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Supplemental Information

Generation of Human Liver Chimeric Mice with Hepatocytes from Familial Hypercholesterolemia Induced Pluripotent Stem Cells

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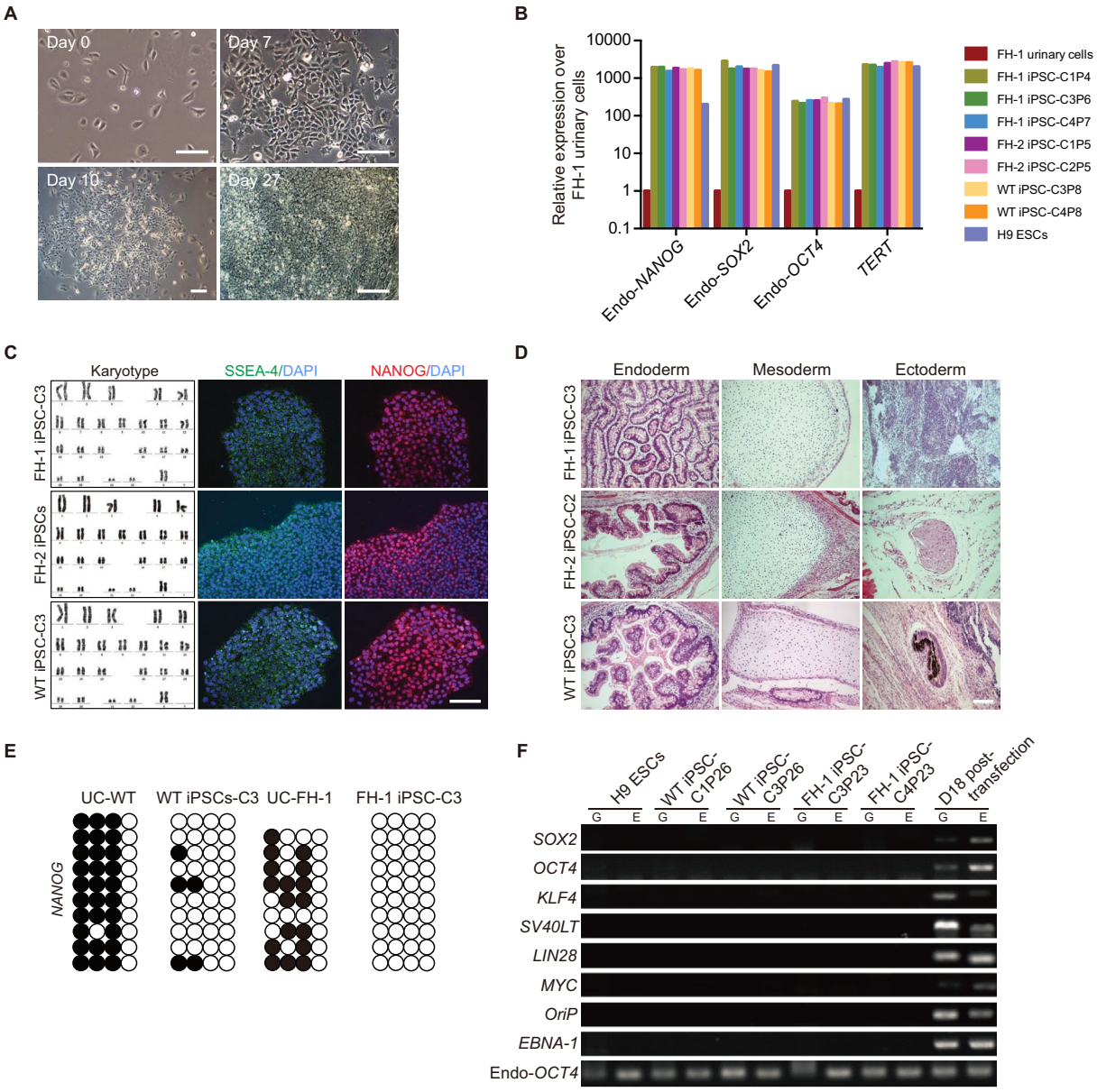
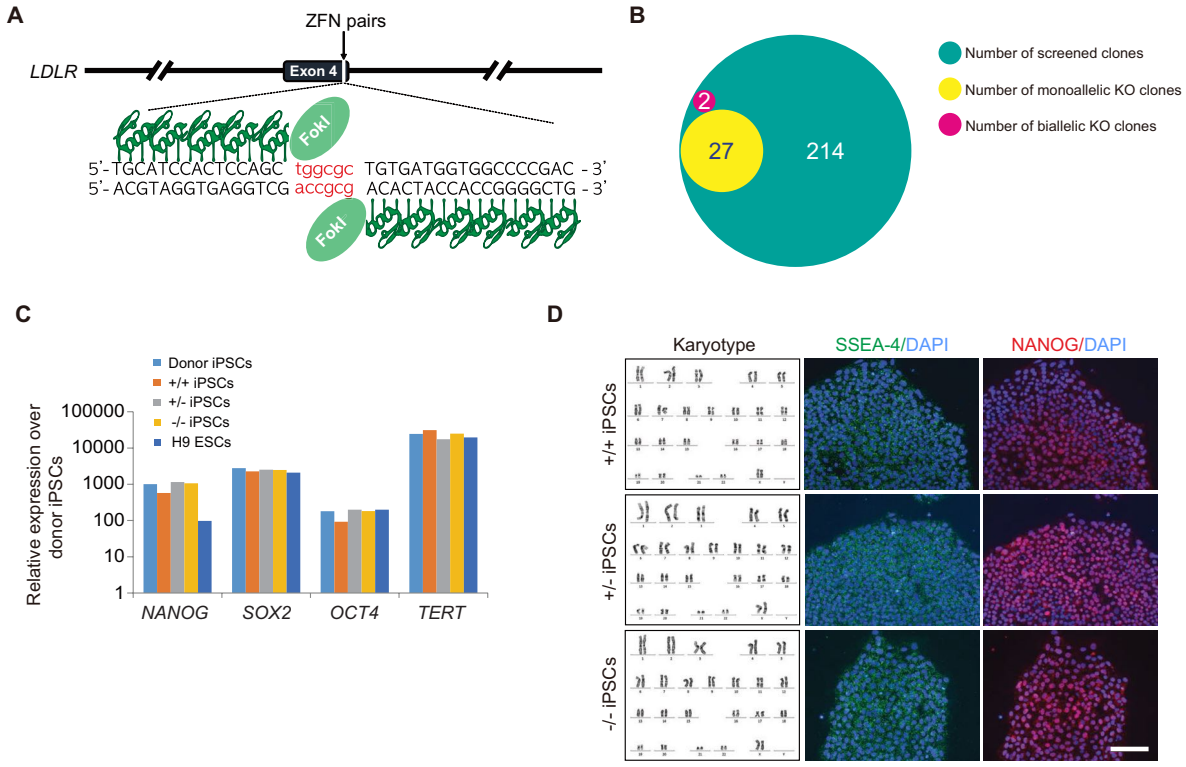
Supplemental Figure S1



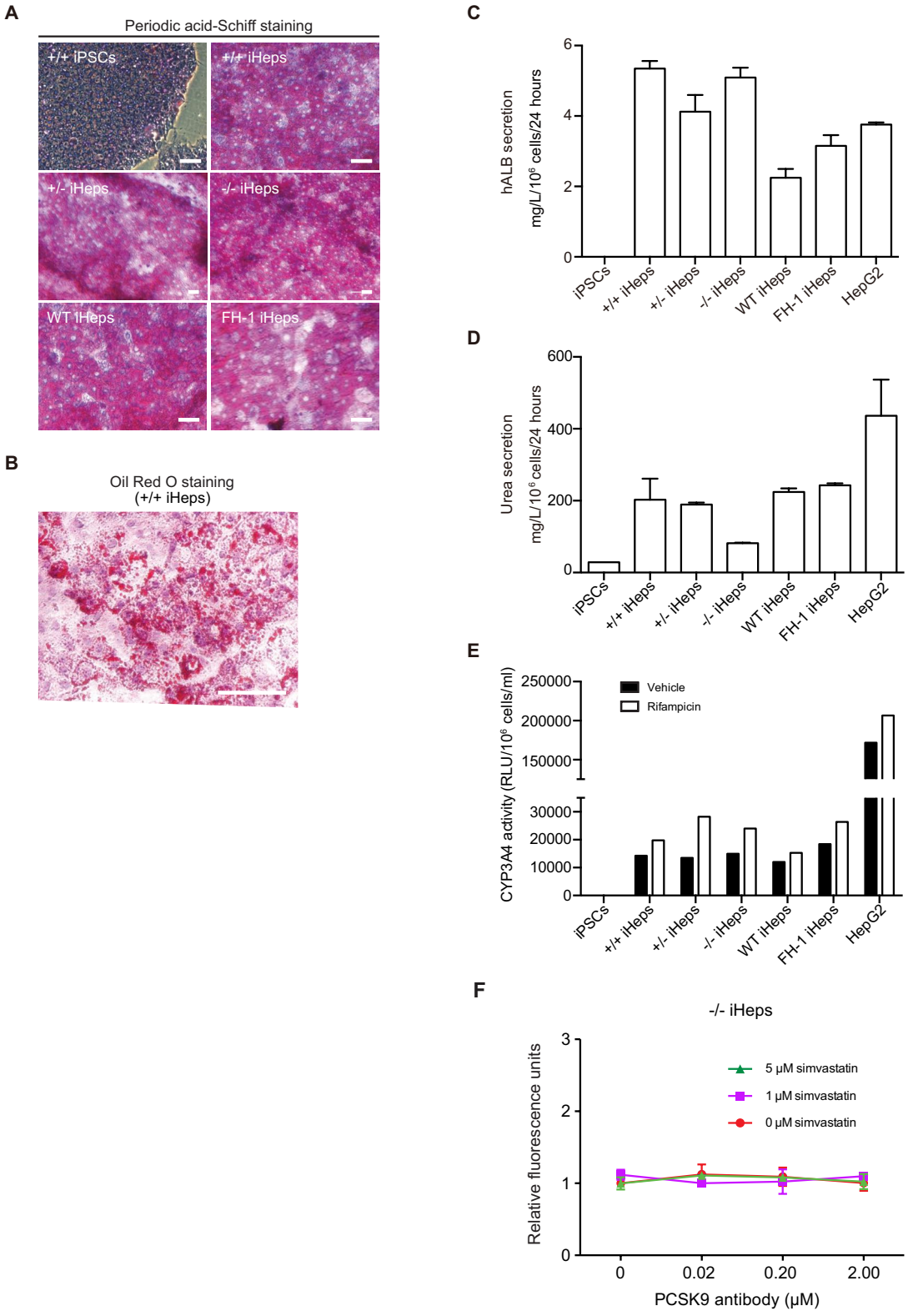
Supplemental Figure S1. Generation of Footprint-Free Patient-Specific iPSCs with Episomal Vectors. Related to Figure 1. A, Representative phase contrast images of different time points of episomal vector-mediated reprogramming, urinary cells were transfected with vectors at day 0. Scale bars represent 200 μm. **B**, RT-qPCR for endogenous (Endo-) *NANOG, OCT4, SOX2*, and *TERT* in the indicated iPSCs. H9 ESCs were used as control, values are referred to non-transfected FH-1 urinary cells. **C**, Phase contrast images of karyotype analysis (1^{st} column) and representative immunofluorescence microscopy photographs for SSEA-4 and NANOG (2^{nd} and 3^{rd} column) of the indicated iPSCs. Scale bars represent 100 μm. **D,** Representative images of hematoxylin/eosin stained sections of teratomas generated by injecting the indicated iPSCs into SCID mice. Scale bars represent 100 μm. **E,** DNA methylation profile of the *NANOG* proximal promoter for the indicated cell types. UC: urinary cells. **F**, Residual and random integration test of episomal DNA in the indicated iPSCs using PCR. G: genomic DNA. E: episomal DNA. Genomic DNA from H9 ESCs and urinary cells transfected with episomal vectors (day 18 post-transfection) were used as negative and positive controls, respectively.

Supplemental Figure S2



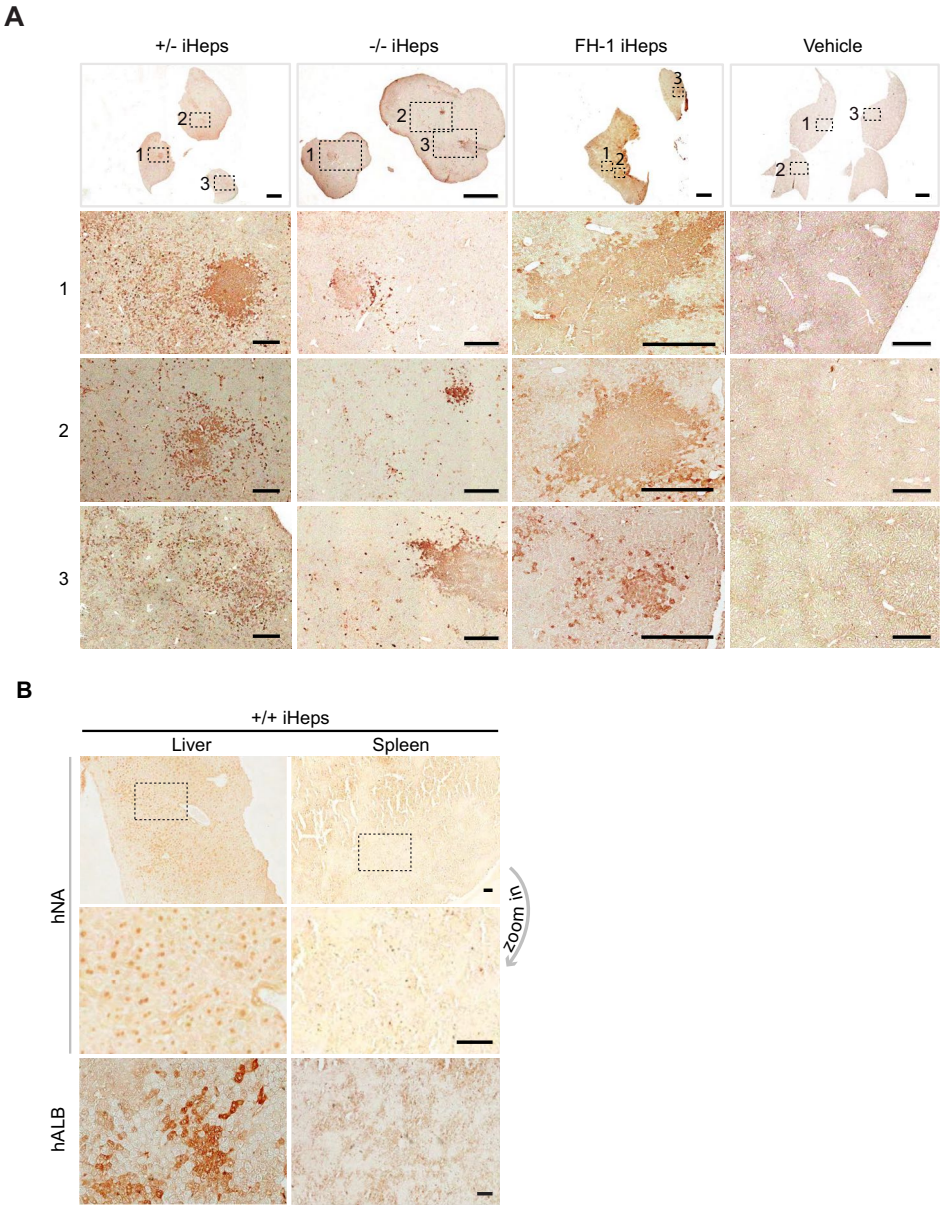
Supplemental Figure S2. Generation of Monoallelic and Biallelic *LDLR* Knockout iPSCs with ZFNs. Related to Figure 1. A, Schematic of the *LDLR* coding region depicting the genomic locus targeted by *LDLR*-ZFNs. **B,** Summary of genome editing experiments with ZFNs and numbers of analyzed subclones. Among a total number of 214 clones screened, 2 were biallelic knockout and 27 were monoallelic knockout. **C**, RT-qPCR for endogenous *NANOG, OCT4, SOX2*, and *TERT* in the indicated iPSCs. H9 ESCs were used as control, values are referred to non-transfected urinary cells. **D**, Phase contrast images of karyotype analysis (1st column), and representative immunofluorescence microscopy photographs for SSEA-4 and NANOG (2nd and 3rd column) of the indicated iPSCs. Scale bars represent 100 μm.

Supplemental Figure S3



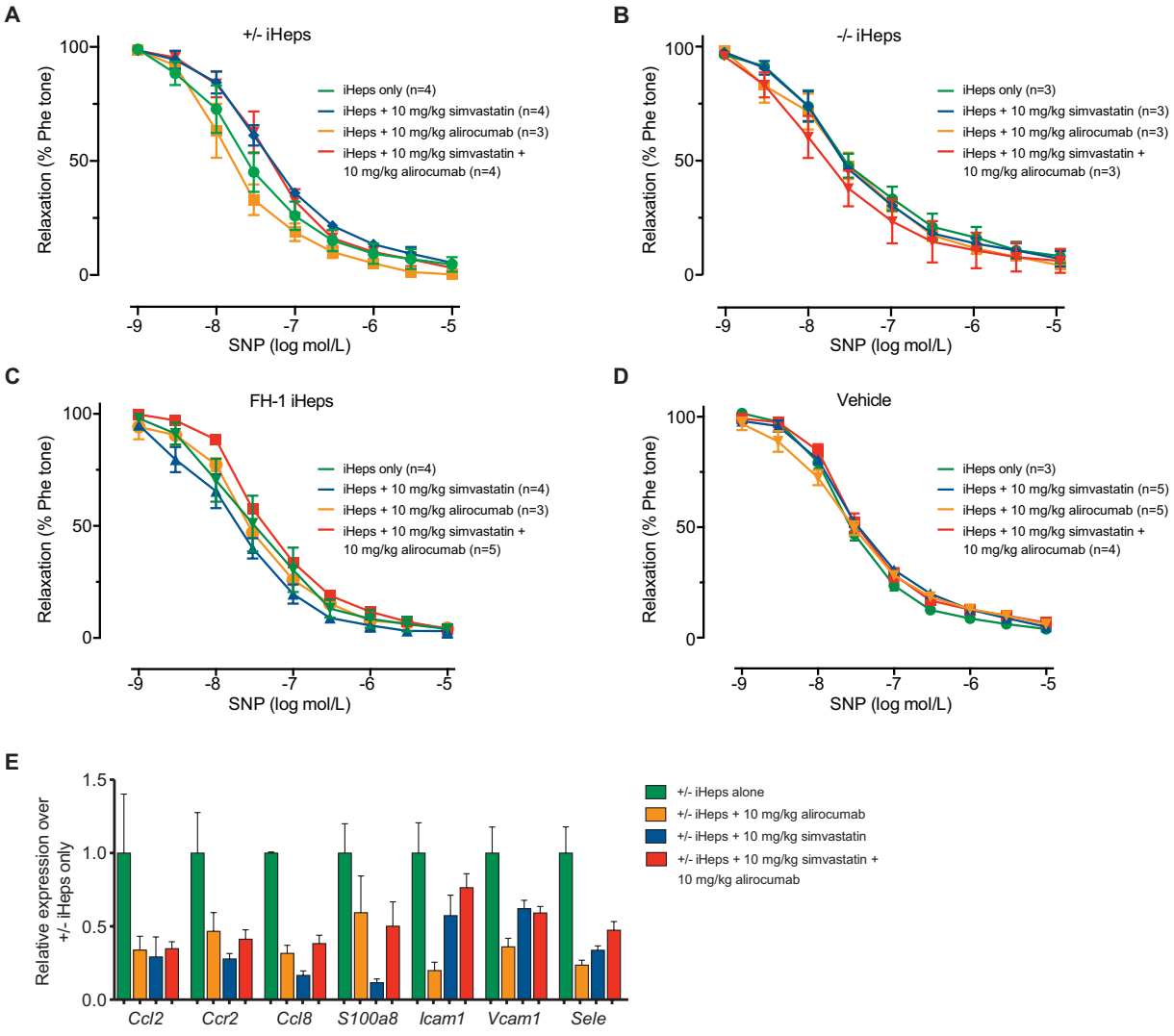
Supplemental Figure S3. Characterization of iHeps. Related to Figure 2. A, Representative images of PAS staining for the indicated iPSC-derived iHeps at day 17 of differentiation. Scale bars represent 50 μm. **B**, Representative images of oil red O staining in +/+ iHeps. Scale bar represents 100 μm. **C**, Bar graph shows hALB secretion level (mg/L/10^6 cells/24 hours) in the indicated iHeps. HepG2 cells and iPSCs were used as positive and negative controls, respectively (also in D and E). A representative experiment with samples measured in triplicate is shown; error bars indicate SD. **D**, Bar graph shows urea secretion (mg/L/10^6 cells/24 hours) in the indicated iHeps. A representative experiment with samples measured in duplicate is shown; error bars indicate SD. **E**, Detection of cytochrome P450 (CYP3A4) metabolic activity (RLU/10^6 cells/ml) of iHeps at day 20 of differentiation after 72 hours of rifampicin induction compared to vehicle. **F,** LDL uptake capacity of -/- iHeps measured with a microplate reader and indicated as relative fluorescence units. Cells were treated with simvastatin and/or PCSK9 antibodies for 16 hours on day 17-21 of differentiation. A representative experiment with samples measured in at least duplicate is shown, error bars indicate SEM.

Supplemental Figure S4



Supplemental Figure S4. iHeps Repopulate *LRG* Mice Liver. Related to Figure 3. A, Top panel, whole-slide scan images of hALB (brown) staining in chimeric mice liver repopulated with the indicated iHeps or vehicle. Scale bars represent 2 mm. Lower panels, zoomed hALB+ areas of the scanned sections are shown as indexed. Scale bars represent 400 μm. **B,** Immunohistochemical staining for hNA (brown) and hALB (brown) in the liver and spleen of *LRG* mice engrafted with +/+ iHeps. Zoomed images for hNA are also shown. Scale bars represent 50 μm.

Supplemental Figure S5



Supplemental Figure S5. Measurement of Endothelium-Independent Vasodilation and Expression of Proinflammatory Markers upon Alirocumab and Simvastatin Treatment. Related to Figure 5. A-D, Endothelium-independent vasodilation in response to SNP in aortae (pre-contracted with Phe) from *LRG* mice fed with HFHC diet. Mice were engrafted with the indicated iHeps or vehicle, and treated with simvastatin (daily) and/or alirocumab (weekly); n indicates number of mice. **E,** RT-qPCR of aortae from *LRG* mice engrafted with +/- iHeps shows the level of gene expression for the indicated cytokines, chemokine receptors, and adhesion molecules related to vascular inflammation.

Supplemental Table S1

List of Primers Used in this Study

RT-qPCR primers for human endogenous pluripotent genes		
Gene	Sequence (5'→3')	
ACTB	Forward	CCCAGAGCAAGAGAGG
	Reverse	GTCCAGACGCAGGATG
NANOG	Forward	TGAACCTCAGCTACAAACAG
	Reverse	TGGTGGTAGGAAGAGTAAAG
OCT4	Forward	CCTCACTTCACTGCACTGTA
	Reverse	CAGGTTTTCTTTCCCTAGCT
SOX2	Forward	CCCAGCAGACTTCACATGT
	Reverse	CCTCCCATTTCCCTCGTTTT
TERT	Forward	CTGGAGCAAGTTGCAAAGC
	Reverse	GTCCATGTTCACAATCGGC
PCR primers for plasmid residual and random integration test		
Gene	Sequence (5'→3')	
MYC	Forward	TCAAGAGGCGAACACACAAC
	Reverse	AGGAACTGCTTCCTTCACGA
EBNA-1	Forward	ATCGTCAAAGCTGCACACAG
	Reverse	CCCAGGAGTCCCAGTAGTCA
KLF4	Forward	CCCACACAGGTGAGAAACCT
	Reverse	CCCCCTGAACCTGAAACATA
LIN28	Forward	AAGCGCAGATCAAAAGGAGA
	Reverse	CCCCCTGAACCTGAAACATA
OCT4	Forward	AGTGAGAGGCAACCTGGAGA
	Reverse	AGGAACTGCTTCCTTCACGA
Endo-*OCT4*	Forward	AGTTTGTGCCAGGGTTTTTG
	Reverse	ACTTCACCTTCCCTCCAACC
OriP	Forward	TTCCACGAGGGTAGTGAACC
	Reverse	TCGGGGGTGTTAGAGACAAC
SOX2	Forward	ACCAGCTCGCAGACCTACAT
	Reverse	CCCCCTGAACCTGAAACATA
SV40LT	Forward	TGGGGAGAAGAACATGGAAG
	Reverse	AGGAACTGCTTCCTTCACGA
RT-qPCR primers for mouse proinflammatory genes		
Gene	Sequence (5'→3')	
Ccl8	Forward	TTCTTTGCCTGCTGCTCATA
	Reverse	AGCAGGTGACTGGAGCCTTA
Ccr2	Forward	ACCAGAAGAGGGCATTGGAT
	Reverse	GCCGTGGATGAACTGAGGTA
Ccl2	Forward	CATCCACGTGTTGGCTCA
	Reverse	GATCATCTTGCTGGTGAATGAGT
S100a8	Forward	TCCTTTGTCAGCTCCGTCTT
	Reverse	AGAGGGCATGGTGATTTCCT
Icam1	Forward	GTGATGCTCAGGTATCCATCCA
	Reverse	CACAGTTCTCAAAGCACAGCG
Vcam1	Forward	CCGGCATATACGAGTGTGAA
	Reverse	GGAGTTCGGGCGAAAAATAG
Sele	Forward	CAGCTTCGTGTACCAATGCA
	Reverse	GGCTTCCATAGTCAGGGTGT
Gapdh	Forward	ATGGTGAAGGTCGGTGTGAA
	Reverse	GAGGTCAATGAAGGGGTCGT
PCR primers for isogenic *LDLR* knockout clone screening		

Gene	Sequence (5’→3’)		
LDLR	Forward		AGCTTCCAGTGCAACAGCTC
	Reverse		AAATCACTGCATGTCCCACA
	Sequencing primer		AGCTTCCAGTGCAACAGCTC
PCR primers for bisulfite sequencing			
Gene	Sequence (5’→3’)		
NANOG promoter	Inner	Forward	TTAATTTATTGGGATTATAGGGGTG
		Reverse	AACAACAAAACCTAAAAACAAACC
	Outer	Forward	TGGTTAGGTTGGTTTTAAATTTTTG
		Reverse	AACCCACCCTTATAAATTCTCAATTA

Supplemental Table S2

List of Antibodies Used in this Study

Primary antibodies			
Name	**Dilution**	**Vendor (cat#)**	
Immunofluorescence staining (IF) and immunohistochemistry (IHC)			
Rabbit anti-A1AT	1:100	Invitrogen (18-0002)	
Rabbit anti-ASGPR	1:100	Santa Cruz (Sc-28977)	
Mouse anti-ASGPR	1:25	Santa Cruz (Sc-166633)	
Goat anti-HNF4A	1:35	Santa Cruz (Sc-6557)	
Goat anti-hALB	1:200	Bethyl Laboratories (A80-129)	
Mouse anti-hNA	1:200	Millipore (MAB1281)	
Rabbit anti-LDLR	1:200	Novus Biologicals (NB110-57162)	
Rabbit anti-NANOG	1:200	Stemgent (09-0020)	
Mouse anti-SSEA-4	1:200	Stemgent (09-0006)	
Western blotting (WB)			
Rabbit anti-LDLR	1:2000	Novus Biologicals (NB110-57162)	
Rabbit anti-ACTIN	1:1000	Sigma Aldrich (A2066)	
Mouse anti-PCSK9	1:1000	Cayman Chemical (10218)	
***In-vitro* drug testing**			
Mouse anti-PCSK9	0.02 μM, 0.20 μM, and 2.00 μM	Cayman Chemical (10218)	
***In-vivo* drug testing**			
Humanized monoclonal antibody anti-human PCSK9	10 mg/kg mouse	Sanofi and Regeneron Pharmaceuticals (SAR236553/REGN727)	
Secondary antibodies			
Name	**Dilution**	**Vendor (cat#)**	**Applications**
Donkey anti-goat Alexa Fluor 594	1:1000	Invitrogen (A11058)	IF
Donkey anti-rabbit Alexa Fluor 488	1:1000	Invitrogen (A21206)	IF/IHC
Goat anti-mouse Alexa Fluor 488	1:1000	Invitrogen (A10667)	IF
Goat anti-rabbit Alexa Fluor 594	1:1000	Invitrogen (A11072)	IF
Rabbit anti-goat Alexa Fluor 488	1:1000	Invitrogen (A21222)	IF/IHC
Rabbit anti-mouse Alexa Fluor 594	1:1000	Invitrogen (A11062)	IF/IHC
Rabbit anti-mouse Alexa Fluor 488	1:1000	Invitrogen (A11059)	IF/IHC
EnVision goat anti-rabbit HRP	No dilution	DAKO (K4003)	IHC
EnVision goat anti-mouse HRP	No dilution	DAKO (K4001)	IHC
Rabbit anti-goat HRP	1:1000	DAKO (P0449)	IHC
Goat anti-mouse HRP	1:5000	Invitrogen (A16072)	WB
Goat anti-rabbit HRP	1:5000	Invitrogen (A16104)	WB

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Knockout iPSC Generation

iPSCs were dissociated into single cells with Accutase (Sigma-Aldrich) and transfected with 8 μg *LDLR*-ZFN encoding mRNAs through nucleofection (Amaxa™ Nucleofector™ I with Human Stem Cell Nucleofector® Kit 2 VPH-5022 under program A23). Cells were maintained for 48-72 hours under 30°C and 5% CO2 transient cold shock conditions (Doyon et al., 2010) in the presence of ROCK inhibitor Y27632 (Sigma-Aldrich), and then transferred to 37°C and 5% CO_2 conditions for recovery. Cell cultures were dissociated into single cells and seeded onto 96-well plates by limited dilution to obtain single cell-derived subclones. Genotype of the individual subclones was confirmed by PCR and sequencing of the *LDLR*-ZFN target site. Clones with biallelic and monoallelic frameshift mutations were selected for further analysis.

RNA Isolation, RT-qPCR Analysis, DNA Methylation Analysis, and Teratoma Formation

Total RNA was extracted from cell lysates or tissue in TRI Reagent® Solution (Invitrogen). cDNA was obtained from 1 mg of total RNA using PrimeScript™ Double Strand cDNA Synthesis Kit (Takara) and RT-qPCR was performed using a Thermal Cycler Dice™ real-time system (ABI7300, ABI) and SYBR Green Premix EX Taq™ (Takara). *ACTB or Gapdh* was used for normalization, and items were measured in triplicate. *NANOG* promoter DNA methylation analysis was performed using bisulfite-assisted genomic sequencing method (Hajkova et al., 2002). All primers used in this study are listed in *Supplemental Table S1*. For teratoma formation, cells were dissociated with Accutase and suspended in 30% Matrigel in Ca^{2+}/Mg^{2+} free PBS (CMF-PBS, Hyclone), then 3 million cells were injected subcutaneously into the flanks of SCID mice. Tumors were dissected and sectioned 7-9 weeks later, and then stained with hematoxylin/eosin (Sigma-Aldrich).

PAS Staining, Immunofluorescence, and Western Blotting

After 17 days of iHep differentiation, PAS staining on iHeps was performed using Periodic Acid-Schiff Kit (Sigma-Aldrich) according to the manufacturer's specifications. Immunofluorescence and Western blotting were performed following standard procedures. For immunofluorescence, cells were fixed in 4% paraformaldehyde (Affymetrix) for 10 minutes at room temperature. Nuclei were stained with DAPI (Sigma-Aldrich). Primary and second antibodies for both procedures are listed in *Supplemental Table S2*.

LDL Uptake Assays

Briefly, iHeps at day 17 of differentiation were washed 3 times with cold CMF-PBS rapidly. Then, ice-cold HCM medium supplemented with 5 μg/ml FL LDL or DiI LDL was added, and the plates were incubated on ice for 5 minutes before transferring to a 37°C and 5% CO_2 incubator for 3.5 hours. Then, cells were washed with ice-cold CMF-PBS for 3 times. For FL LDL monitoring under the fluorescence microscope, iHeps were fixed with 4% paraformaldehyde for 10 minutes at room temperature and then co-stained with anti-HNF4A according to the immunofluorescence protocol described above. For quantification of DiI $LDL^+/ASGPR^+$ iHeps, after DiI LDL capture, iHeps were fixed and permeabilized with Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD), and stained with ASGPR antibodies. Ratios of DiI $LDL^+/ASGPR^+$ iHeps were quantified by flow cytometry.

Oil Red O Staining, Urea Secretion, and CYP3A4 Activity

For Oil Red O staining, iHeps at day 17 of differentiation were washed 3 times with CMF-PBS, and then fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were subjected to Oil Red O (Sigma-Aldrich) staining for 15 minutes after 3 washes with deionized distilled water, and then stained with hematoxylin for 5 minutes after another 3 rounds of washing. Urea secretion was detected directly on culture medium of iHeps at day 17 of differentiation with Urea Assay Kit (Sigma-Aldrich) according to the manufacturer's specifications. Culture medium of iHeps was used as control. CYP3A4 activity was measured using P450-Glo Assays kit (Promega) according to the manufacturer's specifications. Briefly, iHeps at day 17 of differentiation were pretreated with 25 μM rifampicin or vehicle (DMSO) for 72 hours, and then were incubated with fresh medium containing the luminogenic CYP3A4 substrate Luciferin-IPA at 37°C for 1 hour. Then, 25 μl of culture medium were transferred to a 96-well white luminometer-plate, and 25 μl of Luciferin Detection Reagent was added to initiate the luminescent reaction. Luminescence was measured using a CLARIOstar microplate reader (BMG LABTECH) 20 minutes later. Relative light units (RLU)/10^6 cells/ml indicate CYP3A4 activity.

Histology and Immunohistochemistry

Mice were sacrificed by carbon dioxide asphyxiation, and livers were perfused and fixed with 10% formalin solution (Sigma-Aldrich), embedded in paraffin (Leica Biosystems), sectioned and stained with primary antibodies. Secondary antibodies conjugated with fluorescent labels or HRP, which react with DAB detection kit (Invitrogen), were used for immunostaining of chimeric liver sections. Primary and secondary antibodies for immunohistochemistry are listed in *Supplemental Table S2*. Quantification of the percentage of hNA+ cells was based on 10 regions/mouse (randomly selected) and performed by manual counting in Adobe Photoshop (version 2015.0.0). Slides stained with anti-hALB and reacted with DAB were scanned by Aperio ScanScope System (Leica Biosystems). For hALB+ area quantification, images

of whole slides were divided into pieces by snapshot and then quantified by Zeiss AxioVision LE, the edge of the lobes was excluded when doing quantification. Counts were based on no less than 4 sections of liver lobes and lobules per mice, and 3-5 mice per group.

Functional Studies in Myograph

Briefly, after mice were anaesthetized with ketamine/xylazine, the thoracic aortae were dissected in oxygenated ice-cold Krebs-Henseleit solution (mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose). Changes in isometric tension of arteries were measured and recorded in a multi-myograph system (Danish Myo Technology) as described previously (Wong et al., 2010). The aortic ring (~1.5 mm in length) was stretched to basal tension ~3 mN and equilibrated for 1 hour before the experiment. Then, aortic rings were first contracted by 60 M KCl and rinsed in Krebs solution for 3 times. EDV to accumulative concentrations of Ach (10 nM to 10 μM) was examined in Phe (3 μM)-contracted aortic rings. Endothelium-independent relaxations to sodium nitroprusside (SNP) (1 nM to 10 μM) were recorded in endothelium-denuded rings.

SUPPLEMENTAL REFERENCES

Doyon, Y., Choi, V.M., Xia, D.F., Vo, T.D., Gregory, P.D., and Holmes, M.C. (2010). Transient cold shock enhances zinc-finger nuclease-mediated gene disruption. Nat. Methods *7*, 459-460.

Hajkova, P., el-Maarri, O., Engemann, S., Oswald, J., Olek, A., and Walter, J. (2002). DNA-methylation analysis by the bisulfite-assisted genomic sequencing method. Methods Mol. Biol. *200*, 143-154.

Wong, W.T., Tian, X.Y., Xu, A., Ng, C.F., Lee, H.K., Chen, Z.Y., Au, C.L., Yao, X., and Huang, Y. (2010). Angiotensin II type 1 receptor-dependent oxidative stress mediates endothelial dysfunction in type 2 diabetic mice. Antioxid. Redox Signal. *13*, 757-768.