Supplementary Material

Agonist c-Met Monoclonal Antibody Augments the Proliferation of hiPSC-derived Hepatocyte-Like Cells and Improves Cell Transplantation Therapy for Liver Failure in Mice

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Supplementary Methods

○ ***Generation of hiPSC-HLCs stably expressing luciferase (Luc-hiPSC-HLCs)***

To generate Luc-hiPSC-HLCs, the cells were infected with lentivirus carrying the luciferase (Luc) gene and puromycin resistance gene (generated by OBiO Technology, Shanghai, China) according to the manufacturer's instructions. Over 90% of the infected hiPSC-HLCs were positive for luciferase at 3 days post infection. Thereafter, 8 μg/mL of puromycin (Aladdin; #P113126) was added in the culture medium for positive selection. Finally, pure Luc-hiPSC-HLCs were obtained at 2 weeks post infection. The Luc-hiPSC-HLCs were cultured in medium with 1 μg/mL of puromycin.

○ ***Culture of PHHs***

Cryopreserved PHHs were purchased from LONZA (#CC-2591S) and were stored in liquid nitrogen. For the *in vitro* evaluation of 5D5, PHHs were thawed and cultured in similar medium conditions with hiPSC-HLCs. For the engraftment and *in vivo* evaluation of 5D5, PHHs were thawed and directly transplanted to FRGS mice.

○ ***Collection and purification of agonist c-Met mAb 5D5***

The 5D5.11.6 mAb cell line was obtained from ATCC (#HB-11895). The mAb cell line was cultured in RPMI 1640 Medium (GIBCO; #11875-093) with 10% FBS (GIBCO; #10270-106). The cell culture supernatants were collected and purified using an AKTA Purifier (GE Healthcare Life Sciences) and MabSelect SuRe (GE, 17-5438-02). The purified 5D5 antibody (IgG1) was dialyzed to PBS and concentrated using EMD Millipore™ Amicon™ Ultra-15 mL Centrifugal Filter Units (Millipore; #UFC905096) and was stored at -80℃.

○ ***NTBC withdrawal-induced liver failure***

To achieve $Fah^{-/-}$-induced liver injury, NTBC in daily drinking water was gradually reduced from 7.5 to 0 mg/mL from week -2 to -1 post cell transplantation. For mild liver injury, 7.5 mg/mL of NTBC was added in the drinking water for three days at weeks 1, 3, 5 and 7 after cell transplantation (see Fig. 3A). For severe chronic liver injury, 7.5 mg/mL of NTBC was added to the drinking water for three days at weeks 3, 7, 11 and 15 after cell transplantation (see Fig. 6A).

○ ***JO2-induced liver failure***

To achieve acute liver failure, hamster-anti-mouse Fas/CD95 antibody clone JO2 (BD

Biosciences; #554258) was administered to FRGS mice. To induce mild liver injury and kill some of the mouse liver cells, FRGS mice were intraperitoneally injected with 0.2 mg/kg of JO2 at day -1 post cell transplantation (see Fig. 3A). To induce life-threatening acute liver failure, FRGS mice were intraperitoneally injected with 0.2 mg/kg of JO2 at days -1, 2, 5 and 8 post cell transplantation (see Fig. 5A).

○ ***CCl_4-induced liver failure***

To generate chronic liver injury-induced liver cirrhosis, FRGS mice received NTBC cycled off and the administration of CCl_4 twice per week (see Fig. 7A). For NTBC cycled off, NTBC in drinking water was gradually reduced from 7.5 to 0 mg/mL from weeks -6 to -4 post cell transplantation. Next, NTBC was added for three days at weeks -2, 0, 2, 4 and 6 post cell transplantation. Ten percent CCl_4 (Xilong Scientific, China; #1042003) was diluted in olive oil (BBI Life Science, ShangHai, China; #A502795). For CCl_4 administration, the FRGS mice were received intraperitoneally injected with 10% CCl_4 (2 mL/kg) twice per week from weeks -6 to -1 post cell transplantation (see Fig. 7A).

○ ***Agonistic c-Met antibody 5D5 treatment***

For *in vitro* treatment, agonistic c-Met antibody 5D5 dissolved in PBS was directly added to the culture cell medium at the indicated concentrations. For each *in vivo* treatment, 0.5 mg/kg of agonist c-Met mAb 5D5 dissolved in PBS was administered by intraperitoneal injection.

○ ***ELISA***

The levels of hALB and hAAT were measured using ELISA Quantitation Kits according to the manufacturer's protocol (#E80-129, #E88-122, Bethyl Laboratories, Montgomery, Canada). The levels of hAFP were measured using ELISA Quantitation Kits from Wantai, Beijing, China.

○ ***IF staining***

Cells were cultured on slides in a 6-well plate and were fixed by 4% paraformaldehyde (#16005, SIGMA-ALDRICH) for 20 minutes, treated by 0.1% Triton-X100 (#0694, AMRESCO) for 10 minutes, incubated with 20% Bovine Serum Albumin (BSA, #A1933, SIGMA-ALDRICH) for 30 minutes, and then incubated with the first and second antibodies. Cell nuclei was stained by 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, #D1306, Invitrogen) for 3 minutes. After each step, the slides were washed with PBS for three times. The slides were observed by microscope (BX51/IX71, Olympus or AXIO Imager.Z2, ZEISS). The antibodies used in IF staining were detailed showed in the ***Antibody list*** section.

○ ***Detection of cell proliferation assay***

1×10^2 cells were cultured in E-Plates and set in iCELLigence (ACEA Bioscience, San Diego, California, USA) for real-time monitoring of cell proliferation ability. The cell proliferation ability was calculated by the change of cell attachment areas on the bottom of E-Plates between the time point of initial cell attachment and indicated time point.

○ ***Western blot***

The western blotting assays were operated as previously described [1, 2]. Normalization for gray scale of the western blotting results were operated by the Image J software.

○ ***Collection of liver cells by collagenase perfusion***

Cells from repopulated primary recipients were harvested with a standard collagenase perfusion protocol [3]. Briefly, the liver was perfused with calcium- and magnesium-free Earle's balanced salt solution (EBSS) supplemented with 0.5 mM EGTA and 10 mM HEPES for 5 min. The solution was changed to EBSS supplemented with 0.1 mg/ml collagenase IV (#C5138, Sigma-Aldrich) and 0.05 mg/mL DNase I (#2270B, TaKaRa) for 10 min. The liver was gently minced in the second solution and filtered through 70 mm and 40 mm nylon mesh sequentially. After 150g centrifugation for 5 min, the pellet was washed twice at 50g for three min. The number and viability of cells were assessed by Trypan blue exclusion test.

○ ***FACS analysis***

The collagenase perfused liver cells were added in 50 mL tubes, washed twice by PBS via centrifugation at 100 g and resuspended with 5-15 mL PBS. The amount of total collagenase perfused liver cells counted by a Vi-CELL XR instrument (Beckman Coulter). For each mouse, 1×10^6 of collagenase perfused liver cells were used for further FACS analysis. The cells for FACS analysis were incubated at 4℃for 30 minutes with indicated antibodies. They were then rinsed with PBS twice and analyzed with a FACS instrument (Facsaria III, BD). The antibodies used in FACS analysis were detailed showed in the ***Antibody list*** section. For the detection of intracellular markers, such as hALB and Ki67, fixation/permeabilization solution (#554714, BD Bioscience) was used. Dead cells were excluded using fixable viability dye (#L23101, eBioscience). To exclude non-specific reactions, background signals and other interferences, a less than 0.5% positive rate in the FACS analysis was recognized as a negative result.

○ ***IHC, H&E and M&T staining***

Mice liver tissues were fixed in 4% formaldehyde (PH 7.4) for 48 hours. Sections (4 μm)

were applied to poly-L-lysine-coated slides. After the sections were dewaxed, rehydrated and washed, endogenous peroxidases were inactivated with 3% H_2O_2 for 10 minutes. The sections were then incubated overnight with primary antibodies. The sections were subsequently washed with PBS for three times and treated with UltraSensitiveTM SP Kit (Maixin Biotech. Co., Ltd., Fuzhou, China) for the rest steps. Brown staining indicated positive expression. The antibodies used in IHC staining were detailed showed in the ***Antibody list*** section. For H&E and M&T staining were predicted by Kits from Fuzhou Maixin Biotech (#CTS1096, 3CTS4094, #MST8004). The Sections were visualized using an inverted microscope (BX51, Olympus), and digital images were captured using Olympus Cell Sense software.

○ ***Luciferase detection and imaging***

Luciferase signal detection, imaging and analysis were performed as described in our previous study [4]. In brief, mice received Luc-hiPSC-HLCs transplantation were determined at indicated time points. The mice were anaesthetized by isoflurane, received intraperitoneally injection of beetle 150 mg/kg luciferin (#E1605, Promega, Madison, WI, USA) dissolved in PBS, and then detected by IVIS system Lumina II (Xenogen Corporation, Alameda, CA, USA). The bioluminescence signal of luciferase (photons/sec/cm^2/steradian) was also measured.

○ ***Measurement of liver functional markers***

Serum ALT, AST, TBIL, TBA, TP, PT levels were measured using regents form Wantai, Beijing, China, according to the manufacturer's protocol.

○ ***qRT-PCR***

Total RNA was extracted from tissues or purified cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and used for cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative reverse transcription (RT)-PCR was performed on a 7500 Fast Real-Time PCR system. The primers used in this study were showed in the ***Primer list*** section.

Supplementary Table 1. Antibody list

Antibodies	Application	Source	Cat. No.
Purified Anti-Mouse CD95 Clone JO2	Induce liver failure	BD Biosciences	#554254
Anti-human albumin (hALB)	FACS; IF; IHC	Abcam	#ab1024
Anti-Rabbit IgG-TRITC	Second antibody	SIGMA-ALDRICH	#T6778
Anti-Mouse IgG-TRITC			#T5393
Anti-Rabbit IgG-FITC			#F9887
Anti-Mouse IgG-FITC			#F9006
Goat anti-Mouse IgG-HRP		Invitrogen	#31430
Goat anti-Rabbit IgG-HRP			#A16096
Anti-Ki67	IF, FACS	Proteintech	#27309-1
Anti ERK	WB	Abcam	#ab36991
Anti Akt	WB		#ab8805
Anti STAT1	WB		#ab3987
Anti STAT3	WB		#ab119352
Anti-human pERK	WB	CST	#4377S
Anti-human pAkt	WB		#9271S
Anti-human pSTAT1	WB		#9167S
Anti-human pSTAT3	WB		#4113S
Anti-human GAPDH	WB	Proteintech	#60004-1
Anti-human alpha fetoprotein (hAFP)	IHC	Proteintech	#14550-1

Supplementary Table 2. The qRT-PCR primers list

Genes	Primer	
hALB	F	TTTATGCCCCGGAACTCCTTTT
	R	ACAGGCAGGCAGCTTTATCAG
hAAT	F	GCCTATGATGAAGCGTTTAGGC
	R	TTCCAGTAATGGACAGTTTGGGT
hHNF4α	F	AACGGACAGATGTGTGAGTGG
	R	CAGGAGCTTATAGGGCTCAGAC
HNF1α	F	GCCACCTGCTGCCATCCAA
	R	TGCAGCCCGTAGTTTAAAC
hFAH	F	CCTACGGCGTCTTCTCGAC
	R	CTGCAAGAACACTCTCGCCT
hNTCP	F	AAGGACAAGGTGCCCTATAAAGG
	R	ACGATCCCTATGGTGCAAGGA
hGAPDH	F	GGAGTCAACGGATTTGGTCGT
	R	CACTTGATTTTGGAGGGATCTCG
hCEA	F	AGGCACGAGTAACAAGCTCAC
	R	ATGAGGACATAACCAGCCACC
hGOLM1	F	TGGCCTGCATCATCGTCTTG
	R	CCCTGGAACTCGTTCTTCTTCA
hAFP	F	CTTGCACACAAAAAGCCCACT
	R	GGGATGCCTTCTTGCTATCTCAT
hGPC-3	F	CTGCTTCAGTCTGCAAGTATGG
	R	GTGGAGTCAGGCTTGGGTAG
mCOL1A1	F	GCTCCTCTTAGGGGCCACT
	R	ATTGGGGACCCTTAGGCCAT
mCOL1A2	F	GGTGAGCCTGGTCAAACGG
	R	ACTGTGTCCTTTCACGCCTTT
mTIMP-1	F	CGAGACCACCTTATACCAGCG
	R	ATGACTGGGGTGTAGGCGTA
mMMP-2	F	TGTCTTGCGTCTGACACTGC
	R	CTCCTTTGGGCTAGGTATCTCT

Supplementary References

1. Zhang TY, Yuan Q, Zhao JH, Zhang YL, Yuan LZ, Lan Y, et al. Prolonged suppression of HBV in mice by a novel antibody that targets a unique epitope on hepatitis B surface antigen. Gut. 2016; 65: 658-71.
2. Yuan L, Liu X, Zhang L, Li X, Zhang Y, Wu K, et al. A Chimeric Humanized Mouse Model by Engrafting the Human Induced Pluripotent Stem Cell-Derived Hepatocyte-Like Cell for the Chronic Hepatitis B Virus Infection. Front Microbiol. 2018; 9: 908.
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4. Wang W, Pan D, Fu W, Cai L, Ye J, Liu J, et al. A SCID mouse-human lung xenograft model of varicella-zoster virus infection. Antiviral Res. 2017; 146: 45-53.

Supplementary Figures and Legends

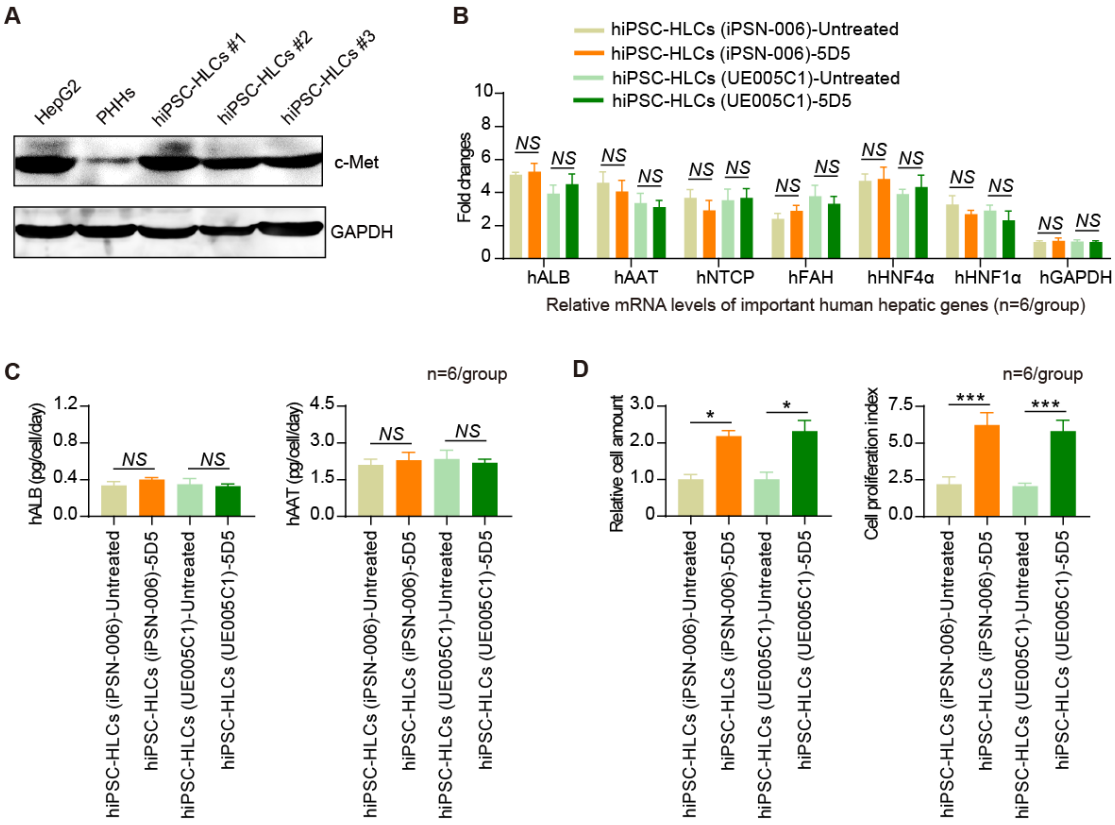


Fig. S1. c-Met protein expression and effects of 5D5 treatment in PHHs and hiPSC-HLCs. **(A)** HepG2 (positive control), PHHs and hiPSC-HLCs derived from three different hiPSCs lines (#1, derived from human fibroblast-induced hiPSCs named GZF2C6; #2, derived from human urethral epithelial cell-induced hiPSCs named UE005C1; #3, derived from human amniotic mesenchymal cells named iPSN-006) were detected by western blot method for expressions of c-Met protein, GAPDH was set as internal reference. All of the hiPSC-HLCs showed significantly higher expression of c-Met protein than the PHHs. **(B)** The relative mRNA levels of important human hepatic genes in hiPSC-HLCs derived from hiPSCs UE005C1 and iPSN-006 treated or untreated with 5D5 for two days were measured by qRT-PCR (n=6/group). **(C)** The hALB (top) and hAAT (bottom) levels in cell culture supernatants with or without 5D5 treatment were measured by ELISA (n=6/group). **(D)** Relative cell numbers of hiPSC-HLCs derived from hiPSCs UE005C1 and iPSN-006 with or without 5D5 treatment, as measured by FACS (left, n=6/group), and cell proliferation index of such cells cultured *in vitro* at two days after 5D5 treatment (right, n=6/group); the untreated cells were set as the control. (*P <0.05, ***P <0.001. *NS*, no significance).

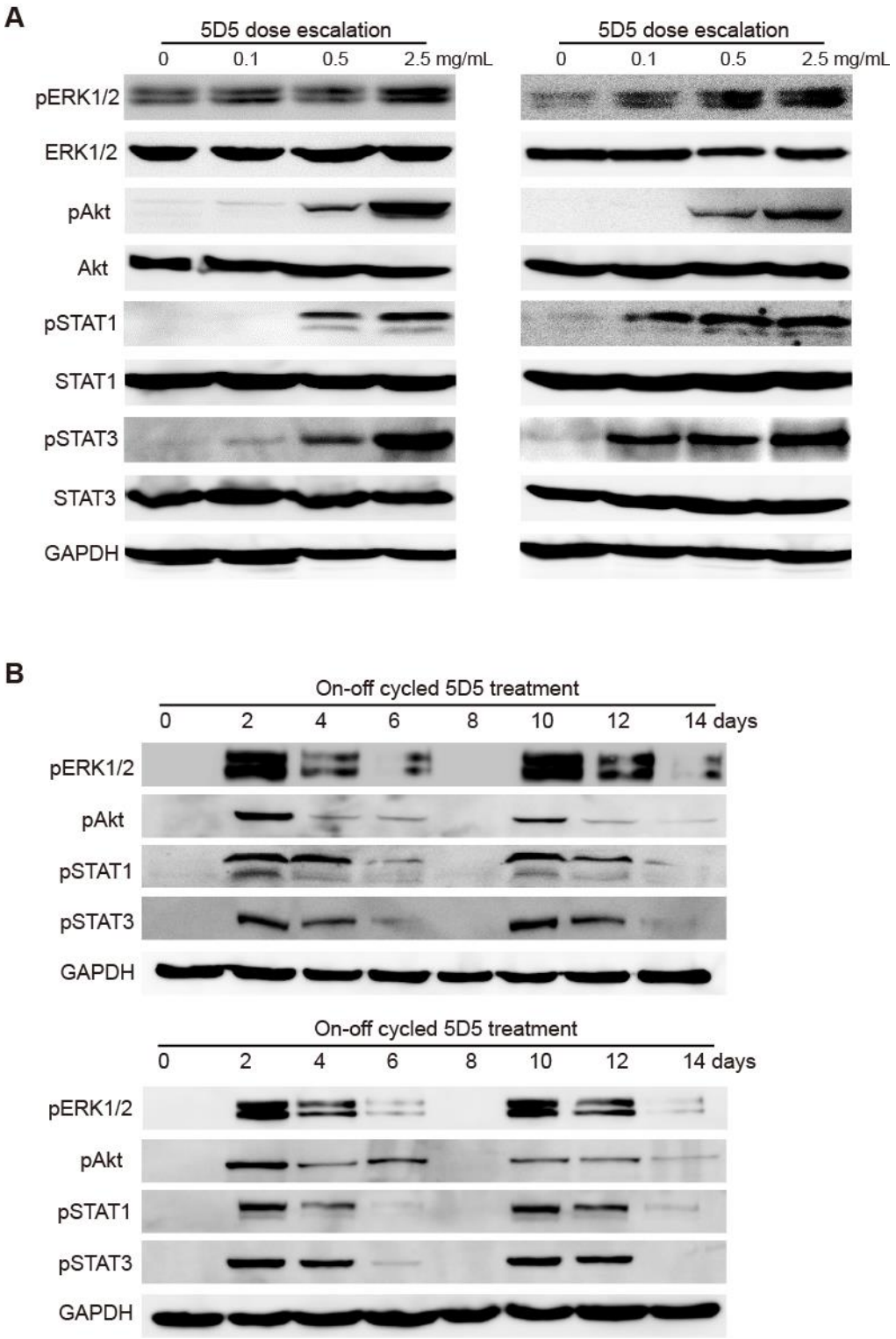


Fig. S2. Repeated experiments for dose escalation and on-off cycle agonist c-Met mAb 5D5 treatment studies in hiPSC-HLCs cultured in vitro. **(A)** Representative western blot assays for the expression of down-stream proteins, including ERK, Akt, STAT1 and STAT3, and their phosphorylation activation in hiPSC-HLCs that received two-day dose escalation 5D5 treatment (from left to right: 0, 0.1, 0.5 and 2.5 mg/mL). **(B)** Representative western blot

assays for phosphorylation activation of down-stream proteins in hiPSC-HLCs that received a 14-day on-off cycle 5D5 treatment (2.5 mg/mL of 5D5 was given from day 0 to 2 and day 8 to 10).

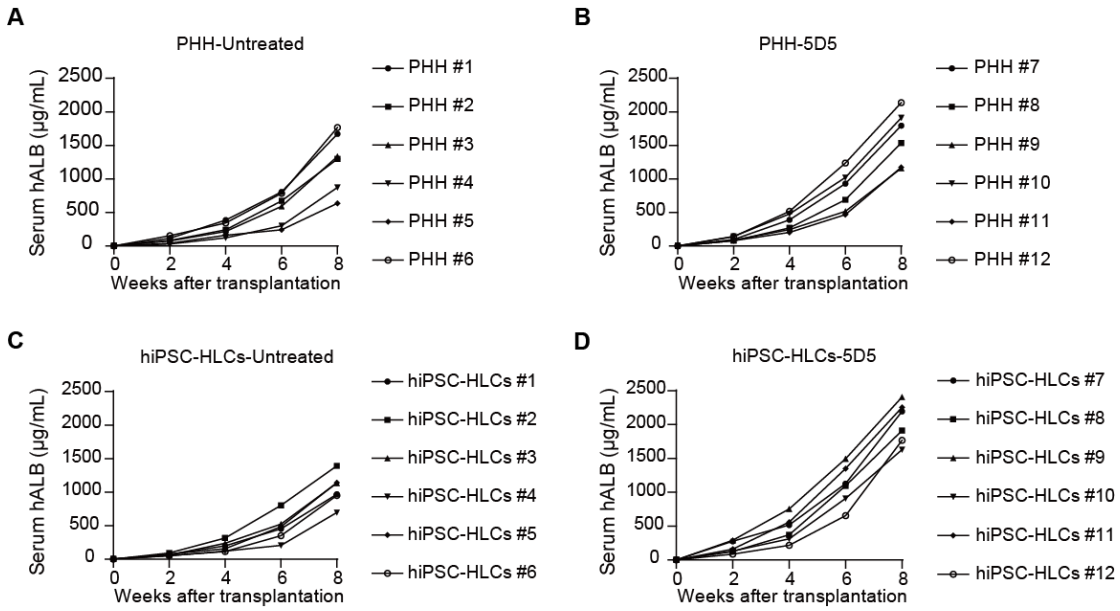
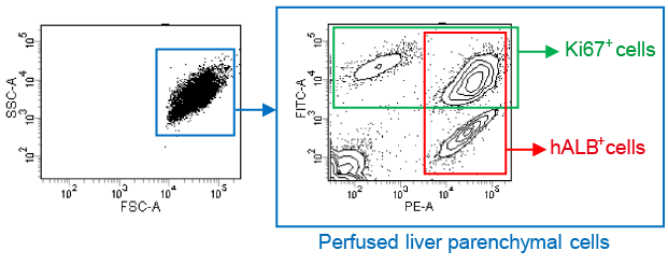


Fig. S3. Serum hALB levels of individual animals. **(A, B)** Detection of the serum hALB levels of FRGS mice that received PHH transplantation with or without 5D5 treatment by ELISA from weeks 0 to 8 after translation. **(C, D)** Detection of the serum hALB levels of FRGS mice that received hiPSC-HLC transplantation by ELISA with or without 5D5 treatment from weeks 0 to 8 after translation.

A



B

Group/Mice	#1	#2	#3	#4	#5	#6
PHHs-Untreated	14.8	13.1	12.9	9.7	8.9	19.5
PHHs-5D5	20.9	16.4	11.2	23.6	10.9	19.9
hiPSC-HLCs-Untreated	25.2	20.5	22.6	12.8	19.7	18.9
hiPSC-HLCs-5D5	42.3	38.2	51.8	39.7	35.8	45.6

C

Group/Mice	#1	#2	#3	#4	#5	#6
PHHs-Untreated	10.7	16.4	27.4	25.7	19.2	21.9
PHHs-5D5	28.9	28.8	26.5	34.3	45.1	39.3
hiPSC-HLCs-Untreated	40.3	55.9	46.2	64.9	38.1	42.9
hiPSC-HLCs-5D5	84.1	93.6	95.9	91.5	78.4	89.5

Fig. S4. Analysis of intrahepatic cells collected from PHH- and hiPSC-HLCs-transplanted mice with or without 5D5 treatment. **(A)** Representative gate scheme for FACS analysis of $Ki67^+$ and $hALB^+$ cells collected from liver of hiPSC-HLC-transplanted mice. For each mouse, 1×10^6 of collagenase perfused liver cells were used for FACS analysis. The ratios of **(B)** $hALB^+$ cells in total liver cells and **(C)** $Ki67^+$ cells in $hALB^+$ liver cells collected from individual PHH- and hiPSC-HLCs-transplanted mice with or without 5D5 treatment at 8 weeks after transplantation (n=6/group).

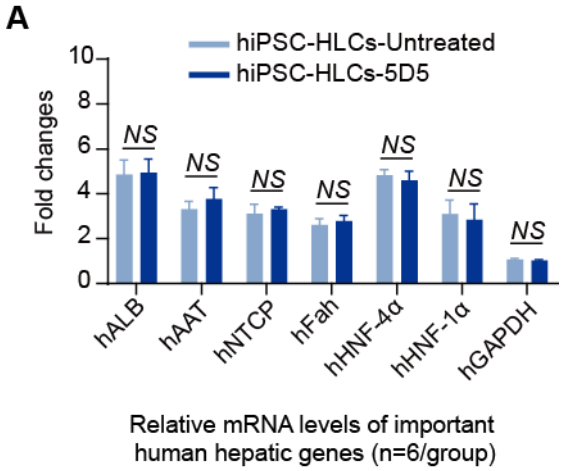


Fig. S5. Analysis of human hepatic gene expression in hALB$^+$ cells collected from hiPSC-HLC-transplanted mice with or without 5D5 treatment. **(A)** Relative mRNA levels of important human hepatic genes, including hALB, hAAT, hNTCP, hFAH, hHNF4α and hHNF1α, in hALB$^+$ cells collected from hiPSC-HLC-transplanted mice with or without 5D5 treatment by qRT-PCR at week 8 after transplantation. The hGAPDH gene was set as the control (n=6/group). (*NS*, no significant difference).

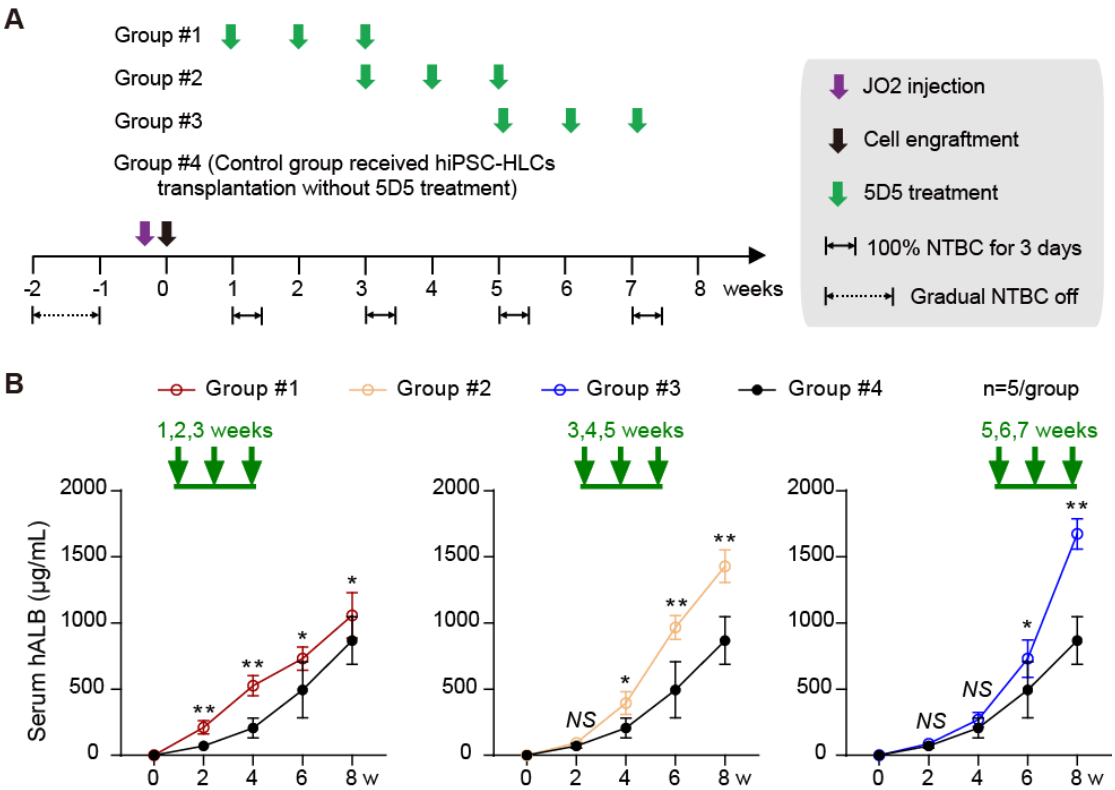


Fig. S6. Administration of 5D5 in implanted hiPSC-HLCs in FRGS mouse liver. **(A)**

Schematic design of 5D5 treatment in different groups after cell transplantation. Mice without 5D5 treatment (group #4) were set as controls. **(B)** Detecting the serum hALB levels of hiPSC-HLCs transplanted mice that received different 5D5 treatment by ELISA from weeks 0 to 8 after cell transplantation (n=5/group). In contrast of the mice without 5D5 treatment (group #4), the 5D5 treated mice showed a rapid increase rate of serum hALB levels (indicated by the line slope) during the 5D5 treatment period and in one week after 5D5 treatment. However, the 5D5 treated mice showed a similar increase rate of serum hALB levels of the mice without 5D5 treatment from 1 to 5 weeks after 5D5 treatment. Overall, these results suggested that the in *vivo* effects of 5D5 were reversible. (*P <0.05, **P <0.01. *NS*, no significant difference).

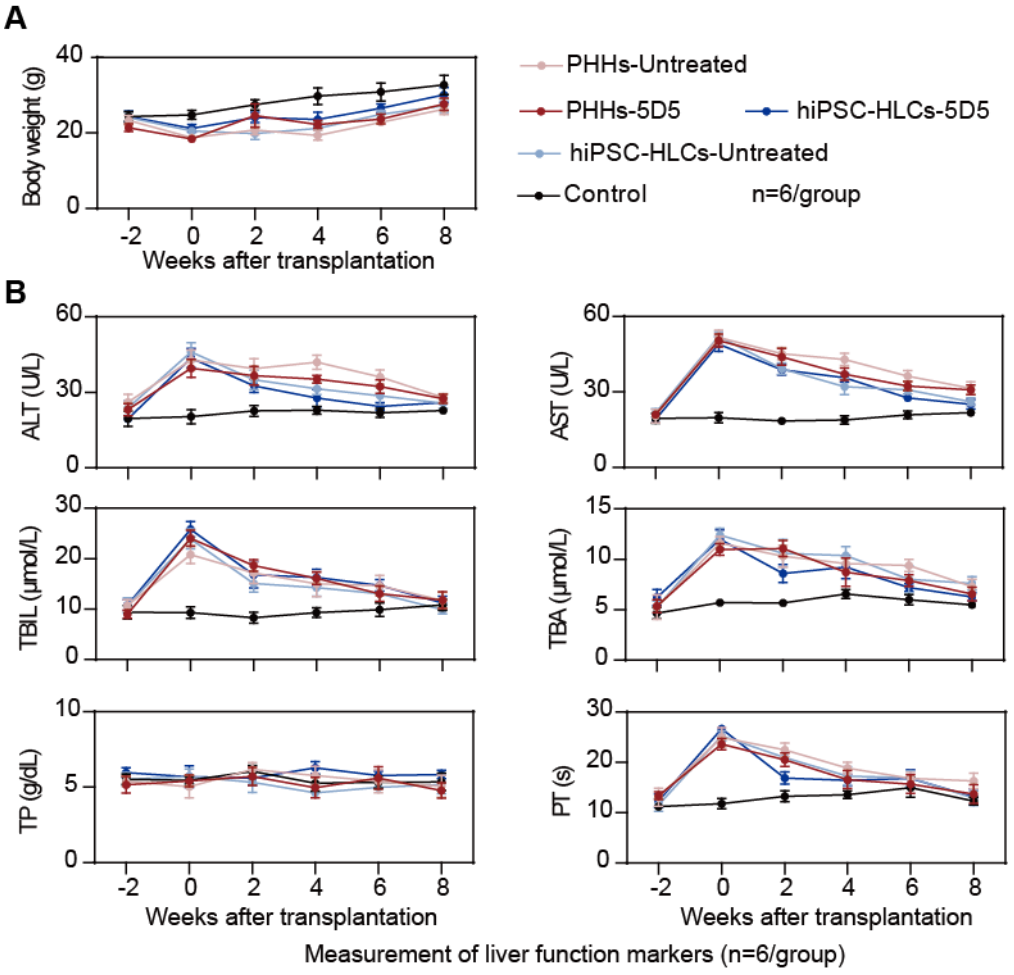


Fig. S7. Preliminary safety analysis of FRGS that received PHH or hiPSC-HLC transplantation with or without 5D5 treatment. **(A)** Body weight and **(B)** liver function markers of FRGS that received PHH or hiPSC-HLC transplantation with or without 5D5 treatment from weeks -2 to 8 after cell transplantation (n=6/group). The liver function

markers included ALT, AST, TBIL, TBA, TP and PT.

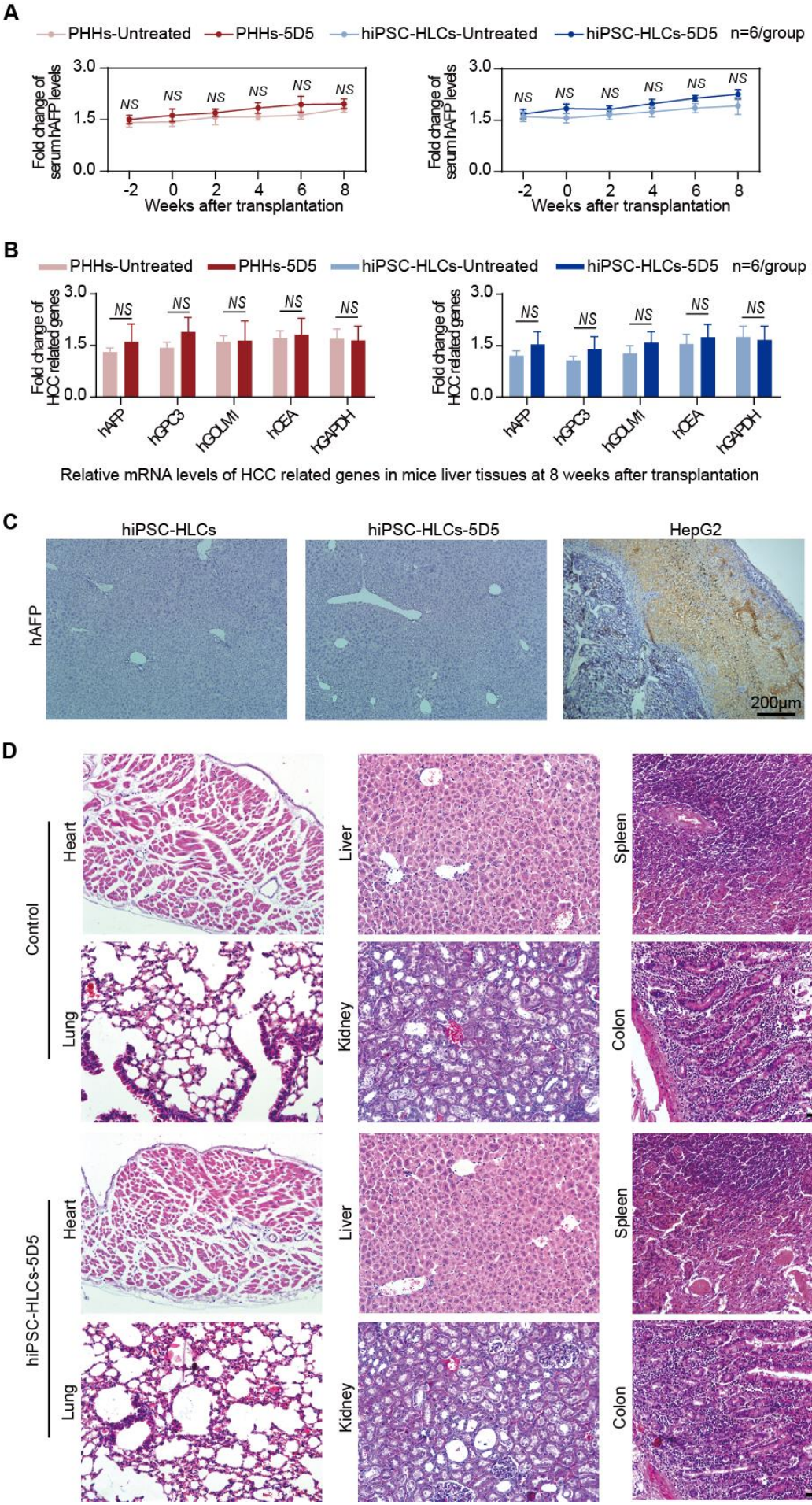

A
PHHs-Untreated
PHHs-5D5
hiPSC-HLCs-Untreated
hiPSC-HLCs-5D5
n=6/group
Fold change of serum hAFP levels
3.0
1.5
0.0
NS
-2
0
2
4
6
8
Weeks after transplantation
B
Fold change of HCC related genes
hAFP
hGPC3
hGOLM1
hCEA
hGAPDH
Relative mRNA levels of HCC related genes in mice liver tissues at 8 weeks after transplantation
C
hiPSC-HLCs
hiPSC-HLCs-5D5
HepG2
hAFP
200μm
D
Control
hiPSC-HLCs-5D5
Heart
Liver
Spleen
Lung
Kidney
Colon
200μm

Fig. S8. Preliminary tumorigenesis analysis of FRGS that received PHH or hiPSC-HLC transplantation with or without 5D5 treatment. **(A)** Detection of the serum hAFP levels of FRGS that received PHH or hiPSC-HLC transplantation with or without 5D5 treatment by ELISA from weeks -2 to 8 after cell transplantation (n=6/group). **(B)** Relative mRNA levels of human hepatocellular carcinoma genes, including hAFP, hGPC3, hGOLM1 and hCEA, in $hALB^+$ cells collected from PHH- or hiPSC-HLC-transplanted mice with or without 5D5 treatment by qRT-PCR at week 8 after transplantation. The hGAPDH gene was set as the control (n=6/group). **(C)** IHC staining for hAFP expression in liver tissues collected from hiPSC-HLC-transplanted mice with or without 5D5 treatment at week 8 after transplantation (bar=200 μm). HepG2 cell-transplanted mice liver tissue was set as positive control. **(C)** H&E staining of tissues collected from the heart, liver, spleen, lung, kidney and colon of hiPSC-HLC-transplanted mice with or without 5D5 treatment at week 8 after transplantation (bar=200 μm). (*NS*, no significant difference).

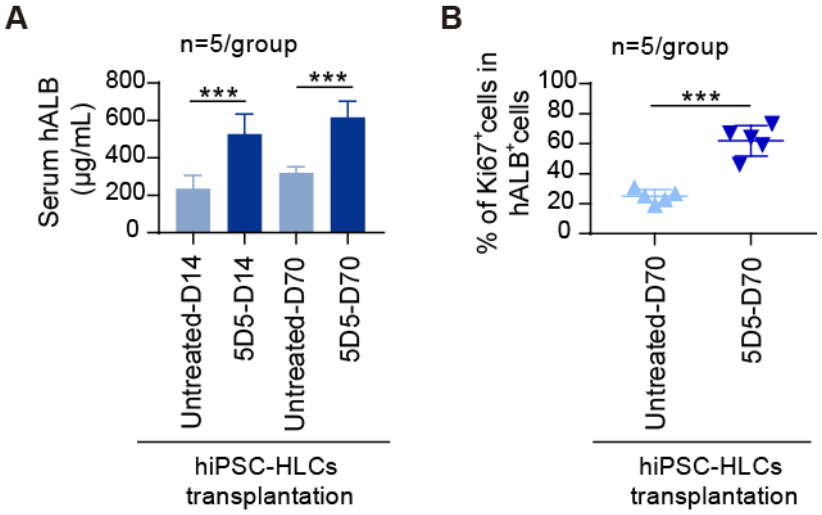


Fig. S9. The serum hALB levels and ratio of $Ki67^+$ cells in $hALB^+$ liver cells of FRGS mice with life-threatening ALF rescued by hiPSC-HLC transplantation combined with agonist c-Met mAb 5D5 treatment (Supplementary data for Figure 5). **(A)** Serum hALB levels of the survived ALF-FRGS mice that received hiPSC-HLCs transplantation with or without agonist c-Met mAb 5D5 treatment at day 14 and 70 after cell transplantation (n=5/group). **(B)** To know the cell proliferation ability *in vivo*, the liver cells of the hiPSC-HLCs transplanted mice were perfused by collagenase at 70 days after transplantation and detected by FACS method to know the ratio of $Ki67^+$ cells in $hALB^+$ liver cells (n=5/group). These results suggested that 5D5 treatment significantly increase the serum hALB levels and the ratio of

Ki67$^+$ cells in hALB$^+$ liver cells of the hiPSC-HLCs transplanted mice with life-threatening ALF (***P <0.001).

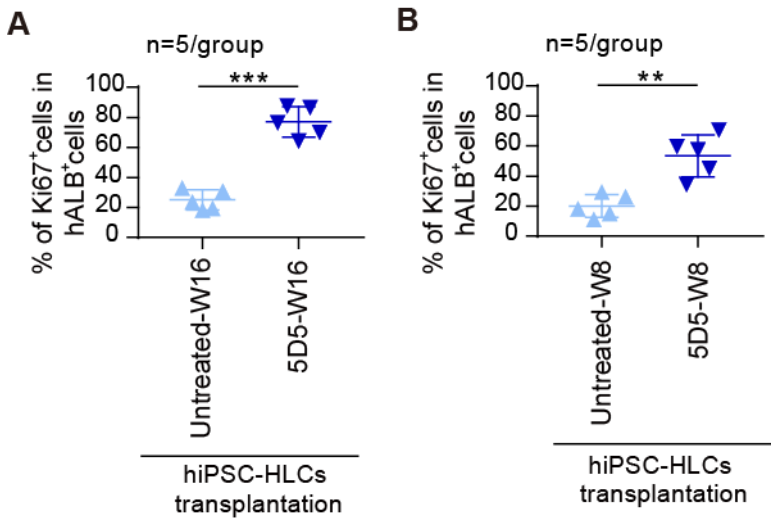


Fig. S10. The ratio of Ki67$^+$ cells in hALB$^+$ liver cells of FRGS mice with life-threatening ESLD rescued by hiPSC-HLC transplantation combined with agonist c-Met mAb 5D5 treatment. To know the cell proliferation ability *in vivo*, the liver cells of the hiPSC-HLCs transplanted mice with **(A)** NTBC-off induced liver failure (Figure 6) were perfused by collagenase and **(B)** CCl_4 induced liver fibrosis (Figure 7) were perfused by collagenase at 16 and 8 weeks after cell transplantation, respectively. These samples were detected by FACS method to know the ratio of Ki67$^+$ cells in hALB$^+$ liver cells (n=5/group). The results suggested that 5D5 treatment significantly increase the ratio of Ki67$^+$ cells in hALB$^+$ liver cells of the hiPSC-HLCs transplanted mice with ESLD (***P <0.001).