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Short Communication

Lentiviral hepatitis B pseudotype entry requires sodium taurocholate co-transporting polypeptide and additional hepatocyte-specific factors

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Hepatitis B virus (HBV) is one of the world's major unconquered infections, resulting in progressive liver disease, and current treatments rarely cure infection. A limitation to discovering new therapies is our limited knowledge of HBV entry and dissemination pathways that hinders the development of *in vitro* culture systems. To address this gap in our understanding we optimized the genesis of infectious lentiviral pseudoparticles (HBVpps). The recent discovery that the bile salt transporter sodium taurocholate co-transporting polypeptide (NTCP) acts as a receptor for HBV enabled us to assess the receptor dependency of HBVpp infection. HBVpps preferentially infect hepatoma cells expressing NTCP, whereas other non-liver cells engineered to express NTCP do not support infection, suggesting that additional hepatocyte-specific factors are required for HBVpp internalization. These results highlight the value of the HBVpp system to dissect the pathways of HBV entry and dissemination.

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Hepatitis B virus (HBV) infection is a major global health problem frequently resulting in progressive, degenerative liver disease, including cirrhosis and hepatocellular carcinoma. Despite the availability of a safe and effective vaccine, and a range of nucleoside analogue antivirals, ~240 million people are chronically infected with HBV (El-Serag, 2012). The high rate of viral turnover has resulted in vaccine-related escape mutants and drug resistance (Billioud *et al.*, 2012; Locarnini & Yuen, 2010). Therefore, development of safe, efficient antiviral strategies remains a key challenge for the treatment of HBV.

A major obstacle to discovering new therapies is the lack of efficient *in vitro* systems or small animal models supporting HBV infection (Thomas & Zoulim, 2012). Our limited knowledge of the host pathways regulating HBV entry and dissemination hinders the development of model systems that recapitulate the dynamics of HBV infection and antigen expression in the liver. HBV infectivity is dependent on the large (L) and surface (S) envelope glycoproteins, with essential domains mapping to the N-terminal 75 aa (Pre-S1) termed the 'receptor binding domain' and to the antigenic 'a' loop of S (reviewed by Baumert *et al.*, 2014). A myristoylated peptide corresponding to the N-terminal Pre-S1 region, Myrcludex B, inhibits HBV infection (Volz *et al.*, 2013) and was used as bait to identify its host interacting partner as sodium taurocholate co-transporting polypeptide (NTCP) (Ni *et al.*, 2014; Yan *et al.*, 2012). NTCP (SLC10A1) is the founding member of the SLC10 family of solute carrier proteins and is exclusively expressed in hepatocytes, where it plays an important role in bile acid uptake.

Although exogenous expression of NTCP in human hepatoma cells renders them susceptible to HBV infection, high levels of viral inocula (100 genome copies per cell) and polyethylene glycol are required to initiate replication (Ni *et al.*, 2014; Yan *et al.*, 2012). As these infections require

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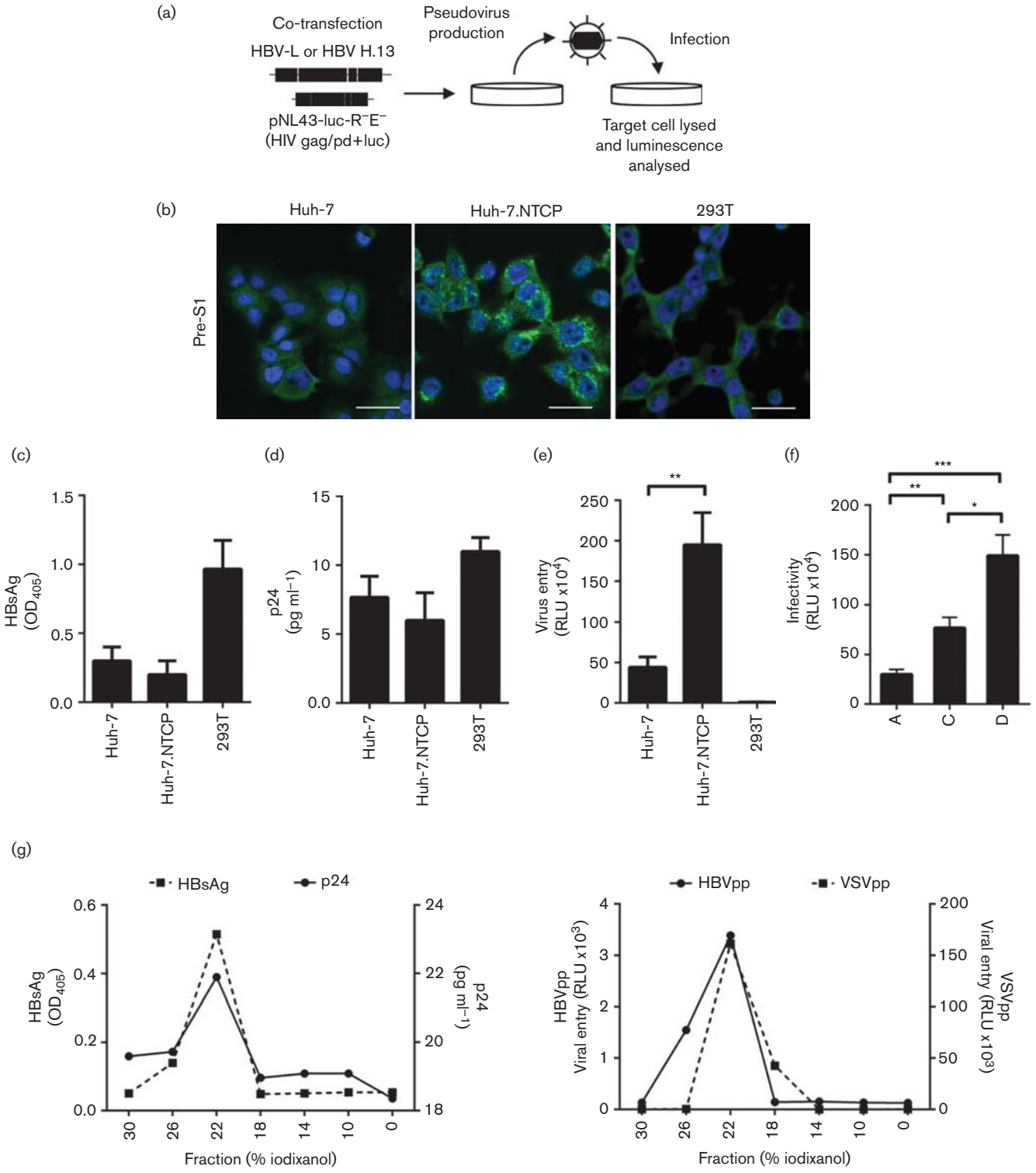

Fig. 1. HBV pseudovirus genesis. (a) HBVpps are produced by co-transfecting pNL4.3-luc-R⁻E⁻, a lentiviral HIV expression vector, with replication-competent HBV genome H1.3 (genotype D) into parental Huh-7, NTCP-expressing hepatoma cells or 293T embryonal kidney cells. (b) The cells were cultured for 72 h, stained with mAb 18/7, specific for Pre-S1, and imaged by confocal microscopy. Bar, 20 μm. (c, d) Extracellular expression of HBsAg or HIV p24 was quantified by ELISA. (e) To evaluate HBV pseudovirus infectivity, the extracellular media (normalized for p24 content) was allowed to infect dHepG2.NTCP cells for 72 h and luciferase activity measured. (f) Huh-7.NTCP cells were co-transfected with HBV genotype A, C or D replication-competent genomes and pNL4.3-luc- R⁻E⁻; 72 h later the extracellular media was assessed for

infection of dHepG2.NTCP cells. (g) HBVpps or VSVpps were separated by density ultracentrifugation for 24 h at 100 000 ***g*** at 4 °C, across a 10–30 % iodixanol gradient, and each fraction assessed for HBsAg and p24 by ELISA or infection of dHepG2.NTCP cells. Results are the mean ± SD of three independent experiments. The no envelope control [500–800 relative light units (RLU)] value was subtracted from the HBVpp RLU value and differences assessed by Student's *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

5–7 days to generate detectable antigen or viral genomes, the current culture systems limit studies on HBV entry.

In order to develop a robust methodology to quantify HBV entry we characterized lentivirus-based pseudoparticles (HBVpps). This system has been used to study the entry pathways of a range of viruses, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), pathogenic avian influenza and Ebola, offering a rapid and reproducible analysis of the viral glycoprotein–receptor interactions (Moller-Tank & Maury, 2015; Temperton *et al.*, 2007; Wright *et al.*, 2009; Zimmer *et al.*, 2014). Previous reports showed that lentiviral HBVpps were infectious for human hepatocytes; however, given the limited knowledge of internalization pathways at the time, the receptor dependency was not studied (Chai *et al.*, 2007; Sung & Lai, 2002). Our studies show that infectious HBVpp genesis is optimal in hepatoma cells, suggesting that liver-specific factors are required for assembly and/or infectivity. HBVpp entry is NTCP-dependent; however, expression of NTCP in non-liver epithelial cells does not confer permissivity to infection, suggesting that additional hepatocyte-specific factors are required. The recent report of a genetic polymorphism (p.Ser267Phe) in NTCP that ablates HepG2 ability to support HBV infection *in vitro* and yet supports HBV infection in man (Peng *et al.*, 2015) suggests that additional host factors are required for HBV entry. Taken together, these results highlight the utility of the pseudovirus approach to dissect the molecular pathways of HBV entry into the liver.

Whilst there are several methods of generating pseudoviruses, we chose a luciferase-based HIV lentiviral system (pNL4.3-luc-R^-E^-), similar to that reported for HCV (Hsu *et al.*, 2003) (Fig. 1a). For our initial studies we generated lentiviral pseudotypes using replication competent HBV genomes to ensure that all three components of the HBV surface proteins were expressed in a ratio and conformation consistent with a native infection. Recent studies show that both HepG2 and Huh-7 cells transduced to express NTCP support HBV replication (Ni *et al.*, 2014; Yan *et al.*, 2012); however, due to the low transfection efficiency observed with HepG2 cells we selected Huh-7 cells to generate lentiviral pseudoparticles. Parental Huh-7 or Huh-7.NTCP cells were co-transfected with equal ratios of lentivirus pNL4.3-luc-R^-E^- and replication-competent 1.3-fold overlength HBV (H1.3) (genotype D) or a control 'empty' plasmid. The extracellular media collected after 72 h were used to infect DMSO-differentiated HepG2.NTCP cells (dHepG2.NTCP). HBV H1.3 and pNL4.3-luc-R^-E^- plasmids were also transfected into human embryonal kidney 293T cells: this cell type is commonly used for pseudoparticle genesis due to its high transfection efficiency; however, it does not support HBV replication (Reese *et al.*, 2013; Tang *et al.*, 2001). All of the transfected cells expressed comparable levels of HBV Pre-S1 (Fig. 1b). Cellular processing and secretion of HBV surface antigen-containing particles (HBsAg) and HIV capsid protein p24 was confirmed by ELISA (Fig. 1c, d). We observed significantly higher levels of HBsAg secretion from 293T compared with Huh-7 and Huh-7.NTCP cells. However, comparable levels of extracellular HIV p24 were detected for all three cell lines (Fig. 1d), consistent with similar levels of lentiviral core secretion.

Having established that both lentiviral capsid and HBV particles are secreted, we measured the infectivity of pseudovirus preparations in dHepG2.NTCP cells. HBVpps produced by hepatoma cells were 30- to 250-fold more infectious than pseudovirus secreted from 293T cells (Fig. 1e), suggesting that hepatocyte-specific factors play a role in the assembly and/or secretion of infectious particles. All pseudotype preparations were normalized for HIV p24 content for infection studies and infectivity values represent the net luciferase activity where the empty control virus activity is subtracted. Interestingly, we noted that HBVpps produced from Huh-7.NTCP cells were eightfold more infectious than virus generated by parental Huh-7 cells (Fig. 1e). HBV Pre-S1 engagement of NTCP has been reported to alter the expression of genes involved in bile acid metabolism (Oehler *et al.*, 2014), and these pathways may play a role in promoting the assembly and/or secretion of pseudoparticles.

To investigate whether pseudoviruses can be generated expressing glycoproteins from diverse HBV genotypes we co-transfected replication-competent HBV clones of genotype A, C and D with pNL4.3-luc-R^-E^- into Huh-7.NTCP and the extracellular virus used to infect dHepG2.NTCP. All three genotypes expressed similar levels of HBsAg and generated infectious pseudovirus; however, genotype D was significantly more infectious than the other clones tested (Fig. 1f). This observation suggests that increased entry efficiency contributes to the enhanced infectivity of genotype D virus observed *in vitro*.

To assess whether HBVpps are assembled we analysed viral preparations by gradient centrifugation, and determined

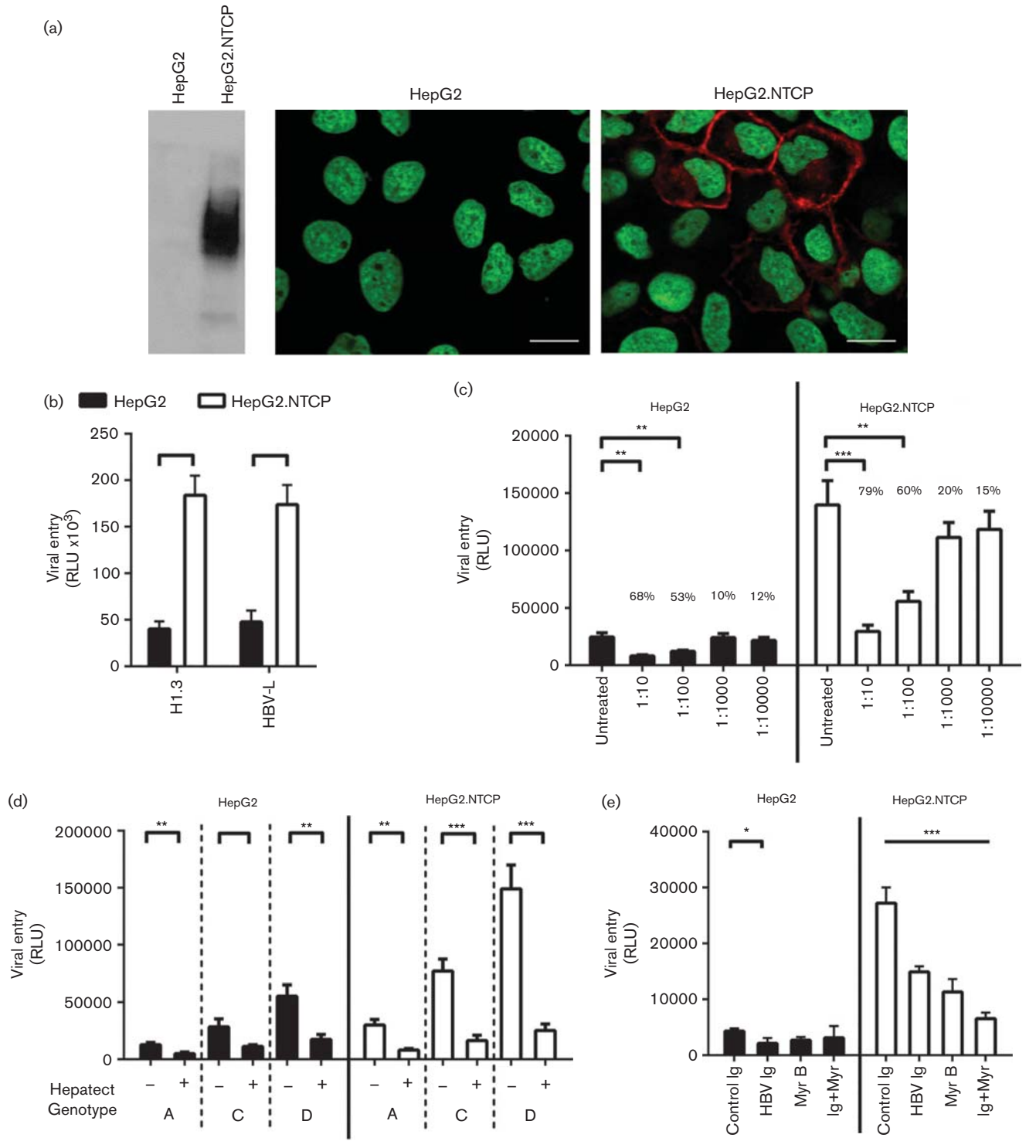

Fig. 2. HBVpp entry is NTCP- and HBs-dependent. (a) HepG2 or HepG2.NTCP cells were tested for NTCP expression by K9 anti-NTCP Western blotting or confocal microscopy. Bar, 10 μm. (b) Huh-7.NTCP cells were co-transfected with replication-competent HBV genotype D or pcDNA3.1 encoding HBV-L and pNL4.3-luc-R$^-$E$^-$; 72 h later the extracellular media were collected and assessed for infection of dHepG2 or dHepG2.NTCP cells. (c) Pseudoparticles generated from HBV-L/pNL4.3-luc-R$^-$E$^-$ transfected Huh-7.NTCP producer cells were incubated with increasing doses of anti-HBs pooled IgG or control irrelevant IgG for 1 h, before infecting dHepG2 or dHepG2.NTCP cells for 72 h; anti-HBs IgG inhibition (%) is shown. (d) Pseudoparticles generated from HBV genotype A, C or D/pNL4.3-luc-R$^-$E$^-$ co-transfected Huh-7.NTCP

producer cells were incubated with anti-HBs (1 : 100 dilution) or control IgG for 1 h prior to infecting dHepG2 or dHepG2.NTCP cells for 72 h. (e) dHepG2 or HepG2.NTCP were treated with Myrcludex B (Myr B) (1 μM), anti-HBs immunoglobulin (Ig) (1 : 10 dilution) or irrelevant immunoglobulin for 1 h before infecting with HBVpp genotype D for 72 h before quantifying infection. Results are the mean ± SD of at least three experiments, with differences assessed using Student's *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

whether HBsAg and lentiviral p24 locate in the same fraction. HBVpps or control pseudoparticles expressing vesicular stomatitis virus G (VSVpp) generated from Huh-7.NTCP cells were fractionated over 10–30 % iodixanol gradients by ultracentrifugation at 100 000 ***g*** for 24 h at 4 °C. Each fraction was assessed for HBsAg/p24 by ELISA and for infectivity in dHepG2.NTCP cells. HBsAg and p24 were detected in the same fractions, consistent with the secretion of intact pseudoparticles and infectivity of HBVpps and control VSVpps associated with fractions between 18 and 22 % iodixanol (Fig. 1g), demonstrating the assembly of HBs-containing lentiviral pseudoparticles.

We assessed the ability of HBVpps to infect parental HepG2 (NTCP null) and those transduced to express NTCP, confirmed by quantitative real-time PCR measurement of NTCP mRNA, anti-NTCP staining by Western blot or confocal microscopy (Fig. 2a) and bile salt uptake (data not shown). NTCP expression significantly enhanced HBVpp infection (Fig. 2b), demonstrating common receptor-dependent pathways with native HBV.

A significant caveat of generating HBVpps using replication-competent genomes is the concomitant production of native HBV that will compete with pseudoparticles for receptors and reduce reporter gene expression. We therefore generated pseudovirus by co-transfecting pcDNA3.1 encoding HBV L with the lentiviral vector and observed similar levels of infection to those generated with full-length HBV (Fig. 2b). To confirm that pseudoparticle infection was dependent on HBV glycoprotein expression we investigated the ability of a clinical-grade polyclonal IgG pooled from vaccinated donors (Hepatect) to neutralize infection. HBVpps were incubated for 1 h with increasing doses of Hepatect or an irrelevant IgG, before inoculating dHepG2 or dHepG2.NTCP cells. We observed a dose-dependent inhibition of HBVpp infection (Fig. 2c), demonstrating that HBVpp infection is HBs-dependent. The low-level HBVpp infection observed in parental HepG2 cells was neutralized by Hepatect, suggesting an alternative NTCP-independent route for pseudoparticle entry. Hepatect also neutralized the infectivity of HBVpps expressing genotype A- and C-encoded glycoproteins (Fig. 2d), confirming HBs-dependent routes of infection for all pseudotypes.

To confirm the role of NTCP in HBVpp entry, we assessed the ability of Myrcludex B to inhibit pseudoparticle infectivity. dHepG2 or dHepG2.NTCP cells were incubated with 1 μM Myrcludex B, Hepatect (1 : 10) or an irrelevant immunoglobulin, or a combination of both for 1 h prior to infecting with HBVpps or VSVpps. Myrcludex significantly inhibited HBVpp infection of dHepG2.NTCP and we noted an additive effect of combining Myrcludex with Hepatect to neutralize HBVpp infection (Fig. 2e). Myrcludex or Hepatect had no effect on VSVpp infection (data not shown).

To investigate whether HBVpps show a restricted tropism for liver-derived cells we measured their ability to infect three liver-derived cell lines (Huh-7, HepG2 and Hep3B) and two non-liver epithelial cell lines [293T (embryonal kidney) and A549 (lung epithelial)] in the presence or absence of exogenous NTCP expression. As a control, we included pseudoparticles that could only infect hepatocytes (HCVpps) or showed broad-range tropism (VSVpps). All cell lines were confirmed to express NTCP by binding fluorescent-labelled Myrcludex (Fig. 3a) or K9 anti-NTCP staining (Fig. 3b). HBVpps only infected the hepatoma lines and NTCP expression increased entry in all cases (Fig. 3c). In contrast, HBVpps did not infect 293T or A549 cells in the presence or absence of NTCP, suggesting that NTCP expression alone is not sufficient to confer HBVpp entry and that additional hepatocyte specific factors are required. HCVpps only infected Huh-7 and Hep3B cells, as previously reported (Hsu *et al.*, 2003), and NTCP expression had no effect (Fig. 3b). As expected given its broad tropism, VSVpps infected all cells types with high efficiency in an NTCP-independent manner, demonstrating efficient HIV LTR-driven reporter activity in all cell lines studied.

In conclusion, we have optimized the genesis of infectious HBVpps, enabling quantitative studies on HBV entry that are independent of downstream HBV replication events. The HBV surface glycoprotein is unlike other viral glycoproteins tested to date (Moller-Tank & Maury, 2015; Temperton *et al.*, 2007; Wright *et al.*, 2009; Zimmer *et al.*, 2014) in its failure to produce infectious pseudoparticles in human embryonal kidney cells (293T), requiring a liver cell background for infectious particles genesis. Our results show that whilst NTCP does play an essential role in HBVpp entry, it is unlikely to be the sole receptor facilitating infection. The recent study by Verrier *et al.* (2015) highlighting a role for Glypican 5, a heparin sulphate moiety that is highly expressed in the liver, in HBV and HDV infection supports our conclusions. Taken together, these results highlight the value of the HBVpp system to dissect the pathways of HBV entry

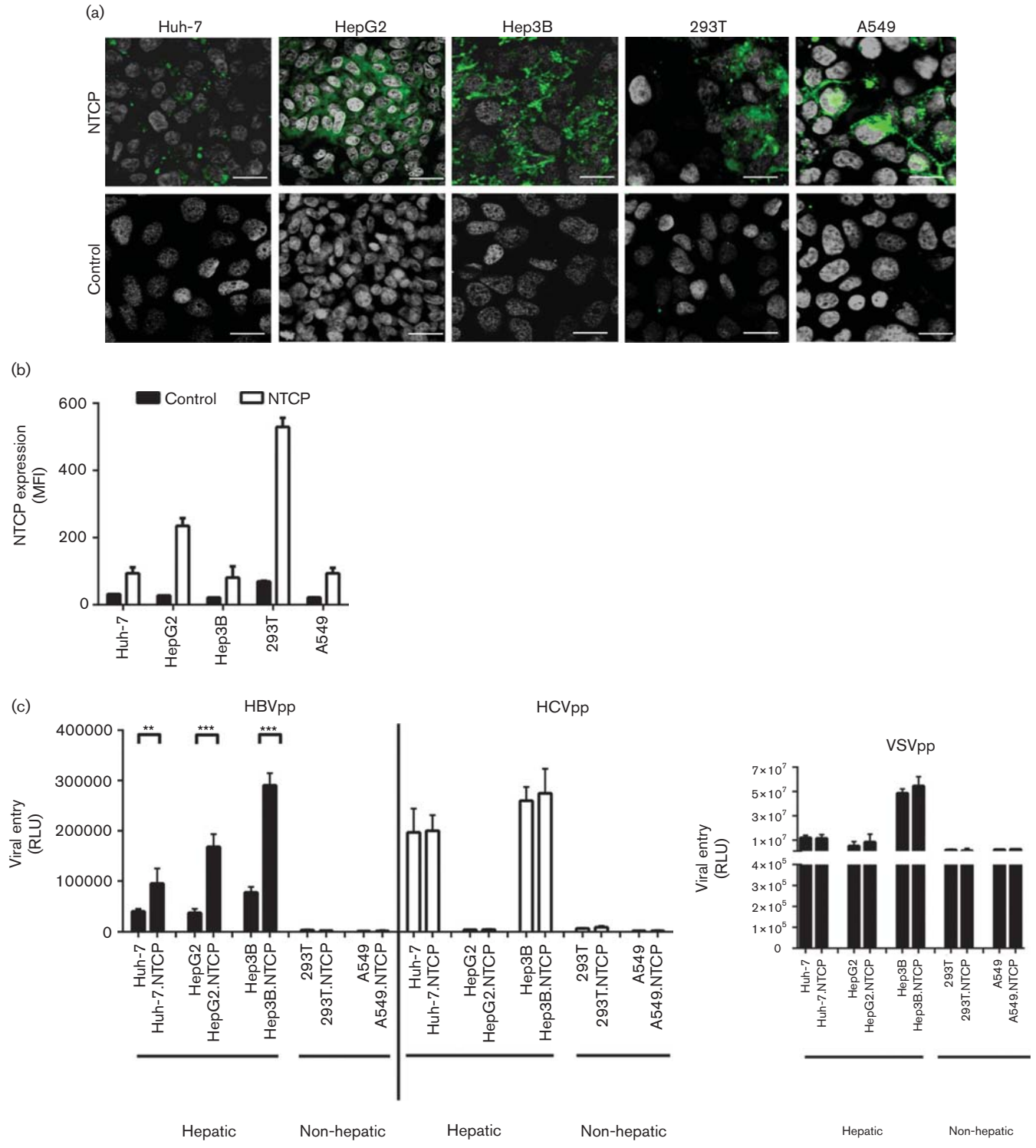

Fig. 3. NTCP expression alone is not sufficient to confer HBVpp infection of non-hepatic cells. (a) Hepatocyte-derived (Huh-7, HepG2, Hep3B) and non-liver embryonal kidney (293T) or lung (A549) epithelial cells were transduced to express NTCP and expression was confirmed by staining with fluorescent-labelled HBV Pre-S1 peptide. (b) NTCP expression was quantified by flow cytometry using K9 anti-NTCP and irrelevant control immunoglobulin, where the data are expressed as mean fluorescence intensity (MFI) of K9 staining and the irrelevant immunoglobulin value subtracted. (c) Parental and NTCP-expressing cell lines were infected with HBVpp generated from HBV-L and pNL4.3-luc-R$^-$E$^-$ transfected Huh-7.NTCP cells,

HCVpps or VSVpps for 72 h. Results are the mean ± SD of three independent experiments where the no envelope RLU value was subtracted from the HBVpp, HCVpp or VSVpp values and significance assessed using Student's *t*-test (**$P<0.01$, ***$P<0.001$).

and dissemination, and will aid in the discovery of new antiviral agents.

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