

Etv2/ER71 induces vascular mesoderm from Flk1⁺PDGFR α ⁺ primitive mesoderm

Hiroshi Kataoka,^{1,2} Misato Hayashi,¹ Reiko Nakagawa,¹ Yosuke Tanaka,¹ Naoki Izumi,¹ Satomi Nishikawa,¹ Martin Lars Jakt,¹ Hiroshi Tarui,³ and Shin-Ichi Nishikawa¹

¹Laboratory for Stem Cell Biology, RIKEN Center for Developmental Biology, Kobe, Japan; ²PRESTO, Japan Science and Technology Agency, Saitama, Japan; and ³Laboratory for Genome Resource and Analysis Unit, RIKEN Center for Developmental Biology, Kobe, Japan

Etv2 (*Ets Variant 2*) has been shown to be an indispensable gene for the development of hematopoietic cells (HPCs)/endothelial cells (ECs). However, how *Etv2* specifies the mesoderm-generating HPCs/ECs remains incompletely understood. In embryonic stem cell (ESC) differentiation culture and *Etv2*-null embryos, we show that *Etv2* is dispensable for generating primitive Flk-1⁺/PDGFR α ⁺ mesoderm but is required for the progression of Flk-1⁺/PDGFR α ⁺ cells into vascular/hematopoietic mesoderm. *Etv2*-null ESCs and embryonic cells were arrested as Flk-1⁺/

PDGFR α ⁺ and failed to generate Flk-1⁺/PDGFR α ⁻ mesoderm. Flk-1⁺/Etv2⁺ early embryonic cells showed significantly higher hemato-endothelial potential than the Flk-1⁺/Etv2⁻ population, suggesting that *Etv2* specifies a hemato-endothelial subset of Flk-1⁺ mesoderm. Critical hemato-endothelial genes were severely down-regulated in *Etv2*-null Flk-1⁺ cells. Among those genes *Scl*, *Fli1*, and *GATA2* were expressed simultaneously with *Etv2* in early embryos and seemed to be critical targets. *Etv2* reexpression in *Etv2*-null cells restored the development of CD41⁺,

CD45⁺, and VE-cadherin⁺ cells. Expression of *Scl* or *Fli1* alone could also restore HPCs/ECs in the *Etv2*-null background, indicating that these 2 genes are critical downstream targets. Furthermore, VEGF induced *Etv2* potently and rapidly in Flk-1⁺/PDGFR α ⁺ primitive mesoderm is committed into Flk-1⁺/PDGFR α ⁻ vascular mesoderm through *Etv2* and that up-regulation of *Etv2* by VEGF promotes this commitment. (*Blood*. 2011;118(26):6975-6986)

Introduction

Based on observations that several single-gene mutations severely affect both endothelial cell (EC) and hematopoietic cell (HPC) lineages, ECs and HPCs are proposed to originate from common precursors called hemangioblasts.^{1,2} In zebrafish, *cloche* is the most classic mutant affecting both ECs and HPCs.^{3,4} Deleting Flk-1, a major signaling receptor for VEGF, in mice causes defects in both ECs and HPCs.^{5,6} However, in vitro differentiation of Flk-1-null embryonic stem cells (ESCs) generates both ECs and HPCs, albeit at much lower efficiencies.^{7,8} These findings demonstrate that Flk-1 is dispensable for the differentiation of hemato-endothelial precursors but is required for the proliferation of these progenitor cells.

Etv2 (*Ets variant 2*), identified in zebrafish as *etsrp71*, has been reported to be a key molecule for investigating EC and HPC development.⁹⁻¹¹ In *etsrp71* mutants, vasculogenesis and myelopoiesis are affected, leaving primitive erythropoiesis relatively intact. Conversely, overexpression of *etsrp71* induces ectopic EC generation indicating a strong vasculogenic potential of *etsrp71*.¹² In mice, *Etv2* disruption results in a lack of Flk-1 expression, leading to the total loss of ECs and HPCs.^{10,13} In ESCs, *Etv2* overexpression up-regulates Flk-1 and subsequent EC generation.¹⁰ From these results, *Etv2* was hypothesized to be induced by bone morphogenic protein (BMP), Notch, and Wnt signaling and then transactivate Flk1. However, the phenotype discrepancy between the *etsrp71* zebrafish mutant and *Etv2*-knockout mice raised the possibility that *Etv2* may have more complex functions than proposed as an activator of Flk-1. Recent studies in zebrafish showed that, in addition to Flk-1, EC or HPC genes can also be up-regulated more robustly by *Etv2*.^{14,15} Using *Etv2* Venus knock-in mice and *Etv2*-null ESC lines (*Etv2-Venus*^{+/-} and *Etv2*^{-/-}, respectively), the role

of *Etv2* and downstream target genes in EC and HPC development from mesoderm were investigated in the present study.

Methods

Etv2-Venus ESCs and mice

Targeting vector for the mouse *Etv2* locus was constructed using the Gateway cassette vector system containing Venus and Cre removable Neo resistance cassette.¹⁶ Genomic sequences corresponding to 111658-119169 (accession number 167978.4) on the 5' side and 119172-122639 (accession number 167978.4) on the 3' side were used as homology arms for recombination, and Venus was subcloned in-frame into the endogenous *Etv2* allele. The resulting targeting construct and TT2 ESCs were used to generate *Etv2*-Venus knock-in mice (CDB0675K). Original and modified *Etv2* loci are depicted in supplemental Figure 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). *Etv2* locus on the other allele was targeted to generate homozygous *Etv2*-null ESCs. These *Etv2*-Venus mice or gene-trap line 141H7 were used to generate *Etv2*-null embryos.¹³

RNA isolation and expression profiling

RNA was purified using the RNeasy MinElute column (QIAGEN) and reverse transcribed by Revatrace (TOYOBO). Real-time PCR was performed with the PowerSyber Green kit (ABI) using ABI 9700. Microarray analysis was performed using Mouse Genome 430 2.0 Array (Affymetrix) using 2 differentiation cultures that were started independently. Oligonucleotide primers for quantitative PCR (Q-PCR) are listed in supplemental Table 3. All microarray data are available in the Gene Expression Omnibus (GEO) under accession numbers GSE31743 and GSE31744.

Submitted May 11, 2011; accepted July 21, 2011. Prepublished online as *Blood* First Edition paper, September 12, 2011; DOI 10.1182/blood-2011-05-352658.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2011 by The American Society of Hematology

Cell culture

ESCs were differentiated on OP9 cells as described previously.¹⁷ Briefly, 10^5 ESCs/25 cm² were seeded onto OP9. To induce Etv2-Venus⁺ cells BMP4, VEGF, and FGF8b were added from day 3 of differentiation. For HPC differentiation, SCF (100 ng/mL), G-CSF, (10 ng/mL), IL-3 (5 U/mL), erythropoietin (5 ng/mL), and VEGF (20 ng/mL) were added. Abs used for flow cytometry were Flk-1 (AVAS12), PDGFR α (APA5), CD41 (MWReg30), ICAM2 (3C4), CD31 (Mec13.3), CD90.1 (Thy1.1; eBioscience), and VE-cadherin (VECAD1; BioLegend). For transgene rescue in Etv2-null cells Etv2, Scl, Fli1, or GATA2 were inserted into the *piggyBac* (PB)-based expression cassette under the control of the Tetracyclin (Tet)-responsive promoter (Guo et al¹⁸ and modified by H. Niwa). Transgene expression was monitored by IRES-tagged CD90.1 staining. These plasmids were cotransfected with PB-CAG-tTA-IRESNeo and PBase expression plasmids (gifts from H. Niwa and Guo et al) using X-fect (Clontech) to obtain Tet-responsive clones. Those cells established as undifferentiated ESC clones in the presence of Tet were differentiated on OP9 without Tet. On differentiation day 3, transgene-expressing cells were sorted by CD90.1, further differentiated on OP9 until day 10, and stained with the indicated Abs. Serum-free culture of Flk-1⁺ mesoderm was performed as described previously.¹⁹

Immunostaining

Etv2 expression in Etv2-Venus mice was detected using rabbit or chicken anti-green fluorescent protein (anti-GFP; Invitrogen; 1:1000) Abs. Whole-mount immunostaining was performed using Abs against Flk-1 (BD Biosciences or R&D Systems), PDGFR α (Cell Signaling Technology), and GATA1 (N6; Santa Cruz Biotechnology).

In situ hybridization

Whole-mount in situ hybridization was performed essentially as described previously.²⁰ Probes for each gene were synthesized using the sequences as follows with the DIG RNA labeling kit. (Roche): GATA2 1589-2059, accession number NM008090; Fli1 1284-1543, accession number NM008026; Scl 860-1428, accession number M59764; and Runx1 2048-2518, accession number NM009821.

EMSA

For the electrophoretic mobility shift assay (EMSA), Etv2 protein was synthesized from pCS2-Etv2 with the TNT in vitro translation kit (Promega). Reticulocyte lysate containing translated Etv2 was incubated with labeled probes with or without competing oligonucleotides (supplemental Table 3) in binding buffer. Electrophoretic mobility shift was analyzed by electrophoresis in 0.5 \times TBE gel and subsequent autoradiography.

ChIP

Mouse EC line F2 or CCE ESCs were transfected with HA-tagged Etv2 and ChIP analysis was performed using the ChIP-IT kit (Active Motif), anti-HA antibody (12CA5 or 3F10; Roche), and the indicated primer sets (supplemental Table 3). Flk-1⁺ cells from HA-Etv2 CCE cells were sorted after differentiation for 4 days.

Luciferase assay

Indicated sequences of the Scl, Fli1, and GATA2 genomes were PCR amplified using KOD-FX polymerase (TOYOBO) and ligated to pGL3-SV40 vector (Promega): Scl, 15199029-15199673 (NT_039264.6); Fli1, 19119650-19120142 (NT_039472.7); and GATA2, 40461620-40463619 (NT_039353.7). Bovine aortic ECs were transfected with luciferase reporter vectors (wild-type and Etv2-binding sites mutated) and control, Etv2, Ets1, or Ets2 expression vectors using Lipofectamine 2000 (Invitrogen). Transfection efficiency was normalized with the pRLCMV-Renilla expression vector. Luciferase activity was measured according to the manufacturer's directions (Promega).

Microscope models for images

Whole-mount embryo samples in PBS were observed in an M205C microscope (Leica Microsystems). Fluorescent images were made with Prolong-Gold medium (Invitrogen) and BZ-9000 (Keyence). Figure 1Bd-e are images made using Entellan (Merck) and visualized on a DMI 6000B microscope (Leica Microsystems). Figure 2Aa shows bright images covered by PBS and imaged using a DMI4000B microscope (Leica Microsystems).

Results

Etv2 is expressed in the Flk-1⁺ cell population in developing embryos and differentiating ESCs

Previous in situ hybridization showed *Etv2* expression in the primitive streak mesoderm, including the blood island, and in developing ECs.¹⁰ To visualize *Etv2* expression in detail, Etv2-Venus mice and differentiated ESCs were analyzed by anti-GFP immunostaining and flow cytometry. Whereas *Etv2* expressions at embryonic day 8.5 (E8.5) or E9.5 by anti-GFP immunostaining were consistent with the previous in situ hybridization report, in the E7.0-E7.5 embryos, the expression pattern was different. Expression of *Etv2* began to appear around E7.0 within the Flk-1⁺ cells in the posterior mesoderm (Figure 1Aai-ii). At this stage, Etv2⁺ cells were seen in a subset of Flk-1⁺ cells (Figure 1Abi-iii). At E7.5, expression was detected in the extra-embryonic mesoderm (Figure 1Ba). Double staining with Flk-1 showed that most of the extra-embryonic Flk-1⁺ cells were Venus⁺, indicating the pivotal role of Flk-1 in generating endothelial and hematopoietic precursors at this stage (supplemental Figure 2A). At E8.5, expression was seen from the anterior to posterior part of the dorsal aorta, and in the endocardium and head mesenchyme region (Figure 1Bb and supplemental Figure 2B-C). However, little or no staining was observed in the extra-embryonic yolk sac area (Figure 1Bbi-iii and supplemental Figure 2B). At E9.5, expression was seen in developing vasculature mainly in the cardinal vein and scattered cells in cranial mesenchyme, but was undetectable in the yolk sac (Figure 1Bc). At E9.5, *Etv2* expression was barely seen in the EC layer of mature vasculature except in the endocardium, indicating its rapid down-regulation in fully differentiated ECs (Figure 1Bd-e).

To understand the relationship between the expressions of Etv2 and other HPC/EC markers, Etv2-Venus^{+/-} embryos were analyzed by flow cytometry using several Abs (Figure 1C). Both at E7.5 and E8.5, the majority of Etv2⁺ (Venus⁺) cells were present as Flk-1⁺ cells. At E7.5, Etv2 and Flk-1 were coexpressed in the majority of Flk-1⁺ cells (76%). At E8.5, Etv2 expression was still seen in > 70% of Flk-1⁺ cells. Whereas approximately 80% of VE-cadherin⁺ cells expressed Etv2⁺, < 10% of CD41⁺ cells expressed Etv2, indicating that Etv2 is down-regulated in cells fated to a primitive HPC lineage. These findings suggest that *Etv2* expression is transient in endothelial and hematopoietic precursors and that it rapidly declines once cells are fully differentiated.

We also analyzed the expression of Etv2 in differentiating Etv2-Venus ESCs on OP9 (Figure 1D). At day 2.5-3 of differentiation we started to see Flk-1⁺ cells before the appearance of significant numbers of Venus⁺ cells. The proportion of Etv2⁺ (Venus⁺) cells leveled off at day 4 or 5 of differentiation, followed by significant reduction after day 6 of differentiation, reflecting the similar pattern of transient Etv2 expression in embryonic development. Etv2 expression in ESC differentiation was also evident only

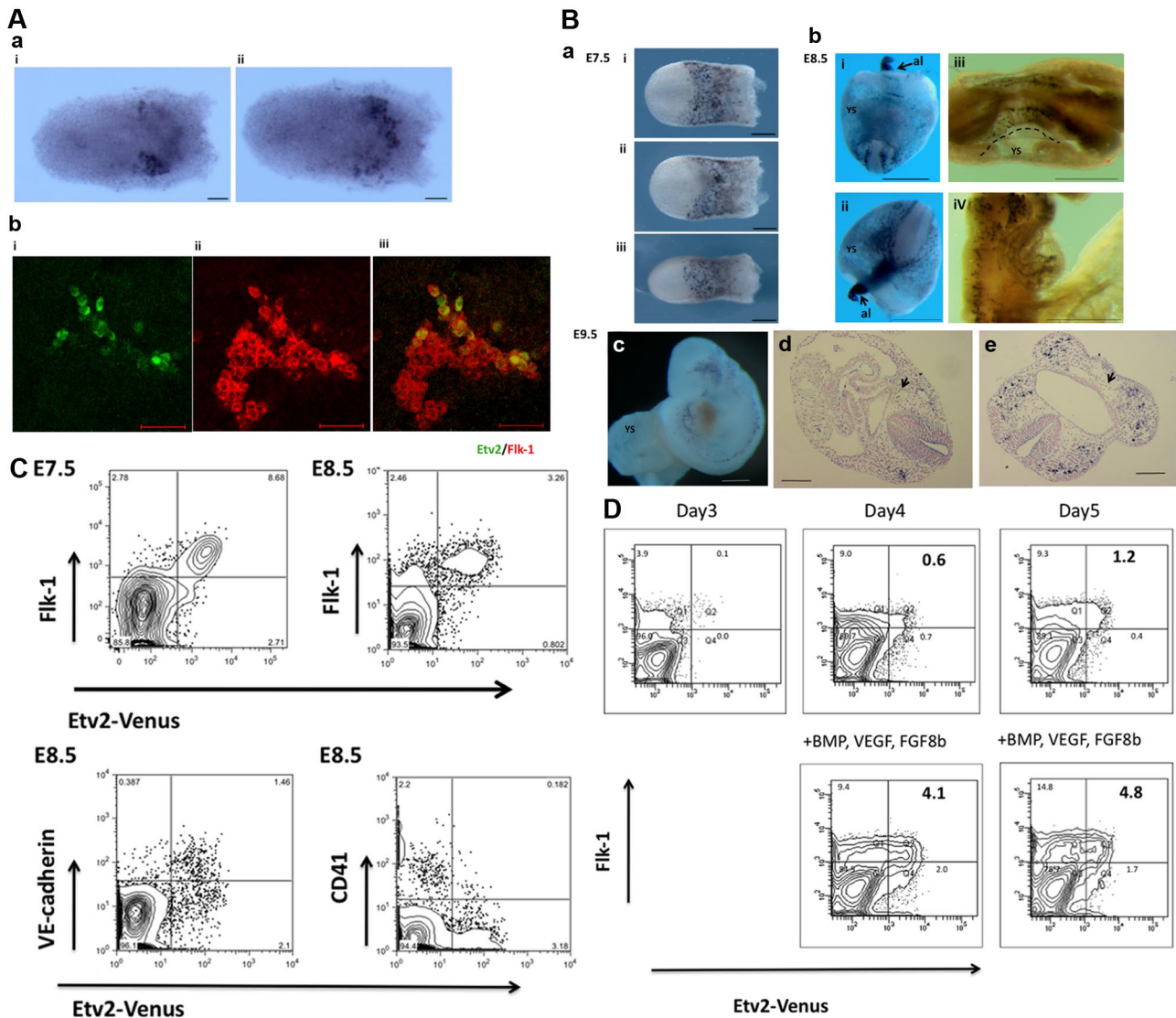


Figure 1. Etv2 expression in developing mouse embryos. (Aa) Etv2 is first detected in the posterolateral mesoderm around E7.0; panel i is the anterior and panel ii is the posterior view. Scale bar indicates 50 μm . (b) Double immunostaining with anti-Flk-1 antibody revealed that Etv2 is expressed in a subset of Flk-1⁺ cells at this stage. Magnified view of the posterolateral mesoderm where Flk-1⁺ cells exists: Etv2-Green (i), Flk-1-Red (ii), and merged (iii). Scale bar indicates 50 μm . (Ba) Etv2 is expressed at E7.5 in the extra-embryonic mesoderm. Etv2 is almost colocalized with Flk-1 at this stage: (i) anterior view, (ii) posterior view, and (iii) lateral view. Scale bar indicates 200 μm . (b) At E8.5 expression was detected along the dorsal aorta, endocardium and allantois (al). Expression in the extra-embryonic yolk sac (YS) region was rapidly down-regulated. Scale bar indicates 500 μm . (c) Expression of Etv2 at E9.5. (d) Expression was detected along the vasculature (arrows). Scale bars in panels b and c indicate 500 μm ; scale bars in panels d and e indicate 200 μm . (C) Flow cytometric analysis of Etv2 expression in developing embryos. (a) Flow cytometry of E7.5 and 8.5 Etv2-Venus^{+/+} embryos. At E7.5, 76% of Flk-1⁺ cells were Etv2⁺. (b) At E8.5, Etv-2 expression was still restricted in the Flk-1⁺ population, with 56% of the Flk-1⁺ cells being Etv2-Venus⁺. (c) At E8.5, 79% of the VE-cadherin⁺ cells retained the Etv2 expression. Approximately 92% of the CD41⁺ HPCs were Etv2-Venus⁻, indicating the rapid down-regulation of Etv2 on HPC differentiation. (D) Etv2 expression in differentiating ESCs. Etv2-Venus ESCs were differentiated on OP9 cells. Mesoderm-inducing factors BMP4, FGF8b, and VEGF were added on day 3 of differentiation. On day 3, when Flk-1⁺ cells started to be seen, only 0.1% were Flk-1⁺/Etv2-Venus⁺ whereas 3.9% were Flk-1⁺/Etv2-Venus⁻. The addition of mesoderm-inducing factors promoted the generation of Etv2⁺ cells in 24 hours on day 4 (Flk-1⁺/Etv2-Venus⁺ cells from 0.6%-4.1%) and day 5 (Flk-1⁺/Etv2-Venus⁺ cells from 1.2%-4.8%). The majority of Etv2-Venus⁺ cells are present as Flk-1⁺.

within the Flk-1⁺ cell population. As seen in the earliest stage of Etv2 expression in embryos, only a fraction of Flk-1⁺ cells were Etv2⁺ (Venus⁺) when Etv2 expression began at day 2.5-3. These observations suggest that Etv2 expression represents a subset of Flk-1⁺ cells being committed to ECs or HPCs.

HPC/EC precursors are preferentially localized in the Etv2⁺ population in early embryos

To investigate whether Etv2⁺ cells are HPC/EC precursors within Flk-1⁺ cells, the Flk-1⁺Etv2⁺ or Flk-1⁺ Etv2⁻ population was sorted from E7.5 or E8.5 embryos and cultured on OP9.

E7.5 and embryo proper parts of E8.5 embryos were analyzed because Etv2⁺ populations in these parts are the cells that start to express Etv2. At later stages, some Etv2⁻ cells might have been committed to HPCs/ECs by prior transient expression of Etv2. After culturing for 10 days, HPCs/ECs were observed only from Flk-1⁺Etv2⁺ fractions in E7.5 embryos, whereas few HPCs/ECs developed from Flk-1⁺Etv2⁻, indicating that HPC/EC precursors are preferentially localized in Flk-1⁺Etv2⁺ populations (Figure 2Aa-b). From the embryo proper parts of E8.5 embryos, ECs were generated from both the Flk-1⁺Etv2⁺ and the Flk-1⁺Etv2⁻ population, however, with much less efficiency

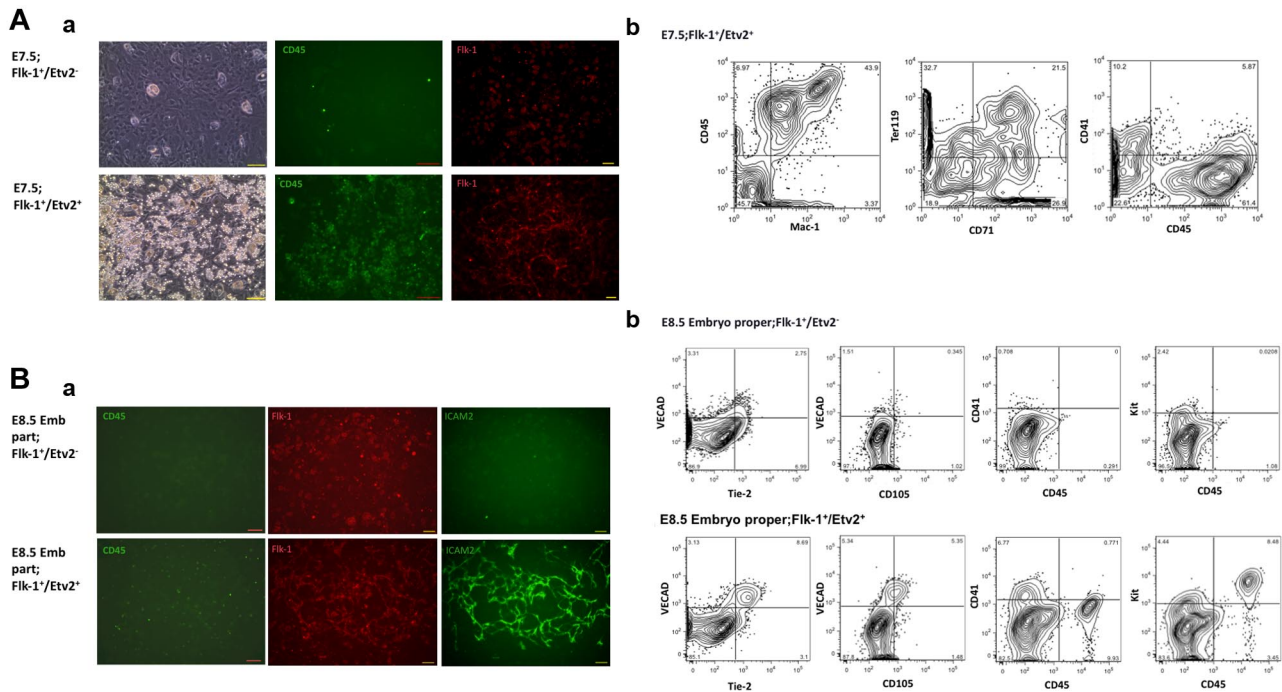


Figure 2. Hemato-endothelial potential in *Etv2*⁺/*Flk-1*⁺. *Flk-1*⁺/*Etv2*⁻ or *Flk-1*⁺/*Etv2*⁺ cells from *Etv2*-Venus^{+/+} embryos (E7.5 embryo; 500 cells/35-mm well, E8.5 embryo proper; 8000 cells/35-mm well) were cultured on OP9 for 10 days with growth factors (erythropoietin, IL-3, G-CSF, SCF, and VEGF). (Aa) HPCs were preferentially observed in E7.5 *Flk-1*⁺/*Etv2*⁺ population (bottom). Similar cells did not develop from *Flk-1*⁺/*Etv2*⁻ cells (top). CD45⁺ cells or *Flk-1*⁺ plexus-like structures were only observed from *Flk-1*⁺/*Etv2*⁺ culture. Scale bar indicates 100 μ m. (b) Flow cytometric analysis of cells generated from *Flk-1*⁺/*Etv2*⁻ or *Flk-1*⁺/*Etv2*⁺ embryonic cells. Mac-1⁺CD45⁺ granulocytes or Ter119⁺ erythroid cells developed from E7.5 *Flk-1*⁺/*Etv2*⁺ fraction. Almost no cells were seen in the same FSC/SSC area containing HPCs from *Flk-1*⁺/*Etv2*⁻ cells cultured in the same condition. (Ba) *Flk-1*⁺/*Etv2*⁻ or *Flk-1*⁺/*Etv2*⁺ cells from E8.5 intra-embryo parts also showed similar results. After 10 days of culture, CD45⁺HPCs or *Flk-1*⁺/*ICAM2*⁺ ECs were seen from *Flk-1*⁺/*Etv2*⁺ cells (bottom), whereas few or no similar cells developed from *Flk-1*⁺/*Etv2*⁻ (top). Scale bar indicates 100 μ m. (b) ECs developed more efficiently from E8.5 embryo proper *Flk-1*⁺/*Etv2*⁺ cells than from *Flk-1*⁺/*Etv2*⁻ cells. CD45⁺c-Kit⁺ cells were seen only from E8.5 embryo proper *Flk-1*⁺/*Etv2*⁺ population.

from *Flk-1*⁺/*Etv2*⁻. CD45⁺ cells were preferentially observed from the *Flk-1*⁺/*Etv2*⁺ population (Figure 2Ba-b). The finding that *Etv2*⁺/*Flk-1*⁺ cells could generate CD45⁺ HPCs or ECs more efficiently than *Etv2*⁺/*Flk-1*⁻ cells suggests that *Etv2* can promote hematopoiesis and vasculogenesis cell autonomously, at least in these early stages.

***Etv2* is dispensable for the differentiation of *Flk1*⁺ cells but indispensable for EC and HPC differentiation**

The preceding results suggest that *Etv2* marks a subset of *Flk1*⁺ cells with hemato-endothelial capacity and that *Flk-1* expression can be partly independent of *Etv2*. To investigate the requirement of *Etv2* for *Flk-1* induction, *Etv2*-null ESCs were differentiated on OP9 cells. Interestingly, from day 4 or 5 of differentiation, *Flk-1*⁺ cells were indistinguishable from *Etv2*^{+/+} and *-/-* ESCs (Figure 3A). *Flk-1*⁺ cells from *Etv2*^{+/+} and *Etv2*^{-/-} ESCs were sorted to confirm that *Etv2* RNA was lost to the background level in *Etv2*^{-/-} cells by Q-PCR (Figure 4Bb). Although initial induction of *Flk-1* was observed in differentiated *Etv2*^{-/-} ESCs, further differentiation into HPCs and ECs was blocked. Flow cytometry revealed that *Etv2*^{-/-} cells were unable to generate VE-cadherin⁺ or CD41⁺ cells (Figure 3A) and failed to down-regulate PDGFR α (Figure 3A). These results demonstrate that *Etv2* is indispensable for the differentiation of VE-cadherin⁺ ECs or CD41⁺ HPCs, but is dispensable for the initial induction of *Flk-1*⁺ cells.

***Etv2* is indispensable for differentiation of *Flk-1*⁺PDGFR α ⁻ vascular mesoderm from the *Flk-1*⁺PDGFR α ⁺ primitive mesoderm**

We showed previously that *Flk1*⁺PDGFR α ⁺ double-positive primitive mesoderm (DP-PM) diverges into *Flk1*⁺PDGFR α ⁻ vascular mesoderm (VSP), which gives rise to ECs and HPCs and *Flk1*⁻PDGFR α ⁺ paraxial mesoderm (PSP).^{17,21} This ESC differentiation model can mimic the initial mesoderm differentiation and part of the primitive hematopoiesis. In this differentiation system, *Etv2*^{-/-} ESCs generated *Flk1*⁺PDGFR α ⁺ primitive mesoderm, however, they failed to further differentiate into *Flk1*⁺PDGFR α ⁻ VSP (Figure 3A). Differentiation into *Flk1*⁻PDGFR α ⁺ PSP was unaffected. These results showed the critical role of *Etv2* in the commitment of the VSP from the *Flk1*⁺PDGFR α ⁺ primitive mesoderm.

To determine whether this observation could be recapitulated in embryos, we analyzed the expressions of PDGFR α and *Flk-1* by flow cytometry (Figure 3B) and immunostaining (Figure 3C) in *Etv2*-null embryos. Whereas previous studies reported almost no *Flk1* expression in *Etv2*-null embryos from the primitive streak stage to E9.5, we detected *Flk-1*⁺ cells in E8.5 embryos by flow cytometry. At E8.5, PSP and VSP commitment was nearly completed in normal embryos. However, the flow cytometric analysis demonstrated that the majority of *Flk-1*⁺ cells in *Etv2*-null embryos coexpressed PDGFR α and failed to generate VE-cadherin⁺ cells, whereas differentiation of *Flk1*⁻PDGFR α ⁺ cells was unaffected (Figure 3B). In control embryos, the DP-PM cells

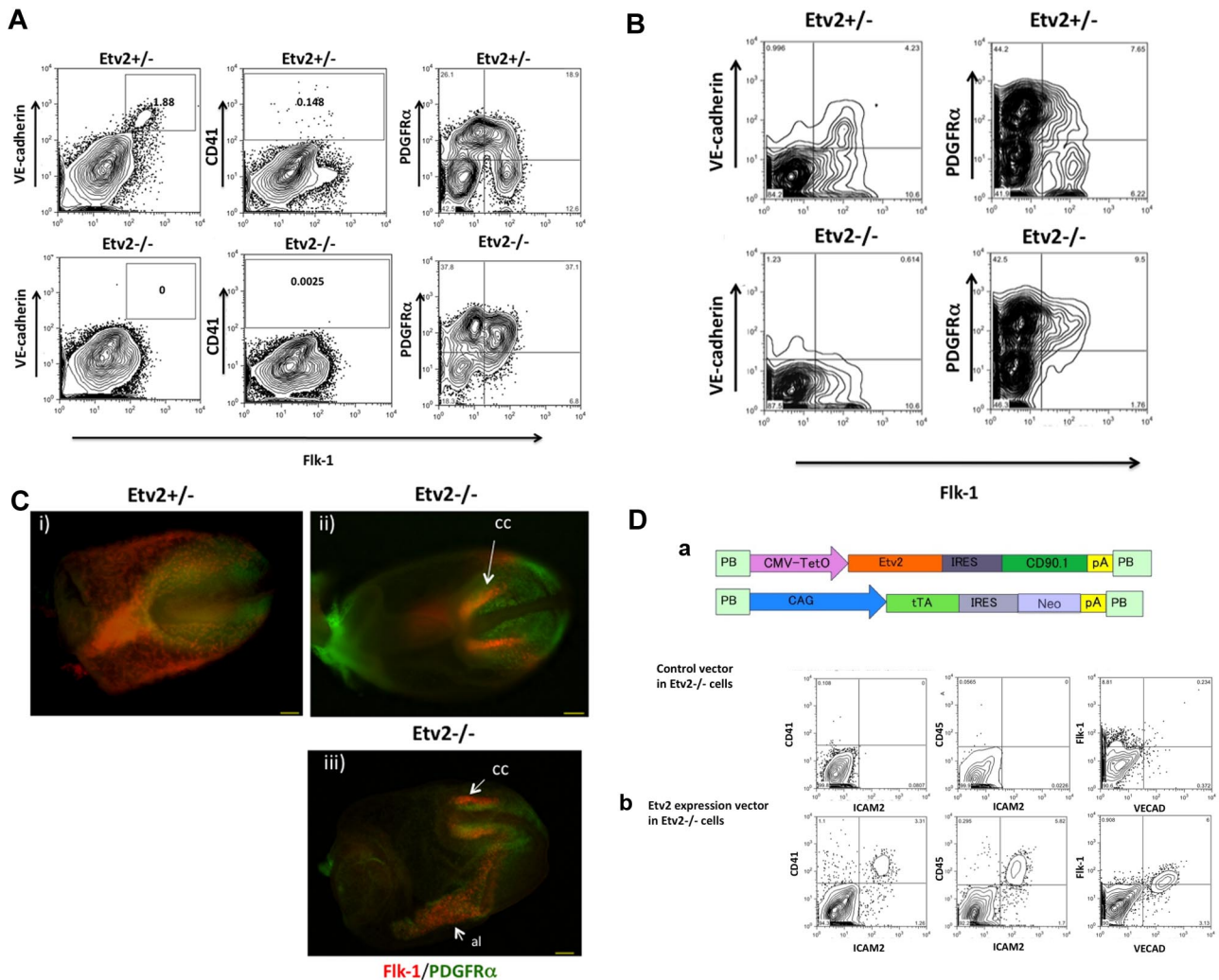


Figure 3. ETV2 is dispensable for the generation of Flk-1⁺ mesoderm but is required for the generation of HPCs/ECs. (A) ETV2^{+/-} cells differentiated on OP9 could generate 1.88% VE-cadherin⁺ or 0.148% CD41⁺ cells (top). ETV2^{-/-} ESCs generated Flk-1⁺ cells but failed to differentiate into VE-cadherin⁺ (0%) nor CD41⁺ cells (0.0025%) (bottom). Flk-1⁺ cells from ETV2^{-/-} ESCs were arrested as PDGFR α ⁺ cells. ETV2^{+/-} Flk-1⁺ cells could down-regulate PDGFR α . (B) In contrast to ETV2^{+/-} (top), E8.5 ETV2^{-/-} cells (bottom) failed to generate Flk-1⁺VE-cadherin⁺ cells (leftpanels). Most of the Flk-1⁺ cells remained as PDGFR α ⁺ in ETV2^{-/-} embryos, failing to become Flk-1 single-positive cells (right). (C) Double immunostaining of ETV2^{+/-} (i) and ETV2^{-/-} (ii) E7.75 mouse embryos by anti-Flk-1 (red) and anti-PDGFR α (green) Abs (iii). In the ETV2^{-/-} embryos, Flk-1 staining in extra-embryonic mesoderm was missing, whereas Flk-1⁺ cells in the cardiac crescent (cc) and allantois (al) were still present. Scale bar indicates 100 μ m. (D) Rescue of ETV2^{-/-} cells by reexpression of ETV2 transgene. (a) Diagrams of ETV2 transgene constructs. PB-based tTA and Tet-inducible promoter-driven transgene vectors were transfected into ETV2-null ESCs with transgene expression monitored by CD90.1 staining. (b) HPCs (CD41⁺ and CD45⁺) and ECs (VECAD⁺ and Flk-1⁺) were generated in ETV2-rescued ETV2^{-/-} cells (bottom), whereas none were generated in cells transfected with empty vector (top).

were already diverged into Flk-1⁻PDGFR α ⁺ or Flk-1⁺PDGFR α ⁻ mesoderm (Figure 3B).

Double immunostaining for Flk-1 and PDGFR α revealed that Flk-1⁺ cells missing in ETV2-null embryos were Flk-1⁺PDGFR α ⁻ cells in the extra-embryonic region where blood islands exist in normal embryos (Figure 3C). Flk-1⁺ cells in ETV2-null embryos were Flk-1⁺PDGFR α ⁺ double positive and localized in allantois, cardiac crescent,²² and a narrow overlapping lateral area between the Flk-1⁺ and PDGFR α ⁺ domains (Figure 3C and supplemental Figure 3). We conclude that ETV2 is the key regulator of the differentiation from primitive to VSP mesoderm.

ETV2 reexpression in ETV2^{-/-} ESCs restores hemato-endothelial differentiation

Results from ETV2^{-/-} ESCs and embryos point to ETV2 as a master regulator of hemato-endothelial fate from the primitive mesoderm. However, it is not clear whether complete loss of HPCs and ECs is

only because of ETV2 deficiency. Although zebrafish *etsrp* morphants were reported to be rescued by injecting *etsrp* mRNA, the effects of ETV2 homolog knock-down on hematopoiesis in zebrafish or *Xenopus* are smaller than in mice.^{23,24} To determine whether ETV2 can rescue the loss of HPCs and ECs in mice, we introduced an ETV2 transgene into ETV2-null ESCs (Figure 3Da). It is assumed that ESC differentiation cannot fully recapitulate the embryonic hematopoiesis, however, it has been used at least to mimic the primitive part of hematopoiesis. ESCs were differentiated in the absence of Tet to induce a transgene for which expression was monitored by CD90.1 staining. Transgene expressers were sorted by CD90.1 after 3 days of differentiation, when key hemato-endothelial factors are assumed to be required for rescue,²⁵ and reseeded on OP9. After a total of 10 days of differentiation, cells transfected with the empty vector failed to generate VECAD⁺ or CD41⁺ cells. In contrast, the ETV2-transfected cells could generate VECAD⁺, CD41⁺, or CD45⁺ cells (Figure 3Db). These findings

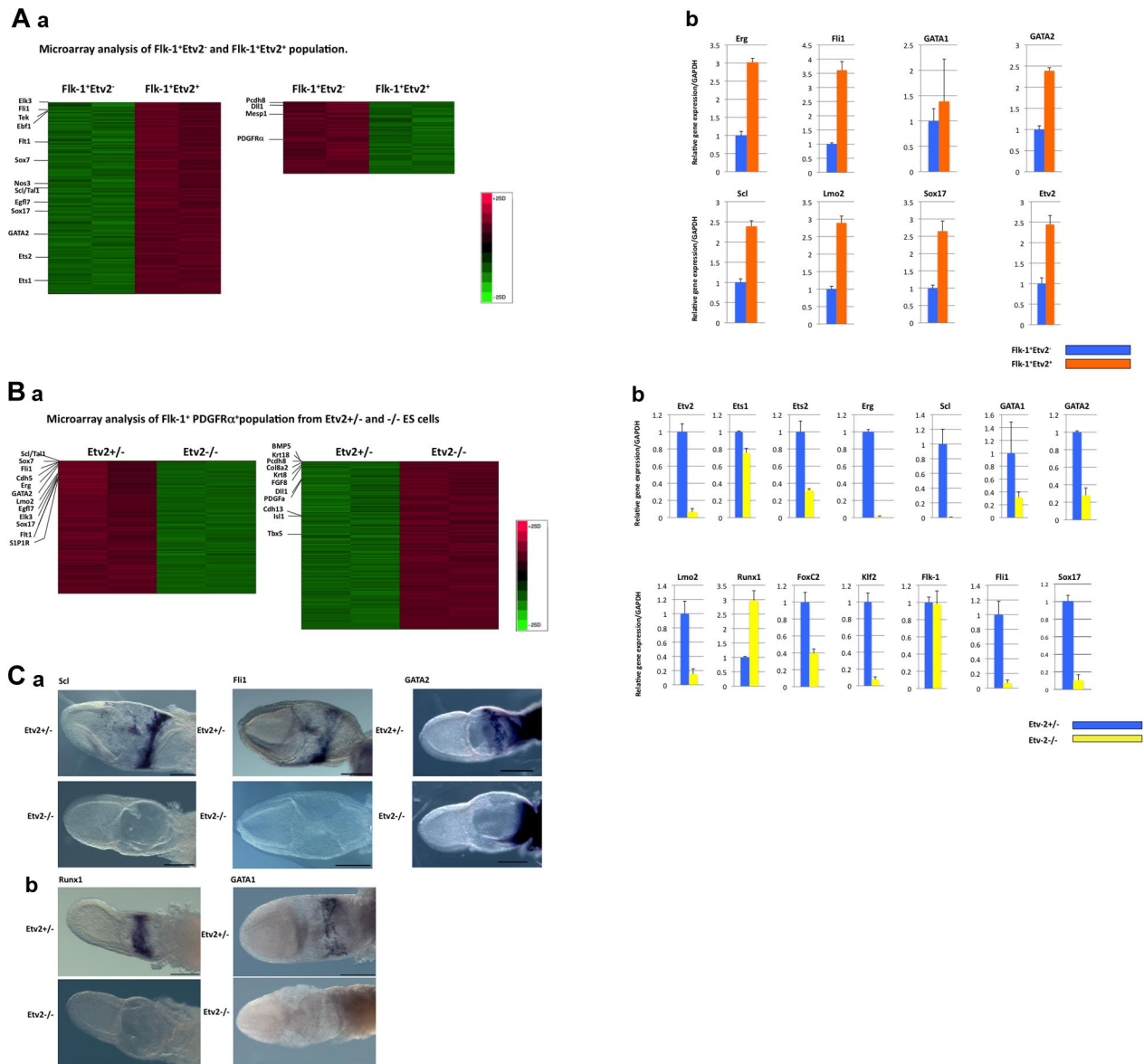


Figure 4. Genes regulated by Etv2. (A) Gene-expression profiling of Flk-1⁺Etv2⁺ and Flk-1⁺Etv2⁻ populations generated from differentiated of Etv2-Venus^{+/-} ESCs for 5 days on OP9. Flk-1⁺Etv2⁺ or Flk-1⁺Etv2⁻ cells were analyzed by (a) microarray and (b) Q-PCR. Genes important for EC and HPC differentiation were preferentially expressed in Etv2⁺ Flk-1⁺ cells. Genes that showed significant changes between Flk-1⁺Etv2⁺ and Flk-1⁺Etv2⁻ populations are listed in supplemental Table 1. (B) (a) Gene-expression analysis of Flk-1⁺PDGFR α ⁺ cells derived from Etv2^{+/-} and ^{-/-} ESCs. After 5 days of differentiation, Flk-1⁺PDGFR α ⁺ cells were subjected to microarray analysis. Genes that showed significant changes between Etv2^{+/-} and ^{-/-} populations are listed in supplemental Table 2. (b) Expression levels of key EC and HPC genes were analyzed by Q-PCR in Etv2^{+/-} and Etv2^{-/-} populations. Genes important for EC and HPC development were down-regulated in Etv2^{-/-} cells. In Q-PCR experiments (Ab and Bb) PCR reactions were performed in triplicate. Data for each gene was normalized by GAPDH and are shown as the -fold change compared with Flk-1⁺Etv2⁺ (Ab) or Etv2^{+/-} (Bb) populations. (C) Expressions of *Scl*, *Fli1*, *GATA2* (a), *Runx1*, and *GATA1* (b) in E7.5 Etv2^{+/-} or Etv2^{-/-} embryos. *Scl*, *Fli1*, and *Runx1*: in situ hybridization; *GATA1*: immunostaining. Unlike expression analysis in ESC-derived Flk-1⁺ cells, *Runx1* was undetectable in Etv2^{-/-} embryos. *GATA1* and *GATA2* were down-regulated but detectable in differentiated Etv2^{-/-} cells but totally undetectable in Etv2^{-/-} embryos. Scale bar indicates 300 μ m.

confirmed that mouse Etv2 by itself reconstitutes initial steps of vasculogenesis and hematopoiesis in the Etv2-null background.

Investigation for genes downstream of Etv2

To identify genes regulated by Etv2, we performed gene-expression profiling using Q-PCR and microarray. In the first experiment, we compared Flk-1⁺Etv2⁺ and Flk-1⁺Etv2⁻ populations in differentiated Etv2-Venus^{+/-} ESCs (Figure 4A). In the second experiment, we compared Flk-1⁺PDGFR α ⁺ populations differentiated from Etv2^{+/-} and Etv2^{-/-} ESCs. Because most of the Flk-1⁺ cells from Etv2^{-/-} cells were PDGFR α ⁺, Flk-1⁺PDGFR α ⁺ cells were sorted for comparison (Figure 4B). Genes up-regulated

in Etv2-Venus⁺ populations but not in Etv2-Venus⁻ include those that are important in vasculogenesis and hematopoiesis (supplemental Table 1). Many key hemato-endothelial genes that were up-regulated in Etv2-Venus⁺ cells in the first analysis showed profoundly reduced expression in the Etv2-null Flk-1⁺ cells in the second analysis (supplemental Table 2). Among the top 192 genes listed in supplemental Table 2, ~ 50 are known to be expressed specifically in HPCs or ECs. The different expression levels of important genes were also confirmed by Q-PCR analyses (Figure 4Ab and 4Bb). Gene lists from both experiments contain an array of genes that are expressed in early HPCs, ECs, or both lineages. Interestingly, *Scl*¹ and *Fli1*,² for which null mice have profound

defects in both EC and HPC differentiation, were completely deficient in *Etv2*-null-derived Flk-1⁺ cells. These 2 genes were also undetectable in *Etv2*-null embryos (Figure 4Ca). *GATA1*²⁶ and *GATA2* expression levels decreased remarkably, but residual expression was detected in *Etv2*-null Flk-1⁺ cells in *in vitro* differentiation. *Runx1*²⁷ expression was almost unaffected in *Etv2*-null ESC-derived Flk-1⁺ cells. However, expressions of these genes were lost in *Etv2*-null E7.5 extra-embryonic mesoderm (Figure 4Cb), presumably because of the loss of the Flk-1⁺ cell population committed to HPCs.

Etv2 target genes are critical in vasculogenesis and hematopoiesis

Among several genes potentially regulated by *Etv2*, we tested *Scl*, *Fli1*, and *Gata2* as critical candidates because of their expressions at sites of early embryonic hematopoiesis and functional importance in EC and HPC differentiation.^{1,2,28,29} We found that these 3 genes were up-regulated in *Etv2*-rescued *Etv2*^{-/-} cells (supplemental Figure 4). Sequences matching reported *Etv2*-binding sites were found within ± 5 kb of the transcription start sites of *Scl*, *Gata2*, and *Fli1* in both humans and mice (Figure 5A).^{11,30,31} Indeed, the *Scl* core enhancer³² has been reported to contain the binding site for *Etv2*.¹¹ EMSA demonstrated the binding of *Etv2* on those conserved sequences of *Gata2* and *Fli1*, as well as *Scl* (Figure 5B). ChIP analysis using ECs (Figure 5C) or ESC-derived (supplemental Figure 5A) Flk-1⁺ cells indicated that *Etv2* can bind to these sequences. Activation of each luciferase reporter for *Scl*, *Fli1*, or *GATA2* was seen by *Etv2* and by *Ets1* and *Ets2* (supplemental Figure 5B). These activations were reduced by mutated *Etv2*-binding sequences in each reporter except *Scl*, presumably because of the existence of other *Ets* motifs. In early embryos, *Etv2* was expressed in E7.5 extra-embryonic mesoderm and in E8.5 dorsal aorta, where *Scl*, *Fli1*, and *Gata2* were expressed (Figure 5D). Expressions of those genes were absent from E7.5 *Etv2*-null embryos, supporting the notion that *Etv2* induces those genes (Figure 4Ca). *Scl*, *Gata2*, and *Fli1* were reported to form a transcription factor (TF) network for HSCs/HPCs.^{33,34} Therefore, it is possible that *Etv2* can induce these 3 genes, resulting in the induction of this TF network. To functionally test whether these genes can rescue at least the primitive part of hematopoiesis downstream of *Etv2*, we introduced *Scl* or *Fli1* into *Etv2*-null cells because these 2 were most severely down-regulated in *Etv2*-null Flk-1⁺ cells (Figure 4Bb). Reexpression of *Scl* or *Fli1* alone in *Etv2*-null cells restored CD41⁺CD45⁺ or VECAD⁺ cells. Transfection of empty control vector did not restore any of these cells (Figure 6A-B). Rescue experiments with *GATA2* so far failed to generate CD41⁺CD45⁺ or VECAD⁺ cells under the same conditions (data not shown). These findings suggest that *Scl* and *Fli1* are critical targets of *Etv2* in the TF network triggered by *Etv2* to generate HPCs and ECs and *GATA2* is less critical.

Although *Scl*, *Gata2*, and *Fli1* induced by *Etv2* were reported to create a core network for HSCs at E10.5-11.5 aorta-gonad-mesonephron (AGM) and E12.5 fetal liver, *Etv2* could not be detected in the vessel wall in AGM and fetal liver by immunostaining or FACS (supplemental Figure 7Aa and supplemental Figure 6). A few Venus⁺ cells in E10.5 embryos were negative for Sca-1, a marker for HSCs (supplemental Figure 7Ab), indicating that *Etv2* was not expressed in AGM HSCs.³⁵ Despite *Etv2* being undetected, *Scl*, *Gata2*, and *Fli1* expressions still persisted in AGM and fetal liver cells.³³ Therefore, it is possible that *Etv2* is required only in the earlier stages to induce the TF network that can be maintained without *Etv2* expression. This idea was also tested

by analyzing expressions of these 3 genes in *Etv2*⁺VE-cadherin⁻, *Etv2*⁺VE-cadherin⁺, *Etv2*⁻VE-cadherin⁺, and VE-cadherin⁻CD41⁺ populations generated in ESC differentiation culture toward hematopoietic and ECs. It is assumed that *Etv2*⁺VE-cadherin⁻P1, *Etv2*⁺VE-cadherin⁺cells-P2, *Etv2*⁻VE-cadherin⁻P3, and VE-cadherin⁻CD41⁺-P4 populations represent immature mesoderm being committed to ECs, EC precursors, differentiated ECs, and differentiated HPCs, respectively (supplemental Figure 7Ba). On differentiation day 5, higher levels of *Scl*, *Fli1*, and *GATA2* were seen in *Etv2*-Venus⁺ than in *Etv2*-Venus⁻ cells, indicating that *Etv2* can trigger the network of these 3 genes. At day 6, as cells differentiated into mature ECs or HPCs, a significant number of VE-cadherin⁺ and CD41⁺ cells appeared in culture (supplemental Figure 7Ba). Q-PCR revealed that *Scl*, *Fli1*, and *GATA2* were expressed at similar levels in *Etv2*⁺VE-cadherin⁺ and *Etv2*⁻VE-cadherin⁺ cells and that *Fli1* was down-regulated in differentiated HPCs represented by VE-cadherin⁻CD41⁺ cells. *Erg*, reported to be important in hemangioblast and HSC development,³⁶ was regulated in a similar manner to *Fli1* (supplemental Figure 7Bb). This expression profile indicates that the *Scl*-*GATA2*-*Fli1* network is induced by *Etv2*, that the established network can be maintained with lower expression of *Etv2*, and that *Fli1* is down-regulated during HSC/HPC generation as cells begin to lose their EC phenotype. To further support the transient requirement of *Etv2*, part of the VE-cadherin⁺ or CD41⁺ cells from rescued *Etv2*-null cells could exist in CD90.1^{low} or CD90.1⁻ fraction after transgene suppression, indicating that cells do not require continuous higher expression of *Etv2* to generate HPCs or ECs (supplemental Figure 8).

Etv2 is an immediate responding gene in VEGF-treated Flk-1⁺ primitive mesoderm

Based on the finding that *Etv2* was dispensable for initial Flk-1⁺ induction but was required for the further differentiation of Flk-1⁺ cells into HPCs/ECs, we reconsidered the way that *Etv2* is induced. Wnt, BMP, or Notch signaling pathways were shown to up-regulate *Etv2*. Although these pathways also contribute to HSC development, we reasoned that *Etv2* induction is correlated with Flk-1 expression. It was reported that VEGF is expressed in the E7.0-E8.5 extra-embryonic endoderm close to the Flk-1⁺ extra-embryonic mesoderm, where *Etv2*⁺ cells were first detected.³⁷ By stimulating ESC-derived Flk-1⁺ mesoderm with VEGF, we found that VEGF potently (~ 5-fold by Q-PCR) induced *Etv2* in these cells (Figure 7A). Because this increase occurred within 1 hour, we considered this to be the direct effect over *Etv2* transcription rather than selective proliferation of *Etv2*⁺ cells. *Fli1* and *Lmo2*, a cofactor of *Scl*, were up-regulated within 2 hours (Figure 7A). VEGF treatment for 24 hours also increased the number of *Etv2*⁺ cells among the Flk-1⁺ population (Figure 7B). These effects of VEGF will generate a positive feedback of VSP specification because *Etv2* can in turn up-regulate Flk-1 expression.

Discussion

Flk-1⁺ cells from ESCs or early embryos represent a broader spectrum of mesoderm precursors than those giving rise only to ECs or to HPCs.³⁸⁻⁴¹ These findings suggest that Flk-1 is not a specific marker for EC/HPC precursors, nor is it absolutely required for their development. *Etv2/ER71* completely blocks the differentiation of HPCs/ECs in mice, which was attributed to the

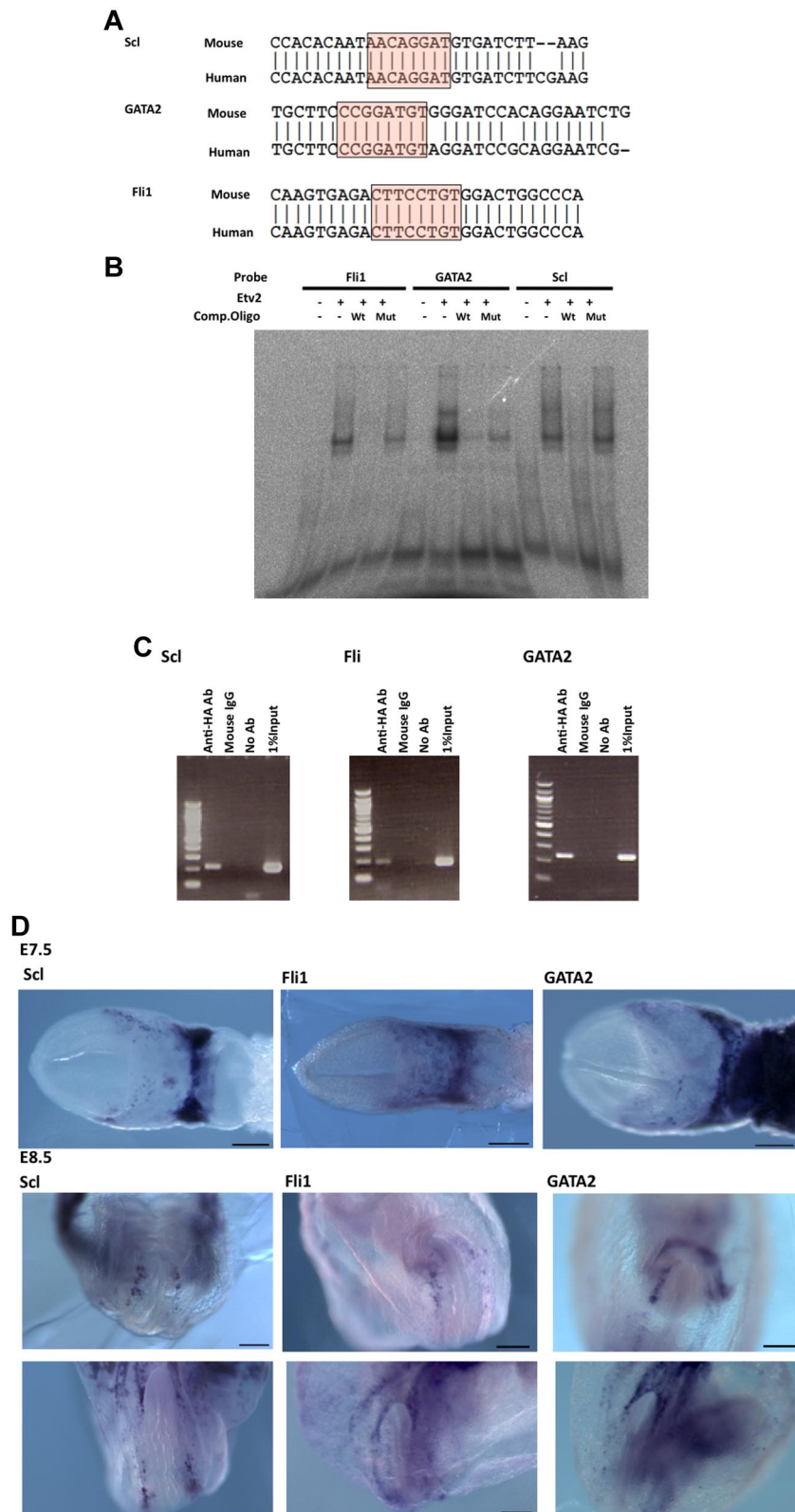
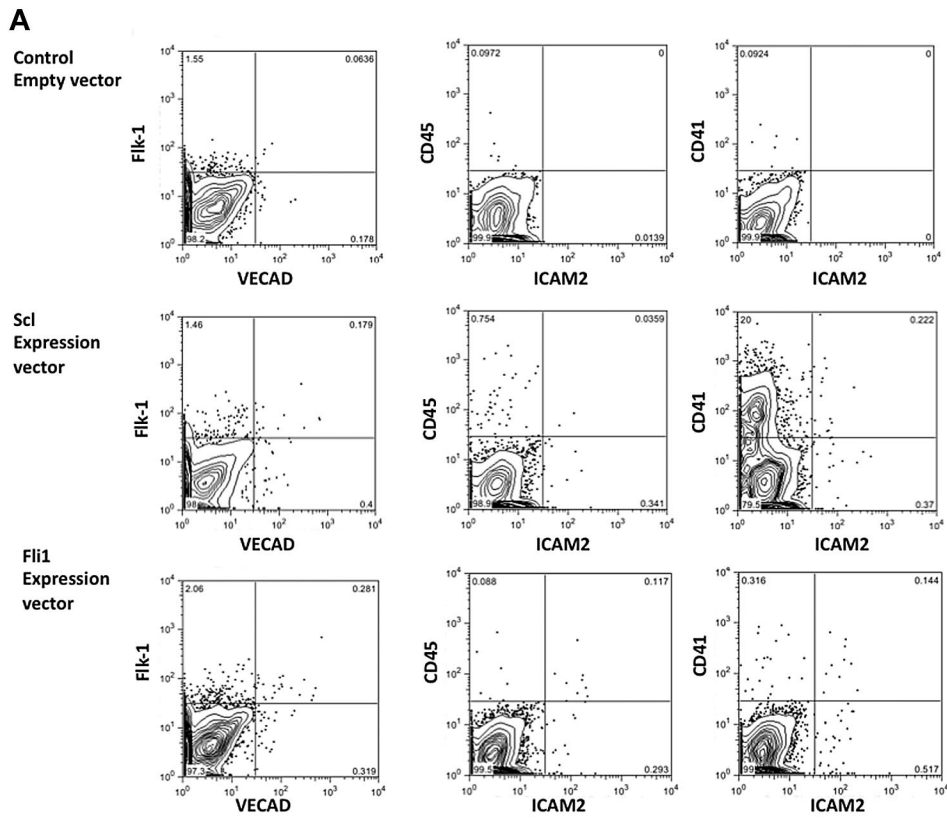


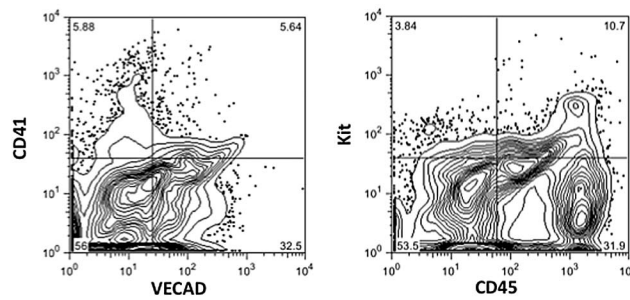
Figure 5. *Scl*, *Fli1*, and *GATA2* are downstream targets of *Etv2*. (A) Alignment of candidate binding sequences containing the Ets binding motif. Putative Ets- or Fox;Ets-binding sequences from human and mouse genomes of *Scl*, *Fli1*, and *GATA2* were aligned. (B) EMSA showing binding of in vitro-translated *Etv2* to oligonucleotide probes containing the Ets-binding sites shown in Figure 6A for *Scl*, *Fli1*, and *GATA2*. Binding was competed by excess wild-type but not by Ets motif-mutated probes. (C) ChIP analysis demonstrating the binding of *Etv2* to target sequences. F2 ECs were transfected with HA-tagged *Etv2* expression vector and subjected to ChIP analysis. Primer set for each analysis was for *Scl*; *Scl*-1_F2/R2, *Fli1*; *Fli1*F11/R11, and *GATA2*; *GATA2*-F3/R3. (D) Expressions of *Scl*, *GATA2*, and *Fli1* at E7.5 and E8.5 in mouse embryos. Expressions of *Scl*, *GATA2*, and *Fli1* were examined by in situ hybridization. These 3 genes are expressed in the extra-embryonic mesoderm of E7.5 (top) and along the dorsal aorta of E8.5 embryos (middle and bottom). Scale bar indicates 200 μ m.

dependence of *Flk-1* transcription on *Etv2*.¹⁰ However, this interpretation does not explain the complete loss of ECs and HPCs in *Etv2*-null mice, which does not happen in *Flk-1*-null cells. We showed that *Etv2* is dispensable for the initial *Flk-1* induction in *Flk-1*⁺*PDGFR* α ⁺ (DP-PM), but critical for the generation of

Flk-1⁺*PDGFR* α ⁻ (VSP) mesoderm. VSP failed to differentiate from DP-PM in *Etv2*-null ESCs and embryos. In contrast, differentiation of *Flk-1*⁻*PDGFR* α ⁺ (PSP) mesoderm seemed normal in the absence of *Etv2*. Although some mesenchymal or muscle-specific genes were up-regulated in *Etv2*^{-/-} cells, no obvious expansion of



B a Scl rescue ;Flk-1+VE-cad+ reculture



b Fli1 rescue ;Flk-1+VE-cad+ reculture

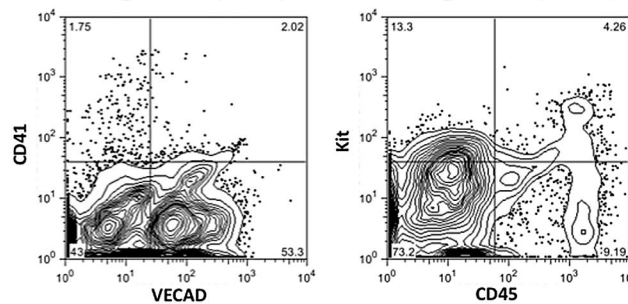


Figure 6. Scl or Fli1 rescues EC and HPC development in *Etv2*-null cells. (A) Expression of Scl or Fli1 alone could restore EC and HPC development in *Etv2*-null cells. Scl or Fli1 was introduced into *Etv2*-null ESCs using a Tet-inducible system. Cells differentiated on OP9 were analyzed by flow cytometry after 10 days. $CD41^+CD45^+$ or $VECAD^+$ cells developed but with lower efficiency compared with *Etv2*-rescued cells (except $CD41^+$ cells in Scl rescue). (B) $VECAD^+$ cells were sorted from Scl- or Fli1-rescued *Etv2*^{-/-} differentiated cells and recultured on OP9 for an additional 8 days in the presence of HPC growth factors. This reculture generated a significant number of $VECAD^+$ or $CD45^+$ cells from both Scl- (a) and Fli1 (b)-rescued *Etv2*-null cells.

the PDGFR α^+ domain was seen in *Etv2*^{-/-} embryos. Therefore, it is possible that up-regulation of mesenchymal or muscle genes in *Etv2*^{-/-} cells may be partly due to the loss of VSP population.

Nonetheless, it is also possible that mesenchymal or muscle genes are derepressed in the absence of *Etv2*, because *Etv2* can work as a transcriptional repressor by interacting with TGA.⁴²

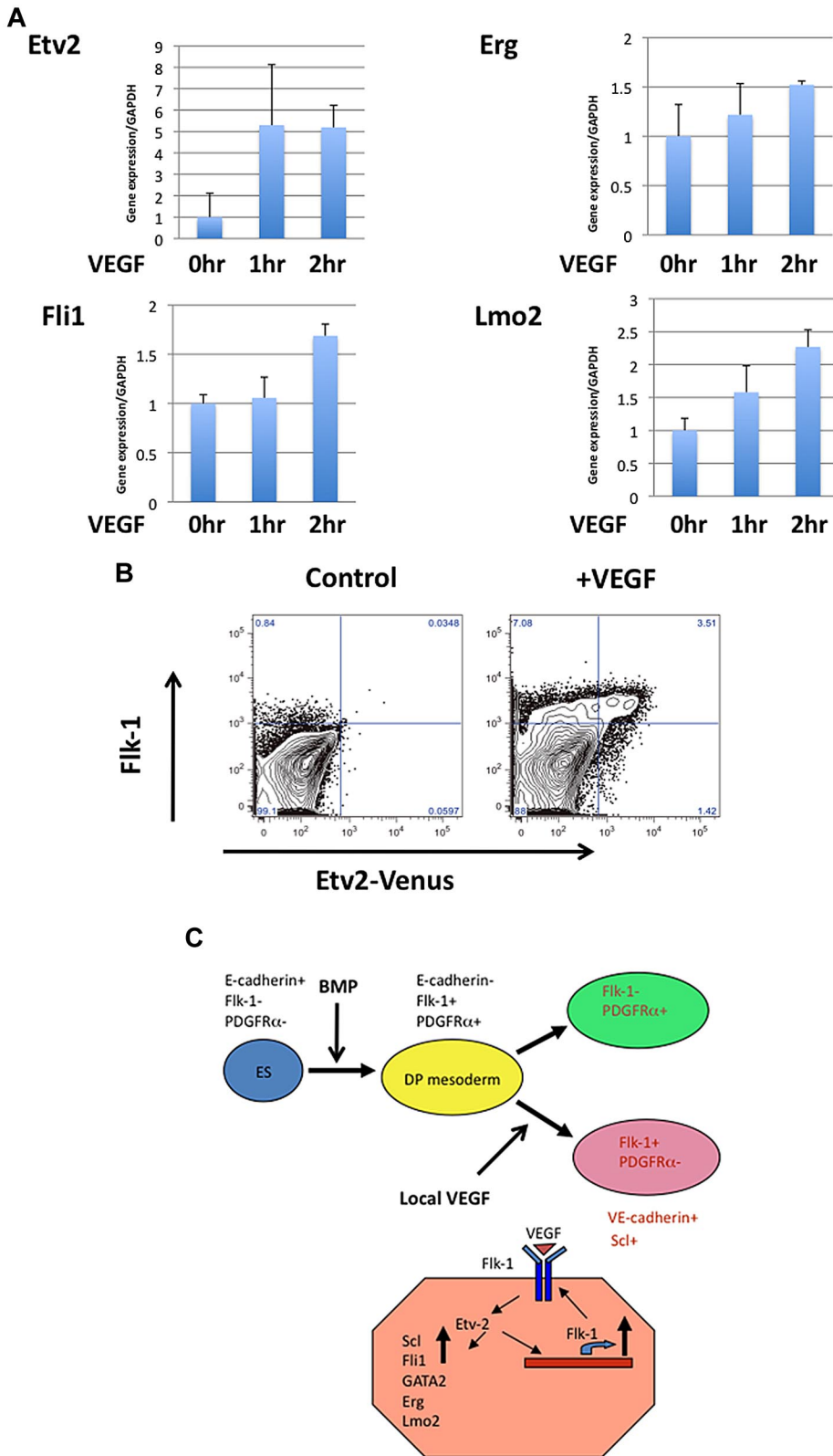


Figure 7. Etv2 is an immediate-response gene in VEGF-treated Flk-1⁺ mesoderm. (A) ESC-derived Flk-1⁺ cells maintained in serum-free medium were treated with VEGF (20 ng/mL) for the indicated periods of time. Gene expressions were quantified by Q-PCR. (B) VEGF (20 ng/mL) treatment for 24 hours significantly increased the Etv2⁺ cells in differentiating ESCs. In addition to an increase in Flk-1⁺ cell numbers, the number of Etv2⁺ cells among Flk-1⁺ cells was also increased. (C) Mesoderm differentiation and role of Etv2 in promoting VSP formation. Primitive mesoderm generated as the Flk-1⁺PDGFR α ⁺ DP-PM population diverges into Flk-1⁺PDGFR α ⁻ VSP and Flk-1⁻PDGFR α ⁺ PSP. Etv2 is required for the progression of DP-PM into VSP. VEGF-Flk-1 signaling promotes VSP induction by up-regulating Etv2 expression. Etv2 triggers VSP induction and subsequent EC and HPC generation by inducing a series of hemat endothelial genes, Scl, GATA2, Fli1, Lmo2, and Erg.

Flk-1⁺PDGFR α ⁺ mesoderm, which normally exists only transiently, was retained longer in Etv2-null-differentiated ESCs and embryos. We also found that VEGF is a potent and rapid inducer of Etv2 in Flk-1⁺ cells. Etv2⁺ cells appeared exclusively in the posterior area of the Flk1⁺ cell cluster around E7.0 (Figure 1A), suggesting that

Flk-1 expression might underlie Etv2 induction. VEGF expression was reported in extra-embryonic endoderm and over the cardiac crescents adjacent to the Flk-1⁺ cells.³⁷ We propose that VEGF induces Etv2 expression in Flk-1⁺ mesoderm and that Etv2 in turn up-regulates Flk-1 to stabilize the VSP fate (Figure 7C).

Comparison of genes expressed in Flk1⁺ cells derived from *Etv2*^{+/-} and *Etv2*^{-/-} ESCs revealed that genes critical in HPC/EC differentiation were up-regulated by *Etv2*, which is consistent with results in *etsrp*-overexpression experiments in zebrafish.^{14,15} Among them, *Scl*, *Fli1*, and *Gata2* were analyzed because of their functional importance in forming a TF network to direct EC/HSC differentiation.^{1,2,28,29} We showed that *Scl*, *Fli1*, and *GATA2* can be up-regulated by *Etv2* and that *Scl* or *Fli1* alone could restore HPCs and ECs in *Etv2*-null cells. Whereas *Fli1* rescue experiments in zebrafish failed to achieve *Runx1* expression,⁴³ in *Etv2*-null ESCs *Fli1* could rescue HPCs to generate *Runx1*-dependent CD45⁺ cells,⁴⁴ suggesting that *Fli1* can be as functional as *Scl*. We did not observe CD41⁺ or CD45⁺ cells in *GATA2*-rescued *Etv2*-null cells. Some VECAD⁺ but ICAM2⁻ cells appeared transiently in *GATA2*-rescued *Etv2*-null cells; however, these cells failed to develop CD41⁺ or CD45⁺ cells. *GATA2* also induced lower levels of *Scl* or *Fli1* expression in *Etv2*-null cells, indicating that there might be partial rescue (data not shown). Our study also suggested a transient requirement for *Etv2* to generate ECs/HPCs. Several endothelial genes considered to be downstream targets of *Etv2* (supplemental Table 2), including VE-cadherin, maintained their expressions in ECs even after *Etv2* down-regulation. Likewise, in experiments to rescue *Etv2*-null cells, some VECAD⁺ or CD41⁺ cells exist as a CD90.1⁻ or CD90.1^{low} population, representing cells of lower *Etv2* expression (supplemental Figure 8). Because ESC differentiation cannot fully recapitulate embryonic hematopoiesis, these insights may not be directly applicable to the in vivo process. However, the transient expression of *Etv2* also observed in developing embryos supports this view. We propose that, as a master regulator, *Etv2* induces several HPC/EC genes, creating a cell-intrinsic status of HPC/EC development that can be maintained even after down-regulation of *Etv2*.

Consistent with its transient requirement, *Etv2* expression was detectable only in the beginning phase of vasculogenesis/hematopoiesis for a restricted period of embryogenesis. *Etv2* was obviously expressed in the E7.5 yolk sac and the E8.5 aorta and posterior part of the embryo (corresponding to splanchnopleure), but was barely detectable in E10.5 AGM and E12.5 fetal liver (supplemental Figure 7A and supplemental Figure 6). It is possible that other *Ets* family members may work in AGM or fetal liver as substitutes.⁴⁵ However, no other *Ets* genes, including *Fli1*, *Ets1*, and *Ets2*, have been reported to cause such severe hematopoietic defects as *Etv2*, indicating the unique role of *Etv2*.⁴⁶⁻⁴⁸ The lack of *Etv2* expression in AGM and fetal liver might indicate the role of *Etv2* mostly in the yolk sac hematopoiesis but not in later stages. Because *Etv2* seems to be required transiently during HPC/EC development and to promote vasculogenesis and hematopoiesis in Flk-1⁺ cells from E7.5 extra-embryonic mesoderm and E8.5 embryo proper parts, it is also possible that transiently expressed *Etv2* initiates hemato-endothelial TFs in precursors present in yolk sac,⁴⁹ splanchnopleure, or dorsal aorta that give rise to AGM and fetal liver HSCs. Defining where *Etv2* specifies HSCs needs further investigation.

AGM ECs have been shown to generate HPCs as hemogenic ECs, whereas some HPCs may be generated directly from common EC/HPC precursors called "hemangioblasts." At present, no molecular marker can clearly distinguish between hemogenic ECs and hemangioblasts. *Etv2* expression elevated in early mesoderm committed to ECs/HPCs but down-regulated in differentiated cells including AGM ECs suggests that it can mark hemangioblasts. However, in zebrafish, *etsrp* (the *Etv2* homolog) deficiency affects EC development more than that of HPCs, indicating that *Etv2*/*Etsrp* expression may represent endothelial precursors rather than common EC/HPC precursors.⁵⁰

Regarding the role of *Etv2* in hematopoiesis, we observed a discrepancy in the expression of *GATA1* and *Runx1* between *Etv2*^{-/-} embryos and in vitro-differentiated Flk-1⁺ cells. *GATA1* and *Runx1* expressions were undetectable in E7.5 extra-embryonic mesoderm of *Etv2*^{-/-} embryos (Figure 4Cb), whereas *Etv2*-null ESCs differentiated on OP9 retained detectable levels of both genes (Figure 4Bb). Impaired *Runx1* expression was also reported in the zebrafish *etsrp* morphant, but not in the *Xenopus Etv2* knock-down.²³ Therefore, *GATA1* and *Runx1* may be influenced by *Etv2* but still be induced without *Etv2* because they may not be direct targets of *Etv2*.^{14,15,23,24} *Runx1* and *GATA1* can promote hematopoietic development from the *Etv2*-induced VSP. Indeed, *GATA1* is known to displace *GATA2* from the same DNA-binding site to disrupt the *Scl/Fli1/Gata2* network and drive the VSP to the primitive erythrocyte fate rather than the EC fate.⁵¹ In this model, *GATA1* and *Runx1* promote hematopoiesis from VSP by antagonizing the EC differentiation by *Scl/Fli1/Gata2* network created by *Etv2*.

In conclusion, *Etv2* is required for the induction of Flk-1⁺ PDGFR α ⁻ VSP but is dispensable for the initial establishment of DP mesoderm. Without *Etv2*, mesoderm cells are trapped as Flk-1⁺ PDGFR α ⁺ DP-PM, failing to differentiate into Flk-1⁺PDGFR α ⁻ VSP. VEGF-Flk-1 induces *Etv2*, creating a positive feedback loop for vascular mesoderm specification (Figure 7C). Flk-1 is not the critical primary target of *Etv2*; instead, more hemato-endothelial specific genes including *Scl* and *Fli1* are important targets. Finally, transient *Etv2* expression can create the TF network to direct the generation of ECs and HPCs, which persists even after *Etv2* is down-regulated (Figure 7C). These insights highlight the possibility that mesoderm derivatives such as ECs, HPCs, and cardiomyocytes can be induced in a more controlled manner from ESC or induced pluripotent stem cells.

Acknowledgments

The authors thank Drs K. Uno, T. Kasukawa, J. Nishio, and C. Imai (Functional Genomics, RIKEN CDB) for microarray analysis; Drs M. Ikeya, Y. Sasai, and H. Niwa (RIKEN CDB) for providing plasmids; Dr M. Hirashima (Kobe University) for helpful comments; and Hiroshi Kiyonari and Kazuki Nakao (RIKEN CDB LARGE) for mice generation.

This work was supported by grants from the PRESTO (Japan Science and Technology Agency), Leading Project for Realization of Regenerative Medicine and Ministry of Education, Culture, Sports, Science and Technology (20590295).

Authorship

Contribution: H.K. and S.-I.N. conceived and designed the experiments and wrote the manuscript and M.H., R.N., N.I., M.L.J., Y.T., S.N., and H.T. performed the experiments.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Hiroshi Kataoka or Shin-Ichi Nishikawa, Laboratory for Stem Cell Biology, RIKEN Center for Developmental Biology, 2-2-3, Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan; e-mail: kataokah@cdb.riken.jp or nishikawa@cdb.riken.jp.

References

- Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt FW, Orkin SH. The T cell leukemia oncogene SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell*. 1996;86(1):47-57.
- Hart A, Melet F, Grossfeld P, et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. *Immunity*. 2000;13(2):167-177.
- Stainier DY, Weinstein BM, Detrich HW, 3rd Zon LI, Fishman MC. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development*. 1995;121(10):3141-3150.
- Patan S. Lycat and cloche at the switch between blood vessel growth and differentiation? *Circ Res*. 2008;102(9):1005-1007.
- Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995;376(6535):62-66.
- Shalaby F, Ho J, Stanford WL, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell*. 1997;89(6):981-990.
- Schuh AC, Faloon P, Hu QL, Bhimani M, Choi K. In vitro hematopoietic and endothelial potential of flk-1(-/-) embryonic stem cells and embryos. *Proc Natl Acad Sci U S A*. 1999;96(5):2159-2164.
- Hidaka M, Stanford WL, Bernstein A. Conditional requirement for the Flk-1 receptor in the in vitro generation of early hematopoietic cells. *Proc Natl Acad Sci U S A*. 1999;96(13):7370-7375.
- Sumanas S, Joriniak T, Lin S. Identification of novel vascular endothelial-specific genes by the microarray analysis of the zebrafish cloche mutants. *Blood*. 2005;106(2):534-541.
- Lee D, Park C, Lee H, et al. ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. *Cell Stem Cell*. 2008;2(5):497-507.
- De Val S, Chi NC, Meadows SM, et al. Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell*. 2008;135(6):1053-1064.
- Sumanas SLS. Ets1-related protein is a key regulator of vasculogenesis in zebrafish. *PLoS Biol*. 2006;4(1):e10.
- Ferdous A, Caprioli A, Iacovino M, et al. Nkx2-5 transactivates the Ets-related protein 71 gene and specifies an endothelial/endocardial fate in the developing embryo. *Proc Natl Acad Sci U S A*. 2009;106(3):814-819.
- Wong KS, Proulx K, Rost MS, Sumanas S. Identification of vasculature-specific genes by microarray analysis of Etsrp/Etv2 overexpressing zebrafish embryos. *Dev Dyn*. 2009;238(7):1836-1850.
- Gomez GA, Veldman MB, Zhao Y, Burgess S, Lin S. Discovery and characterization of novel vascular and hematopoietic genes downstream of etsrp in zebrafish. *PLoS One*. 2009;4(3):e4994.
- Ikeya M, Kawada M, Nakazawa Y, et al. Gene disruption/knock-in analysis of mONT3: vector construction by employing both in vivo and in vitro recombinations. *Int J Dev Biol*. 2005;49(7):807-823.
- Sakurai H, Era T, Jakt LM, Okada M, Nakai S, Nishikawa S. In vitro modeling of paraxial and lateral mesoderm differentiation reveals early reversibility. *Stem Cells*. 2006;24(3):575-586.
- Guo G, Yang J, Nichols J, et al. Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development*. 2009;136(7):1063-1069.
- Hirashima M, Ogawa M, Nishikawa S, Matsumura K, Kawasaki K, Shibuya M. A chemically defined culture of VEGFR2+ cells derived from embryonic stem cells reveals the role of VEGFR1 in tuning the threshold for VEGF in developing endothelial cells. *Blood*. 2003;101(6):2261-2267.
- Wilkinson DG. *In Situ Hybridization: A Practical Approach*. 2nd ed. Oxford University Press; 1999.
- Kataoka H, Takakura N, Nishikawa S, et al. Expressions of PDGF receptor alpha, c-Kit and Flk1 genes clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells. *Dev Growth Differ*. 1997;9(6):729-740.
- Hirata H, Kawamata S, Murakami Y, et al. Coexpression of platelet-derived growth factor receptor alpha and fetal liver kinase 1 enhances cardiogenic potential in embryonic stem cell differentiation in vitro. *J Biosci Bioeng*. 2007;103(5):412-419.
- Salanga MC, Meadows SM, Myers CT, Krieg PA. ETS family protein ETV2 is required for initiation of the endothelial lineage but not the hematopoietic lineage in the *Xenopus* embryo. *Dev Dyn*. 2010;239(4):1178-1187.
- Neuhaus H, Muller F, Hollemann T. *Xenopus* er71 is involved in vascular development. *Dev Dyn*. 2010;239(12):3436-3445.
- Endoh M, Ogawa M, Orkin S, Nishikawa S. SCL/tal-1-dependent process determines a competence to select the definitive hematopoietic lineage prior to endothelial differentiation. *EMBO J*. 2002;21(24):6700-6708.
- Pevny L, Simon MC, Robertson E, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature*. 1991;349(6306):257-260.
- Okuda T, Nishimura M, Nakao M, Fujita Y. RUNX1/AML1: a central player in hematopoiesis. *Int J Hematol*. 2001;74(3):252-257.
- Tsai FY, Keller G, Kuo FC, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature*. 1994;371(6494):221-226.
- Spyropoulos DD, Pharr PN, Lavenburg KR, et al. Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. *Mol Cell Biol*. 2000;20(15):5643-5652.
- Hollenhorst PC, Shah AA, Hopkins C, Graves BJ. Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the ETS gene family. *Genes Dev*. 2007;21(15):1882-1894.
- Wei GH, Badis G, Berger MF, et al. Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. *EMBO J*. 2010;29(13):2147-2160.
- Göttgens B, Nastos A, Kinston S, et al. Establishing the transcriptional programme for blood: the SCL stem cell enhancer is regulated by a multi-protein complex containing Ets and GATA factors. *EMBO J*. 2002;21(12):3039-3050.
- Pimanda JE, Ottersbach K, Knezevic K, et al. Gata2, Fli1, and Scl form a recursively wired gene-regulatory circuit during early hematopoietic development. *Proc Natl Acad Sci U S A*. 2007;104(45):17692-17697.
- Liu F, Walmsley M, Rodaway A, Patient R. Fli1 acts at the top of the transcriptional network driving blood and endothelial development. *Curr Biol*. 2008;18(16):1234-1240.
- de Bruijn MF, Ma X, Robin C, Ottersbach K, Sanchez MJ, Dzierzak E. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity*. 2002;16(5):673-683.
- Nikolova-Krstevski V, Yuan L, Le Bras A, et al. ERG is required for the differentiation of embryonic stem cells along the endothelial lineage. *BMC Dev Biol*. 2009;9:72.
- Miquerol L, Gertsenstein M, Harpal K, Rossant J, Nagy A. Multiple developmental roles of VEGF suggested by a LacZ-tagged allele. *Dev Biol*. 1999;212(2):307-322.
- Ema M, Takahashi S, Rossant J. Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. *Blood*. 2006;107(1):111-117.
- Moretti A, Caron L, Nakano A, et al. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell*. 2006;127(6):1151-1165.
- Nakayama N, Duryea D, Manoukian R, Chow G, Han CY. Macroscopic cartilage formation with embryonic stem-cell-derived mesodermal progenitor cells. *J Cell Sci*. 2003;116(Pt 10):2015-2028.
- Motoike T, Markham DW, Rossant J, Sato TN. Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. *Genesis*. 2003;35(3):153-159.
- Knebel J, De Haro L, Janknecht R. Repression of transcription by TSGA/Jmjd1a, a novel interaction partner of the ETS protein ER71. *J Cell Biochem*. 2006;99(1):319-329.
- Ren X, Gomez GA, Zhang B, Lin S. Scl isoforms act downstream of etsrp to specify angioblasts and definitive hematopoietic stem cells. *Blood*. 2010;115(26):5338-5346.
- Mikkola HK, Fujiwara Y, Schlaeger TM, Traver D, Orkin SH. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood*. 2003;101(2):508-516.
- Liu F, Patient R. Genome-wide analysis of the zebrafish ETS family identifies three genes required for hemangioblast differentiation or angiogenesis. *Circ Res*. 2008;103(10):1147-1154.
- Georgiades P, Rossant J. Ets2 is necessary in trophoblast for normal embryonic anteroposterior axis development. *Development*. 2006;133(6):1059-1068.
- Yamamoto H, Flannery ML, Kupriyanov S, et al. Defective trophoblast function in mice with a targeted mutation of Ets2. *Genes Dev*. 1998;12(9):1315-1326.
- Wei G, Srinivasan R, Cantemir-Stone CZ, et al. Ets1 and Ets2 are required for endothelial cell survival during embryonic angiogenesis. *Blood*. 2009;114(5):1123-1130.
- Samokhvalov IM, Samokhvalova NI, Nishikawa S. Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature*. 2007;446(7139):1056-1061.
- Patterson LJ, Patient R. The "Ets" factor: vessel formation in zebrafish—the missing link? *PLoS Biol*. 2006;4(1):e24.
- Jing H, Vakoc CR, Ying L, et al. Exchange of GATA factors mediates transitions in looped chromatin organization at a developmentally regulated gene locus. *Mol Cell*. 2008;29(2):232-242.