Development 129, 649-659 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV3475

Non-cell autonomous requirement for the *bloodless* gene in primitive hematopoiesis of zebrafish

Eric C. Liao*,†, Nikolaus S. Trede*, David Ransom*, Augustin Zapata‡, Mark Kieran* and Leonard I. Zon*,§

*Division of Hematology/Oncology, Children's Hospital, Department of Pediatrics and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA
†Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
‡Department of Cell Biology, Complutense University, 28040 Madrid, Spain
§Author for correspoondence (e-mail: zon@rascal.med.harvard.edu)

Accepted 30 October 2001

SUMMARY

Vertebrate hematopoiesis occurs in two distinct phases, primitive (embryonic) and definitive (adult). Genes that are required specifically for the definitive program, or for both phases of hematopoiesis, have been described. However, a specific regulator of primitive hematopoiesis has yet to be reported. The zebrafish *bloodless* (*bls*) mutation causes absence of embryonic erythrocytes in a dominant but incompletely penetrant manner. Primitive macrophages appear to develop normally in *bls* mutants. Although the thymic epithelium forms normally in *bls* mutants, lymphoid precursors are absent. Nonetheless, the bloodless mutants can progress through embryogenesis, where red cells begin to accumulate after 5 days post-fertilization (dpf). Lymphocytes also begin to populate the thymic organs by 7.5 dpf. Expression analysis of hematopoietic genes suggests that formation of primitive hematopoietic precursors is deficient in *bls* mutants and those few blood precursors that are specified fail to differentiate and undergo apoptosis. Overexpression of *scl*, but not *bmp4* or *gata1*, can lead to partial rescue of embryonic blood cells in *bls*. Cell transplantation experiments show that cells derived from *bls* mutant donors can differentiate into blood cells in a wild-type host, but wild-type donor cells fail to form blood in the mutant host. These observations demonstrate that the *bls* gene product is uniquely required in a non-cell autonomous manner for primitive hematopoiesis, potentially acting via regulation of *scl*.

Key words: Primitive hematopoiesis, Embryonic, Definitive, Stem cell, Zebrafish, *bloodless*, Non-cell autonomous, *scl*, *gata1*

INTRODUCTION

Studies in vertebrates suggest that hematopoietic progenitors are derived from mesodermal tissue of the developing embryo, under the influence of genes such as *Bmp4* and *Mix1* (Davidson and Zon, 2000; Dzierzak and Medvinsky, 1995; Robertson et al., 1999; Zon, 1995). Subsequently, vertebrate hematopoiesis is characterized by successive waves of development, classified as primitive (embryonic) and definitive (fetal and adult) programs. Primitive and definitive phases of hematopoiesis are often distinguished on the basis of anatomic sites of development, time of initiation, cell types produced and cell morphology. Definitive hematopoiesis produces cells of erythroid, myeloid and lymphoid lineages, whereas primitive hematopoiesis is primarily erythroid with some macrophages produced as well.

Murine primitive hematopoiesis begins in the extra-embryonic mesoderm of the yolk sac around embryonic day 7.5 (E7.5) (Dzierzak and Medvinsky, 1995; Palis et al., 1999; Robb, 1997). In birds, primitive hematopoiesis initiates in yolk sac blood islands that arise in the posterior area opaca at the early somite stage (Dieterlen-Lievre, 1997; Lassila et al., 1982; Peault, 1996; Szenberg, 1977; Zagris, 1986). In amphibians such as *Xenopus*, blood is first formed in the intra-embryonic ventral mesoderm and migrates to form a 'V-shaped' hematopoietic blood island (Kelley et al., 1994; Rollins-Smith and Blair, 1990). In teleosts (bony fish) such as zebrafish, primitive hematopoiesis begins in the intra-embryonic mesodermal tissue called the intermediate cell mass (ICM), which is formed by medial migration of cells in the bilateral lateral plate mesoderm (Detrich et al., 1995; Thompson et al., 1998).

Several studies suggest that the first definitive hematopoietic cells arise from the yolk sac (Godin et al., 1995; Palis et al., 1999; Wong et al., 1986). Studies that correlate stem cell activity from in vitro clonal assays with expression of genes such as *scl* and *gata1* demonstrate that primitive hematopoiesis takes place in the murine yolk sac, where definitive hematopoietic cells also originate (Palis et al., 1999). In mice, yolk sac cells from day 9 embryos can provide long-term multi-lineage reconstitution, which is capable of contributing to mature peripheral blood, thymus, spleen, and bone marrow lymphoid, myeloid and erythroid cell types (Yoder et al., 1997). Subsequently, the site of mouse definitive hematopoiesis moves to the aorta-gonad-mesonephros (AGM) region (Medvinsky and Dzierzak, 1996; Medvinsky et al.,

1993; Muller et al., 1994). Multi-potential hematopoietic progenitors have also been detected in murine para-aortic splanchnopleura (Dieterlen-Lievre and Le Douarin, 1993; Godin et al., 1993).

Factors important for the development of both primitive and definitive hematopoiesis such as *Scl*, *Lmo2*, *Gata1*, *Gata2* and *Flk1* (*Kdr*) have been described (Pevny et al., 1995; Porcher et al., 1996; Robb et al., 1996; Shalaby et al., 1997; Shivdasani et al., 1995; Tsai et al., 1994; Warren et al., 1994). Genes required specifically for definitive but not primitive hematopoiesis, such as *Myb*, *Kit*, *Slf* (*Pou3f4*), *Tel*, *Aml1* (*Runx1)*, *Cbfb* and *Epo* have also been identified (Lin et al., 1996; Mucenski et al., 1991; Ogawa et al., 1993; Okada et al., 1998; Sasaki et al., 1996; Wang et al., 1997; Wang et al., 1996). However, a specific regulator of primitive hematopoiesis has not been reported. A primitive-specific hematopoietic defect in mammals would probably present with in utero lethality, and would not survive to demonstrate normal definitive hematopoiesis. By contrast, zebrafish is uniquely suited to uncover embryonic bloodless phenotypes, as severely anemic zebrafish embryos can be raised to adulthood in laboratory conditions (Brownlie et al., 1998; Liao et al., 2000a).

The development of hematopoietic precursors is regulated by both extrinsic and intrinsic cues. Several studies describe the role of secreted growth factors such as *bmp4* and *stem cell factor* (*Steel*) on the induction of blood and regulation of the hematopoietic stem cells, respectively (Mead and Zon, 1998; Whetton and Spooncer, 1998). Key intrinsic factors such as transcription factors *Scl* and *Lmo2* are required for the formation of hematopoietic stem cells, and *Gata1* is required for the differentiation of stem cells along the erythroid lineage (Orkin, 1996). In zebrafish, *scl* has been demonstrated to be sufficient for specifying hematopoietic progenitors (Gering et al., 1998). Overexpression of *scl* can rescue blood and endothelial cells in the *cloche* (*clo*) mutant, which specifically lacks those two cell types (Liao et al., 1998). With respect to hematopoiesis, the *clo* gene appears to act in a non-cell autonomous manner in the differentiation of embryonic blood cells, where reciprocal transplantation experiments show that wild-type donor cells were less likely to express *gata1* in a mutant environment (Parker and Stainier, 1999). In addition, co-transplantation experiments show that *clo* is required cell autonomously in subsequent proliferation of embryonic blood cells, as wild-type donor cells always contribute a greater number of blood cells than mutant donor cells in the wild-type host.

We report our characterization of *bloodless* (*bls*), a dominant zebrafish mutation producing embryos that are severely anemic at the earliest time point that circulation can be detected. Analysis of the expression of early hematopoietic genes show that decreased number of primitive hematopoietic cells are formed from the lateral plate mesoderm. Those blood precursors that are formed fail to differentiate and undergo apoptosis. In addition to an absence of blood cells during embryogenesis, *bls* mutants also exhibit delayed initiation of lymphopoiesis. However, primitive macrophages develop normally in *bls*, illustrating distinct developmental regulation of erythroid and myeloid lineages during embryogenesis. Overexpression studies with *bmp4*, *scl* and *gata1* suggest that *bls* may regulate primitive hematopoietic cell differentiation or survival, potentially by regulating the expression of *scl*. Cell transplant experiments between wild-type and *bls* mutant animals suggest that the *bls* gene is required in a non-cell autonomous manner for primitive hematopoiesis. Despite the lack of blood cells for the first 4 days of life, hematopoiesis recovers in *bls* mutants as the definitive blood program replaces the primitive wave. We present the first description of a primitive-specific mutant phenotype and show that the gene product is required in a non-cell autonomous manner for embryonic hematopoiesis, potentially regulating *scl* expression.

MATERIALS AND METHODS

Zebrafish strains and maintenance

Zebrafish were raised and maintained as described (Westerfield, 1993), and staged as described (Kimmel et al., 1995). The spontaneous *bloodless* allele, bls^{H75} was obtained from Carl Fulwiler and Walter Gilbert (Harvard University Biolabs), isolated in an insertional mutagenesis screen with a *lacZ* vector. The bls^{H75} allele was outcrossed to the standard wild-type strain (AB), and homozygotes were inbred for five generations. Embryos used for in situ and transplantation experiments were collected from inbred bls^{H75} homozygotes. The spontaneous *cloche* allele, clo^{m39} (Stainier et al., 1995), was obtained from Mark Fishman (Cambridge, MA). Embryos used for in situ and micro-injection experiments were collected from pairwise matings between identified clo^{m39} heterozygotes.

RNA in situ hybridization, biotin-dextran label detection and immunohistochemistry

In situ hybridization and riboprobe synthesis were performed as described (Schulte-Merker et al., 1992), with modifications (Liao, 1998). Antisense riboprobes to *cmyb*, *draculin*, *flk1*, *gata1*, *gata2*, *ikaros*, *ntl*, *rag1*, *scl*, *shh*, *spt* and *tbx6* have been described previously (Detrich et al., 1995; Griffin et al., 1998; Herbomel et al., 1999; Hug et al., 1997; Liao et al., 1998; Thompson et al., 1998; Willett et al., 1999). Digoxigenin or fluorescein-labeled riboprobes were detected with alkaline phosphatase conjugated anti-digoxigenin or anti-fluorescein antibody, respectively. Alkaline phosphatase substrates used to yield crimson, blue and purple are Vector Red, Vector Blue and BCIP/NBT, respectively (Vector Laboratories). To detect biotin-dextran labeled cells, the Vectastain peroxidase kit was used, where colors red and blue were developed using Vector *Nova*Red and VIP, respectively (Vector Laboratories). Whole-mount immunohistochemistry was performed essentially as described previously (Schulte-Merker et al., 1992) with anti-HCK-1 (1:100) followed by anti-rabbit HRP (1:300).

Acridine Orange and *o*-dianisidine staining

Acridine Orange staining of live embryos was performed as described, at five-somite, 15-somite, 23 hpf and 36 hpf (Seiler and Nicolson, 1999). Staining of hemoglobin by *o*-dianisidine was performed as previously described (Detrich et al., 1995). In brief, unfixed embryos were dechorionated and stained for 15 minutes in the dark, with a solution consisting of *o*-dianisidine (0.6 mg/ml), 0.01 M sodium acetate (pH 4.5), 0.65% hydrogen peroxide and 40% (vol/vol) ethanol. Embryos for histological sections were treated with acetone and embedded in Epon-Araldite (Polysciences) plastic resin, for histological sections.

Plasmid micro-injection and expression constructs

Micro-injection of plasmid DNA was performed essentially as described (Westerfield, 1993), using a Nikon pico-injector and a Narishige micro-manipulator. The full-length *bmp4* was a gift from Masataka Nikaido (Hokkaido University, Sapporo, Japan)

and directionally cloned into *Eco*RI and *Xho*I of pCS2+ for overexpression. The expression construct for *tolloid* (pCS2:Ztld-3′MT) was a gift from Patrick Blader (IGBMC, Strasbourg, France), and *gata1* (pCS2+) a gift from Sue Lyons (NIH, Bethesda, MD). The expression constructs for *scl* and GFP control has been described (Liao et al., 1998). Plasmid DNA expression constructs were purified (Qiagen), quantified spectrophometrically, and diluted to 100 ng/μl in sterile double distilled H_2O. Expression constructs of GFP control, *bmp4*, *tolloid*, *scl* and *gata1* were micro-injected into wild-type, *bls* and *clo* embryos at the two- or four-cell stage. Approximately 50-80 pg of DNA was injected into the blastomeres of each embryo. Embryos were fixed at 23 hpf and analyzed by in situ hybridization using *scl*, *gata1* or *globin* antisense riboprobes.

Cell transplantation

Cell transplant studies were carried out as described, with modifications herein specified (Westerfield, 1993). Donor cells were labeled with 1:1 mixture of 5% rhodamine and 5% biotin-dextran, resuspended in 0.2 M KCl. Micro-injection of embryos was performed from one- to four-cell stage, in 1× Danieus media. Donor and host embryos were dechorionated at the 16-cell stage with pronase treatment (4 mg/ml of pronase in 1× Danieus buffer), for exactly 2 minutes, followed by careful and thorough rinses in 1× Danieus buffer. Pronase dechorionation was performed in agarose (1.2% agarose in 1× Danieus) coated petri-dishes, as were all subsequent steps of embryo manipulation. Wild-type embryos used in transplant studies were derived from mating between wild-type AB fish. Mutant embryos used were derived from mating between *bls* homozygote adults, guaranteeing a clutch of embryos with mutant genotype. At the sphere stage, donor cells (25-40) were transplanted into the margin of sphere stage host animals. Donor and host animals were fixed at 23 hpf for in situ hybridization analysis and detection of biotin-dextran-labeled donor cells.

RESULTS

The *bls* mutation causes severe anemia during embryogenesis

The bls^{H75} allele was isolated as a spontaneous mutation. Although the original allele was discovered in a vector-based insertional mutagenesis screen, the mutation did not track with the lacZ vector (data not shown). The *bls* mutation is inherited in a dominant manner with incomplete penetrance, where the bloodless phenotype ranges from complete absence of circulating blood cells to severe anemia (10-20 cells). The *bls* phenotype can be detected morphologically with the onset of circulation at 26 hours post-fertilization (hpf), where the absence of blood cells can be illustrated by *o*-dianisidine staining (Fig. 1). However, as the mutant embryos continue to develop, they begin to accumulate blood cells after 5 dpf. The timing of the accumulation of red cells correlates with the onset of definitive hematopoiesis in zebrafish, which is believed to initiate around 4 dpf (Brownlie et al., 1998; Liao and Zon, 1999; Thompson et al., 1998). By 7.5 dpf, the embryos that were bloodless during embryogenesis become indistinguishable from wild-type (Fig. 1), and can be raised to adulthood. Peripheral blood and kidney of the adult *bls* mutants appear normal, and elaborates full range of blood lineages (data not shown). These studies demonstrate that the *bls* mutation leads to severe embryonic anemia but adult hematopoiesis appears unperturbed.

Unlike a fully penetrant dominant mutation, mating of bls^{H75} heterozygote to wild-type did not yield 50% phenotypic mutant progeny as would be expected. Instead, mating of bls^{H75} heterozygote to wild type produced approximately 15% severely anemic embryos. Likewise, mating between two bls^{H75} heterozygotes produced approximately 60% severely anemic embryos, whereas the expected progeny genotype would have predicted 75% mutants (50% bls^{H75} heterozygotes and 25% bls^{H75} homozygotes). However, as mutant bloodless embryos can be raised to adulthood, consecutive inbreeding of *bls* adults was carried out to obtain homozygous lines of bls^{H75}/bls^{H75} mutants. After five generations of inbreeding, mating of bls^{H75}/bls^{H75} with bls^{H75}/bls^{H75} adults produced 100% of bloodless progeny. All of the studies herein reported were carried out using bloodless embryos derived from mating of inbred *bls* homozygotes.

Analysis of early hematopoietic markers in *bls*

At the five-somite stage, expression of *scl* in the lateral plate mesoderm marks the specification of primitive hematopoietic progenitors (Gering et al., 1998; Liao et al., 1998; Mead et al., 1998). By 23 hpf, *scl* is expressed in the ICM (region overlying the yolk tube and extending slightly caudally, forming a wedge) and an anatomically distinct tailbud derived population, referred to as posterior ICM (Detrich et al., 1995). Expression of *scl* was greatly reduced in the lateral plate mesoderm of *bls* mutants, suggesting that the *bls* gene product participates in the specification of hematopoietic progenitor cells (Fig. 2A). From 18-somite to 23 hpf, *scl* expression in the ICM of *bls* mutants decreased progressively, until only a few cells were detectable in the posterior ICM at 23 hpf (Fig. 2A, arrowhead with asterisk).

Expression of *gata1* can be detected at the five-somite stage in wild-type embryos (Detrich et al., 1995). Similar to *scl*, *gata1* expression at the eight-somite stage was greatly reduced in *bls* mutants, ranging from completely absent to a between five and ten detectable cells (Fig. 2A,B). At 23 hpf, wild-type *gata1* expression is restricted to the anterior ICM and is not found in the posterior ICM (Detrich et al., 1995). In contrast to *scl* expression, *gata1* transcripts were absent in *bls* mutants at 18-somites (Fig. 2B). Both *scl* and *gata1* are absent in the anterior ICM at 23 hpf.

Transcripts of *gata2* were detected in the wedge region of the anterior ICM and the posterior ICM at 23 hpf, albeit qualitatively reduced when compared with wild type (Fig. 2C, arrowhead and arrowhead with asterisk). With the reduced expression of *gata2* in *bls*, it was difficult to determine by double RNA in situ whether the *gata2*-expressing cells co-expressed *scl*. As *gata2* is expressed in both blood and endothelial progenitors, the reduced transcript level could be attributable to decreased number of hematopoietic but not endothelial cells. Additionally, transcripts of the zebrafish *ikaros* gene were detected in the hematopoietic progenitors of the ICM (Fig. 2C). Similar to observations of *scl* and *gata1* expression, *ikaros* expression was absent in the ICM of *bls* mutants at 23 hpf.

As the ICM also includes cells that differentiate into the embryonic angioblasts, we examined *flk1* expression in *bls* (Pardanaud et al., 1996). Expression of *flk1* delineates the embryonic dorsal aorta, axial vein and inter-somitic vessels at 23 hpf (Fig. 2C). Expression of *flk1* in the lateral plate mesoderm at eight somites was also unaffected (data not shown). This finding is corroborated by visual inspection of *bls* mutants, which showed morphologically intact vasculature.

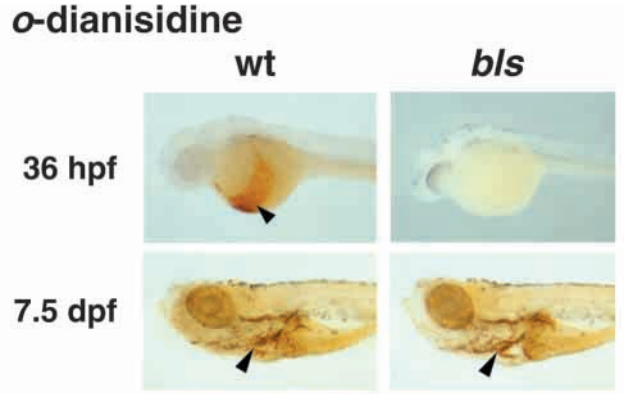

Fig. 1. The *bls* mutation causes severe embryonic anemia that recovers in the larval stage. Wild-type and *bls* embryos stained with *o*-dianisidine, which detects cells containing hemoglobin. All embryos in this and subsequent figures are shown in lateral views, oriented with anterior and dorsal towards left and top of the page, respectively, unless otherwise specified. Blood cells pool in the Duct of Cuvier of wild-type embryos (arrowhead) at 36 hpf, but are absent in *bls* mutants. However, as the bloodless embryos develop beyond 5 dpf, blood cells begin to accumulate. By 7.5 dpf, blood cells can be detected near wild-type levels in the *bls* mutants, as they circulate through the heart and branchial vessels (arrowhead).

Defective hematopoietic progenitors undergo apoptosis in *bls*

The expression of *scl* but not *gata1* at 18 somites suggests that the hematopoietic precursors that were specified early in embryogenesis of *bls* mutants failed to undergo normal differentiation and to maintain *gata1* expression. It has been shown that hematopoietic precursors defective in *gata1* expression undergo apoptosis (Weiss and Orkin, 1995). Consistent with this, staining of apoptotic cells with Acridine Orange demonstrated increased cell death in the ICM of *bls* mutants at 15 somites and 23 hpf (Fig. 3A). In addition to the ICM hematopoietic defects, *bls* embryos also exhibited marked apoptosis of cells lining the dorsal trunk and tailbud margins (Fig. 3A, arrowheads with asterisk).

Given the dorsal position of the Acridine Orange stained cells, we attempted to determine whether they represented dorsal ganglia or Rohon Beard cells. Detection by in situ of neuronal derived dorsal ganglia and Rohon Beard cells with *spt* in situ and HNK-1 antibody, respectively, failed to uncover any abnormality in *bls* (Fig. 3B). Likewise, the expressions of *spt* and *tbx6* in the tailbud were not perturbed by *bls* (Fig. 3B). Of interest, the paraxial mesoderm regulator *spt* is also expressed in the wedge region of the anterior ICM (Fig. 3B, arrowhead), whereas *tbx6* is expressed along the anterior ICM like *gata1* and *ikaros*. The functions of *spt* and *tbx6* in hematopoiesis are unclear, although it has been suggested that the *spt* gene is required for hematopoiesis (Oates et al., 1999; Thompson et al., 1998). Additionally, to exclude the possibility of more global mesoderm abnormality in *bls*, we show that the expression of axial mesoderm markers such as *no tail* (*ntl*) and

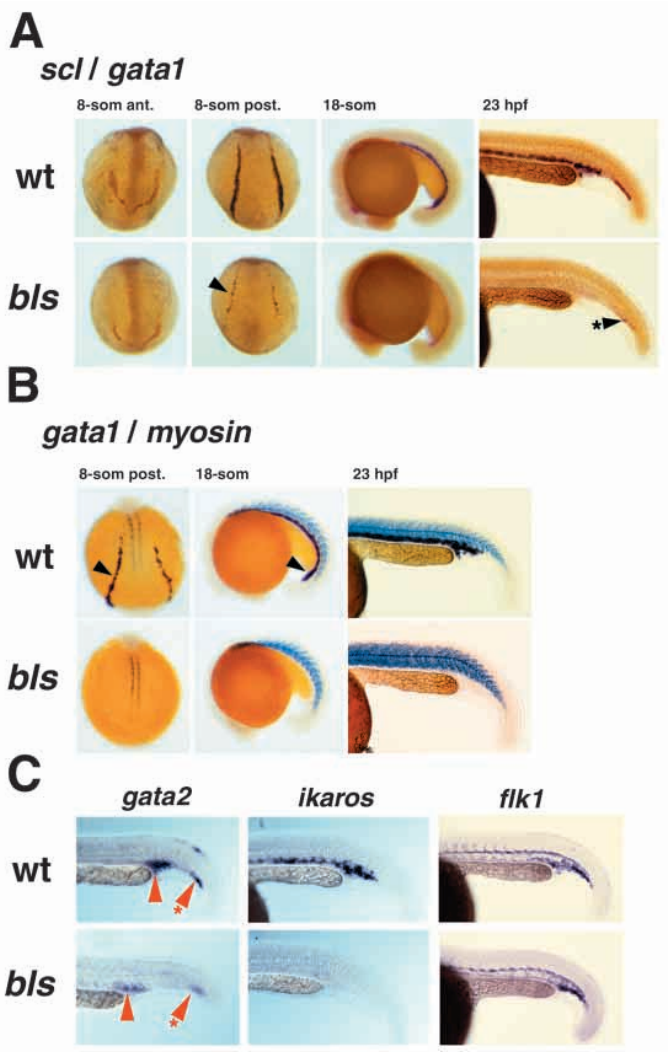

Fig. 2. Disruption of primitive hematopoiesis in *bls* mutants. In situ hybridization analysis of hematopoietic markers *scl*, *gata1*, *gata2*, *ikaros* and the endothelial marker *flk1* in *bls* mutants. (A) Expression of *scl* (red) and *gata1* (blue) in wild-type and *bls* embryos. Cells expressing both *scl* and *gata1* appear purple. At eight somites, *scl* transcripts can be detected in the anterior and posterior lateral plate mesoderm. The expression of *scl* in the anterior lateral plate does not appear to be perturbed in *bls*. By contrast, the expression of *scl* and *gata1* in *bls* is decreased at eight somites, with *scl* expression appearing less reduced than that of *gata1* (arrowhead). By 18 somites, *scl* expression persists in the ICM but *gata1* expression cannot be detected. At 23 hpf, some *scl* transcripts can be detected in the posterior ICM (arrowhead with asterisk) and *gata1* expression remains absent. Note that the *scl*/*gata1* double in situ clearly defines the anterior versus posterior ICM, as the anterior ICM appears dark purple and the posterior ICM appears red. (B) Expression of *gata1* (in dark purple) and *myosin* (in blue) in wild-type and *bls* embryos. In wild-types, *gata1* expression is shown at eight somites in the lateral plate mesoderm (arrowhead). Myosin is expressed in the differentiating somites. *gata1*-expressing cells in the lateral plate mesoderm converge towards the midline by 18 somites to form the ICM, which expresses *gata1* (arrowhead). Expression of *gata1* persists in the hematopoietic progenitors at 23 hpf. By contrast, *gata1* is absent in *bls* embryos, from as early as eight somite stage. (C) Expression of *gata2*, *ikaros*, and *flk1* in wild-type and *bls* embryos. *gata2* transcripts are detectable in *bls*, although the experession appears to be reduced (arroheads and arrowheads with asterisks), whereas *ikaros* expression is absent in *bls*. By contrast, *flk1* expression is undisturbed by *bls*.

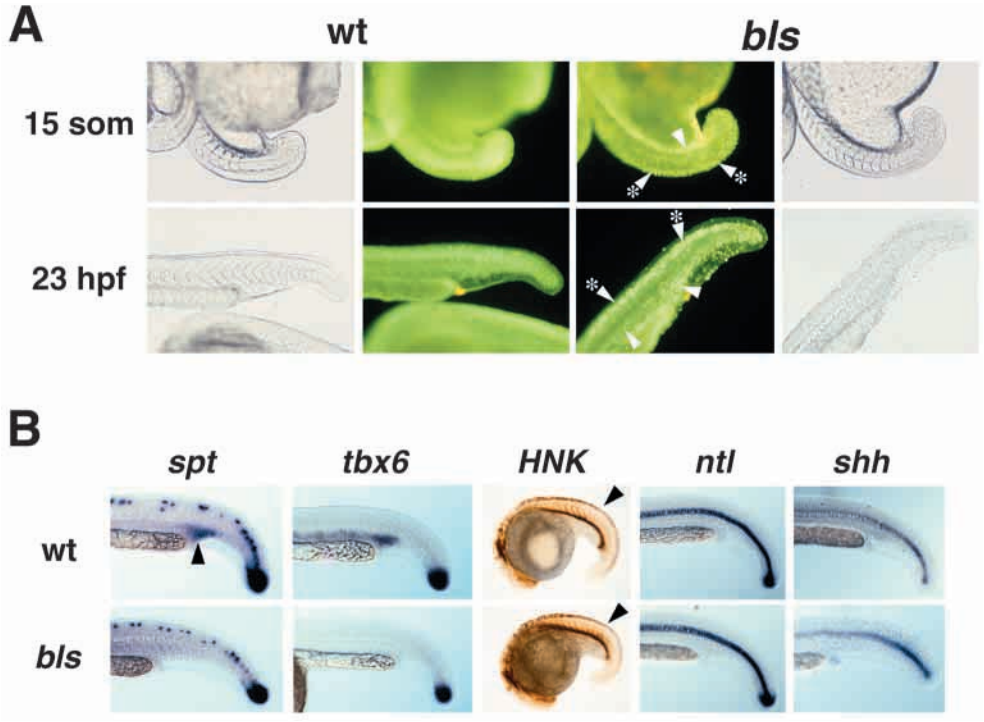

Fig. 3. Apoptosis in the ICM and cells of the dorsal trunk and tailbud. (A) Acridine Orange staining of wild-type and *bls* embryos at 15- somites and 23 hpf detects cells undergoing apoptosis. Images of embryos under light-microscopy (far left and far right) are shown as anatomic references for the fluorescence images (middle left and right). Apoptotic cells appear as yellow dots when stained with Acridine Orange. Low level apoptosis is can be seen in wild-type embryos (sparse yellow dots). However, *bls* embryos exhibit marked apoptosis in the ICM (arrowhead) and dorsal trunk mesenchyme (arrowheads with asterisk). The numerous apoptotic cells line the dorsal trunk of the *bls* embryo, and extend around the tailbud into the ICM. (B) Transcripts of *spt* can be detected in the ICM at 23 hpf in wild-type but not *bls* embryos (arrowhead). Expression of *spt* in the tailbud and spinal neurons are unperturbed by *bls*. Likewise, *tbx6* expression is absent in the ICM of *bls* mutants, but is normally expressed in the tailbud. Neural crest derived Rohon-Beard cells are detected with HNK-1 antibody along the dorsal trunk of both wild-type and *bls* embryos (arrowhead). Axial mesoderm markers *ntl* and *shh* exhibit wild-type expression patterns in *bls* notochord and tailbud.

sonic hedgehog (*shh*) in the notochord and tailbud are undisrupted by *bls* (Fig. 3B). The tissue identity of the dorsal trunk and tailbud margin cells that undergo apoptosis remains uncertain, and the loss of these cells does not appear to affect normal development, as *bls* embryos develop into adult animals that appear grossly normal.

Normal myelopoiesis in *bls*

With the striking deficiency of primitive hematopoietic progenitors in *bls*, we next examined whether primitive myelopoiesis is also affected by the *bls* mutation. Myelopoiesis during zebrafish embryogenesis consists of expansion and differentiation of primitive macrophages (Herbomel et al., 1999). Unlike primitive erythroid cells that arise from the ICM, zebrafish primitive macrophages arise from a distinct rostral anlage that is derived from the anterior paraxial mesoderm (Herbomel et al., 1999; Bennet et al., 2001; Parichy et al., 2000). Primitive macrophages originating from the anterior paraxial mesoderm express genes such as *pu.1* (*spi1*), *cmyb* and *draculin* (*dra*). In addition, cells in the ICM also express *cmyb* and *dra* but not *pu.1* (Fig. 4). When the expression of these genes were examined in *bls* mutants, it was evident that the primitive macrophages were not affected by the mutation. Expression of *pu.1*, *cmyb* and *dra* were at wild-type levels in the primitive macrophages of *bls* mutants (Fig. 4).

Like *gata1*, the expression of *cmyb* was also absent in the ICM of *bls* mutants (Fig. 4B). By contrast, *dra* expression was reduced but not absent in the lateral plate mesoderm (Fig. 4C, asterisk) and persists in a few cells at 20 somites. The reduced expression of *dra* in *bls* is similar to that observed for *scl*. Although the function of *dra* in hematopoiesis remains to be determined, the similarity of reduced *dra* expression with that of *scl* suggests that *dra* may also be an early marker for hematopoiesis (Alan Davidson and L. Z., unpublished).

Initiation of lymphopoiesis is delayed in *bls*

The thymic organs form by 65 hpf and are populated with *rag1*-expressing lymphocytes (Hansen and Zapata, 1998; Trede and Zon, 1998). Expression of *rag1* is absent in *bls* embryos at 4.5 dpf (Fig. 5A). Thymic cytology revealed normal appearing thymic epithelium in *bls* and absence of lymphoblasts (Fig. 5B, arrowhead with asterisk). Interestingly, if mutant animals were raised to 7.5 dpf and then analyzed with *rag1* in situ hybridization, lymphoid cells can be detected in the thymi (Fig. 5A, arrowhead with asterisk). Likewise, histology of thymi of wild-type and *bls* mutants appear similar at 7.5 dpf, populated with lymphocytes (Fig. 5B, arrowheads).

Overexpression of *bmp4*, *scl* and *gata1* in wild-type, *bls* and *clo* embryos

To place *bls* in the context of other known genes that participate in blood formation, we overexpressed *bmp4*, *scl* and *gata1* into wild-type, *bls* and *cloche* (*clo*) zebrafish embryos. Injection of *cmv-GFP* control plasmid did not perturb normal development of the embryos, and did not lead to rescue of *scl* or *gata1* expression in either *bls* or *clo* mutants (Fig. 6A). In the control injected *bls* mutants, *scl* expression is present in the posterior ICM at 23 hpf, as is seen in uninjected embyros. However, *scl* and *gata1* are not detected in the anterior ICM of control injected embryos.

When *bmp4* was injected into wild-type embryos, ventral mesoderm derived ICM was expanded, expressing high levels of *scl* and *gata1* (Fig. 6B). In the *bls* mutants, *bmp4* ventralized animals lacked *scl* expression in the anterior ICM, although the

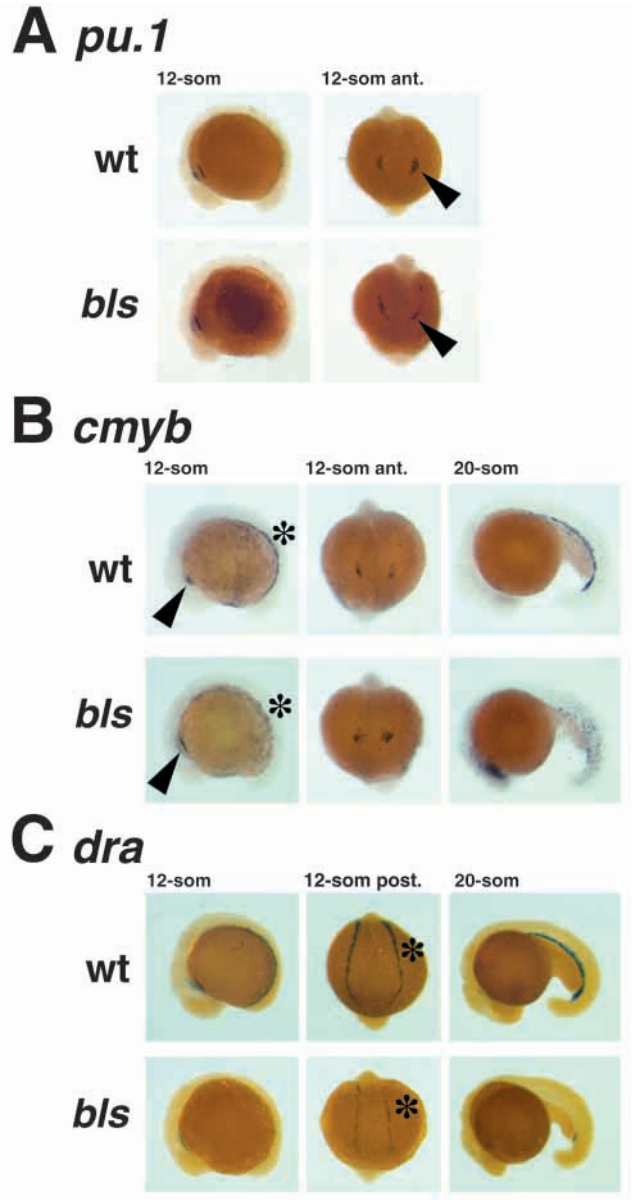

Fig. 4. Primitive macrophages develop normally in *bls*. Primitive macrophages develop from anterior paraxial mesoderm, and express (A) *pu.1*, (B) *cmyb* and (C) *dra*. Expression of *pu.1* is unique to primitive macrophages, whereas *cmyb* and *dra* transcripts are also detected in the ICM at 20 somites. (A) Expression of *pu.1* is not perturbed by *bls*, and can be detected in the anterior paraxial mesoderm at 12 somites. (B) *cmyb* expression in the primitive macrophage is unaffected by *bls* (arrowhead) but its expression in the ICM (asterisk) is absent at 20 somites. (C) Similarly, *dra* expression in the primitive macrophage primordia is unaffected by *bls*. However, *dra* expression in *bls* is significantly reduced in the lateral plate mesoderm at 12 somites (asterisk) and in the ICM at 20 somites, similar to the pattern of *scl* expression.

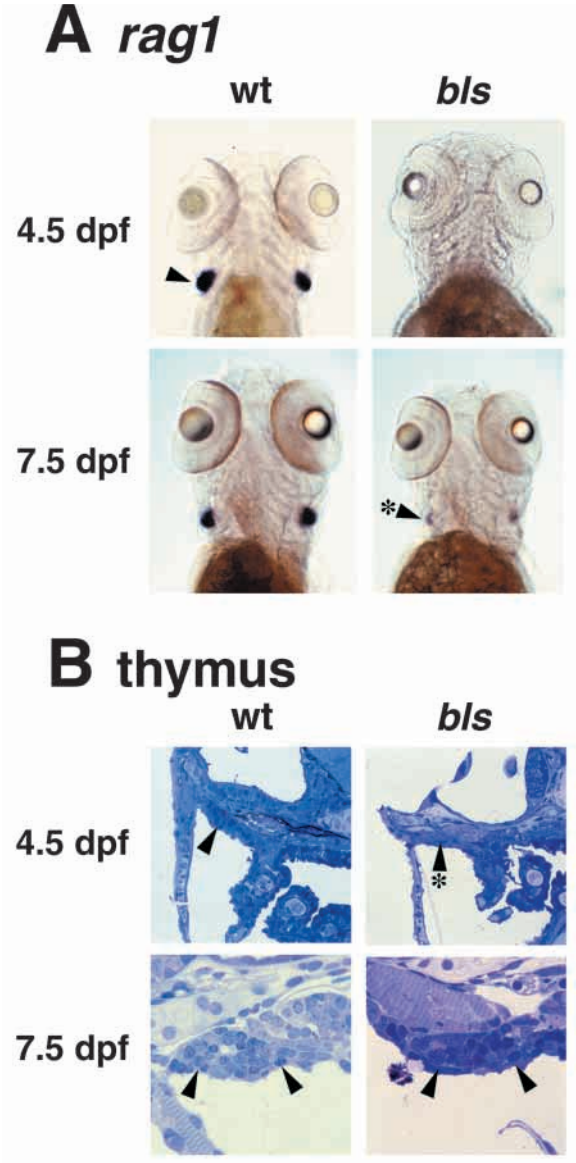

Fig. 5. Initiation of lymphopoiesis is delayed in *bls*. Lymphoid precursors expressing *rag1* can be detected in the thymus around 4 dpf. (A) Lymphoid precursors are present in the thymus of wild-type embryos at 4.5 dpf (arrowhead), but are absent from *bls*. However, if the *bls* embryos are raised to 7.5 dpf, *rag1* expression begins to appear in the thymus, indicating the appearance of lymphoid precursors (arrowhead with asterisk). (B) Coronal section of 4.5 dpf embryos shows that lymphoid cells cluster in the thymus of wild-type embryos (arrowhead), but are absent in the *bls* thymus. Corresponding to the recovery of *rag1* expression at 7.5 dpf, lymphocytes of wild-type morphology can be detected in the thymus of *bls* embryos (double arrowheads).

posterior ICM expression of *scl* was moderately increased (Fig. 6B, arrowhead with asterisk). As the posterior ICM normally expresses *scl* in the *bls* mutant, only the presence of *scl*-positive cells in the anterior ICM is scored as hematopoietic rescue (Table 1). Additionally, only rare *gata1* expressing cells were detected in the ICM of *bmp4* ventralized tails of *bls* animals (Table 1). Furthermore, *bmp4* failed to induce *scl* or *gata1* expression in *clo* mutants (Fig. 6B). In contrast to *bls*, the ventralized posterior ICM of *clo* did not express *scl*. The same results were obtained when the endogenous level of *bmp4* was increased with *tolloid* overexpression, which antagonizes *chordin* degradation of *bmp4* (data not shown) (Blader et al., 1997).

When *scl* was expressed in wild-type animals, the ICM was

slightly expanded (Fig. 6C) (Gering et al., 1998). In *clo* mutants, *scl* expression can lead to partial rescue of hematopoietic progenitors in the anterior ICM, suggesting that *scl* is sufficient to specify hematopoietic progenitors (Fig. 6C, arrowhead with asterisk) (Liao et al., 1998). When *scl* was overexpressed in *bls* mutants, partial rescue of *gata1*-expressing cells was detected in the anterior ICM (Fig. 6C, arrowheads). The *gata1*-expressing cells do differentiate to

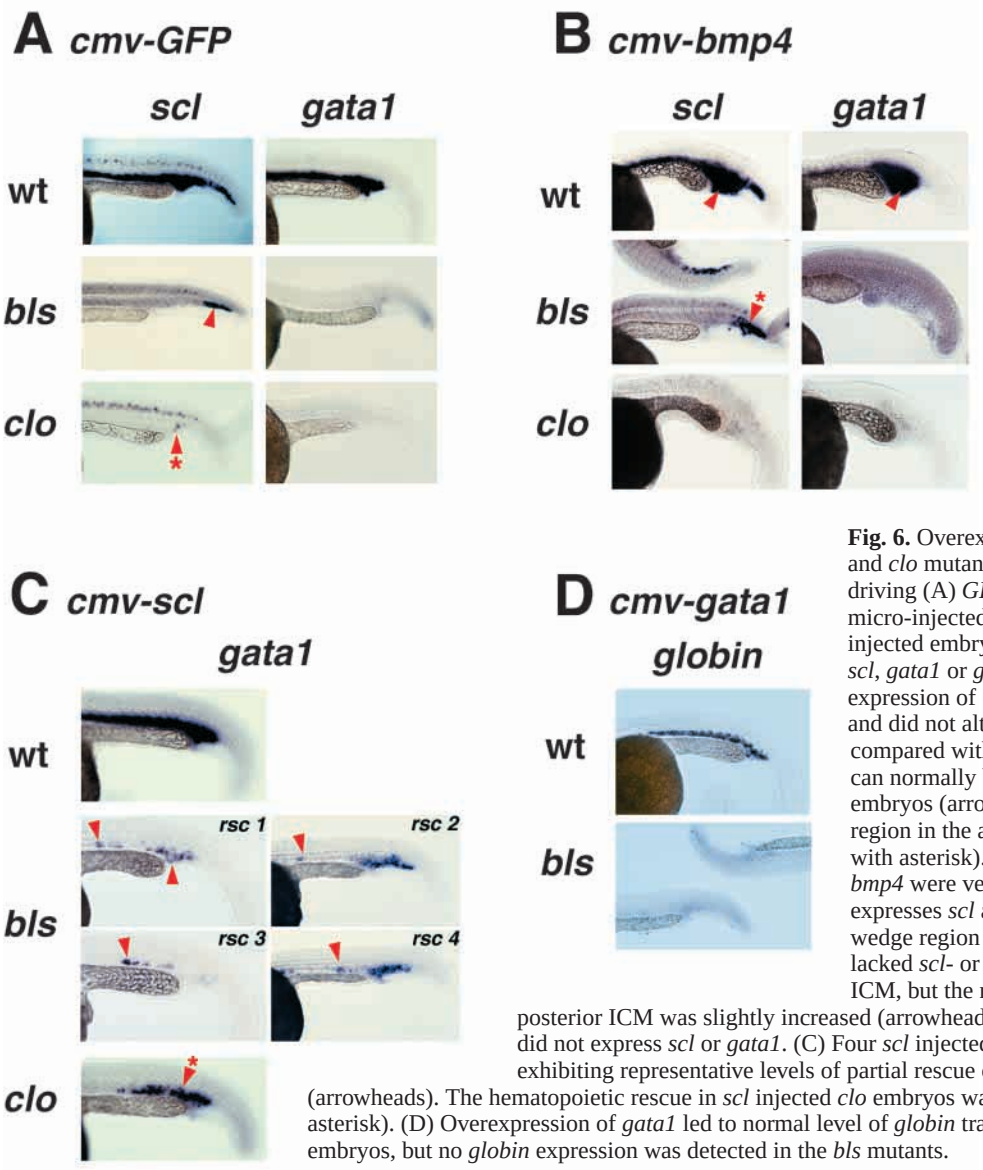

Fig. 6. Overexpression of *bmp4*, *scl* and *gata1* in *bls* and *clo* mutants. Plasmid construct with CMV promoter driving (A) *GFP*, (B) *bmp4*, (C) *scl* and (D) *gata1* were micro-injected into wild-type, *bls* and *clo* embryos. The injected embryos were fixed at 23 hpf and analyzed by *scl*, *gata1* or *globin* in situ hybridization. (A) Plasmid expression of *GFP* did not perturb embryo development and did not alter the expression of *scl* or *gata1* when compared with uninjected embryos. Expression of *scl* can normally be detected in the posterior ICM of *bls* embryos (arrowhead), and in 5-10 cells of the wedge region in the anterior ICM of *clo* embryos (arrowhead with asterisk). (B) Wild-type embryos injected with *bmp4* were ventralized, with an expanded ICM that expresses *scl* and *gata1*. The arrowhead indicates the wedge region of anterior ICM. Ventralized *bls* embryos lacked *scl*- or *gata1*-expressing cells in the anterior ICM, but the number of *scl*-positive cells in the posterior ICM was slightly increased (arrowhead with asterisk). Ventralized *clo* embryos did not express *scl* or *gata1*. (C) Four *scl* injected *bls* embryos are shown (rsc1-4), each exhibiting representative levels of partial rescue of hematopoietic progenitors (arrowheads). The hematopoietic rescue in *scl* injected *clo* embryos was more pronounced (arrowhead with asterisk). (D) Overexpression of *gata1* led to normal level of *globin* transcripts in the anterior ICM of wild-type embryos, but no *globin* expression was detected in the *bls* mutants.

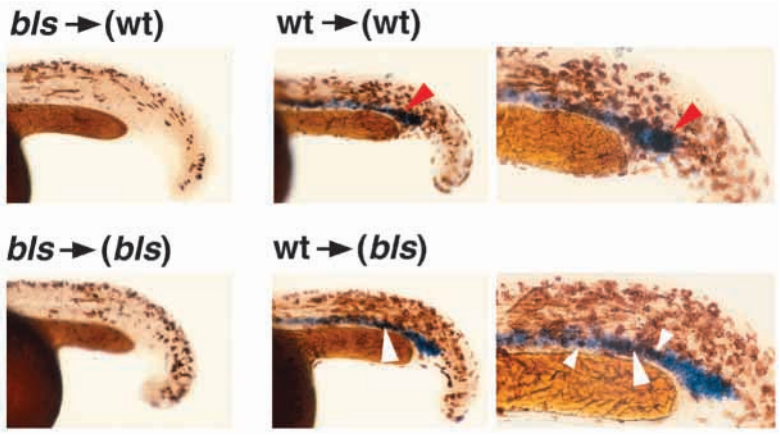

Fig. 7. Non-cell autonomous requirement for *bls* in primitive hematopoiesis. Donor cells labeled with biotin-dextran were transplanted into unlabeled hosts at the sphere stage, to determine whether donor cells can contribute to blood in the host environment. The resulting chimeric animals were analyzed for *gata1* expression. Genotype of the host is indicated outside, and that of the donor within, the parenthesis (host genotype → (donor genotype)). Donor-derived cells were detected by avidine-peroxidase color reaction, and appear brick red. Cells containing *gata1* transcripts appear blue. Donor cells that contribute to blood will be colored with red and blue, appearing dark purple (arrowheads).

Table 1. Overexpression of *bmp4*, *scl* and *gata1* in wild-type, *bls* and *clo* embryos

Injected	In situ	Genotype		
		Wild type	*bls*	*clo*
cmv-GFP	*scl*	221/221	0/136*	0/89
cmv-GFP	*gata1*	187/187	0/98	0/68
bmp4	*scl*	269/269	0/172*	0/117
bmp4	*gata1*	305/305	4/218 (1.8%)	0/81
scl	*gata1*	448/448	58/122 (48%)	54/133 (41%)
gata1	*globin*	63/63	0/93	NA

Plasmid expression constructs were injected into embryos of the indicated genotypes, and analyzed by in situ with *scl*, *gata1* or *globin* antisense riboprobes. Results are given as number of embryos positive for the marker used in the assay/the total number of embryos analyzed.

*Expression of *scl* in the anterior ICM is scored as rescue, because *scl*-positive cells are found in the posterior ICM of control injected and uninjected embryos.

Table 2. Cell transplantation analysis

Donor*	Host	*gata1*-positive donor cells†	Total hosts analyzed
Wild type	Wild type	28 (40%)	69
Wild type	*bls*	0	62
bls	*bls*	0	75
bls	Wild type	68 (32%)	211

*Donor cells (25-40) labeled with biotin-dextran were transplanted in the margin of host animals at the sphere stage (4 hpf).

†Host animals were analyzed by *gata1* in situ, where donor cells were detected by avidine-conjugated peroxidase color reaction. The number of host animals containing donor cells that were *gata1* positive is indicated.

mature red cells as shown by *globin* staining (data not shown). This result is in contrast to *bmp4* overexpression, which was unable to produce *gata1*-positive cells despite ventralization of the ICM. Although the percentage of animals exhibiting partial blood rescue with *scl* overexpression is similar between *bls* and *clo*, the number of cells rescued per embryo is significantly less in *bls* than in *clo* (Table 1). Furthermore, no rescue of blood cells was observed with overexpression of *gata1* (Fig. 6D).

Cell transplantation studies

To determine whether *bls* acts in a cell autonomous or non-cell autonomous manner, reciprocal cell transplantation experiments were carried out between wild-type and *bls* mutant animals (Table 2). Analysis of *gata1* expression in *bls* hosts carrying either *bls* or wild-type donor cells failed to detect any hematopoietic progenitors in the ICM (Fig. 7). By contrast, when *bls* mutant donor cells were transplanted into wild-type hosts, *gata1*-expressing hematopoietic progenitors derived from the mutant donor cells were found in the ICM (Fig. 7, white arrowheads). These studies demonstrate that *bls* acts in a non-cell autonomous manner, such that the mutant cells are competent to differentiate and express *gata1* in a wild-type environment.

DISCUSSION

Our characterization of *bls* demonstrates that the mutation acts at an early developmental window of primitive hematopoiesis. Lacking a functional *bls* gene product, the number of hematopoietic cells specified from the lateral plate mesoderm is severely reduced, illustrated by the decreased number of *scl*-positive cells at eight somites. The primitive blood cells that are formed fail to differentiate, *gata1* expression is not maintained, and the cells undergo apoptosis. Furthermore, although thymic organs form normally in *bls*, they are devoid of lymphocytes at 4 dpf. However, primitive macrophages develop normally in *bls*, as does the embryonic vasculature. Surprisingly, when the bloodless embryos are raised beyond 5 dpf, blood cells begin to accumulate and lymphocytes can be found in the thymi. The mutants can grow to adulthood, with no detectable abnormality in the definitive blood lineages. As such, *bls* is the earliest acting mutation specific to hematopoiesis that is reported in zebrafish. Other zebrafish mutations with early defects in hematopoiesis either affect other organ systems, such as vasculogenesis in *clo*, or have more general mesoderm defects, such as defect in paraxial mesoderm migration in *spt* (Stainier et al., 1995; Thompson et al., 1998). Additionally, *bls* is the only vertebrate mutation that exhibits severe anemia during embryogenesis but not adulthood.

The *bls* gene is required for the differentiation of primitive blood cells

Hematopoietic genes such as *scl* and *gata1* are expressed in the zebrafish lateral plate mesoderm as early as five somites, evidence that hematopoietic precursors have been specified by that time point (Detrich et al., 1995; Gering et al., 1998; Liao et al., 1998; Thompson et al., 1998). The analysis of *scl* and *gata1* expression in *bls* mutants show that the number of hematopoietic cells derived from the lateral plate mesoderm is greatly reduced. Meanwhile, expression of *flk1* is unaffected by *bls*, and the vasculature is intact.

As embryogenesis progresses in *bls*, the number of *scl*- and *gata1*-expressing cells decreases. By 23 hpf, no *scl* or *gata1* transcripts can be detected in the ICM and only a handful of cells expressing *scl* can be found in the posterior ICM. Concurrent with the decreasing number of *scl*- and *gata1*-expressing cells, apoptosis is noted in the ICM. Furthermore, the persistence of *gata2* and *scl* and the absence of *gata1* and *ikaros* in the anterior ICM of the mutant animal suggests that these cells are able to express early hematopoietic genes, but fail to differentiate and express genes associated with progressive hematopoietic lineage differentiation. These observations suggest that the hematopoietic progenitors that do form in *bls* fail to undergo normal differentiation and undergo apoptosis (Weiss and Orkin, 1995). Of note, *scl*, *gata1* and *flk1* transcripts are absent in the lateral plate mesoderm of *clo* mutants (Liao et al., 1997, Liao et al., 1998). Unlike *clo*, some *scl*-positive hematopoietic progenitors are specified in the lateral plate mesoderm. Therefore, *bls* appears to regulate, but is not absolutely required, for the specification of *scl*-expressing blood progenitors. Subsequently, *bls* is involved in maintaining *scl* and *gata1* expression during differentiation of the embryonic blood cells.

Role of *bls* in embryonic lymphoid and myeloid development

Analysis of macrophage markers *pu.1*, *cmyb* and *draculin* demonstrate that primitive macrophages are produced at wild-

type levels in the bilateral rostral paraxial mesoderm. This suggests that distinct hematopoietic programs regulate the development of primitive macrophages from the anterior paraxial mesoderm, and the elaboration of primitive hematopoietic progenitors from the ICM. These different regulatory signals are anatomically segregated, where the ICM may receive signals from the trunk paraxial mesoderm, whereas the rostral macrophage anlage is influenced by anterior cues.

The delay in lymphopoiesis in *bls* suggests that either the gene is required in the development of lymphoblasts, or that the ICM hematopoietic progenitors contribute to the initiation of lymphopoiesis. Moreover, studies in mice underscore the symbiotic dependence between thymic epithelium and lymphoblasts in their mutual development (Manley, 2000; Nehls et al., 1996; Ritter and Boyd, 1993). It is not clear whether this interdependence is also present in the development of lymphoblasts and thymic epithelium in zebrafish. Further work in lineage tracing and electron-microscopic analysis of the thymus in *bls* mutants will better characterize the lymphoid defect.

The posterior ICM of zebrafish embryo

Unlike *gata1* and *ikaros*, the expression of *scl* and *gata2* is not restricted to the anterior ICM and is also found in the posterior ICM at 23 hpf. Fate-mapping experiments have shown that cells of the posterior ICM arise as a result of complex migration from the extending tailbud (Kanki and Ho, 1997). Among vertebrates, *scl* and *gata2* are expressed in both hematopoietic and endothelial cells, whereas *gata1* and *ikaros* are expressed in only hematopoietic cells (Georgopoulos et al., 1992; Orkin, 1995). Additionally, the mutant *clo* lacks *scl* expression in both the anterior and posterior ICM, and fails to produce hematopoietic or endothelial cells. Last, endothelial markers such as *hhex*, *flk1* and *fli1* are highly expressed in the posterior ICM (Liao et al., 1997; Liao et al., 2000b; Thompson et al., 1998). Taken together, these observations suggest that the posterior ICM represents endothelial tissue and not hematopoietic progenitors. Therefore, expression of *scl* in the posterior ICM of *bls* is consistent with its phenotype of compromised hematopoiesis but intact vasculogenesis.

Non-cell autonomous requirement for *bls* in regulating primitive hematopoiesis

Cell transplantation studies showed that *bls* donor cells were able to contribute to *gata1*-expressing cells in the ICM of wild-type hosts. Conversely, *bls* hosts did not support the differentiation of wild-type donor cells to *gata1*-expressing cells. Given the non-cell autonomous action of the *bls* gene, one might speculate that the gene product could be a secreted factor or a cell surface receptor required for proper development of primitive hematopoietic cells. The dominant but incompletely penetrant aspect of the *bls* mutation supports a secreted factor hypothesis, where the proposed cytokine may be limiting during embryogenesis, manifesting in a haplo-insufficient phenotype.

The *clo* mutant has combined defect in hematopoietic and endothelial progenitors, and the *clo* gene is thought to function at the level of the hemangioblast, a proposed transient bi-potential cell that gives rise to endothelial and hematopoietic lineages (Liao et al., 1997; Stainier et al., 1995). Overexpression of *scl* and *hhex* can lead to partial rescue of hematopoietic progenitors in *clo* mutants (Liao et al., 1998; Liao et al., 2000b). Interestingly, the *clo* gene appears to act non-cell autonomously early in the differentiation of embryonic blood cells, before the expression of *gata1* (Parker and Stainier, 1999). Therefore, the relationships between *scl*, *hhex*, *clo* and *bls* in early hematopoiesis deserve further investigation.

Overexpression studies in *Xenopus* have shown that *bmp4* expands ventral mesoderm and induces the expression of *scl* (Mead et al., 1998). Overexpression of *tolloid* leads to ectopic maintenance of endogenous *bmp4*, and leads to ventralized phenotype with expanded *gata1*-expressing cells (Blader et al., 1997). Although the number of cells expressing *scl* is increased in the posterior ICM of *bmp4* ventralized tails of *bls* mutants, no *scl*-expressing cells were observed in the anterior ICM. Only rare *gata1*-expressing cells were seen in the anterior ICM of *bmp4*-injected embryos (1.8% of injected embryos, Table 1). Ventralization of the ICM by *bmp4* in *clo* also did not rescue *scl*- or *gata1*-expressing cells. Failure of *bmp4* to rescue hematopoietic cells in the anterior ICM suggests that the *bls* and *clo* gene products are required for hematopoiesis at a stage downstream of ventral mesoderm induction.

By contrast, *gata1*-expressing cells can be detected in the anterior ICM of *bls* and *clo* mutants that have been injected with *scl*. Either the *gata1*-positive cells are rescued hematopoietic progenitors or they represent blood cells specified from the lateral plate mesoderm. The percentage of *scl* injected embryos with *gata1*-positive blood cells is comparable between *bls* and *clo* mutants (48% and 41%, respectively) (Table 1). However, there are much fewer *gata1*-expressing cells in the anterior ICM of *scl*-injected *bls* animals than similarly injected *clo* mutants (Fig. 6C). This observation implies that *bls* may play additional roles in maintaining *scl* expression during embryogenesis, where the absence of the *bls* gene product attenuates the partial hematopoietic rescue when compared with that observed in *clo*. This view is consistent with expression analysis of *scl* and *gata1*, which show that *bls* mutants are able to produce some *scl*-positive blood progenitors in the lateral plate mesoderm, but such cells fail to express *gata1* and undergo apoptosis. Furthermore, overexpression of *gata1* is not sufficient for rescue of primitive in blood in neither *bls* nor *clo*. This suggests that other downstream target genes of *bls* and *scl* are necessary for primitive hematopoiesis, and that *gata1* represents one such target gene. Taken together with the absence of *scl* induction by *bmp4* in the *bls* mutant, we propose that *bls* acts downstream of *bmp4* in initiating or maintaining expression of *scl*.

The unique role of *bls* as a non-cell autonomous regulator of primitive hematopoiesis exemplifies the utility of zebrafish as a genetic model system for the study of blood development. Possible function of *bls* as a stromal signal in activating and maintaining *scl* expression underscores the importance of extrinsic cues during early hematopoietic differentiation. Moreover, the requirement for *bls* during primitive hematopoiesis but not definitive blood development may provide insight into regulatory cues that differ between embryonic development and adult homeostasis.

Our sincere thanks go to Alan Davidson, Barry Paw and David Trevor for review of this manuscript. Our gratitude goes to Walt

Saganic and Neal White for excellent fish care and husbandry. We thank Jarema Malicki and Kristin Artinger for advice on cell transplantation experiments, Chirs Amemiya for the *ikaros* cDNA, Masataka Nikaido for the *bmp4* cDNA, Sue Lyons for the *gata1* expression construct, and Patrick Blader for the *tolloid* expression construct. E. C. L. has received support from the NIH Medical Scientist Training Program, American Cancer Society Stone Fellowship, and Yamaguchi Award from Children's Hospital Division of Hematology/Oncology. E. C. L. is a Pre-doctoral Fellow and L. I. Z. an Investigator of the Howard Hughes Medical Institute. This work is also supported by grants from the NIH. This work was funded by the Howard Hughes Medical Institute, NIH grant P50 DK49216.

REFERENCES

Bennett, C. M., Kanki, J. P., Rhodes, J., Liu, T. X., Paw, B. H., Kieran, M. W., Langenau, D. M., Delahaye-Brown, A., Zon, L. I., Fleming, M. D. and Look, A. T. (2001). Myelopoiesis in the zebrafish, Danio rerio. *Blood* **98**, 643-651.

Blader, P., Rastegar, S., Fischer, N. and Strahle, U. (1997). Cleavage of the BMP-4 antagonist chordin by zebrafish tolloid. *Science* **278**, 1937-1940.

Brownlie, A., Donovan, A., Pratt, S. J., Paw, B. H., Oates, A. C., Brugnara, C., Witkowska, H. E., Sassa, S. and Zon, L. I. (1998). Positional cloning of the zebrafish sauternes gene: a model for congenital sideroblastic anaemia. *Nat. Genet.* **20**, 244-250.

Davidson, A. J. and Zon, L. I. (2000). Turning mesoderm into blood: the formation of hematopoietic stem cells during embryogenesis. *Curr. Top. Dev. Biol.* **50**, 45-60.

Detrich, H. W., III, Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A., Pratt, S., Ransom, D. and Zon, L. I. (1995). Intraembryonic hematopoietic cell migration during vertebrate development. *Proc. Natl. Acad. Sci. USA* **92**, 10713-10717.

Dieterlen-Lievre, F. (1997). Intraembryonic hematopoietic stem cells. *Hematol. Oncol. Clin. North Am.* **11**, 1149-1171.

Dieterlen-Lievre, F. and Le Douarin, N. M. (1993). Developmental rules in the hemtopoietic and immune system of birds: how general are they? *Semin. Dev. Biol.* **4**, 325-332.

Dzierzak, E. and Medvinsky, A. (1995). Mouse embryonic hematopoiesis. *Trends Genet.* **11**, 359-366.

Georgopoulos, K., Moore, D. D. and Derfler, B. (1992). Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science* **258**, 808-812.

Gering, M., Rodaway, A. R., Gottgens, B., Patient, R. K. and Green, A. R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. *EMBO J.* **17**, 4029-4045.

Godin, I. E., Garcia-Porrero, J. A., Coutinho, A., Dieterlen-Lievre, F. and Marcos, M. A. (1993). Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature* **364**, 67-70.

Godin, I., Dieterlen-Lievre, F. and Cumano, A. (1995). Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc. Natl. Acad. Sci. USA* **92**, 773-777.

Griffin, K. J., Amacher, S. L., Kimmel, C. B. and Kimelman, D. (1998). Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development* **125**, 3379-3388.

Hansen, J. D. and Zapata, A. G. (1998). Lymphocyte development in fish and amphibians. *Immunol. Rev.* **166**, 199-220.

Herbomel, P., Thisse, B. and Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* **126**, 3735-3745.

Hug, B., Walter, V. and Grunwald, D. J. (1997). tbx6, a Brachyury-related gene expressed by ventral mesendodermal precursors in the zebrafish embryo. *Dev. Biol.* **183**, 61-73.

Kanki, J. P. and Ho, R. K. (1997). The development of the posterior body in zebrafish. *Development* **124**, 881-893.

Kelley, C., Yee, K., Harland, R. and Zon, L. I. (1994). Ventral expression of GATA-1 and GATA-2 in the xenopus embryo defines induction of hematopoietic mesoderm. *Dev. Biol.* **165**, 193-205.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Lassila, O., Martin, C., Dieterlen, L. F., Gilmour, D. G., Eskola, J. and Toivanen, P. (1982). Migration of prebursal stem cells from the early chicken embryo to the yolk sac. *Scand. J. Immunol.* **16**, 265-268.

Liao, E. C. and Zon, L. I. (1999). Conservation of themes in vertebrate blood development. In *Cell Lineage and Fate Determination* (ed. S. Moody), pp. 569-582. London: Academic Press.

Liao, W., Bisgrove, B. W., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D. J. and Stainier, D. Y. R. (1997). The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial differentiation. *Development* **124**, 381-389.

Liao, E. C., Paw, B. H., Oates, A. C, Pratt, S. J., Postelthwait, J. H. and Zon, L. I. (1998). SCL/tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev.* **12**, 621-626.

Liao, E. C., Paw, B. H., Peters, L. L., Zapata, A., Pratt, S. J., Do, C. P., Lieschke, G. and Zon, L. I. (2000a). Hereditary spherocytosis in zebrafish riesling illustrates evolution of erythroid beta-spectrin structure, and function in red cell morphogenesis and membrane stability. *Development* **127**, 5123-5132.

Liao, W., Ho, C. Y., Yan, Y. L., Postlethwait, J. and Stainier, D. Y. (2000b). Hhex and scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. *Development* **127**, 4303-4313.

Lin, C. S., Lim, S. K., D'Agati, V. and Costantini, F. (1996). Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev.* **10**, 154-164.

Manley, N. R. (2000). Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. *Semin. Immunol.* **12**, 421-428.

Mead, P. E. and Zon, L. I. (1998). Molecular insights into early hematopoiesis. *Curr. Opin. Hematol.* **5**, 156-160.

Mead, P. E., Kelley, C. M., Hahn, P. S., Piedad, O. and Zon, L. I. (1998). SCL specifies hematopoietic mesoderm in Xenopus embryos. *Development* **125**, 2611-2620.

Medvinsky, A. and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.

Medvinsky, A. L., Samoylina, N. L., Muller, A. M. and Dzierzak, E. A. (1993). An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature* **364**, 64-67.

Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., Jr and Potter, S. S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65**, 677-689.

Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301.

Nehls, M., Kyewski, B., Messerle, M., Waldschutz, R., Schuddekopf, K., Smith, A. J. and Boehm, T. (1996). Two genetically separable steps in the differentiation of thymic epithelium. *Science* **272**, 886-889.

Oates, A. C., Brownlie, A., Pratt, S. J., Irvine, D. V., Liao, E. C., Paw, B. H., Dorian, K. J., Johnson, S. L., Postlethwait, J. H., Zon, L. I. et al. (1999). Gene duplication of zebrafish JAK2 homologs is accompanied by divergent embryonic expression patterns: only jak2a is expressed during erythropoiesis. *Blood* **94**, 2622-2636.

Ogawa, M., Nishikawa, S., Yoshinaga, K., Hayashi, S., Kunisada, T., Nakao, J., Kina, T., Sudo, T. and Kodama, H. (1993). Expression and function of c-Kit in fetal hemopoietic progenitor cells: transition from the early c-Kit-independent to the late c-Kit-dependent wave of hemopoiesis in the murine embryo. *Development* **117**, 1089-1098.

Okada, H., Watanabe, T., Niki, M., Takano, H., Chiba, N., Yanai, N., Tani, K., Hibino, H., Asano, S., Mucenski, M. L. et al. (1998). AML1$^{-/-}$ embryos do not express certain hematopoiesis-related gene transcripts including those of the PU.1 gene. *Oncogene* **17**, 2287-2293.

Orkin, S. H. (1995). Transcription factors and hematopoietic development. *J. Biol. Chem.* **270**, 4955-4958.

Orkin, S. H. (1996). Development of the hematopoietic system. *Curr. Opin. Genet. Dev.* **6**, 597-602.

Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073-5084.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L.-M., Catala, M. and Dierterlen-Lievre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **122**, 1363-1371.

Parichy, D. M., Ransom, D. G., Paw, B., Zon, L. I. and Johnson, S. L. (2000). An orthologue of the kit-related gene fms is required for

development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. *Development* **127**, 3031-3044.

Parker, L. and Stainier, D. Y. (1999). Cell-autonomous and non-autonomous requirements for the zebrafish gene cloche in hematopoiesis. *Development* **126**, 2643-2651.

Peault, B. (1996). Hematopoietic stem cell emergence in embryonic life: developmental hematology revisited. *J. Hematother.* **5**, 369-378.

Pevny, L., Lin, C.-S., D'Agati, V., Simon, M. C., Orkin, S. H. and Costantini, F. (1995). Development of hematopoietic cells lacking transcription factor GATA-1. *Development* **121**, 163-172.

Porcher, C., Wojciech, S., Rockwell, K., Fujiwara, Y., Alt, F. W. and Orkin, S. H. (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* **86**, 1-20.

Ritter, M. A. and Boyd, R. L. (1993). Development in the thymus: it takes two to tango. *Immunol. Today* **14**, 462-469.

Robb, L. (1997). Hematopoiesis: origin pinned down at last? *Curr. Biol.* **7**, R10-R12.

Robb, L., Elwood, N. J., Elefanty, A. G., Kontgen, F., Li, R., Barnett, L. D. and Begley, C. G. (1996). The scl gene product is required for the generation of all hematopoietic lineages in adult mouse. *EMBO J.* **15**, 4123-4129.

Robertson, S., Kennedy, M. and Keller, G. (1999). Hematopoietic commitment during embryogenesis. *Ann. New York Acad. Sci.* **872**, 9-15.

Rollins-Smith, L. A. and Blair, P. (1990). Contribution of ventral blood island mesoderm to hematopoiesis in postmetamorphic and metamorphosis-inhibited xenopus laevis. *Dev. Biol.* **142**, 178-183.

Sasaki, K., Yagi, H., Bronson, R. T., Tominaga, K., Matsunashi, T., Deguchi, K., Tani, Y., Kishimoto, T. and Komori, T. (1996). Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. *Proc. Natl. Acad. Sci. USA* **93**, 12359-12363.

Schulte-Merker, S., Ho, R. K., Hermann, B. G. and Nusslein-Volhard, C. (1992). Then protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021-1032.

Seiler, C. and Nicolson, T. (1999). Defective calmodulin-dependent rapid apical endocytosis in zebrafish sensory hair cell mutants. *J. Neurobiol.* **41**, 424-434.

Shalaby, F., Ho, J., Stanford, W., K.-D., F., Schuch, A., Schwartz, L., Bernstein, A. and Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-990.

Shivdasani, R. A., Mayer, E. L. and Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432-434.

Stainier, D. Y. R., Weinstein, B. M., Deitrich, H. W., III, Zon, L. I. and Fishman, M. C. (1995). cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* **121**, 3141-3150.

Szenberg, A. (1977). Ontogeny of myelopoietic precursor cells in the chicken embryo. *Adv. Exp. Med. Biol.* **88**, 3-11.

Thompson, M. A., Ransom, D. G., Pratt, S. J., MacLennan, H., Kieran, M. W., Detrich, H. W., Vail, B., Huber, T. L., Paw, B. H. and Zon, L. I. (1998). Characterization of primitive and definitive waves of hematopoiesis in wild-type and mutant zebrafish. *Dev. Biol.* **197**, 248-269.

Trede, N. S. and Zon, L. I. (1998). Development of T-cells during fish embryogenesis. *Dev. Comp. Immunol.* **22**, 253-263.

Tsai, F.-Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H. (1994). An early hematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-226.

Wang, L. C., Kuo, F., Fujiwara, Y., Gilliland, D. G., Golub, T. R. and Orkin, S. H. (1997). Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *EMBO J.* **16**, 4374-4383.

Wang, Q., Stacy, T., Binder, M., Martin-Padilla, M., Sharpe, A. H. and Speck, N. A. (1996). Disruption of the CBFa2 gene causes necrosis and hemorrhaging in the central nervous system, and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. USA* **93**, 3444-3449.

Warren, A. J., College, W. H., Carlton, M. B. L., Evans, M. J., Smith, A. J. H. and Rabbits, T. H. (1994). The oncogenic cysteine-rich LIM domain protein Rbtn2 is essential for erythroid development. *Cell* **78**, 45-57.

Weiss, M. J. and Orkin, S. H. (1995). Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc. Natl. Acad. Sci. USA* **92**, 9623.

Westerfield, M. (1993). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Brachydanio rerio*).* Eugene, OR: University of Oregon Press.

Whetton, A. D. and Spooncer, E. (1998). Role of cytokines and extracellular matrix in the regulation of haemopoietic stem cells. *Curr. Opin. Cell Biol.* **10**, 721-726.

Willett, C. E., Cortes, A., Zuasti, A. and Zapata, A. G. (1999). Early hematopoiesis and developing lymphoid organs in the zebrafish. *Dev. Dyn.* **214**, 323-336.

Wong, P. M., Chung, S. W., Chui, D. H. and Eaves, C. J. (1986). Properties of the earliest clonogenic hemopoietic precursors to appear in the developing murine yolk sac. *Proc. Natl. Acad. Sci. USA* **83**, 3851-3854.

Yoder, M. C., Hiatt, K. and Mukherjee, P. (1997). In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc. Natl. Acad. Sci. USA* **94**, 6776-6780.

Zagris, N. (1986). Communication between primary endoderm and mesoderm for erythroblast differentiation in early chick blastoderm. *Exp. Cell Biol.* **54**, 170-174.

Zon, L. I. (1995). Developmental biology of hematopoiesis. *Blood* **86**, 2876-2891.