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Hepatitis C virus infection reduces hepatocellular polarity in a vascular endothelial growth factor dependent manner

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Abstract

Background and aims—Hepatitis C virus (HCV) infection leads to progressive liver disease, frequently culminating in fibrosis and hepatocellular carcinoma. The mechanisms underlying liver injury in chronic hepatitis C are poorly understood. This study evaluated the role of vascular endothelial growth factor (VEGF) in hepatocyte polarity and HCV infection.

Methods—We utilized polarized hepatoma cell lines and the recently described infectious HCV JFH-1 cell culture system to study the role of VEGF in regulating hepatoma permeability and HCV infection.

Results—VEGF negatively regulates hepatocellular tight junction (TJ) integrity and cell polarity by a novel VEGF receptor 2 dependent pathway. VEGF reduced hepatoma TJ integrity, induced a re-organization of occludin and promoted HCV entry. Conversely, inhibition of hepatoma expressed VEGF with the receptor kinase inhibitor Sorafenib or with neutralizing anti-VEGF antibodies promoted polarization and inhibited HCV entry, demonstrating an autocrine pathway. HCV infection of primary hepatocytes or hepatoma cell lines promoted VEGF expression and reduced their polarity. Importantly, treatment of HCV infected cells with VEGF inhibitors restored their ability to polarize, demonstrating a VEGF-dependent pathway.

Conclusion—Hepatic polarity is critical to normal liver physiology. HCV infection promotes VEGF expression that depolarizes hepatoma cells, promoting viral transmission and lymphocyte migration into the parenchyma that may promote hepatocyte injury.

Keywords

HCV; VEGF; tropism; HCC; angiogenesis

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Introduction

Hepatitis C virus (HCV), the sole member of the Hepacivirus genus in the Flaviviridae, poses a global health burden with an estimated 170 million infected individuals. The acute phase of infection is often subclinical and the majority of individuals develop persistent infection with progressive liver pathology, frequently culminating in fibrosis and hepatocellular carcinoma (HCC). HCV infection is the leading indication for liver transplantation in many parts of the world. The mechanisms underlying liver injury in HCV infection are poorly understood with two, non-exclusive, models being proposed. The “immunopathogenic” model argues that disease is largely mediated by the host immune response, while the “cytopathic” model suggests that HCV replication and protein expression may induce cell injury¹. The recent discovery that the JFH-1 strain of HCV can replicate and release infectious particles in cultured cells (HCVcc)²⁻⁴ allows studies to assess the effect(s) of virus replication on hepatocellular properties.

HCV has a short positive sense RNA genome encoding three structural, Core, E1 and E2 glycoproteins (gps) and seven non-structural proteins (p7, NS2-NS5)⁵. The E1E2 gps interact with cell surface receptors to facilitate particle entry via low pH and clathrin-dependent endocytosis⁶. Recent evidence suggests that a number of host cell molecules are important for HCV entry: tetraspanin CD81, scavenger receptor class B member I and several members of the tight junction (TJ) protein family including Claudin-1, -6 and -9⁷ and occludin⁸. Recent data from our laboratory demonstrate that hepatoma polarity limits HCV entry, suggesting that agents which disrupt hepatocyte permeability may promote HCV infection⁹.

Vascular endothelial growth factor (VEGF) was originally discovered for its effect(s) on endothelial cell permeability¹⁰. The critical role of VEGF in pathological angiogenesis has lead to the development and clinical testing of VEGF inhibitors to limit tumour growth¹¹. However, recent research suggests a diversity of roles for VEGF in maintaining normal adult tissue^{12,13}. We demonstrate a role for VEGF in regulating hepatocyte TJ integrity, polarity and permissivity to HCV infection. Neutralization of endogenous HepG2 expressed VEGF promotes polarization and significantly inhibits HCV entry, confirming that an autocrine pathway is in operation. HCV infection increases primary hepatocyte and hepatoma VEGF expression, which reduces their polarity. Importantly, VEGF antagonists restore the ability of infected hepatoma cells to polarize. In summary, our data support a model where HCV upregulation of VEGF expression induces a localized disruption of hepatocellular TJs that promotes viral transmission in the liver, providing a potential therapeutic opportunity for the use of VEGF antagonists to treat chronic hepatitis C infection.

Materials and Methods

Cell lines and antibodies

HepG2 and Huh-7.5 cells (C. Rice, Rockefeller University, NY) were propagated in (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% nonessential amino

acids (NEAA). WIF-B9 cells (D. Cassio, Centre National de la Recherche Scientifique, France) were maintained in Coon's F12 media supplemented with 5% FBS, 1% NEAA and hypoxanthine, aminopterin and thymidine. Primary human hepatocytes (PHH) were isolated and cultured as previously reported¹⁴. Liver sinusoidal endothelial cells (LSEC) were cultured in Endothelial Basal Media supplemented with 10% human serum and HGF. All cells were maintained at 37°C, 5% CO₂, with the exception of WIF-B9 cells that require 7% CO₂. HepG2 cells expressing CD81, AcGFP.CD81 and AcGFP.CLDN1 were generated by lentiviral transduction as previously described¹⁵.

The following primary antibodies were used: anti-multi drug resistant protein 2 (MRP2) (M2 III-6; Abcam Cambridge, UK); anti-NS5A 9E10 (C. Rice, Rockefeller University, USA); anti-CD81 (2s131); anti-occludin (Zymed, CA); anti-CLDN1 JAY.8 (Invitrogen, CA); anti-CLDN1 1C5-D9 (Novus, CO) and anti-ZO-1 (Zymed, CA). Secondary labelled antibodies: Alexa 488 goat anti-mouse IgG; Alexa 488 goat anti-rabbit IgG, Alexa 633 goat anti-mouse IgG and Alexa 633 goat anti-rabbit IgG were purchased from Invitrogen, CA.

Pharmacological treatments and compounds

HepG2 cells were seeded at $4-6 \times 10^4/\text{cm}^2$ on tissue culture plastic or glass coverslips and allowed to polarize for three days before incubating with the following treatments at non-toxic concentrations: recombinant human VEGF-A, placental induced growth factor (PIGF), interferon-gamma (IFN γ) (Peprotech, UK) and VEGF-E (RELIATech, Germany); neutralizing anti-VEGF monoclonal antibody (mAb) VG76e¹⁶; Sorafenib a small molecular inhibitor of tyrosine protein kinases (D. Palmer, University of Birmingham, UK); VEGFR-2 kinase inhibitor Ki8751 (Calbiochem, UK) and anti-VEGF mAb 2c3 (R.Brekken, UT Southwestern Medical Center, USA) that inhibits VEGFR-2 activation¹⁷. Human VEGF was measured by ELISA following the suppliers recommended instructions (Peprotech, UK).

Cell polarity determination

HepG2 and WIF-B9 cells allowed to grow for 3 and 11 days, respectively, to polarize prior to fixing with 3% paraformaldehyde for 30 minutes at room temperature. Cells were permeabilized with 0.1% Triton/0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and stained with anti-MRP2 and Alexa-Fluor 488 goat anti-mouse. Nuclei were visualized using 4', 6'-diamidino-2-phenylindole (DAPI; Invitrogen) and their polarity index determined by counting the number of MRP2 positive apical structures per 100 nuclei using a Nikon Eclipse TE2000-S fluorescence microscope.

Determination of tight junction barrier function

To determine the functionality of TJs and whether they restrict paracellular diffusion of solutes from the bile-canalicular (BC) lumen to the basolateral medium (barrier function), HepG2 cells were incubated with 5mM 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) at 37°C for 10 minutes to allow translocation to BC lumen. After washing extensively with PBS, the capacity of BC to retain CMFDA was enumerated using a fluorescence microscope. WIF-B9 cells were treated with 5mM fluorescein diacetate (CFDA, Sigma, UK) at 37°C for 10 minutes before washing and quantifying the frequency of BC retaining CFDA.

HCVpp generation and infection

Pseudoviruses were generated by transfecting 293T cells with plasmids encoding a HIV provirus expressing luciferase and HCV strain H77 E1E2 region, the murine leukemia virus (MLV) envelope or a no envelope control (Env-), as previously described¹⁸. Virus containing media was added to target cells for 4h, unbound virus removed and the media replaced with DMEM/3% FBS. At 72h post-infection the cells were lysed, luciferase substrate added and luciferase activity measured for 10 seconds in a luminometer (Lumat LB 9507). Specific infectivity was calculated by subtracting the mean Env-pp RLU signal from the HCVpp or MLVpp signals. Infectivity was presented relative to untreated control cells, i.e. the mean luciferase value of the replicate untreated cells was defined as 100%.

HCVcc generation and infection

J6/JFH virus was generated as previously described². Briefly, RNA was transcribed in vitro from full-length genomes using the Megascript T7 kit (Ambion, USA) and electroporated into Huh-7.5 cells. 72 h and 96 h post electroporation supernatants were collected and stored immediately at -80°C. Virus containing media was added to target cells plated as described above and infected cells detected by methanol-fixation and staining for NS5A with mAb 9E10 and Alexa-488 anti-mouse immunoglobulin G. Infection was quantified by enumerating NS5⁺ foci and infectivity defined as the number of focus forming units/ml (FFU/ml).

Immunoprecipitation and western blotting

Polarized HepG2 cells were harvested in lysis buffer (PBS, 1% Brij97, 20mM Tris (pH 7.5), 300mM NaCl, 2mM CaCl₂, 2mM MgCl₂) containing protease and phosphatase inhibitors (Roche, UK). Lysates were clarified by centrifugation (20,000g, 10min), pre-cleared with Protein G-Sepharose (GE Healthcare) and 100µg incubated with protein G-Sepharose beads pre-coated with anti-occludin or irrelevant isotype matched control antibody at 4°C for 90 mins. The beads were collected by centrifugation, washed thoroughly in lysis buffer and the precipitated proteins eluted with Laemmli buffer. Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with anti-occludin, -phosphoserine, -phosphothreonine or -phosphotyrosine (Millipore) (1µg/ml) and horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG antibodies. Proteins were detected by enhanced chemiluminescence (Geneflow, UK).

Quantification of CD81, Claudin-1 and Occludin localization

Parental HepG2 cells and those transduced to express AcGFP tagged CD81 and CLDN1 were allowed to polarize on glass coverslips, fixed in ice-cold methanol and TJs localized by staining for occludin and ZO-1 as previously reported⁹. Coverslips were mounted on glass slides and viewed on a Zeiss Meta Head Confocal Microscope with a 63× water immersion objective. Cells were imaged with the microscope settings optimized for each fluorescent protein to obtain the highest signal to noise ratio. Protein expression at the basolateral, cytoplasmic and TJ locations (arbitrary fluorescence units AFU/pixel) provides 500-1000 measurements per cell. The data from ten cells were normalized and the localized expression calculated.

Statistical analysis

Results are expressed as the mean $\pm$ 1 standard deviation of the mean (SD), except where stated to the contrary. Statistical analyses were performed using Students t-test in Prism 4.0 (GraphPad, San Diego, CA) with a $P < 0.05$ being considered statistically significant.

Results

VEGF regulates hepatocellular TJ integrity and polarity

Hepatocytes in the liver are polygonal and multipolar, with at least two basal surfaces facing the circulation and a branched network of grooves between adjacent cells constituting the apical or bile canalicular (BC) surface. The majority of hepatocyte derived cell lines and primary hepatocytes de-differentiate in culture and fail to develop a polarized phenotype¹⁹. Human HepG2 and human-rat hybrid WIF-B9 hepatoblastoma cell lines polarize and develop apical cysts that are equivalent to BC in the liver. Polarization can be quantified by enumerating MRP2 positive BC per 100 nuclei (polarization index) and TJ integrity assessed by determining the frequency of BC retaining CMFDA or CFDA (Fig.1A). VEGF-A induced a dose-dependent decrease in HepG2 and WIF-B9 polarity and TJ integrity (Fig. 1B-C). Since VEGF is expressed in many tumour derived cell lines, we were interested to investigate whether HepG2 or WIF-B9 cells express VEGF and whether endogenous protein can act in an autocrine manner to regulate cell polarity. Both cell lines express VEGF (Fig. 1D) and neutralization of endogenous VEGF with anti-VEGF VG76e or receptor kinase antagonist Sorafenib for 24h, promoted HepG2 polarization (Fig.1E). Similar data was observed following inhibitor treatment of WIF-B9 cells (data not shown). Neutralization of endogenous HepG2 expressed VEGF had a more significant effect on polarity than expected from the recombinant VEGF-A dose response titrations (Fig.1B), that may represent differences between extracellular and cell-bound forms of VEGF that will not be accurately measured by ELISA or altered expression of VEGF splice variants in hepatoma cells. To ascertain whether primary human hepatocytes (PHH) express VEGF we isolated cells from two independent donors and quantified soluble VEGF levels. PHHs expressed VEGF at comparable levels to WIF-B9, whereas LSEC failed to secrete detectable levels of VEGF (Fig.1D). PHH failed to polarize sufficiently (<3% polarization index) to analyze the effect(s) of VEGF inhibitors on TJ integrity⁹.

VEGF acts through binding two high affinity tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk1/KDR). To investigate the receptor dependency of VEGF mediated effects on hepatocellular TJs, HepG2 cells were pre-treated with the broad spectrum inhibitor Sorafenib or VEGFR-2 antagonist Ki8751²⁰ for 24h followed by VEGF-A or IFN γ for 1h. Treating HepG2 cells with VEGF for a short time period of 1h reduced TJ integrity by 25-40%. Pre-treatment with both inhibitors ablated the effects of VEGF-A on TJ integrity, whilst having no detectable effect(s) on IFN γ induced de-polarization, suggesting a VEGFR-2 dependent pathway (Fig.2A). Furthermore, incubation of VEGF-A with a five-fold molar excess of anti-VEGF mAb 2c3, that blocks VEGF association with VEGFR-2¹⁷, abrogated the effect of VEGF-A but not IFN γ on HepG2 TJ integrity (Fig.2B). Finally, when HepG2 cells were treated with VEGF-E, a specific VEGFR-2 ligand, we noted a

reduction in TJ integrity whereas the VEGFR-1 selective ligand PlGF had no effect, confirming that VEGF regulates TJ integrity via a VEGFR-2 dependent pathway (Fig.2D).

VEGF regulates hepatocellular permissivity for HCV entry

We previously reported that polarization of HepG2-CD81 limits HCV entry⁹. To study the effects of VEGF on HCV infectivity, HepG2 cells were allowed to polarize over three days and treated with VEGF-A or VEGF-E and challenged with HCV J6/JFH. Both treatments led to a significant increase in HCV infectivity (Fig.3A) and concomitant decrease in TJ integrity. VEGF-A had no effect on HCV RNA replication in HepG2 or Huh-7.5 cells (data not shown). To ascertain whether VEGF modulates HCV entry, we studied HCVpp infection that measures HCV glycoprotein specific-receptor dependent internalization. VEGF-A and VEGF-E promoted a significant increase in HCVpp entry and had no detectable effect on MLVpp infection, confirming VEGF modulation of a HCV glycoprotein specific pathway (Fig.3A). To investigate whether endogenous VEGF affects HepG2 permissivity to support HCV replication, polarized HepG2-CD81 cells were treated with VG76e or Sorafenib for 16h and challenged with HCVcc J6/JFH and HCVpp. Both inhibitors significantly reduced HCVcc and HCVpp infection, with no detectable effect on MLVpp entry (Fig.3B).

We recently reported that HCV can transmit by cell-free particle infection of naïve target cells and by cell-to-cell transfer²¹. To assess the role of cell polarity and VEGF in HCV transmission, we monitored the frequency of HCV infected HepG2-CD81 over time in comparison to the permissive Huh-7.5 cell line. We previously reported that HepG2-CD81 cells are approximately 700-fold less permissive at supporting HCV replication than Huh-7.5 cells⁹. Thus, reduced levels of virus inocula were used to infect Huh-7.5 cells in this comparative transmission experiment. The number of NS5A expressing HepG2-CD81 cells in the culture remained constant following 48 and 96h post infection (167 ± 7.0 at 48h to 186 ± 7.4 at 96h). In contrast, the number of NS5A expressing Huh-7.5 cells increased from 579.5 ± 19.5 to 875 ± 27 between 48 and 96h post infection. Similarly, the levels of HCV RNA remained constant in HepG2-CD81 cells. In summary, these data show that HCV can initiate a primary infection of HepG2-CD81 cells, however, there are no second round infection events, most likely explained by the low permissivity of HepG2 cells to support HCV RNA replication and protein expression levels that may be required for particle assembly. Thus, we were unable to study the effect of VEGF antagonists on HCV transmission in polarized HepG2 cells.

HCV infection perturbs hepatocellular polarity in a VEGF-dependent manner

Several recent reports suggest that HCV infection induces VEGF expression in Huh-7 cells^{22, 23}, we confirm and extend these observations, demonstrating that J6/JFH infection of HepG2, Huh-7.5 and PHH induces VEGF expression (Fig.4A). Similar increases in VEGF expression were observed in JFH-1 and H77/JFH infected Huh-7.5 cells (data not shown). To ascertain whether infection perturbs hepatocellular polarity, HepG2-CD81 cells were infected with J6/JFH and monitored for their ability to polarize over a 48h time period. Naïve HepG2-CD81 cells demonstrated a 91.4% increase in polarization over 48h (Fig.4B). HCV J6/JFH infected cells demonstrated a significantly reduced frequency of polarized cells

after 48h (50.5% increase) (Fig.4B). This is particularly noteworthy, if one considers that the frequency of NS5A expressing HepG2-CD81 cells was less than 2% of the population, suggesting a significant bystander effect. Treatment of naïve and infected HepG2-CD81 cells with VG76e or Sorafenib promoted their polarization and abrogated the depolarizing effects of HCV, confirming a VEGF-dependent pathway (Fig.4B).

Effect of VEGF on tight junction protein localization

To define the mechanism(s) of VEGF induced changes in HepG2 TJ integrity and polarity that promote HCV entry, we studied the effect(s) of VEGF-A on occludin, CLDN1, CD81 and ZO-1 localization. We previously reported that occludin and ZO-1 localize as a discrete band surrounding the BC in polarized HepG2 cells. In contrast, CLDN1 and CD81 were detected at the basolateral and apical surfaces⁹. VEGF-A treatment significantly reduced occludin expression at TJs, promoting basolateral and intracellular pools (Fig.5A). In contrast, there was no significant change in CLDN1, CD81 or ZO-1 localization (Fig.5B). Occludin, like many TJ proteins, is a phosphoprotein and phosphorylation/dephosphorylation of both Tyrosine (Tyr) but predominantly Serine (Ser)/Threonine (Thr) residues play an important role in regulating occludin localization and its subsequent role in TJ assembly²⁴⁻²⁶. Western blotting demonstrates that occludin runs predominantly as a single band in lysates prepared from polarized HepG2 cells, whilst VEGF-A treatment results in a second lower molecular weight band that may represent less phosphorylated occludin (Fig.5C). To investigate the effects of VEGF on occludin phosphorylation, we immunoprecipitated occludin from untreated and VEGF-A treated HepG2 cells and probed the precipitates with anti-phosphoserine or -phosphotyrosine by Western blotting. VEGF-A treatment reduced the levels of Ser phosphorylated occludin and had no effect on Tyr phosphorylated occludin levels (Fig.5D).

Discussion

We demonstrate for the first time that VEGF regulates TJ integrity and hepatocellular polarity through a VEGFR-2 dependent pathway (Fig.s 1-2). We previously reported that hepatoma polarization limits the availability of basolateral expressed TJ protein viral receptors and reduces HCV entry⁹, hypothesizing that cytokine or growth factor induced changes in hepatocyte permeability will promote HCV entry. Indeed, VEGF treatment of polarized HepG2 led to a significant increase in HCV entry (Fig.3A). Furthermore, neutralization of hepatoma expressed VEGF with inhibitors targeting extracellular VEGF or intracellular VEGF-R kinases significantly inhibited HCV entry (Fig.3B). HCV infection of primary hepatocytes or hepatoma cells increased VEGF expression and reduced cell polarization. Importantly, VEGF inhibitors restored the ability of infected hepatoma cells to polarize, confirming a VEGF-dependent pathway. Hepatic polarity is critical to the function of the liver and cholestatic disease occurs when TJs, which seal the bile canaliculi, lose function permitting leakage of proteins and bile acids between apical and basal compartments²⁷⁻²⁹. Cholestatic HCV disease occurs in the early post-transplant period and is generally associated with high levels of intrahepatic HCV RNA and may reflect viral-induced cytopathic injury³⁰⁻³². The data presented in this manuscript support a role for viral encoded proteins promoting VEGF-dependent alterations in TJ integrity and hepatocyte polarity.

VEGF was initially recognized as an endothelial specific growth factor that regulated vascular permeability and angiogenesis. The majority of investigations studying the role of VEGF in HCC have characterised the effect of VEGF antagonists on tumour development and metastasis. Indeed, several recent reports have demonstrated that VEGF inhibitors can significantly increase life expectancy of subjects with HCC³³⁻³⁵. A systematic study of VEGF expression in adult VEGF-LacZ mice demonstrated a defined subset of cells expressing VEGF, including hepatocytes in the liver³⁶. Our data support a role for VEGF in regulating hepatocyte polarity, consistent with an earlier report from Schmitt and colleagues³⁷ who demonstrated a Protein Kinase C- α (PKC) dependent pathway underlying VEGF perturbation of HepG2 TJs. The PKC dependency of this pathway is consistent with our earlier report that phorbol ester activation of PKC depolarized HepG2 and increased HCV entry⁹. The observation that PHH secrete VEGF (Fig.1) suggest that this pathway is in operation in non-tumour cells, however given the difficulties in studying primary hepatocyte polarization¹⁹ we were unable to assess the effects of VEGF on hepatocyte polarity.

HCV infection of hepatoma cells has been reported to induce oxidative stress and calcium signalling that stabilizes hypoxia inducible growth factor 1 α (HIF-1 α), a transcription factor that regulates angiogenic cytokines, including VEGF^{22, 23, 38, 39}, suggesting a role for HCV in HCC development and metastasis. Hassan and colleagues recently reported increased VEGF immunostaining of liver biopsies from HCV infected subjects, suggesting a role for HCV core protein in hepatic angiogenesis²³. Several oncogenic viruses, Hepatitis B virus, human papillomavirus-16 and Kaposi's sarcoma herpes virus have been reported to activate HIF-1 α and promote VEGF expression, suggesting an important pathway in tumour development⁴⁰. In addition to the angiogenic properties of elevated VEGF expression, our data support a model where HCV infected hepatocytes secrete VEGF that induce a localized depolarization of hepatocytes that promotes viral transmission between adjacent hepatocytes, consistent with the recent report by Liang and colleagues demonstrating infected foci in the liver of HCV chronically infected subjects⁴¹.

Pro-inflammatory cytokines modulate epithelia and endothelia permeability by a variety of mechanisms⁴². We previously reported that TNF α and IFN γ reduced HepG2 TJ integrity and yet had no demonstrable effect on HCV entry⁹, suggesting independent pathway(s) to perturb TJ protein localization, some of which modulate HCV entry. Our recent data suggest that VEGF inhibitors will promote hepatocellular TJ integrity and reduce HCV infection of naïve cells within the context of an inflamed liver and may have minimal effect(s) on cytokine mediated changes to epithelial permeability.

VEGF has been reported to stimulate the phosphorylation/dephosphorylation of occludin via PKC and mitogen activated protein kinase (MAPK) pathways in a cell type dependent manner^{37,43,44}. VEGF stimulation of brain microvessel endothelial cells leads to a dephosphorylation of occludin, disruption of TJ protein localization and increased permeability⁴⁵. In contrast, VEGF stimulates occludin phosphorylation at Ser490 and increases permeability of bovine retinal endothelial cells^{44,46,47}. Our data demonstrate that VEGF reduces occludin localization at TJs in HepG2 cells, whilst having no significant effect(s) on CLDN1, CD81 or ZO-1 localization (Fig.5A-B). The VEGF-A-dependent

reorganization of occludin is accompanied by an altered serine phosphorylation pattern. In summary, our data support a model where HCV upregulation of VEGF expression disrupts occludin localization, reduces TJ integrity and promotes viral transmission. These data highlight a potential role for VEGF antagonists to help control HCV infection in addition to their known properties of regulating HCC growth and development.

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Abbreviations

BC	bile canaliculi
CLDN1	claudin-1
HCC	hepatocellular carcinoma

HCV	hepatitis C virus
HCVcc	cell culture-derived HCV
HCVpp	HCV pseudotype particles
LSEC	Liver sinusoidal endothelial cells
PHH	primary human hepatocytes
PIGF	placental induced growth factor
MLVpp	murine leukemia virus pseudotype particles
RLU	relative light units
TJ	tight junction
VEGF-A	Vascular Endothelial Growth Factor A

Fig.1. VEGF regulates hepatocellular TJ integrity and polarity

A HepG2 polarity was quantified by fixing the cells in 3% paraformaldehyde and staining for the bile canicular (BC) marker MRP2. Enumerating the frequency of MRP2⁺ BC per 100 DAPI stained nuclei enabled us to determine a polarity index. TJ barrier function was measured by quantifying the frequency of BC retaining CMFDA. Representative images depict apical expressed MRP2 and BC annotated with arrows and their retention of fluorescent CMFDA. Scale bar represents 10 μ M. **B.** HepG2 cells were allowed to polarize for 3 days and treated with VEGF-A for 24h. Treated and untreated cells were stained for MRP2 to quantify their polarity or incubated with CMFDA to measure TJ barrier function. Polarity index and TJ integrity was determined by quantifying the number of MRP2⁺ BC per 100 nuclei that retained CMFDA in five fields of view on three replicate coverslips. **C.** WIF-B9 cells were grown for a minimum of 11 days to develop 'hepatic' polarity and treated with VEGF-A for 24h, polarity and TJ integrity assessed as above. **D.** HepG2, WIF-B9, primary human hepatocytes (PHH) from two independent donors and LSEC were plated and the extracellular media collected from 100,000 cells over a 24h time period and assessed for VEGF using a human VEGF ELISA. **E.** HepG2 cells were allowed to polarize for 3 days and pre-incubated in serum free DMEM for 4h before treating with control DMSO, neutralizing anti-VEGF-A antibody VG76e (1.3 mg/ml) or receptor kinase antagonist Sorafenib (10nM) for 24h. Cells were fixed and their polarity index measured. * $P < 0.01$ (t test).

Fig.2. VEGF regulation of hepatoma TJ integrity and polarity is VEGFR-2 dependent

HepG2 cells were allowed to polarize for 3 days, incubated in serum-free DMEM for 4h followed by: **A.** control DMSO, Sorafenib (10nM) or VEGFR-2 antagonist Ki8571 (10nM) for 24h, followed by treatment with VEGF-A (10ng/ml) or IFN γ (10ng/ml) for 1h; **B.** VEGF-A (10ng/ml) or IFN γ (10ng/ml) pre-treated with irrelevant IgG (5mg/ml) or anti-VEGF 2c3 (5mg/ml) for 1h, or **C.** treated with mock (control), PlGF (50ng/ml), VEGF-E (50ng/ml) or VEGF-A (50ng/ml) for 1h. TJ barrier function was assessed by quantifying the number of CMFDA⁺ BC in a minimum of five fields of view on three replicate coverslips, where * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ (t test).

Fig.3. VEGF regulates HCV entry into polarized HepG2 cells

A. HepG2-CD81 cells at 3 days post-plating were untreated or treated with VEGF-A (10ng/ml) or VEGF-E (10ng/ml) for 1h and challenged with HCVcc J6/JFH (black bars); HCVpp (white bars) or MLVpp (gray bars). * $P < 0.01$ (t test). **B.** HepG2-CD81 cells at 3 days post-plating were untreated or treated with neutralizing anti-VEGF-A antibody VG76e (1.3mg/ml) or receptor kinase antagonist Sorafenib (10nM) for 16h and challenged with HCVcc J6/JFH (black bars); HCVpp (white bars) or MLVpp (gray bars). Infectivity is expressed relative to control. ** $P < 0.001$ (t test).

Fig.4. HCV infection increases VEGF expression that reduces hepatoma polarity

A. HepG2-CD81, Huh-7.5 and primary human hepatocytes (PHH) from two donors were inoculated with mock or HCVcc J6/JFH, pre-titrated to achieve comparable levels of infection in the different target cells. Infected HepG2-CD81, Huh-7.5 and PHHs contained 2.6×10^7 , 2.9×10^7 and 1.9×10^7 HCV RNA copies/ 10^6 cells, respectively. Extracellular media was collected from 100,000 uninfected (white bars) or HCV infected cells (black bars) over a period of 24h and assessed for VEGF expression using a human VEGF-A ELISA. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ (*t* test). **B.** HepG2-CD81 cells were infected with mock or HCV J6/JFH for 4h, unbound virus removed by extensive washing and the cells untreated or treated with anti-VEGF-A antibody VG76e (1.3mg/ml) or Sorafenib (10nM) for 48h. Cells were fixed and the frequency of NS5A+ and polarized cells in duplicate coverslips assessed. Less than 2% of infected HepG2-CD81 cells expressed NS5A antigen at 48h post infection. Relative polarity is presented as the increase in polarized cells over 48h for uninfected (white bars) and J6/JFH infected (black bars) cells. ** $P < 0.001$ (*t* test).

Fig.5. VEGF modulates occludin localization and phosphorylation

A. HepG2 cells were allowed to polarize for 3 days and were untreated (control) or treated with VEGF-A (10ng/ml) for 1h, TJ integrity assessed and occludin (OCLN) localization ascertained by staining with an OCLN-specific antibody. VEGF-A reduced the frequency of CMFDA⁺ BC by 48% and representative images demonstrate a re-organization of OCLN to basolateral membranes. **B.** To quantify the effect of VEGF on OCLN localization and to ascertain the effect on other TJ associated proteins and viral co-receptor CD81, polarized HepG2 cells expressing AcGFP.CLDN1 and AcGFP.CD81 were untreated (control) or treated with VEGF-A (10ng/ml) for 1h and stained with anti-OCLN or anti-ZO-1. OCLN, CLDN1, CD81 and ZO-1 localization at basolateral (black), intracellular (white) and TJ (grey) locations was quantified. **C.** Polarized HepG2 cells were untreated or treated with VEGF-A (10ng/ml) for 1h and OCLN immunoprecipitates subjected to SDS-PAGE and Western blotting with anti- OCLN. **D.** To determine the phosphorylation status of precipitated OCLN following VEGF-A treatment, SDS PAGE separated proteins were probed with anti -phosphoserine or -phosphotyrosine and protein loading confirmed using anti- β actin.