Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo

Julien Y. Bertrand*, Albert D. Kim*, Emily P. Violette, David L. Stachura, Jennifer L. Cisson and David Traver†

Shifting sites of blood cell production during development is common across widely divergent phyla. In zebrafish, like other vertebrates, hematopoietic development has been roughly divided into two waves, termed primitive and definitive. Primitive hematopoiesis is characterized by the generation of embryonic erythrocytes in the intermediate cell mass and a distinct population of macrophages that arises from cephalic mesoderm. Based on previous gene expression studies, definitive hematopoiesis has been suggested to begin with the generation of presumptive hematopoietic stem cells (HSCs) along the dorsal aorta that express c-myb and runx1. Here we show, using a combination of gene expression analyses, prospective isolation approaches, transplantation, and in vivo lineage-tracing experiments, that definitive hematopoiesis initiates through committed erythromyeloid progenitors (EMPs) in the posterior blood island (PBI) that arise independently of HSCs. EMPs isolated by coexpression of fluorescent transgenes driven by the Imo2 and gata1 promoters exhibit an immature, blastic morphology and express only erythroid and myeloid genes. Transplanted EMPs home to the PBI, show limited proliferative potential, and do not seed subsequent hematopoietic sites such as the thymus or pronephros. In vivo fate-mapping studies similarly demonstrate that EMPs possess only transient proliferative potential, with differentiated progeny remaining largely within causal hematopoietic tissue. Additional fate mapping of mesodermal derivatives in mid-somitogenesis embryos suggests that EMPs are born directly in the PBI. These studies provide phenotypic and functional analyses of the first hematopoietic progenitors in the zebrafish embryo and demonstrate that definitive hematopoiesis proceeds through two distinct waves during embryonic development.

KEY WORDS: Erythromyeloid progenitor, Hematopoiesis, Hematopoietic progenitor cells, Zebrafish

INTRODUCTION

The genesis of the blood-forming system is complex, with shifting sites of hematopoiesis occurring during development. The ontogeny of blood cells from multiple hematopoietic organs appears to be a feature common to all organisms with multiple hematopoietic lineages, including both invertebrates and vertebrates (Evans et al., 2003; Hartenstein, 2006). Whereas the specific organs that transiently host blood cell production can be divergent between species, hematopoiesis can be roughly divided into two major waves in vertebrates based on the cell types generated. Primitive hematopoiesis is characterized by a relatively rapid commitment of embryonic mesoderm to monopotent hematopoietic precursors (Keller et al., 1999). These cells give rise to embryonic erythrocytes and macrophages that are respectively required to oxygenate and remodel growing tissues of the embryo (Palis and Yoder, 2001). Definitive hematopoiesis is characterized by the emergence of multipotent hematopoietic stem and progenitor cells (Cumano and Godin, 2007).

The multiple waves of blood cell development have been best studied in the mouse, where hematopoiesis initiates with the formation of primitive erythrocytes and macrophages in the extraembryonic yolk sac (Palis et al., 1999). It is widely believed that definitive hematopoiesis subsequently begins in the midgestation embryo with the formation of HSCs in a region bounded by the aorta, gonads and mesonephros (AGM) (Dzierzak, 2005; Cumano and Godin, 2007). However, recent studies have demonstrated the presence of definitive progenitors within the yolk sac (Yoder et al., 1997a; Yoder et al., 1997b; Palis et al., 1999; Bertrand et al., 2005a; Yokota et al., 2006; Yokomizo et al., 2007) and placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Zeigler et al., 2006) that arise either before, or concomitant with, HSC formation in the AGM region. Several reports have shown that a common marker of definitive hematopoietic stem and progenitor cells in each of these tissues is the integrin, CD41 (Mitjavila-Garcia et al., 2002; Ferkowicz et al., 2003; Mikkola et al., 2003; Ottersbach and Dzierzak, 2005). Before embryonic day (E) 9.5, CD41+ yolk sac cells generate multiple myeloid and erythroid lineages, but lack lymphoid potential in co-culture assays (Yokota et al., 2006). Similar results were obtained at E10.5 using yolk sac cells purified by combined CD41+ c-Kit+ CD45+ expression (Bertrand et al., 2005b). In addition, studies utilizing a transgenic mouse expressing GFP under control of the Gata1 promoter showed that Gata1+ cells purified from E7.5 yolk sacs could generate granulocytes, monocytes, macrophages and erythrocytes upon OP9 stromal cells (Yokomizo et al., 2007). Together, these studies suggest that the definitive hematopoietic program may first generate committed erythromyeloid progenitors in the yolk sac before HSCs arise. The lineage relationships and relative contributions of definitive precursors in the yolk sac to later hematopoietic sites, such as the fetal liver, fetal spleen and bone marrow remain to be clarified.

In the zebrafish, primitive hematopoiesis also produces macrophages and erythrocytes. The first functional hematopoietic cells born in the embryo are primitive macrophages. These cells arise from anterior, cephalic mesoderm then migrate onto the yolk syncitial layer before colonizing embryonic tissues (Herbomel et al., 1999). Primitive erythrocytes develop from bilateral stripes of ventral mesoderm, which, upon migration to the midline, form a structure termed the intermediate cell mass (ICM) (Detrich et al., 1995; Thompson et al., 1998). Endothelial cells encapsulate this...
mass of maturing erythroid precursors to form the cardinal vein and, upon initiation of heart contractions at approximately 24 hours post-fertilization (hpf), primitive erythroblasts enter circulation. Based on the appearance of cells expressing HSC-associated genes such as c-myb and runx1 along the ventral wall of the dorsal aorta, definitive hematopoiesis has been presumed to initiate in this zebrafish equivalent of the AGM region between 28 and 48 hpf (Thompson et al., 1998; Burns et al., 2002; Kalev-Zylinska et al., 2002). Lineage-tracing studies have recently shown that cells residing between the aorta and vein could subsequently colonize the thymus and pronephros, the major definitive hematopoietic organs in zebrafish (Murayama et al., 2006; Jin et al., 2007).

The vertebrate AGM is not a hematopoietic organ per se, because differentiation is not observed in this region (Godin et al., 1999). Rather, the differentiation of embryonic HSCs into multiple definitive lineages occurs only after the seeding of other tissues, such as the fetal liver in mammals. Until recently, it was thought that HSCs born in the zebrafish AGM colonized the pronephros to initiate definitive hematopoiesis (Hsia and Zon, 2005). Recent fate-mapping studies of Murayama et al. showed that presumptive HSCs targeted along the aorta first migrate to a region in the tail they termed caudal hematopoietic tissue (CHT) (Murayama et al., 2006). At earlier stages, before 36 hpf, this region has also been referred to as the posterior ICM (Detrich, 3rd et al., 1995; Thompson et al., 1998), ventral vein region (Liao et al., 1998; Willett et al., 1999) and more conventionally as the posterior blood island (PBI) (Crowhurst et al., 2002; Rombout et al., 2005; Kinna et al., 2006; Renshaw et al., 2006; Jin et al., 2007) based on localization of hematopoietic markers to the ventral portion of the tail immediately caudal to the yolk tube extension. Electron microscopy studies showed that definitive myeloid cells, such as neutrophilic granulocytes, are first detected in this region at 34 hpf (Willett et al., 1999). It is not clear whether these cells migrate here from other hematopoietic sites, or whether they arise in situ from resident stem or progenitor cells. Murayama and colleagues (Murayama et al., 2006) hypothesized that the CHT is generated by migration of HSCs from the AGM to act as a fetal liver equivalent in bridging definitive blood cell production until the pronephros becomes the final hematopoietic site.

In the present study, we describe a previously uncharacterized hematopoietic precursor that arises in the PBI to initiate definitive hematopoiesis. This precursor can be prospectively isolated by flow cytometry as early as 24 hpf based on the expression of fluorescent transgenes. Molecular characterization showed promiscuous expression of erythroid and myeloid genes. Accordingly, functional studies showed that these cells have erythroid and myeloid differentiation potential but lack lymphoid potential. We have therefore termed these cells erythromyeloid progenitors (EMPs). EMPs arise before HSCs were detected in the AGM, and fate-mapping studies suggest that they arise directly from lmo2+ posterior lateral plate mesoderm (LPM) derivatives. Taken together, these results demonstrate that the EMP serves as a transient progenitor that is born independently of HSCs to initiate definitive hematopoiesis in the developing zebrafish embryo.

MATERIALS AND METHODS

Zebrafish stocks and embryos
Zebrafish were mated, staged and raised as described (Westerfield, 1994) and maintained in accordance with UCSD IACUC guidelines. Transgenic lines Tg(gata1:DsRed) (Traver et al., 2003), Tg(CD41:eGFP) (Traver et al., 2003), Tg(lmo2:DsRed) (Zhu et al., 2005) and Tg(lmo2:eGFP) (Zhu et al., 2005) were used. Tg(mpx:eGFP) animals were generously provided by S. A. Renshaw (Renshaw et al., 2006). Hereafter, transgenic lines will be referred to without the Tg(xxx:xxx) nomenclature for clarity. Embryos were processed as described (Westerfield, 1994).

Whole-mount RNA in situ hybridization
Embryos were treated with PTU, then fixed in 4% or 10% paraformaldehyde (PFA). Whole-mount in situ hybridization was carried out as described (Thiese et al., 1993). The zebrafish CD45 homologue was identified on chromosome 22 (EnsEMBL #ENSDARG00000030993), and a 1.2 kb probe generated from genomic DNA by PCR using the following primers: CD45-FP: AATGAAAAGGCTGTAATCGG; CD45-RP: GTCTTCGTGTTCGCGTCG.

Fluorescence in situ hybridization (FISH)
Antisense mRNA probes were prepared as previously reported for gata1, pu.1 (also known as spil – ZFIN) and mpx (Rhodes et al., 2005) using digoxigenin (DIG)- or fluorescein (FITC)-labeled UTPs according to the manufacturer’s instructions (Roche, Palo Alto, CA). Two-color FISH was carried out as previously described (Kosman et al., 2004) with minor modifications. The 90% xylene/10% ethanol wash was omitted and proteinase K treatments were adjusted to 25 minutes for optimal permeabilization. Hybridization with 500 ng DIG-labeled and FITC-labeled probes was performed at 70°C. mpx-FITC probes were detected with a mouse anti-FITC antibody (Roche) followed by a donkey anti-mouse Alexa Fluor 555 antibody (Molecular Probes). Reactions were developed using anti-DIG- and anti-FITC-peroxidase (HRP) antibodies. gata1-DIG was detected with a mouse anti-FITC antibody (Roche) followed by a goat anti-mouse-HRP antibody (Molecular Probes, Carlsbad, CA). Alexa Fluor 488 or 594 tyramide substrates were used according to the manufacturer’s instructions (Molecular Probes) to further amplify gata1-DIG and pu.1-DIG or gata1-FITC signals, respectively. Fluorescence images were acquired on an Olympus Fluoview 1000 confocal microscope at 200× and 400× magnification (Center Valley, PA).

Cell suspension preparation
PTU-treated whole or dissected embryos were dissociated between 24 and 72 hpf. Embryos were treated with 10 mM DTT in E3 medium then transferred to Hank’s balanced salt solution (with calcium) and digested with Liberase Blendzyme II (Sigma Aldrich, St Louis, MO) for at least 1 hour at 33°C. Cell suspensions were then filtered through 40 μm nylon mesh, washed twice and pelleted by centrifugation at 250 g for 5 minutes.

Flow cytometry
Embryonic cell suspensions were prepared as described above and fluorescence-activated cell sorting (FACS) was performed as previously described (Traver et al., 2003) using a FACS Aria flow cytometer (Becton Dickinson, San Jose, CA). Data analyses were performed using FlowJo software (TreeStar, Ashland, OR).

Cytology
Blood cells were collected by tail dissection of embryos, then homogenized using the embryonic cell collection protocol described above. Hematopoietic cells were concentrated by cytocentrifugation at 250 g for 5 minutes onto glass slides using a Shandon Cytospin 4 (Thermo Fischer Scientific, Waltham, MA). Slides were then processed through May-Grünwald and Giemsa stains according to the manufacturer’s instructions (Fluka, Buchs, Switzerland).

Microscopy
Embryos were imaged using a Leica DMi6000 inverted fluorescence microscope (Wetzlar, Germany), a Hamamatsu C7780 digital camera (Hamamatsu, Japan) and Velocity Acquisition and Restoration software (Improvisation, Lexington, MA).

RT-PCR analyses
For RT-PCR analysis, RNA was isolated from cells sorted from lmo2:eGFP and gata1:DsRed dual-positive embryos using Trizol (Invitrogen, Philadelphia, PA). Total RNA (2.5 μg) was reverse transcribed into cDNA using a Superscript III RT-PCR kit (Invitrogen, Philadelphia, PA). The following primers were used: mpx-FP: ATGGATCCAGTTTTCATC - RTGATATCCAGTTTTCATC - TGGTGG, mpx-RP: CAACTTTGCAGGCTGTAATCGG; CD45-FP: ATGGATCCAGTTTTCATC - RTGATATCCAGTTTTCATC - TGGTGG, CD45-RP: CAACTTTGCAGGCTGTAATCGG; pu.1-FP: AATGAAAAGGCTGTAATCGG; pu.1-RP: GTCTTCGTGTTCGCGTCG; gata1-FP: AATGAAAAGGCTGTAATCGG; gata1-RP: GTCTTCGTGTTCGCGTCG.
FP: GTGCATGTGGATGGAACG, l-plastin-RP: CTCCTCGGAGTATGAGTC; CD41-FP: TTACTACGACCTATATCTGGG, CD41-RP: GATGACCTGACATACTGGG; c-myb-FP: AGTTCATTCCGGAAGAACC, c-myb-RP: AGAGCAAGTGGAAATGCGACC; runx1-FP: TTGGGAGCGCCAATAACGACC, runx1-RP: ATATCACCAAGGGCAACC-ACC; elfa-FP: CGGTGACAACATGCTGGAGG, elfa-RP: ACCAGTTCACACAGCACC. Primers for mpx, pu.1 and gata1 transcripts were used as described (Hsu et al., 2004).

Hematopoietic cell transplantation
Single-cell suspensions from lmo2:eGFP; gata1:DsRed transgenic embryos were prepared as described above. EMPs were sorted as described above and transplanted into 36 or 48 hpf wild-type embryos as previously described (Traver et al., 2003).

Fate mapping
One- to eight-cell-stage CD41:eGFP or lmo2:eGFP transgenic embryos were injected with 0.5 nl of a 0:1 or 1:1 mix of caged fluorescein-dextran 10,000 MW and caged rhodamine-dextran 10,000 MW (Molecular Probes, Carlsbad, CA). Uncaging was performed using a 365 nm Micropoint laser system (Photonic Instruments, St Charles, IL) routed through the epifluorescence port and 20× objective of a Leica DMI6000 inverted microscope. Ten GFP+ target cells were uncaged per embryo following laser pulses of 10-20 seconds each. Uncaged embryos injected with rhodamine-dextran were subsequently observed using fluorescence microscopy whereas uncaged FITC was detected by immunohistochemistry, as previously described (Murayama et al., 2006).

Generation of zebrafish kidney stromal (ZKS) cell lines
Kidneys were isolated from wild-type AB* fish by dissection and bleached for 5 minutes in 0.000525% Sodium Hypochlorite (Fisher Scientific, Pittsburgh, PA). Tissue was then rinsed in sterile Dulbecco’s phosphate-buffered saline (PBS; Mediatech, Herndon, VA) and mechanically dissociated by trituration. Dissociated cells were passed through a 0.45 μm filter (BD Biosciences, San Jose, CA) and discarded. The remaining stromal cells were cultured in 12.5% H9262/H11003 supplements from Mediatech). Dissociated cells at 32°C, and split at 1:3 for expansion.

In vitro differentiation assay
EMPs were purified by flow cytometry and plated onto confluent ZKS cells at a density of 1×10^6 cells/well in 12-well plates using 2 ml medium per well. For morphological analyses, wells were gently aspirated and 200 μl removed for cytocentrifugation onto glass slides. Cells were stained with May-Grünwald/Giemsa stains as described above, or for myeloperoxidase activity according to the manufacturer’s instructions (Sigma Aldrich). Light microscopy images were obtained using an Olympus BX51 light microscope, Olympus DP70 camera, and Olympus DP Controller software (Center Valley, PA).

RESULTS
The posterior blood island is a site of hematopoiesis
We wished to determine when and where the first definitive hematopoietic precursors arise in the zebrafish embryo. Early gene expression studies suggested that the most posterior portion of the ICM contained hematopoietic precursors distinct from the primitive erythroblasts that arise in the ICM (Thompson et al., 1998). To determine what types of blood cells are found in the PBI, we performed whole-mount in situ hybridization (WISH) with a panel of hematopoietic genes at 30 and 36 hpf. We first identified the zebrafish homologue of CD45, which in mammals is expressed in all hematopoietic cell lineages except for erythrocytes. Expression of the zebrafish CD45 gene was observed as early as 30 hpf, and positive cells increased in number by 36 hpf in the ventral part of the tail (Fig. 1A,G). Although some studies have treated l-plastin (also known as lcp1 – ZFIN) as a macrophage-specific marker in the

Fig. 1. Genes associated with multilineage hematopoiesis are expressed in the posterior blood island as early as 30 hpf. (A,B) By 30 hpf, expression of the pan-leukocyte markers CD45 and l-plastin are observed throughout the PBI. (G,H) By 36 hpf, the number of cells expressing each gene has increased in the PBI, and expressing cells begin to migrate throughout the embryo. Localized expression of lmo2 is observed in the PBI at 30 hpf (C) and 36 hpf (I). (D,J) Expression of gata1 is also observed in cells within the vascular plexus of the PBI at both timepoints. (E,F,K,L) Expression of genes associated with myelopoiesis is also observed within the PBI, including pu.1 and mpx. Rare pu.1+ cells are observed at 30 hpf in the PBI (E) that increase in number by 36 hpf (K), whereas mpx expression is not observed until 36 hpf (FL), consistent with it being a marker of relatively mature myelomonocytic cell types. Magnification: 100× in whole-embryo images; 300× in PBI images.
zebrafish, it is expressed in all murine leukocytes, including hematopoietic progenitors (Arpin et al., 1994; Bertrand et al., 2005b) and in zebrafish granulocytes (Meijer et al., 2007). Expression of I-plastin was observed in a pattern similar to that of CD45 (Fig. 1B,H), suggesting that both genes similarly mark zebrafish leukocytes.

To assess further the possible presence of hematopoietic progenitors, we analyzed expression of lmo2, gata1 and pu.1. Lmo2 is expressed by both vascular and hematopoietic precursors. In addition to widespread vascular staining in the tail, a small population of lmo2+ cells was observed in the PBI (Fig. 1C,I). Similarly, expression of a canonical marker of erythroid potential, gata1, was observed in small numbers of cells in the PBI at both time points (Fig. 1D,J). These cells did not appear to be within vessels, but rather within the vascular plexus of the PBI. Pu.1 is a transcription factor that acts as a master regulator of myeloid commitment (Tenen et al., 1997). We first observed a small population of pu.1+ cells in the PBI at 30 hpf that increased in number by 36 hpf (Fig. 1E,K). Myeloperoxidase (mpx) is expressed specifically in cells of the myelomonocytic lineages, and is transcriptionally controlled by Pu.1 (Tenen et al., 1997). Accordingly, pu.1 expression precedes transcription of mpx in the PBI by at least 6 hours, because pu.1 and mpx expression are first detected at 30 and 36 hpf, respectively (Fig. 1E,F,K,L). Expression of all genes appeared to localize to the region between the aorta and the caudal vein, just posterior to the yolk tube extension. Taken together, these data indicate that both erythroid and myeloid cells are present in the PBI at 30 hpf and are consistent with the hypothesis that the PBI is an early site of multilineage hematopoiesis.

We next took a cytological approach to determine whether definitive hematopoietic cell types could be recognized morphologically from PBI preparations. Tails were dissected from embryos at time points ranging from 24 to 72 hpf and single-cell suspensions prepared following enzymatic digestion. At 24 hpf, the only hematopoietic lineages observed were erythroblasts and rare monocytes or macrophages (see Fig. S1 in the supplementary material), each probably originating from primitive hematopoiesis. At 30 hpf another cell subset appeared that showed characteristics of early hematopoietic precursors, including blastic morphology, open chromatin structure and basophilic cytoplasm. Eosinophilic and neutrophilic granulocytes were first observed by 36 and 48 hpf, respectively (Fig. S1 in the supplementary material). After 72 hpf, lmo2+gata1+ cells could no longer be detected (not shown).

Before 30 hpf, a population of lmo2+/gata1+ cells was observed by flow cytometry that expressed approximately 15 times lower levels of GFP than lmo2+gata1+ cells (Fig. 2B). Cell sorting showed that the lmo2+/gata1+ fraction consisted entirely of primitive erythroblasts (Fig. 2C, left panel). Unlike purified primitive erythroblasts, purified lmo2+/gata1+ cells showed morphologies characteristic of earlier hematopoietic progenitors (Fig. 2C, middle and right panels). At 30 hpf, two morphological subtypes were present in purified lmo2+/gata1+ cells. Approximately half of this population displayed a morphology characteristic of immature myelomonocytic progenitors, including asymmetric oval or bean-shaped nuclei with lightly stained cytoplasm containing darker granules (Fig. 2C, middle panel). The remaining half displayed a morphology characteristic of immature erythroid progenitors, including centered, round nuclei with stippled staining patterns and basophilic cytoplasm (Fig. 2C, right panel).

**lmo2/gata1+ cells express both erythroid and myeloid genes**

Hematopoietic progenitor cells frequently express lineage markers for multiple mature cell types, reflecting their multilineage differentiation potential (Hu et al., 1997; Miyamoto et al., 2002). We therefore profiled the gene expression pattern of purified lmo2+/gata1+ cells. At 30 hpf, we isolated four populations of cells defined by differential expression of lmo2:eGFP and gata1:dsRed transgenes in double transgenic embryos, including lmo2low/gata1+, lmo2+/gata1+, lmo2+/gata1+ and lmo2+/gata1+ fractions. In addition to expressing high levels of the erythroid-associated gata1 reporter gene, RT-PCR analyses showed that purified lmo2+/gata1+ cells expressed the pan-leukocyte marker, l-plastin, and the myelomonocytic genes, mpx and pu.1 (‘LG’, Fig. 3A). By contrast, expression of these genes in purified primitive erythroblasts (lmo2low/gata1+ cells) was undetectable (‘G’, Fig. 3A). This expression pattern suggests that, despite expression of the gata1:dsRed transgene, the lmo2+/gata1+ population is not committed to the erythroid lineage. Accordingly, these cells also express scl and runx1, genes associated with early hematopoietic progenitors. Expression of the mpx and runx1 genes was found in the lmo2+/gata1+ fraction (‘L’, Fig. 3A). Runx1 is known to be expressed in endothelial cells (Kalev-Zylinska et al., 2002), the most abundant cell type marked by the lmo2:eGFP transgene. The presence of mpx transcripts may be due to low-level expression of the lmo2:eGFP transgene in primitive macrophages (not shown).

We also analyzed CD41 (also known as itga2b – ZFIN) in each isolated subset, since its expression appears to be one of the first markers of mesoderm commitment to definitive hematopoiesis in mammals (Mitjavila-Garcia et al., 2002; Ferkowicz et al., 2003; Mikkola et al., 2003). In 30 hpf embryos, CD41 expression was only...
detected within \textit{lmo2}\textsuperscript{+}\textit{gata1}\textsuperscript{+} cells. This result supports the hypothesis that \textit{lmo2}\textsuperscript{+}\textit{gata1}\textsuperscript{+} cells represent an early definitive hematopoietic progenitor population.

To determine whether individual \textit{lmo2}\textsuperscript{+}\textit{gata1}\textsuperscript{+} cells express multiple lineage markers, as would be expected for a hematopoietic progenitor cell, we performed single-cell expression studies by FACS and FISH. To assess myeloerythroid gene expression at the single-cell level, we analyzed embryos carrying \textit{mpx}\textsuperscript{eGFP} and \textit{gata1}\textsuperscript{DsRed} transgenes. \textit{mpx}\textsuperscript{eGFP} transgenic zebrafish were generated recently and shown to express GFP in embryonic granulocytes (Renshaw et al., 2006). In double transgenic embryos, early expression of the \textit{mpx}\textsuperscript{eGFP} transgene is largely localized to cells also expressing the \textit{gata1}\textsuperscript{DsRed} transgene (Fig. 3B). We have also observed coexpression of the \textit{mpx} and \textit{gata1} transcripts in single cells in the PBI using two-color FISH (Fig. 3C). In murine multipotent progenitors, myeloid versus erythroid fate decisions are largely dependent upon the \textit{Pu.1} and \textit{Gata1} transcription factors, respectively (Nerlov et al., 2000; Zhang et al., 2000; Huang et al., 2007). We observed coexpression of \textit{pu.1} and \textit{gata1} in single cells in the PBI (Fig. 3C). We did not observe coexpression of either \textit{pu.1} and \textit{gata1} or \textit{mpx} and \textit{gata1} in any other anatomical site in the zebrafish embryo (not shown). Taken together, our gene expression analyses strongly support the existence of a definitive, multilineage precursor in the zebrafish PBI.

PBI hematopoietic progenitors lack key characteristics of HSCs

Because \textit{lmo2}\textsuperscript{+}\textit{gata1}\textsuperscript{+} cells displayed many of the features of hematopoietic progenitor cells, we wanted to test their homing, proliferative, and differentiation potentials in functional assays. \textit{lmo2}\textsuperscript{+}\textit{gata1}\textsuperscript{+} cells were purified by flow cytometry from 36 hpf embryos and transplanted into WT embryonic recipients, either stage-matched or at 48 hpf. Transplanted cells homed to the PBI within 24 hours post-transplantation (Fig. 4A). In contrast to transplanted adult whole kidney marrow (WKM) cells which populate the embryonic thymus and pronephros (Traver et al., 2003), embryonic \textit{lmo2}\textsuperscript{+}\textit{gata1}\textsuperscript{+} cells were never observed to seed these hematopoietic organs. Rather, donor-derived cells remained largely within the PBI and, based upon the disappearance of double-fluorescent yellow cells after 1-2 days, appeared to differentiate rapidly (Fig. 4A). Donor-derived cells were observable for approximately one week following transplantation by fluorescent transgene expression. Several reports have demonstrated that rare, definitive hematopoietic precursors arise in the mammalian yolk sac before circulation (Palis et al., 1999; Ferkowicz et al., 2003; Bertrand et al., 2005a; Yokota et al., 2006; Yokomizo et al., 2007). These precursors express CD41 (Ferkowicz et al., 2003; Mikkola et al., 2003; Yokota et al., 2006), which appears to be the first marker of definitive...
hematopoietic commitment from mesoderm. Since CD41 was detected only within lmo2\textsuperscript{+}gata1\textsuperscript{+} cells in 30-36 hpf embryos, we analyzed expression of CD41:eGFP in the PBI of transgenic embryos (Lin et al., 2005). GFP expression was detected as early as 26 hpf, and increased in intensity and in the number of cells labeled embryos (Lin et al., 2005). Based on the similarities to other vertebrate AGMs, the regional fate-mapping results of Murayama et al. (Murayama et al., 2006), and the finding that CD41 marks embryonic HSCs in the mouse (Bertrand et al., 2005b), we hypothesized that CD41:eGFP expression in the AGM may mark the first HSCs to arise in the zebrafish embryo. We therefore performed similar lineage-tracing studies by laser-targeting ten CD41\textsuperscript{+} cells in the AGM. Unlike CD41\textsuperscript{+} cells in the 40 hpf PBI, which never populated the thymus, 10/16 embryos where CD41\textsuperscript{+} cells were targeted in the AGM between 44-48 hpf showed thymic colonization (Fig. 4B). The progeny of AGM cells were also observed to colonize the PBI, but we never observed the progeny of PBI-targeted CD41\textsuperscript{+} cells to colonize the AGM, thymus or pronephros.

Our transplantation and fate-mapping experiments demonstrated that hematopoietic progenitors in the PBI lack lymphoid potential — a hallmark of HSCs. However, PBI progenitors did display transient but relatively robust proliferation potential. To assess the differentiation potential of these cells, we developed in vitro culture assays using a new zebrafish kidney stromal (ZKS) cell line. lmo2\textsuperscript{+}gata1\textsuperscript{+} cells were purified from 30 hpf embryos and deposited onto ZKS layers. Aliquots were removed daily and analyzed for cellular morphology. Compared to the immature morphology of uncultured cells (Fig. 2C), lmo2\textsuperscript{+}gata1\textsuperscript{+} cells rapidly differentiated into erythroid and myelomonocytic cell fates upon 2 days of culture (Fig. 4C). An approximately equivalent ratio of erythroid to myelomonocytic cells was observed on day 2 (see Fig. S2 in the supplementary material). By day 4 of culture myelomonocytic cells predominated, with most erythroid cells presumably having differentiated and died (see Fig. S2 in the supplementary material). Based on morphological analyses, cultured lmo2\textsuperscript{+}gata1\textsuperscript{+} cells
generated multiple myeloid lineages, including neutrophilic granulocytes, monocytes and macrophages (Fig. 4C), whereas parallel experiments using purified \textit{lmo2}^{low}\textit{gata1}^{+} primitive erythroblasts showed only erythroid progeny (not shown). Cells were also removed after 4 days of culture and stained for myeloperoxidase (MPX) activity. Many cells demonstrated robust staining, and nuclear morphology showed that positive cells included both immature myelomonocytic cells and mature granulocytes (Fig. 4C, bottom panel).

Taken together, these functional assays demonstrate that \textit{lmo2}^{+}\textit{gata1}^{+}\textit{CD41}^{+} cells in the PBI represent a population of cells with both erythroid and myeloid differentiation potential. Their proliferation capacity is limited both in transplantation experiments and in vitro, and their progeny remain largely within the PBI. Furthermore, they lack lymphoid potential. We therefore term these cells erythromyeloid progenitors (EMPs).

**EMPs arise independently of HSCs from caudal, \textit{lmo2}^{+} mesoderm**

Collectively, our data suggest that \textit{lmo2}^{+}\textit{gata1}^{+}, or \textit{CD41}^{+} cells in the PBI before 40 hpf are committed erythromyeloid progenitors that lack self-renewal and multilineage differentiation abilities. Our fate-mapping experiments also suggest that \textit{CD41}^{+} cells in the nascent AGM region represent the first HSCs born in the embryo. Although EMPs appear to arise before presumptive HSCs are observed in the AGM, it is possible that EMPs derive from HSCs. To determine whether EMPs are born directly in the PBI or migrate from the AGM, we performed uncaging experiments in mid-somitogenesis embryos. Approximately 10 \textit{lmo2}^{+} cells were targeted in either the medial (bounded by somites 1-10 – the region that will later contain the AGM) or most posterior portion (the region that will later become the PBI) of the stripe of mesodermal-derived tissue marked by an \textit{lmo2}:eGFP transgene in 13- to 15-somite-stage embryos (Fig. 5A,B). Since the number of EMPs peaks at 30 hpf (Fig. 2B), we analyzed uncaged animals at this time point. All eight animals with medial \textit{lmo2}^{+} cells targeted showed marked progeny confined to the anterior trunk region (Table 1), mainly within presumptive vasculature (not shown). None of the eight animals showed marked progeny within the PBI (Fig. 5C). Five out of eight animals with posterior \textit{lmo2}^{+} cells targeted showed marked progeny within the PBI (Table 1). These cells were large and round and localized to the vascular plexus of the PBI (Fig. 5C), consistent with our localization of \textit{lmo2}^{+}\textit{gata1}^{+} EMPs at 30 hpf (Fig. 2A). None of these eight animals showed labeled progeny in the trunk (Table 1).

![Fig. 4. Functional studies demonstrate that gata1^{+} lmo2^{+} CD41^{+} cells are committed erythromyeloid progenitors.](image)
Together, these data suggest that \textit{lmo2$^+$ gata1$^+$} EMPs arise from the most posterior regions of the \textit{lmo2$^+$ (gata1$^+$)} hematopoietic or vasculogenic ‘stripes’ that converge to form the posterior ICM or PBI. These results are consistent with previous studies demonstrating localized expression of ‘definitive’ hematopoietic genes in this region such as \textit{scl} (Liao et al., 2002) and \textit{gata2} (Detrich et al., 1995).

**DISCUSSION**

Our results demonstrate that definitive hematopoiesis initiates in the PBI through a committed progenitor that we have termed the erythromyeloid progenitor (EMP). These progenitors appear to exist transiently, between 24-48 hpf, and can be isolated prospectively by their coexpression of \textit{lmo2} and \textit{gata1} transgenes. Our description of a definitive progenitor in the PBI explains previous gene expression studies, which suggested that a distinct population of cells was present in the caudal most portion of the developing ICM. Based on the expression of \textit{gata2$^+$} cells at the posterior tip of the \textit{gata1$^+$} ICM, Detrich et al. postulated these cells to be undifferentiated, presumptive hematopoietic progenitors (Detrich et al., 1995). Additional early observations showed that cells in this region also expressed \textit{lmo2} (Thompson et al., 1998). The authors hypothesized that these cells may be the founders of a distinct second wave of hematopoiesis. Our identification of the EMP confirms these hypotheses, and characterization of their molecular markers provides the means to isolate them for functional analyses, including lineage differentiation potentials.

The presence of the EMP in the PBI by 24 hpf may help clarify the ontogeny of the definitive myeloid lineages in teleosts. Histological and ultrastructural analyses have described granulocytic precursors in the PBI, both in the zebrafish (Willett et al., 1999; Zapata et al., 2006) and carp (Rombout et al., 2005; Huttenhuis et al., 2006). We observed eosinophilic and neutrophilic granulocytes in zebrafish tail preparations by 36-48 hpf. The first granulocytes produced in the zebrafish embryo may therefore be the daughters of EMPs, and not lineally related to the primitive macrophages that arise from cephalic mesoderm. This model is consistent with the observations of Herbomel and colleagues (Herbomel et al., 1999), and with the single lineage origin of embryonic macrophages produced in other organisms ranging from \textit{Drosophila} to mammals (Lichanska and Hume, 2000; Evans et al., 2003). Based on the robust generation of mononuclear phagocytes by cultured EMPs, it appears that a second wave of macrophage production later occurs in the PBI through monocytic intermediates. The full differentiation potential of the EMP remains to be determined because our in vitro conditions do not appear to support all myeloid/erythroid lineages, including eosinophils and thrombocytes. Isolation of autologous growth factors such as Interleukin-3 and Thrombopoietin will probably be necessary to reveal the complete fate potentials of zebrafish hematopoietic stem and progenitor cells.

The first blood cells born in the mammalian embryo are erythroid cells in the extraembryonic yolk sac. Based on their resemblance to the nucleated erythrocytes of avian and amphibian species, these cells were initially termed primitive, and their enucleated embryonic counterparts definitive (McGrath and Palis, 2005). More recently, these terms have become interchangeable with yolk sac and intraembryonic hematopoiesis, respectively. The correlation of each wave with anatomical sites has led to some confusion because several reports have recently demonstrated the existence of multipotent, definitive hematopoietic progenitors in the extraembryonic yolk sac that generate definitive cell types (Wong et al., 1986; Cumano, 1996; Palis et al., 1999; Bertrand et al., 2005a; Yokota et al., 2006; Yokomizo et al., 2007). Likewise, the nomenclature used for the hematopoietic waves in the zebrafish has often been inconsistent and confusing, with many publications referring to ‘primitive HSCs’, ‘primitive granulocytes’, and so forth.

We propose that the same conventions applied to the murine hematopoietic waves (Keller et al., 1999; McGrath and Palis, 2005) be used to describe primitive versus definitive hematopoiesis in the zebrafish, namely that only ICM-derived erythrocytes and cephalic mesoderm-derived macrophages should be termed primitive. This nomenclature is consistent with observations in each species suggesting that the two primitive lineages appear to arise directly from mesoderm without transiting through a multipotent progenitor (Detrich et al., 1995; Herbomel et al., 1999; Keller et al., 1999; Bertrand et al., 2005a). Conversely, we propose that hematopoietic cells that derive from an oligopotent or multipotent progenitor be termed definitive.
In our current model, hematopoietic development in the zebrafish occurs in four independent waves, each through precursors that arise in different anatomical regions (Fig. 6). Two primitive waves produce macrophages from cephalic mesoderm and erythrocytes from the ICM. Likewise, two definitive waves occur, the first from EMP production in the PBI and the second from HSC generation in the AGM. It was previously proposed by Murayama and colleagues that all hematopoietic cells in the CHT derive from HSCs born in the AGM (Murayama et al., 2006). Our results do not support this hypothesis. Several lines of evidence show that hematopoietic precursors are present in the PBI before presumptive HSCs can be detected in the AGM region beginning at approximately 28 hpf. These include gene expression profiles suggestive of multilineage hematopoiesis in the PBI by 30 hpf, the appearance of definitive myelomonocytic cells within the PBI by 36 hpf, single-cell coexpression of early hematopoietic genes, such as lmo2-gata1 or gata1-pu.1 by 30 hpf, and CD41:eGFP+ cells appearing in the PBI before the AGM. Furthermore, our fate-mapping experiments suggest that EMPs arise directly from the lmo2:eGFP+ descendants of posterior LPM that contribute to the formation of the PBI. The experiments of Murayama et al. were performed after 48 hpf. Our lineage-tracing experiments showed that CD41:eGFP+ cells in the PBI/CHT before 40 hpf lack thymus colonizing potential. After 40 hpf, we observed rare thymic immigrants from targeted CD41:eGFP+ cells in the CHT that increased in number over time. These results suggest that the first CD41:eGFP+ cells in the PBI are EMPs and that CD41:eGFP+ HSCs immigrate into the nascent CHT from the AGM after 40 hpf. It appears that the EMP disappears gradually after this time, with definitive hematopoiesis in the CHT simultaneously arising from incoming HSCs. As previously suggested in the mouse, the generation of committed EMPs in the embryo before HSCs arise has probably evolved to provide a rapid burst of definitive cell types to meet the needs of the growing embryo (Keller et al., 1999; McGrath and Palis, 2005).

In conclusion, we have demonstrated that definitive hematopoiesis initiates through a committed progenitor that is generated independently of HSCs in the posterior blood island of the zebrafish embryo. These findings provide a better understanding of hematopoietic ontogeny in the zebrafish and help to clarify the beginnings of the definitive blood cell lineages in teleosts. The development of prospective isolation approaches and a variety of functional assays to both purify and distinguish EMPs and HSCs now allows the genetic dissection of the cues that pattern each population from mesoderm. Our results also highlight the close conservation between zebrafish and mammals in the temporal development of each hematopoietic wave during development of the blood-forming system.

We thank Amy Kiger, Patricia Ernst and Wilson Clements for critical evaluation of the manuscript; Steve Renshaw for providing mpx:eGFP animals; David Kosman, Bill McGinnis, Samantha Zeitlin and Scott Halley for assistance with FISH techniques and confocal microscopy; Shutian Teng for performing initial CD45 WISH experiments; Shrey Purohit and Jason Reyes for assistance with progenitor cultures; Colin Jamora for microscope use; Adam O’Connor and Karen Ong for assistance with flow cytometry; and Roger Rainville, Evie Wright and Lisa Phelps for excellent animal care. Supported by the Irvington Institute for Immunological Research (J.Y.B.), the Cancer Research Prevention Foundation (D.L.S.), National Institutes of Health grant # DK074482, the March of Dimes Foundation, and the Arnold and Mabel Beckman Foundation (D.T.).

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/23/1417/DC1
conserved mechanisms in Drosophila and vertebrate hematopoiesis. Dev Cell 5, 673-690.


