Oceans of Opportunity: Exploring Vertebrate Hematopoiesis in Zebrafish

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Abstract

Exploitation of the zebrafish model in hematology research has surged in recent years, becoming one of the most useful and tractable systems for understanding regulation of hematopoietic development, homeostasis, and malignancy. Despite the evolutionary distance between zebrafish and humans, remarkable genetic and phenotypic conservation in the hematopoietic system has enabled significant advancements in our understanding of blood stem and progenitor cell (HSPC) biology. The strengths of zebrafish in hematology research lie in the ability to perform real-time *in vivo* observations of hematopoietic stem, progenitor and effector cell emergence, expansion and function, as well as the ease with which novel genetic and chemical modifiers of specific hematopoietic processes or cell-types can be identified and characterized. Further, a myriad of transgenic lines have been developed including fluorescent reporter systems to aid in the visualization and quantification of specified cell types of interest and cell-lineage relationships, as well as effector lines that can be used to implement a wide range of experimental manipulations. As our understanding of the complex nature of HSPC biology during development, in response to infection or injury, or in the setting of hematological malignancy, continues to deepen, zebrafish will remain essential for exploring the spatio-temporal organization and integration of these fundamental processes, as well as the identification of efficacious small molecule modifiers of hematopoietic activity. In this review, we discuss the biology of the zebrafish hematopoietic system, including similarities and differences from mammals, and highlight important tools currently utilized in zebrafish embryos and adults to enhance our understanding of vertebrate hematology, with emphasis on findings that have impacted our understanding of the onset or treatment of human hematologic disorders and disease.

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Introduction

While the zebrafish (*Danio rerio*) was first suggested for use in hematology research in 1963[1], it is only in the last ~20 years that it has truly risen to prominence as one of the preeminent systems for hematopoietic stem cell (HSC) biology, particularly in the areas of development and regeneration. The zebrafish has emerged as a highly tractable model system for scientific research due in large part to the external fertilization of embryos and their optically clear development, allowing for real-time *in vivo* observation of developmental processes. Additionally, the ability of fecund females to lay hundreds of embryos per week enables rapid high-throughput experimentation and strong statistical analysis of phenotypes. Zebrafish are particularly useful for hematology research due to the high conservation of genetic factors regulating blood development as well as the structure and function of hematopoietic cell types, and the ability to visualize circulating erythrocytes with only a dissecting microscope.

Hematopoiesis is Highly Conserved in the Zebrafish Model

As in all other vertebrates analyzed to date, zebrafish hematopoiesis occurs in multiple phases (Figure 1). Primitive hematopoiesis, the first wave of blood development, occurs from ~12 to 24 hours post fertilization (hpf) in two anatomically distinct locations: a section of posterior lateral mesoderm called the inner cell mass gives rise primarily to cells of erythroid lineage[2], while the rostral blood island in the anterior portion of the embryo gives rise to a primitive macrophage population[3,4]. More recent analysis have also suggested that neutrophils and thrombocytes are produced during the window of primitive hematopoiesis; however, their cellular origins and lineage relationships to the primitive erythrocyte and macrophage populations are currently unclear[5,6]. The process of erythropoiesis requires many of the same genes that are utilized during primitive hematopoiesis in other vertebrate species including *scl*[7], *gata1*[8], and *lmo2*[9] while the generation of myeloid cells requires *pu.1* and *cebp1*[10–12]. A transient wave of definitive hematopoietic progenitors has also been recently identified, termed erythromyeloid precursors (EMPs), which are present in the embryo prior to the emergence of true multi-lineage HSCs[13]. These progenitors give rise to both definitive erythroid and myeloid (neutrophilic granulocytes, monocytes, and macrophage) colonies in culture. However, EMPs were never observed to populate the kidney marrow or thymus *in vivo*, indicating a lack of lymphoid potential and supporting their classification as hematopoietic progenitors rather than HSCs. Through a process which appears highly conserved among vertebrate species, definitive HSCs arise from hemogenic endothelium in the ventral wall of the dorsal aorta in the Aorta-Gonad-Mesonephros (AGM) beginning at ~30 hpf in zebrafish[14,15], shortly after the onset of circulation[16]. Following their emergence in the AGM, HSCs migrate to the Caudal Hematopoietic Tissue (CHT), currently thought to be the maturational equivalent of the mammalian fetal liver, where phenotypically labeled cells expand in number. HSPCs subsequently seed the thymus and kidney marrow, the primary sites of adult hematopoiesis in the fish beginning at 3.5–4 dpf [17].

Many of the genetic factors that specify the emergence of definitive HSCs are well-elucidated, and are largely consistent with those required in mammalian systems, indicating

a high degree of evolutionary conservation (Figure 1). A common cascade of transcription factors including sonic hedgehog, followed by Vascular Endothelial Growth Factor (VEGF)[18,19], and Notch[20,21] regulates the development of both arterial-specified endothelium as well as HSCs, reflecting the spatial emergence of HSCs from the ventral endothelial wall of the dorsal aorta in the developing embryo[14,15]. These factors lie upstream of both *scl*[22] and the transcription factor *runx1*, also a key regulator of HSC development, in the fish[23]. In contrast, other signaling pathways appear to modulate *scl/runx1+* HSC formation without impacting specification of the artery: both BMP-[24] and Wnt-signaling[25,26] appear to act in parallel to or intersect with the HH/VEGF/Notch cascade, indicating the process of HSC specification within a specific endothelial population is not a singular linear pathway, but the integrative activity of several regulatory cascades.

Not only are the genetic factors regulating HSC emergence maintained across species, the function of the different blood lineages appears to be highly conserved as well. Although zebrafish erythrocytes remain nucleated throughout their lifespan, they express the same globin genes that are found in mammals[27], indicative of a similar function. Zebrafish also contain thrombocytes (platelets)[28] that, as in mammals, play a role in blood clotting[29]. The cellular components of the innate immune system are also highly conserved; zebrafish contain granulocytes[30] as well as macrophages[4] and neutrophils[31] in the myeloid lineage. Migration of *myeloperoxidase+* granulocytes toward sites of injury and inflammation can be readily observed *in vivo*, confirming that these cells maintain their anti-inflammatory properties in the zebrafish[30]; similarly, macrophages possess the ability to phagocytose apoptotic cells and bacteria[4]. Conservation of myeloid function as well *ex vivo* development has also allowed exploitation of zebrafish as a model for infectious disease: in particular, the progression of tuberculosis, which is difficult to model in mice, has been demonstrated to be recapitulated in zebrafish embryos after infection with mycobacterium; embryos show development of macrophage aggregates and increased expression of Mycobacterium-associated genes[32]. Zebrafish also appear to possess a full component of cells of the adaptive immune system. *Recombination Activating Gene* (*rag*) expressing T-lymphocytes are found in the thymus as early as 3.5dpf by *in situ* hybridization[33–35]; B-cells have similarly been reported, although their site of maturation remains controversial[36,37] and Natural Killer cells are also thought to exist[38,39]. Recent studies have indicated B cells possess similar function including proliferation in response to antigen exposure[37]. Zebrafish have recently been reported to possess dendritic cells that uptake antigens and stimulate T-cell proliferation, indicating conservation of function[40]. In sum, the high conservation of both genetics and function of the hematopoietic cells in zebrafish lends validity to their use in hematology research.

Genetic Manipulation in the Zebrafish Model

Beyond the strong conservation of factors regulating HSC development in the zebrafish, the system is also highly amenable to genetic manipulation, making it useful for identification of novel regulatory factors, as well as gain and loss-of-function studies. While homologous recombination akin to murine models remains difficult to achieve, knockdown of a given gene(s) in the zebrafish system is readily performed using morpholino technology. Morpholinos (MOs) are modified antisense oligonucleotides designed to block either

splicing or translation of a specific mRNA, thereby resulting in a knockdown of function of the target gene due to a lack of translation. MOs are microinjected into zebrafish embryos at the 1-cell stage and they remain effective for the first ~5 days of development (depending on the dose)[41], making the technology useful for its unique ability to rapidly assess the effects of loss of a given gene on vertebrate hematopoietic development. Other significant benefits of the MO system include the ability to titrate the dose of MO injected, making it possible to carefully control the degree of knockdown, often allowing one to bypass embryonic lethal mutations, as well the ease with which suppression of multiple genes can be examined at once via combinatorial injection. However, there are caveats to the use of MOs, including potential toxicity of the MO and/or injection procedure, off-target effects, and the inability to look at the consequences of long-term loss of function of a given gene(s). More recent technologies, including zinc finger nucleases, TALENS and the CRISPR/CAS system, have emerged as promising techniques to make specific, germline-transmissible mutations of genes of interest[42–45]. These techniques make it possible to generate full knockouts (null phenotypes) relatively quickly and will enable better assessment of genetic null phenotypes in the embryo as well as the adult, when MO technology is generally no longer useful. In addition to MO titration, zebrafish are also amenable to use of a variety of inducible promoter-driven transgenes including: heat shock inducible promoters[46], the cre-lox system[47,48], and the Gal4;UAS system[49], that enable induction of gene expression at a specific time or location. More recently, tissue-specific ablation has also been achieved by driving expression of nitroreductase under a relevant promoter, followed by treatment with metronidazole during a time window(s) of interest[50].

The previously discussed benefits of the zebrafish system including external transparent development and large clutch size have historically enabled the isolation of many hematopoietic mutant lines through large-scale mutagenesis screens. Significantly, zebrafish embryos are able to survive for up to 10 days in the absence of blood flow and heartbeat, enabling identification and analysis of mutations that would be impossible to study in mammalian systems due to early embryonic lethality[51]; additionally, as the liver is not a site of embryonic hematopoiesis[17], mutations affecting the gastro-intestinal system will not necessarily impair hematopoietic development. One of the most common methods to introduce mutations into the germline of zebrafish involves the use of N-ethyl N-nitrourea (ENU)[52–54], a mutagen which results in the induction of point mutations. The majority of the hematopoietic mutants isolated from the first ENU screens involved deficits in erythrocyte function (Figure 1). These mutants included well known master erythrocyte regulators, including *gata1* in the *vlad tepes* line[8] and *globin* in *zinfandel* zebrafish[55], confirming the high conservation of hematopoietic regulation across species. More importantly, however, some of the mutations led to the identification of novel factors involved in erythrocyte maturation and function such as ferroportin[56] and mitoferrin[57]; mutations in the *ferroportin* gene have subsequently been identified as one of the most common causes of iron overload in humans[58], making it a prime example of the utility of zebrafish screening for the identification of causal genes relevant to human disease. The zebrafish is also an excellent model for several different forms of anemia, including Hereditary Elliptocytosis, Microcytic Anemia, and Congenital Dyserythropoietic Anemia, making them potentially useful for the identification of compounds or genes that alter the

severity of anemic phenotypes[59–61]. Although the zebrafish genome was duplicated in recent evolutionary history[62], resulting in partial redundancy, this can also be employed as a strength as the two paralogs of the duplicated gene often have similar but not identical expression and function.

Interestingly, zebrafish embryos can survive for several days as a haploid organism. As a result, UV inactivated sperm can be used to fertilize the eggs of females from the F1 generation of an ENU screen, thereby resulting in the formation of haploid embryos. Recessive phenotypes are visible in the F1 generation of haploid embryos, as they are not masked by the wild type copy of the mutated allele, thereby allowing assessment of phenotypes one generation prior to what would normally be possible in traditional zebrafish or mammalian screens[63]. This technique has revealed genes that are important in hematopoiesis including T-cell development[64] and vasculogenesis, a process intimately tied to HSC specification and emergence[65]. Other methodologies, referred to as TILLING (Targeted Induced Local Lesions in Genomes) took advantage of the ease of ENU mutagenesis, combined with recent advances in sequencing and cloning of the zebrafish genome to identify specific mutations in hematopoietic regulatory factors of interest, such as *rag1*[66]. Another common method of inducing mutations in the zebrafish genome involves retroviral insertional mutagenesis[67,68], whereby retroviral DNA is randomly integrated into the genome, resulting in disruption of gene expression and/or function. Analysis of zebrafish mutants from one such insertional mutagenesis screened aided in the identification and sequential organization of a hierarchy of factors required for HSC emergence in the developing vertebrate embryo including *VEGFAa, notch, hdac1, and runx1*[69]. Transposon-mediated gene mutation is also available in zebrafish[70], although it is occasionally noted to exhibit integration biases[71].

As discussed above, one of the most critical genes in the specification of HSCs during development is *runx1*. When *runx1* was mutated in zebrafish, embryos failed to undergo AGM definitive hematopoiesis and showed an absence of definitive lineages at 5 days post-fertilization[72]. While most null animals died between days 11 and 20 post-fertilization, a small fraction survived until adulthood. The reasons for this are not well known, but could be due to the presence of normal erythromyeloid progenitors that fulfill some of the roles of HSCs enabling a portion of the null animals to survive. In addition to *runx1*, the gene *cmyb* is recognized as a conserved HSC marker[73,74]. *cmyb*[t25127] mutant zebrafish embryos show an absence of definitive hematopoiesis but again are able to survive into adulthood; it is believed this is likely to be due to diffusion of oxygen into the fish from the tankwater, making the requirement for oxygen-carrying erythrocyte capacity less critical[75]. However, an alternative *cmyb* mutant line is reported to be lethal by ~10dpf due to a failure in HSPC migration from the AGM[76], suggesting that *cmyb* is indeed required for definitive hematopoietic development in the zebrafish. As the *cmyb* knockout mouse dies at E15[73] prior to birth, direct comparisons of the zebrafish mutant lines may be particularly useful for analyzing the effects of varying degrees of loss of *cmyb* function on hematopoiesis during later developmental stages.

One of the most enigmatic mutants isolated in the large-scale ENU screens of the 1990s was *cloche*[77]. These embryos show broad hematovascular defects, with a strong reduction in the

formation of both the blood and vasculature, as well as heart defects. *cloche* zebrafish are considered to be one of the strongest pieces of evidence for the "hemangioblast", a theoretical bipotential cell population that supports hematopoietic and vascular development. While many attempts to clone the *cloche* gene have been made[78,79], the identity of the exact genetic mutation underlying the *cloche* phenotype remains unknown, as loss of any single gene has been unable to replicate all the phenotypes observed in *cloche* mutant embryos. Beyond its location in a telomeric region, the difficulty of cloning *cloche*, which is recessive[77], raises the intriguing possibility that the mutation may lie an intergenic or non-coding region, such as a miRNA or lncRNA that has the ability to impact the transcription or translation of multiple genes. While simply speculation, a mutation in one of these pleiotropic regulatory regions may explain why the *cloche* phenotype is so penetrant, leading to defects in multiple aspects of hemato-vascular development.

Screens to identify modifiers of hematopoietic phenotypes have also recently been performed using zebrafish; one of these screens utilized a MO-based "knockdown" approach to identify epigenetic factors with specific regulatory roles in hematopoiesis. This relatively high-throughput reverse-genetics screen resulted in the identification of several families of epigenetic regulators that impact either primitive or definitive hematopoiesis, or both, and provides insight into the role of epigenetic regulation in establishing hematopoietic commitment and expansion[80]. Zebrafish were also used in a genetic modifier screen to look for suppressors of the *moonshine* mutant, which has defects in erythropoiesis; this screen resulted in the identification of transcriptional elongation as a regulator of cell fate[81]. Together, these studies suggest that the zebrafish will continue to be useful for identifying novel regulatory factors that modify known hematopoietic phenotypes.

Real-time Imaging of Hematopoietic Stem Cell Emergence During Development

The strong genetic conservation in zebrafish has also enabled engineering of many transgenic reporter lines that express fluorescent proteins under the control of promoter regions of given hematopoietic genes of interest (Figure 2). These transgenic lines have been especially useful for quantifying alterations in hematopoietic cell number, particularly given the lack of cross-reactivity with many existing mammalian antibodies, and for imaging the emergence and migration of HSCs during development *in vivo* in real time, a process which is extremely difficult in mammalian systems [14,15,23]. While many primitive hematopoietic and vascular reporter lines emerged in the late 1990s and early 2000s including *gata1*[82], *lmo2*[83], and *fli1a*[84], the first transgenic HSC-reporter line to be developed was the *Tg(-6.0itga2b (CD41):EGFP)* line, which is commonly used to visualize HSCs after their emergence from the AGM[28,85], particularly in the CHT and kidney marrow. Importantly, while also expressed at high levels on thrombocytes, the utility of CD41 as a blood stem cell marker appears to be conserved as it has also been reported to be present on HSCs during mammalian development[86]. Fluorescent reporters for *runx1*[87] and *cmyb*[74] were subsequently created.

Over the past several years, elegant time-lapse imaging studies have enabled real-time, *in vivo* visualization of HSC emergence from hemogenic endothelium in the AGM. One recent study demonstrated the existence of hemogenic endothelium using an *scl β*-driven GFP

construct and confirmed that *scl* is required for the birth of HSCs in the AGM[88]. Other studies visualized the birth of HSCs from hemogenic endothelium. Co-expression of *cmyb* and *flk1* transgenes was shown by lineage tracing to mark budding HSCs in the vasculature[14] and has been used to enable precise quantification and visualization of HSCs in the embryo. Simultaneously, time-lapse imaging of embryos expressing GFP under the control of the *flk1* transgene was utilized to visualize the process of budding and egress of HSCs from the endothelium into the vasculature, now termed endothelial-to-hematopoietic transition (EHT)[15]. These studies both lent strong support to the theory that HSCs arise from a subset of endothelial cells, termed hemogenic endothelium, found in the vasculature, and the dorsal aorta in particular. This argument was further bolstered by visualization of *runx1* transgenic reporter fish which indicated that *runx1* was expressed in the AGM and in particular in the ventral wall of the dorsal aorta[87], consistent with its expression in hemogenic endothelium in mammalian models[89]. The role of *runx1* in EHT in zebrafish was functionally demonstrated *in vivo* via MO injection, as *runx1* knockdown resulted in a lower rate of budding initiation, as well as cell disruption upon attempting to exit the aortic wall[15], a finding consistent with observations from murine models [90]. Interestingly, both zebrafish imaging studies indicated that HSCs, upon leaving the endothelium, enter into circulation via the vein rather than the artery, a process that appears to be unique in the fish[14,15]; the explanation for this difference could potentially result from the overlapping rather than continuous structure of the venous endothelium that enables easier intravasation of cells[91] or may simply reflect the compact spatial alignment of the major trunk vessels in zebrafish compared with that of mammalian embryos, allowing local chemoattractants from the vein to reach newly produced HSCs immediately upon egress rather than in the circulation. In addition to *in vivo* documentation of the emergence of HSCs performed in the zebrafish system, concurrent studies using explant cultures have indicated a very similar process occurs in the mouse[92], suggesting that this series of events is highly conserved. Transgenic HSC reporter lines can also be used to visualize activity in secondary sites of hematopoiesis including the CHT, thymus, and kidney, enabling their use for lineage tracing as well as analyzing migration, colonization and expansion of HSCs[87,93]. Photoconvertible lineage tracing can also be used to track progeny of a given cell of interest. For instance, use of a photoconvertible Notch Kaede reporter *Tg(EPV.Tp1-Mmu.Hbb:Kaede)* demonstrated that Notch signaling plays at least two temporally distinct roles in HSC development[26]. In the adult, use of GFP+ donor cells has enabled quantification of lineage contribution following kidney marrow transplantation, aiding monitoring and assessment of engraftment kinetics and efficiency[94]. In addition to genetic profiling of labeled embryonic blood populations[79], our recent study has also indicated that transgenic lines (for example CD41) can be utilized to enrich for embryonic HSCs with adult kidney marrow repopulating activity by FACS sorting[95]. Together, these studies confirmed the *in vivo* functional potential of the labeled populations of interest and provide an additional resource for the prospective isolation and analysis of factors regulating HSCs during vertebrate development.

In Vivo High-Throughput Small Molecule Screening in the Zebrafish

Due the to the large numbers of embryos available from pair-wise mating(s) and the external development of the fish, it is relatively easy to do large-scale chemical screening for small molecule modifiers in zebrafish by simple addition of "test" compounds to the fish water.

The first chemical screen was published in 2004 and was aimed at the identification of novel suppressors of the *gridlock* (*grl*) phenotype, caused by a mutation in *hey2*, that leads to aortic coarction and defects in arterial/venous development[96]. The compound discovered, GS4012, was a potent inducer of VEGF and was the first "therapeutic" discovery in zebrafish, resulting in a reduction of the severity of a genetic disorder impacting the hematovascular system.

The first hematopoietic screen used the *Tg(gata1:GFP)* line to identify modifiers of primitive hematopoiesis[97]; subsequent screens have revealed other novel modifiers of both primitive and definitive hematopoiesis[16,74,98]. One screen hit that has shown direct therapeutic potential across species was Prostaglandin E2, which robustly increased the number of *runx1+* HSCs in zebrafish and mice[74], in part through modulation of the Wnt pathway[25]; following translational work using human cells[99], PGE2 became the first compound derived from drug discovery in the zebrafish to reach FDA-approved clinical trials testing in human patients[100]. Other significant discoveries made via the chemical screening approach yielded compounds which could induce hematopoietic differentiation in the AML-ETO model of leukemia[101,102]. A subsequent screen also identified compounds that were specifically toxic to immature T-cells, such as Lenaldekar, a potential chemotherapeutic agent for patients with T-cell Acute Lymphoblastic Leukemia (T-ALL)[103]. While *in vitro* screening of cell populations of interest is certainly a valuable methodology for therapeutic compound identification, *in vivo* screening using the zebrafish model has the advantage of identifying drug toxicity "side effects" from the outset, as well as allow for production and evaluation of any compound metabolites that may be produced, boosting the translational potential of screening hits and speeding the time from discovery to application.

Adult Hematopoietic Assays Adapted for Use in the Zebrafish Model

While the bulk of the hematopoietic studies done in zebrafish have been performed using embryos, a growing number of techniques to study HSC biology in the adult animal are emerging. One of the primary challenges of working with zebrafish to study hematopoiesis in the adult is the lack of antibodies for cell surface markers to enable FACS analysis of HSCs and the different hematopoietic lineages. The first study to identify a simple way to isolate whole kidney marrow (WKM; the site of adult hematopoiesis) and analyze the cellular content of the marrow relied upon flow cytometry using cell size (forward scatter) and granularity (side scatter) to isolate the various lineage fractions[104]; while a similar technique has also been used in the mouse[105], this was a huge advancement for studying hematopoietic cell production and homeostasis in zebrafish. Subsequent studies furthered the functional utility of the WKM dissection and analysis by introducing irradiation-mediated ablation of the hematopoietic system to both enable assessment of KM recovery after injury and to perform adult-to-adult HSC transplantation[106], long considered the best technique to determine the presence of a true multipotent hematopoietic stem cell. Transplantation methodology was further enhanced by the generation of *casper* fish, which lack pigmentation, enabling real-time *in vivo* visualization of HSC homing and engraftment: for example, injection of *beta-actin:GFP* WKM donor cells in irradiated *casper* recipients enabled imaging of the donor cells from 2 hours until at least 5 weeks after

transplantation[107]. Functional potential, including lineage tracing, can also be assessed by transplantation of donor stem cells that carry pan-hematopoietic (CD45) markers[95]. These methodologies have significant advantages over homing and expansion assays in mammalian systems where real-time visualization of is difficult due to the deep interior location of the bone marrow niche. Recent studies focused on the role of the Major Histocompatibility Complex (MHC) have further refined the transplantation protocol and indicated that matching the MHC type between donor and recipient can drastically improve rates of engraftment[94]. Regenerative assays using these techniques have identified several factors, including Notch signaling and PGE2/Wnt, which accelerate hematopoietic recovery after marrow injury, providing clinically relevant insight into the engraftment process following stem cell transplantation and strengthening the connection between regulators of embryonic development and tissue regeneration[20,25]. Importantly, while PGE2 was initially identified as a modifier of HSC regeneration in the zebrafish, it was simultaneously found to play a similar role in the mouse[74], emphasizing the utility of the zebrafish to identify critical pathways in HSC regulation across vertebrates; the Notch pathway has likewise showed utility in transplantation therapies across species[108]. Although zebrafish cells in general have traditionally been difficult to maintain *in vitro*, recent studies indicate that long-term hematopoietic culture of isolated kidney marrow stem and progenitor cells is possible, as both erythroid and myeloid colonies were generated in a clonal methylcellulose assay[109] following identification and isolation of zebrafish specific cytokines such as Erythropoietin (Epo) and Granulocyte Stimulating Colony Factor (GCSF). This advancement opens further avenues of functional analysis and manipulation of hematopoietic stem and progenitor cell biology in the zebrafish. Finally, while not yet examined in detail, the localization of the HSC niche within the kidney marrow rather than bone marrow niche, may itself prove advantageous to elucidation of cell autonomous and/or bone independent regulatory effects on HSCs and hematopoietic progenitors.

One of the most prominent areas of zebrafish hematology research is currently in cancer biology, particularly that of T-ALL, which was the first model of cancer in the fish[110] and is the most frequent childhood hematological malignancy[111]. While the zebrafish has not been a long-standing cancer model, it is quickly gaining traction due to the ease of chemical and genetic screening for modifiers of cancer development or progression[110,112]. Recent studies have led to the identification of the COX/β-catenin[102,113], PTEN[114], S1P1 and ICAM1 pathways[115] as playing key roles in cancer development and progression using zebrafish models of hematologic malignancy. In addition to the cyclooxygenase inhibitors mentioned above[102] for the treatment of AML1-ETO associated AML, screening for chemical modifiers of T-ALL progression also led to the identification of perphenazine, an antipsychotic, as an inducer of apoptosis in malignant cells; importantly, this was true in zebrafish, mouse, and human T-ALL cells[116], indicating the usefulness of the fish for identifying drugs that can impact the course of human cancer.

Concluding Remarks

Over the last 20 years, the use of zebrafish in the field of hematology has undergone a transformation from being an interesting alternative model to being one of the preeminent and tractable vertebrate systems for hematological studies. The amenity of the zebrafish to

genetic manipulation together with the strong evolutionary conservation of the genetic regulation of hematopoietic development, homeostasis, and regeneration makes it a particularly useful system for blood research. However, some weaknesses with the zebrafish model system remain, including the lack of antibody cross reactivity with common cell surface markers for FACS, Western blots and immunohistochemistry[104], differences in the anatomical location of some aspects of hematopoiesis and the evolutionary duplication of a portion of the zebrafish genome, resulting in genes with redundant functions. Nevertheless, great progress is being made in zebrafish biology to help alleviate, or even turn these concerns into strengths (for example, bone-independent HSC regulation), and to ensure the fish remains a useful system for future studies. One of the greatest advantages of the zebrafish system lies in its ability to act as an evolutionary intermediate between the more longstanding invertebrate animal models, such as *Drosophila*, and that of the mouse. The zebrafish maintains many of the advantages of lower organisms, including rapid external development and strong utility for forward genetic screening, while also being a vertebrate, with organ development and regeneration evolutionarily closer to humans at both the cellular and genetic level. Together, the zebrafish has many qualities that make it an extraordinarily useful model system to discover novel hematological findings that will continue to enhance our knowledge of basic HSC biology as well as provide key insights into human health and disease. The zebrafish is likely to be particularly adept in helping to delineate factors that regulate the developmental niche versus those that directly impact HSCs through the use of tissue and lineage specific promoters as well as real-time *in vivo* imaging, both of which are difficult to achieve in a mammalian system. One of the major goals of both the stem cell and regenerative biology fields has been the generation of a transplantable HSCs from either embryonic or iPSC sources for use in clinical applications. However, this goal has thus far proven elusive, suggesting that there is much more to be understood about the *in vivo* mechanisms that integrate to specific HSCs. Finally, the utility of the zebrafish for chemical screening in both a developmental and disease context is likely to continue to be one of the most useful aspect of the model system and will lead to the isolation of novel compounds that can bolster our understanding of embryonic hematopoiesis, as well as the onset and progression of hematological malignancies, and help identify potential therapeutics to aid in the treatment of human blood diseases.

Acknowledgments

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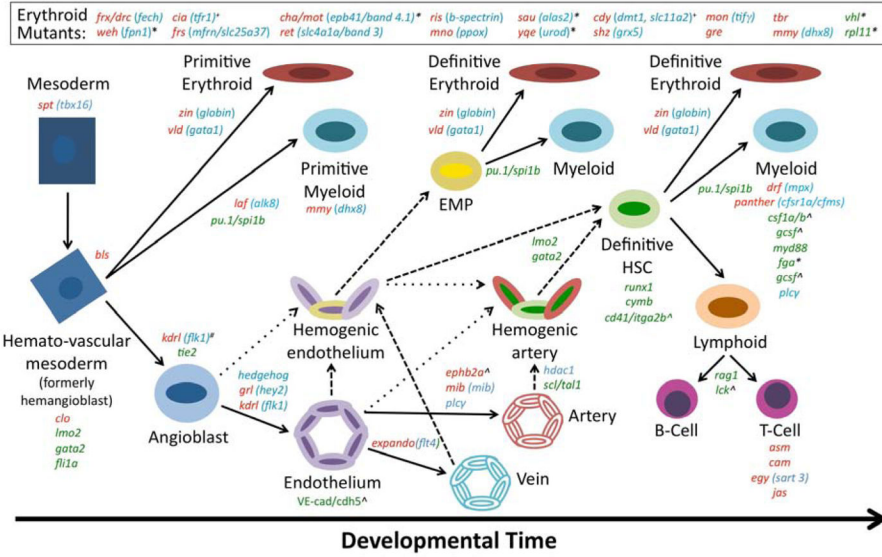
Figure 1. Models for investigating embryonic hematopoiesis in the Zebrafish
Schematic representation of embryonic hematopoietic development showing lineage relationships between the hemato-vascular populations and available zebrafish mutant lines. Red type indicates mutants derived from forward genetic ENU-screening, with blue font illustrating the genetic lesion; green font represents targeted mutants. An * denotes mutants which recapitulate the disease phenotype associated with human gene mutation(s). A ^ indicates a targeted mutation in a conserved gene that has not yet been phenotypically validated. A # indicates a series of genetic alleles are available. A + notes mutants which exhibit an early embryonic phenotype but are viable as adults.
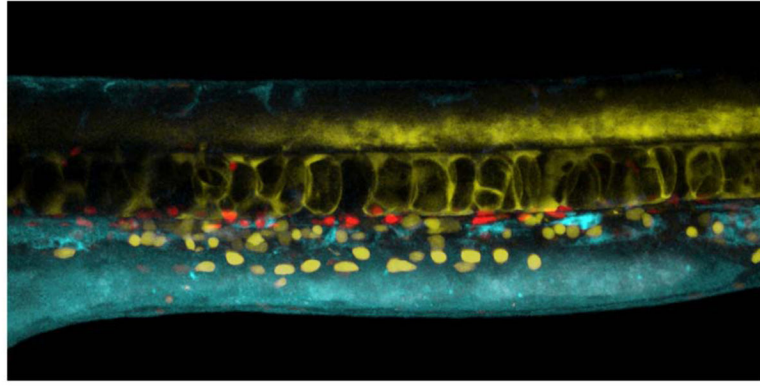
Figure 2. Optical clarity and transgenic tags allow *in vivo* visualization of hematopoiesis in zebrafish embryos

Trunk region of a live zebrafish embryo at 24hpf expressing cmyb:GFP (pseudocolored yellow), tp1:nuclear-mcherry (red), and flk1:ntr-cerulean (blue) (Unpublished image from A. Kim and D. Traver (UCSD); unpublished flk1:ntr-cerulean line from N. Chi (UCSD).

Table 1

Mutations in zebrafish hematopoietic and vascular genes shown in Figure 1 are detailed here. Reference listed is the first to clone the mutation. If the given mutation is unconfirmed, the reference is for the hematovascular expression of the conserved gene.

Gene Name	Mutant (abbreviation)	Original Isolation Method	Cloning/Expression Reference
(unknown)	*cloche (clo)*	ENU screen	(Stainier et al., 1995)
(unknown)	*bloodless (bls)/sort-of-bloodless (sob)*	Insertional Mutagenesis	(Liao et al., 2002)
(unknown)	*grenache (gre)*	ENU screen	(Ransom et al., 1997)
(unknown)	*thunderbird (tbr)*	ENU screen	(Ransom et al., 1997)
(unknown)	*assam (asm)*	ENU-EP screen (targeted: T-cells)	(Trede et al., 2008)
(unknown)	*camomile (cam)*	ENU-EP screen (targeted: T-cells)	(Trede et al., 2008)
(unknown)	*jasmine (jas)*	ENU-EP screen (targeted: T-cells)	(Trede et al., 2008)
(unknown)	*oolong (oln)*	ENU-EP screen	(Trede et al., 2008)
activin A receptor, type 1 like (acvr1l; alk8)	*lost-a-fin (laf)*	ENU screen	(Bauer et al., 2001)
cadherin5/VE-Cadherin (cdh5)			(Helker et al., 2013)
colony stimulating factor 1a/b (csf1a/b)/macrophage colony stimulating factor (mcsf1/2)	Unconfirmed	*csf1a* (ENU screen); *csf1b* (Viral insertion)	(Wang et al., 2008)
colony stimulating factor 3 (csf3)/ granulocyte colony stimulating factor (gcsf)	Unconfirmed	ENU screen	(Liongue et al., 2009)
csfr1a/cfms	*panther*	ENU screen	(Parichy et al., 2000)
dhx8	*mummy (mmy)*	ENU screen	(English et al., 2012)
divalent metal transporter 1 (dmt1)/ slc11a2	*chardonnay (cdy)*	ENU screen	(Donovan et al., 2002)
epb41b/band4.1	*chablis (cha)/merlot (mot)*	ENU screen	(Shafizadeh et al., 2002)
ephrin b2a	Unconfirmed	ENU screen	(Lawson et al., 2001)
ets variant gene 2 (etv2)		ENU screen	(Pham et al., 2007)
ferrochetalase (fech)	*frx/dracula (drc)*	ENU screen	(Childs et al., 2000)
ferroportin1 (fpn1)	*weissherbst (weh)*	ENU screen	(Donovan et al., 2000)
fibrinogen, alpha chain (fga)	Unconfirmed	ENU screen	(Vo et al., 2013)
fli1a			(Liu and Patient, 2008)
flt4	*expando*	ENU screen	(Hogan et al., 2009)
gata1a	*vlad tepes (vld)*	ENU screen TILLING	(Lyons et al., 2002)
gata2a		Zinc-finger nuclease	(Zhu et al., 2011)
globin (locus control region)	*zinfandel (zin)*	ENU screen	(Brownlie et al., 2003)
glutaredoxin 5 (grx5)	*shiraz (shz)*	ENU screen	(Wingert et al., 2005)
hairy/enhancer of split related with YRPW motif 2 (hey2)	*gridlock (grl)*	ENU Screen	(Zhong, 2000)

Gene Name	Mutant (abbreviation)	Original Isolation Method	Cloning/Expression Reference
histone deacetylase 1 (hdac1)	*ascending and descending (add)/ colgate (col)*	ENU Screen; retroviral insertion	(Nambiar et al., 2007; Yamaguchi et al., 2005)
IGM	Unconfirmed	Retroviral	(Danilova et al., 2005)
ikaros (ikzf1)		ENU screen	(Schorpp et al., 2006)
integrin, alpha 2b (itga2b/CD41)	Unconfirmed	ENU Mutagenesis	(Ma et al., 2011)
kdrl/fetal liver kinase 1 (flk1)/VEGF receptor 2 (VEGFR2)		ENU screen	(Covassin et al., 2006)
LIM domain only 2 (lmo2)		ENU screen (targeted)	(Weiss et al., 2012)
lymphocyte specific protein tyrosine kinase (lck)	Unconfirmed	ENU Mutagenesis	(Murayama et al., 2006)
mindbomb	*mindbomb (mib); white tail (wit)*	ENU screen	(Itoh et al., 2003)
mitoferrin/slc25a37	*frascati (frs)*	ENU screen	(Shaw et al., 2006)
myeloid differentiation primary response gene 88 (myd88)		TILLING	(van der Vaart et al., 2013)
myeloperoxidase (mpx/mpo)	*durif (drf)*	ENU screen	(Pase et al., 2012)
phospholipase C, gamma 1 (plcγ1)	*deadbeat (ded)*	2004: retroviral insertion; 2005 and 2009: ENU screen	(Amsterdam et al., 2004; Covassin et al., 2009; Rottbauer et al., 2005)
protoporphyrinogen oxidase (ppox)	*montalcino (mno)*	ENU screen	(Dooley et al., 2008)
pu.1/spi1b		TILLING	(Jin et al., 2012)
recombination activating gene 1 (rag1)		TILLING	(Wienholds et al., 2002)
ribosomal protein L11 (rpl11)		Retroviral Insertion	(Danilova et al., 2011)
runt-related transcription factor 1 (runx1)		TILLING	(Sood et al., 2010)
slc4a1a/band3	*retsina (ret)*	ENU screen	(Paw et al., 2003)
sonic hedgehog (shha)	*sonic you (syu)*	ENU Screen	(Schauerte et al., 1998)
squamous cell carcinoma antigen recognized by T-cells 3 (sart3)	*earl grey (egy)*	ENU-EP screen (targeted: T-cells);	(Trede et al., 2007)
T-box gene 16 (tbx16)	*spadetail (spt)*	ENU screen	(Griffin et al., 1998)
T-Cell acute lymphocytic leukemia 1 (tal1)/stem cell leukemia (scl)		ENU screen (targeted: vessels)	(Bussmann et al., 2007)
TEK tyrosine kinase, endothelial (tie2/ tek)		TILLING	(Gjini et al., 2011)
tif1γ	*moonshine (mon)*	ENU screen	(Ransom et al., 2004)
transcription factor cmyb (cmyb)		2010: ENU screen (targeted: T-cells); 2011: ENU screen	(Soza-Ried et al., 2010; Zhang et al., 2011)
transducin (beta)-like 3 (tbl3)	*ceylon (cey)*	ENU-EP screen (targeted: T-cells)	(Hutchinson et al., 2012; Trede et al., 2008)
transferrin1 (tfr1)	*chianti (cia)*	ENU screen	(Wingert et al., 2004)
uroporphyrinogen decarboxylase (urod)	*yquem (yqe)*	ENU screen	(Wang et al., 1998)
v-ets erthroblastosis virus E26 oncogene homolog 2 (ets2)	Unconfirmed	Retroviral Insertion	(Liu and Patient, 2008)
Vascular Endothelial Growth Factor (VEGF)			(Lawson et al., 2002)
Von Hippel-Lindau (vhl)		TILLING	(van Rooijen et al., 2009)

Gene Name	Mutant (abbreviation)	Original Isolation Method	Cloning/Expression Reference
β-spectrin	*riesling (ris)*	ENU screen	(Liao et al., 2000)
δ-aminolevulinate synthase 2 (alas2)	*sauternes (sau)*	ENU screen	(Brownlie et al., 1998)

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