Hepatitis C virus (HCV) affects 3% of the world’s population and is a leading cause of chronic liver disease, including fibrosis, cirrhosis and hepatocellular carcinoma (Alter, 2007). The recent availability of direct-acting antiviral agents has led to a significant improvement in patient response rates (Scheel & Rice, 2013). However, the cost of such treatments will be prohibitive in many parts of the world, highlighting the need for immune-based therapies for treating chronic hepatitis C and the development of a preventive vaccine. HCV primarily infects hepatocytes in the liver that are highly differentiated and arrested in the G1 stage of the cell cycle (Farquhar & McKeating, 2008). HCV initiates infection by attaching to molecules or receptors at the cell surface and current evidence supports an essential role for scavenger receptor-class B member I (SR-BI), tetraspanin CD81 and occludin expression levels may regulate HCVpp entry. In contrast, we observed a 2-log range in viral replication suggesting that other factors independent of receptor expression levels may regulate HCVpp entry. In contrast, VSV-Gpp infected PHHs at all times post-seeding with comparable luciferase activity (Fig. 1b). Anti-CD81 and anti-SR-BI antibodies inhibited infection, confirming receptor-dependent virus entry (Fig. 1a). HCVpp and HCVcc infected PHHs optimally at 2 days post-seeding, consistent with increased expression of SR-BI and claudin-1 compared with day 1 cells (Fig. 1d–e). However, it is noteworthy that CD81, SR-BI, claudin-1 and occludin expression was optimal at 4 days post-plating, suggesting that other factors independent of receptor expression levels may regulate HCVpp entry. In contrast, VSV-Gpp infected PHHs at all times post-seeding with comparable luciferase activity (Fig. 1b). Anti-CD81 and anti-SR-BI antibodies inhibited infection, confirming receptor-dependent virus entry (Fig. 1a). We noted similar IC_{50} values for both anti-receptor antibodies to neutralize HCVpp infection of PHHs and hepatoma cell lines (data not shown). Having determined the optimal time to infect PHHs after isolation from the liver, we compared the rate of HCVpp and VSVpp infection of PHHs with the permissive Huh-7.5 hepatoma cell line (Blight et al., 2003; Schwarz et al., 2009). Virus was allowed to infect target cells for increasing time periods up to 8 h, unbound virus was removed by washing and luciferase activity was measured after 72 h (Fig. 2). We observed comparable rates of HCVpp [4535 ± 220 and 4728 ± 345 relative light units (RLU) h^{-1}] and VSV-Gpp (220 and 4728 ± 345 RLU h^{-1}).
and VSV-Gpp (265 483 ± 106 122 and 319 216 ± 59 485 RLU h⁻¹) infection of PHHs and Huh-7.5 cells, respectively.

To assess the impact of donor variability on PHH permissivity we compared cells isolated under GLP conditions from five donors for their ability to support HCVpp and HCVcc infection. All PHH preparations showed comparable levels of HCVpp or VSV-G infection (Fig. 3a, b). Similar data were obtained with HCVpp expressing a variety of primary envelope glycoproteins (data not shown).

As controls, we included two commonly used hepatoma cell lines, Huh-7.5 and Hep3B. We noted that the HCVpp-encoded luciferase values for PHHs were approximately
**Fig. 2.** HCV entry kinetics. PHHs and Huh-7.5 cells were seeded at 4×10⁴ cm⁻² on collagen-coated plates and 2 days post-plating were infected with HCVpp or VSV-Gpp. The inoculum was removed at 2 h intervals up to 8 h and cultures were maintained for 72 h. Data are presented as RLU from which a no-envelope pseudotype control value has been subtracted and are representative of three independent experiments. Error bars indicate SD from the mean (n=3).

**Fig. 3.** Cell proliferation augments pseudoparticle infection. PHHs, and Hep3B and Huh-7.5 cells were seeded at 4×10⁴ cm⁻² on collagen-coated plates and 2 days post-plating were infected with HCVpp, VSV-Gpp (a, b) or HCV JFH-1 and SA13/JFH (c) virus for 8 h as detailed in Fig. 1. Data are presented as RLU from which a no-envelope pseudotype control value has been subtracted or as HCV RNA copy number relative to GAPDH and are representative of three independent experiments. (d) Mitomycin C and γ-irradiation block cell growth. Cells were treated with mitomycin C (10 μg ml⁻¹, overnight) or γ-irradiated (32 mSv, 30 min) and proliferation was measured using an MTT assay (Promega) as per the manufacturer’s instructions; data are presented as OD₄₅₀. (e) Mitomycin C or γ-irradiated cells were infected with HCVpp-H77 or VSV-Gpp. Cultures were maintained at 37 °C for 72 h and luciferase activity was measured as described above. Data are presented relative to untreated cells. (f) Control and mitomycin C-treated PHHs (donor 2) and Huh-7.5 cells were studied for their ability to support HCVpp-H77 and VSV-G pseudoviruses expressing green fluorescent protein (GFP) reporter. GFP-expressing cells were detected 72 h post-infection by flow cytometry. *P<0.05, **P<0.01, ***P<0.001 (t-test). Error bars indicate SD from the mean (n=3).
1-log lower compared with both hepatoma cells, suggesting a lower frequency of infected cells in the PHH population. The efficiency of the lentiviral promoter in the different cell types could in part explain the different luciferase signals. However, this is unlikely since the majority of donor primary and hepatoma cells showed comparable VSV-Gpp luciferase signals (Fig. 3b). In contrast to the pseudoparticle infectivity data, we noted significant variability in the ability of PHHs isolated from different donors to support replication of HCV strains JFH-1 and SA13/JFH (Fig. 3c), with levels of HCV RNA in PHHs at least 1-log lower than in HuH-7.5 cells (Fig. 3c).

It is widely accepted that pseudoparticle reporter signals reflect differences in glycoprotein-dependent entry, and luciferase values are commonly used as a read-out in antibody-based neutralization assays and for measuring strain-dependent differences in virus entry. In the liver, hepatocytes are largely quiescent and PHHs show minimal proliferative capacity in vitro (Taub, 2004). In contrast, hepatoma cells have a doubling time in the order of 18–24 h (Sainz et al., 2012b). We hypothesized that proliferation of an infected cell may result in progeny daughter cells bearing the reporter gene, resulting in an increased luciferase signal that does not reflect the initial viral entry event per se. To ascertain whether cell proliferation modulates HCVpp and VSV-Gpp luciferase signals, PHHs and hepatoma cells were arrested with mitomycin C or γ-irradiation and evaluated for pseudoparticle infection. Mitomycin C and γ-irradiation limited hepatoma growth and had no discernible effect on PHH proliferation or viability (Fig. 3d). Both treatments had minimal effect on HCVpp or VSV-Gpp entry into PHHs, consistent with the non-dividing nature of these cells. In contrast, both treatments significantly reduced HCVpp and VSV-Gpp infection of the hepatoma cell lines, resulting in comparable luciferase values to PHHs (Fig. 3e). To confirm our earlier model that HuH-7.5 proliferation affects the number of pseudoparticle-infected cells, we inoculated non-arrested and arrested PHHs and HuH-7.5 cells with HCVpp-INF7 and VSV-Gpp bearing a GFP reporter. Mitomycin C reduced the number of HCVpp and VSV-Gpp-GFP-expressing HuH-7.5 cells but had no impact on the number of infected PHHs (Fig. 3f). Importantly, following mitomycin C treatment the numbers of HCVpp-infected PHHs and HuH-7.5 cells were comparable (Fig. 3f).

In summary, we have shown that PHHs from multiple donors support similar levels of HCVpp entry. In contrast, the same donor hepatocytes showed significant differences in their ability to support HCVcc replication, consistent with recent reports showing donor-dependent differences in innate signalling and type III interferon expression (Li & Lemon, 2013; Marukian et al., 2011; Park et al., 2012). This study raises an interesting question as to whether differences in the frequency of infected hepatocytes and viral RNA reported in chronically infected subjects (Liang et al., 2009) are largely defined at the level of viral replication and innate immune signalling rather than virus entry. We report for the first time that hepatoma proliferation increases HCVpp reporter signals, demonstrating that luciferase values are not solely defined by the HCVpp entry process. This is particularly pertinent when comparing HCV entry into different cell types with varying growth rates or when evaluating the effect of antibodies, growth factors or kinase inhibitors with known effects on cell proliferation (Lupberger et al., 2011; Marukian et al., 2008; Sainz et al., 2012a). In summary, growth-arrested hepatoma cell lines support comparable rates of HCVpp entry into primary hepatocytes, validating their use as a surrogate model system.

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References


