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Supplemental Information

Molecular Signatures of Tissue-Specific Microvascular Endothelial Cell Heterogeneity in Organ Maintenance and Regeneration

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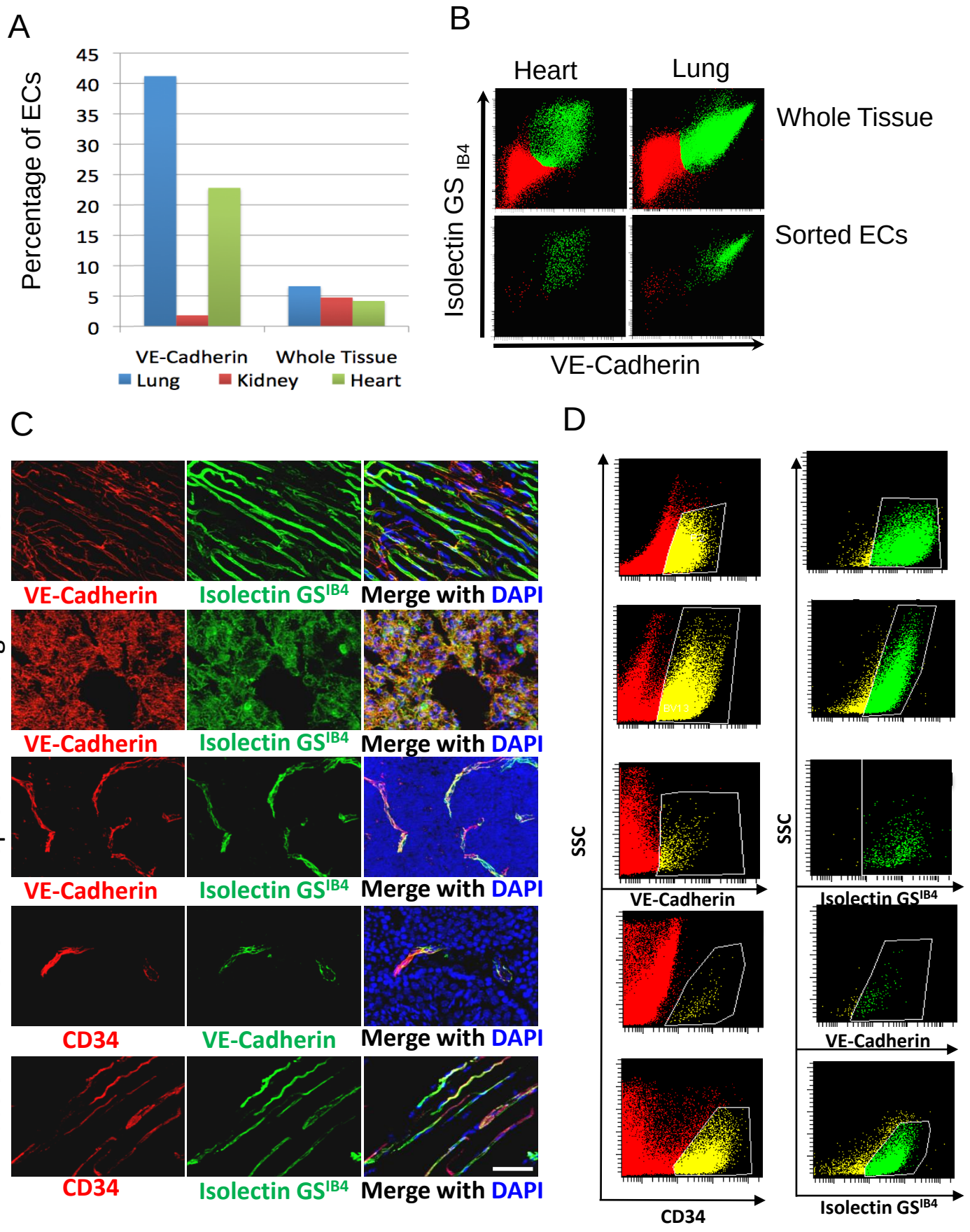
Inventory of Supplemental Information for Nolan et al.

Supplemental Figure 1	Relates to Figure 1. It depicts the purity of various isolation strategies and continues the intravital definitions of the remaining tissues.
Supplemental Figure 2	Relates to Figure 1. It presents autofluorescence and lymphatic EC controls.
Supplemental Figure 3	Relates to Figure 2. This figure shows the fold change of the 116 transcription factors from Figure 2A.
Supplemental Figure 4	Relates to Figure 3. This figure shows additional groupings of genes with deviations in expression patterns between the vascular beds.
Supplemental Figure 5	Relates to Figure 7. The data depicts the phenotype of cultured mESC-ECs and negative staining results for several surface markers on the liver and kidney ECS.
Table 1	The table presents the GEO series number and Accession Numbers for all microarrays.
Supplemental Experimental Procedures	Standard and published protocols are presented here, namely flow cytometry, ChIP analyses, ESC culture and differentiation conditions, mice, de novo motif analysis, and microscopy.
Supplemental References	The references to material covered in the supplemental experimental procedures are included here.

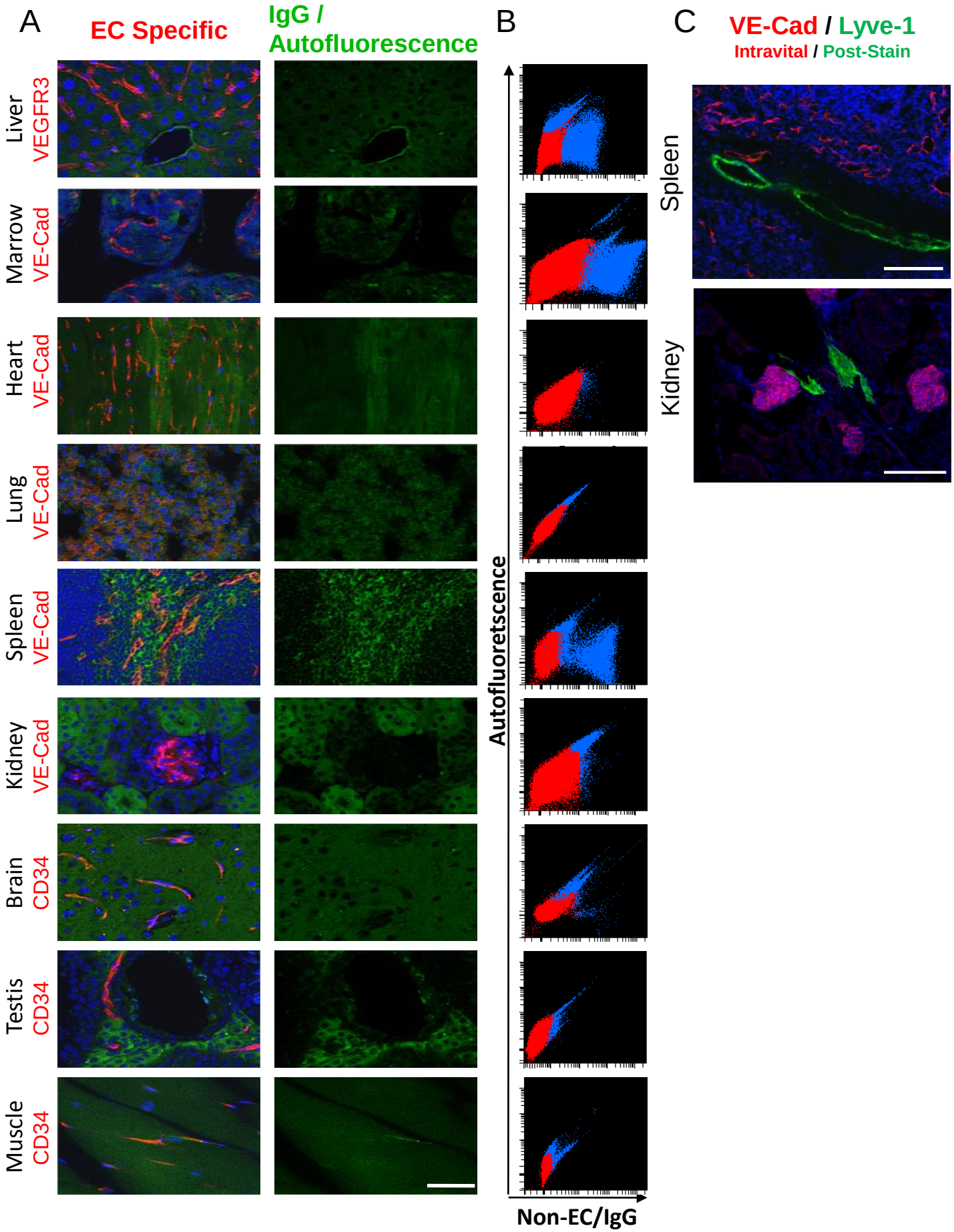
Nolan D, et al. Supplemental Figure 1



Supplemental Figure 1: Purity assessment and phenotypic identification of tissue specific ECs by intravital staining, related to Figure 1.

Wild type (WT) animals were injected with 25 μg unlabeled anti-VE-Cadherin antibody and fluorescently labeled IsolectinGS$_{IB4}$. Anti-Rat IgG microbeads were used to magnetically purify the ECs from the lung, liver, and kidney. Purity was checked by flow cytometry and varied from 3 to 41% (A). In comparison, 25 μg of fluorescently labeled VE-Cadherin antibody along with IsolectinGS$_{IB4}$ was used to isolate ECs from the lung and heart via flow sorting. Purities for flow sorting were consistently over 95% (B). WT animals were co-injected with fluorescently labeled antibodies and IsolectinGS$_{IB4}$. Primary channels (left) provided for the clearest resolution of the ECs, secondary channels (middle) confirmed the cell as endothelial via microscopy. Sections were counter-stained with DAPI (shown in merged images, right) (C). The identical labels from B were applied to flow cytometric analysis. Red cells only include single live cells without highly-autofluorescent, non-specific IgG binding cells, or aggregates of two or more cells. Cells highlighted in yellow are positive for the primary specific EC marker and then interrogated in a secondary channel. Double positive cells are shown in green (D).

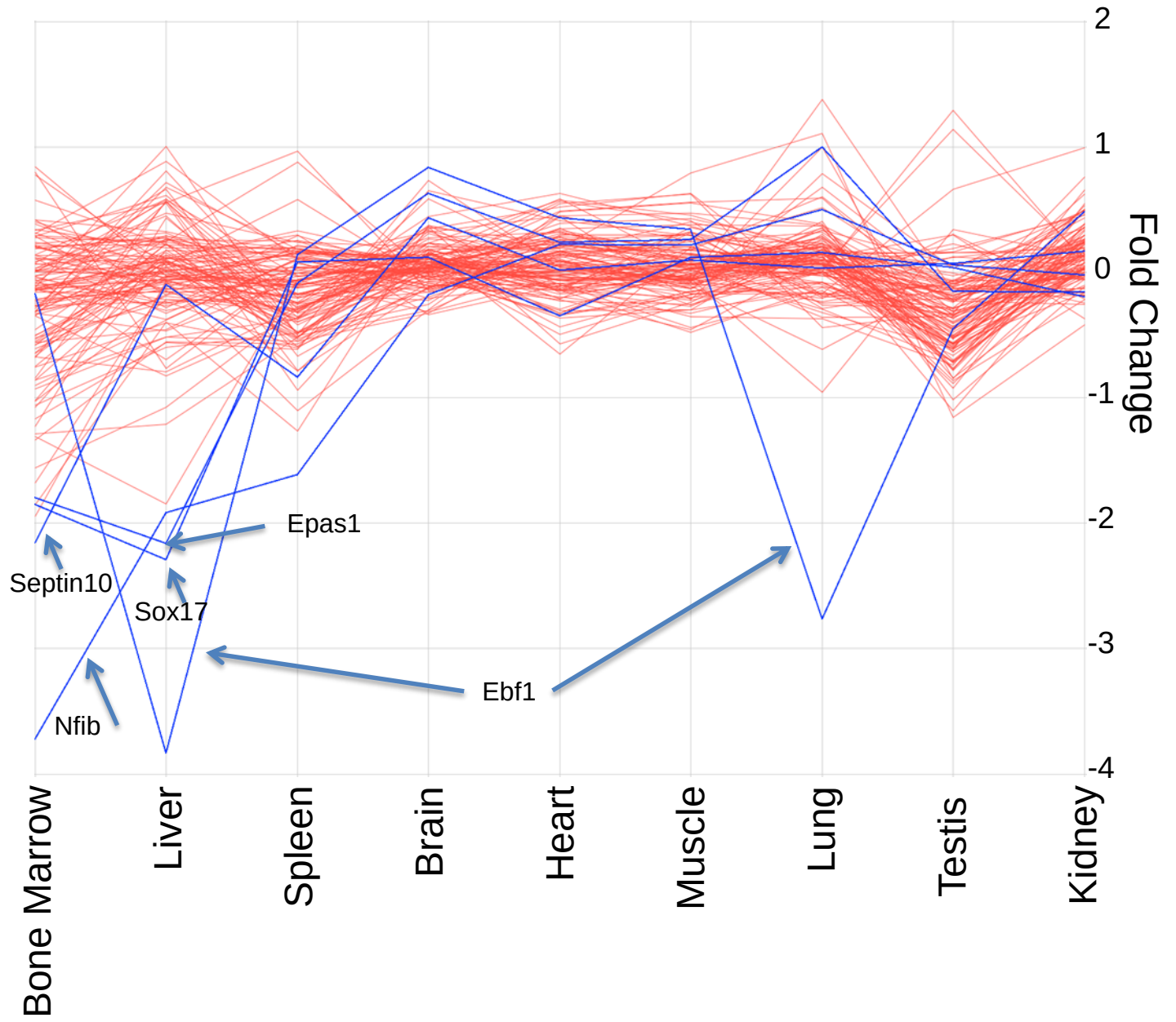
Nolan D, et al. Supplemental Figure 2



Supplemental Figure 2: Specificity of tissue-specific intravital stains by immunofluorescent staining, related to Figure 1.

Representative images of tissues intravitally labeled with EC specific markers are shown (left column). Non-EC specific antibody (rat IgG) was fluorescently labeled and co-injected. Images were enhanced to accentuate autofluorescence and highlight a lack of non-specificity on ECs (A). Flow cytometry of the same organs on a channel without any dye present (y-axis) to identify the autofluorescence. The x-axis measures the nonspecific binding of fluorescently labeled non-EC antibodies (i.e. rat IgG, x axis). Autofluorescent cells and cells non-specifically binding antibodies highlighted in blue were excluded, only cells highlighted in red were considered for further analysis and EC identification (B). Animals intravitally stained with VE-cadherin (red) were post-stained with Lyve-1 (green) antibody after cryopreservation and sectioning demonstrating that the intravital staining method does not label lymphatic ECs (C). Scale bars equal 50μm.

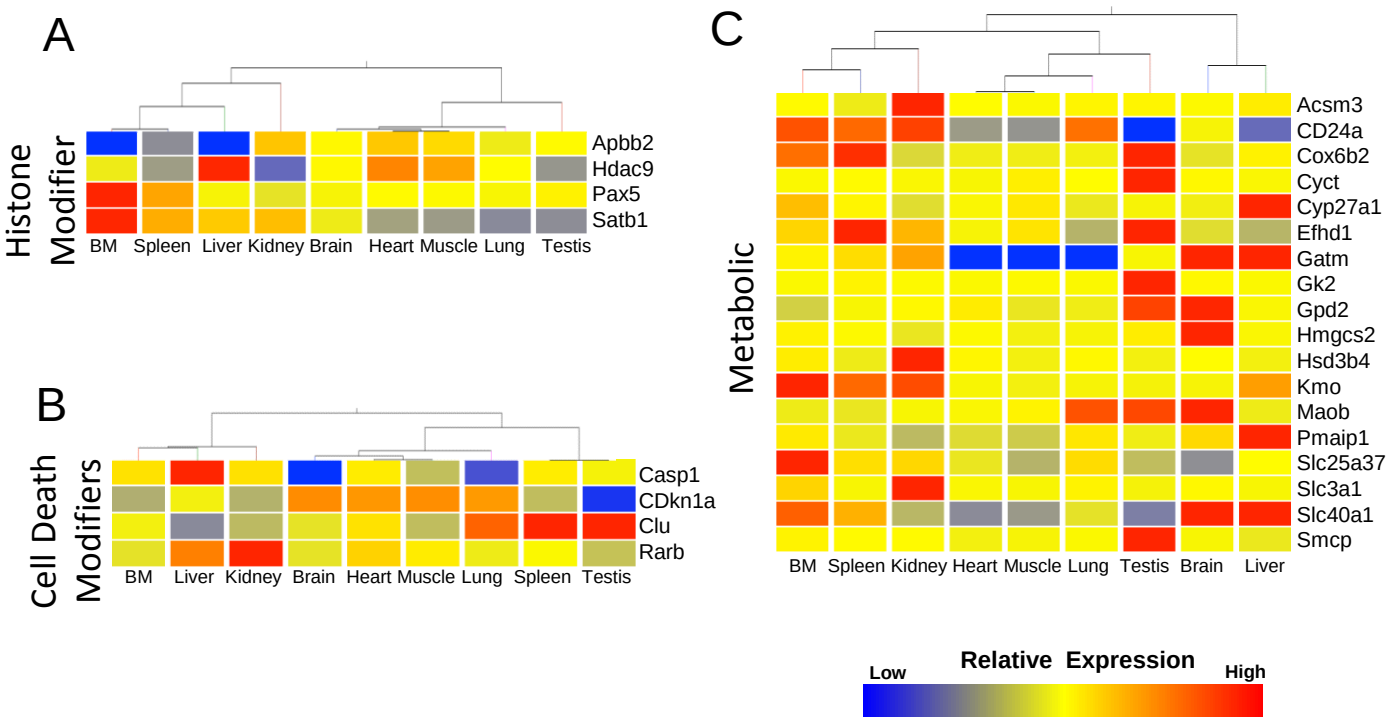
Nolan D, et al. Supplemental Figure 3



Supplemental Figure 3: Deviation of highly expressed transcription factors (TFs), related to Figure 2.

Profile of genes listed in Figure 2A. Any gene that was two fold up or down regulated from the mean is highlighted in blue and labeled.

Nolan D, et al. Supplemental Figure 4

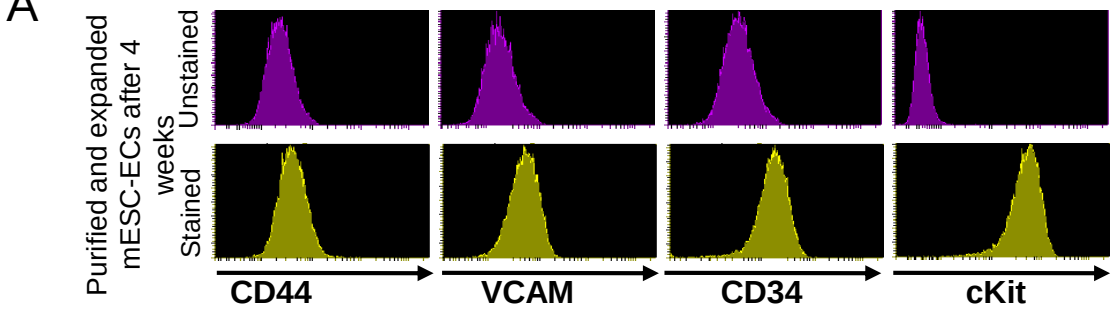


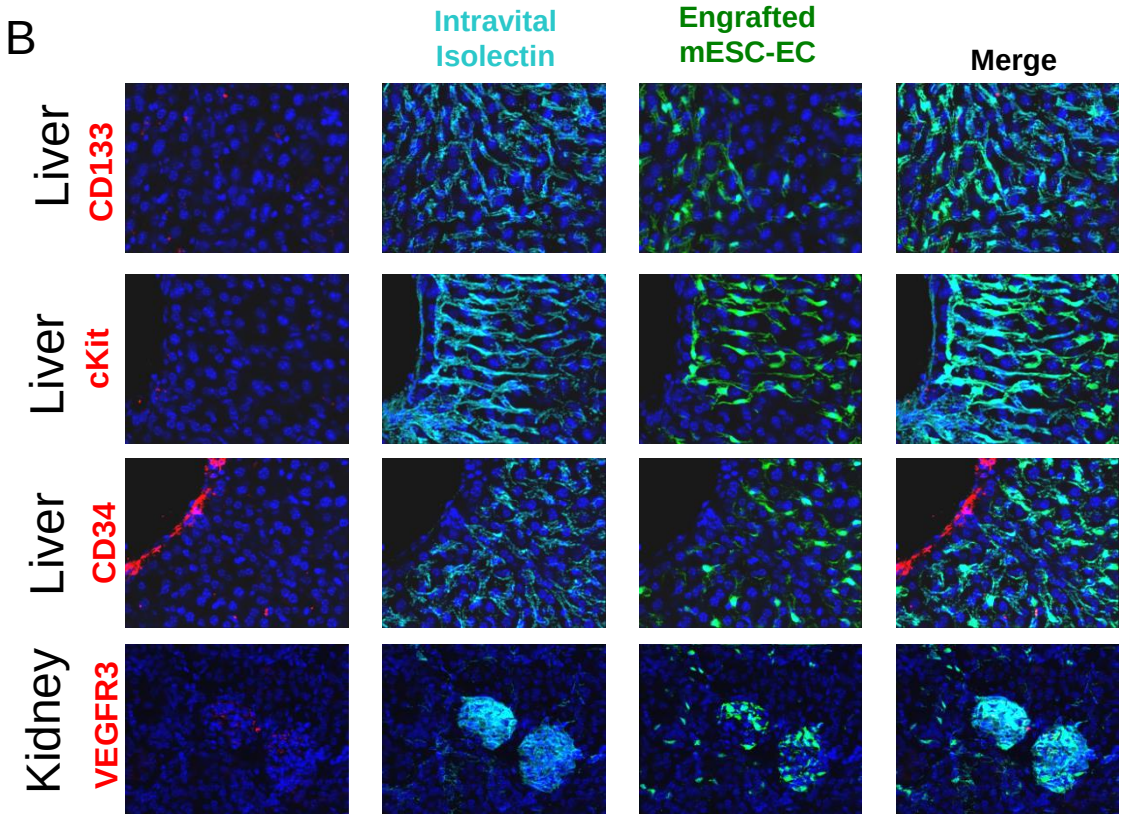
*All genes statistically significant, adjusted P<0.05

Supplemental Figure 4: Hierarchical clustering of genes involved in various aspects of EC biology, related to Figure 3.

The Gene Ontology Database was used to group genes into various aspects of biology. A representative group of genes two fold above or below the mean expression are depicted for histone modifiers (A), cell death modifiers (B), and metabolic components (C) are shown. All genes listed are statistically significant (Benjamini-Hochberg adjusted $P<0.05$).

Nolan D, et al. Supplemental Figure 5





Supplemental Figure 5: Characterization of purified and engrafted mESC-ECs in the liver and kidney, related to figure 7.

As depicted in Figure 7A, mESC-ECs were purified from differentiation cultures and expanded without the influence of any other cell type. After four weeks of culture, mESC-ECs were analyzed for CD44, VCAM, CD34, and c-Kit (A). In addition to the data presented in Figure 7D, liver and kidney sections with functionally engrafted mESC-ECs were stained with CD133, c-Kit, CD34, and VEGFR3 highlighting the distinctions between tissues and the degree of *in vivo* education (B).

Supplemental Experimental procedures

Flow Cytometry and Sorting:

All flow cytometry was performed on a LSRII SORP. All flow sorting was performed on an AriaII SORP. Data analysis was done with BD FACS Diva software (Becton Dickenson). Antibody capture beads were used to calculate compensation (BD Pharmingen). FSC-H vs. FSC-W and SSC-H vs. SSC-W were analyzed to exclude cell doublets. Autofluorescence was monitored for and excluded by removing any signal from the 525/50 filter with signal from the violet laser line. Sample temperatures for sorting were kept at 4^{o}C by a Petlier-cooled sample chamber and cold-water circulator pre and post isolation. A purity mask was used for sorting on a 70μm nozzle to minimize the collection of any non-EC cells or fragments. Cell density was kept high to allow for a 15,000-20,000 events/second sort rate at a flow speed between 1 and 4. All manufacturer recommended quality control tests were performed immediately prior to sorting, namely CS&T beads and Accudrop Beads (BD Pharmingen). Additional antibodies used for flow cytometry were CD133, CSF1R (eBioscience), CD26 (BioLegend), Jag1 (Abcam), CD36, Tie2, CD31, VCAM, Endoglin, c-Kit, CD44, and (BD Pharmingen).

Chip analyses:

Human umbilical vein ECs (HUVECs) were isolated and transduced with lentiviral SFPI1 and gown under puromycin selection. Immunoprecipitations were performed as described (Lee et al., 2006). Briefly, cells were fixed with 1.1% formaldehyde solution and lysed. Chromatin was sonicated with a Daigenode Bioruptor and immunoprecipitated with anti-SFPI1 (Santa Cruz, SC-352) or control antibody. After purification, the amount of immunoprecipitated DNA recovered was measured by real-time quantitative PCR. The primers encompassing the SFPI sites in the CD37 promoter were 5'-CCTTACATGAAGCGGGAGTG-3', and 5'-

CCACACAGAAGAGGTGCTGA-3', the MMP9 promoter were 5'-AGGCTGCTACTGTCCCCTTT -3' and 5'- CTCCCTGACAGCCTTCTTTG -3', and the TNFα promoter were 5'-GCTTGTGTGTCCCCAACTTT-3' and 5-'TGTGCCAACAACTGCCTTTA-3'. Primers designed to amplify a region 3kb away from the SFPI site were used as a negative control and were 5'-AAGGAGGGGAGAGGTGAGAG-3' and 5'-TGTTGGGATAACAGGCATGA-3'. Statistical significance was determined using a one-tailed Student's t-test.

Human embryonic stem cell (hESC) culture and differentiation:

Human ESC culture medium consisted of Advanced DMEM/F12 (Gibco) supplemented with 20% Knockout Serum Replacement (Invitrogen), 1X non-essential amino acids (Gibco), 1X L-Glutamine (Invitrogen), 1X Pen/Strep (Invitrogen), 1X b-Mercaptoethanol (Gibco), and 4 ng/ml FGF-2 (Invitrogen). Human ESCs were maintained on Matrigel™ using hESC medium conditioned by mouse embryonic fibroblasts (MEF, Chemicon). Human umbilical vein ECs were isolated and transduced with the E4ORF1 gene of Adenovirus 5 to generate durable primary endothelial cell (PEC) feeders as previously described (Seandel et al., 2008). One day in advance of plating hESCs on E4ORF1$^+$ PECs, MEF conditioned medium was replaced with hESC culture medium without FGF-2 and supplemented with 2 ng/ml BMP4. The next day, hESCs were plated directly onto an 80% confluent layer of E4ORF1$^+$ PEC in hESC culture medium (without FGF-2, plus 2 ng/ml BMP4) and left undisturbed for 48 hours. At this point, cultures were considered as differentiation day zero and cells were sequentially stimulated with recombinant cytokines in the following order: day 0 to 7 supplemented with 10 ng/ml BMP4; day 2 to point of harvest supplemented with 10 ng/ml VEGF-A; day 2 to point of harvest supplemented with 5 ng/ml FGF-2; day 7 to point of harvest supplemented with 10 mM SB-

431542. Antibodies to analyze hESC-ECs were CD31, CXCR4, c-Kit, CD36 (BD Bioscience) and CD133 (Miltenyi Biotech).

Mice:

All animal experiments were performed using male C57BL/6 mice from Jackson Laboratories aged 8-12 weeks old. Sub-lethal irradiation was achieved with a caesium-137 source supplying 650 Rads. Partial hepatectomy was performed by resecting the three most anterior lobes as previously described (Ding et al., 2010). Each protocol was reviewed and approved by Institutional Animal Care and Use Committee.

De novo motif analysis:

De novo motif discovery was performed using FIRE (Elemento et al., 2007), with 1kb promoters downloaded from UCSC Genome Browser on July 2011. In FIRE, motifs are scored based on the mutual information between motifs and gene groups, and Z-scores shown in the figures indicate the extent to which the motif information is greater than randomized information values. FIRE motifs were compared to known transcription factor binding sites from TRANSFAC and JASPAR using the CompareACE method (Hughes et al., 2000) with threshold 0.8. K-means clustering was performed using Cluster 3.0 with Pearson correlation and 10 random initializations.

Mouse embryonic stem cell (mESC) culture, differentiation and in vivo transplantation studies:

Wild type C57Bl/6 mouse embryonic stem cells (mESCs) were maintained on feeder layers of mitomycin-C-treated mouse embryonic fibroblasts, or on gelatin-coated plates in the presence of 1000 U/ml of leukemia inhibitory factor (LIF) (EMD Millipore). Mouse ESC medium consisted

of Knockout DMEM (Invitrogen), 15% FBS, 1% non-essential amino acids, 1% penicillin/streptomyocin, 1% l-glutamine and 0.1% β-Mercaptoethanol. Embryoid bodies (EBs) were generated on a orbital shaker in Stem Pro-34 medium (Invitrogen) supplemented with 1% l-glutamine, 1% penicillin/streptomycin, 0.5 mM Ascorbic Acid, 0.1% β-Mercaptoethanol and 200 μg/ml bovine holo-transferrin (Sigma Aldrich). Based on previous protocols (Hirashima et al., 1999; James et al., 2010; Kobayashi et al., 2010; Watabe et al., 2003; Yamashita et al., 2000) to induce endothelial cell differentiation, the EBs were supplemented with BMP4 (5ng/ml) (R&D Systems) at day 1. At day 2 the EBs were supplemented with BMP4 (5ng/ml), ActivinA (5ng/ml) (Peprotech) and bFGF (FGF-2) (10ng/ml). At day 3 the EBs were seeded on gelatin-coated plates and supplemented with BMP4 (5ng/ml), FGF-2 (10ng/ml) and VEGF-A (20ng/ml) (Peprotech). Subsequently, the differentiation was continued by adding FGF-2 (10ng/ml) and VEGF-A (20ng/ml) every other day until day 14 from the beginning of the differentiation. At day 14 ECs were sorted as VE-Cadherin positive cells and allowed expansion without competition from the remaining EB-cells (Levenberg et al., 2002, Kobayashi et al., 2010; Park et al., 2004). The endothelial committed cells were then transduced with PGK-GFP and PGK-myrAkt1 lentiviruses. These mouse endothelial cells derived from ESCs (mESC-ECs) were maintained in Stem Pro-34 medium supplemented every other day with FGF-2 (10ng/ml) and VEGF-A (20ng/ml) prior to transplantation into syngeneic mice that have undergone 70% partial hepatectomy, as described previously (Ding et al., 2010). For *in vivo* education studies, 500,000 GFP labeled mESC-ECs were injected intrasplenically (intra-parenchymal technique) immediately after 70% partial hepatectomy (Ding et al., 2010).

Microscopy:

For image acquisition, a Zeiss Observer equipped with an Apotome and Colibri illumination system was used to acquire images of 30μm thick specimens with optical sectioning. hESC-EC images were performed on a Zeiss LSM 710 confocal. All presented images of tissues are MIP processed Z-Stacks. All mouse specimens were counter stained with DAPI to reveal nuclei. Image analysis for brightness and contrast adjustments was performed with Zeiss Axiovision software.

Gene Expression Omnibus Accession Numbers

GEO Series GSE47067

Platforms GPL6244, GPL6246

Accession	Name	
GSM1144153	hESC-EC Prominin A	Human hESC-EC
GSM1144154	hESC-EC Prominin B	
GSM1144155	hESC-EC Prominin C	
GSM1144156	hESC-EC CXCR4 A	
GSM1144157	hESC-EC CXCR4 B	
GSM1144158	hESC-EC CXCR4 C	
GSM1144159	Testis A	Adult Mouse
GSM1144160	Testis B	
GSM1144161	Testis C	
GSM1144162	Spleen A	
GSM1144163	Spleen B	
GSM1144164	Spleen C	
GSM1144165	Muscle A	
GSM1144166	Muscle B	
GSM1144167	Muscle C	
GSM1144168	Brain A	
GSM1144169	Brain B	
GSM1144170	Brain C	
GSM1144171	Lung A	
GSM1144172	Lung B	
GSM1144173	Lung C	
GSM1144174	Heart A	
GSM1144175	Heart B	
GSM1144176	Heart C	
GSM1144177	Glomerulus A	
GSM1144178	Glomerulus B	
GSM1144179	Glomerulus C	
GSM1144180	Bone Marrow Steady State A	
GSM1144181	Bone Marrow Steady State B	
GSM1144182	Bone Marrow Steady State C	
GSM1144183	Liver Steady State A	
GSM1144184	Liver Steady State B	
GSM1144185	Liver Steady State C	

Accession	Name	
GSM1144261	Bone Marrow Steady State A v2	Regenerating Marrow
GSM1144262	Bone Marrow Steady State B v2	
GSM1144263	Bone Marrow Steady State C v2	
GSM1144264	Bone Marrow Day 10 A	
GSM1144265	Bone Marrow Day 10 B	
GSM1144266	Bone Marrow Day 10 C	
GSM1144267	Bone Marrow Day 21 A	
GSM1144268	Bone Marrow Day 21 B	
GSM1144269	Bone Marrow Day 21 C	
GSM1144270	Bone Marrow Day 28 A	
GSM1144271	Bone Marrow Day 28 B	
GSM1144272	Bone Marrow Day 28 C	
GSM1144273	Liver Steady State A v2	Regenerating Liver
GSM1144274	Liver Steady State B v2	
GSM1144275	Liver Steady State C v2	
GSM1144276	Liver Day 2 Post Hepatectomy A	
GSM1144277	Liver Day 2 Post Hepatectomy B	
GSM1144278	Liver Day 2 Post Hepatectomy C	
GSM1144279	Liver Day 4 Post Hepatectomy A	
GSM1144280	Liver Day 4 Post Hepatectomy B	
GSM1144281	Liver Day 4 Post Hepatectomy C	
GSM1144282	Liver Day 6 Post Hepatectomy A	
GSM1144283	Liver Day 6 Post Hepatectomy B	
GSM1144284	Liver Day 6 Post Hepatectomy C	

Relevant References:

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