Regulation of Stem Cells in the Zebra Fish Hematopoietic System

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Hematopoietic stem cells (HSCs) have been used extensively as a model for stem cell biology. Stem cells share the ability to self-renew and differentiate into multiple cell types, making them ideal candidates for tissue regeneration or replacement therapies. Current applications of stem cell technology are limited by our knowledge of the molecular mechanisms that control their proliferation and differentiation, and various model organisms have been used to fill these gaps. This chapter focuses on the contributions of the zebra fish model to our understanding of stem cell regulation within the hematopoietic system. Studies in zebra fish have been valuable for identifying new genetic and signaling factors that affect HSC formation and development with important implications for humans, and new advances in the zebra fish toolbox will allow other aspects of HSC behavior to be investigated as well, including migration, homing, and engraftment.

Stem cells and early progenitors are important for organ formation during development and support tissue function throughout an organism's lifetime. These principles are illustrated in the blood system, where HSCs are needed to maintain a constant pool of progenitors committed to the various blood lineages to replenish the mature blood cells that turn over. Consequently, HSCs, like other stem cells, have the ability to self-renew in order to generate more HSCs and to differentiate along multiple lineage pathways to make erythrocytes, megakaryocytes, monocytes/macrophages, neutrophils, or lymphocytes. Because of their potential therapeutic value, HSCs have been the subject of intense study for many years, but methods for maintaining them in vitro and differentiating them into specific cell types are limited. In addition, HSCs remain difficult to study because their ontogeny is tightly regulated in a spatial and temporal manner, with progenitors of varying potentials arising from different sites.

Research using the zebra fish (Danio rerio) model has provided new insights into some of the major issues regarding the regulation and function of HSCs (Davidson and Zon 2004; Carradice and Lieschke 2008; Orkin and Zon 2008). The advantages of using zebra fish include its high fecundity, rapid growth, and external development of transparent embryos, which facilitates visualization of early embryonic processes. Most importantly, the genes controlling hematopoiesis are highly conserved in fish and mammals. Many blood mutants have been isolated from large-scale genetic screens (Ransom et al. 1996; Weinstein et al. 1996), thereby providing powerful tools for dissecting the molecular aspects of HSC control.

EMBRYONIC HEMATOPOIESIS: PRIMITIVE AND DEFINITIVE WAVES

The onset of hematopoiesis in the early embryo is characterized by the induction of distinct progenitors at various anatomical sites. Hematopoiesis subsequently shifts location during the course of development, as depicted in Figure 1. Blood formation occurs in two major waves. In zebra fish, the primitive wave consists of the formation of primitive erythrocytes (gata1+) in the intermediate cell
Currently, it is thought that blood and vascular cells derive from a common progenitor based on shared marker expression and physical proximity between the two cell types during development. The lack of blood and vascular markers in developing zebrafish cloche (clo) mutants provides evidence for the existence of hemangioblasts (Strainier et al. 1995; Thompson et al. 1998), further supported by recent fate-mapping experiments in the early embryo. Photoactivation of fluorescein dextran in single cells within the ventral mesoderm at shield stage (6 hours postfertilization [hpf]) labeled blood and vascular cells later at 30 hpf (Vogeli et al. 2006).

Definitive HSCs are believed to arise from the hemangioblastic endothelium (Cumano et al. 1996; Medvinsky and Dzierzak 1996). In addition, other data suggest that mesenchymal cells ventral to the dorsal aorta have HSC potential (North et al. 2002). By analogy, HSCs produced within the zebrafish AGM region are believed to be equivalent to those found in the same region in mice, supported by expression of homologous markers and lineage-tracing data. Furthermore, hematopoietic mutants such as clo and spadetail (spt) that disrupt formation of the dorsal aorta show loss of HSC induction (Thompson et al. 1998).

### TRANSCRIPTIONAL REGULATORS OF HSC FORMATION

Specification of hematopoietic cells involves both the action of master blood transcriptional regulators and signaling molecules from the surrounding tissues. The transcription factors important for hematopoiesis in zebrafish are listed in Table 1. Many of them have been implicated in blood development by the blood-specific roles these factors have in other vertebrates or by genetic analysis of mutants isolated from large-scale screens (Fig. 2). Together, they...
form a transcriptional network that directs various aspects of the blood program from specification to differentiation. Runx1 and Lmo2 are expressed in hematopoietic progenitors and endothelial cells, possibly acting together to specify the hemangioblast (Patterson et al. 2007). Runx1 encodes a basic helix-loop-helix transcription factor, whereas Lmo2 is a LIM-domain transcription factor. Runx1 expression is initiated at 10 hfp and is coexpressed with lmo2, gata2, and flk1 in the ALM and PLM (Gering et al. 1998; Thompson et al. 1998). A subset of these runx1+ cells in the PLM becomes gata1+, committing to the erythroid lineage, whereas the lmo2+/gata1- population is believed to form endothelial cells (Gering et al. 1998). In the ALM, pu.1 expression is initiated with the differentiation of myeloid cells (Bennett et al. 2001). Overexpression of runx1 and lmo2 expands the formation of blood and vascular progenitors along the anteroposterior (AP) axis (Gering et al. 2003), although runx1 itself is capable of activating hematopoietic genes independently of lmo2 (Dooley et al. 2005). Once primitive erythroblasts from the ICM enter the circulation, additional markers appear that define the definitive wave.

Targeted disruption of runx1 in mice has demonstrated its requirement for HSC induction in the AGM (Okuda et al. 1996; North et al. 2002). In zebrafish, runx1 and c-myb are expressed in the AGM as well (Thompson et al. 1998; Burns et al. 2002; Gering and Patient 2005). Runx1 is a member of the runt family of transcriptional regulators involved in many developmental processes including blood and bone development in mammals. In zebrafish, runx1 is first expressed in the bilateral stripes of the PLM that then migrate to the midline to form the ICM. It subsequently appears during the definitive wave in the ventral wall of the dorsal aorta (Burns et al. 2002). Runx1 overexpression causes HSC expansion in embryos, whereas morpholino knockdown causes defects in definitive hematopoiesis and vasculoangiogenesis (Burns et al. 2005). Furthermore, c-myb expression is reduced in runx1 morphants (Kalev-Zylinska et al. 2002).

Runx3 was found to regulate primitive and definitive hematopoietic cells but not vascular cells. Depletion of runx3 resulted in decreased runx1 expression in the ventral wall of the dorsal aorta. Conversely, overexpression of runx3 increased primitive scl+ cell numbers and runx1+ cells in the aorta, suggesting that runx3 regulates hematopoietic progenitor numbers and cooperates with runx1 to regulate HSC formation in the AGM (Kalev-Zylinska et al. 2003).

c-myb encodes a proto-oncogene that marks the initiation of HSCs in the ventral wall of the dorsal aorta. In zebrafish, it is expressed in primitive erythroid cells in the ICM region, but it is not required for the primitive wave (Thompson et al. 1998). Expression then shifts to the ventral wall of the dorsal aorta, presumably marking the definitive HSCs in the AGM region. Although these c-myb+ cells have not been transplanted, the lack of c-myb expression in clo and spt mutants provides further evidence for c-myb as a marker of definitive HSCs (Thompson et al. 1998).

The contribution of the cdx-hox pathway to specification of hematopoietic cell fate has been elucidated in zebrafish. cdx genes belong to the caudal family of homeobox transcription factors implicated in the regulation of hox gene expression and in AP patterning. Both cdx1a and cdx4 establish the correct hox expression domains necessary for blood development in zebrafish (Davidson and Zon 2006). Loss of cdx4 gene function in homozygous kugelig (kkg) mutants results in severe anemia with embryos having few runx1+ and gata1+ cells, although the number of flk1+ angioblasts appears to be normal. All hox genes examined (hoxb4, hoxb5a, hoxb6b, hoxb7a, hoxb8a, hoxb10, and hoxa9a) displayed altered expression patterns in kkg mutants, but overexpression of hoxb7a and hox9a could rescue erythropoiesis (Davidson et al. 2003). This pathway regulating blood specification was recently found to be conserved in mouse embryonic stem (ES) cells (Lengerke et al. 2008). Additional transcription factors important for definitive hematopoiesis include fli1a, hhex, and tbx16. fli1a is an ETS-domain transcription factor implicated in proliferation or differentiation of hematopoietic precursors. It is coexpressed in the hemangioblasts of the PLM with gata2, diverging later to mark only the endothelial cells (Brown et al. 2000). Given that its initial expression is normal in clo mutants, fli1a may be the earliest marker of hematangioblasts. Hhex encodes a homeobox-containing protein whose expression begins about 12 hfp in the ALM and PLM. Overexpression enhances blood and endothelial markers but is not essential for their development, which can be compensated by scl (Luo et al. 2000). Tbx16 encodes a T-box transcription factor that regulates mesodermal cell migration, which is defective in spt mutants. As described previously, abnormal somite patterning and accumulation of mesodermal cells perturb vessel formation, which subsequently leads to defective HSC formation as demonstrated by loss of hematopoietic markers gata2, gata1, and runx1 in the PLM (Ho and Kane 1990; Thompson et al. 1998). Overexpression of scl rescues blood formation in spt mutants, indicating that tba16 is upstream of scl in directing HSC formation (Dooley et al. 2005). The analogous expression of these different markers in zebrafish and other vertebrate models suggests that the molecular mechanisms are highly conserved. Once the hematopoietic precursors have been specified, additional blood transcription factors such as gata1, pu.1, and ikaros direct the lineage-specific differentiation of these progenitors into erythroid, myeloid, and lymphoid cell types, respectively. Some of the factors required for HSC forma-
PATHWAYS IN THE INDUCTION OF HSCS

In addition to transcriptional regulators, signal transduction pathways are also important for modulating blood formation. Although distinct hematopoietic precursors are generated in different anatomical sites, common signaling events at each site are expected to lead to blood formation. How these different signaling pathways are coupled to control stem cell induction and development is a subject of ongoing research.

The family of Hedgehog (Hh) proteins are known to be involved in embryonic patterning and cell-fate specification. Based on murine mutants, there was no role for Hh signaling in hematopoiesis (Dyer et al. 2001; Byrd et al. 2002). In zebra fish, Hh was found to be required for definitive but not primitive hematopoiesis (Gering and Patient 2005). It is secreted by midline structures (floor plate, notochord, and hypochord) in the developing embryo. When Hh signaling is inhibited by chemicals or in genetic mutants, embryos showed normal numbers of β-globin/E1 primitive erythrocytes but reduced runx1 definitive stem cells and rag1+ thymocytes, suggesting that Hh is required only for induction of definitive HSCs. Impaired medial migration of flk1+ angioblasts was also observed, indicating the possibility that improper patterning of the aorta is the cause of HSC loss. These effects were similar to those seen with vascular endothelial growth factor (VEGF) and Notch inhibition (Gering and Patient 2005).

Notch signaling has been previously shown to be required for induction of HSCs during embryogenesis in mice (Okuda et al. 1996). The Notch pathway is highly conserved throughout evolution, regulating cell-fate decisions in a wide range of biological processes. Using zebra fish mind bomb mutants that lack an E3 ubiquitin ligase essential for Notch signaling, runx1 was identified as a downstream effector (Burns et al. 2005). Overexpression of notch1a intracellular domain (NICD) expanded the population of c-myb+ and runx1+ cells in the AGM region, and this increase was not due to proliferation or conversion of vein-to-artery identity. This phenotype was recapitulated in runx1 overexpressing embryos (Burns et al. 2005). Given that it rescues the mind bomb phenotype, runx1 may be acting in parallel or downstream from Notch signaling. In addition, runx1 morphants show reduced c-myb+ and ikaros+ cells at 50 hpf and loss of rag1+ thymocytes at 6 days (Gering and Patient 2005).

A new pathway modulating HSC formation by prostaglandins was recently identified in the zebra fish. Prostaglandins are part of the eicosanoid signal transduction pathway, with prostaglandin E2 (PGE2) being the main effector prostaglandin produced in the zebra fish. They are regulated by cyclooxygenases Cox1 and Cox2 (Grosser et al. 2002). When treated with PGE2, zebra fish embryos showed increased runx1+ and cmyb+ cells in the AGM region by in situ hybridization, confocal microscopy, and quantitative polymerase chain reaction (PCR). Chemical or morpholino inhibition of the pathway reduced HSC formation. These results were verified in both colony-forming and limiting dilution competitive transplantation assays in the mouse, demonstrating a functional conservation of prostaglandin signaling not only in inducing HSCs, but also in adult maintenance (North et al. 2007). 16,16-Dimethyl-PGE2 (dmpPGE2), a stable derivative of PGE2, will be tested in a human phase I clinical trial to determine whether it can improve the efficiency of cord blood transplantation (Lord et al. 2007).

MIGRATORY ROUTES OF HSCS TO SECONDARY HEMATOPOIETIC SITES

The ontogeny of HSCs in the AGM region is followed by subsequent colonization of secondary hematopoietic sites, presumably as different niches become available to support HSC growth within the constantly evolving microenvironment of the developing embryo. Lineage tracing in the mouse has been complicated by the inability to stage embryos precisely in utero and to determine the kinetics of conditional recombination activity, making it difficult to identify clearly the anatomic origins of adult HSCs. One advantage of performing in vivo fate mapping in zebra fish is the optical transparency and external development of its embryos.

Live imaging of cells labeled with green fluorescent protein (GFP) driven by HSC-specific promoters, such as CD41 and c-myb, and caged fluorescein-dextran cell-tracing experiments have shown that HSCs from the AGM region migrate to colonize the CHT, thymus, and pronephros (Murayama et al. 2006; Jin et al. 2007; Zhang and Rodaway 2007; Bertrand et al. 2008; Kiss 2008). CD41 marks HSCs in the mouse (Mitjavila-Garcia et al. 2002; Ferkowicz et al. 2003; Mikkola et al. 2003). In zebra fish, both CD41-GFP+ and c-myb-GFP+ cells were observed in the AGM region, consistent with runx1 expression at the same site (North et al. 2007; Kiss 2008). Knockdown of runx1 suppressed the appearance of CD41-GFP+ cells in this region, the CHT, and thymus (Kiss et al. 2008). Transplantation of CD41-GFP+ AGM cells into sibling embryonic recipients demonstrated colonization of the thymus and CHT (Bertrand et al. 2008), although assays have yet to be developed in the zebra fish that can support long-term reconstitution of embryonic donor cells.

Unlike chick and mouse, where AGM HSCs presumably bud off into circulation from intra-aortic clusters, AGM cells in zebra fish enter the circulation through the cardinal vein (CV) to seed the CHT (Kissa et al. 2008). Migration to the CHT requires circulation because CD41-GFP+ and c-myb+ cells were not found in the CHT of silent heart (sih) morphants (Murayama et al. 2006), which lack blood flow due to disruption of cardiac tropomyosin. Seeding of the thymus occurs by both circulation and
migration through the mesenchyme from either the AGM or the CHT. These cells then proliferate and generate rag1+ T-lymphocyte precursors (Murayama et al. 2006; Kissa et al. 2008).

Recently, a novel route was found for HSCs to seed the kidney. Using time-lapse imaging of CD41-GFP and c-myb-GFP transgenic animals, hematopoietic cells were observed crawling along the pronephric tubules from the AGM toward the anterior glomeruli (Bertrand et al. 2008). The migration appears to be circulation-independent given that it remains intact in sih morphants. These migrating cells were found to express runx1 and the pan-hematopoietic marker CD45 as well, suggesting that they are stem cells (Bertrand et al. 2008).

ADVANCES IN ZEBRA FISH FOR THE STUDY OF HSCs

The power of the zebra fish model lies in the ability to perform large-scale genetic screens. Both forward genetic screens, using N-ethyl-N-nitrosourea (ENU) (Haffter et al. 1996; Weinstein et al. 1996) or insertional mutagenesis (Amsterdam et al. 1999), and reverse genetic screens, using targeted induced local lesions in genomes (TILLING) (Wienholds et al. 2002) and morpholinos, have been described. Zebrafish also provide a unique platform for conducting in vivo whole-animal chemical screening to identify novel compounds of therapeutic value. Although a number of hematopoietic mutants have been isolated from previous screens, a small number affect HSCs (Fig. 2); continued screening thus has the potential to generate new mutations that affect other pathways or regulators of HSC development. More precise genetic manipulations in zebra fish can be accomplished with transgenic fish, for example, using the heat shock Cre/lox system to induce tissue-specific gene expression (Feng et al. 2007). Recently, zinc finger nucleases have been used successfully for inducing targeted mutations in the germ line (Meng et al. 2008). The combination of these molecular methods makes it feasible to perform very precise genetic manipulations in zebra fish.

Assays that test stem cell function have also been developed in zebra fish. The major blood lineages (erythroid, lymphoid, myeloid, and precursors) can be segregated by flow cytometry using only the forward scatter and side scatter profiles (Traver et al. 2003); thus, multilineage reconstitution can be measured in irradiation-recovery or transplantation assays (Fig. 3, top left and bottom) (Traver et al. 2004). This method was used to determine the contribution of Notch and prostaglandin signaling to adult hematopoiesis, because these were both identified initially as regulators of HSC induction during embryogenesis (Burns et al. 2005; North et al. 2007). A brief dose of Notch activation or treatment with PGE2 enhanced marrow recovery postirradiation by expanding early multilineage precursors. runx1, imo2, scl, and even fil1 expression were significantly up-regulated in these cells (Burns et al. 2005; North et al. 2007). The effects of PGE2 were verified by limiting dilution transplantation assays of HSCs in the mouse (North et al. 2007). Although the mechanism by which these pathways enhance recovery remains unknown, they are ideal candidates for clinical use because any chemical that can enhance their signaling may have the potential to improve patient recovery posttransplantation by stimulating hematopoietic stem and progenitor cells.

Finally, visualization of HSCs in adult fish is now facilitated due to the recent development of a transparent zebrafish called casper (White et al. 2008). casper fish are doubly mutant for the nacre allele (encoding the misf1a gene) and the roy allele (encoding an unknown pigment gene), which blocks the development melanocytes and iridophores, respectively. As a result, the lack of pigmentation allows internal organs such as the heart, intestinal tube, liver, and gallbladder to be observed in vivo with the naked eye (White et al. 2008). HSC homeostasis could be studied in the casper fish within the context of the endogenous marrow niche, which has been a traditionally difficult process to observe. The ability to use these fish for examining the kinetics of stem cell homing and engraftment in the transplantation setting with resolution down to the single-cell level is currently unparalleled in other model systems (Fig. 3, top left and right).

CONCLUSIONS

Studies of HSCs in zebrafish have complemented investigations in other model organisms and have advanced our
understanding of hematopoiesis. Much of what has been learned about the signaling pathways and transcription factors involved in the development of HSCs in zebrafish and other vertebrates is highly conserved. Given that zebrafish are amenable to large-scale screens, future genetic screens will continue to uncover new mutants with interesting hematopoietic phenotypes, and whole-animal chemical screening will identify new compounds of clinical value. In vivo screens are feasible using reporter lines and mutant and transgenic strains. As zebrafish methods advance, new opportunities for revealing more details of the molecular mechanisms of stem cell regulation will arise. Finally, the knowledge gained about HSCs will likely be applicable to stem cell biology in general.

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