Zebrafish *runx1* promoter-EGFP transgenics mark discrete sites of definitive blood progenitors

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The transcription factor Runx1 is essential for the development of definitive hematopoietic stem cells (HSCs) during vertebrate embryogenesis and is transcribed from 2 promoters, P1 and P2, generating 2 major Runx1 isoforms. We have created 2 stable *runx1* promoter zebrafish-transgenic lines that provide insight into the roles of the P1 and P2 isoforms during the establishment of definitive hematopoiesis. The *Tg*(*runx1P1:EGFP*) line displays fluorescence in the posterior blood island, where definitive erythromyeloid progenitors develop. The *Tg*(*runx1P2:EGFP*) line marks definitive HSCs in the aorta-gonad-mesonephros, with enhanced green fluorescent protein–labeled cells later populating the pronephros and thymus. This suggests that a function of *runx1* promoter switching is associated with the establishment of discrete definitive blood progenitor compartments. These *runx1* promoter–transgenic lines are novel tools for the study of Runx1 regulation and function in normal and malignant hematopoiesis. The ability to visualize and isolate fluorescently labeled HSCs should contribute to further elucidating the complex regulation of HSC development. (Blood. 2009;113:1241-1249)

Introduction

Blood cells in the early vertebrate embryo arise from successive waves of hematopoiesis.[1,2] Within each wave, distinct lineages of hematopoietic cells differing in differentiation potential are generated in embryonic sites that are divergent among vertebrate species.[3] In zebrafish, as in mammals, immediate emergence of hematopoietic cells from mesoderm constitutes primitive hematopoiesis. The mammalian extraembryonic yolk sac gives rise to the earliest macrophages and erythrocytes.[4] Zebrafish primitive macrophages and erythrocytes derived from the lateral plate mesoderm (LPM; equivalent to mammalian yolk sac), specifically from the cephalic mesoderm and intermediate cell mass (ICM), respectively.[5,6] In contrast, a feature of definitive hematopoiesis is cell-lineage generation from multipotent stem cells or progenitors.[1] In recent years, the aorta-gonad-mesonephros (AGM) region has been thought to be the origin of persisting definitive hematopoietic stem cells (HSCs), with expression of genes required for HSC development in that environment.[7,8] In zebrafish, the AGM equivalent is located in the region of the ventral wall of the dorsal aorta (DA), where HSCs are identified by expression of genes, such as *runx1* and *c-myb*.[9,10] Embryonic HSC differentiation to produce multiple lineages proceeds when cells from the AGM migrate to other hematopoietic niches.[11] Zebrafish AGM progenitors first migrate to the posterior ICM, known as the posterior blood island (PBI) or caudal hematopoietic tissue (possible equivalent to mammalian fetal liver), before seeding the pronephros (mammalian bone marrow equivalent) and thymus.[12,13] The idea of having exclusive sites for primitive and definitive hematopoiesis has been challenged by several mammalian studies demonstrating a contribution of yolk-sac progenitors to the formation of definitive erythroid and myeloid lineages, most probably before HSCs arise.[4,14-16] This notion was corroborated in zebrafish by the discovery of erythromyeloid progenitors (EMPs) that arise within the PBI derivative of the LPM before emergence of HSCs in the wall of the DA.[17] Furthermore, recent studies in mutant mice with either a defect in circulation or the migration of hematopoietic progenitors showed that normal hematopoiesis proceeded in the yolk sac, but definitive hematopoietic progenitors were absent in the AGM and fetal liver, raising the possibility of a yolk-sac origin for all definitive blood cells.[18,19] Our understanding of where HSCs arise therefore continues to be challenged, and there is much to learn about how the different hematopoietic microenvironments participate in promoting the adult blood program.

The transcription factor Runx1 is regarded as a key regulator of definitive hematopoiesis, with a conserved gene structure and function in all vertebrates.[20,21] Runx1 is expressed in all sites of hematopoietic development in mammals and zebrafish, and is expressed in HSCs and the endothelial cells from which HSCs are thought to arise.[10,22,23] Homozygous inactivation of Runx1 in mice results in embryonic lethality, with a complete absence of definitive hematopoiesis.[22] Runx1 also functions in the maturation of megakaryocyte and T-cell lineages.[24] The 2 main isoforms of Runx1, generated by alternative usage of the promoters P1 and P2, differ in the 5′ untranslated region (UTR) and the coding sequence of the first exon.[25,26] This alternative promoter usage regulates Runx1 function in several ways. In addition to the differential temporal and spatial pattern of transcription,[27] some of the splice variants generated from P2 lack the transactivation domain and are thought to have dominant-negative functions in inhibiting myeloid differentiation.[26] The short 5′UTR in transcripts from the P1 promoter controls cap-dependent translation, whereas the long 5′UTR in P2 transcripts regulates internal ribosomal entry site–dependent translation.[28]

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With the aim of further advancing our understanding of the role of Runx1 in HSC biology, we generated 2 stable transgenic zebrafish lines expressing enhanced green fluorescent protein (*EGFP*) driven by either the distal P1 or proximal P2 zebrafish *runx1* promoters. We show that segregated and successive emergence of definitive blood progenitors is regulated, in part, by alternative use of *runx1* promoters. At 18 hours postfertilization (hpf), the *Tg*(*runx1P1:EGFP*)–transgenic line first marks EMPs that populate the PBI. By 22 hpf, the *Tg*(*runx1P2:EGFP*)–transgenic line displays EGFP expression within HSCs as they emerge from the zebrafish AGM. This line also later highlights blood cells within the head kidney and thymus.

Methods

Zebrafish maintenance and generation of zebrafish transgenics

Zebrafish (*Danio rerio*) embryos were obtained from natural spawnings and raised in embryo medium (E3) at 28.5°C. All the studies conducted for this manuscript have been reviewed and approved by the University of Auckland Animal Ethics Committee. A 12-kb fragment containing *runx1P1* was identified with the DIG system for nonradioactive Southern blotting (Roche Diagnostics, Indianapolis, IN). The *runx1P1* fragment was cloned into *pBluescript II KS*(+) (*pBSIIKS*+; Stratagene, La Jolla, CA). The 1.5-kb fragment at the 3′ end of the promoter sequence containing the *runx1* coding sequence was removed by partial digest with *Spe*I. To position the *runx1P1* promoter in-frame with *EGFP*, the 1.5-kb sequence adjacent to the coding sequence was polymerase chain reaction (PCR)–amplified with the primers *Eco*RI*runx1P1F and Bam*HI*runx1P1R*, digested with *Eco*RI and *Bam*HI, and then cloned into *pT2KXIGΔin*.[29] This was linearized with *Xho*I (blunted) and *Eco*RI, then ligated with the remaining 10.5-kb *runx1P1* fragment that had been cut with *Sac*II (blunted) and *Eco*RI, generating the *runx1P1::EGFP/pT2K* plasmid that was used for transgenesis. An 8-kb *runx1P2* fragment was isolated by restriction digest with *Sac*I and *Bam*HI and cloned into the *Sac*I/*Bam*HI sites of *pBSIISK*+. The *EGFP* gene from *pIRES-EGFP* (Clontech, Mountain View, CA) was released by *Bam*HI/*Xho*I digestion and cloned into the *Bam*HI/*Xho*I sites of *runx1P2/pBSIISK*+. The constructs were injected into embryos at the one-cell stage and screened for germline integration by random intercrosses. The transgenic founders identified were crossed to establish stable transgenic lines.

Morpholino injections

runx1 morpholinos (MOs): *runx1P1* splice *MO* ATCGCTAGCAAACAACTCACCCCTG, *runx1P1 ATG MO* GGAGCGTCTGCTTGTCCACGTCTTC, and *runx1 runt* domain MO TTTTCAGCATCTCACCTCGTCCGCT (Gene Tools, Philomath, OR) were injected at a dose of 0.25 to 1.0 pmol per embryo.[30] MOs targeting *gata1* and *scl* were used as described.[31,32]

Histology, immunohistochemistry, and in situ hybridization

Juvenile and adult fish were fixed in 4% paraformaldehyde, decalcified in 0.5 M ethylenediaminetetraacetic acid, dehydrated in ethanol, cleared in xylol, and then infiltrated and embedded in paraffin; 5-μm sagittal sections were cut and used for immunohistochemistry for EGFP expression as described,[33] and in situ hybridization for *runx1*. For detection of *runx1* mRNA, sections were deparaffinized and rehydrated in phosphate-buffered saline. Sections were refixed in 4% paraformaldehyde, treated with 0.2 M HCl, 10 mg/mL proteinase K, followed by 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8. Sections were hybridized overnight with a *runx1* DIG riboprobe at 65°C.[10] Unbound probes were washed with 2× saline sodium citrate and 0.2× saline sodium citrate. Sections were blocked, then incubated with anti-DIG–alkaline phosphatase. After washes, the sections were stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate p-toluidine, counterstained with Vector Nuclear Fast Red (Vector Laboratories, Burlingame, CA), and mounted in Entellan (Merck, Whitehouse Station, NJ).

Imaging

Live transgenic embryos were imaged under a Leica MZ16FA fluorescence stereomicroscope with a DC490 camera (Leica Microsystems, Heerbrugg, Switzerland). Histologic sections were imaged with a Leica DMR compound microscope and a DC200 camera. For confocal imaging, embryos were anesthetized in tricaine, mounted in 1% (wt/vol) low-melt agarose in E3, and imaged with an Olympus FV1000 confocal microscope (Tokyo, Japan).

Results

The 2 Runx1 isoforms are differentially expressed during embryogenesis

Transcriptional control of Runx1 from the 2 alternative promoters is thought to be important for modulating Runx1 function. Previous studies of Runx1 isoform expression in cell lines and mouse tissues suggested that HSCs and lymphocytes predominantly express the P1 isoform.[25,27] However, in zebrafish we have previously shown by MO knockdown studies that Runx1P2 is required for definitive hematopoiesis.[10] Support for a role for the P2 isoform in early hematopoiesis and T-cell development has also recently come from mouse studies using a hypomorphic *Runx1* allele with diminished P2 activity.[34] In Figure 1A, we show, by reverse-transcription PCR (RT-PCR), different temporal transcription profiles for the 2 isoforms during zebrafish embryogenesis. Only *runx1P1* is maternally expressed, whereas significant levels of *runx1P2* transcript are detected from 14 hpf.

Cloning of the *runx1* promoters and generation of stable transgenic lines

To generate zebrafish with marked HSCs and to further investigate whether *P1* and *P2* have distinct regulatory functions in blood development, we cloned the 2 zebrafish *runx1* promoters and generated stable transgenic lines expressing EGFP (Figure 1B). Bacterial artificial chromosomes (BACs) that included the *runx1* locus were identified by Southern blotting to the BAC Zebrafish High-Density Filter Library (Incyte Genomics, Wilmington, DE). Four BACs were found to contain *runx1* sequence 14i20, 74j23, 97a02, and 135g16. Although the full mRNA sequence of the *runx1P2* isoform had been cloned, the fragment encoding the N-terminal region of the P1 isoform had not been identified when we commenced the study reported here. We used 5′ rapid amplification of cDNA ends to clone the sequence of *runx1* unique to the P1 isoform. To amplify P1 isoform-specific mRNA sequence, degenerate primers were designed based on the highly conserved N-terminal sequence of mammalian *RUNX* genes. This resulted in a 93-bp sequence that was used to BLAST the Ensembl zebrafish genome (http://www.ensembl.org/Danio_rerio/index.html) to obtain further genomic sequences of the *runx1P1* promoter. Approximately 1700 bp of the sequence upstream of the translational start site was available. Southern blot analysis was used to identify and clone further upstream promoter and enhancer sequences. A 73-bp sequence just upstream of the ATG was used as a probe to hybridize with *runx1P1* sequences in the 97a02 BAC, which had been digested with *Nhe*I 69 bp downstream of the ATG. The 12-kb fragment identified as containing *runx1P1* sequences was cloned (Figure 1B). The 3′ 1.5-kb sequence of the fragment was replaced with a PCR-amplified sequence, without the coding region, and cloned into the *Tol2* vector.[35] The *Tol2 runx1P1::EGFP* vector was coinjected with *transposase* mRNA into one cell–stage embryos. The injected embryos were grown to adulthood and screened for

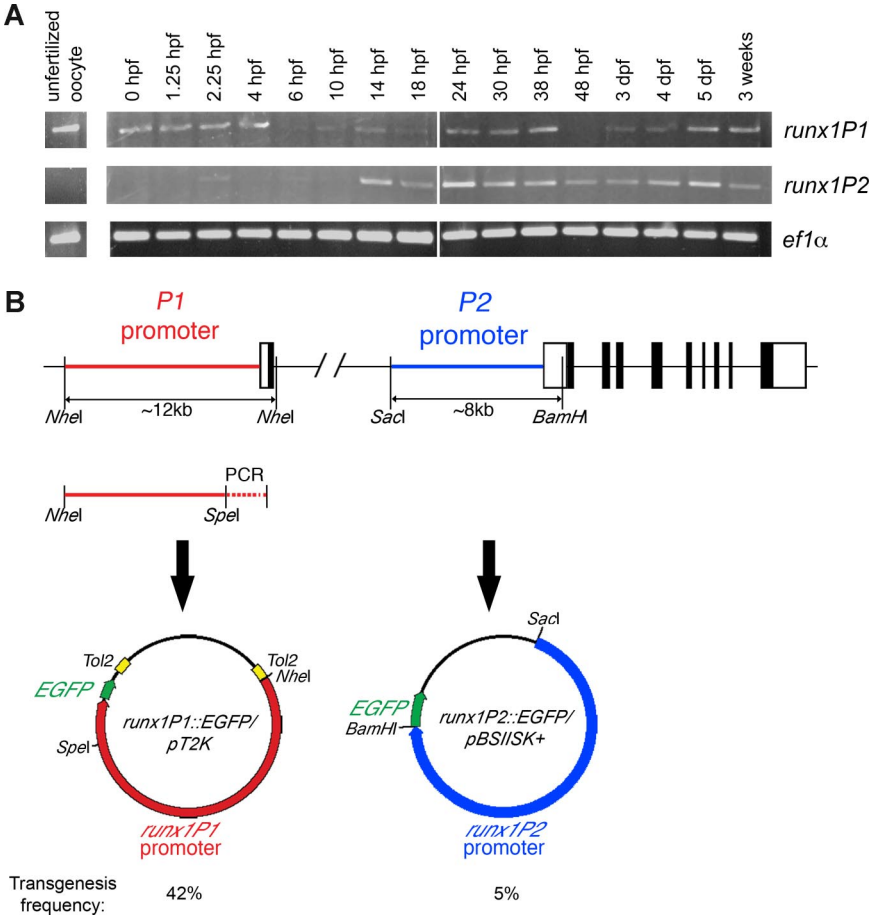

Figure 1. Transcripts encoding the 2 main isoforms of *runx1* are differentially expressed from the 2 alternative promoters, P1 and P2, during development. (A) RT-PCR of *runx1 P1* and *P2* isoforms of RNA extracted from wild-type zebrafish unfertilized oocytes and embryos at different stages, with *ef1*α as a control. The *runx1P1* isoform is maternally expressed, whereas the *P2* isoform is predominantly expressed from 14 hpf. Vertical lines have been inserted to indicate a repositioned gel lane. (B) Genomic organization of the zebrafish *runx1* locus and strategy for generating promoter constructs for transgenesis. The diagram shows the structure of the *runx1* gene and the regions of the promoters used to generate the *runx1::EGFP* transgenics. Exons and UTRs are shown as filled and unfilled boxes, respectively. A 12-kb fragment of the *P1* promoter was cloned by restriction digest and PCR and then inserted into a *Tol2* transposon vector. The frequency of germline integration was 42%. An earlier protocol, with a germline integration frequency here of 5%, was used to generate the *P2*-transgenic line with *EGFP* driven by an 8-kb *Sac*I-*Bam*HI fragment.

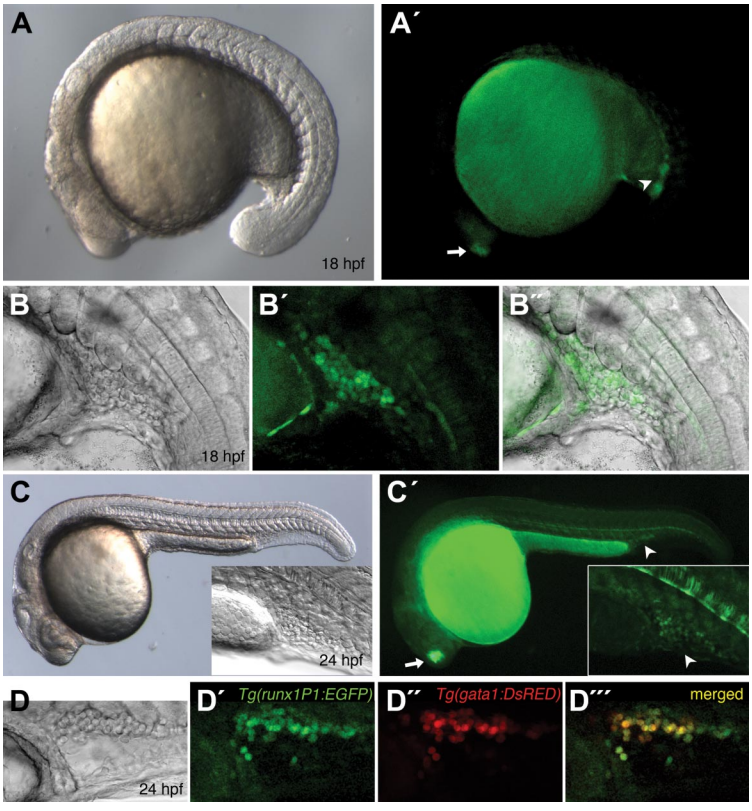

Figure 2. Expression of EGFP in *Tg*(*runx1P1:EGFP*)–transgenic embryos. (A-D) bright-field images, (A′-D′,D″) fluorescent images, (B″) merge of B and B′, and (D‴) merge of D′ and D″. (A,A′) Lateral views at 18 hpf showing EGFP in the posterior LPM (arrowhead) and the olfactory placode (arrow). (B,B′) Confocal images of the LPM showing EGFP-positive hematopoietic cells. (C,C′) Lateral views of 24 hpf embryo with EGFP expression in the PBI (arrowhead), olfactory placode (arrow), and hypochord. (Inset) Confocal images displaying EGFP-positive cells in the PBI (arrowhead). (D,D′,D″,D‴) Lateral view of the PBI of a 24 hpf *Tg*(*runx1P1:EGFP*)/*Tg*(*gata1:DsRED*) double-transgenic embryo, showing coexpression of EGFP and DsRED in EMPs.

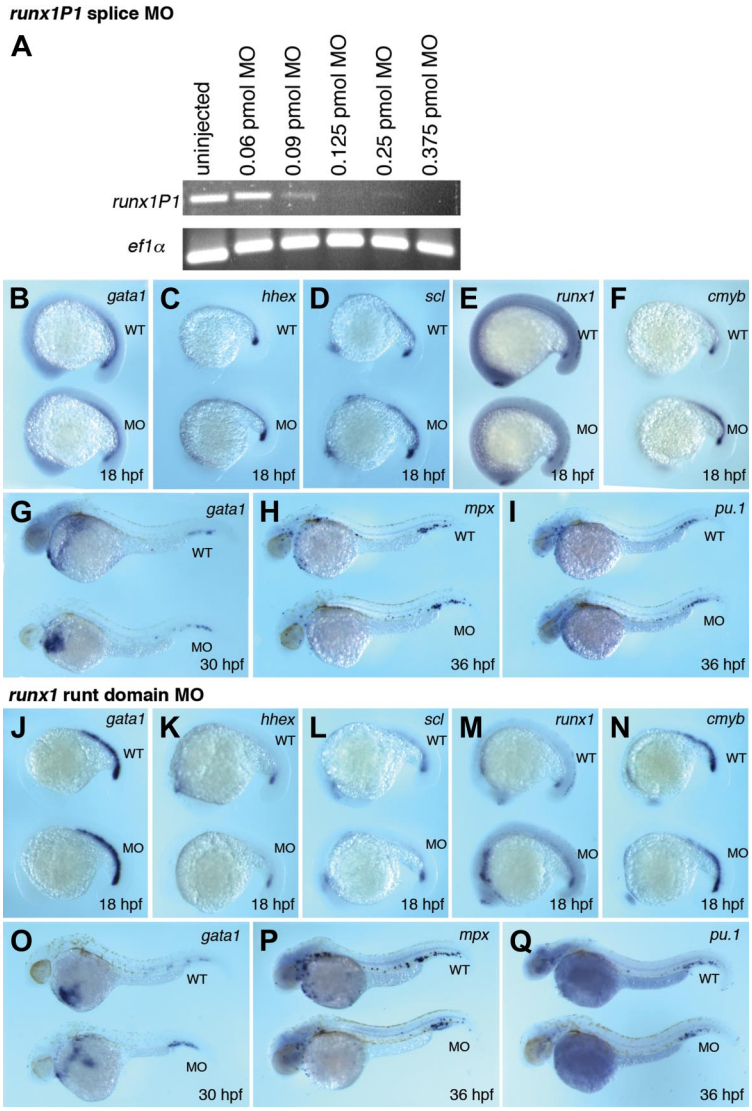

Figure 3. Runx1P1 is not required for primitive or definitive hematopoiesis. (A) RT-PCR confirms deletion of the first exon of *runx1P1* in 24 hpf embryos injected with the *runx1P1* splice MO at doses greater than 0.125 pmol. Markers of primitive erythropoiesis (B-D) and definitive hematopoiesis (E,F) are expressed normally in 18 hpf Runx1P1 morphants. (G-I) The development of EMPs expressing *gata1*, *mpx*, and *pu.1* is also unaffected by Runx1P1 knockdown. (J-N) Injection of a *runx1* runt domain MO targeting both isoforms did not affect expression of primitive or definitive hematopoiesis at 18 hpf. (O-Q) Expression of EMP markers is also unaffected by Runx1 depletion.

germline integration by random intercrosses. Nineteen pairs of fish were successfully crossed, with 15 crosses producing transgenic embryos. A total of 16 transgenic founders were identified from further outcrosses. These transgenic lines displayed fluorescent expression in the same spatial domains.

We have previously shown, by morpholino knockdown studies, that the Runx1 isoform expressed from the P2 promoter is required for definitive hematopoiesis,[10] suggesting that this promoter may be useful in generating a transgenic zebrafish line with labeled definitive blood. Restriction mapping of the 14i20 BAC was used to identify an 8-kb fragment containing *runx1P2* promoter and enhancer sequences. This fragment was subsequently cloned into *pBSIISK+* vector with *EGFP* and used to generate stable transgenic lines. The injected fish were screened by random intercrosses, and 5 transgenic founders were identified from the 92 fish screened. The different lines express EGFP in the same spatial and temporal domains but with different intensity.

The *runx1P1* transgenic labels cells in the PBI

Of the 2 transgenic lines, *Tg(runx1P1:EGFP)* is the first to express EGFP in hematopoietic tissues. Fluorescent cells were visible in the posterior LPM at 18 hpf (Figure 2A,B). Posterior LPM expression of *runx1* is observed at 14 hpf[10]; the delay in EGFP detection was attributed to protein maturation. We detected *EGFP* transcripts in the LPM at 16 hpf (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). EGFP expression was transient and by 24 hpf, when the PBI has formed from the posterior LPM, only weak expression remained (Figure 2C). The ontogeny of these cells resembles that of the definitive EMPs, which express erythroid and myeloid genes such as *gata1* and *pu.1*, respectively.[17] To confirm that the P1 promoter drives expression in EMPs, compound *Tg(runx1P1:EGFP)/Tg(gata1:DsRED)*[36] zebrafish were generated. At 24 hpf, cells in the PBI expressing DsRED also expressed EGFP (Figure 2D). EGFP expression in this domain was not detected at 30 hpf (data not shown), suggesting that *runx1P1* is expressed at an early stage of EMP development but is soon down-regulated.

To examine the expression of *runx1* isoforms in the EGFP-positive cells of *runx1P1*-transgenic embryos, the caudal region of 22 hpf embryos was dissected and dissociated (Figure S2). The EGFP-expressing cells were isolated by fluorescence-activated cell sorting (FACS). RT-PCR on RNA extracted from the sorted cells showed the *runx1P1* isoform is predominantly

expressed. The weak expression of the *runx1P2* isoform possibly arises from EGFP expression in neuronal and somitic tissues.

To determine the function of Runx1P1, transcripts were selectively ablated using either a splice or an ATG MO, followed by expression analysis of a panel of erythromyeloid markers at 18 hpf (primitive hematopoiesis) and 36 hpf (EMPs). Effective inhibition of splicing by the *runx1P1* MO was assessed by RT-PCR (Figure 3A). Knockdown of Runx1P1 did not interfere with the expression of early hematopoietic genes, such as *gata1, hhex*, and *scl* in the ICM at 18 hpf (Figure 3B-D), showing that Runx1P1 is not involved in primitive hematopoiesis. The initiation of definitive hematopoiesis, marked by the expression of *runx1* and *c-myb*, is also unaffected by Runx1P1 depletion (Figure 3E,F). Expression of erythroid and myeloid markers within the PBI at 30 and 36 hpf was also unaltered by ablation of Runx1P1 (Figure 3G-I). This suggests that, whereas *runx1P1* is expressed in the LPM and PBI preceding the emergence of EMPs, it performs a role other than the specification and maintenance of EMPs. Using an MO that targets the splicing of an exon within the runt domain common to both *runx1* isoforms, the development of primitive blood and EMPs was similarly unaffected (Figure 3J-Q). Other tissues that express EGFP in the *runx1P1* transgenic are the olfactory placode and the hypochord dorsal to the PBI, as well as weak expression in neuronal and somitic tissues (Figure 2A,C).

Definitive HSCs are labeled in the *runx1P2* line

In the *Tg*(*runx1P2:EGFP*) line, EGFP was first observed at the 6-somite stage in the notochord (Figure 4A). By the 12-somite stage, distinct neuronal expression was observed (Figure 4B). Hematopoietic EGFP expression is first observed in the ventral wall of the DA or AGM at 22 hpf, recapitulating the *runx1* in situ expression pattern (Figure 4C). This expression before the establishment of circulation suggests that the EGFP-positive cells are generated from the ventral wall of the DA, rather than being seeded from another hematopoietic site in the embryo. EGFP was abundant in the brain and spinal cord (Figure 4C-F). No EGFP was detected in the PBI region at 24 hpf where *runx1* mRNA is expressed from 18 to 24 hpf (Figure 4F), suggesting the P2 isoform is not expressed in EMPs.

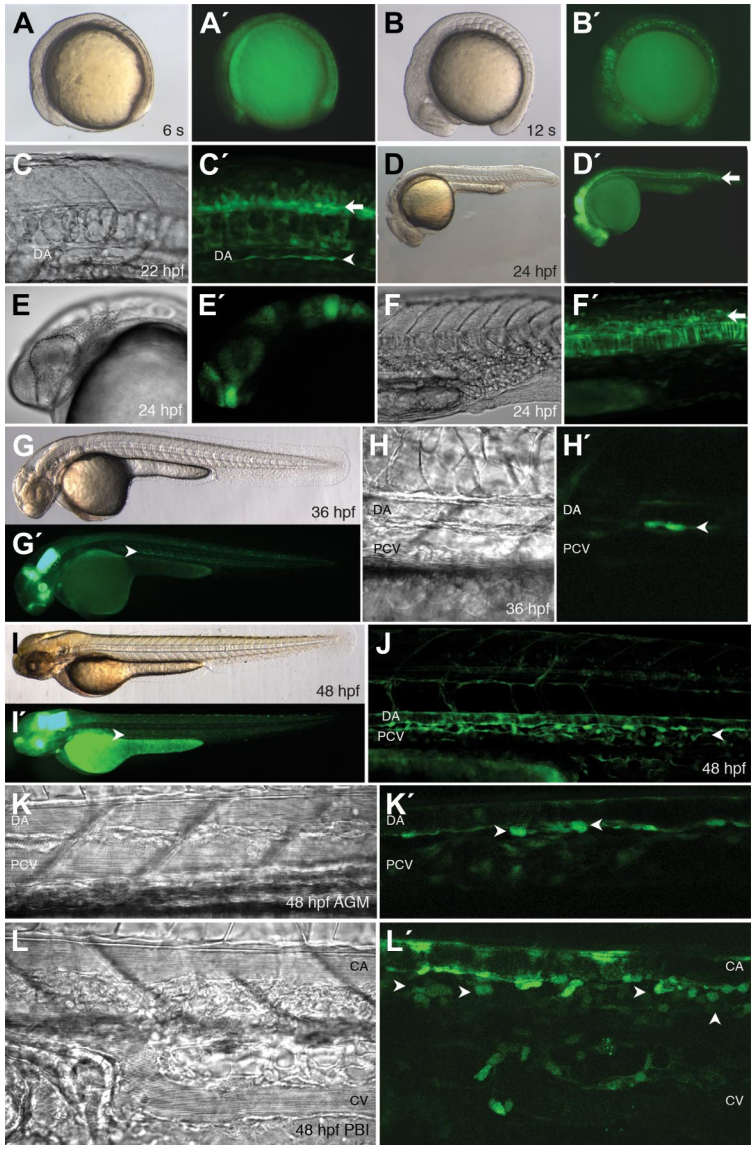

Figure 4. Expression of EGFP in *Tg*(*runx1P2:EGFP*)–transgenic embryos. (A-I,K,L) Bright-field and (A′-I′,J,K′,L′) fluorescent images. (A) Lateral views of 6-somite (s) embryo showing EGFP expression in the notochord. (B) Lateral views of 12-somite embryo. EGFP is expressed in the spinal cord and notochord. (C) Lateral view of the trunk at 22 hpf. EGFP is present in the ventral wall of the dorsal aorta (DA; arrowhead) and the spinal cord (arrow). (D-F) Lateral views at 24 hpf showing EGFP in the brain and spinal cord (arrow, D,E), but not in the PBI (F). (G,H) Lateral views of 36 hpf embryo with EGFP-expressing cells between the DA and PCV along the whole trunk of the embryo (arrowhead). (I-L) Lateral views of 48 hpf embryo with many EGFP-positive cells in the AGM region between the trunk vessels (arrowhead). Round EGFP-positive cells with the morphology of hematopoietic cells are present between the DA and PCV in the AGM (arrowheads, K), and the caudal artery (CA) and caudal vein (CV) in the PBI (arrowheads, L).

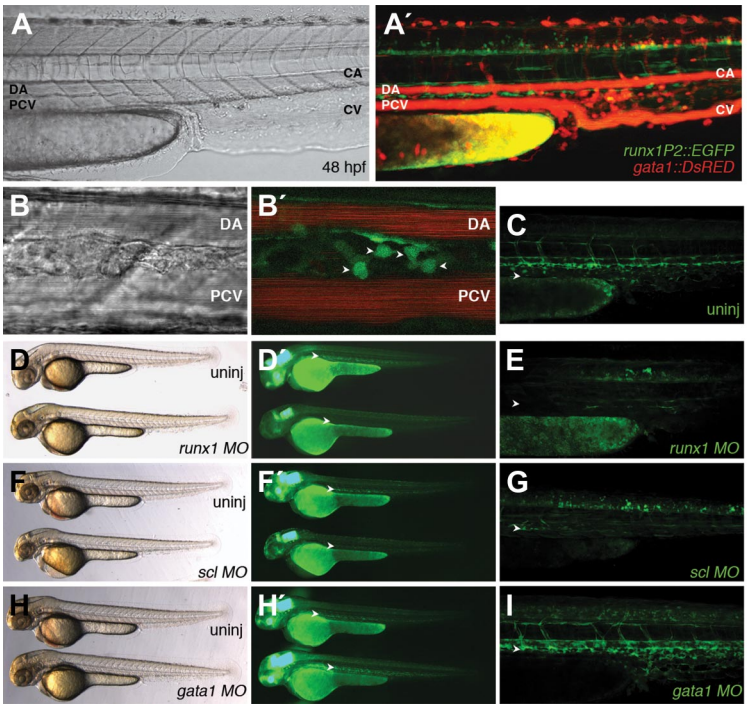

Figure 5. The EGFP-positive cells between the DA and PCV are early hematopoietic cells. (A,B) Lateral views of the trunk of 48 hpf *Tg*(*runx1P2:EGFP*)/*Tg*(*gata1:DsRED*) double-transgenic embryo confirms the localization of round EGFP-expressing cells between the DA and PCV (arrowheads). (C,E,G,I) Lateral views of the trunk regions of uninjected (uninj) and MO-injected 48 hpf *Tg*(*runx1P2:EGFP*) embryos. (D,F,H) Lateral bright-field and (D′,F′,H′) fluorescent images of 48 hpf MO-injected transgenic embryos together with uninjected controls. (D,E) The hematopoietic EGFP-expressing cells are abolished in *runx1* runt domain MO-injected embryos (arrowheads). (F,G) Injection of *scl* MO ablated the EGFP-positive cells between the DA and PCV (arrowheads). (H,I) Injection of *gata1* MO increased EGFP expression in the AGM region compared with controls (arrowheads).

A highly conserved intronic enhancer element that drives *Runx1* expression only in hematopoietic domains in mouse embryos has recently been identified.[37] We performed a similar analysis for zebrafish and Fugu *runx1* but found no homologous regions (Figure S3), suggesting that this enhancer may not be present in teleosts.

By 36 hpf, round cells marked by EGFP were visualized between the DA and posterior cardinal vein (PCV) in the AGM, and the caudal artery (CA) and caudal vein (CV) in the PBI (Figure 4G-L). Crossing the *Tg*(*runx1P2:EGFP*)–transgenic with the *Tg*(*gata1:DsRED*) line,[36] to highlight red fluorescent erythrocytes within vessels, confirmed the localization of EGFP-positive cells in the AGM (Figure 5A,B). These features resemble the hematopoietic progenitors identified by Murayama et al[12] and labeled in the *Tg*(*scl:GFP*) line.[38] As in the *Tg*(*scl:GFP*) line, we also detected EGFP expression in endothelial cells of the trunk vessels by confocal microscopy (Figure 4J). Labeling of these endothelial cells may represent expression at levels undetectable by in situ hybridization or may reflect exclusion of a repressor element required to silence vascular expression. EGFP-positive cells from the trunk and tail of 48 hpf *runx1P2* transgenic embryos were isolated by FACS (Figure S2). RT-PCR showed the EGFP-expressing cells expressed only the *runx1P2* isoform.

Early hematopoietic cells in the AGM express EGFP

To confirm that the fluorescent cells in the AGM were hematopoietic (Figure 5A,B), we used MOs targeting several early hematopoietic transcription factors. We used the *runx1* runt domain MO, and deletion of this important exon was confirmed by RT-PCR (data not shown). Injection of the *runx1* runt domain MO resulted in the almost complete loss of hematopoietic EGFP expression at 48 hpf (Figure 5D,E), but neuronal expression was retained (Figure 5D). The knockdown of Scl affects both primitive and definitive blood, as well as development of the DA, from which the *runx1*-expressing HSCs are thought to emerge.[32,39] As expected, injection of an *scl* MO resulted in loss of the hematopoietic EGFP-positive cells (Figure 5F,G), without affecting the neuronal domains (Figure 5F). Gata1 is required for directing differentiation toward the erythroid lineage at the expense of myeloid specification.[31,33,40] Gata1 knockdown has been shown to increase both the level and duration of *runx1* expression in the ICM, but its effect on definitive hematopoiesis has not been studied. In *Tg*(*runx1P2:EGFP*) embryos injected with a *gata1* MO, EGFP in the AGM at 48 hpf was increased compared with controls (Figure 5H,I). It appears that, as in the ICM, Gata1 negatively regulates the maintenance of definitive progenitors driven toward the myeloid lineage.

The hematopoietic expression in the *runx1P1* transgenic precedes that of *runx1P2* and appears to mark 2 distinct populations of definitive hematopoietic cells. To show that these 2 lineages arise independently, the development of cells in the PBI of 20 hpf *runx1P1* embryos was traced with a photoactivatable rhodamine dextran (Figure S4). Cells in the PBI gave rise to circulating erythrocytes and the caudal vasculature but did not migrate to the AGM.

EGFP-positive hematopoietic cells are present in adults

The migration of HSCs from the PBI has been traced to the thymus and pronephros at 5 days postfertilization (dpf) using photoactivatable fluorescein-dextran.[12,41] We have never detected *runx1* expression by in situ hybridization in the thymus or the pronephros at this stage, possibly because *runx1* expression is reduced to levels undetectable by this technique during HSC differentiation. EGFP stability, with half-life more than 24 hours, might enable blood progenitors to be visualized after migration to the thymus and pronephros. By confocal microscopy, EGFP-positive cells were present in the thymus at 4 dpf and in the pronephros at 5 dpf (Figure 6A,B).

Beyond the embryonic and larval stages, we observed continued EGFP expression in the pronephros and mesonephros

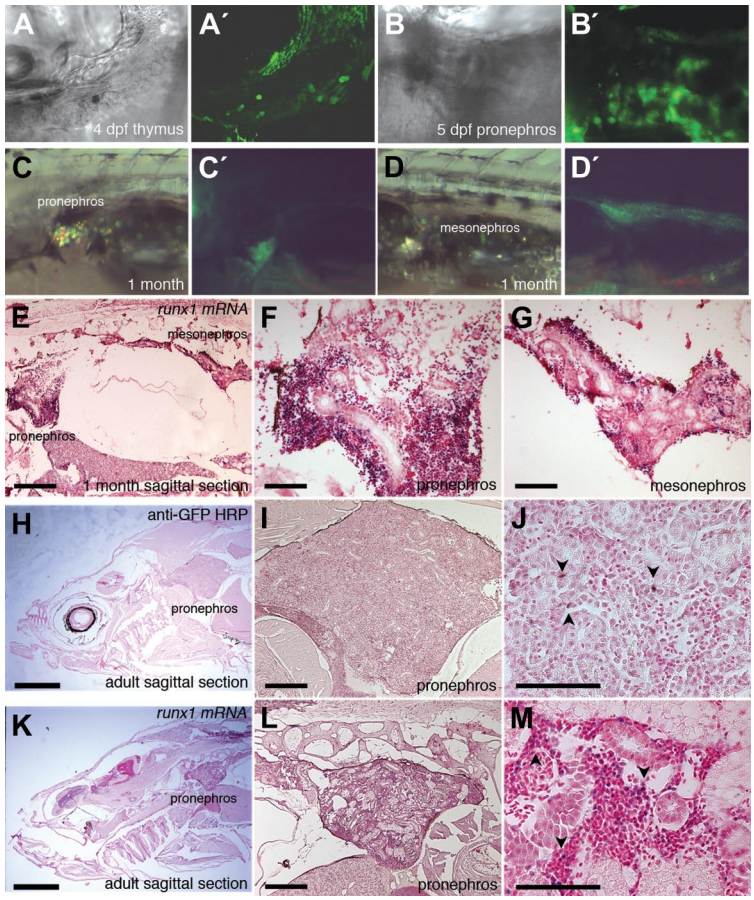

Figure 6. EGFP expression in larval, juvenile, and adult *runx1P2* transgenics. (A-D) Bright-field and (A′-D′) fluorescent images of transgenic larvae and juveniles. (A) Lateral view of the thymus at 4 dpf showing EGFP-positive thymocytes. (B) Dorsal view of 5-dpf transgenic embryo showing EGFP expression in the pronephros. (C,D) Lateral views of 1-month-old juvenile transgenic showing EGFP in the pronephros and mesonephros. (E-G) In situ hybridization for *runx1* mRNA on a sagittal section of a 1-month-old wild-type fish. (F,G) Higher magnification of the pronephros and mesonephros shows *runx1* mRNA localized to blood progenitors around the renal tubules. (H-J) Anti-GFP staining of a sagittal section of an adult *runx1P2* transgenic showing EGFP-positive cells within the pronephros (arrowheads). (K-M) *runx1* in situ hybridization on a sagittal section of the pronephros of wild-type adult. *runx1* is expressed in hematopoietic cells around the renal tubules (arrowheads). Bars represent 200 μm (E,I,L), 50 μm (F,G,J,M), and 1 mm (H,K).

of 1-month-old *runx1P2* transgenics (Figure 6C,D), with the same domain marked by *runx1* in situ hybridization in wild-type fish sections (Figure 6E-G). In *runx1P2* adult transgenics, staining with anti-EGFP demonstrated expression of EGFP in a small subset of cells within the pronephros (Figure 6H-J). This recapitulates the *runx1* in situ hybridization profile on tissue sections (Figure 6K-M). Similar analysis of transgene expression within the *runx1P1* reporter line showed no hematopoietic expression in the adult (data not shown).

Discussion

In the development of the hematopoietic system, different blood lineages develop in overlapping temporal and spatial domains. Determining their origin, potential, and relationship to other lineages has been a challenge. Several recent studies on the lineage relationships between the different hematopoietic populations have produced differing results. In a recent study of zebrafish–definitive hematopoiesis, a population of definitive EMPs were identified in the PBI that arise from the posterior LPM, separately from HSCs from the AGM region.[17] Previously, it was thought that the PBI only contained definitive HSCs that migrate from the AGM to undergo further maturation, before migration to the thymus and pronephros.[12,41,42] It was also suggested that HSCs in the AGM traced at different time points have different differentiation potential,[12] although this was not observed in another study.[41]

The *runx1* promoter–transgenic lines reported in this study mark 2 distinct populations of definitive blood, strongly supporting the notion that distinct lineages of definitive hematopoietic progenitors arise independently in the zebrafish embryo. Together, the 2 lines recapitulate the endogenous expression of *runx1* in hematopoietic tissues.[10] The 2 lineages of definitive blood in zebrafish appear to be analogous to those of mammalian systems. Definitive progenitors from the yolk sac can differentiate into myeloid and erythroid, but not lymphoid, lineages in cocultures, whereas progenitors from the AGM of mouse embryos of the same age have lymphoid potential.[43]

The *Tg*(*runx1P1:EGFP*) line labels EMPs in the PBI, whereas the *Tg*(*runx1P2:EGFP*) line marks definitive progenitors arising from the AGM. In the *runx1P1* line, the onset of *EGFP* expression by in situ hybridization and by fluorescence in the posterior LPM matches expression of *runx1* mRNA in hematopoietic domains at a similar time point.[10] Other transgenic zebrafish lines have been generated that express fluorescent markers in definitive blood cells, including *scl*, *lmo2*, *fli1*, and *gata1*.[36,38,44,45] As all of these transcription factors are expressed in both primitive and definitive blood, fluorescent expression is present along the whole length of the LPM. This is in contrast to the posteriorly restricted expression in the *runx1P1* line, showing expression specific to EMPs and not primitive erythrocytes. In addition, *lmo2* and *fli1* are strongly expressed in the developing vasculature.[44,45]

EMPs are thought to be present in the PBI between 24 and 48 hpf and arise from *lmo2*-expressing cells in the posterior LPM at 13 to 15 somites (16 hpf),[17] the stage at which we first detect *EGFP* mRNA in the *runx1P1* transgenic. This observation, together with the coexpression of *gata1* in this region, suggests that EMPs express the Runx1P1 isoform from an early stage of development in the posterior LPM. However, Runx1P1 does not appear to be required for the specification

or maintenance of EMPs. EMPs are present in the PBI transiently and are later replaced by HSCs derived from the AGM. These 2 waves of definitive hematopoiesis may differ in their dependence on Runx1. The Runx1P1 isoform does not appear to be expressed in definitive HSCs emerging from the AGM region, as no fluorescence was detected in the ventral wall of the DA and *runx1P1* is not expressed at 48 hpf by RT-PCR, a stage when strong expression of *runx1P2* is detected in HSCs in the AGM.

In the *Tg*(*runx1P2:EGFP*) line, putative HSCs in the ventral wall of the DA express EGFP from 22 hpf, corresponding to the stage at which definitive HSCs are thought to first be present in this region. By 36 hpf, fluorescent cells resembling those in *fli1*, *scl*, and *CD41* promoter transgenics are visible in the AGM between the DA and PCV. Although it has been suggested that the PBI contains exclusively EMPs before 40 hpf,[17] cells expressing *runx1P2:EGFP* are found in the AGM along the entire length of the embryo, including the area posterior to the yolk extension designated the PBI. This raises the possibility that a heterogeneous population of definitive blood cells with different developmental potential resides in overlapping spatial domains. This is consistent with the observation that hematopoietic cells expressing the *fli1:EGFP* transgene can be traced from 30 hpf as they migrate to the thymus from either the anterior or posterior region of the AGM.[41]

It has been shown that fluorescent hematopoietic cells in the AGM, expressing either *fli1* or *CD41,* migrate first to the PBI/caudal hematopoietic tissue region and then to the thymus, consistent with an HSC identity.[41,42] This is supported by results here, where the development of fluorescently labeled cells in the AGM in the *runx1P2* transgenic requires the function of transcription factors known to be required for HSC development. Fluorescent cells are found later in the thymus and pronephros, suggesting that in the *runx1P2* transgenic, hematopoietic progenitors are fluorescently marked from the very early stage of development, with persistence of expression as they migrate to other hematopoietic organs for multilineage differentiation.

Several recent zebrafish studies have contributed significant insights into definitive hematopoiesis,[12,17] but understanding the ontogeny of the hematopoietic system is far from complete. These *runx1*-transgenic lines are the first ones characterized that delineate separate waves of definitive hematopoiesis in zebrafish and will be useful tools to further explore HSC biology.

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Authorship

Contribution: E.Y.N.L. and M.V.F. designed and performed the experimental work, analyzed the data, and wrote the paper; J.Y.M.C., M.L.K.-Z., T.M.F., R.S.M., and C.J.H. performed some experiments; and P.S.C. and K.E.C. planned the project, analyzed the data, and edited the manuscript.

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