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Hematopoiesis

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Summary

Hematopoiesis – the process by which blood cells are formed – has been studied intensely for over a century using a variety of model systems. There is conservation of the overall hematopoietic process between vertebrates, although some differences do exist. Over the last decade, the zebrafish has come to the forefront as a new model in hematopoiesis research, as it allows the use of large-scale genetics, chemical screens and transgenics. This comparative approach to understanding hematopoiesis has led to fundamental knowledge about the process and to the development of new therapies for disease. Here, we provide a broad overview of vertebrate hematopoiesis. We also highlight the benefits of using zebrafish as a model.

Key words: Hematopoiesis, Mouse, Zebrafish

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Introduction

Hematopoiesis – the formation of blood cellular components – occurs during embryonic development and throughout adulthood to produce and replenish the blood system. Studying hematopoiesis can help scientists and clinicians to understand better the processes behind blood disorders and cancers. Furthermore, hematopoietic stem cells (HSCs) can be used as a model system for understanding tissue stem cells and their role in ageing and oncogenesis. In this article, and in the accompanying poster, we provide an overview of the process of hematopoiesis, highlighting the sites of hematopoiesis in various organisms, and the factors that regulate HSC emergence and self-renewal.

A general overview of vertebrate hematopoiesis

Blood development in vertebrates involves two waves of hematopoiesis: the primitive wave and the definitive wave (Galloway and Zon, 2003). The primitive wave, which involves an erythroid progenitor, gives rise to erythrocytes and macrophages during early embryonic development (Palis and Yoder, 2001). The

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Overview of hematopoiesis

Factors involved in the emergence of HSCs

Sites of hematopoiesis

HSC renewal

(See poster insert)

primary purpose of the primitive wave is to produce red blood cells that can facilitate tissue oxygenation as the embryo undergoes rapid growth (Orkin and Zon, 2008). In mammals and avians, these erythroid progenitor cells first appear in blood islands in the extra-embryonic yolk sac early in development (Paik and Zon, 2010). The primitive wave is transitory, however, and these erythroid progenitors are not pluripotent and do not have renewal capability. Definitive hematopoiesis, by contrast, occurs later in development, notably at different time points in different species. In most organisms, there is a transient wave of definitive hematopoiesis that occurs in the blood islands and produces progenitors called erythroid-myeloid progenitors (EMPs) (McGrath et al., 2011; Bertrand et al., 2007). Definitive hematopoiesis later involves HSCs, which are multipotent and can give rise to all blood lineages of the adult organism. In vertebrates, definitive HSCs are born in the aorta-gonad-mesonephros (AGM) region of the developing embryo. They migrate to the fetal liver and then to the bone marrow, which is the location for HSCs in adults (Cumano and Godin, 2007).

In humans, hematopoiesis begins in the yolk sac and transitions into the liver temporarily before finally establishing definitive hematopoiesis in the bone marrow and thymus. Experiments with human embryos confirm observations in the hemangioblast, a common precursor for endothelial and hematopoietic cells. In humans, HSCs are present in close proximity to endothelial cells (Tavian et al., 2010), and flow cytometry-sorted vascular endothelial cells from fetal and embryonic human blood-forming tissues cultured over a layer of MS-5 stromal cells underwent hematopoiesis (Tavian et al., 2010). These endothelial cells were sorted from the human embryonic aorta between day 27 and day 40 of development, which is when HSCs are present in this region. Studies using transplantation of HSCs from human embryos into immune-deficient mice have confirmed that the first definitive human HSCs are born in the AGM (Ivanovs et al., 2011). The embryonic origin of hematopoiesis in humans has been reviewed by Tavian et al. (Tavian et al., 2010).

The process of blood development in zebrafish is similar to that occurring in mammals, involving waves of hematopoiesis. During gastrulation, three germ layers are generated – ectoderm, mesoderm and endoderm – and these are then specified into different tissues. Mesoderm is specified into both a dorsal fate, in which somites and the notochord arise, and a ventral fate, in which blood, the vasculature and the pronephros arise. Primitive erythroid progenitors are born in the intermediate cell mass (ICM), which is a tissue derived from the ventral mesoderm (Detrich et al., 1995). The circulation of these primitive cells begins at ~24 hours post-fertilization (hpf). EMP progenitors arise from the posterior ICM region. During the definitive wave, HSCs emerge from the ventral wall of the dorsal aorta beginning at 30 hpf (Thompson et al., 1998; Burns et al., 2002; Kalev-Zylinska et al., 2002). These HSCs migrate to the caudal hematopoietic tissue (CHT) in the posterior region of the tail (Murayama et al., 2006; Jin et al., 2007). By 3 days post-fertilization (dpf), lymphopoiesis occurs in the thymus and one day later the HSCs migrate to the kidney marrow, which is analogous to the bone marrow in mammals.

The hemangioblast: a historical perspective

An 'endothelium with hemogenic properties' was first described as being the precursor to HSCs at the end of the 1800s (reviewed by Adamo and García-Cardeña, 2012). Early observations tied the emergence of these cells to blood flow and vascular development, and, in 1965, Moore and Owen published their observation that all adult hematopoiesis was initiated in extra-embryonic tissues, mainly the yolk sac (Moore and Owen, 1965). Dieterlen-Lievre in 1975 found an intra-embryonic site for hematopoiesis by carrying out experiments in which a quail embryo was transferred into chick blastoderm (yolk sac) (Dieterlen-Lievre, 1975); when HSC formation was traced, there were only HSCs from the quail, indicating that development is intra-embryonic. These experiments led to questions and controversies concerning the origin of HSCs. In 1981, Dieterlen-Lievre and Martin reported that hematopoietic activity in the avian system was only present in the ventrolateral aspect of the aorta (Dieterlen-Lievre and Martin, 1981). In 1993, two groups, Godin and Medvinsky, identified hematopoietic progenitors that appeared in the developing aorta, at the level of the AGM, before appearing in the liver or other hematopoietic organs, demonstrating a link to the endothelium (Godin et al, 1993; Medvinsky et al., 1993). In 1997, Kennedy et al. confirmed these observations *in vitro* by demonstrating that primitive erythrocytes and other hematopoietic lineages come from a common precursor within the embryoid bodies formed from differentiated embryonic stem cells (ESCs) (Kennedy et al., 1997). These experiments were translated into the human ESC system and confirmed by Zambidis in 2008 (Zambidis et al., 2008). These and other observations led to the theory of the 'hemangioblast', which is described as a common precursor for endothelial and hematopoietic cells that retains the ability to give rise to new primitive erythroblasts. Hemangioblasts are different to 'hemogenic endothelium', which gives rise to multilineage HSCs/progenitor cells and is therefore responsible for the production of all blood cell types during definitive hematopoiesis. It is important to note, however, that there are now alternative ways to think of the term 'hemangioblast'. This terminology has been used historically in the literature, but the simple notion of a cell that divides asymmetrically with only two fates is unlikely to be accurate.

The genetic control of hematopoiesis

Genes involved in primitive hematopoiesis

Primitive hematopoiesis is largely regulated by two transcription factors, Gata1 and Pu.1 (now known as Sfpi1 in mouse; Spi1b in zebrafish), that exhibit a cross-inhibitory relationship to regulate primitive erythroid and myeloid fates. Gata1 is a master regulator of erythrocyte development (Cantor and Orkin, 2002); *Gata1*$^{-/-}$ mice die during gestation owing to failed differentiation of pro-erythroblasts into mature erythrocytes. In zebrafish, *gata1*-expressing cells also express erythrocyte-specific hemoglobin, analyzed by benzidine staining, indicating that genes encoding both alpha and beta embryonic globin (*hbbe3*, *hbbe1.1*, *hbae3* and *hbae1*) are expressed in these cells (Detrich et al., 1995). In addition to promoting erythroid-specific gene regulation, Gata1 suppresses myeloid fate; in Gata1 knockdown experiments in zebrafish, blood cells switch to myeloid cells and express myeloid-specific genes, such as *pu.1*, *mpo* (*myeloperoxidase*, now known as *mpx*; a granulocyte-specific gene) and *l-plastin* (*lcp1*). By contrast, Pu.1 is a master regulator of the myeloid cell fate, which includes macrophages and granulocytes (Scott et al., 1994). Similar to the fate switch observed in Gata1 knockdowns, Pu.1 knockdown leads to an increase in *gata1* expression in the anterior lateral mesoderm (ALM) and later these cells express *hbae1*, demonstrating their erythroid switch (Rhodes et al., 2005). As Gata1 and Pu.1 have been shown to interact physically (Cantor et al., 2002), the switch is hypothesized to occur as a result of direct competition between Gata1 and Pu.1 for target genes.

Genes involved in definitive hematopoiesis

Runx1 is a member of the runt family of transcription factors and plays an important role in hematopoiesis (Wang et al., 1996). *Runx1* knockout mice lose definitive erythroid, myeloid and lymphoid cells, indicating the importance of Runx1 in definitive hematopoiesis. In zebrafish, *runx1* expression begins at the five-somite stage in the posterior lateral mesoderm (PLM) and in neural tissues. At 30 hpf, *runx1* is expressed in the dorsal aorta. Zebrafish *runx1* seems to be dispensable in primitive hematopoiesis, but is required for definitive hematopoiesis, as highlighted by experiments in which Runx1 knockdown results in decreased lymphopoiesis (Paik and Zon, 2010). Runx1 knockdown also leads to a decrease in the expression of *cmyb*, which belongs to the myb family of proto-oncogenes (Kalev-Zylinska et al., 2002; Burns et al., 2005; Gering and Patient, 2005). In zebrafish, Cmyb expression begins at around the 10- to 12-somite stage during the primitive wave of hematopoiesis. At around 36 hpf, *cmyb* is expressed in *runx1*-expressing cells in the ventral wall of the dorsal aorta (Gering and Patient, 2005). *cmyb* is also expressed at 2 dpf in the CHT; these cells then migrate to the thymus and the pronephros (early kidney) (Murayama et al., 2006; Jin et al., 2007). Importantly, *Myb* knockout mice die owing to failure of fetal liver erythropoiesis, indicating an essential role for Cmyb in definitive hematopoiesis (Mucenski et al., 1991).

Genes associated with hemangioblast induction

During development, an array of transcription factors coordinates the development of the hemangioblast, the precursor to both primitive erythroid progenitors and endothelial cells. Within the ventral lateral mesoderm of zebrafish embryos, the ALM is a major site of primitive myelopoiesis. By contrast, cells within the PLM contribute predominantly to development of erythrocytes in addition to some myeloid cells (Liao et al., 1998; Thompson et al., 1998; Sumanas et al., 2005; Pham et al., 2007). From the two-somite stage in zebrafish, an early stage of development, cells co-expressing *tal1*, *gata2*, *lmo2*, *fli1* and *etsrp* (*etv2* – Zebrafish Information Network), which encode important transcription factors that control the expression of other genes involved in hemangioblast development, appear in both the ALM and the PLM (Paik and Zon, 2010). These cells are postulated to be hemangioblasts based on evidence indicating the requirement of these transcription factors for both endothelial and hematopoietic differentiation. Gata2, for example, is required for maintenance and proliferation of hematopoietic progenitor cells, as $Gata2^{-/-}$ mice are embryonically lethal and die from severe anemia (Tsai et al., 1994). $Tal1^{-/-}$ embryos lose the ability to undergo primitive erythropoiesis and myelopoiesis in both the zebrafish and mouse (Shivdasani et al., 1995). Endothelial differentiation is affected by Tal1 knockdown, indicating that Tal1 is also required for endothelial differentiation (Dooley et al., 2005; Patterson et al., 2005). Lmo2 acts in parallel with Tal1 and Gata2, and $Lmo2^{-/-}$ mice die due to loss in yolk sac erythropoiesis. Furthermore, Fli1 has been hypothesized to work upstream of Tal1 and Lmo2 (Zhu et al., 2005). Finally, *etsrp* is required for the vascular endothelial and primitive myeloid cells in zebrafish (Sumanas and Lin, 2006; Sumanas et al., 2008). It has a functional homolog in both mammals (Lee et al., 2008; Kataoka et al., 2011) and *Xenopus* (Neuhaus et al., 2010; Salanga et al., 2010). In mouse, knockout of *Etsrp* (*Etv2*) results in complete depletion of endothelium and blood cells, indicating that it plays a role in the induction of bipotent progenitors. These cells co-expressing *tal1*, *gata2*, *lmo2*, *fli1* and *etsrp* in the ALM and PLM of zebrafish have the ability to become either angioblasts (endothelial progenitors) or HSCs, adding further evidence to the hypothesis of the hemangioblast.

Factors regulating HSC self-renewal

There are many factors and pathways that are important for HSC renewal, but owing to space restrictions, we will focus on two important signaling pathways. There is some controversy, but there is general consensus that these pathways are important for the self-renewal of HSCs.

The role of Wnt signaling in HSC function

The Wnt family of molecules, which is known to be crucial for embryonic development (Perrimon et al., 2012), is thought to be important for HSC function. However, studies have found contradictory findings with regard to the importance of Wnt signals for normal lymphopoiesis and hematopoiesis (Reya et al., 2003; Willert et al., 2003; Kirstetter et al., 2006; Scheller et al., 2006; Trowbridge et al., 2006; Koch et al., 2008; Qiang et al., 2008), although most studies have found a positive role for Wnt in HSCs during development and regeneration. Recent findings suggest that these opposing conclusions are due to the different levels of Wnt in different experimental conditions, as reviewed by Luis et al. (Luis et al., 2011; Luis et al., 2012).

The Notch signaling pathway

The Notch pathway controls cell fate specification and pattern formation. Activation of Notch signaling has been shown to promote HSC expansion/self-renewal in both mice and humans in adult hematopoiesis (Guruharsha et al., 2012). Loss-of-function mutations, such as inactivation of Notch receptors, ligands or downstream proteins, do not affect HSC function (Cerdan and Bhatia, 2010). Populations of human cells expressing CD34 (a cell surface marker for HSCs) can be expanded with exposure to Notch ligands, resulting in >100-fold increase in the absolute number of cells, which can subsequently enhance the repopulation of immunodeficient mice. Further studies demonstrated that when Notch ligand-expanded cord blood progenitors were used in a clinical setting, there was a rapid recovery of myeloid cells, indicating rapid engraftment of *ex vivo*-expanded cells in humans (Delaney et al., 2010). Taken together, these data indicate the importance of Notch-dependent regulation of hematopoietic development and suggest that Notch activation may aid in stimulating the production of hematopoietic cells *in vitro* for use in experimental and clinical applications.

The HSC niche

The microenvironment is known to be essential for the regulation and maturation of many stem cells. The adult bone marrow niche of mice is currently the most studied HSC niche. Some studies identify the osteoblast as an important cell that interacts with HSCs in the bone marrow. Mutant mice with disrupted bone morphogenetic protein (BMP) signaling have increased numbers of osteoblasts and HSCs (Calvi et al., 2003; Zhang et al., 2003). There is evidence that vascular cells (and a vascular niche) are also important for HSC regulation (Kiel and Morrison, 2006), further highlighting that HSC activity might depend on its niche. Furthermore, it is likely that the vascular and osteoblastic niches are not physically separated by significant distances. Finally, stromal cells expressing kit ligand are also required for stem cell homeostasis (Ding and Morrison, 2013). It must be noted, however, that although the adult HSC niche is well studied, not much is known about the niches for embryonic HSC development.

The benefits of using zebrafish as a model for hematopoiesis

Zebrafish (*Danio rerio*) have emerged as a useful model organism with which to study hematopoiesis. Zebrafish can produce a large

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number of embryos per mating pair and are sexually mature by 3 months of age. Zebrafish embryos are fertilized externally allowing for *in vivo* visualization of embryogenesis, making them an especially easy model for developmental biologists. Importantly, the programs controlling hematopoiesis in the zebrafish seem to be conserved with mammals, including humans, making them a clinically relevant model system.

Genetic screens

Forward genetic screening in zebrafish has yielded interesting blood-related mutants (reviewed by Hsia and Zon, 2005) that allow researchers to tease apart hematopoietic genes and their function. For example, the *spadetail* mutant (which carries a mutation in the *tbx16* gene) has a defect in mesoderm-derived tissues, including blood, as demonstrated by a decrease in the levels of *tal1*, *lmo2*, *gata2*, *fli1* and *gata1* expression in the PLM, highlighting the importance of *tbx16* during hemangioblast regulation. The *cloche* mutant (which affects an unknown gene) has defects in the very early stages of endothelial and hematopoietic differentiation. This is demonstrated by the complete loss of *tal1*, *lmo2*, *gata1*, *l-plastin*, *mpo* and *flk1* (also known as *vegfr2* and *kdrl*) expression. Other mesoderm-derived tissues, such as the pronephros, were not affected in *cloche* mutants, leading to the hypothesis that this gene is required for hemangioblast specification. The *vlad tepes* mutant (which harbors a nonsense point mutation in the region encoding the C-terminus of Gata1) has revealed the importance of Gata1 in primitive erythropoiesis. These mutants lose expression of some erythroid genes (*gata1* and *hbae1*) after 26 hpf and die between 8 and 15 dpf. These defects can be partly explained by the inability of Gata1 in these mutants to bind to its promoters to induce expression of erythroid genes. The *mind bomb* mutant (which carries a mutation in a gene encoding a ubiquitin E3 ligase) exhibits a defect in the Notch pathway. These defects are caused by an inability to generate Delta (the Notch ligand), as the ubiquitin E3 ligase is required for Delta protein maturation. *mind bomb* mutants show decreased expression of two important definitive hematopoietic genes, *runx1* and *cmyb*, in the dorsal aorta, confirming that the Notch pathway regulates definitive hematopoiesis.

Chemical screens

Chemical screens in zebrafish using biologically active compounds have been useful for identifying novel factors that regulate HSCs. In a recent screen, for example, prostaglandin (PG) E2 was revealed as a compound that could increase stem cell induction in the AGM region, as demonstrated by increased *runx1* and *cmyb* expression. The treatment of sub-lethally irradiated adult zebrafish with dimethyl PGE2 (dmPGE2; a long-acting derivative of PGE2) enhanced hematopoietic recovery. This compound also increased engraftment of murine marrow in competitive transplantation experiments (North et al., 2007). In human clinical trials, the treatment of cord blood cells with dmPGE2 suggested an increase in long-term engraftment, demonstrating for the first time that a compound identified in experiments with zebrafish can be translated into use for humans in the clinic. Further investigation of PGE2, and other novel compounds found in such chemical screens, will hopefully improve the safety and efficacy of bone marrow transplantation for patients with leukemia, lymphoma or blood disorders.

Perspectives and future directions

The insights gained from studying zebrafish, chick, mouse and human have begun to unravel the complex processes required during hematopoietic development. Discoveries such as the HSC-promoting properties of PGE2 demonstrate the power of the zebrafish model. Recent studies indicate that Notch signaling plays an important role in promoting progenitor cell engraftment in *ex vivo*-derived cord blood cells (Delaney et al., 2010). Deciphering the mechanisms of such signaling pathways in the induction and migration of stem and progenitor cells could bring us closer to understanding the mechanisms of HSC homeostasis and response to injury. Recently identified mutations and translocations in human hematopoietic diseases and leukemia can now be modeled in zebrafish to understand the molecular mechanisms of disease progression, and this could lead to new treatments. Overall, the use of chemical and genetic screens in the zebrafish model will help us to understand the underlying mechanisms of HSC development, which is vital for the translation of discoveries into regenerative medicine applications.

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Competing interests statement
L.I.Z. is a founder and stock holder of Fate, Inc. and Scholar Rock, and a scientific advisor for Stemgent.

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A high-resolution version of the poster is available for downloading in the online version of this article at http://dev.biologists.org/content/140/12/2463.full

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