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Supplemental Data

Fate Tracing Reveals the Endothelial Origin of Hematopoietic Stem Cells

Ann C. Zovein, Jennifer J. Hofmann, Maureen Lynch, Wendy J. French, Kirsten A. Turlo, Yanan Yang, Michael S. Becker, Lucia Zanetta, Elisabetta Dejana, Judith C. Gasson, Michelle D. Tallquist, and M. Luisa Iruela-Arispe

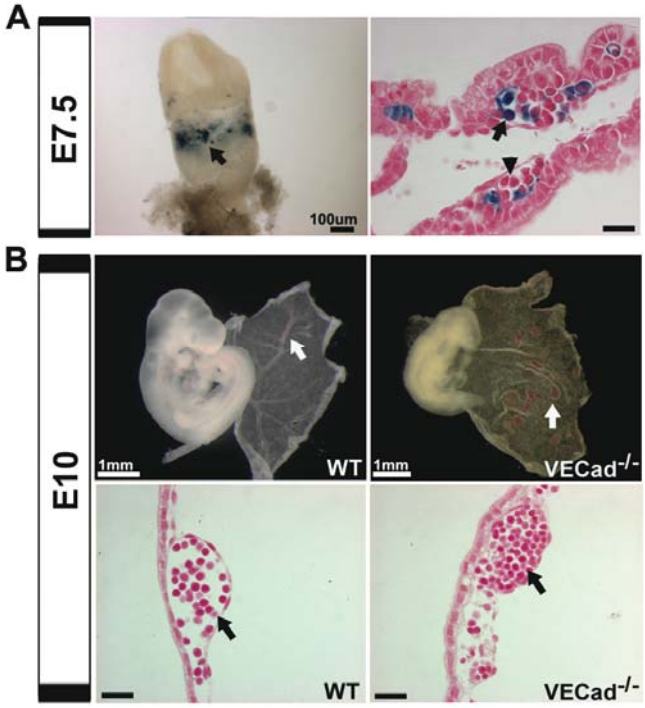
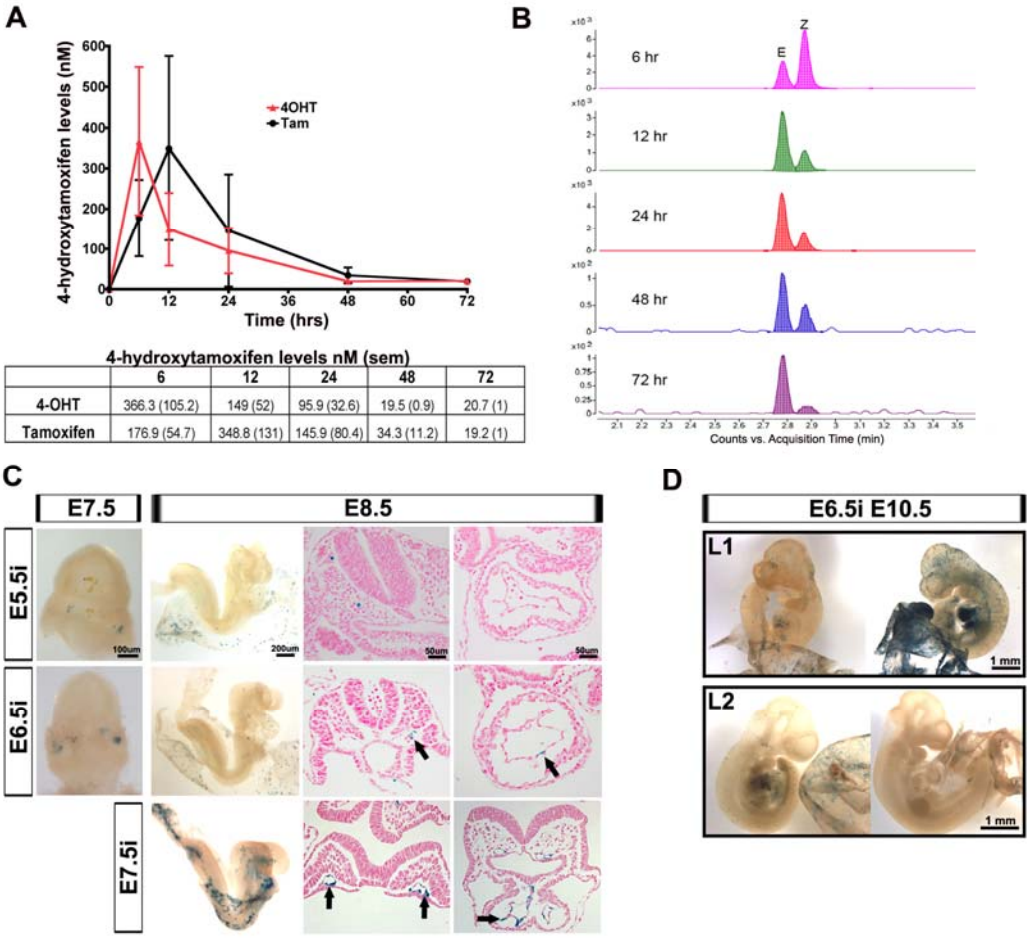


Figure S1: Emergence of early yolk sac population is independent of VE-cadherin. (A) The constitutive VE-cadherin Cre line (crossed to the ROSA26 Cre reporter) demonstrates βgal labeling (blue) within the early yolk sac population at E7.5. Whole mount (left) and histological analysis (right), note both endothelial and hematopoietic populations are labeled (arrows). However, there is also a significant proportion of the hematopoietic population not labeled (right panel, arrowhead). (B, top panels) E10 VE-cadherin null (KO) embryos exhibit defects in yolk sac vascular remodeling compared to WT (arrows). (B, bottom panels) Histological analysis of the VE-cadherin null yolk sac

depicts cells morphologically similar to circulating hematopoietic cells in the WT (arrows). Nuclear counterstain in red. Scale bar = 25μm, unless otherwise specified.



4-hydroxytamoxifen levels nM (sem)

	6	12	24	48	72
4-OHT	366.3 (105.2)	149 (52)	95.9 (32.6)	19.5 (0.9)	20.7 (1)
Tamoxifen	176.9 (54.7)	348.8 (131)	145.9 (80.4)	34.3 (11.2)	19.2 (1)

Figure S2: Tamoxifen kinetics and labeling efficiency. (A) Serum levels of 4-hydroxytamoxifen (nM) after 1mg intraperitoneal injection of either tamoxifen or 4-hydroxytamoxifen (4OHT) in WT (C57BL6/J) females. Peak levels of 4OHT are achieved by 6 hrs when the metabolite is administered directly, while the parent drug (tamoxifen) peaks by 12 hrs. Levels detected by tandem Mass Spectrometry listed as mean +/- sem in table, n=3 each time point. (B) E and Z isomer ratios at various times post dose. (C) Inducible VE-cadherin Cre/R26R after 1mg tamoxifen induction at E5.5 (top row), E6.5 (middle row) and E7.5 (bottom row). E7.5 and E8.5 embryos show βgal labeling (blue). Histological sections of E8.5 paired dorsal aortae (left panels) and endocardium (right panels) with βgal labeling (blue, arrows) depending on the age of

induction. Nuclear counterstain in red. Scale bar as specified per column. (D) Variation in labeling (βgal, blue) within litters is demonstrated by embryos that represent the most and least stained within their respective litter at E10.5 after E6.5 induction of the inducible VE-cadherin Cre/R26R line. L1 – litter 1, L2 – litter 2.

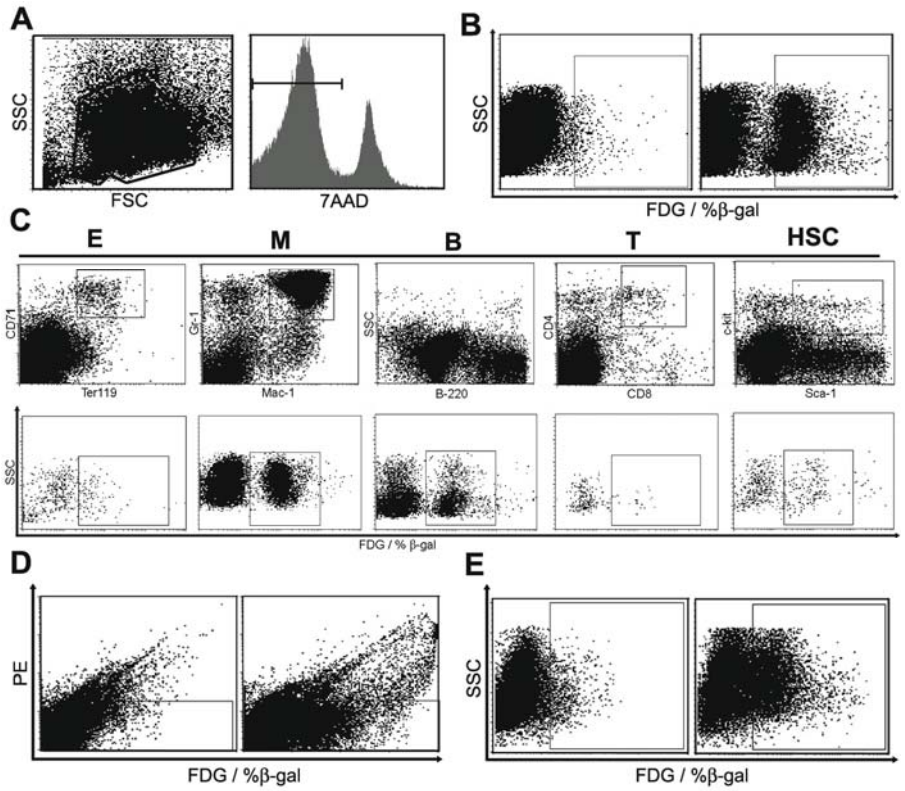


Figure S3: Flow cytometry analysis of fetal liver and adult bone marrow hematopoietic populations. (A) Cells were gated on size and viability. (B) Fluorescein di-β-D-galactopyranoside (FDG) allows measurement of β-galactosidase activity in control adult bone marrow (left) and bone marrow after E9.5 tamoxifen exposure (right). (C) Lineage analysis of bone marrow involved gating the appropriate population per lineage as noted in the top panels (E - erythroid, M - myeloid, B - B cell, T – T cell, HSC – hematopoietic stem cell), and further determining the βgal activity within the gated population (lower panels). (D) As the FDG βgal assay can contribute to autoflourescence in the PE channel, which was increased within the fetal liver population, cells were also negatively gated for PE autofluorescence prior to evaluation of total β-galactosidase activity (for cells not stained with lineage markers). Left panel depicts negative control of

fetal liver population, right panel demonstrates FDG population of E13.5 fetal liver after tamoxifen exposure at E9.5. (E) Fetal liver analysis demonstrating the negative control (left) and fetal liver post E9.5 tamoxifen exposure (right).

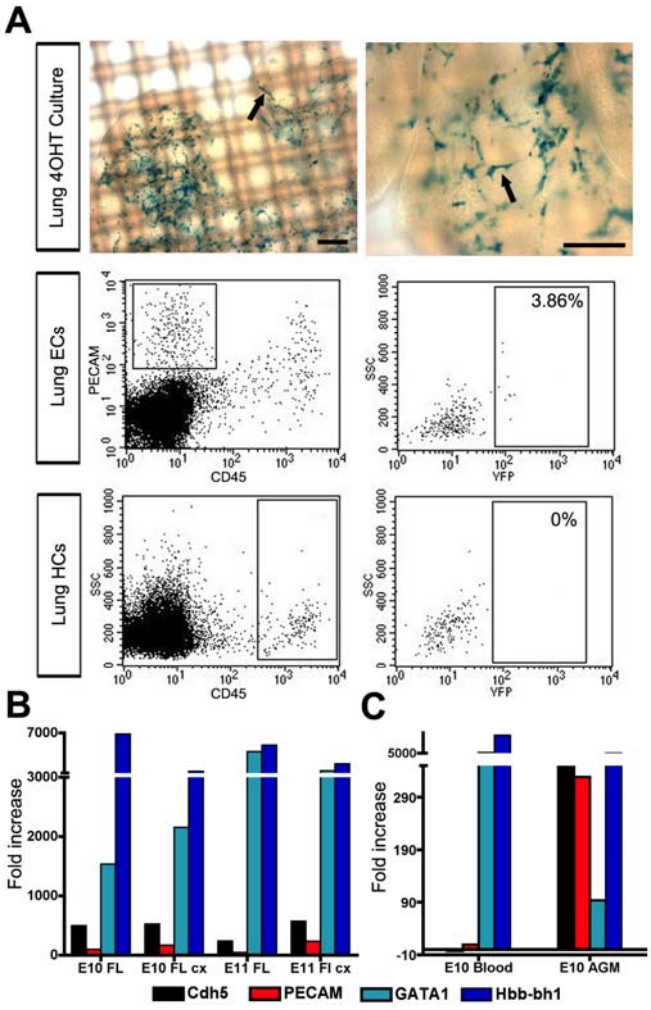


Figure S4: VE-cadherin Cre induction *ex vivo*. (A) Organ cultures of E12.5 lungs induced *in vitro* with 4-hydroxytamoxifen were evaluated for βgal and EYFP expression. Cells morphologically resembling endothelium are labeled, βgal blue (top row, arrows). FACS analysis of endothelial cells (PECAM+CD45-) demonstrate labeled endothelium, but unlabeled hematopoietic cells (CD45+). Scale bars=100μm. (B-C) Wild type (WT) embryonic tissues were evaluated for hematopoietic and endothelial gene expression (as compared to control fibroblasts) by RT-PCR Array. Cdh5: VE-cadherin, PECAM: PECAM-1, GATA1, Hbb-bh1: Hemoglobin Z, beta-like embryonic chain (B) Comparison of gene expression between WT fetal livers and WT fetal livers after *in vitro*

culture (at both E10.5 and E11.5) demonstrates minimal changes. (C) E10.5 peripheral blood is compared to the pooled AGM of the same litter and noted to have negative fold VE-cadherin expression, minimal PECAM expression and high expression of hematopoietic genes GATA1 and Hb-bbh1.

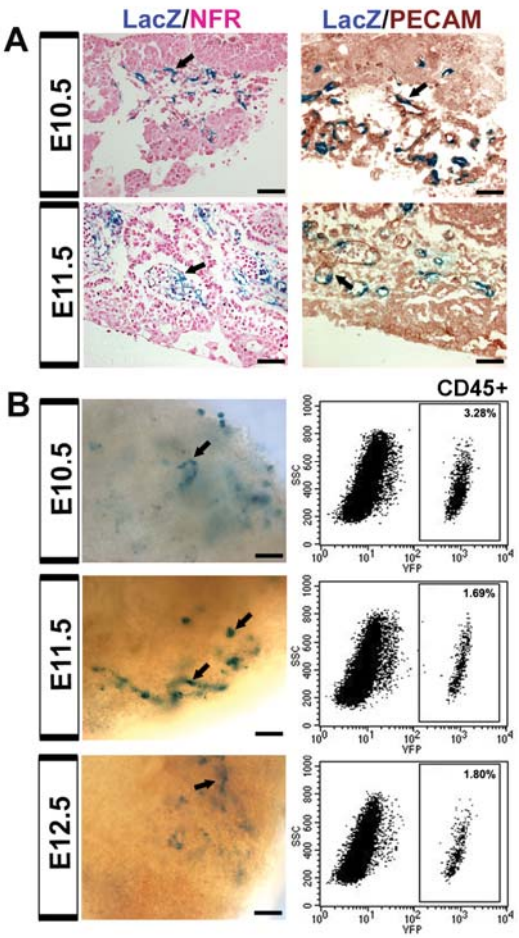


Figure S5: Placental vasculature is also capable of hematopoiesis. (A) Inducible VE-cadherin Cre crossed with ROSA26R LacZ labels placental vasculature at E10.5 and E11.5 after E9.5 tamoxifen induction (arrows). (A, left panels) βgal in blue, nuclear counterstain in red (nuclear fast red: NFR). (A, right panels) PECAM-1 antibody labeling in brown, βgal in blue. Note co-expression of PECAM-1 and βgal (arrows). (B, left panels) Organ cultures of E10.5, E11.5, and E12.5 placentas induced *in vitro* with 4-hydroxytamoxifen were evaluated for βgal expression. Cells morphologically resembling endothelium and hematopoietic cells are labeled (arrows). (B, right panels) Placentas of inducible VE-cadherin Cre crossed with ROSA26R EYFP line were induced *in vitro* as organ cultures, then grown in hematopoietic colony assays for 7-10 days. The cells then underwent FACS analysis for EYFP expression within the CD45+ (hematopoietic) population. Note that at all time points the placenta was capable of hematopoiesis. Scale bars = 50μm.

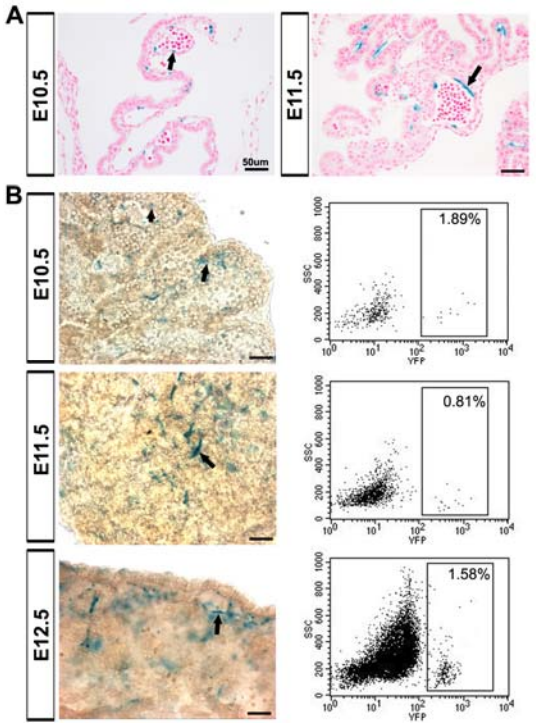


Figure S6: Yolk sac endothelial induction also results in hematopoiesis. (A) Inducible VE-cadherin Cre crossed with R26R LacZ labels yolk sac vessels at E10.5 and E11.5 after E9.5 tamoxifen induction (arrows). (B, left panels) Organ cultures of E10.5, E11.5, and E12.5 yolk sacs induced *in vitro* with 4-hydroxytamoxifen were evaluated for βgal expression. Cells morphologically resembling endothelium and hematopoietic cells are labeled (arrows). (B, right panels) Yolk sac organ cultures also underwent FACS analysis for EYFP expression within the CD45+ (hematopoietic) population and are noted to have some hematopoietic induction (percentage positive is average of two litters). Scale bars = 50μm.

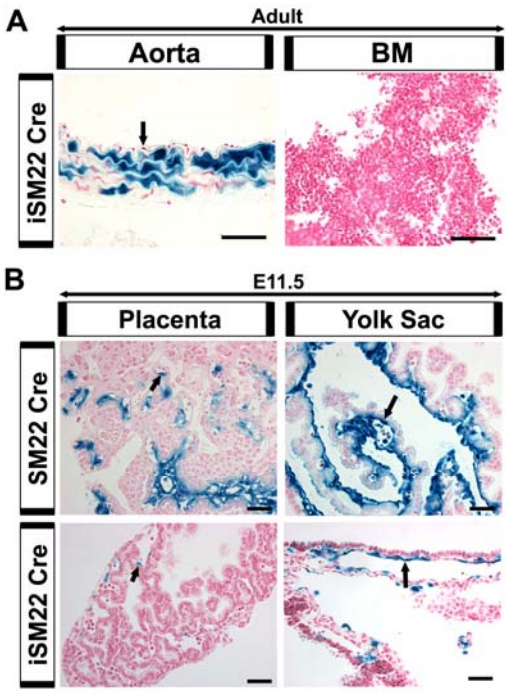


Figure S7: SM22α Cre does not label adult aortic endothelium, but may label embryonic yolk sac and placental vessels. (A) The ability of the SM22α Cre to label the aortic endothelium appears to be restricted to development as adult induction of the tamoxifen inducible SM22α Cre does not result in endothelial labeling (arrow) or bone marrow βgal activity. βgal in blue, nuclear counterstain in red. (B) Both the E11.5 placenta and yolk sac in the constitutive SM22α Cre (top row) and the inducible SM22α Cre (induced at E9.5, bottom row) demonstrate labeling of blood vessels (arrows). βgal in blue, nuclear counterstain in red. Scale bars = 50μm

Supplemental Text

Tamoxifen kinetics and cell labeling efficiency

To understand the population labeled by administrating one dose (1mg) of tamoxifen i.p., we employed several assays. After measuring sera levels of the active metabolite 4-hydroxytamoxifen (4OHT) after tamoxifen administration, we noted a theoretical window with minimally active levels up to 72 hrs post dose (Fig. S2A). However, we also noted differences in 4OHT isomer concentrations (Fig. S2B). There exist two isomers that were detected in the sera: E (cis) and Z (trans) isomers. Of which the Z (trans) isomer, an unstable isomer that converts to other forms in tissues, is a potent anti-estrogen (100X) compared to the E (cis) isomer as measured by fixed ring assays (Murphy et al. 1990). As the Z isomer is a potent anti-estrogen, it may also theoretically have a greater affinity for the mutated estrogen receptor utilized in the tamoxifen inducible Cre system (Feil et al. 1997). Thus, while total sera levels are within the active range by 72 hrs, the Z isomer fraction is significantly less (Fig. S2B) and thus may result in appreciably decreased cell labeling efficiency in comparison to earlier time points when the Z isomer fraction is high.

To investigate actual labeling efficiency, different dosing time points were used in early development. The yolk sac begins exhibiting VE-cadherin expression at E7.5 (Breier et al. 1996), while the intra-embryonic endothelium begins its formation at E8.0-8.5. When tamoxifen is administered at E5.5, there is labeling within the yolk sac at E7.5 (48 hrs later), but intra-embryonic endothelium remains unlabeled at E8.5 (72 hrs), suggesting a 48 hour window of activity (Fig. S2C). This is supported by injection at E6.5 with subsequent yolk sac and intraembryonic labeling at E7.5 and

E8.5 respectively (Fig. S2C). Lastly, there exists variation of labeling between littermates as evidenced in Figure S2D.

Evaluation of the in vitro culture system and VE-cadherin expression in peripheral blood

VE-cadherin protein has well documented expression in the HSCs of the AGM (Fraser et al. 2003) and of the fetal liver (Kim et al. 2005; Taoudi et al. 2005). The fetal liver *in vitro* system was designed to address whether the previously characterized fetal liver HSC protein expression was a result of ongoing VE-cadherin promoter expression (which could nullify our ability to trace earlier populations), or due to historical VE-cadherin promoter activity. To further address this, we have cultured the E12.5 fetal lung, a non-hematopoietic vascular organ that demonstrated endothelial induction does occur without hematopoiesis (Fig. S4A), and thus the AGM, placenta and yolk sac endothelium is special in its "hemogenic capacity". In addition, wild-type (WT) C57BL6/J fetal livers at E10.5 and E11.5 were cultured for 24hrs in 4OHT, and compared to WT fetal livers directly assayed for mRNA. The fetal livers were then evaluated for gene expression of VE-cadherin (and other endothelial and hematopoietic genes). Fold changes in gene expression of all tissues were first compared to cultured mouse embryonic fibroblasts (MEFs) that served as a "negative" control for hematopoietic and endothelial gene expression (Fig. S4B-C). We found that the cultured fetal livers and their wild-type counterparts not only had similar gene expression patterns, but that the VE-cadherin levels were slightly increased in culture (Figure S4B). Lastly, we evaluated VE-cadherin mRNA expression (and other endothelial and hematopoietic genes) in E10.5 WT circulating

blood as compared to the respective AGMs of the same litter, and found no appreciable VE-cadherin gene expression in peripheral blood (Fig. S4C).

Supplemental Experimental Procedures

VE-cadherin Cre early lineages and VE-cadherin KO

VE-cadherin Cre crossed to ROSA26R LacZ lines were mated and E7.5 embryos dissected and evaluated for β-galactosidase expression as described in Experimental Procedures. Mice heterozygous for VE-cadherin deletion (Carmeliet et al. 1999) were mated and embryos at E9.5 and E10.0 dissected and evaluated. Genotyping of embryonic yolk sacs was conducted with the following primers: sense ACCGGATGACCAAGTACAGC antisense GTGGCTGCTTATTCCAGAGC.

Pharmacokinetic evaluation of tamoxifen

4-hydroxytamoxifen (4OHT) sera levels were measured after administration of a one time dose of 1mg tamoxifen or 4OHT i.p. to adult female wild-type mice (n=5-6 per timepoint). 4OHT was prepared similarly to tamoxifen as previously described (Monvoisin et al. 2006), except that 4OHT dissolved more easily in a more dilute solution of 0.5mg per 100µl. Sera was obtained, prepared and measured as described (Plumb et al. 2001), except:10-µL aliquot of sample was injected onto a Zorbax XDB-C18 analytical column (2.1 mm x 100 mm, 1.8 µm particle size; Agilent), maintained at 50ºC, eluted with a 30-60%B gradient over 3 min, followed by 3 min of 95%B, flushing at 0.4 mL/min, where solution A is aqueous formic acid (0.1%) and solution B is acetonitrile. The entire eluate was monitored by MS/MS detection in selected

reaction monitoring of the transition of m/z 388.2>72.1 via an Agilent 6410 triple quadrupole LC/MS system that allowed accurate detection to as low as 0.1nM range. Both tamoxifen and 4OHT sets of serum extracts were 100-fold diluted with 30:70 methanol:water. 1.0 μM 4OHT standard stock solution was diluted to 10, 5.0, 1.0, 0.50 and 0.10 nM with the same dilution buffer.

Lung in vitro assay

Lungs from an E12.5 litter were cultured separately at an air-liquid interface on 40μm filters in media with 4OHT, then pooled and evaluated 24 hrs later for endothelial and hematopoietic induction (as described for peripheral blood and AGM in Experimental Procedures).

Tissue mRNA and Real-time PCR Gene Array

Wild-type (C57BL6/J) fetal livers at E10.5 and E11.5 (n=2 litters each) were cultured separately at an air-liquid interface on 40μm filters in media with 4OHT, then pooled for mRNA analysis. In addition, WT fetal livers of the same ages (n=2 litters each) were dissected and pooled together for comparison. Separately, an E10.5 litter was bled into 1X PBS and the corresponding AGMs dissected and pooled for mRNA analysis. Lastly, mouse embryonic fibroblasts (MEFs from E13.5 embryos, passage 18; a generous gift from Timothy Lane, Department of OB/GYN, David Geffen School of Medicine at UCLA, Los Angeles, California) were plated and collected for control mRNA. Total RNA was isolated from the various tissues using standard protocols (Chomczynski and Sacchi 2006), and transcribed by the SuperArray RT^2 First strand kit (SuperArray Bioscience Corp., Frederick, MD). Genomic DNA contamination was eliminated by Dnase treatment (Dnase I, Invitrogen, Carlsbad,

CA). Mouse Custom RT^2 Profiler PCR Array and PCR Mix were purchased from SuperArray Bioscience Corporation (Frederick, MD). PCR was performed on DNA Engine Opticon 2 RT-PCR System (MJ Research). For data analysis of the PCR array, the ΔΔCt method was used as recommended by supplier; differences in gene expression were calculated as fold-changes between MEFs and WT tissue.

Placenta in vitro assay

Placentas from E10.5, E11.5 (n=3 each) and E12.5 (n=2) were dissected from embryos and the embryonic side dissected away from the maternal. The placentas were then cultured at an air-liquid interface on 40μm filters in 4OHT (as described for fetal livers in Experimental Procedures).

Yolk Sac in vitro assay

Yolk sacs from E10.5, E11.5 and E12.5 (n=2 litters each) were cultured at an air-liquid interface on 40μm filters in 4OHT then pooled and evaluated 24 hrs later for endothelial and hematopoietic induction (as described for peripheral blood and AGM in Experimental Procedures).

Immunohistochemistry

β-galactosidase staining with subsequent PECAM-1 immunohistochemistry conducted as previously described (Alva et al. 2006).

Tamoxifen induction of Adult SM22α Cre

Adult mice 6-8 weeks of age received 1mg of tamoxifen i.p. daily for 28 consecutive days. Aorta and bone marrow were removed and stained for βgalactosidase as described in Experimental Procedures.

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