Hematopoietic stem and progenitor cells (HSPCs) are capable of producing all mature blood lineages, as well as maintaining the self-renewal ability throughout life. The hairy-like organelle, cilium, is present in most types of vertebrate cells, and plays important roles in various biological processes. However, it is unclear whether and how cilia regulate HSPC development in vertebrates. Here, we show that cilia-specific genes, involved in primary cilium formation and function, are required for HSPC development, especially in hemogenic endothelium (HE) specification in zebrafish embryos. Blocking primary cilium formation or function by genetic or chemical manipulations impairs HSPC development. Mechanistically, we uncover that primary cilia in endothelial cells transduce Notch signal to the earliest HE for proper HSPC specification during embryogenesis. Altogether, our findings reveal a pivotal role of endothelial primary cilia in HSPC development, and may shed lights into in vitro directed differentiation of HSPCs.
It has been well established that most vertebrate cells can transmit extracellular signals through a hair-like sensory organelle, called primary cilia. Primary cilia exist in many types of cells including endothelial cells (ECs), epithelia, fibroblasts, and others in vertebrates. Previous studies have revealed that primary cilia have specialized functions, in shear-stress sensation, chemo sensing, differentiation, proliferation, and maintenance of stem cells in a wide array of tissues. Thus, defects in ciliogenesis and function usually lead to ciliopathies, such as autosomal dominant polycystic kidney disease, obesity, and others.

In the cilia system, primary cilia contain a “9 + 0” axoneme (“9” denotes nine parallel doublet microtubules and “0” denotes absence of a central pair of microtubules (MTs)). An intracellular transport (IFT) system, including intracellular transport protein-88 (IFT88 or Polaris), is utilized to elongate MT axoneme. IFT88 knockdown (KD) led to a vascular impairment phenotype. Furthermore, a calcium channel protein, Polycystin1 (PKD1), is localized in cilia, and PKD1 conventional knockout mice are embryonically lethal at E15.5 due to vascular leakages and hemorrhage. Similarly, Polycystin2 (PKD2) KD also caused angiogenesis defects in zebrafish. Interestingly, a recent study demonstrated that primary cilia are present in the ECs of zebrafish blood vessels. Given that a subset of ECs in the dorsal aorta (DA) can develop into hemogenic endothelial cells (HE cells), it is tempting to speculate that the endothelial primary cilia in the DA may participate in HE specification.

During hematopoietic stem and progenitor cell (HSPC) development, HE cells produce HSPCs through the endothelio-hematopoietic transition (EHT) in vertebrates. Nonetheless, our understanding of the precise regulatory mechanisms involved in HE specification is still limited. The transcription factor runx1 is a widely used marker for HE cells at the early embryonic stage. Deficiency of Runx1 results in impairments of EHT and definitive hematopoiesis. Notch signaling is a critical regulator of runx1. In vertebrates, loss of one of Notch receptors, Notch1, causes a decrease of runx1 expression, which subsequently affects definitive hematopoiesis. Furthermore, in the absence of Notch ligands, the definitive hematopoiesis is also disrupted in jagged1 null mice and zebrafish mindbomb mutants. Notch signaling exerts complex regulation in HSPC development through divergent ligands and receptors, as well as multiple inputs. However, very little is known about the upstream factors of Notch signaling and how they initiate Notch activation. Intriguingly, it has been reported that Notch components localize in cilia and Notch signaling can be transmitted through cilia. However, it remains elusive whether cilia can transduce Notch signaling in controlling definitive hematopoiesis in vertebrates.

Here, we use the zebrafish as a vertebrate model and demonstrate that impairment of primary cilia formation or function leads to defects in HSPC development, especially in HE specification. Blocking primary cilia specifically in ECs causes the reduction of HE cells. Mechanistically, we uncover that Notch signaling functions downstream of endothelial primary cilia to specify HE cells properly. Altogether, our findings demonstrate that endothelial primary cilia modulate HSPC development through transducing Notch signaling.

Results

The dynamics of endothelial cilia during embryogenesis. To study the underlying link between cilia and hematopoiesis, primary cilia in the vascular ECs in the aorta- gonad-mesonephros (AGM) region, where the definitive hematopoiesis occurs, were characterized firstly. By visualizing a triple-transgenic line, Tg(βact:Arll13b-GFP/kdrl:mCherry/runx1:en-GFP), which marks cilia, ECs and hematopoietic cells (including HSPCs), respectively, we found that primary cilia were present in ECs in the AGM region at 28 h post fertilization (hpf) (Fig. 1a). Meanwhile, runxl1+kdrl+ HE cells were also ciliated (Fig. 1b), which was supported by analysis of another HE transgenic line, Tg(gfu1:GFP/βact:Arll13b–GFP), at 28 hpf (Fig. 1b). In contrast, the cmyb-labeled HSPCs in the AGM region were non-ciliated (Ac-tubulin labeled cilia) by fluorescence in situ hybridization (FISH) and Ac-tubulin staining (Fig. 1c). Time-course analysis of a double-transgenic line, Tg(βact:Arll13b–GFP/ kdrl:mCherry) showed that the number of primary cilia was reduced from 32 hpf and nearly absent at 52 hpf in the AGM region (Fig. 1d–f). The dynamic changes of primary cilia were consistent with previous observation of cilia in the caudal artery and caudal vein. Interestingly, the dynamic changes of endothelial primary cilia occurred between 24 and 52 hpf, which is the time window critical for HSPC emergence, indicating a possible relationship between HSPC development and primary cilia localized in ECs.

Identification of genes that regulate endothelial cilia. To functionally investigate the requirements of critical genes in ciliogenesis, we first performed KD experiments using antisense morpholino oligonucleotides (MOs) against cilia genes pkd2, kif3a, and ifi88, which have been shown to be involved in cilia formation and function, and a cilia gene fsd1, which is required for ciliogenesis. The efficiency of fsd1 AMO (translational blocking morpholino) and sMO (splice morpholino) was validated by western blotting and RT-PCR, respectively. The specificity of pkd2, kif3a, and ifi88 MOs was validated by GFP reporter assay. The EGFP expression was blocked by co-injection of corresponding MOs at one-cell stage, respectively (Supplementary Fig. 1A). As loss of cilia genes usually causes defects in the left-right asymmetry and body-curvature phenotypes in vertebrates, we first noticed abnormal spaw expression in the lateral plate mesoderm at 18-somite stage, as well as disordered heart looping upon fsd1 KD. In addition, body curvature was observed in the other three types of cilia-impaired embryos (Supplementary Fig. 1B and 1C), consistent with previous reports. The three-dimension (3D) ultrastructure of both endothelial primary cilia in the AGM region and motile cilia in pronephric duct (PD) were characterized by transmission electron microscope (TEM). A canonical “9 + 0” axoneme was observed in the AGM region and a canonical “9 + 2” axoneme was observed in the PD in both control and fsd1 morphants, indicating that the 3D ultrastructure of primary cilia was unaltered (Fig. 2a). Live confocal imaging of endothelial primary cilia in these morphants was performed using Tg(βact:Arll13b–GFP/kdrl:mCherry) double-transgenic line to further examine the cilia phenotype. The results showed that both the number and length of cilia were affected in fsd1 morphants (Fig. 2b–d and Supplementary Fig. 2A–F), as well as in pkd2, kif3a and ifi88 morphants (Fig. 2e, f), compared to controls. The above results demonstrate that cilia genes fsd1, pkd2, kif3a, and ifi88 are indeed required for ciliogenesis in zebrafish embryos.

Ciliogenesis is essential for HSPC development. As primary cilia are present in ECs in the AGM region (Fig. 1a, d–f), from where the earliest HSPCs are derived, our quantitative RT-PCR (qPCR) analysis showed that fsd1 was highly enriched in kdrl+runxl+ cells (HE cells; Supplementary Fig. 3A). These data together indicated a potential role of cilia in hematopoiesis. To test this hypothesis, we first detected the expression level of HSPC markers, runxl1 and cmyb, in cilia-impaired embryos. The decreased expression of HSPC markers was observed in fsd1 morphants at...
wild-type embryos. The cilia length presented in each embryo was the average length of all the cilia in the DA of the AGM region calculated per 200

Data represent the analysis results of one-way ANOVA

the blood

mCherry/+ activated cell sorting of dissected trunk region in Tg (β act:Arl13b–GFP/kdr1:EGFP) line at 28 hpf. White squares indicate the ECs with cilia. White bars denote DA or PCV region. DA dorsal aorta, PCV posterior cardinal vein. Scale bars, 10 µm. b The live confocal imaging of kdr+/runx1+/ or gfi+/ HE cells in Tg (β act:Arl13b–GFP/kdr1mCherry/runx1:en-GFP) (upper panel) or Tg (β act:Arl13b–GFP/gfi1:GFP) at 28 hpf (middle panel). The imaging of gfi+/ HE cells sorted by fluorescence-activated cell sorting of dissected trunk region in Tg (gfi1:GFP/β act:Arl13b–GFP) embryos (lower panel). Yellow arrowheads indicate primary cilia in kdr+/runx1+/ cells; white arrowheads indicate primary cilia in gfi+/ HE cells. Scale bars, 5 µm. c Fluorescence in situ hybridization (FISH) result showing the cmyb expression and Ac-tubulin staining showing the cilia in the aorta-gonad-mesonephros (AGM) region at 48 hpf. Yellow arrowhead indicates primary cilia and the white arrowhead indicates the cmyb+/ HSPC in the AGM region. The cmyb probe was used to examine cmyb expression in Tg (cmyb:EGFP) embryos by FISH. Scale bars, 20 µm. d The live confocal imaging of cilia with kdr1mCherry/β act:Arl13b–GFP double-transgenic line. White arrow denotes the blood flow direction. Scale bar, 5 µm. e The statistical data shows the primary cilia number (e) and length (f) in blood vessels in the AGM region in wild-type embryos. The cilia length presented in each embryo was the average length of all the cilia in the DA of the AGM region calculated per 200 µm. Data represent the analysis results of one-way ANOVA-Sidak test. Error bars, mean ± s.d., n = 10 embryos. ns non-significant, ***P < 0.001

Fig. 1 Ciliogenesis occurs in vascular endothelial cells (ECs) in AGM. a Three-dimension (3D) confocal imaging showing cilia on ECs in the AGM region using Tg (β act:Arl13b–GFP/kdr1mCherry/runx1:en-GFP) line at 28 hpf. White squares indicate the ECs with cilia. White bars denote DA or PCV region. DA dorsal aorta, PCV posterior cardinal vein. Scale bars, 10 µm. b The live confocal imaging of kdr+/runx1+ or gfi+ HE cells in Tg (β act:Arl13b–GFP/kdr1mCherry/runx1:en-GFP) (upper panel) or Tg (β act:Arl13b–GFP/gfi1:GFP) at 28 hpf (middle panel). The imaging of gfi+ HE cells sorted by fluorescence-activated cell sorting of dissected trunk region in Tg (gfi1:GFP/β act:Arl13b–GFP) embryos (lower panel). Yellow arrowheads indicate primary cilia in kdr+/runx1+ cells; white arrowheads indicate primary cilia in gfi+ HE cells. Scale bars, 5 µm. c Fluorescence in situ hybridization (FISH) result showing the cmyb expression and Ac-tubulin staining showing the cilia in the aorta-gonad-mesonephros (AGM) region at 48 hpf. Yellow arrowhead indicates primary cilia and the white arrowhead indicates the cmyb+ HSPC in the AGM region. The cmyb probe was used to examine cmyb expression in Tg (cmyb:EGFP) embryos by FISH. Scale bars, 20 µm. d The live confocal imaging of cilia with kdr1mCherry/β act:Arl13b–GFP double-transgenic line. White arrow denotes the blood flow direction. Scale bar, 5 µm. e The statistical data shows the primary cilia number (e) and length (f) in blood vessels in the AGM region in wild-type embryos. The cilia length presented in each embryo was the average length of all the cilia in the DA of the AGM region calculated per 200 µm. Data represent the analysis results of one-way ANOVA-Sidak test. Error bars, mean ± s.d., n = 10 embryos. ns non-significant, ***P < 0.001

36 hpf, but not in fsl1 mismatch morpholino (misMO)-injected embryos (Fig. 3a and Supplementary Fig. 3B). Consistently, the protein level of Runx1 was also decreased (Fig. 3b).

Furthermore, we generated an fsl1 mutant35 by CRISPR/Cas9 (with a 20-base-pair insertion in the 4th exon of fsl1, Supplementary Fig. 3C) and the reduced expression of runx1 and cmyb in fsl1 mutants (maternal-zygotic mutants, fsl1−/−) mimicked the defects in fsl1 morphants (Fig. 3c, d). To further verify that the HSPC defects were indeed specific to fsl1, we performed rescue experiments by overexpression of human full-length FSD1 mRNA (FSD1 hmRNA, escaping from fsl1 aMO targeting) in fsl1 morphants at one-cell stage. According to whole-mount in situ hybridization (WISH) results, we observed restoration of runx1 expression in FSD1 hmRNA-injected fsl1 morphants, compared to fsl1 morphants alone (Supplementary Fig. 4A).

To investigate whether fsl1 specifically in vascular ECs influences HSPCs, we constructed a plasmid in which the expression of fsl1 fused with an EGFP reporter was driven by ftia1 promoter (ftia1:fsl1-EGFP). Overexpression of fsl1 specifically in ECs could rescue the HSPC defects in fsl1 morphants (Supplementary Fig. 4B and 4C), suggesting that the observed HSPC defects were specific to fsl1 in ECs.

In addition, the other three cilia-dysfunction embryos (pkd2, kif3a, and ift88 morphants) showed similar HSPC defects (Fig. 3e). Furthermore, we injected a sub-effective dose of pkd2 (0.5 ng) or ift88 (0.8 ng) morpholinos, which did not affect HSPC development in wild-type embryos into the pkd2 or ift88 mutants38 to KD the residual mRNA of pkd2 or ift88, respectively (Supplementary Fig. 5A–F). As expected, we observed the decreased expression of runx1 at 36 hpf, compared to controls (Fig. 3f), suggesting that these ciliary genes are indeed required for definitive hematopoiesis.

To further demonstrate the role of cilia in definitive hematopoiesis, ciliobrevin D (CBD) treatment was performed. CBD is a AAA+ ATPase motor cytoplasmic dynein inhibitor and is used to block cilia function39,40. Wild-type embryos were treated with CBD from 10 to 31 hpf, and the results showed that cilia were unaltered (Fig. 4a-c). However, the expression of Notch target genes was reduced (Supplementary Fig. 6A), which suggests that CBD treatment is effective as previously reported40. WISH data showed that expression of runx1 in CBD-treated...
embryos was decreased compared with control group (Fig. 4d). These results imply that cilia are required for HSPC formation.

To gain further evidence that primary cilia in ECs affect HSPC development, impairment of cilia specifically on ECs of blood vessels was achieved by injection of the CRISPR vector fi88a:ift88-cKO (to genetically delete ift88 specifically in ECs) together with Tol2 mRNA (Supplementary Fig. 6B–D)40. Consistent with CBD treatment, the live confocal imaging data showed similar cilia defects (Fig. 4a–c) and the number of HE cells was decreased in endothelial primary cilia-dysfunction embryos (Fig. 4e). Collectively, these results support that cilia in ECs are essential for HSPC development.

Cilia are required for HE specification. To examine when and how exactly HSPC defects occurred in cilia-dysfunction embryos, we next focused on fsd1-deficient embryos. Primitive hematopoietic markers were examined by WISH at 24 hpf and their expression was relatively normal in fsd1 morphants (Supplementary Fig. 7), indicating that fsd1 is dispensable for primitive hematopoiesis. To determine the exact role of fsd1 in definitive hematopoiesis, we examined the time-course expression of runx1 and found that the runx1 expression was decreased from 26 hpf in fsd1 morphants, when HE specification normally occurred in control embryos (Fig. 5a). To confirm the phenotype mentioned above, we examined the other two HE markers, gfi1aa and gata2b. The expression of gfi1aa and gata2b was also decreased in fsd1 morphants at 26 hpf (Fig. 5a), suggesting that HE specification was disrupted in the absence of fsd1. Furthermore, HSPC derivatives such as erythroid cells (gata1), myeloid cells (pu.1) in the CHT, and T cells (rag1) in the thymus were all decreased at 4 days post fertilization (dpf) in fsd1 morphants (Fig. 5b). The defects of HE specification as well as HSPC derivatives were also observed in fsd1 sMO-injected embryos and fsd1 mutants (Supplementary Fig. 3B and 8; Figs. 3a and 5a). Moreover, the number of HE cells was quantified by counting the kdrlCherry+/runx1c:en-GFP+ or kdrlCherry+/cmyb:EGFP+ double positive cells in the AGM region at different developmental stages. In fsd1 morphants, the numbers of HE cells were significantly reduced compared to controls (Fig. 5d–g). Thus, these results support that fsd1 is required for HE specification. Similarly, loss-of-function of pdk2, kif3a, or ift88 and in particular, conditional deletion of ift88 in vascular ECs, all led to impaired HE specification (Fig. 5h–l). Taken together, we conclude that cilia defects in vascular ECs impair HE specification.

Given that HSPCs are originated from the DA and the vessel integrity/identity is essential for HSPC emergence, we next quantified the EC number within DA in the AGM region by using Tg(kdrlCherry/fl1a:nGFP) transgenic line (Supplementary Fig. 9A). In addition, expression of arterial markers dll4, ephrinB2a, venous markers msr, flt4, and also their niche cell markers kdr (pan-EC), myod (somite cell), and pax2.1 (PD) was examined (Supplementary Fig. 9B and 9C). The results showed that both the EC numbers in DA and expression of vessel marker genes were not altered in fsd1 morphants. Similarly, normal blood vessels were observed in the other three cilia defective embryos (Supplementary Fig. 9D–F), albeit with a slightly curved body
Runx1 in expression of HSPC marker blood vessels in alterations of cell proliferation and apoptosis were present in Fig. 10A in Tg(−/−) mutant embryos at one-cell stage. WISH results demonstrated that expression of HSPC markers runx1 and cmyb in DA. To address this issue, antisense MOs often induces a non-specific apoptosis. To explore the possibility that the HSPC defects observed in cilia-impaired embryos might be due to abnormal cell proliferation or apoptosis, we performed BrdU staining and TUNEL assay in Tg(−/−) embryos at 26 and 36 hpf (Supplementary Fig. 10E), indicating that the HE and HSPC emergence, we performed RNA-sequencing (RNA-Seq) analysis showing the expression of HSPC markers (runx1 and cmyb) in the aorta-gonad-mesonephros (AGM) region in control and fsd1 morphants at 36 hpf. The red arrowheads indicate the expression of HSPC markers runx1 and cmyb (red arrowheads) in the DA in fsd1−/−; with a sub-effective dose of pkd2 MO (0.5 ng per embryo, pkd2 MOlow) or ift88 MO (0.8 ng per embryo, ift88 MOlow). The red arrowheads indicate the expression of HSPC marker runx1. Scale bars, 100 µm.

Fig. 3 Loss of cilia genes induces hematopoietic stem and progenitor cell (HSPC) defects. a Whole-mount in situ hybridization (WISH) results of HSPC markers (runx1 and cmyb) in the aorta-gonad-mesonephros (AGM) region in control and fsd1 morphants at 36 hpf. The red arrowheads indicate the expression of HSPC markers runx1 and cmyb in DA. b Western blotting showing the protein level of Runx1 in control and fsd1 morphants at 26 hpf. WISH analysis showing the expression of HSPC markers runx1 and cmyb in the DA in fsd1−/−. c WISH analysis showing the expression of HSPC markers runx1 and cmyb in the DA in fsd1−/−; with a sub-effective dose of pkd2 MO (0.5 ng per embryo, pkd2 MOlow) or ift88 MO (0.8 ng per embryo, ift88 MOlow). The red arrowheads indicate the expression of HSPC marker runx1. Scale bars, 100 µm.

(C) Together, the results suggest that cilia deficiency did not grossly affect the development of blood vessels and neighboring niche cells.

To explore the possibility that the HSPC defects observed in cilia-impaired embryos might be due to abnormal cell proliferation or apoptosis, we performed BrdU staining and TUNEL assay in Tg(flila:EGFP) embryos at 26 and 36 hpf (Supplementary Fig. 10A–D), respectively. The results showed that no obvious alterations of cell proliferation and apoptosis were present in blood vessels in fsd1 morphants. As previously reported, injection of antisense MOs often induces a non-specific p53-dependent apoptosis. To address this issue, fsd1 aMO was injected into fsd1−/−; at one-cell stage. WISH results demonstrated that expression of runx1 was still decreased in fsd1 morphants (Supplementary Fig. 10E), indicating that the HE and HSPC defects were specifically due to fsd1 deficiency. Altogether, these observations support the role of cilia in HE specification, independent of artery formation, niche cells, and EC proliferation or apoptosis.

Cilia regulate HSPC emergence through Notch signaling. To investigate the underlying molecular basis of how cilia impact HSPC emergence, we performed RNA-sequencing (RNA-Seq) with the dissected trunk region from control and fsd1 morphants at 26 hpf. Gene ontology and volcano plot analysis showed that among many developmental pathways, Notch signaling was mainly downregulated in fsd1 morphants, compared to controls (Fig. 6a, b). qPCR analysis further confirmed dramatic reduction of a group of Notch target genes in fsd1 mutants at 26 hpf, when primary cilia defects occurred (Fig. 6c and Supplementary Fig. 2A–C). Previously, it has been reported that Notch-processing enzymes and receptors co-localized with cilia and cilia stimulated Notch signaling in vertebrates. Confocal imaging of the Notch activity reporter line Tg(tp1:mCherry/bact:Arl13b–GFP) showed that tp1+ ECs in the ventral wall of DA are ciliated (Supplementary Fig. 11A). Next, we asked whether Notch signaling is involved in the cilia regulation of HE specification. First, the reduced hey2 expression in DA of AGM region (Supplementary Fig. 11B) was observed. Western blotting analysis showed that the Notch intracellular domain (NICD) in fsd1−/− was decreased (Fig. 6d; Supplementary Fig. 11C). Second, to determine whether the Notch signaling in HE cells was altered, we examined the expression of notch receptor notch1a in kdr−/− runx1+ cells. The qPCR result showed that notch1a expression was decreased in HE cells (Fig. 6e), indicating that HE cells were sensitive to Notch.
Fig. 4  Blocking formation or function of primary cilia impairs hematopoietic stem and progenitor cell (HSPC) development. a Confocal imaging of cilia in the aorta-gonad-mesonephros (AGM) region in kdr/Cherry/pact:Art13b-GFP double-transgenic line with fil1a:ift88-cKO-functional injection, or ciliobrevin D (CBD) treatment at 28 hpf. White arrow denotes the blood flow direction. Scale bar, 5 μm. b, c The quantification of primary cilia number and length in the blood vessels of AGM in a. Data in b, c represent the analysis results of one-way ANOVA-Dunnett test. Error bars, mean ± s.d., n = 10 embryos. ns non-significant, * P < 0.05, **** P < 0.0001. d WISH result of runx1 (red arrowheads) in control and CBD-treated embryos at 31 hpf. The red arrowheads indicate the expression of HSPC marker runx1. Scale bar, 100 μm. e The kdr/Cherry+/cmyb:EGFP+ HE cells (white arrowheads) in the AGM region in fil1a:ift88-cKO-injected embryos (left panel) with quantification (right panel) at 36 hpf. Cmlc2:EGFP-negative embryos were used as a negative control (control). Scale bar, 50 μm. Error bars, mean ± s.d., n = 14 embryos. ** P < 0.01, Student’s t-test

Discussion
Cilia have been extensively studied in a variety of biological processes including cell cycle, mechanosensation, chemosensations, and left-right asymmetry, during embryogenesis and in adulthood. However, its role in developmental hematopoiesis is poorly understood. In this study, we have identified that endotheal primary cilia have essential roles in HE specification during HSPC development in zebrafish embryos. Loss of cilia genes decreased the number and length of primary cilia in blood vessels of the AGM region, which led to a subsequent reduction of Notch signaling. Overexpression of NICD restored the HSPC defects in cilia-impaired embryos. Therefore, loss of primary cilia hindered Notch signaling transduction, which subsequently caused HSPC defects.

Accumulating studies demonstrated that primary cilia are involved in defining stem cell phenotypes by maintaining their ‘stemness’. For example, primary cilia have been shown to influence the recruitment of mesenchymal stem cells (MSCs) through the regulation of transforming growth factor-β (TGFβ) signaling, and can also affect the process of stem cells differentiating into cardiomyocytes. Furthermore, Shh signaling regulates adult neuron stem cell formation through primary cilia. Although previous studies suggested the role of primary cilia in different types of adult stem cells in development, the exact regulatory process and the underlying mechanisms remain incomplete.

Here, our work initially defined a role of primary cilia, in HSPC emergence and the detailed analysis indicated that the bridge between cilia and HSPC development is mediated mainly through Notch signaling in the ECs of DA in zebrafish embryos.

Interestingly, HE specification defects in fsd1 morphants and mutants are independent of artery formation and vessel integrity.
We demonstrated that HE markers, including runx1, gata2b, and gfi1aa, were markedly downregulated, whereas the expression of artery markers dll4 and ephrinB2a, and the EC number of artery and veins remained unaltered. This phenomenon is consistent with recent reports, in which artery identity can be uncoupled from HE specification and HSPC emergence. We speculate that Notch signaling is differentially and dynamically required in these different processes, such as dll4 and ephrinB2a in artery formation and jagged1a in HSPC emergence. Once emerged, HSPCs can give rise to different blood cell lineages, and these processes are tightly controlled by a group of master transcription factors and signaling pathways. Notably, the coordination of cell intrinsic and extracellular signals has great impact on the HSPC formation and developmental hierarchy.

Fig. 5  Cilia are required for hemogenic endothelium (HE) specification. a Whole-mount in situ hybridization (WISH) analysis showing the expression of HE markers, runx1, gata2b, and gfi1aa, in the aorta-gonad-mesonephros (AGM) region at 22, 24, and 26 hpf. Red arrowheads denote the expression of HE markers. b Expression of erythroid marker (gata1), myeloid marker (pu.1) in the caudal hematopoietic tissue (CHT) and T cell marker (rag1) in the thymus at 4 dpf. Red arrowheads mark the corresponding hematopoietic cells. c The expression of HE marker gfi1aa, erythroid marker gata1, and T cell marker rag1 in fsd1−/−. Red arrowheads mark the corresponding hematopoietic cells. d The imaging of kdrCherry+/runx1en-GFP+ HE cells (white arrowheads) in the AGM region in control and fsd1 morphants with quantification (e) at 26 hpf. Error bars, mean ± s.d., n = 15 embryos. **P < 0.01, Student’s t-test. f Confocal imaging of HE cells (kdrCherry+/cmymEGFP+) in control and fsd1 morphants at 36 hpf. White arrowheads denote HE cells. g The quantification of kdrCherry+/cmymEGFP+ HE cells in control and fsd1 morphants at 36 hpf. Error bars, mean ± s.d., n = 10 embryos. ***P < 0.001, Student’s t-test. h, i Representative images of runx1 expression (red arrowheads) in control and cilia-impaired embryos with quantification (j) at 28 hpf. Red arrowheads denote runx1+ cells in the AGM region. j-i High-resolution imaging of HE cells (kdrCherry+/runx1en-GFP+) in control and cilia-impaired embryos with quantification (k, l). White arrowheads indicate kdrCherry+/runx1en-GFP+ cells in the AGM region. Data represent the analysis results of one-way ANOVA-Dunnett test. Error bars, mean ± s.d., n = 12, 9, 9, 9 embryos (i). n = 9, 9, 8, 8 embryos (k). n = 8, 10, 10, 9 embryos (l). ***P < 0.001, ****P < 0.0001. Scale bars, 100 μm.
The well-known communication mode between cells and their niches is mediated by the signaling ligand/receptor pairs. Here, we found that the hairy-like organelle, primary cilium, can also transduce developmental signals into the cells from their niche. In brief, primary cilia act as communication hubs to transduce Notch signaling to emerging HE cells and HSPCs. Although the cilia-transduced signals exert a significant influence on the cell cycle, organ formation, proliferation, differentiation, and embryonic development, the role of cilia in developmental hematopoiesis has never been studied before. Hence, this is the report to show how cilia modulate HSPC development in vertebrates.

The upstream regulatory factors of primary cilia in hematopoiesis are still unknown. Previous studies have shown that...
endothelial primary cell can mediate blood flow in heart 50, mouse hear 51, and zebrafish blood vessels 12. It was reported that blood flow is required for embryonic HSPC development 52,54. Furthermore, we and others recently showed that the Notch target gene (eprhinB2a) was downregulated in blood flow-deficient embryos 52,53. Therefore, it is reasonable to hypothesize that blood flow may lie upstream of primary cellia. Further investigation is required to validate this notion.

In summary, we demonstrated that the hairy-like organelle, primary cellium, is required for HE specification in HSPC development via regulation of Notch signaling. This finding further deepens our understanding on the role of cellia in HSPC development, and may provide insights into developing new strategies in generating functional HSPCs for clinical application.

Methods

Zebrafish strains. Adult Zebrafish including AB strain, Tg(cmlc2:GFP), Tg(fli1a:en-GFP), Tg(gfi1:GFP)55, Tg(fli1a:EGFP) (provided by Steve Wilson), Tg(kdr:mCherry), Tg(cmyb:EGFP), Tg(ptm:mCherry), Tg(fli1a:EGFP) (provided by Anning Meng), Tg(factr133-GFP)56, ifi88 mutant56, pkd2 mutant (provided by Chenglian Zhao), p53 mutant57 (provided by Anning Meng), and fsl1 mutant were maintained in 28.5 °C system water in the Institute of Zoology, Chinese Academy of Sciences. fsl1 mutant with a 20 bp insertion in the fourth exon of fsl1 was generated by CRISPR/Cas9, and the primers for genotyping are listed in Supplementary Table 1. The embryos were all natural spawning of adult zebrafish. This study was approved by the Ethical Review Committee in the Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

Morpholinos. MOs in this study were ordered from Gene tools and were injected into 1–2 cell-stage embryos at the boundary of yolk and cell. The sequences of MOs used in this study are listed in Supplementary Table 2.

Plasmid and mRNA synthesis. We constructed a fli1a:fsd1-EGFP plasmid, which expresses the capped fish fsl1 mRNA in EGS under the control of fli1a promoter within an EGFP reporter. The capped full-length CDS of zebrafish fsl1 was cloned into a pDONER221 plasmid through using Gateway BP reaction (Gateway BP Clonase II Enzyme mix, Invitrogen), and then sub-cloned into pDestToZ2pA2 with a fli1a promoter and an EGFP reporter by Gateway LR reaction (LR Clonase II Plus enzyme; Invitrogen). The full-length fsl1 mRNA (hmRNA) was synthesized from a pCS2 plasmid using M Message Machine SP6 kit (Ambion). The fli1a:fsd1-EGFP plasmid (30 pg) and fsl1 hmRNA (25 pg) were injected into one-cell stage embryos alone or in combination with fsl1 MOs. The GFP-NICD plasmid was generated by cloning the sequence encoding mouse NICD1, fused with GFP sequence in the N-terminal region, into the pCS2 vector.

The fli1a:fsd1-EGFP plasmid (30 pg) and fsl1 hmRNA (25 pg) were injected into one-cell stage embryos alone or in combination with fsl1 MOs. The GFP-NICD plasmid was generated by cloning the sequence encoding mouse NICD1, fused with GFP sequence in the N-terminal region, into the pCS2 vector.

The fli1a:fsd1-EGFP-CKO construct was provided by Massimo M. Santoro (University of Turin, Turin 10126, Italy). In this construct, the fli1a promoter was cloned into the pDestToZ2pA2-U6egRNA (guided RNA) (Addgene #63157) by the gateway system. Then the guide RNA-seq of fli88 was inserted into the above plasmid to generate the final fli1a:fsd1-EGFP-CKO vector58. We validated the efficiency of fli1a:fsd1-EGFP-CKO using T7E1 assay in sorted cellia cells59. We extracted the genomic DNA and amplified the target region by PCR. The PCR products were incubated at 37 °C for
Chemical treatment. Live zebrafish embryos were incubated with DB255 (8 μM, Sigma), CBD (Cisloibrevin D, 10 μM, Merck) from 10 hpf to the examined time point to ensure pharmacologic effect.

Whole-mount in situ hybridization. Whole-mount in situ hybridization (WISH) was performed through standard procedure60. The following probes, including runx1, cmyb, gata2b, gfi1a, gata1, pu.1, sfl, rog1, hey2, ephrinB2a, dll4, msr, flt4, myod, kdr, and pax2.1 were used in this study18,27,59. Nikon SMZ 1500 microscope was used to collect figures.

Western blotting. The dissected trunk regions of zebrafish embryos were collected for extracting protein. Western blotting was performed60 with 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were then transferred into a nitrocellulose membrane and then membranes were blocked by nonfat milk. After blocking, the nitrocellulose membranes were then incubated using anti-Fd1 antibody (Ab) (1:200, monoclonal mouse or polyclonal rabbit anti-FSD1 antibody) was generated using a glutathione-S-transferase fusion protein containing the full-length FSD1, which were expressed in E. coli and then were purified through homegeneity as the antigen. The monoclonal mouse Ab was used in human RPE-1 cells, while the polyclonal rabbit Ab was used in zebrafish experiments), anti-Runx1 Ab (1:200, AS-55593, Ana Spec), anti-NICD Ab (1:1000, ab8923, Abcam, used in human cells), mouse anti-β-actin (1:1000, sc73778, Santa Cruz Biotechnology, used in human cells), and anti-β-actin Ab (1:2000, 4967, Cell Signaling). The figures for western blotting were collected by ImageQuant LAS 4000 (The unscraped uncropped of blots are listed in the Supplementary Figure 12).

Cell sorting and quantitative RT-PCR. ECs, HE cells, and HSCPs from the dissected trunk region of kdr/mCherry/runx:1::GFP transgenic embryos at 26 hpf were sorted by fluorescence-activated cell sorter60. Total RNAs from the sorted cells of wild-type embryos and fsd1 morphants were extracted using QIAGEN RNeasy Micro kit and then were transcribed. Total RNA from the dissected trunk regions was extracted with TRIzol reagent (Ambion) and reversely transcribed. The cdNA was diluted and used for templates. The qPCR was performed52, and the primers used for qPCR are shown in Supplementary Table 4.

RNA-seq. Dissected trunk region of wild-type embryos and fsd1 morphant at 26 hpf were collected for extracting RNAs. The RNAs were then reversely transcribed and amplified cdNA were sequenced on a BGISEQ-500 in Beijing Genomic Institution.

Confocal microscopy. Embryos were mounted on dishes with 1% low-melting agarose. Live embryos were anesthetized before mounting. Confocal images were captured by a Nikon A1 confocal laser microscope. The analysis of the images was carried out by Nikon confocal software, Image J and Imaris.

BrDU assay and TUNEL staining. BrDU (10 mM) was injected into the yolk of flt1a:EGFP transgenic embryos. The embryos were fixed by 4% PFA 2 h later and then treated with standard procedure60. Briefly, the embryos were washed three times with 1× PBST 5 min each and then treated with Proteinase K (10 μg/mL) and 2 N HCl. After blocking with 1% BSA for 1 h, the embryos were incubated with anti-BrDU antibody (1:800, 5-Bromo-2′-deoxyuridine Labeling and Detection Kit, Roche). Before imaging with the confocal microscope, the embryos were washed and incubated with anti-mouse-Ig-fluorescein (1:500, 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit, Roche).

TUNEL staining was performed52. In brief, embryos were fixed by 4% PFA for at least 12 h at 4 °C and then were dehydrated with methanol for at least 4 h at 20 °C. Then the dehydrated embryos were washed three times with 1× PBST 5 min each and then treated with Proteinase K treatment. The embryos were refixed by 4% PFA at room temperature for 20 min and washed three times with 1× PBST 5 min each. The embryos were then incubated in 50 μL mixture (In Situ Cell Death Detection Kit, Fluorescin; Roche) overnight at 4 °C. Finally, the embryos were washed three times with 1× PBST 5 min each and then were captured by confocal microscope.

Transmission electron microscope. Transmission electron microscope (TEM) was performed in control and fsd1 morphants at 26 hpf. In brief, zebrafish embryos were fixed by 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS, pH 7.2) and were then postfixed by 1% osmium tetroxide for 2 h. After washing by ethanol with different gradients, embryos were dehydrated with different gradients of acetone and epoxy resin. Then, embryos embedded in resin were ultrathin-sectioned (60 nm) and the sections were mounted on copper slot grids for further JEM1400 electron microscope imaging.

Cell culture. Human hNTERT-RPE-1 (RPE-1) were cultured in DMEM/F-12 (1:1) medium, supplemented by 10% FBS, 1% penicillin/streptomycin B and 0.01 mg/mL hygromycin. RPE-1 cells were then serum starved for 48 h in Opti-MEM reduced serum media (Life Technologies) for cell generation.

Immunofluorescence. RPE-1 cells were transfected with GFP-NICD plasmid with Lipofectamine LTX Reagent (Life Technologies) according to the manufacturer’s instruction in RPE-1 cells. 6 h post transfection of GFP-NICD plasmid in RPE-1 cells, the medium was changed. RPE-1 cells were transfected with control (siCtrl) or FSD1 siRNA (siFSD1) at 24 h post plasmid transfection. 24 h post siRNA transfection, the RPE-1 cells were starved for 48 h. The cells were then fixed with 4% paraformaldehyde for 10 min at room temperature. After blocking, the RPE-1 cells were incubated with mouse anti-Ac-tubulin (1:400, T0679, Sigma) Ab. The DNA was stained with Hoechst 33342 (1:500, H3570, Invitrogen). The RPE-1 cells were then incubated with second antibodies. The images were captured by Zeiss LSM 880 and were analyzed with Volocity 6.0. The sequences of siRNAs are shown in Supplementary Table 5.

Statistical analysis. Student t-test, one-way ANOVA and two-way ANOVA were used in the statistical analysis. The statistical results were analyzed by GraphPad Prism 6.01 software. Data were represented as mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The RNA-seq data presented in the paper were deposited at NCBI SRA database under accession number SRP140462. Source data is provided as a Source Data file. All the relevant data supported this study are available from the corresponding author upon request.

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Author contributions
Z.L. performed experiments with help from L.W., Y.X. and D.M.; H.T. and H.L. provided Fsd1 antibody and performed experiments in cell culture; Y.K. and C.Z. provided reagents; Z.L. and F.L. conceived the project, analyzed the data, and wrote the paper. All authors read and approved the final manuscript.

Additional information
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