Methods:

Transgenic Reporter and Gene Targeted Animals

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME).VEGFR2-GFP mice were acquired from Dr. Janet Rossant at the Hospital for Sick Children, Toronto, Canada[31]. *Id1*$^{-/-}$ mice were generated as previously described[24] and obtained from Dr. Robert Benezra, Sloan Kettering Institute and Dr. David Lyden, Weill Cornell Medical College, NY.

VEGFR2$^{loxP/loxP}$ mouse was generated by Dr. Thomas N. Sato and experiments with EC-specific inducible VEGFR2 knockout mice were carried out as previously described[6]. Briefly, the *VEGFR2*$^{loxP/loxP}$ mice and then bred with *RosaCre-ER*^{T2} transgenic mice to establish the *RosaCre-ER*^{T2}*VEGFR2*$^{loxP/loxP}$ line and control *ROSA-CreER*^{T2}*VEGFR2*$^{loxP/+}$ to account for potential Cre-mediated toxicity. To induce endothelial-specific knockdown of VEGFR2, *VE-cadherin-CreER*^{T2} mice kindly provided by Dr. Luisa Iruela-Arispa (University of California, Los Angeles, CA) were also crossed with *VEGFR2*$^{loxP/loxP}$ mice to generate *VE-cadherin-CreER*^{T2}*VEGFR2*$^{loxP/loxP}$ mice. To induce VEGFR2 gene ablation, 6-8 week old male mice were treated with tamoxifen at a dose of 250 mg/kg sunflower oil i.p. for 6 days interrupted for 3 days after the third dose. After 3 days of respite, the fourth dose was reinstituted for an additional 3 days, resulting in *ROSA-CreER*^{T2}*VEGFR2*$^{flox/flox}$ (*VEGFR2*$^{fl/fl}$) mice that are deficient in VEGFR2 at both alleles, the control *ROSA-CreER*^{T2}*VEGFR2*$^{fl/+}$ mice or *VE-cadherin-CreER*^{T2}*VEGFR2*$^{fl/fl}$ mice that have EC-specific VEGFR2 knockdown. All animal experiments were carried out under the guidelines set by Institutional Animal Care and Use Committee.

Mouse Liver Regeneration Model

70% partial hepatectomy (PH) model was utilized to induce physiological liver regeneration in mice. most anterior lobes (right medial, left medial, and left lateral lobes), which comprise 70% of the liver were resected, without injuring the blood supply to the caudate and the right lobes. Mice were

by 100 mg/kg intraperitoneal ketamine and 10 mg/kg xylazine. Midline laparotomy was performed in the anesthetized mice. After opening the upper abdomen and the exposure of the liver, the left lobe to be resected was gently lifted while a 5-0 silk suture tie (Roboz, Rockville, MD) was placed underneath the lobe and positioned as proximal to the origin of the lobe as possible. The two ends of the suture were tied over the top of the liver lobe at the base of the lobe near the inferior vena cava. Three knots were tied, and microdissecting scissors was used to cut the tied lobe just distal to the suture. This process was repeated for the other median lobes to perform 70% PH. Then the peritoneum was reapproximated with a running 5-0 silk suture, the skin was closed with a running 4-0 silk suture.

Sham-operated mice underwent laparotomy without liver resection. To characterize the regeneration of liver mass and function, the weight of residual liver lobes were measured and normalized to mouse body weight at various time points days after PH, and plasma bilirubin levels were assayed (Genzyme Diagnostics, Framingham, MA) after 70% PH, respectively. To compare PH model to CCl_4-induced liver injury model, CCl_4 was intraperitoneously injected as previously described[13]. To test liver regeneration promoted by VEGF-A or PlGF, mice were treated with 15 μg/kg of recombinant $VEGF_{164}$ (Biovision, Mountain view, CA) and the same amounts of PlGF (Biovision, CA) 12 hours before the operation and twice a day thereafter. *Id1*$^{-/-}$ mice and wild type littermates were also subjected to similar $VEGF_{164}$ and PBS treatment before and after operation.

Liver Immunofluorescence (IF) and Detection of GFP

VEGFR2-GFP, VEGFR2$^{fl/fl}$, *Id1*$^{-/-}$, and littermate control mice were subjected to PH or sham-operation, perfused with 4% paraformaldehyde, cryoprotected, and snap frozen in OCT. For the analysis of the liver microvasculature, mice were intravenously injected with 2 mg/kg *Griffonia simplicifolia* lectin (isolectin B4, Invitrogen, CA) 5 minutes before sacrifice, as previously described[6]. For IF microscopy, the liver sections (10 μm) were blocked (5% donkey serum/0.3% Triton X-100) and incubated in primary

antibodies: anti-VEGFR3 monoclonal antibody (mAb, mF4-31C1, 10 μg/ml, ImClone, New York, NY), anti-VE-cadherin polyclonal Ab (pAb, 2 μg/ml, R&D Systems, MN), anti-CD34 mAb (553731, 5 μg/ml, BD Biosciences, CA), anti- phospho-Histone H3 (Millipore, Billerica, MA) and anti-HNF4A antibody (Abcam, Cambridge, MA). After incubation in fluorophore-conjugated secondary antibodies (2.5 μg/ml, Jackson ImmunoResearch, PA), sections were counterstained with TOPRO3 or DAPI (Invitrogen, CA).

Liver cell proliferation *in vivo* was measured by BrdU uptake. Briefly, mice received a single dose of BrdU (Sigma) intraperitoneally 60 minutes before death (at a dose of 50 mg/kg animal weight). At the time of death, mice were anesthetized, blood was harvested from the inferior vena cava, and the remaining liver lobes were removed, weighed, and further processed. Cryosections were stained using the BrdU Detection System (BD Biosciences, CA) and fluorophore-conjugated secondary antibodies (2.5 μg/ml, Jackson ImmunoResearch, PA).

Image Acquisition and Image Analysis

IHC images of liver sections were captured with AxioVision software (Zeiss, NY) mounted on Olympus BX51 microscope (Olympus America, NY). IF images were captured on AxioVert LSM510 or 710 confocal microscope (Zeiss). Digital images were analyzed for the density of endothelial marker (VE-cadherin^{+}) and functional perfused vessels (isolectin^{+}) using Image J (NIH, MD). Vessel density was expressed by the percentage of positive component to the total area in each high power field, 400x.

Isolation and Culture of Mouse Cells

Hepatocytes, LSECs, stellate and Kupffer cells were isolated from mice underwent sham-operation and PH, by a two-step collagenase perfusion technique with modifications[32-36]. Briefly, after the inferior vena cava was cannulated and portal vein was cut, the liver was perfused at 5 ml/min via the inferior vena cava with Liver Perfusion Medium (Invitrogen, CA) at 37 ^{o}C for 10 minutes, followed by perfusion with Liver

Digest Medium (Invitrogen, CA) for an additional 10 minutes. The liver was dissociated in Hepatocyte Wash medium (Invitrogen, CA), passed through dacron fabric with 70 μm pores, and separated from the nonparenchymal hepatocyte depleted fraction (NPCs) by low-speed centrifugation (50 g x 5min), which were further purified by percoll gradient centrifugation, using Stock Percoll Solution as previously described[36]. The supernatant containing NPCs was collected and was washed twice at 50 *g* for 5 minutes, pelleted at 350 *g* for 7 minutes, and fractionated with percoll gradient centrifugation (900 g x 20 min) with 75% stock Percoll solution and 35% stock Percoll solution, as previously described[36]. Fraction containing LSECs were enriched, mixed with an equal volume of PBS, and centrifuged at 900 *g* for 7 minutes. The pellet was washed with DMEM (Invitrogen, CA) at 350 *g* for 7 minutes and further labeled by mouse LSEC binding magnetic beads (Miltenyi, Auburn, CA). The purification of LSECs was performed according to the manufacturer's protocol. Purification of stellate and Kupffer cells were carried out as previously described[33-35].

Flow Cytometric Analyses, Identification, and Quantification of LSECs

Purified mAbs were conjugated to Alexa Fluor dyes or Qdots per manufacturer's protocols (Molecular Probes/Invitrogen, CA). Purified hepatocyte-depleted NPCs were analyzed on LSRII-SORP (BD). Data was processed with FACSDiva 6.1 software (BD). Doublets were excluded by FSC-W × FSC-H and SSC-W × SSC-H analysis, single stained channels were used for compensation, and fluorophore minus one (FMO) controls were used for gating. mAbs were purchased from BD except where noted: VE-cadherin (BV13, ImClone, NY); VEGFR3 (mF4-31C1, ImClone, NY); VEGFR2 (DC101, ImClone, NY); CD45 (30-F11, BD Biosciences, CA), CD34 (14-0341, eBioscience, CA).

For quantification of LSECs, the livers were mechanically prepared as above and the number of SECs was quantified by costaining with conjugated antibodies to VEGFR2, VEGFR3, VE-cadherin, CD34.

Number of SECs equals the number of $VEGFR3^{+}CD34^{-}VEGFR2^{+}VE\text{-}cadherin^{+}$ cells. $VEGFR3^{-}CD34^{+}VEGFR2^{+}VE\text{-}cadherin^{+}$ cells were scored as non-SECs.

Determination of Hepatocyte Proliferation in Coculture With ECs

Human LSECs were from ScienCell Research Laboratories (Carlsbad, CA). To selectively knockdown *Id1* in LSECs, *Id1*/Scrambled shRNA Lentiviruses were generated by cotransfecting 15 μg of shuttle lentiviral vector containing *Id1*/Scrambled shRNA, 3 μg of pENV/VSV-G, 5 μg of pRRE, and 2.5 μg of pRSV-REV in 293T cells by Fugene 6 (Roche Applied Science). Viral supernatants were concentrated by ultracentrifugation. These concentrated viral preparations were used to transduce LSECs or hepatocytes. For co-culture studies, 10,000 isolated primary hepatocytes were plated in a 100 mm dish coated with type I collagen, seeded with 500,000 LSECs, or with LSECs treated with *Id1*/scramble shRNA lentivirus, respectively. Culture conditions consisted of Williams' E medium (Invitrogen, CA) supplemented with L-glutamine (2 mmol/L), 1 % fetal bovine serum (FBS), vascular endothelial growth factor-A ($VEGF\text{-}A_{164}$) (5 ng/ml), dexamethasone at 10^{-9} mol/L, streptomycin (100 U/mL), and penicillin (100 U/mL). Cells from each group were harvested after two weeks. To visualize LSECs and hepatocytes, LSECs were marked by mCherry lentivirus (in pCCL backbone) as described above, and hepatocytes were infected with GFP lentivirus. Conditioned medium was also collected from 500,000 LSECs cultured for two weeks, filtered through 0.22 μm filter, and added to 10,000 hepatocytes at 1:2 dilution, in the absence of LSEC coculture. The number of LSECs and hepatocyte were assessed by flow cytometric analysis of mCherry and GFP signals. Hepatocyte proliferation was quantified by comparing the number of retrieved hepatocytes to initially seeded hepatocyte number.

Affymetrix Analysis and Quantitative real-time PCR analysis

RNA was freshly isolated from the liver using RNeasy (Qiagen) and was converted to cDNA using Superscript II (Invitrogen, CA). Microarray was performed using Mouse U133 2.0 (Affymetrix). Details of the methods for RNA quality, sample labeling, hybridization, and expression analysis were according to the manual of Affymetrix Microarray Kit. Quantitative PCR was carried out using Taqman gene expression systems for mouse VEGFR2, VEGFR3, Id1, HGF, Wnt2, Wnt9B and TM (Applied Biosystems, Foster City, CA).

Liver Transplantation of Regenerative LSECs

Multi-lobular 70% PH was carried out in WT ($Id1^{+/+}$) mice and age and sex matched $Id1^{-/-}$ mice. 48 hours after PH, LSECs were isolated from WT ($Id1^{+/+}$ regenerative LSECs) and marked by GFP lentivirus (in pCCL backbone) transduction as described above. The transplantation procedure was modified from previously described[25]. Briefly, 48 hours after PH, $Id1^{-/-}$ mice were anesthetized and placed in the right lateral decubitus position. The left flank was scrubbed with Betadine, and the skin and abdominal wall were incised longitudinally (parallel to the spine). After the spleen was exteriorized, $Id1^{+/+}$ regenerative LSECs were injected into the parenchyma of the spleen through a 27-gauge needle. A splenectomy was performed after the injection. To compare the rescuing effect of $Id1^{+/+}$ regenerative LSECs, $Id1^{-/-}$ and WT mice also subjected to the intrasplenic injection of PBS and splenectomy 2 days after PH (sham transplant). To introduce Wnt2 and HGF expression in LSECs, Wnt2 and HGF cDNA were purchased from Open biosystems (Huntsville, AL) and cloned into lentiviral vector as described above. Infection of LSECs with virus encoding Wnt2 or HGF, or the same amounts of mixed Wnt2 and HGF was carried out together with GFP lentivirus infection.

Data Analysis.

All data are presented as the Mean ± s.e.m. of at least three separate experiments. Differences between groups were tested for statistical significance using Student's *t*-test or analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$.

Supplementary references:

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