Supplementary material

Supplementary Figure 1

Retrorsine improves liver repopulation across different liver chimeric models

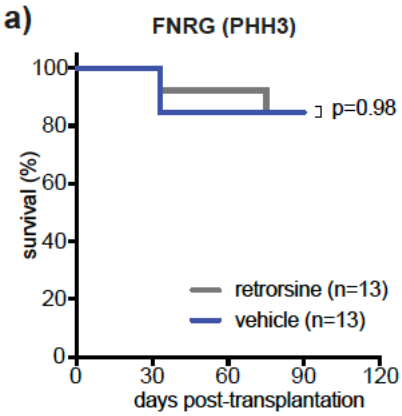
a) Retrorsine has no effect on overall survival during NTBC cycling and hepatocyte repopulation. Log-rank (Mantel-Cox) test between groups of 13 mice.

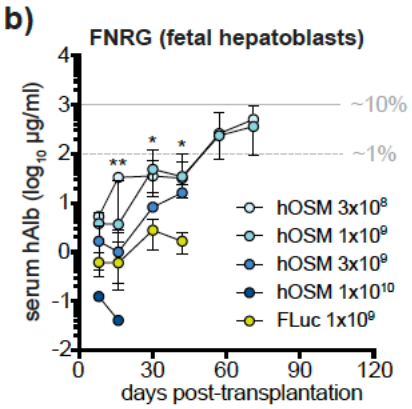
b) Groups of 5 non-preconditioned FNRG mice received various doses of AAV8-hOSM or AAV8-FLuc negative control before transplantation of human fetal hepatoblasts. Low doses of AAV8-hOSM result in higher serum hAlb levels. Median ± range hAlb levels,t-test of time points, * $p<0.05$ of both $3x10^8$ and $1x10^9$ hOSM groups compared to FLuc, ** $p<0.01$ of $3x10^8$ hOSM compared to FLuc.

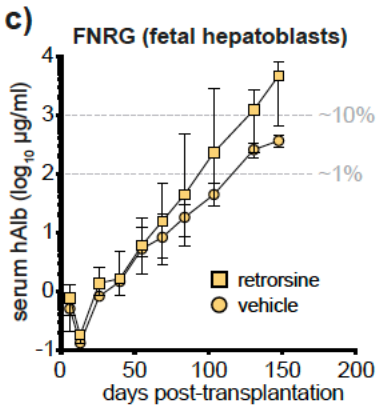
c) In the presence of AAV8-hOSM, groups of 5 retrorsine-preconditioned FNRG mice transplanted with fetal hepatoblasts reach higher serum hAlb levels than vehicle control animals. Median ± range hAlb levels, $p=0.0014$ between groups by mixed design ANOVA.

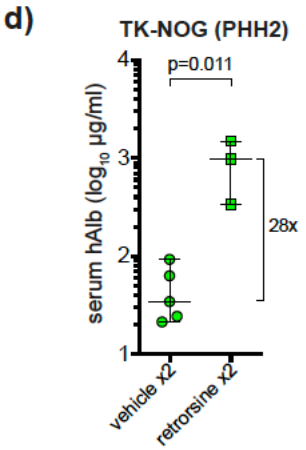
d) Retrorsine preconditioning of PHH2 transplanted TK-NOG mice results in higher peak serum hAlb values. Symbols are individual mice, median ± range, t-test.

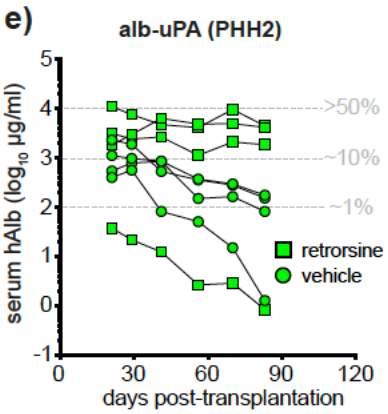
e) Hemizygous alb-uPA mice treated with retrorsine (squares) prior to PHH2 transplantation trend towards higher peak serum hAlb levels and then maintain hAlb above 10^3 µg/ml for longer duration than mice receiving vehicle (circles). Symbols are individual mice, $p=0.0003$ between groups by 2-way ANOVA.


a)
FNRG (PHH3)
p=0.98
survival (%)
retrorsine (n=13)
vehicle (n=13)
days post-transplantation


b)
FNRG (fetal hepatoblasts)
~10%
~1%
hOSM 3x10⁸
hOSM 1x10⁹
hOSM 3x10⁹
hOSM 1x10¹⁰
FLuc 1x10⁹
serum hAlb (log10 µg/ml)
days post-transplantation


c)
FNRG (fetal hepatoblasts)
~10%
~1%
retrorsine
vehicle
serum hAlb (log10 µg/ml)
days post-transplantation


d)
TK-NOG (PHH2)
p=0.011
28x
serum hAlb (log10 µg/ml)
vehicle x2
retrorsine x2


e)
alb-uPA (PHH2)
>50%
~10%
~1%
retrorsine
vehicle
serum hAlb (log10 µg/ml)
days post-transplantation

Supplementary Figure 2

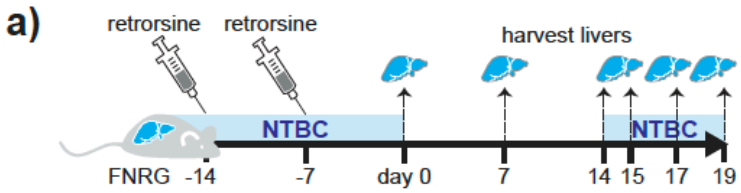
Retrorsine increases mouse hepatocyte Ki67 expression after restarting NTBC

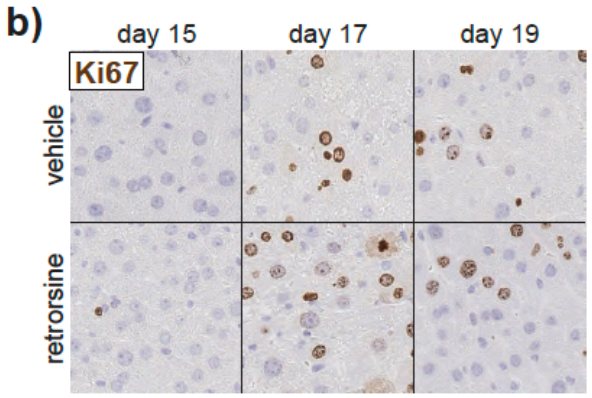
a) Experimental schematic in which FNRG mice received either retrorsine or vehicle prior to NTBC withdrawal and reintroduction. Livers from untransplanted FNRG mice were harvested at indicated times.

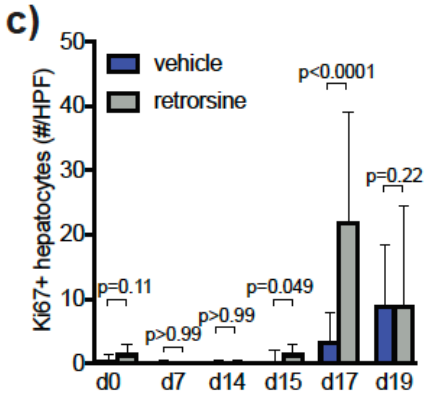
b) Staining for the cell cycle marker Ki67 showed few cycling hepatocytes in either group one day after restarting NTBC but became more abundant in retrorsine than vehicle preconditioned livers two days later.

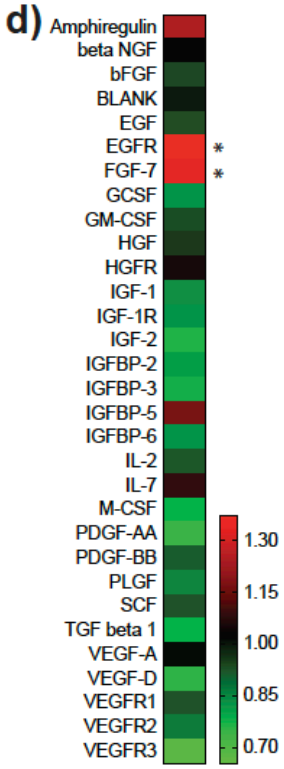
c) Quantification of $Ki67^+$ hepatocytes per high power field (HPF) showed retrorsine to increase the number of $Ki67^+$ hepatocytes after restarting NTBC. Median ±range of 10 HPF of two livers per group.

d) Heatmap of mouse growth factor protein levels in day 14 livers showed several factors being affected by retrorsine preconditioning. Fold change over vehicle control mice, n=3 mice per group, * $p<0.05$.


a)
retrorsine
retrorsine
harvest livers
NTBC
NTBC
FNRG
-14
-7
day 0
7
14
15
17
19


b)
day 15
day 17
day 19
Ki67
vehicle
retrorsine

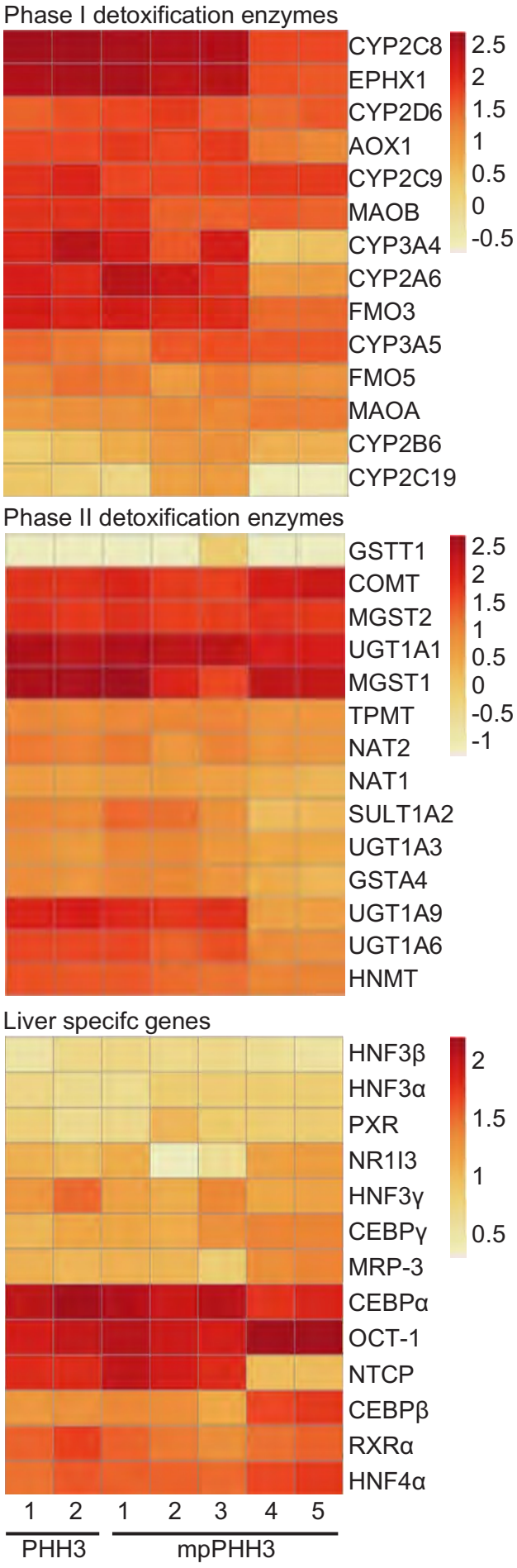

c)
Ki67+ hepatocytes (#/HPF)
vehicle
retrorsine
p=0.11
p>0.99
p>0.99
p=0.049
p<0.0001
p=0.22
d0
d7
d14
d15
d17
d19


d)
Amphiregulin
beta NGF
bFGF
BLANK
EGF
EGFR
FGF-7
GCSF
GM-CSF
HGF
HGFR
IGF-1
IGF-1R
IGF-2
IGFBP-2
IGFBP-3
IGFBP-5
IGFBP-6
IL-2
IL-7
M-CSF
PDGF-AA
PDGF-BB
PLGF
SCF
TGF beta 1
VEGF-A
VEGF-D
VEGFR1
VEGFR2
VEGFR3
1.30
1.15
1.00
0.85
0.70

Supplementary Figure 3

Gene expression profile of mpPHH is similar to cryopreserved PHH

RNAseq analysis of 2 cryopreserved PHH (PHH3) and 3 freshly isolated mpPHH3 from 5 humanized mice. Comparison of transcripts of Phase I detoxification genes (i.e. CYP450), Phase II detoxification genes and various liver-specific genes (nuclear receptors, liver-enriched transcription factors).


Phase I detoxification enzymes
CYP2C8
EPHX1
CYP2D6
AOX1
CYP2C9
MAOB
CYP3A4
CYP2A6
FMO3
CYP3A5
FMO5
MAOA
CYP2B6
CYP2C19
2.5
2
1.5
1
0.5
0
-0.5
Phase II detoxification enzymes
GSTT1
COMT
MGST2
UGT1A1
MGST1
TPMT
NAT2
NAT1
SULT1A2
UGT1A3
GSTA4
UGT1A9
UGT1A6
HNMT
2.5
2
1.5
1
0.5
0
-0.5
-1
Liver specifc genes
HNF3β
HNF3α
PXR
NR1I3
HNF3γ
CEBPγ
MRP-3
CEBPα
OCT-1
NTCP
CEBPβ
RXRα
HNF4α
2
1.5
1
0.5
1 2 1 2 3 4 5
PHH3
mpPHH3

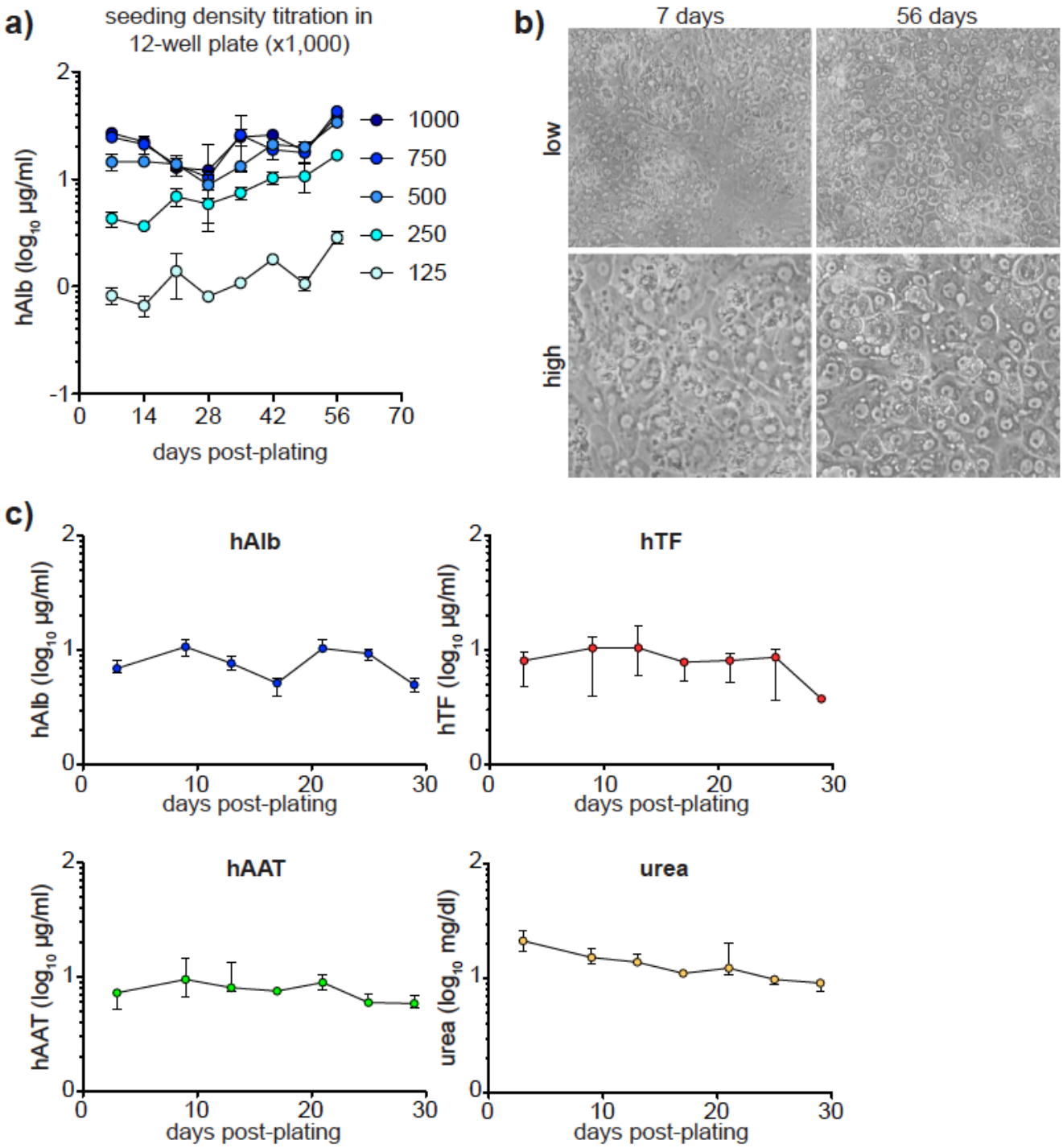
Supplementary Figure 4

Long-term mpPHH cultures are stable for at least 8 weeks

a) Seeding density titration of mpPHH for long-term culture (8 weeks) as shown by secreted hAlb levels over time. Media were changed on days 2, 4 and 7 for a total of 8 weeks. hAlb levels were measured in culture supernatants every seventh day. Numbers in the legend indicate number of cells in thousands seeded in 12-well plates. Data from biological duplicates are represented as mean ± s.d.

b) Representative low and high magnification images from wells seeded with 1 million cells after 7 days and 56 days in culture.

c) Hepatocyte markers measured in culture supernatants after seeding mpPHH on collagen-coated 12-well plates. Media were changed every two days. Human albumin (hAlb), human transferrin (hTF), human alpha-antitrypsin (hAAT) and urea were measured for up to 29 days after seeding. Data from four biological replicates are represented as mean ± s.d.

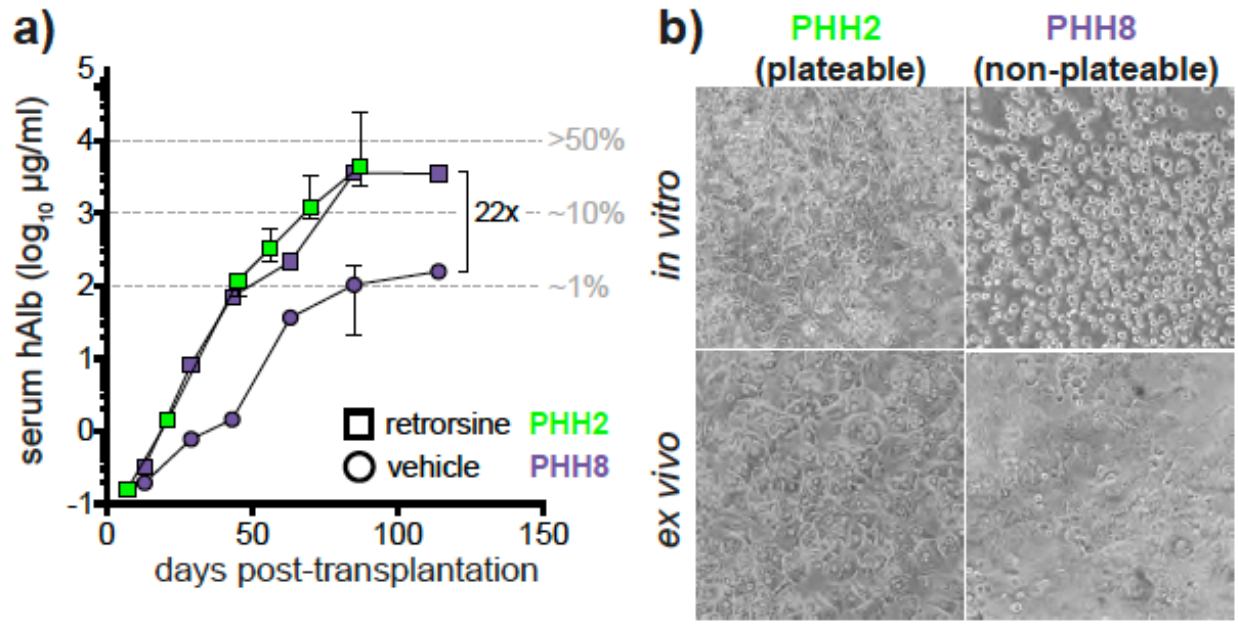

a)
seeding density titration in
12-well plate (x1,000)
hAlb (log10 µg/ml)
days post-plating
1000
750
500
250
125
b)
7 days
56 days
low
high
c)
hAlb
hTF
hAAT
urea
hAlb (log10 µg/ml)
hTF (log10 µg/ml)
hAAT (log10 µg/ml)
urea (log10 mg/dl)
days post-plating

Supplementary Figure 5

Retrorsine can rescue non-plateable PHH donors

a) Plateable PHH2 and non-plateable PHH8 were transplanted into groups of 2-3 FNRG mice, with retrorsine improving hAlb levels 22-fold in mice transplanted with PHH8. Median ± range serum hAlb levels.

b) Brightfield images 3 days after plating illustrate that non-plateable PHH8 only formed cultures *ex vivo* whereas plateable donor PHH2 also formed *in vitro* cultures.


a)
serum hAlb (log10 µg/ml)
5
4
3
2
1
0
-1
0
50
100
150
days post-transplantation
>50%
~10%
~1%
22x
retrorsine PHH2
vehicle PHH8
b)
PHH2
(plateable)
PHH8
(non-plateable)
in vitro
ex vivo

Supplementary Figure 6

mpPHH are suitable for high-throughput applications in 384-well plates

a) mpPHH were seeded in a 384-well plate. Brightfield images of confluent cultures.

b) Cells seeded in 384-well plate were infected with HBV in the presence or absence of HBV entry inhibitor Myrcludex B (500 nM). After washing (1 dpi and 3 dpi) the cells were cultured for an additional 7 days. At 10 dpi culture supernatants were harvested and cells were fixed and stained for HBcAg (red) and nuclei (blue). HBeAg levels in culture supernatants were measured using a chemiluminescence (CLIA) assay. Data from four biological replicates are represented as mean ± s.d.

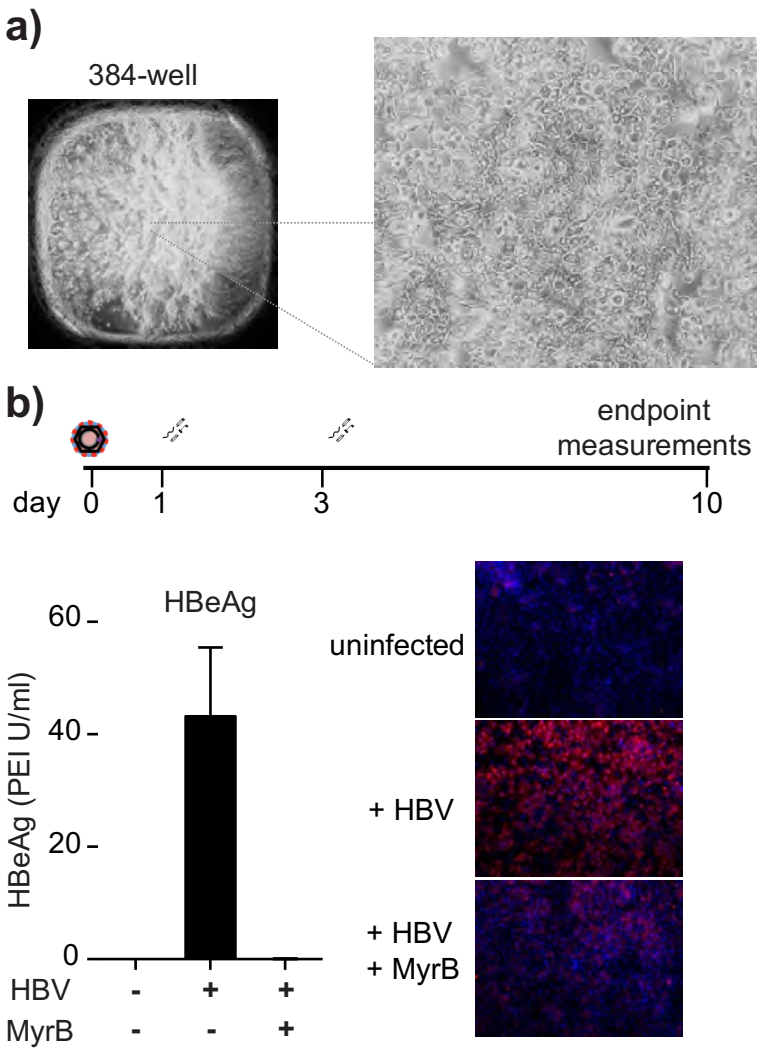

a)
384-well
b)
endpoint
measurements
day 0 1 3 10
HBeAg
60
40
20
0
HBeAg (PEI U/ml)
HBV - + +
MyrB - - +
uninfected
+ HBV
+ HBV
+ MyrB

Figure S6

Supplementary Figure 7

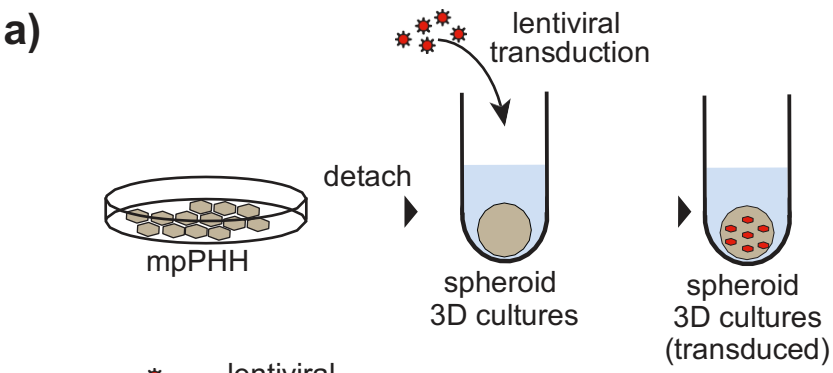
Cultured mpPHH can be transduced, mobilized and then used to form 3D spheroid cultures

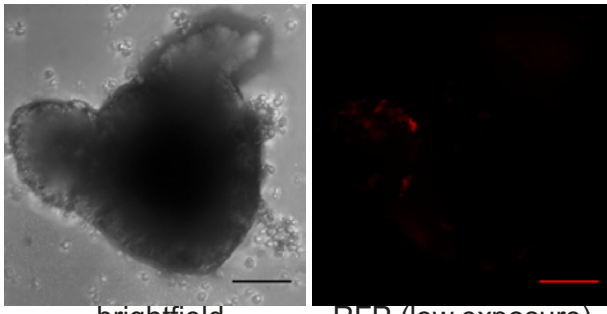
a) mpPHH in 2D cultures were detached and then transduced with RFP-lentiviruses in suspension to form 3D spheroid cultures. Brightfield and RFP fluorescence imaging demonstrates low transduction efficiency.

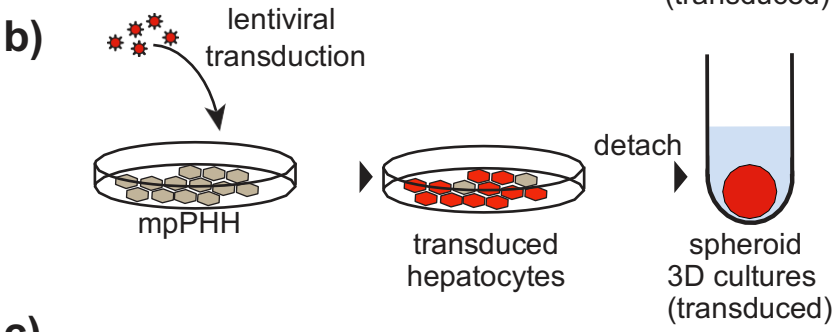
b) mpPHH in 2D cultures were transduced with RFP-lentiviruses then detached and used to form 3D spheroid cultures. Brightfield and RFP fluorescence imaging indicates high transduction efficiency.

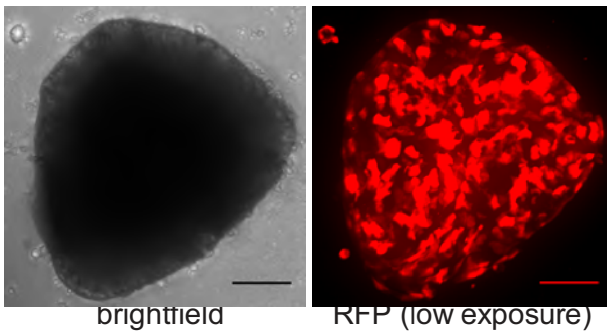
c) Z-stacks of spheroids made by transduced mpPHH (from panel b).

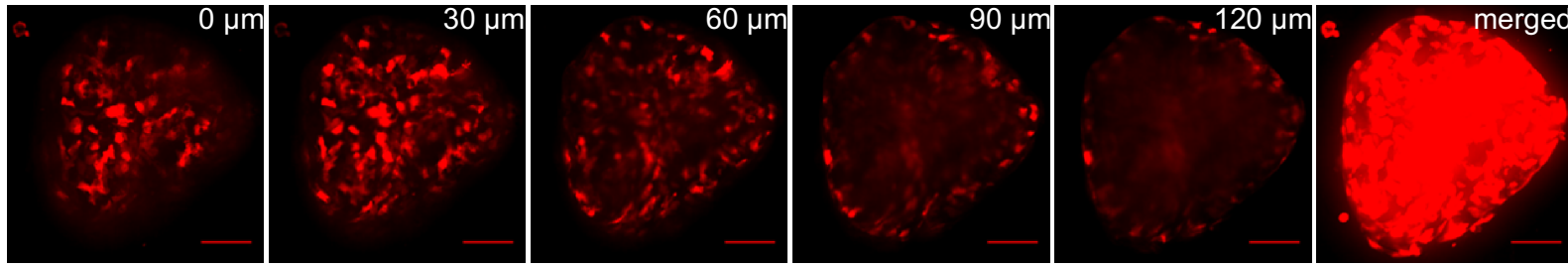
d) Cell seeding titration of RFP-transduced mpPHH (in thousands cells) used to form single spheroid cultures in 96-well plates.


a)
lentiviral
transduction
detach
mpPHH
spheroid
3D cultures
spheroid
3D cultures
(transduced)


brightfield
RFP (low exposure)


b)
lentiviral
transduction
mpPHH
transduced
hepatocytes
detach
spheroid
3D cultures
(transduced)


brightfield
RFP (low exposure)


c)
0 µm
30 µm
60 µm
90 µm
120 µm
merged

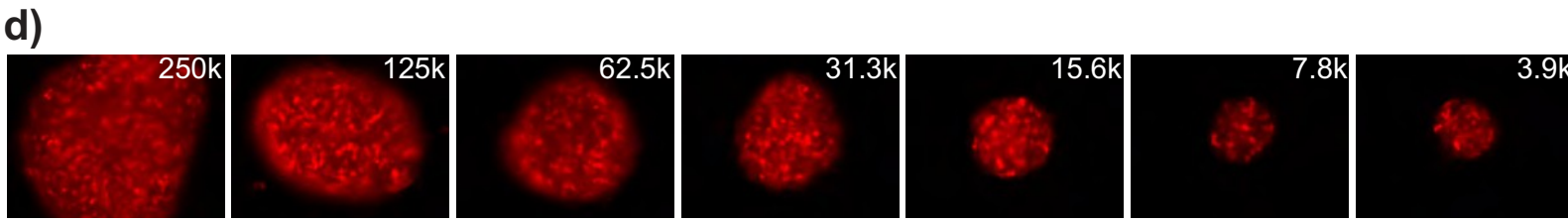

d)
250k
125k
62.5k
31.3k
15.6k
7.8k
3.9k

Figure S7

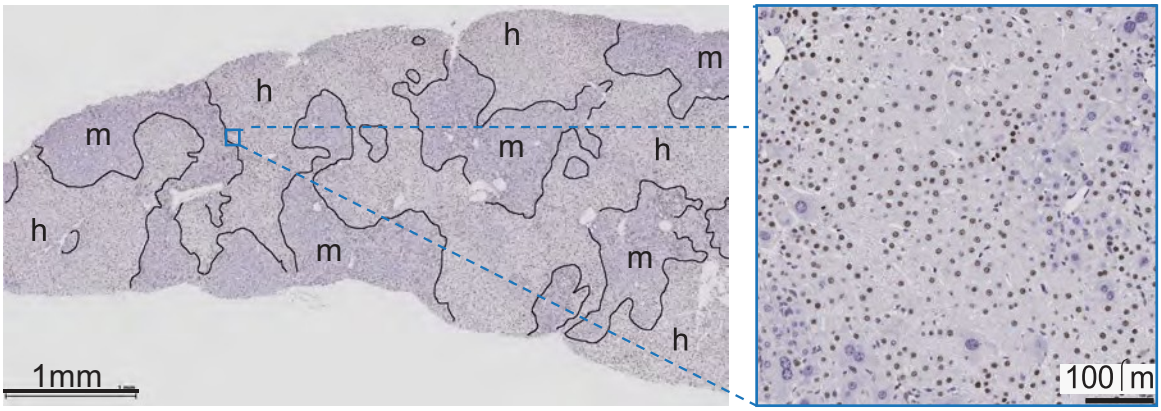
Supplementary Figure 8

Human NuMA staining of a liver slice from a humanized mouse engrafted with RFP-transduced mpPHH

a) Livers from mice from Figure 5f were processed for immunohistochemistry. Areas of human (h) and murine (m) cells are separated with lines. An area with human hepatocytes is shown at high magnification.

b) Cells from Figure 5f were cultured for 2 weeks with media changes every 2 days. A representative image of RFP expression is shown on day 15 post-seeding.

a)



b)

re-plated mpPHH (RFP+)

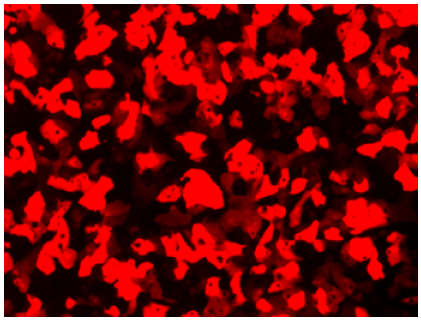


Figure S8