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Sodium Taurocholate Cotransporting Polypeptide Is the Limiting Host Factor of Hepatitis B Virus Infection in Macaque and Pig Hepatocytes

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Infections with the human hepatitis B virus (HBV) and hepatitis D virus (HDV) depend on species-specific host factors like the receptor human sodium taurocholate cotransporting polypeptide (hNTCP). Complementation of mouse hepatocytes with hNTCP confers susceptibility to HDV but not HBV, indicating the requirement of additional HBV-specific factors. As an essential premise toward the establishment of an HBV-susceptible animal model, we investigated the role of hNTCP as a limiting factor of hepatocytes in commonly used laboratory animals. Primary hepatocytes from mice, rats, dogs, pigs, rhesus macaques, and cynomolgus macaques were transduced with adeno-associated viral vectors encoding hNTCP and subsequently infected with HBV. Cells were analyzed for Myrcludex B binding, taurocholate uptake, HBV covalently closed circular DNA formation, and expression of all HBV markers. Sodium taurocholate cotransporting polypeptide (Ntcp) from the respective species was cloned and analyzed for HBV and HDV receptor activity in a permissive hepatoma cell line. Expression of hNTCP in mouse, rat, and dog hepatocytes permits HDV infection but does not allow establishment of HBV infection. Contrarily, hepatocytes from cynomolgus macaques, rhesus macaques, and pigs became fully susceptible to HBV upon hNTCP expression with efficiencies comparable to human hepatocytes. Analysis of cloned Ntcp from all species revealed a pronounced role of the human homologue to support HBV and HDV infection. *Conclusion:* Ntcp is the key host factor limiting HBV infection in cynomolgus and rhesus macaques and in pigs. In rodents (mouse, rat) and dogs, transfer of hNTCP supports viral entry but additional host factors are required for the establishment of HBV infection. This finding paves the way for the development of macaques and pigs as immunocompetent animal models to study HBV infection *in vivo*, immunological responses against the virus and viral pathogenesis. (HEPATOLOGY 2017;66:703-716).

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Hepatitis B, which is caused by the human hepatitis B virus (HBV), is a widespread viral infection, with around 240 million chronic active carriers worldwide.[1] Chronic HBV infection results in progressive liver disease leading to cirrhosis and hepatocellular carcinoma, often decades after infection. HBV is a partially double-stranded, DNA-containing, enveloped para-retrovirus that

Abbreviations: AAV8, adeno-associated virus 8; cccDNA, covalently closed circular DNA; cDNA, complementary DNA; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HBcAg, hepatitis B virus core antigen; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HDAg, hepatitis D antigen; HDV, hepatitis D virus; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; hNTCP, human sodium taurocholate cotransporting polypeptide; MyrB, Myrcludex B; Ntcp, sodium taurocholate cotransporting polypeptide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG8000, polyethylene glycol 8000; PHH, primary human hepatocyte; qPCR, quantitative polymerase chain reaction; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; rcDNA, relaxed circular DNA; TC, taurocholate; YFP, yellow fluorescent protein.

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Potential conflict of interest: Stephan Urban is a coapplicant and coinventor for patents protecting Myrcludex B as an HBV/HDV entry inhibitor.

belongs to the family *Hepadnaviridae*. Members of this family are found in many species, including rodents and birds.[2]

Hepatitis D virus (HDV) is an RNA satellite virus that replicates in HBV-infected individuals. HDV requires HBV as a helper virus, because it depends on the HBV-encoded envelope proteins for assembly and dissemination within its human host.[3] Chronic HBV/HDV coinfections cause the most severe form of viral hepatitis. Although HBV vaccines protect from infection of both HBV and HDV, treatment options for the 240 million chronic HBV carriers are limited to nucleoside analogues and interferon-α, with little curative outcome.[4] Unfortunately, nucleoside analogue regimens, which diminish reverse transcription of HBV pregenomic RNA, have no effect on HDV replication. Following the establishment of susceptible cell lines, expressing the HBV/HDV receptor human sodium taurocholate cotransporting polypeptide (hNTCP),[5,6] novel drugs are currently being identified and preclinically evaluated.[4] The development of such drugs into clinical proof-of-concept studies is still profoundly impeded by the lack of appropriate animal models. It is therefore of high importance to generate such models e.g., by identification of host-specific factors that overcome species-specific restrictions of distinct replication steps or by the identification of species that are only restricted by the respective sodium taurocholate cotransporting polypeptide (Ntcp) receptor.

HBV shows a peculiar host range, infecting only humans, chimpanzees, and, under certain experimental conditions, the tree shrew *Tupaia belangeri*.[7] Laboratory animals such as mice, rats, and macaques are resistant to HBV and HDV infection.[8] The chimpanzee, therefore, remains the only immunocompetent model for HBV and HDV. Several studies to understand the clearance of a natural infection have been obtained in that model.[9] However, because the experimental use of human primates is ethically no longer acceptable, novel models are required to understand how chronic infections can be cured and immunological control of the virus can be obtained with upcoming drugs.[10] The discovery of hNTCP as a limiting host factor for HBV and HDV infection in human hepatoma cells raised the hope to develop hNTCP transgenic animals that become fully susceptible to HBV. Such mice weakly support HDV infection[11,12] but are still constrained for HBV infection.

There is accumulating evidence that the distinct species specificity of HBV and HDV is related to hNTCP-dependent and hNTCP-independent steps in the early infection events of both viruses. Differences in later steps such as virus assembly play minor roles; for example, HBV-transgenic mice[13] are fully competent to produce infectious virions. Using the HBV/HDV entry inhibitor Myrcludex B (MyrB),[14] a synthetic L-protein–derived lipopetide that interacts with hNTCP and thereby interferes with the formation of a functional receptor complex, we have shown previously that HBV/HDV can bind hepatic Ntcp from some nonsusceptible species (e.g., mouse, rat, dog). Curiously, MyrB and thus HBV/HDV is unable to interact with some forms of Ntcp, such as that from cynomolgus macaques, rhesus macaques, and pigs.[15,16] These peculiar binding properties could be mapped to distinct sequence elements within the Ntcp homologues in humans, mice, and macaques (Supporting Fig. S6); Ntcp from mice but not from cynomolgus macaques binds MyrB, because of a crucial sequence element located at position 157-165.[5,6] However,

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mouse Ntcp cannot mediate efficient virus entry due to a sequence alteration within amino acids 84-87.[5,17] Only the human homologue hNTCP supports both binding and successful infection. When hNTCP is expressed in cell lines of murine origin, these cells become HDV-susceptible to a limited level but remain essentially refractory to HBV,[5,17,18] reflecting the observations *in vivo*. These restrictions can be overcome through fusion of hNTCP-expressing mouse cells with nonsusceptible human HepG2 hepatoma cells, indicating a lacking host factor in murine cells.[18]

To systematically investigate hNTCP-mediated species restrictions in laboratory animals that might serve as candidates for an immunocompetent model for HBV/HDV, we expressed hNTCP in primary hepatocytes from mice, rats, dogs, cynomolgus macaques, rhesus macaques, and pigs and analyzed their ability to support HBV replication. We found that hepatocytes from macaques and pigs become fully susceptible to both HDV and HBV, indicating that their endogenous Ntcp homologues are the only factor restricting replication of HBV.

Materials and Methods

PRIMARY HEPATOCYTE ISOLATION AND CULTURE

Primary mouse (C57BL/6) and rat (Han Wistar) hepatocytes were isolated from 10- to 14-week-old animals in a two-step perfusion method using an ethylene glycol tetraacetic acid (EGTA)-containing buffer [2 mM glutamine, 0.5% glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 2 mM EGTA diluted in phosphate-buffered saline (PBS)], followed by perfusion with 0.3 mg/mL collagenase type IV (Sigma-Aldrich) (in 2 mM glutamine, 0.5% glucose, 25 mM HEPES, 3 mM $CaCl_2$ diluted in Williams E medium) as described previously.[15] Primary human hepatocytes (PHHs) were isolated from liver resections of patients undergoing partial hepatectomy as described previously.[19] All tissue donors provided written informed consent for the experimental use of their liver specimen. The protocol was approved by the ethics commission of Hannover Medical School. Primary cynomolgus macaque (*Macaca fascicularis*), rhesus macaque (*Macaca mulatta*), pig (Göttingen minipig), and dog (beagle) hepatocytes were obtained from the commercial vendor BioreclamationIVT. As a second independent source, primary cynomolgus hepatocytes were isolated freshly from livers of cynomolgus macaques as described previously.[20] Primary rat hepatocytes were cultured in Williams E medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 5 μg/mL insulin, 50 μM hydrocortisone hemisuccinate, and 1.5% dimethyl sulfoxide (DMSO). All other primary hepatocytes were cultured in HCM hepatocyte medium (Lonza) supplemented with 1.5% DMSO.

VIRAL TRANSDUCTION/ INFECTION

Primary hepatocytes were seeded in 24-well plates pretreated with 50 μg/mL rat tail collagen (Corning) overnight. After attachment of the cells (usually after 6-8 hours), transduction was initiated by inoculation of the cells with 50,000 genome equivalents (ge) per cell of adeno-associated virus 8 (AAV8)-hNTCP or AAV8-yellow fluorescent protein (YFP) in the presence of 4% polyethylene glycol 8000 (PEG8000) (Sigma-Aldrich). After 16 hours, cells were washed and incubated with fresh medium. Three days after transduction, cells were infected with HBV or HDV by inoculation with 750 ge/cell HBV or 0.1, 1, or 10 IU/cell HDV (determined by way of quantitative reverse-transcription polymerase chain reaction [qRT-PCR] using the World Health Organization HDV International Standard [Paul-Ehrlich-Institut, Langen, Germany] for normalization) in medium containing 4% PEG8000. For entry inhibition, cells were preincubated with 500 nM MyrB for 30 minutes. The same concentration was present in the infection inoculum as well as during the first day after removal of the inoculum. After 16 hours, the infection inoculum was removed, the cells were washed three times with PBS, and fresh medium was added. For quantification of HBV infection, secreted hepatitis B virus e antigen (HBeAg) was determined in the cell culture supernatant by the ADVIA Centaur XPTM automated chemo-luminescence system (Siemens), and hepatitis B surface antigen (HBsAg) was quantified as international units by way of enzyme-linked immunosorbent assay (ELISA) (Architect, Abbott).

COVALENTLY CLOSED CIRCULAR DNA QUANTIFICATION

DNA samples from total lysates of infected cells were prepared using the NucleoSpin Tissue Kit

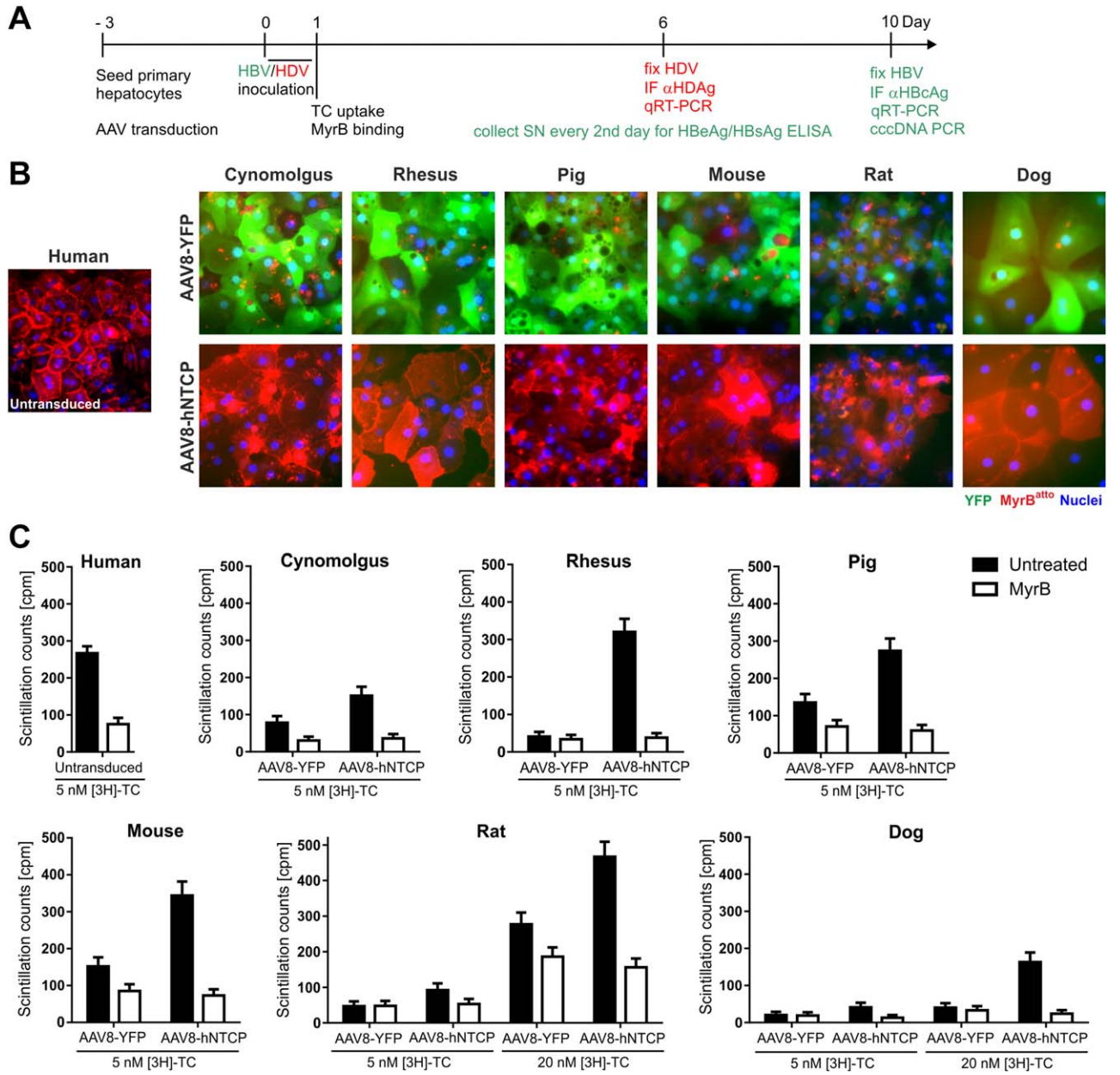

FIG. 1. Transgene expression efficiency of AAV8-transduced primary hepatocytes from different species. (A) Experimental layout: Primary hepatocytes from different species were seeded in 24-well plates and inoculated with AAV8 (carrying either YFP or hNTCP) in the presence of 4% PEG8000 for 16 hours. (B) Four days after transduction, cells were incubated with atto565-labeled MyrB for 30 minutes at 37°C, washed, and imaged for bound $MyrB^{atto}$ (red), YFP transgene expression (green), and nuclei (blue). Only an overlay of all three colors is shown (magnification ×400). See Supporting Fig. S3 for single images. PHHs, which were not transduced, served as positive control. (C) Four days after transduction, cells were incubated with 5 nM or 20 nM [3H]-labeled TC for 15 minutes at 37°C in the presence or absence of 2 μM MyrB and washed extensively, before uptake of [3H]-TC was measured by way of liquid scintillation counting of the cell lysate.

(Macherey-Nagel). Briefly, cells were lysed in provided T1 buffer and incubated with proteinase K and B3 buffer at 70°C for 1 hour, and the following steps were performed according to the protocol. For quantification of covalently closed circular DNA (cccDNA), DNA samples were incubated with T5 exonuclease

(New England Biolabs) (reaction: 5 μL of the eluted DNA, 1 μL 10× reaction buffer, 5 units T5 Exo, and 3.5 μL water) at 37°C for 1 hour to digest genomic DNA and viral relaxed circular DNA (rcDNA). The enzyme was inactivated at 70°C for 20 minutes and products were immediately subjected to quantitative polymerase chain reaction (qPCR) in PerfeCTa qPCR Toughmix (Quanta Biosciences) with cccDNA-specific primers (forward: 5′-GTGGTTATCCTG CGTTGAT-3′; reverse: 5′-GAGCTGAGGCGG TATCT-3′) and a probe (5′-FAM-AGTTGGCGA GAAAGTGAAAGCCTGC-TAMRA-3′) using an optimized two-step program (denaturation: 95°C for 15 minutes; annealing and extension: 95°C for 5 seconds and 63°C for 70 seconds for 50 cycles). A plasmid carrying a head-to-tail dimer of the full-length HBV genome was used for calibration.[21]

CLONING OF HOMOLOGOUS hNTCP SEQUENCES

Total RNA was extracted from freshly isolated primary hepatocytes using the Nucleospin RNA Kit (Macherey Nagel) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (ABI) according to the manufacturer's protocol. PCR primers were designed based on database sequences for the different hNTCP homologues (see Supporting Table S1 for details). The hNTCP coding regions were amplified from the complementary DNA (cDNA) by way of PCR using the HotStarTaq Plus DNA polymerase (Qiagen) and ligated by way of the introduced restriction sites into a pWPI plasmid that encodes green fluorescent protein under an internal ribosomal entry site, to monitor transfection efficiencies.[5] All sequences were verified by way of Sanger sequencing.

AAV/HBV/HDV production, taurocholate uptake, MyrB binding, and immunofluorescence staining were performed as described previously[5,18,22]; see the Supporting Information for details.

Results

To analyze the susceptibility of different primary hepatocytes toward HBV and HDV, we seeded freshly prepared or frozen cells from cynomolgus or rhesus macaques, pigs, mice, rats, and dogs in 24-well plates at densities of 2.2×10^5 (mice) or 3.5×10^5 (all others) cells/well. Eight hours after plating, the cells were transduced with equal genome equivalents of AAV8 encoding YFP (transduction control) or hNTCP.[5] This construct promotes taurocholate (TC)-transporter and HBV/HDV receptor function to the transduced cells. The experimental setup is depicted in Figure 1A. Nontransduced PHHs, the current gold standard for *in vitro* infections with HBV and HDV, were used as a control. One day after seeding, cells were inspected by way of microscopy to ensure the formation of confluent monolayers of nondividing hepatocytes, which is optimal for efficient infection (Supporting Fig. S1).[23] For dog hepatocytes, only approximately 60% confluency could be achieved. To study a possible contribution of the endogenous forms of Ntcp to the effects mediated by the transduced hNTCP, we performed a kinetic analysis. As depicted exemplarily for primary mouse hepatocytes in Supporting Fig. S2, endogenous Ntcp in the primary hepatocytes is rapidly lost under the chosen culture conditions: after isolation, mouse Ntcp transcripts were degraded with a half-life of 3.7 hours, whereas mouse Ntcp protein functionality decreased with a half-life of 27.2 hours as measured by TC uptake assay. Therefore, we estimate that at the time of infection or transduction analysis experiments, activity of endogenous Ntcp was <10% compared with freshly isolated hepatocytes. This result is consistent with previous studies performed in human hepatocytes.[24]

TRANSDUCTION OF PRIMARY HEPATOCYTES BY AAV8

Four days after AAV8 transduction, transgene expression was analyzed using a MyrB-atto binding test (Fig. 1B) and a TC uptake assay (Fig. 1C). All YFP-transduced primary cells showed YFP expression in >80% of the parenchymal cells, indicating high transduction efficiencies with the AAV8 vector in hepatocytes of all species, whereas hNTCP-transduced cells show no YFP signal (see Supporting Fig. S3 for single-channel images). Binding of fluorescently labeled MyrB to hNTCP resulted in a red fluorescence signal in PHHs, mostly at the cell membrane but also in the cytoplasm (Fig. 1B, left). The hNTCP-transduced hepatocytes showed specific MyrB binding in the majority of cells, whereas the YFP-transduced cells only showed a few unspecific dot-like signals, which possibly reflected unspecific binding of the lipopeptide (Fig. 1B, right panels). Because hNTCP is a bile salt transporter with preference for conjugated bile salts, we analyzed the uptake of [^{3}H]-TC in the cells

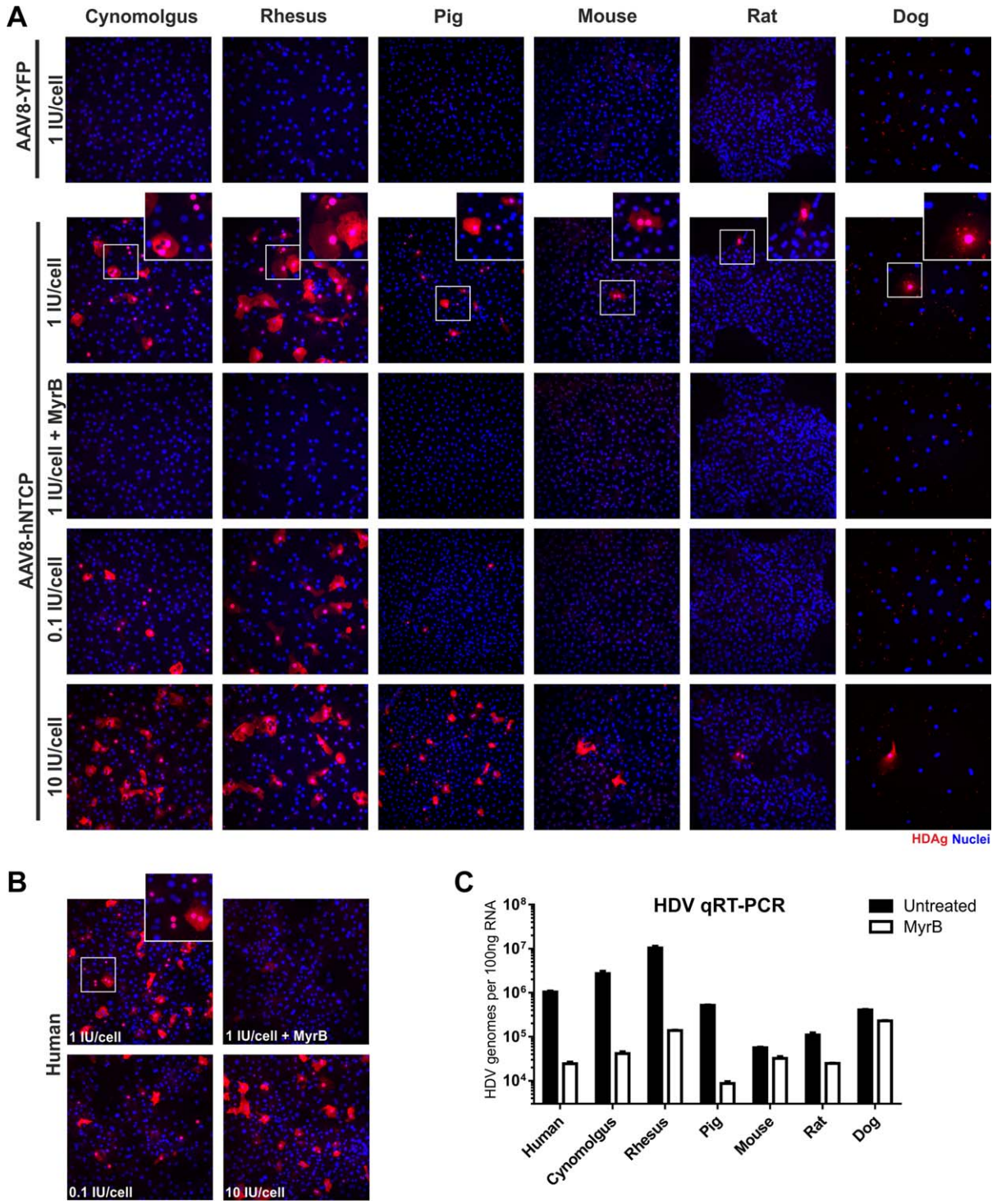

FIG. 2. HDV infection of hNTCP-transduced primary hepatocytes from different species. (A) Three days after AAV8 transduction, cells were inoculated with HDV at 0.1, 1, or 10 IU/cell in the presence or absence of 500 nM MyrB. (B) PHHs were directly infected without previous transduction. Six days after infection, cells were fixed and immunostained with an antibody against HDAg (red, magnification ×200 [insets, magnification ×400]). (C) Three days after AAV8-hNTCP transduction, cells were infected with HDV at 1 IU/cell in the presence or absence of 500 nM MyrB. Six days after infection, total RNA was extracted and reverse-transcribed, and intracellular HDV genomes were quantified by way of qPCR with HDV-specific primers and probe. Note that PHHs were directly infected with HDV without previous transduction.

by liquid scintillation counting as described previously[22] (Fig. 1C). hNTCP-transduced cells showed 2- to 7-fold higher TC uptake rates compared with YFP-transduced cells, indicating that the ectopically expressed hNTCP receptor was correctly sorted to the hepatocyte surface and functions as a transporter and that the endogenous forms of Ntcp do not play a significant role in TC transport. Furthermore, hNTCP-dependent TC transport could be inhibited by MyrB, indicating accurate interaction of the HBV preS-domain with the ectopically expressed receptor on the surface of all parenchymal cells. For rat and dog hepatocytes, initial TC uptake rates were low; therefore, we increased the concentration of [^{3}H]-TC by 4-fold to enhance comparability between YFP- and hNTCP-transduced cells. Taken together, primary hepatocytes from all species when complemented with human hNTCP support binding of MyrB and transport of TC.

hNTCP-TRANSDUCED HEPATOCYTES GAIN SUSCEPTIBILITY TO HDV

To test their susceptibility to HDV, AAV-hNTCP transduced hepatocytes were inoculated at day 3 post transduction with 0.1, 1, or 10 IU/cell of HDV for 16 hours. Six days later, infection was analyzed by way of immunofluorescence staining of hepatitis D antigen (HDAg) (Fig. 2A,B). HDAg-positive cells were found among all hNTCP-transduced cells. No HDAg-expressing cells were observed in YFP-transduced cells or in cells treated with MyrB during virus inoculation. Strikingly, the number of HDAg-positive cells was higher in human, macaque, and pig hepatocytes when compared with hepatocytes from mice, rats, and dogs, indicating that the latter still exhibit an additional limitation to HDV infection. Whereas infection with the minimal 0.1 IU/cell already resulted in clearly detectable infection events per view field in human, macaque, and pig cells, no infected cells could be detected for the other species (mice, rats, dogs) at this infectious dose. The lower degree of infection was consistent with the quantification of intracellular HDV genomes after infection with a 10-fold higher dose of HDV (Fig. 2C). Whereas human, macaque, and pig hepatocytes showed very high levels of intracellular HDV RNA, which can be blocked by MyrB, rat and dog hepatocytes showed generally over 50-fold fewer replicative intermediates. Moreover, the difference to the MyrB-treated cells was significantly lower compared with the other species.

HBV INFECTION OF MACAQUE AND PIG HEPATOCYTES AFTER hNTCP TRANSDUCTION

To investigate whether ectopic expression of hNTCP in the primary hepatocytes allows infection with HBV, cells were inoculated with HBV in the presence or absence of MyrB. Cell supernatants were collected every second day starting from day two post infection for quantification of secreted HBsAg (Fig. 3A). A strong time-dependent increase of secreted HBsAg could be observed in the supernatants of hNTCP-transduced, non–MyrB-competed human, cynomolgus macaque, rhesus macaque, and pig hepatocytes. Remarkably, the levels of secreted HBsAg measured by quantitative assays in international units were comparable between human, cynomolgus macaque, and rhesus macaque hepatocytes (reaching up to 73 IU between days 8 and 10 after HBV infection in the rhesus macaque samples). Pig hepatocytes showed an approximately 10-fold lower secretion of HBsAg, but a strong increase over time was still evident. HBsAg was not produced when cells were transduced with AAV8-YFP or when hNTCP-transduced cells were treated with MyrB. This indicates that viral entry exclusively proceeds via the transduced receptor. No increase in secreted HBsAg occurred in hNTCP-transduced mouse, rat, or dog hepatocytes. The determined levels were comparable with those measured for MyrB-competed or YFP-transduced control cells and reflect the decrease of HBsAg input after medium exchange. The kinetics of HBsAg secretion was substantiated through quantification of secreted HBeAg (Supporting Fig. S4). Again, comparable levels of HBeAg secretion occurred in PHHs and transduced cells from nonhuman primates. Mouse, rat, and dog hepatocytes showed no specific signals, whereas pig hepatocytes showed a delayed increase to high levels. Ten days (or in the case of rats, 8 days) after HBV infection, cells were fixed and stained with an antibody recognizing hepatitis B virus core antigen (HBcAg) (Fig. 3B). Comparable numbers of HBcAg-positive cells were found in human, cynomolgus macaque, rhesus macaque, and pig hepatocytes but not in mouse, rat, or dog hepatocytes. Treatment with MyrB blocked infection, verifying hNTCP as the responsible host factor.

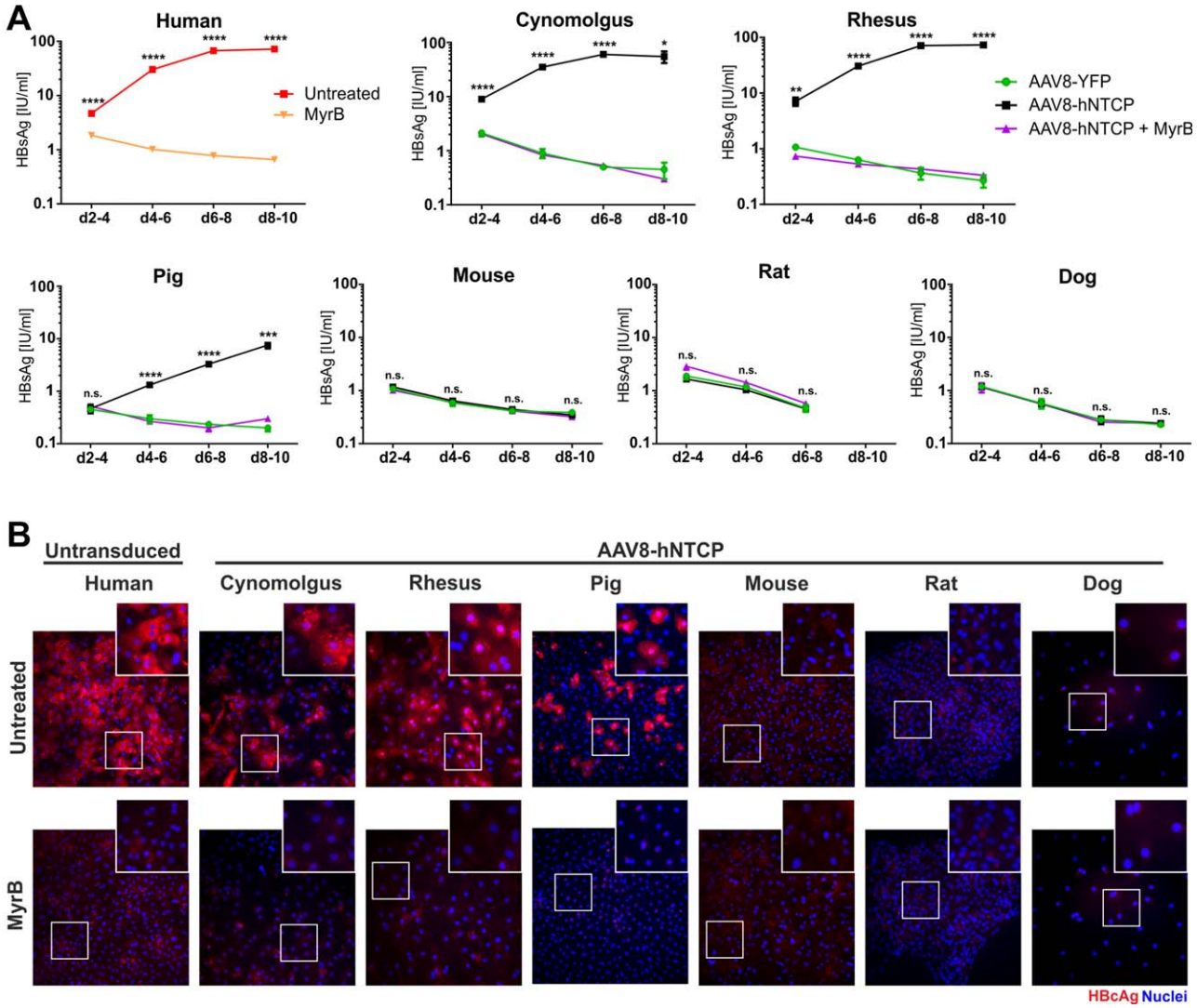

FIG. 3. HBV infection of hNTCP-transduced primary hepatocytes from different species. Three days after AAV8 transduction, cells were inoculated with HBV at a multiplicity of genome equivalents of 750 in the presence or absence of 500 nM MyrB. PHHs were directly infected without previous transduction. (A) After washing of the cells at day 1 and 2 after infection, supernatant was collected every second day, and secreted HBsAg was quantified by way of ELISA. Each data point represents the mean of three biological replicates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s., not significant. (B) Ten days after infection (8 days for rat, as the cells started to detach after 8 days), cells were fixed and immunostained with an antibody recognizing HBcAg (red, magnification ×200 [insets, magnification ×400]).

In addition to the levels of secreted antigens and intracellular expression of HBcAg, we also quantified intracellular transcripts by qRT-PCR (Fig. 4A). Consistent with the results from antigen secretion, HBV RNA levels in noncompeted infections of human, cynomolgus macaque, rhesus macaque, and pig hepatocytes were approximately 40- to 400-fold higher compared with MyrB-inhibited infections. No differences between the MyrB-competed and noncompeted infections were observed for mouse, rat, or dog hepatocytes. Remarkably, dog hepatocytes showed significantly increased levels of cell-associated HBV RNA. A possible explanation could be a higher unspecific absorption of RNA-containing particles present in the inoculum. Such particles have been described as major constituents of cell culture–derived viral particles.[25] To investigate whether cccDNA is established in these cells, we extracted total intracellular HBV DNA, digested the relaxed circular DNA pool by T5 exonuclease, and quantified the nuclease-

resistant fraction using cccDNA-specific primers. This method has been used reliably before[26] and has been verified in comparison with Southern blot analyses (Bingqian Qu, personal communication). cccDNA could only be detected in infected hepatocytes from human, cynomolgus macaque, rhesus macaque, and pig hepatocytes (Fig. 4B). As expected, MyrB treatment completely inhibited cccDNA formation. In line with previous findings,[13] no cccDNA could be detected in mouse, rat, or dog hepatocytes. In conclusion, our data show that hNTCP receptor-mediated HBV infection is possible in primary hepatocytes from macaque and pig, resulting in cccDNA formation and accumulation of comparable levels of viral transcripts, secreted antigens, and release of virus DNA (Supporting Fig. S5).

Ntcp FROM MACAQUE AND PIG SUPPORTS NEITHER BINDING NOR ENTRY OF HBV

So far, only Ntcp from cynomolgus monkeys,[6] mice,[5,17] and *Tupaia*[6] were analyzed in *in vitro* systems for their ability to support HBV infection. Regarding their preS binding activities, hNTCP and Ntcp from mouse bind MyrB, whereas Ntcp from cynomolgus monkey is binding deficient.[6,15,16] It was thus interesting to study and characterize the ability of all Ntcp homologues used in this study to support infection in cells under more defined conditions. We cloned the respective cDNAs from the reverse-transcribed messenger RNA of the different primary hepatocytes into a eukaryotic expression vector. HuH7 cells were transfected with the resulting constructs and inoculated 2 days later with HBV/HDV or analyzed for TC uptake and MyrB binding (Fig. 5A). Equal transfection efficacies were verified by fluorescence microscopy, since the plasmids additionally encode green fluorescent protein. Similar TC uptake rates were obtained for all Ntcp homologues but not for the empty vector control, indicating that all cloned Ntcps were functionally expressed and localized to the plasma membrane (Fig. 5B). TC uptake was inhibited by MyrB (2 μM) to background levels for human, mouse, rat, and dog Ntcp verifying that the peptide binds and efficiently inhibits the transporter function. In contrast, inhibition of the Ntcp transporters from cynomolgus macaque, rhesus macaque, and pig was only 20%-30%, indicating a profound defect in MyrB to bind and thereby inhibit their activity. This fitted to the results of a direct binding assay using atto565-labeled MyrB

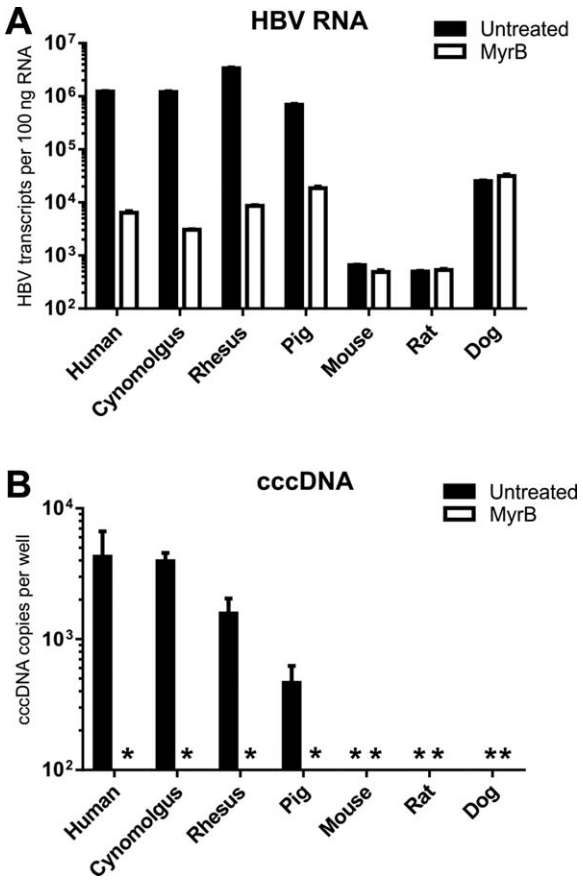

FIG. 4. Quantification of viral nucleic acids after HBV infection. AAV8-hNTCP–transduced primary hepatocytes were infected with HBV as described in Figure 3 (PHHs were infected without previous transduction). Cells were lysed at day 10 after infection (day 8 for rats). (A) Total RNA of the cells was extracted and reverse-transcribed, and the total HBV transcripts were quantified by way of qPCR using primers that bind in the viral X open reading frame. Lower limit of quantification: 10^2 transcripts. (B) Total DNA was extracted, and genomic DNA as well as viral rcDNA were digested using T5 exonuclease. The remaining viral cccDNA was quantified using primers and a probe that spanned the gap region. Values below the limit of quantitation are marked by asterisks (*).

(Fig. 5C). No binding was observed for cells expressing cynomolgus macaque, rhesus macaque, and pig Ntcp, whereas specific membrane association of the peptide occurred for the other Ntcp homologues (human, mouse, rat, and dog).

To test the capability of the different Ntcp homologues to support infection, the transfected HuH7 cells were inoculated with HDV (Fig. 5D,E) or HBV (Fig. 5F), and infection markers were quantified. As expected, transfection of hNTCP fully supported both HDV and HBV infection, whereas cynomolgus Ntcp

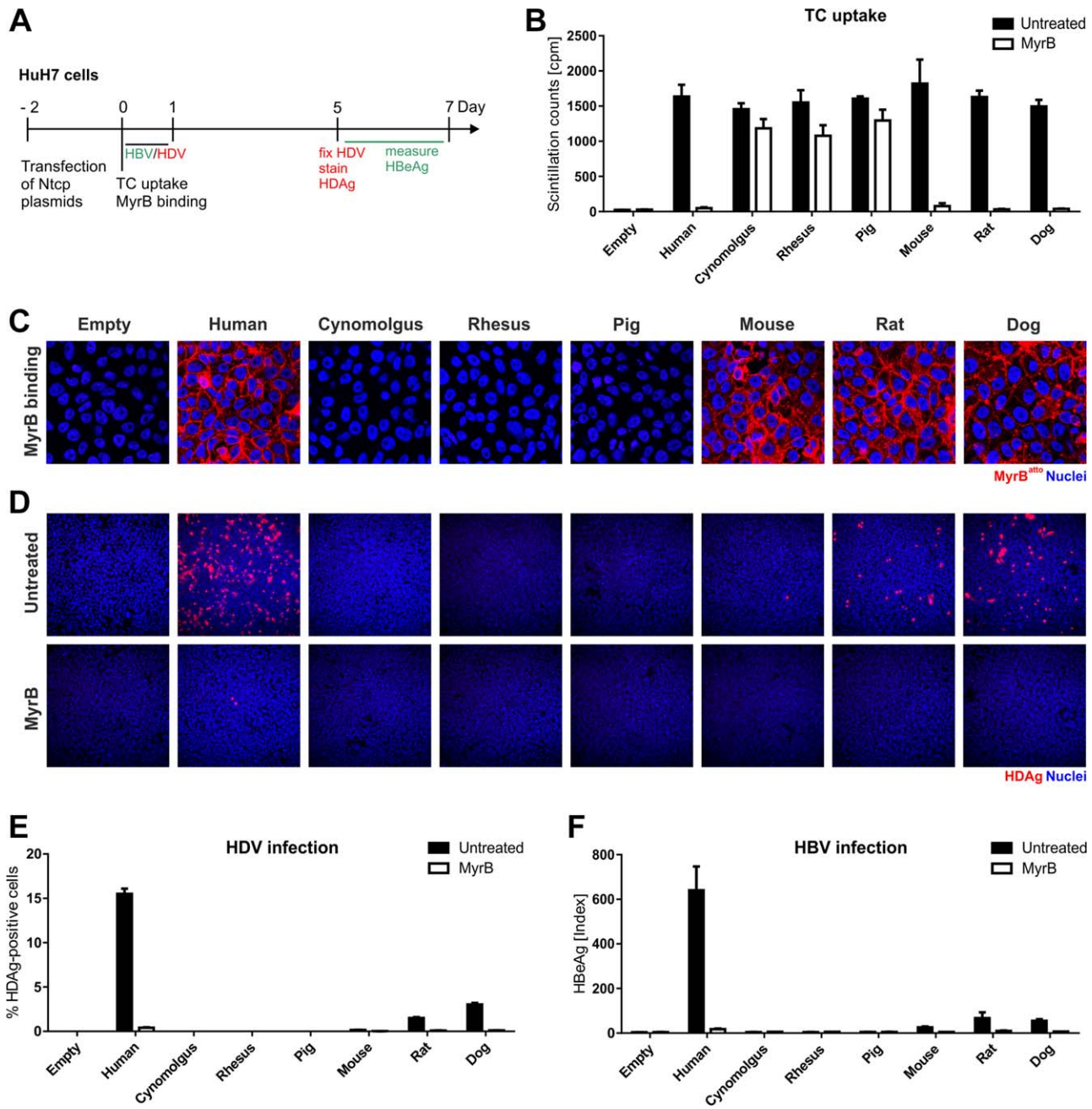

FIG. 5. Characterization of hNTCP homologues from different species. All Ntcp expression plasmids were cloned from the cDNA of primary hepatocytes. (A) Experimental design of the Ntcp characterization: HuH7 cells were transfected with plasmids encoding Ntcp from different species and 2 days after transfection, cellular, or infection assays were performed. (B) Cells were incubated with [3H]-labeled TC for 15 minutes at 37°C in the presence or absence of MyrB and washed extensively, before uptake of [3H]-TC was measured by liquid scintillation counting of the cell lysate. (C) Cells were incubated with atto565-labeled MyrB for 30 minutes at 37°C, washed, and fixed. MyrB binding was analyzed using confocal microscopy. (D) Cells were infected with HDV in the presence or absence of MyrB. Five days after infection, cells were fixed and immunostained with an antiserum against HDAg. (E) HDV immunostaining was quantified by automated image acquisition with 16 images per well. HDAg-positive cells and total nuclei were quantified automatically using ImageJ software. (F) Cells were infected with HBV in the presence or absence of MyrB. Supernatant of the infected cells was collected from day 5 to day 7 after infection, and secreted HBeAg was quantified by ELISA.

did not support infection with either virus. The same was true for rhesus and pig Ntcp. Surprisingly, mouse, rat, and dog Ntcp supported infection at a low level of 2%, 10%, and 14% compared with hNTCP. This limited effect could still be blocked by MyrB, indicating that entry proceeded through the respective Ntcp homologue. Taken together, Ntcp from cynomolgus macaques, rhesus macaques, and pigs, although being able to transport TC, are deficient in HBVpreS/MyrB-binding and mediating HBV or HDV infection. Ntcp from mice, rats, and dogs can mediate infection with both viruses to very low levels, and hNTCP is the only receptor homologue to fully support both HBV and HDV binding and infection.

Discussion

HBV is a virus with notable species specificity. Successful infection of chimpanzees but restriction of infection in macaques suggest an adaptation of the virus to evolutionary related species. However, the finding that HBV can infect *Tupaia belangeri*[27] implies a more complex adaptation of the virus to its host. Because none of the susceptible species can be applied for *in vivo* immunological studies, we still lack immunocompetent animal models to investigate the pathogenesis and the effect of new antiviral drugs that control infection and may lead to "functional cure." The discovery of hNTCP as the *bona fide* receptor for HBV led to the establishment of susceptible cell culture systems for HBV and HDV and allows gaining molecular insights into receptor-mediated restrictions of the host specificity of both viruses. Understanding these restrictions related to virus entry is key for the development of immunocompetent animals.

In this study, we systematically compared primary hepatocytes from different laboratory animals with respect to their ability to support HBV infection after expression of the human receptor hNTCP. We found that hepatocytes from cynomolgus macaque, rhesus macaque, and pig become fully susceptible to HBV, supporting entry and all subsequent steps such as nucleocapsid release, transport of nucleocapsids and nuclear import of rcDNA, cccDNA formation, unrestricted RNA transcription, and production of viral proteins. Through characterization of cloned Ntcp homologues of the different species, we found that hNTCP is the only homologue that efficiently mediates viral entry. Our finding implies that homologues from naturally susceptible species such as *Tupaia belangeri*, chimpanzee, gorilla, orangutan, and bats may also fulfill this function; however, it remains an open question whether minor species-specific differences exist. The fact that macaque and pig hepatocytes replicate HBV with efficacy similar to that of PHHs upon hNTCP expression indicates that no further constraints by intracellular dependency factors or species-specific restriction factors are present. In contrast, mouse hepatocytes expressing hNTCP are still resistant to HBV. This indicates the requirement of additional factors in these cells, an observation that has also been observed in mouse liver cell lines.[5,17,18,28] Although the specific replication step at which this restriction occurs is still poorly understood, a recent study suggests that it occurs after hNTCP-mediated entry but before cccDNA transcription and that it is due to a missing host dependency factor rather than presence of an active murine restriction factor.[18] This missing host factor is probably not species-specific, because one peculiar mouse liver cell line (AML12) supports the full HBV replication cycle, including cccDNA formation after hNTCP expression.[26,29] To overcome this peculiar limitation in murine cells, more thorough investigations of the AML12 cell line and particularly its differences to other murine cell lines will be crucial.

In this study, we addressed the question whether hepatocytes of other commonly used laboratory animals underlie the same limitation as mouse hepatocytes. As expected, hNTCP transduction rendered mouse but also rat and dog hepatocytes susceptible to HDV infection.[5,17,18,28] Accordingly, immunocompetent mouse models for HDV infection could be established once hNTCP is complemented. He et al.[11] reported the successful *in vivo* HDV infection of hNTCP-transgenic mice. However, the study also revealed several restraints: 1) only very young mice could be infected, 2) high virus inoculates were needed, 3) infection efficiency was very low, and 4) infection was transient and cleared after 20 days. In a recent publication, exchange of amino acids 84, 86, and 87 in endogenous mouse Ntcp also resulted in a gain of HDV susceptibility[12]; however the efficacy was still lower than that observed in PHH-transplanted uPA-SCID mice.[30] These findings fit very well to the data reported here (Fig. 2) in that HDV at a very high inoculation dose is required to obtain only low infection rates (compared with PHHs). This indicates an additional constraint in mouse hepatocytes also for HDV, which is not as strict for HBV but still pronounced. This limitation presumably relates to virus entry events

(e.g., attachment to HSPGs or interference with binding to endogenous mouse Ntcp or to innate immune responses triggered by HDV). Notably, it only occurs in hepatocytes of those species that support binding of the HBV preS domain, for unknown reasons. Because this HDV-related effect in hNTCP-expressing mouse hepatocytes likely also affects the efficacy of HBV entry, it is important to elucidate this limitation for the future development of immunocompetent mouse or rat models for HBV.

One concern related to our study was whether HBV binding-competent Ntcp from non–HBV-susceptible species like mouse, rat, and dog interfere with the efficacy of infection when coexpressed with hNTCP. This objection could be excluded in this study, because the endogenous Ntcp levels of the used primary hepatocytes declined substantially within the first 3 days of cultivation, as is evident through analysis of the TC transporter activity of nontransduced cells after plating (Supporting Fig. S2). However, we cannot rule out that coexpression of hNTCP and a binding-competent endogenous Ntcp in the same hepatocyte *in vivo* (e.g., by hNTCP expression in an Ntcp-competent animal) or by partial transplantation of PHHs into mouse livers (e.g., uPA-SCID mice) might also limit the efficacy of infection.

In the past, the chimpanzee was the only immuno competent animal model for HBV infection,[31,32] and these animals have been widely used to study cytolytic and noncytolytic mechanisms of HBV clearance during acute infection.[9] Due to ethical considerations, however, chimpanzees are no longer available as experimental animals; therefore, nonhuman primates such as macaques or other suitable animals are desirable for future HBV *in vivo* studies. Although *in vitro* and *in vivo* data demonstrate that Ntcp of macaques cannot interact with the HBV preS domain, due to an Ntcp sequence variation at amino acids 157-165[5,6,16] (see also Supporting Fig. S6), a naturally occurring transmissible HBV infection (genotype D) was reported among *Macaca fascicularis* from Mauritius Island.[33] The viral serum titers in these animals were very low, which may indicate marginal replication levels (if at all) in the liver.[33] Our finding that *trans*-complementation of macaque hepatocytes by hNTCP enables replication raises the question whether non–Ntcp-mediated genome delivery to hepatocytes occurs in these animals or whether other nonhepatic reservoirs of HBV exist. The discrepancy between the presence of a nonfunctional Ntcp receptor in macaques and the low-level maintenance of HBV markers without elimination of the virus by the immune system demands further investigation.

Our data demonstrate that macaque hepatocytes, after hNTCP transduction, become fully susceptible to HBV infection with infection rates indistinguishable from PHHs. This is consistent with a previous observation that baculovirus-mediated transduction of HBV over-length genomes into macaque hepatocytes initiates HBV replication, including the formation of cccDNA.[20] The fact that no other host-limiting factor is present indicates that macaques presumably represent an immunocompetent animal model for HBV when hNTCP is complemented *in trans* (e.g., after AAV8-mediated gene transfer or rendering the animal transgenic for hNTCP). Safe and efficient liver-specific transduction of macaques using recombinant AAV or adenoviral vectors has been shown using various transgenes.[34-36] Thus, further studies should focus on applying such hNTCP-encoding vectors for macaques and should perform HBV infection studies similar to those described in chimpanzees. Novel macaque-based animal models could be used to study the mounting of immune responses against infection, immune pathogenesis, evaluation of new therapies, and ideally the mechanisms of viral persistence, as has been described in persistently infected chimpanzees.[37]

An unexpected finding was that hNTCP transduction rendered pig hepatocytes susceptible to HBV. Infection efficacy and the kinetics of viral markers differed significantly compared with PHHs or transduced macaque hepatocytes but still reached high levels when infection proceeded. In a few recent reports, an HBV-related virus has been described in domestic pigs[38,39]; however, its characterization remains incomplete, and important markers such as cccDNA are missing. Because pigs are commonly used as animal models for hepatitis E virus infections,[40] a further characterization of a possible endogenous pig hepadnavirus would be important, especially with respect to the comparative analysis of receptor-independent viral determinants of replication. Whether pigs could be used as infection models after hNTCP expression remains to be tested.

Taken together, our data provide the basis for the rapid development of nonhuman primate animal models for HBV based on hNTCP-transduced macaques or small pigs. In contrast to murine or rat models, macaques and pigs are only restricted in their susceptibility by the lack of a functional hNTCP receptor and do not require complementation with additional host factors.

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