Supplemental Figures

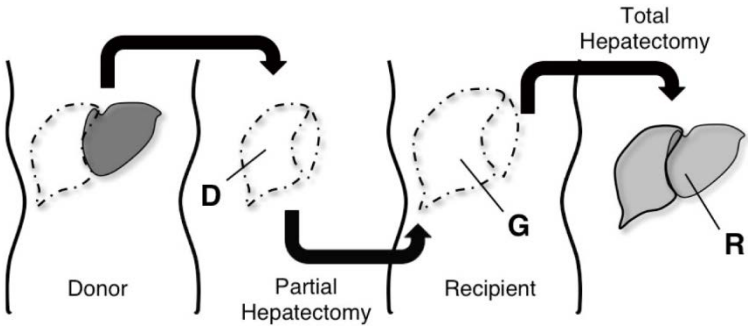

Figure S1: Schematic representation of living donor liver transplantation (LDLT) LDLT is a surgical procedure in which a living donor undergoes physical partial hepatectomy. The transected liver is then transplanted into a recipient body.

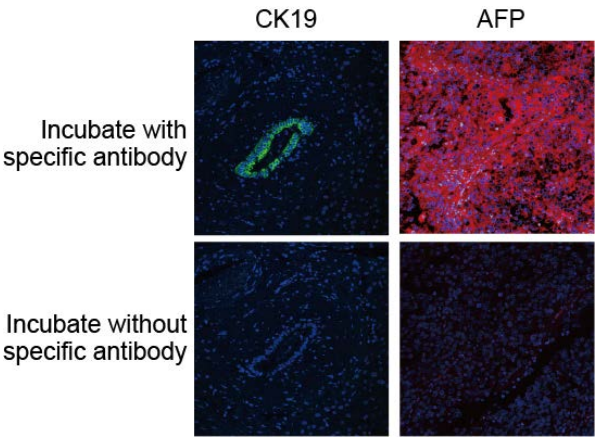

Figure S2: Positive and negative controls for CK19 and AFP immunostaining An anti-CK19 antibody was used to stain an intact human liver section for the detection of cholangiocytes. An anti-AFP antibody was used to stain a human hepatocellular carcinoma (all tumor cells are positive in the cytoplasm).

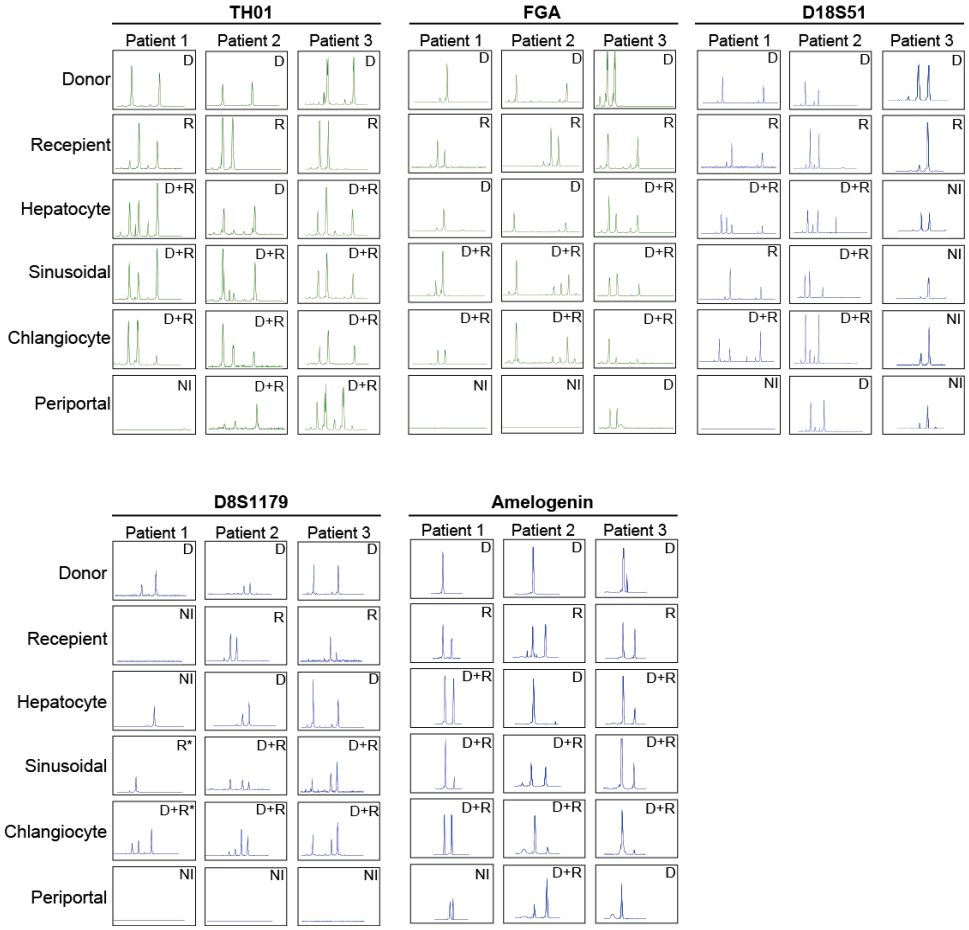

Figure S3: STR analysis for all 5 loci D, Donor; R, Recipient; NI, Not Informative; *, non-Donor allele.

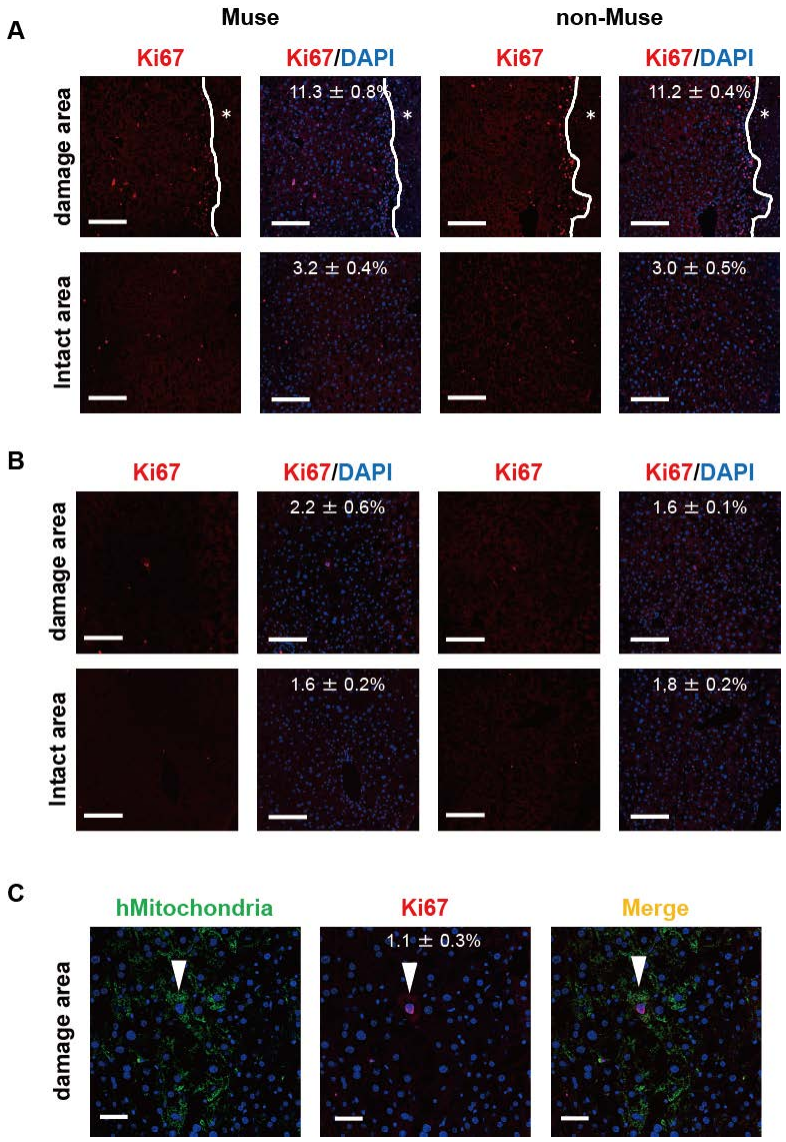

Figure S4: Hepatocyte proliferation in the damaged area of the liver Ki67 immunostaining and DAPI staining on the marginal zone of the liver at day 2 (A) and 4 weeks (B) after cellular transplantation. The average frequency and standard error of $Ki67^+$ cells is indicated in the respective conditions. $Ki67^+$ cells among human mitochondria-positive cells at 4 weeks after cellular transplantation (C). The frequency was calculated from multiple views containing at least 600 cells/view. Asterisks in (A), damaged area; Bar, 50μm.

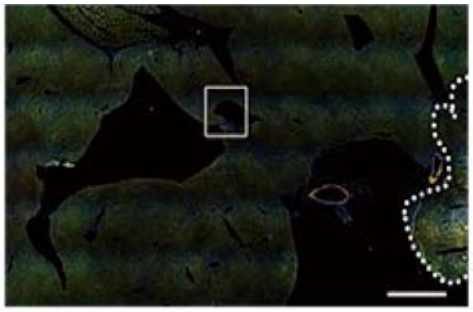

Figure S5: Low-power view of a mouse liver sample used for immunohistochemistry of human liver progenitor markers after Muse cell transplantation The area of the inset is approximately 4 mm away from the transection line (dotted line).

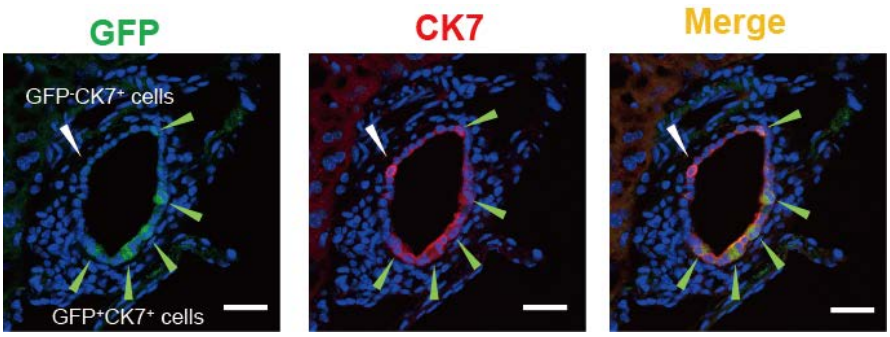

Figure S6: Immunostaining of a Muse cell transplanted liver (4 weeks)
An anti-CK7 antibody reacts with both mouse and human CK7. GFP+/CK7+ cells suggesting Muse-derived cells integrated into the mouse bile duct, which also included GFP-/CK7+ mouse cholangiocytes.

Supplemental Table

Table S1: Primer sequences used for human samples

Gene	Position	Sequences
GFP	sense	5’-AGTCCGCCCTGAGCAAAGA-3’
	antisense	5’-TCCAGCAGGACCATGTGATC-3’
β-actin	sense	5’-ACTGCTCTGGCTCCTAGCAC-3’
	antisense	5’-ACATCTGCTGGAAGGTGGAC-3
human-specific PTGER2	sense	5’-GCTGCTTCTCATTGTCTCGG-3
	antisense	5’-GCCAGGAGAATGAGGTGGTC-3’
mouse-specific PTGER2	sense	5’-CCTGCTGCTTATCGTGGCTG-3’
	antisense	5’-GCCAGGAGAATGAGGTGGTC-3’
CD45	sense	5’-GGAGCCCGCTGAGACTTGAATC-3’
	antisense	5’-GGAGCGAGAGTGGCAGAGGAC-3’
Vimentin	sense	5’-AAAACCTTGAACCCGAACATGA-3’
	antisense	5’-CTCCTGGACTCCCAAAATCTGT-3’
GAPDH	sense	5’-AATCCCATCACCATCTTCCA-3’
	antisense	5’-TGGACTCCACGACGTACTCA-3’

Reference: (1).

Table S2: List of antibodies used for immunohistochemistry

Antibody against	Provider	Cat No.	Host	Dilution	Application
AFP	DAKO	A0008	Rabbit	1:1500	LDLT
AFP	Nichirei	422221	Mouse	Predilute	Xenograft
Albumin	Bethyl	A80-229A	Goat	1:100	Xenograft
α1-antitrypsin	Thermo	RB367-A	Mouse	1:200	Xenograft
CD31	DAKO	M0823	Mouse	1:50	LDLT
CD68	abcam	ab955	Mouse	1:100	Xenograft
CD68	DAKO	M0814	Mouse	1:100	LDLT
CK7	Thermo	MS-1352	Mouse	1:200	Xenograft
CK7	Millipore	MAB3226	Mouse	1:100	Mouse
CK7	DAKO	M7018	Mouse	1:100	LDLT
CK19	Novocastra	NCL-CK19	Mouse	1:100	LDLT
CK19	Thermo	MS-198	Mouse	1:100	Xenograft
DLK	Santa Cruz	sc-376755	Mouse	1:100	Xenograft
GFP	abcam	ab6556	Rabbit	1:500	Xenograft
HepPar1	DAKO	M7158	Mouse	1:200	LDLT
Ki67	Thermo	RM-9106	Rabbit	1:200	Xenograft
Lyve-1	Oriental Yeast	LYVE1-MCA	Rat	1:300	Xenograft
Mitochondria	abcam	ab3298	Mouse	1:100	Xenograft
Neurofilament-M	Millipore	AB1987	Rabbit	1:200	Muse
OV-6	Santa Cruz	sc-101863	Mouse	1:100	Xenograft
SMA	Thermo	MS-113	Mouse	1:500	Muse

Table S3: Clinical characteristics of LDLT cases

Case #	Indication of LDLT	Recipient		Donor		Rejection (Days)	IS withdrawal (Days)	Graft biopsies (Days)
		Age	Sex	Age	Sex			
1	HCV-LC-HCC	57	M	27	M	No	No	0, 64, 340
2	BA	2	F	29	F	No	No	0, 23, 38, 371, 826
3	HCV-LC-HCC	56	F	30	M	Yes (305)	No	0, 20, 76, 305, 780
4	NBNC-LC-HCC	56	F	57	M	Yes (38)	No	0,24, 38, 63, 74, 87, 101
5	HCV-LC-HCC	49	M	55	F	No	No	0, 70, 501
6	PBC	57	F	32	F	No	No	0, 8, 36, 71, 191
7	AIH-LC-HCC	57	F	32	F	No	No	0, 170
8	PSC	37	F	37	M	No	No	0, 26, 173, 595, 738
9	HCV-LC-HCC	59	M	27	F	No	No	0, 80, 415, 482, 494
10	HCV-LC-HCC	53	F	25	M	No	No	0, 25, 183
11	PBC	49	M	45	F	No	No	0, 166, 290
12	HCV-LC-HCC	56	M	30	M	Yes (134)	No	0, 10, 111, 134, 200, 274, 552
13	PBC	51	F	24	M	Yes (10,166)	No	0, 10, 166
14	HCV-LC	46	M	35	F	No	No	0, 24, 162
15	HCV-LC	51	M	48	F	No	No	0, 44
16	HCV-LC-HCC	57	F	33	F	No	No	0, 28
17	HCV-LC	60	F	32	F	No	No	0, 58, 226
18	HCV-LC	47	F	22	F	No	No	0, 45, 103, 178
19	NBNC-LC	55	M	20	M	No	No	0, 35
20	HBV-LC	66	M	38	F	No	No	0, 26, 87

HCV, Hepatitis C virus; LC, Liver cirrhosis; HCC, Hepatocellular carcinoma; BA, BA, Biliary atresia; NBNC, Non-B non-C hepatitis; PBC, Primary biliary cirrhosis; AIH, Autoimmune hepatitis; PSC, Primary sclerosing cholangitis; HBV, Hepatitis B virus; and IS, Immunosuppresive drugs.

Table S4: The numbers of cells with recipient origin Y chromosomes in each cell type

	Hepatocyte		Sinusoid		Cholangiocyte		Periportal	
	Y count	Y%	Y count	Y%	Y count	Y%	Y count	Y%
Patient 4	0/194	0.0	18/56	32.1	3/44	6.8	38/129	29.5
Patient 18	0/148	0.0	14/46	30.4	16/63	25.4	19/44	43.2
Patient 19	1/85	1.2	20/42	47.6	NA	0.0	NA	0.0
Average		0.4		36.7		16.1		36.4

NA, Not Available.

Table S5: Average frequency of recipient origin in each cell type

	STR (% recipient allele)		FISH (% Y chromosome)
	D8S1179	FGA	
Hepatocyte	NI	NI	0.4
Sinusoidal	36.5	27.4	36.7
Cholangiocyte	18.9	16.2	16.1
Periportal	NI	NI	36.4

NI, Not Informative. STR data was obtained from Patient 2. FISH data was the average of three patients (Patient 4, Patient 18, and Patient 19).

Supplemental Methods

Laser microdissection

Laser microdissection (LMD) was performed for all 20 cases to isolate the indicated cell types using an LMD7000 System (Leica Microsystems, Watzlar, Germany). Approximately 300 cells were collected for each hepatocyte and sinusoidal cell populations, and 30 to 50 cells were collected from the cholangiocyte and periportal area populations. DNA was extracted from cells using the QIAamp® DNA Micro Kit according to the manufacturer's protocol (Qiagen, Limberg, The Netherlands).

Genotyping by STR markers

Formalin-fixed paraffin-embedded (FFPE) tissues were prepared as 5 μm-thick sections for DNA extraction using the WaxFree™ DNA Paraffin Sample DNA Extraction Kit (TrimGen, Sparks, MD). Four loci of STR (TH01, FGA, D18S51, and D8S1179) and one sex-related locus (amelogenin) were used for genotyping with the PowerPlex® S5 System (Promega, Madison, WI). The templates amplified from each DNA fragment were analyzed with an Applied Biosystems 3130 Genetic Analyzer (Life Technologies, Grand Island, NY). For PCR amplification of DNA from the whole tissue slice, the final volume of the reaction was 25 μL, which contained 5 μL of PowerPlex® S5 5× Master Mix, 2.5 μL of PowerPlex® S5 10× Primer Pair Mix, 0.5 μL of template DNA, and 17 μL of distilled water. The PCR cycling parameters were 96°C for 2 min followed by 30 cycles of 94°C for 30 s, 60°C for 2 min, and 72°C for 90 s. A final extension was performed at 60°C for 45 min. For laser microdissection (LMD) samples with the LMD7000 (Leica Microsystems, Watzlar, Germany), the PCR amplification mix consisted of 5 μL of PowerPlex® S5 5× Master Mix, 2.5 μL of the PowerPlex® S5 10× Primer Pair Mix, 2 μL of template DNA, and 15.5 μL of distilled water. The PCR cycling parameters for LMD samples were 96°C for 2 min followed by 33 cycles of 94°C for 30 s, 60°C for 2 min, and 72°C for 90 s. A final extension was performed at 60°C for 45 min. The resulting histograms from each DNA fragment was quantified using Peak Scanner™ Software v1.0 (Applied BioSystems, Waltham, MA).

RT-PCR for microdissected human samples

Cells of each liver component (hepatocytes, cholangiocytes, sinusoidal cells, and periportal cells) on a FFPE tissue section were obtained using LMD and deparaffinated according to the protocol of RNeasy® FFPE kit (Qiagen, Limberg, The Netherlands). RNA was subsequently extracted from each liver component. Five μl of the extracted RNA was subjected to the first strand cDNA synthesis using random hexamers (Invitrogen, Carlsbad, CA) and ReverTra Ace (Toyobo, Osaka, Japan). After cDNA

preparation, quantitative PCR was performed with a LightCycler nano (Roche, Basel, Switzerland) using the FastStart Essential DNA Green Master mix (Roche). The PCR product was electrophoresed and the resulting band intensity was quantified with Image J (http://imagej.nih.gov/ij/). Primer sequences are shown in Table S1.

PPHx

Mice were anesthetized by inhalation of isoflurane (2.5% v/v). After a 1 cm midline abdominal skin and muscle incision was made starting at the xiphoid process, the left lobe was gently pulled out. In contrast to a conventional 2/3 PH that simply ligates to remove the median and left lateral lobes (2), our PPH model employs a harmonic scalpel (Harmonic Focus®, Ethicon Endo-Surgery, Somerville, NJ); (3), which is an ultrasonically-activated surgical device for tissue dissection without lobular ligation, removing approximately 20–30% of the entire liver (Figure 3A). The average length of the liver transection line of the left lobe was approximately 10 mm to create a residual liver volume of approximately 70-80%.

RT-PCR for Muse cells

Total RNA from Muse cells, non-Muse cells, and M-clusters was purified using the NucleoSpin RNA XS (MACHEREY-NAGEL, Duren, Germany), and the first strand cDNA was synthesized using Oligo(dT)20 primer (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen). After cDNA preparation, quantitative PCR was performed with a 7500 Fast real time PCR system (Applied Biosystems, Foster, CA) using the following TaqMan gene expression assays (Applied Biosystems): Oct3/4 (Hs00999632_g1), Sox2 (Hs01053049_s1), Nanog (Hs04260366_g1), and beta-actin (Hs01060665_g1). Data were processed using the ΔΔCt method (4). RT-PCR for the OCT4A isoform was performed according to the methods previously described by Atlasi et al (5).

Species-specific DNA analysis

The species-specific DNA analysis was performed according to the method introduced by Alcoser et al. (1). Four weeks after Muse or non-Muse cell transplantation, the livers were harvested and DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen). The PCR reaction mix consisted of 25 μL of AmpliTaqGold® PCR Master Mix (Life Technologies), 5 μL of template DNA, 5 μL of primers, and 15 μL of distilled water. The PCR cycling parameters were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A final extension was performed at 72°C for 7 min. The primer sequences are presented in Table S1.

Immunocytochemistry and immunohistochemistry

Human LDLT biopsy samples were fixed with 10% formalin for examination. A pair of primary antibodies was used for colorimetric staining: mouse anti-CK7 monoclonal antibody (DAKO Japan, Tokyo, Japan), and mouse anti-CD68 monoclonal antibody (DAKO). The primary antibodies were detected with EnVision™ (DAKO) with the final precipitation by either 3,3'-diaminobenzidine or Vector VIP substrate (Vector Laboratories, Burlingame, CA). Another pair of primary antibodies was used for immunofluorescent staining: mouse anti-human CK19 IgG monoclonal antibody (Novocastra Labolatories, Leica Microsystems K.K., Tokyo, Japan) and rabbit anti-human AFP polyclonal antibody (DAKO). All primary antibodies were detected with one of the following secondary antibodies: goat anti-mouse IgG-Alexa-488 antibody (Life Technologies) or goat anti-rabbit IgG-Alexa-594 antibody (Life Technologies). Positive and negative controls for CK19 and AFP antibodies are shown in Supporting Figure S2.

For alkaline phosphatase staining of a single Muse cell-derived cluster, a Leukocyte Alkaline Phosphatase kit was used according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). For cells expanded from a single Muse cell-derived cluster on gelatin, the following primary antibodies were used for staining: mouse anti-CK7 monoclonal antibody (Millipore, Billerica, MA), mouse anti-smooth muscle actin monoclonal antibody (Thermo Fisher, Waltham, MA), and rabbit anti-neurofilament-M polyclonal antibody (Millipore).

For SCID mouse liver analysis, animals were fixed with Periodate-Lysine Paraformaldehyde (PLP) solution (0.01 M NaIO4/0.075 M Lysine/2% PFA at pH 6.2) under deep anesthesia by cardiac perfusion at 2 days, and the liver was removed at 4 weeks after transplantation. The liver was then fixed with PLP again for an additional five hours. The following primary antibodies were used for staining: rabbit anti-GFP IgG polyclonal antibody (Abcam, Cambridge, UK); Ki67 antibody; mouse anti-human hepatocyte (HepPar1) IgG monoclonal antibody (DAKO); rabbit anti-Ki67 polyclonal antibody (Thermo Fisher); mouse anti-CK7 IgG monoclonal antibody (Thermo Fisher); mouse anti-human CK19 IgG monoclonal antibody (Thermo Fisher); rat anti-human Lyve-1 IgG monoclonal antibody (Oriental Yeast); mouse anti-CD68 IgG monoclonal antibody (Abcam); goat anti-human albumin IgG polyclonal antibody (Bethyl Laboratories); rabbit anti-α-1-antitrypsin IgG polyclonal antibody (Thermo Fisher); mouse anti-human oval cell marker (OV-6) IgG monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX); mouse anti-human delta-like protein (DLK) IgG monoclonal antibody (Santa Cruz Biotechnology); mouse anti-human AFP IgG monoclonal antibody (Nichirei, Tokyo, Japan); and mouse anti-human mitochondria antibody (Abcam). The primary antibodies were detected by one of the following procedures: horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research, West Grove, PA) followed by 3,3'-diaminobenzidine; donkey anti-rabbit IgG-biotin polyclonal antibody (Jackson ImmunoResearch) followed by streptavidin-Alexa-488 (Life Technologies); donkey anti-rabbit IgG-Alexa-488; goat

anti-rat IgG-Alexa-568; and donkey anti-mouse IgG-Alexa-568 antibody (Life Technologies). All slides were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). The images were obtained by laser confocal microscopy (Nikon Corporation, Tokyo, Japan). All antibodies used in this study are listed in Supporting Table S2.

FISH

The presence of X and Y chromosomes in constituent cells in the human liver biopsy specimens was evaluated using a CEP X Spectrum Orange/Y Spectrum Green DNA Probe Kit (Abbott Laboratories, North Chicago, IL). Five μm sections were immersed in 2 M HCl for 20 min, in deionized water for 3 min, and in 2× SSC for 5 min. The slides were then placed in sodium thiocyanate for 30 min at 80°C and rinsed in deionized water for 1 min and twice in 2× SSC for 5 min. After incubation in protease solution at 37°C for 1 h, the enzymatic reaction was stopped by placing slides in two changes of 2× SSC for 5 min each. The slides were air dried, treated with formamide solution at 72°C for 5 min and dehydrated with alcohol. A total of 10 μL of a DNA probe mixture containing the CEP X DNA probe (directly labeled with Spectrum Orange, which is specific for the DXZ1 alpha Satellite DNA locus on Xp11.1-q11.1) and the CEP Y DNA probe (directly labeled with Spectrum Green, which is specific for the DYZ3 satellite III DNA locus on Yq12) was applied to the sections. The fluorescence images of the FISH results were acquired with the Metafer-Metacyte microscope scanning system (Karl-Zeiss and MetaSystems, Altlussheim, Germany). These images were compared with the light microscopic features of the corresponding H&E- and immunohistochemically-stained serial sections.

For the Muse cell transplantation model, 10 μm thick frozen tissue sections were fixed with 4% paraformaldehyde/PBS for 30 min at 4°C. They were rinsed in PBS, and treated with 0.1% pepsin (Wako, Osaka, Japan)/0.1M HCl for 5 min at 37°C. The slides were dehydrated with 70%, 85%, and 100% ethanol for 2 min at room temperature and air dried. The mixture of mouse genomic DNA specific probe (SPM-20, Chromosome Science Labo Inc., Sapporo, Japan) labeled with Green-dUTP (Abbott Molecular Probes, Abbott Park, IL) and human genomic DNA specific probe (SPH-20, Chromosome Science Labo Inc.) labeled with Red-dUTP (Abbott Molecular Probes) were added onto the slides. Sections were covered by coverslips, denatured for 10 min at 80°C, and hybridized in a humid chamber overnight at 37°C. Sections were then washed with 2× SSC for 5 min at 37°C and removed coverslips carefully. They were washed with preheated 50% formamide/2× SSC for 20 min at 37°C, with 1× SSC for 15 min at room temperature, and then counterstained with DAPI. The samples were analyzed by laser scanning confocal microscope (C2si, Nikon, Tokyo, Japan). The images were compared with the corresponding fluorescence images of the serial sections stained with GFP and HepPar-1.

Histological examination

Archived formalin (10%)-fixed-paraffin-embedded human liver biopsy tissue blocks

were obtained from 20 LDLT cases of both donor and recipient performed at Iwate Medical University Hospital. Five μm sections of all samples were mounted on glass slides for hematoxylin and eosin (H&E) staining. For SCID mice, paraformaldehyde (4%)-fixed-paraffin-embedded SCID mice liver tissue blocks were cut into 3 μm sections for H&E staining.

Statistical analysis

Either JMP 10.0 (SAS Institute) or Prism 6 (GraphPad Software) was used for statistical analysis. Quantitative values of each experimental group were analyzed using either student's t-test or Mann-Whitney U test, depending on the subject groups. Unless otherwise noted, all mean values are described with ± one standard deviation (SD).

Supplemental References

1. Alcoser SY, Kimmel DJ, Borgel SD, Carter JP, Dougherty KM, Hollingshead MG. Real-time PCR-based assay to quantify the relative amount of human and mouse tissue present in tumor xenografts. BMC Biotechnol. 2011; 11: 124.
2. Mitchell C, Willenbring H. A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. Nature protocols. 2008; 3: 1167-70.
3. Arru M, Pulitano C, Aldrighetti L, Catena M, Finazzi R, Ferla G. A prospective evaluation of ultrasonic dissector plus harmonic scalpel in liver resection. Am Surg. 2007; 73: 256-60.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284: 143-7.
5. Atlasi Y, Mowla SJ, Ziaee SA, Gokhale PJ, Andrews PW. OCT4 spliced variants are differentially expressed in human pluripotent and nonpluripotent cells. Stem cells. 2008; 26: 3068-74.