Regular Article

HEMATOPOIESIS AND STEM CELLS

Notch1 acts via *Foxc2* to promote definitive hematopoiesis via effects on hemogenic endothelium

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Key Points

- *Notch1* induction promotes specification of hemogenic endothelial cells during embryonic stem cell differentiation.
- *Foxc2* functions downstream of *Notch* in specification of hemogenic endothelium in mouse and zebrafish embryos.

Hematopoietic and vascular development share many common features, including cell surface markers and sites of origin. Recent lineage-tracing studies have established that definitive hematopoietic stem and progenitor cells arise from vascular endothelial–cadherin$^+$ hemogenic endothelial cells of the aorta-gonad-mesonephros region, but the genetic programs underlying the specification of hemogenic endothelial cells remain poorly defined. Here, we discovered that *Notch* induction enhances hematopoietic potential and promotes the specification of hemogenic endothelium in differentiating cultures of mouse embryonic stem cells, and we identified *Foxc2* as a highly upregulated transcript in the hemogenic endothelial population. Studies in zebrafish and mouse embryos revealed that *Foxc2* and its orthologs are required for the proper development of definitive hematopoiesis and function downstream of *Notch* signaling in the hemogenic endothelium. These data establish a pathway linking *Notch* signaling to *Foxc2* in hemogenic endothelial cells to promote definitive hematopoiesis. (*Blood*. 2015;125(9):1418-1426)

Introduction

Generating hematopoietic stem cells (HSCs) from embryonic stem cells (ESCs) remains challenging despite considerable efforts. Although genetic modification with *HoxB4* and *Cdx4* enables hematopoietic progenitors derived from murine embryoid bodies (EBs) to reconstitute multilineage hematopoiesis in primary and secondary mice, these ESC-derived HSCs remain distinct from bone marrow–derived HSCs.[1,2] Live imaging of hematopoietic differentiation from ESCs has shown that CD41$^+$ cells arise from hemogenic endothelial cells that express vascular endothelial (VE)–cadherin or tyrosine kinase with Ig and EGF homology domains-2 and later express the hematopoietic marker CD45.[3,4] In vivo lineage tracing in mice using a tamoxifen-inducible VE-cadherin Cre transgene has shown that pulse induction during the aorta-gonad-mesonephros (AGM) stage of hemogenesis abundantly labels fetal liver, bone marrow, and thymic hematopoietic cells, and constitutive induction marks the vast majority of adult blood cells. These reports strongly indicate that definitive hematopoietic cells, which replace transient primitive hematopoietic cells during embryo development, arise from hemogenic endothelium.[5-8]

Notch signaling has been implicated in cell-fate decisions and differentiation of various cell types, including endothelial cells and blood cells.[9-11] Upon ligand activation, the intracellular domain of *Notch* (ICN or NICD) is cleaved at the plasma membrane and translocates to the nucleus where it binds to the transcription factor *CSL* (for *CBF1/Su(H)/Lag-1*, also known as *Rpbsuh* or *RBP-jκ*) to activate expression of downstream targets such as Hes and Hey genes.[12] Organ culture of the *Notch1* null E9.5 para-aortic splanchnopleura, which later develops into the AGM, has revealed marked impairment of vascular network formation and hematopoietic cell development, whereas colony-forming cell (CFC) activity was preserved in the yolk sac.[13-15] In situ hybridization of para-aortic splanchnopleura/AGM from E9.5 and E10.5 wild-type embryos showed that *Notch1* expression was restricted to the ventral wall of the dorsal aorta.[15] These studies suggest that *Notch1* is a key regulator of hemogenic endothelial cells.

The forkhead box (*Fox*) family of transcription factors is an evolutionarily ancient gene family that has expanded to more than 40 members in mammals.[16] In mice, *Foxc1* and *Foxc2* are essential for arterial specification before the onset of circulation by directly inducing

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transcription of a *Notch* ligand, Delta-like 4.[17-19] A recent study has also shown that *Foxc2* binds to the VE-cadherin enhancer and directly activates its transcription.[20] Although the roles of *Foxc* genes are well established in angiogenic remodeling, there is currently no link between *Fox* genes and HSC emergence.

In this study, we generated ESCs with a doxycycline (Dox)–inducible intracellular domain of *Notch1* (ICN1) and analyzed the effect of induction during EB differentiation. ICN1 induction expanded VE-cadherin$^+$ hemogenic endothelial cells and enhanced hematopoietic potential. Expression analysis of the ICN1-induced VE-cadherin$^+$ population showed the upregulation of *Foxc2*, which we discovered using genetic analyses in both zebrafish and mice functions downstream of *Notch* signaling in hemogenic endothelium. Thus, we demonstrate that the *Notch* pathway promotes the maturation of hemogenic endothelium via *Foxc2*, establishing *Foxc2* as a key factor in promoting definitive hematopoiesis.

Materials and methods

ESC culture, cloning, and EB differentiation

Ainv15 murine ESCs were maintained on mouse embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle medium with 15% heat-inactivated fetal calf serum (IFS) (HyClone Laboratories, Logan, UT), 1000 U/mL leukemia inhibitory factor, 0.1 mM nonessential amino acids, 2 mM penicillin/streptomycin/glutamate, and 100 μM β-mercaptoethanol at 37°C/5% CO_2. Dox-inducible ICN1 embryonic stem cell line was generated after subcloning ICN1 complementary DNA (cDNA; generously provided by David Scadden[21]) into plox vector (*Mlu*I and *Xba*I sites with blunt ligation) and targeting Ainv15 ESCs with pSALK-Cre.[22]

Murine Foxc2 cDNA with a flag tag at the 3′ terminus was cloned downstream of the tetO minimal promoter of pBS31' vector and coelectroporated with pCAGGS FLPε plasmid into murine KH2 embryonic stem cell line at 500 V and 25 μF using Gene PulserII.[23] The colonies were selected in hygromycin at 140 μg/mL for 10 days, and the resistant clones were subjected to Foxc2 screening by Immunoblotting.

ESCs were differentiated into EBs after removing MEFs and cultured in Iscove modified Dulbecco medium (IMDM) with 15% fetal calf serum (Stem Cell Technologies, Vancouver, BC), 200 μg/mL holo-transferrin, 50 μg/mL ascorbic acid, 2 mM penicillin/streptomycin/glutamate, and 450 μM monothioglycerol as described previously.[24]

Immunoblotting

Immunoblotting was performed with sodium dodecyl sulfate– polyacrylamide gel electrophoresis and transfer system (Bio-Rad). ICN1 was detected with anti-Notch1 antibody (sc-6014; Santa Cruz Biotechnology). The membranes were incubated with horseradish peroxidase–conjugated secondary antibodies and developed using the Enhanced Chemiluminescence kit.

Methylcellulose CFC, OP9 colony, and HE culture

Day 6 EBs were dissociated by treatment of collagenase IV (2 mg/mL), hyaluronidase (10 mg/mL), DNase (160 U/mL), and trituration with enzyme-free dissociation buffer (Invitrogen). Cells were mixed into methylcellulose media (M3434, StemCell Technologies) and put in nonadherent 30-mm^2 nontreated dishes (StemCell Technologies). Colonies were counted at day 4 (EryP) and day 8 (others) or day 10. OP9 cells were maintained in α-minimum essential medium with 20% IFS and 2 mM penicillin/streptomycin/glutamine. Day 6 EB-derived cells were cultured on OP9 cells in IMDM with 10% IFS, 100 ng/mL human fms-related tyrosine kinase 3 ligand, 100 ng/mL human stem cell factor, 40 ng/mL human thyroid peroxidase, and 40 ng/mL murine vascular endothelial growth factor (PeproTech, Rocky Hill, NJ), and 2 mM penicillin/streptomycin/glutamine, and colonies were counted 4 to 6 days later. For hematopoietic and endothelial (HE) culture, day 6 EB-derived cells were cultured in IMDM containing 10% fetal calf serum, 10% equine serum, 5 ng/mL murine vascular endothelial growth factor, 10 ng/mL insulin-like growth factor 1, 2 U/mL erythropoietin, 10 ng/mL basic fibroblast growth factor, 50 ng/mL interleukin 11, 100 ng/mL murine stem cell factor, 100 μg/mL endothelial cell growth supplement, 2 mM penicillin/streptomycin/glutamate, and 450 μM monothioglycerol on matrigel-coated plates.[25]

Fluorescence-activated cell sorter, sorting, and real-time RT-PCR

Day 6 EB-derived cells were stained with fluorescein isothiocyanate anti-CD41, purified rat anti-mouse CD144, phycoerythrin mouse anti-rat IgG2a, allophycocyanin anti-CD45, phycoerythrin-Cy7 anti-CD49d (BD Biosciences, San Jose, CA), and 7AAD (Sigma-Aldrich). Cells were analyzed by using FACSCalibur/Canto or sorted by Aria. For real-time reverse transcription–polymerase chain reaction (RT-PCR), cells were harvested into TRIzol (Invitrogen) and total RNA was isolated with treatment of DNase I (Ambion, Austin, TX). After preparing cDNA (Superscript II, Invitrogen), quantitative PCR was performed using SYBR Green (Strategene, La Jolla, CA) on MX3000P with indicated primers,[24,26] and the expression was normalized to β-actin (ICN1, forward: ATGCTGGAGGACCTCATCAACTCA, reverse: TGAACAATGTGGATG CCGCAGTTG; *Foxc2*, forward: AACCCAACAGCAAACTTTCCC, reverse: GCGTAGCTCGATAGGGCAG, PrimerBank ID 8850213a1).

Further experimental procedures are described in supplemental Experimental Methods (available on the *Blood* Web site).

Results

Promotion of hematopoiesis with ICN1 induction during mouse EB differentiation

Notch signaling is involved in multiple steps of tissue specification and progenitor cell maturation during embryo development.[9,27] To test the effect of *Notch1* signaling on early blood lineage development, we cloned the ICN1 into the plox vector, and targeted Ainv15 ESCs to generate the Dox-inducible ICN1 line (iICN1).[21,22] After confirming ICN1 induction with Dox (Figure 1A), we differentiated ESCs into EBs and observed the effects of ICN1 induction over specific time periods on the number of hematopoietic CFCs at day 6 (Figure 1B). ICN1 induction on single days from days 3 to 5 resulted in increased colony numbers, and a 2-day induction (ICN 3-5) resulted in higher colony numbers than either single-day or 3-day induction; a similar pattern was observed for hematopoietic colonies that form on OP9 stroma (Figure 1C). These results show that timed induction of *Notch* signaling promotes hematopoietic development of EBs.

Promotion of hemogenic endothelial cells with ICN1 induction

Recent lineage-tracing experiments in the mouse have shown that definitive hematopoietic populations arise from hemogenic endothelial cells that express VE-cadherin and can be detected in the AGM region.[5,6,28] CD41 has been reported to mark embryonic hematopoietic precursors prior to CD45 expression.[29,30] Continuous single-cell imaging has shown that the VE-cadherin$^+$ population derived from fetal liver kinase 1 (Flk1)$^+$ cells can give rise to CD41$^+$ and eventually CD45$^+$ cells.[3] When day 6 EB cells were analyzed for CD41 and VE-cadherin expression by flow cytometry, ICN1 induction from days 3 to 5 (ICN 3-5) increased the VE-cadherin single positive (VE SP) population up to twofold (Figure 2A) compared with uninduced control (ICN 0). The majority of the VE SP population (60% to 75%) was also positive for α4-integrin, another marker of hemogenic endothelial cells required for proliferation and differentiation of multilineage hematopoietic progenitors in the embryo.[31] In day 6 EBs, there was little change in Flk1, CD45, tyrosin-protein kinase kit, stem cell antigen-1, or

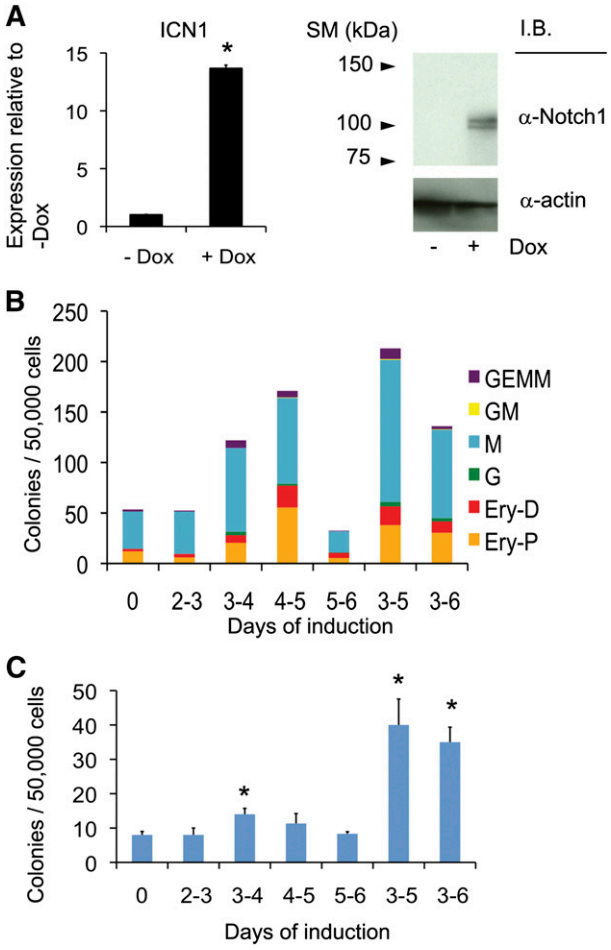

Figure 1. ICN1 induction during EB differentiation enhances hematopoietic development. (A) Induction of ICN1 24 hours after adding Dox (0.5 μg/mL) during ESC culture is shown by real-time RT-PCR (left) and immunoblotting (right). (B) Methylcellulose (M3434) CFC counts from day 6 iICN1 EB-derived cells with indicated day(s) of ICN1 induction by Dox (0.5 μg/mL) are shown. A representative of 3 independent experiments is shown. (C) OP9 stromal cell colony counts from day 6 iICN1 EB-derived cells with indicated day(s) of ICN1 induction with Dox (0.5 μg/mL) are shown. n = 3; *$P < .05$ by Student *t* test in comparison with noninduced control.

CD41 positive populations following ICN1 induction (data not shown). When we induced ICN1 during blast colony development, in which the hemogenic endothelial core first develops and hematopoietic blast colony cells bud off from the core, development stopped at the core level with a dramatic decrease in core colony formation (supplemental Figure 1A). However, the cells harvested from the ICN1-induced blast colony assay generated more hematopoietic colonies compared with the same number of cells from the control (supplemental Figure 1B). These results suggest that ICN1 induction promotes hematopoietic development through the promotion of hemogenic endothelium.

When VE SP cells were sorted from day 6 EBs and put into HE culture, $CD45^+$ cells were generated after 4 days of culture. From the same number of VE SP cells, more $CD45^+$ cells were produced from ICN1-induced EBs (ICN 3-5) than controls (Figure 2B). The absolute numbers of $CD45^+$ cells produced from the same number of VE SP cells in days 2, 4, and 6 HE culture showed 42% to 46% increase in cultures of ICN1-induced cells (Figure 2C; supplemental Table 1). With an assumption that a single VE SP cell generates a single $CD45^+$ cell and proliferation rates of cells in HE culture are not different, we estimated the frequency of HE cells as $1/5.446 \times 10^4$ from control VE SP cells and $1/2.589 \times 10^4$ from ICN1-induced VE SP cells by limiting dilution analysis (supplemental Figure 2). Cytospin of day 10 HE culture showed a significant increase in hematopoietic cells from ICN1-induced VE SP cells, whereas no differences were seen between control and ICN1-induced CD41 single positive (CD41 SP) or double positive (DP) cells (Figure 2D). Moreover, day 10 HE culture of VE SP cells exhibited tube-forming ability (data not shown). When we analyzed the cell cycle in day 4 EBs following ICN1 induction from day 3, we observed an approximately twofold increase of $BrdU^+$ VE-$cadherin^+$ cells in ICN1-induced EBs (20.3% to 38.3%), which suggests that the increase in VE-$cadherin^+$ cells in day 6 EBs following ICN1 induction could have been contributed by an increase in cell proliferation (supplemental Figure 3). Taken together, our data suggest that inducing *Notch1* signaling during EB differentiation promotes proliferation and hematopoietic specification of hemogenic endothelial cells.

To understand whether ICN1 induction impacts hematopoietic differentiation in a cell-autonomous manner, we mixed murine ROSA-GFP ESCs, which constitutively express green fluorescent protein (GFP),[32] with iICN1 ESCs, which are GFP^-, to form EBs, and analyzed day 6 EBs with or without ICN1 induction from days 3 to 5. Flow cytometric analysis of day 6 EBs showed that GFP^- cells showed the increase of VE SP population with ICN1 induction but GFP^+ cells showed no change (supplemental Figure 4A-B). EBs made from iICN1 ESCs alone or ROSA-GFP ESCs alone showed the same results. When colony-forming activity was measured with sorted GFP^+ or GFP^- cells

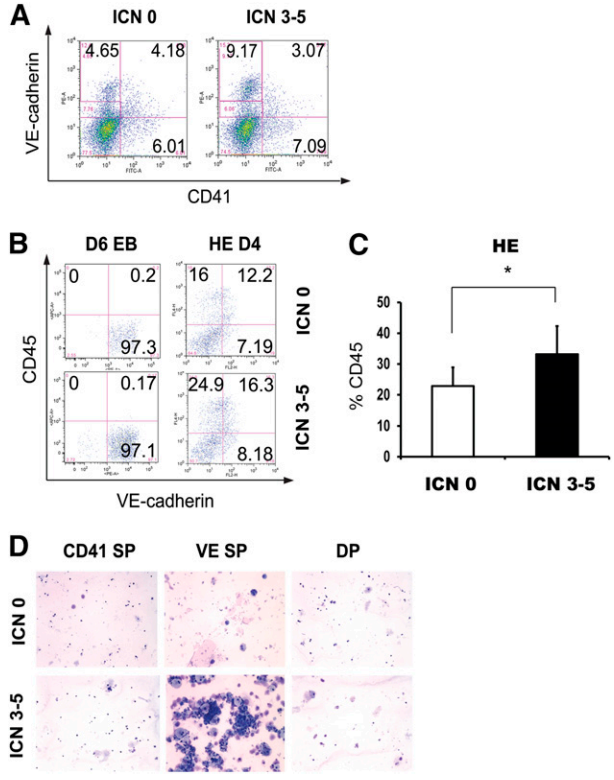

Figure 2. ICN1 induction promotes VE-$cadherin^+$ hemogenic endothelial cell specification. (A) Flow cytometric analysis of day 6 iICN1 EBs with (ICN 3-5) or without (ICN 0) Dox induction (0.5 μg/mL) from days 3 to 5 is shown. (B) $CD45^-$ VE SP cells were sorted from day 6 iICN1 EBs with (ICN 3-5) or without (ICN 0) Dox induction (0.5 μg/mL) from days 3 to 5, and the same number of sorted cells was subjected to HE culture. Flow cytometric analysis of day 4 HE culture is shown. (C) The cumulative average of $CD45^+$ cells of days 2, 4, and 6 HE culture is shown. n = 3; *$P < .05$ by Student *t* test in comparison with noninduced control. (D) Cytospins of day 10 HE culture of $CD45^-$ CD41 SP, VE SP, and DP cells from day 6 iICN1 EBs with (ICN 3-5) or without (ICN 0) Dox induction (0.5 μg/mL) from days 3 to 5 are shown. ICN1 was not induced in HE culture. Representatives of 3 independent experiments are shown.

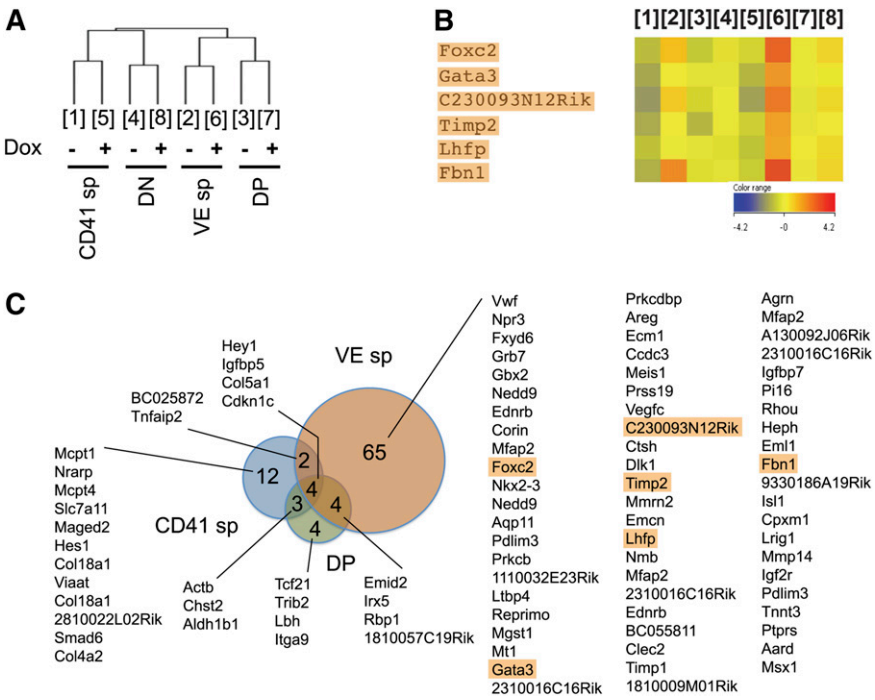

Figure 3. Foxc2 expression is upregulated in VE SP population after ICN1 induction. (A) CD45$^-$ CD41 SP, VE SP, DP, and double negative (DN) populations were sorted from day 6 iICN1 EBs with or without Dox induction (0.5 μg/mL) from days 3 to 5 during EB differentiation and subjected to microarray analysis for gene expression. Hierarchical clustering of populations is shown (Euclidean metric). The numbers, which are applied afterward, represent the corresponding sorted populations. (B) Genes with a similar expression pattern to Gata3 are indicated (Spearmann metric with cutoff 0.95 followed by Pearson metric with cutoff 0.9). Expression levels relative to average gene expression are plotted. (C) Genes with an increase in expression by twofold or higher after ICN1 induction in CD41 SP, VE SP, and DP population are listed from high to low expression. Genes showing an expression pattern similar to Gata3 are marked by a rectangular shade. Data were collected from 3 independent experiments.

from mixed EBs, we observed an increase of colony-forming activity with ICN1 induction in GFP$^-$ cells but not in GFP$^+$ cells (supplemental Figure 4C). Moreover, we observed the same patterns with EBs made from iICN1 ESCs alone or ROSA-GFP ESCs alone. These results indicate that ICN1 works in a cell-autonomous manner in promoting hematopoietic differentiation.

Upregulation of *Foxc2* with ICN1 induction in hemogenic endothelial cells

Despite the long history of detecting hemogenic endothelial cells by surface marker expression, relatively little is known regarding the global gene expression profiles of these cells.[7,33-38] To identify changes in the molecular signature of hemogenic endothelial cells following Notch induction, we isolated RNA from CD41 SP, VE SP, DP, and double negative populations of day 6 EBs and performed microarray analyses. Hierarchical clustering of global gene expression patterns showed that the relationship among these populations was unchanged by ICN1 induction (ICN 3-5) (Figure 3A). When genes whose expression levels were equal or higher than the twofold of the average gene expression level were plotted, the VE SP population showed the greatest number of genes upregulated by ICN1 induction (Figure 3B). Among upregulated genes in the VE SP population, *Gata3* drew our attention because of its known roles in both endothelial and hematopoietic development.[39,40] We then searched for genes with a similar expression pattern to *Gata3* across different populations with or without ICN1 induction, and we identified *Foxc2*, along with *C230093N12Rik*, *Lhfp*, *Timp2*, and *Fbn1*. The increase of *Foxc2* expression after ICN1 induction in the VE SP population was higher than that of *Gata3* (Figure 3C). These results suggest that *Foxc2*, previously known to be involved in endothelial development, may have a role in the specification of hemogenic endothelium.

Defective definitive hematopoiesis in *Foxc2* null mouse embryos

To test whether *Foxc2* is involved in the specification of definitive hematopoiesis during embryo development, we analyzed *Foxc2* null mouse embryos for hematopoietic phenotypes in comparison with wild-type littermates. *Foxc2* null mouse embryos die perinatally or prenatally between E12 and E17 and display abnormal aorta morphology.[41,42] When we measured the expression of *Gata1*, *Gata2*, and *Gata3* in the liver of E13.5/E14.5 embryos, *Gata3*, but not *Gata1* or *Gata2*, showed a decrease in E14.5 *Foxc2*$^{-/-}$ embryos compared with the control embryos (supplemental Figure 5). When the AGM of E10.5 *Foxc2*$^{-/-}$ embryos was stained with runt-related transcription factor 1 (*Runx1*), a definitive hematopoietic marker,[43] we noted a significant decrease of Runx1-positive cells compared with control littermates (Figure 4A). Peripheral blood analysis showed a decrease in the ratio of definitive, enucleated erythrocytes (EryD) to primitive, nucleated erythroblasts (EryP) in E13.5 and E14.5 *Foxc2*$^{-/-}$ embryos (Figure 4B), and quantitative RT-PCR of peripheral blood showed a decrease in the *β-major* to *β-H1* ratio (~52% in E13.5 and ~40% in E14.5, Figure 4C). These results suggest that *Foxc2* is an important factor in definitive hematopoietic development.

Defective definitive hematopoiesis in *foxc1a/b* morphant zebrafish

To confirm the involvement of *Foxc2* in definitive hematopoiesis, we further investigated *foxc1a* and *foxc1b,* the *Foxc2* orthologs in zebrafish. In zebrafish embryos, *foxc1a* expression was detected in both artery and vein, whereas *foxc1b* expression was detected in artery at 24 hours postfertilization (hpf). At 36 hpf, *foxc1a* and *foxc1b* are expressed in both the artery and vein (data not shown). The *foxc1a* and *foxc1b* morpholinos were injected into 1-cell–stage fertilized eggs. Although injection of high doses of *foxc1a* and *foxc1b* morpholinos results in defective intersomitic vessel sprouts and axial vessel formation, low-dose knockdown did not significantly affect artery or vein development.[20] We confirmed that low-dose knockdown had little effect on the vascular development by injecting *foxc1a* and *foxc1b* morpholinos into fertilized eggs of transgenic (Tg) (*flk1:GFP*) zebrafish (Figure 5A). Low-dose knockdown of *foxc1a* and *foxc1b* in wild-type zebrafish embryos did not significantly alter the artery (*ephB2a*,

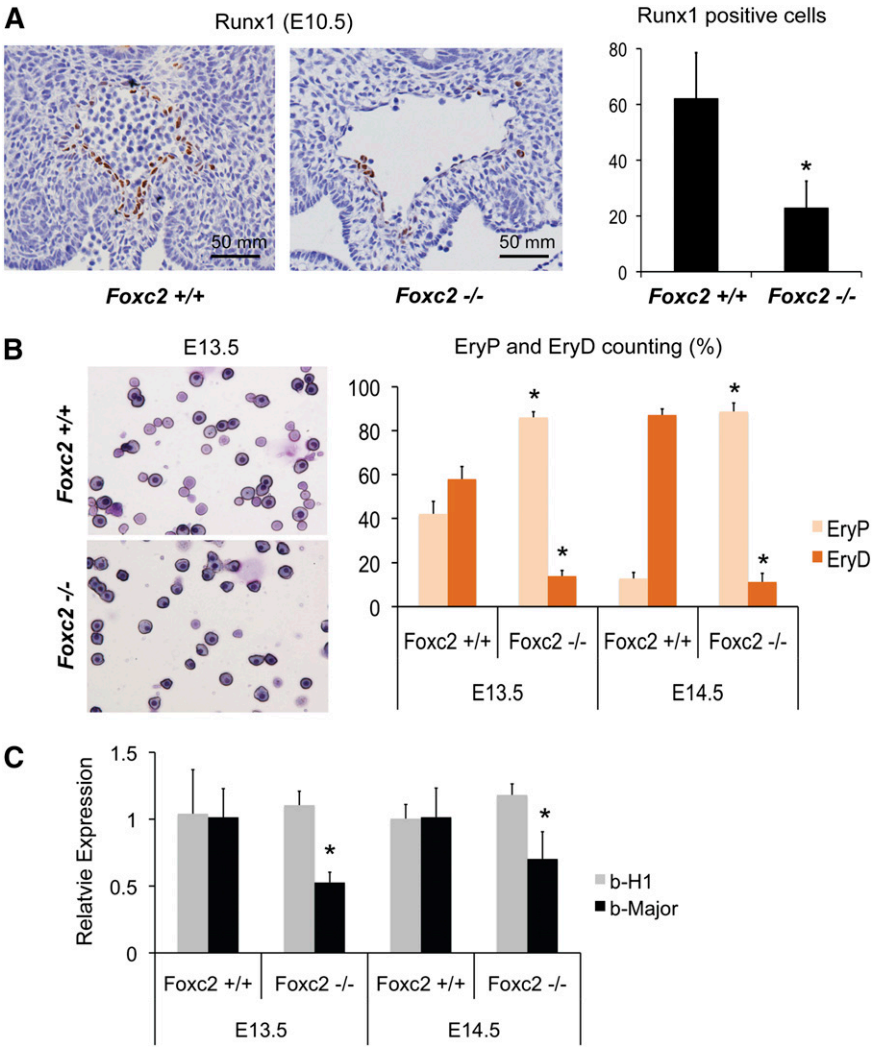

Figure 4. *Foxc2*$^{-/-}$ mouse embryos show defective definitive hematopoiesis. (A) Representative AGM sections of E10.5 *Foxc2*$^{-/-}$ mouse embryos and wild-type littermates with anti-Runx1 antibody staining are shown (left panel). The dorsal aorta region is shown. Quantification of Runx1-positive cells are shown on the right (3 sections per embryo; *Foxc2*$^{+/+}$, n = 4; *Foxc2*$^{-/-}$, n = 5; Volocity version 5.1.0, Improvision Ltd.). (B) Peripheral blood smears of a representative E13.5 *Foxc2*$^{-/-}$ embryo and a wild-type littermate are shown on the left, and the percentages of primitive, nucleated erythroblast and definitive, enucleated erythrocytes are shown on the right. (C) Real time RT-PCR of peripheral blood is shown. Gene expressions relative to wild-type controls are plotted. *$P < .05$ by Student *t* test in comparison with *Foxc2*$^{+/+}$.

notch3, and *deltaC*) or vein (*ephB4a*) marker expression (supplemental Figure 6). In zebrafish embryos, *runx1* and *c-myb* mark hemogenic endothelial cells where definitive hematopoiesis originates.[44] When *foxc1a* and *foxc1b* morpholinos were injected into fertilized eggs of wild-type zebrafish, *runx1* staining (24 hpf) and *c-myb* staining (36 hpf) in arteries significantly decreased (Figure 5B). In addition, at 4 dpf, *foxc1a/b* morphant zebrafish showed the decrease of *rag1* staining, which is a definitive hematopoiesis marker, in thymi (Figure 5B). These results suggest that *Foxc2* and its orthologs are required for the development of hemogenic endothelium and definitive hematopoiesis.

Promotion of hemogenic endothelial cells with *Foxc2* induction

To investigate the effect of *Foxc2* signaling on the specification of hemogenic endothelial cells, we generated a Dox-inducible iFoxc2 embryonic stem cell lines using the flippase recognition target-mediated targeting in KH2 ESCs.[23] *Foxc2* induction after 48 hours of Dox treatment was confirmed by real-time RT-PCR and immunoblotting in comparison with KH2 ESCs, 3T3 cells, and MEFs (Figure 6A, data not shown). Similar to ICN1 induction, *Foxc2* induction from days 3 to 5 during EB differentiation increased the VE SP population when day 6 EBs were subjected to flow cytometric analysis (Figure 6B). When the same number of VE SP cells was sorted and subjected to subsequent HE culture, cells that had experienced *Foxc2* induction during EB differentiation showed increased CD45$^+$ cell generation, which is consistent with the effects of ICN1 induction, whereas the noninduced control cells showed no change (Figure 6C). Differences in the basal frequency of the VE-cadherin$^+$ population in the iICN, Rosa-GFP, and iFoxc2 cell lines could in part be because of the leaky expression of the transgene or the different cellular background.[45] The absolute numbers of CD45$^+$ cells from VE SP cells with *Foxc2* induction are summarized in supplemental Table 1 (160% increase with *Foxc2* induction). These results suggest that *Foxc2* induction promotes the hematopoietic specification of hemogenic endothelial cells during EB differentiation.

Foxc2 acts downstream of *Notch* in hemogenic endothelium

To further investigate the relationship between *Notch* signaling and *Foxc2* in specification of hemogenic endothelium, we analyzed zebrafish embryos following *Notch* and *foxc1a/b* pathway perturbation. Prior studies showed that constitutive *Notch* activation using a heat-inducible bitransgenic system resulted in expansion of *c-myb*$^+$ definitive hematopoietic progenitors.[44] To test whether *foxc1a/b* are required to mediate this *Notch* gain-of-function phenotype, we injected low-dose *foxc1a/b* morpholinos into Tg(*hsp70:gal4*); Tg(*uas:NICD*) embryos and performed heat induction as previously described. The *foxc1a/b* morphants showed relatively intact vascular development and significant decrease of *c-myb* expression at 36 hpf in wild-type embryos

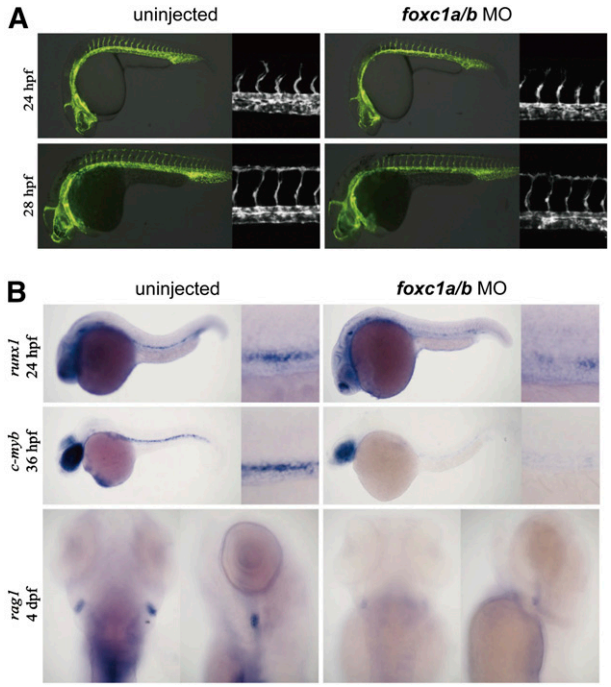

Figure 5. *foxc1a* and *foxc1b* are required for definitive hematopoiesis in zebrafish. (A) Low-dose *foxc1a/b* (4/4 ng) morpholinos were injected into fertilized eggs of Tg(*flk1:GFP*) zebrafish. Fluorescence images of uninjected control and injected zebrafish are shown; (*flk1:GFP*) at 24 hpf, 66/75 intact; (*flk1:GFP*) at 28 hpf, 49/54 intact. (B) Low-dose *foxc1a/b* (4/4 ng) morpholinos were injected into fertilized eggs of wild-type zebrafish. In situ hybridization images with *runx1*, *c-myb*, and *rag1* probes at indicated time points are shown. *runx1*, 17/35 decreased; *c-myb*, 11/26 decreased; *rag1*, 17/28 decreased.

as shown previously. Heat-induction of *NICD* expanded *c-myb* expression in uninjected controls but did not restore *c-myb* expression in *foxc1a/b* morphants, which suggests that *foxc1a/b* activity is necessary to mediate *Notch* signaling (Figure 7A; supplemental Figure 7A). However, we were unable to robustly rescue the *mind bomb* (*mib*) mutant zebrafish, which has defective *Notch* signaling and hemogenic endothelium, by injecting *Foxc2* messenger RNA (supplemental Figure 8), indicating that *Foxc2* is not the sole mediator of *Notch* signaling. Taken together, the results in zebrafish suggest that *foxc1a/b* are necessary but not sufficient to act downstream of *Notch* signaling for hemogenic endothelial specification.

To confirm that *Foxc2* functions as a downstream effector of *Notch* signaling, we analyzed mouse embryos deficient in the *Notch* ligand *Jagged1*. These embryos have impaired hematopoiesis in the AGM but normal artery identity.[46] *Jagged1* null embryos showed decreased *Runx1* expression, as previously reported (supplemental Figure 7B). *Foxc2* expression was detected along the endothelium in the AGM of wild-type and heterozygous littermates, whereas *Jagged1* null embryos showed significant decrease of *Foxc2* expression in the endothelium and the ventral mesenchyme in the AGM (Figure 7B). Given that the ventral side of the AGM is hemogenic and originates from the lateral plate mesoderm, the decrease of *Foxc*2 expression in the ventral side of the AGM of *Jagged1* null embryos correlates with the observation in zebrafish in which *Foxc2* orthologs function downstream of *Notch* for specification of hemogenic endothelium. Taken together, the results from differentiating EBs and embryos of mouse and zebrafish suggest that *Foxc2* plays a key role in definitive hematopoiesis by regulating hemogenic endothelial cell development downstream of *Notch* signaling.

Discussion

The emergence of definitive hematopoietic stem and progenitor cells from hemogenic endothelium has been well documented in the developing murine embryo, but little is known about the molecular pathways that mediate this critical developmental specification.[5,6,47,48] Our study shows that *Notch1* induction increases the VE-cadherin$^+$ hemogenic endothelial population in differentiating murine ESCs, and that *Foxc2* is a downstream mediator of *Notch* signaling in definitive hematopoiesis in both zebrafish and mouse embryos.

Definitive hematopoietic precursors first appear in the aorta and are also detected in vitelline and umbilical arteries, but not in veins, which suggests that HSC generation is associated with artery specification.[7]

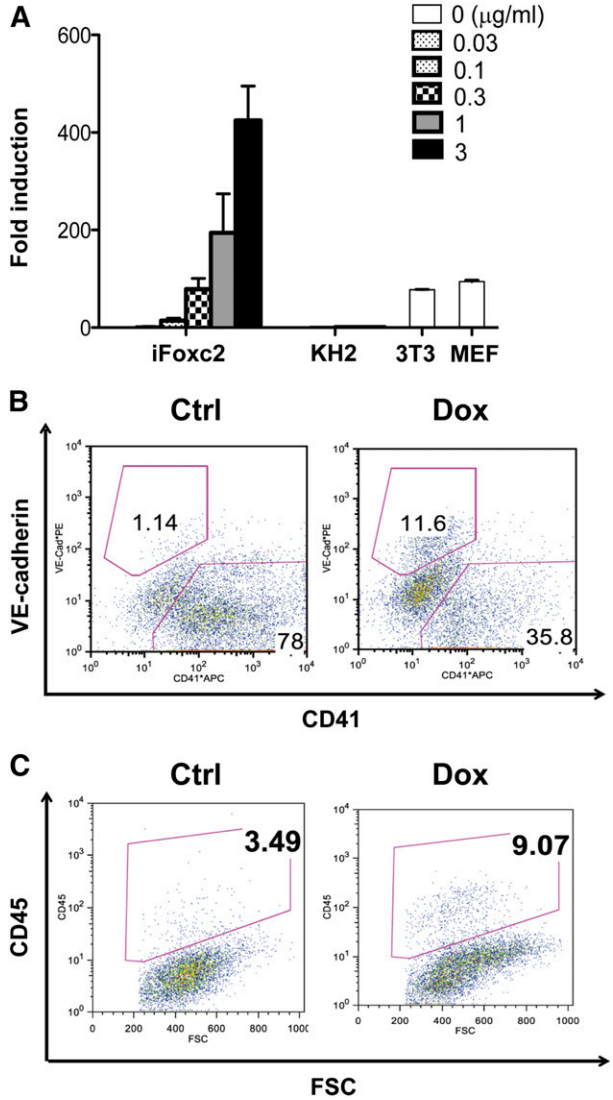

Figure 6. *Foxc2* induction during EB differentiation increases VE-cadherin$^+$ CD41$^-$ population and enhances CD45$^+$ cell generation from VE-cadherin$^+$ CD41$^-$ cells. (A) Real-time RT-PCR analysis for *Foxc2* expression in iFoxc2 cells 48 hours after Dox induction in comparison with KH2, 3T3, and MEF cells is shown. (B) Flow cytometric analysis of day 6 iFoxc2 EBs with or without Dox induction (0.5 μg/mL) from days 3 to 5 is shown. (C) VE-cadherin$^+$CD41$^-$ cells were sorted from day 6 iFoxc2 EBs with or without Dox induction (0.5 μg/mL) from days 3 to 5, and the same number of VE-cadherin$^+$CD41$^-$ cells was subjected to HE culture. Flow cytometric analysis of day 4 HE culture is shown. *Foxc2* was not induced in HE culture. The absolute numbers of CD45$^+$ cells are summarized in supplemental Table 1.

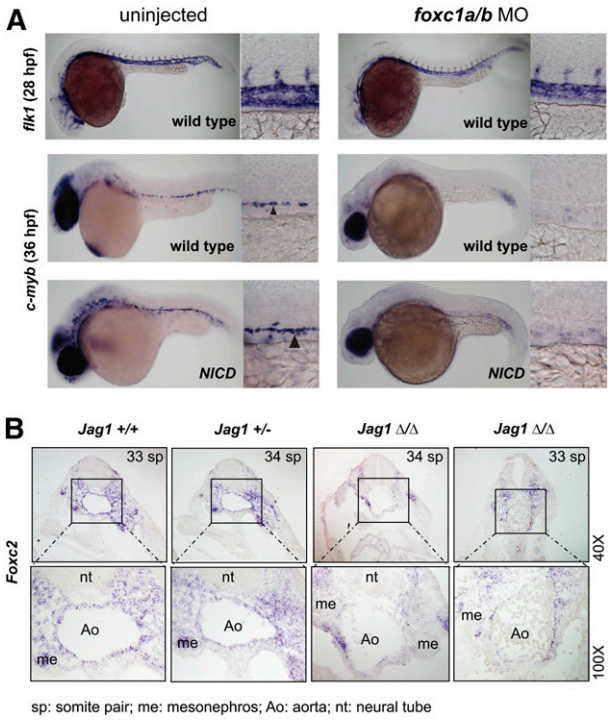

Figure 7. *Foxc2* is a downstream mediator of *Notch* signaling in AGM hematopoiesis. (A) Low-dose *foxc1a/b* morpholinos (4/4 ng) were injected into fertilized zebrafish eggs from a wild-type cross or from a cross of *hsp70*:gal4 and uas:*NICD*. Heat shock was performed on embryos from *hsp70*:gal4 and uas:*NICD* cross between 8 and 12 somite stages at 37°C for 30 minutes to induce *NICD* expression. Embryos were incubated at 28°C. Wild-type crosses were harvested at 28 hpf and 36 hpf, and *hsp70*:gal4 and uas:*NICD* crosses were harvested at 36 hpf. In situ hybridization with *flk1* (wild-type at 28 hpf) or *c-myb* (wild-type and *NICD* at 36 hpf) is shown. Arrowhead indicates positive *c-myb* staining. Morpholino injection decreased (7 partial + 23 complete/30) *c-myb* expression, and *NICD* induction did not rescue the decrease (1 partial + 6 complete/7). (B) *Jagged1* null mouse embryos and littermates (E10.5) were subjected to in situ hybridization with *Foxc2* probe. Transverse sections of dorsal aorta in the AGM region are shown in a dorsal to ventral orientation. Two representative Jagged1 null embryos are shown (n = 4).

Notch and *Foxc* genes are known to play critical roles in determining arterial cell fate during embryo development.[17] In this study, we demonstrated that induction of *Notch1* signaling increased the hemogenic endothelial population within differentiating cultures of ESCs and also enhanced their hematopoietic potential. We then identified the *Foxc2* transcription factor as a chief candidate for mediating *Notch1* signaling, as it showed the highest increase after *Notch1* induction in a similarity search with *Gata3*, a master hematopoietic regulatory gene for the hemogenic endothelial population. Induction of *Notch* signaling has been shown to enhance development of *c-myb*-positive hematopoietic progenitors in the zebrafish.[44] In this study, we show that in the setting of *Notch* induction, morpholino knockdown of the zebrafish orthologs *foxc1a* and *foxc1b* markedly reduced detection of *c-myb*-positive hematopoietic progenitors without disrupting vascular integrity, thereby implicating *FoxC* genes as downstream targets in the *Notch* pathway. Moreover, mouse embryos deficient in the *Notch* ligand *Jagged1* showed markedly reduced levels of *Foxc2* staining in the aortic endothelium, and *Foxc2* null embryos showed reduced levels of *Runx1*-positive hematopoietic elements, thereby demonstrating that *Notch* signaling promotes hematopoietic specification through the *Foxc2* transcription factor.

HSCs can be derived from ESCs (ESC-HSCs) by ectopic expression of *HoxB4* and *Cdx4*, but ESC-HSCs do not faithfully recapitulate adult HSCs in their function or surface phenotype.[2] According to transcriptome analysis of hematopoietic stem and progenitors from developing embryos, differentiating ESCs, and adult mice, ESC-HSCs cluster most closely with fetal liver and adult bone marrow HSCs but reveal the absence of a transcriptional response to *Notch* signaling.[1] Weighted Gene Coexpression Network Analysis clustering algorithm assigns *Foxc2* to a "HSC-specifying" module (module 19) (http://hsc.hms.harvard.edu). A recent report in which hematoendothelial cells were generated from human ESCs showed that *Foxc2* was enriched in hemogenic endothelial cells compared with nonhemogenic endothelial cells.[49] In addition, *Etv2*, which cooperates with *Foxc2* in regulating endothelial genes, was shown to cooperate with *Gata2*, a well-known *Notch1* downstream target, to regulate endothelial and hematopoietic lineage development.[20,50] Thus, the *Notch-Foxc2* axis may be important in the emergence of not only definitive hematopoietic progenitors but also definitive HSCs. However, when the VE-cadherin$^+$ population of day 6 EBs with *Notch1* induction was transplanted into mouse neonates, no meaningful long-term engraftment was detected (data not shown). Even with enhanced hematopoietic potential following *Notch1* induction, the day 6 EB-derived VE-cadherin$^+$ population needs additional developmental maturation to achieve engraftment in the mouse. Further investigation of *Notch1* signaling and *Foxc2* in hematopoietic development may help identify essential differences between ESC-HSCs and adult HSCs.

Hemogenic sites in early embryo development overlap with vasculogenic sites, including the yolk sac, allantois, vitelline, and umbilical vessels, and the emergence of definitive hematopoiesis appears to correlate with angiogenic remodeling of the primitive vascular plexus.[51-55] Inside the embryo, vasculogenic sites include the aorta, lung, spleen, and pancreas, but among them, only the aorta is known to have hemogenic activity. It will be of high interest to analyze the molecular and cellular mechanisms of this anatomic distinction and to look further into the transient character of hemogenic endothelial cells. Clonogenic analysis of hemogenic endothelial cells in the culture condition that supports outgrowth of endothelial and hematopoietic cells will facilitate more accurate estimation of hemogenic endothelial cell frequency.[56] In the era of reprogramming, it may be possible to convert nonhemogenic endothelial cells to hemogenic endothelial cells via ectopic expression of transcription factors like *Foxc2*, which may give us a new strategy for generating autologous HSCs.

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Authorship

Contribution: I.H.J. and Y.-F.L. designed the research and performed experiments; L.Z., P.L.W., S.M.D., N.A., J.G., M.L., P.G.K., and E.K.D. performed experiments; J.H.K., T.K., T.M.S., L.I.Z., A.B., C.E.B., and G.Q.D. provided resources and comments; and I.J. and G.Q.D. wrote the manuscript.

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Supplemental Data

	HE D2		HE D4		HE D6		HE D4	
	ICN 0	ICN 3-5	ICN 0	ICN 3-5	ICN 0	ICN 3-5	Foxc2 0	Foxc2 3-5
Total viable cells	1712	1825	1530	1906	1055	2020	5107	6273
CD45+ cells	278	423	432	784	253	711	178	569

Table S1. Absolute number of CD45$^+$ cells generated from VE-cadherin$^+$ cell in HE culture. The numbers of CD45$^+$ cells generated among viable cells in HE cultre are summarized (related to Fig. 2B and Fig. 6C).

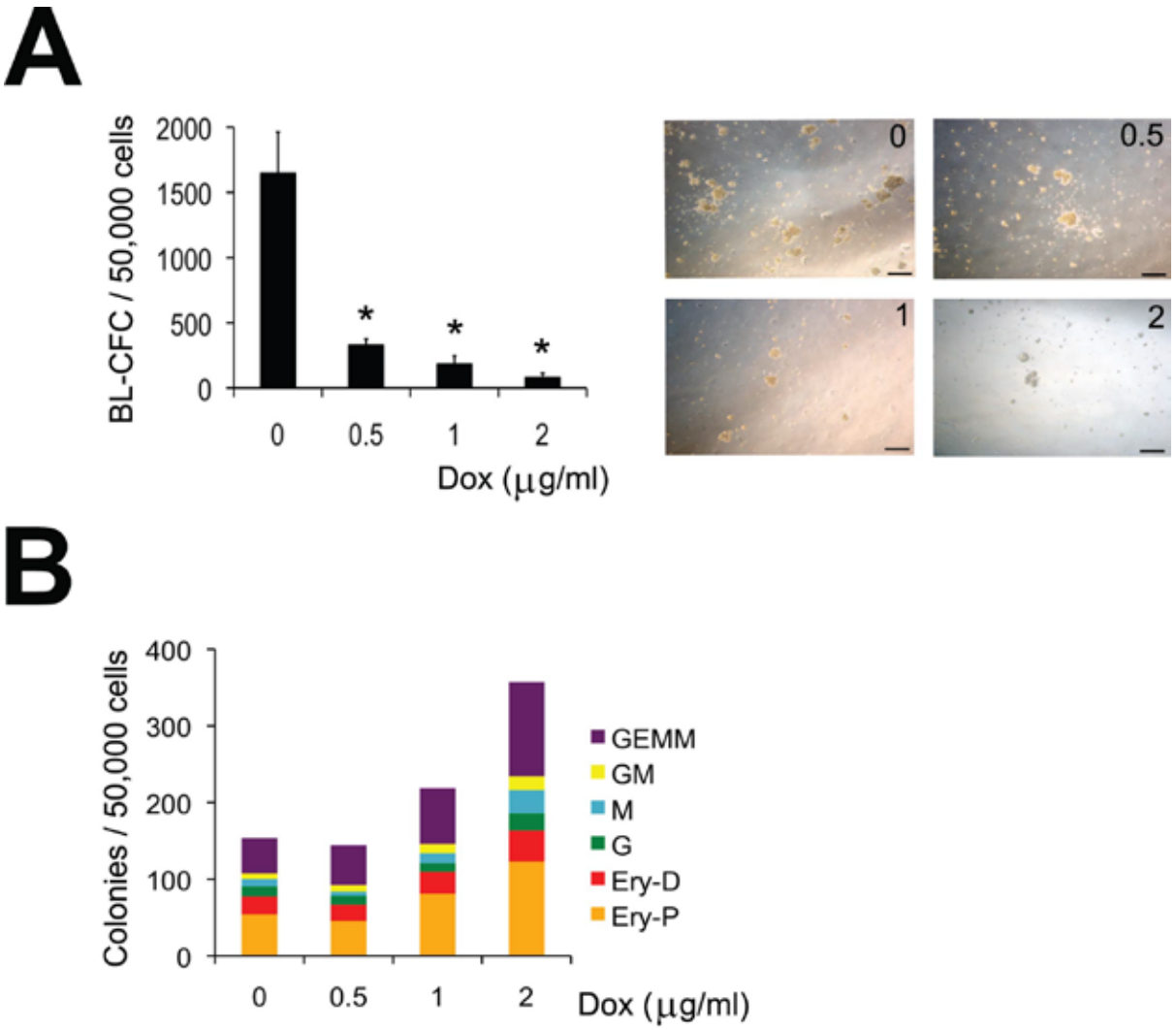
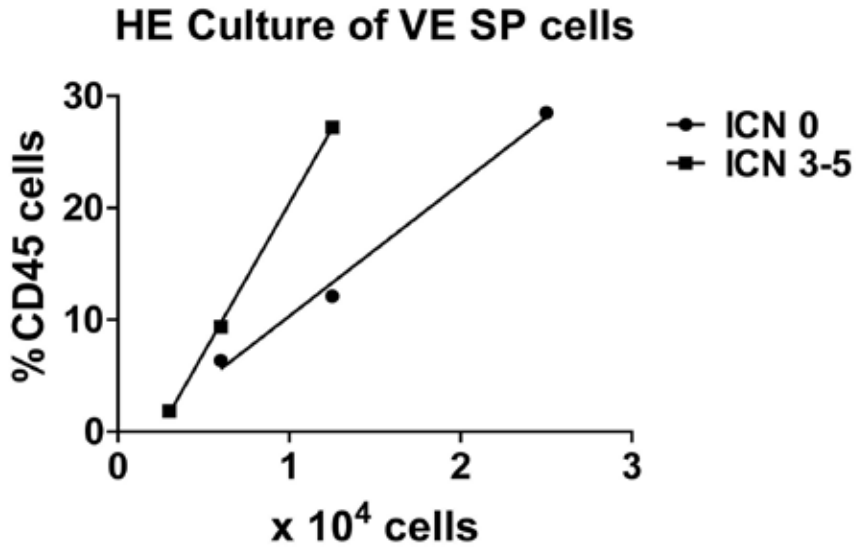


Figure S1. ICN1 induction inhibits blast colony formation but promotes hematopoietic development. (A) Day 3 EB-derived cells were placed into blast colony forming cell (BL-CFC) assay with or without adding doxycycline. Blast colony count and representative images are shown. (*, $p < 0.05$ by Student's t-test in comparison with non-induced control) (B) Cells harvested from BL-CFC assay were washed to remove doxycycline and subjected to hematopoietic colony assay. Doxycycline label indicates the treatment during BL-CFC assay.



Best-fit values	ICN 0	ICN 3-5
Slope	11.85 ± 1.079	26.81 ± 0.5563
Y-intercept when X=0.0	-1.528 ± 1.781	-6.413 ± 0.4557
X-intercept when Y=63	5.446	2.589

Figure S2. HE cell frequency increases with ICN1 indcution. VE SP cells from day 6 EB with or without ICN1 induction were plated into HE culture with indicated cell numbers. The frequency of HE cell was estimated by calculating plating cell number that generates 37% negative cultures after linear regression (GraphPad Prism 5.03).

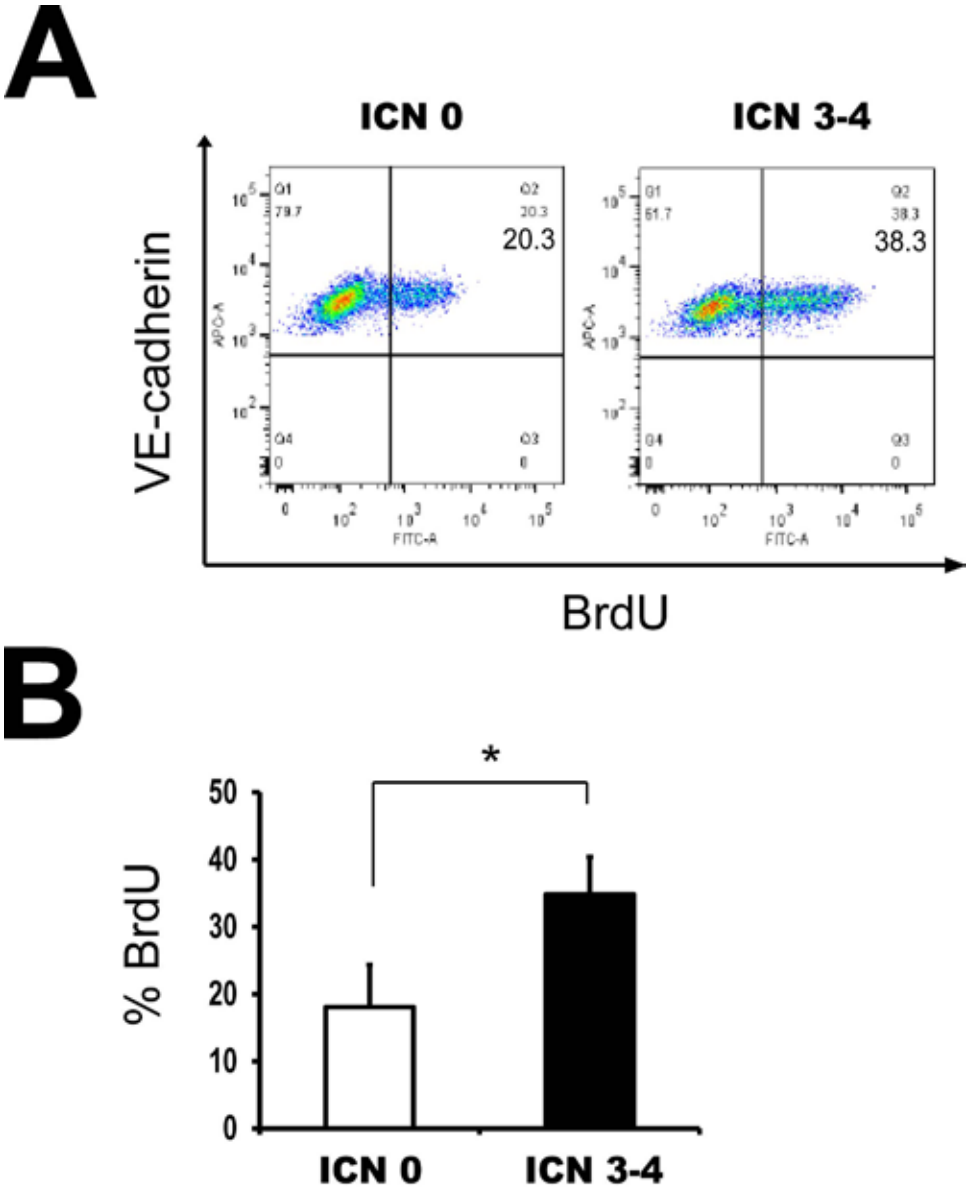


Figure S3. Proliferation of VE-cadherin$^+$ cells increases with ICN1 induction. (A) Flow cytometric analysis of BrdU uptake in VE-cadherin$^+$ cells of day 4 EBs with or without ICN1 induction from day 3 is shown. (B) Quantification of BrdU$^+$VE-cadherin$^+$ cells in day 4 EBs with or without ICN1 induction from day 3 is shown. (n=3, *, $p < 0.05$ by Student's t-test in comparison with non-induced control)

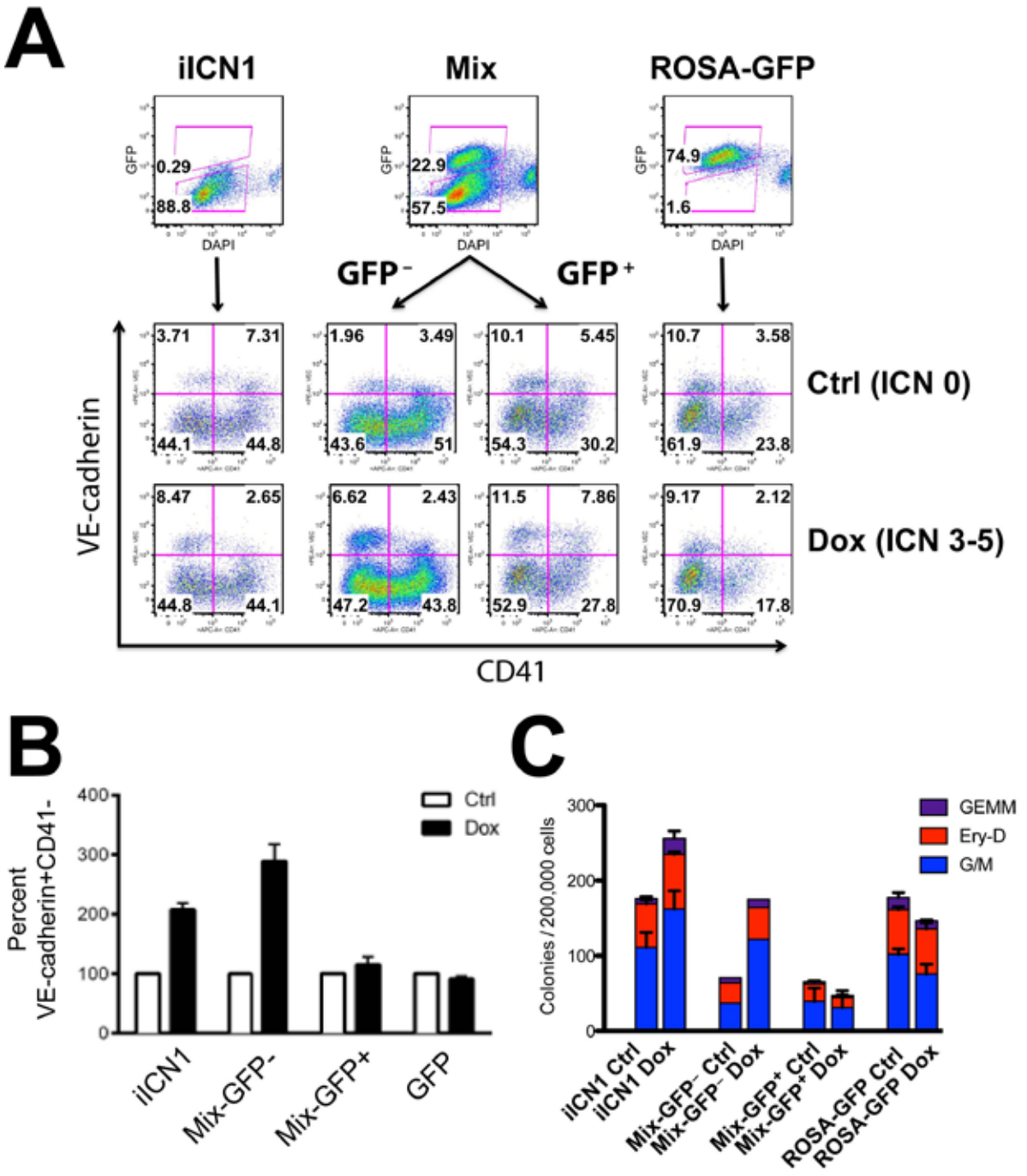


Figure S4. ICN1 induction up-regulates the expression of VE-cadherin in a cell-autonomous manner. (A) iICN1, ROSA-GFP (GFP), and a mixture of both type of ES cells (Mix) were cultured with or without Dox (0.5 ㎍/ml) from d3 to 5 during EB differentiation. Day 6 EB-derived cells were analyzed for VE-cadherin and CD41 expression by flow

cytometry. (B) The quantitative analysis of percent VE-cadherin^{+} CD41^{-} cells is shown after normalization to uninduced control. (C) Day 6 EB-derived cells of the indicated treatment groups were sorted for GFP^{+} and GFP^{-} and subjected to methylcellulose colony assay. Colony counts after 10 days of culture are shown.

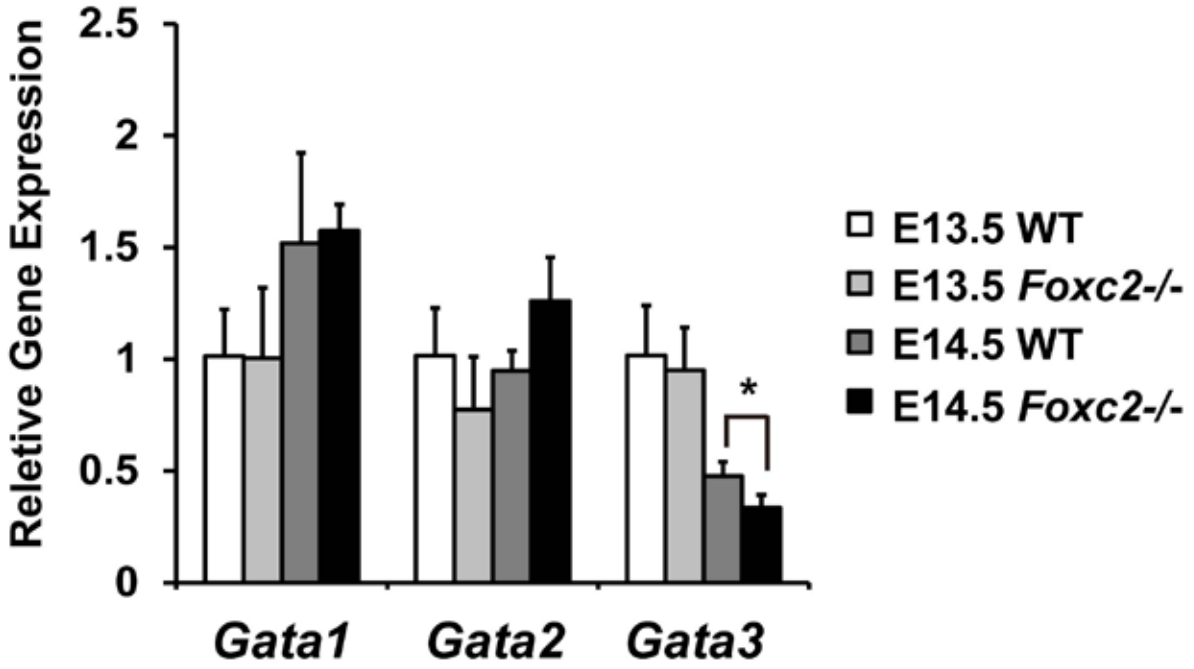


Figure S5. *Gata3* but not *Gata1* or *Gata2* decreases in the fetal liver of *Foxc2-/-* embryos. Fetal liver of E13.5 or E14.5 *Foxc2-/-* embryos were subjected to reatl time RT-PCR analysis with *Gata1*, *Gata2*, or *Gata3* probes in comparison with wild type controls. (*, $p < 0.05$ by Student's t-test)

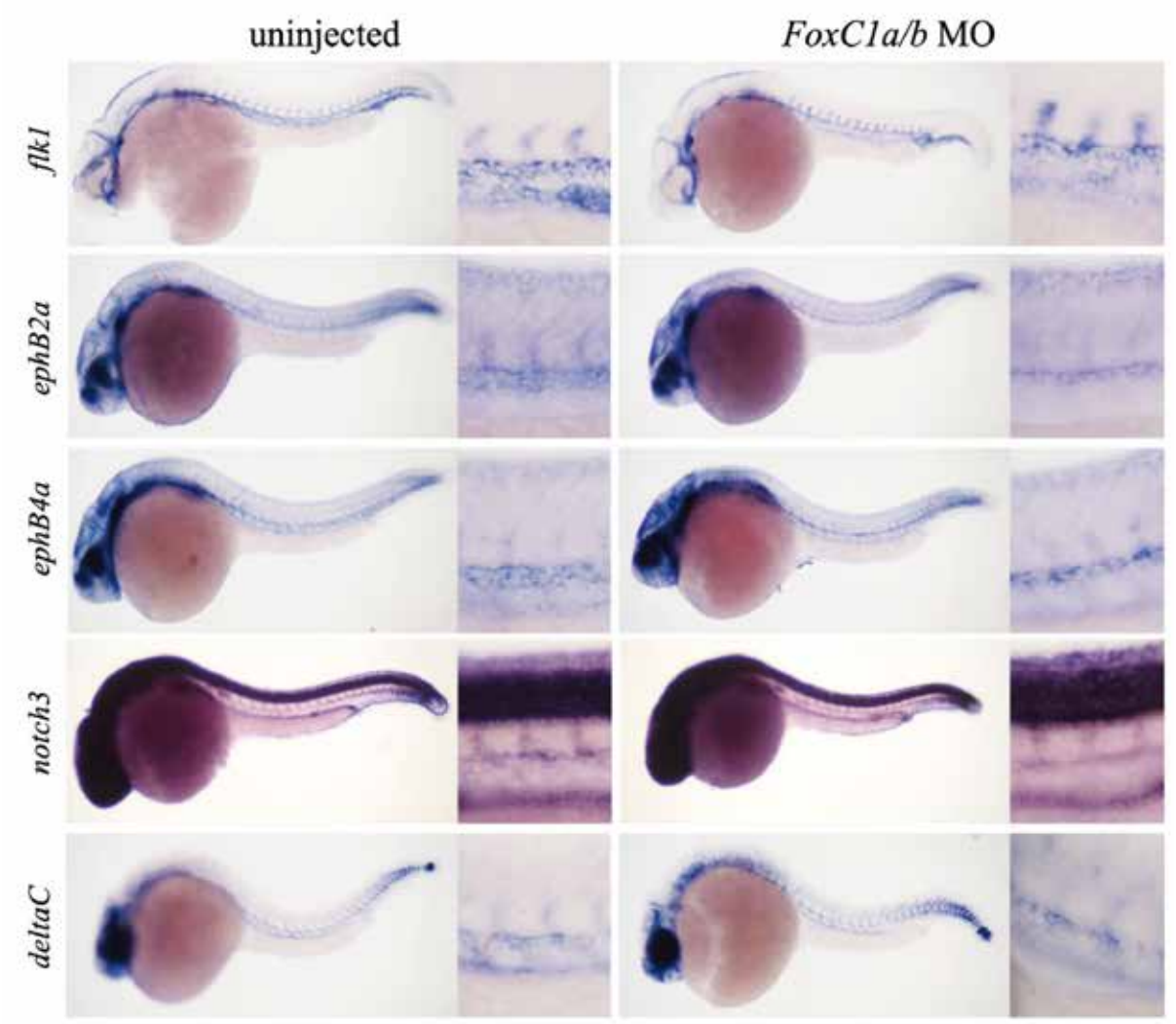


Figure S6. Low dose injection of *foxc1a/b* morpholinos does not affect the vascular development. .Low dose *foxc1a/b* morpholinos (4/4 ng) were injected into the fertilized eggs of zebrafish embryos. *In situ* hybridization images with vascular probes of 30 hpf uninjected control embryos and morphants are shown. Vascular marker (*flk-1*), arterial markers (*ephB2a*, *notch3* and *deltaC*), and venous marker (*ephB4a*) are shown.

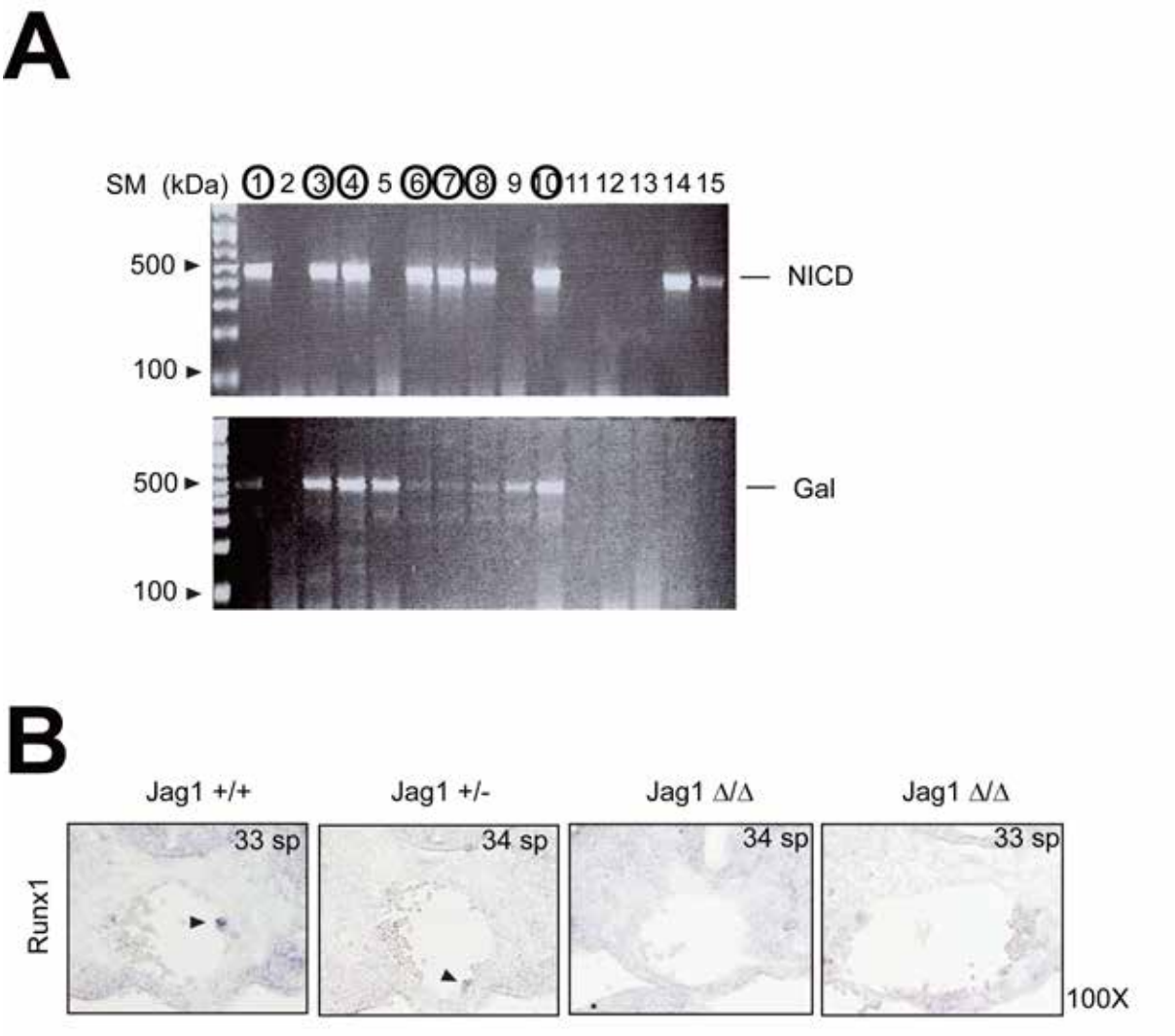


Figure S7. *Foxc2* is a downstream mediator of *Notch* signaling in AGM hematopoiesis. (A) Genotyping PCR results for *nicd* (450 bp) and *gal* (450 bp) zebrafish embryos are shown. (B) *In situ* hybridization images with *Runx1* probe on serial sections of *Jagged1* null and litter mate mouse embryos (E10.5) from figure 7B are shown.

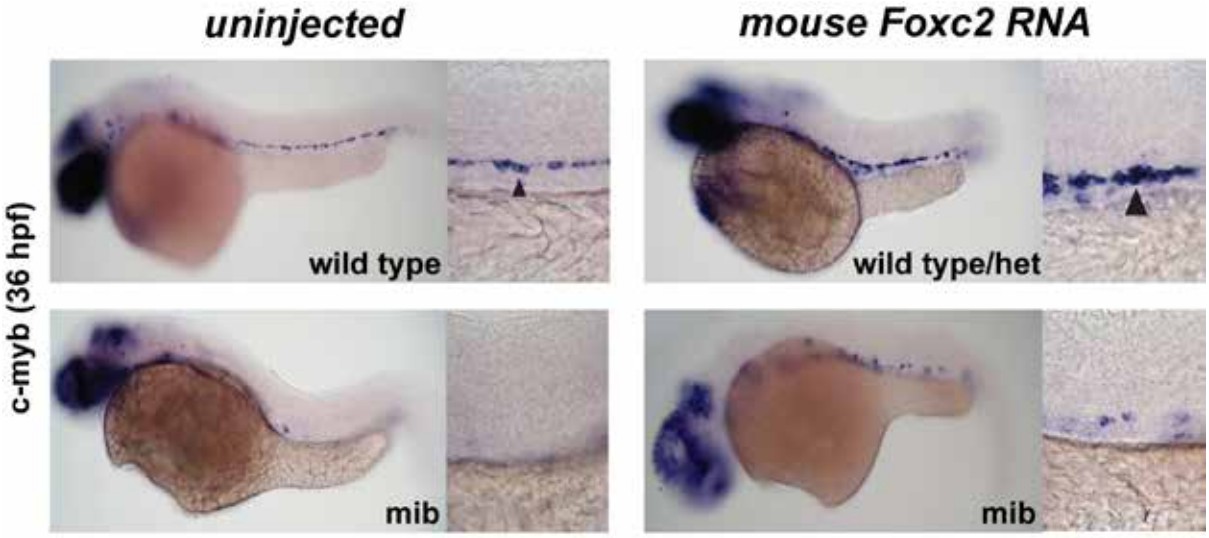


Figure S8. *Foxc2* mRNA injection does not rescue *mib* zebrafish mutant. Mouse *Foxc2* mRNA (100 pg) was injected to yolk of 1-2 stage embryos of *mib/+* cross. Embryos were incubated at 28°C and harvested at 36 hpf. Whole mount *in situ* hybridization is shown. Arrowhead indicates positive *c-myb* staining (wild type/het n=50 and *mib* n=19 were analyzed after mouse *Foxc2* RNA injection).

Supplemental Experimental Methods

Blast colony forming cell (BL-CFC) assay

Day 3 EBs were dissociated by trypsin treatment and cells were mixed into BL-CFC media containing 1% methylcellulose, 10% FCS, 25% D4T conditioned medium, 5 ng/ml mVEGF, 100 ng/ml mSCF, 200 μg/ml holo-transferrin, 25 μg/ml ascorbic acid, 450 mM monothioglycerol, and colonies were counted 4 days later.

Cell cycle analysis

Day 4 EBs were labeled with 10 mM BrdU (559619, BD Bioscieces) for 2 hr followed by dissociation and incubation with VE-cadherin antibody (562242, BD Biosciences). Cells were subjected to flow cytometric analysis in the presence of 7-AAD (BD FACSCanto II).

Microarray analysis

For microarray, RNAs from independent biological triplicates were isolated with RNeasy kit (QIAGEN), and the hybridization was performed on Mouse Ref-8 Expression BeadChip (illumina) at Molecular Genetics Core (Children's Hospital Boston, MA). Data were analyzed by GeneSpringGX. Microarray data are deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE19951.

Real-time RT-PCR of fetal liver

Feta liver was harvested with TRIzol and subjected to real-time RT-PCR with *Gata* primers (*Gata1*, forward: CATTGGCCCCTTGTGAGGCCAG, reverse: CGCTCCAGCCAGATTCGACCC; *Gata2*, forward: CTGCAACACACCACCCGATA, reverse: GGAGCGAGCCTTGCTTCTC, Primer Bank ID: 31982351a3; *Gata3*, forward: ATCCGCCCTATGTGCCCGAGTA, reverse: ATGTGGCTGGAGTGGCTGAAGG).

Morpholinos, mRNA, *in situ* hybridization, and immunohistochemistry.

Zebrafish were maintained at 28°C, and the antisense morpholino oligonucleotides for *foxc1a* and *foxc1b* were prepared as previously described.[1] Morpholinos were injected into the yolk of 1-2 cell stage embryos. Whole-mount *in situ* hybridization and heat-shock of Tg(*hsp70:gal4*);Tg(*uas:NICD*) embryos were performed as previously described.[2] For immunohistochemisty, E10.5 mouse embryos were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned (5 μm). Sections were deparaffinized in xylene, rehydrated through a downgraded series of alcohol solutions. After washing the sections with tap water and TBS, tissues were blocked with 5% normal goat serum at room temperature for 1 hr and then incubated with the rabbit monoclonal anti-Runx1 antibody (1:100; Epitomics) overnight at 4°C. Sections were washed with TBS and incubated for 1 hr at room temperature with biotinylated goat anti-rabbit secondary antibody (1:500; Jackson Immuno Research). After rinsing the sections in TBS, the antigen was visualized using ABC kit (Vector laboratories), developed in DAB substrate (Vector laboratories). The slides were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene and mounted with Permount (Fisher). Jagged1 null mouse embryos[3] and littermates were age-

matched by somite counting, genotyped by PCR and subjected to *in situ* hybridization as described previously.[4]

Statistical Analysis

Quantitative data are presented as the mean ± SD from representative experiments ($n \geq 3$). Statistical significances were calculated with the unpaired Student's t-test; p values < 0.05 were considered significant.

Supplemental References

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