An *in vivo* chemical genetic screen to identify new regulators of hematopoietic stem cell expansion

Master Thesis

For the attainment of the academic degree

Master of Science

From the University of Applied Sciences FH Campus Wien

Submitted by:
Rafaela Vostatek, BSc

Personal identity code
c1810544009

Supervisor:
Dr. Anne Ellett
Boston Children's Hospital – Harvard Medical School
Boston
USA

Submitted on:
17.02.2021

Abstract

Each year about 25,000 hematopoietic stem cell transplantations (HSCTs) are performed to treat various non-malignant and malignant conditions. The number of transplantations is unlikely to significantly decrease during the coming years and the supply of hematopoietic stem cells (HSCs) is limited. Problems exist such as minor cell numbers in the donated tissue or donor incompatibility, which may lead to unmatched transplants and carry the risk of rejection or graft-versus-host disease (GvHD). An improvement of the success of bone marrow transplantation is necessary and new methods, like the expansion of stem cells from induced pluripotent stem cells (iPSCs), should be implemented to generate and expand hematopoietic stem cells *in vitro* to overcome these potential problems. With this in mind, the Zon Laboratory generated a new transgenic zebrafish line (Tg(Runx1:GFP) which expresses the mouse stem cell-specific Runx1 enhancer driving GFP and marks HSCs during the process of definitive hematopoiesis. The Zon laboratory also identified the compound Ro5-3335, which can expand HSCs during a specific time frame of development, although the mechanism behind this activity is not yet clearly understood. Through the usage of different transgenic fish embryos, including Runx:GFP and Runx:mcherry, the effect of this compound was characterized, and its activity further investigated. Additionally, a small chemical library of selected promising compounds was screened to identify other possible drugs which stimulate the expansion of HSCs during embryonic development. My findings revealed new chemicals that expand HSCs and could have a positive therapeutic effect on strategies to improve bone marrow transplantations following further investigation.

Key terms: Chemical genetic screen, Hematopoietic stem cells, Zebrafish,

Abstract

Um verschiedene bösartige und gutartige Erkrankungsbilder zu behandeln, werden jedes Jahr über 25.000 hämatopoetische Stammzellentransplantationen (HSCTs) durchgeführt. In den nächsten Jahren ist es sehr unwahrscheinlich, dass die Anzahl der Transplantationen geringer wird und daher ist die Versorgung mit hämatopoetischen Stammzellen (HSC) limitiert. Dabei bleibt das Risiko, dass eine zu geringe Anzahl von Zellen im Spendergewebe vorhanden ist oder eine Spenderinkompatibilität auftritt, welche das Risiko einer Abstoßung oder einer Transplantat-gegen-Wirt-Reaktion (GvHD) auslöst. Daher ist eine Verbesserung des Erfolges einer Knochenmarkstransplantation unumgänglich und neue Methoden, wie die Expansion von Stammzellen aus induzierten pluripotenten Stammzellen (iPSCs) sollte eingeführt werden um die genannten Probleme zu umgehen und hämatopoetische Stammzellen *in vitro* zu generieren und erweitern. Um einen Schritt in die richtige Richtung zu wagen, generierte das Zon Labor eine neue transgene Zebrafischlinie (Tg(Runx1:GFP), welche den maus-spezifischen Runx1 Enhancer, während des Prozesses von definitiver Hämatopoese, exprimiert und mit GFP markiert. Das Zon Labor hat bereits die Chemikalie Ro5-3335 entdeckt, welche hämatopoetische Stammzellen während einer bestimmten Entwicklungsspanne, erhöht; wobei der Mechanismus noch nicht ganz verstanden wird. Durch die Verwendung von verschiedenen transgenen Zebrafischembryonen, wie Runx:GFP und Runx:mcherry, wurde der Effekt dieser Chemikalie charakterisiert und weiter untersucht. Zusätzlich wurde eine chemische Datenbank mit vielversprechenden Chemikalien untersucht, um weitere mögliche Medikamente zu entdecken, welche die Vervielfältigung von hämatopoetischen Stammzellen während der embryonischen Entwicklung stimuliert. Die identifizierten Chemikalien wurden weiter untersucht und validiert durch zusätzliche Untersuchungen (Untersuchung 2. Grades) wie in in situ Hybridisierung und Dosis-Wirkungsassays. Meine Entdeckung zeigten neue Strategien, um hämatopoetische Stammzellen zu vervielfältigen und diese hat möglicherweise nach weiteren Untersuchungen einen positiven Effekt auf die Methode der Knochenmarkstransplantationen in der Zukunft.

.

Preface

The master thesis aims to sum up the master's internship during the fourth and fifth semester of the master's program 'Molecular Biotechnology' (February 2020 – December 2020). The internship was executed in the group of Professor Leonard I. Zon, MD at the Boston Children's Hospital, which is part of the Harvard Medical School. Due to the COVID-19 pandemic the internship had to be paused between mid of March and mid of August. A lot of setbacks happened but the lab work could be resumed in August and substantial progress had been achieved. The internship was supported by the Austrian Marshall Plan Foundation.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

%	Percent
°C	Grad Celsius
µL	Microliter
µM	Micromolar
AGM	Aorta-gonad-mesonephros
AML	Acute myeloid leukemia
bHLH	Basic helix-loop-helix
CBfβ	Core-binding factor subunit beta
CHT	Caudal hematopoietic tissue
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dpf	Days post-fertilization
E3	E3 Buffer (Embryonic water)
EB	Embryoid body
EHT	Endothelial-to-hematopoietic transition
EMP	Erythroid-myeloid progenitor
EP2	Ptger2
EP4	Ptger2
ESC	Embryonic Stem Cells
FACS	Fluorescence-Activated Cell Sorting
GFP	Green Fluorescent Protein
GvHD	Graft-versus-host disease
HE	Hematopoietic Epithelium
HLA	Human leukocyte antigen
Hpf	Hours post-fertilization
HC	Hematopoietic cell
hPSC	human pluripotent stem cell
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and Progenitor cells
ICM	Intermediate cell mass
MHC	Major Histocompatibility Complex
mL	Milliliter

mM	Millimolar
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nM	Nanomolar
No	Number
o/n	Overnight
PGE2	Prostaglandin E2
RNA	Ribonucleic acid
RT	Room temperature
SR1	StemRegenin 1
SSC	Saline Sodium Citrate
TALEN	Transcription activator-like effector nuclease
TF	Transcription Factor
TNFα	Tumor necrosis factor
V	Volume
Vegf	Vascular epithelial growth factor
X	time

1. INTRODUCTION

Each year about 25,000 hematopoietic stem cell transplantations (HSCTs) are performed to treat various non-malignant and malignant conditions, such as leukemia, lymphoma, congenital metabolic defects, immunodeficiency illnesses, myeloproliferative and myelodysplastic syndromes and hemoglobinopathies (Gyurkocza, Rezvani, & Storb, 2010; Hatzimichael & Tuthill, 2010). The number of required transplantations is unlikely to significantly decrease during the coming years and the supply of hematopoietic stem cells (HSCs) is limited. Problems exist such as low cell numbers in the donated tissue or donor incompatibility, which may lead to unmatched transplants that carry the risk of rejection or graft-versus-host disease (GvHD) (Gyurkocza et al., 2010; Hatzimichael & Tuthill, 2010; Perlin, Robertson, & Zon, 2017). Actions are necessary to achieve a higher success of transplantations and new methods must be established to expand and generate hematopoietic stem cells *in vitro* which could surmount these potential problems (**Figure 1**) (Perlin et al., 2017).

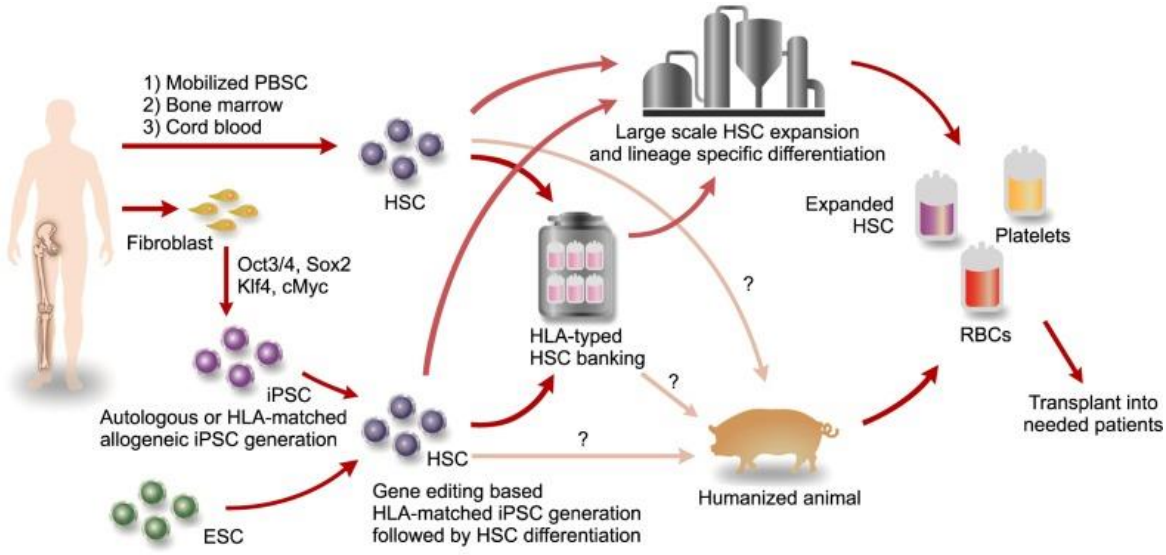

Figure 1 Finding new approaches to expand HSCs. HLA-matched iPSC derived HSCs and *ex vivo* expanded HSCs could be a solution to overcome the HLA-matched HSC shortage. Additionally, the usage of humanized animals for blood production could be applicable. (Park, Yoo, & Kim, 2015)

1.1 Overview of Hematopoiesis

The emergence of cellular components of the blood is known as hematopoiesis. This process begins during embryonic development and continues throughout adulthood. In vertebrates, the development of blood consists of two main waves, the primitive wave and the definitive wave (Galloway & Zon, 2003). The goal of the primitive wave is the production of red blood cells (erythrocytes) and primitive macrophages (Palis & Yoder, 2001). Red blood cells are capable of facilitating tissue oxygenation during the rapid growth stage of the embryo and primitive macrophages provide a functional innate immune response (Jagannathan-Bogdan & Zon, 2013; Stuart H. Orkin & Leonard I. Zon, 2008; Paik & Zon, 2010). During early mammalian development, these erythroid progenitor cells arise in blood islands in the yolk sac and are neither pluripotent nor able to renew (Paik & Zon, 2010). Later in development, a transient definitive wave of hematopoiesis occurs in the blood islands, which leads to production of erythroid-myeloid progenitors (EMPs) (Bertrand et al., 2007; McGrath et al., 2011). These cells give rise to definitive erythroid and myeloid cells but lack lymphoid potential. At a later time point of definitive hematopoiesis, true multipotent hematopoietic stem cells emerge (Cumano & Godin, 2007; Jagannathan-Bogdan & Zon, 2013; Paik & Zon, 2010). In vertebrates, these definitive HSCs arise from the aorta-gonad-mesonephros (AGM) region and then migrate to the intermediate stem cell niche in the fetal liver, where they divide prior to migration to the bone marrow or the thymus, which are the main HSC reservoirs in adults. **(Figure 3)** (Cumano & Godin, 2007; Jagannathan-Bogdan & Zon, 2013; Paik & Zon, 2010).

Throughout the lifespan of an organism, millions of blood cells die and are ultimately replaced by the appropriate type and number of new cells. This phenomenon is achieved by definitive HSCs, which have the ability to differentiate into all of the different blood cell lineages, within both the myeloid and lymphoid subgroups. T cells, lymphoid dendritic cells, B cells and natural killer cells are part of the lymphoid blood lineage and all other cells belong to the myeloid lineage (**Figure 2**) (Schroeder, 2010). When HSCs are ready to divide, they are capable of generating at least one HSC potent daughter cell in the process of self-renewal, and this daughter cell maintains the stem cell pool (Schroeder, 2010). Other multipotent hematopoietic progenitors are also able to generate daughter cells, which have the same differentiation potential but only for a limited time period. However, this is not true for HSCs, the self-renewal capability of which lasts long enough to sustain the hematopoietic system throughout life (Schroeder, 2010).

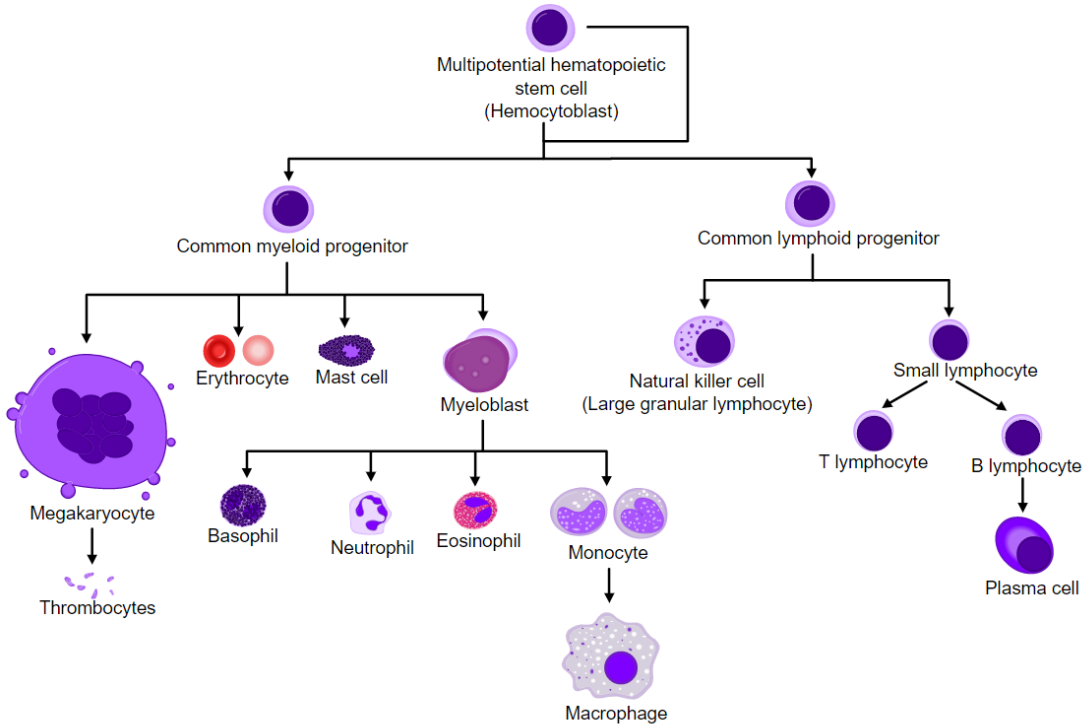

Figure 2 A Simplified Overview of Hematopoiesis. HSCs differentiate into various cell lineages, either in the subgroup of myeloid or lymphoid cells. T cells, lymphoid dendritic cell lineages, B cells and natural killer cells are part of the lymphoid lineage whereas erythrocytes, mast cells, myeloblasts and megakaryocytes belong to the myeloid lineage (A. Rad and Mikael Häggström, 9 December 2016)

One of the main attributes of HSCs is their transplantation ability: upon harsh removal from their original niche environment and injection into a recipients blood stream, they survive, maintain their self-renewal potential, find their way to the host niche, and proceed with their regeneration function to contribute to the hematopoietic system. Their phenomenal flexibility to reconstitute blood in a new host is probably derived from their developmental migratory behavior, as they originally bud off the AGM niche into the blood stream and continue to migrate to new and distant sites throughout development. Additionally, HSCs are easy to manipulate, making them one of the best understood adult mammalian stem cells and one of the most successfully used for stem cell therapy (Schroeder, 2010).

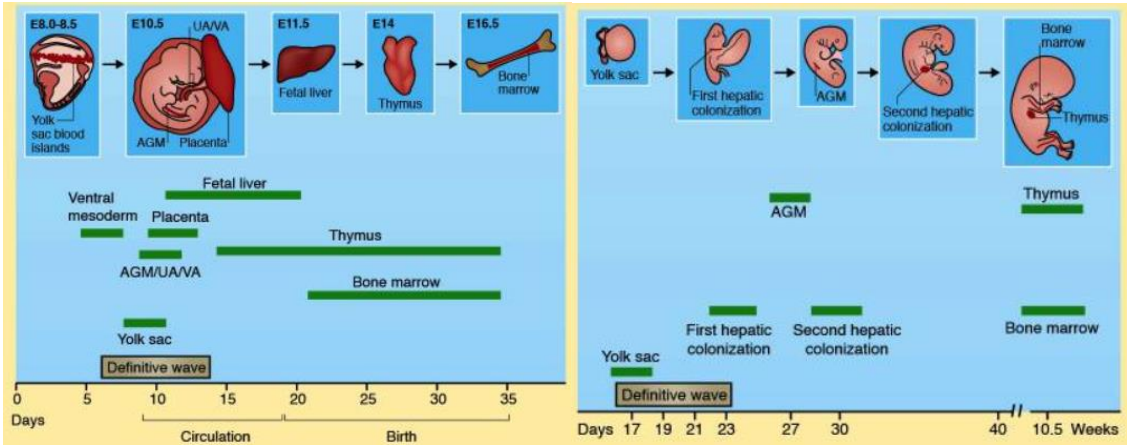

Figure 3 Hematopoiesis in mouse and human.

(Left) In the ventral yolk sac blood islands of the mouse, the first hematopoietic progenitors occur, which are derived from the ventral mesoderm. The circulation of these progenitors begins leading to colonization of other hematopoietic organs. At a timepoint of 10.5 days after fertilization, generation of definitive blood precursors initiates. The placenta, umbilical artery (UA), AGM, vitelline artery (VA) and yolk-sac derived cells are responsible for this initiation. Together, they colonize the fetal liver and expansion and maturation ensues. From the fetal liver, the bone marrow gets colonized and remains as the main adult hematopoietic niche (Jagannathan-Bogdan & Zon, 2013).

(Right) Hematopoiesis in the human embryo occurs in the yolk sac and after the onset of blood circulation it proceeds transiently to the developing liver. Before the thymus and bone marrow colonization occurs at 10.5 weeks, a second hepatic colonization event proceeds (Jagannathan-Bogdan & Zon, 2013).

1.2 Overview of hematopoiesis in zebrafish

Over the past few decades, the zebrafish model has been widely used to study hematopoiesis and hematopoietic disorders due to its many unique attributes. For example, embryos are fertilized externally and develop in a fast manner. Different genetic modifications can be performed with relative ease using multiple techniques, allowing the creation of mutants and transgenic reporter lines. In addition, the transparency of the embryos facilitates the visualization of early development using fluorescent reporter lines. These characteristics have enabled the discovery of many cell types, genes and cellular behaviors that are important and conserved in hematopoiesis (Perlin et al., 2017).

In zebrafish, blood development is similar to that in mammals, with distinct waves of hematopoiesis occurring. During the gastrulation process, the ectoderm, mesoderm and endoderm are established which further differentiate into various tissue types. Throughout the dorsal mesoderm, the notochord and somites develop, while the pronephros and vasculature arise from the ventral mesoderm. Additionally, the intermediate cell mass (ICM) develops from the ventral mesoderm, which is the location from which the primitive erythroid progenitors emerge. (Detrich et al., 1995b; Jagannathan-Bogdan & Zon, 2013). These primitive cells start to circulate at a

timepoint of 24 hours post-fertilization (hpf). In zebrafish, the EMP progenitors are born in the posterior ICM region. At approximately 30 hpf, the definitive phase starts, which results in the development of HSCs from the ventral wall of the dorsal aorta and continues with the migration of the HSCs to the caudal hematopoietic tissue (CHT), which is located in the posterior region of the tail of the zebrafish embryo and is equivalent to the mammalian fetal liver **(Figure 4)** (Burns et al., 2002; Kalev-Zylinska et al., 2002; Thompson et al., 1998). Lymphopoiesis starts after three days post-fertilization (dpf) in the thymus and at day four the HSCs move to the kidney marrow, the equivalent of the mammalian bone marrow. (Jagannathan-Bogdan & Zon, 2013; H. Jin, Xu, & Wen, 2007; Murayama et al., 2006; Perlin et al., 2017).

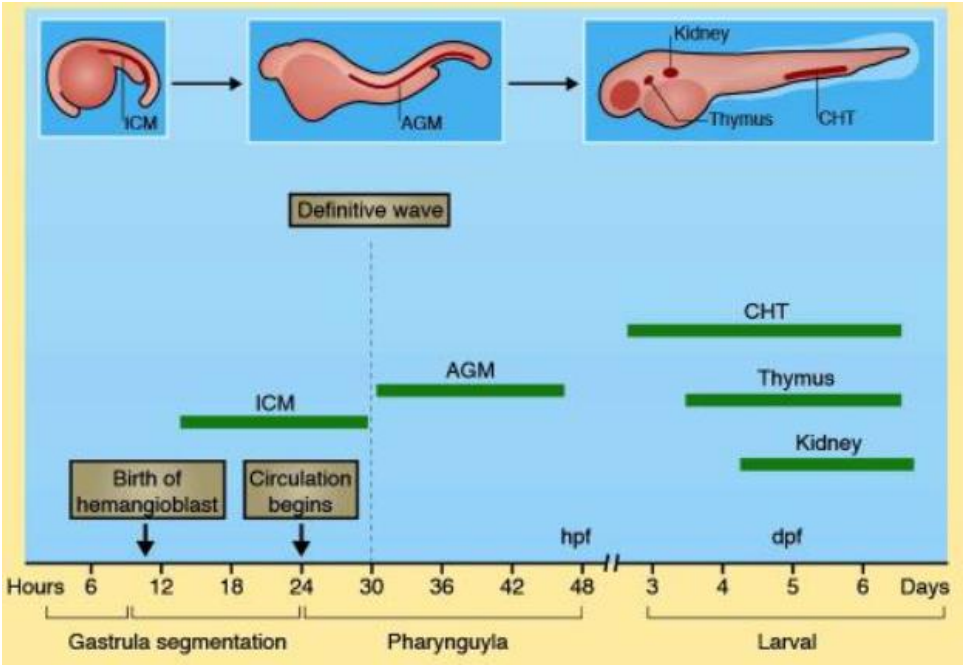

Figure 4 Sites of hematopoiesis in zebrafish.

The ventral mesoderm-derived tissue 'intermediate cell mass (ICM)' is the place of origin of primitive erythroid progenitors, which start to circulate at approximately 24 hpf. Within the AGM of the ventral wall of the dorsal aorta, definitive HSCs emerge at around 30 hpf and migrate to the CHT within the posterior region of the zebrafish tail. Lymphopoiesis starts after three days post-fertilization (dpf) in the thymus and at day four the HSCs move to the kidney marrow, the analog of the mammalian bone marrow (Jagannathan-Bogdan & Zon, 2013)

1.2.1 Early specification of the hematopoietic system in zebrafish

The hematopoietic system of zebrafish derives from different regions of the lateral and ventral mesoderm, along with cardiac and endothelial lineages. At 9 hpf the zebrafish draculin *(drl)* gene can be detected, which is the earliest marker of blood progenitors

(Mosimann et al., 2015; Mucenski et al., 1991; Robertson, Avagyan, Gansner, & Zon, 2016). After gastrulation there are several transcription factors that are expressed in blood progenitors, including the GATA binding factor *gata2*, the basic helix-loop-helix transcription factor *scl*, and the binding partner of LIM-domain *lmo2* (Dooley, Davidson, & Zon, 2005; Robertson et al., 2016). Blood progenitor cells additionally express the vascular markers *fli1* and *kdrl*, and these are also expressed in the putative zebrafish hemangioblast, which has the ability to specify into endothelial or hematopoietic cells. There is an ongoing debate regarding the existence of either one or two precursors that give rise to endothelial and blood lineages. A previous histological study using chick embryos in the 1920s revealed the association of erythroblasts and endothelial cells, which together form the blood islands (Murray, 1932). Many years later, in the 1990s, a bipotent progenitor thought to be an *in vitro* equivalent of the hemangioblast was found in embryonic stem cell cultures (K. Choi, Kennedy, Kazarov, Papadimitriou, & Keller, 1998). Different *in vivo* studies showed that single cells can develop into both hematopoietic and endothelial cells and contribute to the presence of the hemangioblast (K. Choi et al., 1998; Huber, Kouskoff, Fehling, Palis, & Keller, 2004; Lugus, Park, Ma, & Choi, 2009; Robertson et al., 2016). However, there is also evidence of a blood island containing more heterogenous cell populations, which may indicate that the progenitor cells are committed to either a vascular or hematopoietic fate (K. Choi et al., 1998; Huber et al., 2004; Lugus et al., 2009; Robertson et al., 2016). Other studies revealed a linear pathway, describing that the hemangioblast gives rise to an intermediate cell type with hemogenic potential, which further differentiates to blood (Lancrin et al., 2009). Another study using cell fate mapping showed that bipotent cells exist in zebrafish, but not all endothelial and hematopoietic progenitors develop from these bipotent cells (Vogeli, Jin, Martin, & Stainier, 2006). Nevertheless, there is a need for additional studies to further define the contribution and importance of bipotent cells in vasculogenesis and hematopoiesis (Hsia & Zon, 2005; Robertson et al., 2016).

The transcription factors *gata2*, *scl* and *lmo2* have an important role in hematopoietic development and their functions are highly conserved among different species (Ciau-Uitz, Monteiro, Kirmizitas, & Patient, 2014). *Lmo2* and *scl* are essential for hematopoiesis and the deletion of these in zebrafish embryos leads to defects in angiogenesis and loss of primitive and definitive hematopoietic cell (HC) lineages (Dooley et al., 2005; Hsia & Zon, 2005; Patterson et al., 2007; Robertson et al., 2016; Shivdasani, Mayer, & Orkin, 1995; Yamada et al., 1998). In contrast, an increased

production of endothelial and hematopoietic precursors, at the expense of pronephric and somatic duct tissue, can be seen when *scl* is ectopically expressed (Dooley et al., 2005; Gering, Rodaway, Göttgens, Patient, & Green, 1998; Gering, Yamada, Rabbitts, & Patient, 2003). In the zebrafish *cloche* mutant, *scl* expression is decreased, which leads to defects in endothelial and blood cell development (E. C. Liao et al., 1998; Stainier, Weinstein, Detrich, Zon, & Fishman, 1995). This mutant was identified in a large-scale mutagenesis screen; however, researchers were faced with a significant challenge when it came to cloning the underlying gene. Studies have indicated that the hemangioblast may be affected by the *cloche* mutation, as in addition to *scl*, many other important genes for downstream vascular and hematopoietic development are affected, such as *fli1*, *etv2*, *hhex*, *kdrl*, and *gata1* (W. Liao et al., 1997; Patterson et al., 2007; Robertson et al., 2016; Stainier et al., 1995; Sumanas & Lin, 2006). Recently, 20 years after the mutant was identified, the PAS-domain-containing bHLH (Basic helix-loop-helix) transcription factor *npas4l*, acting upstream of *etv2* and *sc1*, was revealed to be encoded by the *cloche* gene (Reischauer et al., 2016). The revelation of this master regulator of hematopoietic and endothelial fates will expedite the identification of important additional regulators in early blood development and could be fundamental for *in vitro* generation of HSCs from pluripotent stem cells and regenerative medicine (Robertson et al., 2016).

Hemangioblast specification also involves other genes such as *Etv2*, *Flk1* and *Hhex* and mammalian studies have shown that deletion of these genes leads to abnormalities in blood and endothelial cells (Guo et al., 2003; D. Lee et al., 2008; Shalaby et al., 1997).

1.2.2 **Genetic Regulation of Primitive Hematopoiesis**

There are two transcription factors which contribute largely in primitive hematopoiesis, *Gata1* and *Pu1*. *Pu1* is also known as *Spi1b* in zebrafish and *Sfp1* in mouse. *Gata1* and *Pu1* have a cross-inhibitory relationship in which they regulate the primitive myeloid and erythroid fates. The transcription factor *Gata1* is the main regulator of erythroid development (Cantor & Orkin, 2002). In zebrafish, cells expressing *Gata1* also express erythrocyte-specific hemoglobin (Detrich et al., 1995a; Jagannathan-Bogdan & Zon, 2013). *Gata1* not only promotes gene regulation in erythrocytes, but also suppresses myeloid fate. In contrast to *Gata1*, the main regulator of the myeloid cell fate, is *Pu1* (Cantor & Orkin, 2002) which regulates production of granulocytes and macrophages. Due to the interaction between *Gata1* and *Pu1*, they are in direct

competition for target genes (Jagannathan-Bogdan & Zon, 2013; Scott, Simon, Anastasi, & Singh, 1994).

1.2.3 **Transcriptional control of definitive hematopoiesis in zebrafish**

HSCs develop in the hemogenic endothelium, which is a defined area in the ventral wall of the dorsal aorta alike to the mammalian AGM. At around 30 hours post fertilization the first definitive, multi-lineage hematopoietic stem cells begin to emerge (Kissa, Murayama, Zapata, Cortés, et al., 2008). The development of hematopoietic potential in a sub-population of endothelial cells in the AGM can be visualized via live cell imaging in transgenic zebrafish. One of the earliest HSC markers is the transcription factor *runx1*, which is needed for the development of definitive HSCs and regulates endothelial-to-hematopoietic transition (EHT), but the exact dynamics behind this is still unclear. During EHT, the endothelial cells start to express *runx1*, then bud from the vessel wall to enter the circulation (Kissa & Herbomel, 2010; Lam, Hall, Crosier, Crosier, & Flores, 2010; Robertson et al., 2016). This process can be observed in zebrafish using transgenic HSPC reporter lines combined with an endothelial marker. In the time frame of 28 to 32 hours post fertilization, the endothelial cells, which surround the ventral wall of the dorsal aorta, start to contract and change their shape resulting in spherical forming buds which extend into the aortic lumen (Bertrand, Chi, et al., 2010; Kissa & Herbomel, 2010; Perlin et al., 2017). Simultaneously, the expression of other specific HSPC genes occurs, including *cd41* and *cmyb*. HSPCs bud off from the subaortic space and migrate into the blood circulation through the dorsal wall of the cardinal vein and further transmigrate to the stem cell niche in the caudal hematopoietic tissue (Kissa & Herbomel, 2010; Kissa, Murayama, Zapata, Cortés, et al., 2008; Perlin et al., 2017).

Studies showed that HSCs do not survive in zebrafish embryos and the process of EHT is aborted when *runx1* is absent (Boisset et al., 2010; Burns et al., 2002; Kissa & Herbomel, 2010; Lam et al., 2010; North et al., 2002; Robertson et al., 2016). Similarly, *Runx1*$^{-/-}$ mice lose definitive lymphoid, erythroid and myeloid cells (Jagannathan-Bogdan & Zon, 2013; Paik & Zon, 2010; Q. Wang et al., 1996). In zebrafish, the first expression of *runx1* starts in the neural tissue at the five-somite stage, within the posterior lateral mesoderm (PLM). Despite this early expression, *runx1* is not required for the primitive wave of hematopoiesis but is essential for the definitive wave. *Runx1*-expressing cells also express *cmyb* at a timepoint of 36 hpf (Paik & Zon, 2010). *Cmyb* belongs to the *myb* family of proto-oncogenes and is another conserved marker of

HSCs (Burns, Traver, Mayhall, Shepard, & Zon, 2005; Gering & Patient, 2005; Jagannathan-Bogdan & Zon, 2013; Kalev-Zylinska et al., 2002; Paik & Zon, 2010). (Burns et al., 2005; Gering & Patient, 2005; Jagannathan-Bogdan & Zon, 2013; Kalev-Zylinska et al., 2002; Paik & Zon, 2010). It's expression begins at the ten to twelve somite stage, during the primitive wave of hematopoiesis. Cells expressing *cmyb* have been observed to migrate from the AGM to the pronephros, also known as the early kidney, and the thymus (Gering & Patient, 2005; H. Jin et al., 2007; Murayama et al., 2006). Cmyb is also important for definitive hematopoiesis and c*myb*$^{-/-}$ mice die due to fetal liver erythropoiesis failure (Jagannathan-Bogdan & Zon, 2013; Mucenski et al., 1991)

Various other transcription factors are needed for hemogenic endothelium specification and the development of definitive HSCs (**Figure 5**), including *scl*, as mentioned above (Dooley et al., 2005; Patterson et al., 2007; Robb et al., 1996; Robertson et al., 2016; Shivdasani et al., 1995). An interesting fact about *scl* is that two isoforms can be found in zebrafish due to a whole genome duplication event, and both are required during definitive hematopoiesis (Qian et al., 2007). Firstly, the *sclβ* isoform is expressed in endothelial cells in the AGM, which reach hematopoietic potential and continue with EHT. *Sclα* is expressed during the budding process of nascent HSCs and is likely to be important for HSC maintenance in the AGM, whereas *sclβ* is known in hemogenic endothelium specification (Jing et al., 2015; Zhen, Lan, Yan, Zhang, & Wen, 2013). Adenosine signaling is an important regulator of *sclβ*+ endothelium (Jing et al., 2015) and occurs through the A2b receptor and the cAMP-PKA pathway to induce production of the chemokine Cxcl8. This leads to *sclβ* expression in the hemogenic endothelium, which in turn facilitates the emergence of HSCs. Scl interacts as part of the Hedgehog-Notch-Scl signaling axis in the EHT. Hedgehog is required for hemogenic endothelial patterning, and mutations in this signaling protein lead to malfunctions in the production of HSCs, interestingly without affecting primitive hematopoiesis (Gering & Patient, 2005; Kim et al., 2013; Robertson et al., 2016). Notch is another important signaling factor that plays a prominent role in the hemogenic endothelium whilst controlling the expression of *runx1*. The zebrafish notch signaling mutant, *mindbomb*, has normal EMP and primitive blood production, but is unable to specify HSCs (Bertrand, Cisson, Stachura, & Traver, 2010; Burns et al., 2005). Transient Notch activation can rescue the mutant phenotype, as it induces *runx1* expression. Increase in HSC budding events have been linked to expression of constitutively active Notch (Burns et al., 2005; Lin et al., 2015; Robertson et al., 2016).

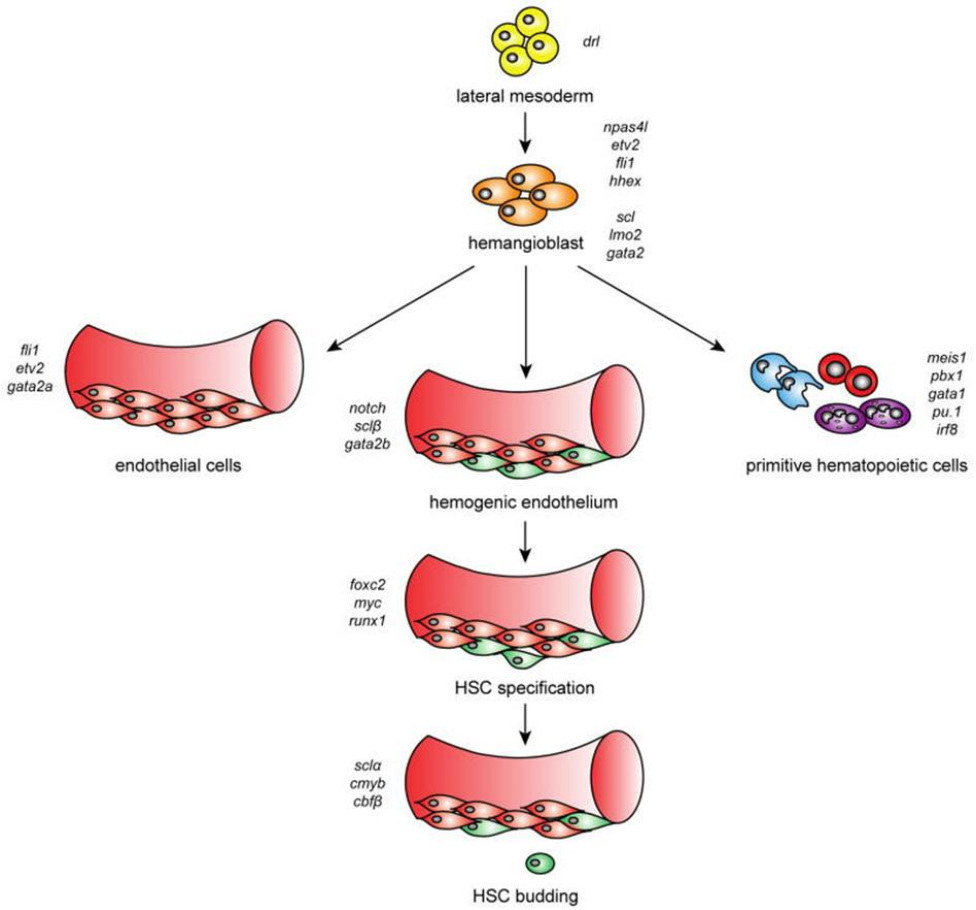

Figure 5 Control of transcription in the development of hematopoietic stem cells in zebrafish.

In zebrafish, the hemangioblast develops from the lateral mesoderm and continues with the differentiation into hemogenic endothelium, primitive hematopoietic cells or endothelial cells. Hematopoietic stem cells are specified in the hemogenic endothelium and then bud into the circulation. Multiple transcription factors are important during each of the individual steps (Robertson et al., 2016).

Notch is regulated via several distinct mechanisms, including non-autonomous signals from other cell types, which influence HSC development. For instance, the pro-inflammatory signaling molecule TNFα is released from primitive neutrophils and takes part in the regulation of HSC development via NFκB and Notch activation (Espin-Palazon et al., 2014; Robertson et al., 2016). Furthermore, secreted growth factors, such as angiopoietin-like proteins, contribute to cleavage of the Notch receptor, which leads to it's activation and consequently the transcription of downstream target genes (Lin et al., 2015). Notch can also be regulated at the epigenetic level. For instance, the 5-methylcytosine dioxygenases Tet2 and Tet3 are important for the activation of Notch. Tet2 and Tet3 contribute to the demethylation of DNA and the acquisition of hemogenic potential in aortic endothelium, by controlling Notch signaling in the ventral wall of the dorsal aorta that is necessary for the development of HSCs (C. Li et al., 2015;

Robertson et al., 2016). Another study in zebrafish showed that non-canonical Wnt16 signaling is also needed. This pathway serves as indirect regulation of HSC emergence and it helps to control the expression of Notch ligands. This is of great importance, because it implies that HSC fate is determined very early in development via Notch signaling, even prior to AGM formation. HSC precursors expressing *jam1a* migrate over the ventral somite where they interact with Jam2a junctional adhesion molecules, facilitating Notch signaling between the somite and the precursor cells (Clements et al., 2011; Kobayashi et al., 2014; Robertson et al., 2016).

A previous study in Gata2$^{-/-}$ mice showed the importance of GATA2 in primitive and definitive hematopoiesis, and also revealed it as a key regulator of SCL expression. In zebrafish there are two GATA2 orthologs, *gata2a* and *gata2b (Emerald Butko et al., 2015; Robertson et al., 2016; Tsai et al., 1994)*, which vary in their expression pattern and function. When *gata2a* is mutated in zebrafish, there are malfunctions in the circulation and endothelial integrity, demonstrating the role of *gata2a* in vascular development (E. Butko et al., 2015). In turn, *gata2b* is only expressed in a subset of endothelial cells, showing its importance in the emergence of definite HSCs. In mice, *Gata2* is prominent for its contribution in the regulation of the hemogenic endothelial-specific *Runx1* enhancer and for the survival of HSCs (Emerald Butko et al., 2015; Robertson et al., 2016). Additionally, in a recent study in zebrafish it was found that *gata2* is a main regulator of EHT upstream of *runx1* and a marker of hemogenic endothelium. *Gata2b-/-* embryos revealed a reduction of *runx1* expression in the AGM, and that deletion of a critical upstream enhancer leads to a loss of functional HSC generation, especially when the deletion occurs in endothelial cells (Emerald Butko et al., 2015; Gao et al., 2013; Kanz, Konantz, Alghisi, North, & Lengerke, 2016; Lim et al., 2012; Robertson et al., 2016).

As already mentioned, previous studies in zebrafish demonstrated the importance of epigenetic regulators in the emergence of definitive HSCs. A chromatin factor screen, performed by the Zon lab, revealed 29 factors which affected the number of HSCs in the AGM and, even previously unknown factors such as *jmjdlc*, *nap1l4a*, *brd8a* and *cbx6b* were discovered (Huang et al., 2013; Robertson et al., 2016). Downstream of *runx1* and *notch*, Dnmt3bb.1, a histone-modifying enzyme DNA methyltransferase is expressed. Zebrafish lacking this methyltransferase show a reduced number of HSCs, owing to a decreased expression of *cmyb* and decreased methylation of the *cmyb* locus (Gore et al., 2016; Robertson et al., 2016). Additionally, another forward genetic screen in zebrafish unveiled a different DNA methyltransferase with a key function in

the regulation of the downstream target gene *cebpa*, which is important for maintenance of HSCs (Liu et al., 2015; Robertson et al., 2016).

As previously discussed, HSCs bud from the hemogenic endothelium after undergoing EHT and enter the sub-aortic space. The process behind this is not understood entirely, but Cbfβ seems to contribute in the release of HSCs (Bresciani et al., 2014; Jagannathan-Bogdan & Zon, 2013; Kissa & Herbomel, 2010; Robertson et al., 2016).

In a recent zebrafish study (Bresciani et al., 2014), two independent cbfb zebrafish knockout mutants (*cbfb* -/-) were produced, which showed comparable hematopoietic phenotypes. The *cbfb* knockout embryos maintained primitive hematopoiesis and erythro-myeloid progenitors, yet entirely lacked all definitive blood lineages. CBFβ is believed to be an obligate partner of RUNX1, and the loss of definitive hematopoietic lineages in both *Cbfb*-/- and *Runx1*-/- mice implies that the heterodimer of RUNX1/CBFβ is needed for HSC formation. However, data from zebrafish indicates that cbfb and runx1 are required at different steps in early HSC formation. The emergence of nascent, *runx1*+/*c-myb*+ HSCs from the AGM was unaltered in *cbfb* knockout mutants (Bresciani et al., 2014). This is further supported by pharmacologic inhibition of the interaction between Runx1 and Cbfβ in wild type zebrafish embryos using Ro5-3335. Analogous to the *cbfb* knockout mutants, the development of nascent HSCs from the ventral walls was unaffected by treatment with Ro5-3335. However, HSCs did not appear to reach the CHT, suggesting that Cbfβ inhibition prevents the release of nascent HSCs from the AGM (Bresciani et al., 2014).

1.3 Stem cell niche and their interactions

As discussed previously, following EHT, the HSCs bud off from the hemogenic endothelium and flow in the circulation to sites of hematopoiesis (Stuart H. Orkin & Leonard I. Zon, 2008; Perlin et al., 2017). A critical process is the homing and settling down in the niche, which has to take place in HSC transplants as well as in development (Heazlewood, Oteiza, Cao, & Nilsson, 2014; Perlin et al., 2017). In mammals, the adult bone marrow niche consists of various types of osteoblast, macrophages, stromal cells, osteoclasts, sympathetic nerves, endothelial cells and megakaryocytes (Birbrair & Frenette, 2016; Mendelson & Frenette, 2014; Morrison & Scadden, 2014). A lot of research has been done to identify the different signals and cell types responsible for HSC homeostasis in the bone marrow niche. Endothelial and stromal cells of the vascular niche have a particularly important role, along with the key cytokine KIT ligand (KITLG, also known as Stem Cell Factor), and the chemokine

CXCL12 (also known as stromal derived factor-1), which both support the maintenance of HSCs within, and localization of HSCs to, the perivascular niche (**Figure 6A**) (Ding & Morrison, 2013; Ding, Saunders, Enikolopov, & Morrison, 2012; Greenbaum et al., 2013; Kisanuki et al., 2001; Morrison & Scadden, 2014); Perlin et al. (2017). These factors are being used in efforts to mimic the niche *in vitro* in order to improve our understanding of how it works. Hematopoietic progenitors can already be generated through the cultivation of stromal cells with AGM- and ESC-derived cells, but these techniques need further improvement for better efficiency (K. D. Choi et al., 2012; Ledran et al., 2008; Perlin et al., 2017; Sturgeon, Ditadi, Clarke, & Keller, 2013). One study investigating the reprogramming of non-hematopoietic endothelial cells to engraftable hematopoietic cells revealed that this process was successful only when the cells were in contact with a vascular niche, leading to the conclusion that supportive and inductive signals from endothelial cells are required (Perlin et al., 2017; Sandler et al., 2014).

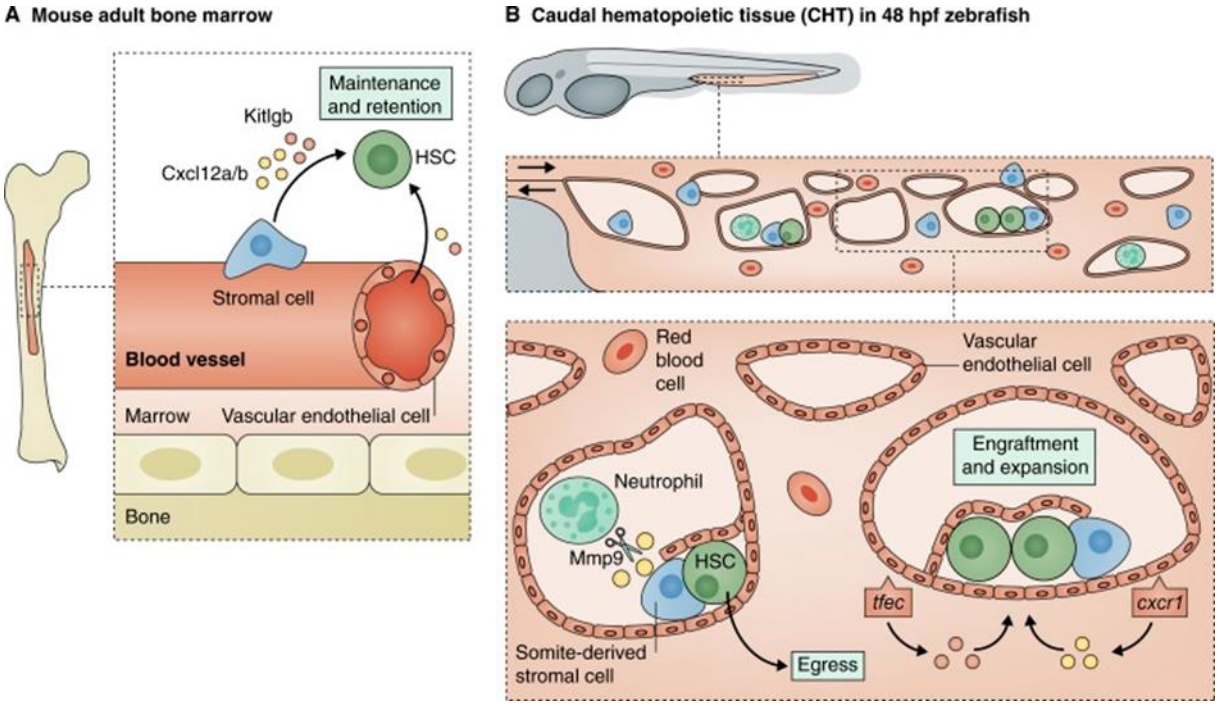

Figure 6 Endothelial and stromal cells of the vascular niche have an important role in the modulation of the two key factors KITLG and CXCL12 and the regulation of HSCs.

A. The vascular niche of the mammalian bone marrow is comprised of various types of endothelial and perivascular stromal cells. Both cell types are important for the expression of factors KITLG and CXCL12 which contribute to the location, maintenance, retention and quiescence of HSCs. The expression of both factors are ~1000x higher in stromal cells than in endothelial cells. **B.** At 48 hpf, HSPCs populate the CHT in zebrafish. The expression of KITLG and CXCL12 is influenced by various factors in the CHT, including the chemokine receptor Cxcr1 and the transcription factor Tfec, which are expressed by vascular

endothelial cells and are also important for engraftment and expansion of HSPCs. Neutrophils express Mmp9 which further cleaves Cxcl12 resulting in HSPCs leaving the vascular niche to colonize secondary niches (Perlin et al., 2017).

In the zebrafish niche, HSPCs interact with various cell types, including macrophages, endothelial cells, neutrophils and stromal cells (Tamplin et al., 2015), all of which are important for migration and engraftment of HSCs and support hematopoiesis (Campbell et al., 2015; Perlin et al., 2017; Stachura et al., 2009; Tamplin et al., 2015; Wolf et al., 2017). The transparency of the zebrafish embryo makes it an excellent model for live imaging of interactions in the intimate HSC niche, which can be performed in a continuous, long-term and non-invasive manner. It is possible to track HSCs from their place of origin in the AGM as they migrate to the CHT niche and further to the kidney niches and adult thymus (Kissa, Murayama, Zapata, Cortes, et al., 2008; Lassailly, Foster, Lopez-Onieva, Currie, & Bonnet, 2013; Lo Celso et al., 2009; Murayama et al., 2006; Perlin et al., 2017). The Zon lab established a new transgenic zebrafish line which specifically labels HSPCs with GFP, using the *Runx1+23* enhancer region isolated from the mouse intron upstream of Runx1 (Perlin et al., 2017; Tamplin et al., 2015). This made it possible to observe dynamic cell-cell interactions in a high-resolution manner during HSPC homing and niche engraftment in unperturbed embryos. During this imaging, various interactions of HSPCs with endothelial cells were discovered during their engraftment in the CHT. As previously described, the migration of HSPCs occurs through the circulation to the CHT niche, followed by adhesion to the endothelium and extravasation across the endothelial cell wall. High-resolution imaging revealed the fascinating process of ‘endothelial cell cuddling’, in which the arrival of HSPCs in the CHT triggers remodeling of the endothelial cells such that they surround the HSPC in a niche pocket (Perlin et al., 2017; Tamplin et al., 2015). Within the niche pocket, the stem cell division axis is orientated by the interaction of HSPCs with mesenchymal stromal cells. The same phenomena could also be observed in fetal livers dissected from mice, in which a similar surrounding of HSPCs by endothelial cells was observed, leading to the assumption that this is a conserved cell-cell interaction. Nevertheless, further studies are required to define the effects of the interaction of stromal and endothelial cells in the niche and their influence on HSCs (Perlin et al., 2017; Tamplin et al., 2015).

Much research is underway to try to figure out which signals and cells are important in the development of HSCs. Two independent studies discovered somite-derived cell types that are needed for both induction of HSCs in the AGM and retention of HSPCs

in the CHT (Murayama et al., 2015; Nguyen et al., 2014; Perlin et al., 2017). One study (Nguyen et al., 2014) revealed the importance of the endotome; cells from which contribute to the formation of endothelial cells in the dorsal aorta of the AGM and express *cxcl12b*. The homeobox gene, *meox1*, is expressed in a subset of somite cells and was identified to play a role in the determination of their endothelial fate. Mutants with a null mutation in *meox1* are known as *choker* mutants and have increased Cxcl12b signaling, leading to expansion of endotome cells and HSC induction. In this study it was also revealed that the generation of HSCs *in vitro* might be facilitated by the presence of somite-derived endothelial cells, which function as niche cells to promote HSC induction (Nguyen et al., 2014; Perlin et al., 2017). Another study highlighted the somite-derived stromal niche cell population in the fetal CHT as essential for the maintenance, expansion and differentiation of HSPCs (Murayama et al., 2015; Perlin et al., 2017). The gene 'nascent polypeptide-associated complex' is required for the maturation and survival of stromal cells. In the absence of stromal cells, homing of HSPCs to the CHT still occurs, however, stromal cell derived *naca* is needed for the differentiation and expansion of the stem cells and for their retention in the niche (**Figure 6B**) (Murayama et al., 2015). The unfolded protein response gets activated in the absence of *naca*, which leads to apoptosis of somite-derived stromal cells. These discoveries revealed a new role of somite-derived cells in the differentiation and expansion of HSCs in the fetal niche and HSC specification in the AGM (Murayama et al., 2015; Perlin et al., 2017). Further studies are necessary to determine the signals and cells that are important in the mammalian stem cell niche (Perlin et al., 2017).

Another transcription factor, *Tfec*, was discovered to enhance the expansion of HSCs in mice, though the mechanism remains unknown (Mahony, Fish, Pasche, & Bertrand, 2016; Perlin et al., 2017). A study in zebrafish showed that expression of this transcription factor is conserved (Mahony et al., 2016; Perlin et al., 2017). Caudal endothelial cells are expressed in the region where the CHT niche develops. Within this vascular niche, *tfec* functions non-autonomously, and the expansion of HSCs is regulated via the controlled expression of cytokines, such as *kitlgb*. Other endothelial cell-derived niche signals are also essential for HSPC development. Gene expression profiling of CHT endothelial cells revealed that HSPC niche colonization is enhanced by the chemokine *cxcl8* and its receptor *cxcr1*, which play a role in vascular CHT niche remodeling (Blaser et al., 2017; Perlin et al., 2017). This signaling axis regulates cuddling events, the duration that HSPCs are present in the niche, and the number of

divisions. Moreover, the increased expression of *cxcr1* leads to *cxcl12a* expression which further increases the vascular niche volume. This nonautonomous activity of *cxcr1* leads to enhanced niche engraftment of HSPCs (Blaser et al., 2017; Perlin et al., 2017). The chemokine CXCL12 regulates HSC migration, which in turn is modulated by matrix metalloproteinase 9 (MMP9) signaling in the bone marrow of adults (F. Jin et al., 2008; Perlin et al., 2017). This metalloproteinase degrades CXCL12 in plasma from mobilized bone marrow in humans to promote $CD34^+$ HSC migration (F. Jin et al., 2008; Perlin et al., 2017). In the fetal niche of zebrafish, *mmp9* gets released from neutrophils and regulates HSPC homeostasis by mediating *cxcl12* expression (Perlin et al., 2017; Theodore et al., 2017). When HSPCs leave the CHT they also need the support of *mmp9* for secondary niche colonization. When Mmp9 signaling is absent, HSPCs are retained in the CHT and the vascular composition is altered. Blocking the migration defects through overexpressing *cxcl12b* and knocking down the signaling of *cxc12a*, revealed that *mmp9* is needed for modulation of this signaling axis which further contributes to the regulation of homeostasis in HSPCs (Perlin et al., 2017; Theodore et al., 2017). In summary, the different studies mentioned above indicate that the expression of *cxcl12* and *kitlgb* is important for the control of HSPCs maintenance and migration, while *mmp9* and the signaling of *cxcr1* and *tfec* are needed for regulation of the vascular HSC niche. For HSCT patients, the modulation of these signaling molecules and cell-cell interactions could lead to a great therapeutic benefit (Perlin et al., 2017).

1.4 Understanding hematopoiesis using chemical and genetic manipulation in zebrafish

As already mentioned, the zebrafish is an amenable model for genetic manipulation. Morpholino knockdown, genetic screens, CRISPR, TALENs and DNA or RNA microinjection overexpression are methods which have been performed in this animal model with great outcomes (Avagyan & Zon, 2016; Perlin et al., 2017). In particular, a breakthrough genetic screen in zebrafish in the 1990s discovered novel genes which play an important role in the development of hematopoiesis, many of which have a role in human diseases (de Jong & Zon, 2005; Driever et al., 1996; Haffter et al., 1996; Perlin et al., 2017; Ransom et al., 1996). A prominent example of such a discovery is the ferroportin 1 transporter; the isolation of the gene encoding this transporter from a zebrafish mutant was found to be the same mutation present in humans with an iron-overload disorder (Donovan et al., 2000; Gordeuk et al., 2003). Moreover, other mutations related to human diseases were also discovered in insertional mutagenesis

screens, for example in the case of Diamond-Blackfan anemia (Kissa, Murayama, Zapata, Cortés, et al., 2008; Perlin et al., 2017).

1.4.1 **Using zebrafish for drug discovery**

Over the last few decades, the zebrafish model has risen to the forefront of human disease modelling and small molecule drug discovery. It has exclusive advantages for drug screening in a high-throughput fashion, in particular over *in vitro* screening assays, as the usage of a whole-organism system permits awareness of embryonic lethality and toxicity at an early stage of drug development (Robertson et al., 2016). Due to the high fecundity of zebrafish and external fertilization of the embryos, a huge number of embryos can be obtained and screened rapidly (Avagyan & Zon, 2016; Perlin et al., 2017). Moreover, their small size allows the arraying of a large number of larvae into multi-well plates, and their transparency assists phenotype-based screening by enabling direct visualization of changes induced by compound treatment. This system makes it possible to discover novel signaling pathways associated with human disease, which can be manipulated for therapeutic benefit (Robertson et al., 2016).

Various chemical screens of known, approved drugs and other small molecule libraries have revealed that essential hematopoietic signaling is conserved between zebrafish and mammals. Such screening has revealed several pathways which have an influence on the development of HSCs and homeostasis and also identified compounds which could have a promising effect in the treatment of human disease. A prominent example of successful screening is that of prostaglandin E2 (PGE_2), which induces an increase in the number of HSPCs in the zebrafish AGM and also contributes to the recovery of zebrafish marrow after an irradiation injury (North et al., 2007). The screening assay measured the effect on the number of *runx1*/*cmyb*-positive HSPCs in the zebrafish embryo, which was assessed by whole mount in situ hybridization. From three different compound libraries, ~2500 compounds were screened, with 5% of the compounds causing toxicity in zebrafish embryos (Hoggatt, Singh, Sampath, & Pelus, 2009; Robertson et al., 2016). Two compounds with opposing effects on HSPCs were also identified; linoleic acid which increased HSPCs in the CHT and celecoxib which decreased the number of HSPCs in the CHT. These two compounds target prostanoids and their effector, PGE2. As well as the increasing effects on *runx1*/*cmyb* HSPCs in zebrafish embryos, PGE2 also promotes HSC engraftment in adult zebrafish transplantations. These findings were recapitulated in mouse transplantation assays, where a two hour pulse of PGE2 increased the long-

term reconstitution potential of HSPCs (Hoggatt et al., 2009; Robertson et al., 2016). A clinical trial in which patients receive transplants of umbilical cord blood has shown encouraging results. Cord blood treated with PGE2 led to an early and sustained chimerism followed by long-term engraftment of the blood and accelerated recovery of neutrophils in ten out of twelve patients (Cutler et al., 2013). There are several ongoing studies to decipher the mechanism of PGE2 and how it regulates HSPCs. Two of four prostaglandin receptors are expressed on HSCs in zebrafish. The knockdown of these two receptors, Ptger2 (EP2) and Ptger4 (EP4), results in reduced numbers of *runx1/cmyb*-positive cells in the AGM, however, this effect is not rescued by PGE2 treatment (North et al., 2007). After binding to EP2 or EP4, PGE2 activates the GSK3/β-catenin pathway, and it also interacts with Wnt to contribute to the stabilization of ß-catenin (Fujino, West, & Regan, 2002; Goessling et al., 2009). This led to the conclusion that PGE2 may increase HSC number via β-catenin-mediated expression of survival genes and cell cycle proliferation through the inhibition of cell death. Another recent study showed the influence of cannabinoid receptor-2, which contributes to the regulation of HSC development via the activity of PGE2 and P-selectin. However, the exact mechanisms underlying how PGE2 affects HSCs are still under debate (Esain et al., 2015; Robertson et al., 2016).

A prominent example of combining chemical screening approaches with transgenic technology used the zebrafish line Tg(hsp70:AML1-ETO, which displays characteristics of human acute myeloid leukemia (AML) (Yeh et al., 2008). This line was used to perform a suppressor screen for compounds that inhibit the AML1-ETO induced switch from erythropoiesis to granulopoiesis, and led to the discovery that β-catenin and PGE2 pathways are important modulators of AML1-ETO-regulated hematopoietic differentiation (Yeh et al., 2009; Zhang et al., 2013). This was recently confirmed in human and mouse leukemia cells (Zhang et al., 2013).

Chemical screens have mostly been done in zebrafish embryos, but it is also possible to perform screens in adult zebrafish or directly treat hematopoietic cells *ex vivo* prior to re-infusion. Due to the difficultly of drug administration, it remains challenging to conduct pharmacologic screens in adults, but a promising technique was recently developed using serial gavage (Collymore, Rasmussen, & Tolwani, 2013). An alternative approach would be the harvesting of fluorophore marked hematopoietic cells for *ex vivo* treatment and reinfusion into conditioned recipients. This approach has already been used to screen for compounds that may improve HSC engraftment (P. Li et al., 2015; Robertson et al., 2016).

1.4.2 **Using zebrafish to study HSC transplantation**

Another advantage of the zebrafish model is the ability to perform HSC transplantation. In 2003, the first case of hematopoietic stem cell transplantation in zebrafish was documented, when it was revealed using flow cytometry that the lymphoid fraction from dissociated kidney marrow contains long-term reconstituting HSCs (Traver et al., 2003). Using transgenic cd41:GFP donor fish for transplantation, it was observed that some HSCs express cd41:GFP at low levels (Ma, Zhang, Lin, Italiano, & Handin, 2011). Another study showed HSC activity in Runx1:GFP and Runx1:mCherry cells isolated from the kidney marrow (Tamplin et al., 2015). Unfortunately, it has proven difficult to develop monoclonal antibodies for the identification of HSCs, despite efforts by various research groups (Robertson et al., 2016).

There are some limiting factors that make it more difficult to study HSC transplantation in zebrafish than in mammals. In comparison to mammals, zebrafish are not isogenic and there is not sufficient knowledge about their relevant major histocompatibility complex (MHC) loci, which are more diverse in zebrafish than in human (de Jong et al., 2011; de Jong & Zon, 2012; Dirscherl, McConnell, Yoder, & de Jong, 2014; Dirscherl & Yoder, 2014; McConnell, Restaino, & de Jong, 2014). Due to this lack of MHC matching there is the possibility of graft failure via rejection (Robertson et al., 2016). Adequate results have been achieved without MHC matching, but the question remains if MHC matching would improve the efficiency (Ma et al., 2011; Tamplin et al., 2015). The generation and maintenance of multiple transgenic donor strains with matched MHC requires significant resources, and additionally, such congenic strains have the disadvantage of a lack of fecundity (de Jong et al., 2011; LaFave, Varshney, Vemulapalli, Mullikin, & Burgess, 2014; Shinya & Sakai, 2011). Some alternative strategies have been explored, including chemical treatments, modulating the dose of irradiation, and subjecting recipients to cyclosporine A (Glass, Hui, Blazar, & Lund, 2013; Robertson et al., 2016; Shayegi et al., 2014). The usage of genetically immunocompromised recipients is also of interest to researchers (Hess & Boehm, 2016; Hess, Iwanami, Schorpp, & Boehm, 2013).

A good knowledge about the mechanisms which contribute to the engraftment of HSCs *in vivo* is necessary to guarantee the success of bone marrow transplantation in patients with immunodeficiency syndrome, blood cancers and bone marrow failure. To limit the transfusion of blood products and decrease the risk of infection, the improvement of the engraftment of HSC after transplantation is very critical (P. Li et al.,

2015; Robertson et al., 2016). Approaches to achieve this goal include post-transplantation treatment of patients to promote a more supportive environment for the incoming stem cells, attempts to try to increase the number of transplanted stem cells, or ways to enhance the engraftment capacity of stem cells prior to transplant through the improvement of seeding or homing to the bone marrow niche. Various methods to increase HSC number are currently being tested in clinical trials, where patients receive transplants of umbilical cord blood stem cells. A chemical screen in zebrafish revealed new compounds which increase the engraftment of HSCs in transplantation assays and could be translatable to human systems (P. Li et al., 2015; Robertson et al., 2016). In this particular study, increased engraftment was assessed by fluorescence intensity in transparent *casper* zebrafish (White et al., 2008). During the time of definitive hematopoietic initiation, zebrafish embryos were treated with the lipid mediator 11,12-epoxyeicosatrienoic acid (11,12-EET), which caused an increase in Runx1-positive HSPCs in the AGM, suggesting that it acts at the hemogenic endothelium level (Forsberg et al., 2010; Robertson et al., 2016). Further dissection of the mechanism revealed that multiple activator protein 1 (AP-1) family transcription factors were involved, including *junbl*, *fosl2* and *junb*, which showed increased expression after 11,12-EET treatment. Additionally, it was revealed that the AP-1 program plays an important role in cell-cell signaling during cell migration, which could explain why 11,12-EET has a positive effect on the migration and homing of HSPCs. This study led to the conclusion that 11,12-EET may play a critical role in the improvement of engraftment in human HSC transplantation assays (Forsberg et al., 2010; Robertson et al., 2016).

Despite the challenge of the lack of characterized antibodies and surface markers to enable isolation of zebrafish HSCs by fluorescence-based cell sorting (FACS) (Robertson et al., 2016), advances have been made using transgenic zebrafish, for instance, those which express fluorescent proteins driven by HSPC-specific promoters during HSPC engraftment and early development. The transplantation of HSCs in zebrafish has led to insights in the roles of various signaling pathways (Ma et al., 2011; Tamplin et al., 2015; Traver et al., 2003), and there is a great interest in the use of zebrafish as a xenograft model (Hess & Boehm, 2016; Robertson et al., 2016; Staal, Spaink, & Fibbe, 2016).

1.5 Engineering of HSCs for therapeutic purposes

Various attempts have been made to engineer ‘designer HSCs’ which could be useful for different therapies and research applications. An engineered HSC should have the same properties as native HSCs in terms of hemostasis, long-term self-renew ability, and the capability to produce the full repertoire of differentiated progeny cells with innate and acquired immunity. The emergence of human embryonic stem cells in research has made it possible to attempt to engineer HSCs for use in HSCT (Chadwick et al., 2003; Kaufman, Hanson, Lewis, Auerbach, & Thomson, 2001; Rowe, Mandelbaum, Zon, & Daley, 2016; Schmitt, Bruyns, & Snodgrass, 1991; Vodyanik, Bork, Thomson, & Slukvin, 2005; Wiles & Keller, 1991). Many different approaches have been implemented to try to differentiate human and mouse ESCs into hematopoietic lineages. However, only limited success has been made in the production of definitive HSPCs over the past two decades (Rowe et al., 2016).

The study of Takahashi et al. (Takahashi, Okita, Nakagawa, & Yamanaka, 2007), made a breakthrough in stem cell research, showing that somatic cells can be reprogrammed to iPSCs, which would theoretically enable the production of autologous patient-specific HSCs in an unlimited manner (Takahashi et al., 2007). The development of CRISPR/Cas9-mediated gene repair may also help to aid the engineering of HSCs from iPSCs (Hendriks, Warren, & Cowan, 2016; Rowe et al., 2016). Multiple studies have used transcription factor-mediated reprogramming approaches to try to differentiate iPSCs, ESCs or more differentiated cells into HSCs, and have shown some success in generating hematopoietic progenitor cells that can produce mature blood cells with some efficiency (S. Doulatov et al., 2013; Elcheva et al., 2014; Kennedy et al., 2012; Sturgeon, Ditadi, Awong, Kennedy, & Keller, 2014). Nevertheless, further studies are necessary to generate true, definitive HSCs and to determine the potential therapeutic value and long-term self-renewal capacity of HSCs derived from different pluripotent sources (Rowe et al., 2016).

1.5.1 Directed differentiation of blood stem cells

The engineering of HSCs involves many challenges that have to be overcome to create cells that are comparable to endogenous adult HSCs. Recapitulating the precise timing of tissue development might be key. The development of hematopoiesis *in vivo* occurs in a sequentially manner, both in the functionality of stem and progenitor cells and in their distribution and anatomical location, from early embryogenesis to adulthood. During development and throughout life, maturation of distinct hematopoietic

phenotypes is dictated by the needs of the host (Benz et al., 2012; S. H. Orkin & L. I. Zon, 2008; Rebel, Miller, Eaves, & Lansdorp, 1996; Rowe et al., 2016). We have considerable knowledge of the stage-specific regulators of hematopoiesis, including epigenetic factors, morphogen signals and transcription factors (Dzierzak, 2002; Eaves, 2015; S. H. Orkin & L. I. Zon, 2008). Understanding the various stages of developmental hematopoiesis and the timing of events in order to simulate them in directed differentiation approaches towards engineering HSCs are imperative to enhance their therapeutic value and functionality (Rowe et al., 2016).

The complexity of hematopoietic ontogeny is an obstacle in the generation of engraftable HSPCs. As mentioned earlier, hematopoietic cells develop during mid-gestation in multiple waves from hemogenic endothelial cells (Bertrand, Chi, et al., 2010; Boisset et al., 2010). Protocols have been developed to imitate hematopoietic ontogeny by calibrating the addition of morphogens such as Notch ligands, BMP4 and Activin A. These promote HE formation and imitate the temporal waves of hematopoietic progenitor emergence, but the yield of transplantable cells is very few or none at all (K. D. Choi et al., 2012; Sergei Doulatov et al., 2013; Kennedy et al., 2012). Due to the variety of protocols and hPSC (human pluripotent stem cell) line heterogeneity, engraftment assays using hPSC derived cells in immunodeficient mice have not been commonly adopted (Sergei Doulatov et al., 2013). Efforts have yielded only minor numbers of transplantable cells and no solution to expand these cells, creating barriers to move forward with practical models and explore the potential of *in vivo* engraftment assays using disease-relevant iPSCs (Sergei Doulatov et al., 2013; Ledran et al., 2008; L. Wang et al., 2005).

1.5.2 **Transcriptional reprogramming approaches to generate HSCs**

Reprogramming using transcription factors might be a promising approach. The advantage of using transcription factors rather than the more traditional principles of using morphogen signals in these engineering studies is that transcription factors have the potential to overcome the epigenetic and phenotypic barriers that are imposed by the developmental ontogeny (Kennedy et al., 2012; Rowe et al., 2016; Sturgeon et al., 2014). A recent study discovered that the combination of transcription factors Fos, Gata2, Etv6 and Gfi1b, promoted reprogramming of mouse fibroblasts into hematopoietic cells but these lacked all the necessary attributes of HSPCs (Pereira et al., 2013). Using more closely related lineages could be favorable in order to minimize the epigenetic distance to the desired cell type and encourage more precise cell fate

alteration (Sergei Doulatov et al., 2013). Enhancing HE specification into transplantable HSPCs could be an appropriate approach, which would take advantage of known cues in the development. A disadvantage is the insufficient knowledge of endothelial to hematopoietic transition which makes it challenging to design rational interventions. The reversion of committed hematopoietic progenitors to a more immature state could be an alternative, which would combine transcription-based reprogramming with directed differentiation to try to generate HSPCs. It has been hypothesized that key regulators of HSCs revealed by gene expression profiling might re-activate the stem cell properties in more mature progenitors (Doulatov et al., 2010; Sergei Doulatov et al., 2013; Laurenti et al., 2013).

The first demonstration of transcriptional re-specification was performed in mouse ESCs with HoxB4. This transcription factor was isolated either from yolk sac or from EB (Embryoid body) differentiation, and induced long-term multi-lineage engraftment capacity and self-renewal when expressed in lineage-restricted hematopoietic progenitors (Kyba, Perlingeiro, & Daley, 2002). Unfortunately, HOXB4 does not function similarly in human systems, and further research is necessary to define the differences in species (Amsellem et al., 2003; Sergei Doulatov et al., 2013; Lee, Kim, Sheih, & Moore, 2008; L. Wang et al., 2005). Here, the Daley lab (Sergei Doulatov et al., 2013) wanted to discover factors which are tailored for hPSCs. They identified transcription factors from ETS- and HOX-families in hPSCs, including ERG and HOXA9. ERG and HOXA9 contribute as inducers of multi-lineage potential and self-renewal in hematopoietic progenitors. Furthermore, erythroid and myeloid engraftment is achieved through the addition of MYB and SOX4, enabling the development of *in vivo* models of hematopoietic diseases using human iPSCs (Sergei Doulatov et al., 2013).

Over the last few years, huge progress has been made to refine the engineering of customized HSCs for application in research, however, there has been no breakthrough in approaches of differentiation and reprogramming. As mentioned previously, patient-specific HSCs must possess the characteristics of true endogenous HSCs (Rowe et al., 2016). Advancing our understanding of HSC development *in vivo* will help to engineer new methods to recapitulate definitive HSC differentiation *in vitro*. The transcriptional and epigenetic profiles of normal HSCs need to be further characterized and a more in-depth understanding of their prenatal counterparts is necessary to thoroughly define the required factors that specify their specialized state. Engineered HSCs and their progeny cells must be evaluated with stage-specific

markers to reveal their potential value in cellular therapies. The usage of cross-species approaches and chemical genetic screening could bring additional understanding of normal HSC biology to aid the generation of human HSCs *ex vivo*. The zebrafish is a particularly powerful model for defining the factors that promote HSC expansion and specification, as high-throughput chemical screening methods can be used. A combination of the relevant transcription factors, morphogens and chemical factors that are needed for normal specification of HSCs, along with manipulated epigenetic factors, could make the engineering of adult HSCs an achievable goal.

1.5.3 *Ex vivo* strategies for the chemical expansion of HSCs

Strategies to expand human HSCs rely on high-throughput screens of chemical compounds and small molecule libraries. These approaches have led to the identification of various molecules that have undergone evaluation in clinical studies to promote the expansion of HSCs from umbilical cord blood (UCB)-CD34+ cells in *ex vivo* cultures.

In one study, the aryl hydrocarbon receptor (AhR) inhibitor StemRegenin-1 (SR-1), in combination with a cytokine cocktail and a serum-free expansion media, was found to increase the number of UCB-CD34+ cells by 50-fold and the number of HSCs which were able to long-term establish engraftment in immunodeficient mice by 17-fold. Insights into the mechanism underlying the *ex vivo* expansion by AhR pathway inhibition with SR1 treatment are still to be determined (Boitano et al., 2010). Even though evidence clearly implicates the AhR pathway in hematopoiesis and HSC metabolism, further studies must be conducted to clearly understand the role of AhR in human HSC self-renewal (Casado, 2016; Jackson et al., 2017). A clinical study in phase I/II was performed with one unchanged UCB unit and the progeny of CD34+ cells from another UCB unit, in which cells were treated with SR1 for 15 days *ex vivo*, resulting in fast platelet and neutrophil recovery (Wagner et al., 2016)

Another potent small molecule is UM171, a pyrimidoindole derivative, which triggers robust *ex vivo* expansion of human HSCs with a significant marrow repopulating capacity. UM171 appears to enhance expansion of CD34+ CD45RA- HSCs from cord blood, which have a primitive phenotype, independently of the AhR pathway, although the mechanism responsible is not yet understood. UM171 is a powerful agonist of HSC renewal and, compared to SR1, leads to a higher degree of primitive-like human HSC expansion (Fares et al., 2014). Currently, UM171 is being tested with single UCB expanded grafts in a clinical trial. Preliminary results are hopeful and show that UCB-

CD34+ cells expanded with UM171 for seven days establish swift engraftment, full donor chimerism and clinical advantages associated with decreased infection and transplant-related mortality (Cohen et al., 2018).

The deacetylase inhibitor valproic acid (VPA) induces cellular reprogramming of UCB-CD34+ cells causing primitive HSCs to expand *ex vivo*. These expanded HSCs are able to establish multilineage hematopoiesis in primary and secondary NGS mice (Chaurasia, Gajzer, Schaniel, D'Souza, & Hoffman, 2014; Iancu-Rubin & Hoffman, 2015). Noteworthy, this reprogramming is accompanied by the gain and retention of a transcriptome and primitive mitochondrial profile, which strictly mirrors that of primary human HSCs (Papa et al., 2018). A novel and attractive approach is the *ex vivo* expansion of HSC with VPA due to the high degree of short- and long-term HSC expansion. The expansion of HSC *ex vivo* with VPA occurs rapidly and only needs seven days of culture, which limits the degree of differentiation, bacterial contamination and the risk of genetic instability (Arulmozhivarman et al., 2017).

Another HSC expansion approach was recently published from Wilkinson et al. (Wilkinson et al., 2019), which describes the alteration of already existing methods for HSC *in vitro* cultures. In this approach the serum albumin was replaced with the polymer, polyvinyl alcohol (PVA). This method demonstrated a several hundred-fold expansion of functional murine HSCs *in vitro* over one month. This relatively straightforward technique is now suggested to work for human HSCs and should be accessible to clinical translation (Wilkinson et al., 2019).

Despite the success of multiple studies, we still do not have a well-established and robust system for generating engraftable human HSCs that can be used for therapeutic purposes. With this in mind, we designed this Masters project to identify novel ways to expand HSCs using the zebrafish model, which might in future be applicable to human systems.

1.6 Aims of the project

This Master thesis project was conducted with two goals:

1. To characterize the effect of Ro5-3335 on HSC expansion during hematopoiesis in the zebrafish embryo.

For this first aim, I intended to further investigate the effect and the mechanism of Ro5-3335. This is a chemical that was identified in a previous chemical screen for inducers of Runx1:GFP in the Zon lab, which shows the ability to expand HSCs *in vivo* by increasing the number of Runx1:GFP cells in the CHT at 54 hpf following treatment from 24 hpf. Due to the COVID-19 pandemic, my time in the lab was very limited, so I chose to focus primarily on the second aim of my project.

2. To identify new compounds that promote HSC expansion using an *in vivo* chemical screening approach and evaluate their effects on hematopoiesis using high-resolution spinning disk confocal microscopy of zebrafish transgenics and gene expression studies.

The second aim of the project was to screen additional compounds with the potential to promote HSC expansion *in vivo* using the transgenic Runx1:GFP zebrafish line, using Ro5-3335 as a positive control. A ‘mini-library’ of compounds was designed by selecting compounds with known biology targets from existing chemical libraries in the Zon lab. Based on literature searching, I hypothesized that these selected compounds would have the potential to promote HSC expansion. Runx1:GFP zebrafish embryos were treated with the selected compounds at 24 hpf and screened at the time-point of 54 hpf via high resolution spinning disk confocal microscopy. The number of HSCs in the CHT were quantified using Imaris software.

2. MATERIALS AND METHODS

2.1 Animal care

Zebrafish (Danio rerio) lines were maintained as previously described (Westerfield, 2007). All animals were housed at Boston Children's Hospital and handled according to approved Institutional Animal Care and Use Committee (IACUC) protocols. Embryos were generated by incrossing Runx1:GFP (Runx1+23:eGFP) or Runx1:mCherry (Runx1+23:NLS-mCherry) adult zebrafish, which express the mouse stem cell-specific *Runx1* enhancer driving GFP or mCherry, to label hematopoietic stem cells during the process of definitive hematopoiesis (Tamplin et al., 2015). A male and a female zebrafish were transferred to a mating tank and kept separated with a divider overnight for around 20 hours. The next morning, the dividers were removed, and embryos were collected within one hour. The embryos were transferred to E3 media and maintained at 28.5°C. After four hours, the embryos were cleaned to remove unfertilized embryos and other debris. If necessary, they were sorted into several petri dishes, containing no more than 80 embryos per dish.

2.2 Compound library selection

The Zon Lab has access to various chemical libraries with known biological targets. Almost 4,000 compounds were prescreened for increased expression of a RUNX1 reporter in human hemogenic endothelial cells (unpublished data, Schlaeger lab, Boston Children's Hospital). From this screen, a library of 160 compounds with a positive effect on RUNX1 expression was generated. From these, a 'mini-library' of 24 compounds was assembled and ranked based on known literature and unpublished data from the Zon Lab.

2.3 Optimization of drug volume and well plate size for compound screen

To determine the optimum conditions for the screening assay, several different combinations of well plate size, final volume and number of fish were investigated, with the goal to identify the condition in which healthy embryos could be maintained whilst using as little compound solution as possible. Runx1:GFP embryos were incubated in 24 and 48-well plates at 24 hpf, with varying volumes of E3 media from 200 to 650uL.

2.4 Compound screen protocol

For the compound screen (**Figure 7**), a 48-well plate was prepared for drug treatment just prior to 24 hpf. Ro5-3335 was used as positive control at a final concentration of 5 µM (stock concentration = 10mM). The final volume of the required drug solution per well was 300 µL, therefore a 1:10 dilution in E3 was made and from this solution 1.5 µL were used for the control. The same dilutions and volume were used for the vehicle control, DMSO. The to be tested compounds were used with a final concentration of 10 µM (stock concentration 9 or 10 mM). Therefore, 0.3 µL (10 mM) or 0.33 µL (9 mM) compound were used.

For preparation of the well plate, 150 µL E3 was added in the appropriate number of wells (one for Ro5-3335 and one for the DMSO vehicle control, plus the required number of wells for the compounds being screened). Then 1.5 µL from the 1:10 dilution of Ro5-3335 or DMSO or 0.3 µL or 0.33 µL compound was added to the appropriate well. Afterwards the plate was mixed by swirling carefully.

At 24 hpf, healthy, stage-matched Runx1:GFP or Runx1:mCherry embryos were selected for drug treatment. At this timepoint a heartbeat should be noticeable. Eight embryos were selected per treatment group and then transferred to a well using a P1000 pipette. Prior to pipetting, the very end of the pipette tip was cut using scissors, to avoid damage to the embryos. The embryos were transferred in 150 µL to make a final volume of 300 µL in each well. Finally, the plate was placed at 28.5°C in the incubator.

At 54 hpf, the embryos were dechorionated using forceps and then anesthetized with 160 µL of 25x Tricaine. They were transferred to a 6-well plate with glass coverslips, and mounted in 750 µL of 0.8% low melting point agarose, which was prewarmed to 37°C. Embryos were imaged individually using a high-resolution spinning disk confocal microscope, to capture the CHT region at 20x magnification. Images were acquired in both brightfield and the appropriate fluorescence channel (GFP or mCherry). The number of HSCs in the CHT niche were quantified using automated IMARIS software and graphically represented using GraphPad Prism 9 software.

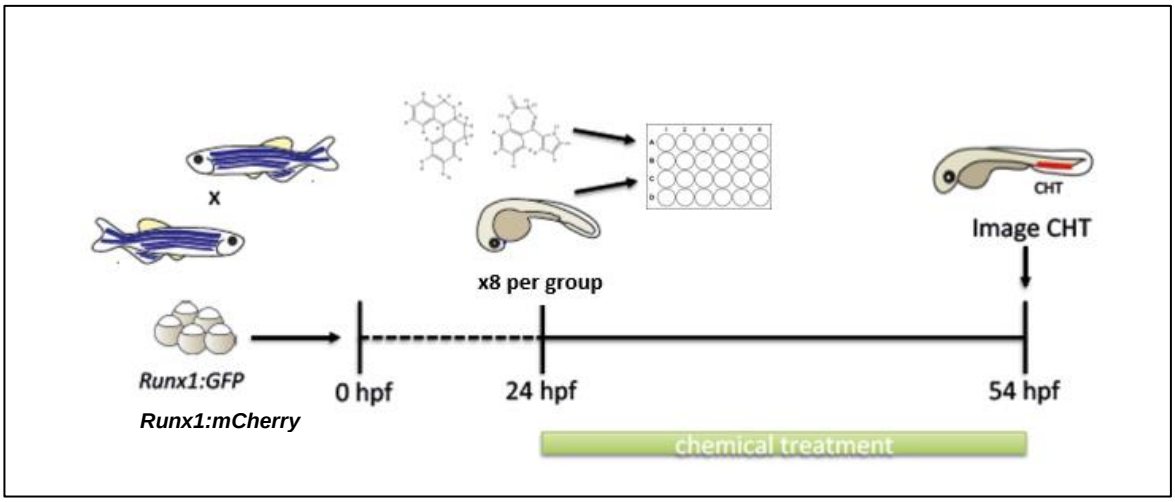

Figure 7 Schematic workflow of screen for expanders of Runx1+ zebrafish HSCs.

2.4.1 **Spinning Disk Confocal Microscopy**

Images of the mounted zebrafish embryos were acquired using a spinning disk confocal microscope (Nikon). For Runx1:GFP embryos, the 488 nm excitation laser was used, with the emitted light channeled through a 493-556nm filter. For Runx1:mCherry embryos, the 555 nm excitation laser was used, with the emitted light channeled through a 562-624 nm filter. As a standard, the exposure was usually set around 30ms for the brightfield channel and 500ms for the fluorescence channels. The EM Gain Multiplier was set between 10 and 300, dependent on the brightness of the cells, which is variable between clutches. The laser power was usually at 100% but turned down if the image was overexposed at 30ms. Images were acquired with the 20x objective. Within each individual experiment, acquisition settings were kept the same for every embryo.

2.4.2 **IMARIS analysis**

Runx1+ cells were quantified using Imaris 9.3.0 software. The CHT was defined as the region of interest (**Figure 8**) and the spots function was used to detect the number of cells within this region (**Figure 9**). The XY diameter of detectable spots (cells) was defined as 7 µm, and the mean intensity threshold was defined by judging the appropriate level of fluorescence of the correct cells by eye. Cells were then counted in an automated manner using the same parameters for every fish within the same experiment. For Runx1:GFP assays, only the high expressing cells GFP+ were included in the analysis. For Runx1:mCherry assays, both high and low expressing mCherry+ cells were counted, by setting the appropriate mean intensity thresholds to define these cells within each experiment.

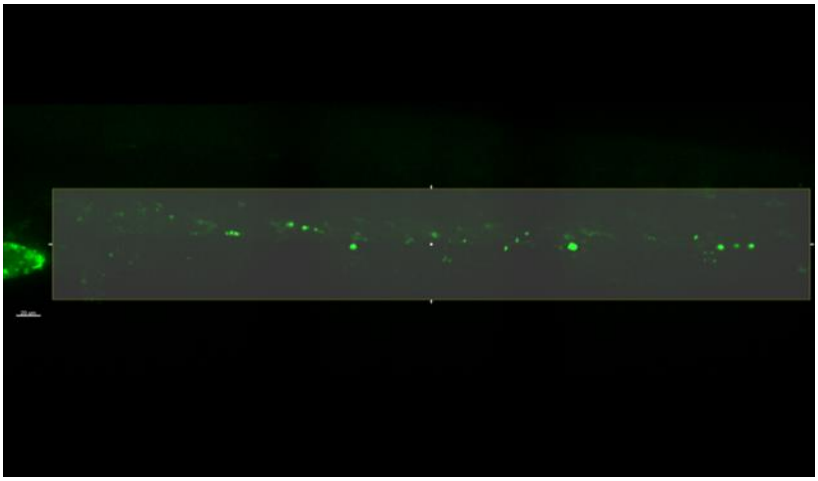

Figure 8 Yellow square represent the CHT of the zebrafish. The to be analyzed section, the CHT, was chosen and within the square, the cell count algorithm is processing the data.

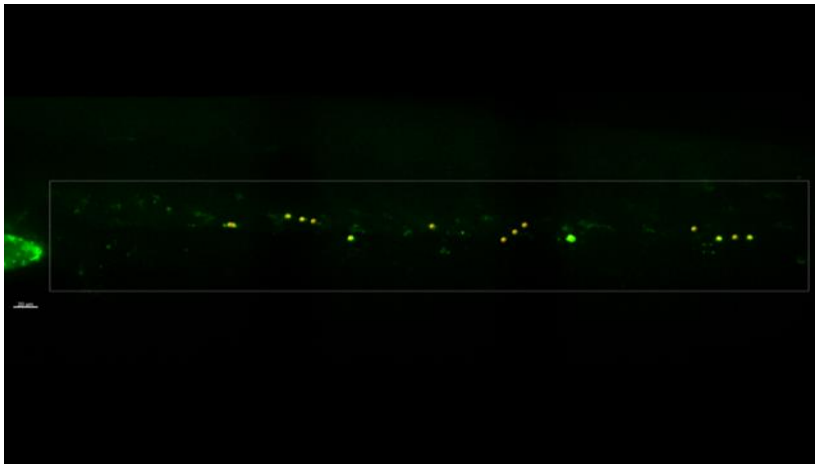

Figure 9 Yellow dots represent HSCs in the zebrafish. After adjusting the intensity for the cell counting, the cells within the right intensity range were counted.

2.4.3 Statistical analysis

After inputting the cell number data to GraphPad Prism 9 software in order to graphically represent the results, they were statistically analyzed using a one-way ANOVA. This analysis assumes Gaussian distribution and equal standard deviations. For data generated in Runx1:GFP, the Dunnett's post-test was used by assigning DMSO as the control column. For data generated in Runx1:mCherry, Bonferroni's multiple comparison post-test was used on selected pairs of columns, to enable comparison between the relevant control for both the high and low mCherry populations of cells. The adjusted p-value was added to each graph displaying the combined data from two individual experiments (n=2).

3. RESULTS

3.1 Assembly of a small diverse compound library

The Zon lab has access to over 4,000 chemicals from a diverse range of compound libraries, including known FDA-approved drugs, bioactives and natural products. In order to create a manageable number of compounds to study, I chose to focus on a small library of 160 compounds, which had already been preselected by the Schlaeger lab at Boston Children's Hospital (data not shown) using a screening assay that tested for increased expression of a RUNX1 reporter in human hemogenic endothelial cells. Given the limited time I was able to spend in the lab due to the COVID-19 pandemic, it would not have been possible for me to screen all of these, so I assembled my own 'mini-library' containing 24 compounds (**Table 1**), which I prioritized based on their known activity from thorough literature searching.

Table 1 Selected Compound Library. A compound library containing 24 compounds that I predicted may expand hematopoietic stem cells *in vivo*. Compounds were selected from a library containing 160 compounds, which were pre-selected by screening for increased expression of a RUNX1 reporter in human hemogenic endothelial cells (unpublished data, Schlaeger lab, Boston Children's Hospital).

ID	Concentration	Name	Known Target	Known Effects In Stem Cells
1	5mM	16,16-dimethyl Prostaglandin E2	EPR agonist	Increased formation of hematopoietic stem and progenitor cells
2	10 mM	IWR-1-endo	Wnt inhibitor	Promotes self-renewal and maintains pluripotency of hESCs
3	10 mM	SR1	AhR antagonist	Promotes ex vivo expansion of CD34+ hHSCs
4	10 mM	Cyclic Pifithrin alpha	p53 inhibitor	Capable of increasing the efficiency of inducing pluripotent stem cells
5	9 mM	Splitomicin	Sir2p inhibitor	Inhibition of platelet aggregation
6	10 mM	IWP-2	Wnt inhibitor	Inhibit embryonic stem cell self-renewal
7	10 mM	Pyrintegrin	B1-integrin agonist	Promote embryonic stem cells survival
8	10 mM	A83-01	ALK-5 inhibitor	Inhibits TGF-β-induced epithelial-to-mesenchymal transition
9	10 mM	Kenpaullone	ATP-competitive inhibitor of CDKs	Replaces Klf4 in the generation of iPSCs
10	9 mM	Suramin	Multiple	Blocks the binding of IGF-I, EGF, PDGF, and TGF-beta
11	10 mM	Pluripotin	RasGAP and ERK1 inhibitor	A dual kinase and GTPase inhibitor that promotes self-renewal
12	10 mM	PD0325901	MEK inhibitor	Increases the efficiency of reprogramming human somatic cells to iPS cells
13	10 mM	XAV939	Tankyrase inhibitor	Used in the inhibition of wnt signalling in hiPSCs
14	1 mM	L-Thyroxine sodium salt pentahydrate	Thyroid receptor	Hormones, which stimulate growth and development
15	10 mM	DAPT	gamma-secretase inhibitor	Inhibits Notch signaling, cancer cell growth, angiogenesis, and differentiation of hiPSC
16	10 mM	2-phospho-l-ascorbic acid	HGF stimulator	Used in cell differentiation and tissue engineering applications
17	9 mM	Sirtinol	SIRT inhibitor	Inhibits the physiological regulators of platelet aggregation
18	10 mM	IDE-1	TGFb activator	Induces differentiation of mouse or human pluripotent stem cells
19	10 mM	LDN-193189	ALK inhibitor	Potent inhibitor of the bone morphogenetic (BMP) pathway
20	10 mM	SB431542	ALK inhibitor	Stimulates proliferation, differentiation and sheet formation of ESC-derived endothelial cells
21	10 mM	Purmorphamine	Hedgehog agonist	Activates the Hedgehog pathway
22	10 mM	Dorsomorphin	AMPK inhibitor	Selective inhibitor of Bone morphogenetic protein (BMP) signaling.
23	9 mM	Aminoresveratrol sulfate	Multiple	Lifespan extension in model systems, protection of the cardiovascular apparatus
24	10 mM	Cyclopamine	Hedgehog inhibitor	Steroid alkaloid that inhibits the Hedgehog pathway

3.2 Optimization of the screening assay

Due to the limited availability of the compounds, I needed to perform optimization experiments to determine the most appropriate well plate size, treatment volume per well, and number of fish per well. Previous experiments using a similar method to assess the effect of Ro5-3335 in Runx1:GFP embryos were performed using 6 well plates, with twelve zebrafish embryos in a final solution volume of 4 mL. As mentioned in the introduction, Ro5-3335 is a CBFβ inhibitor that was identified by the Zon Lab as

an expander of Runx1:GFP cells. When exposed to Ro5-3335 from 24 to 54 hpf, Runx1:GFP embryos have a significantly increased number of HSCs in the CHT region compared to DMSO treated embryos (**Figure 10**). I used this approach as a basis upon which to design the screening assay for my Masters project, and used Ro5-3335 as a positive control compound.

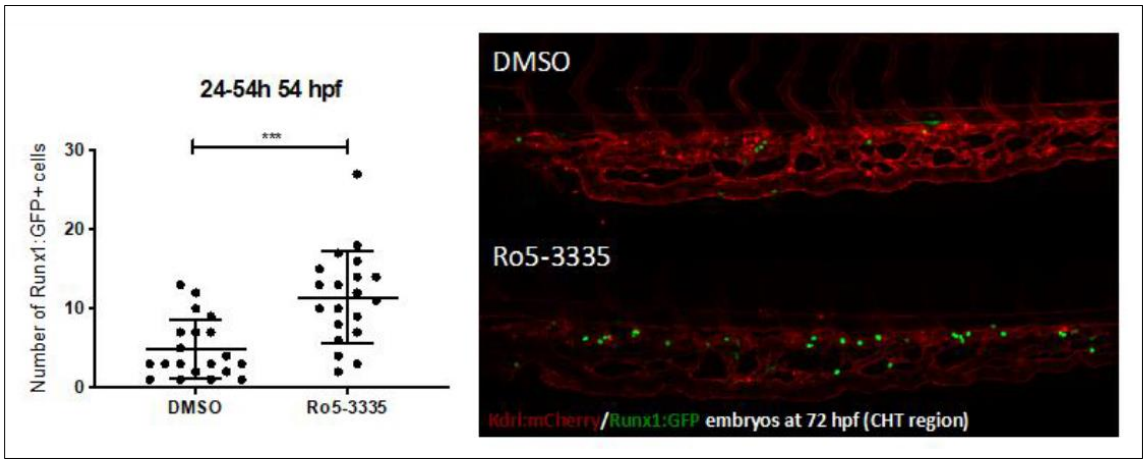

Figure 10 Ro5-3335 is a positive control that expands Runx1:GFP+ HSCs in the CHT at 54 hpf.

To determine the smallest possible volume of chemical solution that I could use for the screen without causing embryo toxicity due to overcrowding, I tested several combinations of volume and fish number in both 24 and 48-well plates (**Table 2**). Embryos were arrayed into plates with E3 solution at 24 hpf and toxicity was assessed 30 hours later, at 54 hpf. An optimal combination was the usage of a 24 well plate with 10 fish in 650 µL compound solution or a 48 well plate with 8 fish in 300 µL compound solution. To conserve the compounds, I chose to use a 48 well plate format with 300 µL final volume for the screening assay.

Table 2 Optimization of drug volume and fish number for compound screen. Two combinations were ideal for the compound screen (orange).

24 well plate (No/V)	48 well plate (No/V)
6 fish/300 µL	6 fish/200 µL
6 fish/ 500 µL	6 fish/ 300 µL
6 fish/ 650 µL	6 fish/ 500 µL
8 fish/ 300 µL	8 fish/ 200 µL
8 fish/ 500 µL	8 fish/ 300 µL
8 fish/ 650 µL	8 fish/ 500 µL
10 fish/300 µL	10 fish/ 200 µL
10 fish/500 µL	10 fish/ 300 µL
10 fish/ 650 µL	10 fish/ 500 µL
12 fish/ 300 µL	
12 fish/ 500 µL	
12 fish/ 650 µL	

3.3 A chemical genetic screen for HSC expanders

For the chemical genetic screen, Runx1:GFP adult zebrafish were paired, and the generated zebrafish embryos were treated with the selected compound at 24 hpf in 48 well plates, at a concentration of 10 µM. For each individual experiment, I screened between one to four compounds, alongside DMSO as a vehicle control and Ro5-3335 at 5 µM as a positive control. At 54 hpf, the zebrafish embryos were mounted in 0.8% low-melting point agarose and imaged using a high resolution spinning disk microscope. The number of HSCs in the intermediate stem cell niche were quantified using Imaris software and graphically represented using GraphPad Prism 9 software. During my limited time in the lab, I tested twelve different compounds from my mini-library. Here, I will present my results from selected compounds that induced an interesting range of phenotypes on HSCs *in vivo*.

3.3.1 **Compounds that significantly increased HSCs in zebrafish**

One of the first compounds I tested was the β1-integrin agonist, Pyrintegrin (**Figure 11**). The experiment was performed as described in the previous paragraph.

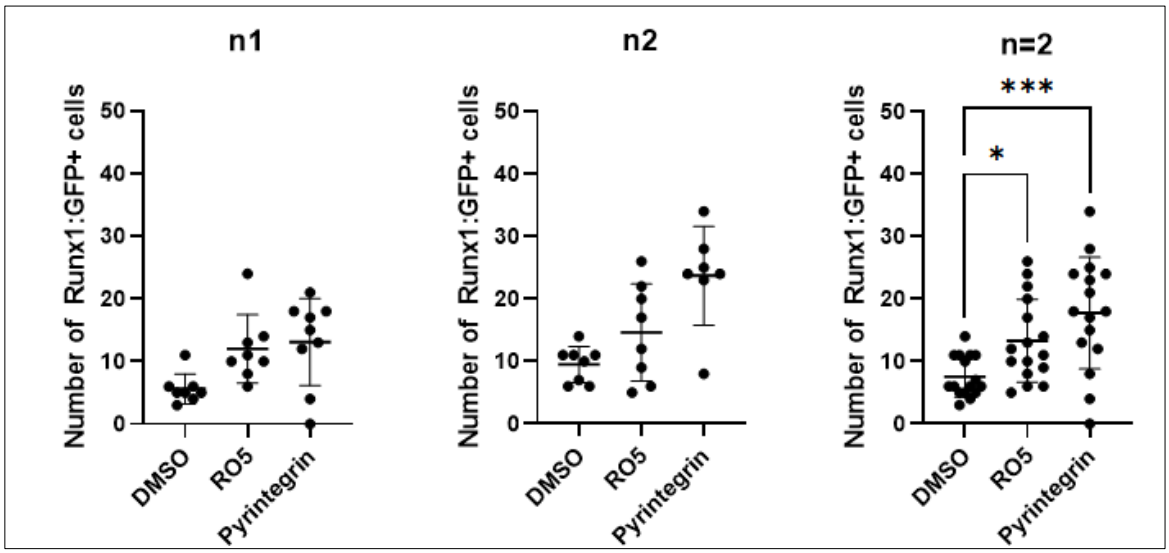

Figure 11 Pyrintegrin increases Runx1:GFP+ HSCs in the zebrafish CHT. Runx1:GFP embryos were treated with Pyrintegrin at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. Two independent experiments were carried out (n1 and n2) and the combined data is represented as n=2. One-way-ANOVA with Dunnett's multiple comparison post-test analysis indicates that the combined results are significant (*= $P \leq 0.05$, ***= $P \leq 0.001$).

In these experiments, I observed one of the highest increases in Runx1:GFP cell number in the presence of the positive control, Ro5-3335, compared to other sets of experiments. This suggests that the experiment worked very well and the Pyrintegrin data is reliable. In the first experiment (n1), I also observed an expanded number of cells with Pyrintegrin. In the second experiment (n2), an even higher number of HSCs was observed in the presence of Pyrintegrin. The combined data (n=2) shows a significantly increased number of Runx1:GFP cells with Pyrintegrin. Example images of treated embryos are shown in **Figure 12**.

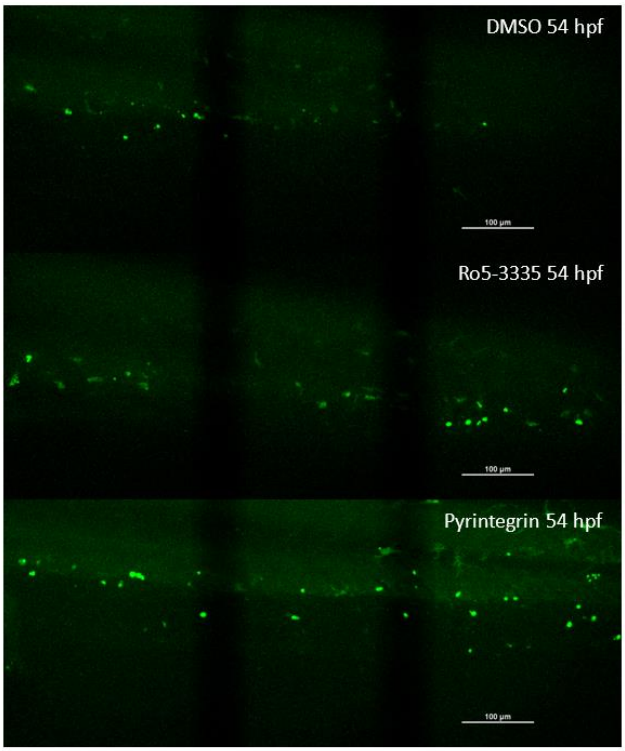

Figure 12 Pyrintegrin treatment leads to the highest number of Runx1:GFP cells. Runx1:GFP embryos were treated at 24 hpf. At 54 hpf, the zebrafish embryos were mounted in agarose and imaged using a high resolution spinning disk microscope. DMSO (top) was used as a vehicle control and Ro5-3335 (middle) as a positive control. The representative zebrafish embryo treated with Pyrintegrin (bottom) shows the highest number of cells and the expression level is very bright, followed by the zebrafish embryo treated with Ro5-3335. Cell number is lowest in the DMSO embryo. Green dots (GFP) represent HSCs. Scale bar: 100 µm.

I also tested the AhR antagonist from my mini-library, StemRegenin1 (SR1) (**Figure 13**). In these experiments, I observed a similar increase in Runx1:GFP cell number in the presence of the positive control, Ro5-3335, compared to the set of experiments in which I tested Pyrintegrin. In the first experiment (n1), I observed the highest number of cells with SR1. In the second experiment, the number of cells with SR1 was lower than in the first experiment, but still showed an expansion. The combined data (n=2) shows a significantly increased number of Runx1:GFP cells. Example images of treated embryos are shown in **Figure 14**.

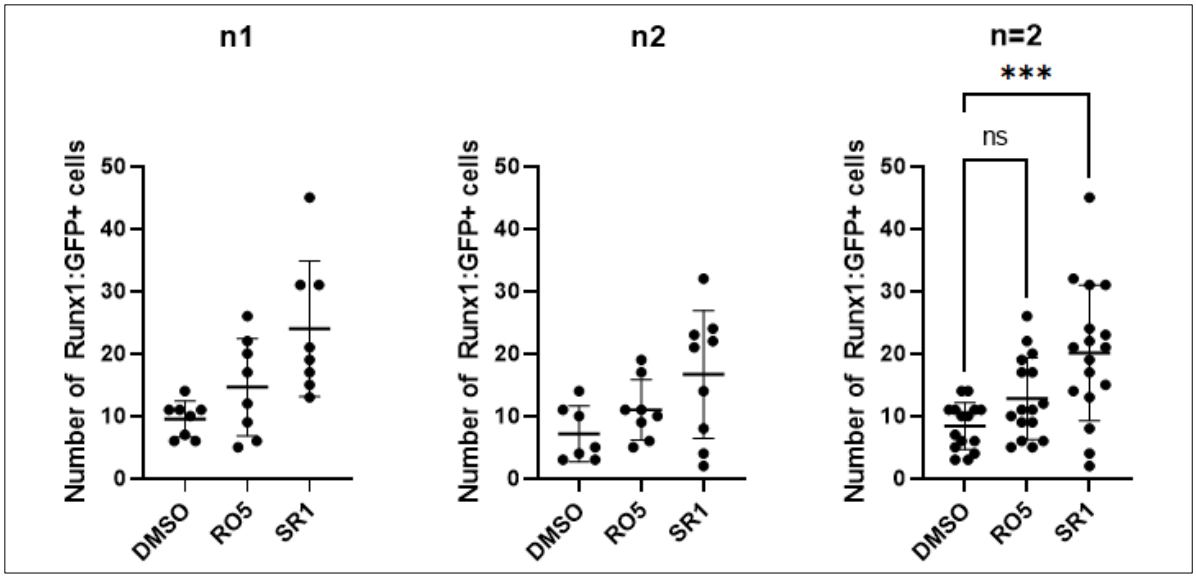

Figure 13 StemRegenin1 (SR1) increases Runx1:GFP+ HSCs in the zebrafish CHT. Runx1:GFP embryos were treated with SR1 at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. Two independent experiments were carried out (n1 and n2) and the combined data is represented as n=2. One-way-ANOVA with Dunnett's multiple comparison post-test analysis indicates that the combined results of SR1 are significant (***= P ≤ 0.001, ns=not significant (P ≤ 0.05)).

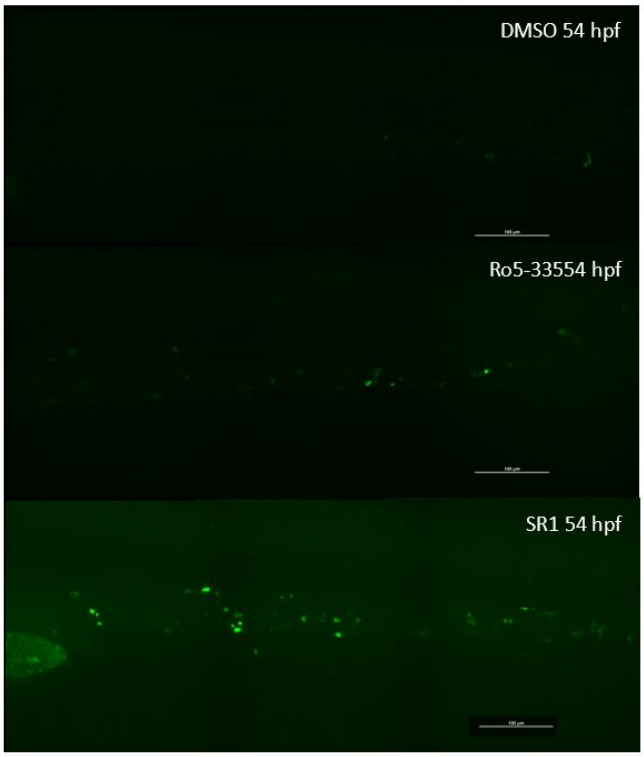

Figure 14 StemRegenin1 (SR1) treatment results in the highest number of Runx1:GFP cells. Runx1:GFP embryos were treated at 24 hpf. At 54 hpf, the zebrafish embryos were mounted in agarose

and imaged using a high resolution spinning disk microscope. DMSO (top) was used as a vehicle control and Ro5-3335 (middle) was used as a positive control. The representative zebrafish embryo treated with SR1 (bottom) shows the highest number of cells and the expression level is very bright, followed by the zebrafish embryo treated with Ro5-3335. In the middle image just a few bright GFP cells can be seen, the other cells are very dim. Cell number and brightness were lowest in the DMSO embryo (top). Green dots (GFP) represent HSCs. Scale bar: 100 µm.

3.3.2 **The effect of Wnt inhibition on HSCs in the zebrafish CHT**

My selected library contained a number of compounds with known effects on the Wnt pathway. These included the Wnt pathway inhibitor, IWR-1-endo (**Figure 15**). In the first experiment with this compound (n1), I did not observe a difference between any of the treatment groups. In the second experiment, there was a trend towards a higher number of cells with both Ro5-3335 and IWR-1-endo.The combined data (n=2) showed a minimally increased number of cells with IWR-1-endo, however, the results are not statistically significant. Example images of treated embryos are shown in **Figure 16**.

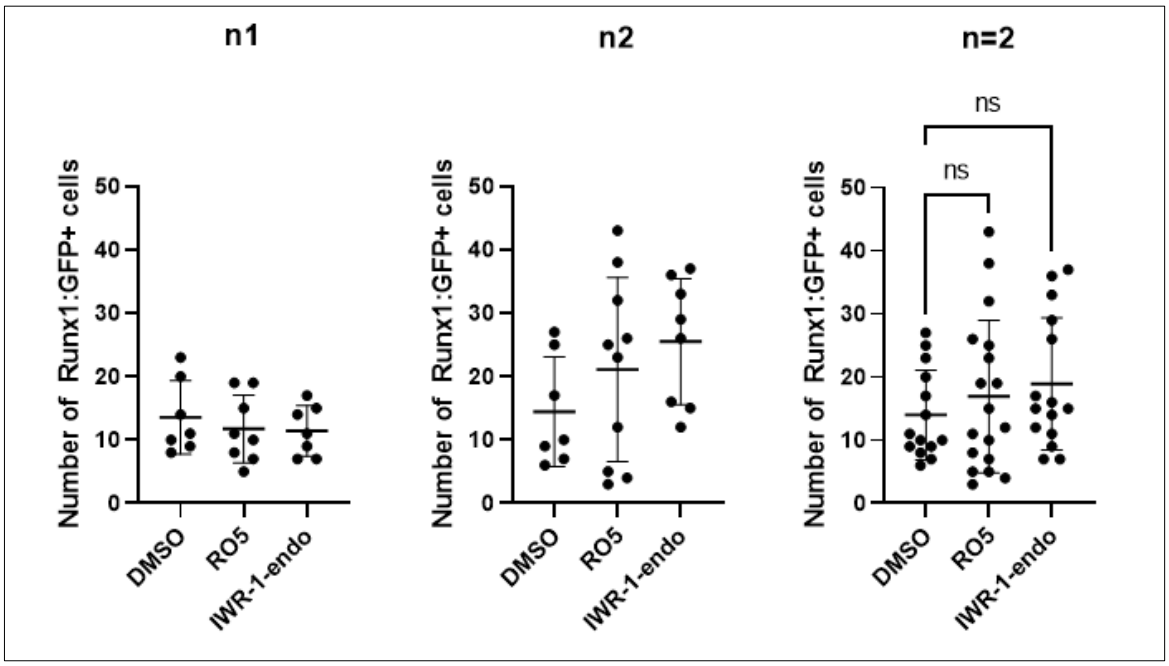

Figure 15 IWR-1-endo does not significantly affect HSC number in the CHT. Runx1:GFP embryos were treated with IWR-1-endo at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. Two independent experiments were carried out (n1 and n2) and the combined data is represented as n=2. One-way-ANOVA with Dunnett's multiple comparison post-test analysis indicates that the combined results are not significant (ns=not significant ($P > 0.05$)).

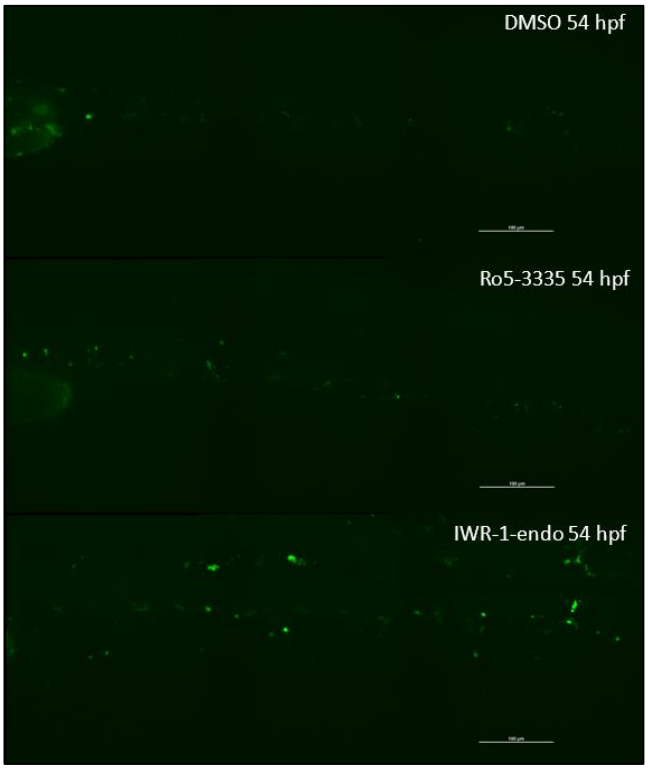

Figure 16 IWR-1 may increase Runx1:GFP cells in the zebrafish CHT. Runx1:GFP embryos were treated at 24 hpf. At 54 hpf, the zebrafish embryos were mounted in agarose and imaged using a high resolution spinning disk microscope. DMSO (top) was used as a vehicle control and Ro5-3335 (middle) as a positive control. In some IWR-1-endo treated embryos, the number of Runx1:GFP cells was high (bottom), but this was not statistically significant. Green dots (GFP) represent HSCs. Scale bar: 100 µm.

I also tested IWR-1-endo in Runx1:mCherry embryos (**Figure 17**). This transgenic line also labels HSCs in zebrafish, but the expression is more promiscuous than in the Runx1:GFP line. A large number of progenitor cells (referred to as Runx1:mCherry low cells) are labeled with mCherry, and these tend to be smaller and dimmer than the HSCs, which are the brightest and largest mCherry+ cells (referred to as Runx1:mCherry high cells). In the first experiment (n1), I observed a higher number of both Runx1:mCherry high and low cells with IWR-1-endo treatment. In the second experiment, there was a trend towards a higher number of Runx1:mCherry low cells with IWR-1-endo, but no change in Runx1:mCherry high cells. The combined data (n=2) showed a statistically significant increased number of Runx1:mCherry low cells with IWR-1-endo, suggesting that this compound may expand progenitor cells.

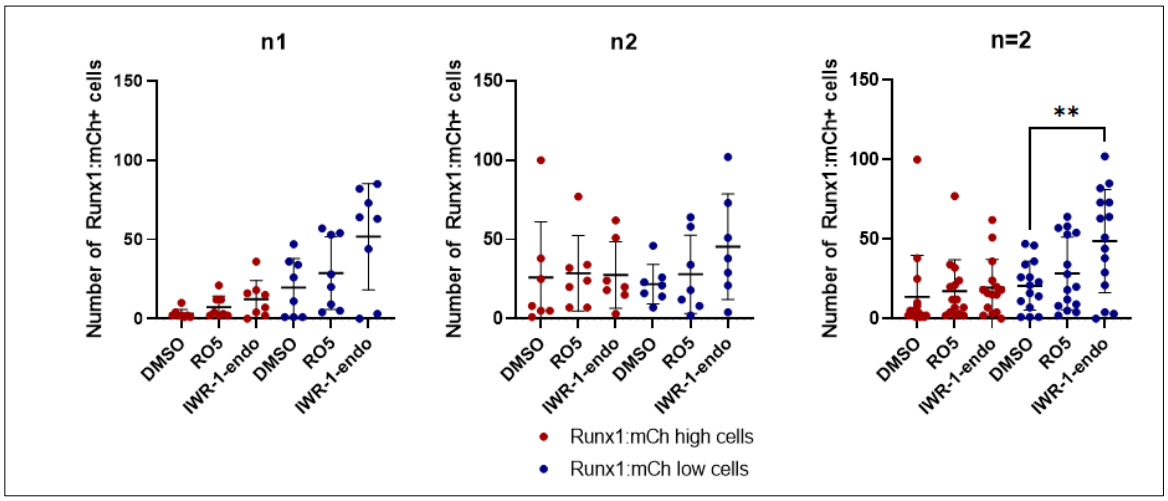

Figure 17 IWR-1-endo treatment causes a significant increase in Runx1:mCherry low cells. Runx1:mCherry embryos were treated with IWR-1-endo at 24 hpf. DMSO was used as vehicle control and Ro5-3335 as positive control. Red dots represent the number of Runx1:mCherry+high cells per zebrafish embryo and blue dots indicate the number of Runx1:mCherry+ low cells per zebrafish embryo. Two independent experiments were carried out (n1 and n2) and the combined data is represented as n=2. One-way-ANOVA with Bonferroni's multiple comparison post-test analysis indicates that the combined results of Runx1:mCherry high cells are not significant whereas the combined results of Runx1:mCherry low cells have a significance of $P \leq 0.01$ (ns=not significant; ($P > 0.05$), **=$P \leq 0.01$).

Another Wnt inhibitor from my mini-library, IWP-2, was also investigated in both Runx1:GFP and Runx1:mCherry assays. In these experiments, I observed more of an increase in Runx1:GFP cell number in the presence of the positive control (**Figure 18**), Ro5-3335, compared to the first set of experiments in which I tested IWR-1-endo. I did not see an increase with IWP-2 in either experiment, suggesting that this compound does not expand Runx1:GFP HSCs in zebrafish. However, there was a high level of variation in cell number between fish, as can be seen by the wide distribution of the data, making it difficult to interpret the results.

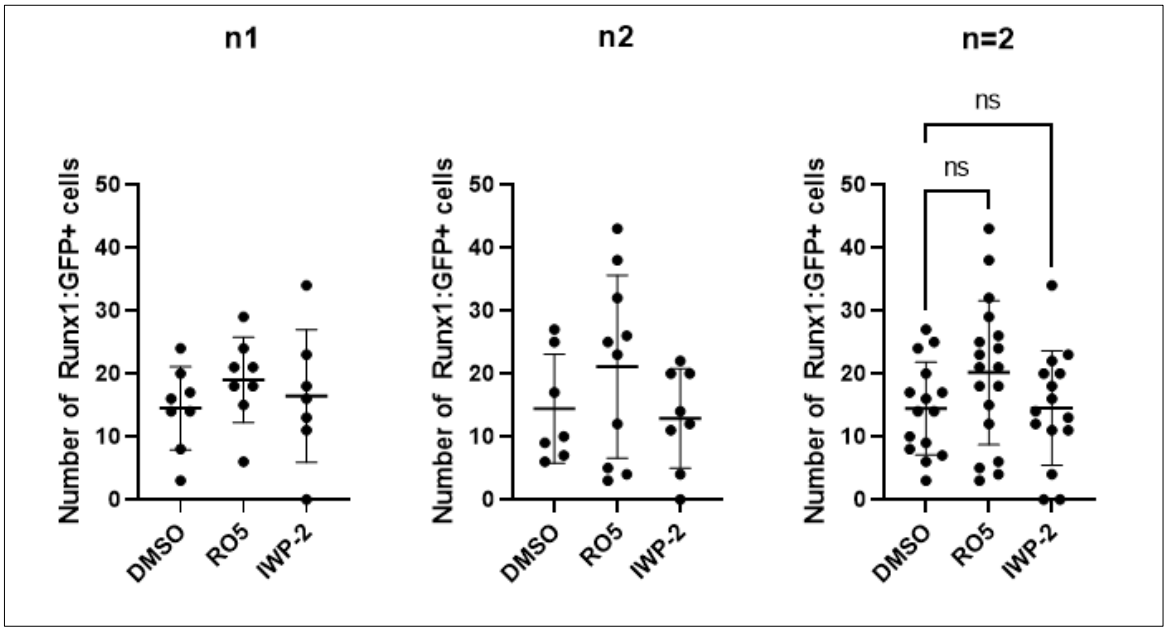

Figure 18 IWP-2 has no significant effect on HSCs in the zebrafish CHT. Runx1:GFP embryos were treated with IWP-2 at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. Two independent experiments were carried out (n1 and n2) and the combined data is shown as n=2. The Ro5-3335 control group had the highest number of cells, although this was not statistically significant in these experiments. IWP-2 treatment did not lead to an increase in Runx1:GFP+ cells. One-way-ANOVA with Dunnett's multiple comparison post-test analysis indicates that the combined results are not significant (ns=not significant ($P > 0.05$)).

I also screened IWP-2 in Runx1:mCherry embryos (**Figure 19**). In the first experiment (n1), I observed a significant increase in Runx1:mCherry low cells and a slight increase in Runx1:mCherry high cells. In the second experiment (n2), similar results could be observed. The combined data (n=2) showed no significant increase in the number of Runx1:mCherry high cells but a significant increase with Runx1:mCherry low cells with IWP-2, suggesting that this compound may expand progenitor cells. Example images of treated embryos are shown in **Figure 20**.

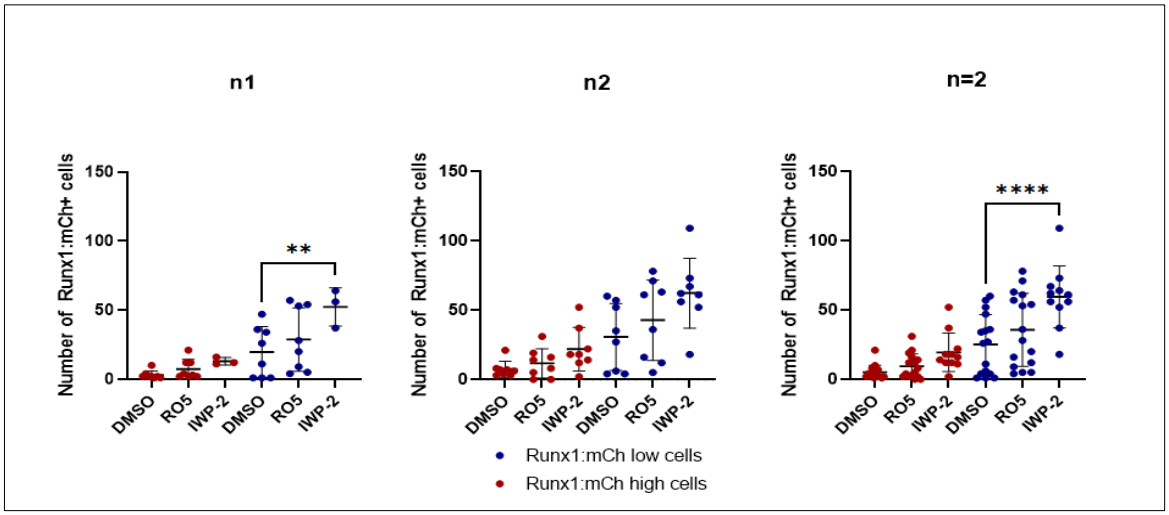

Figure 19 IWP-2 treatment significantly increase Runx1:mCherry low cells. Runx1:mCherry embryos were treated with IWP-2 at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Red dots represent the number of Runx1:mCherry+ high cells per zebrafish embryo and blue dots indicate the number of Runx1:mCherry+ low cells per zebrafish embryo. Two independent experiments were carried out (n1 and n2) and the combined data is represented as n=2. One-way-ANOVA with Bonferroni's multiple comparison post-test analysis indicates that the combined results of Runx1:mCherry high cells are not significant whereas the combined results of Runx1:mCherry low cells have a significance of $P \leq 0.0001$ (****= $P \leq 0.0001$).

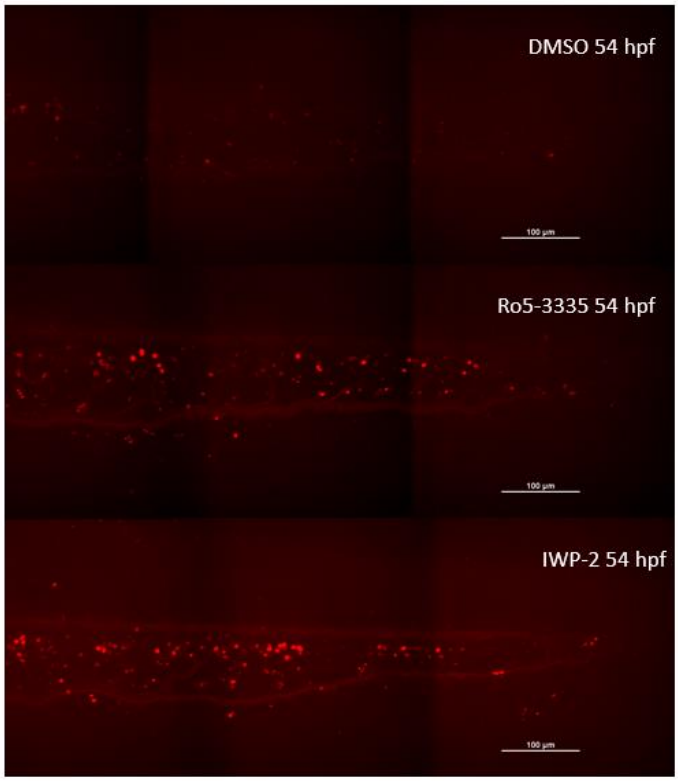

Figure 20 IWP-2 treatment increases Runx1:mCherry cells in the zebrafish CHT. Runx1:mCherry embryos were treated at 24 hpf. At 54 hpf, the zebrafish embryos were mounted in agarose and imaged

using a high resolution spinning disk microscope. DMSO (top) was used as a vehicle control and Ro5-3335 as a positive control (middle). A representative zebrafish embryo treated with IWP-2 (bottom) showed an increased number of Runx1:mCherry+ cells compared to the DMSO vehicle control. Red dots (mCherry) represent HSCs. Scale bar: 100 µm.

3.3.3 **Other compounds that may increase progenitor cells but not HSCs**

In my screen, I identified a number of compounds that did not expand Runx1:GFP cells but did have an effect in Runx1:mCherry embryos, particularly on the Runx1:mCherry low expressing cells. These included Suramin, a drug traditionally used to treat African sleeping sickness with multiple potential targets including the growth factors IGF-1, EGF and TGFβ (Babokhov, Sanyaolu, Oyibo, Fagbenro-Beyioku, & Iriemenam, 2013; Chamberlain, Shah, & Ferguson, 1995). In Runx1:GFP embryos, there was no difference in cell number between DMSO and Suramin in either experiment, as shown in the combined n=2 data (**Figure 21**). However, these results may not be valid because I did not observe an effect with the positive control in these experiments.

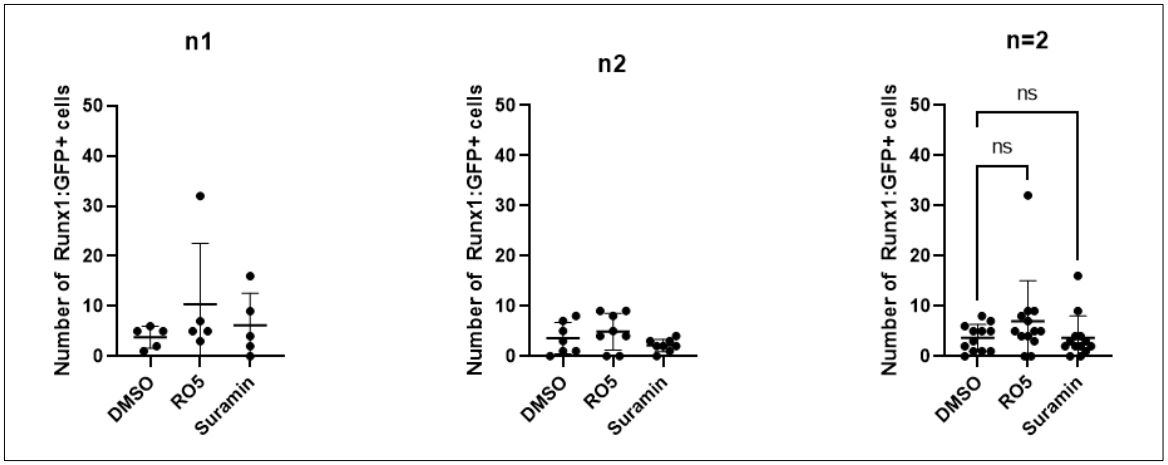

Figure 21 Suramin does not significantly affect HSC number in the CHT. Runx1:GFP embryos were treated with Suramin at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. Two independent experiments were carried out (n1 and n2) and the combined data is represented as n=2. One-way-ANOVA with Dunnett's multiple comparison post-test analysis indicates that the combined results are not significant (ns=not significant; ($P > 0.05$)).

I also tested Suramin in Runx1:mCherry embryos, but did not have time to do this more than once (**Figure 22**). In this experiment, I observed what appeared to be a higher number of both Runx1:mCherry high and low cells, however, only the increase in Runx1:mCherry low cells was significant. Additional experiments would need to be completed to confirm the effect. Example images of treated embryos are shown in **Figure 23**.

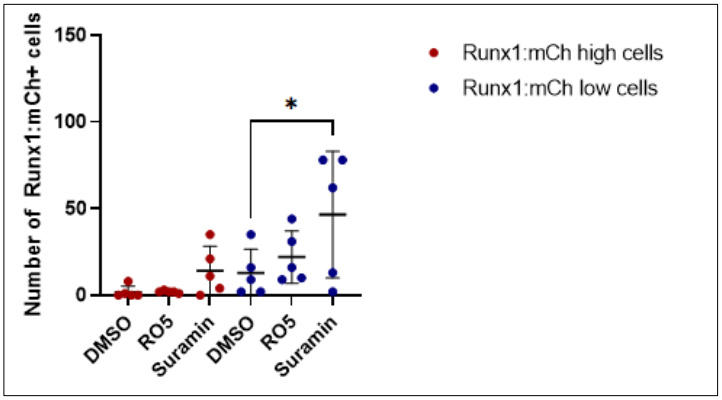

Figure 22 Suramin causes a significant increase in Runx1:mCherry low cells. Runx1:mCherry embryos were treated with Suramin at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Red dots represent the number of Runx1:mCherry+ high cells per zebrafish embryo and blue dots indicate the number of Runx1:mCherry+ low cells per zebrafish embryo. One experiment was carried out. One-way-ANOVA with Bonferroni's multiple comparison post-test analysis indicates that the results of Runx1:mCherry high cells are not significant (ns=not significant; ($P > 0.05$)) but there is a significant effect on Runx1:mCherry low cells (*=$P \leq 0.05$).

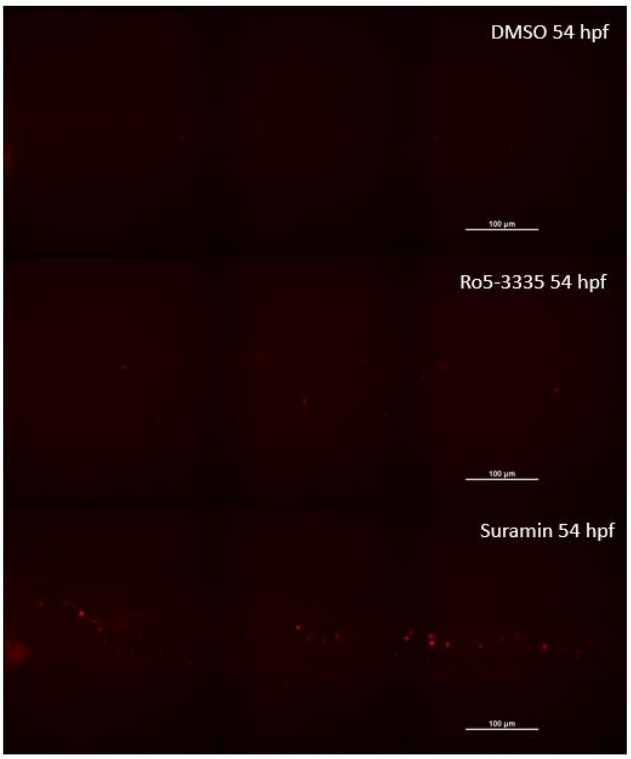

Figure 23 Suramin may increase Runx1:mCherry high and low cells. Runx1:mCherry embryos were treated with at 24 hpf. At 54 hpf, the zebrafish embryos were mounted in agarose and imaged using a high resolution spinning disk microscope. DMSO (top) was used as a vehicle control and Ro5-3335 (middle) as a positive control. Embryos treated with Suramin (bottom) showed an increased number of Runx1:mCherry+ cells with both high and low levels of mCherry expression. Red dots (mCherry) represent HSCs. Scale bar: 100 µm.

I also tested the RasGAP and Erk1 inhibitor, Pluripotin. In the first experiment with Runx1:GFP embryos (n1) I observed the highest cell number with Ro5-3335, but there was no difference between DMSO and Pluripotin (**Figure 24**). In the second experiment, I did not observe a difference between any of the treatment groups. The combined data (n=2) showed only a small, non-significant increase in cell number with Ro5-3335, again making it difficult to interpret the reliability of the experiment.

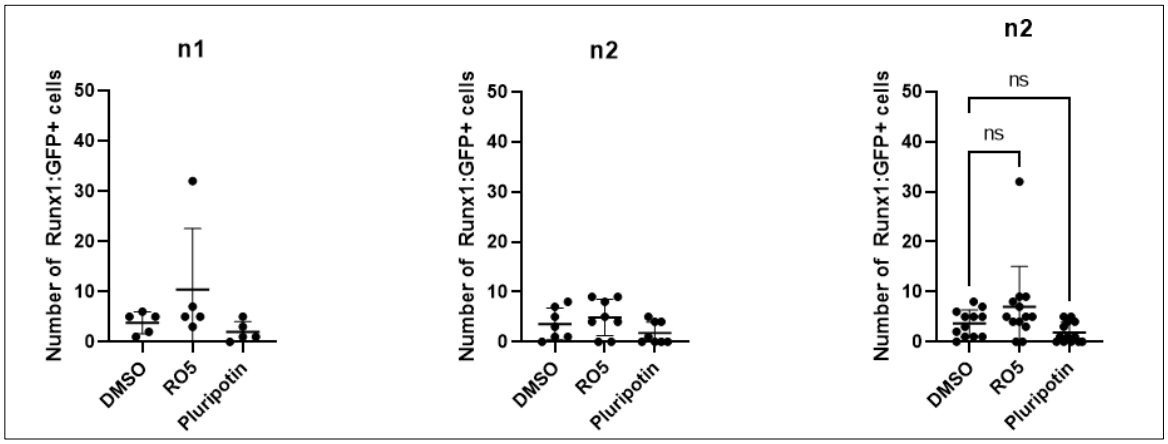

Figure 24 Pluripotin does not significantly affect HSC number in the CHT. Runx1:GFP embryos were treated with Pluripotin at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. Two independent experiments were carried out with Pluripotin (n1 and n2) and the combined data is represented as n=2. One-way-ANOVA with Dunnett's multiple comparison post-test analysis indicates that the combined results are not significant (ns=not significant; ($P > 0.05$)).

However, when I tested Pluripotin in Runx1:mCherry embryos, I observed a significantly higher number of Runx1:mCherry low cells (**Figure 25**). Example images of treated embryos are shown in **Figure 26**.

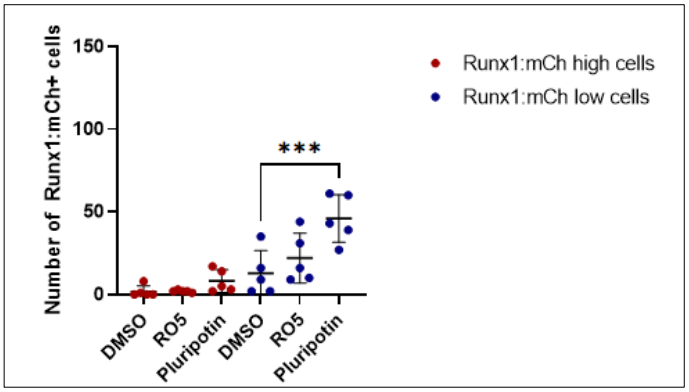

Figure 25 Pluripotin treatment leads to a significant increase in Runx1:mCherry low cells . Runx1:mCherry embryos were treated with Pluripotin at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Red dots represent the number of Runx1:mCherry+ high cells per

zebrafish embryo and blue dots indicate the number of Runx1:mCherry+ low cells per zebrafish embryo. One experiment was carried out. One-way-ANOVA with Bonferroni's multiple comparison post-test analysis indicates that the results of Runx1:mCherry high cells are not significant (ns=not significant; (P > 0.05)), but there was a significant increase in Runx1:mCherry low cells (***=P≤ 0.001).

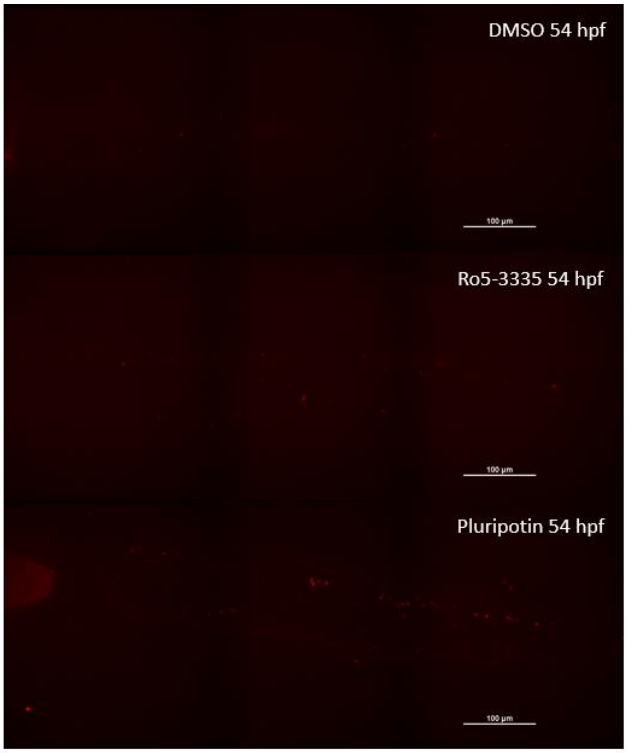

Figure 26 Pluripotin increases Runx1:mCherry low cells in the CHT. Runx1:mCherry embryos were treated at 24 hpf. At 54 hpf, the zebrafish embryos were mounted in agarose and imaged using a high resolution spinning disk microscope. DMSO (top) was used as a vehicle control and Ro5-33335 as a positive control (middle). Embryos treated with Pluripotin (bottom) showed a higher number of Runx1:mCherry+ cells, in particular those with low level expression. Red dots (mCherry) represent HSCs. Scale bar: 100 µm.

1.3.4 Compounds tested only once in Runx1:GFP embryos

Due to time restrictions, there were a number of compounds that I tested only once in Runx1:GFP zebrafish embryos. I screened the MEK inhibitor PD0325901 (left), the tankyrase inhibitor XAV939 (middle) and the thyroid inhibitor L-thyroxin (right) (**Figure 27**). In the one experiment with PD0325901, I did not observe a difference between any of the treatment groups. In the treatment with XAV939, there was a trend towards a higher number of cells with both Ro5-3335 and XAV939. The screen with L-thyroxin showed no effect with L-thyroxin. None of the results were statistically significant, but this is not unusual for experiments that were only performed once.

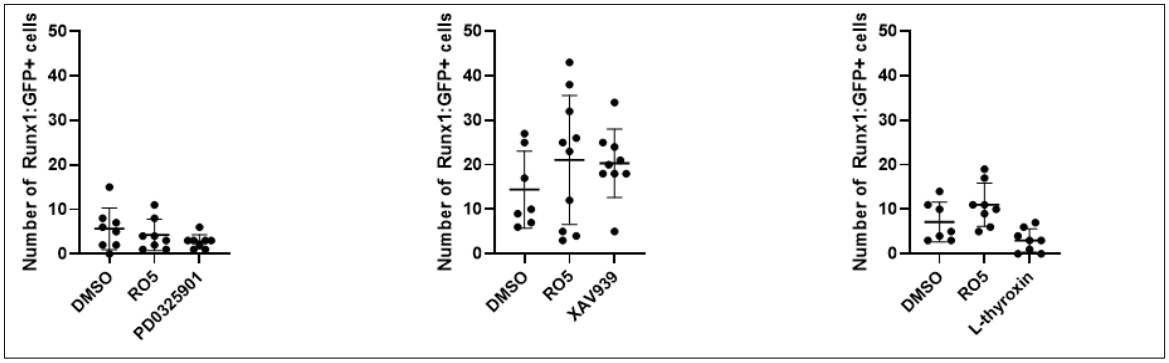

Figure 27 PD0325901, XAV939 and L-thyroxin may have no effect on Runx1:GFP cells. Runx1:GFP embryos were treated with PD0325901, XAV939 or L-thyroxin at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. One independent experiment was carried out with each compound. One-way-ANOVA with Dunnett's multiple comparison post-test analysis indicates that the results of PD0325901, XAV939 and L-thyroxin are not significant (ns=not significant ($P \leq 0.05$)).

Similarly, I also screened the p53 inhibitor Cyclin Pifithrin α (left), the Sir2p inhibitor Splitomicin (middle) and the ATP-competitive inhibitor of CDKs Kenpaullone (right) in Runx1:GFP embryos (**Figure 28**). There may have been a small increase in cell number with cyclic pifithrin α, but this was again difficult to interpret due to the lack of effect of Ro5-3335 and the low number. With Splitomicin, there was a small increase with Ro5-3335 only. Kenpaullone had no effect, but there was a clear increase in Runx1:GFP cells with Ro5-3335. As in the previous experiments that were performed only once, the results were not statistically significant.

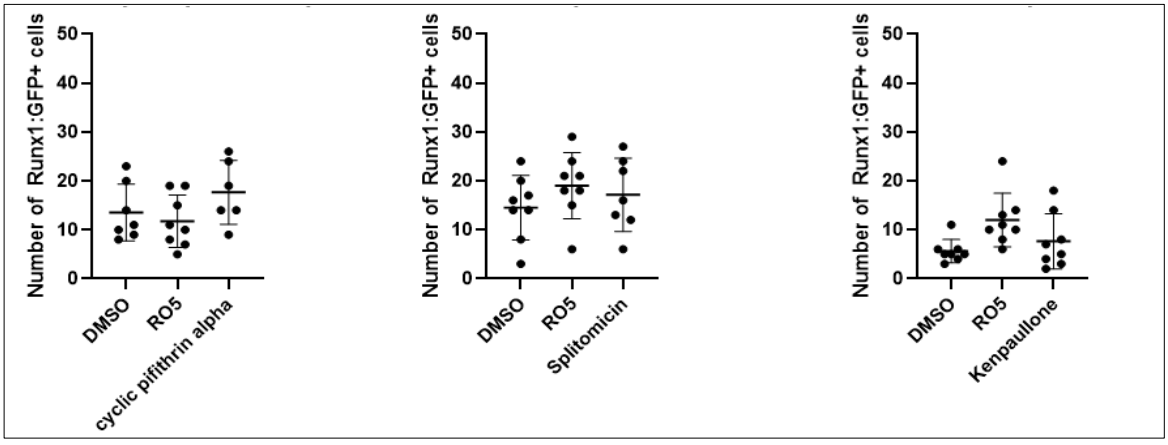

Figure 28 Cyclic Pifithrin α, Splitomicin and Kenpaullone may have no effect on Runx1:GFP cells. Runx1:GFP embryos were treated with Cyclic Pifithrin α, Splitomicin or Kenpaullone at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Dots represent the number of Runx1:GFP+ cells per zebrafish embryo. One independent experiment was carried out with each compound. One-way-

ANOVA with Dunnett's multiple comparison post-test analysis indicates that the results of Cyclic Pifithrin α, Splitomicin and Kenpaullone are not significant (ns=not significant (P ≤ 0.05)).

3.3.4 Compounds tested only once in Runx1:mCherry embryos

I screened two compounds once in Runx1:mCherry zebrafish embryos; the p53 inhibitor cyclic pifithrin α (left) and the Sir2p inhibitor Splitomicin (right) (**Figure 29**). I did not observe a significant difference or any particularly noticeable trends between any of the treatment groups in either experiment.

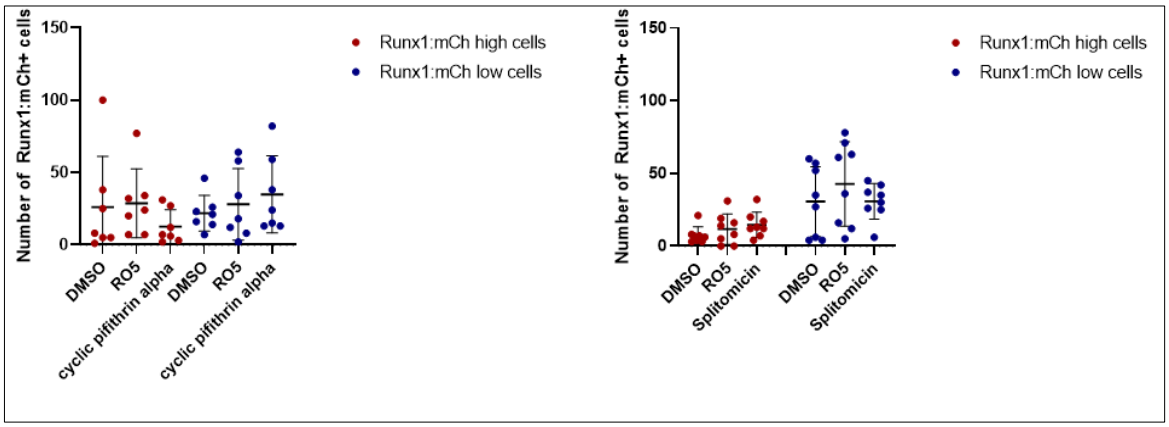

Figure 29 Cyclic Pifithrin α and Splitomicin have no effect on Runx1:mCherry cells. Runx1:mCherry embryos were treated with cyclic Pifithrin α or Splitomicin at 24 hpf. DMSO was used as a vehicle control and Ro5-3335 as a positive control. Red dots represent the number of Runx1:mCherry+ high cells per zebrafish embryo and blue dots indicate the number of Runx1:mCherry+ low cells per zebrafish embryo. One independent experiment was carried out with cyclic Pifithrin α and Splitomicin. One-way-ANOVA with Bonferroni's multiple comparison post-test analysis indicates that the results were not significant (ns=not significant; (P > 0.05)).

3.3.5 **Compound screen summary**

The performed screens and results are summarized in a table for better visualization (**Table 3**).

Table 3 Summary of individual screens. The table displays the mini-library of compounds and if they had an effect on Runx1:GFP+ and/or Runx1:mCherry high and/or Runx1:mCherry low cells. Two compounds showed a significantly positive effect on Runx1:GFP+ cells and four compounds on Runx1:mCherry low cells.

ID	Name	Runx1:GFP n	Potential effect on Runx1:GFP	Runx1:mCh n	Potential effect on Runx1:mCherry high cells	Potential effect on Runx1:mCherry low cells
1	16,16-dimethyl Prostaglandin E2	0	-	0	-	-
2	IWR-1-endo	2	None	2	None	Increase
3	SR1	2	Increase	0	-	-
4	Cyclic Pifithrin alpha	1	None	1	None	None
5	Splitomicin	1	None	1	None	None
6	IWP-2	2	None	2	None	Increase
7	Pyrintegrin	2	Increase	0	-	-
8	A83-01	0	-	0	-	-
9	Kenpaullone	1	None	0	-	-
10	Suramin	2	None	1	None	Increase
11	Pluripotin	2	None	1	None	Increase
12	PD0325901	0	-	0	-	-
13	XAV939	1	None	0	-	-
14	L-Thyroxine sodium salt pentahydrat	1	None	0	-	-
15	DAPT	0	-	0	-	-
16	2-phospho-l-ascorbic acid	0	-	0	-	-
17	Sirtinol	0	-	0	-	-
18	IDE-1	0	-	0	-	-
19	LDN-193189	0	-	0	-	-
20	SB431542	0	-	0	-	-
21	Purmorphamine	0	-	0	-	-
22	Dorsomorphin	0	-	0	-	-
23	Aminoresveratrol sulfate	0	-	0	-	-
24	Cyclopamine	0	-	0	-	-

4. DISCUSSION AND CONCLUSION

In this chemical genetic screen, I identified some compounds that expanded zebrafish hematopoietic stem and progenitor cells *in vivo*. The results are intriguing, but the compiled hypotheses and conclusions require further validation in future experiments.

Due to the pandemic, not all planned experiments could be executed, and it was impossible to screen the number of compounds initially desired. The original screening library contained 160 different compounds, but was narrowed down to 24 compounds, a size which was thought to be ideal for testing in the remaining time. Unfortunately, just 12 different compounds could be tested in this chemical genetic screen. There were some additional unexpected events that occurred and prevented screening of the entire library, for example the adult zebrafish pairs didn't lay enough embryos during the first few weeks I was in the lab. There were also issues with the Runx1:GFP zebrafish line, as the GFP expression wasn't satisfying in some screening attempts and the embryos did not have adequate cell numbers to reliably quantify. The Zon lab is making attempts to rescue this transgenic line by reconstituting it from frozen sperm, reinjecting the original plasmid and also exploring the use of other potential Runx1 enhancers.

I originally planned to test all the compounds in both Runx1:GFP and Runx1:mCherry transgenic zebrafish embryos. Runx1 is one of the earliest HSC markers in zebrafish and is needed for the development of definitive HSCs. The Zon lab established both of these transgenic zebrafish lines, which are driven by the same murine Runx1+23 enhancer (Tamplin et al., 2015). Theoretically, each line should label the same population of cells, however this isn't the case. In the Runx1:mCherry zebrafish line, more cells are labeled, which are likely the progenitor population. This is most likely due to the transgene being inserted into a different part of the genome when the line was generated, which could result in the influence of different transcription factors that might affect its expression levels. During analysis of the screens performed in Runx1:mCherry zebrafish embryos, the positive cells were divided into Runx1:mCherry high and low cells. The difference between high and low depends on the level of expression of the fluorescent protein (mCherry). It is assumed that cells with higher intensity are likely to be more 'stem-like' and when these cells start to divide and differentiate into progenitor cells, they will express lower levels of mCherry. Unfortunately, there were also some issues with overall expression levels in the Runx1:mCherry embryos, so I was only able to test a few compounds in this line.

These fish laid fewer embryos than the Runx1:GFP transgenic line and the expression levels of Runx1:mCherry+ cells differed immensely. In some experiments (data not shown) the results were very variable and sometimes no expression at all could be observed. It is possible that some of the adult fish were heterozygous for the transgene; hence some embryos did not express any mCherry labeled cells. In both the Runx1:GFP and Runx1:mCherry assays, there was a high level of variability in cell number leading to large standard deviations and a lack of statistical significance in many experiments. This is not unusual for this type of *in vivo* zebrafish assay. With some compounds, the results were very different between each individual experiment, making it difficult to make a robust conclusion. Given more time, I would have repeated all of the experiments to increase the number, which likely would have made my results more reliable.

The most interesting compound identified in my screen was Pyrintegrin. This chemical is known to promote embryonic stem cell survival, a characteristic which makes it interesting to examine the effect on HSCs specifically (Shah et al., 2017). Pyrintegrin treatment caused a significant increase in Runx1:GFP HSCs, to a level even greater than the positive control, Ro5-3335 (**Figure 11**). This makes it a particularly intriguing compound for further study. Unfortunately, Pyrintegrin could not be tested in Runx1:mCherry zebrafish embryos. Two attempts were made but no mCherry expression could be observed, likely for the reasons described above. In summary, Pyrintegrin appears to expand Runx1:GFP HSCs *in vivo*, but these observations need to be validated further. Given that Pyrintegrin is a β1-integrin agonist, it is possible that this effect on HSCs may be a result of increased migration to or retention in the CHT or increased self-renewal. Pyrintegrin binds to and activates β1-integrin receptors on the cell surface. Integrins are molecules on the cell surface which mediate signal transduction through the cell membrane either by binding to ligands in the ECM or receptors on other cells such as VCAM-1. This activates intracellular signaling which results in effects on migration, proliferation, survival, and gene expression (Howe & Addison, 2012). The ECM is a network of secreted proteins, which regulates and supports various cellular processes and is an important part of the hematopoietic microenvironment within the niche (Rozario & DeSimone, 2010). The ECM, signaling factors and other surrounding cells influence HSC function (Davis & Senger, 2005). Integrin activation has also been shown to affect HSC proliferation and stemness (Orford & Scadden, 2008).

The AhR antagonist StemRegenin 1 (SR1) is known to promote *ex vivo* expansion of CD34+ human HSCs and the generation of CD34+ hematopoietic progenitor cells from primate iPSCs (Casado, 2016; StemCellTechnologies). The treatment of zebrafish embryos with SR1 induced an increase in the number of Runx1:GFP+ cells, suggesting that this compound may also expand HSCs *in vivo*. There was a high level of variability within these experiments, however, SR1 did appear to enhance HSC number more effectively than Ro5-3335, making it another interesting compound to pursue further (**Figure 13**). Unfortunately, the effect of SR1 also could not be investigated in Runx1:mCherry zebrafish embryos, as there was no mCherry expression in the embryos tested. In summary, treatment with SR1 leads to a higher number of Runx1:GFP cells, but further experiments are necessary to confirm these findings.

The Wnt inhibitor IWR-1-endo is known to promote self-renewal and maintenance of human embryonic stem cells when combined with the GSK3 inhibitor CHIR99021 (Chen et al., 2009; StemCellTechnologies), making it an interesting candidate for the chemical genetic screen. With this compound, the 2 independent experiments in Runx1:GFP zebrafish embryos resulted in different outcomes; one treatment with IWR-1-endo led to enhanced cell number and the other experiment showed no difference between this compound and the DMSO control. Due to these opposing results, the combined data did not show a significant increase in Runx1:GFP. However, I would be hesitant to conclude that there is no effect as it is possible that the first experiment did not work, particularly as no effect was observed with the positive control either. In the IWR-1-endo embryo shown in **Figure 15**, there is a clear increase in the number of Runx1:GFP cells, which is reflective of the data from the second experiment. Interestingly, the screens performed in Runx1:mCherry zebrafish embryos showed a significantly increased number of Runx1:mCherry+ low cells, suggesting an expansion of progenitor cells (**Figure 17**). As with the previous compounds, the experiments with IWR-1-endo must be repeated several times to make a firm conclusion about the effect on HSPCs *in vivo*. Nevertheless, the chance of this compound being a potential enhancer of HSCs remains.

I also tested another Wnt inhibitor, IWP-2, which is known to promote cardiomyocyte differentiation but actually inhibit embryonic stem cell self-renewal, in contrast to IWR-1-endo (PeproTech; Xu et al., 2016). As might be expected, treatment of Runx1:GFP zebrafish embryos with IWP-2 had no effect on HSC number (**Figure 18**). However, the finding in Runx1:mCherry zebrafish embryos was interesting, in which IWP-2 significantly increased the number of both Runx1:mCherry+ high and low cells (**Figure**

19). These experiments need to be repeated, but it is possible that this compound may have an effect on differentiating progenitor cells.

I also tested other compounds that may increase progenitor cells but not HSCs, which could be assessed by an increase in Runx1:mCherry low expressing cells. For example, Suramin showed no effect on Runx1:GFP cells but did have an effect on Runx1:mCherry low expressing cells (**Figure 22**). This phenotype was also true with Pluripotin, which is a RasGAP and Erk1 inhibitor. There seemed to be no effect in Runx1:GFP cells but an increase in Runx1:mCherry low cells was observed (**Figure 25**). However, in both screens the positive control Ro5-3335 showed no effect on Runx1:GFP cells, so these results may not be valid and need to repeated. Unfortunately, the experiments with Runx1:mCherry zebrafish embryos were only performed once, therefore it is difficult to make a robust conclusion on the effect of these two compounds. These experiments need to be repeated, but it may be possible that the compounds increase progenitor cells.

I also screened some other compounds only once in Runx1:GFP or Runx1:mCherry zebrafish embryos. In Runx1:GFP embryos, these included the MEK inhibitor PD0325901, the tankyrase inhibitor XAV939, the thyroid inhibitor L-thyroxin, the p53 inhibitor Cyclin Pifithrin α, the Sir2p inhibitor Splitomicin and the ATP-competitive inhibitor of CDKs Kenpaullone. The experiments with PD0325901, L-thyroxin, Splitomicin and Kenpaullone showed no effect on HSCs. The experiment with PD0325901 is difficult to interpret, because Ro5-3335 did not have an effect in this screen (**Figure 27**). There is also the possibility that there was a problem with the expression of Runx1:GFP cells in these particular zebrafish embryos. The experiment with Cyclin Pifithrin α may show a trend towards an increase in Runx1:GFP cells, but here it was also difficult to make a robust conclusion, because treatment with Ro5-3335 showed no increasing effect in this screen (**Figure 28**). The treatment with XAV939 might be promising, because it showed an increase on HSC number compared to the treatment with DMSO, even though this did not reach the level of the effect with Ro5-3335 in this experiment (**Figure 27**). In particular this result is interesting because XAV939 is also reported to act as a Wnt/β-catenin pathway inhibitor (Stakheev et al., 2019). Wnt is known to function in HSCs, by promoting their development, expansion and maintenance, depending on the age of the animal (reviewed by (Lento, Congdon, Voermans, Kritzik, & Reya, 2013). In other experiments and contexts, Wnt is reported to inhibit self-renewal of HSCs (Kirstetter, Anderson, Porse, Jacobsen, & Nerlov, 2006; Scheller et al., 2006). These characteristics make XAV939 an interesting candidate

compound, which may promote expansion of HSCs in the CHT of zebrafish embryos upon further investigation.

None of the compounds that were tested only once showed a significant result, but this is not unusual in this type of zebrafish assay when the number is very low. All of these experiments need to be repeated to make a conclusion about the effect on HSCs or their progenitors.

4.1 Experimental outlook

As mentioned, not all planned experiments could be executed due to time constraints. There is still a lot of work to do to validate my findings. It would be interesting to test the other compounds from my library and repeat the screens with the compounds that had the most significant results in order to prove my conclusions. If the compounds can be validated in additional experiments, it would be interesting to perform dose-response assays to determine the optimum concentration of each chemical for the expansion of HSCs. Also, the impact of changing the treatment duration and start time could be investigated. Furthermore, time-lapse movies of the most promising compounds should be acquired to enable accurate observation and quantification of cell divisions in the CHT, and also budding of nascent HSCs in the AGM. This would help to indicate when and where the drugs are having their effects. It is possible that they could be enhancing HSC specification in the AGM, or self-renewal and proliferation in the CHT. Another approach would be to examine the effects of the compounds on other blood lineages using other transgenic zebrafish lines. For instance, the effect of the different drugs could be characterized on macrophages, thrombocytes, neutrophils and T-cells, because it is important that the expanded HSCs function appropriately and have the potential to differentiate into all of the required blood lineages. It would be possible to assess this with alternative transgenic zebrafish lines and also by examining the expression of different blood cell markers by in situ hybridization. By continuation of the screen with the missing compounds, there will be the chance to find other interesting compounds that may expand HSCs *in vivo*. However, it could be also possible to find compounds that have the opposite effect, leading to a reduction of HSCs in the CHT.

4.2 Summary

In this chemical genetic screen, some interesting discoveries were made, however not all results were conclusive, and a variety of further experiments and repetition needs to be done. Of particular interest, two compounds, Pyrintegrin and SR1, showed very promising significant effects on HSC expansion and further investigation would determine their precise mechanism of action. It is also intriguing that a number of compounds in my library target the Wnt pathway, and these are worthy of further investigation. Screening of the remaining compounds in the library that I did not have time to test could also identify additional expanders of Runx1+ HSCs. These findings could ultimately be useful for the expansion of human HSCs *ex vivo* and could have important therapeutic implications for bone marrow transplantation. They may also reveal new insights into the molecular signaling pathways that are important for regulating definitive hematopoiesis and HSC self-renewal.

Bibliography

A. Rad and Mikael Häggström, M. D. (9 December 2016). Hematopoiesis simple. In https://upload.wikimedia.org/wikipedia/commons/f/f0/Hematopoiesis_simple.svg (Ed.), (Vol. 1,800 × 1,200 pixels). https://en.wikipedia.org/wiki/Hematopoietic_stem_cell.

Amsellem, S., Pflumio, F., Bardinet, D., Izac, B., Charneau, P., Romeo, P. H., . . . Fichelson, S. (2003). Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nat Med, 9*(11), 1423-1427. doi:10.1038/nm953

Arulmozhivarman, G., Kräter, M., Wobus, M., Friedrichs, J., Bejestani, E. P., Müller, K., . . . Bornhäuser, M. (2017). Zebrafish In-Vivo Screening for Compounds Amplifying Hematopoietic Stem and Progenitor Cells: - Preclinical Validation in Human CD34+ Stem and Progenitor Cells. *Sci Rep, 7*(1), 12084. doi:10.1038/s41598-017-12360-0

Avagyan, S., & Zon, L. I. (2016). Fish to Learn: Insights into Blood Development and Blood Disorders from Zebrafish Hematopoiesis. *Human gene therapy, 27*(4), 287-294. doi:10.1089/hum.2016.024

Babokhov, P., Sanyaolu, A. O., Oyibo, W. A., Fagbenro-Beyioku, A. F., & Iriemenam, N. C. (2013). A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathogens and global health, 107*(5), 242-252. doi:10.1179/2047773213Y.0000000105

Benz, C., Copley, M. R., Kent, D. G., Wohrer, S., Cortes, A., Aghaeepour, N., . . . Eaves, C. J. (2012). Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell, 10*(3), 273-283. doi:10.1016/j.stem.2012.02.007

Bertrand, J. Y., Chi, N. C., Santoso, B., Teng, S., Stainier, D. Y., & Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature, 464*(7285), 108-111. doi:10.1038/nature08738

Bertrand, J. Y., Cisson, J. L., Stachura, D. L., & Traver, D. (2010). Notch signaling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. *Blood, 115*(14), 2777-2783. doi:10.1182/blood-2009-09-244590

Bertrand, J. Y., Kim, A. D., Violette, E. P., Stachura, D. L., Cisson, J. L., & Traver, D. (2007). Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development (Cambridge, England), 134*(23), 4147-4156. doi:10.1242/dev.012385

Birbrair, A., & Frenette, P. S. (2016). Niche heterogeneity in the bone marrow. *Ann N Y Acad Sci, 1370*(1), 82-96. doi:10.1111/nyas.13016

Blaser, B. W., Moore, J. L., Hagedorn, E. J., Li, B., Riquelme, R., Lichtig, A., . . . Zon, L. I. (2017). CXCR1 remodels the vascular niche to promote hematopoietic stem and progenitor cell engraftment. *The Journal of experimental medicine, 214*(4), 1011-1027. doi:10.1084/jem.20161616

Boisset, J. C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., & Robin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature, 464*(7285), 116-120. doi:10.1038/nature08764

Boitano, A. E., Wang, J., Romeo, R., Bouchez, L. C., Parker, A. E., Sutton, S. E., . . . Cooke, M. P. (2010). Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science, 329*(5997), 1345-1348. doi:10.1126/science.1191536

Bresciani, E., Carrington, B., Wincovitch, S., Jones, M., Gore, A. V., Weinstein, B. M., . . . Liu, P. P. (2014). CBFβ and RUNX1 are required at 2 different steps during

the development of hematopoietic stem cells in zebrafish. *Blood, 124*(1), 70-78. doi:10.1182/blood-2013-10-531988

Burns, C. E., DeBlasio, T., Zhou, Y., Zhang, J., Zon, L., & Nimer, S. D. (2002). Isolation and characterization of runxa and runxb, zebrafish members of the runt family of transcriptional regulators. *Exp Hematol, 30*(12), 1381-1389. doi:10.1016/s0301-472x(02)00955-4

Burns, C. E., Traver, D., Mayhall, E., Shepard, J. L., & Zon, L. I. (2005). Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev, 19*(19), 2331-2342. doi:10.1101/gad.1337005

Butko, E., Distel, M., Pouget, C., Weijts, B., Kobayashi, I., Ng, K., . . . Traver, D. (2015). Gata2b is a restricted early regulator of hemogenic endothelium in the zebrafish embryo. *Development (Cambridge, England), 142*(6), 1050-1061. doi:10.1242/dev.119180

Butko, E., Distel, M., Pouget, C., Weijts, B., Kobayashi, I., Ng, K., . . . Traver, D. (2015). Gata2b is a restricted early regulator of hemogenic endothelium in the zebrafish embryo. *Development (Cambridge, England), 142*(6), 1050-1061. doi:10.1242/dev.119180

Campbell, C., Su, T., Lau, R. P., Shah, A., Laurie, P. C., Avalos, B., . . . Stachura, D. L. (2015). Zebrafish embryonic stromal trunk (ZEST) cells support hematopoietic stem and progenitor cell (HSPC) proliferation, survival, and differentiation. *Exp Hematol, 43*(12), 1047-1061. doi:10.1016/j.exphem.2015.09.001

Cantor, A. B., & Orkin, S. H. (2002). Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene, 21*(21), 3368-3376. doi:10.1038/sj.onc.1205326

Casado, F. L. (2016). The Aryl Hydrocarbon Receptor Relays Metabolic Signals to Promote Cellular Regeneration. *Stem Cells Int, 2016*, 4389802. doi:10.1155/2016/4389802

Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A., & Bhatia, M. (2003). Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood, 102*(3), 906-915. doi:10.1182/blood-2003-03-0832

Chamberlain, J., Shah, M., & Ferguson, M. W. (1995). The effect of suramin on healing adult rodent dermal wounds. *Journal of anatomy, 186 (Pt 1)*(Pt 1), 87-96.

Chaurasia, P., Gajzer, D. C., Schaniel, C., D'Souza, S., & Hoffman, R. (2014). Epigenetic reprogramming induces the expansion of cord blood stem cells. *J Clin Invest, 124*(6), 2378-2395. doi:10.1172/jci70313

Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C.-W., . . . Lum, L. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol, 5*(2), 100-107. doi:10.1038/nchembio.137

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C., & Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development (Cambridge, England), 125*(4), 725-732.

Choi, K. D., Vodyanik, M. A., Togarrati, P. P., Suknuntha, K., Kumar, A., Samarjeet, F., . . . Slukvin, II. (2012). Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. *Cell reports, 2*(3), 553-567. doi:10.1016/j.celrep.2012.08.002

Ciau-Uitz, A., Monteiro, R., Kirmizitas, A., & Patient, R. (2014). Developmental hematopoiesis: ontogeny, genetic programming and conservation. *Exp Hematol, 42*(8), 669-683. doi:10.1016/j.exphem.2014.06.001

Clements, W. K., Kim, A. D., Ong, K. G., Moore, J. C., Lawson, N. D., & Traver, D. (2011). A somitic Wnt16/Notch pathway specifies haematopoietic stem cells. *Nature, 474*(7350), 220-224. doi:10.1038/nature10107

Cohen, S., Roy, J., Lachance, S., Marinier, A., Barabé, F., Delisle, J.-S., . . . Sauvageau, G. (2018). Single UM171 Expanded Cord Blood Transplant is

Feasible, Safe, and Permits Transplantation of Better HLA Matched Cords with Very Low Transplant Related Mortality. *Biology of Blood and Marrow Transplantation, 24*(3), S190-S191. doi:10.1016/j.bbmt.2017.12.301

Collymore, C., Rasmussen, S., & Tolwani, R. J. (2013). Gavaging adult zebrafish. *Journal of visualized experiments : JoVE*(78). doi:10.3791/50691

Cumano, A., & Godin, I. (2007). Ontogeny of the hematopoietic system. *Annu Rev Immunol, 25*, 745-785. doi:10.1146/annurev.immunol.25.022106.141538

Cutler, C., Multani, P., Robbins, D., Kim, H. T., Le, T., Hoggatt, J., . . . Shoemaker, D. D. (2013). Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. *Blood, 122*(17), 3074-3081. doi:10.1182/blood-2013-05-503177

Davis, G. E., & Senger, D. R. (2005). Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res, 97*(11), 1093-1107. doi:10.1161/01.RES.0000191547.64391.e3

de Jong, J. L., Burns, C. E., Chen, A. T., Pugach, E., Mayhall, E. A., Smith, A. C., . . . Zon, L. I. (2011). Characterization of immune-matched hematopoietic transplantation in zebrafish. *Blood, 117*(16), 4234-4242. doi:10.1182/blood-2010-09-307488

de Jong, J. L., & Zon, L. I. (2005). Use of the zebrafish system to study primitive and definitive hematopoiesis. *Annu Rev Genet, 39*, 481-501. doi:10.1146/annurev.genet.39.073003.095931

de Jong, J. L., & Zon, L. I. (2012). Histocompatibility and hematopoietic transplantation in the zebrafish. *Adv Hematol, 2012*, 282318. doi:10.1155/2012/282318

Detrich, H. W., 3rd, Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A., . . . Zon, L. I. (1995a). Intraembryonic hematopoietic cell migration during vertebrate development. *Proceedings of the National Academy of Sciences of the United States of America, 92*(23), 10713-10717. doi:10.1073/pnas.92.23.10713

Detrich, H. W., 3rd, Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A., . . . Zon, L. I. (1995b). Intraembryonic hematopoietic cell migration during vertebrate development. *Proceedings of the National Academy of Sciences of the United States of America, 92*(23), 10713-10717. doi:10.1073/pnas.92.23.10713

Ding, L., & Morrison, S. J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature, 495*(7440), 231-235. doi:10.1038/nature11885

Ding, L., Saunders, T. L., Enikolopov, G., & Morrison, S. J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature, 481*(7382), 457-462. doi:10.1038/nature10783

Dirscherl, H., McConnell, S. C., Yoder, J. A., & de Jong, J. L. (2014). The MHC class I genes of zebrafish. *Dev Comp Immunol, 46*(1), 11-23. doi:10.1016/j.dci.2014.02.018

Dirscherl, H., & Yoder, J. A. (2014). Characterization of the Z lineage Major histocompatability complex class I genes in zebrafish. *Immunogenetics, 66*(3), 185-198. doi:10.1007/s00251-013-0748-z

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S. J., Moynihan, J., . . . Zon, L. I. (2000). Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature, 403*(6771), 776-781. doi:10.1038/35001596

Dooley, K. A., Davidson, A. J., & Zon, L. I. (2005). Zebrafish scl functions independently in hematopoietic and endothelial development. *Dev Biol, 277*(2), 522-536. doi:10.1016/j.ydbio.2004.09.004

Doulatov, S., Notta, F., Eppert, K., Nguyen, L. T., Ohashi, P. S., & Dick, J. E. (2010). Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nat Immunol, 11*(7), 585-593. doi:10.1038/ni.1889

Doulatov, S., Vo, L. T., Chou, S. S., Kim, P. G., Arora, N., Li, H., . . . Daley, G. Q. (2013). Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. *Cell Stem Cell, 13*(4), 459-470. doi:10.1016/j.stem.2013.09.002

Doulatov, S., Vo, L. T., Chou, S. S., Kim, P. G., Arora, N., Li, H., . . . Daley, G. Q. (2013). Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. *Cell Stem Cell, 13*(4), 459-470. doi:10.1016/j.stem.2013.09.002

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., . . . Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development (Cambridge, England), 123*, 37-46.

Dzierzak, E. (2002). Hematopoietic stem cells and their precursors: developmental diversity and lineage relationships. *Immunol Rev, 187*, 126-138. doi:10.1034/j.1600-065x.2002.18711.x

Eaves, C. J. (2015). Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood, 125*(17), 2605-2613. doi:10.1182/blood-2014-12-570200

Elcheva, I., Brok-Volchanskaya, V., Kumar, A., Liu, P., Lee, J. H., Tong, L., . . . Slukvin, I. (2014). Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators. *Nature communications, 5*, 4372. doi:10.1038/ncomms5372

Esain, V., Kwan, W., Carroll, K. J., Cortes, M., Liu, S. Y., Frechette, G. M., . . . North, T. E. (2015). Cannabinoid Receptor-2 Regulates Embryonic Hematopoietic Stem Cell Development via Prostaglandin E2 and P-Selectin Activity. *Stem cells (Dayton, Ohio), 33*(8), 2596-2612. doi:10.1002/stem.2044

Espin-Palazon, R., Stachura, D. L., Campbell, C. A., Garcia-Moreno, D., Del Cid, N., Kim, A. D., . . . Traver, D. (2014). Proinflammatory signaling regulates hematopoietic stem cell emergence. *Cell, 159*(5), 1070-1085. doi:10.1016/j.cell.2014.10.031

Fares, I., Chagraoui, J., Gareau, Y., Gingras, S., Ruel, R., Mayotte, N., . . . Sauvageau, G. (2014). Cord blood expansion. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science, 345*(6203), 1509-1512. doi:10.1126/science.1256337

Forsberg, E. C., Passegué, E., Prohaska, S. S., Wagers, A. J., Koeva, M., Stuart, J. M., & Weissman, I. L. (2010). Molecular signatures of quiescent, mobilized and leukemia-initiating hematopoietic stem cells. *PLoS One, 5*(1), e8785. doi:10.1371/journal.pone.0008785

Fujino, H., West, K. A., & Regan, J. W. (2002). Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *J Biol Chem, 277*(4), 2614-2619. doi:10.1074/jbc.M109440200

Galloway, J. L., & Zon, L. I. (2003). Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. *Curr Top Dev Biol, 53*, 139-158. doi:10.1016/s0070-2153(03)53004-6

Gao, X., Johnson, K. D., Chang, Y. I., Boyer, M. E., Dewey, C. N., Zhang, J., & Bresnick, E. H. (2013). Gata2 cis-element is required for hematopoietic stem cell generation in the mammalian embryo. *The Journal of experimental medicine, 210*(13), 2833-2842. doi:10.1084/jem.20130733

Gering, M., & Patient, R. (2005). Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell, 8*(3), 389-400. doi:10.1016/j.devcel.2005.01.010

Gering, M., Rodaway, A. R., Göttgens, B., Patient, R. K., & Green, A. R. (1998). The SCL gene specifies haemangioblast development from early mesoderm. *Embo j, 17*(14), 4029-4045. doi:10.1093/emboj/17.14.4029

Gering, M., Yamada, Y., Rabbitts, T. H., & Patient, R. K. (2003). Lmo2 and Scl/Tal1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. *Development (Cambridge, England), 130*(25), 6187-6199. doi:10.1242/dev.00875

Glass, T. J., Hui, S. K., Blazar, B. R., & Lund, T. C. (2013). Effect of radiation dose-rate on hematopoietic cell engraftment in adult zebrafish. *PLoS One, 8*(9), e73745. doi:10.1371/journal.pone.0073745

Goessling, W., North, T. E., Loewer, S., Lord, A. M., Lee, S., Stoick-Cooper, C. L., . . . Zon, L. I. (2009). Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell, 136*(6), 1136-1147. doi:10.1016/j.cell.2009.01.015

Gordeuk, V. R., Caleffi, A., Corradini, E., Ferrara, F., Jones, R. A., Castro, O., . . . Pietrangelo, A. (2003). Iron overload in Africans and African-Americans and a common mutation in the SCL40A1 (ferroportin 1) gene. *Blood Cells Mol Dis, 31*(3), 299-304. doi:10.1016/s1079-9796(03)00164-5

Gore, A. V., Athans, B., Iben, J. R., Johnson, K., Russanova, V., Castranova, D., . . . Weinstein, B. M. (2016). Epigenetic regulation of hematopoiesis by DNA methylation. *eLife, 5*, e11813-e11813. doi:10.7554/eLife.11813

Greenbaum, A., Hsu, Y. M., Day, R. B., Schuettpelz, L. G., Christopher, M. J., Borgerding, J. N., . . . Link, D. C. (2013). CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature, 495*(7440), 227-230. doi:10.1038/nature11926

Guo, Y., Chan, R., Ramsey, H., Li, W., Xie, X., Shelley, W. C., . . . Hromas, R. (2003). The homeoprotein Hex is required for hemangioblast differentiation. *Blood, 102*(7), 2428-2435. doi:10.1182/blood-2003-02-0634

Gyurkocza, B., Rezvani, A., & Storb, R. F. (2010). Allogeneic hematopoietic cell transplantation: the state of the art. *Expert review of hematology, 3*(3), 285-299. doi:10.1586/ehm.10.21

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., . . . Nüsslein-Volhard, C. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development (Cambridge, England), 123*, 1-36.

Hatzimichael, E., & Tuthill, M. (2010). Hematopoietic stem cell transplantation. *Stem cells and cloning : advances and applications, 3*, 105-117. doi:10.2147/SCCAA.S6815

Heazlewood, S. Y., Oteiza, A., Cao, H., & Nilsson, S. K. (2014). Analyzing hematopoietic stem cell homing, lodgment, and engraftment to better understand the bone marrow niche. *Ann N Y Acad Sci, 1310*, 119-128. doi:10.1111/nyas.12329

Hendriks, W. T., Warren, C. R., & Cowan, C. A. (2016). Genome Editing in Human Pluripotent Stem Cells: Approaches, Pitfalls, and Solutions. *Cell Stem Cell, 18*(1), 53-65. doi:10.1016/j.stem.2015.12.002

Hess, I., & Boehm, T. (2016). Stable multilineage xenogeneic replacement of definitive hematopoiesis in adult zebrafish. *Sci Rep, 6*, 19634. doi:10.1038/srep19634

Hess, I., Iwanami, N., Schorpp, M., & Boehm, T. (2013). Zebrafish model for allogeneic hematopoietic cell transplantation not requiring preconditioning. *Proceedings of*

the National Academy of Sciences of the United States of America, 110(11), 4327-4332. doi:10.1073/pnas.1219847110

Hoggatt, J., Singh, P., Sampath, J., & Pelus, L. M. (2009). Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood, 113*(22), 5444-5455. doi:10.1182/blood-2009-01-201335

Howe, G. A., & Addison, C. L. (2012). β1 integrin: an emerging player in the modulation of tumorigenesis and response to therapy. *Cell adhesion & migration, 6*(2), 71-77. doi:10.4161/cam.20077

Hsia, N., & Zon, L. I. (2005). Transcriptional regulation of hematopoietic stem cell development in zebrafish. *Exp Hematol, 33*(9), 1007-1014. doi:https://doi.org/10.1016/j.exphem.2005.06.013

Huang, H.-T., Kathrein, K. L., Barton, A., Gitlin, Z., Huang, Y.-H., Ward, T. P., . . . Zon, L. I. (2013). A network of epigenetic regulators guides developmental haematopoiesis in vivo. *Nature cell biology, 15*(12), 1516-1525. doi:10.1038/ncb2870

Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J., & Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature, 432*(7017), 625-630. doi:10.1038/nature03122

Iancu-Rubin, C., & Hoffman, R. (2015). Role of epigenetic reprogramming in hematopoietic stem cell function. *Curr Opin Hematol, 22*(4), 279-285. doi:10.1097/moh.0000000000000143

Jackson, C. S., Durandt, C., Janse van Rensburg, I., Praloran, V., Brunet de la Grange, P., & Pepper, M. S. (2017). Targeting the aryl hydrocarbon receptor nuclear translocator complex with DMOG and Stemregenin 1 improves primitive hematopoietic stem cell expansion. *Stem Cell Res, 21*, 124-131. doi:10.1016/j.scr.2017.04.007

Jagannathan-Bogdan, M., & Zon, L. I. (2013). Hematopoiesis. *Development (Cambridge, England), 140*(12), 2463-2467. doi:10.1242/dev.083147

Jin, F., Zhai, Q., Qiu, L., Meng, H., Zou, D., Wang, Y., . . . Zhou, B. (2008). Degradation of BM SDF-1 by MMP-9: the role in G-CSF-induced hematopoietic stem/progenitor cell mobilization. *Bone Marrow Transplant, 42*(9), 581-588. doi:10.1038/bmt.2008.222

Jin, H., Xu, J., & Wen, Z. (2007). Migratory path of definitive hematopoietic stem/progenitor cells during zebrafish development. *Blood, 109*(12), 5208-5214. doi:10.1182/blood-2007-01-069005

Jing, L., Tamplin, O. J., Chen, M. J., Deng, Q., Patterson, S., Kim, P. G., . . . Zon, L. I. (2015). Adenosine signaling promotes hematopoietic stem and progenitor cell emergence. *The Journal of experimental medicine, 212*(5), 649-663. doi:10.1084/jem.20141528

Kalev-Zylinska, M. L., Horsfield, J. A., Flores, M. V., Postlethwait, J. H., Vitas, M. R., Baas, A. M., . . . Crosier, K. E. (2002). Runx1 is required for zebrafish blood and vessel development and expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. *Development (Cambridge, England), 129*(8), 2015-2030.

Kanz, D., Konantz, M., Alghisi, E., North, T. E., & Lengerke, C. (2016). Endothelial-to-hematopoietic transition: Notch-ing vessels into blood. *Ann N Y Acad Sci, 1370*(1), 97-108. doi:10.1111/nyas.13030

Kaufman, D. S., Hanson, E. T., Lewis, R. L., Auerbach, R., & Thomson, J. A. (2001). Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 98*(19), 10716-10721. doi:10.1073/pnas.191362598

Kennedy, M., Awong, G., Sturgeon, C. M., Ditadi, A., LaMotte-Mohs, R., Zúñiga-Pflücker, J. C., & Keller, G. (2012). T lymphocyte potential marks the

emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. *Cell reports, 2*(6), 1722-1735. doi:10.1016/j.celrep.2012.11.003

Kim, P. G., Albacker, C. E., Lu, Y. F., Jang, I. H., Lim, Y., Heffner, G. C., . . . Daley, G. Q. (2013). Signaling axis involving Hedgehog, Notch, and Scl promotes the embryonic endothelial-to-hematopoietic transition. *Proceedings of the National Academy of Sciences of the United States of America, 110*(2), E141-150. doi:10.1073/pnas.1214361110

Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E. W., & Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat Immunol, 7*(10), 1048-1056. doi:10.1038/ni1381

Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A., & Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol, 230*(2), 230-242. doi:10.1006/dbio.2000.0106

Kissa, K., & Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature, 464*(7285), 112-115. doi:10.1038/nature08761

Kissa, K., Murayama, E., Zapata, A., Cortes, A., Perret, E., Machu, C., & Herbomel, P. (2008). Live imaging of emerging hematopoietic stem cells and early thymus colonization. *Blood, 111*(3), 1147-1156. doi:10.1182/blood-2007-07-099499

Kissa, K., Murayama, E., Zapata, A., Cortés, A., Perret, E., Machu, C., & Herbomel, P. (2008). Live imaging of emerging hematopoietic stem cells and early thymus colonization. *Blood, 111*(3), 1147-1156. doi:10.1182/blood-2007-07-099499

Kobayashi, I., Kobayashi-Sun, J., Kim, A. D., Pouget, C., Fujita, N., Suda, T., & Traver, D. (2014). Jam1a-Jam2a interactions regulate haematopoietic stem cell fate through Notch signalling. *Nature, 512*(7514), 319-323. doi:10.1038/nature13623

Kyba, M., Perlingeiro, R. C., & Daley, G. Q. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell, 109*(1), 29-37. doi:10.1016/s0092-8674(02)00680-3

LaFave, M. C., Varshney, G. K., Vemulapalli, M., Mullikin, J. C., & Burgess, S. M. (2014). A defined zebrafish line for high-throughput genetics and genomics: NHGRI-1. *Genetics, 198*(1), 167-170. doi:10.1534/genetics.114.166769

Lam, E. Y., Hall, C. J., Crosier, P. S., Crosier, K. E., & Flores, M. V. (2010). Live imaging of Runx1 expression in the dorsal aorta tracks the emergence of blood progenitors from endothelial cells. *Blood, 116*(6), 909-914. doi:10.1182/blood-2010-01-264382

Lancrin, C., Sroczynska, P., Stephenson, C., Allen, T., Kouskoff, V., & Lacaud, G. (2009). The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature, 457*(7231), 892-895. doi:10.1038/nature07679

Lassailly, F., Foster, K., Lopez-Onieva, L., Currie, E., & Bonnet, D. (2013). Multimodal imaging reveals structural and functional heterogeneity in different bone marrow compartments: functional implications on hematopoietic stem cells. *Blood, 122*(10), 1730-1740. doi:10.1182/blood-2012-11-467498

Laurenti, E., Doulatov, S., Zandi, S., Plumb, I., Chen, J., April, C., . . . Dick, J. E. (2013). The transcriptional architecture of early human hematopoiesis identifies multilevel control of lymphoid commitment. *Nat Immunol, 14*(7), 756-763. doi:10.1038/ni.2615

Ledran, M. H., Krassowska, A., Armstrong, L., Dimmick, I., Renström, J., Lang, R., . . . Lako, M. (2008). Efficient hematopoietic differentiation of human embryonic

stem cells on stromal cells derived from hematopoietic niches. *Cell Stem Cell, 3*(1), 85-98. doi:10.1016/j.stem.2008.06.001

Lee, D., Park, C., Lee, H., Lugus, J. J., Kim, S. H., Arentson, E., . . . Choi, K. (2008). ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification. *Cell Stem Cell, 2*(5), 497-507. doi:10.1016/j.stem.2008.03.008

Lee, G. S., Kim, B. S., Sheih, J. H., & Moore, M. (2008). Forced expression of HoxB4 enhances hematopoietic differentiation by human embryonic stem cells. *Mol Cells, 25*(4), 487-493.

Lento, W., Congdon, K., Voermans, C., Kritzik, M., & Reya, T. (2013). Wnt signaling in normal and malignant hematopoiesis. *Cold Spring Harbor perspectives in biology, 5*(2), a008011. doi:10.1101/cshperspect.a008011

Li, C., Lan, Y., Schwartz-Orbach, L., Korol, E., Tahiliani, M., Evans, T., & Goll, M. G. (2015). Overlapping Requirements for Tet2 and Tet3 in Normal Development and Hematopoietic Stem Cell Emergence. *Cell reports, 12*(7), 1133-1143. doi:10.1016/j.celrep.2015.07.025

Li, P., Lahvic, J. L., Binder, V., Pugach, E. K., Riley, E. B., Tamplin, O. J., . . . Zon, L. I. (2015). Epoxyeicosatrienoic acids enhance embryonic haematopoiesis and adult marrow engraftment. *Nature, 523*(7561), 468-471. doi:10.1038/nature14569

Liao, E. C., Paw, B. H., Oates, A. C., Pratt, S. J., Postlethwait, J. H., & Zon, L. I. (1998). SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev, 12*(5), 621-626. doi:10.1101/gad.12.5.621

Liao, W., Bisgrove, B. W., Sawyer, H., Hug, B., Bell, B., Peters, K., . . . Stainier, D. Y. (1997). The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. *Development (Cambridge, England), 124*(2), 381-389.

Lim, K. C., Hosoya, T., Brandt, W., Ku, C. J., Hosoya-Ohmura, S., Camper, S. A., . . . Engel, J. D. (2012). Conditional Gata2 inactivation results in HSC loss and lymphatic mispatterning. *J Clin Invest, 122*(10), 3705-3717. doi:10.1172/jci61619

Lin, M. I., Price, E. N., Boatman, S., Hagedorn, E. J., Trompouki, E., Satishchandran, S., . . . Zon, L. I. (2015). Angiopoietin-like proteins stimulate HSPC development through interaction with notch receptor signaling. *eLife, 4*. doi:10.7554/eLife.05544

Liu, X., Jia, X., Yuan, H., Ma, K., Chen, Y., Jin, Y., . . . Zhu, J. (2015). DNA methyltransferase 1 functions through C/ebpa to maintain hematopoietic stem and progenitor cells in zebrafish. *Journal of hematology & oncology, 8*, 15-15. doi:10.1186/s13045-015-0115-7

Lo Celso, C., Fleming, H. E., Wu, J. W., Zhao, C. X., Miake-Lye, S., Fujisaki, J., . . . Scadden, D. T. (2009). Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature, 457*(7225), 92-96. doi:10.1038/nature07434

Lugus, J. J., Park, C., Ma, Y. D., & Choi, K. (2009). Both primitive and definitive blood cells are derived from Flk-1+ mesoderm. *Blood, 113*(3), 563-566. doi:10.1182/blood-2008-06-162750

Ma, D., Zhang, J., Lin, H. F., Italiano, J., & Handin, R. I. (2011). The identification and characterization of zebrafish hematopoietic stem cells. *Blood, 118*(2), 289-297. doi:10.1182/blood-2010-12-327403

Mahony, C. B., Fish, R. J., Pasche, C., & Bertrand, J. Y. (2016). tfec controls the hematopoietic stem cell vascular niche during zebrafish embryogenesis. *Blood, 128*(10), 1336-1345. doi:10.1182/blood-2016-04-710137

McConnell, S. C., Restaino, A. C., & de Jong, J. L. (2014). Multiple divergent haplotypes express completely distinct sets of class I MHC genes in zebrafish. *Immunogenetics, 66*(3), 199-213. doi:10.1007/s00251-013-0749-y

McGrath, K. E., Frame, J. M., Fromm, G. J., Koniski, A. D., Kingsley, P. D., Little, J., . . . Palis, J. (2011). A transient definitive erythroid lineage with unique regulation of the β-globin locus in the mammalian embryo. *Blood, 117*(17), 4600-4608. doi:10.1182/blood-2010-12-325357

Mendelson, A., & Frenette, P. S. (2014). Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nat Med, 20*(8), 833-846. doi:10.1038/nm.3647

Morrison, S. J., & Scadden, D. T. (2014). The bone marrow niche for haematopoietic stem cells. *Nature, 505*(7483), 327-334. doi:10.1038/nature12984

Mosimann, C., Panáková, D., Werdich, A. A., Musso, G., Burger, A., Lawson, K. L., . . . Zon, L. I. (2015). Chamber identity programs drive early functional partitioning of the heart. *Nature communications, 6*, 8146-8146. doi:10.1038/ncomms9146

Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., . . . Potter, S. S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell, 65*(4), 677-689. doi:10.1016/0092-8674(91)90099-k

Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H. F., . . . Herbomel, P. (2006). Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity, 25*(6), 963-975. doi:10.1016/j.immuni.2006.10.015

Murayama, E., Sarris, M., Redd, M., Le Guyader, D., Vivier, C., Horsley, W., . . . Herbomel, P. (2015). NACA deficiency reveals the crucial role of somite-derived stromal cells in haematopoietic niche formation. *Nature communications, 6*, 8375. doi:10.1038/ncomms9375

Murray, P. D. F. (1932). The development in viitro of the blood of the early chick. *Proc. R. Soc. Lond. B.*, 497-521.

Nguyen, P. D., Hollway, G. E., Sonntag, C., Miles, L. B., Hall, T. E., Berger, S., . . . Currie, P. D. (2014). Haematopoietic stem cell induction by somite-derived endothelial cells controlled by meox1. *Nature, 512*(7514), 314-318. doi:10.1038/nature13678

North, T. E., de Bruijn, M. F., Stacy, T., Talebian, L., Lind, E., Robin, C., . . . Speck, N. A. (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity, 16*(5), 661-672. doi:10.1016/s1074-7613(02)00296-0

North, T. E., Goessling, W., Walkley, C. R., Lengerke, C., Kopani, K. R., Lord, A. M., . . . Zon, L. I. (2007). Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature, 447*(7147), 1007-1011. doi:10.1038/nature05883

Orford, K. W., & Scadden, D. T. (2008). Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet, 9*(2), 115-128. doi:10.1038/nrg2269

Orkin, S. H., & Zon, L. I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. *Cell, 132*(4), 631-644. doi:10.1016/j.cell.2008.01.025

Orkin, S. H., & Zon, L. I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. *Cell, 132*(4), 631-644. doi:10.1016/j.cell.2008.01.025

Paik, E. J., & Zon, L. I. (2010). Hematopoietic development in the zebrafish. *Int J Dev Biol, 54*(6-7), 1127-1137. doi:10.1387/ijdb.093042ep

Palis, J., & Yoder, M. C. (2001). Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Exp Hematol, 29*(8), 927-936. doi:10.1016/s0301-472x(01)00669-5

Papa, L., Zimran, E., Djedaini, M., Ge, Y., Ozbek, U., Sebra, R., . . . Hoffman, R. (2018). Ex vivo human HSC expansion requires coordination of cellular

reprogramming with mitochondrial remodeling and p53 activation. *Blood Adv, 2*(20), 2766-2779. doi:10.1182/bloodadvances.2018024273

Park, B., Yoo, K. H., & Kim, C. (2015). Hematopoietic stem cell expansion and generation: the ways to make a breakthrough. *Blood research, 50*(4), 194-203. doi:10.5045/br.2015.50.4.194

Patterson, L. J., Gering, M., Eckfeldt, C. E., Green, A. R., Verfaillie, C. M., Ekker, S. C., & Patient, R. (2007). The transcription factors Scl and Lmo2 act together during development of the hemangioblast in zebrafish. *Blood, 109*(6), 2389-2398. doi:10.1182/blood-2006-02-003087

PeproTech. IWP-2. Retrieved from https://www.peprotech.com/de/iwp2

Pereira, C. F., Chang, B., Qiu, J., Niu, X., Papatsenko, D., Hendry, C. E., . . . Moore, K. (2013). Induction of a hemogenic program in mouse fibroblasts. *Cell Stem Cell, 13*(2), 205-218. doi:10.1016/j.stem.2013.05.024

Perlin, J. R., Robertson, A. L., & Zon, L. I. (2017). Efforts to enhance blood stem cell engraftment: Recent insights from zebrafish hematopoiesis. *The Journal of experimental medicine, 214*(10), 2817-2827. doi:10.1084/jem.20171069

Qian, F., Zhen, F., Xu, J., Huang, M., Li, W., & Wen, Z. (2007). Distinct functions for different scl isoforms in zebrafish primitive and definitive hematopoiesis. *PLoS Biol, 5*(5), e132. doi:10.1371/journal.pbio.0050132

Ransom, D. G., Haffter, P., Odenthal, J., Brownlie, A., Vogelsang, E., Kelsh, R. N., . . . Nüsslein-Volhard, C. (1996). Characterization of zebrafish mutants with defects in embryonic hematopoiesis. *Development (Cambridge, England), 123*, 311-319.

Rebel, V. I., Miller, C. L., Eaves, C. J., & Lansdorp, P. M. (1996). The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. *Blood, 87*(8), 3500-3507.

Reischauer, S., Stone, O. A., Villasenor, A., Chi, N., Jin, S. W., Martin, M., . . . Stainier, D. Y. (2016). Cloche is a bHLH-PAS transcription factor that drives haemato-vascular specification. *Nature, 535*(7611), 294-298. doi:10.1038/nature18614

Robb, L., Elwood, N. J., Elefanty, A. G., Köntgen, F., Li, R., Barnett, L. D., & Begley, C. G. (1996). The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *Embo j, 15*(16), 4123-4129.

Robertson, A. L., Avagyan, S., Gansner, J. M., & Zon, L. I. (2016). Understanding the regulation of vertebrate hematopoiesis and blood disorders - big lessons from a small fish. *FEBS letters, 590*(22), 4016-4033. doi:10.1002/1873-3468.12415

Rowe, R. G., Mandelbaum, J., Zon, L. I., & Daley, G. Q. (2016). Engineering Hematopoietic Stem Cells: Lessons from Development. *Cell Stem Cell, 18*(6), 707-720. doi:10.1016/j.stem.2016.05.016

Rozario, T., & DeSimone, D. W. (2010). The extracellular matrix in development and morphogenesis: a dynamic view. *Dev Biol, 341*(1), 126-140. doi:10.1016/j.ydbio.2009.10.026

Sandler, V. M., Lis, R., Liu, Y., Kedem, A., James, D., Elemento, O., . . . Rafii, S. (2014). Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. *Nature, 511*(7509), 312-318. doi:10.1038/nature13547

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M. M., Birchmeier, W., Tenen, D. G., & Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat Immunol, 7*(10), 1037-1047. doi:10.1038/ni1387

Schmitt, R. M., Bruyns, E., & Snodgrass, H. R. (1991). Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. *Genes Dev, 5*(5), 728-740. doi:10.1101/gad.5.5.728

Schroeder, T. (2010). Hematopoietic stem cell heterogeneity: subtypes, not unpredictable behavior. *Cell Stem Cell, 6*(3), 203-207. doi:10.1016/j.stem.2010.02.006

Scott, E. W., Simon, M. C., Anastasi, J., & Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science, 265*(5178), 1573-1577. doi:10.1126/science.8079170

Shah, B. S., Chen, M., Suzuki, T., Embree, M., Kong, K., Lee, C. H., . . . Mao, J. J. (2017). Pyrintegrin Induces Soft Tissue Formation by Transplanted or Endogenous Cells. *Sci Rep, 7*(1), 36402. doi:10.1038/srep36402

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., . . . Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell, 89*(6), 981-990. doi:10.1016/s0092-8674(00)80283-4

Shayegi, N., Meyer, C., Lambert, K., Ehninger, G., Brand, M., & Bornhäuser, M. (2014). CXCR4 blockade and sphingosine-1-phosphate activation facilitate engraftment of haematopoietic stem and progenitor cells in a non-myeloablative transplant model. *Br J Haematol, 164*(3), 409-413. doi:10.1111/bjh.12641

Shinya, M., & Sakai, N. (2011). Generation of highly homogeneous strains of zebrafish through full sib-pair mating. *G3 (Bethesda), 1*(5), 377-386. doi:10.1534/g3.111.000851

Shivdasani, R. A., Mayer, E. L., & Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature, 373*(6513), 432-434. doi:10.1038/373432a0

Staal, F. J., Spaink, H. P., & Fibbe, W. E. (2016). Visualizing Human Hematopoietic Stem Cell Trafficking In Vivo Using a Zebrafish Xenograft Model. *Stem Cells Dev, 25*(4), 360-365. doi:10.1089/scd.2015.0195

Stachura, D. L., Reyes, J. R., Bartunek, P., Paw, B. H., Zon, L. I., & Traver, D. (2009). Zebrafish kidney stromal cell lines support multilineage hematopoiesis. *Blood, 114*(2), 279-289. doi:10.1182/blood-2009-02-203638

Stainier, D. Y., Weinstein, B. M., Detrich, H. W., 3rd, Zon, L. I., & Fishman, M. C. (1995). Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development (Cambridge, England), 121*(10), 3141-3150.

Stakheev, D., Taborska, P., Strizova, Z., Podrazil, M., Bartunkova, J., & Smrz, D. (2019). The WNT/β-catenin signaling inhibitor XAV939 enhances the elimination of LNCaP and PC-3 prostate cancer cells by prostate cancer patient lymphocytes in vitro. *Sci Rep, 9*(1), 4761-4761. doi:10.1038/s41598-019-41182-5

StemCellTechnologies. IWR-1-endo. Retrieved from https://www.stemcell.com/iwr-1-endo.html

Sturgeon, C. M., Ditadi, A., Awong, G., Kennedy, M., & Keller, G. (2014). Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nat Biotechnol, 32*(6), 554-561. doi:10.1038/nbt.2915

Sturgeon, C. M., Ditadi, A., Clarke, R. L., & Keller, G. (2013). Defining the path to hematopoietic stem cells. *Nat Biotechnol, 31*(5), 416-418. doi:10.1038/nbt.2571

Sumanas, S., & Lin, S. (2006). Ets1-related protein is a key regulator of vasculogenesis in zebrafish. *PLoS Biol, 4*(1), e10. doi:10.1371/journal.pbio.0040010

Takahashi, K., Okita, K., Nakagawa, M., & Yamanaka, S. (2007). Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc, 2*(12), 3081-3089. doi:10.1038/nprot.2007.418

Tamplin, O. J., Durand, E. M., Carr, L. A., Childs, S. J., Hagedorn, E. J., Li, P., . . . Zon, L. I. (2015). Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. *Cell, 160*(1-2), 241-252. doi:10.1016/j.cell.2014.12.032

Theodore, L. N., Hagedorn, E. J., Cortes, M., Natsuhara, K., Liu, S. Y., Perlin, J. R., . . . North, T. E. (2017). Distinct Roles for Matrix Metalloproteinases 2 and 9 in Embryonic Hematopoietic Stem Cell Emergence, Migration, and Niche Colonization. *Stem Cell Reports, 8*(5), 1226-1241. doi:10.1016/j.stemcr.2017.03.016

Thompson, M. A., Ransom, D. G., Pratt, S. J., MacLennan, H., Kieran, M. W., Detrich, H. W., 3rd, . . . Zon, L. I. (1998). The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol, 197*(2), 248-269. doi:10.1006/dbio.1998.8887

Traver, D., Paw, B. H., Poss, K. D., Penberthy, W. T., Lin, S., & Zon, L. I. (2003). Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat Immunol, 4*(12), 1238-1246. doi:10.1038/ni1007

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., . . . Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature, 371*(6494), 221-226. doi:10.1038/371221a0

Vodyanik, M. A., Bork, J. A., Thomson, J. A., & Slukvin, II. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood, 105*(2), 617-626. doi:10.1182/blood-2004-04-1649

Vogeli, K. M., Jin, S. W., Martin, G. R., & Stainier, D. Y. (2006). A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature, 443*(7109), 337-339. doi:10.1038/nature05045

Wagner, J. E., Jr., Brunstein, C. G., Boitano, A. E., DeFor, T. E., McKenna, D., Sumstad, D., . . . Bleul, C. C. (2016). Phase I/II Trial of StemRegenin-1 Expanded Umbilical Cord Blood Hematopoietic Stem Cells Supports Testing as a Stand-Alone Graft. *Cell Stem Cell, 18*(1), 144-155. doi:10.1016/j.stem.2015.10.004

Wang, L., Menendez, P., Shojaei, F., Li, L., Mazurier, F., Dick, J. E., . . . Bhatia, M. (2005). Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *The Journal of experimental medicine, 201*(10), 1603-1614. doi:10.1084/jem.20041888

Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H., & Speck, N. A. (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America, 93*(8), 3444-3449. doi:10.1073/pnas.93.8.3444

Westerfield, M. (2007). *THE ZEBRAFISH BOOK* (5th Edition ed.).

White, R. M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., . . . Zon, L. I. (2008). Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell, 2*(2), 183-189. doi:10.1016/j.stem.2007.11.002

Wiles, M. V., & Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development (Cambridge, England), 111*(2), 259-267.

Wilkinson, A. C., Ishida, R., Kikuchi, M., Sudo, K., Morita, M., Crisostomo, R. V., . . . Yamazaki, S. (2019). Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation. *Nature, 571*(7763), 117-121. doi:10.1038/s41586-019-1244-x

Wolf, A., Aggio, J., Campbell, C., Wright, F., Marquez, G., Traver, D., & Stachura, D. L. (2017). Zebrafish Caudal Haematopoietic Embryonic Stromal Tissue (CHEST) Cells Support Haematopoiesis. *Sci Rep, 7*, 44644. doi:10.1038/srep44644

Xu, Z., Robitaille, A. M., Berndt, J. D., Davidson, K. C., Fischer, K. A., Mathieu, J., . . . Moon, R. T. (2016). Wnt/β-catenin signaling promotes self-renewal and inhibits the primed state transition in naïve human embryonic stem cells. *Proceedings*

of the National Academy of Sciences, 113(42), E6382. doi:10.1073/pnas.1613849113

Yamada, Y., Warren, A. J., Dobson, C., Forster, A., Pannell, R., & Rabbitts, T. H. (1998). The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America, 95*(7), 3890-3895. doi:10.1073/pnas.95.7.3890

Yeh, J. R., Munson, K. M., Chao, Y. L., Peterson, Q. P., Macrae, C. A., & Peterson, R. T. (2008). AML1-ETO reprograms hematopoietic cell fate by downregulating scl expression. *Development (Cambridge, England), 135*(2), 401-410. doi:10.1242/dev.008904

Yeh, J. R., Munson, K. M., Elagib, K. E., Goldfarb, A. N., Sweetser, D. A., & Peterson, R. T. (2009). Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. *Nat Chem Biol, 5*(4), 236-243. doi:10.1038/nchembio.147

Zhang, Y., Wang, J., Wheat, J., Chen, X., Jin, S., Sadrzadeh, H., . . . Yeh, J. R. (2013). AML1-ETO mediates hematopoietic self-renewal and leukemogenesis through a COX/β-catenin signaling pathway. *Blood, 121*(24), 4906-4916. doi:10.1182/blood-2012-08-447763

Zhen, F., Lan, Y., Yan, B., Zhang, W., & Wen, Z. (2013). Hemogenic endothelium specification and hematopoietic stem cell maintenance employ distinct Scl isoforms. *Development (Cambridge, England), 140*(19), 3977-3985. doi:10.1242/dev.097071

5. ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my research supervisors Leonard I. Zon, M.D. and Anne Ellett, PhD for their continuous support.

Len, thank you for the possibility of doing my Master thesis in your lab. You have an awesome group and it was a pleasure working with them. You are a great mentor and I am really grateful for your patience, motivation and enthusiasm during that time. You always took your time and assured that we are doing fine. That is not a matter of course, but you did it anyway! I could not have imaged being in a better group than yours, especially during that difficult year.

But this is also true for you, Anne. You're a great supervisor and you made this stay unique. Your guidance helped me a lot with my research and writing of this thesis. Your meetings, be it via Zoom or in person, were a bright spot in a difficult time. Your sincerity and motivation inspired me deeply and showed me to continue with research and to believe in myself. Keep your wonderful personality because it was a privilege and honor to work under your guidance!

Besides my supervisors, I also would like to thank the entire Zon lab. It was a great experience working with you. You were a part of this great experience and you showed me that there is no need to be afraid to ask for help. In particular, I am really grateful to Hannah for helping with the organization of the tons of documents. Your humor put a smile on my face several times, thank you!

My sincere thanks also to my University FH Campus Wien, especially to my advisor FH-Prof. Dr. Thomas Czerny and Dr. Susanne Zhanial. I am grateful for your guidance and support during my stay abroad.

In addition, a thank you to the Harvard International Office for the support and help during the Pandemic. I am also appreciative for the financial support of the Marshall Plan Foundation, the reason which made it possible to fulfill a dream.

I am extremely grateful for my parents and my family for their love and help during the year. It was not easy to be apart during these uncertain times, but you supported my decision and build me up mentally.

My deepest gratitude also to my friends, which guided me through difficult decision and helped in every possible situation. Thank you for being there, even if not physically. In particular, for Michelle for the long Zoom talks and for making birthdays even amazing

abroad.

One of my greatest supporters during that time was my husband. We overcame a lot of obstacles throughout this journey, but we have grown closer together (again). Thank you for this amazing experience and your help during this time. I could not have imaged experiencing this stunning journey with a better person. Taking your name was the best decision in my life, followed shortly by this journey. I am looking forward to our next adventures!

Last but not least, I am also thankful for myself. I didn't go nuts during the last year and overcome a lot of hurdles. Honestly, I can say the experience was not the same as I had imagined, but I never would want to miss it. I grew in many ways and now I am a strengthened version of myself and my ambition is greater than before.

Erklärung:

Ich erkläre, dass die vorliegende Diplomarbeit/Masterarbeit von mir selbst verfasst wurde und ich keine anderen als die angeführten Behelfe verwendet bzw. mich auch sonst keiner unerlaubter Hilfe bedient habe.

Ich versichere, dass ich diese Diplomarbeit/Masterarbeit bisher weder im In- noch im Ausland (einer Beurteilerin/einem Beurteiler zur Begutachtung) in irgendeiner Form als Prüfungsarbeit vorgelegt habe.

Weiters versichere ich, dass die von mir eingereichten Exemplare (ausgedruckt und elektronisch) identisch sind.

Declaration:

I hereby declare that the submitted Master thesis was written by myself and that I did not use any aids other than those indicated, none of which are unauthorised.

I assure that I have not previously submitted this Master thesis or its contents in any form for assessment as part of an examination either in Austria or abroad.

Furthermore, I assure that all copies submitted by myself (electronic and printed) are identical.

Date: 17.02.2021 Signature: .....

Appendix

Appendix I – Figure Licenses

Permissions and licenses were obtained for every figure used in this thesis. A list of said licenses can be found below:

Figure 1

The figure was obtained from (Park et al., 2015) which is licensed under Creative Commons Version 4.0 CC BY-NC which can be found at http://creativecommons.org/licenses/by-nc/4.0.

Figure 2

Reprinted by permission from The Company of Biologists Ltd: Development "Hematopoiesis" by Madhumita Jagannathan-Bogdan, Leonard I. Zon

License Number	1097190-1
License Date	Feb 13, 2021
Licensed Content Publisher	The Company of Biologists Ltd
Licensed Content Publication	Development
Licensed Content Title	Hematopoiesis
Licensed Content Author	Madhumita Jagannathan-Bogdan, Leonard I. Zon
Licensed Content Date	May 28, 2013

Figure 3

Reprinted by permission from Development: COMPANY OF BIOLOGISTS "Hematopoiesis" by Madhumita Jagannathan-Bogdan, Leonard I. Zon

License Number	1097190-1
License Date	Feb 13, 2021
Licensed Content Publisher	Development
Licensed Content Publication	COMPANY OF BIOLOGISTS
Licensed Content Title	Hematopoiesis
Licensed Content Author	Madhumita Jagannathan-Bogdan, Leonard I. Zon
Licensed Content Date	May 28, 2013

Figure 4

Reprinted by permission from John Wiley and Sons: FEBS Letters “Understanding the regulation of vertebrate haematopoiesis and blood disorders – big lessons from a small fish by Leonard I. Zon, John M. Gansner, Serine Avagyan, et al

License Number	5007011352145
License Date	Feb 13, 2021
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	FEBS Letters
Licensed Content Title	Understanding the regulation of vertebrate hematopoiesis and blood disorders – big lessons from a small fish
Licensed Content Author	Leonard I. Zon, John M. Gansner, Serine Avagyan, et al
Licensed Content Date	Sep 25, 2016

Figure 5

The figure was obtained from (Perlin et al., 2017) which is licensed under Creative Commons Version 4.0 CC BY-NC-SA which can be found at https://creativecommons.org/licenses/by-nc-sa/4.0/ .

Figure 6

The figure was obtained from (A. Rad and Mikael Häggström, 9 December 2016) which is licensed under Creative Commons Version 3.0 CC BY-SA which can be found at https://creativecommons.org/licenses/by-sa/3.0/ .

Appendix II

Buffers

50 x E3 buffer (embryo water)

Table 6 50 x Embryo water

	1 L
NaCl	14.61 g
KCl	0.63 g
$CaCl_2$ $2H_2O$	2.43 g
$MgSO_4$	1.99 g
Deionized water	1 L

Can be stored at room temperature (~20–25° C) for months. Dilute to 1X in deionized water just prior to use.