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Article

Estrogen Defines the Dorsal-Ventral Limit of VEGF Regulation to Specify the Location of the Hemogenic Endothelial Niche

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SUMMARY

Genetic control of hematopoietic stem and progenitor cell (HSPC) function is increasingly understood; however, less is known about the interactions specifying the embryonic hematopoietic niche. Here, we report that 17β-estradiol (E2) influences production of *runx1*+ HSPCs in the AGM region by antagonizing VEGF signaling and subsequent assignment of hemogenic endothelial (HE) identity. Exposure to exogenous E2 during vascular niche development significantly disrupted *flk1*+ vessel maturation, *ephrinB2*+ arterial identity, and specification of *scl*+ HE by decreasing expression of *VEGFAa* and downstream arterial Notch-pathway components; heat shock induction of *VEGFAa*/*Notch* rescued E2-mediated hematovascular defects. Conversely, repression of endogenous E2 activity increased somitic *VEGF* expression and vascular target regulation, shifting assignment of arterial/venous fate and HE localization; blocking E2 signaling allowed venous production of *scl*+/*runx1*+ cells, independent of arterial identity acquisition. Together, these data suggest that yolk-derived E2 sets the ventral boundary of hemogenic vascular niche specification by antagonizing the dorsal-ventral regulatory limits of VEGF.

INTRODUCTION

Hematopoietic stem cells (HSCs) are characterized by a lifelong ability to self-renew and give rise to each differentiated blood lineage. In mice, the first definitive HSCs arise at embryonic day 10.5 from hemogenic endothelium (HE) in the ventral wall of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region (Dzierzak and Speck, 2008). Whereas signaling cascades that control specification of arterial identity are well elucidated (Lawson et al., 2001, 2002), less is known about the factors regulating the dorsal/ventral limits of HE identity. Recent studies have implicated several pathways in patterning the AGM and subsequent HSC emergence, acting in part via polarized expression of signaling elements on the dorsal side (Clements et al., 2011; Wilkinson et al., 2009). However, the mechanism by which these signaling cascades integrate to specify the limits of HE remains an open question, as does the identity of ventral-derived signals (Jaffredo et al., 2013). Additionally, data from both zebrafish (Burns et al., 2005; Kim et al., 2013) and mice (Robert-Moreno et al., 2008) indicate that production of HSCs within arteries may not be required for HE specification. Following niche assignment, the transcription factor *Runx1* is recognized for its critical and highly conserved role in HSC development (North et al., 2002; Okuda et al., 1996; Wang et al., 1996), where it is required for HSCs to "bud" from HE (Chen et al., 2009). In zebrafish, hematopoietic stem and progenitor cells (HSPCs) first emerge in an analogous region of the dorsal aorta between 30–36 hr postfertilization (hpf), and *runx1* mediates a similar role in their production (Kissa and Herbomel, 2010). Our group has previously identified regulators of vertebrate HSC development via an in vivo chemical screening approach in zebrafish (Goessling et al., 2009, 2011; North et al., 2007, 2009); in that screen, estrogens and estrogen-related compounds were found to have a potent impact on the formation of *runx1*+ HSCs.

Estrogen is a cholesterol-derived steroid hormone synthesized from testosterone by the enzyme CYP19A1 (Aromatase). There are three primary forms of estrogen found in the vertebrate phylum: estrone, estradiol, and estriol. 17β-Estradiol (E2), commonly referred to as "estrogen," is the most potent. Classically, E2 acts as a transcription factor upon binding to cytoplasmic nuclear hormone receptors, estrogen receptor 1 (Esr1; ERα) or Esr2 (ERβ), which subsequently translocate to the nucleus and bind estrogen response elements (EREs) in regulatory regions of estrogen-responsive genes (Heldring et al., 2007). In zebrafish, because of a partial genome duplication, in addition to *esr1*, there are two *esr2* receptors: *esr2a* and esr2*b* (Menuet

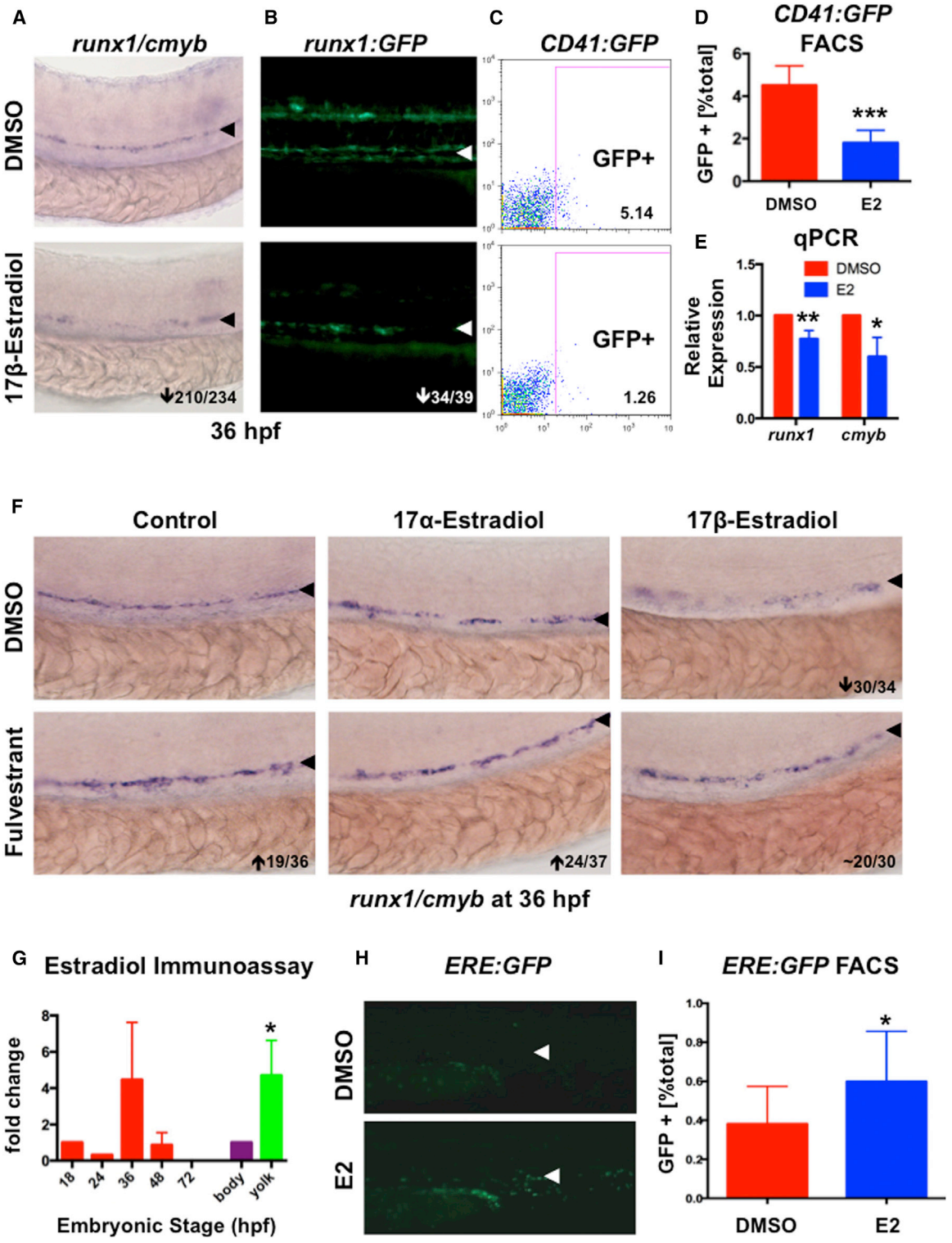

Figure 1. Exposure to E2 Impairs Formation of HSPCs in the AGM

(A) E2 exposure from five somites to 36 hpf decreased *runx1/cmyb* expression in the AGM (210/234).

(B) E2 diminished runx1:GFP+ cells in the AGM (34/39).

(C) FACS analysis of *CD41:GFP* embyros (representative samples shown) confirmed that E2 reduced HSCs.

(D) CD41:GFP was significantly decreased by E2 (n = 20, two-tailed t test, $p < 0.001$; error bars indicate SD).

(E) qPCR quantified reduced *runx1* and *cmyb* expression by E2 (mean of triplicate experiments ± SEM; one-tailed t test *runx* $^{**}p < 0.01$, *cmyb* $^{*}p < 0.05$).

(F) The pan-Esr antagonist FULV blocked the effect of E2; the isomer 17α-estradiol (10 μM) had no effect (n $\geq$ 30/treatment).

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et al., 2002). E2 is also a ligand for a less well-characterized G protein-coupled receptor (GPER; also called GPR30) (Liu et al., 2009; Revankar et al., 2005). Although the role of E2 in reproductive organ development is established (Wilson and Davies, 2007), less is known of its impact on the formation of other organ systems.

Endogenous E2 levels are highly variable during mammalian gestation. E2 levels are low during early pregnancy but increase throughout gestation, peaking just prior to delivery (Tulchinsky et al., 1972). It is unclear whether the developing embryo is exposed to increasing concentrations of E2; indeed, several pieces of evidence suggest mechanisms are in place to limit E2 exposure to the conceptus. Expression of 17β-hydroxysteroid dehydrogenase type 2, which degrades E2, varies between umbilical arteries and veins and may protect the developing embryo from deleterious effects of excess maternal E2 (Simard et al., 2011). Surfeit estrogen can have a negative impact on maintenance of pregnancy, indicating a need for careful control over E2 levels during gestation (Mahendroo et al., 1997). Based on the presumed importance of controlled E2 exposure during embryogenesis, there are increasing concerns regarding the presence of estrogenic substances in the environment. Diethylstilbestrol (DES), a synthetic estrogen previously prescribed as an antiabortifactant, was found to increase risk of vaginal and cervical cancer, as well as male genital defects, in offspring whose mothers took the drug (Harris and Waring, 2012). Maternal hormonal use in the first trimester of pregnancy is associated with increased risk of infant acute leukemia, indicating in utero exposure to estrogenic compounds may influence fetal hematopoietic homeostasis (Pombo-de-Oliveira et al., 2006). As little is known about the impact of estrogens on hematopoiesis during embryogenesis, we sought to prospectively determine the effect of E2 and related compounds on HSC formation.

Here, we demonstrate that exposure to excess E2 from early somitogenesis until 24 hpf, the window of hemogenic endothelial (HE) specification, significantly decreased the formation of *runx1*+ AGM HSPCs. In contrast, later exposure, during HSC specification and budding, enhanced HSPC number. HSPC loss after early E2 exposure was mediated via esr2 and resulted from a failure to specify HE in the dorsal aorta. Defects in both VEGF and Notch signaling, required for the establishment of arterial identity and hemogenic niche formation, were noted following E2 treatment; hyperactivation of VEGF rescued *runx1* expression through induction of the Notch pathway, indicating that disruption of this signaling cascade underlies the observed hematovascular alterations. Exposure to xenoestrogens ethinylestradiol and genistein partially replicated E2-mediated phenotypes, decreasing *runx1*+ HSPC expression and altering vascular niche specification. Significantly, antagonism of intrinsic estrogen signaling enhanced the expression and regulatory function of *VEGF*, increasing the zone of *scl*+ HE specification, independent of *ephrinB2*+ arterial assignment, and enhancing the production of HSPCs. Together, these data suggest endogenous E2 acts as a previously unappreciated morphogen, opposing the action of VEGF to control the assignment of the embryonic HE niche.

RESULTS

17β-Estradiol Decreases Formation of HSCs

E2 and estrogen-related compounds were identified in a prior screen for modulators of HSC formation (North et al., 2007) (Figure S1A available online). To confirm and characterize the impact of hormonal regulation on hematopoiesis, zebrafish embryos were exposed to 17β-estradiol (E2) from five somites (12 hpf) until 36 hpf, the window of hematopoietic initiation in zebrafish (Figure S1B). Markedly decreased expression of the conserved HSPC markers *runx1* and *cmyb* was observed in the AGM after E2 treatment relative to sibling controls (Figure 1A) by whole-mount in situ hybridization (WISH); with the chorion intact, this effect was dose dependent over a range of 0.1 to 10 μM E2 in the fish water, whereas phenotypic changes were seen at 0.01 μM E2 with the chorion removed (Figure S1C). Using a transgenic *runx1*-reporter line (*Tg*(*runx1P1:EGFP*)), E2-mediated reduction in runx1+ cells was confirmed in vivo (Figure 1B); fluorescence-activated cell sorting (FACS) quantification of the *CD41:GFP* line (*Tg*(*−6.0itga2b:EGFP*)), marking mature HSCs, revealed a similar decrease (Figures 1C and 1D; $p < 0.001$). These findings were corroborated by whole-embryo quantitative PCR (qPCR) (Figure 1E; *runx* $p < 0.01$, *cmyb* $p < 0.05$). To determine if the effect of E2 was receptor mediated, embryos were treated with a pan-Esr antagonist, Fulvestrant (FULV; 15 μM), in the presence and absence of E2 (Figure 1F). Exposure to FULV alone increased *runx1*/*cmyb* and restored expression in the majority of E2-treated embryos. Identical results were observed with ZK164015 (ZK; 10 μM) (Figure S2A). Notably, exposure to 17α-estradiol, the inactive isomer of E2, did not affect HSPCs (Figure 1F), nor did the GPER agonist, G1, or antagonist, G15 (Figure S2A). Finally, no changes were observed in the gross development of other major embryonic organ systems (Figure S1D), demonstrating that E2 acts via cytoplasmic nuclear hormone receptors to impact HSPC formation during this window of embryogenesis.

Zebrafish Possess Endogenous Estrogenic Activity and the Ability to Respond to E2 Signaling

To examine endogenous E2 content during hematopoiesis, a modified ELISA assay was performed. Zebrafish embryos showed higher E2 levels early in development (0–48 hpf) than in larval stages (72–120 hpf) (Figure 1G). The decrease in E2 concentration during embryogenesis suggested that endogenous estrogen may be contained in the yolk; following manual deyolking at 18 hpf, E2 levels were found to be 4.7-fold higher in yolk compared to the embryo body ($p < 0.05$). Consistent with maternal contribution of E2, enzymes involved in E2 synthesis were undetectable by qPCR until ~20 hpf (Figure S2D). As

(G) Estradiol EIA revealed that endogenous estrogens are present during hematopoiesis; E2 was enriched 4.7-fold in the yolk versus body at 18 hpf (mean of triplicate experiments ± SEM; two-tailed t test, *$p < 0.05$).
(H) *ERE:GFP* fish show estrogenic activity in the AGM.
(I) GFP expression (as seen in H) was increased 1.57-fold upon E2 exposure ($n = 15$, two-tailed t test, $p < 0.05$; error bars indicate SD).
The number of embryos with altered expression over the total number analyzed is shown (bottom right).

endogenous E2 was present, expression of the *esrs* was also assessed. Whole-embryo qPCR confirmed published results (Chandrasekar et al., 2010): *esr1* was expressed at low levels immediately after fertilization but increased starting at 12 hpf. In contrast, *esr2a* and *esr2b* were more robustly expressed immediately after fertilization and subsequently declined, consistent with maternal deposition (Figure S2B); after 12 hpf, expression of *esr2a* and *esr2b* increased, suggestive of embryonic transcriptional initiation. *esr2a* was previously shown to be expressed ubiquitously via WISH during the time frame of hematopoietic development, whereas neither *esr1* nor *esr2b* were observed at detectable levels until >36 hpf (Bertrand et al., 2007). To identify if hematovascular populations in the AGM express *esrs*, we analyzed publicly available microarray data (Weber et al., 2005): both endothelial cells and emerging HSPCs express *esrs* to varying degrees, indicating that they can respond to E2 regulation during embryogenesis (Figure S2C).

To determine if E2 signaling was active in the AGM, we examined activity in transgenic embryos expressing GPF under control of the E2 response element *[Tg(5xERE:GFP)]* (Gorelick and Halpern, 2011). At 36 hpf, endogenous E2 activity was observed over the yolk extension, with the strongest signal situated to the ventral side of the embryo (Figure 1H). Addition of 10 μM E2 increased whole-embryo ERE:GFP levels 1.57-fold, as quantified by FACS, within the physiological range of estrogen activity (Figure 1I; $p < 0.05$), and consistent with recent reports (Hao et al., 2013); expression of *vitellogenin-1* (*vtg1*), a classic estrogen target, showed a similar 2-fold induction ($p < 0.001$) (Figure S2E). *ERE:GFP* WISH revealed E2 activity over the yolk in the ventral aspect of the trunk as early as 18 hpf (Figure S2F). To ensure that GFP expression was due to endogenous E2 and to identify the receptor(s) responsible for the HSPC phenotype, we utilized morpholino (MO)-mediated knockdown. Injection of *esr* MOs (Froehlicher et al., 2009; Griffin et al., 2013; Pang and Thomas, 2010) reduced ERE:GFP levels similarly to ZK exposure (Figures S3A and S3B). No effects on HSPCs were observed for *esr1* knockdown (Figure 2A). Whereas injection of the *esr2a* MO increased *runx1*/*cmyb* expression, E2 remained capable of reducing HSPCs. *esr2b* knockdown had only mild effects on HSPCs; however, coinjection of *esr2a* and *esr2b* MOs blocked the effect of E2, indicating that these isoforms are the primary mediator of the HSPC phenotype. These results were corroborated using receptor-selective compounds for Esr1 (PPT [agonist] and MPP [antagonist]) and Esr2 (DPN [agonist] and PHTPP [antagonist]) (Figure S3C; data not shown). As microarray analysis indicated *esr2a* was present in endothelium (Figure S2C), ERE:GFP activity was examined in *lmo2:dsRed* embryos (Figure 2B). Occasional ERE:GFP+ cells were embedded in the aortic vasculature of controls at 36 hpf, and double-positive cell numbers increased following E2 exposure; WISH indicated vascular (*fli1*+) localization and response to E2-stimulation could be detected by 24 hpf (Figure S3E). Strong E2-responsive ERE:GFP colocalization was also noted in the kidney, ventral to the aortic vasculature and adjacent to maternal yolk deposits, using the *Tg*(*cdh17:mCherry*) reporter (Figure 2C); by WISH, kidney expression and E2 responsiveness was observed at 24 hpf (Figure S3D). Together, these data indicate E2 is present and can elicit receptor-mediated activity in the AGM during hematovascular development.

E2 Exposure Impairs Formation of the Hemogenic Arterial Hematopoietic Niche

To determine how effects on HSPCs were mediated, we examined the impact of E2 exposure during two major developmental milestones of HSC production: from ~12–24 hpf, the hemogenic vascular niche is established, and from ~24–36 hpf, post-circulation onset, HSCs are specified and begin to bud from HE in the artery. E2 exposure during these discrete time windows caused a biphasic effect: treatment from ~12–23 hpf resulted in the absence of *runx1*/*cmyb* expression, whereas treatment from ~26–36 hpf increased HSPCs (Figure 3A); DPN mimicked both aspects of this phenotype, suggesting each was mediated by esr2 (Figure S4A). As the negative impact of E2 from 12–24 hpf was the dominant phenotype, we sought to further characterize effects on the development of the hemogenic vascular niche. Analysis of embryos expressing GFP under control of the angiopoietin receptor promoter, *tie2 (Tg(Tie2:EGFP)*, indicated normal circulation and proper formation of the dorsal aorta and cardinal vein after E2 exposure (Figure 3B); in contrast, tie2:GFP+ intersomitic vessels (ISVs) were strongly reduced. Expression of the VEGF receptor *flk1* (*kdrl*, *VEGFR2*) was decreased in the major vessels of most E2-exposed embryos, with ISV expression reduced or absent at 36 hpf, as confirmed by qPCR (Figures 3C and 3H; $p < 0.01$); however, flk1+ cell number, as seen by FACS of the *flk1:GFP* line *(Tg(kdrl:EGFP*) was not significantly altered (Figure 3G), confirming gross vascular structure was unaffected by exogenous E2. To determine if reductions in *flk1*+ ISVs were indicative of defective vessel maturation, markers of artery-vein specification were examined: WISH and qPCR showed *ephrinB2* was robustly decreased, whereas *flt4*, a venous marker, increased (Figures 3D, 3E, and 3H; *ephrinB2* $p < 0.001$; *flt4* $p < 0.05$). To assess if the subset of HE was negatively impacted, *scl*-expressing cells in the transgenic *Tg*(*-6tal1:EGFP*) reporter were examined: following E2-exposure, arterial expression of *scl* was almost absent, as confirmed by qPCR ($p < 0.05$) (Figures 3F and 3H). To determine if scl loss was responsible for the dramatic reduction in HSPCs, E2-treated embryos were injected with *scl* mRNA (Figure 3I); whereas 86% of E2-exposed embryos showed *runx1* loss, *scl*-injection rescued expression in 77% of treated embryos. Together, these data indicate E2 alters the production HSPCs via regulation of aortic vessel maturation and tissue specification.

As early E2 exposure had a potent negative effect on HE, we sought to determine if the later effects of E2 on HSPCs were likewise due to niche regulation. A positive response to E2 treatment from 26–36 hpf was confirmed by qPCR for *runx1* and *cmyb* (Figure S4B; $p < 0.05$), but no impact was found on *ephrinB2* or *flt4* (data not shown), suggesting at later stages E2 may act directly on HSPCs. As E2 is a known cell-cycle regulator, several indicators of proliferation were examined: both *cyclinD1* and *cmyc* were increased by E2 exposure from 26–36 hpf (Figure S4C, *cyclinD1* $p < 0.01$, *cmyc* $p < 0.001$); phosphohistone H3 (Figure S4D, $p < 0.01$) and BrdU incorporation (Figure S4E) were similarly enhanced, together indicating elevations in *runx1*+ HSPCs, following later E2 treatment, coincide with increased cell proliferation, consistent with recent reports (Nakada et al., 2014). In contrast, BrdU was not altered by early E2 exposure. These data indicate E2 only

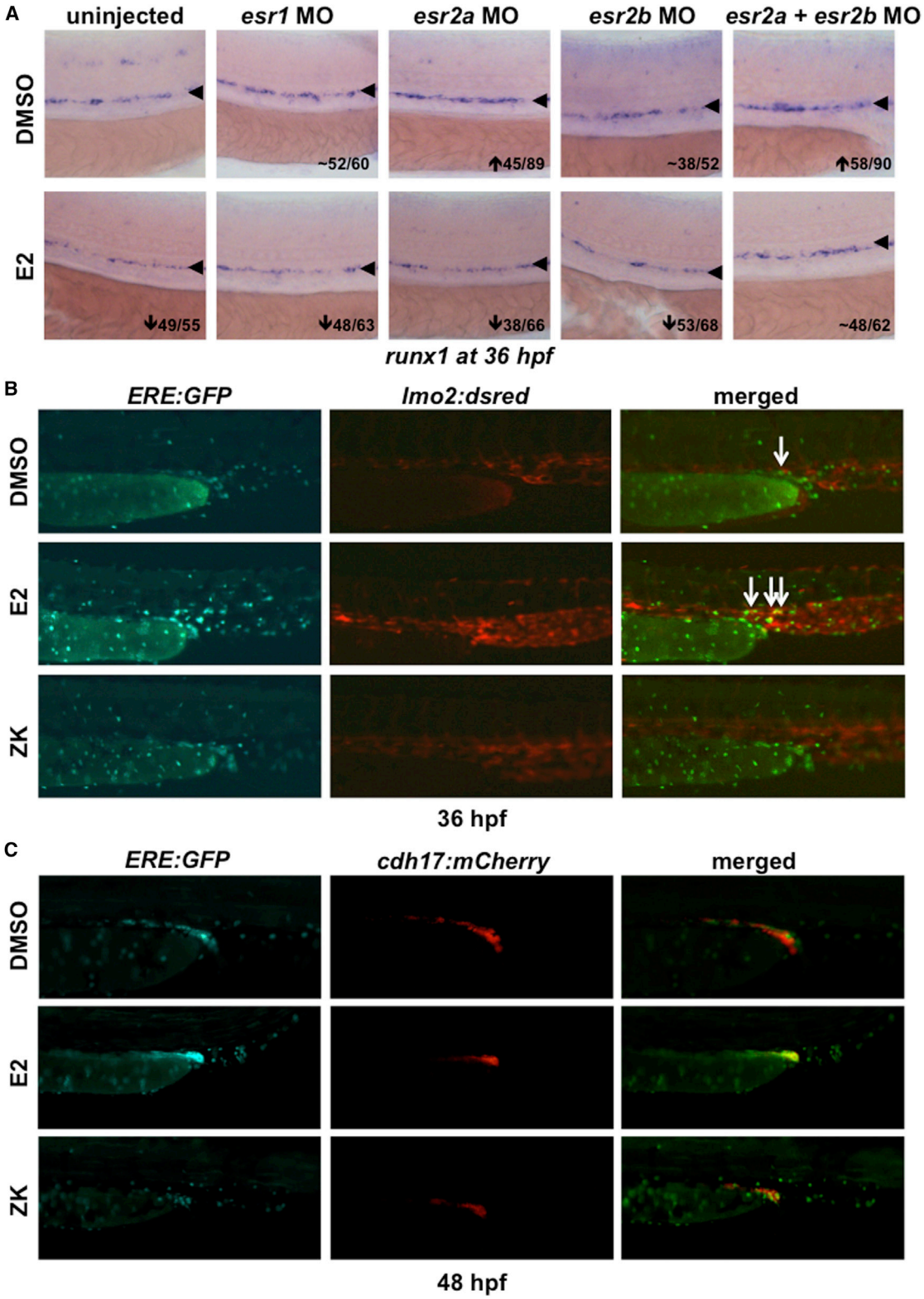

Figure 2. ERE:GFP Embryos Display Estrogen Activity in the Developing AGM

(A) MO knockdown showed that the effect of E2 on HSPCs is mediated through *esr2a+b*; *esr1*-MO had no impact on *runx1/cmyb* ($n \geq 30$).

(B) *ERE:GFP* embryos show low expression in the trunk vasculature, labeled by *lmo2:dsRed*; ERE activity expands following E2 exposure and is alleviated by treatment with ZK164015 ($n \geq 20$).

(C) *ERE:GFP* embryos exhibit activity in the developing mesonephros, as indicated by *cdh17:mCherry* at 48 hpf, which responds to altered E2 signaling ($n \geq 20$).

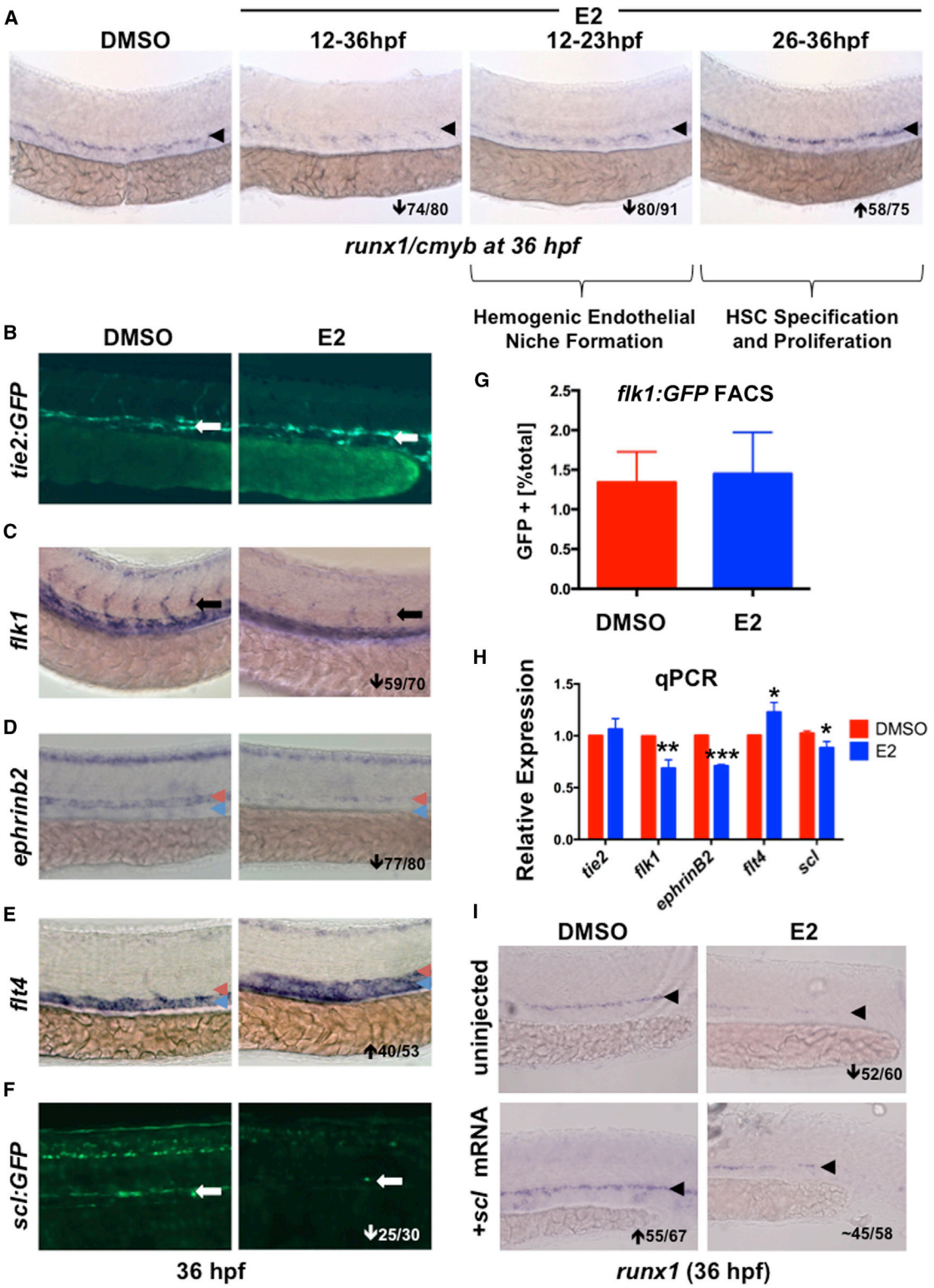

Figure 3. E2 Exposure Impairs Formation of the Hemogenic Endothelial Niche

(A) E2 had a biphasic effect on HSPCs: treatment during hemogenic niche specification (12–23 hpf) decreased *runx1*, whereas exposure during HSC specification and budding (26–36 hpf) increased HSPCs (n ≥ 75/treatment).

(B) Treatment with E2 had no impact on axial vessel formation as assessed by *tie2:GFP* (n ≥ 20).

(C) E2 disrupts ISV formation as indicated by *flk1* WISH (59/70).

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affects the vascular niche for a limited time (12–24 hpf), independent of proliferation, implying it plays a role in specifying HE.

E2 Exposure Disrupts the VEGF/Notch Pathway, Leading to Vascular Defects

Specification of the arterial trunk vasculature, where the hemogenic vascular endothelium is located, occurs through precise stepwise induction of known signaling pathways: Hedgehog (HH), Vascular Endothelial Growth Factor (VEGF), and Notch (Lawson et al., 2001, 2002). As Notch was the most proximal to HSCs, it was tested for response to E2 exposure. At 18 hpf, after 6 hr of treatment, no changes in global expression of the Notch ligand *deltaC* were noted, indicating Notch signaling initiates correctly (Figure 4A); this finding was corroborated using *Notch:GFP* (*Tg(EPV:Tp1-MmuHbb:EGFP)*) reporter embryos (Parsons et al., 2009) and *ephrinB2* WISH (Figures S5A and S5B). At 24 and 36 hpf, however, marked decreases in arterial *deltaC* expression were observed, as confirmed by qPCR (Figures 4A and 4C; $p < 0.05$); a similar pattern of regulation was observed for *notch3* (Figures 4B and 4C; $p < 0.05$). The *Notch:GFP* reporter likewise showed strongly reduced arterial and intersomitic activity (Figure 4D); inhibition was also noted for the notch target *hey2* and the VEGF-regulated arterial marker and notch pathway component *notch1b* (Figures S5C and S5D). Hyperactivation of the Notch pathway, downstream of ligand binding, was used to determine if restoration of Notch signaling could rescue E2-mediated HSPC defects. Heat shock induction of the Notch intracellular domain (NICD) *(hs:Gal4; UAS:NICD)* in controls resulted in increased *runx1* expression in both the artery and the vein (23/37), matching published results (Burns et al., 2005), whereas NICD activation in E2-treated fish recovered *runx1*+ HSPCs (20/31) (Figure 4E). *mindbomb* (*mib*) mutants have defective Notch signaling (Itoh et al., 2003); *mib*+/− embryos, compared to controls, showed a previously unreported reduction in *runx1* expression (Figure S5E), which was exacerbated after E2 treatment (22/41), mimicking the homozygous phenotype. These data indicate synergy between E2 exposure and loss of Notch activity and imply deficits in Notch signaling cause HSPC abnormalities induced by exogenous E2.

VEGF acts upstream of Notch in vascular specification. E2 exposure caused decreased somitic expression of *VEGFAa* at both 18 and 24 hpf, as confirmed by qPCR (Figures 4F and 4G; $p < 0.001$). Heat-shock-inducible *VEGFAa* embryos (*hs:VEGFAa* [Wiley et al., 2011]) were utilized to determine if hyperactivation of VEGF could rescue E2-mediated HSPC defects. *VEGFAa* induction increased *runx1* (31/37) in controls (Figure 4H), consistent with our recent observations (Harris et al., 2013) and compensated for the deleterious effect of E2 (16/33). However, when Notch activity was simultaneously blocked by the γ-secretase inhibitor DAPT (20 μM), VEGF overexpression failed to rescue the effect of E2 on HSPCs (38/38) (Figure 4H); this Notch dependence was confirmed by *Notch:GFP* WISH, where increased activity was observed following *VEGF* induction (Figures S5F and S5G). *tbx20* expression is unaffected in *mib* mutants, indicating it is regulated upstream of Notch signaling in arterial specification (Lawson et al., 2001). Arterial *tbx20* was reduced by E2 (23/25); induction of *VEGFAa* in E2 treated embryos partially rescued *tbx20* expression (16/25), whereas *NICD* could not (Figure S6A) (4/26). In contrast, effects of later E2 exposure were independent of Notch or VEGF (Figures S4F and S4G). To assess if HH-signaling, upstream of VEGF, was also impacted by E2, we examined expression of *shh* and its targets: *shh* was unaltered, whereas decreases in *patched2* (*ptch2*) and *GLI-Kruppel family member 1* (*gli1*) were observed by qPCR (Figures S6B and S6C; $^{*}p < 0.05$, $^{**}p < 0.01$), although no changes in muscle or somite development were noted. To see if E2-mediated HH regulation caused *VEGFAa*, loss, embryos were treated with the smoothened agonist SAG: SAG enhanced *ptch2* expression and blunted the effects of E2. SAG likewise enhanced *VEGFAa* in controls; however, it had no impact on *VEGFAa* in E2-treated embryos, indicating E2 antagonizes VEGF independently of HH (Figure S6D). Together, these data suggest E2-mediated alterations in hemogenic niche specification are caused by loss of *VEGFAa* expression and downstream activity.

Xenoestrogens Partially Mimic the Effects of 17β-Estradiol on HSCs

To determine if estrogens could limit HSPC production in the absence of deficiencies in maternal or fetal E2 regulation, we examined the impact of environmentally derived xenoestrogens (XE). Three compounds, genistein (GEN), a component of soy products, ethinylestradiol (EE), an estrogen found in hormonal contraceptives, and Bisphenol A (BPA), a hardening agent used in the manufacturing of plastics, were selected as representatives of the larger class of XEs. Upon XE treatment, *runx1/cmyb* expression was reduced in all cases, as confirmed by qPCR (Figures 5A and 5F). Importantly, XE-mediated HSPC loss could be rescued by treatment with FULV, indicating the effects were mediated, at least in part, by Esr signaling (Figure 5A). GEN and EE treatment also partially replicated the vascular niche defects observed with E2: at nontoxic doses, no major abnormalities were seen in ISV formation by *fli1/flk1* staining (Figure 5B); however, arterial Notch:GFP was reduced by both GEN and EE (Figure 5C). Exposure to GEN and EE likewise led to alterations in arterial marker expression and expression of *scl*, whereas BPA had no effect on these targets (Figures 5E and 5F). Further, a noticeable reduction in *VEGFAa* expression was caused by EE (Figure 5D). Whereas changes following XE treatment were neither as severe nor as penetrant as those from excess E2, the same signaling cascades were affected,

(D) Arterial *ephrinb2* was robustly decreased by E2 (77/80); red arrowheads indicate artery, and blue arrowheads indicate vein.
(E) Venous *flt4* was increased after E2 exposure (40/53).
(F) scl:GFP was reduced by E2 treatment (25/30).
(G) FACS of *flk1:GFP*+ embryos (n = 32) revealed no change in endothelial cell number by E2 (error bars indicate SD).
(H) qPCR confirmed decreased expression of *flk1*, *ephrinB2*, and *scl* following E2 treatment (mean of triplicate experiments ± SEM; one-tailed t test; *flt4* $^{*}p < 0.05$, *flk1* $^{**}p < 0.01$, and *ephrinB2* $^{***}p < 0.001$).
(I) Injection of *scl* mRNA increased *runx1* expression in the AGM and rescued loss following E2 treatment (n > 55 per treatment).

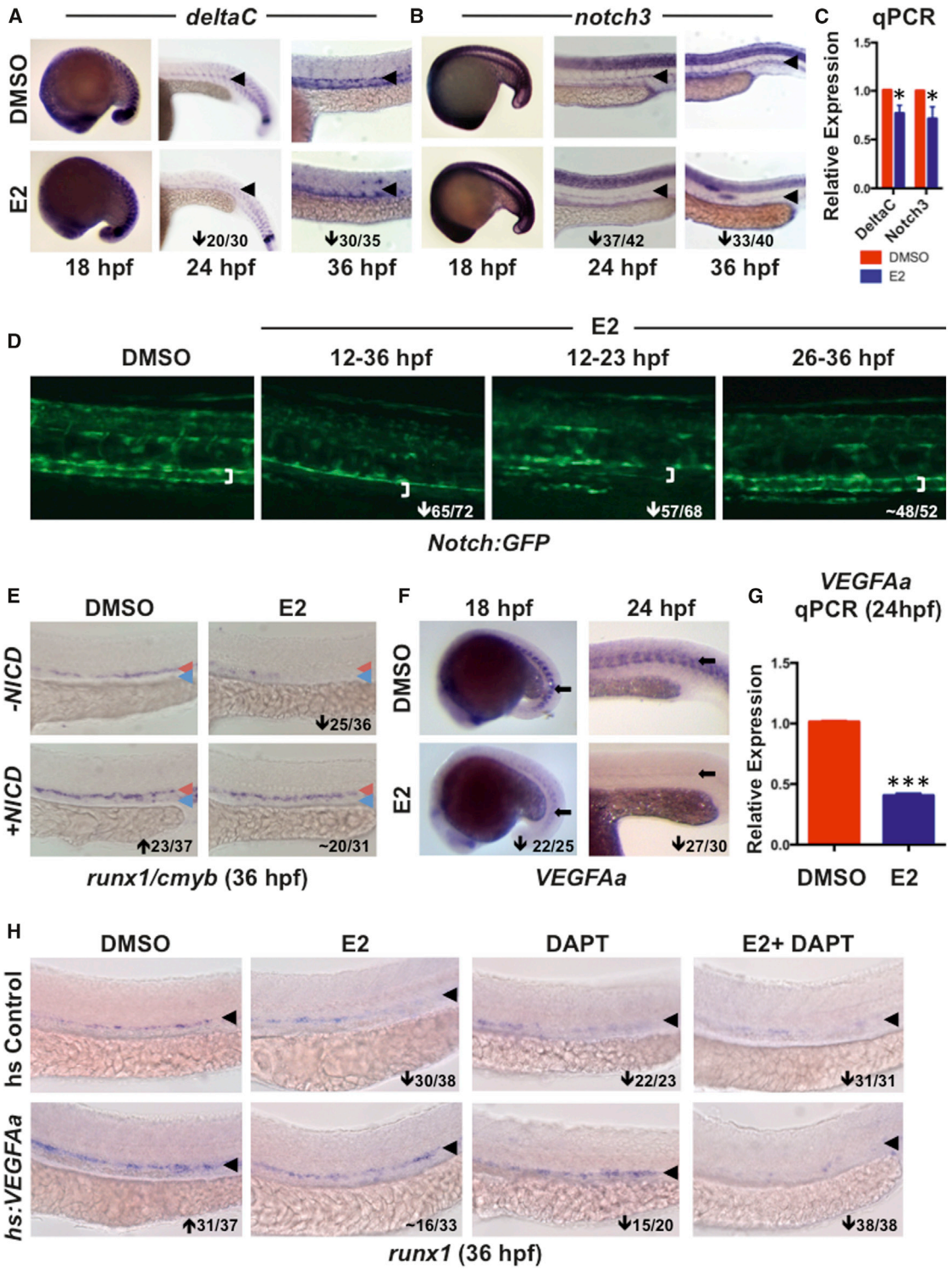

Figure 4. Exogenous E2 Signaling Disrupts VEGF/NOTCH Cascades in the AGM

(A) E2 exposure had no impact on *deltaC* expression by WISH at 18 hpf but strongly reduced arterial expression at 24 and 36 hpf (n > 30/treatment); an arrowhead indicates artery.

(B) Expression of *notch3* was similarly regulated by E2 at 18, 24, and 36 hpf (n > 30/treatment); an arrowhead indicates artery.

(C) qPCR confirmed E2-mediated reductions in *deltaC* and *notch3* expression at 36 hpf (mean of triplicate experiments ± SEM; one-tailed t test *deltaC*, *notch3* $^{*}p < 0.05$).

(D) Decreased Notch:GFP was observed in the artery (white bracket indicates artery walls) after E2 treatment from 12–36 or 12–23 hpf; no change occurred with exposure from 26–36 hpf (n ≥ 52).

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implying exposure to XEs may have a negative impact on HSPC specification.

Antagonism of Estrogen Signaling Expands the Zone of VEGF Regulation

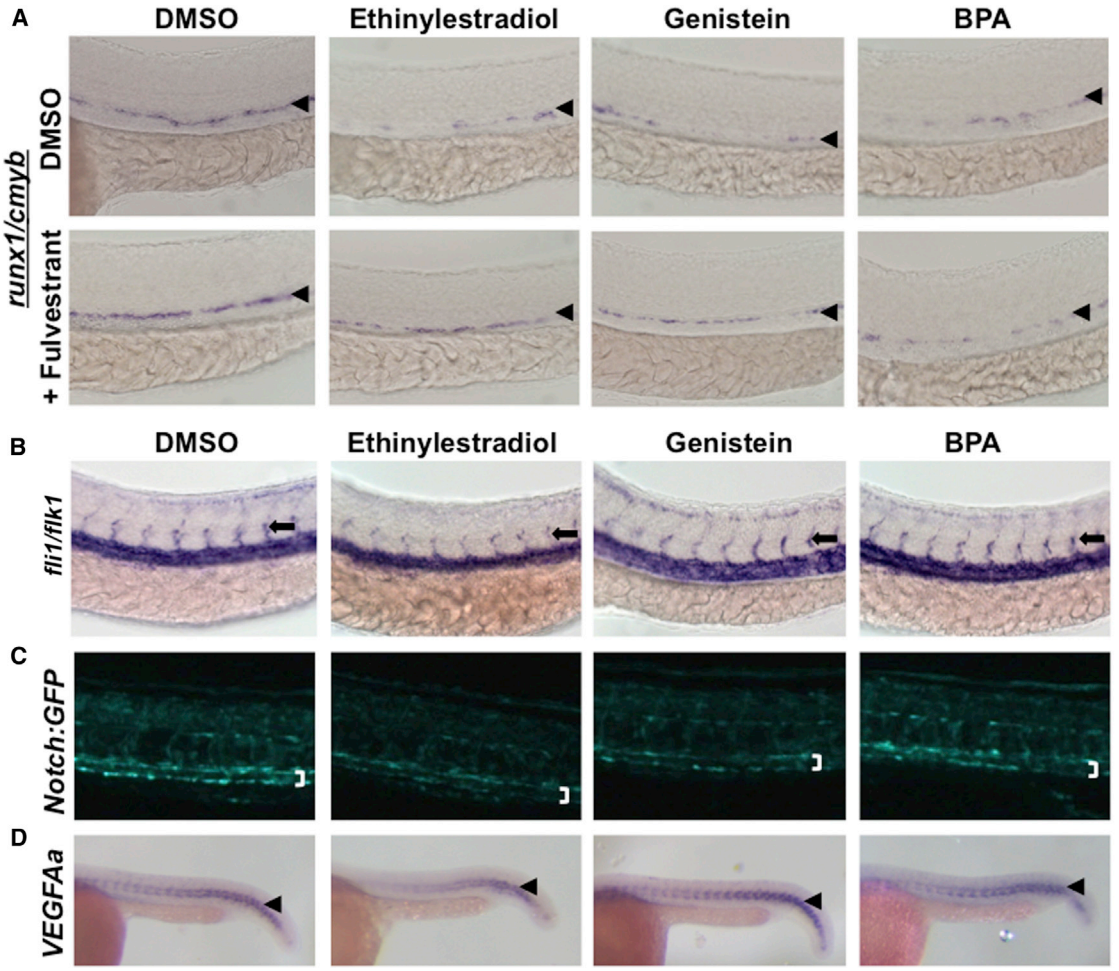
Having determined exogenous E2 and related XEs negatively impact HSC development via inhibition of the VEGF/Notch cascade and subsequent formation of HE within the aortic vasculature, we next wanted to see if intrinsic levels of E2 play a functional role in niche specification. In contrast to excess E2, embryos exposed to the pan-Esr antagonist ZK showed increased somitic expression of *VEGFAa* at 24 hpf (Figure 6A); downstream *Notch:GFP* activity was also enhanced by ZK (Figure S7F). In other species, both Notch and VEGF family members contain functional EREs (Buteau-Lozano et al., 2002; Soares et al., 2004); however, no consensus EREs were observed up to 8 kb from the zebrafish *VEGF* transcriptional start site (data not shown). To confirm that nascent E2 could nevertheless regulate VEGF activation, we examined embryos expressing GFP under the regulation of 1.2 kb of the *VEGF* promoter (He and Chen, 2005) (Figure 6B); E2 strongly reduced *VEGF* activation, whereas ZK broadened the zone of expression. To determine if VEGF activity was also impacted, known transcriptional targets *dll4*, *dusp5*, and *notch1b* (Bellou et al., 2009; Liu et al., 2003) (Figures 6C, 6D, and S7C) were assessed: inhibition of E2-signaling by ZK strongly enhanced expression of each VEGF target compared to controls, suggesting VEGF-mediated transcriptional regulation is actively antagonized by endogenous E2 in hematovascular development. Consistent with that finding, ZK-treated embryos exhibited phenotypes reminiscent of those observed with *VEGF* overexpression in the ventral aspect of the AGM, including widening of the major vessels and ectopic or disorganized ISV sprouting (Figures S7A and S7B) (Lawson et al., 2002; Wiley et al., 2011).

Endogenous E2 in the yolk is spatially positioned in a location proximal and just ventral to that of the developing AGM. To determine if antagonism of VEGF activity by nascent E2 impacted dorsal/ventral patterning of trunk vasculature, we next examined the boundaries of artery-vein specification following Esr antagonism. Whereas *ephrinB2* within the *fli1+* endothelial population was reduced by exogenous E2, it was noticeably enhanced by ZK treatment (Figure 6E); similar results were observed with *deltaC* and the Notch target *hey2* (Figures S7D and S7E). Expression of VEGF-regulated *tbx20* was likewise increased by ZK, including expansion to both walls of the aorta from its characteristic position on the roof (Wilkinson et al., 2009) (Figure 6F); enhanced arterial marker expression was confirmed by qPCR (Figure S7K; *p < 0.05, **p < 0.01). In contrast, whereas the dorsal limit of venous *flt4* was heightened by E2 toward the *col2a+* hypochord, within the arterial space, expression was diminished and patchy in embryos exposed to ZK (Figure 6G). Identical findings were observed for *dab2*, where reduced expression was seen in the vein after ZK treatment (Figure 6H), indicating the zone of VEGF-regulation was affected by modulation of E2 activity.

Loss of E2-Mediated VEGF Regulation Alters Hemogenic Potential of the Vein

Hemogenic vascular endothelium is normally situated within arteries, such as the dorsal aorta; we next sought to determine if loss of E2-mediated antagonism of VEGF activity would be sufficient to convert the functional potential of venous endothelium. Expression of the HE marker *scl* was elevated in ZK treated embryos, with induction in the vein in a subset of embryos (Figure S7G). This result was confirmed using *scl:GFP/flk1:dsRed* embryos where ZK increased the number of double positive vascular cells (Figure 7A). Arterial *runx1* expression was also increased via ZK (Figures 7B and 7D; *p < 0.05) and localized to the vein in some embryos. This phenotype, reminiscent of that previously reported for NICD (Burns et al., 2005), was also seen after genetic suppression of *esr2* signaling via MO knockdown (Figure S7I). Although reassignment of arterial fate as marked by *ephrinB2* (Figure 6E) was not observed with ZK, the percentage of embryos with venous *runx1* expression was proportional to those exhibiting Notch:GFP in the vein (Figure S7F); this finding was congruent with *scl* mRNA-mediated recovery of HSPCs following E2 exposure, where *runx1* expression was restored (Figure 3I) in the absence of *ephrinB2* rescue (Figure S7J). To confirm that alterations in E2 signaling had functional consequences for HSC production, we assessed HSC content using *CD41:GFP+* and *flk1:dsRed+/cmyb:GFP+* embryos, previously shown to contain functional HSCs by embryo-to-adult transplantation (Harris et al., 2013) and lineage tracing (Bertrand et al., 2010), respectively. Following ZK exposure, the number of mature CD41:GFP+ HSCs in the AGM region was significantly increased (Figures 7E and S7H; $p < 0.05$). Analysis of flk1:dsRed+/cmyb:GFP+ cells budding from the trunk vasculature was similarly elevated (Figures 7C and 7F). To demonstrate that the increase in HSPCs mediated by ZK was due to elevated VEGF activity, we utilized previously described VEGF pathway mutants: $kdrl^{y17}$ embryos exhibit normal vasculogenesis but defective VEGF-mediated ISV sprouting, whereas $plcg1^{y13}$ mutants show no ISV production, indicative of a block of increasing severity in VEGF activity (Covassin et al., 2009). Control and ZK-treated embryos were sorted based on fli1:GFP+ ISV expression (Figure S7L). $kdrl^{y17}$ mutants displayed reduced *runx1* expression; however, ZK exposure boosted HSPC number as determined by WISH (Figure 7G). In contrast, whereas $plcg1^{y13}$ mutants also displayed a virtual absence of *runx1+* expression, the block in HSPC formation could not be alleviated by ZK (Figure 7H), indicating VEGF activity was the target of E2 regulation. These data lead to a model (Figure 7I) whereby maternally deposited E2 functions to pattern the HSC niche via antagonism of the ventral limit of VEGF regulation, allowing for the ''appropriate'' assignment of HE and subsequent HSPC production.

(E) *NICD* induction increased expression of *runx1/cmyb* and rescued HSPCs in E2-treated embryos ($n \geq 31$/treatment).
(F) Expression of *VEGFAa* was strongly decreased in zebrafish following E2 treatment at both 18 and 24 hpf ($n \geq 25$/treatment); an arrow points to somite staining.
(G) qPCR confirmed that expression of *VEGFAa* was decreased at 24 hpf (mean of triplicate experiments ± SEM; one-tailed t test ***p < 0.001).
(H) Induction of *VEGFAa* increased *runx1* expression and rescued the effect of E2 exposure; treatment with DAPT decreased expression of *runx1* and blocked the VEGF-mediated rescue of HSPCs ($n \geq 20$/treatment); an arrowhead indicates artery.



E

	ephrin B2	%	*flt4*	%	*notch3*	%	*deltaC*	%
Ethinyl Estradiol	↓16/37	43	↑11/34	32	↓11/32	34	↓9/34	26
Genistein	↓22/34	65	↑10/30	33	↓12/37	32	↓5/35	14
BPA	↓3/35	8	↑3/37	8	↓4/38	10	No effect	-

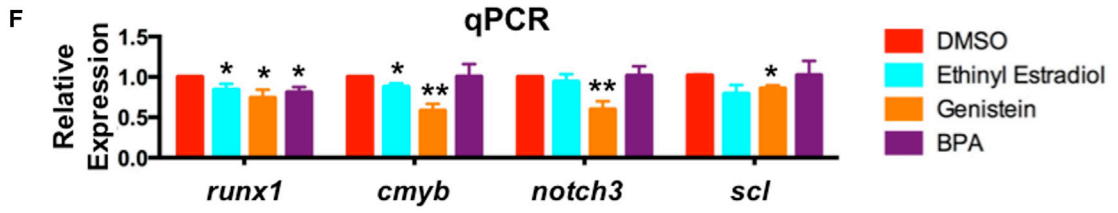


Figure 5. Xenoestogens Partially Replicate the Effects of E2 on HSPC Formation

(A) Exposure to xenoestrogens ethinyl estradiol (EE), genistein (GEN), or Bisphenol A (BPA) decreased *runx1/cmyb* expression and could be partially blocked by cotreatment with FULV (n ≥ 25/treatment).

(B) Treatment with GEN reduced the expression of *fli1/flk1* in the intersomitic vessels (n ≥ 25/treatment); an arrow points to ISVs.

(C) Treatment with GEN (15/25) and EE (12/23) decreased Notch:GFP expression.

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DISCUSSION

In this paper, we identify estrogens as previously uncharacterized regulators of embryonic hematovascular niche patterning. Estrogens are considered the most ancient of the steroid hormones and have remarkable ability to act as morphogens, signaling molecules and transcription factors simultaneously, thus controlling a myriad of downstream processes through a combination of their relative concentration, receptor distribution, and availability of direct genetic targets (Heldring et al., 2007; Thornton, 2001). We propose that in zebrafish embryos (Figure 7I, top panel), endogenous E2 maternally deposited in the yolk diffuses to define the ventral boundary of somite-derived VEGF regulation. The opposing dorsal-ventral gradients result in the artery receiving higher VEGF and lower E2 relative to the vein and enables "proper" vasculature patterning with coincident localization of the hemogenic vascular niche to the artery. When embryos are exposed to excess levels of E2 (Figure 7I, middle panel), or related XEs, the gradient is disrupted and additional estrogen is present to antagonize the VEGF/Notch cascade in all cell types expressing Esrs. In this scenario, the total region of VEGF (and Notch) activity decreases, leading to loss of arterial markers in the developing vasculature, with a concomitant enhancement in venous identity, and reductions in HE specification. In contrast, when Esr signaling is inhibited (Figure 7I, lower panel), the functional impact of the gradient of E2 from the yolk is lessened, allowing the domain of VEGF regulation to increase: expression of VEGF targets—including notch components—are enhanced, repression of venous markers is observed, and genes required for HE specification are elevated, jointly causing occasional mislocalized production of *runx1*+ HSPCs in the vein independent of full arterial reassignment (Figure 7B). This study highlights the ability of E2 to act simultaneously as a morphogen, signaling molecule and (presumably) transcription factor to pattern an embryonic niche outside of the urogenital system. Further, this role in setting the limits of VEGF regulation may help resolve discrepancies in the necessity of arterial gene expression for hemogenic HSPC production.

Fine-tuned regulation of estrogen availability during early development is highly conserved in placental mammals: maternal estrogen levels are quite low early in pregnancy but steadily increase until delivery (Tulchinsky et al., 1972). Our data suggest that E2 levels may be actively suppressed during early embryonic development in part to avoid negative impact on vasculature specification and maturation, including HE localization. Although there are clear anatomical differences between yolk-supported and placental vertebrate embryos, recent studies have indicated that gene networks and cellular movements establishing vascular progenitor fate are VEGF concentration dependent (Kohli et al., 2013) and more similar across species than previously appreciated (Lindskog et al., 2014). Beyond myeloproliferative disorders in a subset of adults, few hematopoietic phenotypes have been noted, thus far, in mice with homozygous *Esr1* or *Esr2* mutations (Krege et al., 1998; Lubahn et al., 1993); this finding is consistent with our data indicating that early *esr2* antagonism enhances, rather than negatively impacts, HSPC production. Likewise, our observations concerning exposure to exogenous E2 are reminiscent of prior reports of intrauterine hemorrhaging and eventual death observed in embryos of *Steroid 5α-Reductase*-deficient dams, which have maternally derived elevations in E2 (Mahendroo et al., 1997); in future studies, it will be interesting to specifically examine how E2 modulation impacts hemogenic vascular endothelial specification and HSC induction in mammalian models.

In zebrafish, we can attribute the negative impact of exogenous E2 on HSC formation to repression of VEGF-Notch signaling during the window of hemogenic endothelium development. Although E2 has long been recognized as having the ability to modulate both VEGF and NOTCH expression in other contexts, its impact on the development of the hematovascular system during embryogenesis is unique. Indeed, whereas ERE sites in *NOTCH* and *VEGF* are functional in mammals, most evidence from breast cancer studies suggests that E2 acts to induce both pathways rather than repress them, as we observed (Buteau-Lozano et al., 2002; Soares et al., 2004). Notably, however, E2 has been reported to antagonize VEGF signaling in hypoxic conditions (Miyamoto et al., 2002). Although we found no evidence of a consensus ERE in the *VEGF* promoter up to 8 kb from the start site (data not shown), increasing evidence suggests the hematopoietic niche is a hypoxic environment in the embryo and adult; we recently showed Hypoxia-Inducible Factor 1 alpha (HIF1α) is a critical regulator of hematopoietic development (Harris et al., 2013). Additional studies will be needed to determine whether hypoxia and/or the HIF complex are interacting with E2 in this context to directly or indirectly impact *VEGF* production and downstream function. Our findings on the timing of E2-mediated HE regulation indirectly support the hypoxia model, as the negative impact of E2 on VEGF is only observed prior to the start of circulation and maturation of oxygen-carrying erythrocytes beginning at 24 hpf. It will be important to determine if transitioning out of this relatively hypoxic state postcirculation onset is the reason E2 displays differential effects in later hematovascular development. Also of potential consequence to the findings reported here, recent investigations in *Xenopus* indicate different VEGF isoforms are required for the establishment of arterial versus hemogenic fate during development (Leung et al., 2013); although it has not yet been demonstrated whether this will also be the case in zebrafish or mammalian embryos, it will be valuable to determine if estrogens could play a role in transcript selectivity.

Finally, the observation that common XEs, such as genistein and ethinylestradiol, can similarly impact vascular patterning and the subsequent specification of HSPCs suggests environmentally relevant estrogenic substances may have an unexpected impact on hematovascular development in vivo. Although the XEs tested were not as potent as E2, as a group they are present at increasingly elevated concentrations in our

(D) *VEGFAa* was noticeably decreased by EE (15/26); arrowheads indicate somite region.
(E) Summary of changes in the expression of markers of vascular identity *ephrinB2*, *flt4*, *notch3*, and *deltaC* following treatment with XEs ($n \geq 25$).
(F) qPCR confirmed decreases in *runx1*, *cmyb notch3*, and *scl* by XE exposure (mean of triplicate experiments ± SEM; one-tailed t test *$p < 0.05$; **$p < 0.01$).

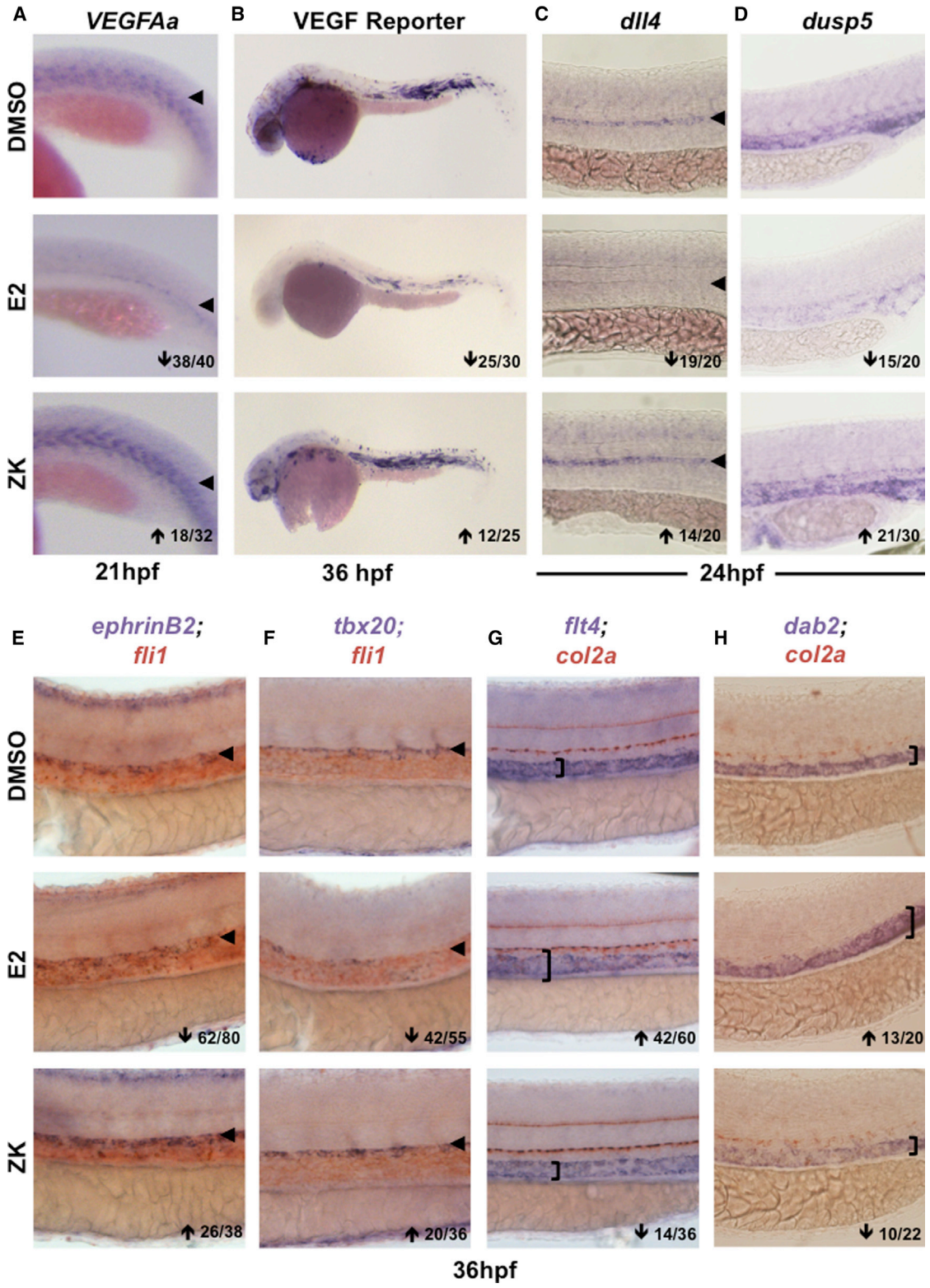

Figure 6. Blocking Esr Signaling Increases VEGF Activity and Alters Trunk Vasculature Patterning

(A) *VEGFAa* expression was enhanced after inhibition of endogenous estrogen activity via the pan-Esr antagonist ZK164015 (18/32).

(B) *gfp* expression in *VEGF:GFP* reporter embryos was reduced by E2 and enhanced by ZK (n > 25).

(C) The VEGF target gene *dll4* was decreased after E2 exposure but increased by ZK (n > 20).

(D) *dusp5* levels were reduced by E2 treatment but elevated after Esr antagonism (n > 20).

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environment; the specific representative examples tested may have had difficulty activating zebrafish Esrs at a level sufficient to elicit a major response, as differences in receptor affinity compared with endogenous E2 have been previously described (Barkhem et al., 1998). The fact that XEs could affect the HSC niche raises important questions about the long-term deleterious effects of in utero exposure on hematopoietic development and homeostasis. Although not specifically examined here, pregnant mice fed a diet rich in genistein had pups with increased erythropoiesis and granulopoiesis at 12 weeks of age (Vanhees et al., 2011). Genistein can also induce rearrangements in the Mixed Lineage Leukemia (MLL) gene in human CD34+ cells, resembling those commonly seen in infant leukemias (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2007), and epidemiological studies show exposure to dietary flavonoids during pregnancy is linked to infant leukemia development (Ross, 2000). Our data indicate that concentration and timing may significantly alter the outcome of estrogenic exposure.

There is increasing evidence that hematopoietic cells continue to be responsive to estrogenic regulation throughout their lifetime. During gestation, maternal blood volume grows by ~50% to accommodate the growing oxygenation and nutritional needs of the fetus (Hytten, 1985), with recent investigations indicating this is due in part to E2-mediated enhancement in HSPC proliferation (Nakada et al., 2014). Further, guinea pigs administered exogenous E2 showed increased blood volume comparable to that seen during pregnancy, indicating it may be a critical regulator of hematopoietic output in sexually mature adults (Davis et al., 1989). Reduced E2 levels, as a result of ovariectomy in rats, can lead to hematopoietic dysfunction, characterized by increased extramedullary hematopoiesis (Qiu et al., 2012). However, exposure to excess E2 has also been associated with alterations in hematopoietic cell number (Perry et al., 2000). Together with our data, these studies indicate E2 regulates of several aspects of hematopoiesis and suggest spatiotemporal alterations in concentration of this previously underappreciated, but well conserved, molecule will have significant impact beyond embryogenesis. Here, we specifically demonstrate maternally derived E2 has a central role in establishing dorsal-ventral patterning of the hematovascular system, acting as a ventral-limiting factor to specify the hemogenic vascular niche.

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry

Zebrafish were maintained in accordance with the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee protocols. Zebrafish lines utilized were previously described and are listed in the Supplemental Experimental Procedures.

Chemical Treatments and Evaluation

Embryonic zebrafish exposures were performed from five somites until 36 hpf in multiwell plates, unless otherwise specified. The following compounds (Cayman Chemicals, unless otherwise indicated) were used: 17β-estradiol (E2) (8–10 μM), 17α-estradiol (10 μM), 2,3-*bis*(4-hydroxyphenyl)propionitrile (DPN) (10 μM), propylpyrazole triol (PPT) (20 μM), fulvestrant (FULV; ICI-182,780) (15 μM), genistein (GEN) (7.5 μM), ethinyl estradiol (EE) (6 μM), bisphenol A (BPA) (30 μM [Sigma]), DAPT (GSI-IX) (20 μM), G1 (1 μM), G15 (10 μM), ZK164015 (ZK) (10 μM [Tocris]), SAG (10 μM), SU1498 (10 μM [Calbiochem]), and screen hits (10 μM). WISH was performed using previously published probes and methods (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). Qualitative phenotypes (≥3 replicate clutches) were scored as the number altered/number analyzed per treatment. *plcg1*$^{y13/y13}$ and *kdrl*$^{y17/y17}$ mutants were identified posttreatment, and evaluated by genotyping as previously described (Covassin et al., 2006, 2009). FACS analysis of transgenic lines (pools of three embryos × >10 biological replicates), phospho-Histone H3 (pHH3) labeling (Abcam), and 5-bromo-2′-deoxyuridine (BrdU) incorporation (Sigma) were performed as previously described (Harris et al., 2013; North et al., 2007). Images were acquired using a Zeiss Axio Imager A1 or Zeiss Discovery V8/Axio Cam MRC and Axiovision LE software (Carl Zeiss).

Morpholino and mRNA Injections

MOs (Supplemental Experimental Procedures; Gene Tools) were injected into one-cell-stage embryos and treated and analyzed as detailed above; the *ERE:GFP* line was used to confirm specificity. *scl* mRNA was synthesized using the manufacturer's directions for Ambion mMessage mMachine as previously described (Kim et al., 2013) and injected (150 pg) into one-cell-stage embryos. Treatments and analysis were performed as described above.

Quantitative Real-Time PCR

RNA was isolated from pooled (n = 30) embryos at 24 or 36 hpf and isolated using an RNAqueous Kit (Ambion). cDNA was made using the SuperScript III First-Strand Synthesis kit (Invitrogen). qPCR reactions (primers listed in the Supplemental Experimental Procedures) were performed using an ABI 7900 machine and analyzed as previously described (North et al., 2007) using the ddCt method with either *18s* or *tbp* as the reference gene.

Heat Shock Modulation of Notch and VEGF Signaling

Embryos were treated with test compounds prior to and post-heat shock as indicated. *hs:VEGFAa* and *hs:Gal4 x UAS:NICD* expression was induced by incubation in a 38°C circulating water bath for 10 or 7 min, respectively, at ~16 hpf. *hs:VEGFAa* fish were identified by cardiac GFP expression, and *hs:Gal4 x UAS:NICD* fish were identified by genotyping post-WISH analysis.

Estradiol Immunoassay

E2 content was measured using an Estradiol EIA Kit (Cayman Chemical). Embryos (n = 30) were pooled and homogenized in 100 μl of EIA buffer and diluted to 300 μl total volume; 50 μl was used per reaction in accordance with the manufacturer's instructions. For yolk content analysis, the yolk was manually removed by pipetting (10 μl tip), and embryos were pelleted for 30 s at 300 rpm. Yolk extract (supernatant) was collected, and the pellet (body) was disaggregated and resuspended in 300 μl EIA buffer as above. Analysis was performed in accordance with instructions from Cayman Chemical.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.04.012.

AUTHOR CONTRIBUTIONS

K.J.C., V.E., M.C.D., C.C.C., G.F., S.N., W.K., J.M.H., S.L., and M.C. performed embryo exposures, MO injection, FACS, qPCR, and WISH. K.J.C. and M.K.G. conducted estradiol immunoassays. D.G. and M.H. provided

(E) Arterial *ephrinB2* expression in the *fli1+* vasculature was enhanced after ZK-mediated inhibition of estrogen signaling (26/38).
(F) *tbx20* expression in the roof of the aorta was substantially increased by ZK (20/36).
(G) Venous *flt4* was expanded dorsally toward the *col2a+* hypocord by E2 but reduced in expression by ZK exposure (14/36).
(H) The venous marker *dab2* was similarly regulated as determined by WISH (n > 20).

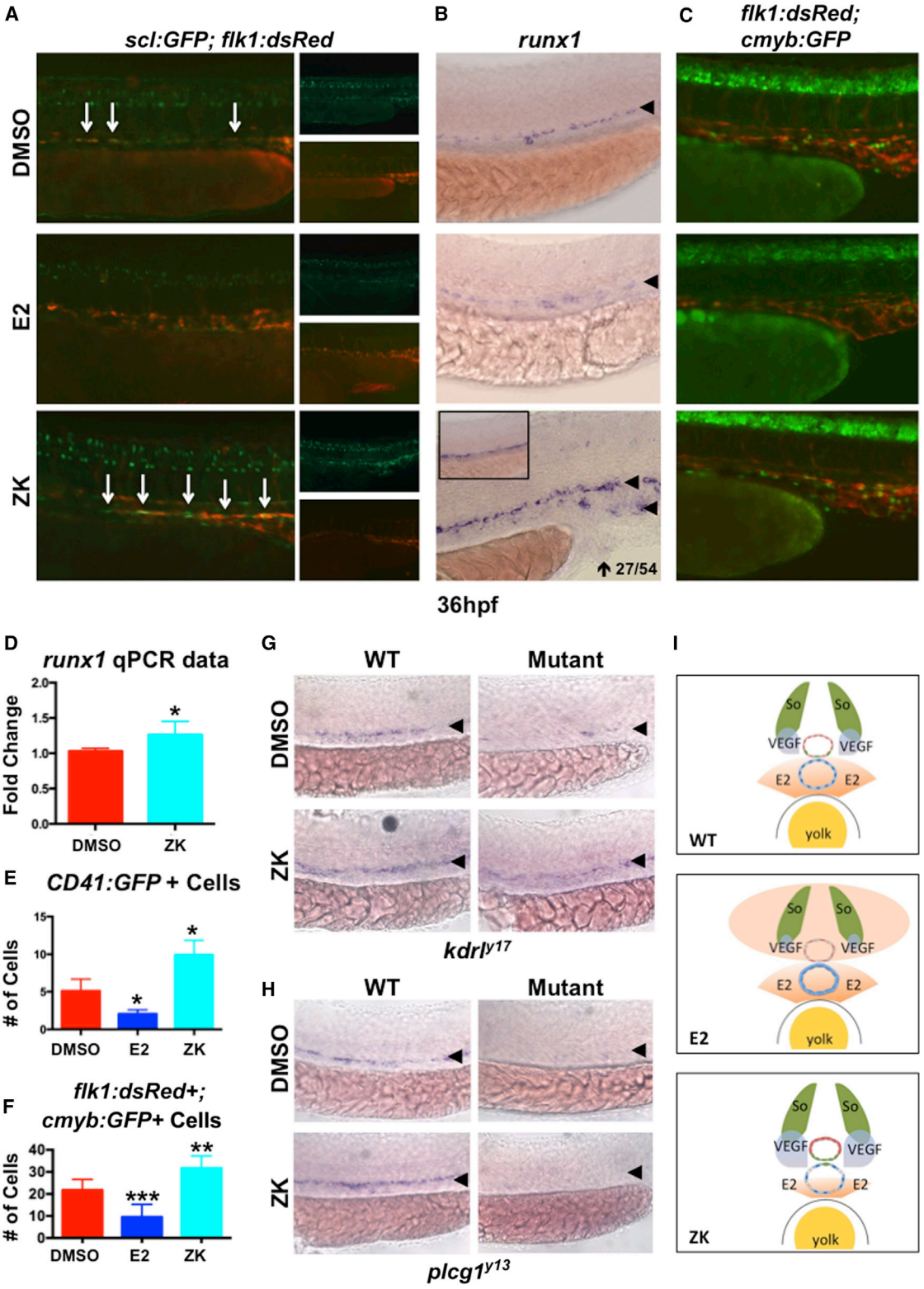

Figure 7. E2-Mediated VEGF Antagonism Regulates Hemogenic Endothelial Specification

(A) Hemogenic endothelium, as assessed by the dual expression of *scl:GFP* and *flk1:dsRed*, was reduced by exogenous E2 treatment but was enhanced following Esr antagonism by ZK (n > 15).

(B) *runx1* expression increased (27/54) after estrogen receptor inhibition, with mislocalization to the vein (10/54).

(C) Production of flk1:dsRed+; cmyb:GFP+ HSCs was enhanced by ZK (n ≥ 10).

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Tg(5xERE:GFP) zebrafish, and N.L. provided *plcg1*$^{y13/y13}$ and *kdrl*$^{y17/y17}$ zebrafish. K.J.C., W.G., and T.E.N. designed experiments, evaluated results, and wrote the manuscript.

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(D) Quantification of changes in *runx1* expression (mean of triplicate experiments ± SEM; one-tailed t test *p < 0.05).
(E) Quantification of the number of *CD41*+ cells after E2 treatment and antagonism (one-tailed t test *p < 0.05; error bars indicate SD).
(F) The number of flk1:dsRed+; cmyb:GFP+ HSCs was decreased by E2 and enhanced after ZK treatment (one-tailed t test **p < 0.01, ***p < 0.001; error bars indicate SD).
(G) *kdrl*^{y17} mutant embryos reveal loss of *runx1*, which can be partially restored by reduction of E2-mediated VEGF antagonism after ZK treatment (n > 20/condition).
(H) WISH analysis in *plcg1*^{y13} mutant embryo shows complete loss of *runx1* expression in the presence or absence of ZK (n > 20/condition).
(I) Model: in wild-type embryos (upper panel), opposing gradients of E2 and VEGF specify the arterial/venous boundaries of hemogenic endothelium and subsequent emergence of HSPCs (red circle, artery; blue circle, vein). Excess E2 (middle) disrupts this gradient, further antagonizing VEGF and causing artery, hemogenic endothelium, and HSPCs to fail to form properly. In contrast, inhibition of esr activity (lower panel) allows the range of VEGF regulation to increase, resulting in ectopic HE specification and HSPC production in the vein.

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