A blood flow–dependent *klf2a*-NO signaling cascade is required for stabilization of hematopoietic stem cell programming in zebrafish embryos

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Blood flow has long been thought to be important for vessel development and function, but its role in HSC development is not yet fully understood. Here, we take advantage of zebrafish embryos with circulation defects that retain relatively normal early development to illustrate the combinatorial roles of genetic and hemodynamic forces in HSC development. We show that blood flow is not required for initiation of HSC gene expression, but instead is indispensable for its mainte- nance. Knockdown of *klf2a* mimics the *silent heart* (*sih/tnnt2a*) phenotype while overexpression of *klf2a* in *tnnt2a* mor- phant embryos can rescue HSC defects, suggesting that *klf2a* is a downstream mediator of blood flow. Furthermore, the expression of NO synthase (*nos*) was reduced in *klf2a* knockdown embryos, and ChIP analysis showed that endoge- nous Klf2a is bound to the promoters of *nos* genes in vivo, indicating direct gene regulation. Finally, administration of the NO agonist S-nitroso N-acetylpenicilla- mine (SNAP) can restore HSC develop- ment in *tnnt2a* and *klf2a* morphants, sug- gesting that NO signaling is downstream of Klf2a which is induced by hemody- namic forces. Taken together, we have demonstrated that blood flow is essential for HSC development and is mediated by a *klf2a*-NO signaling cas- cade in zebrafish. (*Blood*. 2011;118(15): 4102-4110)

Introduction

The origin and migratory path of definitive HSCs has been delineated in zebrafish embryos, and is highly conserved in higher vertebrates.[1-5] In zebrafish, HSCs are derived from the ventral wall of the dorsal aorta via an endothelial-hematopoietic transition (EHT), migrate to the caudal hematopoietic tissue (CHT), and finally home to definitive hematopoiesis sites in the thymus and kidney to produce T cells and all other blood lineages during adulthood.[3,6-9] The specification and maintenance of the HSC program during early embryogenesis are tightly controlled by a complex of extrinsic and intrinsic signaling.[3,6] However, the interplay between intrinsic genetic factors and local hemodynamic cues has not been fully understood. For example, it remains to be addressed how hemodynamic factors contribute significantly to the remodeling of artery and vein and then HSC development, and how physiologic factors are integrated with genetic factors. Most relevant knockout mice die of circulation defects before HSC emergence in the aorta-gonad-mesonephros (AGM).[10] Therefore, it is difficult to separate the roles played by genetic and physiologic factors during artery-vein specification and then HSC development.

Recent evidence suggests that NO is responsible for HSC development in zebrafish and in mice. By chemical screening, North et al found that a group of chemical blood flow modulators can regulate HSC induction in zebrafish, mainly via flow-induced NO signaling, which is consistent with the finding reported in mice.[11,12] However, how blood flow exerts its effect on the endothelial NO signaling is still obscure. Interestingly, the endothe- lial gene *klf2*, an immediate early responder to blood flow, has been demonstrated to be involved in blood vessel remodeling and heart valve development in zebrafish.[13-15] Thus, an attractive hypothesis would be that flow-induced *klf2* expression regulates NO signal- ing[16] and thereby HSC development.

Here, we took advantage of available zebrafish blood flow– deficient embryos *silent heart/tnnt2a*, *heart-of-glass/heg*, and *santa/ krit1* combined with chemical treatment, to test this hypothesis in a comprehensive manner. Our findings show that blood flow is not required for the initiation of HSC gene expression, but is essential for its maintenance in zebrafish. Blood flow–induced expression of *klf2a*, a zinc-finger transcription factor, directly regulates the NO signaling pathway, and thereby controls artery/vein maturation and continued HSC programming.

Methods

Fish strains and embryos

Zebrafish embryos were obtained by natural spawning of adult AB strain zebrafish. Embryos were raised and maintained at 28.5°C in system water and staged as described.[17] *fli1a*:GFP transgenic line was kindly provided by Steve Wilson (King's College London, London, United Kingdom). The *flk1*GFP/*gata1*:dsRed transgenic line was kindly provided by Stefan Schulte-Merker (Hubrecht Institute, Utrecht, The Netherlands). *fli1a*:nGFP line and *moonshine* were kindly provided by Anming Meng (Tsinghua University, Beijing, China). This study was approved by the Faculty of Clinical Medicine, Local Ethical Review Committee, University of Oxford, and the Ethical Review Committee in the Institute of Zoology, Chinese Academy of Sciences, China.

Morpholinos, mRNA synthesis, and microinjection

Standard morpholinos (MOs) and antisense MOs were purchased from GeneTools and prepared as 1mM stock solutions using dH$_2$O. Standard MOs were used as negative controls in all MO injection experiments alongside with gene-specific MOs. The sequences of gene-specific MOs are described in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Capped fish *klf2a* full-length mRNA for injection was synthesized from *Not*I-digested pCS2 expression plasmid using the mMessage mMachine SP6 kit as instructed by the manufacturer (Ambion). For fish embryo injections, MOs (2-10 ng) and capped mRNA were injected alone or in combination, into 1-2 cell-stage zebrafish embryos at the yolk/blastomere boundary.

Chemical treatment

NO staining was performed by incubating zebrafish embryos with 5μM cell-permeant DAF-FM-DA (diamino-fluorophore 4-amino-5-methylamino-2′-7′-difluoro-fluorescein diacetate; Molecular Probes) for 2 hours in the dark at 28°C.[18] Zebrafish embryos were placed in the NO-donor SNAP (S-nitroso N-acetylpenicillamine; Sigma-Aldrich) from bud stage at a concentration of 10μM in 0.1% DMSO. During the next 24 hours, the embryos were examined periodically to ensure that the pharmacologic effect remained constant over time.[19]

In situ hybridization

Whole-mount in situ hybridization (WISH) for zebrafish embryos was performed as described previously with probes including *scl*, *gata1*, *runx1*, *cmyb*, *dll4*, *dlc*, *gata1*, *klf2a*, *nos1*, *hif2α*, and *rag1*.[20]

Transverse sectioning

The embryos were mounted with the JB-4 Embedding Kit (Polysciences) before sectioning with a Leica RM2255 vibratome into 5- to 10-μm thick slices.

Quantitative RT-PCR

Total RNA from the trunk region of control and MO-injected embryos (20-40 embryos pooled for each sample) at 24 and 36 hours postfertilization (hpf) was extracted by TriReagent and was reversely transcribed. The cDNAs were all diluted 5 times to be used as templates. The PCR primers used and the expected product length are summarized in supplemental Table 2. The GoTaq qPCR Master Mix (Promega) was used in quantitative PCR on the Bio-Rad CFX96 Real-Time PCR system. All the experiments were repeated 3 times with triplicates. We analyzed the data using the ΔΔCt method. Data were represented as mean ± SD and *t* test was performed for comparison between control and experimental groups.[21] $P < .05$ indicates significant difference.

TUNEL assay

Embryos were dechorionated and fixed in 4% paraformaldehyde overnight at 4°C then washed with PBS buffer twice and permeabilized with 0.1% sodium citrate and 0.1% Triton X for 2 minutes on ice. After washing 3 times with PBST for 5 minutes each and incubated with 90 μL of labeling solution plus 10 μL of enzyme solution (In Situ Cell Death Detection Kit, Fluorescein; Roche) at 37°C for 4 hours or at 4°C overnight. They were washed 3 times with PBST for 5 minutes each and the images were examined by confocal microscopy.

ChIP assay

ChIP analysis was carried out with wild-type embryos of 36 hpf. Embryos were lyzed using lysis buffer (10mM Tris-HCl pH 8.0, 10mM NaCl 0.5% NP-40) and Klf2a polyclonal Ab (Millipore) were used for immunoprecipitation with rabbit serum as negative control. After extensive washing, the DNA was eluted using elution buffer (25mM Tris-HCl, 10mM EDTA, 0.5% SDS) and the eluted DNA was assayed by PCR. The primers specific and unspecific to the Klf2 binding site in the upstream regions of *nos1*, *nos2a*,

and *nos2b* were designed by searching for the conserved CACCC box and the primers unspecific to the Klf2-binding sites were included as negative controls. At least 3 pairs of specific primers and one pair of control primers for each gene were used in ChIP PCR. The primers and the expected product length were summarized in supplemental Table 3. The PCR program was as follows: 94°C, 5 minutes; 94°C, 30 seconds; 55°C, 30 seconds; 72°C, 15 seconds, 35 circles (nos2b-3F/R with 28 circles); 72°C, 10 minutes. All the PCR products were ∼ 200 bp and were evaluated on a 2% agarose gel.

Western blot analysis

Zebrafish embryos at 36 hpf were washed with fish water and the trunk region was dissected out after manual dechorionation, then homogenized with 1-mL syringe and needle in lysis buffer (10mM Tris-HCl pH 8.0, 10mM NaCl 0.5% NP-40) containing protease inhibitor (Roche) at 1× concentration (1 tablet in 2 mL of redistilled water as 25× concentration) for 30 seconds. Lysate was centrifuged at 12 000*g* for 2 minutes at 4°C and the resulting supernatant was loaded as protein samples. After 12% SDS-PAGE separation, proteins were transferred to nitrocellulose membranes. After blocking with 5% nonfat milk for 3 hours, nitrocellulose membranes were incubated with Klf2a polyclonal Ab (1:1000; Millipore) or β-actin Ab (1:1000; Cell Signaling) overnight. Membranes were washed in 1× TBST (Tris buffer solution plus Tween 20: 136.8mM NaCl, 24.8mM Tris, 0.5% [v/v] Tween 20, pH7.4) 3 times with each for 15 minutes, and incubated for 3 hours with anti–rabbit secondary Ab (1:5000; Jackson ImmunoResearch Laboratories). After extensive washing membranes were detected by a chemiluminescent HRP substrate (Millipore).

Confocal microscopy

Confocal images were acquired with a Zeiss LSM 510 META confocal laser microscope and 3-dimensional (3D) projections were generated using Zeiss LSM software (Carl Zeiss Inc).[20]

Results

Primitive hematopoiesis is normal in *tnnt2a* morphants

To address whether primitive hematopoiesis is affected in the absence of flow, we used 2 different methods in *silent heart* (*sih/tnnt2a*) embryos which have no heartbeat and therefore no circulation.[22] First, we applied a *flk1*:GFP/*gata1*:dsRed double transgenic line. *flk1*:GFP marks all endothelial cells in blood vessels, while *gata1*:dsRed marks erythroid lineage derived from primitive wave of hematopoiesis during embryogenesis. As shown in Figure 1B, both endothelial cells and RBCs developed normally in *tnnt2a* morphants, albeit the latter were accumulated in the trunk region because of lack of heartbeat. Second, we examined expression of primitive hematopoietic markers, *scl* (stem cell leukemia), a primitive hematopoietic progenitor marker, *gata1*, an erythroid marker, by WISH. Expression of *scl* and *gata*1 in the intermediate cell mass (ICM), the primitive hematopoiesis site in zebrafish embryo, was relatively normal at 24 hpf in *tnnt2a* morphants, compared with that in controls (Figure 1C-F). Taken together, these experiments demonstrate that primitive hematopoiesis is not altered in flow-deficient *tnnt2a* morphants.

HSC programming is initiated but not maintained in *tnnt2a* morphants

To determine whether blood flow is required for HSC development, we examined *runx1* expression in *silent heart* (*sih/tnnt2a*) embryos (Figure 2A-D).[22] At 24 hours, *runx1* expression in the dorsal aorta region was normal in *tnnt2a*MO-injected embryos, compared with
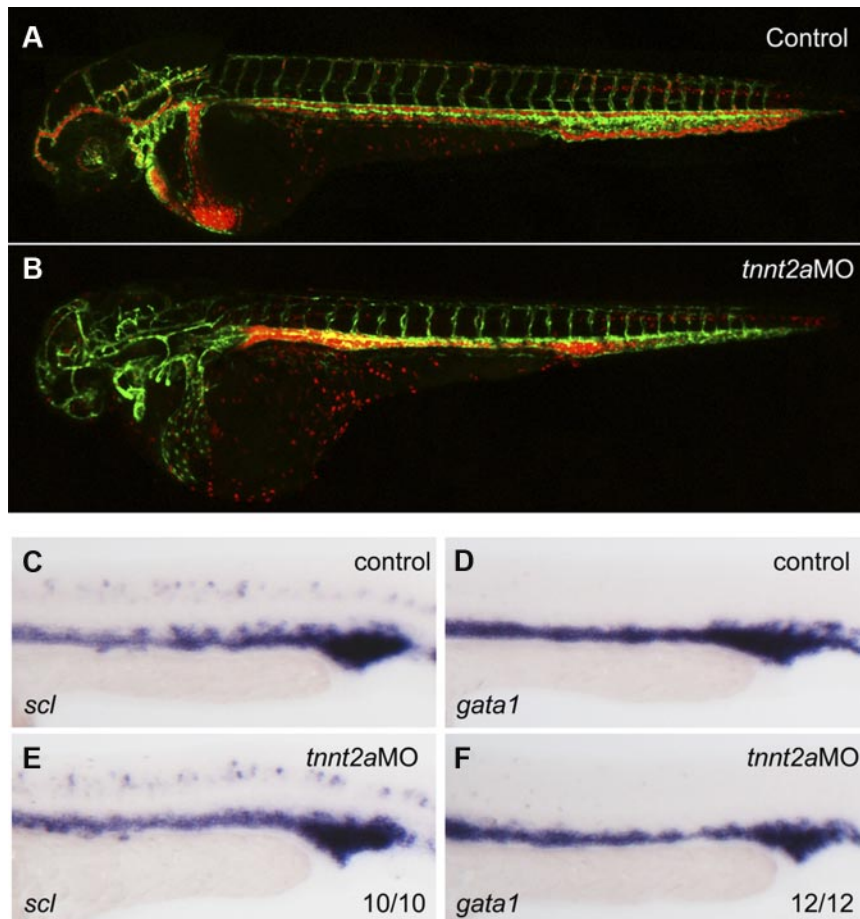
Figure 1. Primitive hematopoiesis is normal in *tnnt2a* morphants. (A-B) Confocal images of control MO (A) and *tnnt2a*MO (B) injected *flk1*:GFP/*gata1*:dsRed transgenic embryos at 2 dpf. (C-F) *scl* and *gata1* expression in the intermediate cell mass (ICM) was normal in *tnnt2a* morphants at 24 hpf. Anterior is to the left and dorsal is up.

that in the controls, suggesting that the HSC program was initiated normally (Figure 2B). However, from 36 hpf onward, *runx1* expression was not maintained in *tnnt2a* morphants (Figure 2D). Quantitative RT-PCR analysis showed that *runx1* expression in the trunk region was comparable between control and *tnnt2a*MO embryos at 24 hpf (Figure 2K), then subsequently decreased at 36 hpf in *tnnt2a*MO embryos (Figure 2L), further supporting the WISH results. TUNEL assay showed that there were more apoptotic cells in the *tnnt2a* morphants (supplemental Figure 1), potentially at least partly explaining the down-regulation of HSC genes.

To examine whether HSCs or their derivatives appear in the CHT or thymus/kidney, we performed WISH with, *cmyb,* a conserved HSC marker, and *rag1,* a T-cell marker, in *tnnt2a* morphants (Figure 2E-J). Similar to *runx1*, *cmyb* expression in the dorsal aorta was also down-regulated in *tnnt2a*MO-injected embryos (Figure 2F). Expression of *cmyb* was significantly decreased in the CHT at 2 days postfertilization (dpf), when HSC derivatives can first be detected there,[1,2] and onward (Figure 2H arrow; data not shown). Expression of the thymic T-cell marker, *rag1*, was also nearly abolished (Figure 2J). These results show that the loss of HSCs in the dorsal aorta seen in the absence of blood flow is permanent. Taken together, these data indicate that blood flow in the trunk is critical for maintenance of HSC programming during zebrafish embryogenesis.

Two other heartbeat defective morphants mimic the phenotype of *tnnt2a*MO-injected embryos

To confirm the role of blood flow in HSC development, we applied alternative methods to block heartbeat and confer circulation

defects. *heart of glass/heg1* and *santa/krit1* encode 2 proteins that are vital for patterning concentric growth of the heart.[23,24] Loss of function of these 2 proteins individually, by mutations or MO injection, leads to heartbeat defects and virtually no circulation in the body. In *heg1* and *krit1* morphants, expression of *runx1* and *cmyb* was relatively normal at 24 hpf (supplemental Figure 2), but significantly decreased in the AGM region at 36 hpf (Figure 3B,C,E,F). In addition, *cmyb* expression in the CHT and *rag1* expression in the thymus were also down-regulated or even abolished in these 2 morphants (Figure 3H,I,K,L), compared with controls. We therefore conclude that when circulation was inhibited by 3 different perturbations, that is, *tnnt2a*, *heg1*, and *krit1* morphants, the HSC program was not maintained in the DA in zebrafish.

Arterial development is disrupted in *tnnt2a*-deficient embryos

Because the dorsal aorta is thought to be the source of the first definitive HSCs,[7,8] we assessed arterial development in *tnnt2a*-deficient embryos. Time-course studies showed that the lumen was formed at 24 hpf in *tnnt2a*MO-injected embryos, but shrunk after 30 hpf (supplemental Figure 3; Figure 4G-H), which coincides with simultaneously down-regulated expression of 2 arterial markers, that is, early specification marker, *dll4* and late differentiation marker, *ephrinB2* (Figure 4A-F). Quantitative RT-PCR analysis (Figure 4I-J) with the dissected trunk region of control and *tnnt2a*MO embryos at 24 and 36 hpf, respectively, further supports that *dll4* expression in the dorsal aorta was specifically decreased, as shown by WISH results. Thus, the smaller lumen did not result merely from lack of inflation but apparently from a reduced number
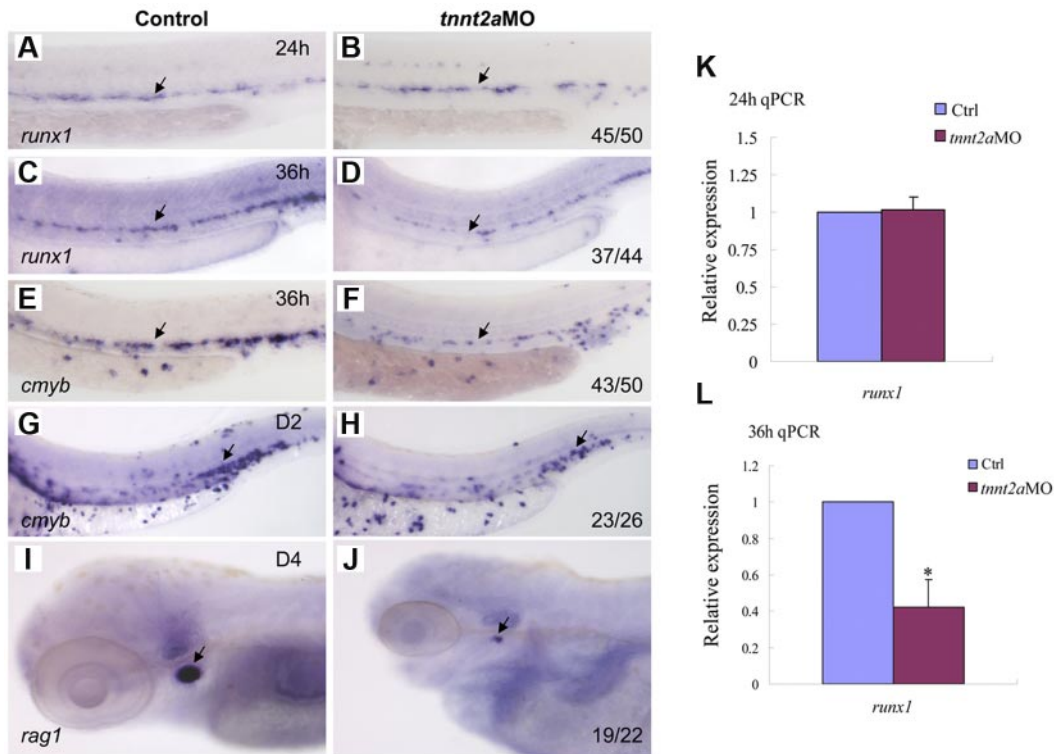
Figure 2. Blood flow is not required for the initiation of HSC gene expression, but is essential for its maintenance in zebrafish. Expression of *runx1* and *cmyb* at 24 and 36 hpf in control MO (A,C,E), *tnnt2a*MO (B,D,F) injected embryos. Arrows mark *runx1* and *cmyb* expression in HSCs. (G-J) The maintenance and homing of HSCs were affected in *tnnt2a* morphants. Expression of *cmyb* in CHT in control MO (G) and *tnnt2a*MO (H) injected embryos at 2 dpf. Expression of *rag1* in thymus in control MO (I) and *tnnt2a*MO (J) injected embryos at 4 dpf. (G-H) Arrows indicate CHT. (I-J) Arrows indicate thymus. Anterior is to the left and dorsal is up. CHT, caudal hematopoietic tissue. (K,L) Quantitative RT-PCR analysis of *runx1* and *cmyb* expression in the dissected trunk region of control and *tnnt2a*MO embryos at 24 and 36 hpf (mean ± SD, t test, *$P <$ .05, n = 3).

of endothelial cells in the DA (supplemental Figures 3-4). In mammals, HSCs are kept quiescent in the hypoxic niche, the endosteal zone of the BM.[25] To test whether the heartbeat stop causes hypoxia in *tnnt2a*MO injected embryos, we examined *hif2α* expression but did not observe any alteration, suggesting no involvement of hypoxia (data not shown). Taken together, these data support that blood flow affects arterial cell maturation as well as HSC formation in zebrafish.

klf2a is a mediator of blood flow in the control of artery and HSC development

In vitro cell-culture studies showed that a kruppel-like transcription factor, *klf2*, is an immediate mediator of hemodynamic forces created by blood flow.[14,26-29] Klf2 regulates flow-dependent endothelial phenotypes in cultured endothelial cells from zebrafish and mice.[14,30] Mice lacking *klf2* die during embryogenesis, defective in assembly of the vessel wall, which leads to massive hemorrhaging.[31] Zebrafish *klf2a* is expressed in trunk vessels and pronephric ducts during embryogenesis (supplemental Figure 5).[32] Zebrafish *moonshine* (*mon*) mutant has no red blood cells during embryogenesis.[33] *klf2a* expression in *mon* embryos is similar to that in controls, further suggesting its specific expression in endothelial cells (Figure 5C).

To determine whether zebrafish *klf2a* expression is affected by blood flow, we monitored *klf2a* expression in *tnnt2a*MO-injected embryos. As shown in Figure 5B, *klf2a* expression specifically in the vessel was significantly decreased in *tnnt2a*MO-injected embryos (red arrow), while the strong *klf2a* expression in the cloaca was not altered (Figure 5B, blue arrow). This observation was further supported by quantitative RT-PCR and Western blot of

dissected trunk regions (Figure 5D-E), in which *klf2a* expression in the trunk region was down-regulated in *tnnt2a*MO-injected embryos compared with controls. These data strongly suggest that *klf2a* may be a downstream effector of blood flow. To address whether *klf2a* is involved in HSC development, we examined *runx1* and *cmy*b expression in the AGM and/or the CHT in *klf2a*-deficient embryos generated by antisense MO knockdown. Similar to *tnnt2a* knockdown, *runx1* expression in the AGM was only modestly changed at 24 hpf (Figure 6B) but was significantly reduced at 36 hpf (Figure 6D) in *klf2a*-deficient embryos. *cmy*b expression in the AGM and CHT was also attenuated, respectively (Figure 6F,H). Quantitative RT-PCR analysis of *runx1* and *ephrinB2* expression in the dorsal aorta of control and *klf2a*MO embryos at 24 and 36 hpf was consistent with the WISH data (Figure 6K-L, supplemental Figure 6). To further confirm that HSC numbers were reduced, we monitored expression of the thymic T-cell marker, *rag1,* and found that it was nearly abolished in *klf2a*MO-injected embryos (Figure 6J). Moreover, overexpression of *klf2a* mRNA partially rescued *runx1* and *dll4* expression in the DA region in *tnnt2a* MO embryos (Figure 7I; supplemental Figures 7D, 8), strongly supporting that *klf2a* is a major player downstream of blood flow. These data demonstrate that flow-induced *klf2a* expression is required for artery maturation and maintenance of HSC programming in zebrafish.

NO signaling is directly regulated by Klf2a during HSC development

Several studies have shown that *klf2* activates the expression of vascular genes including endothelial NO synthase (eNOS).[16,30,34] NO is involved in several physiologic and pathologic events. In
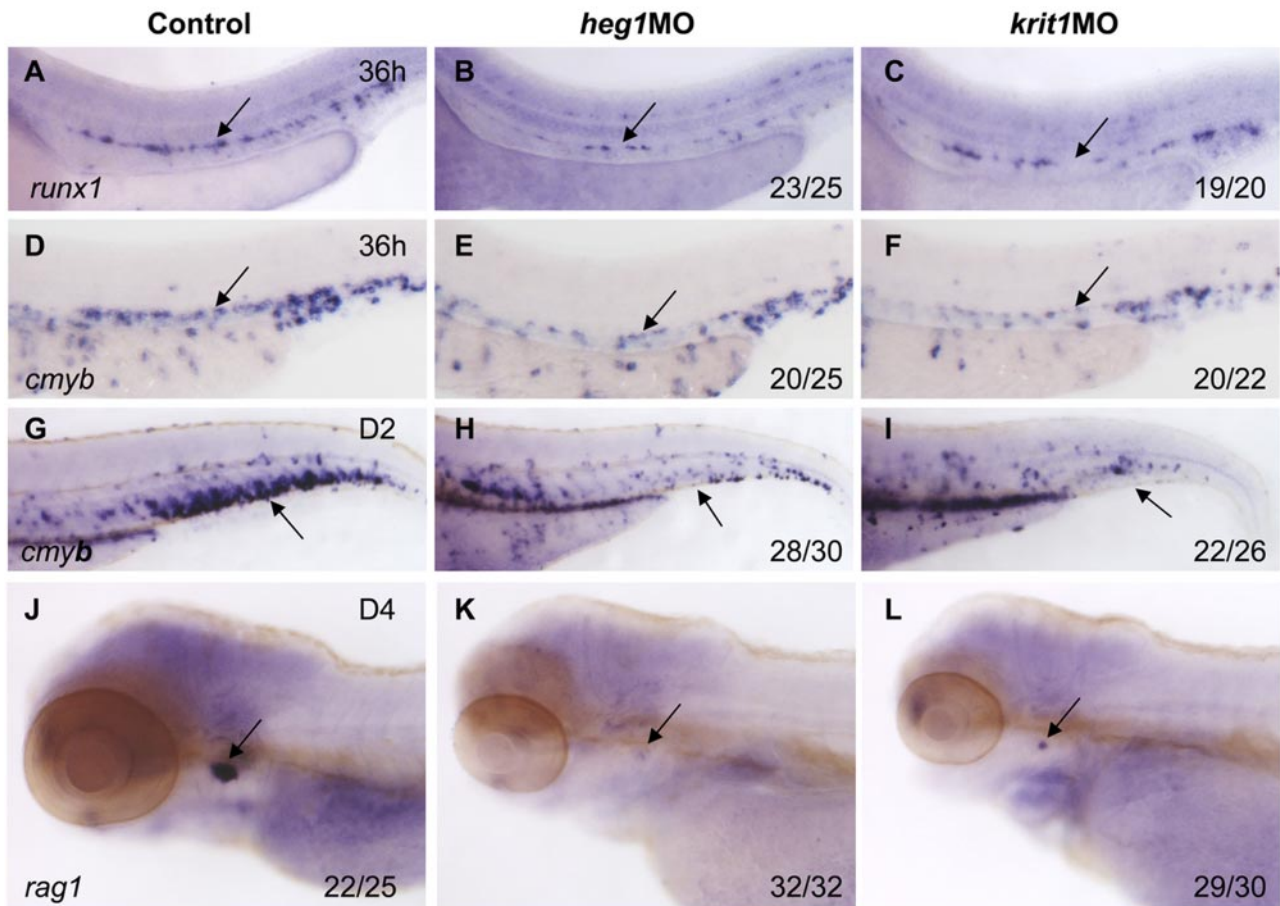
Figure 3. Heart of glass/*heg1* and *santa/krit1* embryos display similar HSC phenotypes to *silent heart* embryos. Expression of *cmyb* (A-C) or *runx1* (D-F) in HSCs in the AGM region (A-F) or CHT (G-I) in control MO (A,D,G), *heg1*MO (B,E,H), and *krit1*MO (C,F,I) injected embryos. Expression of *rag1* in the thymus in control MO (J), *heg1*MO (K), *krit1*MO (L) injected embryos. (A-F) Arrows mark AGM HSCs, 36 hpf. (G-I) Arrows mark CHT, 2 dpf. (J-L) Arrows mark T cells, 4 dpf. Anterior is to the left and dorsal is up.

particular, NO produced by the eNOS is a fundamental determinant of cardiovascular homeostasis, including systemic blood pressure, vascular remodeling, and angiogenesis.[35] In cultured endothelial cells, shear stress and other factors have been shown to increase eNOS expression.[36] To determine whether NO signaling was altered in *tnnt2a*- and *klf2a*-deficient embryos, we first used NO imaging to visualize NO production in live embryos. DAF-FM staining showed that NO production was reduced in the *tnnt2a*- and *klf2a*-deficient embryos (Figure 7B-C red arrows), compared with that in controls. To further test whether NO signaling was involved in the blood flow response required for HSC development, we examined *nos1* and *nos2a/2b* expression in *klf2a*-deficient embryos. *nos1* and *nos2b* expression was significantly decreased in *klf2a* morphants at 36 hpf (Figure 7D). To test whether *nos1* and *nos2a/2b* were directly regulated by Klf2a, ChIP analysis was carried out. *nos1* and *nos2b* but not *nos2a* were specifically enriched in the Klf2a Ab but not in serum-incubated samples (Figure 7E), suggesting that endogenous Klf2a bound to the promoters of *nos*1 and *nos2b* in vivo. This strongly indicates that *klf2a* regulates NO signaling directly. Finally, addition of a NO agonist, SNAP, slightly increased *runx1* expression in wild-type embryos (supplemental Figure 8E) and rescued *runx1* and *dll4* expression in the AGM of *tnnt2a* (Figure 6G,J; supplemental Figures 8B,E) and *klf2a*-deficient embryos (Figure 7H,K, supplemental Figure 7C,F), suggesting that NO signaling acts down-

stream of blood flow controlling artery maturation and definitive HSC development. Taken together, these results demonstrate that blood flow induces expression of *klf2a*, which in turn, directly activates NO signaling to transduce the flow-dependent response to endothelial cells necessary for HSC development and artery maturation in zebrafish (Figure 7L).

Discussion

In the present study, we provide evidence for a novel *klf2a*-NO signaling cascade in integrating blood flow with HSC development in zebrafish embryos. This conclusion is based on the following findings: First, blood flow promotes continued HSC program development, but not its initiation. Second, HSC maintenance defects in the blood flow–deficient embryos were closely associated with artery maintenance defects. Third, Klf2a is the immediate downstream effector of blood flow in regulating dorsal aorta and HSC development. Fourth, Klf2a directly regulates *nos1* and *nos2*, and thereby promotes the role of NO signaling in HSC development. These data lead us to propose a model in which blood flow controls dorsal aorta and HSC program maintenance through the *klf2a*-NO signaling cascade in zebrafish (Figure 7L).

The importance of mechanical forces in cardiovascular development has been largely neglected. However, recent data have
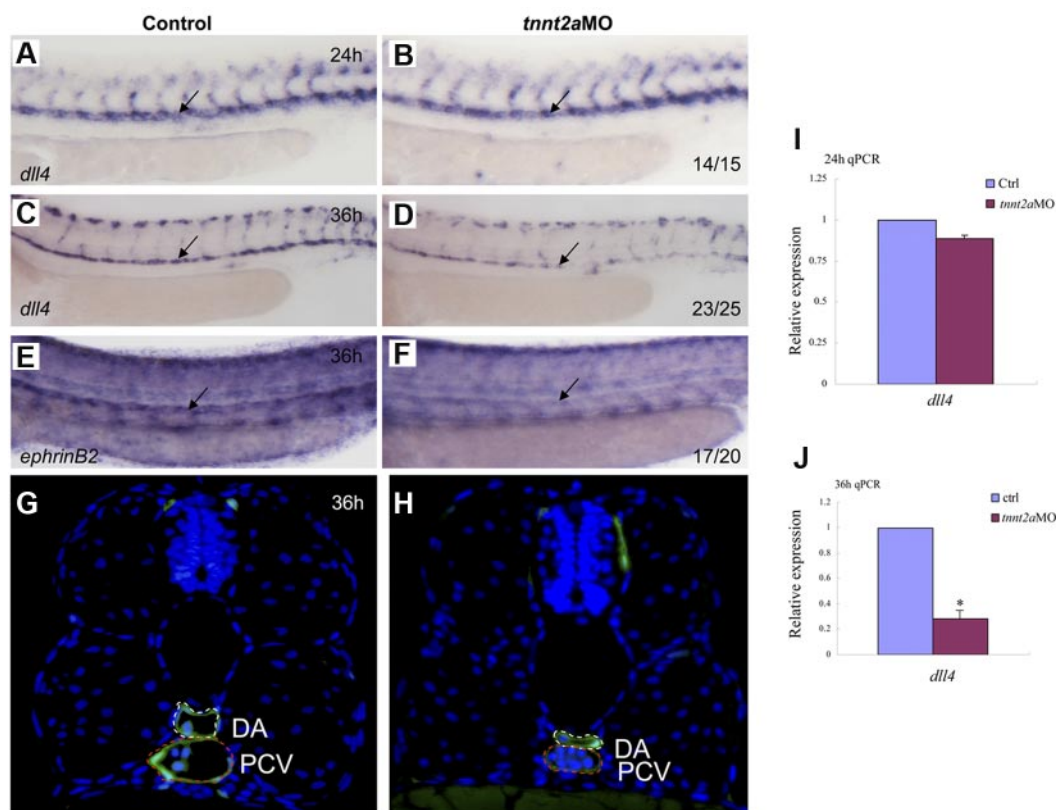
Figure 4. Artery maturation is attenuated in *tnnt2a* morphants. Expression of *dll4* (A-D) or *ephrinB2* (E,F) in the dorsal aorta in control MO and *tnnt2a*MO-injected embryos at 24 and 36 hpf, respectively. Anterior is to the left and dorsal is up. (G-H) Transverse section of control MO (G) and *tnnt2a*MO (H) injected *fli1a*:GFP embryos at 36 hpf. White dotted line denotes dorsal aorta; red dotted line denotes cardinal vein. (I-J) Quantitative RT-PCR analysis of *dll4* expression in the dissected trunk region of control and *tnnt2a*MO embryos at 24 and 36 hpf, respectively (mean ± SD, *t* test, *$P < .05$, n = 3).

identified fluid blood flow as a dominant modifier of cardiogenesis, vessel remodeling, vessel integrity, and HSC development.[11,13,15,35,37,38] However, some questions remained concerning the role of blood flow in HSC development. Previously, North et al and Lam et al showed that blood flow is required for HSC development in zebrafish embryos.[9,11] They looked at *runx1/cmyb* expression in 28- or 36-hpf zebrafish embryos, 6 or 14 hours after the start of HSC gene expression. The earliest expression of *runx1* in HSC progenitors is detected at 22-24 hpf in zebrafish.[39,40] In another 2 studies, Jin et al and Murayama et al showed relatively

normal *cmyb* expression in HSCs at 30 hpf and also later stages in *tnnt2a*MO-injected embryos.[1,2] To reconcile the obvious discrepancy, we have systematically checked several HSC markers, including *runx1*, *cmyb*, and *ikaros* at various stages, by using in situ hybridization and real time RT-PCR (Figure 2 and data not shown). Our data show that *runx1* in fact initiated normally at 24 hpf, but it was not maintained after 36 hpf (Figure 2). This indicates that blood flow is not required for the initiation of HSC gene expression in the DA, but is essential for its continued expression, therefore providing a more precise description of the role of blood flow in
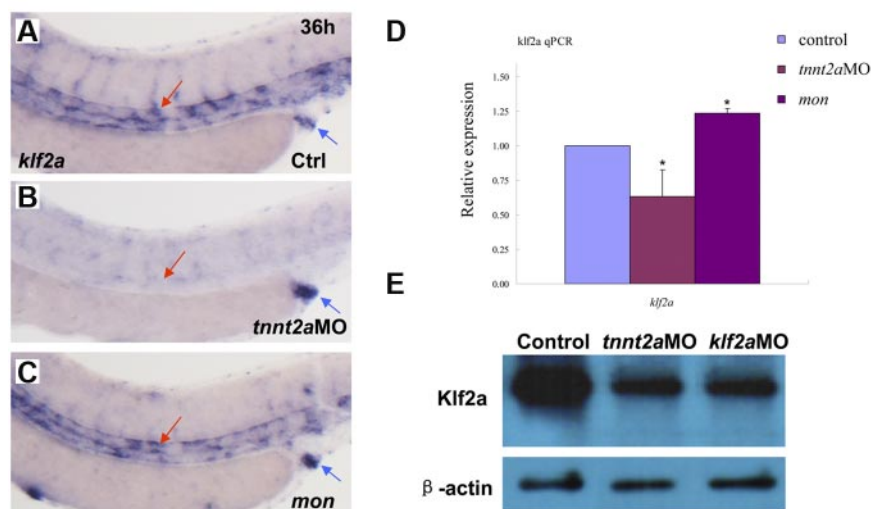

Figure 5. *klf2a* expression is reduced in *tnnt2a* morphants, but maintained in *moonshine* mutants. (A-C) WISH showed that klf2a expression in the trunk region was reduced in the *tnnt2a*MO-injected embryos, but maintained in *moonshine* mutants. (D) Quantitative RT-PCR analysis of *klf2a* expression in the dissected trunk region of control and *tnnt2a*MO embryos at 24 and 36 hpf, respectively (mean ± SD, *t* test, *$P < .05$, n = 3). (E) Western blot showed Klf2a protein was significantly down-regulated in *tnnt2a* and *klf2a*MO-injected embryos.
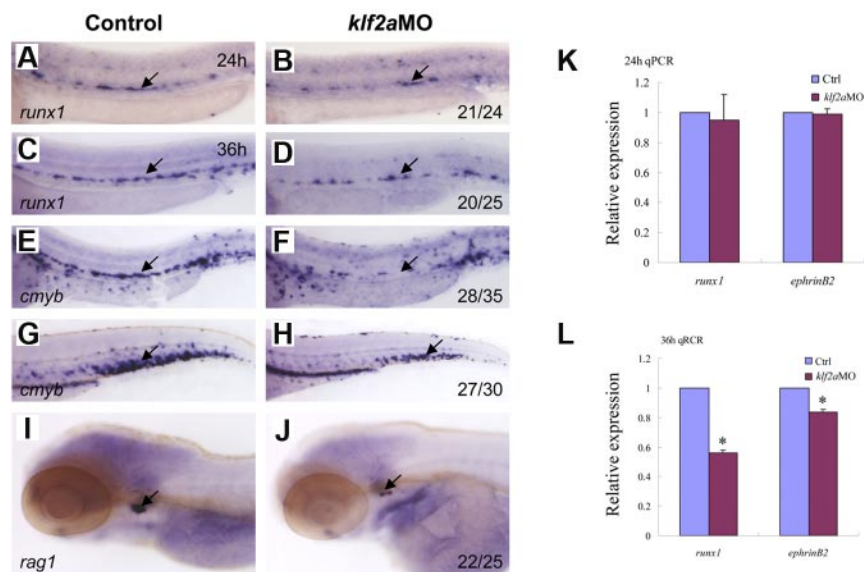
Figure 6. *klf2a* is downstream of *tnnt2a* in the regulation of artery maturation and maintenance of HSC programming. Expression of *runx1* (A-D) and *cmyb* (E-H) in HSCs in control MO (A,C,E,G) and *klf2a*MO (B,D,F,H) embryos at 24, 36, and 48 hpf, respectively. (I-J) Expression of *rag1* in thymus in control MO (I) and *klf2a*MO (J) injected embryos at 4dpf. (K-L) Quantitative RT-PCR analysis of *runx1* and *eph-rinB2* expression in the dissected trunk region of control and *klf2a*MO embryos at 24 and 36 hpf, respectively (mean $\pm$ SD, t test, *$P < .05$, n = 3).

HSC development. Our findings, together with those from others, add HSC development to a growing list of developmental processes requiring fluid dynamic inputs for proper growth and patterning, and further demonstrate that physiologic cues are part of the embryonic developmental program.

The kruppel-like factor (KLF) family of transcription factors is widely involved in vessel development in vertebrates. Specifically, *klf2*, responds to blood flow immediately, then exerts its function in angiogenesis and heart valve formation in zebrafish and in human vascular endothelial cells.[13,15,26,27,30] Previous studies showed that induction of *klf2* by fluid shear stress was mediated by several factors, including PCAF (P300/cAMP-response element-binding protein-associated factor), PI3K, Histone acetylation and MEF2 (myocyte enhancer factor-2) in HUVECs.[29] Recently, Nicoli et al
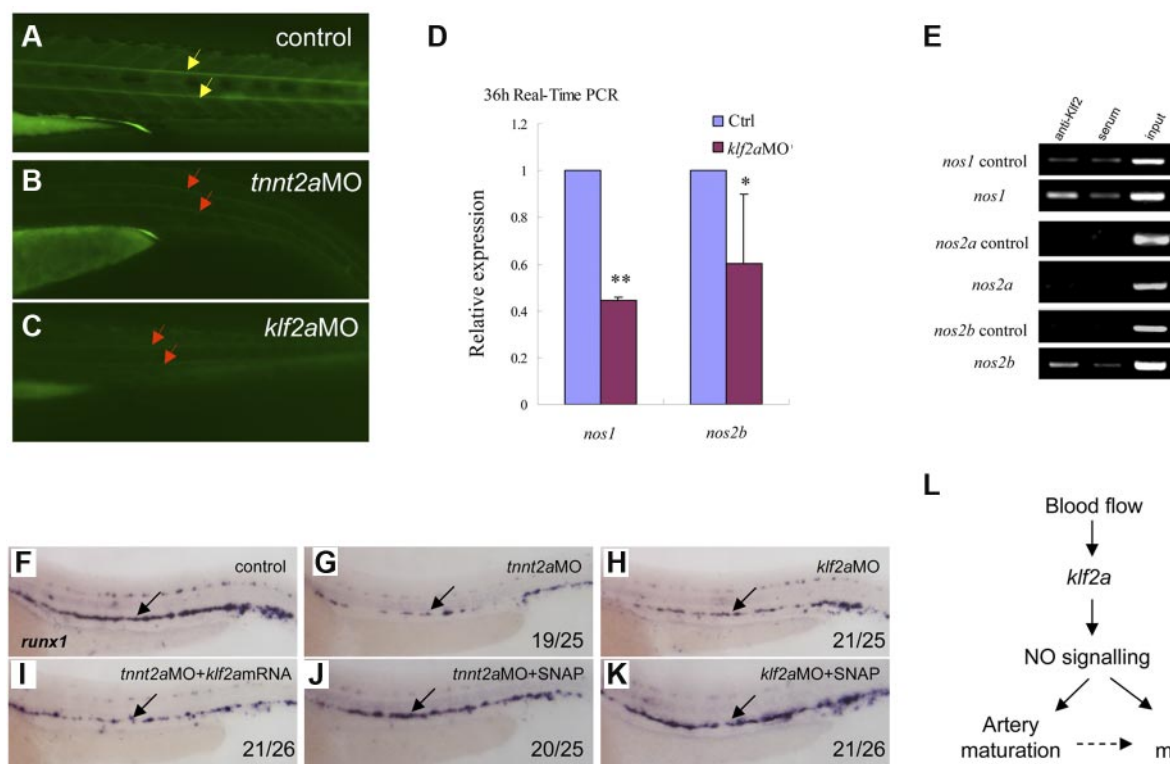

Figure 7. NO signaling is directly regulated by *klf2a* in the control of artery maturation and maintenance of HSC programming in zebrafish. (A-C) DAF-FM imaging showed reduced NO production in *tnnt2a*- and *klf2a*MO-injected embryos at 36 hpf. Yellow arrows mark normal NO production in the notochord area; red arrows mark reduced NO production. (D) Quantitative RT-PCR analysis of *nos1* and *nos2* expression in the dissected trunk region of control and *klf2a*MO embryos at 36 hpf (mean $\pm$ SD, t test, *$P < .05$, **$P < .01$, n = 3). (E) ChIP assay showing that Klf2a directly binds to *nos1* and *nos2b* promoter region. (F-K) Rescue of *runx1* expression in *tnnt2a*- and *klf2a*MO-injected embryos by *klf2a* mRNA or SNAP treatment. (F,G,I) Expression of *runx1* in HSCs of *tnnt2a*MO-injected embryos (G) was partially rescued by coinjection of *klf2a* mRNA (I) at 36 hpf. (J-K) SNAP treatment at tailbud stage can rescue *runx1* expression in *tnnt2a*MO (G,J) and *klf2a*MO (H,K) injected embryos. (L) Model of blood flow-induced *klf2a*-NO signaling in HSC maintenance.

showed that in zebrafish, hemodynamic force-induced *klf2a* acts upstream of miR-126 which in turn represses *spred1*, the repressor of Vegf signaling, to promote aortic arch angiogenesis.[15] Recent *Xenopus* studies showed that *klf2* regulates expression of the VEGF receptor *flk1* directly in endothelial cells.[41] Together, these suggest Vegf signaling might also be involved in flow-dependent HSC development. Therefore, although our data show that in zebrafish, the *klf2a*-NO signaling cascade controls continued HSC development as well as dorsal aorta maturation, we cannot rule out a role for *klf2a*-induced Vegf signaling during this process.

HSC emergence has been tightly associated with the dorsal aorta in the AGM region in vertebrates. Recent evidence has demonstrated that HSCs are directly derived from hemogenic endothelium through an endothelial-hematopoietic transition in zebrafish embryos in vivo and in mouse AGM ex vivo.[7,8,42] Thus, the dorsal aorta acts as the requisite de novo site or microenvironment for definitive hematopoiesis to occur. Our data shows that blood flow-dependent *klf2a*-NO signaling affects both dorsal aorta maturation and HSC program maintenance, but not its initiation. Whether this blood flow-induced signaling cascade regulates both dorsal aorta and HSC maintenance programs, or primarily controls dorsal aorta maturation which in turn affects HSC maintenance still needs to be clarified.

Taken together, our studies demonstrate a requirement for blood flow in blood stem cell program maintenance during zebrafish embryogenesis. Because the signaling cascade comprising the components of NO signaling and *klf2* are highly conserved across the vertebrates, they might be considered as candidates for disease genes contributing to later HSC (BM) failure in mammals.[43]

Authorship

Contribution: L.W., P.Z., Y.W., and Y.G. performed experiments; R.P. revised the manuscript; and F.L. designed and performed the experiments, analyzed the data, and wrote the manuscript.

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