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Developmental HSC Microenvironments: Lessons from Zebrafish

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Abstract

Hematopoietic stem cells (HSCs) possess the ability to maintain the blood system of an organism from birth to adulthood. The behavior of HSCs is modulated by its microenvironment. During development, HSCs acquire the instructions to self-renew and differentiate into all blood cell fates by passing through several developmental microenvironments. In this chapter, we discuss the signals and cell types that inform HSC decisions throughout ontogeny with a focus on HSC specification, mobilization, migration, and engraftment.

Keywords

Zebrafish; Hematopoietic niche; Blood development; Hematopoietic stem cell

4.1 Introduction

Hematopoietic stem cells (HSCs) are self-renewing, multi-potent cells with the capacity to give rise to all mature blood lineages. HSCs first acquire these skills during embryonic

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development as they move among a variety of niches, each providing different signals that help educate embryonic HSCs. Much of the earlier work on HSC development focused on the intrinsic factors dictating fate choices (reviewed in Orkin and Zon (2008)), but studies over the last decade have uncovered a wealth of information on the cell types and signals of the microenvironment that inform HSC decisions during ontogeny.

The use of the zebrafish (*Danio rerio*) to understand early patterning and organogenesis has exploded since the seminal genetic screens initiated by Christiane Haffter (1996). Since that time, over 600 papers have been published studying zebrafish hematopoiesis. Zebrafish possess many advantages that make it an excellent model to study developmental hematopoiesis. Hematopoietic cell types and gene programs are highly conserved from zebrafish to humans. The small size and extensive fertility of zebrafish are ideal attributes for performing unbiased forward genetic and chemical screens in a vertebrate model. Combined with the natural optical clarity of embryos, the use of transgenic zebrafish that express fluorescent proteins in a cell-type or tissue-type specific manner greatly facilitate live imaging of cells within their native microenvironment (reviewed in Zhang and Liu (2011)). Additionally, with the advent of CRISPR/Cas9 genome editing (Hwang et al. 2013) and tol2-based transgenesis (Kawakami et al. 2004), reverse genetic approaches are now also commonly used in zebrafish. These advantages have made zebrafish a rapidly emerging model system for the study of hematopoiesis.

In all vertebrates including zebrafish, hematopoiesis occurs in sequential waves (Fig. 4.1a). The earliest hematopoietic cells emerge during the primitive wave, which gives rise to mostly erythroid and myeloid cells arising from the lateral mesoderm during the first 24 h post fertilization (hpf) (equivalent to blood formation in the mammalian yolk sac) (reviewed in Robertson et al. (2016)). The final wave gives rise to definitive HSCs that persist into adulthood to maintain hematopoiesis throughout the organism's life. HSCs originate from the posterior lateral mesoderm (PLM). The cells emerge from specialized endothelial cells termed hemogenic endothelium found within the ventral wall of the dorsal aorta (the equivalent to the aorta-gonad-mesonephros (AGM) in mammals, Fig. 4.1b). Induction of HSCs is first detectable by expression of the transcription factors *gata2b* and *runx1* in the hemogenic endothelium around 24 h post fertilization (hpf) (Burns et al. 2002; Butko et al. 2015; Kalev-Zylinska et al. 2002). The process of HSC conversion from endothelium is termed the endothelial-to-hematopoietic transition (EHT) and involves the budding of HSC from the aortic endothelium (Bertrand et al. 2010; Boisset et al. 2010; Kissa and Herbomel 2010). Around 48–72 hpf, nascent HSCs migrate from the dorsal aorta (DA) via the circulation to an intermediate hematopoietic organ known as the caudal hematopoietic tissue (CHT) (the fetal liver equivalent in mammals) (Kissa et al. 2008; Murayama et al. 2006) (Fig. 4.1b). The CHT is the first site where HSC expand and differentiate into mature blood cells. The majority of HSCs then re-enter circulation and seed their final destination in the kidney marrow (equivalent to the bone marrow in mammals).

Each stage of HSC development is regulated by extrinsic cues from the local and systemic microenvironment. In this chapter, we will review the newest findings on the niche factors critical for early hemogenic endothelial induction as well as HSC specification, migration, and expansion. Understanding the key signals during ontogeny is not only important to

developmental biologists, but could also have great clinical significance. Many of the players in embryonic niches are also important in adult HSC biology, thus new discoveries from development could enlighten the microenvironmental requirements necessary for maintaining adult HSC homeostasis. Moreover, uncovering how HSCs are normally produced in the embryo will help improve attempts to generate patient-specific HSCs from pluripotent stem cells in vitro (reviewed in Kyba and Daley (2003)).

4.2 Somite-Derived Niche Signals Promoting HSC Production

HSCs arise from specific mesoderm positioned in the posterior aspect of the embryo and lateral to somitic mesoderm termed the posterior lateral mesoderm (PLM) (Ho and Kimmel 1993). Recent work has demonstrated that this juxtaposition is critical for the early events of hemogenic endothelial and HSC specification. Specifically, several groups demonstrated that signals and cells emanating from the somite are required for proper HSC formation within the zebrafish embryo (Clements et al. 2011; Kim et al. 2014; Kobayashi et al. 2014; Lee et al. 2014; Nguyen et al. 2014; Pillay et al. 2016; Pouget et al. 2014).

During embryogenesis, PLM cells begin as bilateral strips along the lateral aspect of the embryo and then migrate medially (reviewed in Davidson and Zon (2004)). After migration, the endothelial and hemogenic endothelial progenitors within the PLM will form the dorsal aorta. During this journey, cells in the PLM make direct physical contact with the somites, a connection that Kobayashi and colleagues showed were essential for proper HSC formation (Kobayashi et al. 2014). The appropriate interaction between these cells is needed for proper transmission of Notch signaling, an important pathway for several steps of HSC formation (reviewed in Butko et al. (2016)). The Notch signaling pathway is well known to play a fundamental role in regulating cell fate decisions among adjacent cells through signaling between a transmembrane Notch receptor and membrane-spanning ligands on neighboring cells (Artavanis-Tsakonas et al. 1999). Thus, direct cell contact is the main modality for transmission of Notch signaling. In zebrafish, PLM cells expressing the cell-adhesion factor *Jam1a* interact with somite cells expressing *Jam2a* en route to the DA (Kobayashi et al. 2014). Knockdown of *jam1a* led to a decrease in Notch signaling and a decrease in HSC formation, but upon forced activation of Notch, specifically in endothelial precursors, HSC levels could be rescued.

Several additional studies have implicated Notch signaling in the early somitic niche. The non-canonical Wnt ligand, Wnt16, is highly expressed in somites and promotes HSC formation in a non-cell autonomous manner (Clements et al. 2011) (Fig. 4.2). Mechanistically, Wnt16 regulates the expression of two Notch ligands, *dlc* (*delta-c*) and *dld* (*delta-d*), which promotes Notch signaling for HSC induction. The relevant Notch receptor was later shown to be Notch3, which is expressed within the dorsal aorta but also earlier in the somites (Kim et al. 2014). Three out of four Notch receptors (Notch1a, Notch1b, and Notch3) are important for HSC formation, but only Notch3 function is needed during the stage when the PLM receives somite-derived signals. Epistasis analysis between Notch3 and Wnt16 demonstrated that Wnt16 lies upstream of Notch3 function within the somite (Kim et al. 2014).

Fibroblast growth factor (FGF) signaling provides a bridge between Wnt16 and Notch function during HSC emergence (Lee et al. 2014). Specifically, FGF signaling is required in the developing zebrafish somite for HSC formation during mid- somitogenesis (14–17 hpf), but not in the pre-endothelial PLM (Fig. 4.2). During this timeframe, FGF signaling informs HSC specification by relaying signals between Wnt16 and Dlc via the activity of its receptor, Fgfr4. Slightly later in development at the 23 somite-stage (~20.5 hpf), FGF signaling is a crucial player in establishing the HSC microenvironment around the dorsal aorta by regulating BMP pathway activity in the sub-aortic mesenchyme (Pouget et al. 2014). By modulating BMP pathway activity via transcriptional inhibition of *bmp4* and activation of the BMP antagonists, *noggin2* and *gremlin1a*, FGF provides a carefully regulated axis which functions as a developmental switch. Collectively, these data indicate that FGF provides a precise temporal mechanism to both activate and inhibit its own signaling activity to modulate HSC formation.

Work from the Currie laboratory found that not only does the somite provide signals as the PLM migrates, but cells derived from the somite can migrate and form a supportive niche within the dorsal aorta (Nguyen et al. 2014). Zebrafish *choker* mutants are loss-of-function for the *meox1* gene, which encodes a homeobox factor normally expressed in the early somite. Interestingly, loss of *meox1* resulted in an increase in HSC levels within the dorsal aorta. Nguyen and colleagues demonstrated that *choker* mutants also have an expansion of endotomal cells—a specialized subset of somitic cells that they showed give rise to endothelial cells that line the DA (Fig. 4.2). Although the somite-derived cells are known to contribute to the DA in other organisms (Jaffredo et al. 2013), findings from this study were the first evidence of its existence in zebrafish. Important to the hematopoietic field is the discovery that the endotomal cells are not hemogenic endothelial cells, but rather form an endothelial niche that promotes HSC induction from adjacent endothelial cells in a Cxcl12 (CXC chemokine ligand 12)-dependent manner. As a complement to these findings, Pillay et al. further characterized somite-derived signaling factors and found that retinoic acid (RA) is required prior to DA formation to regulate components of the Notch and Cxcl12 chemokine signaling pathways (Pillay et al. 2016).

The above studies demonstrate that the somite is a dynamic and integral niche for the earliest events of HSC formation in zebrafish. As HSCs originate in similar anatomical locations in mammals, it is highly probable that the influence of the somite on HSC development will be conserved from zebrafish to mammals. The recent identification of somite-derived cells within the developing dorsal aorta supports this idea (Jaffredo et al. 2013). Undoubtedly, our understanding of the influence of the somitic environment on HSC development is at its infancy.

4.3 Vascular Forces Influence HSC Emergence

HSCs emerge within blood vessels, but the functional relevance of this HSC birth site had long been a mystery (Dieterlen-Lievre 1975; Medvinsky and Dzierzak 1996; Walmsley et al. 2002). Research in multiple vertebrate systems has revealed that the environment of the vasculature, specifically the dorsal aorta, is a vital niche for emerging HSCs. Signals important for vessel growth and those that are key for vascular tone are not only critical for

proper vascular development, but in many instances, play an additional role in HSC induction and survival.

VEGF (Vascular Endothelial Growth Factor) is a pro-angiogenic signal that promotes sprouting of intersegmental blood vessels and HSC formation (Connolly et al. 1989; Habeck et al. 2002). During development, there are a variety of inputs that regulate VEGF, keeping it at the appropriate level. Moreover, VEGF signaling feeds into additional downstream pathways important for HSC production. Carroll et al. uncovered estrogen as a titrator of Vegf signaling within the dorsal aorta endothelial niche in zebrafish (Carroll et al. 2014) (Fig. 4.2). In mammals, endogenous estrogen levels are generally low during early pregnancy, but increase as gestation goes on (Tulchinsky et al. 1972). There are several pieces of evidence suggesting that there are mechanisms intentionally in place to limit the amount of estrogen exposure on the fetus, including the expression of 17 β -hydroxysteroid dehydrogenase type 2, which degrades estrogen (E2) in the umbilical arteries and veins (Simard et al. 2011). During the first trimester of pregnancy maternal use of estrogen hormone supplementation is associated with an increased risk of infant acute leukemia, indicating that in utero exposure to estrogenic compounds may negatively impact fetal hematopoiesis (Pombo-de-Oliveira et al. 2006). Carroll and colleagues uncovered one mechanism to explain the negative impact of E2 on embryonic hematopoiesis (Carroll et al. 2014). They showed that excessive exposure to E2 from early somitogenesis (~12 hpf) until 24 hpf, the window of hemogenic endothelial specification, significantly decreased the formation of *runx1*+ AGM HSCs. Activation of Notch signaling via overexpression of *vegfa* rescued hemogenic endothelium specification and HSC formation defects from excess E2. Overall, they show that maternally deposited E2 antagonizes the ventral limit of Vegf and downstream Notch signaling, allowing for the correct assignment of hemogenic endothelium and subsequent HSC formation (Carroll et al. 2014).

Prior work showed that TGF β (Transforming Growth Factor β) signaling also regulates VEGF levels (reviewed in Holderfield and Hughes (2008) and Massague and Gomis (2006)). New work from the Patient lab showed that Vegf signaling could also regulate the TGF β pathway (Monteiro et al. 2016) (Fig. 4.2). During zebrafish development, Vegfa signaling through its receptors Kdr (Kinase-insert domain receptor) and Kdr-like promote the expression of the TGF β ligands *tgfb1a* and *tgfb1b* in endothelial cells. These ligands bind Tgf β Receptor 2 (Tgf β R2) to induce the signaling cascade. Monteiro and colleagues demonstrate that decreasing levels of Tgf β R2 resulted in a severe decrease in expression of the HSC markers *runx1* and *gata2b* at 26–28 hpf but had no effect on endothelial or arterial markers, suggesting that HSC emergence is impaired at a step after dorsal aorta specification (Monteiro et al. 2016). Expression of the Notch ligand *jagged1a* (*jag1a*) downstream of Tgf β R2 activation was shown as a critical mediator needed for expression of the HSC transcription factors *runx1* and *cmyb*.

These data suggest that TGF β signaling is a positive regulator of HSC induction, but another group found that excessive TGF β could suppress HSC formation. In a study of the transcriptional elongation mutant *spt5^{m806}* (*suppressor of Ty-5 homolog*), Yang and colleagues showed that loss of transcriptional pausing lead to elevated TGF β signaling, which was detrimental to HSC formation (Yang et al. 2016). Treatment of *spt5^{m806}* mutants

with the TGF β inhibitors SB505124 or LY2157299 restored HSCs in these embryos. Additionally, they showed that elevating TGF β signaling in wild-type embryos via expression of a constitutively-active TGF β R1/ALK5 actually diminished HSC levels (Yang et al. 2016). The differences between these studies could be the differences between TGF β R1 and TGF β R2 signaling or could demonstrate that HSC emergence requires a balanced level of signaling. In adult HSCs, there are noted differences in how distinct HSC subtypes respond to different levels of TGF β ligands (reviewed in Blank and Karlsson (2015)). It is also known that excess TGF β R1 signaling can lead to hematologic defects including cytopenias as observed in myelodysplastic syndromes (Zhou et al. 2008). TGF β receptors can act as homodimers or heterodimers (reviewed in Blank and Karlsson (2015)). The different responses to TGF β receptor perturbation could therefore represent differences in receptor dimerization, downstream signaling, levels, or a combination of all three.

In addition to VEGF-regulated signaling, the biomechanical forces present in blood vessels were discovered to control embryonic HSC formation. Seminal work from the Zon, Daley, and Garcia-Cardena labs showed that blood flow-induced physical forces are critical to promote HSC production from endothelial cells both in murine and zebrafish models (Adamo et al. 2009; North et al. 2009) (Fig. 4.2). Specifically, they found that shear stress stimulated endothelial production of nitric oxide (NO), which induced HSC emergence. Through studies in zebrafish mutants devoid of a robust heartbeat and therefore possessing poor blood flow, Wang et al. later went on to show that the Kruppel-like transcription factor Klf2a directly regulates the NO signaling pathway allowing for HSC induction (Wang et al. 2011). KLF2 (or the zebrafish paralog Klf2a) is a zinc-finger transcription factor expressed in endothelial cells that is a known mediator of hemodynamic forces created by blood flow (Dekker et al. 2002; Lee et al. 2006). Several previous studies showed that KLF2 activates the expression of endothelial NO synthase (eNOS), which is a fundamental determinant of cardiovascular homeostasis, including systemic blood pressure, vascular remodeling, and angiogenesis (Dimmeler et al. 1999; Groenendijk et al. 2007; Lin et al. 2005; Parmar et al. 2006). In zebrafish development, Liu and colleagues showed that *klf2a* expression was induced by blood flow, and that Klf2a indeed regulated expression of the eNOS genes *nos1* and *nos2b* in vivo. They demonstrated that activation of the NO signaling pathway was an important downstream mediator of Klf2a as treatment with the NO donor SNAP (S-Nitroso-*N*-Acetyl-d,l-Penicillamine) partially rescues artery maturation and HSC production in *klf2a*-deficient embryos (Wang et al. 2011).

HSCs remain adjacent to blood vessels in the adult niche (reviewed in Crane et al. (2017)). In particular HSCs in the bone marrow are in close proximity to arterioles, which have different blood flow properties compared to venous or sinusoidal vessels (Kunisaki et al. 2013). Of note, HSC localization in the murine liver corresponds to the change in blood flow that occurs in the portal system after birth (Khan et al. 2016). These findings indicate that biomechanical forces are not only influencing HSC emergence, but likely play a function later in the life of a HSC.

4.4 Inflammatory Signaling Regulates HSC Emergence

Adult HSCs can proliferate in response to inflammatory cues from systemic infection or myeloablation and differentiate to replace lost effector immune cells (Baldrige et al. 2010; Essers et al. 2009; Feng et al. 2008; Kobayashi et al. 2015; Takizawa et al. 2011, 2012). This response of HSCs is not a secondary outcome to the loss of immune cells, but rather a direct response to inflammatory cytokines (Baldrige et al. 2010; Essers et al. 2009). More recently, similar cytokine signaling pathways have been found to play a critical role during embryonic HSC production independent of infection, a process termed sterile inflammation (Espin-Palazon et al. 2014; He et al. 2015; Li et al. 2014; Lim et al. 2017; Sawamiphak et al. 2014; Stachura et al. 2013). Specifically, roles for cytokines including tumor necrosis factors (TNFs) like TNF α and TNF β , interferons (IFN) like IFN α and IFN γ , interleukins (IL) like IL-6 and growth factors like granulocyte colony stimulating factor (G-CSF) appear to be essential for definitive HSCs (Fig. 4.2).

NF κ B (Nuclear factor κ -light-chain enhancer of activated B core) is a transcription factor stimulated by a variety of pro-inflammatory cytokines such as TNFs and toll-like receptor (TLR) agonists such as the bacterial pathogen-associated molecular pattern (PAMPs) lipopolysaccharide (LPS). TNF α binds to its receptors TNFR1 or TNFR2 to activate NF- κ B (reviewed in Aggarwal et al. (2012) and Faustman and Davis (2010)). In zebrafish, Espin-Palazon et al. showed that Tnf α signaling specifically through Tnfr2 is required for definitive hematopoiesis (Espin-Palazon et al. 2014). Prior work showed Tnfr2 is also important for vascular development, but the hematopoietic requirement of Tnfr2 could be uncoupled from the vascular role by titrating the amount of knockdown of *tnfr2* (Espin et al. 2013). Tnf α -induced expression of *jag1a* activated Notch1a signaling required within endothelial cells to promote HSC formation. He et al. showed that NF κ B-stimulation through Tlr4 is also utilized in both zebrafish and mice during definitive hematopoietic induction (He et al. 2015). Like Tnfr2, Notch signaling is a major downstream effector of Tlr4-regulated HSC production.

Type I and II Interferons are released in response to infection and stress and signal through the Interferon α or Interferon γ receptors, respectively (reviewed in Baldrige et al. (2011)). Work from the Speck, North, and Stainier labs demonstrated that both Type I and II Interferons are employed during zebrafish and murine development to promote HSC formation (Li et al. 2014; Sawamiphak et al. 2014). Expression of the ligand *ifn γ* and the Interferon γ receptor *crfb17* (*cytokine receptor family b17*) are positively regulated by Notch signaling and blood flow (Sawamiphak et al. 2014). These data place the Interferon γ pathway downstream from early endothelial niche signals, and likely downstream of the effects of Tnf α - and Tlr4- mediated NF κ B signaling.

Myeloid effector cells are some of the main producers of pro-inflammatory cytokines, thus the researchers examining the role of inflammatory signaling in embryonic HSC formation also assessed the contribution of myeloid cells on HSC development (Espin-Palazon et al. 2014; He et al. 2015; Li et al. 2014). Primitive myelopoiesis precedes HSC emergence, thus early neutrophils and macrophages are present to interact with precursors to HSCs. Ablation of all primitive myelopoiesis via decreasing the levels of the master myeloid transcription

factor *pu.1* resulted in lower HSC formation, suggesting these cells could be early HSC niche cells and a key source of pro-inflammatory cytokines during development (Espin-Palazon et al. 2014; He et al. 2015; Li et al. 2014). Expansion of primitive neutrophils increased HSC numbers in a *Tnfa*-dependent manner, while the effects of Interferon γ could be more dependent on primitive macrophages (Espin-Palazon et al. 2014; Li et al. 2014).

In addition to classic myeloid effector cytokines, other inflammatory pathways were also recently identified to modulate HSC formation. Through a chemical screen, the Zon lab found that adenosine signaling could increase HSC numbers (Tamplin et al. 2015) (Fig. 4.2). The elevation in HSC levels was not due to expansion of existing HSCs, but rather an increased production of HSCs from hemogenic endothelium (Jing et al. 2015). Extracellular adenosine binds to the transmembrane adenosine receptors A_1 , A_{2A} , A_{2B} , or A_3 (reviewed in Sousa and Diniz (2017)). The A_{2b} adenosine receptor was shown to be the main receptor important for the effect on HSC formation. They showed that endothelial-expressed A_{2b} activates the cAMP (cyclic Adenosine monophosphate)–PKA (Protein Kinase A) pathway, which up-regulates expression of the chemokine *cxc18* (*il-8*), which in turn promotes the emergence of HSCs from the endothelium (Jing et al. 2015). CXCL8, also known as neutrophil chemotactic factor, induces chemotaxis and phagocytosis in neutrophils at sites of infections, and plays a major role in immunity and inflammation (reviewed in Kobayashi (2008)). Later in the chapter we will discuss an additional role for Cxcl88 signaling in HSC migration.

Adult HSCs must respond to an ever-changing milieu in animals exposed to life stressors, such as hematologic injuries and infections. It was long thought that this ability arose later in life when animals first encounter these stressful situations, but new data from studies of zebrafish embryogenesis indicate that HSCs are exposed to and utilize pro-inflammatory signals early in life. In addition to the utilization to promote HSC formation, it is possible that the early exposure to pro-inflammatory signaling is part of a HSC's "education" and that perturbation of these pathways in early life could have long-lasting impacts on adult HSC function.

4.5 Niche Signals from the Nervous System and Neural Crest

Groundbreaking work from the Frenette lab was among the first to demonstrate that the nervous system provided regulatory signals to the bone marrow HSC niche (Mendez-Ferrer et al. 2008). This early work showed a function specifically for nerves from the peripheral nervous system (PNS), which are derived from the neural crest (NC) (reviewed in Bronner and Simoes-Costa (2016)). NC cells are a migratory neural-ectoderm-derived cell population that, depending on their location within the developing embryo, gives rise to neurons, pigment cells, glia and endocrine cells of the parasympathetic and sympathetic nervous system. New work in the zebrafish uncovered a role for trunk NC in HSC formation (Damm and Clements 2017) (Fig. 4.2). Using time-lapse confocal microscopy in transgenic fluorescence reporter lines, Damm and Clements were able to demonstrate that NC precursors, in particular those arising from the trunk NC, migrate to and physically associate with the DA just prior to the initiation of HSC production. This ability of the NC to directly migrate from the neural tube to the DA is dependent on platelet-derived growth factor

(PDGF) signaling. Perturbing the signaling cascade or the migratory path of the trunk NC removed these cells from the HSC microenvironment and negatively impacted HSC emergence. Future studies will be informative in discerning the role of the embryonic NC on HSC specification signals, but the close proximity of NC to emerging HSCs in the developing zebrafish suggest that short-range signaling through PDGF contributes to the HSC niche. Recently, Lim and colleagues found that PDGF acts downstream of Hif1 α and that it induces a pro-proliferative effect on HSCs via IL-6 signaling (Lim et al. 2017). Together these studies suggest NC precursors could crosstalk with pro-inflammatory signals during HSC emergence.

In addition to neural crest inputs, recent studies suggest that the central nervous system (CNS) also provides environmental cues to HSCs (Kwan et al. 2016; Pierce et al. 2017)}. Kwan et al. uncovered an early role for the hypothalamic-pituitary-adrenal (HPA) axis in HSC emergence (Kwan et al. 2016). Through a prior chemical screen for regulators of HSC development, they identified serotonin as a positive regulator of stem cell formation (North et al. 2007). Serotonin is produced both in the CNS and the periphery and acts as a neurotransmitter to both CNS and PNS neurons (reviewed in Barnes and Sharp (1999)). Within the CNS, serotonin stimulates neurons in the hypothalamus activating the HPA cascade. This axis is a relay system among three endocrine glands that results in the balanced production of many hormones regulating diverse body processes, including digestion, the immune system, and energy storage and expenditure (reviewed in Del Rey et al. (2008)). One of the main hormones released by the adrenal gland in response to HPA stimulation is glucocorticoid. In zebrafish developmental hematopoiesis, GCs are the main downstream effectors mediating the positive effects of serotonin on HSC formation. Studies in the murine system demonstrate that the HPA axis and GC production can also modulate HSC mobilization, indicating a conserved role for the CNS in HSC regulation (Pierce et al. 2017).

4.6 Cellular Constituents Involved in HSC Engraftment of the CHT Niche

After their emergence, HSCs migrate to different locations at discrete developmental time points where they receive necessary inputs as part of their “education” to become fully functioning adult HSCs. Movement to these different niches is necessary as conditions that hinder HSC seeding of developmental microenvironments result in hematopoietic defects (reviewed in Karpova and Bonig (2015)). The stages involved in HSC migrations during development are similar to the steps in adult HSC mobilization and engraftment: (1) escape the current niche, (2) travel to and seed the next niche, and (3) grow and differentiate within the new niche. Studies of developmental HSC migrations and niche interactions thus hold great potential to inform human HSC transplant biology.

Visualization of HSC movements within and in between their native microenvironments is greatly enhanced in transparent zebrafish. Murayama and colleagues were among the first to image the migration of HSCs from the DA to the CHT, and established this anatomical location as the secondary niche for HSCs (Murayama et al. 2006). Since then, many groups have uncovered cellular constituents and vital signals regulating the stages of CHT engraftment. To understand the first step, how HSCs escape the DA niche, Travnickova et al.

used in vivo real time imaging to examine the dynamic interactions between emerging HSCs and macrophages derived from the primitive wave of hematopoiesis and demonstrated the importance of this niche component in facilitating HSC mobilization (Travnickova et al. 2015) (Fig. 4.3a). By secreting matrix metalloproteinases (Mmps), these primitive macrophages are able to remodel the extracellular matrix, allowing HSCs to migrate through the AGM stroma, and begin to mobilize towards the CHT. Coupled with the studies on inflammatory signaling, these studies demonstrate that depending on developmental time point, primitive macrophages could therefore promote HSC production and expansion as well as migration.

To gain insight into the cellular events critical for CHT seeding, Tamplin et al. used high-speed, time-lapse imaging to monitor the dynamic interplay between HSCs and the CHT niche elements (Tamplin et al. 2015). They observed extensive vascular remodeling once HSCs enter into the CHT microenvironment. In a process they termed “endothelial cuddling,” a cluster of ECs surrounds a single HSC (Fig. 4.3b). Within this “cuddle” niche, HSC are also in contact with a single stromal cell, and this interaction appeared to regulate the plane of cell division of the HSC. To uncover pathways regulating HSC-CHT interplay, they also performed a chemical screen and identified a novel drug lycorine, which is characterized as a putative anti-inflammatory molecule. Treatment of zebrafish with lycorine promoted HSC-CHT niche interactions by inducing significant gene expression changes that alter the adhesive properties of ECs and ultimately lead to a long-term increase in HSC number from early development to adulthood.

The CHT niche is comprised of two main cell types: the venous endothelial cells of the caudal vein (CV) plexus and stromal fibroblast reticular cells (FRCs). The origin of the FRCs was unknown until a recent study by Murayama et al., where they discovered that they in fact originate from the ventral border of the caudal somites, through an epithelial-to-mesenchymal transition (EMT) mechanism (Murayama et al. 2015) (Fig. 4.3b). In zebrafish *olaca* mutants, definitive hematopoiesis is greatly diminished due to a drastically compromised ability of HSCs to remain and differentiate within the CHT. However, this was not due to a defect within the HSCs, but rather in the CHT niche and its defective maturation of FRCs. The defective gene in *olaca* mutants is the nascent polypeptide-associated complex alpha subunit (NACA) gene whose function is to bind to nascent polypeptides on the ribosome and act as a chaperone system to ensure proper folding of emerging proteins (Kirstein-Miles et al. 2013; Luring et al. 1995). Its deficiency in mammals has been associated with ER stress and the UPR, which can ultimately trigger apoptosis (Hotokezaka et al. 2009). Given this role, the authors investigated the role of ER stress-induced UPR/apoptosis during CHT niche formation in *olaca* mutants and observed that chemical treatment with chaperones rescued HSC levels and reduced cell death within the CHT niche (namely the stromal cells). Along with the work demonstrating the role of somites in the emergence of HSCs (Kobayashi et al. 2014; Nguyen et al. 2014), this work demonstrates a second contribution of somitic mesoderm-derived stromal cells within the CHT niche. Many non-hematopoietic mesodermal cells within bone marrow are derived from somitic mesoderm. Thus, although adult HSCs within the bone marrow are no longer in contact with developing somites or muscle, a conserved crosstalk between these two mesodermal compartments likely remains important into adulthood.

4.7 Signals that Regulate CHT Engraftment

The best characterized players in the adult HSC niche are the chemokine CXCL12 and the growth factor Stem Cell Factor (SCF) (reviewed in Crane et al. (2017)). CXCL12-mediated signaling is one of the main targets for therapeutic mobilization of HSCs (reviewed in Karpova and Bonig (2015)). Chemokine downregulation also plays a role in mobilizing HSCs from their initial site within the DA (Zhang et al. 2011). Zhang et al. showed that HSC egression from the DA was impaired in a zebrafish mutant *cmyb^{hkkz3}*, due to upregulated expression of *Cxcl12* (Fig. 4.3a). The *cmyb^{hkkz3}* mutants can initiate definitive hematopoiesis normally, but the HSCs accumulate in the DA and don't egress to begin the migration and seeding process, collectively indicating that C-Myb is required to down-regulate *Cxcl12* signaling and promote HSC mobilization. Based on this model, high levels of *cxc112* expression within the HSC niche are required to prevent nascent HSCs from mobilizing too early from the DA. As HSCs develop and mature, *cxc112* expression decreases to facilitate their release and migration to the CHT. Interestingly, high levels of *cxc112* and *cmyb* are observed in the *meox1/choker* mutants that have expanded endotomal- contribution to the DA, suggesting the somite-derived cells could play a role in the crosstalk of C-Myb and *Cxcl12*.

Cbfb, a non-DNA-binding subunit of the core-binding factor (Cbf), is also required for the release of HSCs from the AGM into circulation (Bresciani et al. 2014) (Fig. 4.3a). Similar to the *cmyb^{hkkz3}* mutant, *cbfb* knockout zebrafish mutants show defects in HSC escape from the DA niche. In *cbfb* mutants, nascent HSCs emerge unaffected, but do not seed the CHT. This uncouples the role of CFBF from its interacting partner RUNX1, a key transcription factor that regulates the emergence of HSCs from the DA, and provides it an independent and temporally regulated function in HSC development. The downstream mediator of this egress defect in *cbfb* mutants is unknown, but given the importance of chemokine signaling in directing HSC migrations, it is possible that Cbfb regulates expression of chemokines in a model analogous to cMyb-Cxcl12 identified by Zhang et al.

As with egression of HSCs from the DA to the CHT, chemokine signaling is also a vital component of the hematopoietic niche that enhances CHT colonization. By analyzing the CHT of 72 hpf zebrafish embryos, Blaser et al. observed that the chemokine *Cxcl8* and its receptor *Cxcr1* are positive regulators of HSC colonization (Blaser et al. 2017) (Fig. 4.3b). Mechanistically, *Cxcr1* signals in a positive feedback loop to enhance CHT residency time and endothelial cell "cuddling" (an HSC- niche interaction described above), resulting in an increased mitotic rate and expanded pool of HSCs. Using a parabiotic zebrafish system they were able to show that *Cxcr1* acts non-autonomously to promote HSC engraftment by directly altering the vascular niche.

Cytokine signaling from granulocyte-colony-stimulating factor (G-Csf) has also been reported to positively regulate the expansion of embryonic HSCs in the developing CHT (Stachura et al. 2013). The G-Csf receptor *gcsfr* is expressed starting as early as 6 hpf and up through 72 hpf, indicating it is active during both waves of hematopoiesis. The two ligands of *Gcsfr*, *gsfa* and *gsfb* are expressed at low levels at 6 hpf with increasing levels over time during development through 72 hpf. By modulating *Gcsf* levels through gain and loss of

function experiments, Stachura et al. demonstrated that the number of HSCs in the CHT at 48 hpf directly correlated with Gcsf levels (Fig. 4.3b). Clinically, G-CSF is used to promote granulopoiesis and to promote HSC mobilization, thus it is possible that embryonic Gcsf could also play a role in developmental HSC migrations.

WNT (Wingless/INT) signaling is a well characterized pathway in regeneration and stem cell formation, but recent work from the Traver and Willert labs has demonstrated a new role for the WNT pathway in HSC migration to secondary sites such as the CHT (Grainger et al. 2016). In their study, Grainger and colleagues depicted how early Wnt signals, specifically Wnt9a, from the developing aorta, prior to 20 hpf, are required for HSCs to undergo an expansion event at 31 hpf. HSC loss when *wnt9a* levels are diminished persists to later stages of embryonic hematopoiesis, including CHT seeding, which the authors show is due to an accumulation of G1-arrested endothelial cells, preventing the initial HSC amplification needed to drive later expansion and seeding.

4.8 Conclusions and Perspectives

During development HSCs acquire the skills they will need throughout the life of an organism: self-renew, differentiate, regenerate, and migrate. These attributes are mediated via the integration of stem cell intrinsic programs and extrinsic microenvironmental signals. Using the imaging and genetic approaches afforded in the zebrafish system, researchers have defined niche components involved in hemogenic endothelial induction as well as HSC emergence, proliferation, mobilization, and engraftment. Although each stage of HSC development occurs in distinct anatomical locations, common themes are emerging among all of the developmental niches. Myeloid effector cells, endothelial cells, somite-derived and neural crest-derived cells serve as niche constituents beginning in the earliest stages of HSC ontogeny and into adulthood. The HSC-supportive cells are also developing and will therefore provide different signals depending on context. For example, granulocytes and macrophages provide inflammatory signaling which promotes HSC emergence, but macrophages also aid in HSC mobilization from the DA via secretion of MMPs (Espin-Palazon et al. 2014; He et al. 2015; Li et al. 2014; Travnickova et al. 2015).

The preserved juxtaposition of HSCs and niche constituents from emergence through adulthood also suggests that the signals employed during development have conserved roles in adult HSC biology. For example, interferon signaling is involved in HSC specification and then re-used in adulthood to promote HSC proliferation and differentiation (Baldrige et al. 2010; Essers et al. 2009; Li et al. 2014). The chemokine CXCL12 is a well-known regulator of HSC mobilization and proliferation in adults (Karpova and Bonig 2015), and it has similar conserved roles in HSC development (Nguyen et al. 2014; Zhang et al. 2011). Although the developmentally- defined biomechanical forces of blood flow are not known to play a role in adult HSCs, the enrichment of HSCs in the bone marrow positioned adjacent to arterioles, which have different blood flow properties than sinusoids, suggests that it is possible that biomechanical properties have a yet to be determined role in adult HSC homeostasis. Thus, despite the seemingly different characteristics of adult and developing HSCs, studies of both microenvironments provide complementary and surprisingly conserved information about the extrinsic regulation of HSCs.

The mounting data on the HSC niche clearly shows that it is a highly complex microenvironment with many revelations still to be learned. The discoveries on the contribution of the nervous system and neural crest cells to the HSC niche highlight the need for researchers to keep an open mind when conceptualizing the microenvironment. Zebrafish are a great system to make unbiased and novel discoveries regarding the niche. Conditional and inducible cell ablation strategies will allow researchers to determine which niche constituents are used and when. Tissue-specific mutagenesis approaches will now permit the assignment of specific signals to specific cell types. Lineage-tracing can provide information about the origins of niche cells from development into adulthood. By capitalizing on these advanced experimental strategies in zebrafish, researchers should be able to translate findings in the developmental HSC niche into approaches to benefit the treatment of blood diseases as well as in vitro HSC production.

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Fig. 4.1.

The developmental timing and location of HSC development. **(a)** Timeline showing when and where primitive and definitive hematopoietic induction occurs in zebrafish. **(b)** Schema showing the location of the AGM/DA (*left*) and CHT (*right*) within zebrafish. Larger diagrams of the boxed regions are shown on the bottom. *ICM*, inner cell mass, *AGM* aorta-gonad-mesonephros, *DA* dorsal aorta, *CHT* caudal hematopoietic tissue, *CA* caudal artery, *CV* caudal vein, *CVP* caudal vein plexus

Fig. 4.2.

The HSC specification niche. **(a)** Diagram showing the position within the zebrafish shown in the panel **(b)** cross-section. **(b)** Diagram of a cross-section within the dorsal aorta HSC specification niche in a zebrafish embryo. The legend is shown to the right. **(c)** The bottom panel highlights the most recent findings on the signaling pathways involved in the HSC specification niche. *HSC* hematopoietic stem cell, *NC* notochord, *DA* dorsal aorta, *CV* caudal vein, *vegfa* vascular endothelial growth factor a, *FGF* fibroblast growth factor, *TGF β 1* a/b-transforming growth factor β 1 a/b, *Tnfa* tumor necrosis factor α , *IFN* interferon, *Cxcl8*-*CXC* chemokine ligand 8, *Gcsf* granulocyte-colony stimulating factor

Fig. 4.3.

Signals and cell types regulating developmental HSC mobilization and engraftment. **(a)** The left diagram shows the position within the zebrafish shown in the cross-section (*right*). The right panel highlights the most recent findings on the niche cells and signaling pathways involved in HSC mobilization/migration from the DA. Expression of the transcription factors c-Myb and Cbfb in emerging HSCs is important to mediate egress from the DA. Appropriate levels of Cxcl12 are required for this process, and are regulated by endotomal-derived endothelial cells and HSC expression of c-Myb. Macrophages serve as escorts that facilitate HSC migration into the CV. **(b)** The left diagram shows the position within the zebrafish shown in the cross-section (*right*). The right panel highlights the most recent findings on the niche cells and signaling pathways involved in HSC engraftment in the CHT niche. Within the CHT, HSCs are surrounded by endothelial cells and contact a stromal cell, which provides cues on the orientation of the division plane. The stromal cells are derived from the somite and are critical for HSC maintenance in the CHT niche. Secreted factors, such as Cxcl8 and G-csf modify HSC interactions and proliferation within the niche. *HSC* hematopoietic stem cell, *NC* notochord, *DA* dorsal aorta, *CV* caudal vein, *Cxcl12-CXC* chemokine ligand 12, *Cbfb* core binding protein beta, *CA* caudal artery, *CVP* caudal vein plexus, *CHT* caudal hematopoietic tissue, *Cxcl8-CXC* chemokine ligand 8, *Gcsf* granulocyte-colony stimulating factor