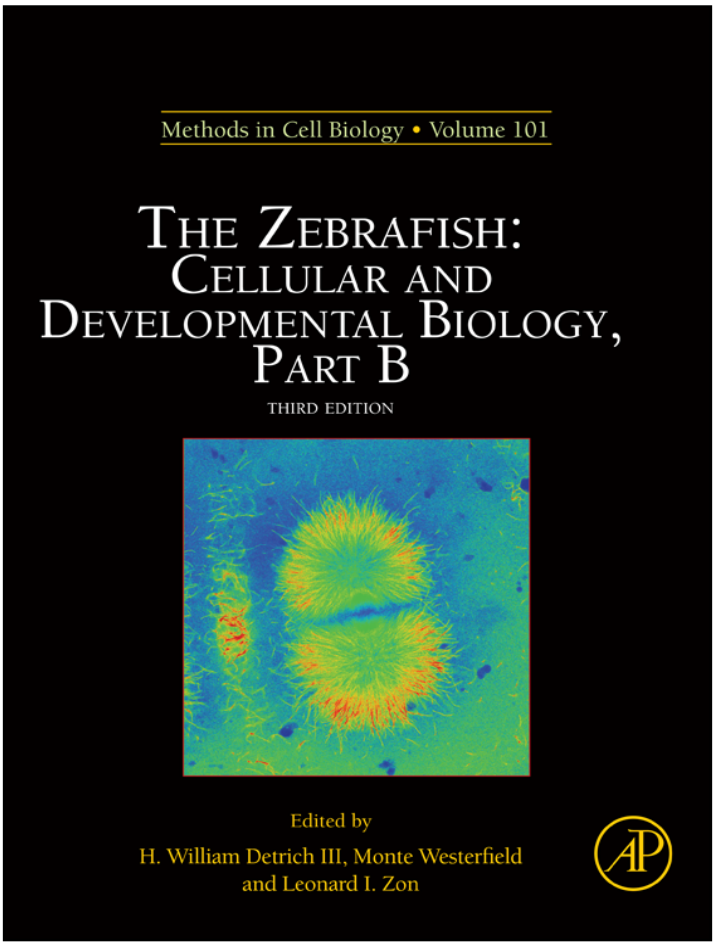

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CHAPTER 4

Cellular Dissection of Zebrafish Hematopoiesis

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Abstract

The zebrafish is an excellent model system to study vertebrate blood cell development due to a highly conserved hematopoietic system, optical transparency, and amenability to both forward and reverse genetic approaches. The development of functional assays to analyze the biology of hematopoietic mutants and diseased animals remains a work in progress. Here we discuss recent advances in zebrafish hematology, prospective isolation techniques, cellular transplantation, and culture-based assays that now provide more rigorous tests of hematopoietic stem and

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progenitor cell function. Together with the proven strengths of the zebrafish, the development and refinement of these assays further enable efforts to better understand the development and evolution of the vertebrate hematopoietic system.

I. Introduction

Over the past two decades, the development of forward genetic approaches in the zebrafish has provided unprecedented power in understanding the molecular basis of vertebrate blood development. Establishment of cellular and hematological approaches to better understand the biology of resulting blood mutants, however, has lagged behind these efforts. In this chapter, recent advances in zebrafish hematology will be reviewed, with an emphasis on prospective strategies for isolation of both embryonic and adult hematopoietic stem cells (HSCs) and the development of assays to rigorously test their function.

II. Zebrafish Hematopoiesis

Developmental hematopoiesis, in both mammals and teleosts, occurs in four sequential waves (Fig. 1). The first two waves are termed "primitive," and each generates transient precursors that respectively give rise to embryonic macrophages and erythrocytes (Keller *et al*., 1999; Palis *et al*., 1999). The next two waves consist of definitive hematopoietic precursors, defined as multipotent progenitors of adult cell types. The first to arise are erythromyeloid progenitors (EMPs), which give rise to erythroid and myeloid lineages (Bertrand *et al*., 2005b, 2007; Palis *et al*., 1999, 2001), followed by multipotent HSCs, which are endowed with the potential to both self-renew and generate all adult hematopoietic cell types [reviewed in Cumano and Godin (2007)].

A. Primitive Hematopoiesis

Primitive hematopoiesis has been extensively studied in the mouse, where primitive macrophages and erythroid cells are generated in the extraembryonic yolk sac (YS) (Bertrand *et al*., 2005b; Palis *et al*., 1999). In the zebrafish, primitive macrophages develop in an anatomically distinct area known as the rostral blood island (RBI) (Fig. 1A). Transcripts for *tal1* (also known as *scl)*, *lmo2*, *gata2a*, and *fli1a* are found in the RBI between the three- and five-somite stages (ss) (Brown *et al*., 2000; Liao *et al*., 1998; Thompson *et al*., 1998). This is quickly followed by expression of *spi1* (also known as *pu.1*) in a subset of these precursors (Bennett *et al*., 2001; Lieschke *et al*., 2002). Between 11 and 15 somites, *spi1*$^{+}$ macrophages are detectable, and they migrate toward the head midline (Herbomel *et al*., 1999; Lieschke *et al*., 2002; Ward *et al*., 2003) and across the yolk ball (Fig. 1C). Some of these precursors enter circulation, while others migrate into the head (Herbomel *et al*.,

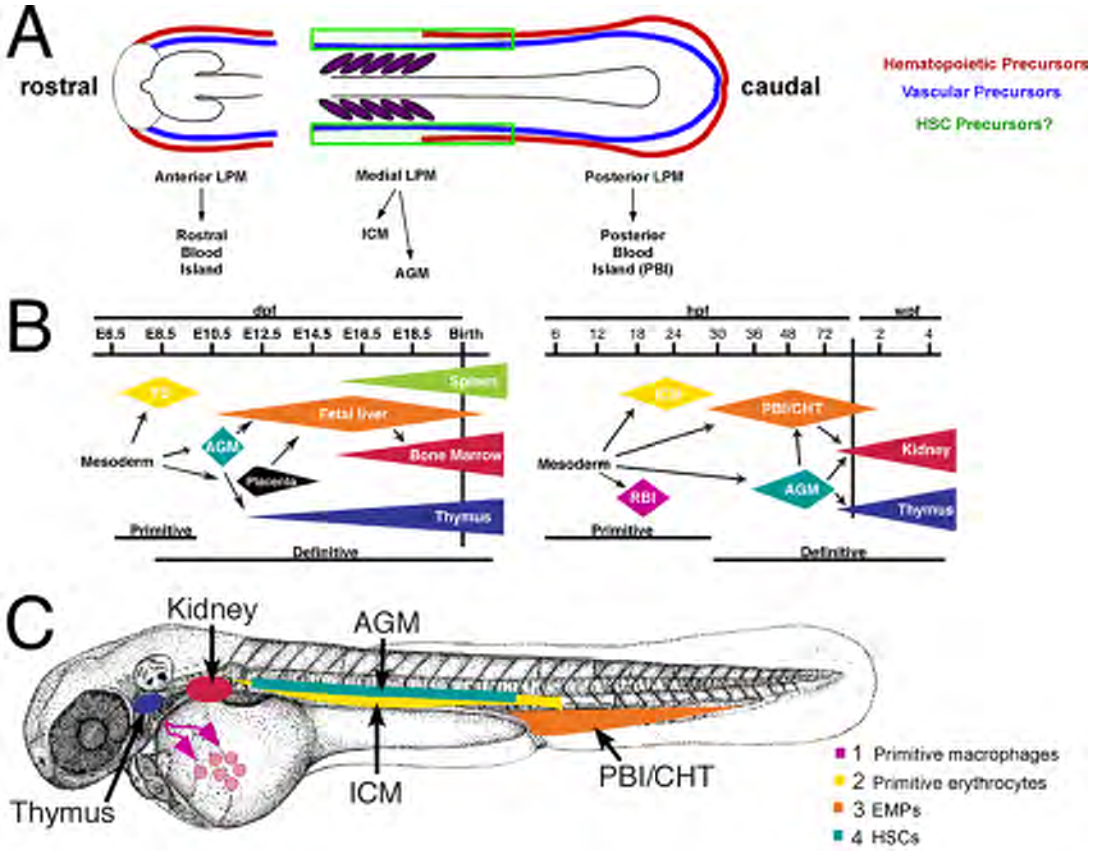

Fig. 1 Model of hematopoietic ontogeny in the developing zebrafish embryo. (A) Different regions of lateral plate mesoderm give rise to anatomically distinct regions of blood cell precursors. Anatomical regions of embryo responsible for generation of hematopoietic precursors (red), vasculature (blue), and pre-HSCs (green) are highlighted. Cartoon is a five-somite stage embryo, dorsal view. (B) Timing of mouse and zebrafish hematopoietic development. In mouse (left), primitive hematopoiesis initiates in the yolk sac (YS; yellow), producing primitive erythroid cells and macrophages. Later, definitive EMPs emerge in the YS. HSCs are specified in the aorta, gonad, and mesonephros (AGM, teal) region. These HSCs eventually seed the fetal liver (orange), the main site of embryonic hematopoiesis. Adult hematopoiesis occurs in the thymus (blue), spleen (green), and bone marrow (red). Zebrafish hematopoiesis is similar: temporal analogy to mouse hematopoiesis shown in (B, right), spatial locations shown in (C). Numbers in (C) correspond to timing of distinct precursor waves. (C) Embryonic hematopoiesis occurs through four independent waves of precursor production. First, primitive macrophages arise in cephalic mesoderm, migrate onto the yolk ball, and spread throughout the embryo (purple, 1). Then, primitive erythrocytes develop in the intermediate cell mass (ICM; yellow, 2). The first definitive progenitors are EMPs, which develop in the posterior blood island (PBI; orange, 3). Later, HSCs arise in the AGM region (teal, 4), migrate to the CHT (later name for the PBI, orange), and eventually seed the thymus and kidney (blue; red). Similar hematopoietic events in mouse and fish are color-matched between right and left panels of (B). Hematopoietic sites (B) and locations (C) are also color matched. (hpf: hours postfertilization, dpf: days postfertilization, wpf: weeks postfertilization, E: embryonic day, RBI: rostral blood island.) (See Plate no. 6 in the Color Plate Section.)

1999). By 28–32 h postfertilization (hpf), macrophages are found in circulation and dispersed throughout the embryo.

Primitive erythroid cell generation begins in the murine YS blood islands at day 7.5 postcoitum (E7.5) (Fig. 1B) [reviewed in Palis *et al*. (2010)]. These blood islands consist of nucleated erythroid cells that express embryonic *globin* genes surrounded by endothelial cells. Although it was previously believed that mammalian primitive erythroid cells uniquely remained nucleated (similar to the nucleated erythrocytes of birds, fish, and amphibians), it is now accepted that mammalian primitive red blood cells do, in fact, enucleate into reticulocytes and prenocytes (Fraser *et al*., 2007; Kingsley *et al*., 2004; McGrath *et al*., 2008). The zebrafish has an equivalent anatomical site to mammalian blood islands, known as the intermediate cell mass (ICM), where two stripes of mesodermal cells expressing *tal1*, *lmo2*, and *gata1a* converge to the midline of the zebrafish embryo and are surrounded by endothelial cells that become the cardinal vein. (Al-Adhami and Kunz, 1977; Detrich *et al*., 1995) (Fig. 1A and B). Although the ICM is intraembryonic in zebrafish, it has a cellular architecture similar to the mammalian YS blood islands (Al-Adhami and Kunz, 1977; Willett *et al*., 1999).

The development of transgenic zebrafish expressing fluorescent markers under the control of early mesodermal, prehematopoietic promoters (see Table I) now allow testing of primitive fate potentials by prospective isolation strategies and the functional assays outlined in this chapter.

B. Definitive Hematopoiesis

Similar to primitive hematopoiesis, definitive hematopoiesis initiates through two distinct precursor subsets. In the mouse, multilineage hematopoiesis is first evident in the YS (Bertrand *et al*., 2005b; Palis *et al*., 1999, 2001; Yoder *et al*., 1997a, 1997b) and placenta (Gekas *et al*., 2005; Ottersbach and Dzierzak, 2005) by E9.5. Multilineage precursors in both tissues can be isolated and distinguished by the expression of CD41, an integrin molecule that labels early hematopoietic progenitors. $CD41^{+}$ cells differentiate into both myeloid and erythroid lineages, but conspicuously lack lymphoid potential (Bertrand *et al*., 2005a; Yokota *et al*., 2006). These studies suggest that the definitive hematopoietic program in the developing mouse begins with committed EMPs. Recent studies in the zebrafish have demonstrated evolutionary conservation of EMPs as the first definitive precursor formed, and expanded upon findings in the mouse YS. EMPs can be isolated from the zebrafish posterior blood island (PBI) between 26 and 36 hpf (Fig. 1C) by their co-expression of fluorescent transgenes driven by the *lmo2* and *gata1a* promoters (Fig. 2A) (Bertrand *et al*., 2007). *In vitro* differentiation experiments (Fig. 2B and C) and *in vivo* transplantation assays have shown these cells capable of only erythroid and myeloid differentiation (Bertrand *et al*., 2007). Studies performed in *mindbomb* mutant zebrafish lacking Notch signaling showed that EMP specification and differentiation are not affected by loss of the Notch pathway (Bertrand *et al*., 2010b),

whereas HSCs are absent (Burns *et al*., 2005), further distinguishing these two definitive progenitors.

The zebrafish allows the discrimination of EMPs from (HSCs, both of which have similar cell-surface markers and differ only in their differentiation and self-renewal potentials (Bertrand *et al*., 2005a,; 2005b). Unlike the murine system, these two progenitors arise in separate anatomical locations (Bertrand *et al*., 2007), and are therefore easily distinguishable. Importantly, fate-mapping studies in the zebrafish

Table I
List of relevant transgenic zebrafish lines currently available for hematopoietic studies, indicating the promoter:gene expressed and the cell population(s) identified

Transgene	Tissue	Transgene	Tissue
lmo2:GFP (Zhu *et al*., 2005)	Prehematopoietic, vasculature	*kdrl:EGFP* (Cross *et al*., 2003)	Pre-hematopoietic, vasculature
lmo2:mCherry[*]	Prehematopoietic, vasculature	*kdrl:DsRed* (Jin *et al*., 2007b)	Pre-hematopoietic, vasculature
lmo2:DsRed (Lin *et al*., 2005)	Prehematopoietic, vasculature	*fli1a:EGFP* (Lawson and Weinstein, 2002)	Pre-hematopoietic, vasculature
itga2b:GFP (Lin *et al*., 2005)	EMPs, HSCs, thromobocytes	*fli1a:DsRed* (Jin *et al*., 2007b)	Pre-hematopoietic, vasculature
itga2b:mCherry[*]	EMPs, HSCs, thromobocytes	*gata3:AmCyan* (Bertrand *et al*., 2008)	Kidney
itga2b:CFP[*]	EMPs, HSCs, thromobocytes	*rag2:EGFP* (Langenau *et al*., 2003)	Immature B and T cells
ptprc:DsRed (Bertrand *et al*., 2008)	Pan-leukocyte	*lck:EGFP* (Langenau *et al*., 2004)	Mature T cells
ptprc:CFP[*]	Pan-leukocyte	*il7r:mCherry*[*]	Lymphoid precursors, T cells
ptprc:AmCyan[*]	Pan-leukocyte	*mhcII:GFP*[*] (Wittamer *et al*., 2011)	B cells, macrophages, dendritic cells
gata1a:GFP (Long *et al*., 1997)	Red blood cells	*mhcII:AmCyan*[*]	B cells, macrophages, dendritic cells
gata1a:DsRed (Traver *et al*., 2003b)	Red blood cells	*lyz:EGFP* (Hall *et al*., 2007)	Neutrophils
cmyb:GFP (Bertrand *et al*., 2008)	HSCs, neural	*lyz:DsRed* (Hall *et al*., 2007)	Neutrophils
mpx:EGFP (Renshaw *et al*., 2006)	Neutrophils	*runx1P1:GFP* (Lam *et al*., 2008)	EMPs
gata2a:EGFP (Traver *et al*., 2003b)	Eosinophils	*runx1P2:GFP* (Lam *et al*., 2008)	HSCs
Ighm:EGFP[*]	B cells	*ccr9a:cfp*[*]	Lymphoid precursors
Ighz:EGFP[*]	B cells	*mpeg1:GAL4* (Ellett *et al*., 2010)	Embryonic Macrophages

This list is not comprehensive; other transgenic animals are being constantly generated, but these are a few of the essential tools currently being used in zebrafish hematopoiesis laboratories.

[*] Unpublished transgenic animals generated in the Traver laboratory.

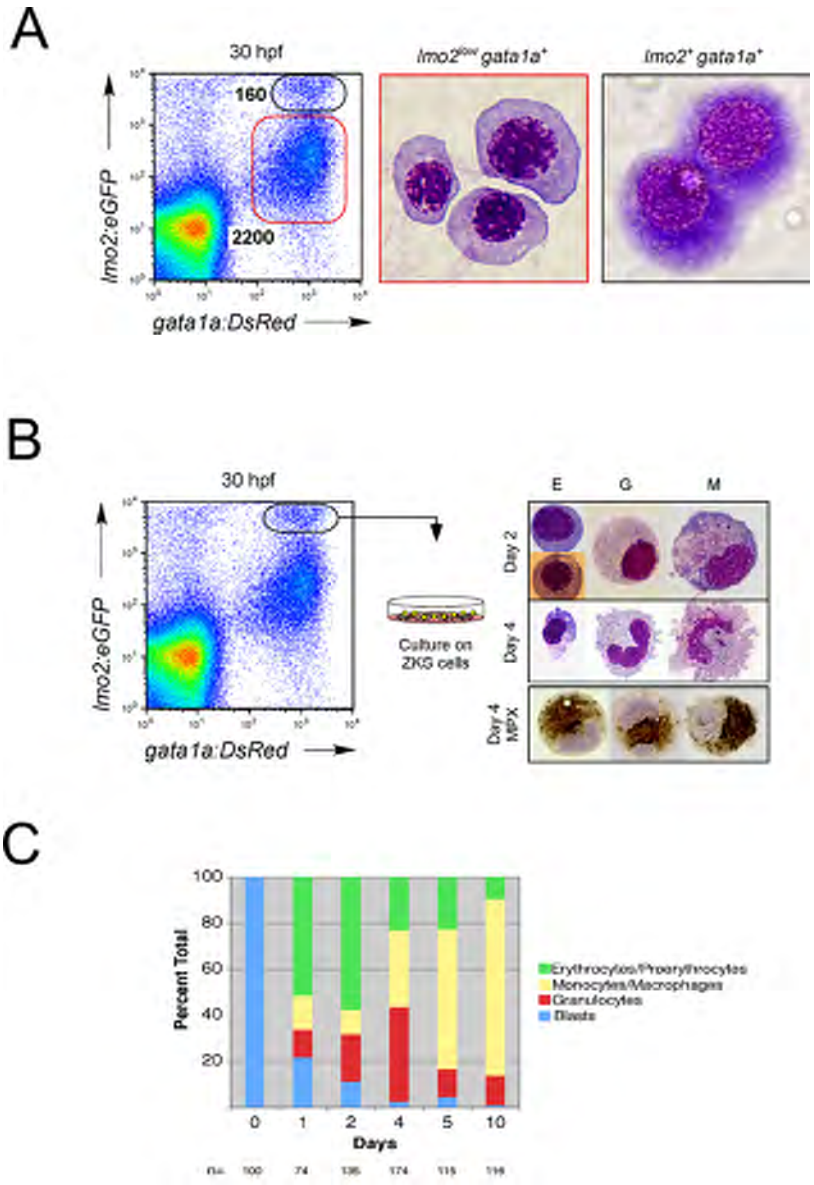

Fig. 2 Functional *in vitro* differentiation studies demonstrate that *gata1*$^{+}$*lmo2*$^{+}$ cells are committed erythromyeloid progenitors (EMP). (A) Purified EMP at 30 hpf (*lmo2*$^{+}$*gata1a*$^{+}$, black gate) have the immature morphology of early hematopoietic progenitors. As a comparison, purified primitive erythroblasts are shown (*lmo2*^{low}*gata1a*$^{+}$, red gate). Magnification, 1000×. (B) Short-term *in vitro* culture of *lmo2*$^{+}$*gata1a*$^{+}$ cells atop ZKS cells demonstrate erythroid (E), granulocytic (G), and monocytic/macrophage (M) differentiation potentials. Cultured cells were stained with May-Grünwald/Giemsa and for myeloperoxidase (MPX) activity. *lmo2*^{low}*gata1a*$^{+}$ cells only differentiated into erythroid cells (not shown).

have demonstrated EMPs to arise from posterior mesodermal derivatives that express the *lmo2* gene. This finding, in combination with lineage tracing studies demonstrating EMPs to completely lack T lymphoid potential, indicate that EMPs and HSCs are unique populations and independently derived during development.

The final wave of hematopoiesis culminates with the formation of HSCs, which self-renew and give rise to all definitive blood cell lineages, including lymphocytes. It has been demonstrated that HSCs arise in an area of the mid-gestation mouse bounded by the aorta, gonads, and mesonephros (AGM) at E10–10.5 (Fig. 1B) (Cumano and Godin, 2007; Dzierzak, 2005). Many studies have also suggested that transplantable HSCs are present in the YS on E9 (Lux *et al*., 2008; Weissman *et al*., 1978; Yoder *et al*., 1997a, 1997b) and later in the placenta by E11 (Gekas *et al*., 2005; Ottersbach and Dzierzak, 2005). While these results suggest that HSCs may arise in distinctly different locations in the developing mouse embryo, it is now clear that HSCs originate from arterial endothelium. Recent studies in the E10.5 mouse (Boisset *et al*., 2010) and 36–52 hpf zebrafish embryo (Bertrand *et al*., 2010a; Kissa and Herbomel, 2010) demonstrated directly the birth of HSCs from aortic endothelium via confocal imaging. A commonality among all vertebrate embryos thus seems to be the generation of HSCs from hemogenic endothelium lining the aortic floor (Ciau-Uitz *et al*., 2000; de Bruijn *et al*., 2002; Jaffredo *et al*., 1998; North *et al*., 2002; Oberlin *et al*., 2002). Similar studies will need to be performed to determine whether or not additional embryonic sites can likewise generate HSCs autonomously, including the YS and placenta. In all locations, however, it is clear that HSCs are present only transiently; by E11, the fetal liver (FL) is populated by circulating HSCs (Houssaint, 1981; Johnson and Moore, 1975) and becomes the predominant site of blood production during midgestation, producing the first full complement of definitive, adult-type effector cells. Shortly afterward, hematopoiesis is evident in the fetal spleen, and occurs in bone marrow throughout adulthood (Keller *et al*., 1999).

The zebrafish possesses an anatomical site that closely resembles the mammalian AGM (Fig. 1B and C). Between the dorsal aorta and cardinal vein between 28 and 48 hpf, *cmyb*$^{+}$ and *runx1*$^{+}$ blood cells appear in intimate contact with the dorsal aorta (Burns *et al*., 2002; Kalev-Zylinska *et al*., 2002; Thompson *et al*., 1998). Lineage tracing of CD41^{+} HSCs derived from this ventral aortic region show their ability to colonize the thymus (Bertrand *et al*., 2007; Kissa *et al*., 2008) and pronephros (Bertrand *et al*., 2008; Murayama *et al*., 2006), which are the sites of adult hematopoiesis (Jin *et al*., 2007a; Murayama *et al*., 2006). After 48 hpf, blood production appears to shift to the caudal hematopoietic tissue (CHT) (Fig. 1C), and later to the pronephros, which serves as the definitive hematopoietic organ for the remainder of life.

The development of transgenic zebrafish expressing fluorescent markers under the control of definitive hematopoietic promoters such as *itga2b* (also known as *cd41), cmyb, and runx1* (see Table I) now allows testing of fate potentials by prospective isolation strategies and functional assays outlined later in this chapter.

C. Adult Hematopoiesis

Previous genetic screens in zebrafish were successful in identifying mutants that affected primitive erythropoiesis. The screening criteria used in these screens scored visual defects in circulating blood cells during early embryogenesis; mutants defective in definitive hematopoiesis but displaying normal primitive blood cell development were therefore likely missed. Current screens aimed at identifying mutants with defects in the generation of definitive HSCs in the AGM should reveal new genetic pathways required for multilineage hematopoiesis. Recent studies in zebrafish show that nearly all adult hematopoietic cells derive from HSCs born from aortic endothelium (Bertrand *et al*., 2010a), consistent with findings in the murine system (Chen *et al*., 2009; Zovein *et al*., 2008). Therefore, mutational screens designed to identify defects in hemogenic endothelium may yield information about the full repertoire of hematopoietic regulation, specification, maintenance, and differentiation over the organism's lifespan. Understanding the biology of mutants isolated using these approaches, however, first requires the characterization of normal, definitive hematopoiesis and the development of assays to study the biology of zebrafish blood cells more precisely. To this end, we have established several tools to characterize the definitive blood-forming system of adult zebrafish.

Blood production in adult zebrafish, like other teleosts, occurs in the kidney, which supports both renal functions and multilineage hematopoiesis (Zapata, 1979). Similar to mammals, T lymphocytes develop in the thymus (Trede and Zon, 1998; Willett *et al*., 1999) (Fig. 3A), which exists in two bilateral sites in zebrafish (Hansen and Zapata, 1998; Willett *et al*., 1997). The teleostean kidney is a sheath of tissue that runs along the spine (Fig. 3B, E); the anterior portion, or head kidney, shows a higher ratio of blood cells to renal tubules than does the posterior portion (Zapata, 1979), termed the trunk kidney (Fig. 3B, C). All mature blood cell types are found in the kidney and morphologically resemble their mammalian counterparts (Fig. 3G, Fig. 4), with the exceptions that erythrocytes remain nucleated and thrombocytes perform the clotting functions of platelets (Jagadeeswaran *et al*., 1999). Histologically, the zebrafish spleen (Fig. 3D) has a simpler structure than its mammalian counterpart in that germinal centers have not been observed (Zapata and Amemiya, 2000). The absence of immature precursors in the spleen, or any other adult tissue, suggests that the kidney is the predominant hematopoietic site in adult zebrafish. The cellular compositions of whole kidney marrow (WKM), spleen, and blood are shown in Fig. 3F-H. Morphological examples of all kidney cell types are presented in Fig. 4.

Analysis of WKM by fluorescence activated cell sorting (FACS) showed that several distinct populations could be resolved by light scatter characteristics (Fig. 5A). Forward scatter (FSC) is directly proportional to cell size, and side scatter (SSC) proportional to cellular granularity (Shapiro, 2002). Using combined scatter profiles, the major blood lineages can be isolated to purity from WKM following two rounds of cell sorting (Traver *et al*., 2003b). Mature erythroid cells were found exclusively within two FSC^{low} fractions (Populations R1 and R2,

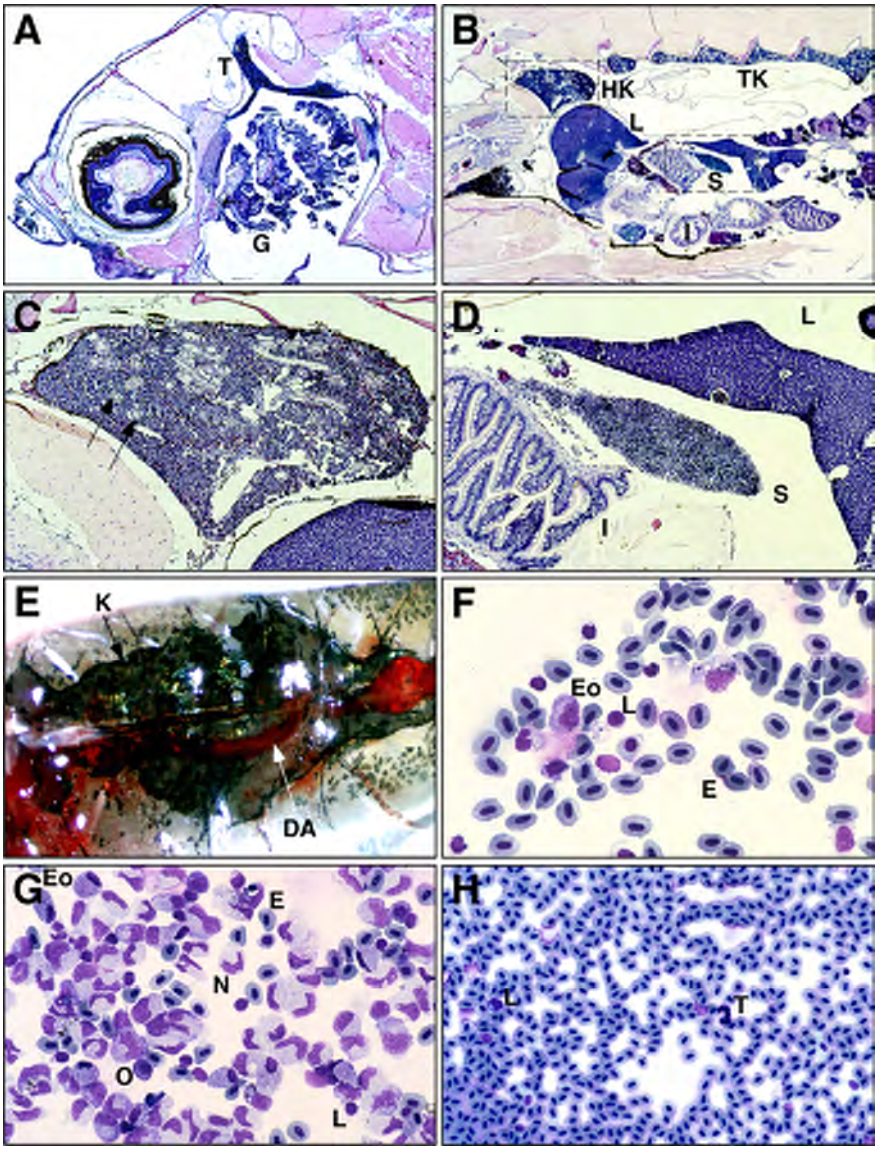

Fig. 3 Histological analyses of adult hematopoietic sites. (A) Sagittal section showing location of the thymus (T), which is dorsal to the gills (G). (B) Midline sagittal section showing location of the kidney, which is divided into the head kidney (HK), and trunk kidney (TK), and spleen (S). The head kidney shows a higher ratio of blood cells to renal tubules (black arrows), as shown in a close up view of the HK in (C). (D) Close up view of the spleen, which is positioned between the liver (L) and the intestine (I). (E) Light microscopic view of the kidney (K), over which passes the dorsal aorta (DA, white arrow). (F) Cytospin preparation of splenic cells, showing erythrocytes (E), lymphocytes (L), and an eosinophil (Eo). (G) Cytospin preparation of kidney cells showing cell types as noted above plus neutrophils (N) and erythroid precursors (O, orthochromic erythroblast). (H) Peripheral blood smear showing occasional lymphocytes and thrombocytes (T) clusters amongst mature erythrocytes. (A–D) Hematoxylin and eosin stains, (F–H) May-Grünwald/Giemsa stains.

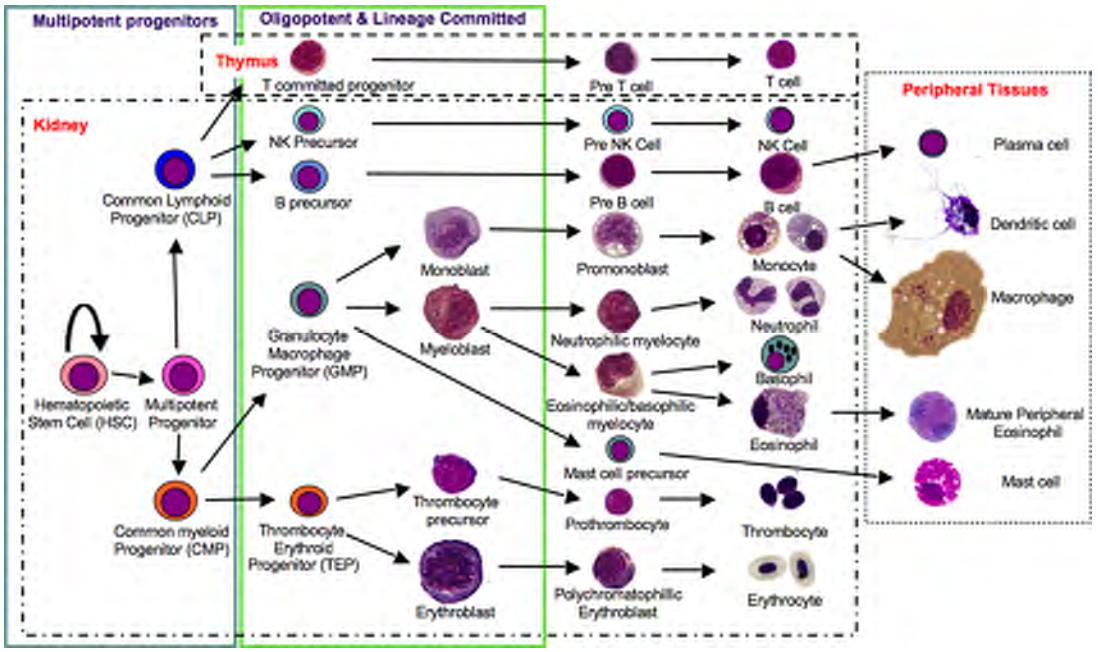

Fig. 4 Proposed model of zebrafish definitive hematopoietic differentiation. Isolated, cytospun, and stained blood cells from the zebrafish kidney, thymus, and peripheral tissues and their proposed upstream progenitors. Proposed lineage relationships are based on those demonstrated in clonogenic murine studies. Multipotent and lineage restricted progenitors likely reside in the kidney marrow, but their existence has never been experimentally proven due to a paucity of *in vitro* assays.

Fig. 5A, D), lymphoid cells within a FSC^{int} SSC^{low} subset (Population R3, Fig. 5A, E), immature precursors within a FSC^{high} SSC^{int} subset (Population R4, Fig. 5A, F), and myelomonocytic cells within only a FSC^{high} SSC^{high} population (Population R5, Fig. 5A, G). Interestingly, two distinct populations of mature erythroid cells exist (Fig. 5A, R1, R2 gates). Attempts at sorting either of these subsets reproducibly resulted in approximately equal recovery of both (Fig. 5D). This likely resulted due to the elliptical nature of zebrafish red blood cells, because sorting of all other populations yielded cells that fell within the original sorting gates upon re-analysis. Examination of splenic (Fig. 5B) and peripheral blood (Fig. 5C) suspensions showed each to have distinct profiles from WKM, each being predominantly erythroid. It should be noted that, due to differences in the fluidics and beam size, erythroid cells are not discretely detectable on BD FACScan, FACS Caliber, or FACS Aria I and II flow cytometers. However, FACS Vantage and LSR-II flow cytometers have a different fluidics system and are well suited to these analyses. Sorting of each scatter population from spleen and blood showed each to contain only erythrocytes, lymphocytes, or myelomonocytes in a manner identical to those in the kidney. Immature precursors were not observed in either tissue. Percentages of cells within each scatter population closely matched those obtained by morphological cell counts,

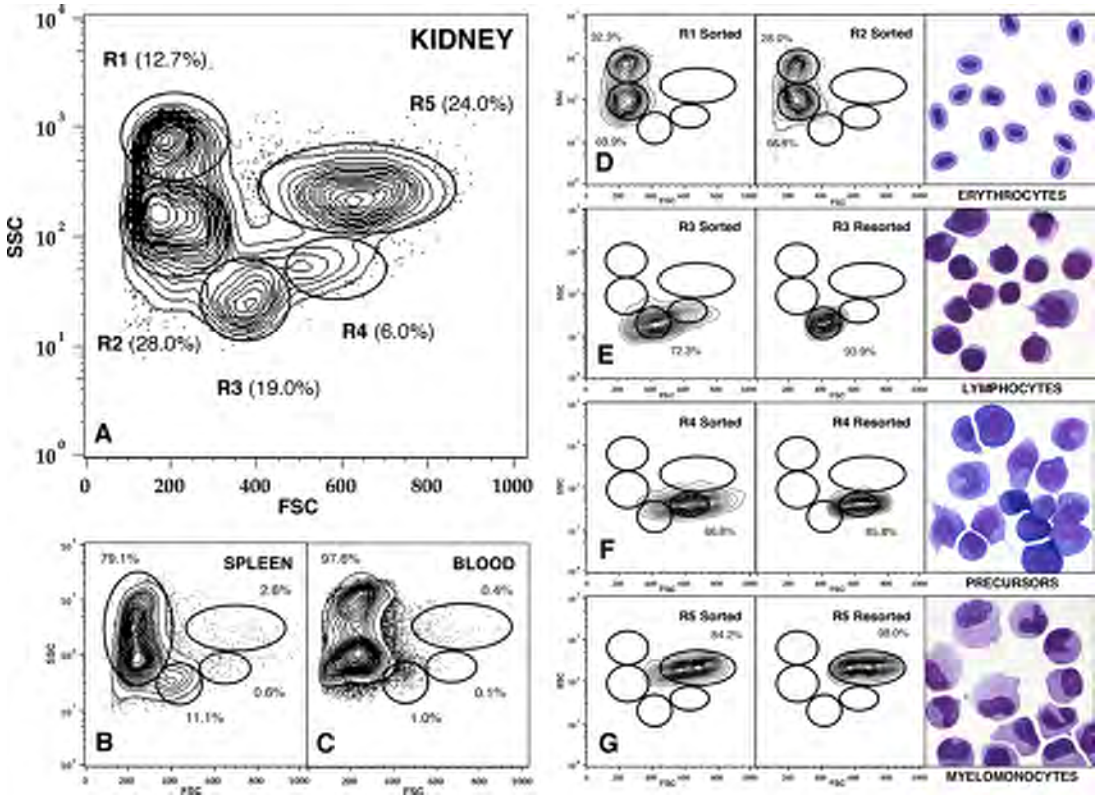

Fig. 5 Each major blood lineage can be isolated by size and granularity using FACS. (A) Scatter profile for WKM. Mature erythrocytes are found within R1 and R2 gates, lymphocytes within the R3 gate, immature precursors within the R4 gate, and myeloid cells within the R5 gate. Mean percentages of each population within WKM are shown. Scatter profiling can also be utilized for analyzing spleen (B) and peripheral blood (C). Purification of each WKM fraction by FACS (D–G). (D) Sorting of populations R1 or R2 yields both upon re-analysis. This appears to be due to the elliptical shape of erythrocytes (right panel). (E) Isolation of lymphoid cells. (F) Isolation of precursor fraction. (G) Isolation of myeloid cells. FACS profiles following one round of sorting are shown in left panels, after two rounds in middle panels, and morphology of double-sorted cells shown in right panels (E–G).

demonstrating that this flow cytometric assay is accurate in measuring the relative percentages of each of the major blood lineages.

Many transgenic zebrafish lines have been created using proximal promoter elements from genes that demonstrate lineage-affiliated expression patterns in the mouse. These include *gata1a:GFP* (Long *et al*., 1997), *gata2a:EGFP* (Jessen *et al*., 1998; Traver *et al*., 2003b), *rag2:EGFP* (Langenau *et al*., 2003), *lck:EGFP* (Langenau *et al*., 2004), *spi1:EGFP* (Hsu *et al*., 2004; Ward *et al*., 2003), and *itga2b:EGFP* (Lin *et al*., 2005; Traver *et al*., 2003b) stable transgenic lines. In the adult kidney, we have demonstrated that each of these animals expresses green

fluorescent protein (GFP) in the expected kidney scatter fractions (Traver *et al*., 2003b). For example, all mature erythrocytes express GFP in *gata1a:GFP* transgenic animals, as do erythroid progenitors within the precursor population. High expression levels of Gata2 are seen only within eosinophils, Rag2 and Lck only within cells in the lymphoid fraction, and Spi1 in both myeloid cells and rare lymphoid cells. The development of *itga2b:EGFP* transgenic animals has demonstrated that rare thrombocytic cells are found within the kidney, with thrombocyte precursors appearing in the precursor scatter fraction and mature thrombocytes in the lymphoid fraction. Without fluorescent reporter genes, rare populations such as thrombocytes cannot be resolved by light scatter characteristics alone. By combining the simple technique of scatter separation with fluorescent transgenesis, specific hematopoietic cell subpopulations can now be isolated to a relatively high degree of purity for further analyses.

FACS profiling can also serve as a diagnostic tool in the examination of zebrafish blood mutants. The majority of blood mutants identified to date are those displaying defects in embryonic erythrocyte production (Traver *et al*., 2003a). Most of these mutants are recessive and many are embryonic lethal when homozygous. Most have not been examined for subtle defects as heterozygotes. Several heterozygous mutants such as *retsina*, *riesling*, and *merlot* showed haploinsufficiency as evidenced by aberrant kidney erythropoiesis (Traver *et al*., 2003b). All mutants displayed anemia with concomitant increases in erythroid precursors. These findings suggest that many of the gene functions required to make embryonic erythrocytes are similarly required in their adult counterparts at full gene dosage for normal function.

III. Hematopoietic Cell Transplantation

In mammals, cellular transplantation has been used extensively to functionally test putative hematopoietic stem and progenitor cell populations, precursor/progeny relationships, and cell autonomy of mutant gene function. To address similar issues in zebrafish, several different varieties of hematopoietic cell transplantation (HCT) have been developed (Fig. 6).

A. Embryonic Donor Cells

Although scatter profiling has proven very useful in analyzing and isolating specific blood lineages from the adult kidney, it cannot be used to enrich blood cells from the developing embryo. To study the biology of the earliest blood-forming cells in the embryo, we have made use of transgenic zebrafish expressing fluorescent proteins. As discussed above, hematopoietic precursors appear to be specified from mesodermal derivatives that express *lmo2*, *kdrl* (also known as *flk1*), and *gata2a*. The proximal promoter elements from each of these genes have been shown to be sufficient to recapitulate their endogenous expression patterns. Using germline transgenic animals expressing GFP under the control of each of these promoters,

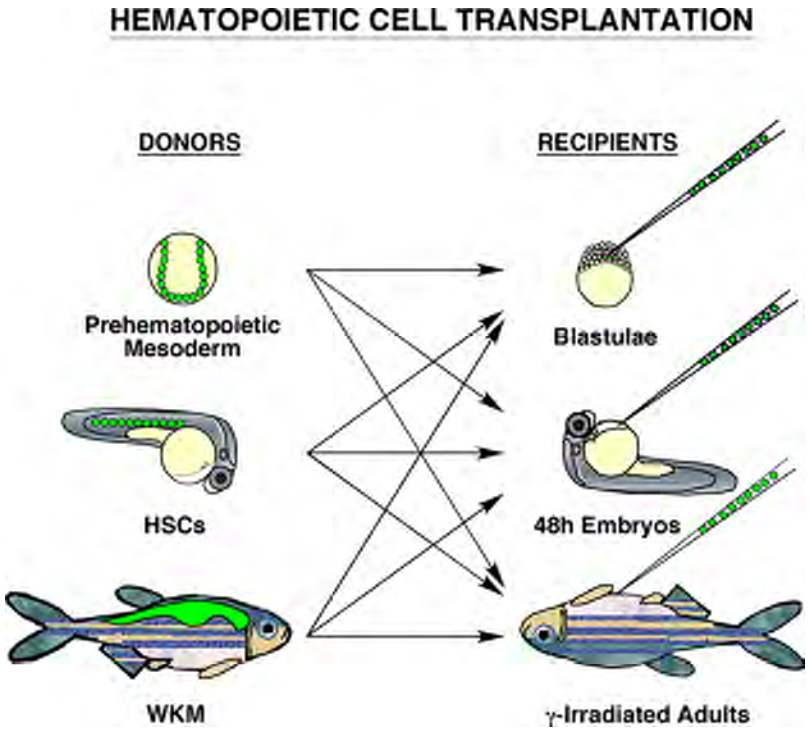

Fig. 6 Methods of hematopoietic cell transplantation in the zebrafish. See the text for experimental details.

blood cell precursors can be isolated by flow cytometry from embryonic and larval animals for transplantation into wild-type recipients. For example, GFP^+ cells in *lmo2:EGFP* embryos can be visualized by FACS by 8–10 somites (Traver, 2004). These cells can be sorted to purity and tested for functional potential in a variety of transplantation (Fig. 6) or *in vitro* culture assays (see Fig. 2).

We have used two types of heterochronic transplantation strategies to address two fundamental questions in developmental hematopoiesis. The first is whether cells that express Lmo2 at 8–12 somites have hemangioblastic potential, i.e., can generate both blood and vascular cells. We reasoned that purified cells should be placed into a relatively naive environment to provide the most permissive conditions to assess their full fate potentials. Therefore, we attempted transplantation into 1000 cell stage blastulae recipients. Transplanted cells appear to survive this procedure well and GFP^+ cells could be found over several days later in developing embryos and larvae. By isolating GFP^+ cells from *lmo2:EGFP* animals also carrying a *gata1a:DsRed* transgene, both donor-derived endothelial and erythroid cells can be independently visualized in green and red, respectively. Using this approach we have shown that $Lmo2^+$ cells from 8 to 12 ss embryos can generate robust regions of donor

endothelium and intermediate levels of circulating erythrocytes (D. Traver, C. E. Burns, H. Zhu, and L. I. Zon, unpublished results). We are currently generating additional transgenic lines that express DsRed or mCherry under ubiquitous promoters to test the full fate potentials of $Lmo2^+$ cells upon transplantation. Additionally, although these studies demonstrate that $Lmo2^+$ cells can generate at least blood and endothelial cells at the population level, single-cell fate-mapping studies need to be performed to assess whether clonogenic hemangioblasts can be identified *in vivo*.

The second question addressed through transplantation is whether the earliest identifiable primitive blood precursors can generate the definitive hematopoietic cells that arise later in embryogenesis. It has been previously reported that the embryonic lethal *vlad tepes* mutant dies from erythropoietic failure due to a defect in the *gata1a* gene (Lyons *et al*., 2002). This lethality can be rescued by transplantation of WKM from wild-type adults into mutant recipients at 48 hpf (Traver *et al*., 2003b). We therefore tested whether cells isolated from 8 to 12ss *lmo2:EGFP* embryos could give rise to definitive cell types and rescue embryonic lethality in *vlad tepes* recipients. Following transplantation of GFP^+ cells at 48 hpf, approximately half of the cells in circulation were GFP^+ and the other half were $DsRed^+$ 1 day posttransplantation. Three days later, analyses of the same animals showed that the vast majority of cells in circulation were $DsRed^+$, apparently due to the differentiation of $Lmo2^+$ precursors to the erythroid fates. Compared to untransplanted control animals which all died by 12 days postfertilization (dpf), some mutant recipients survived for 1–2 months following transplantation. We observed no proliferation of donor cells at any time point following transplantation, however, and survivors analyzed over 1 month post-transplantation showed no remaining cells in circulation (D. Traver, C. E. Burns, H. Zhu, and L. I. Zon, unpublished results). Therefore, these data indicate that mutant survivors were only transiently rescued by short-lived, donor-derived erythrocytes. Thus, within the context of this transplantation setting, it does not appear that Lmo2 + hematopoietic precursors can seed definitive hematopoietic organs to give rise to enduring repopulation of the host blood forming system.

1. Protocol for Isolating Hematopoietic Cells from Embryos

This simple physical dissociation procedure is effective in producing single cell suspensions from early embryos (8–12 ss) as well as from embryos as late as 48 hpf.

1. Stage and collect embryos. We estimate that approximately 200 cells can be isolated per 10–12 ss *lmo2:EGFP* embryo. It is recommended that as many embryos are collected as possible since subsequent transplantation efficiency depends largely upon cell concentration. At least 500–1000 embryos are recommended.
2. Transfer embryos into 1.5 ml eppendorf centrifuge tubes. Add embryos until they sediment to the 0.5 ml mark. Remove embryo medium since it is not optimal for cellular viability.

3. Wash 2X with 0.9X Dulbecco's PBS (Gibco; 500 ml 1X Dulbecco's PBS + 55 ml ddH_2O).
4. Remove 0.9X PBS and add 750 µl ice-cold staining medium (SM; 0.9X Dulbecco's PBS + 5% FCS). Keep cells on ice from this point onward.
5. Homogenize with blue plastic pestle and pipette a few times with a p1000 tip.
6. Strain resulting cellular slurry through a 40 µm nylon cell strainer (Falcon 2340) atop a 50 ml conical tube. Rinse with additional SM to flush cells through the filter.
7. Gently mash remaining debris atop strainer with a plunger removed from a 28-gauge syringe.
8. Rinse with more SM until the conical tube is filled to the 25 ml mark (this helps to remove the yolk).
9. Centrifuge for 5 min @ 200*g* at 4 °C. Remove supernatant until 1–2 ml remain.
10. Add 2–3 ml SM; resuspend by pipetting.
11. Strain again through 40 µm nylon mesh into a 5 ml Falcon 2054 tube. It is important to filter the cell suspension at least twice before running the sample by FACS. Embryonic cells are sticky and will clog the nozzle if clumps are not properly removed beforehand.
12. Centrifuge again for 5 min @ 200*g* at 4 °C. Repeat steps 10–12 if necessary.
13. Remove supernatant and resuspend with 1–2 ml SM depending upon number of embryos used.
14. Propidium iodide (PI) may be added at this point to 1 µg/ml to exclude dead cells and debris on the flow cytometer. When using, however, bring samples having PI only and GFP only to set compensations properly. Otherwise, the signal from PI may bleed into the GFP channel resulting in false positives. Alternatively, add 1:1000 Sytox Red (excited by 633 nm laser), or Sytox Blue (excited by the 405 nm laser) for dead cell discrimination, as they have no spectral overlap with GFP.

Embryonic cells are now ready for analysis or sorting by flow cytometry. It is often difficult to visualize GFP^+ cells when the expression is low or the target population is rare, so one should always prepare age-matched GFP-negative embryos in parallel with transgenic embryos. It is then apparent where the sorting gates should be drawn to sort *bona fide* GFP^+ cells. If highly purified cells are desired, one must perform two successive rounds of sorting. In general, sorting GFP^+ cells once yields populations of approximately 50–70% purity. Two rounds of cell sorting generally yields >90% purity as observed with 10ss *lmo2:EGFP* cells (Traver, 2004). Cells should be kept ice-cold during the sorting procedure.

2. Transplanting Purified Cells into Embryonic Recipients

After sorting, centrifuge cells for 5 min at 200*g* and 4 °C. Carefully remove all supernatant. Resuspend cell pellet in 5–10 µl of ice-cold SM containing 3U heparin and 1U DnaseI to prevent coagulation and lessen aggregation. Preventing the cells

from aggregating or adhering to the glass capillary needle used for transplantation is critical. Mix the cells by gently pipetting with a 10 μl pipette tip. Keep on ice. For transplantation, we use the same needle-pulling parameters used to make needles for nucleic acid injections, the only difference being the use of filament-free capillaries to maintain cell viability. We also use the standard air-powered injection stations used for nucleic acid injections.

3. Transplanting Cells into Blastula Recipients

1. Stage embryonic recipients to reach the 500–1000 cell stage at the time of transplantation.
2. Prepare plates for transplantation by pouring a thin layer of 2% agarose made in E3 embryo medium into a 6 cm Petri dish. Drop transplantation mold [similar to the embryo injection mold described in Chapter 5 of The Zebrafish Book (Westerfield, 2000) but having individual depressions rather than troughs] atop molten agarose and let solidify.
3. Dechorionate blastulae in 1–2% agarose-coated Petri dishes by light pronase treatment or manually with watchmaker's forceps.
4. Transfer individual blastulae into individual wells of transplantation plate that has been immersed in 1X HBSS (Gibco). Position the animal pole upward.
5. Using glass, filament-free, fine-pulled capillary needles (1.0 mm OD) backload 3–6 μl of cell suspension after breaking needle on a bevel to an opening of ~20 μm. Load into needle holder and force cells to injection end by positive pressure using a pressurized air injection station.
6. Gently insert needle into the center of the embryo and expel cells using either very gentle pressure bursts or slight positive pressure. Transplanting cells near the marginal zone of the blastula leads to higher blood cell yields since embryonic fate maps show blood cells to derive from this region in later gastrula stage embryos.
7. Carefully transfer embryos to agarose-coated Petri dishes using glass transfer pipettes.
8. Place into E3 embryo medium and incubate at 28.5 °C. Many embryos will not survive the transplantation procedure, so clean periodically to prevent microbial outgrowth.
9. Monitor by fluorescence microscopy for donor cell types.

4. Transplanting Cells into 48 hpf Embryos

1. All procedures are performed as above except that dechorionated 48 hpf embryos are staged and used as transplant recipients.
2. Fill transplantation plate with 1X HBSS containing 1X penicillin/streptomycin and 1X buffered tricaine, pH 7.0. Do not use E3, as it is suboptimal for cellular viability. Anesthetize recipients in tricaine and then array individual embryos into

individual wells of transplantation plate. Position head at bottom of well, yolk side up.

3. Load cells as above. Insert injection needle into the sinus venosus/duct of Cuvier and gently expel cells by positive pressure or gentle pressure bursts. Take care not to rupture the YS membrane. A very limited volume can be injected into each recipient. It is thus important to use very concentrated cell suspensions in order to reconstitute the host blood-forming system. If using WKM as donor cells, concentrations of 5×10^5 cells/μl can be achieved if care is taken to filter and anticoagulate the sample.
4. Allow animals to recover at 28.5 °C in E3. Keep clean and visualize daily by microscopy for the presence of donor-derived cells.

B. Adult Donor Cells

Whereas the first HSCs transdifferentiate from embryonic aortic endothelium, multilineage hematopoiesis is not fully apparent until the kidney becomes the site of blood cell production. The kidney appears to be the only site of adult hematopoiesis, and we have previously demonstrated that it contains HSCs capable of the long-term repopulation of embryonic (Traver *et al*., 2003b) and adult (Langenau *et al*., 2004) recipients. For HSC-enrichment strategies, both high-dose transplants and limiting dilution assays are required to gauge the purity of input cell populations. In embryonic recipients, we estimate that the maximum number of cells that can be transplanted is approximately 5×10^3, and the precise quantitation of transplanted cell numbers is difficult. To circumvent both issues, we have developed HCT into adult recipients.

For transplantation into adult recipients, myeloablation is necessary for successful engraftment of donor cells. We have found γ-irradiation to be the most consistent way to deplete zebrafish hematopoietic cells. The minimum lethal dose (MLD) of 40 Gy specifically ablates cells of the blood-forming system and can be rescued by transplantation of one kidney equivalent (10^6 WKM cells). Thirty-day survival of transplanted recipients is approximately 75% (Traver *et al*., 2004). An irradiation dose of 20–25 Gy is sublethal, and the vast majority of animals survive this treatment despite having nearly total depletion of all leukocyte subsets 1 week following irradiation (Traver *et al*., 2004). We have shown that this dose is necessary and sufficient for transfer of a lethal T cell leukemia (Traver *et al*., 2004), and for long-term (>6 month) engraftment of thymus repopulating cells (Langenau *et al*., 2004). We do not yet know the average relative chimerism of donor to host cells when transplantation is performed following 20–25 Gy. That this dose is sufficient for robust engraftment, for long-term repopulation, and yields extremely high survival suggests that 20–25 Gy may be the optimal dose for myeloablative conditioning prior to transplantation. Improvement in short-term engraftment and long-term survival of transplant recipients will also likely require matching of MHC loci between donor and host genotypes.

1. Protocols for Isolating Hematopoietic Cells from Adult Zebrafish

Anesthetize adult animals in 0.02% tricaine in fish water.

For blood collection, dry animal briefly on tissue then place on a flat surface with head to the left, dorsal side up. Coat a 10 μl pipette tip with heparin (3 U/μl) then insert tip just behind the pectoral fin and puncture the skin. Direct the tip into the heart cavity, puncture the heart, and aspirate up to 10 μl blood by gentle suction. Immediately perform blood smears or place into 0.9X PBS containing 5% FCS and 1 U/μl heparin. Mix immediately to prevent clotting. Blood from several animals may be pooled in this manner for later use by flow cytometry. Red cells may be removed using a red blood cell hypotonic lysis solution (Sigma; 8.3 g/l ammonium chloride in 0.01 M Tris–HCl, pH 7.5) on ice for 5 min. Add 10 volumes of ice-cold SM then centrifuge at 200*g* for 5 min at 4 °C. Resuspended blood leukocytes can then be analyzed by flow cytometry or cytocentrifuge preparations.

For collection of other hematopoietic tissues, place fish on ice for several minutes following tricaine. Make a ventral, midline incision using fine scissors under a dissection microscope.

For spleen collection, locate spleen just dorsal to the major intestinal loops and tease out with watchmaker's forceps. Place into ice cold SM. Dissect any nonsplenic tissue away and place on a 40 μm nylon cell strainer (Falcon 2340) atop a 50 ml conical tube. Gently mash the spleen using a plunger removed from a 28-gauge insulin syringe and rinse with SM to flush cells through the filter. Up to 10 spleens can be processed through each filter. Centrifuge at 200*g* for 5 min at 4 °C. Filter again through 40 μm nylon mesh if using for FACS.

For kidney collection, remove all internal organs using forceps and a dissection microscope. Take care during dissection because ruptured intestines or gonads will contaminate the kidney preparation. Using watchmaker's forceps, tease the entire kidney away from the body wall starting at the head kidney and working toward the rear. Place into ice-cold SM. Aspirate vigorously with a 1 ml pipetteman to separate hematopoietic cells (WKM) from renal cells. Filter through 40 μm nylon mesh, wash, centrifuge, and repeat. Perform last filtration step into a Falcon 2054 tube if using for FACS. It is important to filter the WKM cell suspension at least twice before running the sample. PI may be added at this point to 1 μg/ml to exclude dead cells and debris on the flow cytometer. When using, however, compare to samples having PI only and GFP only (if using) to set compensations properly. Otherwise, the signal from PI may bleed into the GFP channel resulting in false positives. Alternatively, add 1:1000 Sytox Red (excited by the 633 nm laser) or Sytox Blue (excited by the 405 nm laser) for dead cell discrimination, as they have no spectral overlap with GFP.

2. Transplanting Whole Kidney Marrow

After filtering and washing the WKM suspension three times, centrifuge cells for 5 min at 200*g* and 4 °C. Carefully remove all supernatant. Resuspend cell pellet in 5–10 μl of ice-cold SM containing 3U heparin and 1U DnaseI to prevent coagulation

and lessen aggregation. Preventing the cells from aggregating or adhering to the glass capillary needle used for transplantation is critical. Mix the cells by gently pipetting with a 10 μl pipette tip. Keep on ice. For blastulae and embryo transplantation, perform following previous protocols. Between 5×10^2 and 5×10^3 cells can be injected into each 48 hpf embryo if the final cell concentration is approximately 5×10^5 cells/μl.

3. Transplanting Cells into Irradiated Adult Recipients

For irradiation of adult zebrafish, we have used a ^{137}Cesium source irradiator typically used for the irradiation of cultured cells (Gammacell 1000). We lightly anaesthetize five animals at a time and then irradiate in sealed Petri dishes filled with fish water (without tricaine). We performed careful calibration of the irradiator using calibration microchips to obtain the dose rate at the height within the irradiation chamber nearest to the ^{137}Cesium point source. We found the dose rate to be uniform among calibration chips placed within euthanized animals in Petri dishes under water, under water alone, or in air alone, verifying that the tissue dosage via total body irradiation (TBI) was accurate.

Transplantation into circulation is most efficiently performed by injecting cells directly into the heart. We perform intracardiac transplantation using pulled filament-free capillary needles as above, but we break the needles at a larger bore size of approximately 50 μm. The needle assembly can be handheld and used with a standard gas-powered microinjection station. We have also had limited success transplanting cells intraperitoneally using a 10 μl Hamilton syringe. Engraftment efficiency for WKM is only marginal using this method, but transplantation of T cell leukemia or solid tumor suspensions is highly efficient following irradiation at 20 Gy (Traver *et al*., 2004).

4. Irradiation

1. Briefly anaesthetize adult zebrafish in 0.02% tricaine in fish water.
2. Place five fish at a time into 60 mm × 15 mm Petri dishes (Falcon) containing fish water. Wrap dish with Parafilm and irradiate for length of time necessary to achieve desired dose.
3. Return irradiated animals to clean tanks containing fish water. We have successfully transplanted irradiated animals from 12 to 72 h following irradiation. Using a 20 Gy dose, the nadir of host hematopoietic cell numbers occurs at approximately 72 h postirradiation.

5. Transplantation

1. Prepare cells to be transplanted as above, taking care to remove particulates/contaminants by multiple filtration and washes. When using WKM as donor cells, we typically make final cell suspensions at 2×10^5 cells/μl. Keep cells on ice.

2. Anaesthetize an irradiated animal in 0.02% tricaine in fish water.
3. Transfer ventral side up into a well cut into a sponge wetted with fish water. Under a dissection microscope, remove scales covering the pericardial region with fine forceps.
4. Fill injection needle with ~20 μl of cell suspension. Force cells to end of needle with positive pressure and adjust pressure balance to be neutral. Hold needle assembly in one hand while placing gentle pressure on the abdomen of the recipient with the index finger of the other hand. This will position the heart adjacent to the skin and allow visualization of the heartbeat. Insert needle through the skin and into the heart. If the needle is positioned within the heart, and the pressure balance is neutral, blood from the heart will enter the needle and the meniscus will rise and fall with the heartbeat. Inject approximately 5–10 μl by gentle pressure bursts.
5. Return recipient to fresh fish water. Repeat for each additional recipient. Do not feed until the next day to lessen chance of infection.

IV. Enrichment of Hematopoietic Stem Cells

The development of many different transplantation techniques now permits the testing of cell autonomy of mutant gene function, oncogenic transformation, and stem cell enrichment strategies in the zebrafish. For HSC enrichment strategies, fractionation techniques can be used to divide WKM into distinct subsets for functional testing via transplantation. The most successful means of HSC enrichment in the mouse has resulted from the subfractionation of whole bone marrow cells with monoclonal antibodies (mAbs) and flow cytometry (Spangrude *et al*., 1988). We have attempted to generate mAbs against zebrafish leukocytes by repeated mouse immunizations using both live WKM and purified membrane fractions followed by standard fusion techniques. Many resulting hybridoma supernatants showed affinity to zebrafish WKM cells in FACS analyses (Fig. 7). All antibodies showed one of two patterns, however. The first showed binding to all WKM cells at similar levels. The second showed binding to all kidney leukocyte subsets but not to kidney erythrocytes, similar to the pattern shown in the left panel of Fig. 7A. We found no mAbs that specifically bound only to myeloid cells, lymphoid cells, etc when analyzing positive cells by their scatter profiles. We reasoned that these nonspecific binding affinities might be due to different oligosaccharide groups present on zebrafish blood cells. If the glycosylation of zebrafish membrane proteins were different from the mouse, then the murine immune system would likely mount an immune response against these epitopes. To test this hypothesis, we removed both O-linked and N-linked sugars from WKM using a deglycosylation kit (Prozyme), and then incubated these cells with previously positive mAbs. All mAbs tested in this way showed a time-dependent decrease in binding, with nearly all binding disappearing following 2 h of deglycosylation (Fig. 7A). It thus appears that standard immunization approaches using zebrafish WKM cells elicit a strong immune response against

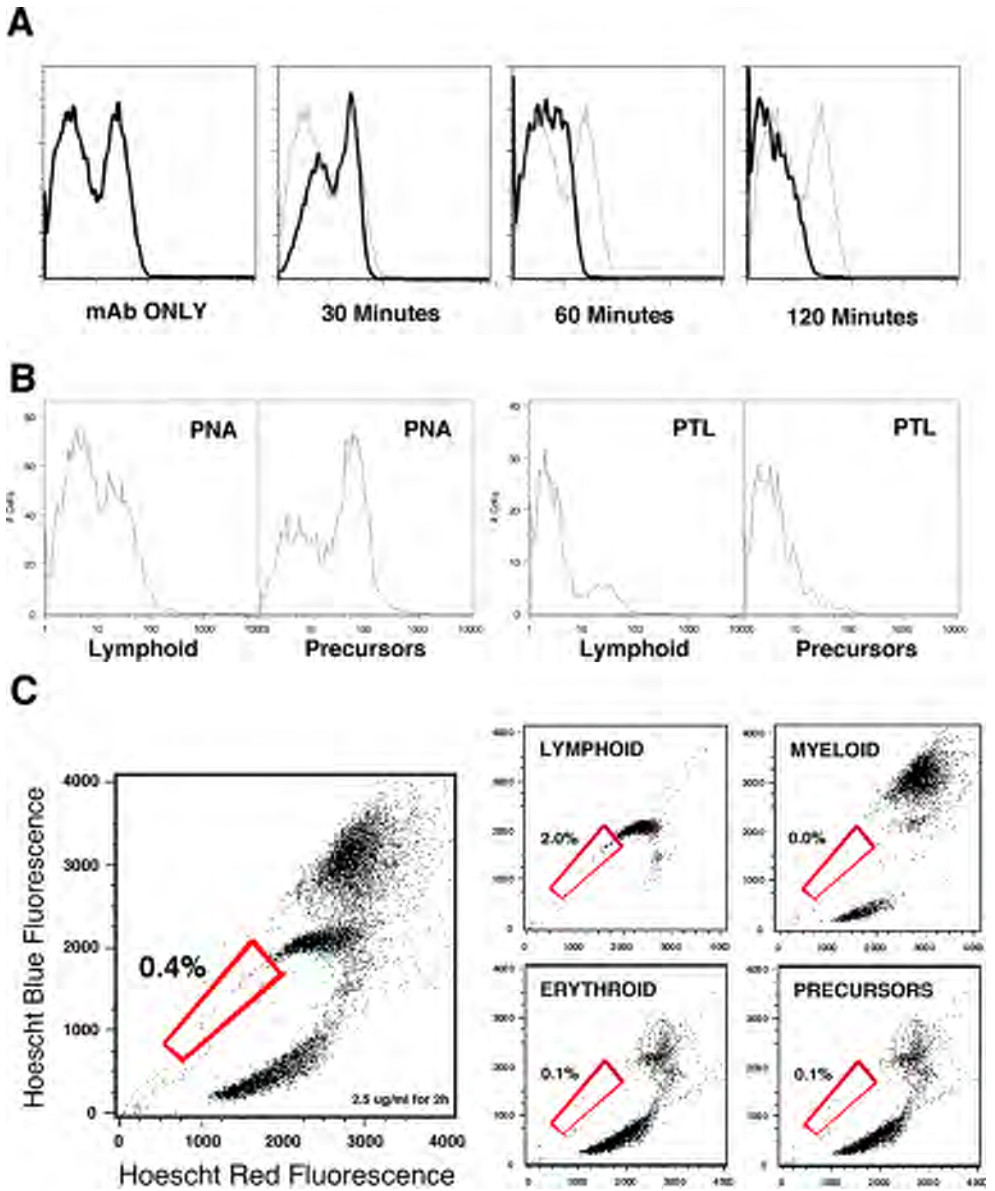

Fig. 7 Potential methods of stem cell enrichment. (A) Mouse monoclonal antibodies generated against zebrafish WKM cells react against oligosaccharide epitopes. De-glycosylation enzymes result in time-dependent loss of antibody binding (bold histograms) compared to no enzyme control (left panel and grey histograms). (B) Differential binding of lectins to WKM scatter fractions. Peanut agglutinin splits both the lymphoid and precursor fraction into positive and negative populations (left panels). Potato lectin shows a minor positive fraction only within the lymphoid fraction (right panels). (C) Hoechst 33342 dye reveals a side population (SP) within WKM. 0.4% of WKM cells appear within the verapamil-sensitive SP gate (left panel). Only the lymphoid fraction, where kidney HSCs reside, contains appreciable numbers of SP cells (right panels).

oligosaccharide epitopes. This response is likely to be extremely robust, because we did not recover any mAbs that reacted with specific blood cell lineages. Similar approaches by other investigators using blood cells from frogs or other teleost species have yielded similar results (L. du Pasquier, M. Flajnik, personal communications). In an attempt to circumvent the glycoprotein issue, new series of immunizations using deglycosylated kidney cell membrane preparations may be effective.

Previous studies have shown that specific lectins can be used to enrich hematopoietic stem and progenitor cell subsets in the mouse (Huang and Auerbach, 1993; Lu *et al*., 1996; Visser *et al*., 1984). In preliminary studies, we have shown that FITC-labeled lectins such as peanut agglutinin (PNA) and potato lectin (PTL) differentially bind to zebrafish kidney subsets. As shown in Fig. 7B, PNA binds to a subset of cells both within the lymphoid and precursor kidney scatter fractions. Staining with PTL also shows that a minor fraction of lymphoid cells binds PTL, whereas the precursor (and other) scatter fractions are largely negative (Fig. 7B). We are currently testing both positive and negative fractions in transplantation assays to determine whether these different binding affinities can be used to enrich HSCs.

We have previously demonstrated that long-term HSCs reside in the adult kidney (Traver *et al*., 2003b). We therefore isolated each of the kidney scatter fractions from *gata1a:EGFP* transgenic animals and transplanted cells from each into 48 hpf recipients to determine which subset contains HSC activity. The only population that could generate GFP^+ cells for over 3 weeks in wild type recipients was the lymphoid fraction. This finding is in accord with mouse and human studies that have shown purified HSCs to have the size and morphological characteristics of inactive lymphocytes (Morrison *et al*., 1995).

Another method that has been extremely useful in isolating stem cells from whole bone marrow is differential dye efflux. Dyes such as rhodamine 123 (Mulder and Visser, 1987; Visser and de Vries, 1988) or Hoechst 33342 (Goodell *et al*., 1996) allow the visualization and purification of a "side population" (SP) that is highly enriched for HSCs. This technique appears to take advantage of the relatively high activity of multidrug resistance transporter proteins in HSCs that actively pump each dye out of the cell in a verapamil-sensitive manner (Goodell *et al*., 1996). Other cell types lack this activity and become positively stained, allowing isolation of the negative SP fraction by FACS. Our preliminary studies of SP cells in the zebrafish kidney demonstrated a typical SP profile when stained with 2.5 μg/ml of Hoechst 33342 for 2 h at 28 °C (Fig. 7C). This population disappears when verapamil is added to the incubation. Interestingly, the vast majority of SP cells appear within the lymphoid scatter fraction (Fig. 7C). Further examination of whether this population is enriched for HSC activity in transplantation assays is warranted.

Finally, there are many other methods to enrich hematopoietic stem and progenitor cells from WKM including sublethal irradiation, cytoreductive drug treatment, and use of transgenic lines expressing fluorescent reporter genes (see Table I). We have shown following 20 Gy doses of γ-irradiation that nearly all hematopoietic lineages are depleted within 1 week (Traver *et al*., 2004). Examination of kidney cytocentrifuge preparations at this time shows that the vast majority of cells are immature

precursors. That this dose does not lead to death of the animals demonstrates that HSCs are spared and are likely highly enriched 5–8 days following exposure. We have also shown that cytoreductive drugs such as cytoxan and 5-fluorouracil have similar effects on kidney cell depletion, although the effects were more variable than those achieved with sublethal irradiation (A. Winzeler, D. Traver, and L. I. Zon unpublished). Because HSCs are contained within the kidney lymphoid fraction, they can be further enriched by HSC-specific or lymphocyte-specific transgenic markers. Possible examples of transgenic promoters are *lmo2*, *gata2a*, or *cmyb* to mark HSCs and *ccr9a*, *il7r*, *rag2*, *lck*, or B-cell receptor genes to exclude lymphocytes from this subset (see Table I).

V. *In vitro* Culture and Differentiation of Hematopoietic Progenitors

Hematopoiesis is one of the best-studied models of developmental differentiation because of the multitude of experimental methods developed over the past 60 years to assess the proliferation, differentiation, and maintenance of its cellular constituents. Stem and progenitor cell transplantation into lethally irradiated animal recipients (Ford *et al*., 1956; McCulloch and Till, 1960) were the first *in vivo* assays to be developed, followed shortly thereafter by the clonal growth of bone marrow progenitors *in vitro* (Bradley and Metcalf, 1966). Although these techniques have been substantially refined over the past decades, they still remain the foundation for analyzing the hierarchical organization of vertebrate hematopoietic stem and progenitor cells.

In vitro cultures to assess hematopoietic stem and progenitor cell biology generally fall into two categories: growth of progenitor cells on a supportive stromal cell layer, and clonal growth of cells in a semisolid medium with the addition of supplemental cytokines or growth factors. Most stromal culture assays largely derive from the modification and refinement of Dexter cultures (Dexter *et al*., 1977a, 1977b), whereby stromal cells from hematopoietic organs support the differentiation of HSCs and their downstream progenitors. These early studies were instrumental for the development of cobblestone-area-forming-cell (CAFC) (Ploemacher *et al*., 1991) and long-term culture initiating cell (LTC-IC) assays, which have been utilized to examine murine (Lemieux *et al*., 1995) and human (Sutherland *et al*., 1991) multilineage hematopoietic differentiation. The development of stromal cells from the calvaria of macrophage colony stimulating factor (M-CSF)-deficient mice (Nakano *et al*., 1994, 1996) were instrumental for examining hematopoietic differentiation of embryonic stem (ES) cells down the hematopoietic pathway, and for differentiation of hematopoietic precursors into multiple mature blood cell types. With refinement, these OP9 cells have proven to be an efficient tool to study T-cell lineage commitment and development (Schmitt *et al*., 2004; Schmitt and Zuniga-Pflucker, 2002), once an extremely difficult process to study.

To assess the progenitor capacity of normal and mutant zebrafish hematopoietic cells functionally, we created primary zebrafish kidney stromal (ZKS) cells derived

from the main site of hematopoiesis in the adult fish. Culture of hematopoietic progenitor cells on these stromal cells resulted in their continued maintenance (Stachura *et al*., 2009) and differentiation (Bertrand *et al*., 2007, 2010b; Stachura *et al*., 2009). It also allowed investigation and rescue of a genetic block in erythroid maturation, confirming the utility of these assays (Stachura *et al*., 2009). Finally, the ZKS culture system has been utilized to investigate the molecular events underlying the progression of T-lymphoblastic lymphoma (T-LBL) to acute T-lymphoblastic leukemia (T-ALL) (Feng *et al*., 2010).

A. Stromal Cell Culture AssaysFS

To create a suitable *in vitro* environment for the culture of zebrafish hematopoietic cells, we isolated the stromal fraction of the zebrafish kidney, the main site of hematopoiesis in the adult fish (Zapata, 1979). The benefit of utilizing hematopoietic stromal layers is two-fold. First, performing culture assays in the zebrafish has been hampered by a paucity of defined and purified hematopoietic cytokines. Most zebrafish cytokines have poor sequence homology to their mammalian counterparts, and as a consequence, have not been well described, characterized, or rigorously tested. Secondly, some hematopoietic cell types, especially T cells, require physical cell-cell interaction for their differentiation.

1. Generation of ZKS Cells

To create ZKS cells, kidney was isolated from AB* wild-type fish as described above (also see Stachura *et al*., 2009). The kidney tissue was sterilized by washing for 5 min in 0.000525% sodium hypochlorite (Fisher Scientific), then rinsed in sterile 0.9X Dulbecco's PBS. Tissue was then mechanically dissociated by trituration and filtered through a 40 μm filter (BD Biosciences). Flow-through cells (WKM) were discarded, and the remaining kidney tissue was cultured in vacuum plasma-treated vented flasks (Corning Incorporated Life Sciences) at 32 °C and 5% CO_2.

2. Maintenance and Culture of ZKS Cells

ZKS cells are maintained in the following tissue culture medium:

500 ml	L-15
350 ml	DMEM (high glucose)
150 ml	Ham's F-12
150 mg	Sodium bicarbonate
15 ml	HEPES (1 M stock)
20 ml	Penicillin/streptomycin (5000 U/ml penicillin, 5000 μg/ml streptomycin stock)
10 ml	L-glutamine (200 mM stock)
100 ml	Fetal bovine serum (FBS)
2 ml	Gentamicin sulfate (50 mg/ml stock)

Medium is made by first adding sodium bicarbonate to the mixture of L-15, DMEM, and Ham's F-12. Warm the medium to 37 °C, and allow the sodium bicarbonate to dissolve. Then, add other medium components and filter-sterilize with 0.22 μm vacuum apparatus.

All medium components are available from Mediatech. We utilize FBS from the American Type Culture Collection, but one can use FBS from other sources. It is important to note, however, that different manufacturing lots of FBS investigated in the laboratory differ wildly in their support of hematopoietic progenitor differentiation and proliferation. Once a manufacturing lot is tested and shown to be supportive, we recommend buying a large quantity to minimize experimental variation.

ZKS cells are maintained at 32 °C and 5% CO_2 in a humidified incubator. Cells are grown in vacuum plasma treated 75 cm^2 vented flasks (T-75; Corning Incorporated Life Sciences) in 10 ml of medium until 60–80% confluent before passaging. Medium is then removed, and 2 ml trypsin–EDTA (0.25%; Invitrogen) is added to cover the stromal cells. Allow cells to incubate for 5 min at 32 °C. Add 8 ml medium to cells to stop trypsinization, pipetting up and down to achieve a single cell suspension. Spin cells for 5 min at 300*g*, aspirate supernatant and resuspend pellet gently in 10 ml of medium. Take 1 ml of the cell solution, add 9 ml of medium, and move to a new flask. Cells should not be split more than 1:10 to passage, as they are somewhat density-dependent.

ZKS cells may be frozen and thawed at a later time. Even though we have never experienced senescence or a decrease in hematopoietic differentiation capacity of ZKS cells in culture, it is useful to perform critical experiments with similar passages of cells to avoid experimental variation. To freeze ZKS cells, first trypsinize a T-75 flask. Spin cells for 5 min at 300*g*, aspirate supernatant, and resuspend pellet gently in 2 ml of medium. Prepare freezing medium (500 μl of tissue culture-certified DMSO and 1.5 ml of FBS) and aliquot 500 μl into four cryopreservation tubes keeping everything on ice. Add 500 μl of cells to each tube, invert to gently mix, and place tubes into isopropanol-jacketed freezing chamber. Place freezing chamber at –80 °C for 24 h. Remove tubes from freezing chamber and place into liquid nitrogen for long-term storage.

To thaw ZKS cells at a later date, remove tube from nitrogen, and quickly warm in 37 °C water bath. Wear eye protection; if nitrogen seeped into the freezing tubes, the rapid warming may cause the tube to violently rupture. Remove liquid from tube, add slowly to 10 ml of medium in a 15 ml conical tube, and spin at 300*g* for 5 min. Carefully aspirate all of the medium to remove all traces of DMSO. Resuspend cells in 10 ml of fresh medium and place into T-75 flask. Early the next morning change the medium and determine whether the cells are ready to be passaged or require another day to recover from thawing.

As with all tissue culture, strict attention to sterility and cleanliness should be adhered to at all times. All procedures should be performed in a tissue culture laminar flow hood, and it is recommended that vented, filtered flasks be utilized to prevent airborne contamination during culture in incubators.

3. Protocols for *in vitro* Proliferation and Differentiation Assays

Purify prospective progenitors by FACS as described above. Plate cells on confluent ZKS at a density of 1×10^4 cells/well in a 12-well tissue culture plate, using 2 ml complete medium per well. Lower density of progenitors is not recommended; if using fewer cells, reduce the size of the tissue culture well and volume of medium. If testing or investigating the effects of growth factors or small molecules, add them to the medium, being sure to have a vehicle only control as well as different concentrations of your experimental factor. 24-well tissue culture plates are extremely useful in this regard, as one can easily plate out a multitude of experimental conditions on one plate.

A. Morphological assessment of hematopoietic cells after *in vitro* culture

   a. Gently aspirate hematopoietic cells from the ZKS cultures, taking care not to disturb the stromal underlayer.
   b. Cytocentrifuge up to 200 μl of the hematopoietic cells at 250*g* for 5 min onto glass slides using a Shandon Cytospin 4 (Thermo Fischer Scientific). It is possible to concentrate the cells before cytocentrifugation at 300*g* for 10 min. Cytocentrifugation of over 200 μl of cell suspensions is not recommended.
   c. Perform May-Grünwald/Giemsa staining by allowing slides to air-dry briefly. Then, submerge slide in May-Grünwald staining solution (Sigma Aldrich) for 10 min. Transfer slide to 1:5 dilution of Giemsa stain (Sigma Aldrich) in dH_2O for an additional 20 min. Rinse slide in dH_2O, and allow to air dry. Coverslip slide with cytoseal XYL mounting medium (Richard-Allan Scientific) and Corning no.1 18 mm square cover glass (Corning). Allow slides to completely dry before visualization on upright microscope, especially if using an oil-immersion lens.

B. Proliferation assessment of hematopoietic cells after *in vitro* culture

   a. Gently aspirate hematopoietic cells as above.
   b. Count cells with use of a bright line hemacytometer (Hausser Scientific) using trypan blue dye (Invitrogen) exclusion to assess viability.

C. RT-PCR analysis of hematopoietic cells after *in vitro* culture

   a. Gently aspirate hematopoietic cells as above.
   b. Isolate RNA from hematopoietic cells using either Trizol (Invitrogen) or RNAeasy kit (Qiagen).
   c. Generate cDNA with oligo dT primers and superscript RT-PCR kit (Invitrogen).
   d. Perform PCR with the desired zebrafish DNA primers.

D. Cell labeling and cell division determination of hematopoietic cells after *in vitro* culture

   a. Prior to plating cells on ZKS monolayer, wash cells twice with 0.1% bovine serum albumin (BSA) to remove FBS from the medium.

b. Resuspend cells in 0.1% BSA and 2 μl/ml of 5 mM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) at room temperature for 10 min, in the dark.
c. Wash cells with complete medium supplanted with an additional 10% FBS twice.
d. Save 1/10 of the culture and perform FACS (Day 0 time point). Culture remaining cells in complete medium as described above.
e. For analysis, remove hematopoietic cells from culture at desired time points as described above and FACS. CFSE is read in the FL-1 channel (on FACS caliber) or with most GFP filters (FACS Aria I and II, LSR-II), and will decrease in fluorescence intensity as cells divide. Compare divisions to Day 0 time point with FloJo software (TreeStar, Ashland, OR, USA). We recommend using the BD LSR-II flow cytometer, as the different scatter profile of mature cells is easily distinguished and directly comparable to profiles shown in Fig. 5.

B. Clonal Methylcellulose-based Assays

Although stromal *in vitro* culture methods have been instrumental for the investigation of hematopoiesis, culturing bulk populations of progenitor cells on stroma cannot distinguish between homogeneous multipotent progenitor populations or heterogeneous lineage-restricted populations without performing limiting dilution assays. The development of clonal *in vitro* cultures by Metcalf and colleagues allowed not only the growth of murine bone marrow progenitors (Bradley and Metcalf, 1966), but also the study and quantitation of progenitor numbers during hematological disease (Bradley *et al*., 1967) and exposure to irradiation (Robinson *et al*., 1967). These assays were utilized to investigate the ontogeny of the developing murine hematopoietic system (Moore and Metcalf, 1970), and refined to study human hematopoietic progenitors dysregulated during leukemogenesis (Moore *et al*., 1973a, 1973b). Importantly, the utilization of clonal assays was instrumental for the identification and validation of CSFs, secreted proteins that stimulate the differentiation of specific hematopoietic lineages. The ability to isolate, recombinantly produce, and test these factors was a huge advance in hematological research, allowing the sensitive analysis of progenitor differentiation, proliferation, and restriction in the murine and human blood system.

This capability to grow progenitors *in vitro* to test their differentiation capacity in an unbiased manner has greatly advanced the current understanding of hematopoietic lineage restriction. The isolation of putative lineage-restricted daughter cells by FACS coupled with *in vitro* clonal analysis was pivotal in identifying multipotent (Akashi *et al*., 2000; Kondo *et al*., 1997), oligopotent (Akashi *et al*., 2000), and monopotent progenitor (Mori *et al*., 2008; Nakorn *et al*., 2003) intermediates downstream of HSCs in the murine system.

While zebrafish likely possess multipotent, oligopotent, and monopotent progenitor cells, their existence has never been proven and remains speculative (see Fig. 4). To investigate whether these cells exist in zebrafish, we developed assays to

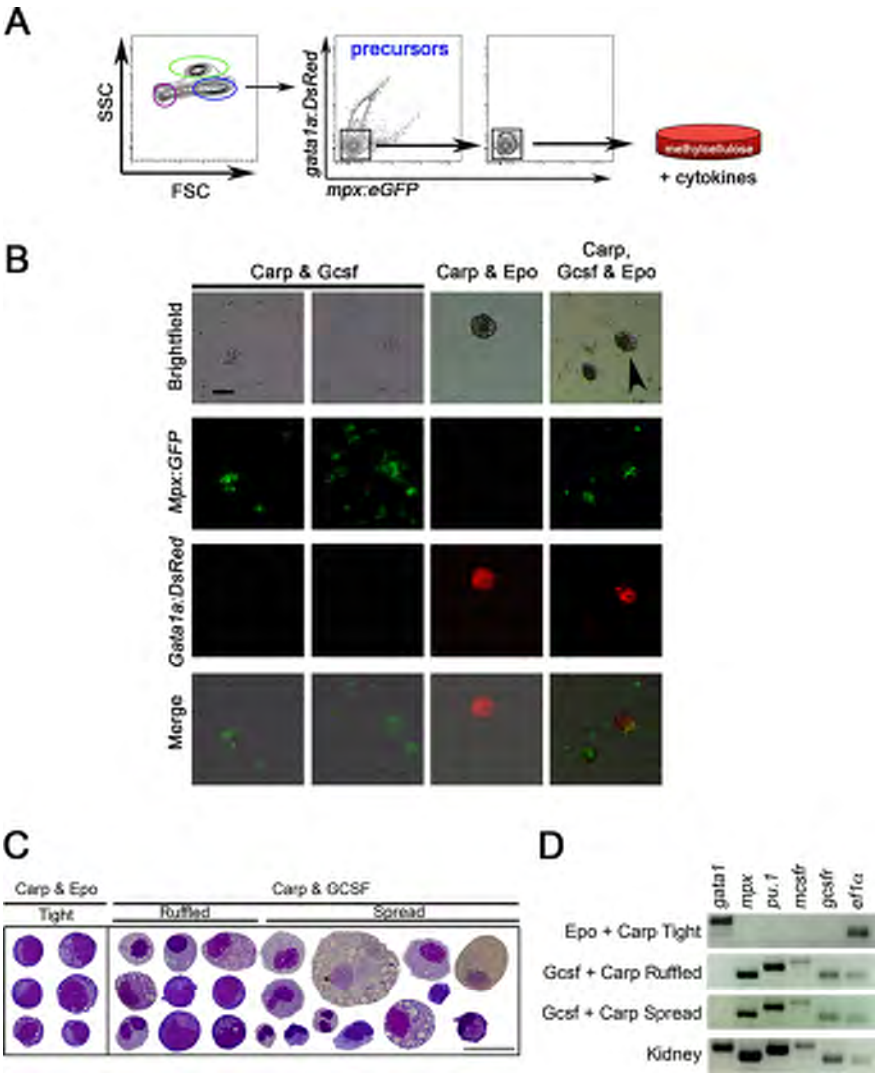

Fig. 8 Recombinantly generated and purified Gcsf and Epo encourage myeloid and erythroid differentiation, respectively, from zebrafish hematopoietic progenitors in clonal methylcellulose assays. (A) Experimental schematic for isolation and culture of *mpx:GFP*$^{-}$, *gata1a:DsRed*$^{-}$ cells from the precursor (blue) fraction of adult WKM. (B) Brightfield images (top row), *mpx:GFP* fluorescence (second row), *gata1a:DsRed* fluorescence (third row), and merged images (bottom row) of colonies grown in various growth factor conditions from the precursor fraction of WKM (conditions listed along top row of images). All images in B taken at 100×. Scale bars in top left panels are 50 μm. Arrowheads in top right panels denote mixed colonies. (D) Colonies isolated from precursor fraction methylcellulose cultures cytospun and stained with May Grünwald/Giemsa. Tight colonies were isolated from cultures with only carp serum and Epo (left column), while ruffled and spread colonies were isolated from cultures containing carp serum and Gcsf (right column). All images were taken at 1000×, and scale bar in bottom right is 20 μm. (E) RT-PCR analysis of colonies isolated from precursor fraction of methylcellulose cultures. Colony morphology is listed on left, and genes assayed are listed along top of gel images. (See Plate no. 8 in the Color Plate Section.)

investigate progenitors in the zebrafish in a clonal manner by modifying existing methylcellulose culture techniques, exogenously adding the recently identified zebrafish recombinant cytokines erythropoietin (Epo) (Paffett-Lugassy *et al*., 2007) and granulocyte Gcsf (Liongue *et al*., 2009) to quantitate the number of myeloid and erythroid progenitors in adult kidney marrow scatter fractions (Fig. 8) (Stachura *et al*., 2011). This level of precision allows the further testing of prospective hematopoietic progenitors in normal and mutant zebrafish, allowing more careful investigation of lineage determination and its conservation among vertebrate animals. In addition, it allows comparison of hematopoietic progenitor cells and their response to cytokines, furthering our understanding of cytokine signaling. Furthermore, the ability to examine blocks in hematopoietic differentiation, aberrant gene expression, and proliferative regulation is now possible in mutant fish already (and currently being) generated. Finally, it also allows the rapid screening of small molecules, blocking antibodies, and other drug compounds that may affect lineage differentiation, maturation, and proliferation.

1. Methylcellulose

To develop a clonal assay to further enumerate and characterize progenitor cells in the zebrafish, we utilized methylcellulose; a semi-solid, viscous cell culture medium used in murine and human progenitor studies. The nature of methylcellulose culture allows individual progenitor cells to develop isolated colonies within the medium, where they can be enumerated after several days in culture. In addition, the use of methylcellulose allows examination of colony morphology and subsequent isolation for further characterization by morphological examination and gene expression.

2. Methylcellulose Stock Preparation

Prepare 2.0% methylcellulose by adding 20 g of methylcellulose powder (Sigma Aldrich) to 450 ml of autoclaved H_2O and boiling for 3 min. Allow mixture to cool to room temperature before adding 2× L-15 medium powder (Mediatech). Then, adjust the weight of the methylcellulose mixture to 1000 g with sterile water. Methylcellulose should be allowed to thicken at 4 °C overnight before being aliquoted and stored at –20 °C.

3. Methylcellulose Clonal Assays

Complete methylcellulose medium:

10 ml	2.0% methylcellulose stock
4.9 ml	DMEM (high glucose)
2.1 ml	Ham's F-12
2 ml	FBS
300 μl	HEPES (1 M stock)
200 μl	Penicillin/streptomycin (5000 U/ml and 5000 μg/ml stock, respectively)
200 μl	L-glutamine (200 mM stock)
40 μl	Gentamicin sulfate (50 mg/ml)

To perform experiments in triplicate, add 3.5 ml of complete methylcellulose to sterile round bottom 14 ml tubes (Becton Dickinson) with 5 ml syringes and 16 gauge needles for each condition. Cells of interest (prospective progenitors) should be isolated, counted, and resuspended in 100 μl of ZKS medium and added to complete methylcellulose, along with cytokines, small molecules, or other agents to be investigated.

For myeloid differentiation, add 1% carp serum and 0.3 μg/ml recombinant zebrafish Gcsf to methylcellulose medium. For erythroid differentiation, add 1% carp serum and 0.1 μg/ml recombinant zebrafish Epo to methylcellulose medium. Cytokines and additives should not total more than 10% of the total volume, as the medium will not be viscous enough to discern individual colonies.

To observe separable, individual colonies, cells should be resuspended at 1×10^4–5×10^4 cells/ml. Tightly cap tubes, and gently vortex solution to mix. In triplicate, aliquot 1 ml of solution into 35 mm Petri dishes (Becton Dickinson). Swirl Petri dishes to distribute the methylcellulose culture evenly, and place plates in a humidified 15 cm dish (made by placing a plate of sterile dH_2O inside the 15 cm dish) at 32 °C and 5% CO_2. Plates should be removed 7 days after plating for microscopic examination, colony isolation, and gene expression analyses.

As with all tissue culture, strict attention to sterility and cleanliness should be adhered to at all times. All procedures should be performed in a tissue culture laminar flow hood.

4. Enumeration of Colony Forming Units (CFUs)

CFUs are a measurement of how many progenitors are present in a given population of cells; if an individual cell has the capability to proliferate and divide into mature blood cells under certain growth conditions, it will make an individual colony. For example, if 100 putative myeloid progenitor cells are plated under conditions suitable for myeloid differentiation and one myeloid colony arises, 1:100 of the cells plated was a myeloid CFU.

To perform enumeration of CFUs, observe and count colonies on an inverted microscope after 7 days in culture. Counting with a 5× or 10× objective (50×–100× magnification) is recommended, with the aperture closed down slightly to grant high contrast, which aids in the visualization of colonies. Be careful not to disturb the dish; even though methylcellulose is viscous, excessive movement of the plates will cause colonies to move, complicating further analysis. Depending on the cytokines, growth factors, and other culture additives, colony shape, size, and color will be different; be sure to note and record all of this information. Inclusion of a transgenic marker will aid in identification of colony type and morphology as shown in Figure 8 B and C, whereby erythroid colonies express DsRed driven by the erythroid-specific *gata1a* promoter, and myeloid colonies express GFP driven by the myeloid-specific *mpx* promoter.

If cultures are to be returned to incubator do not remove Petri plate lids, and take care to wipe down surfaces with 70% ethanol before starting experiment.

5. Picking and Analyzing Colonies from Methylcellulose

Hematopoietic colonies can be carefully plucked from methylcellulose cultures with a pipetteman, preferably a p20 with a fine tip. Pay attention to pick only individual colonies, placing them into 1.5 ml eppendorf tubes with 200 μl of PBS. Pipette up and down gently in the PBS to remove traces of methylcellulose from your tip, and to break up the colony. It is possible to pool colonies of similar morphology for analyses, especially when large cell numbers are required. Colonies may be cytospun and stained with May-Grünwald/Giemsa (Sigma Aldrich) as described above. In addition, colonies may be subjected to RT-PCR analysis for mature lineage gene transcripts as described above.

VI. Conclusions

Over the past decade, the zebrafish has rapidly become a powerful model system to elucidate the molecular mechanisms of vertebrate blood development through forward genetic screens. In this chapter, we have described the cellular characterization of the zebrafish blood forming system and provided detailed protocols for the isolation, transplantation, and culture of hematopoietic cells. Through the development of lineal subfractionation techniques, transplantation technology, and *in vitro* hematopoietic assays, a hematological framework now exists for the continued study of the genetics of hematopoiesis. By adapting these experimental approaches that have proven to be powerful in the mouse, the zebrafish is uniquely positioned to address fundamental questions regarding the biology of hematopoietic stem and progenitor cells.

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