Hepatitis Delta virus persists during liver regeneration and is amplified through cell division both in vitro and in vivo

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Supplementary Material and Methods

Generation of HepG2-hNTCP and HepG2-hNTCP-RGB cells. The cDNA coding for hNTCP was cloned into the third-generation self-inactivating HIV-1 derived lentiviral vector LeGO-iG2-Puro+, co-expressing the fluorescent marker eGFP linked to a puromycin resistance by a 2A-sequence[1]. Production of lentiviral particles was described in detail earlier[2] and protocols are available online (http://www.LentiGO-Vectors.de). Transduction of HepG2 cells was done by adding viral-particle containing supernatant to the cells and subsequent selection with 1 µg/mL puromycin. Successful drug selection was determined by quantifying eGFP fluorescence in flow cytometry. Of note, the HepG2-hNTCP cells have already been eGFP-positive prior to the RGB marking, but the comparatively low brightness of eGFP from the hNTCP-expressing lentiviral vector does not interfere noticeably with the rendition of the RGB marking used to monitor clonal cell expansion by immunofluorescence.

To apply RGB marking for HepG2-hNTCP, we plated 50,000 cells per well in a 24-well plate and transduced them simultaneously with equal amounts of three lentiviral vectors, each expressing a fluorescent protein of a specific color linked to a puromycin resistance by a 2A-sequence: LeGO-Cer2-Puro+ (expressing Cerulean, blue), LeGO-V2-Puro+ (expressing Venus, green) and LeGO-C2-Puro+ (expressing mCherry, red). Cells were analyzed by flow cytometry, and the well with the desired transduction rate of 50% to 70% per color was selected for further experiments.

HepG2-hNTCP and HepG2-hNTCP-RGB cells were cultured in DMEM + GlutaMax-I (Invitrogen, Darmstadt, Germany) supplemented with 10% FBS (Invitrogen), 5 mg/ml gentamicin, 125 µg/ml amphotericin B (Invitrogen) and passaged once a week.

Virological measurements. Viral nucleic acids were extracted from mouse serum samples using the QiAmp MinElute Virus spin kit (Qiagen, Hilden, Germany) and from HepG2-hNTCP cells and liver tissue by using the RNeasy Micro and Mini RNA purification kit (Qiagen), respectively. HDV viremia and intracellular HDV RNA was determined via reverse transcription and qRT-PCR using the ABI Fast 1-Step Virus Master (Applied Biosystems, Carlsbad, USA) on an ABI Viia7 (Applied Biosystems). In detail, 5 µl RNA was denatured at 95°C for 10 min, immediately cooled down at -20°C and reverse transcribed at 50°C for 5 min with HDV specific primers and probes[3]. After inactivation of the reverse transcriptase at 95°C for 20 s, amplification was performed under the following conditions: 40 cycles at 95°C for 3 s and 60°C for 30 s [4]. The plasmid pBluescript II SK(þ), containing one copy of the HDV genome, was used as a standard for HDV cDNA quantification[5]. To determine HBV viremia and HBV RNA in the cell culture supernatant HBV-specific primers and probes (Taqman Gene Expression Assay Pa03453406_s1, Applied Biosystems) and the ABI Fast Advanced Master (Applied Biosystems) were used under the following conditions: Initial step 95°C 20 s; 40 cycles at 95°C for 3 s and 60°C for 30 s. HBsAg quantification was performed using the Architect HBsAg assay (Abbott Ireland Diagnostics, Sligo, Ireland). For genomic and antigenomic HDV RNA quantification, intracellular RNA was reverse transcribed using biotinylated genomic and antigenomic HDV RNA primers[3] and the ABI Fast 1-Step Virus Master (Applied Biosystems). Reverse transcription was performed at 50°C for 5 min on an ABI Viia7 (Applied Biosystems) and enzymes were inactivated at 95°C for 20 s. Biotinylated cDNA was purified with the MinElute PCR Purification Kit (Qiagen, Venlo, Netherlands) and isolated with dynabeads specifically interacting with biotin (Dynal Kilobase Binder Kit, Invitrogen, Carlsbad, USA) following the manufacturer's instructions. For qRT-PCR purified biotinylated cDNA bound to dynabeads, HDV-specific primers and probes[3] and the ABI Fast Advanced Master (Applied Biosystems) were used under the conditions described above. Steady-state levels of intracellular viral RNA amounts were normalized using human-specific GAPDH primers (Taqman Gene Expression Assay Hs99999905_m1, Applied Biosystems).

In vivo, copies of intrahepatic HDV RNAs were normalized to ng liver RNA. Three liver specimen per mouse were used for total HDV RNA quantification and one liver specimen per mouse with detectable average HDV RNA levels was chosen for determination of genomic and antigenomic HDV RNAs.

HDV RNA genome sequencing. For HDV sequencing, the genomic regions R1 (location: 305-1285, product size: 980 bp, according to the numeration of Wang et al.[6] and R2 (location: 885-327, product size: 1121 bp) were amplified and sequenced using the primers R1fw 305-327: ccagaggacccttcagcgaac, R1rv 1285-1261: gaaggaaggccctcgagaacaaga, R2fw 885-908: catgccgacccgaagaggaaag, R2rv 327-306: gttcgctgaaggggtcctctg)[7] and Red-Taq Polymerase (Sigma-Aldrich, St. Louis, USA). PCR products were purified (PCR purification kit, Qiagen, Hilden, Germany), and both strands were sequenced on an ABI Prism 377 automated sequencer (PE Applied Biosystems, Foster City, USA) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). Sequencing was performed at all time points of the *in vitro* experiments and shown are representative time points (day 5, 16, 37, 51, 86, 100 p.i.).

Immunofluorescence. Cryostat sections of chimeric mouse livers and cultured cells were stained as previously described[8]. Briefly, liver sections and cultured cells were fixed for 10 min with acetone or 4% paraformaldehyde, respectively, and incubated with mouse anti-CK18 (1:400; Dako, Glostrup, Denmark), human anti-delta (anti-HDAg-positive human serum 1:8000), rabbit anti-HBcAg (1:1000; Dako), or mouse anti-Ki67 (1:100; Dako). Specific signals were visualized with Alexa 488-, 546- or 633-labeled secondary antibodies (Invitrogen, Darmstadt, Germany) as recommended by manufactures. The TSA Fluorescein System (Perkin Elmer, Jügesheim, Germany) was used to enhance HBcAg staining[8]. Nuclear staining was achieved by Hoechst 33258 (1:20,000; Invitrogen, Darmstadt, Germany). Stained sections were then mounted with fluorescent mounting media (Dako) and

analyzed by fluorescence microscope BZ9000 (Keyence, Osaka, Japan) using the same settings for different experimental groups.

In vitro, cell counting of HDAg-positive or double positive Ki67-HDAg cells (nuclei) was performed using three different visual sections per time point and by using the BZ-II Analyzer Software (Keyence). Each counted field contained approximately 2500 cells (nuclei). The amount of HDAg-positive cells was estimated at every time point throughout the experiment and Ki67-positive uninfected and HDV infected cells were counted at d23 and d44 post infection. *In vivo*, HDAg-positive or double positive Ki67-HDAg cells (nuclei) were counted manually in three liver specimen per mouse at 3 days and 2 weeks post transplantation.

Statistics. Statistical analyses were performed with the GraphPad Prism 5 software. For group-wise comparisons, the nonparametric Mann-Whitney U test was applied. P values <0.05 were considered statistically significant.

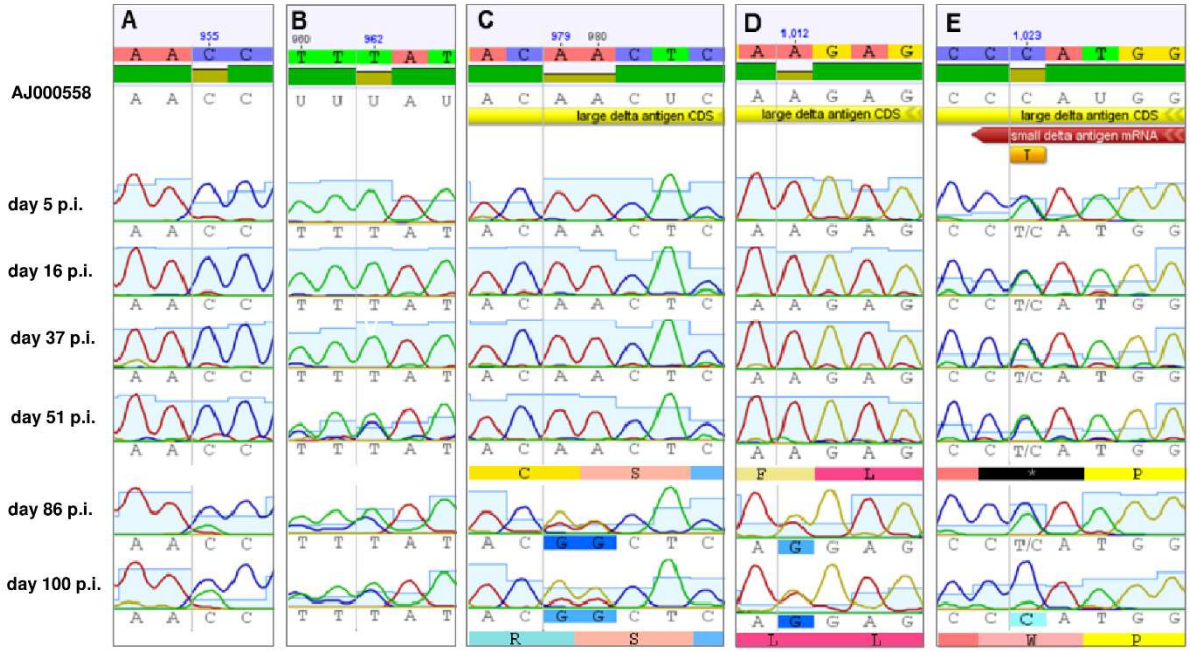
Supplementary Results

Sequencing of HDV RNA. To determine whether specific nucleotide changes accumulated during HDV infection in HepG2-hNTCP cells, we sequenced the HDV RNA genome using the specific primers R1 and R2 at all time points (see **Supplementary Material and Methods**). Interestingly, only very few nucleotide changes occurred on the virus genome during cell proliferation *in vitro* and these were mostly determined at later time points of the experiment. Five different mutations occurred at around day 86 p.i. and were also detected at all later time points (**suppl. figure 1**). At position 955 (according to the numeration of Wang et al.[6]) cytosine changed into thymidine (**suppl. figure 1A**), at position 962 thymidine changed into cytosine (**suppl. figure 1B**), at position 979 and 980 adenosine changed into guanine (**suppl. figure 1C**) and at position 1012 adenosine changed into guanine (**suppl. figure 1D**). The single point mutations at position 979 and 1012 lie within the region coding for the two HDAgs and result in two amino acid substitutions (979: cysteine/C to arginine/R,

1012: phenylalanine/F to leucine/L). Interestingly, cysteine-211 at position 979 is part of a four amino acid long isoprenylation site at the end of the large HDAg (CRPQ), which is crucial for interaction between the large HDAg and HBsAg that is required for HDV assembly[9]. At position 1023, the documented RNA editing site leads to either a stop codon (ATC) for the small HDAg, or to a tryptophan codon (ACC) for the large HDAg. After several weeks in culture, we observed a shift in the ratio of the two forms, beginning with the co-existence of both variants at a similar ratio and ending with the predominance of RNAs encoding only the large HDAg (**suppl. figure 1E**). Previous *in vitro* studies described that an accumulation of the large HDAg leads to the inhibition of HDV replication[10, 11]. Therefore, the predominance of edited HDV genomes in our experiment at day 86 p.i. could have been responsible for the decrease of HDV RNA loads and lower amount of HDAg-positive cells determined towards the end of the experiment, at a time when cells underwent extensive cell proliferation (cell dilution 1:1.4x10^7).

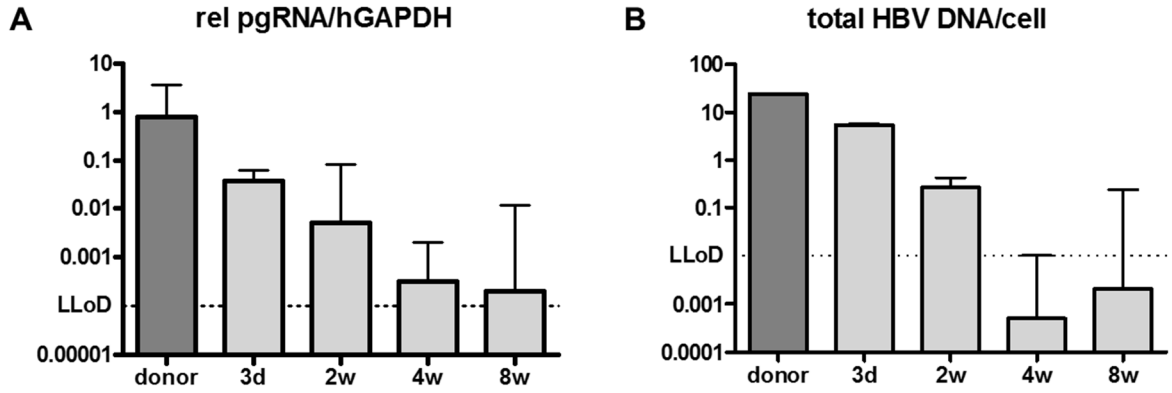
Supplementary Figures and Legends

Supplementary figure 1



Suppl. figure 1. HDV RNA genome sequencing. 5 different mutations occured in HDV mono-infected HepG2-hNTCP cells at around day 86 p.i.. and remained also at later time points (here shown: day 100 p.i.). **A)** At position 955 (according to the numeration of Wang et al.[6]) cytosine changed into thymidine. **B)** At position 962 thymidine changed into cytosine. **C)** At position 979 and 980 adenosine changed into guanine leading to an amino acid substitution from cysteine/C, which is required for isoprenlyation and assembly of HDV, to arginine/R. **D)** At position 1012 adenosine changed into guanine leading to an amino acid substitution from phenylalanine/F to leucine/L. **E)** At position 1023, which is the RNA editing site where either the small HDAg (stop codon, ATC= *) or the large HDAg (no stop codon, ACC=tryptophan/W) is generated, a shift from the co-existance of both variants to the RNA which encodes only for the large HDAg was observed.

Supplementary figure 2



Suppl. figure 2. Intrahepatic HBV markers. Intrahepatic levels of HBV pgRNA relative to hGAPDH (**A**) and total HBV DNA per ß-globin (per cell) (**B**) in serial transplanted and donor mice. Levels of circulating HBsAg and cccDNA per cell were below the detection limit at all time points after transplantation.

Supplementary References

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