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HEMATOPOIESIS AND STEM CELLS

Fev regulates hematopoietic stem cell development via ERK signaling

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Key Points

- Fev is required for endothelium-based HSC emergence.
- Fev directly regulates ERK signaling to regulate HSC development cell-autonomously.

Reprogramming of somatic cells to desired cell types holds great promise in regenerative medicine. However, production of transplantable hematopoietic stem cells (HSCs) in vitro by defined factors has not yet been achieved. Therefore, it is critical to fully understand the molecular mechanisms of HSC development in vivo. Here, we show that Fev, an ETS transcription factor, is a pivotal regulator of HSC development in vertebrates. In *fev*-deficient zebrafish embryos, the first definitive HSC population was compromised and fewer T cells were found in the thymus. Genetic and chemical analyses support a mechanism whereby Fev regulates HSC through direct regulation of ERK signaling. Blastula transplant assay demonstrates that Fev regulation of HSC development is cell autonomous. Experiments performed with purified cord blood show that *fev* is expressed and functions in primitive HSCs in humans, indicating its conserved role in higher vertebrates. Our data indicate that Fev-ERK signaling is essential for hemogenic endothelium-based HSC development. (*Blood*. 2013;122(3):367-375)

Introduction

Hematopoietic stem cells (HSCs) can give rise to all blood lineages, including erythroid (erythrocyte), myeloid (macrophage, neutrophil, monocyte), and lymphoid (B cell and T cell). HSC transplantation has been successfully applied in the clinic to treat hematological diseases such as leukemia. However, the shortage of sources (mainly compatible bone marrow) has restricted its application worldwide. Production of transplantable HSCs in vitro holds great promise for solving this issue but has not been successfully achieved from embryonic or induced pluripotent stem cells or by defined factors. Therefore, a full understanding of molecular mechanisms of HSC development in vivo is critical for regenerative medicine application of HSCs expanded ex vivo or in vitro. During embryogenesis, the first HSCs are believed to be derived from the ventral wall of the dorsal aorta (ie, hemogenic endothelium) through endothelial-hematopoietic-transition (EHT) in the embryos of zebrafish, *Xenopus*, chick, and mammals.[1-8] However, the underlying molecular mechanism of hemogenic endothelium-derived HSC differentiation remains to be determined.

Several transcription factors have been shown to be essential for HSC development.[9] Runx1 is a known master regulator of HSC specification that is expressed in the ventral wall of the dorsal aorta, the underlying mesenchyme, and intra-aortic hematopoietic clusters in mice.[10,11] Loss-of-Runx1 in mice completely abolished HSC formation in the dorsal aorta[10]; however, it did not affect the formation of the dorsal aorta from which HSCs are derived. *cmyb* is expressed in long-term HSCs and *cmyb*-null mice failed to develop adult hematopoiesis in the fetal liver, but the commitment to definitive hematopoiesis can occur in the absence of cMyb.[12] Therefore, identification of upstream regulators of Runx1 and cMyb is essential for understanding the origin of HSC development from hemogenic endothelium. Several ETS transcription factors act individually or combinatorially to regulate dorsal aorta[13,14] and/or HSC development and functions.[15-17] In *Xenopus* embryos, we previously showed that the ETS factor Tel1/ETV6 specifies the first HSCs in the dorsal aorta by regulating the expression of *vegfa* in both the lateral plate mesoderm and the somites, suggesting cell-autonomous and non-cell–autonomous roles for Tel1 in HSC development.[16] In zebrafish, we identified a new ETS gene, *fev* (also known as *pet1* in mammals), which belongs to the same subgroup as *fli1* and *erg*.[18] *Fev* was initially reported to be expressed in serotonergic neurons in vertebrates and in mice lacking Pet-1 the majority of serotonin-producing (5-hydroxytryptamine) neurons fail to differentiate and the level of 5-hydroxytryptamine is decreased by 70% to 80%.[19-21] However, whether *fev* plays a role during HSC development remains unknown.

Here, we demonstrate that Fev, an ETS transcription factor, is a pivotal regulator of HSC development in zebrafish and humans. Fev acts upstream of ERK signaling to regulate HSC specification and function, which is distinct from its well-known role in serotonergic neuron development in vertebrates.

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Methods

Zebrafish husbandry

Zebrafish strains including AB, *fli1a*:GFP (generously provided by S. Wilson), *fli1a*-nuclear-GFP (*fli1a*:nGFP) and *cmyb*:GFP (generously provided by A. Meng), and *kdrl*:mCherry (from B. Zhang laboratory in Peking University) transgenic lines were raised and maintained at 28.5°C in system water and staged as previously described.[22] This study was approved by the Ethical Review Committee in the Institute of Zoology, Chinese Academy of Sciences, China and was conducted in accordance with the Declaration of Helsinki.

Morpholinos, mRNA synthesis, and microinjection

The detailed protocols for these assays are described in supplemental Methods.

Chemical treatment

Zebrafish embryos were incubated with fish water containing LY294002 or U0126 (final concentration 10 μM) from bud stage, while embryos incubated with fish water containing 0.1% dimethylsulfoxide (vehicle alone) served as control. During the next 24 h, the embryos were periodically examined to ensure that the pharmacological effect remained constant over time. Chemicals including LY294002 and U0126 were purchased from Sigma.

Whole mount in situ hybridization

Whole mount in situ hybridization with zebrafish embryos was performed using a ZF-A4 in situ hybridization machine (Zfand, China) with probes, including *fev*, *runx1*, *cmyb*, *dll4*, *ephrinB2*, *flt4*, *msr*, *erk1/2*, and *rag1* as previously described.[18,23]

Immunofluorescence

Anti-phosphorylated ERK (Cell Signaling) staining was performed on transverse sections as previously described.[24]

RT-PCR and qPCR

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR) were performed as described[23] and are described in supplemental Methods. Three independent experiments were carried out and in each experiment, 30 to 50 embryos were used for each sample. The PCR primers used and the expected product lengths are summarized in supplemental Table 2.

Western blot, TUNEL assay, and BrdU labeling, reporter assay, and Fev polyclonal antibody production

The detailed protocols for these assays are described in the supplemental Methods.

ChIP assay

Chromatin immunoprecipitation (ChIP) analysis was carried out with the trunk tissues of wild-type 36 hours post fertilization (hpf) embryos, and the eluted DNA (precipitated by Fev polyclonal antibody) was assayed by PCR as previously described.[23,24] The primers specific to the Fev binding site within the upstream regulatory regions of *erk2* were designed by searching for the conserved binding sites and primers nonspecific for these binding sites were designed and used as control primers. The primers and the expected product length are summarized in supplemental Table 3. Rabbit purified immunoglobulin G and nonspecific primers were included as negative controls.

Blastula transplantation

Tg(*cmyb*:GFP) embryos were injected with *fev*MO or control MO with dextran tetramethylrhodamine (Red, Invitrogen) at the one-cell stage. And at the shield stage, 30 to 50 cells were removed from the donor embryos' ventral marginal region and transplanted into the stage-matched host embryos. Host embryos were then examined and analyzed by confocal microscopy at 36 hpf.

Human cord blood assays

Cord blood (CB) from anonymous healthy donors was provided from Shanghai Cord Blood Bank in accordance with local ethics procedures, and the experiments related to human CB were approved by the local ethics committee. More details are described in supplemental Methods.

Confocal microscopy

Confocal images were acquired with a Zeiss LSM 510 META confocal laser microscope, and 3D projections were generated using Zeiss LSM software (Carl Zeiss).[18]

Statistical analysis

For statistical analysis, Student's unpaired 2-tailed *t* test was used for all comparisons.

Results

Fev is required for HSC emergence

During zebrafish early embryogenesis, *fev* expression was initially detected bilaterally in the lateral plate mesoderm at the 5s stage and in blood/endothelial cells (ECs) at 24 hpf (Figure 1A), suggesting a role in vessel formation and hematopoiesis. To examine whether *fev* is required for HSC development, we used an antisense ATG MO to knock down *fev* expression in zebrafish. Immunoblotting analysis indicated that compared with the controls, endogenous Fev protein was markedly reduced in the morphants (Figure 1B). Whereas primitive hematopoiesis[18] and vasculogenesis were relatively normal in *fev*MO-injected embryos (supplemental Figure 1), expression of the earliest HSC markers, *runx1* and *cmyb*, was reduced at 24 hpf and 36 hpf (Figure 1C). qPCR analysis of *runx1* and *cmyb* expression (Figure 1D) was consistent with WISH results, supporting the idea that the development of HSCs was disrupted significantly with *fev* knockdown. To further examine whether HSCs or their derivatives appear in the caudal hematopoietic tissue (CHT; the zebrafish equivalent of fetal liver in mouse) or thymus, we analyzed the expression of *cmyb* and the early T-cell marker, *rag1*, in *fev* morphants. *cmyb* expression in the CHT at 48 hpf and *rag1* expression in the thymus at 4 days post fertilization (dpf) (Figure 1C) were remarkably attenuated. Furthermore, the population of *cmyb*:GFP labeled HSCs[1] in the CHT region at 48 hpf and in the pronephros at 4 dpf and *cmyb*:GFP labeled T cells in the thymus at 4 dpf were much reduced in *fev* morphants (Figure 1E). Quantitative analysis showed that the number of GFP^+ cells remarkably decreased in the CHT, thymus, and kidney in *fev* morphants compared with that in controls (Figure 1F).

The impaired HSC and T-cell development in *fev* morphants could be due to abnormal cell proliferation or excessive apoptosis. To test these possibilities, we employed BrdU labeling for cell proliferation and TUNEL staining for apoptosis assays (supplemental Figure 2). BrdU labeling experiments showed that decreased cell proliferation occurred in *fev* morphants (supplemental Figure 2A-B), suggesting that the HSC defects in *fev* morphants might be due to slightly reduced cell numbers. In addition, the TUNEL assay showed more apoptotic cells in *fev* morphants (supplemental Figure 2C) compared with the controls, indicating that the increased apoptosis might contribute to the HSC defects as well. To further explore this possibility, we used *p53*MO, which was previously demonstrated to prevent *p53*-dependent

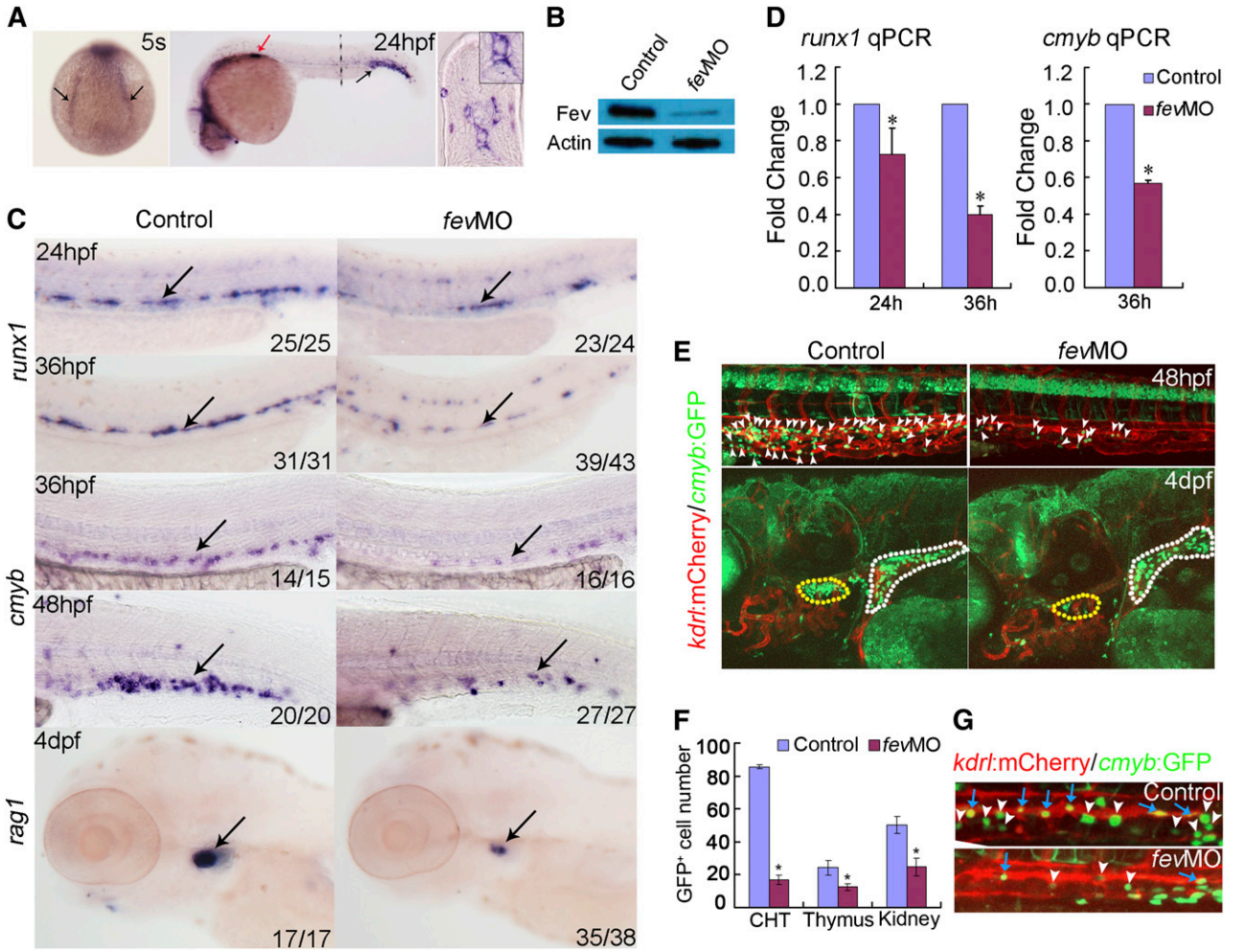


Figure 1. Fev is required for HSC development. (A) Whole-mount in situ hybridization shows expression of *fev* in lateral plate mesoderm (arrows) at 5-somite stage and in pancreas (red arrow) and blood vessels (black arrow) at 24 hpf. The right panel shows a cross-section of 24 hpf embryo in the trunk region at the location marked by the dotted line, showing *fev* expression in vessels. (B) Validation of *fev*MO (3 ng/embryo) by western blot in control and *fev* morphant embryos at 24 hpf. (C) The *runx1* data, expression of *runx1* at 24 and 36 hpf in embryos injected with control MO or *fev*MO (top panels). Arrows mark *runx1* expression in HSCs. The *cmyb* data, expression of *cmyb* at 36 and 48 hpf in CHT of embryos injected with control MO (middle panels). The *rag1* data, expression of *rag1* at 4 dpf in thymus of embryos injected with control MO or *fev*MO (bottom panels). Arrows indicate CHT or thymus. Anterior is to the left and dorsal is up. (D) qPCR analysis of *runx1* and *cmyb* expression in the dissected trunk region of embryos injected with control or *fev*MO at 24 and 36 hpf (mean ± SD, n = 3,*$P < .05$). (E) Population of the GFP$^+$ cells (hematopoietic stem/progenitor cells) in the CHT region was reduced in *cmyb*:GFP/*kdrl*:mCherry double transgenic embryos injected with *fev*MO compared with those injected with control MO (top panels). White arrowheads mark HSCs/HSPCs. Population of the GFP$^+$ cells in the pronephros and T cells in the thymus at 4 dpf were much reduced in *cmyb*:GFP/*kdrl*:mCherry double transgenic embryos injected with *fev*MO (bottom panels). The thymus and kidney are circled in yellow and white, respectively. (F) Quantification of GFP$^+$ cells in the CHT, thymus, and kidney between control and fev morphant embryos in E. Note that 5 control embryos and 5 *fev* morphants were used for counting GFP$^+$ cells in the CHT, thymus, and kidney regions. (G) Hemogenic endothelium analysis in the AGM region using a *cmyb*:GFP/*kdr1*:mCherry double transgenic line at 36 hpf. Blue arrow, *kdrl*$^+$*cmyb*$^+$ cells (hemogenic ECs); white arrowhead, *kdrl*$^-$*cmyb*$^+$ cells (emerging HSCs). CHT, caudal hematopoietic tissue.

apoptosis.[25,26] As shown in supplemental Figure 2C-D, *p53*MO knockdown can efficiently prevent ectopic apoptosis in *fev* morphants but failed to restore *runx1* and *cmyb* expression in the aorta-gonad-mesonephros (AGM) region at 24 and 36 hpf, respectively. Taken together, these data suggest that the impaired HSC development in *fev* morphants was most likely attributed to the specification and/or proliferation defects, but not apoptosis.

To verify that the HSC defects were indeed specific to *fev* gene activity, we generated *fev* mutants by TALEN (supplemental Methods).[27] We obtained 3 different indel mutants for the *fev* gene, and the most severe allele contained an 8-bp indel in which endogenous Fev protein levels were nearly abolished (supplemental Figure 3A-D). We examined the expression of aforementioned HSC and T-cell markers in this mutant and obtained similar results to those in *fev* morphants (supplemental Figure 3E-F). Western blot results further confirmed that protein levels of Fev and Runx1 were similarly reduced in *fev* morphants and *fev* TALEN mutants (supplemental Figure 3G). Taken together, these data indicate that *fev* is required for HSC emergence.

Hemogenic endothelium-based dorsal aorta is affected in *fev*-deficient embryos

The dorsal aorta functions as a prerequisite site for definitive hematopoiesis, because the first definitive HSCs are derived from the ventral wall of the dorsal aorta (ie, hemogenic endothelium) through the EHT process in fish[1,2] or in the AGM in mammals.[3] To monitor the hemogenic endothelium-mediated EHT process, we took advantage of a *cmyb*:GFP/*kdr1*:mCherry double transgenic line. As shown in Figure 1G, both *cmyb*$^+$*kdr1*$^+$ cells (ie, hemogenic endothelium)

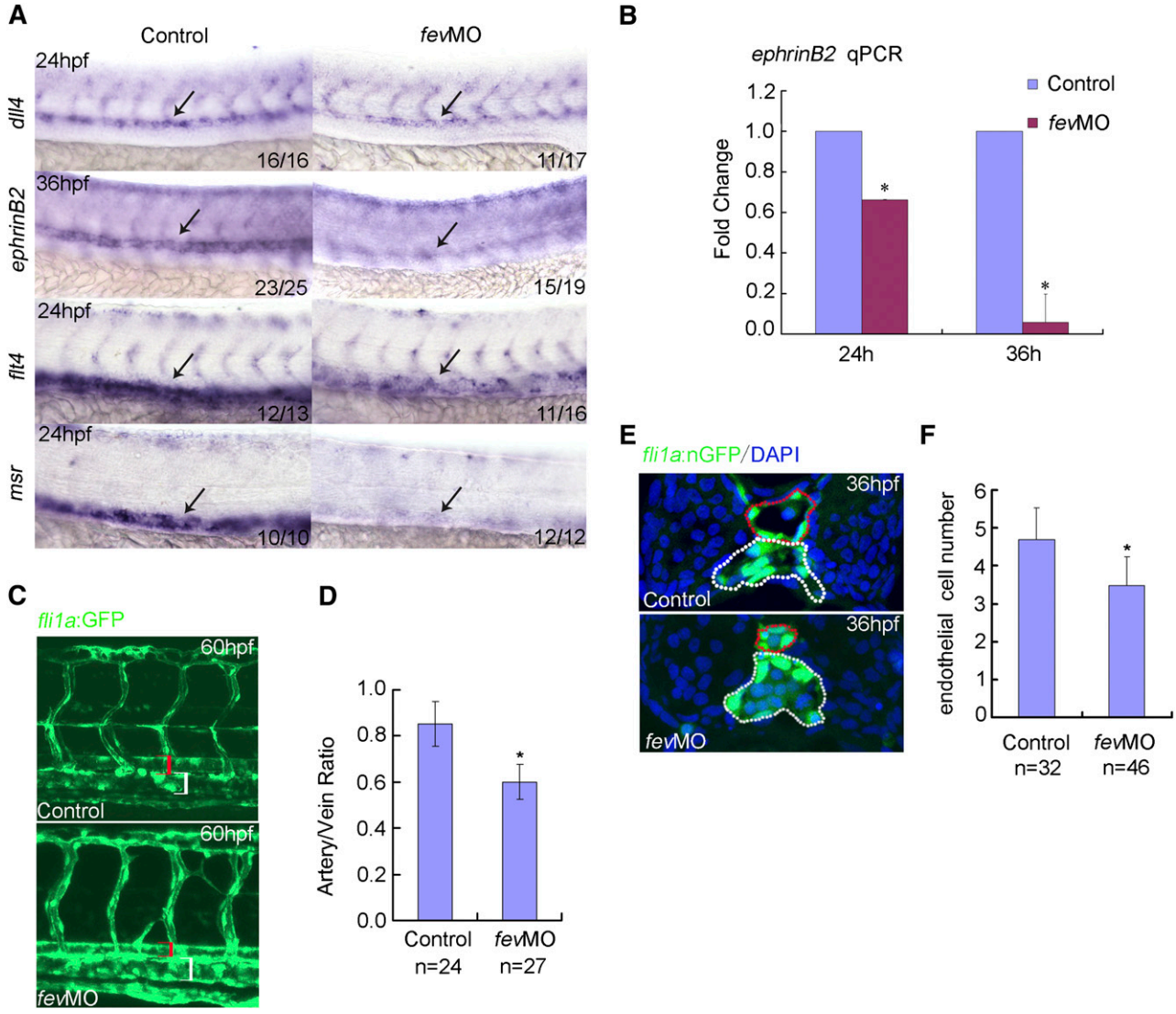


Figure 2. Hemogenic endothelium-based dorsal aorta is affected in *fev*-deficient embryos. (A) Expression of *dll4* or *ephrinB2* in the dorsal aorta of embryos injected with control MO or *fev*MO at 24 and 36 hpf, respectively. Expression of *flt4* and *msr* in the cardinal vein of embryos injected with control MO or *fev*MO at 24 hpf. Anterior is to the left and dorsal is up. (B) qPCR analysis of *ephrinB2* expression at 24 and 36 hpf in the dissected trunk region of embryos injected with control MO or *fev*MO (mean ± SD, n = 3, $^*P < .05$). (C) Confocal microscopy of *fli1a*:GFP embryos at 60 hpf injected with control MO or *fev*MO. Red bracket denotes dorsal aorta, white bracket denotes cardinal vein. (D) Quantification of the artery/vein ratio of the lumen size between controls and *fev* morphants (mean ± SD, $^*P < .05$). (E) Transverse section of *fli1a*:nGFP embryos injected with control MO or *fev*MO at 36 hpf. Dorsal aorta and cardinal vein are denoted by dotted circles in red and white, respectively. Nuclei were shown by 4,6 diamidino-2-phenylindole staining. (F) Statistics of EC numbers of the dorsal aorta wall on transverse sections in controls embryos and *fev*-morphants (mean ± SD, $^*P < .05$).

and $cmyb^+$ $kdr1^-$ cells (ie, HSCs formed through EHT) were reduced in *fev* morphants, suggesting hemogenic endothelium-derived HSCs were compromised. *runx1* is initially expressed in a small subset of endothelium, then is restricted to hemogenic endothelium and HSCs thereafter in fish and mice.[7,10] We further performed a detailed time-course assay for *runx1* expression in *fev* morphants. It is clear that the earliest expression of *runx1* in ECs at 22 hpf was not affected in *fev* morphants. However, from 24 hpf onwards, *runx1* expression in hemogenic endothelium and the HSCs derived through EHT was increasingly compromised (supplemental Figure 4). Previous studies from us and others showed that when Shh, VEGF, Notch, or BMP signaling was disrupted, programming of artery and/or HSC was affected.[7,28,29] Therefore, we next evaluated the expression of these signaling components and arterial-venous markers in *fev*-deficient embryos. Interestingly, there were no obvious alterations in the expression of Shh signaling, BMP signaling, or VEGF ligand (supplemental Figure 5). However, expression of *dll4*, an early artery marker, was modestly reduced at 24 hpf, and expression of *ephrinB2*, a more differentiated artery marker, dramatically decreased at 36 hpf (Figure 2A). qPCR analyzed at 24 and 36 hpf further suggests that the artery program was disrupted with *fev* knockdown (Figure 2B). Interestingly, expression of the venous markers, *flt4*, which encodes the VEGF receptor 3, and *msr* was also down-regulated in *fev* morphants (Figure 2A). To further examine the vascular defects, the vessel structure of *fli1a*:GFP embryos was visualized using confocal microscopy after knocking down *fev*. It was clearly shown that the lumen size of the dorsal aorta was significantly reduced, whereas the lumen size of the vein in the morphants was still comparable with that in the controls (Figure 2C-D). We further examined the EC numbers in the trunk vessels using *fli1a*:nGFP embryos. A transverse section of the trunk region showed that in control embryos, there are on average 4.7 cells within the dorsal aorta

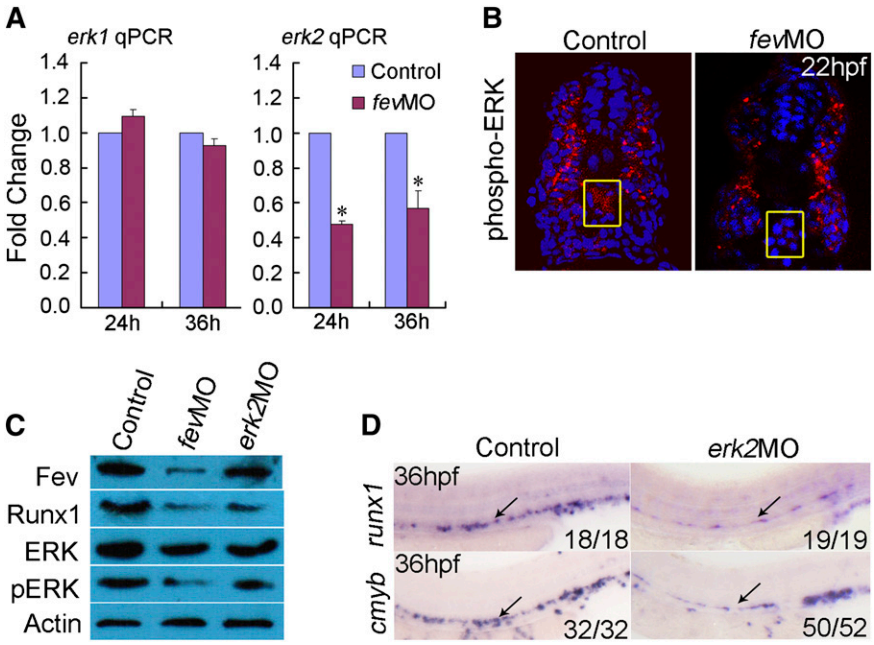


Figure 3. Fev acts upstream of ERK signaling to establish the HSC fate. (A) qPCR analysis of *erk1* and *erk2* expression at 24 and 36 hpf in the dissected trunk region of embryos injected with control MO or *fev*MO showed that *erk2* but not *erk1* expression in the trunk region is reduced in *fev* morphants (mean ± SD, n = 3,*$P < .05$). (B) Immunofluorescence shows pERK1/2 expression in the dorsal aorta is reduced in *fev* morphants (n = 5) compared with the controls (n = 5). Note that the somitic expression of pERK1/2 is not affected in *fev* morphants. Yellow squares indicate the dorsal aorta area. (C) Western blot showing that Runx1 protein is significantly downregulated at 36 hpf in embryos injected with fevMO or erk2MO. In addition, total ERK1/2 and pERK1/2 proteins are strongly down-regulated at 36 hpf in embryos injected with fevMO or erk2MO. Fev is downregulated in fev morphants but not altered in erk2 morphants. (D) runx1 and cmyb expression in the dorsal aorta is reduced at 36 hpf in embryos injected with erk2MO. Anterior to the left, lateral view, arrows mark the AGM region.

wall at 36 hpf, whereas there are about 3.4 ECs in the morphants, with statistical significance at a level of $P < .05$ (Figure 2E-F). These data suggest that *fev* deficiency disrupted the hemogenic endothelium-based dorsal aorta and that the reduced lumen size of the dorsal aorta was likely attributed to fewer ECs in the morphants.

Fev acts upstream of ERK signaling to establish the HSC fate

The unaltered Shh and BMP signaling in *fev* morphants suggests other signaling might be involved in the defects caused by *fev* deficiency (supplemental Figure 5). The VEGF-Notch signaling cascade is required for both artery and HSC specification in zebrafish.[7,28] The down-regulation of *dll4* and *eprhinB2* (a target of Δ/Notch signaling) expression, together with unchanged *vegfa* expression in *fev* morphants (Figure 2A; supplemental Figure 5D), suggests that downstream components of VEGF signaling might be affected as well. The MAPK signaling proteins ERK 1/2 are major downstream effectors of signaling cascades initiated by VEGF and other growth factors, which are involved in many eukaryotic cellular processes.[30] Importantly, activated ERK signaling in the vascular niche is required for maintenance and lineage-specific differentiation of HSCs.[31] However, it is unclear whether ERK also plays a role in regulating HSC formation in the dorsal aorta. We therefore examined the expression of *erk1* and *erk2* in the *fev* morphants. qPCR showed that expression of *erk2* but not *erk1* was significantly down-regulated in *fev* morphants at 24 and 36 hpf, respectively (Figure 3A). Using immunofluorescence, we showed that phosphorylated ERK1/2 was reduced specifically in the dorsal aorta area but not in the somite in *fev* morphants (Figure 3B yellow squares). Immunoblotting analysis further demonstrated that the levels of total ERK1/2 protein and phosphorylated ERK1/2 were both reduced in *fev* morphants (Figure 3C). To determine whether *erk2* expression is required for HSC development in zebrafish, we knocked down *erk2* expression by an antisense MO at a dose optimized to avoid any early gastrulation defects.[30] As shown in Figure 3D, expression of *runx1* and *cmyb* in the AGM region was down-regulated in *erk2* morphants, suggesting that *erk2* expression is also required for HSCs in the AGM region after gastrulation. It is interesting to note that although Runx1 decreased in *fev* or *erk2* morphants, the expression of Fev was unchanged in the *erk2* morphants (Figure 3C), suggesting that *fev* drives *erk2* expression. To determine whether *fev* expression is also regulated by MAPK signaling, we applied MEK inhibitor U0126 and AKT inhibitor LY294002, respectively, to treat wild-type embryos at the tail bud stage. The expression of *fev* was not affected by any of the inhibitors examined at 24 hpf (supplemental Figure 5E), indicating that Fev indeed acts upstream of VEGF-ERK signaling. Collectively, these results indicate that in zebrafish, Fev acts upstream of ERK signaling to establish the HSC program.

Fev directly regulates ERK signaling

To verify the hierarchy of this pathway, we performed epistasis experiments using HSC markers, *runx1*, *cmyb*, and the T-cell marker *rag1* as phenotypic readouts. Overexpression of *erk2* partially rescued the reduced expression of *runx1* and *cmyb* in AGM and *rag1* in the thymus in *fev* morphants (Figure 4A). qPCR and western-blot analysis agreed with the WISH results (Figure 4B-C). To determine whether Fev is located in the nucleus as a functional transcription factor, HEK293T cells overexpressing *fev* were subjected to cellular subfractionation analysis. As expected, Fev is only detected in the nuclear fraction extracts (supplemental Figure 6A). Interestingly, ChIP assay with the trunk region of zebrafish embryos at 36 hpf demonstrated that Fev binds to the promoter of *erk2* in vivo, suggesting a direct regulation of *erk2* by Fev (Figure 4D-E). Reporter assay in HEK 293 cells using the promoter constructs of *erk2*, which contains conserved wild-type or mutated Fev binding sites, indicated that Fev positively regulated *erk2* expression in a dose-dependent manner (Figure 4F). Moreover, overexpression experiments showed that Fev can up-regulate *erk2* and *runx1* expression and ERK2 can up-regulate *runx1* expression (supplemental Figure 6C-D). To further demonstrate that Fev regulates HSCs specifically, we performed mRNA rescue experiments. Modified mRNA, *fev* misRNA (with the mutated atgMO target sequence without changing amino acid coding), was generated and co-injected with *fev*MO into the one-cell stage embryos. Western blot showed that *fev* misRNA can efficiently restore Fev expression in *fev* morphants. Strikingly, overexpression of

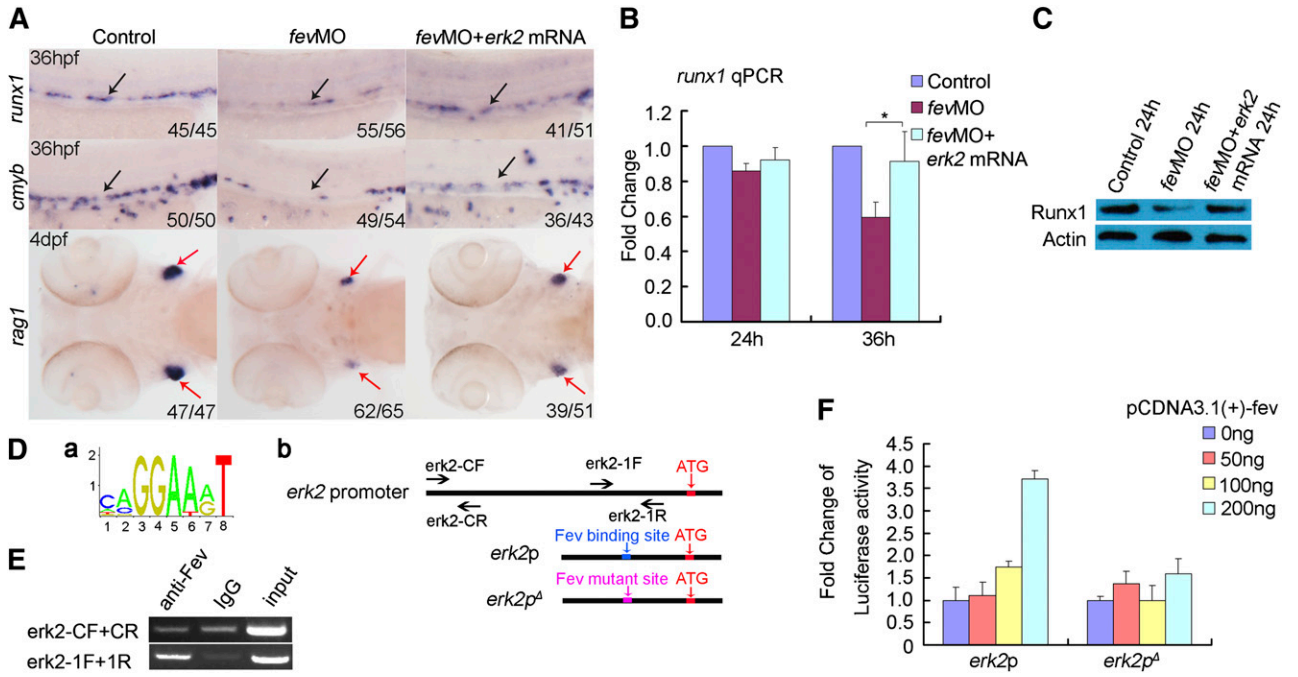
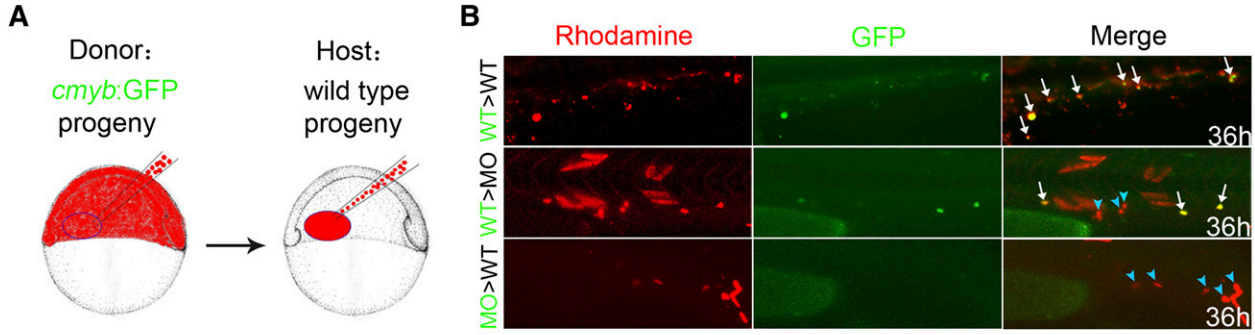


Figure 4. Fev directly regulates ERK signaling. (A) *erk2* mRNA can partially rescue the expression of *runx1*, *cmyb*, and *rag1* in the AGM and thymus of *fev* morphants. The AGM region and thymus are indicated by arrows in black and red, respectively. Anterior to the left. (B) qPCR analysis of *runx1* expression in control embryos, *fev* morphants, and rescued embryos by overexpression of *erk2* mRNA (mean ± SD, n = 3, $^*P < .05$). (C) Western-blot analysis of Runx1 expression in *fev* morphants and rescued embryos. (D) The Fev core binding motif and the *erk2* promoter. a, The conserved core binding site of Fev. b, The schematic structure of the *erk2* promoter. CF/CR are control forward and reverse primers, which are used to amplify the *erk2* promoter region without the conserved Fev binding site. *erk2*-1F/1R represent primers used to amplify the promoter region with the conserved Fev binding site. *erk2*p means the *erk2* promoter construct with the wild-type Fev binding site; *erk2*p$^{\Delta}$ means the *erk2* promoter construct with mutated Fev binding site. (E) ChIP assay showed that Fev directly binds to the *erk2* promoter region. (F) Reporter assay with the promoter constructs of *erk2*, which contain the conserved or mutated ETS binding sites, cotransfected with pCDNA3.1(+)-*fev* plasmid.

fev misRNA in *fev* morphants restored ERK, phosphorylated ERK (pERK), and Runx1 expression at 24 hpf (supplemental Figure 6B). Moreover, expression of ERK, pERK, and Runx1 was comparably decreased in *fev* morphants and *fev* TALEN mutants (supplemental Figure 3G). Taken together, these data indicate that the Fev-ERK cascade regulates HSC development.



C

Summary of transplantation results

Donor *cmyb*:GFP	Host	Number of host embryos with Rhodamine	Number of host embryos with Rhodamine$^+$ hematopoietic cells in the CHT	Number of host embryos with donor-derived GFP$^+$ HSC cells in the CHT	Number of host embryos with donor-derived GFP$^-$ hematopoietic cells in the CHT
wild type	wild type	174	49	12	37
wild type	*fev* morphants	160	44	14	30
fev morphants	wild type	189	74	0	74

Figure 5. Fev regulation of HSC development is cell autonomous. (A) Transplantation scheme: rhodamine-labeled donor cells at the ventral marginal zone of *cmyb*:GFP transgenic donor embryos were transplanted into wild-type recipients at the blastula stage. (B) HSC reconstitution in the CHT of recipient embryos at 36 hpf. Green, *cmyb*: GFP$^+$ cells; red, rhodamine. White arrows, GFP$^+$ HSCs contributed by donor cells; light blue arrowheads, donor-derived GFP$^-$ hematopoietic cells. (C) Summary of transplantation results. Note that only host embryos with donor-derived rhodamine$^+$ hematopoietic cells in the CHT were included here.

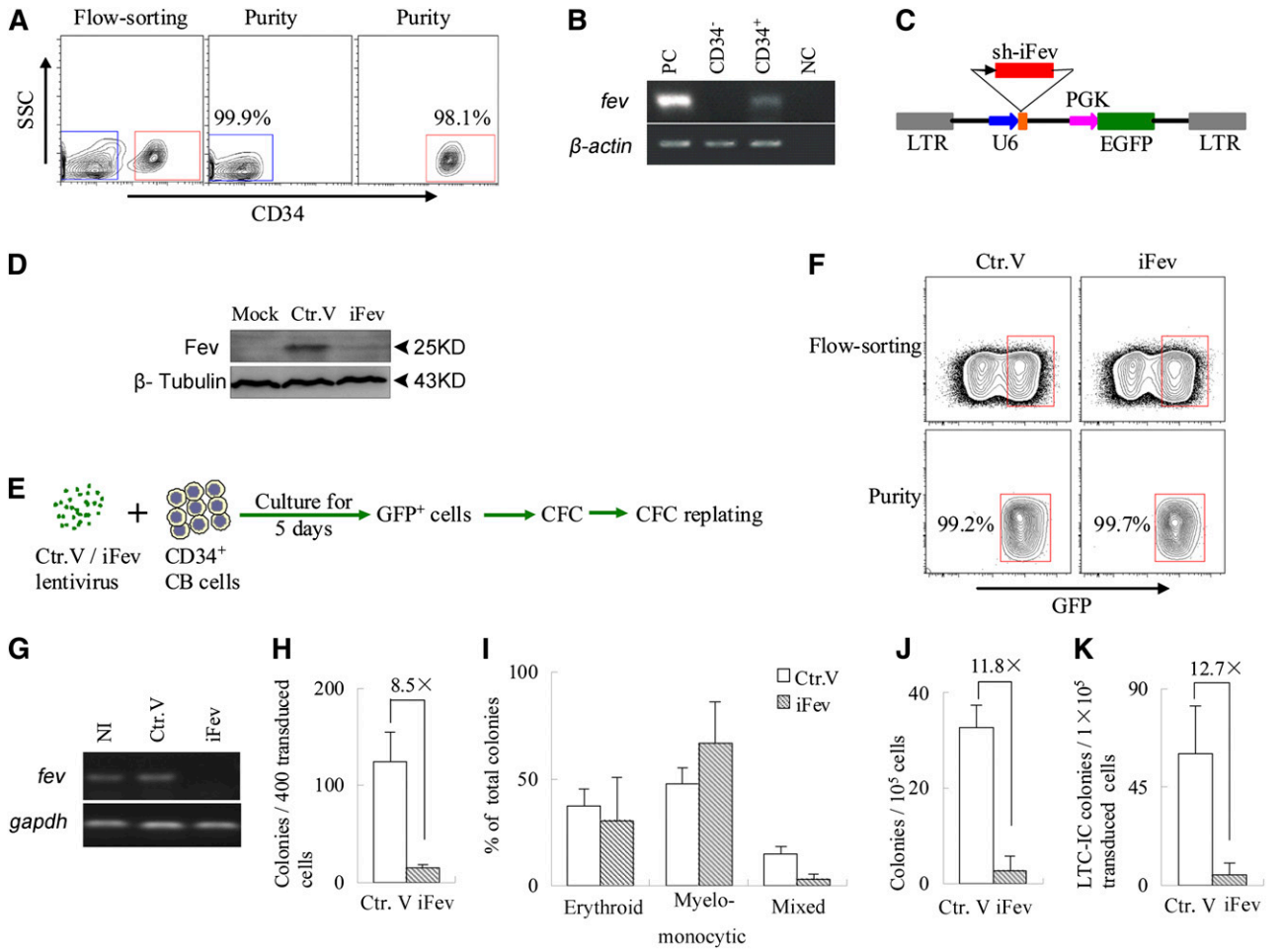


Figure 6. *fev* is expressed and functions in human primitive HSCs. (A) Flow-sorting and purity detection of $CD34^+$ and $CD34^-$ fractions from CD34-enriched CB cells. (B) RT-PCR detection of *fev* expression in fractions of (A). (C) Construction of *fev*-knockdown vectors (iFev or control virus [Ctr.V]). (D) Efficiency validation of *fev*-knockdown by Western-blot analysis in 293T cells permanently expressing fev. Mock: lysis of 293T cells. (E) The scheme of in vitro functional assays. CB $CD34^+$ cells transduced with Ctr.V or iFev lentivirus were cultured in stem cell expansion medium for 5 d and then GFP^+ cells were flow-sorted for CFC assay. (F) Flow-sorting and purity detection of GFP^+ cells from 5-d cultured cells of (C). (G) RT-PCR detection of *fev* in the flow-sorted cells of (D), indicating the efficiency of *fev* knockdown. (H) Results of methylcellulose CFC assay of Ctr.V and iFev cells. iFev cells had an 8.5-fold decrease in total colonies (124.5 ± 29.9 vs 16.4 ± 3.4, n = 5 independent experiments, $P = 0.0008$). (I) The proportion of progenitors in total colonies, including erythroid CFU-E (colony forming unit-erythroid), myelo-monocytic CFU-G (colony forming unit-granulocyte), CFU-M (colony forming unit-macrophage), and CFU-GM (colony forming unit-granulocyte, macrophage), and mixed CFU-GEMM (colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte) (n = 5 independent experiments; erythroid: $P = 0.4$; myelo-monocytic: $P = 0.06$; mixed: $P = 0.0006$). (J) Results of CFC replating of primary colonies of (F). iFev cells showed an 11.8-fold decrease in total colonies (32.7 ± 4.8 vs 2.8 ± 3.2, n = 5 independent experiments, $P = 0.003$). (K) Long time culture-initiating cell frequency of Ctr.V and iFev cells measured by limit-dilution assay. iFev cells showed a 12.7-fold decrease (60.5 ± 21.8 vs 4.8 ± 5.6, n = 5 independent experiments, $P = 0.004$). PC, positive control in HEK293T cells stably expressing *fev*; NC, non-template control.

Cell-autonomous regulation of HSC development by Fev

Fev expression in blood/ECs indicates that *fev* might act cell autonomously during HSC development (Figure 1A). To further determine whether Fev is required cell autonomously for specification of HSCs, we performed blastula transplant experiments. Donor cells labeled by rhodamine from *cmyb*:GFP transgenic embryos were transplanted at the blastula stage into nontransgenic recipients. A subset of rhodamine-labeled cells from the ventral marginal zone gives rise to GFP^+ HSCs in the recipients. Rhodamine fluorescence indicated that 12 of 49 wild-type recipient embryos had a few GFP^+ HSCs derived from control donor cells, whereas none of the donor cells from *fev* morphants gave rise to GFP^+ HSCs in the wild-type recipient embryos (Figure 5). Conversely, 14 of 44 recipient *fev* morphants had GFP^+ HSCs derived from control donor cells. We could not formally exclude the possibility that wild-type donor cells also repopulate the CHT niche, because we indeed noticed that there were a few *cmyb*:GFP^-Rhodamine$^+$ cells in this area, some of which were donor-derived GFP^-hematopoietic cells (Figure 5B light blue arrowheads; Figure 5C). Taken together, these data suggest that Fev is required for HSC development cell autonomously.

Fev is expressed and functions in purified human CB cells

Fev (also called Pet1 in mammals) is highly conserved in vertebrates.[19,20] Therefore we asked whether *fev* is expressed and functions in human HSCs. Umbilical vein CB cells were used to address the issue. CB $CD34^+$ cells have been well characterized as HSCs by in vitro colony-forming cell (CFC) assays.[32-34] Here, we used this assay to assess functional roles of *fev* in human hematopoiesis.

We first examined the expression profile of *fev* in CB cells using RT-PCR. CB cells were flow-sorted as $CD34^+$ and $CD34^-$ populations (Figure 6A). *fev* expression was consistently detected in $CD34^+$ but not in $CD34^-$ cells for all samples examined in this study (Figure 6B).

To determine the function of *fev* in CB cells, a lentiviral, vector-mediated, knockdown system was constructed (Figure 6C). The short

hairpin oligonucleotides of *fev* interference RNA (iFev) were cloned into the pLKO.1 lentiviral vector driven by a U6 promoter, and the expression was reported by enhanced GFP. Fev knockdown efficiency was validated by western blot in 293T-*fev* cells, which stably express *fev* protein (Figure 6D). We set up in vitro experiments as schemed in Figure 6E. CB $CD34^+$ cells were transduced with iFev or control virus and cultured for 5 d under a well-characterized condition for maintaining self-renewal potential of stem cells[33,35] before GFP^+ cells were flow sorted with high purity (Figure 6F). RT-PCR analysis indicated that *fev* expression was efficiently knocked down in iFev-transduced cells but not in control virus-infected cells (Figure 6G). The purified cells were inoculated for in vitro functional assays. A CFC assay showed that *fev* knockdown led to an ~8.5-fold reduction of total colony number (Figure 6H). Colonies were categorized into erythroid CFU-E, myelo-monocytic CFU-G, CFU-M, and CFU-GM, and mixed CFU-GEMM. Calculation of their proportions in total colonies showed that the primitive mixed CFCs were compromised more severely, whereas more mature cells were not significantly affected by *fev* knockdown (Figure 6I). CFC replating experiments suggested self-renewal defects in which reduction of colony-forming ability appeared to be more obvious when primary colony cells were collected and replated in new methylcellulose (Figure 6J). To further demonstrate this, we applied a quantitative limiting dilution analysis to evaluate the frequency of long time culture-initiating cells, the most primitive human progenitor assessable in vitro. We found that loss of *fev* reduced long time culture-initiating cell readout (Figure 6K). Taken together, these data imply that self-renewal of human primitive HSCs was impaired in the absence of Fev in vitro.

Discussion

In this study, we have shown that an ETS transcription factor, Fev (also called Pet1 in mammals), is required for HSC development and function. *fev* deficiency in zebrafish disrupted HSC emergence; therefore, the morphants had fewer T cells in the thymus. This phenotype was due to a cell-autonomous intrinsic defect in HSC specification. This effect is executed by a novel Fev-ERK signaling targeting the hemogenic endothelium-derived HSCs. Moreover, Fev is also expressed and functions in primitive HSCs in humans, indicating its conserved role in higher vertebrates.

During vertebrate embryogenesis, once the dorsal aorta is formed, a subset of ECs become specialized or "reprogrammed" with the expression of *runx1*, a pivotal transcription factor required for HSC generation.[36] This specialized region in the ventral wall of dorsal aorta is called "hemogenic endothelium" from which the earliest HSCs are derived through an EHT process.[1-3] Determining how the earliest HSCs arise from hemogenic endothelium and are maintained in adulthood is currently of great interest and may lead to new therapies. HSC fate decisions during development must be tightly controlled by both cell-intrinsic and -extrinsic regulatory mechanisms.[4,11] So far, Runx1 is the only known cell-intrinsic regulator of hemogenic endothelium-derived HSCs and is required for only the initiation of EHT, not before or thereafter.[36] Very recently, thrombin receptor, which is a prototypical G protein-coupled receptor, has been reported to negatively regulate the EHT process as a cell-extrinsic microenvironmental factor in mESCs and zebrafish.[37] Thrombin receptor inhibition might increase the rate of HSC induction through accelerating EHT.[37] Here, we demonstrated that loss-of-function of *fev* or *erk2* attenuates, while gain-of-function up-regulates, the expression of *runx1* specifically in the AGM, suggesting they are functionally upstream of *runx1*, and acts earlier than *runx1* during hemogenic endothelium formation. The reduced ECs in the dorsal aorta and the following decrease in HSC numbers in *fev* morphants or mutants further indicate that Fev-ERK signaling can promote both the number of ECs that are specified to HSC fate and HSC induction through EHT.

Previous studies showed that *fev* is expressed in serotonergic neurons and required for serotonin synthesis in mammals.[20] Serotonin is a monoamine neurotransmitter that has multiple functions in the central nervous system, gastrointestinal tract, and cardiovascular system.[38] In humans, serotonin can enhance ex vivo expansion of CB $CD34^+$ stem/progenitor cells, suggesting that serotonin might act as a paracrine factor to facilitate crosstalk between hematopoiesis and the neural system.[38] However, our data, including *fev* expression in hematopoietic tissues in zebrafish and purified $CD34^+$ CB cells in humans and blastula transplantation, strongly demonstrated that Fev acts cell autonomously to control HSC emergence through ERK signaling during the onset of definitive hematopoiesis. VEGF signaling has been reported to be an autocrine factor to regulate adult HSC survival in bone marrow.[39] Our previous work has also shown that in *Xenopus* as well as in zebrafish, VEGF signaling is essential for HSC emergence both cell autonomously and non-cell autonomously.[7,16] However, how the downstream core effector of VEGF signaling, ERK, regulates HSC development during vertebrate embryogenesis (for example, what are the direct target genes of ERK signaling in hemogenic endothelium and HSCs) remains to be determined.

In summary, the work presented here elucidates a novel role for *fev*, a previously uncharacterized ETS gene expressed in blood/vessels, which acts as a pivotal regulator of definitive HSC development in zebrafish and humans. In particular, our data reveal that Fev directly regulates ERK signaling during hemogenic endothelium formation and then HSC emergence, thus providing a new mechanism of how ETS factors control HSC development through a novel gene regulation. This provides some insights for new strategies to induce or expand transplantable HSCs in vitro or ex vivo for treatment of hematological diseases.

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Authorship

Contribution: L.W., T.L., L.X., Y.G., Y.W., and C.D. performed experiments; D.H. and F.L. designed the research, analyzed data, and wrote the manuscript; and G.-Q.C., S.L., R.P., and B.Z. participated in the preparation of the manuscript.

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