocytes in acinar zone-3 are exposed to hypoxia, which induces survival by hypoxic/‘anaerobic’ (non-mitochondrial) metabolism. Zone-3 hepatocytes are characterized by the expression of Glutamine Synthetase (GS), β-Catenin and Hypoxia-Inducible Factor (HIF)-1 [2-5]. Expression of the GS gene in zone-3 hepatocytes is stimulated by C/EBPβ [6].

We have shown that TGFα induces phosphorylation of mouse C/EBPβ on Thr217 as well as hepatocyte proliferation [7]. The C/EBPβ-Thr217 phosphoacceptor is highly conserved through evolution [7,8]. We reported that expression of a catalytically inactive mutant RSK, which behaves as a dominant negative, blocks hepatocyte proliferation induced by TGFα, indicating that RSK activity is important for this effect [7].

Phosphorylation of C/EBPβ on Thr217 is required for the stimulation of hepatocyte proliferation by TGFα since hepatocytes expressing a C/EBPβ-Ala217 mutant, lacking the critical phosphoacceptor, are refractory to the stimulation of hepatocyte proliferation by TGFα. Also, expression of the phosphorylation-mimic C/EBPβ−Glu217 transgene was sufficient to induce hepatocyte proliferation in the absence of TGFα. In contrast, expression of the C/EBPβ−Ala217 transgene blocked hepatocyte proliferation in the presence of TGFα [7]. Thus, C/EBPβ PhosphoThr217 plays an active role in inducing hepatocyte proliferation after treatment with TGFα.

In this study we asked whether Hepatitis C Virus infection in cultured human hepatocytes stimulates C/EBPβ-Thr266 phosphorylation (the human homologue phosphoacceptor) and...
whether it is required for hepatocyte proliferation and a zone-3 phenotype.

**Conclusion**

In this study, we assessed the effects HCV infection in the highly-differentiated primary human hepatocyte culture that we have developed for the efficient replication of intact HCV virions. We used primary human hepatocytes (Invitro technologies; Baltimore, MD) cultured on a collagen matrix and infected on day-3 after plating the cells, with serum from a patient with a chronic HCV genotype 1 infection as described previously [9]. The matrix was prepared with 20% fetal calf serum for not more than 18 hr; the hepatocyte-specific media was given for at least 24 hr prior to the HCV infection; the hepatocytes were >85% confluent until the time of infection; and hepatocyte media was changed every 72 hr [9].

This normal human hepatocyte culture system is permissible to the infection with, and physiologically significant amplification of, naturally occurring HCV [9].

We found that HCV genotype 1 infection after 72 hr (viral load ~ 7 x10^6 IU/uL) stimulated a zone-3 phenotype since the cultured hepatocytes expressed HIF-1α and GS [2,5]. HCV infection also induced phosphorylated-C/EBPβ-Thr266 (the human homologous phosphoacceptor [7,8,10]) in these cultured hepatocytes. Control uninfected, normal quiescent cultured human hepatocytes did not express phosphorylated C/EBPβ-Thr266 with pVHL and Axin (Figure 1 and 2). Further, the interaction between β-catenin and HIF-1 promotes cellular adaptation to hypoxia [13].

Collectively, these results strongly suggest that C/EBPβ-Thr266 phosphorylation is indispensable for the activation of GS, HIF-1α and β-catenin, and the induction of zone-3 phenotype in HCV-infected human hepatocytes. These novel findings support the activation of β-catenin and HIF-1α by phosphorylated-C/EBPβ-Thr266.

**Figure 1:** Phosphorylation of human C/EBPβ-Thr266 induced by HCV infection is indispensable for the zone-3 phenotype. Primary human hepatocytes infected with HCV (viral load ~ 7 x10^6 IU/uL after 72) also express phosphorylation of C/EBPβ-Thr266 and a zone-3 phenotype (HIF-1α; GS). Blocking phosphorylation of C/EBPβ-Thr266 with the dominant negative peptide (100 nM for 24 hr) prevents expression of the zone-3 phenotype. Nuclear stain and the merge images are shown.

**Figure 2:** Phosphorylation of human C/EBPβ-Thr266 induced by HCV infection is indispensable for the expression of β-Catenin and hepatocyte proliferation. Experiments were performed as described in Figure 1. Normal or HCV-infected primary human hepatocytes express unphosphorylated C/EBPβ-Thr266. HCV-infected primary human hepatocytes express β-Catenin and a marked increase in zone-3 hepatocyte proliferation (ki67 + hepatocytes). Blocking phosphorylation of C/EBPβ-Thr266 with the dominant negative peptide (100 nM for 24 hr in 72-hr HCV-infected human hepatocytes) prevents expression of the zone-3 β-Catenin and hepatocyte proliferation. Nuclear stain and merge images are shown.
Thr266 in zone-3 and their inhibition by the peptide blocking the zone-3 phenotype.

We hypothesize that the induction of hepatocyte proliferation confers a survival advantage to the HCV infection. The HCV-induced hepatocyte proliferation is mediated in cultured human hepatocytes by C/EBPβ-Thr266 phosphorylation and the adoption of a zone-3 hepatocyte phenotype.

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References