Supplemental Methods

Animals. For timed matings, the day a vaginal plug was detected (mouse) or the presence of sperm in the vaginal lavage (rat) were considered as e0.5 of the pregnancy. Females were age matched and between 8-20 weeks of age. Females were sacrificed at e11.5 and e13.5 for placenta dissection.

C57Bl/6J, *B6;129S-Tg(Prox1-tdTomato)*$^{12Nrud/J}$ and *B6.129S4-Il2rg*^{tm1Wjl} mice were purchased from Jackson Labs (Bar Harbor, ME) and mated to WT *C57Bl/6J* males.

Flt4$^{Chy/+}$ mice carrying the kinase-dead *Flt4* point mutant allele (MRC Harwell) were genotyped using 5'-CTGGCTGAGTCCCTAACTCG-3' forward and 5'-CGGGGTCTTTGTAGATGTCC-3' reverse primers, followed by a restriction enzyme digestion with BglII (1). *Flt4*$^{Chy/+}$ heterozygous and wild-type littermate control female mice on NMRI background were mated to wild-type NMRI male mice to induce timed pregnancies. All animal experiments were approved by the Animal Experimentation Review Board of the Semmelweis University.

Gestationally-timed rat placentation sites were obtained from a Holtzman Sprague Dawley rat colony maintained at the University of Kansas Medical Center.

Generation of Vegfc-floxed mice. The *Vegfc* gene was targeted in SV129 embryonic stem cells by replacing exon 3 with a loxP-flanked exon 3, and inserting a FRT-flanked neomycin resistance cassette upstream of the 5'-loxP site to allow selection of positively targeted clones. Correctly targeted ES cells were injected into blastocysts, and chimeric offspring were crossed with C57BL/6 mice to generate germline offspring. Germline offspring were then crossed with *Flpe* mice to remove the neomycin-resistance cassette. Mice were genotyped using the following PCR primers, which flank the downstream loxP-site: 5'-AATGTTTCGATGATGTGGGTTT-3' (forward) and 5'- TGCTGTATATGCTGTCGATGAT-3'

(reverse). The floxed allele gives a band size of 249 bp, while the wildtype allele gives a band size of 215 bp. *Vav1*-Cre mice were a gift from Dr. Thomas Graf.

Preeclampsia Phenotyping. Kidney samples were collected from pregnant *Flt4*$^{Chy/+}$ and WT mice at e18.5. Urine samples were collected from pregnant *Flt4*$^{Chy/+}$ and WT mice at e16.5 and control non-pregnant mice, respectively. Mice were placed into metabolic cage with ad lib water for 24 hours starting at e16.5 until e17.5. Urine samples were then collected and stored at -80°C until thawed for analysis.

Systolic Blood Pressure was measured using IITC Life Science Model 29 NIBP amplifier with a B60- ¼" sensor, ¼" cuffs and custom-made mouse holders. For recording and analyzing data Biopac Student Lab PRO 3.7.7 via Biopac MP45 data acquisition system were used. To minimize inter-channel and cuff variation all measurements for each mouse was done in the same channel with the same cuffs. Mice were kept immobilized in a holder on a heated platform set to 37 ºC prior to commencement and throughout the measurement session. Systolic blood pressures of pregnant *Flt4*$^{Chy/+}$ and WT mice from timed matings with WT males were measured every day from e13.5 at the same time each day, except e16.5 when mice were put into metabolic cage for collecting urine samples. On e16.5 systolic blood pressure of pregnant mice was measured at 10:00. Non-pregnant mice were measured for at least 4 days. Before every measurement 5 pre-conditioning measurements were performed. Blood pressure measurements were performed up to 10 times each day, with a maximum cuff pressure of 200 mmHg. At least 1 minute was between the measurements to provide full regeneration of the walls of the tail artery. An average of all accepted measurements was taken as the day's systolic blood pressure.

Histological Analysis. For H&E and immunofluorescence staining, placentation sites and kidneys were fixed in 4% paraformaldehyde (PFA) overnight at 4°C then washed in PBS for 5 min 3 times. For paraffin sections, tissues were first dehydrated and then embedded in paraffin. For

cryosections, tissue was cryopreserved in 30% sucrose overnight at 4°C, embedded in O.C.T. Compound (Scigen). Sections were permeabilized in PBS with 0.3% Triton X 100 for 15 min. Additional, antibody manufacturer-recommended steps were followed. Sectioned placentas were incubated with 1 or more of the following antibodies: Rabbit anti-PROX1 (AngioBio, 11-002), Goat anti-PROX1 (R&D Systems, AF2727), Rabbit anti-LYVE1 (Fitzgerald, 70R-LR005), Goat anti-VEGFR3 (R&D Systems, AF349), Rat anti-CD31 (BD-Biosciences, 550274), FITC-conjugated Dilocho Biflorus Agglutinin (DBA) Lectin (Vector Labs, FL-1031), Mouse anti-α-Smooth Muscle Actin (Sigma-Aldrich, clone 1A4), Syrian Hamster anti-Podoplanin (Developmental Studies Hybridoma Bank, 8.1.1), Rabbit anti-RFP (Rockland Immunochemicals, 600-401-379), Chicken anti-GFP (Aves Labs, GFP-1020), Rabbit anti-Neuropilin-2 (Cell Signaling Technologies, 3366P), Rat anti-Endomucin (eBioscience, eBioV.7C7 (V.7C7)), Rabbit anti-MAPK p44/42 (ERK1/2) (Cell Signaling Technology, 9102), and Rabbit anti-Phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204) (Cell Signaling Technology, 4370). After 3 x 5 minute PBS washes, sections were incubated in the dark for 2 h at room temperature with the following antibodies: Cy2-, Cy3-, or Cy5 conjugated Donkey anti-Rabbit IgG antibody (Jackson ImmunoResearch, 711-225-152, 711-165-152, 711-175-152); Cy3- or Cy5-conjugated Donkey anti-Goat IgG antibody (Jackson ImmunoResearch, 705-165-147, 705-175-147); Cy2-conjugated Goat anti-Syrian Hamster IgG antibody (Jackson ImmunoResearch, 107-225-142); Cy3-conjugated Donkey anti-Chicken IgG antibody (Jackson ImmunoResearch, 703-165-155). Nuclei were visualized by incubating sections with Bisbenzimide H 33258 (Hoechst, Sigma). Then sections were washed 5 x 5 min in PBS and mounted in ProLong Gold Antifade Reagent (Invitrogen) under a glass coverslip. H&E-stained slides were imaged using a Leitz Dialux 20 with QImaging Micropublisher 5.0 RTV color CCD camera. Fluorescence IHC images were taken on a Nikon E800 fluorescence microscope with a Hammamatsu Orca CCD camera on Metamorph software (Molecular Devices Corp.) Images of DBA-stained uNK cells were subjected to 2D deconvolution to show intracellular granularity.

Before analysis, the identity of the slide samples were blinded by an individual not involved in the analysis. The identity of samples was not revealed until data from all samples were collected.

Measurements for mean fluorescent intensity (MFI) were performed in ImageJ 1.51n while blind to sample ID. The MFI is a readout of the mean pixel intensity of a channel within a selected area. For measurements of endothelial VEGFR3 and p-ERK MFI, the endothelium was specifically selected for measurement with hand-traced borders in ImageJ 1.51n, eliminating intraluminal signal and signal outside the endothelium from the analysis. Smooth muscle coverage was determined by hand-tracing a line 360 degrees around a vessel through the smooth muscle cell layer. The fluorescent intensity was determined for all points along that line, and any points along the line above a threshold signal for alpha-smooth muscle actin were considered to be positive for smooth muscle coverage.

uNK cell isolation and culture. uNK cells were isolated from e12.5 placentas of *C57Bl/6J* mice using a modified version of the DBA-coated magnetic bead isolation protocol described in Croy et al. 2010 (2). Briefly, CELLection M450 magnetic beads (Thermo Fisher Scientific) were washed in PBS/Tween, then resuspended in 1:50 biotinylated DBA-lectin (Vector Laboratories) for 30 min at room temperature and washed in PBS/Tween again. Placentas were dissected into Hank's balanced salt solution with 1000 IU DNase I (Worthington Biochemical), and then cells were dissociated by finely chopping tissue with a sterile razor blade and pipetting for 2 min. Debris was removed by passing cells through a 100 μm and then 70 μm mesh nylon filter.

DBA-coated magnetic beads were added to cell suspensions at approximately 1 bead per 2 cells and mixed for 15 min on an orbital rotator at 4°C. Bead-bound uNK cells were immobilized with a magnet and the unbound Non-uNK cell solution was moved to a fresh tube. To the bead-bound uNK cells, 500 μL of PBS with 2% BSA and 0.1 M N-acetyl-d-galactosamine (Sigma) was added and mixed at 4°C for 5 min to separate uNK cells from beads. These uNK isolation steps

were repeated again with the Non-uNK cell solution using fresh DBA-coated magnetic beads, and then repeated once more with the resulting Non-uNK cell solution to maximize uNK cell yield.

Culture wells were coated in 2 µg/mL sterile fibronectin (MP Biomedicals), and uNK cells were plated at approximately 6 x 10^4 cells per 1.0 cm^2 surface area. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 200 ng/mL IL-15, and 100 ng/mL CXCL10 in a humidified 5% CO_2 incubation chamber at 37°C. After the first 6 h, the medium was replaced, and then every 24 h thereafter (for a maximum of 5 days) the uNK-conditioned media was collected and stored at -20°C until the time of experimentation.

Cell culture. Human neonatal dermal LECs (HMVEC-dLyNeo-Der, Lonza) were used within 6 passages and maintained in EGM-2MV bullet kit medium (Lonza). For experiments measuring p-VEGFR3 (Tyr1230/1231) cells were seeded at 1 x 10^5 cells on glass coverslips in 24-well plates and grown to 80%–100% confluency. Prior to treatments, the LEC culture medium was switched to low serum medium (0.5% FBS in RPMI 1640 with Penicillin-Streptomycin) and cultured overnight. The next morning, the medium was replaced with fresh low serum medium containing 1 µM sunitinib (LC Laboratories) inhibitor pre-treatments or vehicle for 2 h prior to treatment. Treatments were prepared in low serum medium with 1 tablet/10 ml PhosSTOP (Sigma) to maximize phosphorylation detection. Pre-treatments did not contain PhosSTOP. Where indicated treatments contained 500ng/ml VEGFC (Abcam) and/or 1 µM sunitinib (LC Laboratories) and cells were treated for 1 minute. Following treatment, cells were fixed in 4% PFA supplemented with 1 tablet/10ml PhosSTOP for 10 min and then washed 3 x 5 min in PBS.

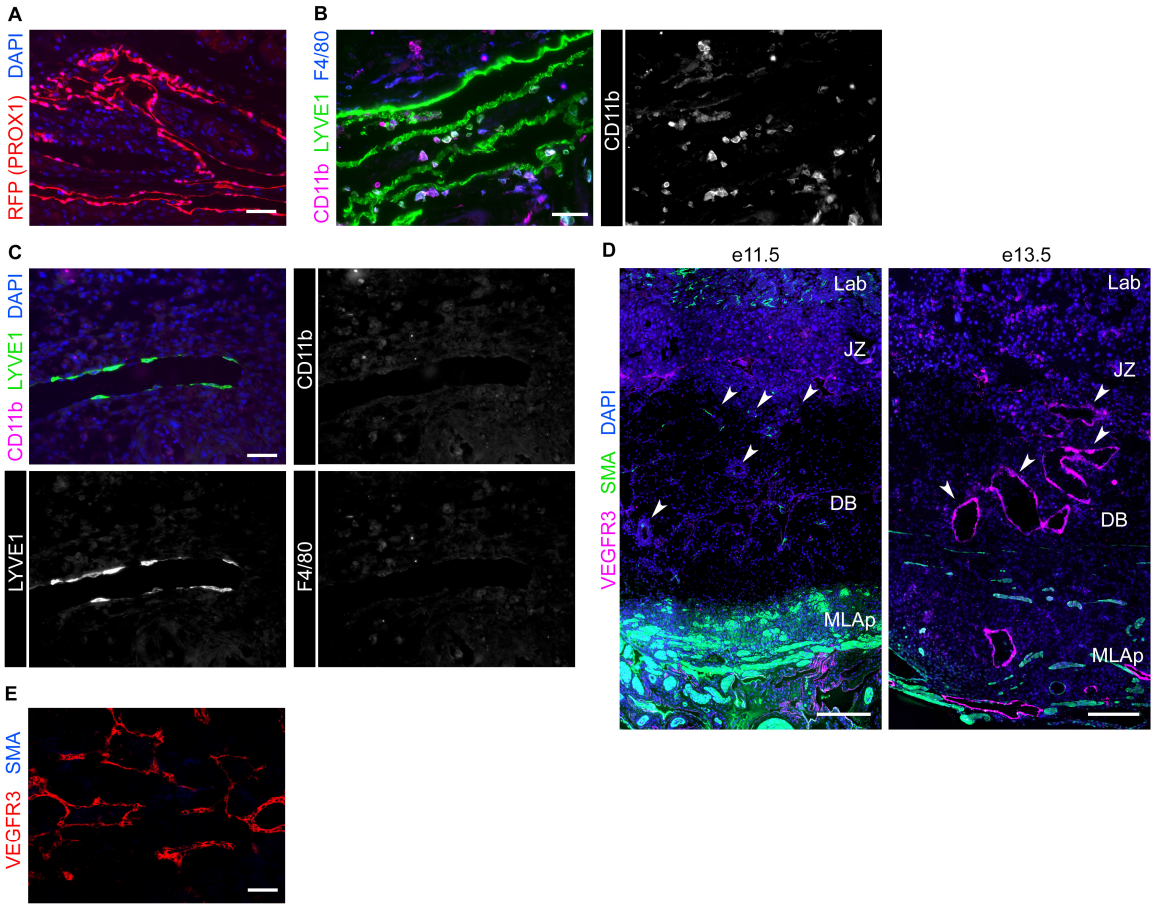
For p-VEGFR3 (Tyr1230/1231) experiments, wells were incubated with Rabbit anti-Phospho-VEGFR3 (Tyr1230/1231, Cell Applications). After 3 x 5 minute PBS washes, sections were incubated in the dark for 2 h at room temperature with Cy3-conjugated anti-Rabbit IgG antibody (Jackson ImmunoResearch). Nuclei were visualized by incubating sections with Bisbenzimide H 33258 (Hoechst, Sigma). Then sections were washed 3 x 5 min in PBS and

glass coverslips were removed from wells and mounted in ProLong Gold Antifade Reagent (Invitrogen) on a glass slide. MFI of p-VEGFR3 (Tyr1230/1231) was calculated by measuring the the whole image in ImageJ 1.51n

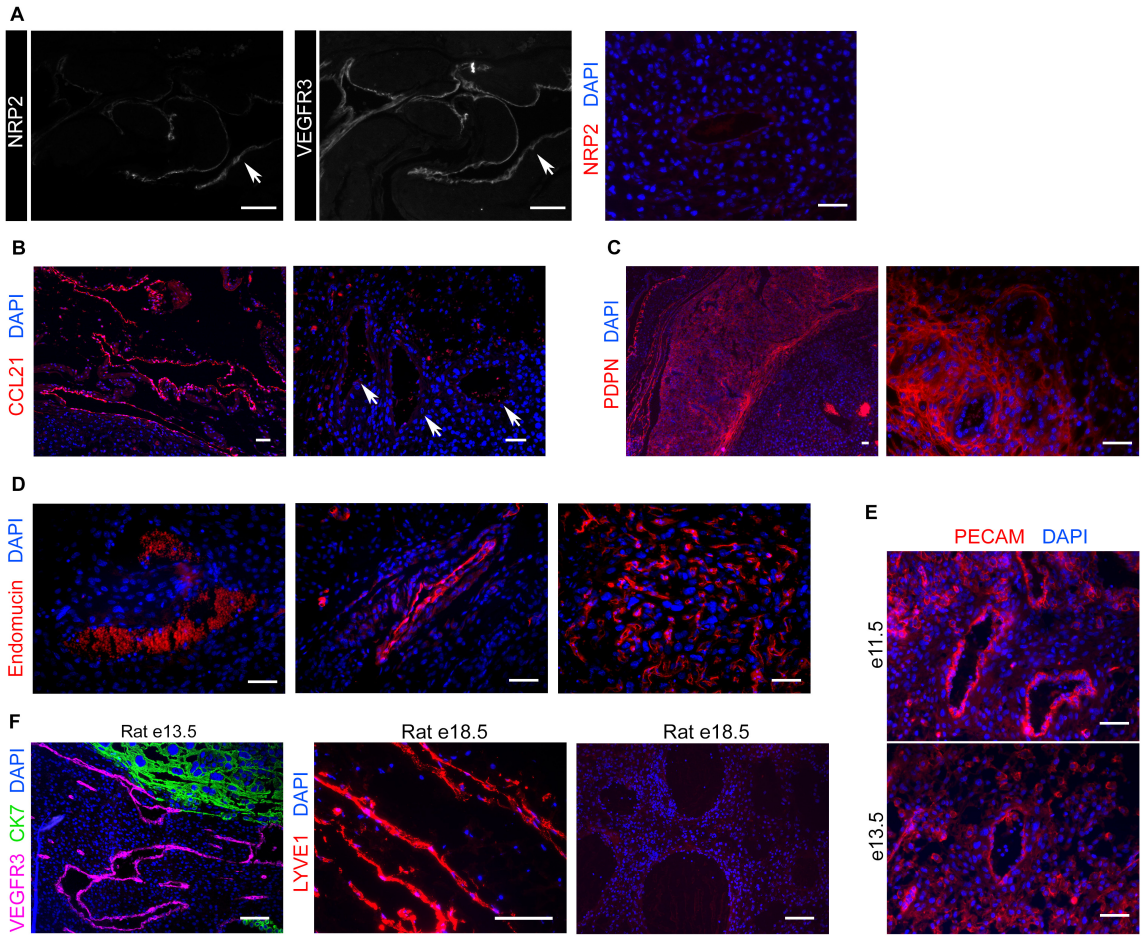
Statistical Analysis. Measurements for mean fluorescent intensity (MFI) were performed in ImageJ 1.51n while blinded to sample ID. In PLA assays, quantification of the number of foci per cell were determined by BlobFinder v3.2. Statistical analyses were performed using GraphPad Prism v5.01. One-way ANOVA with Bonferroni posttest were used to determine statistical significance for multiple comparisons with a single variable. Two-way ANOVA with Bonferroni posttest were used to determine statistical significance for multiple comparisons with two variables (i.e. genotype and embryonic day), otherwise statistical significance was determined by independent 2-tailed *t* test. A P value less than 0.05 was considered significant. In all graphs the red horizontal line represents the mean.

Study Approval. All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

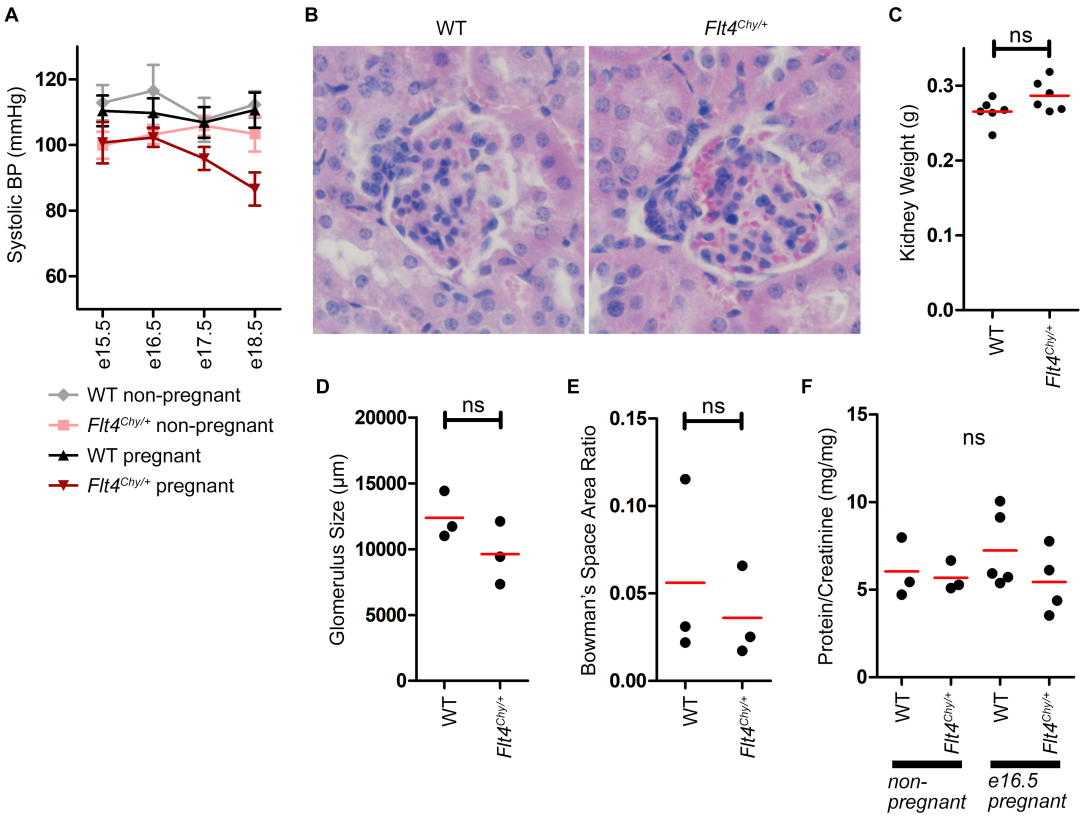
Supplemental Figures



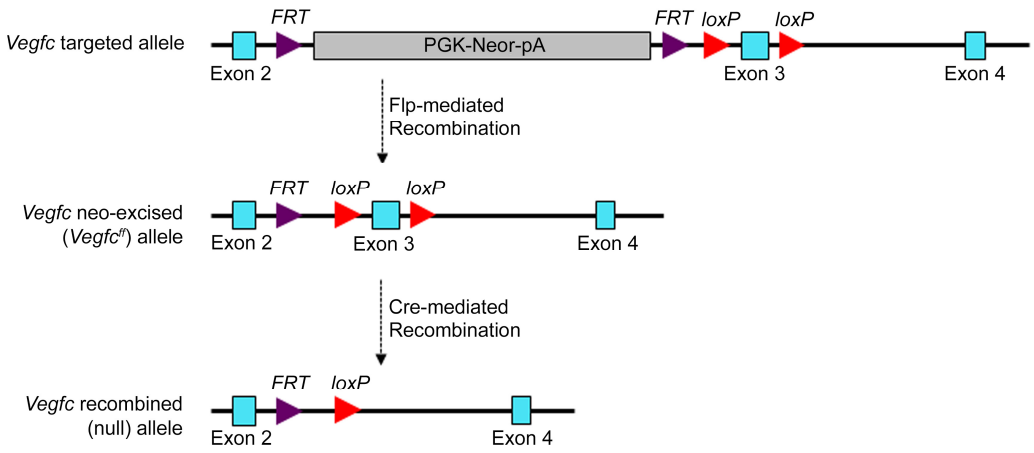
Supplemental Figure 1: Remodeled SAs express a subset of lymphatic markers. (**A**) RFP is detected in the myometrium lymphatics of *Tg(Prox1-tdTomato)*$^{12Nrud/J}$ reporter mice at e13.5. Scale bar = 20 μm. (**B-D**) Placental tissue from *C57Bl/6J* mice at e13.5. (**B**) Positive control staining of myeloid marker CD11b and macrophage marker F4/80 in the myometrium. Scale bar = 20 μm. (**C**) LYVE1+ cells in the SA endothelium do not co-localize with CD11b nor F4/80. Scale bar = 20 μm. (**D**) Low power images of VEGFR3 expression in WT placentas generated by photo merging (panorama) multiple high power images. Arrow heads point to SAs. Mesometrial lymphoid aggregate of pregnancy (MLAp), decidua basalis (DB), junctional zone (JZ), labyrinth (Lab). Scale bar = 200 μm. (**E**) As previously reported, VEGFR3 is expressed in the vascular zone of the decidua, sometimes referred to as vascular sinuses (3). Scale bar = 20 μm. All images are representative of multiple observations from at least 3 litters each, with 1-5 placentas from each litter.



Supplemental Figure 2: Remodeled SAs do not express all lymphatic markers.
(**A**) NRP2 (a known interactor of VEGFR3), is expressed in the myometrium lymphatics identified by VEGFR3. Arrows indicate the same vessel in adjacent sections. SAs do not express NRP2. (**B**) CCL21 is expressed in myometrium lymphatics (left), but is not expressed in SAs which are indicated by arrows (right). (**C**), Myometrium lymphatic and stromal staining of PDPN is detected (left) as has been previously reported in humans (4), but PDPN is not expressed in the SA endothelium (right). (**D**) Endomucin, a marker of fenestrated endothelium is not detected in SAs (left), however it is detected in myometrium lymphatics (middle) and in the vasculature of the placental labyrinth (right). (**E**) PECAM-1 is expressed in SAs from e11.5 to e13.5. (**F**) The vascular sinus folds in rat placentation sites are positive for VEGFR3 (left). LYVE1 is expressed in myometrium lymphatics (middle). Rat spiral arteries do not express LYVE1, dissimilar to that observed in mice (right). All scale bars = 20 μm. All images are representative of multiple observations from at least 3 litters each, with 1-3 placentas from each litter.



Supplemental Figure 3: *Flt4*$^{Chy/+}$ mice do not exhibit symptoms of preeclampsia. (**A**) Blood pressure measurements by tail cuff method from e15.5 to e18.5 pregnant and non-pregnant mice. (n = 5-6 non-pregnant mice per genotype, n = 8-13 pregnant mice per genotype) (**B**) Representative images of glomeruli from WT and *Flt4*$^{Chy/+}$ mice. (**C-E**) Quantification of kidney weights (n = 6 mice per genotype; unpaired t-test), glomerulus size (n = 3 mice per genotype; unpaired t-test), and Bowman's Space area normalized to glomerulus area (n = 3 mice per genotype; unpaired t-test) from e18.5 pregnant mice. (**F**) Protein content in the urine normalized to creatinine of e16.5 pregnant and non-pregnant mice. (n = 3-5 mice per genotype; two-way ANOVA) In all graphs the red horizontal line represents the mean.



Supplemental Figure 4: A map of the targeted *Vegfc* allele used to generate *Vegfc*$^{fl/fl}$ mice.

Supplemental References

1. Karkkainen MJ, et al. A model for gene therapy of human hereditary lymphedema. *Proceedings of the National Academy of Sciences of the United States of America.* 2001;98(22):12677-82.
2. Croy BA, Zhang J, Tayade C, Colucci F, Yadi H, Yamada AT. Analysis of uterine natural killer cells in mice. *Methods Mol Biol.* 2010;612(465-503.
3. Kim M, et al. VEGF-A regulated by progesterone governs uterine angiogenesis and vascular remodelling during pregnancy. *EMBO Mol Med.* 2013;5(9):1415-30.
4. Wang Y, Sun J, Gu Y, Zhao S, Groome LJ, Alexander JS. D2-40/podoplanin expression in the human placenta. *Placenta.* 2011;32(1):27-32.