Reagents
bFGF was purchased from Millipore. Recombinant human VEGF-A 165, VEGF-A 121, VEGF-B, VEGF-C, PlGF, SDF-1α (CXCL12), SCF (Kit ligand), IL-3, Flt-3L, EPO, G-CSF, GM-CSF, BMPs 2, 4, 6 and M-CSF are from R&D Systems. IL-6 and TPO are from Invitrogen. Antibodies: PECAM/CD31 (Dako: M0823); p-Erk1/2, Erk1/2, p-Akt, Akt, p-Pak2, Pak2, p-Pak4, Pak4, p-cRaf, cRaf, p-bRaf, bRaf, p-Src, Src, (Cell Signaling Antibodies); MT1-MMP, CD34, Runx1/AML1 (Epitomics); α2 Integrin (BD Bioscience); PDGF-BB, IL-6, VEGF-A (mAb and pAb), VEGFR2, SCF, IL-3, SDF-1α, IL-3Rα, and c-Kit (R&D Systems); CXCR4, Laminin (Sigma); Collagen IV (Millipore). QH1 antibodies were purchased from Developmental Studies Hybridoma Bank (F. Dieterlen; under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242). Quail eggs were purchased from Ozark Egg Company. AMD3100 was purchased from Sigma; Rp-cCAMPS and Rp-cGMP were purchased from BioMol; Imatinib was purchased from Cayman Chemical; ISCK03, SB 203580, Y27632, GM6001 were purchased from Calbiochem.

SiRNA suppression in ECs
SiRNA suppression protocols in ECs were carried out essentially as described1. Both SMARTpool (Dharmacon/Thermo Scientific) and single (Invitrogen) siRNA sequences were used. SMARTpool siRNAs were used at a final concentration of 100 nM, while single siRNAs were used at a final concentration of 20 nM. Following treatment, cells were collected and used for 3D collagen assays, designated for cell lysates or RNA extracted for further analysis. Single siRNA sequenced used are as follows: c-Kit #1: 5′-ATAAGCAGTTGCCTCAACAACCTTC-3′; 5′-GAAGGTTG TTGAGGCAACTGCTTAT-3′; c-Kit #2: 5′-CCGGTCGATTCTAAGTTCTAAGTTCT ACAAGA-3′; 5′-TCTTGTAGAACTTAGAATCGACCGG-3′; Runx1 #1: 5′-CCTCTC TGCAGAACTTTCCAGTCGA-3′; 5′-TCGACTGGAAAGTTCTGCAGAGAGG-3′

PCR analysis
Cells were suspended in 3D collagen type I matrices and allowed to assemble to pre-determined time points. Collagen gels were removed, collagenase treated, and cells lysed using the Ambion ToTALLY RNA kit (#1910) to isolate total RNA. RNA was produced following the manufactures recommendations. cDNA was produced using the Stratagene AccuScript High Fidelity 1st Strand cDNA Synthesis Kit, and RT-PCR and analysis of differentially expressed genes was performed. Primer sets were made (300–400bp) in both a gene and species-specific manner. IL-3Rα: 5′-CTCGACTCTCCAGC GGTTC-3′; 5′-CTGCAGATCACGAAGACACAGACCAG-3′; c-Kit: 5′-GTGCTTCT ACCGGGAAAC-3′; 5′-TTCAATTCCATGAGACGG-3′; CXCR4: 5′-TCTTAACTGG CATTGTGG-3′; 5′-TCATCTGCCTCACTGACG-3′; VEGFR2: 5′-GTGCTTCTACCG GGAAAC-3′; 5′-TTCAATTCCATGAGACGG-3′; CSFR: 5′-GTGGCCAGCAACGTG CAGGGCAAGCAC-3′; 5′-CAGCACACAAAGGGTGCCAGAGC-3′; Flt3: 5′-GACGG ACATAAGGTGCTGTGCCAG-3′; 5′-CGAGAGAAGGTCCACGTACATCTG-3′; CD34: 5′-CCTGTGTCTCAACATGGC-3′; 5′-CCCTGTCCTTCTTAAACTC-3′; G3PDH: 5′-GACCTCAGTCCATTGCTTAG-3″ 5′-GGATGGCTACTCCTTCTATC-3′

Electron microscopy
Cultures were fixed in 3% EM glutaraldehyde for 1 hour (EM Grade, Sigma-Aldrich) and processed for electron microscopy. Images were acquired using a Hitachi S4700 Field Emission scanning electron microscope (FESEM) (Hitachi, LTD).

Histology and paraffin sections
Quail embryos were fixed in 4% formalin and processed for embedding in paraffin. Standard H&E staining was performed using 10 μm mounted cross sections for analysis.

Microscopy, imaging, and data analysis
Time-lapse videomicroscopy and fluorescence imaging was performed using a fluorescence inverted microscope (Eclipse TE2000-E; Nikon) and the analysis software MetaMorph (Molecular Devices). Data analysis was done by tracing the tube area of individual high powered fields of culture assays using MetaMorph software and Microsoft Excel to do statistical analysis.

Growth Factors Tested as Substitutes:			
	SCF	IL-3	SDF-1α
Flt-3 Ligand	+	-	-
M-CSF	+	-	-
GM-CSF	-	-	-
G-CSF	-	-	-
EPO	-	-	-
IL-6	-	-	-
IL-8	-	-	-
TPO	-	-	-
VEGF-A	-	-	-
BMP-2	-	-	-
BMP-4	-	-	-
BMP-6	-	-	-
TGF-β1	-	-	-
Angiopoietin 1	-	-	-
Angiopoietin 2	-	-	-
SHH	-	-	-

Abbreviations/Symbols: IL (Interleukin); BMP (Bone Morphogenic Protein); TGF (Transforming Growth Factor); SHH (Sonic Hedgehog); EPO (Erythropoietin); TPO (Thrombopoietin); M-CSF (Macrophage Colony Stimulating Factor); GM-CSF (Granulocyte Macrophage Colony Stimulating Factor); G-CSF (Granulocyte Colony Stimulating Factor); Flt (fms-like tyrosine kinase)

+ : Growth Factor has the ability to replace the indicated hematopoietic cytokine

- : Growth Factor does not have the ability to replace the indicated hematopoietic cytokine

Table S1

Figure S1. Human endothelial tube morphogenesis in 3D extracellular matrices are models of vasculogenesis and angiogenic sprouting and reveal separable and sequential priming and tube morphogenic events

Two models of EC tube formation were utilized to assess the factors controlling vasculogenic plexus formation with pericyte recruitment and angiogenic sprouting of ECs. These models are carried out in 3D collagen type I matrices and in serum free defined media (and in the absence of phorbol esters). In some cases, ECs were primed with VEGF-A, FGF-2 or both and after 16 hr, were utilized in morphogenic assays (A). The vasculogenic assays incorporate only SCF, IL-3 and SDF-1α into the matrix with the cells, while the angiogenic assays also incorporate VEGF-A or BMP-4 into the matrix along with these three hematopoietic cytokines (B). For the vasculogenic assay, all cells are suspended in the collagen matrix and allowed to undergo morphogenesis for a period of 3 or 5 days. Over this time period, ECs interconnect, form lumens and can recruit pericytes. In the angiogenic assay, ECs are seeded onto the surface of the collagen matrix and allowed to sprout over a period of 24 hours. FGF-2 is present in the media in all cases. After reaching the indicated time points the assays are fixed or extracted for protein or mRNA and these samples are analyzed.

Figure S2. Addition of exogenous VEGF-A, inhibition of endogenous VEGF-A or VEGFR2 does not alter EC tube morphogenesis, however neutralization of pro-morphogenic hematopoietic cytokines or their receptors alters EC tube formation in 3D collagen matrices

(A) VEGF-A was provided to ECs as a morphogenic stimulus either alone or in combination with hematopoietic cytokines and average EC vessel area was assessed (n≥ 10; p≤ 0.01) and representative images are shown (B). (C/D) Neutralizing antibodies to VEGFR2 (C) or VEGF-A ligand (D) were added along with neutralizing antibodies to c-Kit (C) and SCF (D) (n≥ 10; p≤ 0.01). (E) Blocking antibodies for SDF-1α, IL-3 and SCF were identified and added to EC morphogenic assays at 20 μg/ml. As shown, all combinations lead to inhibition of tubulogenesis, however the combination of IL-3 and SCF was the most potent and was therefore used for *in vivo* studies (n≥ 10; p≤ 0.01). *Denotes significance from control.

Figure S3. siRNA suppression of c-Kit and chemical inhibitors of c-Kit and CXCR4 block EC tube morphogenesis in response to hematopoietic cytokines

(A) SiRNA suppression of c-Kit in human ECs was performed using two individual siRNAs and were compared to control luciferase siRNA treatment. EC tube formation assays were performed with SCF/IL-3/SDF-1a in 3D collagen matrices and after 72 hr, cultures were fixed and quantitated for total EC tube area. Cell lysates were prepared to confirm that the siRNAs suppressed c-Kit expression using Western blot analysis. (B) A dose response curve of AMD3100 was administered to EC cultures *in vitro* to determine the minimum dose of inhibitor that maximally blocked EC tube formation in 3D collagen matrices (n≥ 10; p≤ 0.01). A dose of 100 nM was determined to be an optimal dose and was utilized *in vivo* at this dose. (C) A similar dose response curve was generated for Imatinib with a similar finding that 100 nM was an optimal dose to elicit blocking effects on EC tubulogenesis (n≥ 10; p≤ 0.01).

Figure S4. VEGF and FGF prime EC tube morphogenic responses to hematopoietic cytokines from both human ECs and embryonic quail vessels
(A) ECs were primed for 16 hours in the presence of FGF-2 alone, VEGF-A alone, the combination of VEGF-A/FGF-2 or under control conditions. EC morphogenic assays were then established in the presence of the hematopoietic cytokines and average EC tube area was measured (n≥ 10; p≤ 0.01) and representative images are shown (B). *Denotes significance over control; +Denotes significance over the FGF-2 and VEGF-A alone conditions. Bar equals 100 μm. (C) E6 Quail vitelline vessels were primed with VEGF-A/FGF-2 versus control and then were explanted in 3D collagen matrices in the presence of hematopoietic cytokine morphogenic stimuli. After 120 hr, cultures were fixed and stained with QH-1 antibodies to label quail ECs. Arrows denote the original border of the vessel explant. Bar equals 100 μm.

Figure S5. Administration of chemical inhibitors to CXCR4 and c-Kit or blocking antibodies to SCF and IL-3 during quail development leads to hemorrhage phenotypes and defects in vascular morphogenesis
(A) Chemical inhibitors and neutralizing antibodies were administered to quail embryos at day 1 or 3 of development and embryos were allowed to develop until day 4 or 6, respectively, to assess the phenotypes of treated versus control embryos. Embryos at these time points were paraffin embedded, cross-sectioned and sections were stained with H&E versus immunostained with the EC specific marker, QH1, to visualize blood vessels. White arrowheads indicate QH1 positive vessels, while white arrows indicate enlarged vessels with areas of vessel wall disruption and the black arrows indicate areas of hemorrhage and/or blood within markedly enlarged vessels. Bar equals 50 μm. (B) The table shows the hemorrhage and survival rates of quail embryos treated with AMD3100 alone, Imatinib alone, and their combination as well as anti-SCF blocking antibodies alone, anti–IL-3 antibodies alone and their combination from 1–4 days of development. (C) Representative images of control and hemorrhage phenotypes observed during the 1–4 day time frame are shown for control versus anti-SCF/IL-3 conditions as well as H&E analysis of paraffin embedded sections of these embryos. White arrowheads indicate the borders of a large axial vessel, while the black arrows indicate sites of vascular hemorrhage only observed in the antibody treated embryos. Bar equals 50 μm.
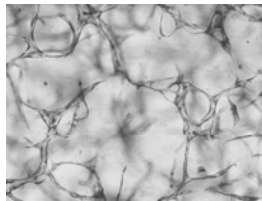
A

Priming Cues

16 hours; VEGF-A/FGF-2 →

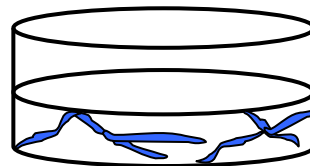**Cells used for Vasculogenic or Angiogenic Assays**
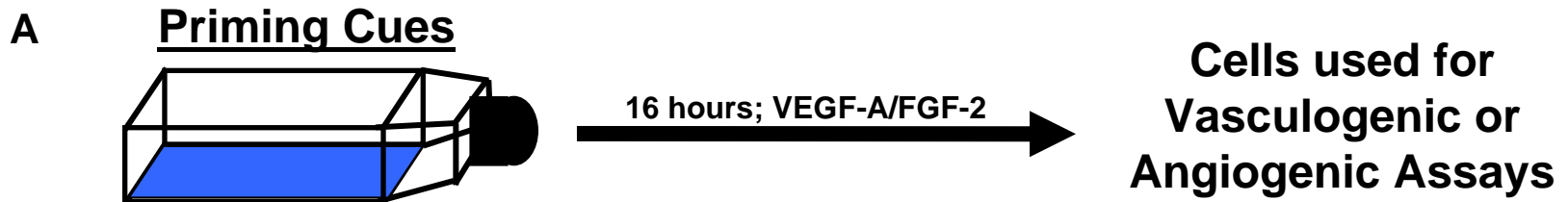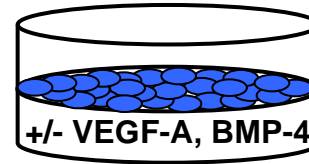
B

Vasculogenic Assay
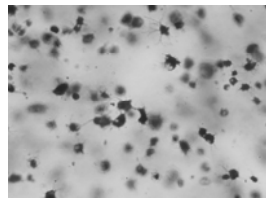
0 hours
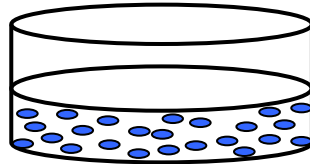
3-5 Days

72 hours

Media:
RSII/AA/bFGF

Gel: SCF/IL3/SDF-1α

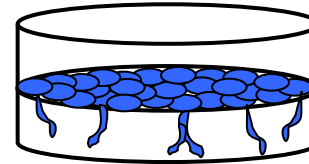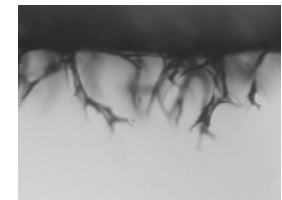**Angiogenic Assay**

+/- VEGF-A, BMP-4
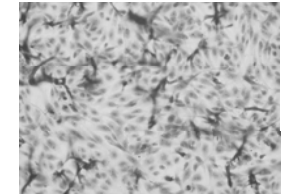
Monolayer: 24 hrs

24 hours

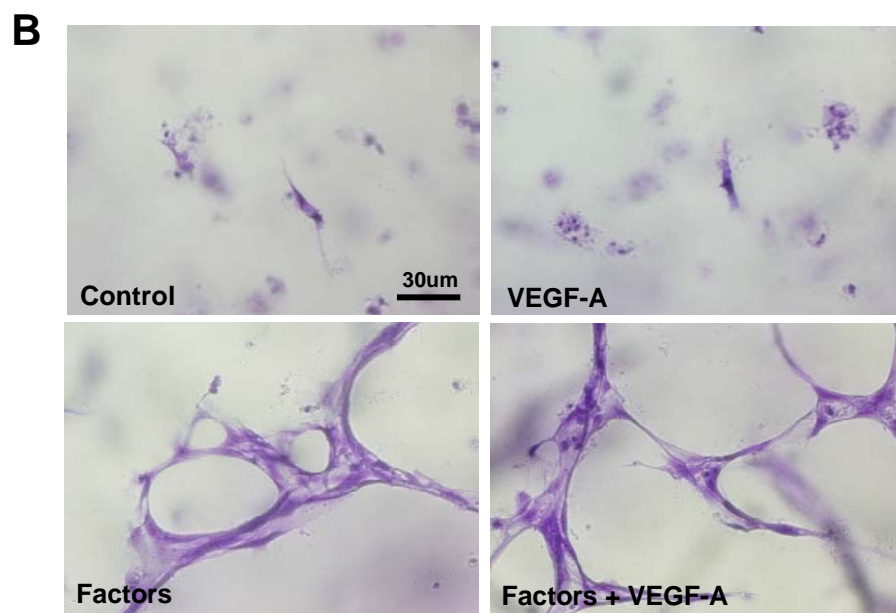Bottom View: 24 hours

Side View: 24 hours

Figure S1

Figure S2

Figure S3

A

B

1ˢᵗ step Priming

2ⁿᵈ step + Factors

C

<u>**Figure S4**</u>

A

6d	6d	6d	4d

Control

α-SCF/IL-3
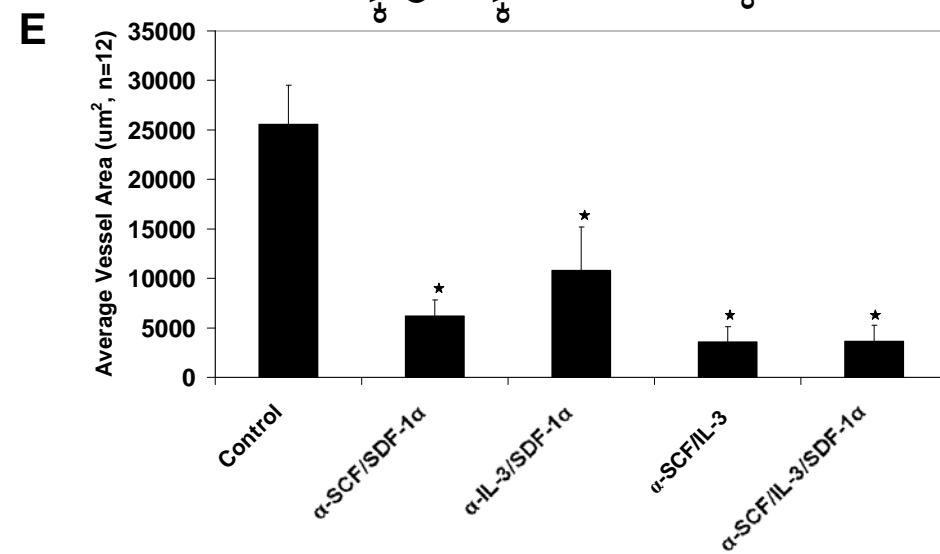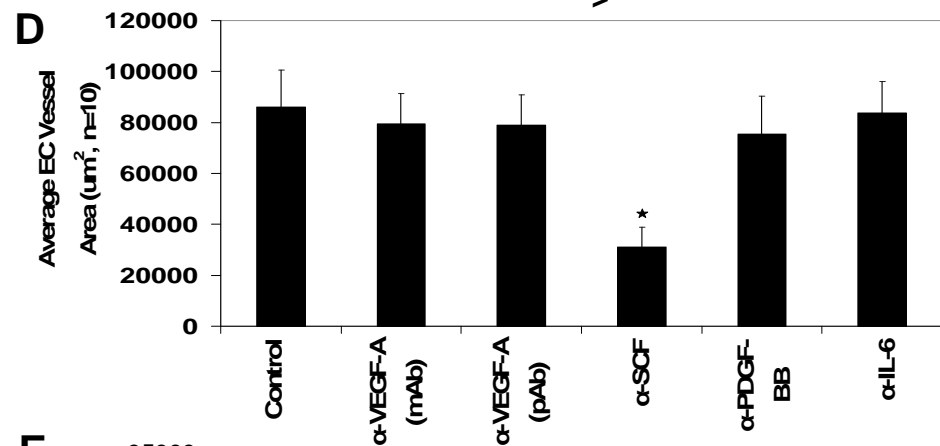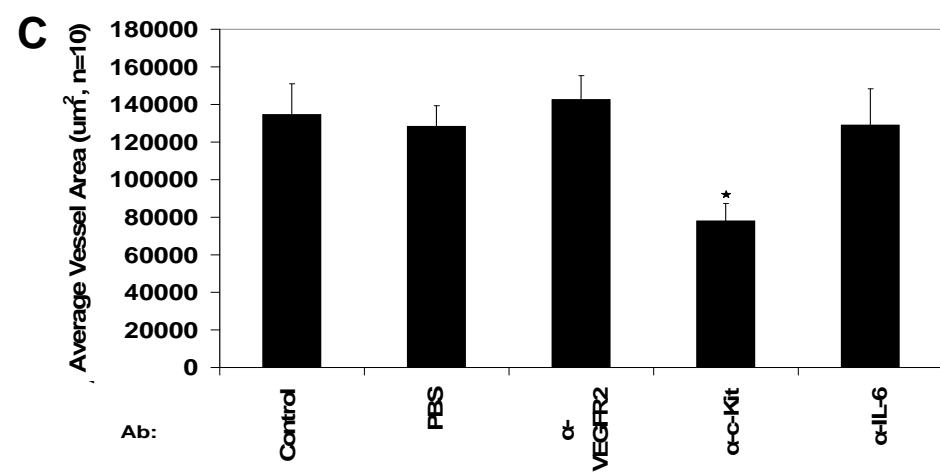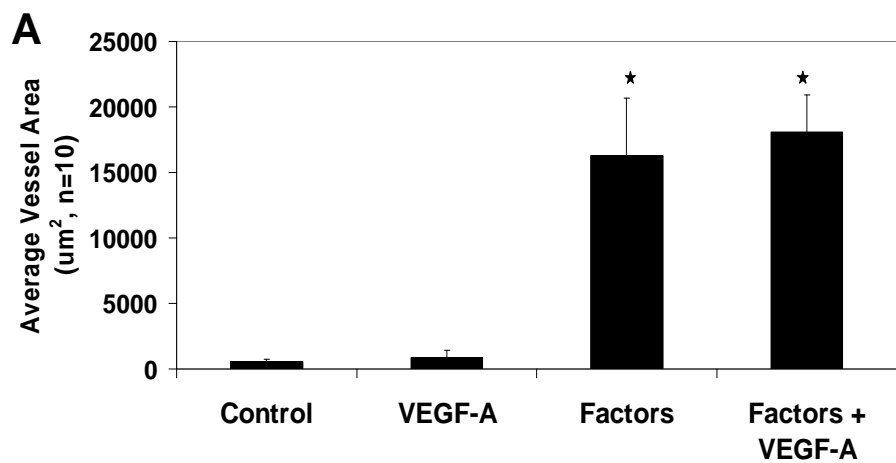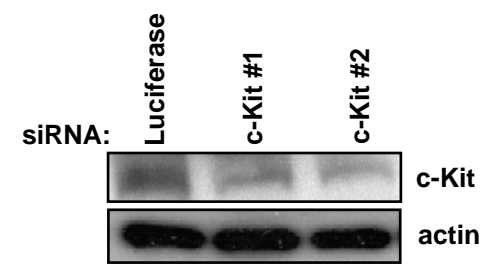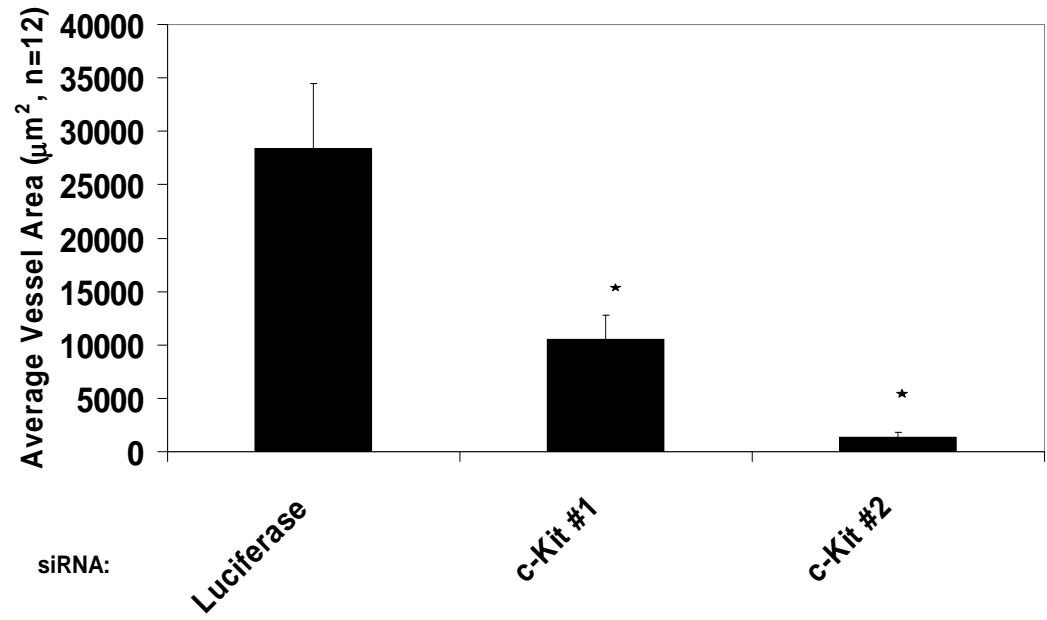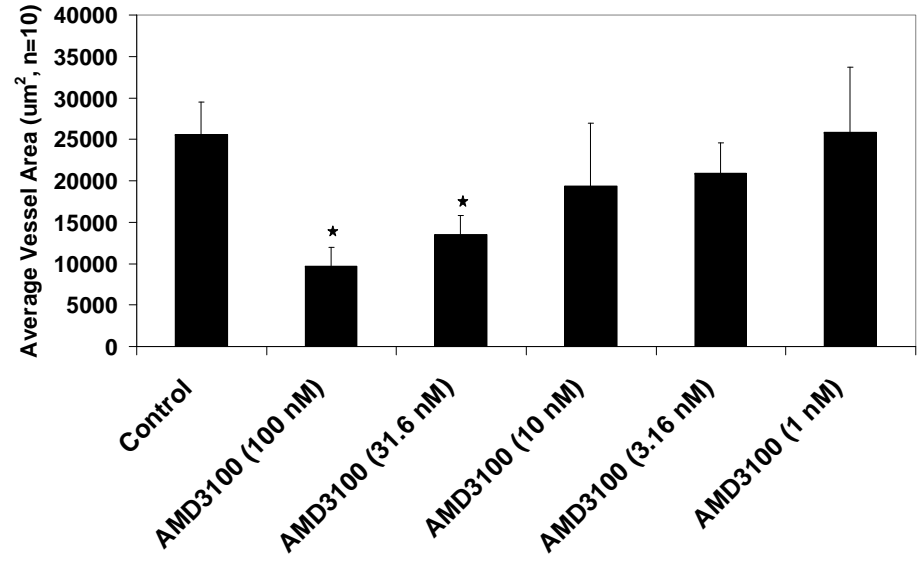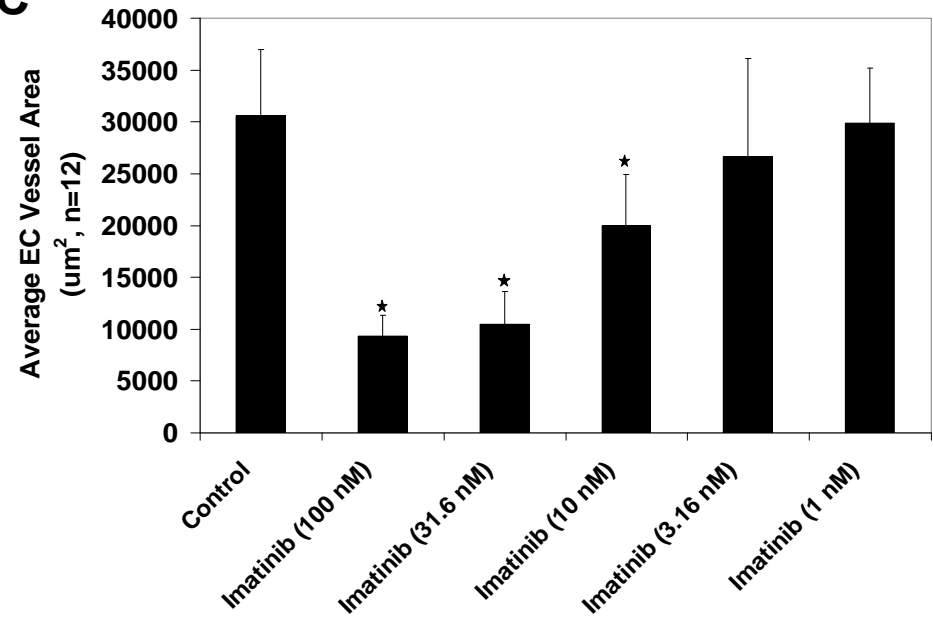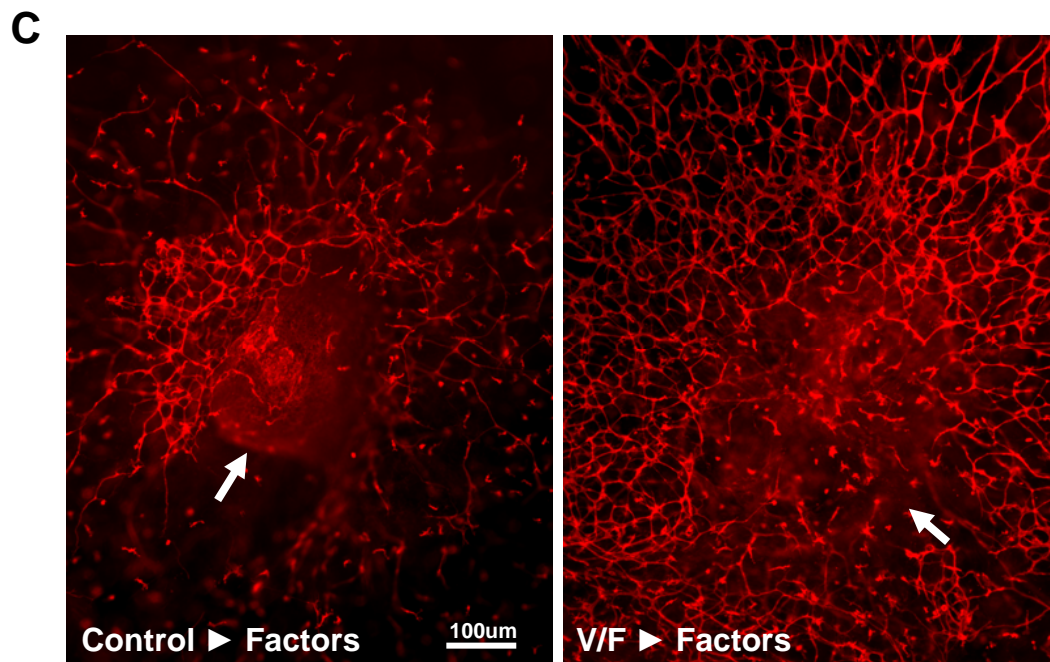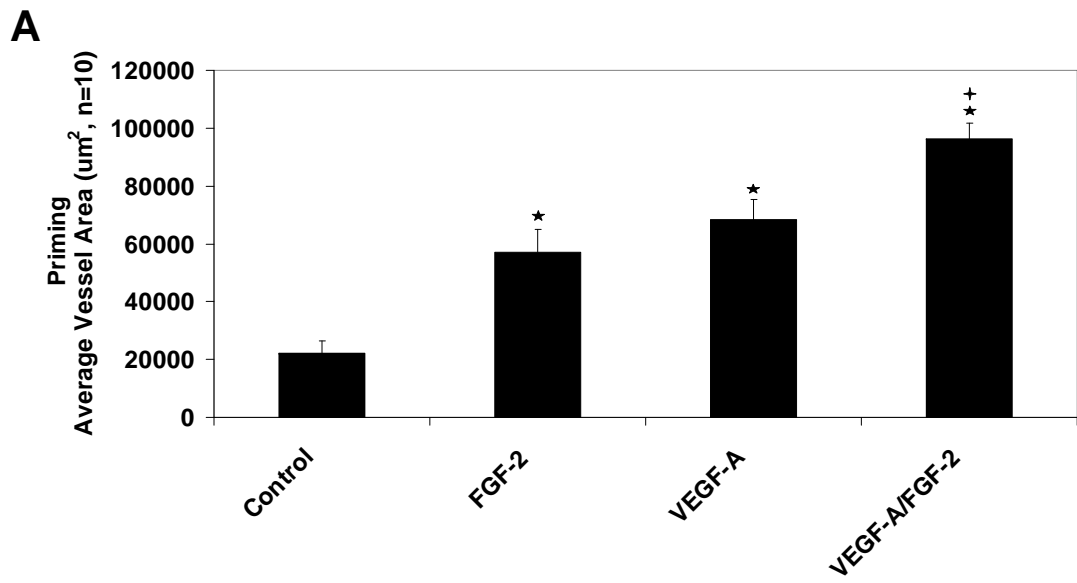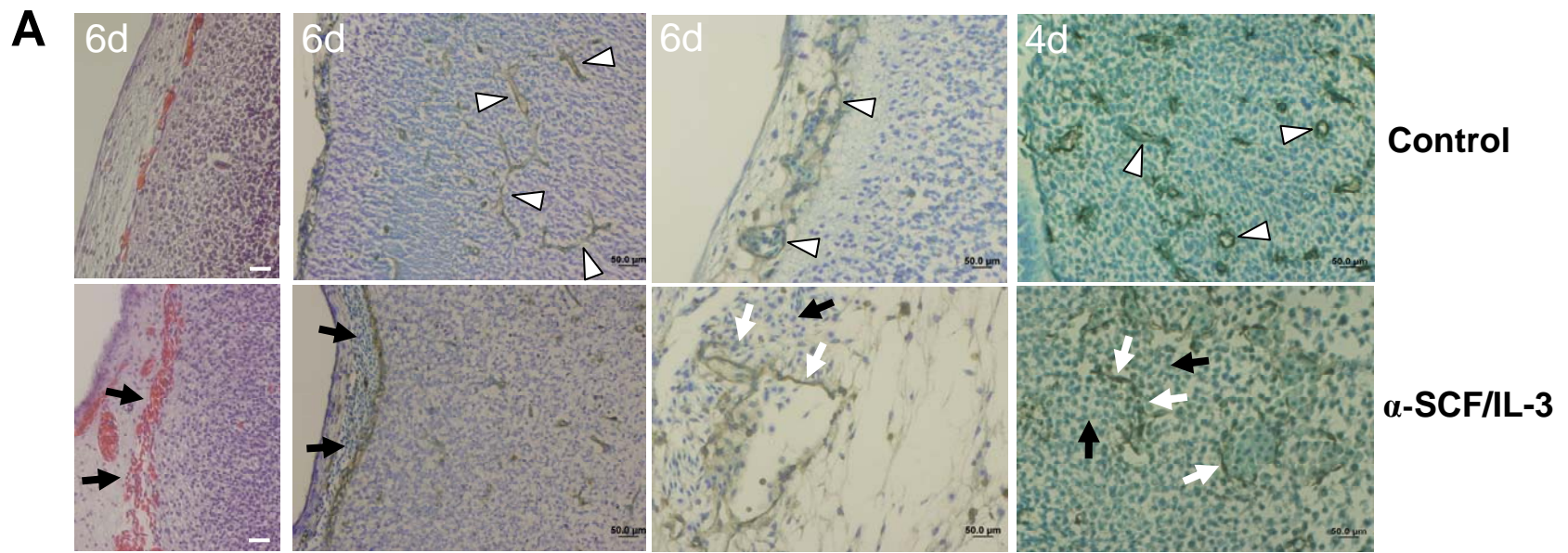
B
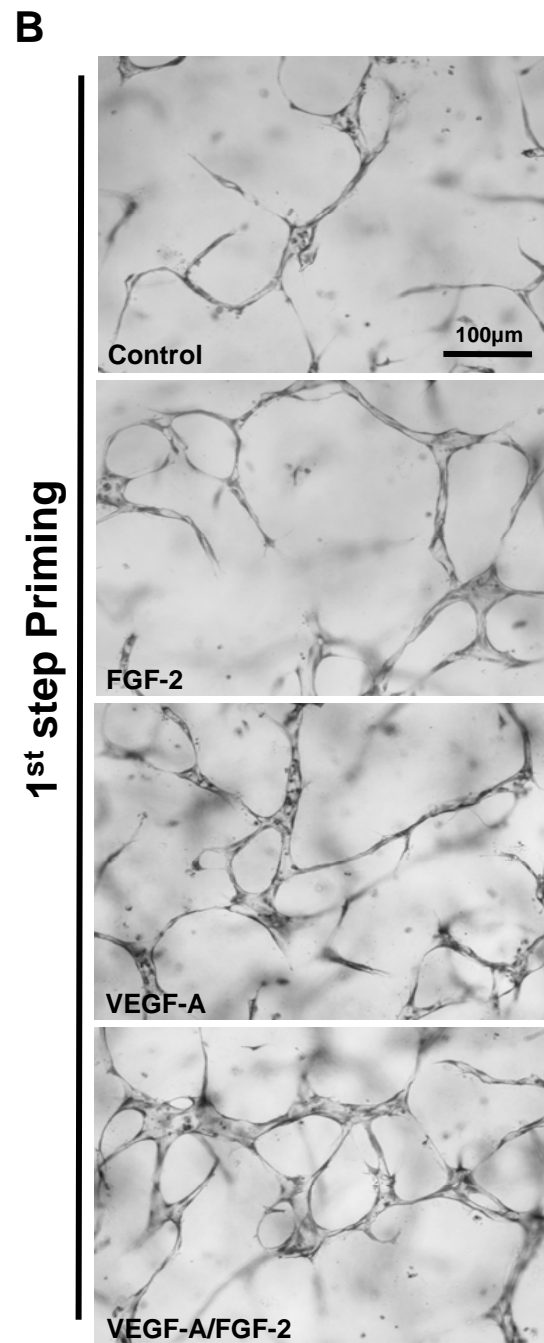
Quail Injected with Drugs during Embryonic Development (at day 1):			
	Cranial Hemorrhage	Abdominal Hemorrhage	Survival
DMSO Control	0/9	0/9	8/9
Imatinib	0/9	2/9	7/9
AMD3100	0/9	4/9	6/9
Imatinib/AMD3100	3/9	9/9	4/9
α-IL-3	0/9	0/9	8/9
α-SCF	0/9	2/9	8/9
α-IL-3/SCF	4/9	9/9	5/9

C

Control	Control	α-SCF/IL-3	α-SCF/IL-3

Figure S5