Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo

Lena Allweiss, Tassilo Volz, Katja Giersch, Janine Kah, Giuseppina Raffa, Joerg Petersen, Ansgar W. Lohse, Concetta Beninati, Teresa Pollicino, Stephan Urban, Marc Lütgehetmann, Maura Dandri

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Supplementary Methods

Mouse experiments

All animal experiments and procedures were performed in accordance with the European Union directive 86/609/EEC and approved by the Ethical Committee of the city and state of Hamburg in accordance with the principles of the Declaration of Helsinki. The generation of human liver chimeric mice was conducted as previously described[1]. Briefly, thawed human hepatocytes obtained from a single human donor were injected intrasplenically into 3-week-old USB mice anesthetized with isofluoran. Levels of human chimerism were determined by measuring human serum albumin (HSA) in mouse serum with the Human Albumin ELISA kit (Immunology Consultants Lab, Portland, USA). HBV infection was established upon a single intraperitoneal injection of HBV-containing serum (genotype D, HBeAg-positive). To perform serial human hepatocyte transplantations, HBV-infected donor mice with high levels of human chimerism and viremia were chosen for liver cell isolation by a retrograde two-step collagen perfusion. Briefly, a pre-perfusion of 25 ml of 0.5mM EGTA in Leffert´s buffer (10mM HEPES, 3mM KCl, 0.13M NaCl, 1.9mM NaH_2PO_4-H2O, 10mM D-Glucose) was followed by digestion with 150 ml collagenase type 2 (0.6 mg/ml) and 0.0279% $CaCl_2$ in Leffert´s buffer. Subsequently, hepatocytes were enriched and washed through centrifugation at 50 g. One million isolated hepatocytes were transplanted into naïve USB mice as described above. Before liver isolation, the caudate process of the mouse livers was removed to serve as a reference for future analyses. Some mice received subcutaneous injections of Myrcludex-B (2 µg/g body weight) daily for nine weeks[2], or Lamivudine (Zeffix®, GlaxoSmithKline, Brentford, United Kingdom) supplemented in the drinking water (20mg/100ml). Liver specimens removed at the time of sacrifice were snap-frozen in 2-methylbutane for histological and molecular analyses.

Serological and intrahepatic measurements

Quantitative PCR measurements were performed on the ViiA™ 7 Real-Time PCR System with probes and primers from the TaqMan® Gene Expression Assay System and the Taqman® Fast Advanced Master Mix (Life Technologies, Carlsbad, California, USA) unless otherwise indicated[3]. Viral DNA was extracted from serum samples using the QiAamp MinElute Virus Spin Kit (Qiagen, Hilden,

Germany) and quantified with HBV-specific primers and probes (TaqMan® Gene Expression assay ID: Pa03453406_s1). HBsAg quantification was performed on the Abbott Architect platform (quantitative HBsAg kit, Abbott, Ireland, Diagnostic Division) as recommended by the manufacturer[4].

DNA and RNA were extracted from liver specimens using the Master Pure DNA purification kit (Epicentre, Madison, USA) and the RNeasy RNA purification kit (Qiagen), respectively [1]. Intrahepatic viral loads were quantified in total extracted DNA with the help of primers and probes specific for total HBV DNA (TaqMan® Gene Expression assay ID: Pa03453406_s1) and cccDNA[5]. For normalization, the number of human hepatocytes was estimated by measuring human hemoglobin beta (assay ID Hs00758889_s1) while human genomic DNA (Roche Applied Science, Mannheim, Germany) was used as a standard curve for quantification.

CccDNA levels were determined in extracted liver DNA after digestion with plasmid-safe ATP-dependent DNase (Epicentre) using the following conditions: 1µg total DNA was incubated with 30 U of enzyme at 37°C in a total volume of 200 µl for 2 h. After heat inactivation (30 min at 70°C) and precipitation, qPCR with cccDNA-selective primers and probe [5] was performed. To enhance the specificity of the PCR, the forward primer was used in a final concentration of 100 nmol/l and the reverse in final concentration of 800 nmol/l. The cycling program consisted of an initial denaturation at 95°C for 10 min and 40 cycles of step 1 (95°C for 1 sec) and step 2 (65°C for 1 min). To verify the cccDNA quantification by the use of alternative procedures aiming at reducing rcDNA contaminants, 1 µg of total DNA was digested with two alternative nucleases: Either 10 U of T5 exonuclease were used for 30 min at 37°C or a combination of 20 U of exonuclease I and 25 U of exonuclease III for 2 h at 37°C (personal communication Jianming Hu at the International HBV meeting 2016). In addition, cccDNA was isolated by Hirt extraction as previously reported[6]. Briefly, 50 mg of liver tissue was homogenized and lysed in the presence of 0.67% SDS and precipitated by an overnight incubation in high salt buffer (final concentration 0.5 M KCl) and subsequent centrifugation. Viral DNA in the supernatant was extracted with phenol/chloroform followed by an ethanol precipitation. After this, Hirt extracted DNA samples were treated with plasmid-safe ATP-dependent DNase or T5 exonuclease. Non-digested and digested Hirt samples were cleaned up with the Geneclean Spin Kit (MP Biomedicals, Illkirch, France)

and subjected to qPCR as described above. For normalization, 1/100 of the liver homogenate was removed before Hirt extraction and was used instead for total DNA extraction with the Master Pure DNA purification kit and human hemoglobin beta qPCR as described above.

Viral and genomic RNAs were reverse transcribed and amplified in a one-step procedure using the TaqMan® Fast Virus 1-Step Master Mix as recommended by the manufacturer (Life Technologies). Primers and probes specific for total HBV RNA (assay ID Pa03453406_s1) and pregenomic HBV RNA (including precore HBV RNA)[5] were used for reverse transcription and amplification while the expression of the human housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, assay ID Hs99999905_m1) was used for normalization. All intrahepatic measurements were performed on three distinct liver pieces isolated per mouse.

Mathematical calculations

The total amount of PHHs present in an entire mouse liver at the time of sacrifice was calculated with the following equation:

$$Cells_{liver} = \frac{N_{cells}}{m_{DNA}} * k * m_{liver}$$

where N is the amount of PHHs in one PCR assay as determined by measuring the single copy gene hemoglobin beta, m_{DNA} is the amount of DNA present in one PCR assay, m_{liver} is the mass of the liver and k is a constant which equals 1.5 mg. This constant takes into account the fact that, in general, from every gram of liver 1.5 milligram of DNA is extracted. Similarly, the total amount of cccDNA molecules was calculated with the equation:

$$cccDNA_{liver} = \frac{N_{cccDNA}}{m_{DNA}} * k * m_{liver}$$

where N_{cccDNA} is the amount of cccDNA molecules determined in one PCR assay. The number of cell doublings between two time points was calculated as follows:

$$Cell\ doublings = \ln(\frac{Cells_{liver}\ at\ t_2}{Cells_{liver}\ at\ t_1})$$

and the cell doubling time during a given time t was estimated with the equation:

$$doubling\ time = \frac{t}{cell\ doublings}$$

The half-life of cccDNA was calculated on the linear regression of the logarithmized values (cccDNA/PHH or cccDNA/liver) determined from day 3 or 5 until day 30 after transplantation of experiment 1 and 2 together following the equation:

$$t_{1/2} = (log_{10}0.5)/m$$

where m is the slope of the regression line.

Immunofluorescence and RNA in situ hybridization

Human hepatocytes in acetone or paraformaldehyde fixed frozen liver sections were visualized with human-specific antibodies recognizing keratin 18 (Santa Cruz Biotechnology, Dallas, TX, USA), calnexin (Cell Signaling Technology, Danvers, MA, USA) or SP100 nuclear antigen (kindly provided by H. Will). HBV-infected hepatocytes were identified with an HBcAg-specific (Dako Diagnostika, Glostrup, Denmark) or a human, recombinant, HBsAg-specific antibody (HBD-7, Humabs BioMed, Bellinzona, Switzerland), proliferating hepatocytes with antibodies to Ki-67 (Dako), and apoptotic hepatocytes with antibodies to active caspase 3 (Biosciences, Franklin Lakes, NJ, USA). The differentiation status of hepatocytes was assessed with antibodies recognizing hepatocyte nuclear factor 4 alpha (HNF4A, Santa Cruz Biotechnology), keratin 7 (Dako), catenin beta 1 (Santa Cruz Biotechnology) and epithelial cell adhesion molecule (EPCAM, Acris Antibodies, San Diego, CA, USA). Specific signals were visualized with Alexa Fluor 488, 546, or 633 labeled secondary antibodies (Invitrogen, Carlsbad, CA, USA) or the TSA Fluorescein System (Perkin Elmer, Jügesheim, Germany). Nuclear staining was achieved with Hoechst 33258 (Invitrogen). Stained sections were analyzed by fluorescence microscopy (Biorevo BZ-9000, Keyence, Osaka, Japan) using the same settings for all groups. Positive PHHs were quantified on 1-3 sections per mouse depending on the amount of PHHs present in the section.

RNA in situ hybridization was performed on paraformaldehyde-fixed, cryo-preserved liver sections using the RNAScope Fluorescent Multiplex Kit (Advanced Cell Diagnostics, ACD, Hayward, CA, USA) as previously described[7]. Specific target probes recognizing the pregenomic region (sequence range 2271-2630; assay number 442741-C2) were used in parallel with probes for the S region of the

HBV genome (sequence range 141 - 1402; RNAScope assay number 448791) or human gapdh transcripts (RNAScope assay number 442201), respectively.

Gene expression analysis

Mouse and human specific gene expression levels of genes involved in liver regeneration and with anti-HBV activity were determined by quantitative PCR as previously described[3, 5]. Measurements were either performed on oligo dT, reverse transcribed liver RNA using the Taqman® Fast Advanced Master Mix (Life Technologies) or - to increase sensitivity – in a one-step procedure using the TaqMan® Fast Virus 1-Step Master Mix. Primer and probe sets were purchased from the TaqMan® Gene Expression Assay System and are listed in Supplementary Table 1.

Analysis of HBV DNA integrations

For the detection of HBV DNA integrations, genomic DNA potentially containing viral integrations was separated from episomal rcDNA and cccDNA molecules via gel electrophoresis as previously described[8]. QPCR with primers and probes recognizing the HBV S region (TaqMan® Gene Expression assay ID: Pa03453405_s1) was performed to compare the amount of viral sequences before and after gel separation. Artificial mixtures of genomic DNA from non-infected human-chimeric USB mice livers and rcDNA extracted from infectious mouse serum served as a negative control and DNA from Hep3B cells harboring known HBV integrations as a positive control.

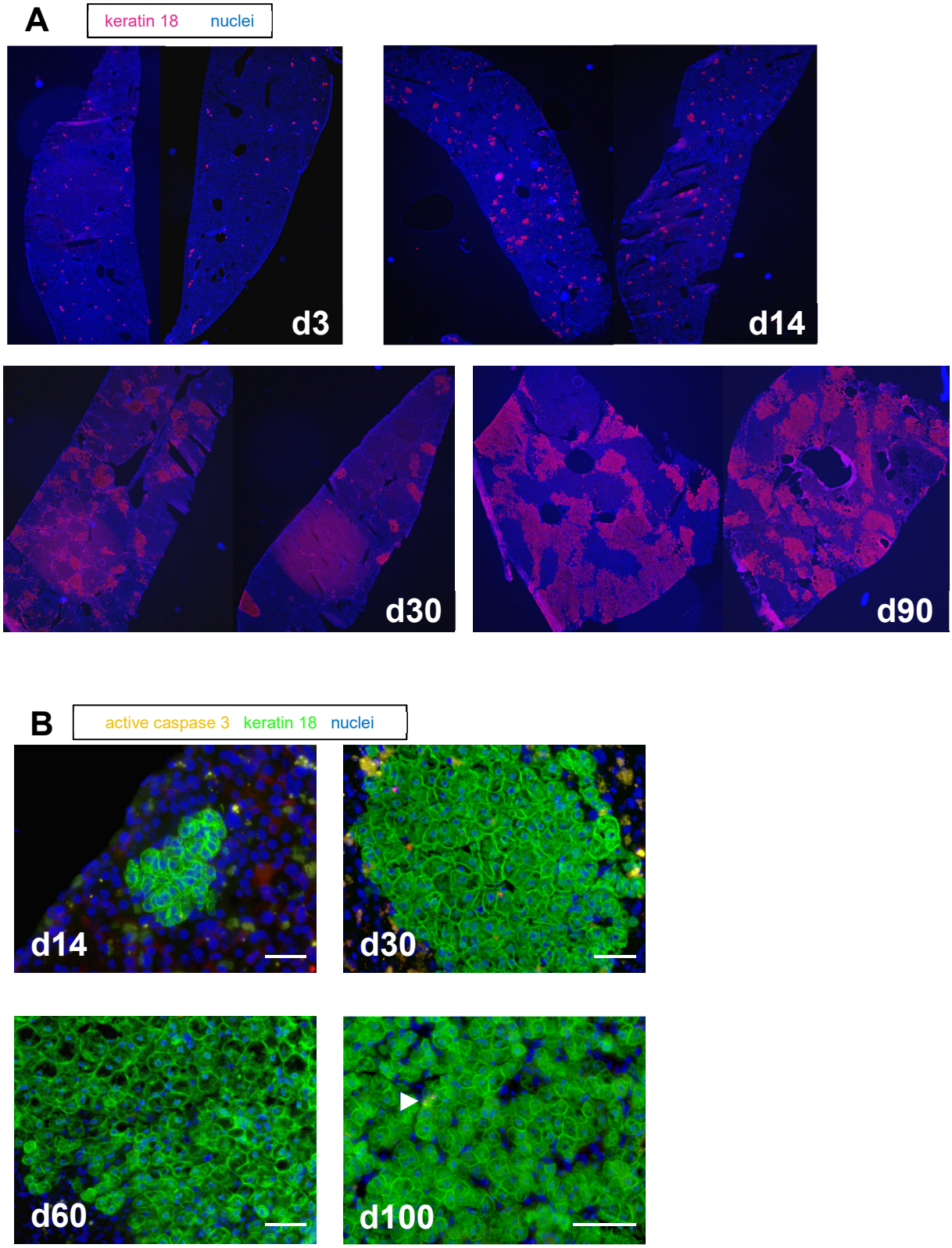
To characterize HBV integrations and sites of viral integration, the Alu-PCR technique[9-11] was coupled to deep-sequencing analysis. For Alu-PCR, primers specific for the human Alu sequence and for core, X , PreS, and S viral genomic regions were used as described[12]. Dual indexed libraries were generated using the Nextera XT library preparation technology according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Purified PCR products were diluted to 0.2 ng/µl and a total of 10 ng was used for library generation in a 96-well plate format. Transposome-mediated simultaneous DNA fragmentation and adapter ligation (tagmentation) was performed at 55°C for 5 minutes. After the tagmentation reaction, indexing specific PCR primers were added two per well for unique dual indexing of the libraries for multiplex sequencing. Limited cycle-number PCR was performed to amplify the

libraries and incorporate the index sequences. Double-stranded libraries were quality checked on a High Sensitivity DNA Agilent chip run on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) for size and molarity determination. PhiX control libraries (Illumina) were used to increase diversity of base calling during sequencing. Denatured and diluted libraries were sequenced on the Illumina MiSeq benchtop sequencer using a paired-end 2x250 bp (v2) cycle protocol according to the manufacturer's instructions. For data analysis, sequence reads obtained in FASTQ format by the Illumina Miseq were first processed using the “Align, assemble and analyze reads” software in the Illumina BaseSpace® (https: basespace.illumina.com). Next, single contigs were analyzed using nucleotide blast (http:// blast.ncbi.nlm.nih.gov/Blast.cgi) and mapped to host and viral genomes.

Supplementary Table 1

Gene name	Mouse-specific primer and probe set	Human-specific primer and probe set
	TaqMan® Gene Expression assay ID	
Interleukin 6	Mm00446190_m1	Hs00985639_m1
Tumor necrosis factor	Mm00443258_m1	Hs99999043_m1
Hepatocyte growth factor	Mm01135184_m1	Hs00300159_m1
Transforming growth factor alpha	Mm00446232_m1	Hs01034455_m1
Transforming growth factor beta 1	Mm00441724_m1	Hs00171257_m1
Interferon alpha 2	Mm00833961_s1	Hs00265051_s1
Interferon beta 1	Mm00439552_s1	Hs00277188_s1
Interferon gamma	Mm01168134_m1	Hs00989291_m1
Lymphotoxin beta	Mm00434774_g1	Hs00242737_m1
APOBEC3B	not determined	Hs00358981_m1

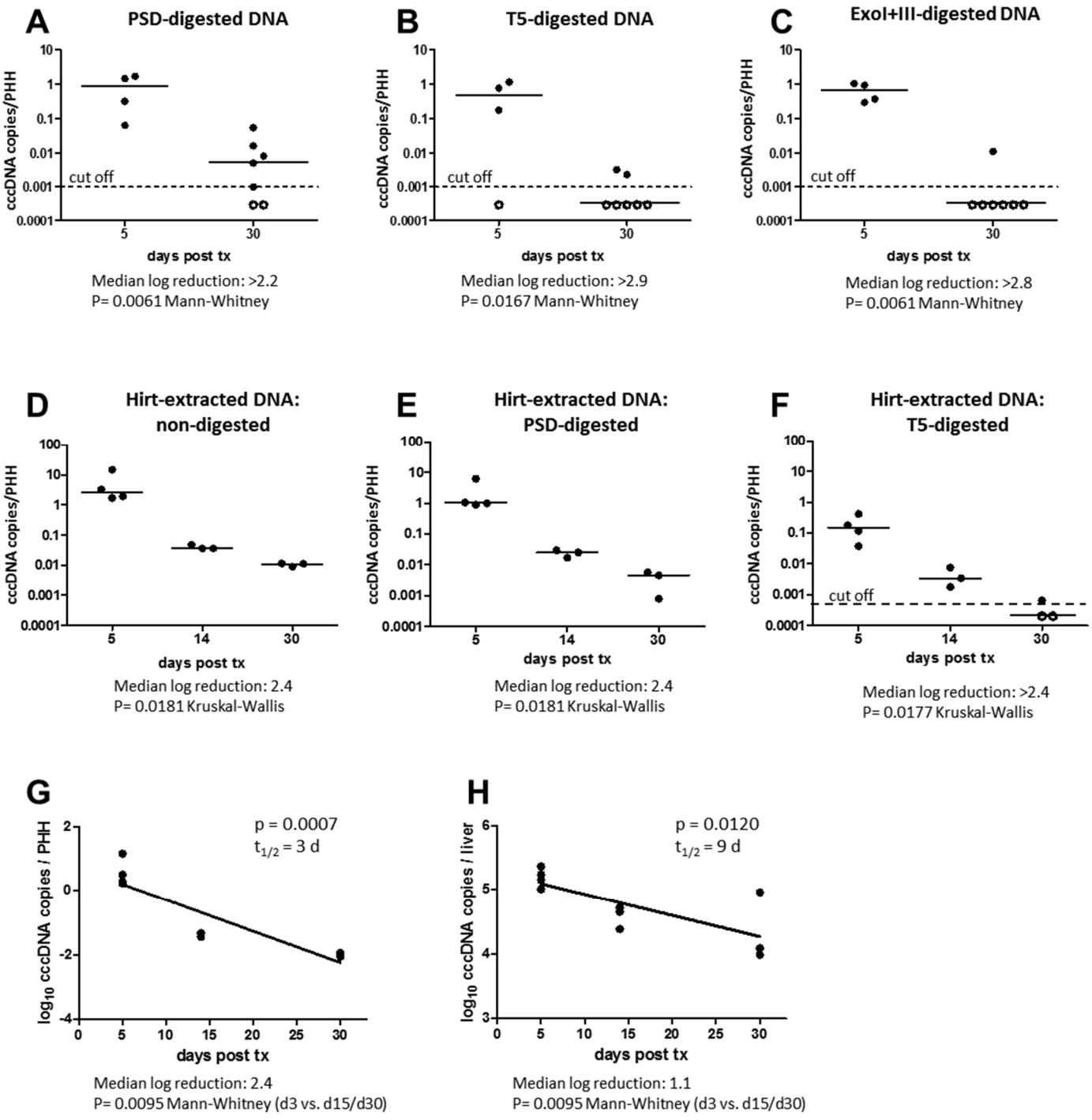
Supplementary Figure 1



Supplementary figure 1 Assessment of proliferation and cell death characteristics in recipient USB mice after serial transplantation. (A) Immunofluorescent staining of human hepatocytes in full liver

sections of recipient USB mice using keratin 18 (red) as a human specific marker. Nuclei are shown in blue. Shown are representative overview pictures (2x magnification) for two mice per time point: 3 days, 14 days, 30 days and 90 days post Tx as indicated in the lower right-hand corner of the photographs. (B) Immunofluorescent staining for active caspase 3 (red/yellow when merged with green), keratin 18 as a marker for human hepatocytes (green) and nuclei (blue). The panel shows one representative picture of a liver section for each time point: 14 days, 30 days, 60 days and 100 days post Tx. Arrow head indicates one apoptotic cell. Scale bars = 50 μm.

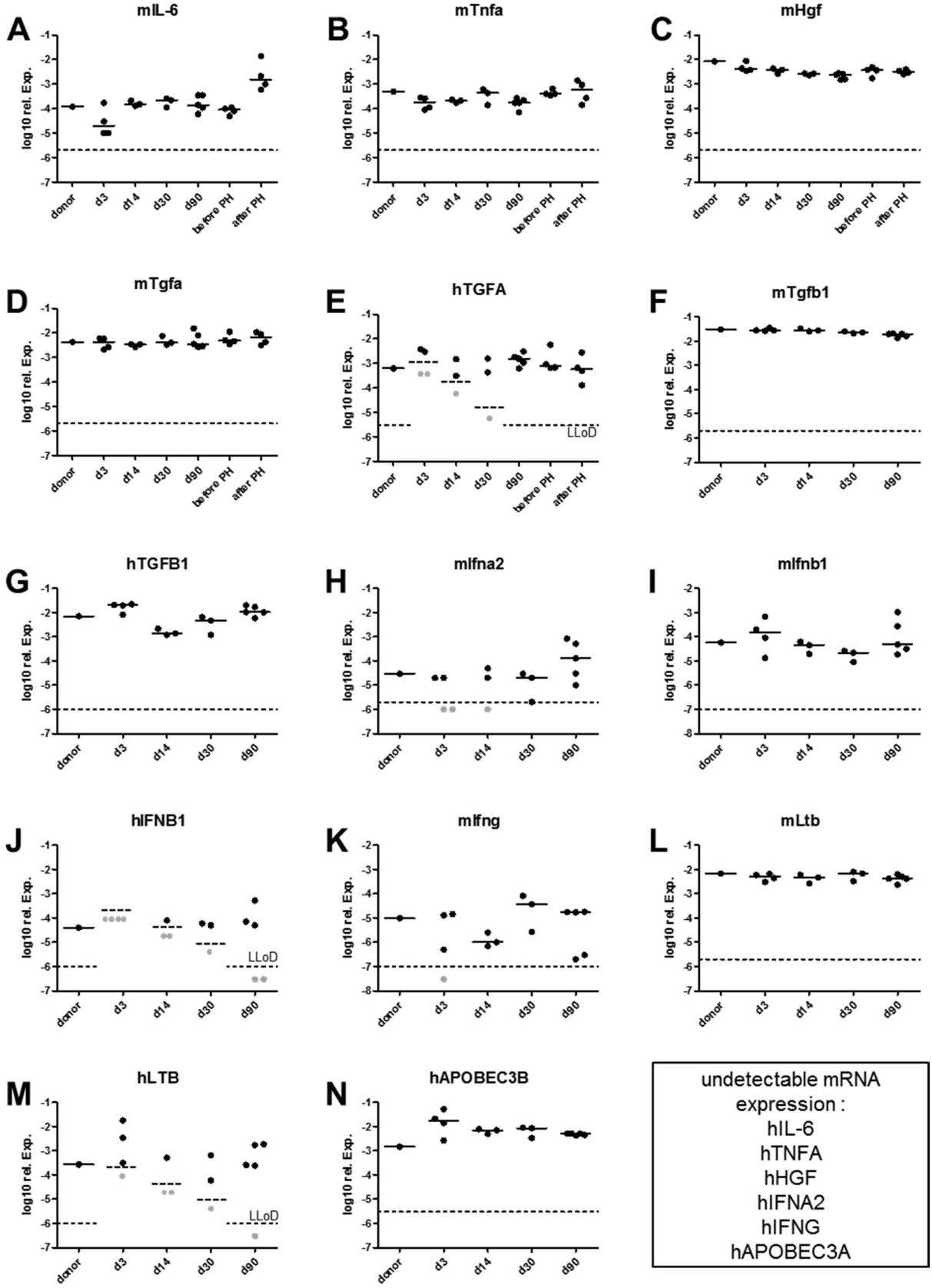
Supplementary Figure 2



Supplementary figure 2 Quantification of cccDNA levels using different nuclease digestion and DNA extraction methods. (A-C) The dot blots depict PCR measurements of cccDNA copy number normalized to the amount of human hepatocytes using whole cell DNA extracted from one piece of liver per mouse (as depicted in Figure 3). Results in A show cccDNA values after plasmid-safe ATP-dependent DNase (PSD) digestion, B depicts values after digestion with T5 exonuclease and C shows results after

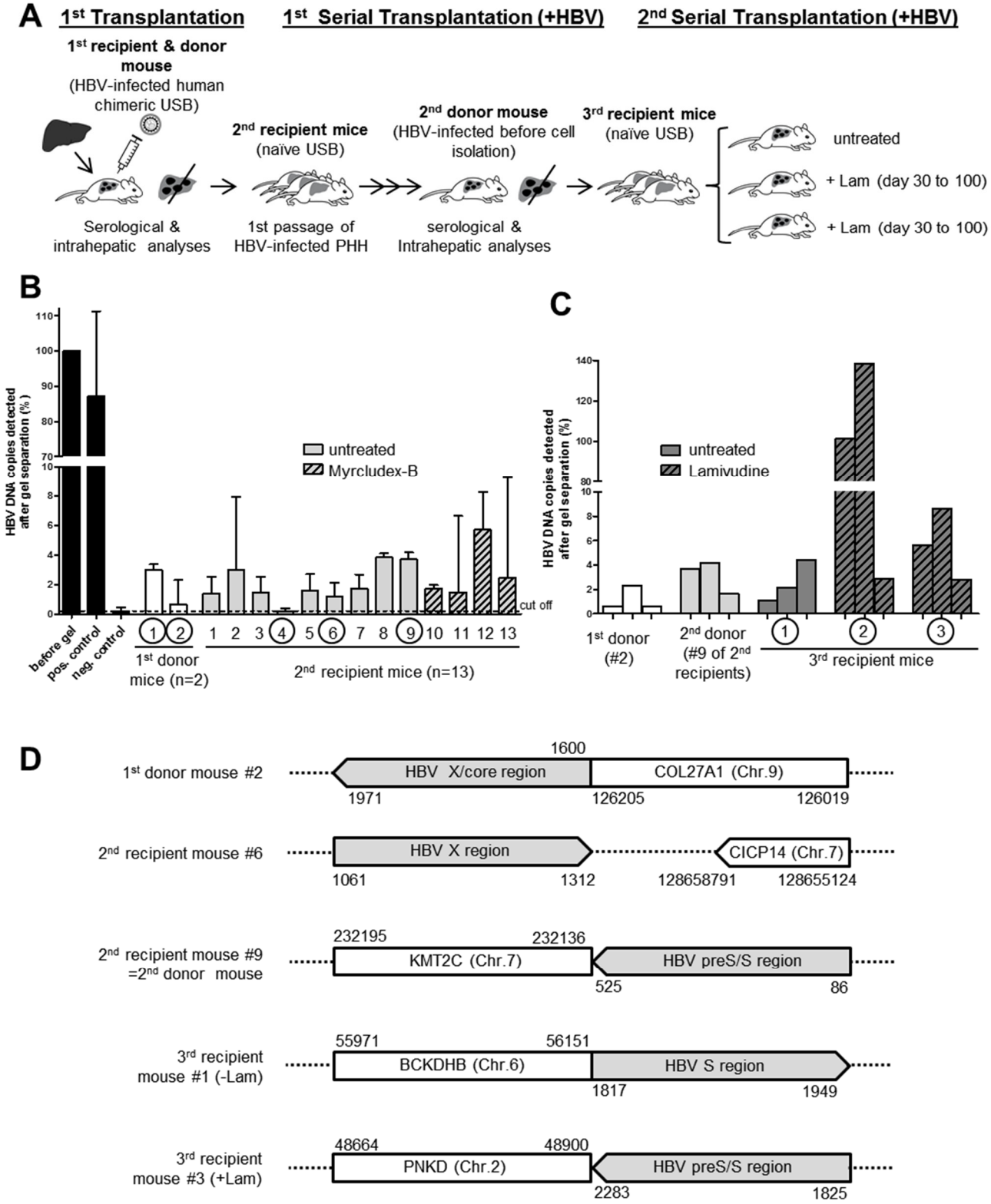
digestion with exonuclease I and III (as described in the supplementary methods). (D-F) Dot blots of PCR measurements of cccDNA copy number normalized to the amount of human hepatocytes of DNA extracted with the Hirt method in mice from experiment 2. Results in D show cccDNA counts without prior nuclease digestion, E depicts cccDNA values after PSD digestion and F depicts values after digestion with T5 exonuclease. (G, H) Logarithmized values of cccDNA copies/PHH (G) and cccDNA copies scaled up per liver (H) determined at day 3, 14 and 30 after serial transplantation using Hirt-extracted samples. Regression analysis was used to calculate the half-life of cccDNA amounts per human hepatocyte and mouse liver, respectively (as described in the supplementary methods). The significance of the deviation from zero of the regression line is shown in the graph (F test). Each dot represents a single mouse. Open circles symbolize undetectable PCR measurements. Bars indicate the median.

Supplementary Figure 3



Supplementary Figure 3 Expression levels of genes involved in liver regeneration and anti-HBV activity. Gene expression was analyzed in the livers of the donor mouse and the recipient mice after serial transplantation from experiment 2 (see Figure 3). Every dot blot depicts the expression of one gene - either murine or human-specific - as indicated on top of each graph. Each data point represents one mouse; bars indicate the median. Grey dots symbolize undetectable measurements. Whenever the expression of a certain gene was undetectable in all mice, the respective gene symbol is listed in the box at the lower right corner. The lower limit of detection (LLoD) is indicated as a broke line. Please note that the LLoD for human genes depends on the amount of PHH present in the mouse livers. Since few cells are present at early time points after transplantation but increase through proliferation over time, the detection limit is high at these early time points but sensitivity of measurements increases with the increase of repopulation. As reference measurement for genes involved in liver regeneration (IL-6, TNF, HGF, TGF alpha) HBV-infected mice before and 3 or 5 days after partial hepatectomy were included. The expression of human TGF beta, human LT beta, murine IFN gamma and human and murine IFN beta was determined through a one-step procedure using liver RNA in order to increase sensitivity while the remainder of genes was determined through a two-step procedure using cDNA.

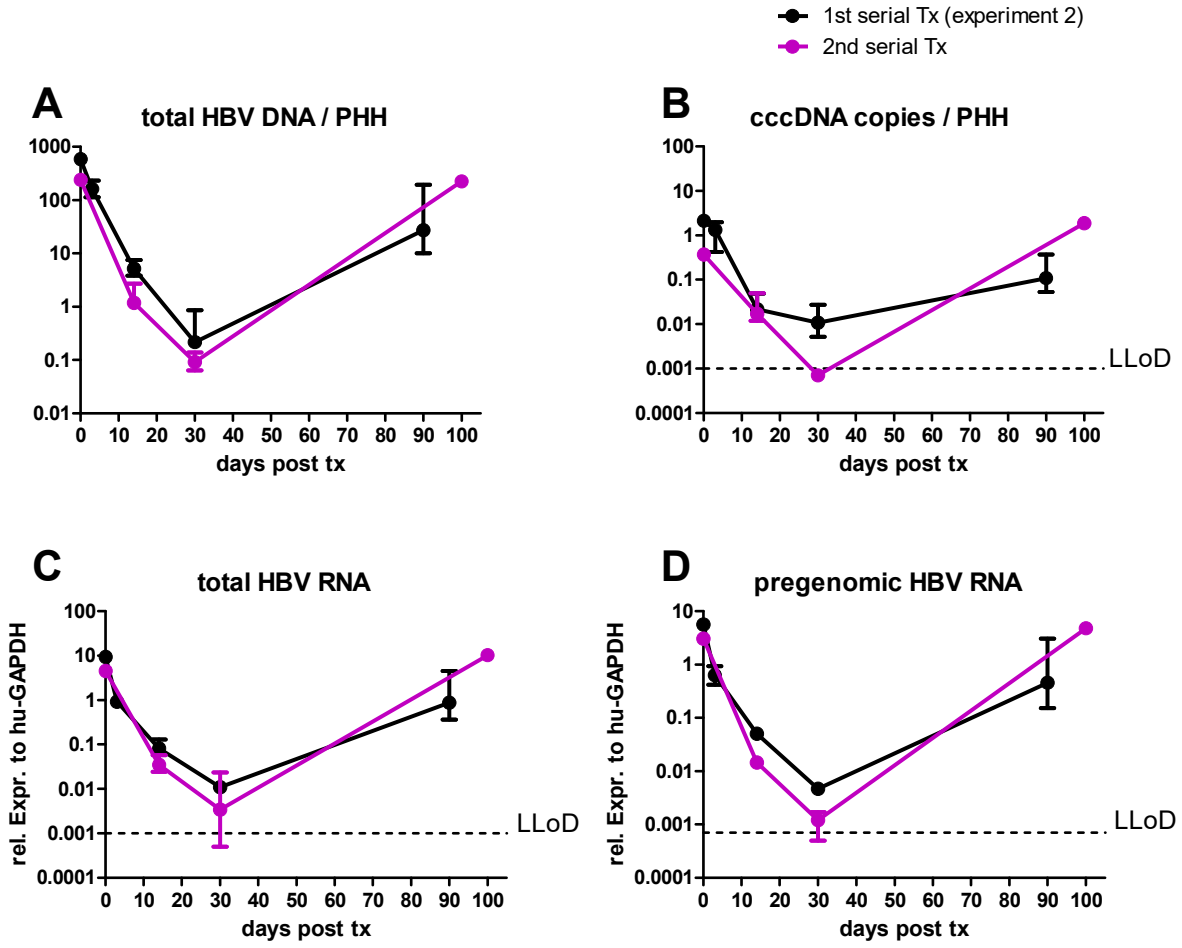
Supplementary Figure 4



Supplementary figure 4 Analysis of HBV DNA integrations in serially passaged HBV-infected PHHs. (A) Experimental procedures used to induce proliferation of HBV-infected PHHs. To perform a second serial transplantation, 130 days post transplantation one mouse was chosen as a new donor (2nd donor).

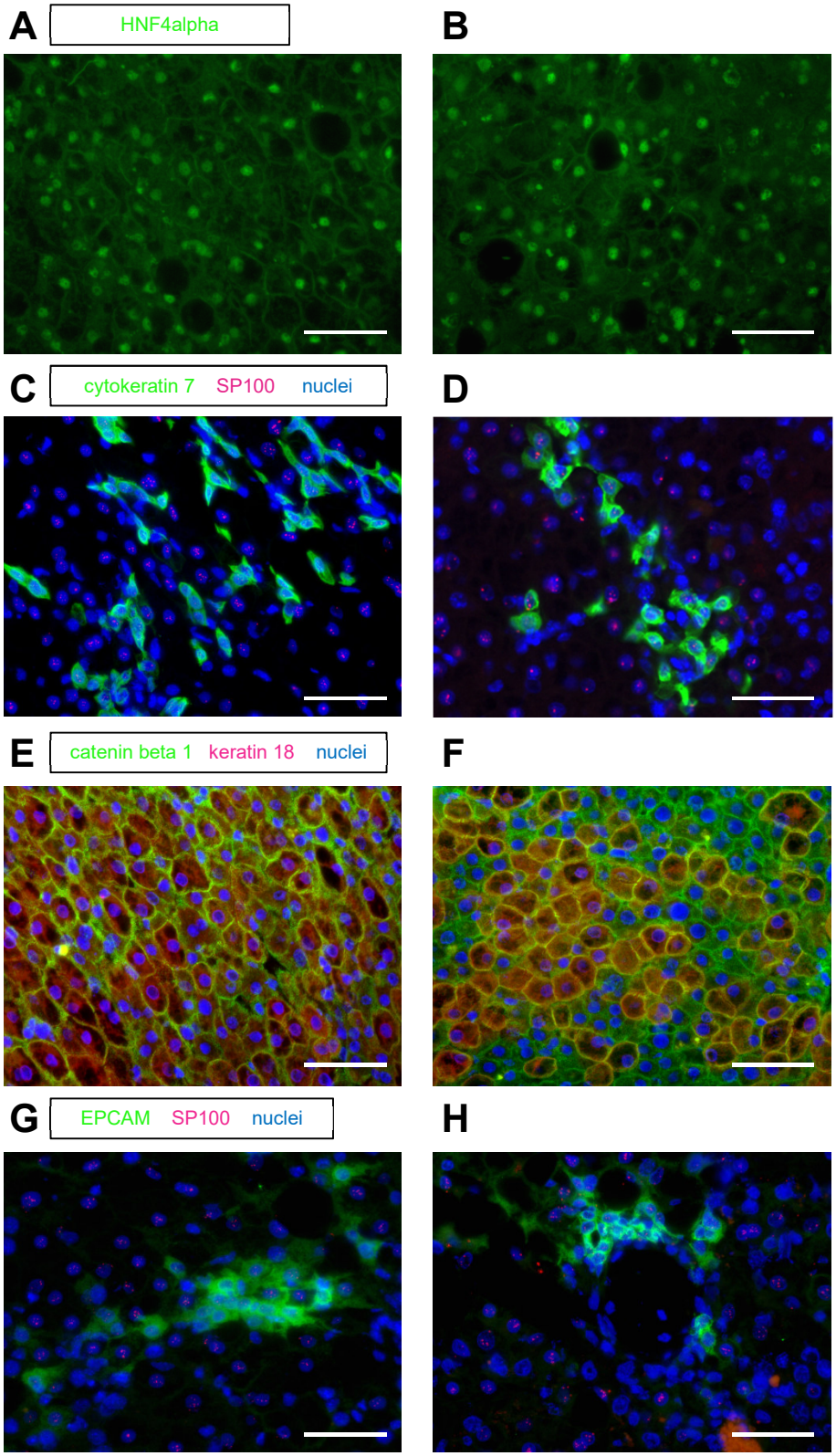
(B) Integration analysis based on gel separation of three liver pieces/mouse. Bars depict median HBV DNA copies and range in percent relative to the amounts present in the same sample before gel electrophoresis. Positive control = Hep3B cells harboring known HBV integrations; negative control = artificial mixtures of genomic DNA from non-infected human-chimeric USB mice livers and rcDNA extracted from infectious mouse serum, which was used to determine the cut off. Hatched bars represent Myrcludex-B treated mice. (C) Integration analysis based on gel separation showing the distribution of integrations in the mouse livers by displaying the results of the three distinct liver pieces for each mouse separately. Hatched bars indicate Lamivudine treatment. (D) Illustrations of HBV DNA integrations identified by Alu-PCR and deep sequencing. Grey arrows depict the direction and numbering according to the *Eco*RI site of genotype D3 of the integrated HBV sequence; white boxes represent the host gene and nucleotide position at the integration site. Labeling of mice corresponds to B and C where circles indicate the mice used for this analysis. COL27A1 = collagen, type XXVII, alpha 1; CICP14 = capicua transcriptional repressor pseudogene 14; KMT2C = lysine (K)-specific methyltransferase 2C; BCKDHB = branched chain keto acid dehydrogenase E1, beta polypeptide; PNKD = paroxysmal nonkinesigenic dyskinesia.

Supplementary Figure 5



Supplementary figure 5 Changes of intrahepatic viral markers induced by a second serial transplantation of HBV-infected PHHs. (A-D) Viral markers of the second donor mouse (time point 0) and the groups of recipient mice sacrificed at different time points after the second serial transplantation (purple curve) are displayed in comparison to the first serial transplantation experiment (black line: experiment 2 as depicted in Figure 3). Data points represent the median with range for 1-5 mice per time point and experiment. The point and connecting line graphs display total HBV DNA/PHH (A), cccDNA copies/PHH (B), total HBV RNA species (C) and pregenomic HBV RNA (D) normalized to the expression of the human housekeeping gene *GAPDH*.

Supplementary Figure 6



Supplementary figure 6 Analysis of the hepatocytes´ differentiation status after two rounds of serial transplantation. Immunofluorescent staining of hepatocyte nuclear factor 4 alpha (HNF4A) in green in a liver section of the first donor mouse, i.e. a mouse without proliferation of HBV-infected PHHs (A)

and a recipient mouse sacrificed at day 100 after the second transplantation, i.e. after two rounds of proliferation of HBV-infected PHHs (B). Immunofluorescent staining of cytokeratin 7 (KRT7) in green, SP100 as a human marker in red and nuclei in blue again in the first donor mouse (C) and a second recipient (D). Immunofluorescent staining of catenin beta 1 (CTNNB1) in green and keratin 18 (KRT18) as a human marker in red and nuclei in blue again in the first donor mouse (E) and a second recipient (F). Immunofluorescent staining of epithelial cell adhesion molecule (EPCAM) in green, SP100 as a human marker in red and nuclei in blue again in the first donor mouse (G) and a second recipient (H). Scale bars = 50 μm.

Supplementary References

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