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Hematopoietic Stem Cell Development Is Dependent on Blood Flow

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A

α-adrenergic	β-adrenergic	Ca^{2+} Flux	Na^{+}/K^{+} Flux	Nitric Oxide	Angiotensin	Other
↓ Ergotamine	↑ Atenolol	↓ BayK8644	↑ Strophanthidin	↓ Proadifen	↓ Enalapril	↑ Hydralazine
↓ Ephedrine	↑ Pronethalol	↑ Fendiline	↑ Lanatoside C	↑ Histamine	↓ Captopril	↑ Todralazine
↓ Methoxamine	↑ Propranolol	↑ Nicardipine	↓ Nerifolin	↓ Chrysin		
↓ Mephentermine	↑ Pindolol	↑ Nifedipine	↑ Peruvoside	↓ Ambroxol		

B

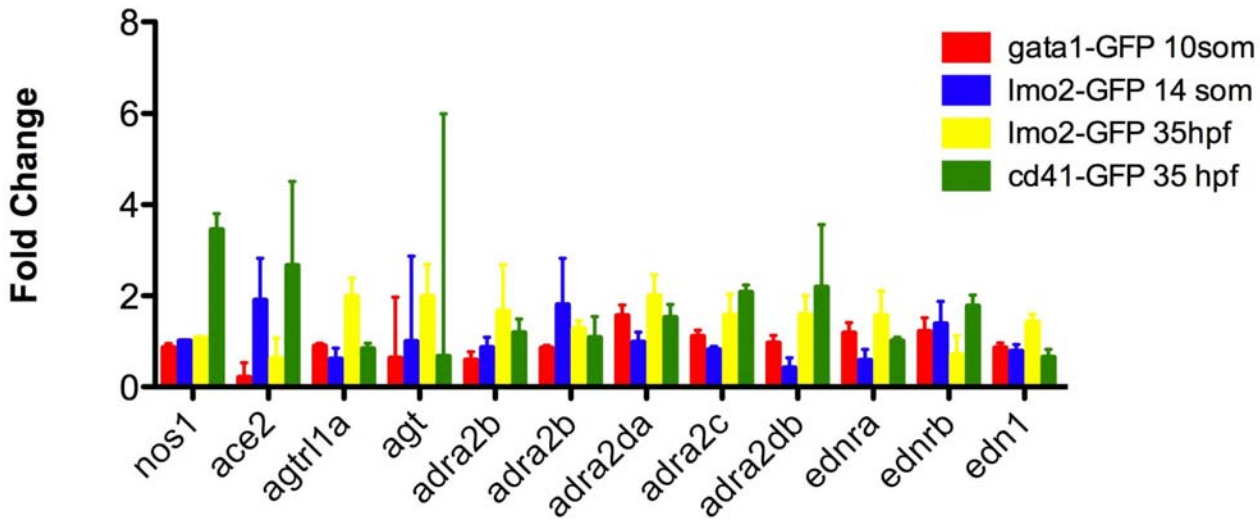


Figure S1. Modulation of blood flow affects HSC formation in zebrafish.
Zebrafish were exposed to chemicals (10 μM) from 10 somites to 36 hpf and subjected to *in situ* hybridization for *runx1/cmyb*.
(A) Summary of the effects of drugs included in the original chemical screen libraries and their mechanism of action.
(B) Stage-specific regulation of genes involved in blood flow regulation in the hematopoietic and endothelial compartments of the developing zebrafish embryo. Cell populations were isolated by FACS in transgenic zebrafish embryos and subjected to microarray analysis. *nos1* is upregulated in the HSC compartment at 36 hpf.

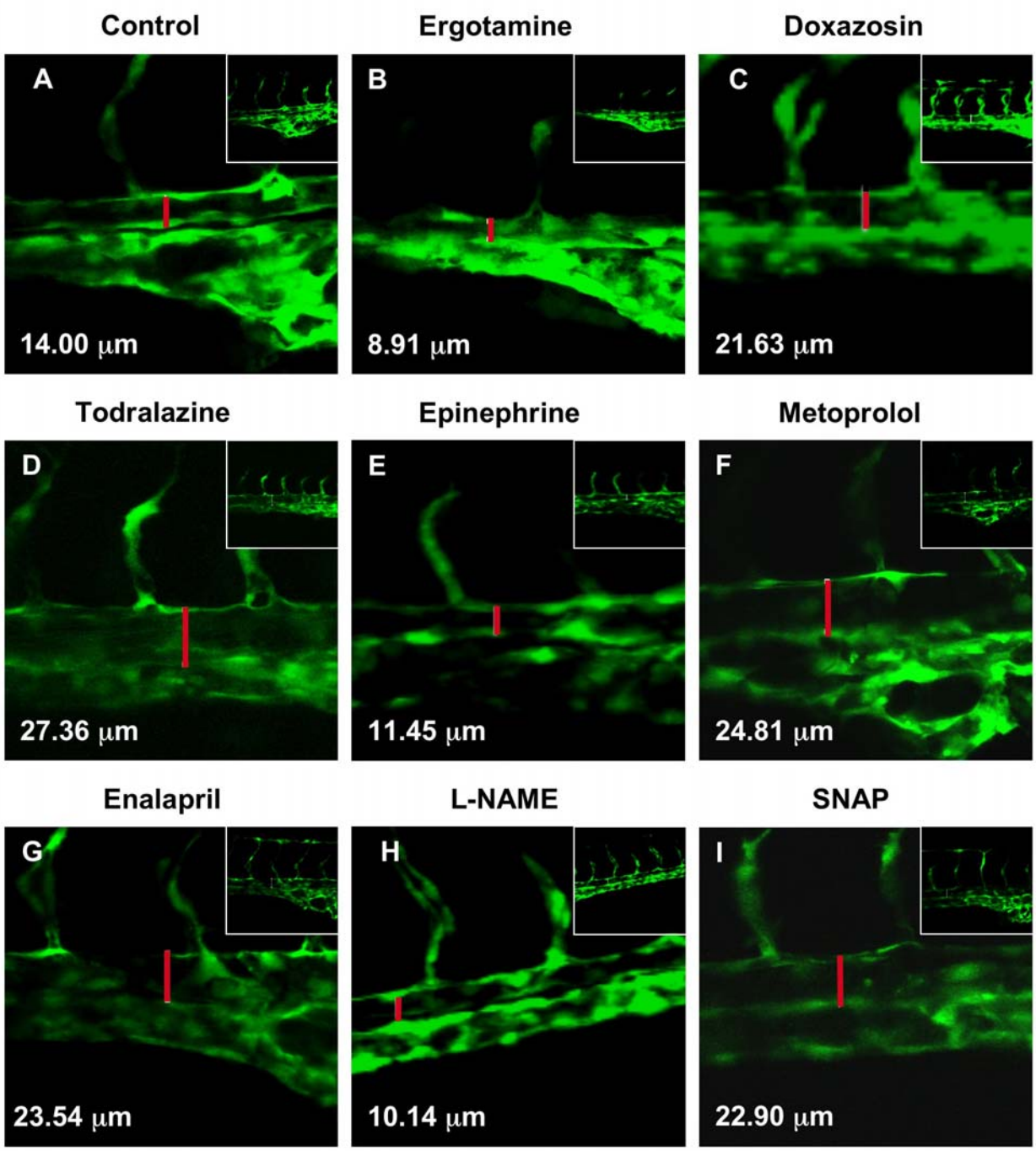


Figure S2. Nitric Oxide mediates the effect of blood flow on HSCs.
(A-I) Effect of chemical modifiers of blood flow on vascular diameter *in vivo*. Transgenic *fli:GFP* fish were treated with chemicals (10 μM) from 10 somites to 36 hpf and imaged by confocal microscopy. Microscopy images with measurement of the diameter of the dorsal aorta in representative samples of drug-treated embryos. The inset shows a lower magnification image to visualize the entire tail region. The red bars indicate the width of the dorsal aorta.

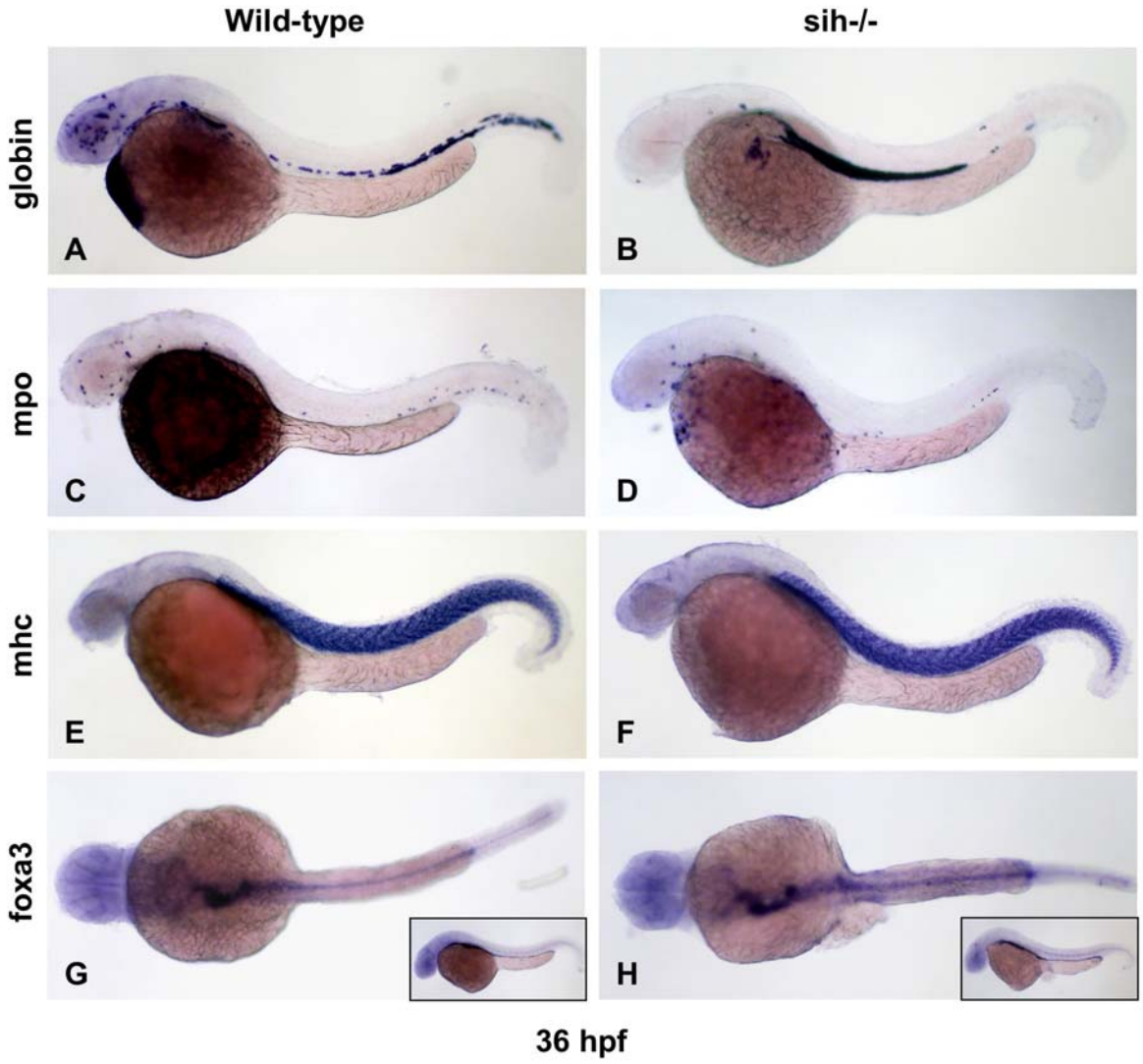


Figure S3. The silent heart mutation does not affect primitive hematopoiesis or mesodermal and endodermal development at 36 hpf.
(A-D) *In situ* hybridization (n>25 per treatment) for *globin* and *myeloperoxidase (mpo)* demonstrates pooling of blood cells due to the absent heartbeat, but no quantitative changes for primitive erythropoiesis or myelopoiesis in *sih* mutants.
(E,F) Somite formation as depicted by *in situ* hybridization for *myosin heavy chain* (*mhc*) is normal in *sih* mutants, indicating that other mesodermal organs develop normally.
(G,H) Endoderm development, visualized by *foxa3* expression, is not affected in *sih* mutants.

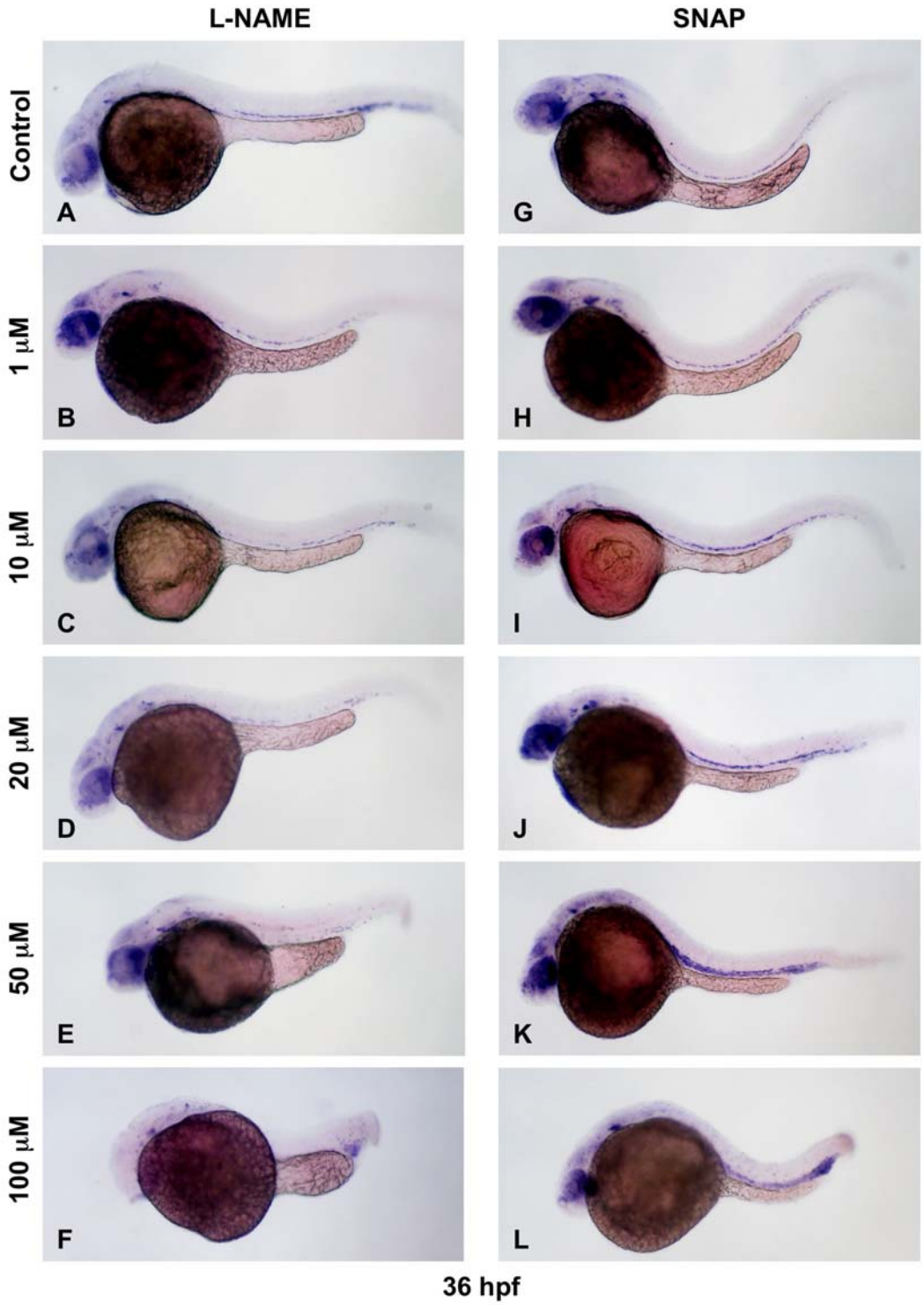
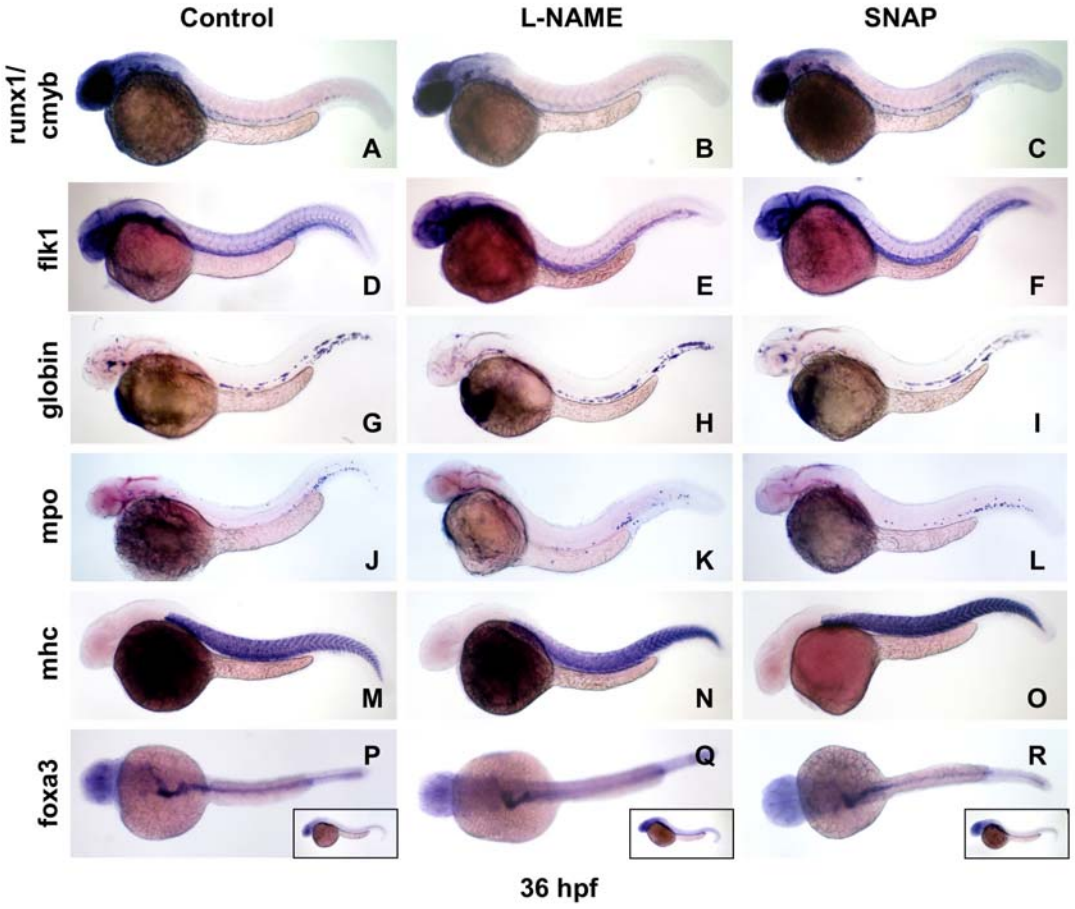


Figure S4. Modulation of NO has dose-dependent effects on HSC formation.
(A-L) Embryos (n>25 per treatment) were exposed to increasing doses of L-NAME (**B-F**) or SNAP (**H-L**) from 10 somites to 36 hpf. With increasing L-NAME dose, HSC formation was progressively diminished. Similarly, SNAP lead to a dose-dependent increase of *runx1/cmyb* expression. Doses >20 μM for each drug led to gross morphological abnormalities.



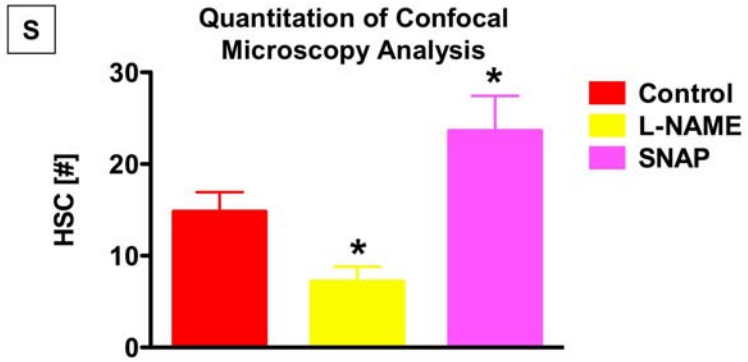


Figure S5. NO modulation does not affect primitive hematopoiesis or development of mesodermal and endodermal structures.

(A-R) Embryos (n>25 per treatment) were exposed to control, L-NAME or SNAP from 10 somites to 36 hpf.

(A-F) Expression of the vascular marker *flk1* is minimally altered by NO modulation.

(G-R) Primitive erythro- *(globin)* and myelopoiesis (*mpo*) as well as early muscle (*mhc*) and endoderm (*foxa3*) development are not affected by changes in NO signaling.

(S) Quantitation of HSC number in confocal microscopy analysis of *cmyb:GFP; lmo2:dsRed* embryos (**Fig.3Q-S**) reveals significant changes in response to NO modulation (* sig vs. control, ANOVA, $p<0.001$, n=5).

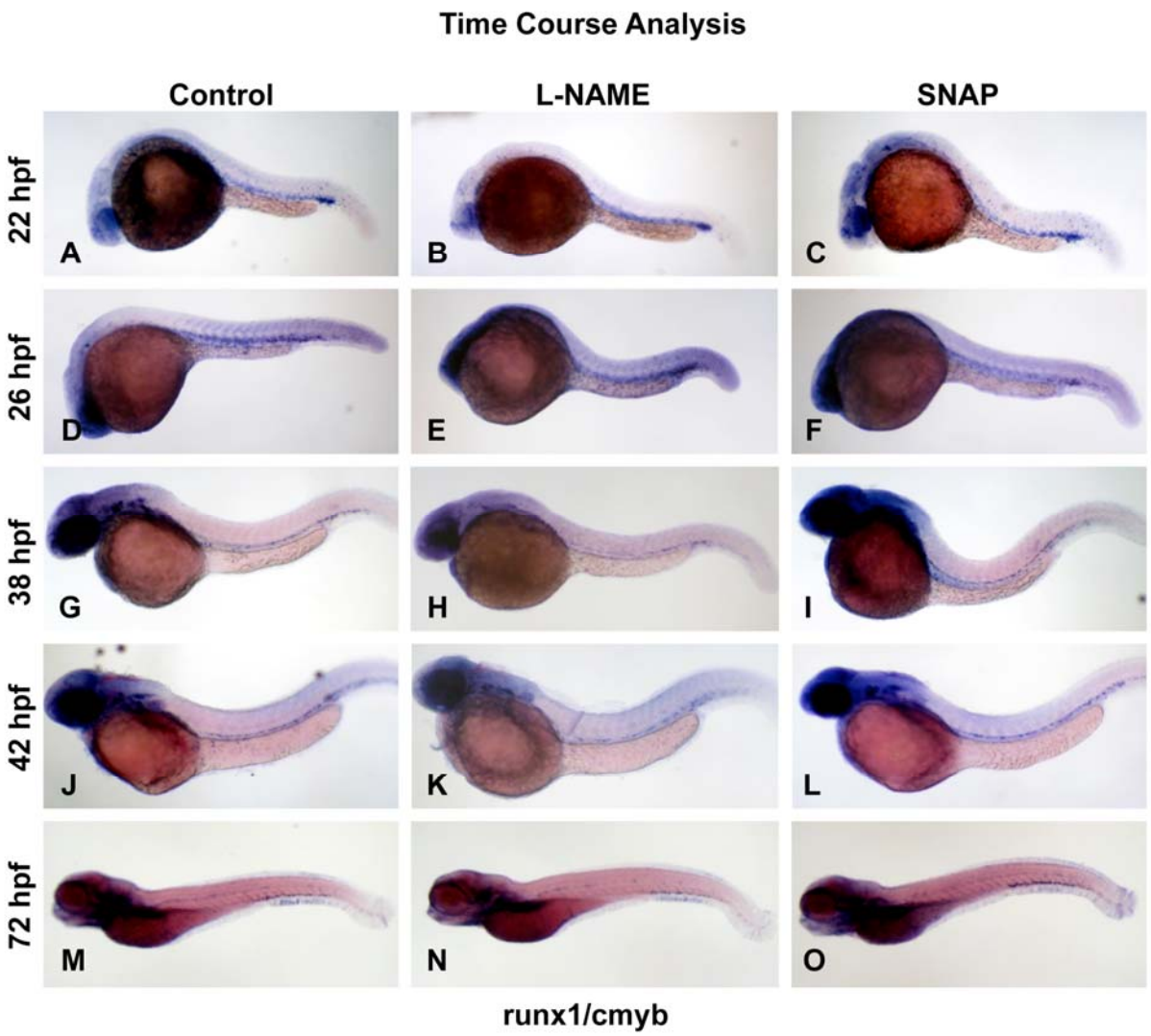


Figure S6. Time course analysis reveals time-specific effect of NO modulation on HSCs.

(A-O) Embryos were exposed to L-NAME and SNAP from 10 somites until fixation (22 – 72 hpf).

(A-F) SNAP exposure does not increase the expression of HSC markers at early stages.

(G-O) nos inhibition by L-NAME does not cause a delay in HSC development that can be compensated for at later developmental stages.

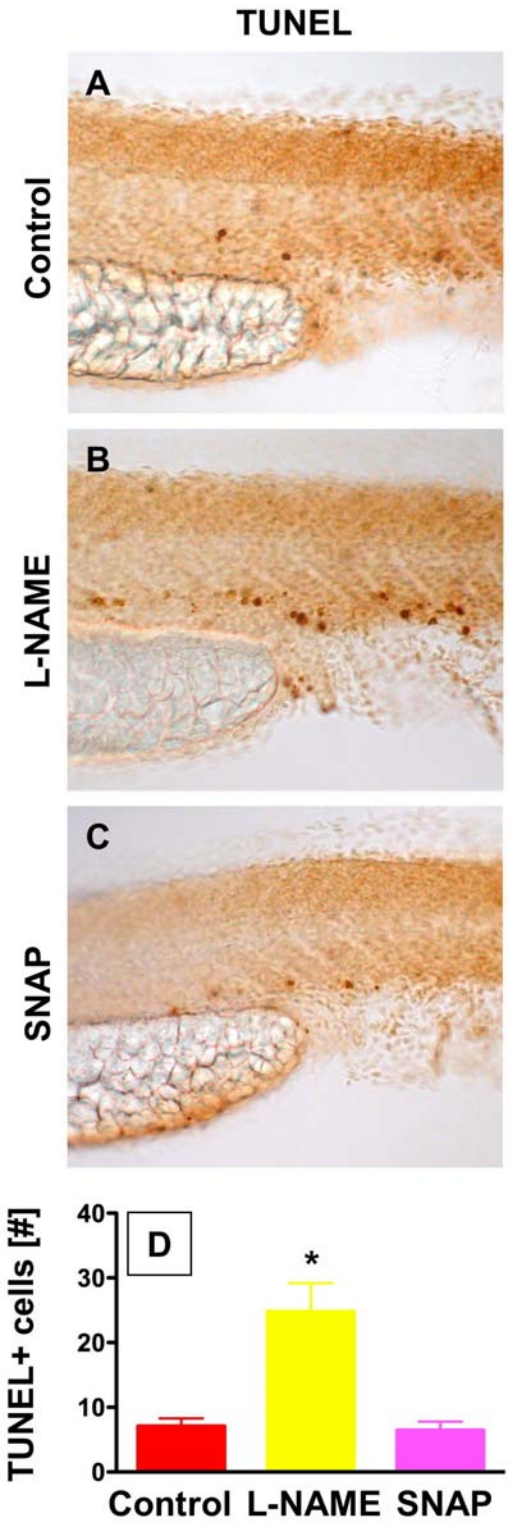


Figure S7. Nos inhibition increases apoptosis within the AGM
(A-D) Zebrafish embryos were exposed to L-NAME and SNAP from 10 somites to 36 hpf and processed for TUNEL staining. L-NAME treatment significantly enhanced the number of apoptotic cells in the zebrafish AGM tail region. * sig vs. control, $p<0.001$, ANOVA, n=10.

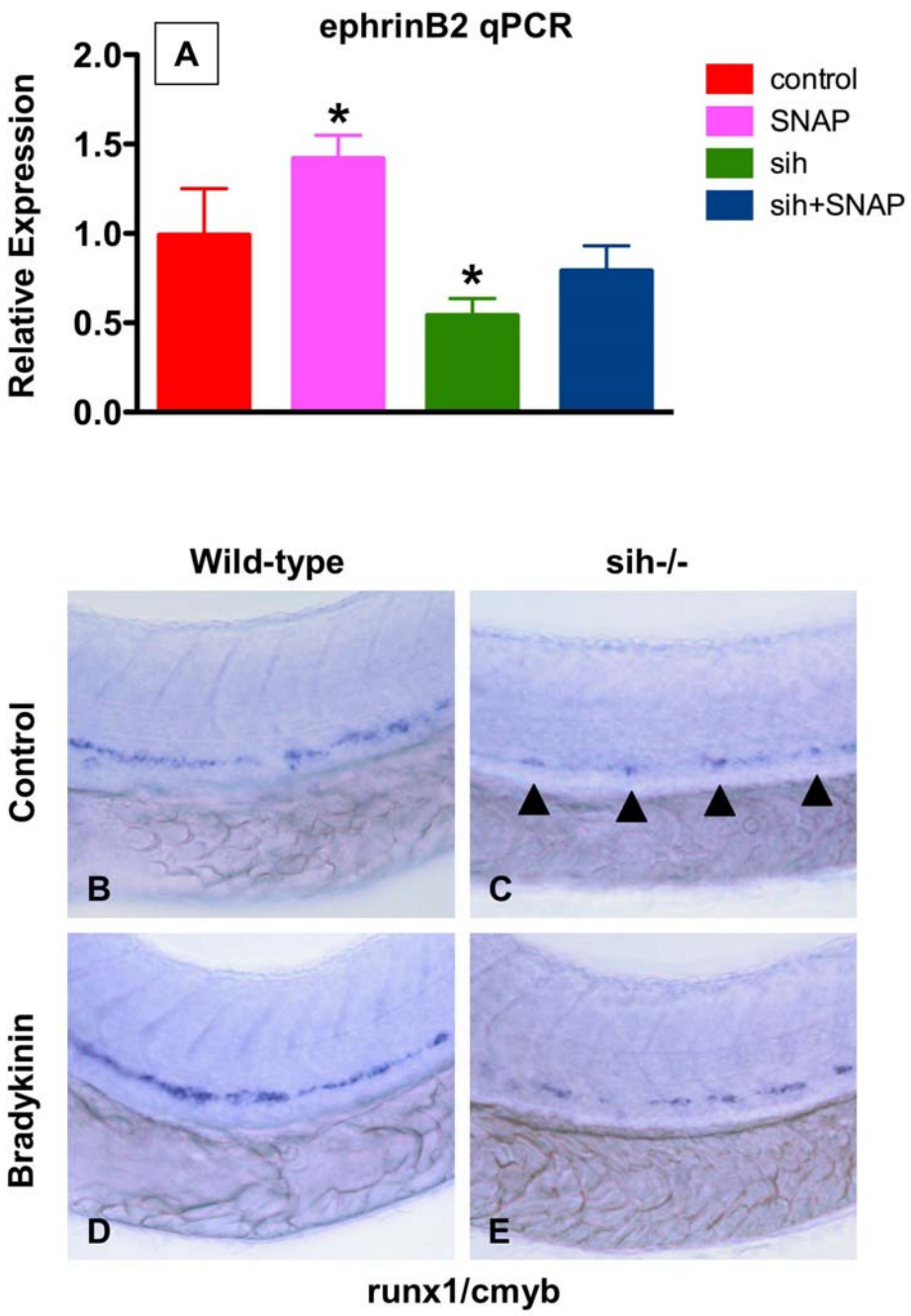


Figure S8. NO signaling affects vascular and HSC development.
(A) qPCR for *ephrinB2* in WT and *sih-/-* in the presence and absence of SNAP. * sig vs. control, $p<0.05$, ANOVA, n=5.
(B-E) Effect of bradykinin (10 μM) on *runx1/cmyb* expression in wild-type and *sih-/-* embryos at 36 hpf. *runx1/cmyb* positive cells are highlighted by arrowheads.

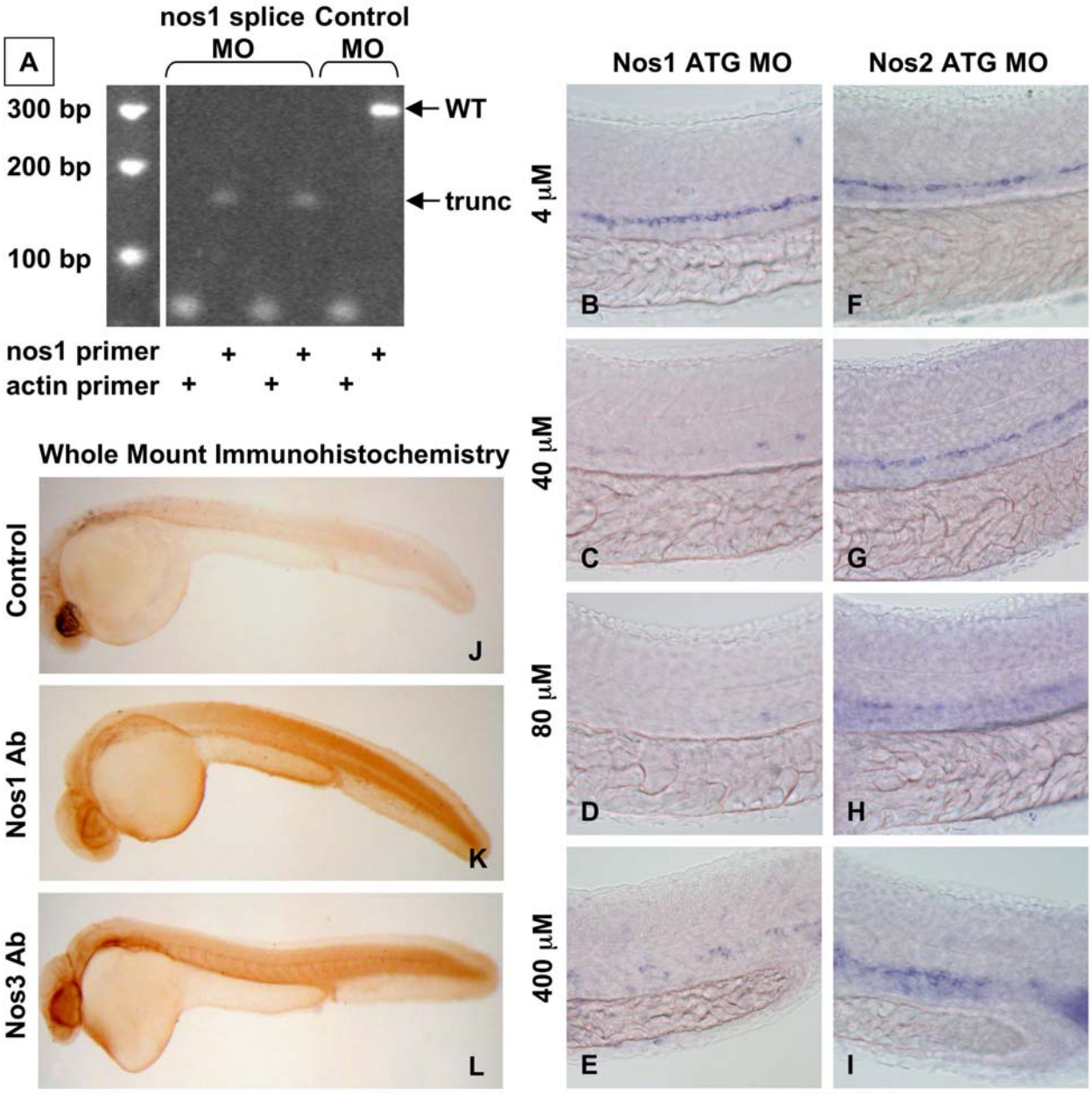


Figure S9. The effect of *nos1* MO inhibition is dose-dependent.
(A) RT-PCR performed on cohorts of 20 pooled *nos1* splice site MO (40 μM) and control MO injected embryos. The control injected embryos exhibited the expected fragment length (300bp), while the PCR product after splice site MO injection is shorter as expected. Actin is shown as a control.
(B-E) Increasing *nos1* knockdown by increasing doses of MO caused progressive decrease in *runx1/cmyb* expression.
(**F-I)** *nos2* knockdown did not affect HSC formation.
(J-L) Immunoreactivity to both anti-mouse Nos1 and Nos3 antibody was present in zebrafish embryos at 36 hpf. Nos3 reactivity was found in the vasculature, neural tube and endodermal tissues.

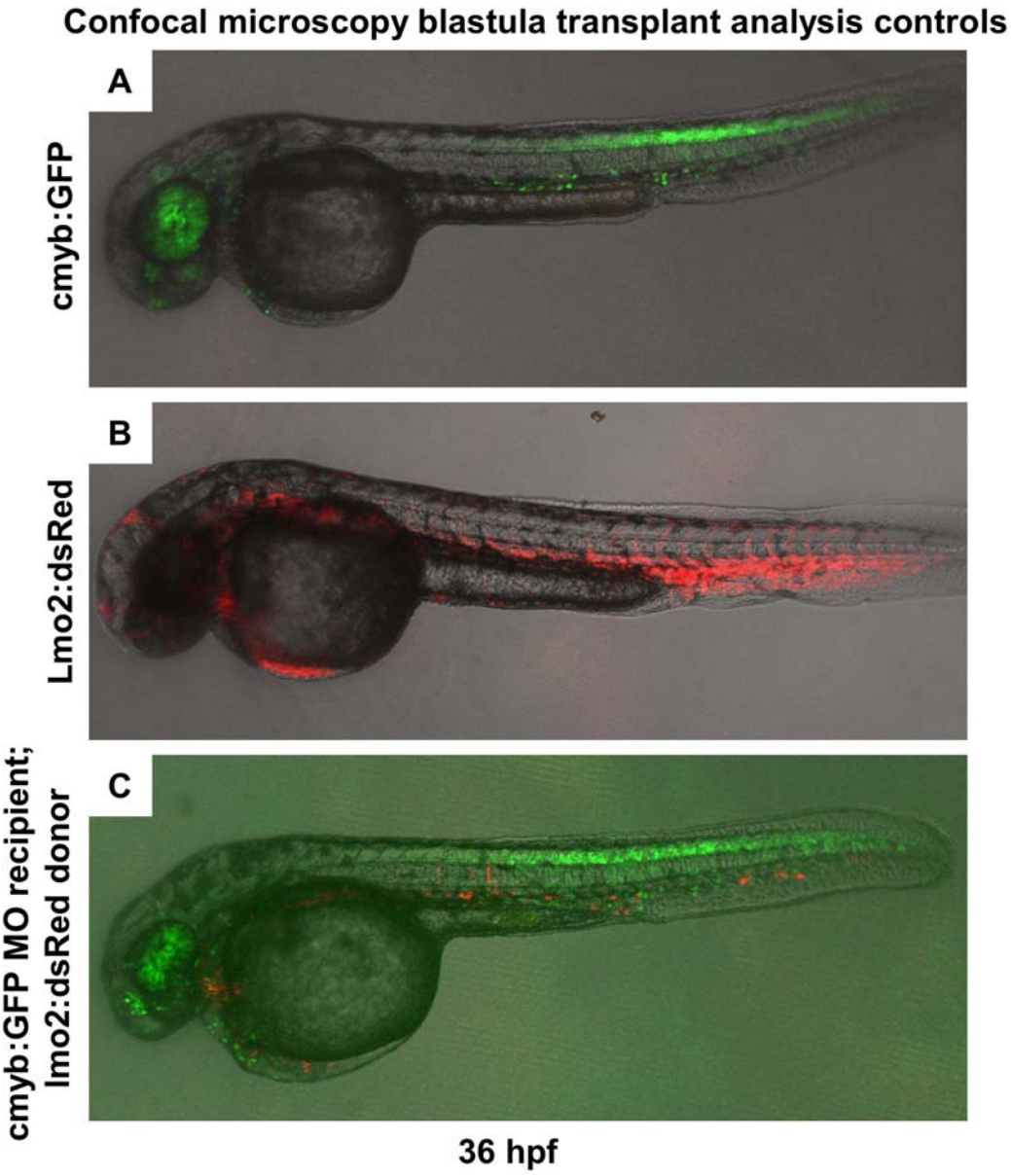


Figure S10. Blastula transplant controls.
(A) Uninjected control *cmyb:GFP* embryo.
(B) Uninjected control *lmo2:dsRed* embryo.
(C) Recipient *cmyb:GFP* embryos injected with *nos1* MO had a grossly normal phenotype normal and express *cmyb:GFP* robustly in the eye, and neural crest; greatly reduced expression was found in the HSC compartment. Donor-derived endothelial cells could be seen in red fluorescence.

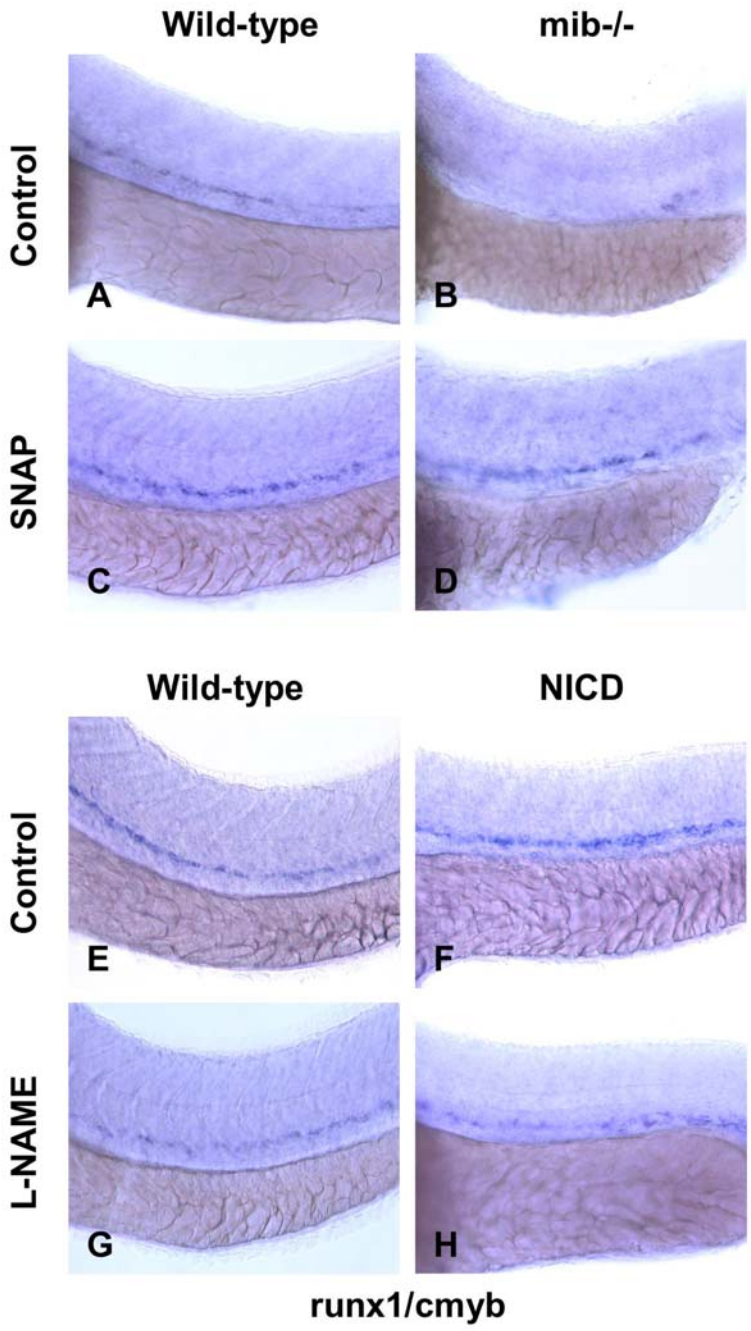


Figure S11. NO modifies the effects of notch signaling on HSC formation.
Zebrafish embryos were assessed by *in situ* hybridization for *runx1/cmyb* at 36 hpf.
(**A-D)** wild-type and *mib-/-* mutant embryos were exposed to DMSO and SNAP (10 μM) from 10 somites to 36 hpf. NO rescued the HSC defect in *mib-/-* embryos.
(**E-H)** Inhibition of NO by L-NAME (10 μM) diminished the enhancing effect of constitutive notch activation in NICD transgenic zebrafish embryos.

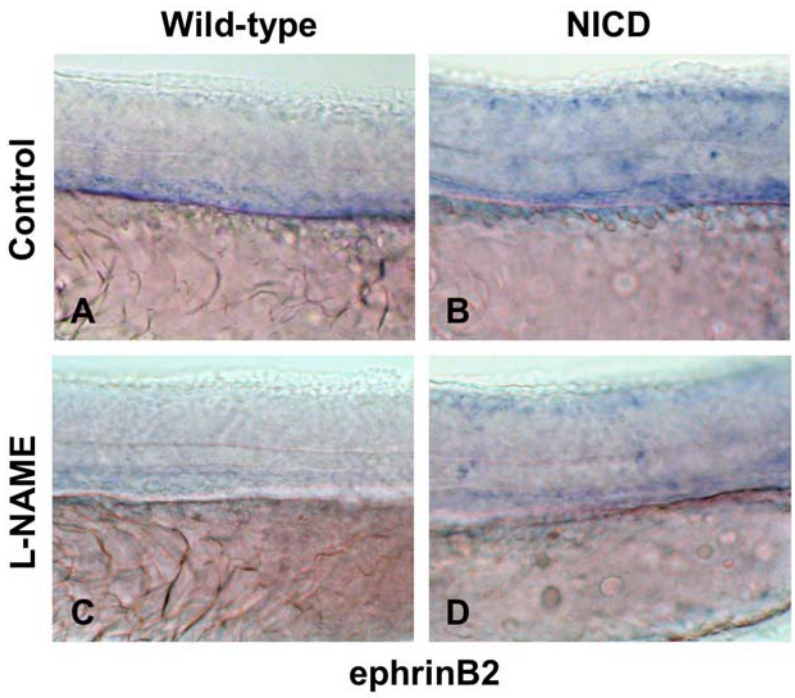


Figure S12. NICD-mediated elevation of ephB2 is blocked by nos inhibition.
Zebrafish embryos were assessed by *in situ* hybridization for *ephB2* at 36 hpf (n>25/condition).
(A-D) The effect of L-NAME on *ephB2* expression in WT and NICD transgenic embryos; L-NAME treatment blocked the notch-mediated increase in *ephB2* staining.

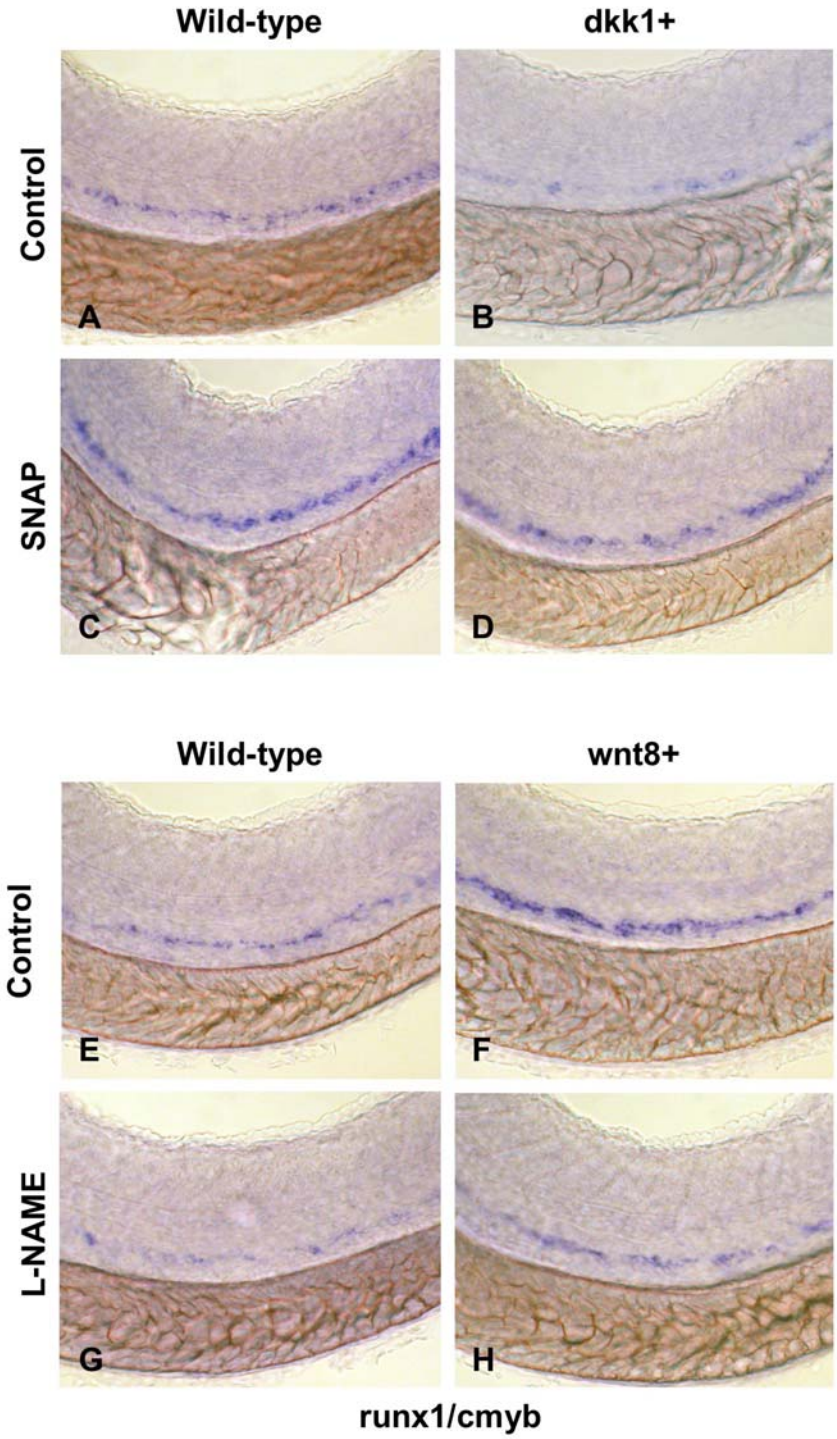
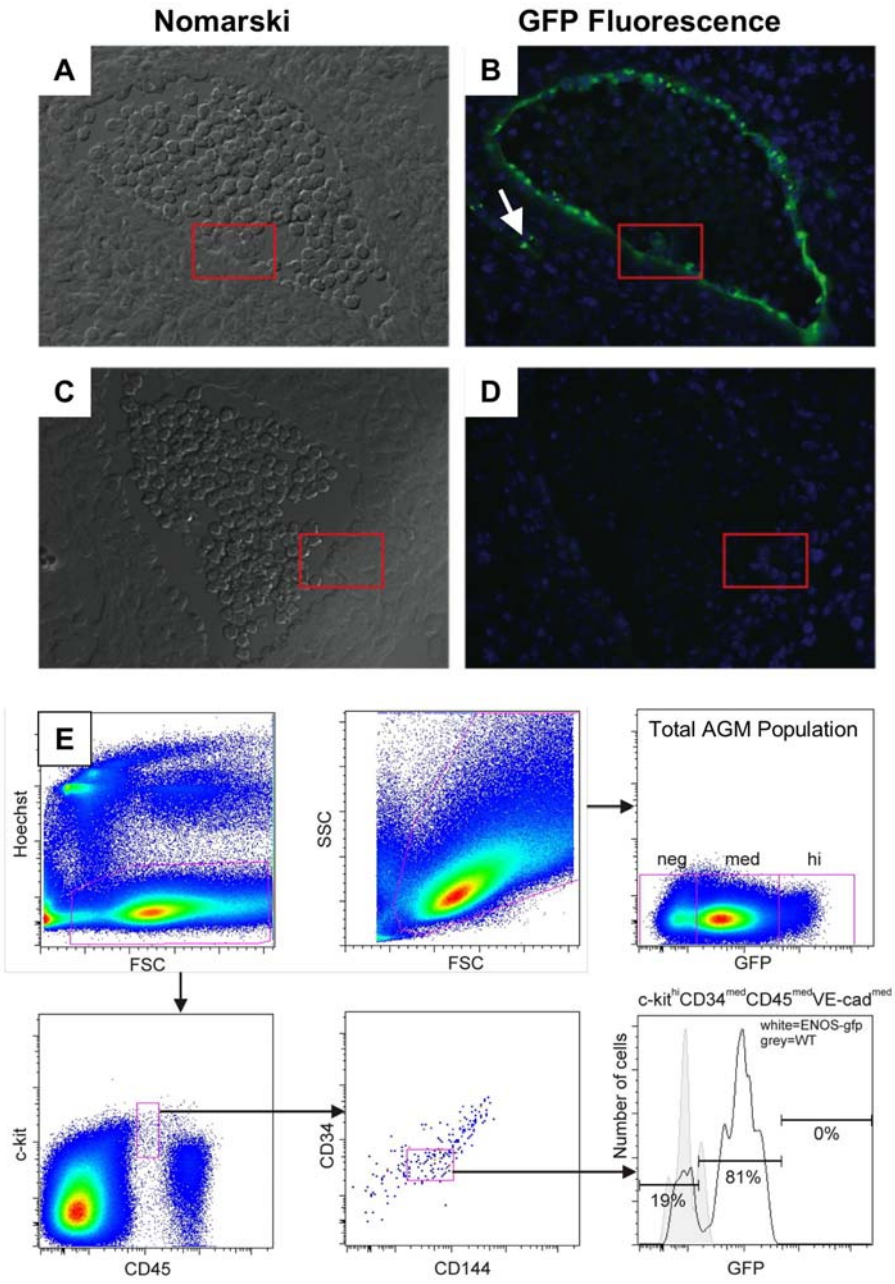


Figure S13. NO modifies the effects of wnt signaling on HSC formation.
(A-H) Inducible wnt pathway transgenic embryos were subjected to heatshock at 38°C for 20 mins at 10 somites and then exposed to chemicals (10 μM) until 36 hpf and subjected to *runx1/cmyb in situ* hybridization.
(**A-D)** *dkk1* induction diminished HSC number, which can be rescued by SNAP.
(**E-H)** L-NAME inhibited the *wnt8*-mediated enhancement of HSCs.



F	# repopulated/ # total (% repopulated)		
Tissue	**Nos3^{neg}**	**Nos3^{lo}**	**Nos3^{hi}**
e11.5 AGM	1/16 (6%)	3/15 (20%)	0/7 (0%)

Figure S14. Nos3 expression characterizes the transplantable HSC population in the AGM

(A-D) DIC and green fluorescence microscopy of sections through the aorta of *Nos3:GFP* transgenic **(A,B)** and wild-type control **(C,D)** mouse embryos at e8.5.

Individual cell nuclei are indicated by DAPI staining. Hematopoietic clusters are highlighted by a red box. The arrow indicates a subaortic patch of HSCs. The arrowhead indicates the lack of GFP fluorescence within erythrocytes in the lumen of the vessel.

(E) Representative multicolor flow cytometric analysis of E11.5 *Nos3:GFP* transgenic AGM cells. Single cell suspensions, gated for live (Hoechst negative) mononuclear cells (SSC; FSC), were analyzed for HSC marker expression. The top right panel shows fractionation of AGM cells fall into Nos3:GFP negative, medium and high expression groups. The bottom right panel shows a histogram plot of GFP expression in *Nos3:GFP* transgenic (black outlined curve) and wild type (grey curve) E11.5 AGM cells gated on viable, mononuclear C-kit^{hi}CD45^{med}CD34^{med}VE-cadherin^{med} cells. 9.4±8.9% were negative for Nos3:GFP expression, 90.5±8.2% expressed Nos3:GFP to an intermediate level and no cells exhibited high levels of Nos3:GFP. Three independent experiments were performed using a total of 40 *Nos3:GFP* transgenic embryos and 25 wild-type embryos.

(F) Nos3:GFP^{lo} expressing AGM cells contain the transplantable population. Suspension of AGM cells were sorted into Nos3:GFP negative, intermediate, and high fractions. Donor-derived cells in recipient peripheral blood at 4 months post transplantation were detected by PCR, with >10% donors marked cells considered positive.

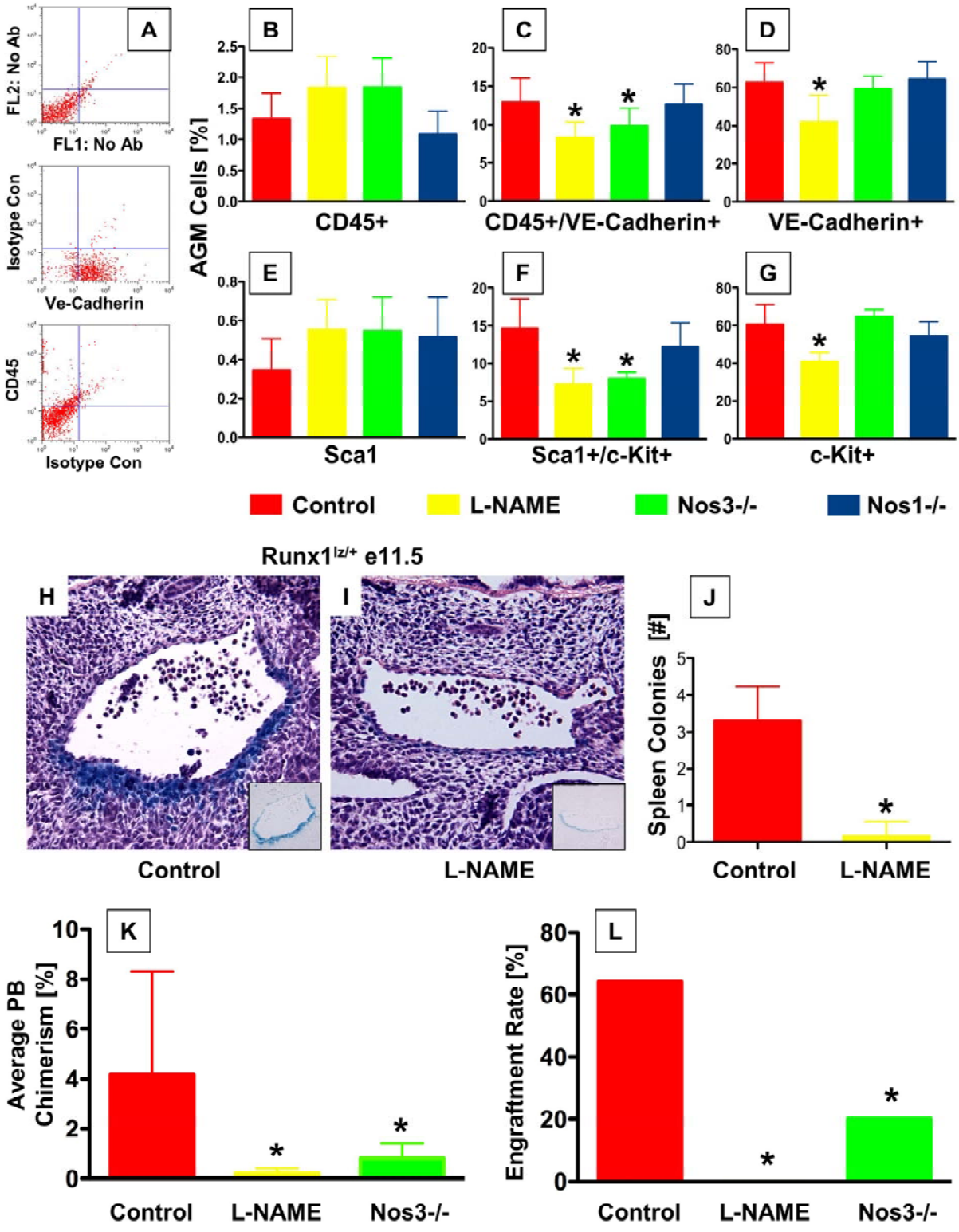


Figure S15. Inhibition of NO signaling decreases phenotypic and functional characteristics of AGM HSCs

(A-G) Summary of FACS analysis of subdissected AGM at E11.5.

(A) Representative control FACS plots showing (top to bottom) an unstained AGM cell suspension; a FL1+ (VE-cadherin), FL2 isotype control; a FL2+ (CD45); FL1 isotype control.
(C,F) The CD45+/VE-Cad+ and sca1+/ckit+ cell populations are significantly diminished in L-NAME and *Nos3-/-* embryos. * sig vs. control, $p<0.05$, ANOVA, $n\geq8$.
(D,G) The VE-Cad+ and c-Kit+ populations were significantly decreased in L-NAME treated embryos. * sig vs. control, $p<0.05$, ANOVA, $n\geq8$.
(H,I) Histological analysis of *Runx1:lacZ* mice revealed lack of hematopoietic clusters and reduced Runx1+ cells in the AGM of embryos from L-NAME treated females. Serial sections through the entire aorta of ≥10 embryos per genotype/treatment were analyzed.
(J) L-NAME treatment of pregnant females decreased functional embryonic progenitors, as measured in spleen colony formation on day 8 post AGM transplantation into irradiated mice. * sig vs. control, $p<0.001$, t-test, $n\geq10$.
(K,L) Diminished NO signaling in the AGM of either L-NAME exposed or *Nos3-/-* e11.5 embryos caused a decrease in transplantable HSCs, assessed both by average chimerism or engraftment >1% at 6 weeks post transplantation. * sig vs. controls, $p<0.05$, ANOVA, $n\geq5$.

Table S1.

PCR primer sequences

beta actin F	5'-GCTGTTTTCCCCTCCATTGTT
beta actin R	5'-TCCCATGCCAACCATCACT
runx1 F	5'-CGTCTTCACAAACCCTCCTCAA
runx1 R	5'-GCTTTACTGCTTCATCCGGCT
cmyb F	5'-TGATGCTTCCCAACACAGAG
cmyb R	5'-TTCAGAGGGAATCGTCTGCT
flk1 F	5'-CGAACGTGAAGTGACATACGG
flk1 R	5'-CCCTCTACCAAACCATGTGAAA
ephrin B2 F	5'-CAAGGACAGCAAATCGAATG
ephrin B2 R	5'-TGAGCCAATGACTGATGAGG
nos1 F	5'-CTCCATTCAGAGCCTTCTGG
nos1 R	5'-CCGACAACCAAACACCAAG
nos2 F	5'-AGGCACTCGTGGCTATCAAT
nos2 R	5'-ATGCTGCATGAAGGACTCG
nos1 splice junct F	5'-TGGGGTGGAGGATAACAATG
nos1 splice junct R	5'-ACAGCCTTGGTAGGAGAAACTC