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# Hematopoietic development in the zebrafish

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**ABSTRACT The model organism *Danio rerio*, also known as the zebrafish, is an excellent system for studying the developmental process of hematopoiesis. It is an ideal model for *in vivo* imaging, and it is useful for large-scale genetic screens. These have led to the discovery of previously unknown players in hematopoiesis, as well as helped our understanding of hematopoietic development. In this review, we will summarize hematopoiesis in the zebrafish and discuss how genetic approaches using the zebrafish system have helped to build our current knowledge in the field of hematopoiesis.**

KEY WORDS: *hematopoietic stem cell, HSC, hemangioblast, primitive hematopoiesis, definitive hematopoiesis*

In the last decade, zebrafish rose as a new genetic system to analyze hematopoietic development. The zebrafish system has a number of unique advantages compared to other vertebrate model organisms. Its embryos are externally fertilized and transparent, enabling *in vivo* visualization of early embryonic processes ranging from birth of hematopoietic stem cells (HSCs) in the mesoderm to migration of blood cells. In addition, large production of embryos makes phenotype-based forward genetics feasible (de Jong and Zon, 2005). For example, 26 complementation groups with blood defects were identified from two seminal large-scale mutagenesis projects performed in the 1990s (Ransom *et al.*, 1996; Weinstein *et al.*, 1996). Cloning and characterization of these mutants helped us analyze hematopoietic ontogeny and blood-related disease mechanisms. Most importantly, even though sites of hematopoiesis are very different in fish and mammals, the genetic program governing hematopoiesis was found to be highly conserved, which made new knowledge gained from the zebrafish field applicable to mammalian hematopoiesis (Davidson and Zon, 2004).

## Overview of vertebrate hematopoiesis

All vertebrate organisms experience waves of hematopoiesis in their lifetime (Galloway and Zon, 2003). During mammalian and avian development, the first HSCs appear from the blood islands in the extraembryonic yolk sac, giving rise to erythrocytes and macrophages that are required for growing tissues of the embryos (Palis and Yoder, 2001). This primitive wave is only transient, and the successive definitive wave starts intraembryonically in the aorta-gonad-mesonephros (AGM) region. In contrast to primitive HSCs, the definitive HSCs are multipotent, giving rise to all different lineages of blood. Subsequently, HSCs born from the AGM then migrate to the fetal liver where they will proliferate and ultimately seed the bone marrow, which is the adult hematopoietic organ (Cumano and Godin, 2007).

Zebrafish also have waves of hematopoiesis, which occur in a spatially unique manner compared to other vertebrate model organisms (Fig. 1). Its primitive HSCs are born intraembryonically in ventral mesoderm derived tissue called the intermediate cell mass (ICM) (Detrich *et al.*, 1995). During this wave, the anterior part of the embryo generates myeloid cells, while the posterior part generates mostly erythrocytes and some myeloid cells. From 24 hours post-fertilization (hpf), these primitive blood cells start to circulate throughout the embryo. Subsequently, the definitive HSCs emerge from the ventral wall of the dorsal aorta (Thompson *et al.*, 1998; Burns *et al.*, 2002; Kalev-Zylinska *et al.*, 2002), and these HSCs migrate to the posterior region in the tail called the caudal hematopoietic tissue (CHT) (Murayama *et al.*, 2006; Jin *et al.*, 2007). From 3dpf, lymphopoiesis initiates in the thymi. Eventually by 4dpf, HSCs seed the kidney marrow, which is equivalent to bone marrow in mammals. Despite these unique characteristics, zebrafish and other vertebrate animals share genetic pro-

*Abbreviations used in this paper:* AGM, aorta-gonad-mesonephros region; ALM, anterior lateral mesoderm; AMV, avian myeloblastosis virus; CHT, caudal hematopoietic tissue; dpf, days post-fertilization; etsrp, ets1-related protein; hpf, hours post-fertilization; HSC, hematopoietic stem cell; PLM: posterior lateral mesoderm; scl, stem cell leukemia; YSL, yolk synctial layer.

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grams that regulate hematopoiesis. In the following sections, we will explain each step of hematopoiesis and provide an overview of important transcription factors and zebrafish mutants.

## Patterning of ventral mesoderm

Although the first circulating blood cells are visible by 24hpf during zebrafish development, the processes required for HSC generation are already underway from 5hpf, when gastrulation takes place. During the gastrula period, embryos develop three germ layers - ectoderm, mesoderm and endoderm. Among these three germ layers, blood and angioblasts (endothelial progenitors) originate from the mesoderm (Kimmel *et al.*, 1990; Warga and Nusslein-Volhard, 1999).

In zebrafish, the mesoderm arises from the equatorial region above the yolk synctial layer (YSL). The YSL secretes morphogens such as transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) family members to induce mesoderm and endoderm (Fig. 2A) (Holley, 2006). Mesoderm is first induced by *nodal*, one of the TGFβ family members. The importance of *nodal* signaling in mesoderm induction is seen in mutants called *squint* and *cyclops*. These animals have mutations in the *nodal-related 1* and *2* genes and the *squint;cyclops* double mutants are devoid of all mesoderm and endoderm derived tissues except for the tail somites (Feldman *et al.*, 1998).

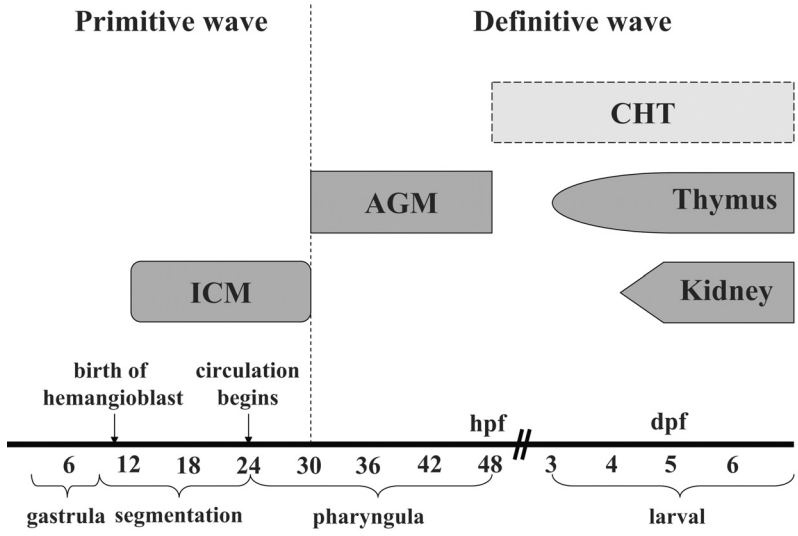


**Fig. 1. The timeline of zebrafish hematopoiesis.** *(Galloway and Zon, 2003; Davidson and Zon, 2004). ICM=intermediate cell mass; AGM=aorta-gonad-mesonephros region (ventral wall of dorsal aorta); CHT=caudal hematopoietic tissue; hpf=hours post-fertilization; dpf=days post-fertilization.*

Once the three germ layers are defined by *nodal* signaling, mesoderm is further specified into either dorsal fate (notochord, somites) or ventral fate (blood, vasculature, pronephros). The bone morphogenetic protein (BMP) pathway is one of the most crucial players in this dorsoventral decision (Fig. 2A). Like *nodal*, BMPs are members of the TGFβ superfamily. Among multiple BMPs in the zebrafish, *bmp2b* and *bmp7* are especially important in ventral mesodermal patterning (Kondo, 2007). The *swirl* (*bmp2b*) and *snailhouse* (*bmp7*) mutants have a severely dorsalized phenotype and fail to produce ventral mesodermal tissues such as blood and pronephros (Kishimoto *et al.*, 1997; Nguyen *et al.*, 1998; Schmid *et al.*, 2000). On the other hand, when *bmp2b* is overexpressed during the gastrula period, this results in expansion of erythrocytes (Lengerke *et al.*, 2008). These studies show how *bmp2b* and *bmp7* are necessary and sufficient for blood specification.

## Emergence of the hemangioblast

Starting early during the segmentation period, ventral lateral mesoderm surrounding paraxial mesoderm is further specified into blood, angioblast, and kidney progenitors. Among ventral lateral mesoderm, the anterior lateral mesoderm (ALM) is a major site of primitive myelopoiesis, while the posterior lateral mesoderm (PLM) gives rise to predominantly erythrocytes, as well as some myeloid cells.

From the 2 somite stage, cells co-expressing *scl*, *gata2*, *lmo2*, *fli1*, and *etsrp* transcription factors appear bilaterally in both the ALM and PLM (Fig. 2B) (Liao *et al.*, 1998; Thompson *et al.*, 1998; Sumanas *et al.*, 2005; Pham *et al.*, 2007). These cells have the potential to become either HSCs or angioblasts, but not kidney progenitors. This close relationship between HSCs and angioblasts has led to a hypothesis regarding the presence of a common ancestor between them, called the “hemangioblast”. This hypothesis was further supported by a zebrafish mutant *cloche* (described below) that completely lacks both blood and vessels but not other mesodermal organs, and by a single cell-resolution fate map study in zebrafish embryos (Stainier *et al.*, 1995; Vogeli *et al.*, 2006). In this fatemap study, it was discovered that there are rare population of cells during early gastrulation that can give rise to HSCs and angioblasts but not to other mesodermal lineages such as heart progenitors (Vogeli *et al.*, 2006).

## Transcription factors involved in hemangioblast induction

### *stem cell leukemia (scl)*

*scl* is a basic helix-loop-helix (bHLH) transcription factor which was first discovered at a chromosomal translocation site in leukemic T cells (Brown *et al.*, 1990; Chen *et al.*, 1990). In the mouse embryo it is expressed in hemogenic sites including yolk sac blood islands, fetal liver, and the dorsal aorta (Kallianpur *et al.*, 1994). In addition, *Scl-/-* embryos are not able to initiate primitive erythropoiesis, indicating its requirement in primitive hematopoiesis (Shivdasani *et al.*, 1995).

The *scl* gene has also been extensively studied in the zebrafish hematopoiesis field. During zebrafish development, *scl* is expressed from the 2 to 3 somite stage in the hemangioblast population together with *lmo2*, *gata2* and *fli1* (Liao *et al.*, 1998). *scl* expression in the ALM persists until the cells in the ALM migrate medially, while its expression in the PLM stays on and is later found in the ICM (Fig. 3A). Additionally, *scl+* cells appear in the dorsal aorta during definitive hematopoiesis (Liao *et al.*, 1998).

The function of *scl* in zebrafish hematopoiesis was studied by

knockdown approach through morpholino injection. In accordance with the mouse *Scl* knockout data, knockdown of *scl* in zebrafish leads to complete loss of primitive erythropoiesis and myelopoiesis. Also, *scl* knockdown leads to loss of *c-myb* and *runx1* expression in the dorsal aorta, which implies its importance in definitive hematopoiesis (Dooley *et al.*, 2005; Patterson *et al.*, 2005). In addition to HSC formation, endothelial differentiation is severely disrupted in *scl* knockdown embryos. For example, the expression of vascular genes in the dorsal aorta is decreased and intersomitic vessels fail to form in *scl* knockdown embryos (Dooley *et al.*, 2005; Patterson *et al.*, 2005). These results indicate that *scl* is also required for endothelial differentiation.

### *gata2*

*gata2* is a zinc finger transcription factor which is required for proliferation and maintenance of hematopoietic progenitor cells (Tsai and Orkin, 1997). In mice, *Gata2* expression is observed in both intraembryonic and extraembryonic sites of hematopoiesis. The importance of *Gata2* in primitive hematopoiesis was shown with the early embryonic death of *Gata2-/-* mice from severe anemia (Tsai *et al.*, 1994).

In zebrafish, *gata2* is expressed from the 2 somite stage as two stripes both in the ALM and PLM. Just as *scl*, *lmo2*, and *fli1*, *gata2* expression stays on in the ICM (Detrich *et al.*, 1995; Patterson *et al.*, 2005; Patterson *et al.*, 2007). Although *gata2* seems to be expressed in the hemangioblast population, *gata2* knockdown in zebrafish results in mild defects during primitive hematopoiesis. For example, erythroid specific genes *gata1*, *biklf*, *globin*, and *alas2* expression stay on in *gata2* knockdown embryos (Galloway *et al.*, 2005). These results indicate that *gata2* may not be essential for zebrafish primitive hematopoiesis, or that other transcription factors may compensate loss of *gata2*.

### *lmo2*

lmo2 is a LIM domain transcription factor that physically bridges scl and gata2 when they form a DNA-binding complex (Wadman *et al.*, 1997). Like *scl* and *gata2*, *lmo2* is expressed in hematopoietic progenitors, erythrocytes, and endothelial cells (Fig. 3B). Targeted knockout of *Lmo2* in mice results in embryonic death due to loss of yolk sac erythropoiesis (Warren *et al.*, 1994).

In zebrafish, *lmo2* is expressed in the ALM and PLM from the 2 to 3 somite stage, and in the ICM in later stages (Thompson *et al.*, 1998; Zhu *et al.*, 2005). Knockdown of *lmo2* leads to complete loss of PLM hematopoiesis including primtive erythropoiesis. In addition, *lmo2* knockdown partially reduces expression of myeloid gene expression in the ALM. *lmo2* knockdown does not affect either *scl* or *gata2* expression (Patterson *et al.*, 2007). This knockdown experiment brings up two possibilities regarding the role of *lmo2* in the hemangioblast. First, it may indicate that *lmo2* is required for maintaining the hemangioblast population and for differentiation of the hemangioblast into erythrocytes and myeloid cells (Patterson *et al.*, 2007). Conversely, it could also indicate that *lmo2* acts in parallel to *scl* and *gata2*, and that all three genes are required for erythroid and myeloid differentiation.

### *fli1*

*Fli1* encodes an ETS-domain transcription factor that was first identified by cloning the integration site of the Friend murine leukemia virus in transformed murine erythroleukemia cell lines (Ben-David *et al.*, 1991). The zebrafish homologue of *Fli1*, *fli1,* starts to be expressed from the 1 somite stage in the ALM and PLM (Thompson *et al.*, 1998). Its expression stays on in the ICM and later in vascular cells.

When overexpressed, *fli1* can induce ectopic *scl* and *lmo2* expression both in the ALM and in the PLM (Liu *et al.*, 2008). Since both *scl* and *lmo2* have several ETS -domain transcription binding sites in their promoters, this led to a hypothesis that *fli1* may work upstream of *scl* and *lmo2* (Zhu *et al.*, 2005). This hypothesis has been difficult to address since morpholino knockdown of single ETS domain transcription factors or a combination of multiple ETS domain transcription factors was insufficient to downregulate either *scl* or *lmo2* (Zhu *et al.*, 2005).

### *ets1-related protein (etsrp)*

*etsrp* is an *ets* domain containing gene which was first identified duing a search for novel vascular genes affected in *cloche* mutant animals (Sumanas *et al.*, 2005). During zebrafish development, *etsrp* is expressed bilaterally in both ALM and PLM from the 2 somite stage. Its expression stays on throughout the segmentation period, and it is detected in the ICM and intersomitic vessels by 24hpf (Sumanas and Lin, 2006; Pham *et al.*, 2007).

The function of *etsrp* was studied by knocking down the gene

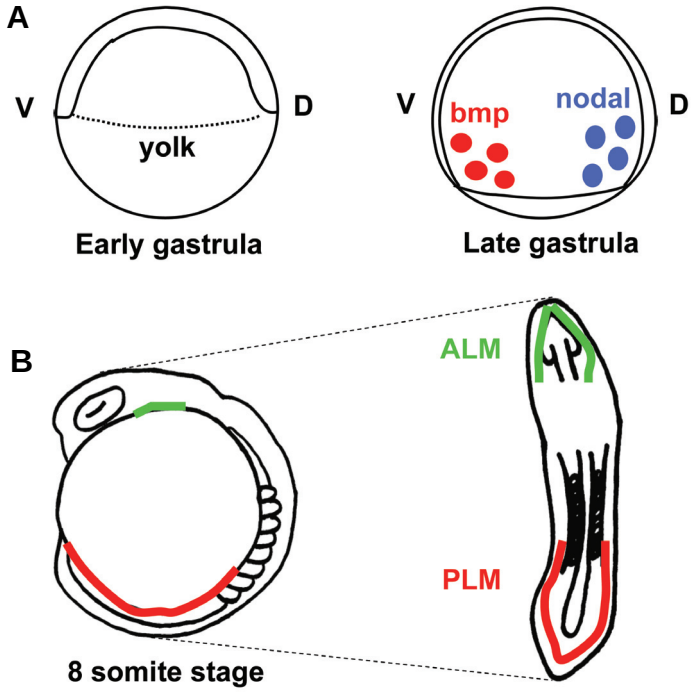


**Fig. 2. Patterning of ventral mesoderm and the emergence of hemangioblast. (A)** *Mesoderm patterning during gastrula period. The bmp and nodal proteins that are secreted from the yolk pattern the mesoderm from early gastrula period. bmps specify ventral fate, while nodals regulate initial mesoderm specification and dorsalization.* **(B)** *Emergence of hemangioblast from lateral plate mesoderm. By the segmentation period, the hemangioblast population arises from both ALM (green) and PLM (red). The schematic shows the 8 somite stage embryo and its corresponding flat mounted form, in which the yolk of the embryo is removed. ALM=anterior lateral mesoderm; PLM=posterior lateral mesoderm; V=ventral; D=dorsal.*

through morpholino injection and by characterizing the *etsrp* mutant animal called *y11* (Sumanas and Lin, 2006; Pham *et al.*, 2007). In both studies, *etsrp* was shown to be required for vascular development. For instance, loss of *etsrp* affects expression of endothelial genes including *flk1/vegfr2* and *flt4* (a venous specific gene). In addition, overall vascular structure is severely disrupted when *etsrp* is knocked down (Sumanas and Lin, 2006; Pham *et al.*, 2007).

In contrast to studies in vascular biology, the role of *etsrp* in hematopoiesis is yet unclear. In *etsrp* knockdown studies, it was shown that *etsrp* specifically regulates ALM myelopoiesis (Sumanas and Lin, 2006; Sumanas *et al.*, 2008). In these studies, *etsrp* knockdown led to loss of *scl* in the ALM, and to loss of myeloid genes including *pu.1* and *l-plastin* in the ALM. On the other hand, PLM hematopoiesis including erythropoiesis and myelopoiesis was unaffected in these animals (Sumanas and Lin, 2006; Sumanas *et al.*, 2008). Unlike the knockdown studies, the *y11* mutant animal had decreased *gata1* expression in the PLM (Pham *et al.*, 2007). This discrepancy could be due to differences in the level of *etsrp* gene expression in knockdown embryos and *y11* mutant animals. There was residual *etsrp* expression in the knockdown embryos, but no expression was detected in *y11* mutant animals (Sumanas and Lin, 2006). In these cases, it is possible that ALM myelopoiesis is more sensitive to the level of *etsrp* gene expression compared to PLM hematopoiesis.

## Primitive hematopoiesis

From the 4 somite stage, the hemangioblast population diverges into either blood cells or angioblasts. Just as primitive myeloid cells emerge from the head of a mammalian embryo, hemangioblasts that are located in the zebrafish ALM become *pu.1*+ myeloid progenitors or *flk1/vegfr2*+ endothelial cells (Bennett *et al.*, 2001; Lieschke *et al.*, 2002). These *pu.1*+ myeloid progenitors then migrate to the midline at the 14 somite stage and start to express myeloid specific genes such as *l-plastin* (*leucocyte-specific plastin*; a monocyte/macrophage specific gene) (Herbomel *et al.*, 1999; Bennett *et al.*, 2001; Lieschke *et al.*, 2002). In parallel, the majority of the hemangioblast population in the PLM becomes *gata1*+ erythroid progenitors, *pu.1*+ myeloid progenitors or endothelial cells (Davidson and Zon, 2004). These bilateral cells in the PLM start to converge to the midline from the 12 somite stage, and eventually form the ICM (Detrich *et al.*, 1995). With the start of the heartbeat at 24hpf, the primitive erythroid progenitors enter circulation and develop into mature erythrocytes, expressing erythroid specific genes such as *alas2*, *carbonic anhydrase*, and *globin* (Brownlie *et al.*, 2003).

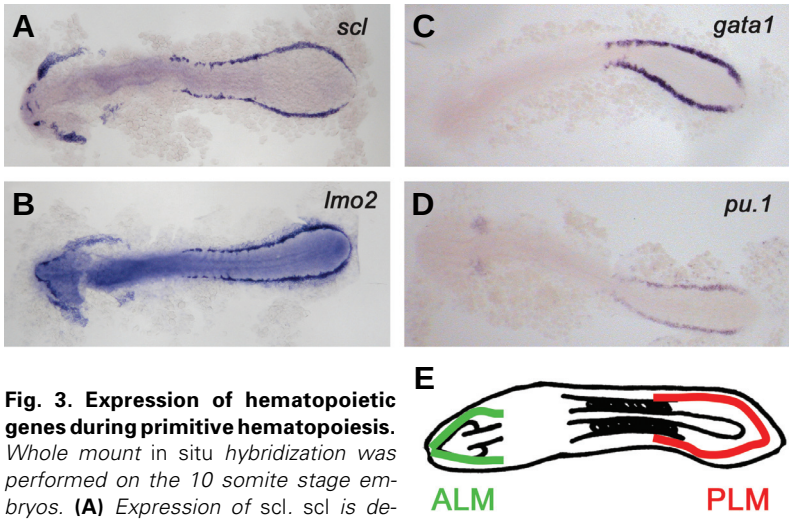


**Fig. 3. Expression of hematopoietic genes during primitive hematopoiesis.** *Whole mount* in situ *hybridization was performed on the 10 somite stage embryos.* **(A)** *Expression of* scl. scl *is detected both in the ALM and PLM.* **(B)** *Expression of* lmo2. lmo2 *is expressed both in the ALM and PLM.* **(C)** *Expression of* gata1. *Erythropoietic gene* gata1 *appears mainly in the PLM region.* **(D)** *Expression of* pu.1. *Myeloid regulating gene* pu.1 *is expressed both in the ALM and in the PLM.* **(E)** *Schematic of flatmounted embryo. ALM=anterior lateral mesoderm; PLM=posterior lateral mesoderm.*

## Early transcription factors involved in primitive hematopoiesis

### *gata1*

*gata1*, a zinc finger transcription factor, is a master regulator in erythrocyte development. It has been shown to physically interact with other transcription factors including FOG1 (Friend of GATA1), EKLF (Erythroid Kruppel Like Factor), and PU.1 to name a few (Cantor and Orkin, 2002). Gene targeting studies in mice have initially addressed the essential role of *Gata1* in erythropoiesis, since *Gata1-/-* mice die during gestation due to failure to differentiate proerythroblasts into mature erythrocytes (Fujiwara *et al.*, 1996). In zebrafish, *gata1* is expressed from the 5 somite stage in the PLM, along with *bikf* (blood island enriched Kruppel-like factor) in the *scl*+*lmo2*+*gata2*+ population (Detrich *et al.*, 1995; Thompson *et al.*, 1998; Kawahara and Dawid, 2000) (Fig. 3C). From the 12 somite stage, these *gata1*+ cells migrate medially and start to express erythroid specific genes including *globin* (Detrich *et al.*, 1995).

In addition to regulating erythroid specific gene transcription, *gata1* suppresses myeloid fate in the ICM. In *gata1* knockdown embryos, blood cells in the ICM switch their fate to myeloid cells, expressing *pu.1*, *mpo* (myeloperoxidase; granulocyte specific gene) and *l-plastin* that are normally not detected in erythrocytes (Galloway *et al.*, 2005). There seems to be a cross-inhibitory mechanism between *gata1* and *pu.1*, as *pu.1* knockdown has a reciprocal effect on ALM myeloid cells (Rhodes *et al.*, 2005) (see below).

### *pu.1*

*pu.1* is an ETS-domain transcription factor which gets its name from having a PU box binding domain on its C-terminus. It is a master regulator of myeloid cell development, including the development of granulocytes and macrophages (Scott *et al.*, 1994). As a transcription factor, it not only regulates expression of myeloid genes including *mpo* and *l-plastin*, but also its own transcription (Chen *et al.*, 1995). In zebrafish, it is expressed from the 6 somite stage in the ALM among the *scl*+*lmo2*+*gata2*+ population, and also appears in the ICM from the 10 somite stage (Fig. 3D). This specification of *pu.1+* cells relies on the bmp signaling pathway. When bmp signaling is repressed by dominant negative bmp receptor, *pu.1+* cells fail to appear even though hemangioblast population is present (Hogan *et al.*, 2006). After their emergence, *pu.1*+ cells in the ALM migrate towards the midline, then over the

yolk (Lieschke *et al.*, 2002). These *pu.1*+ cells in the ALM and ICM give rise to granulocytes expressing *mpo* and macrophages expressing *l-plastin* (Herbomel *et al.*, 1999; Bennett *et al.*, 2001).

Just as *gata1*+ cells in the ICM can become myeloid cells in the absence of *gata1*, *pu.1*+ cells have the potential to become erythrocytes. *pu.1* knockdown embryos have ectopic *gata1*+ cells in the ALM, and these cells later express *alpha-globin*, indicating differentiation into erythrocytes (Rhodes *et al.*, 2005). In addition to this 'autonomous' potential of *pu.1*+ cells, there also seems to be a non-autonomous cue from the ALM environment driving *pu.1*+ cells to become myeloid cells. When ALM *pu.1*+ cells are transplanted into the ICM of another embryo, these inital ALM cells now become erythroid cells (Rhodes *et al.*, 2005). These examples show there is an interplay between *gata1* and *pu.1* transcription factors during primitive hematopoiesis to balance erythroid and myeloid populations.

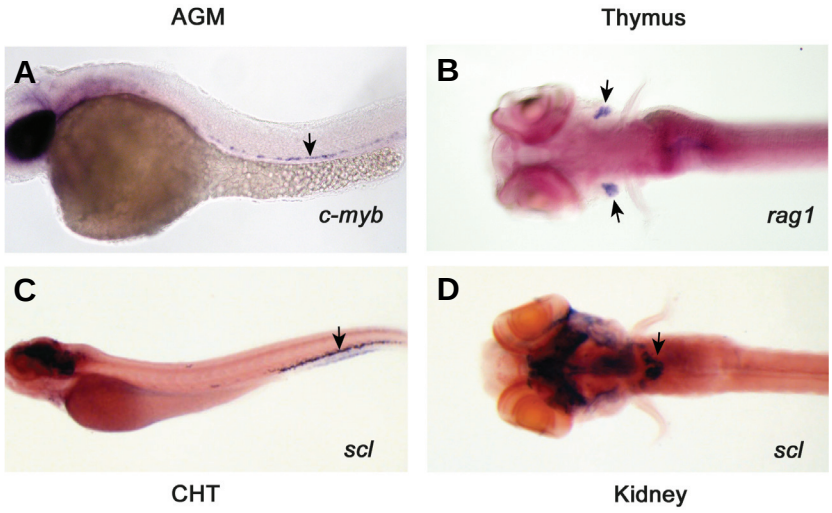


**Fig. 4. Sites of definitive hematopoiesis.** *Whole mount* in situ *hybridization was performed on different stages of embryos.* **(A)** c-myb *expression at 36hpf. The* c-myb*+ definitive HSCs appear at the dorsal aorta region (AGM).* **(B)** scl *expression at 4dpf. The definitive HSCs migrate to the posterior part of the embryo, starting from 48hpf.* **(C)** rag1 *expression at 7dpf. Lymphopoiesis starts in the thymi from 3dpf.* **(D)** scl *expression at 7dpf. The HSCs appear in the pronephros (kidney) from 4dpf, which is equivalent to mammalian bone marrow. AGM=aorta-gonad-mesonephros; CHT=caudal hematopoietic tissue.*

## Definitive hematopoiesis

The second wave of hematopoiesis, also known as the definitive wave, initiates in the zebrafish embryo from 30hpf. Unlike primitive HSCs, definitive HSCs have the potential to become all blood lineages including lymphocytes. Like in mammals, the first definitive HSCs arise from the ventral region of the dorsal aorta and express *runx1* and *c-myb* transcription factors (Fig. 4A) (Burns *et al.*, 2002; Kalev-Zylinska *et al.*, 2002). From 48hpf, these *c-myb*+ cells appear in the posterior part of the embryo, known as the caudal hematopoietic tissue (CHT) (Murayama *et al.*, 2006; Jin *et al.*, 2007). The structure of the CHT highly resembles that of a kidney marrow as it has many sinusoids that slow down the blood flow, and this could help seeding of HSCs (Murayama *et al.*, 2006). The *c-myb*+ cells later appear in the thymus from 3dpf and in the pronephros from 4dpf (Fig. 4C and 4D) (Murayama *et al.*, 2006; Jin *et al.*, 2007). Thymi and kidney marrow serve throughout the life of a zebrafish, generating adult hematopoietic cells.

HSCs born in the AGM are thought to take two independent routes when they seed the pronephros. The first route involves the CHT, where HSCs first seed the CHT, and then migrate to the pronephros, as shown by photoactivatable tracer experiments (Murayama *et al.*, 2006; Jin *et al.*, 2007). The second route does not involve the CHT. In this case, HSCs in the AGM directly migrate to the pronephros by moving through pronephric tubules as addressed by *in vivo* imaging of the zebrafish *c-myb* reporter line (Bertrand *et al.*, 2008).

## Transcription factors involved in definitive hematopoiesis

### *runx1*

*runx1* is a member of the runt family of transcription factors, which was shown to play roles in many developmental processes from neurogenesis to hematopoiesis (Wang *et al.*, 1996b). *RUNX1* has been a focus of many studies since it often appears as a fusion oncogene in leukemia patients (Lutterbach and Hiebert, 2000). In mice, *Runx1* is expressed in the ventral region of the dorsal aorta that will become the AGM (North *et al.*, 1999). Also, *Runx1* knockout mice fail to develop definitive erythroid cells, myeloid cells, and lymphoid cells, demonstrating requirement for *Runx1* in definitive hematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1996b).

In zebrafish, *runx1* is expressed starting from the 5 somite stage both in the PLM and in the neural tissues. The PLM expression colocalizes with *scl*, indicating *runx1* is also expressed in the hemangioblast population. As definitive hematopoiesis starts, *runx1* is detected in the dorsal aorta (Burns *et al.*, 2002; Kalev-Zylinska *et al.*, 2002; Gering and Patient, 2005). The *runx1* knockdown does not affect primitive erythropoiesis revealing a dispensable role of *runx1* in primitive hematopoiesis (Kalev-Zylinska *et al.*, 2002; Gering and Patient, 2005). In accordance with murine experiments, *runx1* is indispensable for definitive hematopoiesis as *c-myb* expression decreases with *runx1* knockdown in the dorsal aorta and later lymphopoiesis is also affected (Kalev-Zylinska *et al.*, 2002; Burns *et al.*, 2005; Gering and Patient, 2005).

### *c-myb*

The myb family of proto-oncogenes encodes transcription factors which were initially discovered in the acutely oncogenic avian myeloblastosis virus (AMV). *c-myb*, one of the first myb members to be identified, is predominantly expressed in immature hematopoietic cells, and its expression decreases as these cells differentiate (Klempnauer *et al.*, 1982; Westin *et al.*, 1982; Gonda and Metcalf, 1984). The *c-Myb-/-* mouse dies prematurely during gestation due to a failure in fetal liver erythropoiesis despite having normal yolk sac hematopoiesis (Mucenski *et al.*, 1991). This led to the conclusion that *c-Myb* has a role in definitive hematopoiesis.

In zebrafish, *c-myb* is detected from the 10 to 12 somite stage, when primitive hematopoiesis takes place. However, as shown in mammals, *c-myb* is not required for primitive hematopoiesis, as loss of *c-myb* in zebrafish does not affect expression of *gata1*, the master regulator of primitive erythropoiesis (Thompson *et al.*, 1998). Around 36hpf, *c-myb* expression is detected in the ventral wall of the dorsal aorta in the *runx1*+ cells (Gering and Patient, 2005). From 2dpf, these *c-myb*+ cells appear in the CHT, and eventually migrate to the thymus (from 3dpf) and pronephros (from 4dpf) (Murayama *et al.*, 2006; Jin *et al.*, 2007).

### *ikaros*

The *ikaros* gene encodes a lymphoid transcription factor which is essential for B and T lymphoid lineage specification (Wang *et al.*, 1996a). It is characterized by having six zinc fingers, which are used in DNA binding and protein-protein interaction (Sun *et al.*, 1996). In mice, *Ikaros* is detected in all lymphoid cells and in both primitive and definitive hematopoietic precursors (Georgopoulos *et al.*, 1997).

During zebrafish development, *ikaros* is detected from the primitive wave as two stripes in the ALM and PLM, similar to *scl* and *gata1* (Willett *et al.*, 2001). Then from 2dpf, *ikaros* is detected in the pharyngeal arch area, indicating *ikaros*+ cells are migrating towards the thymi. By 3dpf, the *ikaros*+ cells finally appear in the thymi, which are located just ventral to the developing ear (Willett *et al.*, 2001). These cells start to express *rag1* (*recombination activating gene-1*) by 4dpf, indicating lymphoid differentiation is taking place (Willett *et al.*, 1997). The role of *ikaros* in zebrafish lymphopoiesis has been characterized in the *ikaros* mutant, which will be described below.

## Zebrafish hematopoiesis mutants

Forward genetic screening efforts in zebrafish have yielded many interesting blood related mutants. These mutants were utilized to characterize many aspects of hematopoiesis and to study the role of known blood specific transcription factors. We selected 8 zebrafish mutants to discuss and categorized them into four groups by their defects in hematopoietic ontogeny (Fig. 5).

### *Mesoderm patterning mutants*

*spadetail (tbx16)*

*spadetail* has a mutation in the *tbx16* gene, which is a member of the T-box transcription factors (Griffin *et al.*, 1998). The T-box transcription factors are downstream of the FGFs, which are secreted from the YSL. The *spadetail* mutant has a defect in many mesoderm-derived tissues including blood, despite having normal trunk development (Ho and Kane, 1990; Griffin *et al.*, 1998). The hematopoietic defects are represented by decreased level of *scl*, *lmo2*, *gata2*, *fli1*, and *gata1* expression in the PLM (Thompson *et al.*, 1998; Rohde *et al.*, 2004).

Blastula transplant experiments have shown that *tbx16* regulates hematopoiesis in both autonomous and non-autonomous ways (Rohde *et al.*, 2004). When blastomere cells are transplanted from *spadetail* mutant to wildtype animals, the donor cells are unable to give rise to blood cells, indicating the autonomous role of *spadetail* in hematopoiesis. On the other hand, when wildtype cells are transplanted into *spadetail* mutant embryos, erythropoiesis only occurs when wildtype cells contribute to both trunk mesoderm and lateral mesoderm (Rohde *et al.*, 2004). This suggests that trunk mesoderm requires tbx16 for inducing hematopoietic progenitors in the lateral mesoderm. Thus, in addition

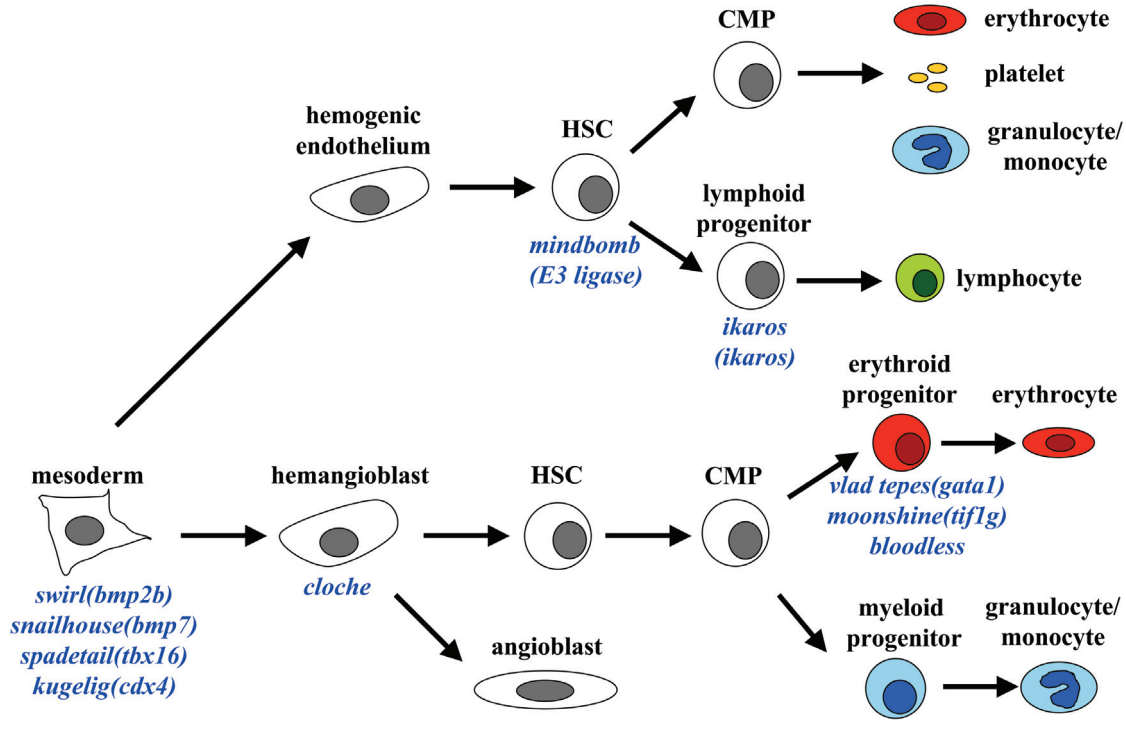


**Fig. 5. Overview of zebrafish mutants corresponding to the hematopoietic stage of development.** *HSC=hematopoietic stem cell; CMP=common myeloid progenitor.*

to autonomous function, *spadetail* is required nonautonomously during zebrafish hematopoiesis.

*kugelig (cdx4)*

*kugelig* has a mutation in the *cdx4* gene, and it was first identified by its shortened tail (Hammerschmidt *et al.*, 1996). *cdx4* is a member of the caudal related family of transcription factors, a family of genes that have been studied for their roles in anterior-posterior patterning (Mlodzik *et al.*, 1985; Edgar *et al.*, 2001). In wildtype zebrafish embryos, *cdx4* expression is detected during gastrulation in the area right above the YSL which will later become ventral mesoderm (Davidson *et al.*, 2003). During segmentation, *cdx4* is expressed posteriorly, and its expression transiently overlaps with that of *scl* at the 3 somite stage. All of these expression patterns are lost in *kugelig* mutant embryos (Davidson *et al.*, 2003). In addition, the mutant embryos show low level of *scl* and *gata1* at the 5 somite stage and low level of *runx1* at 36hpf, indicative of the role of *cdx4* in primitive and definitive hematopoiesis. Despite these defects, other mesoderm derived tissues including the pronephros and vasculature are relatively normal in *kugelig* (Davidson *et al.*, 2003; Davidson and Zon, 2006).

Genetically, *cdx4* is downstream of a newly identified bHLH transcription factor *mespa*. The *mespa* morphant has hematopoietic defects which are rescuable by *cdx4* overexpression (Hart *et al.*, 2007). At the same time, *cdx4* is upstream of posterior *hox* genes, namely *hoxb7a* and *hoxa9a*. When *hoxb7a* and *hoxa9a* are overexpressed in *kugelig* mutants, *scl* and *gata1* expression is recovered, although the tail defect is still present. This result indicates the hematopoietic defect shown in *kugelig* is due to the downregulation of *hox* genes in the hematopoietic cell population, rather than due to defects in gross posterior body patterning (Davidson *et al.*, 2003).

***Hemangioblast specification mutant***

*cloche (unknown gene)*

*cloche* was first discovered during a search for spontaneous mutations in an Indonesian fish farm. *cloche*, meaning 'bell' in French, has a swollen heart due to loss of endocardium (Stainier *et al.*, 1995). The mutation in the *cloche* gene perturbs the very early stage of hematopoietic and endothelial differentiation as shown with the complete disappearance of *scl*, *lmo2*, *gata1, l-plastin*, *mpo*, and *flk1/vegfr2* (Stainier *et al.*, 1995; Liao *et al.*, 1998; Thompson *et al.*, 1998). Through blastula transplantation assays, *cloche* was shown to regulate hematopoiesis in both autonomous and non-autonomous ways (Parker and Stainier, 1999).

Despite complete absence of hematopoietic and endothelial cells, other mesoderm derived tissues, including the pronephros, are unaffected in *cloche* (Davidson and Zon, 2004). This led to a hypothesis that the *cloche* gene is required for hemangioblast specification. Unfortunately, the gene responsible for this mutant has not been cloned to date, due to its telomeric location. Until now, there have been numerous efforts to identify the *cloche* gene by candidate gene searches. Although these studies have not been able to identify *cloche*, they provided fruitful information about the relationship between other hematopoietic transcription factors and *cloche*. For example, forced expression of *scl* in cloche mutant animals was able to rescue both erythrocyte and vasculature defects, putting *cloche* upstream of *scl* (Liao *et al.*, 1998; Liao *et al.*, 2000). On the other hand, *fli1* expression was minimally affected in *cloche*, putting *fli1* upstream of *cloche* (Thompson *et al.*, 1998*).*

*tal1 (scl)*

The zebrafish mutant *tal1* was identified during a genetic screen looking for genes that affect vasculature development (Bussmann *et al.*, 2007). These animals have a nonsense mutation in the third exon of the *scl* gene. This mutation leads to the loss of the HLH domain required for DNA binding, and the loss of the C-terminus domain which is important in interacting with lmo2 (Patterson *et al.*, 2007). As previously shown by *scl* knockdown experiments, the *tal1* mutant animals have severe defects in both hematopoietic and endothelial development. They do not initiate either primitive or definitive hematopoiesis, as displayed by loss of *gata1*, *pu.1* and *runx1* expression. In addition, they have defects in endocardial development, due to a failure in endocardial precursor migration (Bussmann *et al.*, 2007). These *tal1* mutant animals are anticipated to provide a new tool for studying the role of *scl* in hematopoiesis.

***Primitive erythroid specification/maintenance mutants***

*vlad tepes (gata1)*

The importance of the *gata1* transcription factor in primitive erythropoiesis was shown by a zebrafish mutant called *vlad tepes* (Weinstein *et al.*, 1996). *vlad tepes* mutants have a nonsense point mutation in the basic domain located C-terminus to the zinc finger, and they die between 8-15dpf. These mutants initially express some erythroid genes including *gata1* and *alpha-globin*, but these genes are not detected past 26hpf (Lyons *et al.*, 2002). Part of this defect can be explained by the fact that the mutated form of gata1 loses its ability to bind to promoters of *gata1* target genes such as *globin* (Lyons *et al.*, 2002). In addition, as shown with *gata1* knockdown experiments, the ICM "erythrocytes" in *vlad tepes* mutants switch fate to myeloid cells and express myeloid genes instead (Galloway *et al.*, 2005).

*moonshine (tif1γ)*

The *moonshine* mutant has a mutation in a chromatin remodeling factor called *tif1γ* (*transcriptional intermediate factor1γ*) (Ransom *et al.*, 2004). It first got its name moonshine from having an excessive amount of pigment cells in the dorsal part of the body (Kelsh *et al.*, 1996). The *tif1γ* gene is expressed maternally, and by early segmentation period, it is expressed throughout the embryo, and highly enriched in the PLM (Ransom *et al.*, 2004).

In addition to its pigment characteristics, the *moonshine* mutant has specific defects in primitive and definitive erythropoiesis but normal ALM myelopoiesis. In these embryos, embryonic erythroid specification is present, as shown with normal *gata1* and *scl* expression at the 5 somite stage. The proerythroblast cells however, are progressively lost by apoptotic cell death from the 12 somite stage. As a result, expression of *gata1*, *scl,* and *gata2* genes are completely absent by 22hpf (Ransom *et al.*, 2004).

Two groups have proposed that mammalian Tif1 γ interacts with SMADs which are downstream of TGFβ pathway. However, the mechanism still remains unclear, since one group proposed Tif1 γ may downregulate SMAD4 through ubiquitination, whereas the other group suggested Tif1 γ may compete with SMAD4 for

binding R-SMAD complex (Dupont *et al.*, 2005; He *et al.*, 2006).

*bloodless (unknown gene)*

The *bloodless* mutant zebrafish was first isolated as a spontaneous mutation during an insertional mutagenesis screen. The *bloodless* mutants do not have circulating erythrocytes until 5dpf, but survive through this anemic stage by relying on oxygen diffusion. Once the blood is recovered, these fish survive to adulthood (Liao *et al.*, 2002).

In the mutant embryos, the *scl*+ population in the PLM is present during early segmentation, but it is completely lost through apoptosis by 23hpf. In accordance with this, *gata1*+ cells are also absent in PLM. On the other hand, ALM myelopoiesis is unaffected and other mesoderm derived tissue such as vasculature and somites are also intact in the *bloodless* mutant. This indicates that the *bloodless* gene solely affects primitive erythropoiesis. By 5dpf, blood cells, possibly from a definitive origin, start to circulate in the mutant embryos, and lymphopoiesis starts at 7dpf (Liao *et al.*, 2002). The mechanism of the *bloodless* gene is still unclear, and cloning of this gene will help elucidate the difference between the primitive and definitive waves.

***Definitive hematopoiesis mutant***

*mindbomb (ubiquitin E3 ligase)*

The *mindbomb* mutant, which was first studied for its defects in neural development, has a defect in the *notch* pathway (Itoh *et al.*, 2003). The *notch* pathway is composed of the transmembrane *notch* receptor and its ligand *delta*. When the *notch* pathway is activated by *delta*, the intracellular domain of the *notch* receptor (nicd) translocates into the nucleus, inducing target gene transcription (Gridley, 2007). *mindbomb* has a defect in generating delta, due to loss of the *ubiquitin E3 ligase* gene required for delta protein maturation (Itoh *et al.*, 2003).

In addition to its neural defects, *mindbomb-/-* embryos have defects in definitive hematopoiesis but normal primitive hematopoiesis (Burns *et al.*, 2005). *mindbomb* mutant embryos have a reduction in *runx1* and *c-myb* expression in the dorsal aorta despite having intact vasculature. When the *notch* pathway is hyperactivated by overexpression of nicd, the number of *runx1*+*c-myb*+ cells significantly increases in the AGM area. However, when *runx1* is downregulated by morpholino injection, ectopic nicd can no longer induce *c-myb* expression in the AGM. In addition, loss of *c-myb* expression in mindbomb mutant embryo is rescued by *runx1* overexpression. In conclusion, these experiments indicate the *notch* pathway regulates definitive hematopoiesis, which is mediated by the *runx1* transcription factor (Burns *et al.*, 2005).

*ikaros (ikaros)*

The *ikaros* mutant zebrafish was identified during a search for animals with an absence of lymphopoiesis at 5dpf. *ikaros* mutant animals have a nonsense mutation in the last exon of the *ikaros* gene, leading to a truncation of two last zinc fingers which are required for protein-protein interaction (Schorpp *et al.*, 2006).

Characterization of the *ikaros* mutant suggests that there may be two phases of lymphopoiesis during zebrafish development. The embryonic lymphopoiesis, which happens between 3dpf and 14dpf, is dependent on the *ikaros* gene, as shown with absence of *rag1* expression in the *ikaros* mutant during that period. On the other hand, *rag1*+ cells in *ikaros* mutant animals appear in thymi from 14dpf, and the mutant fish survive through adulthood. These results indicate that the *ikaros* gene is absolutely required for embryonic lymphopoiesis, but can be compensated during adult lymphopoiesis by an unknown mechanism (Schorpp *et al.*, 2006).

## Conclusion

Forward genetics screens, targeted gene knockdown by morpholino, and gene overexpression approaches have so far built our knowledge of hematopoietic genes during zebrafish development. In addition to these traditional genetic approaches, a chemical genetic screen was able to isolate many unknown players in hematopoiesis including the prostaglandin pathway (North *et al.*, 2007).

In recent years, the zebrafish field has seen a dramatic increase in the number of available tools. Development of assays such as irradiation recovery and kidney marrow transplantation has provided new ways to examine zebrafish hematopoiesis (Traver *et al.*, 2003; Traver *et al.*, 2004). In addition, a recent success in the reverse genetics approach using zinc finger nucleases and a transposon strategy for generating transgenic zebrafish will help in analyzing roles of additional genes in larval and adult hematopoiesis (Kawakami, 2004; Meng *et al.*, 2008). Finally, a recent generation of 'transparent' adult zebrafish will help our understanding of other aspects of hematopoiesis, including HSC migration and homing processes (White *et al.*, 2008).

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