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Supplemental Information

An Expandable, Inducible Hemangioblast State Regulated by Fibroblast Growth Factor

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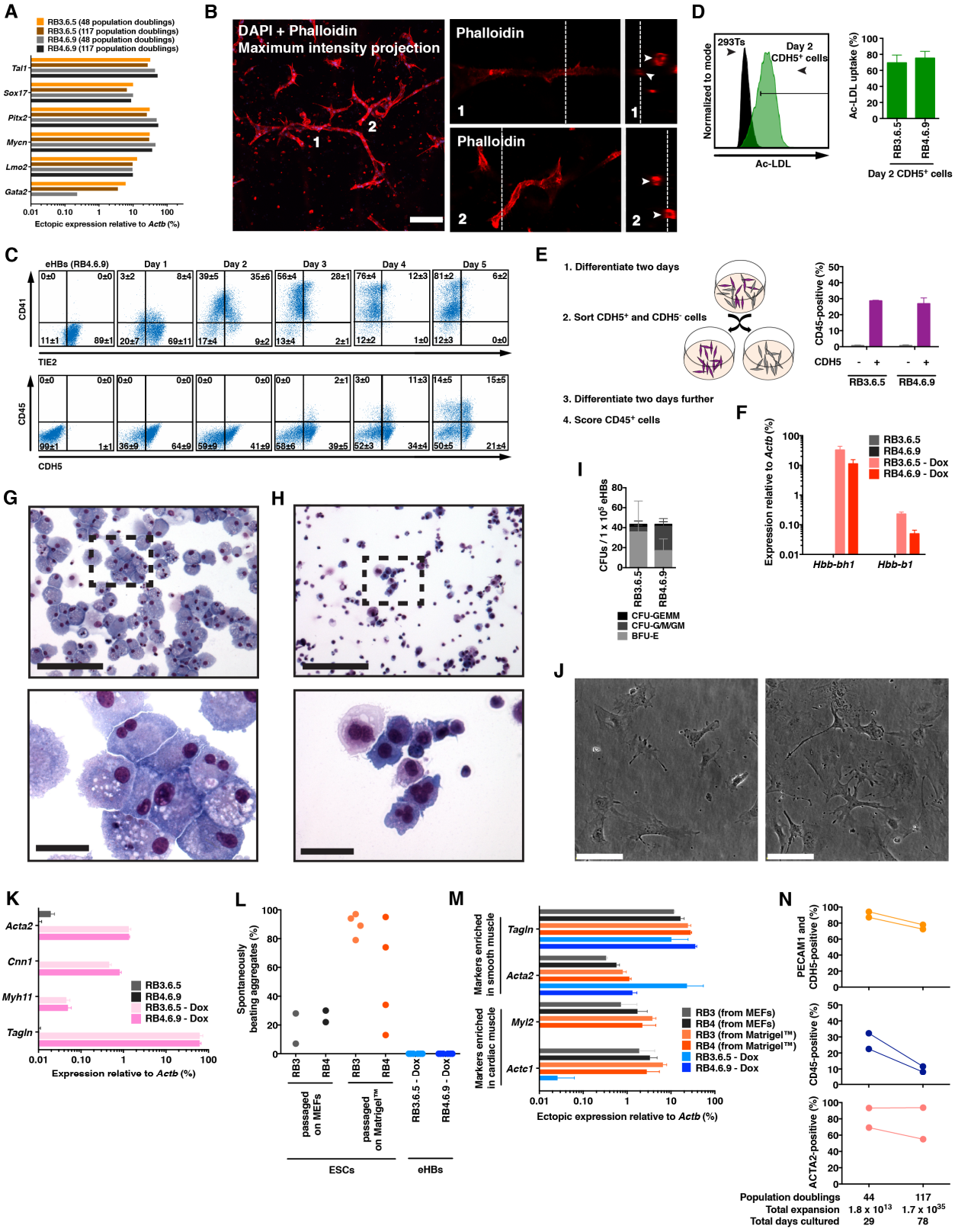
A
RB3.6.5 (48 population doublings)
RB3.6.5 (117 population doublings)
RB4.6.9 (48 population doublings)
RB4.6.9 (117 population doublings)
Tal1
Sox17
Pitx2
Mycn
Lmo2
Gata2
Ectopic expression relative to Actb (%)
B
DAPI + Phalloidin
Maximum intensity projection
Phalloidin
Phalloidin
D
293Ts
Day 2 CDH5+ cells
Normalized to mode
Ac-LDL
Ac-LDL uptake (%)
RB3.6.5
RB4.6.9
Day 2 CDH5+ cells
C
eHBs (RB4.6.9)
Day 1
Day 2
Day 3
Day 4
Day 5
CD41
TIE2
CD45
CDH5
E
1. Differentiate two days
2. Sort CDH5+ and CDH5- cells
3. Differentiate two days further
4. Score CD45+ cells
CD45-positive (%)
CDH5
RB3.6.5
RB4.6.9
F
Expression relative to Actb (%)
RB3.6.5
RB4.6.9
RB3.6.5 - Dox
RB4.6.9 - Dox
Hbb-bh1
Hbb-b1
I
CFUs / 1 x 10^5 eHBs
RB3.6.5
RB4.6.9
CFU-GEMM
CFU-G/M/GM
BFU-E
G
H
J
K
Acta2
Cnn1
Myh11
Tagln
RB3.6.5
RB4.6.9
RB3.6.5 - Dox
RB4.6.9 - Dox
Expression relative to Actb (%)
L
Spontaneously beating aggregates (%)
RB3
RB4
RB3
RB4
RB3.6.5 - Dox
RB4.6.9 - Dox
passaged on MEFs
passaged on Matrigel™
ESCs
eHBs
M
Markers enriched in smooth muscle
Markers enriched in cardiac muscle
Tagln
Acta2
Myl2
Actc1
RB3 (from MEFs)
RB4 (from MEFs)
RB3 (from Matrigel™)
RB4 (from Matrigel™)
RB3.6.5 - Dox
RB4.6.9 - Dox
Ectopic expression relative to Actb (%)
N
PECAM1 and CDH5-positive (%)
CD45-positive (%)
ACTA2-positive (%)
Population doublings 44 117
Total expansion 1.8 x 10^13 1.7 x 10^35
Total days cultured 29 78

Figure S1, related to Figure 2: Characterization of the expandable hemangioblasts
(A, F, K, M) Measurements of gene expression by qPCR, normalized to *Actb*. The y-axis is on a $\log_{10}$ scale, with a threshold of detection cutoff of 0.01%. (A) Ectopic gene expression in two eHB lines. Estimated population doublings are based on the average doubling time (16 hours) as determined in Figure 5B. (B) Lumenized tubes. Cultured as described in Figure 2C except cells were embedded in Matrigel™ for 20 days. The left image is a maximum intensity projection of a Z-stack of images, viewed along the X-Y plane. White bar = 100 μm. The numbers indicate regions of interest that correspond to the images in the middle, which are of a single Z position viewed along the X-Y plane. The dashed lines in the middle images indicate the position of the corresponding images on the right, which are the projected image viewed from the Y-Z plane. The arrows point to lumen. (C) Hematopoietic differentiation timecourse conducted and depicted as described in Figure 2D, except that here the eHB line RB4.6.9 is employed. (D, E) Further analysis of the CDH5-positive cells arising two days after the withdrawal of doxycycline, as described in Figure 2D and S1C. (D) Measurement of the ability of the cells to take up acetylated LDL. Left, an example histogram. 293T cells served as a negative control. The bar indicates the cells considered positive for Ac-LDL uptake. Note the bar is different from the bar used in Figure 2B to measure "efficient" uptake. In contrast, the bar employed here measures total uptake. Right, the average percent of cells ± S.D. taking up acetylated LDL from two independent experiments of each line. (E) Left, experimental design. eHBs were differentiated as in (C) for two days and then sorted based on their CDH5 expression by FACS (gated conservatively to ensure high purity). Sorted CDH5-positive or CDH5-negative cells were cultured for two days further, and then the percentage of cells positive for CD45 in each population was determined by FACS. Right, the average percent of cells ± S.D from two independent experiments of each line. (F) Measurements of the expression of hemoglobin in cells described in Figure 2E by qPCR. Results are the average expression ± S.D from three independent experiments of each line. (G, H) Giemsa staining of unsorted differentiated blood cells from the eHB line RB3.6.5. The boxed areas in the top images are shown below at higher magnification. Top image black bars = 200 μm. Bottom image black bars = 40 μm. (G) Macrophages differentiated for nine days. (H) Megakaryocytes differentiated for four days. (I) Hematopoietic colony forming units (CFUs) formed in methylcellulose (M3434, StemCell Technologies). Results are the average efficiency ± S.D from three independent experiments of each line. (J) Example images of smooth muscle cells differentiated as described in Figure 2H. Cells derived from the eHB line RB3.6.5 are shown on the left; cells derived from the eHB line RB4.6.9 are shown on the right. White bars = 100 μm. (K) Measurements of the expression level of smooth muscle markers in cells described in Figure 2H by qPCR. Results are the average expression ± S.D from three independent experiments of each line. (L) Quantification of spontaneously beating aggregates. A total of six experiments were performed, two with control ESCs that had been passaged on MEFs, and four with control ESCs that had been briefly passaged on Matrigel™. There were surviving aggregates from the eHB line RB4.6.9 in all six experiments to score. There were surviving aggregates from the eHB line RB3.6.5 in only two experiments (survival correlated positively with passage number), in the remaining four experiments they died out before the end of the assay. (M) Measurements of the expression level of smooth and cardiac muscle markers by qPCR of cultures from (L). Results are the average expression ± S.D from at least two independent experiments of each line. (N) Analysis of the endothelial (top graph, three days differentiation), blood (middle graph, four days differentiation), or smooth muscle (bottom graph, three days differentiation) potential of two independent eHB lines (RB3.6.5 and RB4.6.9) as determined by the indicated markers. Cells were tested for their ability to give rise to these cell types after culturing for the indicated lengths in Vm containing doxycycline. Assays were conducted as described in Figure 1 or 2. Estimated population doublings and total expansion values are based on the average doubling time (16 hours) as determined in Figure 5B.

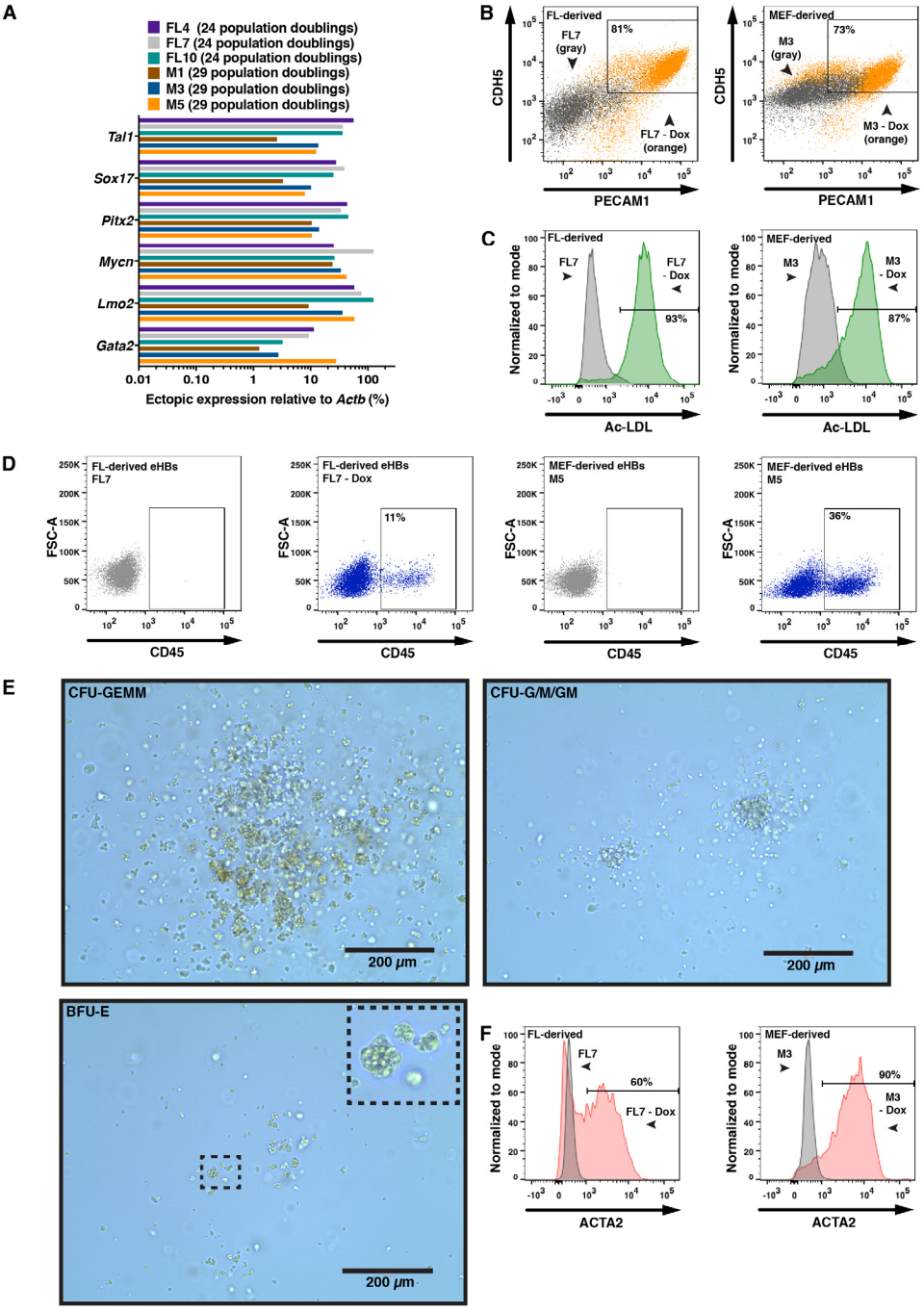

Figure S2, related to Figure 3: The eHBs can be derived from different cell sources
(A) Measurements of ectopic gene expression in six eHB lines by qPCR, normalized to *Actb*. The y-axis is on a $\log_{10}$ scale. Estimated population doublings are based on the average doubling time (16 hours) as determined in Figure 5B. (B, C, D, F) Example FACS dot plots or histograms of FL- or MEF- derived eHBs and their differentiated progeny ("- Dox"). Refer to Figure 3 to see the compiled results from all the tested lines. The eHB lines FL7 and M3 are presented because they tended to

form the median among the lines, except (D) in which M5, the only MEF-derived eHB to appreciably generate CD45-positive cells, is shown. The percentage of differentiated cells considered positive in each assay is shown. Assays were conducted as described in Figure 1 or 2. (B, C) Endothelial assays (three days differentiation). (D) Blood assays (four days differentiation). (E) Example blood colonies derived from the eHB line FL10. Images taken six days after plating in methylcellulose (nine days after the withdrawal of doxycycline). In the lower image, the small dotted box indicates the location of the inset, zoomed in. (F) Smooth muscle assays (three days differentiation).

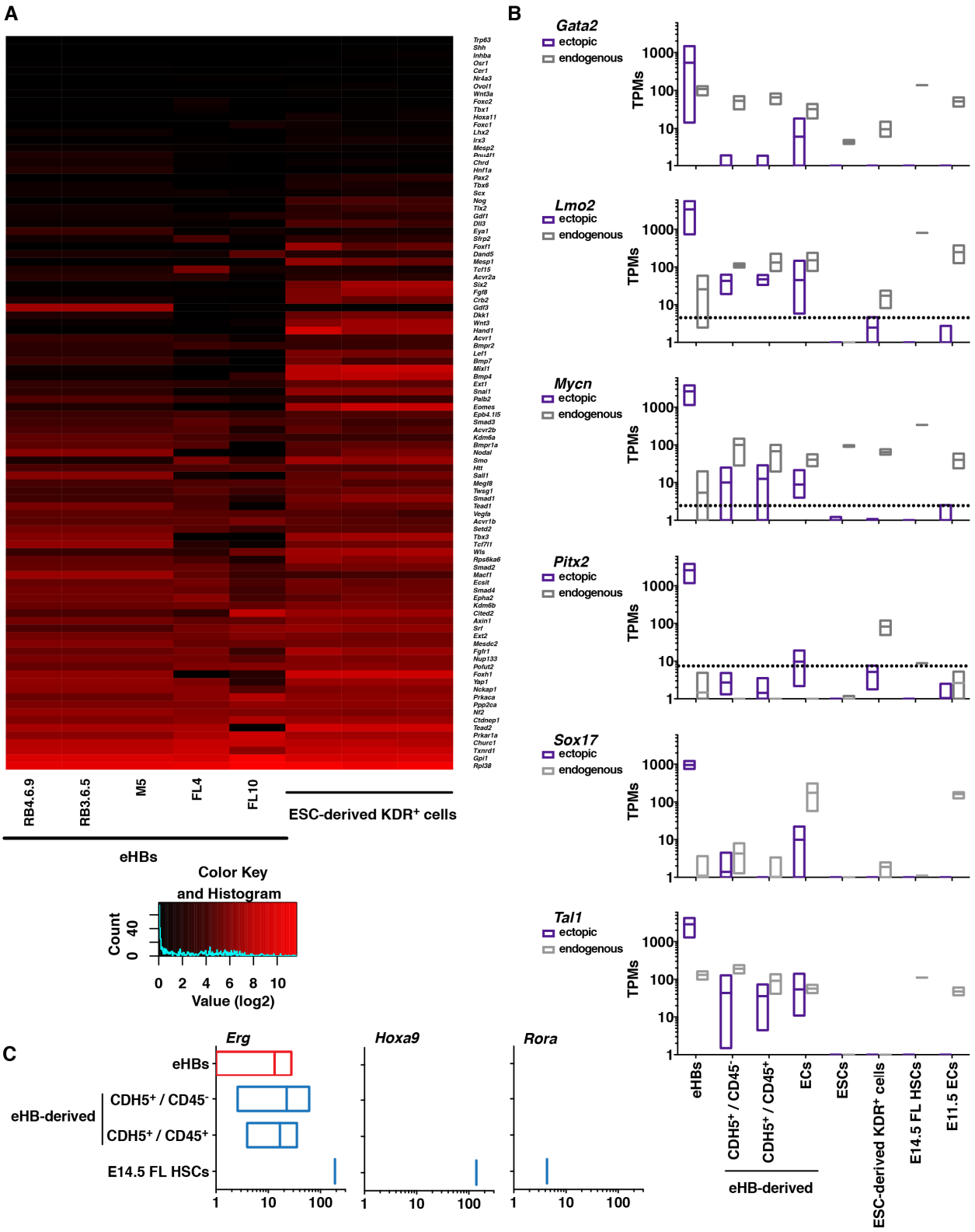
A
RB4.6.9
RB3.6.5
M5
FL4
FL10
ESC-derived KDR+ cells
eHBs
Color Key and Histogram
Count
Value (log2)
B
Gata2
Lmo2
Mycn
Pitx2
Sox17
Tal1
ectopic
endogenous
TPMs
eHBs
CDH5+ / CD45-
CDH5+ / CD45+
ECs
ESCs
ESC-derived KDR+ cells
E14.5 FL HSCs
E11.5 ECs
eHB-derived
C
Erg
Hoxa9
Rora
eHBs
eHB-derived
CDH5+ / CD45-
CDH5+ / CD45+
E14.5 FL HSCs

Figure S3, related to Figure 4: RNA-seq uncovers the developmental state of the eHBs
The data are compiled from the RNA-seq experiments performed in Figure 4. (A) Heatmap of genes associated with mesoderm development. Represented as the $\log_2$ values of the TPM measurements. (B, C) Expression levels of genes. The box sizes represent the minimum to maximum values, with the mean depicted as a horizontal line within each box. The y-axes are on a $\log_{10}$ scale, with a threshold of detection cutoff of 1 TPM. (B) The six transcription factors either ectopically or endogenously expressed. The horizontal dotted lines provide a measure of the background noise: the extent to which the algorithm (RSEM) apparently fails to distinguish endogenous from ectopic transcripts, as noted by the presence of ectopic signals in cells that do not express the ectopic genes (the four cell types furthest to the right). (C) Hematopoietic factors key to the ability to generate CFUs.

eHB cell line	Origin	Gender	Passage number	Abnormal clone(s) detected	Frequency	Associated figures
RB2.6.3	ESC	Male	5	XO	5 of 22 cells	Not characterized in detail
RB2.6.6	ESC	Male	6	XO	2 of 20 cells	Not characterized in detail
				XYY	2 of 20 cells	
RB3.6.5	ESC	Male	6	None detected	-	Figure 2, 4, 5, S1, S3
			9	XYY	2 of 22 cells	
RB3.6.10	ESC	Male	5	XYY	2 of 20 cells	Not characterized in detail
RB4.6.9	ESC	Male	7	None detected	-	Figure 2, 4, 5, S1, S3
RB4.6.3	ESC	Male	5	XO	4 of 20 cells	Not characterized in detail
M1	MEF	Female	9	Trisomy 2	4 of 20 cells	Figure 3, S2
M3	MEF	Male	9	XYY	20 of 20 cells	Figure 3, S2
M5	MEF	Female	9	None detected	-	Figure 3, 4, S2, S3
FL4	Fetal liver	Female	6	None detected	-	Figure 3, 4, 5, S2, S3
FL7	Fetal liver	Male	7	None detected	-	Figure 3, 4, 5, S2
FL10	Fetal liver	Male	7	None detected	-	Figure 3, 4, 5, S2, S3

Table S1, related to Figure 2, 3, 4, 5
The karyotypes of example eHB lines, with the threshold of detection of an abnormal clone defined as two or more analyzed cells exhibiting the same abnormality. The passage number indicates when the cells were analyzed.

Movie S1, Related to Figure 5: The effect of fibroblast growth factor on the eHBs. Cells were maintained with doxycycline in Vm. Cells were observed for 48 hours and images were taken every 30 minutes. The yellow line corresponds to 0.10 mm. The eHB line RB3.6.5 is presented.

Movie S2, Related to Figure 5: The effect of fibroblast growth factor on the eHBs. Cells were maintained with doxycycline in N2B27 medium without any additional growth factors. Cells were observed for 48 hours and images were taken every 30 minutes. The yellow line corresponds to 0.10 mm. The eHB line RB3.6.5 is presented.

Movie S3, Related to Figure 5: The effect of fibroblast growth factor on the eHBs. Cells were maintained with doxycycline in N2B27 medium supplemented with FGF2. Cells were observed for 48 hours and images were taken every 30 minutes. The yellow line corresponds to 0.10 mm. The eHB line RB3.6.5 is presented.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice, mES isolation and culture, MEF isolation and culture

The B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (Jackson, stock number 002014) mice were intercrossed with the B6.Cg-*Gt(ROSA)26Sor*$^{tm1(rtTA*M2)Jae}$/J (Jackson, stock number 006965) mouse strain to obtain progeny (referred to here as RosaBoy) homozygous for both the CD45.1 allele and the R26-M2rtTA allele. RosaBoy females and males were time mated (the morning of the day a vaginal plug was discovered was considered embryonic day 0.5). Embryos were obtained on embryonic day 3.5 from pregnant females, and embryonic stem cells were isolated and expanded from them, following a procedure adapted from previous reports (Bryja et al., 2006; Chu et al., 2011). Three independent embryonic stem cell lines, RB2, RB3, and RB4, were expanded in culture and verified to be free of clonal karyotypic abnormalities (Wicell). They were maintained in culture on irradiated mouse embryonic fibroblast feeders in Knockout DMEM (Life Technologies) supplemented with 10% FBS (StemCell, catalog number 06952), non-essential amino acids (Life Technologies), GlutaMAX™ (Life Technologies), 100 µM -mercaptoethanol (Life Technologies), 3 µM CHIR99021 (Stemgent), 1 µM PD0325901 (Stemgent), and 1000 U/mL LIF (Millipore). Mouse embryonic fibroblasts (MEFs) for reprogramming were derived from embryonic day 12.5 embryos of the B6.Cg-*Gt(ROSA)26Sor*$^{tm1(rtTA*M2)Jae}$/J (Jackson, stock number 006965) strain and cultured in MEF medium [DMEM supplemented with 10% FBS (Hyclone) and non-essential amino acids].

Differentiation of murine ES cells to hemogenic endothelium

Conducted as previously described (Chiang and Wong, 2011). Murine ES cells were cultured for two days in Matrigel™ (BD)-coated plasticware in N2B27 medium [50% N-2 medium (Neurobasal® medium and N-2 supplement), 50% B-27 medium (DMEM/F12 and B-27® supplement, minus vitamin A), non-essential amino acids, GlutaMAX™, 100 µM -mercaptoethanol, and 25 µg/mL BSA fraction V, all reagents from Life Technologies]. The medium was then changed to N2B27 medium supplemented with 4 ng/mL Activin A (R&D Systems), 3 µM CHIR99021 (Stemgent), 5 ng/mL human BMP4 (R&D Systems), and 12.5 ng/mL human FGF2 (synthesized and purified in our lab). After two additional days, cells were replated in Vm [N2B27 medium supplemented with 20 ng/mL murine VEGF (R&D Systems or Peprotech), 250 µM 8-Bromoadenosine 3•,5•-cyclic monophosphate sodium salt (Sigma), 4 µM SB431542 (Sigma or Cellagen), 20 ng/mL human BMP4, and 12.5 ng/mL human FGF2], and cultured further. When performing assays with introduced doxycycline inducible factors, the same procedure was followed, except the ES cells were electroporated as described below prior to plating in N2B27 medium, and 2 µg/mL doxycycline (Sigma) was added to Vm to activate gene expression in the ESC-derived mesoderm cells (day four of the differentiation).

Electroporation

Murine ES cells, embryonic fibroblasts [either cell type dissociated with TypLE (Life Technologies)] or fetal liver cells were suspended N2B27 medium supplemented with 25 mM HEPES. Depending on the cell type, 2-10 x 10^5 cells in 0.5 mL medium were mixed with 30 µg DNA (purified using the HiSpeed Plasmid Maxi Kit, Qiagen) and 1 µg hyPBase mRNA (Yusa et al., 2011) (synthesized using mMESSAGE mMACHINE T7 Ultra Kit, Life Technologies from a PCR product encoding the T7 promoter, hyPBase, and BGH polyA sequences). Cells were then placed in 0.4 cm cuvettes (Biorad) and electroporated with Gene Pulser II (Biorad) at 250 volts, 500-1000 µFarads, and infinite resistance.

Induction of eHBs from MEFs or fetal liver cells
B6.Cg-*Gt(ROSA)26Sor*$^{tm1(rtTA*M2)Jae}$/J MEFs and RosaBoy fetal liver (FL) cells were employed. Total fetal liver cells were obtained from day 14.5 embryos as described below, except that they were unsorted. Both MEF and primary FL cells were electroporated as described above and placed on Matrigel™ -coated plasticware. The MEFs were cultured in MEF medium containing 2 µg/mL doxycycline and the fetal liver cells were cultured in Stemline II (Sigma) supplemented with 100 ng/mL SCF, 100 ng/mL IL-3 (R&D Systems or Peprotech), and 2 µg/mL doxycycline. The cells were gradually transitioned to N2B27 medium containing 12.5 ng/mL FGF2 and 2 µg/mL doxycycline (BFD) over the following days by removing about half the medium each day and replacing it with fresh BFD.

Quantitative PCR (qPCR) analysis
Total RNAs were purified using RNeasy kits (Qiagen) with either on-column DNAse treatment or genomic DNA removal columns, and the purified RNAs were reverse transcribed with SuperScript VILO (Life Technologies). TaqMan® realtime qPCR reactions (20 µL total volumes) were performed in wells containing TaqMan® Universal Master Mix II with or without UNG (Life Technologies), 1 µL of the cDNA reaction, and 1 µL of the following primer/probe sets (all from Life Technologies): Mm00607939_s1 (*Actb*), Mm01611268_g1 (*Hbb-b1*), Mm00433932_g1 (*Hbb-bh1*), Mm00487032_m1 (*Cnn1*), Mm00725412_s1 (*Acta2*), Mm00441661_g1 (*Tagln*), Mm00443013_m1 (*Myh11*), Mm01333821_M1 (*Actc1*), and Mm00440384_M1 (*Myl2*).
SYBR® Green realtime qPCR reactions (also 20 µL total volumes) were performed in wells containing 1X *Power* SYBR® Green master mix (Life Technologies), 1 µL of the cDNA reaction and 0.3 µM of the following primers listed below. The expression levels of the ectopic genes were detected using a common reverse primer complementary to the vector sequence and a forward primer complementary to the particular gene sequence. The *Actb* primer sequences have been previously reported (Pathak et al., 2014).

Gene	Primer	Sequence (5` to 3`)
Actb	Forward	CCA CCA TGT ACC CAG GCA TT
Actb	Reverse	AGG GTG TAA AAC GCA GCT CA
Ectopic factor	Reverse	GCT CTA GAG TCG GTG ACT AGT AT
Gata2	Forward	CCT CCT CCA GTC TCT CCT TT
Lmo2	Forward	CCT TCT CAT CAA CTC CGA CAT AG
Mycn	Forward	CAG CAG CAG TTG CTA AAG AAG
Pitx2	Forward	CCT GAG TGC TTG CCA GTA T
Sox17	Forward	TCG AAC AGT ATC TGC CCT TTG
Tal1	Forward	CTC CAT CCT GCC CTG CT

Reaction signals were measured on a ViiA 7 (Life Technologies) and analyzed by the ViiA 7 v1.1 - v1.2.3 software. When normalizing signals to *Actb*, the amplification efficiency was assumed to be

100% (e.g. 1 CT = 2 fold increase). The expression level of each gene was then described as a percentage of the expression level of *Actb*.

Small molecule inhibitors
The following small molecules were employed: PD173074 (Mohammadi et al., 1998) (Stemgent), PI828 (Gharbi et al., 2007) (Tocris), PD0325901 (Barrett et al., 2008) (Stemgent), U0126 (Favata et al., 1998) (Sigma). All were dissolved in DMSO and used at the concentrations indicated in Figure 5.

Endothelial differentiation of eHBs
Cells were dissociated by incubation and trituration in HEH [0.5 mM EDTA (Fisher) in 1x HBSS supplemented with 10 mM HEPES (both from Life Technologies)] and replated at 1 x 10^5/10 cm^2 on Matrigel™ -coated plasticware in Vm and cultured for three days. To form capillary-like networks, cells were plated at 1.5 x 10^6/60 cm^2 in Vm for seven days and then dissociated and embedded in Matrigel™ (4.6 mg/mL, BD, 354230) at a concentration of 10 x 10^6 cells/mL. The three-dimensional (3D) Matrigel™/cell constructs were formed in µ-Slide Angiogenesis wells (Ibidi, 81506). The 3D constructs were cultured in Vm supplemented additionally with 50 ng/mL murine VEGF and human FGF2 for ten or twenty days and then fixed in paraformaldehyde and stained with Alexa Fluor® 568 Phalloidin (Life Technologies, A12380) and DAPI.

Blood differentiation of eHBs
All experiments were conducted at 1.5% O_2 in a hypoxic glove box (Coy Labs) unless otherwise specified. Cells were dissociated by incubation and trituration in HEH and replated at 0.5-1 x 10^5/10 cm^2 on Matrigel™ -coated plasticware. To generate CD45-positive cells, or for the hematopoietic differentiation timecourses in Figure 2D or S1C, eHBs were placed in N2B27 medium supplemented with SCIF [3 µM CHIR99021 (Stemgent), 100 ng/mL murine SCF, 100 ng/mL murine IL-3, 100 ng/mL murine FLT3L (the latter three from R&D Systems or Peprotech) (Ruiz-Herguido et al., 2012; Taoudi et al., 2008)] (SCIF medium) for four days unless otherwise noted. For erythrocyte differentiation, a previously established protocol was adapted (Sturgeon et al., 2012). Cells were placed in N2B27 medium supplemented with 5 ng/mL murine VEGF (R&D Systems or Peprotech), 3 µM CHIR99021, 1 ng/mL Activin A (R&D Systems), 1 ng/mL human BMP4 (R&D Systems), and 50 µg/mL ascorbic acid (Sigma) for two days. The medium was then changed to N2B27 medium supplemented with 5 ng/mL murine VEGF, 1 ng/mL Activin A, 1 ng/mL human BMP4, 50 µg/mL ascorbic acid, and 100 ng/mL murine EPO (R&D Systems) and cells were cultured for two additional days. For macrophage differentiation, portions of a previously reported protocol were employed (Choi et al., 2011). Cells were differentiated as described above in SCIF medium for three days, then transferred to N2B27 medium supplemented with 200 ng/mL GM-CSF (Peprotech) on low adherence plates (StemCell Technologies) and cultured for two days at 21% (atmospheric) O_2, and finally transferred to gelatinized plates in IMDM (Life Technologies) supplemented with 10% FBS (Hyclone), 10 ng/mL IL-1 (R&D Systems) and 20 ng/mL M-CSF (Peprotech) and cultured for four days at 21% O_2. For megakaryocyte differentiation, cells were placed in Stemline II medium (Sigma) supplemented with SCIF and 100 ng/mL murine TPO (Peprotech or R&D) and cultured for two days. The medium was then replaced with Stemline II medium containing the same supplements except for CHIR99021, and the cells were cultured for two additional days. Giemsa stains (GS500, Sigma) were performed following the manufacturer's instructions on slides prepared using a Cytospin™ 2 (Shandon). For CFU-C assays, cells growing sub-confluently were placed in N2B27 medium (fetal liver- or MEF-derived lines) or Stemline II medium (ES- or MEF-derived lines) supplemented with SCIF and cultured for three days. Cells were pelleted and gently resuspended in methylcellulose medium (M3434, StemCell Technologies), cultured for three days at 1.5% O_2, and then transferred to 21% O_2. Colonies were scored six days after plating in methylcellulose.

Smooth muscle differentiation of eHBs
Cells were dissociated by incubation and trituration in HEH and replated at 5 x 10^5/10 cm^2 on plasticware coated with 5 µg/10 cm^2 murine collagen IV (Fisher Scientific) in smooth muscle medium

[MEM supplemented with GlutaMAX™, 50 μM -mercaptoethanol (all from Life Technologies), and 10% FBS (Hyclone)], conditions adapted from a previously described protocol (Xiao et al., 2007).

Cardiomyocyte differentiation of ESCs and eHBs
Two previously described protocols were combined and adapted (Sargent et al., 2009; Zhang et al., 2012). The ESCs employed for these assays were either maintained on MEFs as described above or briefly passaged (four to six days) on Matrigel™ in N2B27 medium supplemented with 3 μM CHIR99021, 1 μM PD0325901 and 1000 U/mL LIF, to remove MEF feeders in a manner akin to a previous report (Chiang and Wong, 2011). Cells were dissociated and plated in AggreWell™ plates (StemCell Technologies) at 1000 cells/microwell (except for the ESCs passaged on MEFs, which were plated at 300 cells/microwell) in DMEM supplemented with 10% FBS (StemCell Technologies) and GlutaMAX™. The eHB cultures additionally contained 2 μg/mL doxycycline for the first four days of the protocol. Cells were cultured in the AggreWell™ plates for two days and then the resulting aggregates were gently transferred to low adherence plates (StemCell Technologies) and cultured on a Nutator mixer (24 to 25 RPM, BD) for another four days. The aggregates were then plated on gelatinized plates in the same differentiation medium as above but now additionally supplemented with 100 nM PD173074 and cultured for four more days. Aggregates were scored and lysed ten days from the start of the protocol.

FACS analysis and cell sorting
Cells were stained in cold HBSS+ [1x HBSS (Life Technologies), 2% FBS (HyClone), and 10 mM HEPES (Life Technologies)]. Protocols using this staining buffer can be found at https://www.bcm.edu/research/labs/goodell/index.cfm?PMID=17588. All antibodies, which recognize murine epitopes, were used at a working dilution of 1:100, unless otherwise stated. The following antibodies were purchased from eBioscience: anti-CD42D-APC (17-0421-80), anti-CD71-PE (12-0711-83), anti-TER119-FITC (11-5921), anti-TIE2-PE (12-5987-83), anti-CD16/32 (14-0161-85), anti-CD11B-PE/Cy7 (25-0112-82), and anti-CDH5-biotin (13-1441-80 or -82) [along with streptavidin-PE (12-4317-87)]. The following antibodies were purchased from BD: anti-PECAM1-APC (561814), anti-CD45-FITC (553080), and anti-CDH5-PE (562243). The following antibodies were purchased from Biolegend: anti-CD150-PE/Cy7 (115914), anti-CD41-FITC (133904), anti-CD45.1-APC (110714), and anti-CD48-APC (103412). The following antibodies were purchased from StemCell Technologies: anti-CD201-PE (60038PE) and anti-CD45-FITC (10709). An anti-KDR-APC antibody (used at a 1:20 dilution) was purchased from R&D (FAB4432A). An anti-ACTA2-FITC antibody was purchase from Sigma (F3777). The expression of CDH5 on the emerging blood progeny of the eHBs is low (Figure 2D, S1C), thus for assays with these cells we find that the use of the biotinylated CDH5 antibody coupled to streptavidin-PE, in a manner similar to a previous report (Rybtsov et al., 2011), gives an improved separation of the positive and negative cells in contrast to the antibody directly conjugated to PE. To measure acetylated LDL uptake, cells were incubated for two hours with acetylated LDL conjugated to Alexa Fluor® 488 (Life Technologies). Live stained cells were resuspended for analysis or sorting in HBSS+ with either propidium iodide or DAPI for the exclusion of dead cells. To stain for ACTA2, a protocol for intracellular staining was adapted from one previously described (Xie et al., 2013). The cells were fixed in 4% paraformaldehyde in PBS at 37C for at least 30 minutes, washed with HBSS+, permeabilized in ice-cold 90% methanol for at least one hour, and then blocked and stained in HBSS+ with anti-ACTA2-FITC. Cells were examined on either a FACSAria IIIu or a FACSCanto II (BD) and analyzed using FACSDiva software version 6.1.3 (BD) and FlowJo (Tree Star) software version 9.5.1 and version X 10.0.7r2. Cells were sorted on the FACSAria IIIu. Controls for fluorescence compensation and FACS gating included cells stained with single fluorochromes, and to serve as negative controls, stained cells known to not express the marker(s), such as 293T cells or the parental cell lines.

Fetal liver HSC isolations
Fetal liver HSCs were obtained by a procedure adapted from a previous report (Kent et al., 2009). Embryonic day 14.5 fetal livers from B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ mice were dissected on ice in

HBSS+ and dissociated by gently mashing through a 40 µm filter (BD). Prior to staining, the non-specific binding of antibodies was blocked by incubating cells on ice in HBSS+ with rat serum (diluted 1:100) and anti-mouse CD16/32 (diluted 1:1000). Cells were then stained on ice in HBSS+ with anti-CD150-PE/Cy7 and subsequently with anti-Cy7 microbeads (Miltenyi Biotec). Cells bound by the microbeads were positively selected using an AutoMACS Pro (Miltenyi Biotec). These cells were then additionally stained on ice with anti-CD201-PE, anti-CD45-FITC, and anti-CD48-APC. Fetal liver HSCs, defined as $CD150^{+}/CD201^{+}/CD48^{-}/CD45^{+}$ were then sorted by FACS into Trizol (Life Technologies) for analysis by RNA-seq.

Primary endothelial cell isolations

Embryos of B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ mice were isolated on embryonic day 11.5, along with their yolk sacs. The embryos or yolk sacs were dissociated in 0.25% collagenase (dissolved in HBSS supplemented with 20% FBS and 10 mM HEPES) at 37C for 25-30 minutes, triturated, and then passed through a 40 µm filter (BD) to yield single cell suspensions, a process adapted from a previously described protocol (Morgan et al., 2008). The cells were stained on ice in HBSS+ with anti-CDH5-PE and anti-PECAM1-APC. Populations enriched for endothelial cells were defined as $CDH5^{+}/PECAM1^{+}$, and these cells were sorted by FACS into Trizol for analysis by RNA-seq.

RNA-seq preparation, sequencing, and analysis

The murine ESC lines were depleted of MEF feeders by culture on Matrigel™ for four days as described above in “Cardiomyocyte differentiation of ESCs and eHBs.” Total RNAs were purified from Trizol (Life Technology) over RNeasy columns (Qiagen) with DNAse treatment. To make cDNA libraries for sequencing, at least 10 ng of total RNA was reverse transcribed and prepared for sequencing using the Tru-seq RNA Sample Prep kit v2 (Illumina) following the manufacturer's instructions, including the recommended 15 cycles of PCR for amplification. The cDNA libraries were sequenced on an Illumina HiSeq 2500. Base pairs were called by the software Casava v1.8.2 (Illumina). Reads were mapped to a mm10-based transcriptome reference, using the software Bowtie v0.12.9 (Langmead et al., 2009). The reference included all Refseq mRNA transcripts annotated with “NM_” identifiers, augmented with mitochondrial transcripts, hand-selected ENSEMBL transcripts for *Gata2*, *Lmo2*, *Mycn*, *Pitx2*, *Sox17*, and *Tal1* and the predicted transcripts from the ectopic vectors (Macias et al., 1996) encoding these six genes. The average number of aligned reads (± standard deviation) for the dataset was $2.9 \times 10^{7} \pm 1.3 \times 10^{7}$. Mapped reads were used to calculate TPMs using the software RSEM v1.2.3 (Li and Dewey, 2011). TPMs calculated by RSEM for non-mitochondrial transcripts were renormalized to a scale of one million non-mitochondrial transcripts before any further analysis. The software R v3.0.2 was employed either for principal component analysis (PCA) using the packages prcomp and scatterplot3d or for hierarchal clustering using hclust (with “complete” algorithm) and heatmap.2. The package heatmap.2 was also used to generate the heatmap for Figure S3A. The genes presented in Figure S3A were chosen from lists provided by AmiGO 2 (http://amigo.geneontology.org/amigo/landing) for Mus musculus using the terms “mesoderm development” (GO:0007498) “mesoderm formation” (GO:0001707), and “mesoderm morphogenesis” (GO:0048332). The PCA was performed on the $\log_2$(TPM) values of all genes whose expression was greater than two TPM for at least one replicate. A description of principle component analysis can be found elsewhere (Ringner, 2008). The hierarchal clustering was performed on the Spearman correlations between samples, using only genes whose expression was greater than eight TPM for at least one sample, with a $\log_2$ fold ratio greater than two between the highest and lowest expressers. In hierarchical clustering, the lengths of the branches are qualitatively proportional to the difference between samples. The GEO accession number for the RNA-seq data reported in this paper is GSE60896.

Cell imaging

Time lapse and single images were taken on a BioStation CT microscope (Nikon) and analyzed using CL Quant software version 3.00. Time-lapse images were converted into QuickTime movie files using ImageJ software version 1.43u, 50% image scaling, five frames per second, Sorenson

compression, and maximum quality. ImageJ is freely available at http://rsbweb.nih.gov/ij/. ImageJ software was used to convert TIFF images into JPEG images using default settings, except for Figure S1J, in which the brightness and contrast of the images was adjusted for visualization upon reduction in size by changing the minimum and maximum displayed values to 35 and 115 respectively. Single images were also taken on an Evos FL Auto microscope using software revision 23486 and 26049 or on a Zeiss Axio Scope.A1 using iVision-Mac software V4.5.0 (BioVision Technologies). Single slice, fluorescent, confocal images were taken on a Nikon Eclipse Ti inverted microscope configured with Nikon A1R Confocal and analyzed using NIS-Elements C software V4.20.00 (Build 967).

Other software used
The software Mstat version 5.5.1 (N. Drinkwater, University of Wisconsin) was employed for statistical analysis. It is freely available at http://www.mcardle.wisc.edu/mstat. To generate figures and text, the following software packages were used: Microsoft Word, Excel, and Powerpoint for Mac v14.3.6 – v14.4.4; Adobe Illustrator CSS v15.0.0 – v15.0.2; Endnote vX7.0.1; and Prism 6 for Mac v6.0c.

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