Functional Interactions in the Hematopoietic Stem Cell Niche: Stem Cells, Endothelial Cells, and Macrophages

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Glossary

Commonly used abbreviations:

HSC Hematopoietic stem cell
HSPC Hematopoietic stem and progenitor cell
HSCT Hematopoietic stem cell transplantation
CHT Caudal hematopoietic tissue
AGM Aorta-gonad-mesonephros
hpf hours post-fertilization
FACS Fluorescence-activated cell sorting (applied in flow cytometry)

Transgenic promoter-driven fluorescent reporter lines:

*Runx1* (also called *Runx1*+23) labels HSCs
*CD41* labels HSPCs
*Flk1* (also known as *Kdr* or *VEGFR2*) labels endothelial cells
*Mpeg1* labels macrophages
*LCR* labels erythrocytes
*Hsp70l* is a heat-shock inducible promoter
Abstract

The hematopoietic stem cell (HSC) niche is a specialized environment that supports the maintenance and regulation of hematopoietic stem and progenitor cells (HSPCs). The various cell types that comprise the niche and the molecular mechanisms underlying their supportive functions remain incompletely understood. During zebrafish development, HSCs engraft in a vascular plexus in the tail called the caudal hematopoietic tissue (CHT), which provides a niche for the HSCs. This tissue, analogous to the mammalian fetal liver, provides a model to study interactions between HSCs and their microenvironment. To study the vascular niche, a gain-of-function screen was performed by overexpressing genes differentially expressed in endothelial cells and assaying for HSCs in the CHT by in situ hybridization. Overexpression of the receptor CXCR1 or its ligand CXCL8 were both found to independently increase HSC colonization of the CHT. Using parabiotic animals overexpressing CXCR1 in one animal and fluorescently labeled HSCs circulating from the other animal, CXCR1 was also demonstrated to act cell autonomously to the sinusoidal vascular niche rather than the HSC. In parallel experiments, live cell imaging of macrophages and HSCs in the CHT revealed frequent intimate cell-cell interactions between the two populations. Quantification of the data showed that approximately 17% of HSPCs in the CHT are interacting with a macrophage at any point in time during development. A macrophage-HSPC co-culture system re-capitulated the result that roughly 15-20% of HSPCs are interacting with a macrophage at steady state. To test whether these interactions may play a functional role, macrophages were depleted from the zebrafish embryo using clodronate liposomes, and HSC numbers were assayed,
revealing a significantly decreased number of HSCs in the CHT in the setting of macrophage loss. Together, these findings suggest a role for the CXCL8/CXCR1 signaling pathway in the HSC vascular niche as well as a functional role for macrophage-HSC interactions in the CHT, which may have important implications for the design of new therapeutic approaches for improving transplantation-based treatment of hematopoietic disorders.
Lay Summary

Hematopoietic stem cells (HSCs) are long-lived cells resident in the bone marrow that have the potential to produce all blood cell lineages. These HSCs reside in a complex microenvironment comprised of multiple cell types that play key roles in regulating HSC behaviors, but their exact roles and the molecular mechanisms governing their roles are still incompletely understood. Two cell types present in the HSC niche are endothelial cells, which comprise the inner layer of blood vessels and capillaries, and macrophages, which are immune cells.

Here, I use the zebrafish embryo model system to study HSCs and their interactions with endothelial cells and macrophages. During zebrafish development, HSCs engraft in a tissue in the tail of the fish called the caudal hematopoietic tissue (CHT), which forms the HSC niche. Zebrafish are transparent during early development, so HSC interactions with their surrounding environment can be easily visualized and studied.

Screening through genes expressed in endothelial cells, overexpression of CXCR1, a receptor, was found to increase HSCs in the CHT of the zebrafish. Overexpression of the ligand for this receptor, CXCL8, recapitulated this effect. CXCR1 was found to be acting through the niche by expanding the volume of the niche, perhaps allowing for better engraftment of HSCs.

When investigating macrophages, I found that macrophages express a variety of cellular adhesion molecules. Using live cell imaging in the zebrafish, intimate cell-cell interactions between macrophages and HSCs were observed. In quantifying this data, roughly 17% of HSCs are interacting with a macrophage at any given point in time. Using a cell culture system, macrophages were found to preferentially
adhere to HSCs rather than a control cell type. Furthermore, selective depletion of macrophages in the fish resulted in an overall decrease in HSC numbers, suggesting that macrophages play a functional role in supporting HSCs in the CHT.

Together, these studies have important implications for HSC homing and engraftment. These types of HSC behaviors are critical in bone marrow transplant settings, a treatment often used for acute leukemias. A better understanding of the interactions between HSCs and their supporting cells may pave the way for improvements to transplantation therapies and better patient outcomes in the future.
Introduction

Hematopoietic stem cells (HSCs) are long-lived, quiescent cells that carry the potential to produce all blood cell lineages. These HSCs reside in a complex microenvironment comprised of multiple cell types that supports the HSCs and hematopoiesis in general. The HSC niche is comprised of many different cell types including sympathetic neurons, osteoblasts, macrophages, adipocytes, stromal cells, endothelial cells, and perivascular cells [1, 2]. Together, these niche cells help to regulate hematopoiesis via various signaling pathways and environmental maintenance. The specific roles and signaling pathways for many of the cell types in the HSC niche are incompletely understood and present themselves as an important topic of research.

During homeostasis or periods of increased demand on the hematopoietic system (pregnancy, infection, development), the HSC niche, HSCs, and progenitor cells function in a regulated manner to produce proper amounts of mature blood cells to meet the demands of the organism. In the setting of hematologic malignancy, an HSC or more mature blood cell can transform into a leukemic stem cell, which divides in an unregulated manner. In many of these cases, hematopoietic stem cell transplantation (HSCT) may be required. In HSCT, a patient receives high doses of chemotherapy and radiation followed by an infusion of HSCs. These HSCs home to and engraft in the recipient bone marrow niche where they expand and reconstitute the recipient’s hematopoietic system by producing mature blood cells.

For example, pediatric acute lymphoid leukemia (ALL) remains the most common form of childhood cancer [3]. While ALL has become a curable disease in
the vast majority of cases, a sizable percentage (20-25%) of patients will relapse after their first course of chemotherapy and require HSCT [4, 5]. Half of these relapse patients go on to ultimately die from disease or complications of treatment [5]. In adults, acute myeloid leukemia (AML) is the most common form of leukemia [6]. In cases of relapse and high-risk AML, allogeneic HSCT is the best treatment option. Unfortunately, outcomes remain poor with more than half of patients succumbing to their disease [7].

Indeed, HSCT is a potentially curative treatment for hematologic malignancies, and high risk or relapsed ALL or AML are among the most common indications for HSCT in pediatric and adult patient populations. HSCT, however, carries significant morbidity and mortality in the form of immunosuppression and infection, graft rejection, and graft versus host disease. HSCT success is largely predicate on donor HSC engraftment in the recipient bone marrow niche, followed by a rapid reconstitution of the immune system. Consequently, a better understanding of HSC homing and engraftment in the bone marrow niche is critically important for improving HSCT and could lead to better outcomes in pediatric and adult patients alike.

Zebrafish have emerged as an excellent model to study HSCs and the HSC microenvironment during hematopoietic development. The Zon laboratory has utilized the zebrafish as an HSC model system to progress quickly from bench to bedside. Using a chemical screen, prostaglandin E2 (PGE2) was discovered to significantly boost HSC engraftment and survival in the developing zebrafish [8]. HSCT experiments in adult zebrafish, mice, humaized mouse models, and non-human primates went on to confirm the effect of PGE2 on HSC engraftment [8, 9]. PGE2 has been shown to effectively increase umbilical cord blood derived
HSCs for use in adult patients undergoing HSCT for leukemia [10]. These encouraging results stemming from study of the zebrafish embryo suggest that further work in this model system could provide significant contributions to understanding and therapy in human biology.

Figure 1: Schematic and timeline depicting HSC migration during zebrafish development. HSCs born in the aorta-gonad-mesonephros (AGM) enter circulation to colonize the caudal hematopoietic tissue (CHT) before finally migrating to the adult niches of the kidney and thymus (adapted from [11]).

Definitive hematopoiesis in the zebrafish embryo begins at 24 hours post-fertilization (hpf) in the aorta-gonad-mesonephros (AGM). HSCs arise from the aorta endothelium and enter circulation where they home to and engraft in a vascular plexus in the tail of the animal known as the caudal hematopoietic tissue (CHT) [11, 12]. This engraftment process in the CHT begins at roughly 36 hpf, via seeding from the AGM, during which HSCs divide and interact with the other cell types present in the CHT [12]. The CHT functions as the hematopoietic equivalent of the mammalian fetal liver, acting as the main hub of hematopoietic activity before the HSCs migrate to the thymus at kidney where they will reside.
Zebrafish embryos present a unique opportunity to study HSC biology because the embryos are transparent during early development, allowing direct visualization of HSC behaviors and the surrounding microenvironment. The Zon lab has generated transgenic reporter lines that specifically label HSCs using the $\text{Runx1}$ promoter enhancer element (also called $\text{Runx1+23}$) driving fluorescent reporter genes (GFP or mCherry), thereby enabling HSC tracking using fluorescence confocal microscopy in the zebrafish embryo [13]. As HSCs enter the CHT, distinct steps in engraftment can be observed by live cell imaging. First HSCs adhere to the lumen of the sinusoids and migrate across the endothelium where they are engaged by endothelial cells and stromal cells [13]. The endothelial cells then remodel to surround the the HSC and form a pocket where the HSC interacts with stromal cells, which are thought to be involved in regulating HSC cell division [13].

By taking advantage of live cell imaging in the zebrafish embryo, cell-cell interactions in the HSC niche can be more closely examined. These techniques would allow for in vivo observation of HSC behaviors at a cellular level. A thorough understanding of the HSC niche and signaling pathways therein may pave the way to new therapeutics and improvements to HSCT that could improve patient outcomes in the future.
CXCR1 and CXCL8 in the vascular HSC niche

Introduction

The vascular HSC niche has been well established as a critical supporting microenvironment for HSCs. Endothelial cells and perivascular cells have been shown to be required for HSC maintenance and regeneration [1, 14, 15]. Within the vascular niche there are two distinct compartments: the arteriolar niche and the sinusoidal niche.

The arteriolar niche has been shown to contain perivascular stromal cells expressing CXCL12/SDF1 that maintain HSC quiescence [16]. The sinusoidal niche contains endothelial cells and perivascular stromal cells that contribute a large number of signalling molecules including Notch, CXCL12/SDF1, BMP, and SCF [15, 17, 18]. The sinusoidal niche has been shown to be critical for long term HSC repopulation after myeloablation and splenic hematopoiesis in other conditions of hematopoietic stress (chemotherapy, blood loss, pregnancy) [14, 19]. Deeper insight into the sinusoidal niche is important for development of new therapies seeking to improve hematopoietic reconstitution after or during conditions of stress hematopoiesis such as chemotherapy or stem cell transplantation.

Previous zebrafish studies in the lab have identified HSCs adhering and interacting with sinusoidal endothelial cells in the CHT [13]. To identify genes that may play a significant role in endothelial cell-HSC signaling, endothelial cells and HSCs were sorted from transgenic zebrafish embryos at 72 hpf as well as adult kidney marrow using markers for endothelial cells and HSCs (flk1:hRAS-dsRed
and Runx1+23:GFP, respectively) [13]. Gene expression profiling on all four populations was done by microarray (Affymetrix Zebrafish 1.0 ST array) to identify genes differentially expressed between HSCs and endothelial cells [20].

Figure 2: Gene set enrichment analysis profiles in the chemokine gene set in embryonic and adult endothelial cells and HSCs. Genes enriched in endothelial cells are plotted to the left and genes enriched in HSCs are plotted to the right (adapted from [20]).

Gene set enrichment analysis and ingenuity pathway analysis were used by Brad Blaser in the lab to narrow down the list of differentially expressed genes to 210 cDNAs encoding growth factors, chemokines, cell adhesion molecules, and other secreted factors that may play a role in HSC maintenance (Figure 2) [20]. Using this list of genes, a gain-of-function genetic screen was then carried out to identify genes that could increase HSC colonization of the CHT in zebrafish embryos. Using this screening method, a number of genes were found that increase HSCs in the CHT, including Wnt5a, ephb4b, and CXCR1. Additional studies then demonstrated that overexpression of the ligand for CXCR1, CXCL8, also increased HSCs in the CHT. We were then able to demonstrate that CXCR1 acts cell autonomously in the HSC niche to increase HSC numbers and also CHT volume, implicating an important
role for CXCL8/CXCR1 signaling in the HSC microenvironment.
Materials and Methods

Zebrfish

Wildtype AB zebrafish and casper mutants were used in this study along with Runx1+23:NLS-mCherry and flk1:hRAS-mCherry transgenic lines. Animals were housed at Boston Children’s Hospital and utilized in accordance with institutional animal care and use committee protocols.

Plasmid cloning

Candidate zebrafish genes were amplified from kidney marrow total RNA using the Superscript III RT kit (ThermoFisher) in conjunction with gene-specific primers. PCR products were cloned into the pentr SD Topo middle entry vector (Invitrogen). These middle entry vectors were cloned with a 5’ Hsp70l promoter into a Tol2 expression vector by Gateway reaction.

Microinjection

A total of 25 pg of DNA along with 200 pg of Tol2 mRNA was injected in a 1 nL volume into zebrafish embryos at the single cell stage (roughly 20-30 minutes post-fertilization) using a Harvard Apparatus microinjection system. The 25 pg of DNA consisted of 20 pg of candidate gene Tol2 expression vector mixed with 5 pg of Hsp70l:GFP as a read out for embryos with high mosaicism.

Heat shock induction of gene expression

Embryos were transferred to a 96 well PCR plate at 36 hpf: 1 embryo per well in 100 μl of E3 embryo media. The embryos were then heated in a PCR machine at 40°C for 30 minutes at 36 hpf and 48 hpf. The embryos were kept in the zebrafish incubator at 28.5°C when not incubating at 40°C. The embryos were then removed
from the 96 well PCR plate after the second heat shock at 48 hpf by gently flushing the well with E3 embryo media and transferred to a standard zebrafish dish.

**Drug treatment**

Zebrafish embryos were treated by adding SB225002 (Cayman Chemicals, Ann Arbor, Michigan) at a final concentration of 0.5 μM in E3 embryo media. The SB225002 stock was dissolved in DMSO. An equal volume of DMSO was added to the E3 embryo media for control fish groups.

**In situ hybridization**

Zebrafish embryos were euthanized by tricaine overdose. They were then fixed in 4% paraformaldehyde overnight. *In situ* hybridization for Runx1 and c-myb was performed using standard techniques [21]. Embryos were scored in a blinded process where embryo staining was assessed in a semi-quantitative manner by scoring the CHT staining intensity. Zebrafish groups were then unblinded and the scores were compared using the Wilcoxon Rank Sum test. At least 20 embryos were scored from each group.

**Zebrafish parabiosis**

Parabiotic zebrafish were generated by surgically stiching two zebrafish blastulae (see Supplemental Materials) [22, 23]. In brief, Casper zebrafish were injected with *Hsp70l:CXCR1* and *Hsp70l:GFP* plasmids at the single cell stage (or *Hsp70l:GFP* alone as a control). At roughly 3-4 hours post-fertilization, these embryos were dechorionated and surgically fused to stage-matched *Runx1+23:NLS-mCherry* zebrafish embryos while embedded in methylcellulose and covered in high-calcium Ringer’s solution with added antibiotics. A large majority of these fish would develop to have a fused head or body, two separate
tails, and a single shared circulation. These parabionts would then undergo heat shock and confocal imaging.

**Confocal microscopy**

Zebrash embryos were anesthetized with tricaine and embedded in low melting point 0.8% agarose on glass bottom 6-well plates for imaging. Images were acquired using a Yokogawa spinning disk confocal, Nikon inverted Ti microscope, and Andor iXon EMCCD camera using NIS elements software.

**Digital image analysis**

Image analysis was performed using Imaris software (Bitplane). Cells were counted in the CHT via the software after setting appropriate quality thresholds to reduce background noise. The same thresholds and imaging parameters were used between control and experimental treatment groups. To compute the volume of the CHT, a 3 dimensional isosurface was automatically generated in Imaris that contained only the sinusoidal area of the CHT. This digital isosurface was then used to compute the volume of the CHT.
Results

Establishing an overexpression screen to assess candidate endothelial genes in HSC colonization of the CHT

A gain-of-function overexpression screening method was developed to search for potential genes that increase HSC colonization in the CHT. The screening method expressed candidate genes during HSC colonization of the CHT. HSC numbers were then assayed in the CHT after transgene induction.

Firstly, to achieve temporal control of gene expression, candidate genes were cloned downstream of the \textit{Hsp70l} heat-shock inducible promoter in a Tol2 expression vector [24]. DNA was then injected into the embryo along with Tol2 transposase mRNA, which promotes integration into the genomic DNA. By heat-shocking the fish at 40°C for 30 minutes at 36 hpf and 48 hpf, the candidate gene was robustly expressed with high mosaicism in the embryo (Figure 3). This developmental window is critical because HSCs migrate from the AGM and begin to colonize the CHT at 36 hpf until 72 hpf.

![Figure 3: Robust GFP expression with high mosaicism lasting until 72 hpf induced by 30 minute heat shock at 40°C at 36 and 48 hpf.](image)

Co-injection of \textit{Hsp70l:GFP} along with the candidate gene heat-shock construct was used as a screening tool to allow for positive selection of transgenic embryos with high levels of transgene expression and mosaicism. Control embryos from the same clutch were injected with \textit{Hsp70l:GFP} alone as a negative control.
To assess HSC numbers in the CHT, the fish were fixed at 72 hpf and \textit{in situ} hybridization was performed for \textit{Runx1} and \textit{c-myb} mRNA expression, which is specific to HSPCs \cite{8}. The fish were blinded and scored semi-quantitatively for staining in the CHT.

Together, these methods allowed for screening of 57 candidate genes generated from the gene set enrichment analysis on genes differentially expressed in endothelial cells.

\textbf{Figure 4:} (A) Wnt5a, (B) ephb4b, and (C) CXCR1 overexpression results in significantly increased \textit{Runx1/c-myb} staining in the CHT at 72 hpf ($n \geq 15$, $p < 0.01$, $p < 0.01$, $p = 0.03$, respectively).
Screen reveals genes that increase CHT colonization

After screening through 57 candidate genes, 3 genes—Wnt5a, ephb4b, and CXCR1—were found to increase Runx1/c-myb staining in the CHT at 72 hpf ($p < 0.01$, $p < 0.01$, $p = 0.03$, respectively) (Figure 4). This results in a screen hit rate of roughly 5%.

Both Wnt5a and ephb4 have been shown to play roles in hematopoiesis and HSC maintenance and regulation [25–27]. While their roles are still incompletely understood, this validates the screening methodology as an unbiased approach to finding genes involved in the HSC niche. CXCR1 has also not been fully characterized in the context of hematopoiesis or the HSC niche, making it an exciting gene for further study.

CXCR1 and its ligand CXCL8 enhance HSC/HSPC colonization in the CHT

To follow up the hit of CXCR1 (also called IL8R), a similar overexpression heat shock experiment was performed with its ligand CXCL8 (IL8). Hsp70l promoter driven expression of CXCL8 recapitulated increased Runx1/c-myb staining in the CHT at 72 hpf ($p = 0.02$) (Figure 5). Together, these gain-of-function experiments suggest a novel role for the CXCR1/CXCL8 signaling pathway in HSC colonization of the CHT during development.

It is also worth noting that the murine model system has been shown not to express CXCL8, and thus, murine CXCR1 homologs do not natively function as CXCL8 receptors except when exposed to human ligand [28, 29]. In contrast, CXCR1 and CXCL8 are expressed in humans, a wide variety of other mammals, and zebrafish. This positions the zebrafish embryo as an excellent model system to study the biology of CXCR1/CXCL8 signaling in the context of HSC maintenance.
Figure 5: CXCL8 overexpression results in significantly increased *Runx1/c-myb* staining in the CHT at 72 hpf (*n* ≥ 20, *p* = 0.02).

and the HSC niche.

**Selective inhibition of CXCR1/CXCR2 shows a trend of decreased HSCs in the CHT**

As a loss-of-function experiment, embryos were treated with 0.5 μM SB225002, a selective inhibitor of CXCR1/CXCR2, at both early (36-60 hpf) and late (108-132 hpf) time points in CHT colonization. The embryos were fixed at 132 hpf and *in situ* hybridization for *Runx1/c-myb* was performed to assess HSC numbers. While the statistics were not significant at a *p* < 0.05 threshold, drug treatment at early and late time points did show a trend of decreased staining in the CHT (*p* < 0.25 and *p* < 0.15, respectively) (Figure 6). This result may suggest that CXCR1 plays
Figure 6: Selective inhibition of CXCR1/CXCR2 by drug at early and late time points in CHT colonization show a trend towards decreased Runx1/c-myb CHT staining by in situ hybridization at 132 hpf (n ≥ 10 with p < 0.25 and n ≥ 30 with p < 0.15, respectively).

some role, albeit limited, in native hematopoiesis during embryogenesis.

CXCR1 acts autonomously to the niche rather than the HSC

Up to this point, CXCR1 has been expressed systemically in a mosaic fashion driven by the Hsp70l promoter. It may be the case, however, that CXCR1 expression in the HSC compartment directly leads to HSC expansion rather than acting through the endothelial cell niche. Thus, to test whether CXCR1 is acting autonomously to the HSC or to the niche, parabiotic zebrafish were generated (see Supplemental Materials) such that a ‘donor’ fish’s HSCs could be tracked colonizing a genetically modified ‘recipient’ fish’s niche.
For this experiment, the ‘donor’ fish was a Runx1+23:NLS-mCherry fish with red fluorescent HSCs fused at 4 hpf to a ‘recipient’ fish injected with the Hsp70l:CXCR1 construct or with Hsp70l:GFP as a negative control (Figure 7). Gene expression was induced in the recipient embryos by heat shock at 36 hpf and 48 hpf as with previous experiments; gene expression was localized to only the recipient fish but not the donor fish (Figure 7). All parabionts analyzed in this study were fused in the anterior region with morphologically normal tails (and CHTs) as well as shared circulation, evidenced by a presence of mCherry+ cells in the recipient CHT.

![Recipient and Donor](image)

**Figure 7:** Shown is a parabiont at 60 hpf comprised of an Hsp70l:GFP injected embryo (left) fused to an uninjected Runx1+23:NLS-mCherry transgenic embryo (right) (mCherry expression is too faint to visualize at low magnification). GFP expression is cleanly localized to only the recipient fish.

The shared circulation of the parabionts allowed the HSCs from the single donor animal to circulate into both niches and engraft in both CHTs (Figure 8). The parabiotic animals were imaged at 72 hpf. Donor HSCs were quantified by imaging the CHTs of the donor and recipient animals and counting mCherry+ cells (Figure 9).

High CXCR1 expression in the recipient fish resulted in a significantly increased
Figure 8: Depicted is a schematic of Runx1:mCherry HSCs originating in the donor fish, entering circulation, and arriving in the recipient fish which may or may not express Hsp70l:CXCR1.

The total number of HSCs per parabiont: 6.6 HSCs in the GFP control compared to 25.9 HSCs in the CXCR1 overexpressing niche (Control n = 6, CXCR1 n = 5, p < 0.01) (Figure 10). This finding indicates that the total donor HSC pool is expanded in the presence of CXCR1.

When counting the number of HSCs in the recipient in each experimental group, the CXCR1 overexpression group had significantly increased colonization in the recipient compared to the control recipient (14.3 ± 4.2 compared to 3.7 ± 1.7, p = 0.04) (Figure 11). Similarly, the CXCR1 overexpression group had significantly increased colonization in the donor compared to the GFP control donor (11.6 ± 1.8 compared to 2.9 ± 1.6, p < 0.01) (Figure 11).

Since the CXCR1 overexpression is localized to the recipient animal (and not the donor HSCs), these results imply that CXCR1 is acting autonomously in the
Figure 9: Representative images of the CHT of donor and recipient halves of parabiotic zebrafish imaged at 72 hpf. Top 2 panels: the recipient expresses GFP (control, GFP fluorescence not shown) in the niche. Bottom two panels: the recipient expresses CXCR1 in the niche. *Runx1+23:NLS-mCherry* HSCs are colored in red, scale bar=50μm.

niche to increase the total HSC pool rather than autonomously to the HSC. This is consistent with the initial screening hypothesis that the candidate factor is differentially expressed in the endothelial cell niche and perhaps functional in the
Figure 10: Total HSCs from the whole parabiont (donor and recipient) in Hsp70l:GFP compared to Hsp70l:CXCR1 ($p < 0.01$).

endothelial cell niche.

**CXCR1 acts locally to increase the size of the endothelial cell niche**

While the total HSC pool is expanded in the CXCR1 overexpression parabionts, it is unclear why there are increased HSCs resident in both the recipient CHT and the donor CHT (Figure 11). This result suggests two distinct possibilities: either the expansion of the donor HSC pool occurred locally in the transgenic recipient niche and HSCs re-circulated back to the donor niche or downstream signaling in the transgenic recipient niche resulted in secretion of unknown circulating factors that acted on the donor niche as well.

To test this, a similar parabiotic experiment was performed where both donor
Figure 11: HSC numbers in the CHT compared in donor animals and in recipient animals in \textit{Hsp70l:GFP} and \textit{Hsp70l:CXCR1} experimental groups ($p < 0.01$ and $p = 0.04$, respectively).

and recipient animals were from the \textit{flk1:hRAS-mCherry} transgenic line, which marks endothelial cells. The recipient animal was again injected with either the \textit{Hsp70l:CXCR1} construct or \textit{Hsp70l:GFP} as a negative control. The fish were then imaged and software was used to reconstruct the CHT in 3 dimensions and quantify the volume of the CHT. The ratio between the volume of the recipient CHT and the volume of the donor CHT was then computed for each experimental group.

The CXCR1 overexpression parabionts had a higher ratio of recipient CHT
Figure 12: CXCR1 overexpression parabionts had a higher ratio of recipient CHT volume to donor CHT volume compared to GFP controls ($p = 0.01$).

Volume to donor CHT volume compared to GFP controls (1.265 compared to 0.896, $p = 0.01$) (Figure 12). First of all, these data demonstrate direct structural changes in the endothelial cell niche in response to CXCR1 overexpression. This confirms the study design and gene screening methodology looking at genes specifically expressed in the endothelial cell niche. Indeed, the data suggest that CXCR1 acts locally to expand the recipient endothelial cell niche by roughly 20%. Secondly, this predominantly local effect also suggests that there is not a circulating factor effecting both niches, but rather that the recipient niche is expanding, thereby improving HSC colonization and increasing the total HSC pool, which is then re-circulating back to the donor CHT.
Discussion

Interactions between HSCs and the vascular niche play key roles in the maintenance and regulation of hematopoiesis under stress conditions as well as in steady state. During early development, high demand and rapid proliferation in the HSC compartment reflect the demands of hematopoietic stresses in adults (toxins, infection, etc.). This makes the embryonic zebrafish CHT a powerful model system for understanding HSC response and niche interactions.

In this study, an unbiased screening technique was used to discover novel signaling molecules in the vascular HSC niche. Both the receptor CXCR1 and its ligand CXCL8 were shown to independently increase HSC colonization during development. CXCR1 was demonstrated to act cell autonomously to the sinusoidal vascular niche rather than the HSC. CXCR1 overexpression also resulted in an expansion of the size of the niche. Experiments by Brad Blaser in the lab have gone on to show that CXCR1/CXCL8 signaling enhances HSC colonization in the niche by increasing HSC residency time, allowing for more HSC mitotic events, thereby expanding the total stem cell pool [20]. He has also shown that in vitro treatment of endothelial cells with recombinant CXCL8 induces CXCL12, survivin, VEGFA, and CXCL8 itself, perhaps leading to a positive feedback loop resulting in vascular growth and priming of the vascular niche for HSC colonization [20]. Finally, he has generated a \textit{flk1:CXCR1} transgenic line to demonstrate that CXCR1 acting in the endothelial niche results in increased CHT volume [20].

These data lead to the following model of HSC-niche dynamics in early development: the nascent CHT expresses low levels of CXCR1. CXCL8 signaling from supporting cells and/or HSPCs results in induction of an autocrine/paracrine positive feedback loop in the sinusoidal niche, consisting of continued CXCL8
production inducing CXCL12, survivin, VEGFA and other vascular growth factors. This signaling cascade then results in rapid growth, expansion, and maturation of the vascular niche in preparation for continued HSC arrival to the sinusoidal vascular niche. This remodeling then provides a microenvironment poised for HSC colonization and continued HSC cell division. Once the HSCs colonize the CHT, they then continue to expand as well as produce progenitors and myeloid cells which can continue to sustain CXCL8 signaling for niche maintenance.

Roles for CXCL8 and CXCR1 have been well characterized in inflammation and infection; however, their roles in hematopoiesis are just starting to be investigated [30–32]. In a separate study in the lab, loss of CXCL8 has been demonstrated to decrease HSC specification in the AGM [33]. Similarly, other inflammatory signals such as TNFα have been shown to play a role in HSC specification in the AGM [34]. Thus, it is not difficult to imagine that many of these same inflammatory pathways may play other roles downstream in colonization of the HSC niche. Further mechanistic studies are required to completely understand the role of CXCL8 and CXCR1 in hematopoiesis, including in adult hematopoiesis, malignancy, and transplant contexts.

For many patients with myeloproliferative disorders and leukemias, HSCT can be the only curative therapeutic treatment. HSCT requires ablation of the host marrow and leaves patients in a severely immunosuppressed state before the graft can reconstitute the immune system. Remodeling of the HSC vascular niche is critical for engraftment in these settings, and secreted signaling factors like CXCL8 may present druggable targets for potential pharmacologic applications. Indeed, one can imagine that modulation of CXCL8/CXCR1 signaling may induce remodeling in the sinusoidal vascular niche that may improve HSC engraftment and reconstitution.
of mature blood lineages. A better understanding of the CXCL8/CXCR1 pathway in the context of HSC engraftment and hematopoietic stress may provide insights toward potential therapies that can improve outcomes for patients receiving HSCT.
Introduction

The zebrafish CHT presents a unique opportunity to study HSC engraftment. Due to the highly stereotyped development of the zebrafish embryo, HSCs are known to leave the AGM to colonize the CHT beginning at 36 hpf. The CHT runs anterior-posterior through the ventral region of the tail as a thin vascular plexus. This anatomic localization and well characterized developmental time window facilitates the identification of genes expressed within the CHT during HSC colonization using recently developed methods for spatial transcriptomics.

In collaboration with Jan Philipp Junker and Alexander van Oudenaarden, Elliott Hagedorn in the lab has used a newly developed technology called RNA tomography (or Tomo-seq) to investigate the CHT. Tomo-seq combines cryosectioning and RNA-seq techniques to generate a spatial anatomic map of transcriptome-wide gene expression [35]. Using this technique, 72 hpf zebrafish embryo tails were snap frozen, and 8 μm thick cryosections were taken along the dorsal-ventral axis. RNA was isolated from each tissue section and barcoded (Figure 13A). Next generation sequencing was then used to compare gene expression profiles between sections. This allowed for anatomic resolution of gene expression profiles.

The unique anatomy of the CHT combined with known gene expression profiles of cell types known to be in the CHT and hierarchical clustering techniques enabled identification of roughly 300 genes that cluster anatomically in that region (Figure 13B) [36]. These genes included signaling molecules, adhesion
receptors, proteases, and cytoskeletal regulatory proteins.

Figure 13: (A) Schematic depicting zebrafish embryo cryosectioning and tissue isolation for RNA-seq. (B) Heat map of hierarchical clustering of gene expression data from individual slices shows an anatomic signature for CHT-specific gene expression.

Of these different classes of genes, I was particularly interested in cellular adhesion molecules. For example, one interesting gene from the Tomo-seq analysis shown to be enriched in the CHT was itgb2 (Figure 14). Integrins have long been studied as key players in cellular adhesion, particularly in immune cells and inflammatory states [37, 38]. Integrins are heterodimers with one alpha subunit and one beta subunit. The integrin dimers sit in the plasma membrane on the
surface of the cell and interact with their ligands on other cell types and in the extracellular matrix.

Some preliminary studies have demonstrated roles for integrins in HSC engraftment and homing [39, 40]. $\beta 7$ integrin knock out animals have been shown to have decreased HSC engraftment potential [39]. Small molecule targeting of integrins has also been demonstrated to mobilize the stem cell compartment [40]. Clinical reports of the integrin blocking monoclonal antibody natalizumab (used to treat multiple sclerosis) have revealed that the drug can mobilize CD34+ HSPCs in humans [41]. All of these studies point to integrins playing a role in HSC homing or engraftment, implicating an important role for integrins in HSC-niche interactions, but the mechanisms and specific roles for integrins in the HSC niche are still incompletely understood.

Additionally, prior work in the lab had demonstrated interactions between macrophages and HSPCs in the AGM (Figure 15). In roughly 70% of these interacting pairs, the HSPCs exited the AGM within 30 minutes to migrate to the

**Figure 14:** Anatomic gene expression profile of *itgb2* taken from Tomo-seq of the tail of a 72 hpf embryo showing CHT enrichment.
Figure 15: Imaging of the AGM (outline in blue) at 43 hpf demonstrates macrophages (mpeg1:mCherry, red) in close association with HSPCs (CD41:eGFP, green) (adapted from [42]).

CHT [42]. Imaging of stromal cells and HSPCs in the CHT also revealed possible interactions between macrophages and HSCs (Figure 16). Consequently, it was hypothesized that macrophages played a role in regulating HSPC behaviors in both the CHT and AGM. Moreover, it was hypothesized that these macrophage surface interactions may be mediated by surface molecules such as integrins.

Figure 16: Imaging of the CHT demonstrates macrophages (CXCL12a:DsRed low) in close association with HSPCs (Runx1:GFP) (adapted from [42]).

In our experiments below, we found that RNA-seq of sorted macrophages demonstrated an enrichment for cellular adhesion molecules. Further live cell imaging also showed intimate surface cell-cell interactions between macrophages and HSCs in the CHT. Co-culture experiments using macrophages and HSPCs revealed that macrophages-HSC interactions have increased specificity compared
to macrophage interactions with other cell types. Finally, macrophage depletion in zebrafish embryos resulted in decreased HSPC numbers in the CHT, suggesting a functional role for macrophages in the niche.
Materials and Methods

Zebrfish

Wildtype AB zebrafish and casper mutants were used in this study along with Runx1+23:NLS-mCherry, Runx1+23:GFP, CD41:GFP and mpeg1:mCherry transgenic lines. Animals were housed at Boston Children’s Hospital and utilized in accordance with institutional animal care and use committee protocols.

Fluorescence-assisted cell sorting (FACS) on zebrafish embryos

Zebrafish embryos were raised until 72 hpf and sacrificed by tricaine overdose. The embryos were then chopped with a razor blade and resuspended in PBS. Liberase was added 1:65 and the embryos were incubated at 37°C for 20 minutes. FBS was used to quench the liberase and cells were filtered and washed in FACS tubes with 40μm mesh filter tops. FACS was performed by the flow cytometry core at Boston Children’s Hospital.

RNA-seq on macrophages

Macrophages were sorted from 72 hpf mpeg1:mCherry transgenic embryos using FACS. RNA was isolated using Trizol and GenElute LPA. The cDNA library was prepared using Ribogone kit (Clontech) and the SMARTer Universal Low RNA Kit (Clontech). DNA was sequenced using a Hiseq 2500, and reads were aligned using Tophat 2.0. FPKM values were computed using Cufflinks.

Clodronate liposome injection

Clodronate liposomes were used to deplete macrophages according to established protocols [43]. Clodronate liposomes were injected into circulation in 48 hpf zebrafish embryos through the Duct of Cuvier using a Harvard Apparatus
microinjection system. Dosage was titrated based on pressure and injection timing using control mpeg1:mCherry zebrafish embryos. PBS-loaded liposomes were injected for controls. Embryos selected for analyses were those with proper dosing and no clotting in the tail due to liposomes.

In situ hybridization

Zebrafish embryos were euthanized by tricaine overdose. They were then fixed in 4% paraformaldehyde overnight. In situ hybridization for Runx1 was performed using standard techniques [21]. Embryos were scored in a blinded process where embryo staining was assessed in a semi-quantitative manner by scoring the CHT staining intensity. Zebrafish groups were then unblinded and the scores were compared using the Wilcoxon Rank Sum test.

Live cell confocal microscopy

Zebrafish embryos were anesthetized with tricaine and embedded in low melting point 0.8% agarose on glass bottom 6-well plates for imaging. Images were acquired using a Yokogawa spinning disk confocal, Nikon inverted Ti microscope, and Andor iXon EMCCD camera using NIS elements software.

Macrophage-HSPC co-cultures

Macrophages and HSPCs were FACS sorted from mpeg1:mCherry and cd41:GFP embryos using methods detailed above. A 1536-well plate was prepared by coating the wells with 0.1% gelatin and suctioning off the excess. Cells were mixed in a 1:1 ratio and resuspended in culture media. Media used was RPMI with 10% FBS, 10% embryo extract, and 1x Primocin primary culture antibiotic. Embryo extract was made by homogenizing 24 hpf embryos, spinning down the debris, and keeping the supernatant. For the co-cultures, cells were resuspended to
a concentration of 2000 cells per 8μl. 8μl of cells were plated into each gelatin coated well of the 1536-well plate, resulting in 1000 HSPCs and 1000 macrophages per well.

**Imaging macrophage-HSPC co-cultures**

Co-cultures were imaged using 2 modalities. In the first modality, images of the wells were acquired using a Yokogawa spinning disk confocal, Nikon inverted Ti microscope, and Andor iXon EMCCD camera using NIS elements software. In the second modality, images were acquired using the Yokogawa Cell Voyager 7000 automated microscope. In both systems, the plates were imaged for 16 hours and kept at 28.5°C (appropriate for zebrafish culture systems).

**Digital image analysis**

Image analysis was performed using Imaris software (Bitplane) for live embryos and Matlab via custom script for co-culture experiments. With Imaris, cells were counted in the CHT via the software after setting appropriate quality thresholds to reduce background noise. Co-localization of macrophages and HSPCs was done in ImarisXT using the ‘coloc’ package. Co-culture experiments were analyzed using a custom Matlab script that allows for thresholding and calculation of cell-cell contact (see Supplemental Materials).

**CRISPR/Cas9 generation of mutants**

Guide RNA design was done using CHOP-CHOP by selecting multiple gRNAs that are predicted to have activity in early exons. Oligos were designed with SP6 promoter region in front of the target sequence, which was then annealed to a separate oligo with the constant sequence. T4 DNA polymerase was used to fill in the non-overlapping regions. Transcription of gRNAs was then done using Ambion
MEGAscript SP6 kit. Clean up was done with Zymo RNA columns and eluted in H2O. Injection mixes were prepared 2:2:1 with Cas9 protein (Pna Bio), gRNA, and phenol red (as coloring to see the mix). The mix was injected in a 1nL volume into single cell embryos using a Harvard Injection apparatus. Guides were validated using high resolution melt curves, and alleles were identified using Sanger sequencing.

**Mouse bone marrow harvest and FACS**

Mice were sacrificed with CO2, and femurs, tibias, and pelvic bones were collected. The bones were crushed using an autoclaved mortar and pestle, allowing the bone marrow cells to enter suspension. The cells were collected and filtered. The cells were then lineage depleted using the magnet assisted cell sorting Lineage Cell Depletion Kit (Miltenyi Biotec). The flow through cells were stained with lineage positive (TER-110, CD45R, CD3ε, CD11b, Gr-1), Sca-1, and c-Kit antibodies before FACS. Lineage- Sca-1+ c-Kit+ cells (LSK) were collected as HSPCs. The cells in the Miltenyi columns were eluted and stained for F4/80 with an isotype negative control. FACS was used again to isolate F4/80+ bone marrow macrophages.

**Mouse cell staining and culture conditions**

Mouse cells (macrophages and HSPCs) were stained with Vybrant DiO and DiI fluorescent cell-labeling dyes (Thermo Fisher). The cells were cultured at 37°C using StemSpan media supplemented with 10% FBS.

**Nanowell co-culture system**

The nanowell chips were obtained via collaboration with Chris Love’s lab at MIT (Koch Institute) [44]. Nanowell chips are made from Silgard and contain 30 by 30 µm wells. Chips were plasma treated to remove hydrophobicity and kept in
FBS. The chips were placed in a 4-well plate and washed with PBS and then culture media. Cooled 1% agarose gel was pipetted onto the ends of the chip to affix it’s position in the plate. Cells were resuspended in 500μl of media and dispensed onto the center of the chip. The chip was then left to rest while the cells randomly settle into the wells. Additional media was then added to prevent the chip from drying, and the chip was imaged using confocal microscopy.
Results

Itgb2 expressed in the CHT

To validate candidate CHT genes from the Tomo-seq experiment, \textit{in situ} hybridization probes were made to genes thought to be expressed in the CHT. \textit{In situ} hybridization was then done on 36, 48, and 72 hpf zebrafish embryos using these probes. The cellular adhesion molecule itgb2 was found to be specifically expressed in the CHT (Figure 17).

![itgb2

Figure 17: \textit{In situ} hybridization to itgb2 reveals expression in the CHT at 72 hpf.

Macrophages specifically express cellular adhesion molecules

To determine which cell types in the CHT are expressing genes from the Tomo-seq, RNA-seq was performed on sorted cell populations from transgenic zebrafish lines. \textit{mpeg1:mCherry} embryos were raised to 72 hpf, where they were then sacrificed and put into single cell suspension. Macrophages were sorted by FACS and low-input RNA-seq was performed comparing the sorted macrophages to the rest of the embryo.

The RNA-seq data revealed that macrophages specifically express a wide variety of cellular adhesion molecules, including itgb2 (Figure 18A). Fluorescence microscopy showed a large number of macrophages present in the CHT
(Figure 18B). Together with the in situ staining for itgb2 (Figure 17), this suggested that perhaps there is a specific population of macrophages resident in the CHT expressing these cellular adhesion molecules. Indeed, while numerous macrophages are observed all along the yolk extension (Figure 18B), itgb2 expression is limited only to the CHT (Figure 17).

<table>
<thead>
<tr>
<th>Zebrfish Gene</th>
<th>Neg. Population (FPKM)</th>
<th>mpeg1: mCherry (FPKM)</th>
<th>mpeg1: mCherry vs Neg. (log2 FC)</th>
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<tr>
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Figure 18: (A) RNA-seq on sorted macrophage populations in 72 hpf embryos reveals enriched expression of numerous cellular adhesion molecules. (B) Macrophages (red) are resident in the CHT at 72 hpf.

**Macrophages interact directly with HSCs in the CHT**

Live cell imaging was used to visualize macrophages (mpeg1:mCherry) and HSCs (Runx1:GFP) in the CHT at 56 hpf. High magnification time lapse imaging
revealed intimate cell-cell interactions where the macrophage appears to groom the surface of the HSC (Figure 19). These interactions can last for periods of 30-45 minutes.

![Figure 19: Shown are stills from a live cell imaging time lapse movie of the CHT taken at 56 hpf. mpeg1:mCherry marks a macrophage (red) as it interacts closely with an HSC marked by Runx1:GFP (green). The macrophage can be seen interrogating the surface of the HSC over the course of 30 minutes.](image)

These interactions occur with relatively high frequency within the CHT *in vivo*. CD41:GFP; mpeg1:mCherry transgenic zebrafish with fluorescently labeled HSPCs and macrophages were time lapse imaged from 36-52 hpf. Imaging data was reconstructed 3 dimensionally and interacting HSPC-macrophage pairs were enumerated using Imaris software (Bitplane). This data revealed that roughly 12-17% of HSPCs are interacting with a macrophage at any given point in time (Figure 20).

**Macrophages interact directly with HSCs in co-culture**

In order to observe and quantify these interactions in a controlled environment, a macrophage-HSC co-culture system was developed. Transgenic
Figure 20: *In vivo* HSPC-macrophage interacting pairs were quantified in 3D timelapse live cell imaging of the CHT in embryos with fluorescently labeled HSPCs and macrophages from 36-52 hpf. Shown is the percentage of total HSPCs that are interacting with a macrophage at any given time point. Shaded is the 95% confidence interval.

Embryos with fluorescently labeled macrophages and HSPCs were sacrificed at 72 hpf. Embryos were then put into single cell suspension. FACS sorted HSPCs and macrophages were then co-cultured in RPMI media with FPS and embryo extract (see Methods for details). Time lapse imaging was then used to assess cell-cell interactions.

Macrophage-HSPC interactions in culture closely resemble interactions *in vivo*. Macrophages can be seen cupping the HSPC in a close surface interaction (Figure 21A). Moreover, these interactions are numerous in co-culture and can be
Figure 21: (A) High magnification image of a macrophage (mpeg1:mCherry) interacting with an HSPC (cd41:GFP) in culture. (B) Many interacting pairs of macrophages and HSPCs can be identified in co-culture. Time lapse movies of the co-cultures show that macrophages do not display chemotactic behavior in co-culture. Rather, both cell types drift randomly in culture, but when they collide, the macrophage latches onto the HSPC.

**Macrophage-HSPC interaction has specificity**

It may be possible that macrophages do not have particular affinity for HSCs in these interactions, but that macrophages simply adhere to any cell type. To
address this possibility, a co-culture experiment was performed. Macrophages, HSPCs, and erythrocytes were all isolated from 72 hpf transgenic embryos via FACS (mpeg1:mCherry, CD41:GFP, and LCR:GFP, respectively). Macrophages were then plated at equal density with either HSPCs or erythrocytes. The plates were then time lapse imaged for 16 hours and the data was analyzed via a custom Matlab script that detects pairs of interacting cells (see Supplemental Materials).

![Graph showing interaction between CD41+ cells and macrophages vs LCR+ cells. The graph shows that CD41+ cells interact more often with macrophages compared to LCR+ cells. The shaded area represents the 95% confidence interval.]

**Figure 22:** When plated at 1:1 density with macrophages, CD41+ cells (HSPCs) interact more often with macrophages compared to LCR+ cells (erythrocytes). Shaded is the 95% confidence interval.

When HSPCs are co-cultured with macrophages, roughly 15-20% of HSPCs are interacting with a macrophage at any time point past 5 hours (Figure 22). In
contrast, when erythrocytes are co-cultured with macrophages, less than 10% of them are interacting with macrophages at steady state (Figure 22). This data is aggregate from 4 replicates of the HSPC co-culture and 3 replicates of LCR co-culture; the shaded 95% confidence intervals do not overlap, showing a significant difference in interaction frequency (Figure 22). Together, these data suggest that macrophages do not simply attach randomly to any cell type, but that they specifically interact with HSPCs.

The peak interaction percentage takes roughly 10 hours to reach, perhaps due to the non-chemotactic nature of HSPC-macrophage interactions. It may take 10 hours for enough macrophages and HSPCs to randomly collide. This peak percentage closely mirrors the *in vivo* interaction percentage measured in the CHT (Figure 20). This suggests that the culture conditions may be sufficient to replicate *in vivo* HSPC-macrophage interactions.

**Figure 23:** Macrophages marked by *mpeg1:mCherry* (red) are depleted 6 hours after clodronate liposome injection (right). Macrophages are unaffected by PBS liposome injection (left). Zebrafish embryos are healthy with normal morphology after liposome injection.

**Macrophage-HSC interactions play a functional role**

A loss-of-function experiment was performed to test whether macrophage-HSC interactions play a functional role in the CHT. Macrophages were selectively depleted from 48 hpf zebrafish embryos by injection of clodronate liposomes [43].
liposomes are specifically taken up by macrophages, which are then killed. Control embryos from the same clutch were injected with PBS liposomes as a negative control. The clodronate liposomes effectively deplete nearly all the macrophages in the embryo over the course of 6 hours (Figure 23).

Clodronate liposomes were injected into CD41:GFP embryos, which have fluorescently labeled HSPCs. Macrophage depletion in these embryos resulted in decreased GFP$^+$ cells in the CHT ($p < 0.01$) (Figure 24). There were still circulating GFP$^+$ cells in the macrophage depleted embryos, suggesting that while the HSPCs are present, they are unable to properly engraft in the CHT.

Figure 24: Representative pictures of CD41:GFP embryos injected PBS-loaded liposomes (control) versus clodronate liposomes (macrophage depleted), demonstrating decreased decreased GFP$^+$ cells in the CHT ($p < 0.01$).
Clodronate liposomes were also injected into wildtype embryos at 48 hpf. The fish were fixed at 72 hpf. In situ hybridization was then performed for Runx1, a specific HSC marker. The results show significantly decreased Runx1 staining in the CHT of the embryos injected with the clodronate as compared to PBS controls ($p = 0.02$) (Figure 25).

![Image](image_url)

**Figure 25**: Significantly decreased Runx1 staining in the CHT by in situ hybridization at 72 hpf in embryos injected with clodronate liposomes at 48 hpf compared to PBS liposome controls ($p = 0.02$).

A similar experiment was performed using Runx1:NLS-mCherry embryos, which specifically label HSCs in zebrafish embryos. The embryos were injected with clodronate liposomes at 48 hpf and time lapse imaging was performed starting at 60 hpf until 72 hpf. Macrophage depletion resulted in significantly decreased HSCs in the CHT over time (Figure 26,27).

Together, these experiments reveal a functional role for macrophages in HSC biology. HSCs fail to properly colonize the CHT in the absence of macrophages, which appear to play a retention role in the HSC niche. It is then not unreasonable to hypothesize that the cell surface interactions and cellular adhesion molecules may be involved in this process.
Generation of mutants and transgenics

In order to more carefully examine these interactions from a mechanistic standpoint, fish were obtained or generated with mutant cellular adhesion molecules. Using the CRISPR/Cas9 system, \(itgb2\) mutants were generated with a 19bp deletion in exon 6. F1 fish have been in-crossed and homozygous F2 mutants are growing up. \(Itga4\) mutants have also been obtained in collaboration with Weijun Pan’s lab in Shanghai. The \(Itga4\) mutant has been shown by their group to have decreased HSC colonization of the CHT. Both \(itgb2\) and \(itga4\) mutants have been outcrossed to fluorescent reporter lines (\(mpeg1:mCherry\), \(Runx1:mCherry\), \(cd41:GFP\)) to better observe macrophage and HSC behaviors in vivo;
Figure 27: Average number of Runx1:mCherry cells (HSCs) in the CHT over a 12 hour time lapse (60-72 hpf) with either clodronate (red) or PBS (blue) liposome injection at 48 hpf. Shaded regions specify the 95% confidence interval (n = 12).

heterozygotes are currently growing up in the fish facility.

Additionally, gRNAs to other CHT specific genes from Tomo-seq with high expression in macrophages have been developed. Nrros (also called lrcc33) and sepp1a are both genes involved in regulation of reactive oxygen species (ROS) and were highly expressed in the CHT and in macrophages. Macrophages can generate ROS as a means to combat infectious pathogens. It may be possible that these ROS regulator genes are present in macrophages in the CHT to maintain a low-ROS environment for the HSCs. I have designed, validated, and injected gRNAs for nrros and sepp1a. F0 mutant embryos are currently growing up. Further experiments will need to be performed to characterize all of these mutant lines and look for phenotypes in HSC engraftment.
**Nanowell co-culture system**

In collaboration with the Chris Love lab at MIT (Koch Institute), nanowell culture chips were used to better isolate HSC-macrophage interactions in a controlled environment. Nanowell chips consist of thousands of 30 by 30 μm wells [44]. A mixture of fluorescently labeled cells can then be dropped over the wells. The cells will then randomly settle into the wells, and by plating cells at the appropriate density, large numbers of wells with 2 cells can be obtained.

![Figure 28: (A) Low magnification and (B) high magnification of 30μm nanowells plated with F4/80+ macrophages (red) and lineage- cells (green).](image)

In a pilot experiment, mouse bone marrow HSPCs (Lineage-, Sca-1+, c-Kit+) and macrophages (F4/80+) were obtained from mouse bone marrow by FACS. To test the nanowells, lineage- cells and F4/80+ macrophages were stained using fluorescent dyes, mixed, plated onto the nanowells, and imaged (Figure 28). A large number of doublets of cells could be detected. In an independent experiment, the nanowells were also tested in conjunction with the cell picking robot in the Love lab. The robot is able to suck out the contents of individual wells, allowing possible analyses with single cell RNA-seq or other techniques on HSPCs that have interacted with macrophages compared to those that have not. These nanowell
techniques can be used with mutant zebrafish or mouse lines to further interrogate macrophage-HSC interactions and signaling pathways in future experiments.
Discussion

The zebrafish CHT presents a developmentally and anatomically stereotyped tissue where HSC-niche interactions can be readily visualized and studied. By using Tomo-seq, members of the lab were able to generate an anatomic map of gene expression to localize CHT specific genes. Combining this technique with RNA-seq on sorted macrophage populations, I was able to narrow down the list of CHT enriched genes to those highly enriched in macrophages.

Cell adhesion molecules were found to be differentially expressed in the macrophage population in the CHT. Live cell imaging of the macrophages and HSCs in the CHT revealed frequent intimate cell-cell interactions between the two populations. Quantification of the data showed that approximately 17% of HSPCs in the CHT are interacting with a macrophage at any point in time during development. A macrophage-HSPC co-culture system was then developed to study these interactions in a more controlled environment devoid of other cell types in the CHT. The co-culture experiment re-capitulated the result that roughly 15-20% of HSPCs are interacting with a macrophage at steady state. The co-culture experiment was also able to demonstrate that macrophages have an increased affinity for HSPCs as compared to a negative control cell type (erythrocytes), in which less than 10% of cells were engaged with a macrophage at any given point in time. To test whether these interactions may play a functional role, macrophages were depleted from the zebrafish embryo and HSC numbers were assayed, revealing a significantly decreased number of HSCs in the CHT in the setting of macrophage loss. Together these experiments demonstrate a specificity of macrophage-HSC interactions in the CHT as well as a functional role.

Macrophage depletion studies in mammalian models suggest that macrophages
play a role in retaining HSCs in the bone marrow and spleen [45–48]. In particular, one study showed that VCAM-1 expressed on splenic macrophages is important for splenic HSC retention [47]. VCAM-1 is a known ligand of the α4β1 integrin VLA-4. From the zebrafish macrophage RNA-seq data, both integrins α4 (itga4) and β1 (itgb1b) are expressed in macrophages (Figure 18A). Moreover, Julie Perlin in the lab has recently shown VCAM-1 promoter driven GFP co-localizes with mCherry+ cells in the Runx1:mCherry transgenic zebrafish line marking HSCs [49]. This may imply that in zebrafish, VLA-4 is present on the macrophage and VCAM-1 is present on the HSC, the opposite of mammalian systems. Microglia in the brain have also been shown to play roles in pruning of synapses by surface interactions as well as presentation of TGFβ by Lrrc33 [50, 51]. TGFβ inhibition has also been shown to expand the HSPC pool in the CHT by increasing cell divisions and preventing HSCs from entering a quiescent state [13]. It would not then be surprising if macrophages in the HSC niche were similarly utilizing surface interactions for signaling and HSC regulation.

Taking these studies into account, the current working model for macrophage-HSC interactions in the niche is as follows. HSCs begin to colonize the CHT starting at 36 hpf. When a macrophage in the CHT encounters an HSC, it anchors itself to the surface via VLA-4 on the macrophage interacting with VCAM-1 on the HSC. The macrophage is then able to present signaling molecules, perhaps including TGFβ via Lrrc33, to promote HSC quiescence and retention within the niche, avoiding HSC exhaustion by uncontrolled cell division.

Moreover, existing monoclonal antibodies directed at integrin subunits have already made large impacts in both multiple sclerosis and inflammatory bowel disease. By modulating integrin binding in the HSC niche, one could begin to
target HSC engraftment and proliferation. Understanding these cell-cell interactions is paramount to creating therapies targeted toward the HSC niche.

**Future directions**

The integrin mutants will allow for further investigation into the direct interactions between macrophages and HSCs. Co-culture can be performed using mutant macrophages to quantify whether HSC-macrophage binding is integrin dependent and to identify the specific subunits involved. Moreover, use of integrin inhibitors can be used to see if they recapitulate the effect. *In vivo* live cell imaging can also be done to quantify interactions between macrophages and HSCs in the CHT as well as quantify any changes in HSC numbers or behaviors. Similarly, *Lrrc33* mutants can be imaged to assess HSC numbers and behaviors. Nanowells could also be used with single cell RNA-seq to identify differences in the transcriptome between HSCs that have and have not interacted with macrophages. This can also be performed with mutant macrophages to better understand the roles of *Lrrc33* and integrins in this interaction. By understanding the role of macrophages in the HSC niche, therapies could be developed that target macrophages in the niche to promote HSC retention or engraftment in the setting of HSCT or even alter blast dynamics in the setting of blood cancers.
Conclusions

Interactions between HSCs and the HSC niche play key roles in the maintenance and regulation of hematopoiesis. By taking advantage of the zebrafish model system, I was able to directly observe HSCs in their microenvironment, leading to novel findings in both the vascular niche as well as HSC interactions with macrophages.

By utilizing an unbiased screening technique on genes enriched in endothelial cells, overexpression of the receptor CXCR1 was found to increase HSC colonization of the CHT. Moreover, overexpression of its ligand CXCL8 recapitulated this same effect. CXCR1 was also demonstrated to act cell autonomously to the sinusoidal vascular niche rather than the HSC. Finally, CXCR1 overexpression was demonstrated to increase the volume of the sinusoidal niche. Together, these findings implicate a novel role for the CXCL8/CXCR1 signaling pathway in the HSC vascular niche.

Macrophages were found to differentially express cellular adhesion molecules in developing zebrafish embryos. Live cell imaging of the macrophages and HSCs in the CHT revealed frequent intimate cell-cell interactions between the two populations. Quantification of the data showed that approximately 17% of HSPCs in the CHT are interacting with a macrophage at any point in time during development. A macrophage-HSPC co-culture system was then developed to study these interactions in a more controlled environment devoid of other cell types in the CHT. The co-culture experiment re-capitulated the result that roughly 15-20% of HSPCs are interacting with a macrophage at steady state. The co-culture experiment was also able to demonstrate that macrophages have an increased affinity for HSPCs as compared to a negative control cell type (erythrocytes), in which less than 10% of
cells were engaged with a macrophage at any given point in time. To test whether these interactions may play a functional role, macrophages were depleted from the zebrafish embryo and HSC numbers were assayed, revealing a significantly decreased number of HSCs in the CHT in the setting of macrophage loss. Together these experiments demonstrate a specificity of macrophage-HSC interactions in the CHT as well as a functional role for these interactions.

Both CXCL8/CXCR1 signaling in the vascular niche as well as macrophage-HSC interactions are still not fully understood, and more work needs to be done to better characterize these interactions. By continuing to study the HSC niche, a better understanding of HSC dynamics and engraftment may lead to improvements in HSCT and better outcomes for patients in the future.
References


42. Hagedorn, E. Unpublished data.


49. Perlin, J. Unpublished data.


Supplemental Materials

Generation of parabiotic zebrafish embryos

Parabiotic zebrafish were generated according to published protocols [22, 23]. Parabiotic embryos were dechorionated at 4 hpf and surgically stitched in methylcellulose using a glass needle. In collaboration with Elliott Hagedorn in the lab, I was able to improve the efficiency of generation of parabiotic embryos by increasing the degree of wounding during surgery as well as revisiting prior surgeries that did not sufficiently take (Figure 29) [23]. This resulted in a nearly 100% efficiency of generating fused embryos.

By stitching embryos animal pole to animal pole, reliable head to head fusions were generated that had shared circulation (Figure 30) [23]. By injecting a heat shock construct, temporal control of transgene expression was also achieved, allowing for transgene expression in one embryo but not the other (Figure 7).
Figure 29: High magnification images show zebrafish embryos oriented with their animal poles touching each other. (A) Prior to surgical stitching, (B) minimal wounding, (C) more substantial wounding. (D) After 20 minutes, the embryos have been successfully fused. Scale bar represents 250 µm.
Figure 30: Dextran conjugated fluorescent dye was injected into a parabiont at 72 hpf. The dye was swept into circulation, lighting up the vasculature of both embryos, demonstrating a shared circulation.
Co-culture image analysis via Matlab

In order to analyze co-culture time lapse data sets and quantify cell-cell interactions in a high throughput fashion, I wrote a custom Matlab image analysis script complete with graphical user interface (GUI) to allow quantification of interacting pairs of cells in two fluorescence channels.

![Image Analysis GUI](image_analysis_gui.png)

**Figure 31:** Example of the image analysis GUI in the custom Matlab script.

The segmentation works by allowing the user to apply different filters to a sample user-selected mono image for each channel using a GUI (Figure 31). The user can apply a minimum filter, gaussian blur, fill holes, filter by area size of cells, use local maxima with voronoi diagrams to split cell doublets, and apply a user-defined threshold. After applying these filters, the image is converted to a binary image where everything below threshold is considered background and everything
above threshold is considered a cell (Figure 32). While the user adjusts the various filters and thresholds, a live preview binary image is displayed on the left along with an update of the current number of detected objects in the image. After the user is satisfied with the thresholds and filters for each preview image in each fluorescence channel, those same settings are then applied to the entire data set (every time point and every well in the plate).

![Figure 32: Example of a co-culture image after segmentation and what the computer detects as cells in each fluorescence channel.](image)

Interacting pairs are counted by dilating one channel with a small structuring element and then counting regions of overlap. Phagocytosed cells are excluded by counting regions of overlap that share greater than 95% area with the HSPC underlying the overlapping region. These types of interactions are excluded because
they are not the type of surface interactions present in time lapse CHT data but are likely an artifact in culture of a macrophage eating a dying cell (Figure 19).

The data for each time point and each well is saved into a data table and plots are automatically generated enumerating the number of total cells of each type, percentage of cells interacting, number of interacting pairs, and number of phagosomes. The analysis settings including filter and threshold settings are also exported.