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Zebrafish Blood Stem Cells

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ABSTRACT

Within the past two decades, the zebrafish (*Danio rerio*) has become an excellent model to study the development of hematopoietic stem cells (HSCs). All vertebrates including zebrafish have primitive and definitive waves of hematopoiesis, but self-renewing pluripotent HSCs are only produced by the definitive wave. The primitive wave occurs in two intraembryonic locations called the intermediate cell mass (ICM) and the anterior lateral mesoderm (ALM). Primitive erythropoiesis is in the ICM, whereas myelopoiesis initiates in the ALM. After circulation starts at 24 h post-fertilization, hematopoiesis shifts to the posterior blood island (PBI) for a brief period. The definitive wave starts in the aorta-gonad-mesonephros (AGM). There are three different HSC migration and colonization events that begin 2 days post-fertilization: AGM progenitor cells migrate to (1) the caudal hematopoietic tissue (CHT), which is an intermediate site of blood development; (2) the thymus, which is a site of lymphocyte maturation; and (3) the developing kidney marrow, which is the larval and adult location for production of all hematopoietic cell types, and is comparable to the bone marrow of mammals. Many of the transcription factors and signaling pathways that regulate the formation of HSCs in a zebrafish are conserved with mammals. Large-scale forward and reverse genetic screens have identified zebrafish blood and HSC mutants that represent models for known human diseases. Along with the technological advancements in the field of zebrafish research, future HSC studies in zebrafish will help us illuminate the genetic network controlling the development and function of stem cells in all vertebrates. J. Cell. Biochem. 108: 35–42, 2009.

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The zebrafish (*Danio rerio*) has emerged as an ideal model organism for studying hematopoiesis because it combines many of the attributes of other developmental models. For example, as in frogs, zebrafish eggs are fertilized externally and can be observed and manipulated at all developmental stages, from a single-celled embryo to a multi-cellular adult. Like the mouse, zebrafish has a short generation interval of 2–3 months, but zebrafish are smaller and produce many more offspring in a shorter time (~200 eggs per week). More importantly, the early stages of zebrafish development occur more rapidly than mouse, and the early embryos can be easily observed because they are optically transparent. Lineage tracing to study the migration of hematopoietic stem cells (HSCs) in the embryo can be done easily in the zebrafish [Bertrand et al., 2008]. The zebrafish has proven itself as an ideal organism for large-scale forward genetic screens, which have produced many informative mutants with hematopoietic defects [Driever and Fishman, 1996; Haffter et al., 1996]. The genes and molecular signaling pathways controlling hematopoiesis are highly conserved between fish and mammals.

The similarities between hematopoiesis in zebrafish and mammals is not only limited to the conservation of genes, and both share all major blood cell types that are generated from common lineages of hematopoietic stem cells [Traver et al., 2003]. This is an important advantage over invertebrate models that lack the complex hematopoietic and adaptive immune system of vertebrates. The primary sites of hematopoiesis change during development of vertebrates (e.g., from yolk sac to liver to bone marrow in mammals), providing the optimal microenvironments for blood development at different stages of life. Here we review the two waves of hematopoiesis, primitive and definitive, that are common to all vertebrates including the zebrafish (Fig. 1). We also discuss the molecular pathways involved in the production of HSCs and several technological advancements that have enhanced the study of HSCs in the zebrafish.

PRIMITIVE HEMATOPOIESIS

The initial embryonic wave of blood production, the primitive wave, predominantly produces erythrocytes and some primitive macrophages in zebrafish [reviewed in de Jong and Zon, 2005]. In

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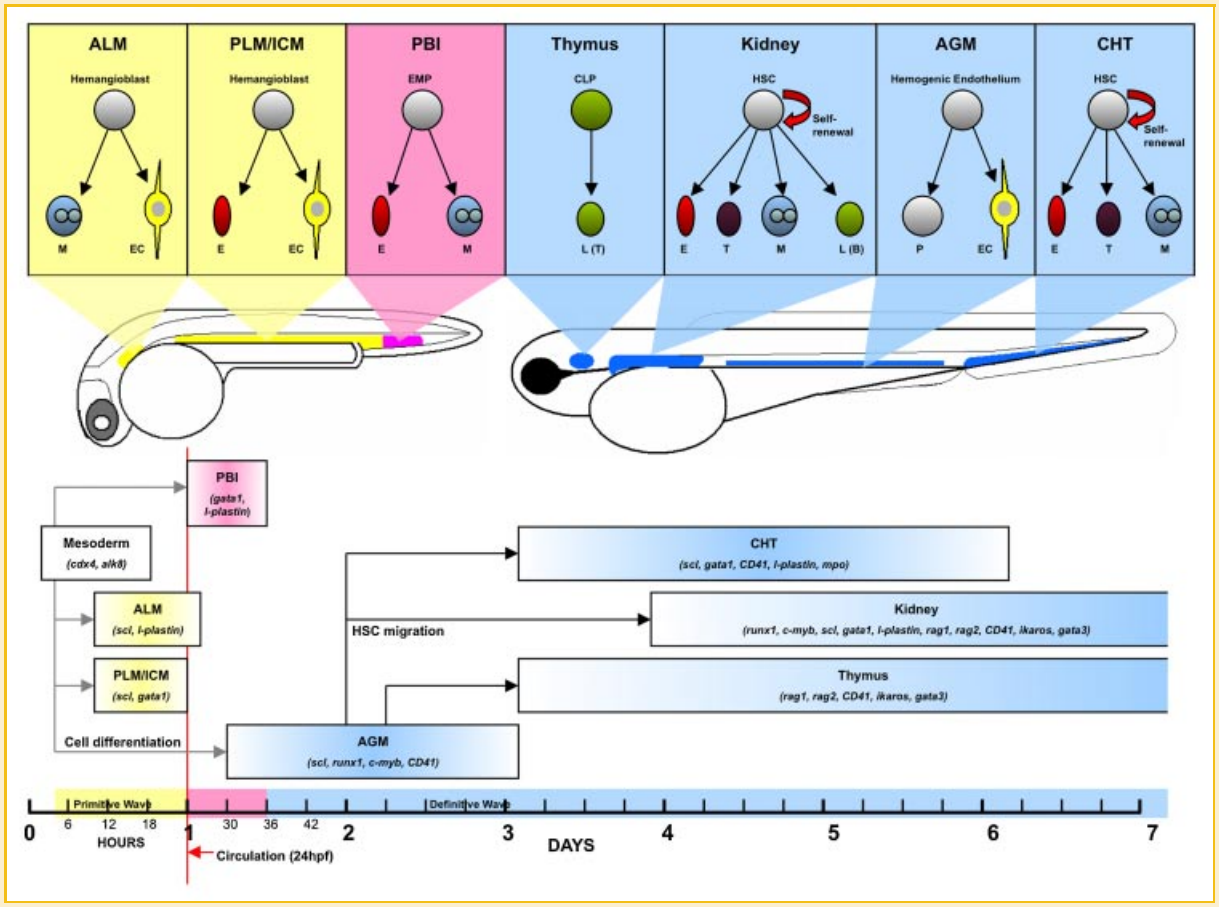

Fig. 1. Spatial and temporal representation of hematopoiesis in the zebrafish. Hematopoiesis at different sites is depicted in the top panel. The primitive wave of hematopoiesis (in yellow) consists of presumptive hemangioblasts that give rise to myeloid (M) and endothelial cells (EC) in the anterior lateral mesoderm (ALM) and to erythroid (E) and ECs in the posterior lateral mesoderm (PLM), which later becomes the intermediate cell mass (ICM). The first hematopoietic progenitor cells with multilineage potential are found in the posterior blood island (PBI); these erythromyeloid progenitors (EMPs) give rise to erythroid and myeloid cells in a transient definitive wave (in pink). The definitive wave of hematopoiesis (in blue) that contains long-term self-renewing hematopoietic stem cells (HSCs) originates in the aorta-gonad-mesonephros (AGM). The hemogenic endothelium in the AGM differentiates into ECs and hematopoietic progenitor cells (P) that migrate to and colonize other sites of definitive hematopoiesis. The AGM progenitor cells seed the caudal hematopoietic tissue (CHT) and kidney and proliferate to population of HSCs with self-renewal potential. Based on the expression patterns of blood-specific markers, erythroid, myeloid, and thromboid (T) lineages are found in the CHT. Kidney marrow contains all the different blood lineages, including the lymphoid B cell (L(B)) lineage. The common lymphoic progenitors (CLP) mature into lymphoid T cells (L(T)) in the thymus. The locations of different sites of hematopoiesis are depicted in a 24 h post-fertilization (hpf) embryo (left) and a 72 hpf larva (right). The timeline for expression of blood-specific markers at different sites is shown in the bottom panel. Important blood-specific transcription factors and markers are listed in each box. Mesoderm cells (white box and gray arrows) differentiate into four independent populations of blood progenitor cells in the ALM, PLM/ICM, PBI, and AGM. Blood circulation begins around 24 hpf (red line and arrow). The AGM progenitors begin to enter the circulation around 33 hpf, and by 48 hpf, the CHT and kidney are simultaneously seeded by these progenitors (black arrows). The CLPs from the AGM begin to migrate to the thymus around 54 hpf. The CHT serves as an intermediate site of definitive blood development, then from larval stage and into adulthood the kidney is the primary site of hematopoiesis. The thymus is the site of maturation of lymphoid T cells, however the CLPs, like other blood progenitors in the adult, originate in the kidney. ALM, anterior lateral mesoderm; PLM, posterior lateral mesoderm; ICM, intermediate cell mass; PBI, posterior blood island; AGM, aorta-gonad-mesonephros; CHT, caudal hematopoietic tissue; HSC, hematopoietic stem cell; M, myeloid; EC, endothelial cell; E, erythroid; EMP, erythromyeloid progenitor; CLP, common lymphoic progenitor; L(T), lymphoid T cell; L(B), lymphoid B cell; T, thromboid; P, hematopoietic progenitor cell; hpf, hour post-fertilization. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mammals and birds, the site of primitive hematopoiesis is found in the extraembryonic yolk sac, while in zebrafish the site is found at two intraembryonic locations: the anterior lateral mesoderm (ALM) and the posterior lateral mesoderm (PLM) that later forms the intermediate cell mass (ICM). Primitive myelopoiesis takes place in the ALM, whereas primitive erythropoiesis occurs in the ICM [Detrich et al., 1995; Davidson et al., 2003]. The zebrafish *spadetail* mutant has normal myelopoiesis in the ALM, but fails to make erythroid cells in the ICM, indicating the independent genetic regulation of progenitors in these two regions [Thompson et al., 1998].

HEMANGIOBLAST IN THE ALM AND ICM

Zebrafish and mammals have a similar transcription profile in hematopoietic tissues, in which highly conserved transcriptional factors regulate the blood development. At the 2-somite stage, or 10 h post-fertilization (hpf), two stripes in both the ALM and PLM express the *stem cell leukemia* (*scl*) gene that encodes a basic

helix–loop–helix transcription factor, indicating initiation of HSC and angioblast (vascular precursor) formation [Davidson et al., 2003]. Other hematopoietic transcription factors, such as *lmo2* and *gata2*, and vascular transcription factors, such as *fli1* and *flk1*, are also coexpressed with *scl* in the ALM and PLM cells, suggesting the existence of a bipotent hemangioblast [Dooley et al., 2005]. Fate-mapping using a caged fluorescent dye also shows that a single cell in the ventral mesoderm can give rise to both hematopoietic and endothelial cells in the ICM [Vogeli et al., 2006]. The zebrafish mutant *cloche* lacks all scl^+ cells in both the ICM and ALM, and has a severe deficit in both hematopoietic and endothelial lineages, suggesting that the gene encoded by the *cloche* locus acts upstream of differentiation of the hemangioblast [Thompson et al., 1998].

ERYTHROPOIESIS IN THE INTERMEDIATE CELL MASS

The ICM is formed from the PLM cells adjacent to the developing somites. These PLM cells express both early vascular and blood markers, they migrate medially to fuse at the midline and form the ICM by 18 hpf, and later differentiate into proerythroblasts and endothelial cells in the trunk vasculature. The zinc-finger transcription factor, *gata1* is crucial for primitive erythropoiesis. Starting from 4 to 5 somite stage (12 hpf), *gata1* is expressed in a subset of scl^+ cells in the PLM that will eventually give rise to the first circulating blood cells [Detrich et al., 1995; Thompson et al., 1998]. Therefore the fates of PLM cells are already determined as early as the 4-somite stage to become either hematopoietic or vascular cells.

Approximately 300 $gata1^+$ proerythroblasts in the ICM enter the circulation around 24 hpf and mature into erythrocytes. These primitive erythrocytes are morphologically different from adult zebrafish erythrocytes, which have less cytoplasm and a large elongated nucleus. All zebrafish erythrocytes are nucleated, compared to mammalian erythrocytes that only retain their nucleus in the primitive wave but not in the adult. Weinstein et al. [1996] showed that primitive zebrafish erythrocytes are the only circulating red blood cells for the first 4 days post-fertilization (dpf) by performing transfusion experiments with rhodamine-labeled circulating erythroid cells. In this experiment, the rhodamine-labeled 36 hpf donor cells are injected into the sinus venosus of the recipient embryos, and the declination of donor cells in circulation begins around 6 dpf as the definitive erythrocytes of the host start to populate the circulation. This observation suggests that blood cells from the primitive wave are left in the circulation for more than a week after fertilization even though primitive erythropoiesis stops around 24 hpf, indicated by diminishing levels of hematopoietic transcription factors in the ICM [reviewed in Hsia and Zon, 2005].

MYELOPOIESIS IN THE ANTERIOR LATERAL MESODERM

The second site of primitive hematopoiesis, the ALM, is the site for production of myeloid progenitors. Similar to cells in the ICM, the cells in the ALM contribute to both blood and vascular development and express similar transcription factors such as *fli1*, *gata2*, *lmo2*, and *scl*. There is no *gata1* expression in the ALM since there is no production of proerythroblasts. The myeloid-specific transcription factor *pu.1* (*spi1*) is first detected in a subset of scl^+ cells in the ALM in 3 somite embryos (11 hpf). These $pu.1^+/scl^+$ myeloid progenitors migrate rostrally during 14–16 hpf, laterally across the yolk sac around 22–24 hpf, and mature into macrophages and granulocytes [Bennett et al., 2001].

Using lineage specific markers, two distinct populations of primitive myeloid cells are detected in the ALM. The myeloid-specific marker, actin-binding protein, *l-plastin*, is expressed in all myeloid cells, but a major enzyme of granulocytes, myeloperoxidase (*mpo*), is only expressed in granuloid lineages. mpo^+ population marks granulocytes while the $mpo^-/l\text{-}plastin^+$ myeloid cell population represents monocytes and macrophages [Bennett et al., 2001].

Starting from 26 hpf, the function of the primitive macrophages can be observed in the ducts of Cuvier, where they engulf apoptotic erythroid cells. By 28 hpf $l\text{-}plastin^+$ macrophages are found throughout the whole embryo, and by 2 dpf macrophages will migrate to sites infected with bacteria [Herbomel et al., 2001] and engulf microinjected carbon particles in the circulation [Leschke et al., 2001].

Pu.1 is coexpressed with *gata1* in the ICM from 16 hpf to 20 hpf, and the equilibrium between these two factors regulates the production of primitive erythroid and myeloid cells. Loss of *gata1* expression in zebrafish embryos results in transformation of erythroid precursors into myeloid precursors [Galloway et al., 2005], and knockdown of *pu.1* expression induces red blood cell formation in the ALM [Berman et al., 2005]. Longer *pu.1* expression in the ICM due to the absence of *gata1* leads to the expansion of myeloid cells population at the expense of the total number of erythroid cells, and vice versa. This suggests that *gata1*and *pu.1* regulate balance of erythroid or myeloid cells in primitive hematopoiesis by promoting or suppressing their respective populations [Berman et al., 2005; Galloway et al., 2005].

THE TRANSIENT SITE OF HEMATOPOIESIS IN THE POSTERIOR BLOOD ISLAND (PBI)

The primitive hematopoiesis in the ALM and ICM lasts for less than 24 h and is followed by another transient wave of hematopoiesis in the posterior blood island (PBI), which is also known as the caudal hematopoietic tissue (CHT). Although these terms have been used interchangeably, in this current review we will use PBI in association with the earlier transient wave in the region (24–36 hpf), and CHT to refer to later stages. This wave is also known as the first wave of definitive hematopoiesis since a common progenitor gives rise to multiple blood lineages [Bertrand et al., 2007].

After circulation begins around 24 hpf, *gata1* expressions decrease in the ICM but not in the PBI, indicating a shift in the site of blood development. The expression of *pu.1* disappears in the ICM at 22 hpf and in the ALM at 28 hpf [Bennett et al., 2001], but then reappears in the PBI around 30 hpf [Bertrand et al., 2007]. Between 1 and 2 dpf, cells with erythroid and myeloid potential are found in the PBI, but these erythromyeloid progenitors (EMPs) do not have the self-renewing property of the definitive HSCs. Fate mapping studies show that these EMPs of the PBI originate from an independent lineage of posterior lateral mesoderm cells and can only differentiate into two blood lineages [Bertrand et al., 2007]. Therefore, these bipotent EMPs represent a transient wave between primitive and definitive hematopoiesis.

DEFINITIVE HEMATOPOIESIS

Multipotent HSCs capable of self-renewing and generating all the different blood lineages arise during definitive hematopoiesis. In mammals, these HSCs are first produced in the ventral wall of the dorsal aorta in a region known as the aorta-gonad-mesonephros (AGM), next in the fetal liver, and finally in the bone marrow. Comparable transitions in the production sites of HSCs are also observed in zebrafish. HSCs are first detected in the equivalent to the AGM region, which in zebrafish has also been referred to as the "DP joint," a thin mesenchyme between the dorsal aorta and the posterior cardinal vein [Murayama et al., 2006]. Next HSCs are found in the CHT, the intermediate site of hematopoiesis that may be equivalent to mammalian fetal liver. A subset of HSCs from the AGM also migrate directly to the thymus for lymphopoiesis [Kissa et al., 2008]. Finally, HSCs migrate from the AGM and CHT to seed the kidney marrow, the equivalent to mammalian bone marrow, and the site of larval and adult HSC production [Murayama et al., 2006; Bertrand et al., 2008].

HEMOGENIC ENDOTHELIUM AND HSC/PROGENITOR IN THE AGM

The transcription factor *runx1* is a key regulator of definitive hematopoiesis and is expressed in HSCs and all sites of adult blood development in mammals and zebrafish. Lack of *runx1* expression in mice leads to complete absence of definitive hematopoiesis and embryonic lethality [North et al., 1999]. In zebrafish, shortly after *gata1*$^+$ cells of the ICM enter circulation around 24 hpf, expression of *runx1* is detected in the HSCs and endothelial cells of the AGM. Like the hemangioblast of the ICM, the putative hemogenic endothelium is thought to be the common progenitor of HSCs and endothelial cells in the AGM. An alternative model suggests that the HSCs bud off from AGM mesenchyme rather than hemogenic endothelial cells in the ventral wall of the aorta. However, a recent fate tracing study in mouse shows that the mesenchymal cells are incapable of hematopoiesis [Zovein et al., 2008], and a single-cell imaging study shows that mouse hemogenic endothelial cells give rise to blood cells [Eilken et al., 2009]. Therefore, the AGM endothelial cells that express the HSC-specific marker, *runx1*, are presumably the hemogenic endothelial cells that give rise to the earliest definitive HSCs.

While the expression of *gata1*, *gata2*, and *scl* in the ICM begins to diminish after 24 hpf, definitive wave specific markers such as *runx1* and *c-myb* and other blood markers including *lmo-2*, *gata2*, and *scl* begin to express in the AGM [Thompson et al., 1998; Bertrand et al., 2008; Kissa et al., 2008]. The AGM HSCs give rise to progenitors that will colonize the CHT, thymus, and kidney. After 36 hpf, the PBI region undergoes considerable remodeling and becomes the CHT. By 3 dpf, the expression of *scl* is detected in the CHT, and it is followed by expressions of *gata1*, *αe1-globin*, *l-plastin* [Murayama et al., 2006; Zhang and Rodaway, 2007; Jin et al., 2009]. Since the expression of *gata1* and *l-plastin* are first detected in the CHT rather than the AGM, the first site of definitive erythropoiesis and myelopoiesis is located in the CHT.

Fate mapping and cell tracing studies of HSCs reveal several novel migration routes from the AGM to the CHT, thymus, and pronephros [Bertrand et al., 2008; Kissa et al., 2008]. A subset of the *runx1*$^+$ cells in the AGM also express CD41, the earliest known surface marker of nascent HSCs. Endothelial cells in the AGM do not express CD41; thus, transgenic zebrafish carrying the *CD41* promoter driving expression of *green fluorescent protein* (*gfp*) can be used to study the migration of HSCs by live imaging and timelapse microscopy [Kissa et al., 2008]. CD41 is also a platelet or thromboid-specific marker, but the GFP$^+$ thrombocytes are easily distinguishable from the GFP$^+$ HSCs in CD41-gfp transgenic zebrafish because GFP expression of thrombocytes (GFP^{high}) is much stronger than that of HSCs (GFP^{low}). In addition, GFP^{high} thrombocytes are first found in the CHT at 48 hpf while GFP^{low} HSCs are first detected in the AGM around 33 hpf. Due to these differences in timing, location, and intensity of GFP expression, GFP^{low} HSCs in the AGM are used to trace the migration routes of HSCs. After their first detection at 33 hpf, GFP^{low} AGM cells begin to enter the circulation through the axial vein but not through the dorsal aorta, as previously thought. By 48–56 hpf, GFP^{low} cells have reached and seeded the CHT, and by 54–56 hpf, GFP^{low} cells can be seen to colonize the nascent thymus, 16–18 h before the first detection of lymphoid markers in the thymus. Surprisingly, GFP^{low} cells that colonize the thymus do not take a direct circulating route to thymus; instead they migrate through the mesenchyme from widely diverse locations, both rostral and caudal to the thymus, indicating that they use more remote veins for migration [Kissa et al., 2008]. GFP^{low} AGM cells migrate slowly to the developing kidney along the pronephric tubules from the posterior AGM towards the anterior glomeruli between 48 and 96 hpf [Bertrand et al., 2008]. In mice, migration of HSCs from the AGM to fetal liver and bone marrow happens simultaneously [Delassus and Cumano, 1996]. Similarly in zebrafish, the CHT and pronephros are seeded by the AGM HSCs around the same time point during development [Bertrand et al., 2008; Kissa et al., 2008].

The seeded AGM HSCs or progenitors proliferate to form more HSCs in the CHT and kidney. By 3 dpf, the expression of HSC markers diminishes in the AGM, and the site of hematopoiesis shifts to the CHT. Around 6 dpf, the blood development wanes in the CHT as the kidney marrow becomes the lifelong and primary site of definitive hematopoiesis. Much like mammalian adult hematopoiesis that takes place in and around the fat and stroma cells of the bone marrow, HSCs are found intercalated between the renal tubules in the kidney marrow of the adult zebrafish [Traver et al., 2003]. Flow cytometry of kidney marrow and peripheral blood smears reveal that all the circulating hematopoietic blood cell types including erythrocytes, granulocytes, monocytes, lymphocytes, and thrombocytes are present in zebrafish [Traver et al., 2003; reviewed in Carradice and Lieschke, 2008].

ERYTHROPOIESIS

The definitive wave of erythropoiesis begins at 3.5 dpf in the CHT, as indicated by the *αe1-globin* and *gata1* expression in this region [Zhang and Rodaway, 2007; Jin et al., 2009]. The definitive erythrocytes slowly replace the circulating primitive erythrocytes, and *αe1-globin* expression is first detected in the pronephros around 5 dpf, as the site of erythropoiesis gradually transitions from the CHT to the kidney [Jin et al., 2009]. In the adult zebrafish, erythropoiesis is in the interstitium of the anterior and posterior kidneys

[Al-Adhami and Kunz, 1977]. The morphology of erythroid precursors is very similar in mammals and zebrafish. However, like in avian and reptilian erythrocytes, the mature zebrafish red blood cells are nucleated and elliptical in shape, where as mammalian erythrocytes have a classic biconcave discoid shape and do not retain their nuclei.

Hematopoietic transcription factors such as *scl* and *gata1* regulate erythropoiesis [Davidson et al., 2003; Dooley et al., 2005] along with erythropoietin signaling pathway involving erythropoietin receptor [Paffett-Lugassy et al., 2007] and JAK kinases [Ma et al., 2007]. The microRNA, *miR-451*, is found to play a role in red blood cell maturation [Pase et al., 2009], and erythropoiesis is controlled by many blood genes including *tif1γ* and *biklf* [Ransom et al., 2004; Galloway et al., 2008]. Like other higher vertebrates, zebrafish hemoglobin is a quaternary structure ($\alpha_2\beta_2$), and globin switching in erythrocytes from embryonic globin chains to the adult globin chains occurs between 15 and 30 dpf (Hsia and Zon, unpublished). As in mammals, zebrafish spleen functions as a reservoir for red blood cells, where erythrocytes are stored and destroyed [reviewed in Carradice and Lieschke, 2008].

MYELOPOIESIS

The definitive wave of myelopoiesis is observed around 3 dpf in the CHT by *l-plastin* expression; this expression is not seen in *runx1*^{w84x} mutants that lack definitive hematopoiesis [Jin et al., 2009]. The site of myelopoiesis switches from the CHT to the kidney when the developing kidney starts expressing *l-plastin* around 4 dpf [Galloway et al., 2005]. In an adult zebrafish, all the different myeloid lineages (macrophage/monocyte and granulocyte) differentiate from the HSCs in the kidney marrow.

The expression of *l-plastin* marks maturing macrophages and monocytes. Macrophage/monocyte specialization in tissues is observed from fate mapping of individual leukocytes as they migrate to different tissues. Based on morphology, macrophages from different tissues have different physical and cellular properties. Macrophages that reach the brain become microglia [Herbomel et al., 2001], while kidney and spleen macrophages contain phagosomes with erythrocyte and other cellular debris [Leschke et al., 2001]. Using fluorescent reporter transgenic zebrafish lines that express myeloid-specific marker, *lysozyme C*, Hall et al. [2007] showed the ability of macrophages migrating to inflammatory sites and phagocytosing bacteria in real-time.

Zebrafish have three granulocyte lineages as in mammals: neutrophils, eosinophils, and basophils. The site of granulopoiesis in zebrafish is located in the kidney interstitium [Bennett et al., 2001]. Similar to humans, zebrafish neutrophils express the granulocyte-specific marker, myeloperoxidase (*mpo*), but the mature adult zebrafish neutrophils have segmented nuclei with two or three lobes compared with the four or five lobes found in human cells. Zebrafish eosinophils have very large granules and do not have the bilobed nuclei seen in human eosinophils, and they stain positive for periodic acid-Schiff [Bennett et al., 2001; Leschke et al., 2001]. The third type of granulocytes, basophil/mast cells, expresses the mast cell-specific marker, carboxypeptidase A5 along with *mpo* and *pu.1*, and they are structurally similar to mammalian mast cells [Dobson et al., 2008]. The function and ontogeny of the zebrafish eosinophils and basophil/mast cells have not been fully described in literature.

LYMPHOPOIESIS

The adaptive immune system first evolved in jawed vertebrates nearly 450 million years ago. Mammals and zebrafish have similar lymphopoiesis pathways that produce T and B cells with recombination-activating gene (RAG)-mediated recombination of antigen receptors. Like in mice and humans, zebrafish T cells are matured in the thymus. The HSCs from the AGM first migrate to developing thymus around 54–56 hpf, and the immature lymphoblasts are first detected in the thymus around 65 hpf. By 4 dpf, T cell specific markers, such as *gata3*, *ikaros*, *lck*, *rag-1* and *rag-2*, begin to express in the thymus, and mature lymphocytes are found in thymic epithelium by 7 dpf [Trede et al., 2001]. It is hypothesized that a subpopulation of *ikaros*$^{+}$ HSCs from the AGM represents the progenitors that later migrate to the thymus. Throughout the lifetime of a zebrafish, kidney HSCs give rise to common lymphoic progenitors, and committed lymphoid T cell precursors reach maturation in the thymus. However, zebrafish B cell maturation takes place in the kidney marrow, as human B cell production occurs in the bone marrow. One of the challenges of studying lymphopoiesis in zebrafish is that antibodies specific for blood-cell lineages have yet to be developed, making the identification of distinct T cell populations difficult at present.

THROMBOPOIESIS

Zebrafish thrombocytes are nucleated and are easily distinguished by their dense nuclear chromatin and cytoplasmic projections. Like mammalian platelets, zebrafish thrombocytes function to maintain homeostasis by facilitating clot formation and express the platelet-specific marker, CD41, also know as glycoprotein(Gp)IIb or integrin, alpha 2b. Aggregation in peripheral blood smears, adhesion at the endothelium after injury, and agglutination without secretion in response to ristocetin (an antibiotic that induces platelet aggregation in mammals) are all similarly observed in zebrafish thrombocytes and human platelets [Jagadeeswaran et al., 1999]. Along with the expression of the GpIIb/IIIa complex, GpIb is also expressed on the surface of zebrafish thrombocytes as in mammals. The CD41-gfp transgenic line is used to trace the formation and migration of thrombocytes, and as noted above, CD41-gfp animals have two distinct populations of GFP^{+} cells; CD41 GFP^{low} cells are the HSCs or earlier progenitors, while CD41 GFP^{high} cells are the mature thrombocytes. Tracing the migration of the CD41 GFP^{high} cells, thrombocytes are first detected in the CHT around 2 hpf and enter the circulation by 3 dpf. As in erythropoiesis and myelopoiesis, the site of thrombopoiesis shifts from the CHT to the kidney around 5 dpf [Lin et al., 2005].

PATHWAYS INVOLVED IN THE DEVELOPMENT OF HSCs

The hematopoiesis in zebrafish is initiated and regulated by signal transduction pathways including VEGF, BMP, Hedgehog, PGE2-Wnt, and Notch-Runx pathways. Vascular endothelial growth factor (VEGF) is a potent growth factor that is involved in development of endothelial and hematopoietic cells [He and Chen 2005]. Reduction

of VEGF activity is known to reduce hematopoietic and endothelial differentiation and VEGF ligands are required for attracting myeloid cells at specific sites. The zebrafish *vegf* promoter contains a number of Smad binding sites that respond to bone morphogenetic protein (BMP)-activation, and overexpression of BMP induces an expansion of the myeloid population in the PBI, indicating regulation of the VEGF signaling pathway by the BMP pathway [He and Chen, 2005].

The family of Hedgehog (Hh) proteins plays a role in cell-fate specification and embryonic patterning. In zebrafish, Hh is found to be required for definitive but not primitive hematopoiesis [Gering and Patient, 2005]. In a developing embryo, Hh proteins are released from midline structures such as floor plate, notochord, and hypochord, and when Hh signaling is disrupted by chemicals, the number of *runx1*$^{+}$ HSCs and *rag1*$^{+}$ thymocytes decreases and the migration of *flk1*$^{+}$ angioblasts is impaired. It is believed that inhibition of Hh signaling results in improper patterning of the aorta, which leads to HSC loss in the AGM. Since, the number of primitive erythrocytes is not affected by inhibition of Hh pathway, Hh is required only for induction of definitive HSCs [Gering and Patient, 2005].

The Notch-Runx pathway is also a signal transduction pathway that is involved in regulation of HSC production and artery formation during embryogenesis. Morpholino knockdown of *runx1* and inhibition of Notch signaling pathway both result in disruption of HSC development in the AGM [Burns et al., 2005], and the *runx1*^{w84x} mutants lack all the definitive hematopoietic lineages [Jin et al., 2009]. The *mindbomb* mutant lacks an E3 ubiquitin ligase that is required for Notch signaling, and overexpression of *runx1* rescues the HSC production in these mutants, suggesting that *runx1* lies downstream or parallel to Notch signaling. Similarly, overexpression of *notch1a* intracellular domain (NICD) increases the population of *c-myb*$^{+}$ and *runx1*$^{+}$ cells in the AGM [Burns et al., 2005].

The prostaglandin (PG) E2-Wnt pathway has been recently identified as a crucial pathway that regulates the homeostasis of HSCs [North et al., 2007; Goessling et al., 2009]. Prostaglandin E2 (PGE2) is a main effector prostanoid in the zebrafish and is regulated by cycloxygenases Cox1 and Cox2. Wnt activation in HSCs requires PGE2, and zebrafish embryos treated with PGE2 or chemicals that enhance PGE2 synthesis increase HSC numbers, indicating the therapeutic potential of PGE2 treatment in the bone marrow of transplanted human patients [North et al. 2007; Goessling et al., 2009]. All of these signal transduction pathways need to function together properly for normal development of HSCs and blood lineages.

ADVANCEMENTS AND FUTURE OF ZEBRAFISH IN HSC STUDY

The study of HSCs in zebrafish has shed new light on many aspects of vertebrate HSC development. The molecular properties of many transcription factors and regulatory pathways in HSCs have been defined and redefined by zebrafish studies. The zebrafish has become an excellent model system to study HSCs because of the many technological advancements and innovations that can be applied to the HSC system. A major breakthrough in zebrafish research came with large-scale forward-genetic mutagenesis screens. Mutations are introduced in the fish germline using potent chemical mutagens, such as ENU (*N*-ethyl-*N*-nitrosourea) [Driever and Fishman, 1996; Haffter et al., 1996], or retroviral vectors that are injected into blastula-stage embryos to induce insertional mutations [Amsterdam et al., 1999]. The same scale of genetic screen is difficult to conduct in other vertebrate model systems due to the high cost, space limitations, and maintenance issues. Blood mutants from first zebrafish mutagenesis screens [Thompson et al., 1998], such as *cloche* and *spadetail*, carry mutations in genes involved in HSC development and have become invaluable resources for the HSC studies. Many novel genes that are important in hematopoiesis have been positionally cloned using these blood mutants, and their biochemical pathways have been investigated.

Secondly, many innovative reverse-genetic approaches to study HSCs have been developed in the zebrafish. Producing transgenic zebrafish lines to express a gene of interest under tissue-restricted gene promoters or inducible promoters has been one of the most common ways to manipulate and study a specific gene. DNA constructs containing fluorescent protein coding genes (e.g., GFP or DsRed) driven by any number of defined tissue-specific promoters, can be introduced to follow the temporal and spatial expression of a gene or cell type. These tools have been used to follow migration routes of HSCs during development [Bertrand et al., 2008; Kissa et al., 2008]. Heat or chemically inducible conditional Cre/lox-regulated transgenic zebrafish lines are also being generated to study effects of over-expression of a gene in a specific tissue at a given time point, and to establish a system for long-term lineage analysis [Langenau et al., 2005; Hans et al., 2009].

Gene knockdown of an mRNA transcript by anti-sense morpholino is widely used in the zebrafish [Nasevicius and Ekker, 2000]. Morpholinos persist in the embryos for 1–5 days after injection into one-cell embryos, and inhibit proper gene translation by targeting either the translation initiation site or RNA splice sites. Although morpholinos can only be used to evaluate gene function during the first few days of development, they often closely recapitulate mutant phenotypes, and can be applied rapidly if a specific genetic mutation is not available. Morpholinos have been successfully applied to study of the blood system as evidenced by the loss of primitive and definitive hematopoietic cell lineages in *scl* morphants [Dooley et al., 2005].

Recently, a new permanent gene knockout technology has been developed in zebrafish using engineered zinc finger nucleases (ZFNs) [Meng et al., 2008]. Engineered ZFNs introduce heritable mutations in the genome, and creation of knockout fish lines for HSC related genes has become possible with this technology. The conditional knockout technology that is widely available in mouse is not yet available in the zebrafish, but will undoubtedly become possible in the future.

Target induced local lesions in genomes (TILLING) is another reverse-genetic approach to produce mutants in specific genes. Standard chemical mutagenesis, as used in the forward-genetic screens, is used in TILLING to produce many random mutations in the germ line of treated animals. The genome from F1 progeny are sequenced for mutations in a gene of interest, and a mutant line for a specific gene is created by incrossing the progeny of the F1 fish

carrying the mutation in the gene. In TILLING screens, each gene of interest can have a series of mutations ranging from null to hypomorphic alleles; thus, an allelic series of mutations that modify gene function by different degrees can be studied. Many gene knockout zebrafish lines have been created using this approach, and many more will be identified using next generation sequencing technologies. The zebrafish community is currently organizing a TILLING project to generate knockout lines for all essential zebrafish genes. It is likely that more genes will be discovered that play important roles in hematopoiesis and HSC biology.

Recently, transplantation of whole kidney marrow or HSC transplantation has been developed in zebrafish [Traver et al., 2004]. The whole kidney marrow of the donor fish is isolated, different blood cell lineages are sorted, and the HSCs are transplanted into the circulation of irradiated recipient fish via intracardiac or retro-orbital injection. The survival and engraftment rates of the transplanted fish are carefully monitored to analyze the homing and transplantability of the HSCs. More recently, with the improvement of the zebrafish genome assembly, major histocompatibility complex (MHC) loci of the zebrafish have been identified and are matched between the donors and the recipients in the HSC transplantation experiments (Burns and Zon, unpublished).

Chemical screens have also been conducted in zebrafish to find novel drugs that can affect HSC formation or blood development. Many zebrafish HSC or blood mutants mirror human diseases and can be used as disease models to seek more effective treatment for human patients. Due to the small size of zebrafish, and their ability to absorb chemicals from surrounding water, chemical screens can be conducted in a high-throughput manner. PGE2 as a regulator of HSC number was first discovered in a zebrafish chemical screen [North et al. 2007], and post-chemotherapy or irradiation patients taking derivative of PGE2 is a potential to accelerate recovery of their hematopoietic system. Many more chemical screens are ongoing in zebrafish, and we can expect that other compounds will be found for treatment of blood diseases.

Lastly, a double pigmentation mutant named *casper* has recently been generated that is transparent as an embryo as well as an adult [White et al., 2008]. Creating transgenic lines in *casper* background, and conducting HSC transplantation assays using *casper*, bring the zebrafish to a new level of manipulating and studying HSCs. With the use of the *casper* zebrafish, stem cell engraftment, homing assays, and monitoring the effectiveness of chemical treatment on HSCs can be conducted in vivo.

All of these technological advancements in zebrafish have been a major driving force in the vertebrate HSC research. As more mutant lines are made, more pathways are mapped, and more potential chemicals are discovered, HSC research in zebrafish will help us to understand the genetic network or molecular aspects controlling the development and function of stem cells in all vertebrates and contribute to treatment of human hematopoietic diseases.

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