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Novel Insights into Haematopoietic Stem Cell Regulation and Function

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Novel Insights into Haematopoietic Stem Cell Regulation and Function

Novel Insights into Haematopoietic Stem Cell Regulation and Function

Sarah Warsi

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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FACULTY OPPONENT

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Abstract

The haematopoietic stem cell (HSC) is a somatic stem cell essential for life long haematopoiesis; HSCs have the capability to differentiate into all mature types of blood cells and can also undergo self-renewal to maintain their own numbers. They also have the potential to reconstitute a complete haematopoietic system following HSC transplantation, also known as bone marrow transplantation, which is used to treat many haematopoietic disorders. To obtain successful long-term engraftment of HSCs, a large number of cells need to be transplanted. This limiting factor in the clinical use of HSC transplantation could be overcome by methods that expand and maintain HSCs *ex vivo*. Despite continuous progress in this field, efficient HSC expansion has had limited success in part due to limited knowledge on HSC regulation. Better understanding of how HSC fate options are governed in the HSCs' natural environment can contribute to the development of future expansion protocols and improved HSC therapies.

HSCs reside in the bone marrow and the fate of each HSC is tightly regulated by both intrinsic and extrinsic factors. The studies presented in this thesis have identified novel intrinsic regulatory factors for haematopoietic stem cell self-renewal and quiescence. In summary, our results demonstrate the importance of preserved SLFN2 and BMP signalling for proper HSC functionality. We show that loss of SLFN2 function in HSCs causes a defect in reconstitution potential of the HSCs by perturbing cell cycle status and stress response (Paper I). SLFN2 is known to regulate cell quiescence and apoptosis in other cell types and our data now shows that it plays a similar role in HSCs. Furthermore, we show that loss of BMP signalling also leads to a defect in HSC reconstitution potential, in part mediated via TJP1 (Paper II), which is a previously known regulator of self-renewal in other stem cells. Our study thus establishes a previously unknown role for BMP signalling in adult HSCs and demonstrates a putative connection between BMP and TJP1 in HSCs.

HSC transplantation is today the only curative treatment for many haematopoietic disorders, but it is associated with many risks for the patients. Prior to HSC transplantation patients today undergo extensive conditioning, often involving irradiation or chemotherapy, which are independently associated with increased morbidity and mortality. As haematopoietic disorders often originate from the HSC itself, the HSCs are in many respects less fit than HSCs in a healthy individual. The work in this thesis also shows, using a mouse model of Diamond-Blackfan anaemia, that HSC transplantation can be successful even without conditioning or with a reduced conditioning regimen (Paper III), i.e. that the less fit HSCs can be out-competed by healthy HSCs.

Taken together, we have identified novel factors that affect HSC function and fate options and provided insight into HSC transplantation in haematopoietic disorders.

Key words Haematopoiesis, haematopoietic stem cells, stem cell regulation, HSC transplantation

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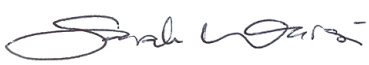
Signature  Date 2022-08-19

Novel Insights into Haematopoietic Stem Cell Regulation and Function

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On the cover: Abstract haematopoiesis

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Till min familj

"The most difficult thing is the decision to act,
the rest is merely tenacity."

Amelia Earhart

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List of publications

Papers in this thesis

This dissertation is based on the articles listed below.

Paper I

Warsi S, Dahl M, Smith EMK, Rydström A, Mansell E, Sigurdssom V, Sjöberg J, Soneji S, Rörby E, Siva K, Grahn THM, Liu Y, Blank U, Karlsson G, Karlsson S. Schlafen2 is a critical regulator of adult murine hematopoietic stem cells. Haematologica. 2022. In press.

Paper II

Warsi S*, Blank U*, Dahl M, Grahn THM, Schmiderer L, Andradottir S, Karlsson S. BMP signaling is required for postnatal murine hematopoietic stem cell self-renewal. Haematologica. 2021. 106(8):2203-2214. (*equally contributing authors)

Paper III

Dahl M, Warsi S, Liu Y, Debnath S, Billing M, Siva K, Flygare J, Karlsson S. Bone marrow transplantation without myeloablative conditioning in a mouse model for Diamond-Blackfan anemia corrects the disease phenotype. Exp Hematol. 2021. 99:44-53.

Papers not included in this thesis

Listed below are additional studies in which I have been involved during my doctorate studies.

Safi F, Dhapola P, Warsi S, Sommarin M, Erlandsson E, Ungerbäck J, Warfvinge R, Sitnicka E, Bryder D, Böiers C, Thakur RK, Karlsson G. Concurrent stem- and lineage-affiliated chromatin programs precede hematopoietic lineage restriction. Cell Rep. 2022. 39(6):110798.

Grahn THM, Niroula A, Végvári Á, Oburoglu L, Pertesi M, Warsi S, Safi F, Miharada N, Capellera-Garcia S, Siva K, Liu Y, Rörby E, Nilsson B, Zubarev RA, Karlsson S. S100A6 is a critical regulator of hematopoietic stem cells. Leukemia. 2020. 34(12):3439.

Rörby E, Billing M, Dahl M, Warsi S, Andradottir S, Miharada K, Siva K, Jönsson JI, Blank U, Karlsson G, Karlsson S. The stem cell regulator PEDF is dispensable for maintenance and function of hematopoietic stem cells. Sci Rep. 2017. 7(1):10134.

Dahl M*, Smith EMK*, Warsi S, Rothe M, Ferraz MJ, Aerts JMFG, Golipour A, Harper C, Pfeifer R, Pizzurro D, Schambach A, Mason C, Karlsson S. Correction of pathology in mice displaying Gaucher disease type 1 by a clinically-applicable lentiviral vector. Mol Ther Methods Clin Dev. 2020. 20:312-323.
(*equally contributing authors)

Liu Y, Dahl M, Debnath S, Rothe M, Smith EM, Grahn THM, Warsi S, Chen J, Flygare J, Schambach A,Karlsson S. Successful gene therapy of Diamond-Blackfan anemia in a mouse model and human CD34+ cord blood hematopoietic stem cells using a clinically applicable lentiviral vector. Haematologica. 2022. 107(2):446-456.

Hochgerner M, Bauer T, Zyulina V, Glitzner E, Warsi S, Konkel J, Tam-Amersdorfer C, Chen W, Karlsson S, Sibilia M, Strobl H. BMPR1a is required for the optimal TGFβ1-dependent CD207+ Langerhans cell differentiation and limits skin inflammation via CD11c+ cells. J Invest Dermatol. 2022. In press.

Haraldsson A, Wichert S, Engström PE, Lenhoff S, Turkiewicz D, Warsi S, Engelholm S, Bäck S, Engellau J. Organ sparing total marrow irradiation compared to total body irradiation prior to allogeneic stem cell transplantation. Eur J Haematol. 2021. 107(4):393-407.

Abbreviations

BMPR	bone morphogenetic protein receptor
CD	cluster of differentiation
CFU	colony forming unit
cGy	centigray
Cre	cyclization recombinase
CSF	colony-stimulating factor
DBA	Diamond-Blackfan anaemia
FACS	fluorescence activated cell sorting
Flt3	fms-related tyrosine kinase 3
GFP	green fluorescent protein
GMP	granulocyte/macrophage progenitor
HSC	haematopoietic stem cell
HSPC	haematopoietic stem/progenitor cell
GvHD	graft versus host disease
IL	interleukin
KD	knockdown
KO	knockout
LMPP	lymphoid-primed multipotent progenitor
LRP	lineage restricted progenitor
LSK	lineage– sca1+ c-kit+
LT-HSC	long term HSC
MPP	multipotent progenitor
PB	peripheral blood
Sca1	stem cell antigen 1

SCF	stem cell factor
shRNA	short hairpin ribonucleic acid
SLAM	signalling lymphocyte activating molecule
SLFN	schlafen
SP	side population
ST-HSC	short term HSC
TGF-β	transforming growth factor beta
TJP	tight junction protein
TPO	thrombopoietin
WT	wild type
5FU	5-fluorouracil

Nomenclature

Here follows a short note on certain nomenclature in this dissertation:

Mouse gene symbols are italicized with the first letter in upper-case (e.g. *Slfn2*). Human genes are italicized with all letters in upper-case (e.g. *SLFN2*). Both murine and human proteins are written non-italicized with all letters in upper-case (e.g. SLFN2).

Latin terms are italicized, such as *in vivo.*

The term long-term HSC (LT-HSC) refers to a cell with the ability to reconstitute all haematopoietic lineages at least four months after transplantation. Phenotypically the cell is usually defined as LSK and either CD34- Flt3+ or CD48- CD150+ or CD105+ CD150+. In this dissertation it is sometimes specified which phenotypic type of LT-HSC is discussed and sometimes the HSC is discussed in general without such specification. In certain assays, e.g. following 5FU treatment, LT-HSCs are defined using other combinations of phenotypic markers; this is specified where relevant.

Preface

My doctorate studies began in late 2011 in Stefan Karlsson's lab and almost simultaneously, in early 2012, I commenced studies in medicine at Lund University. Combining these two pursuits, the PhD and the MD, these past circa ten years has been challenging in the best of ways. Though I am now (finally) near the end, this end is only a new beginning. I will not and cannot say what will begin, as I have learnt that life and research are very similar in a way; it is good to have a plan and it is good to know that you will need to change that plan a thousand times.

This dissertation focuses on haematopoietic stem cells (HSCs), i.e. blood stem cells. These are the rare cells that reside in the bone marrow and have the astonishing potential to not only maintain life-long haematopoiesis but also regenerate an entire haematopoietic system through HSC transplantation. The latter is of immense importance as this capacity is why HSC transplantation can cure many blood disorders. HSCs are the most studied stem cells of the adult body and the field dates back at least 60 years – from when Till and McCulloch provided the first experimental proof of a multipotent HSC – though the concept of an HSC had already surfaced in earlier studies by Florence Sabin and E. Donnall Thomas. The study of HSCs was facilitated by the almost concurrent invention of fluorescence activated cell sorting (FACS), for which Leonard and Leonore Herzenberg are recognized as two main contributors. FACS is a method that I have used extensively in my research and a method without which the field of haematopoiesis research would not have been able to progress to where it is today. Though a lot of progress has been made, much remains unknown about the HSC and more knowledge is needed to reach the goals of growing HSCs in a laboratory and availability of donor HSCs to all patients in need of HSC transplantation.

The overall aim of my thesis work has been to expand the current knowledge on HSC regulation and HSC transplantation. Our research investigated the proteins Schlafen2 (Article I) and bone morphogenetic protein (Article II) and presented novel findings that proved they are necessary for HSC quiescence and self-renewal, respectively. We also evaluated pre-transplantation conditioning regimens in a disease mouse model of Diamond-Blackfan anaemia (Article III) and showed that HSC transplantation can be successful even without any conditioning. These studies have added to the combined knowledge base on regulation of HSC fate options and HSC transplantation. My hope is that, in the future, this could be useful in the development of novel clinical transplantation therapies or HSC expansion protocols.

Sarah Warsi

Lund, August 2022

Abstract

The haematopoietic stem cell (HSC) is a somatic stem cell essential for life long haematopoiesis; HSCs have the capability to differentiate into all mature types of blood cells and can also undergo self-renewal to maintain their own numbers. They also have the potential to reconstitute a complete haematopoietic system following HSC transplantation, also known as bone marrow transplantation, which is used to treat many haematopoietic disorders. To obtain successful long-term engraftment of HSCs, a large number of cells need to be transplanted. This limiting factor in the clinical use of HSC transplantation could be overcome by methods that expand and maintain HSCs *ex vivo*. Despite continuous progress in this field, efficient HSC expansion has had limited success in part due to limited knowledge on HSC regulation. Better understanding of how HSC fate options are governed in the HSCs' natural environment can contribute to the development of future expansion protocols and improved HSC therapies.

HSCs reside in the bone marrow and the fate of each HSC is tightly regulated by both intrinsic and extrinsic factors. The studies presented in this thesis have identified novel intrinsic regulatory factors for haematopoietic stem cell self-renewal and quiescence. In summary, our results demonstrate the importance of preserved SLFN2 and BMP signalling for proper HSC functionality. We show that loss of SLFN2 function in HSCs causes a defect in reconstitution potential of the HSCs by perturbing cell cycle status and stress response. SLFN2 is known to regulate cell quiescence and apoptosis in other cell types and our data now shows that it plays a similar role in HSCs. Furthermore, we show that loss of BMP signalling also leads to a defect in HSC reconstitution potential, in part mediated via TJP1, which is a previously known regulator of self-renewal in other stem cells. Our study thus establishes a previously unknown role for BMP signalling in adult HSCs and demonstrates a putative connection between BMP and TJP1 in HSCs.

HSC transplantation is today the only curative treatment for many haematopoietic disorders, but it is associated with many risks for the patients. Prior to HSC transplantation patients today undergo extensive conditioning, often involving irradiation or chemotherapy, which are independently associated with increased morbidity and mortality. Milder conditioning regimens could therefore benefit patients. As haematopoietic disorders often originate from the HSC itself, the HSCs are in many aspects less fit than HSCs in a healthy individual. The work in this thesis also shows, using a mouse model of Diamond-Blackfan anaemia, that HSC transplantation can be successful even without conditioning or with a reduced conditioning regimen, i.e. that the less fit HSCs can be out-competed by healthy HSCs.

Taken together, we have identified novel factors that affect HSC function and fate options and provided insight into HSC transplantation in haematopoietic disorders.

Background

Haematopoiesis

Blood is one of the most dynamic and regenerative tissues of the body and haematopoiesis is the process by which blood is formed. Blood has several crucial functions such as red blood cell mediated oxygen transport from the lungs to the rest of the body, white blood cells combating infections, and platelets forming clots to stop bleeding (Table 1) (Boron and Boulpaep, 2009). Most mature blood cells have a short life span and need to be replaced regularly. At normal homeostatic conditions the body is supplied with an estimated 300 billion to one trillion new blood cells each day throughout life (Notta *et al.*, 2016; Ogawa, 1993). Additionally, following situations such as blood loss the haematopoietic system needs to increase its production to replenish the systemic circulation. The production of all these blood cells is dependent on a rare population of primitive cells, the haematopoietic stem cells (HSCs), which reside in the bone marrow. These cells are a type of tissue-specific multipotent somatic stem cell in the adult body and they have the capability to differentiate into blood cells of all lineages. They also have self-renewal capacity, i.e. the capability to produce a daughter cell that is also a stem cell. Through self-renewal, HSCs can maintain their numbers throughout an individual's life. For decades, haematopoiesis has been described to occur through several steps where HSCs generate progenitors with decreasing self-renewal capacity and increasingly restricted differentiation capacity until the progenitors themselves finally produce or differentiate to mature blood cells; this process forms the haematopoietic hierarchy (Figure 1). Although there are many divergent theories on the exact structure of the haematopoietic hierarchy (Cheng *et al.*, 2020; Ema *et al.*, 2014), the classical model advocates that the first delineation occurs at the stage of differentiation into the common lymphoid progenitor (CLP), which forms white blood cells of the adaptive immune system, versus the common myeloid progenitor (CMP), forming the majority of the innate immune system (Akashi *et al.*, 2000; Kondo *et al.*, 1997). This classical model is opposed by several later studies showing myelo-lymphoid branching points further downstream (Ceredig *et al.*, 2009; Kawamoto and Katsura, 2009) and a recent paper on human HSCs further challenged current dogma by suggesting a much simpler "two-tier" hierarchy with only multipotent or unipotent progenitor cells (Notta *et al.*, 2016), though this model is yet to be investigated in the murine system. The classical haematopoietic hierarchy depicts only short term (ST) and long term (LT) HSCs, which contribute to haematopoiesis for a few weeks or a whole

lifetime respectively. However, the notion of a so-called intermediate term HSC was later introduced, adding a new dimension to the field of haematopoiesis research (Ema *et al.*, 2014).

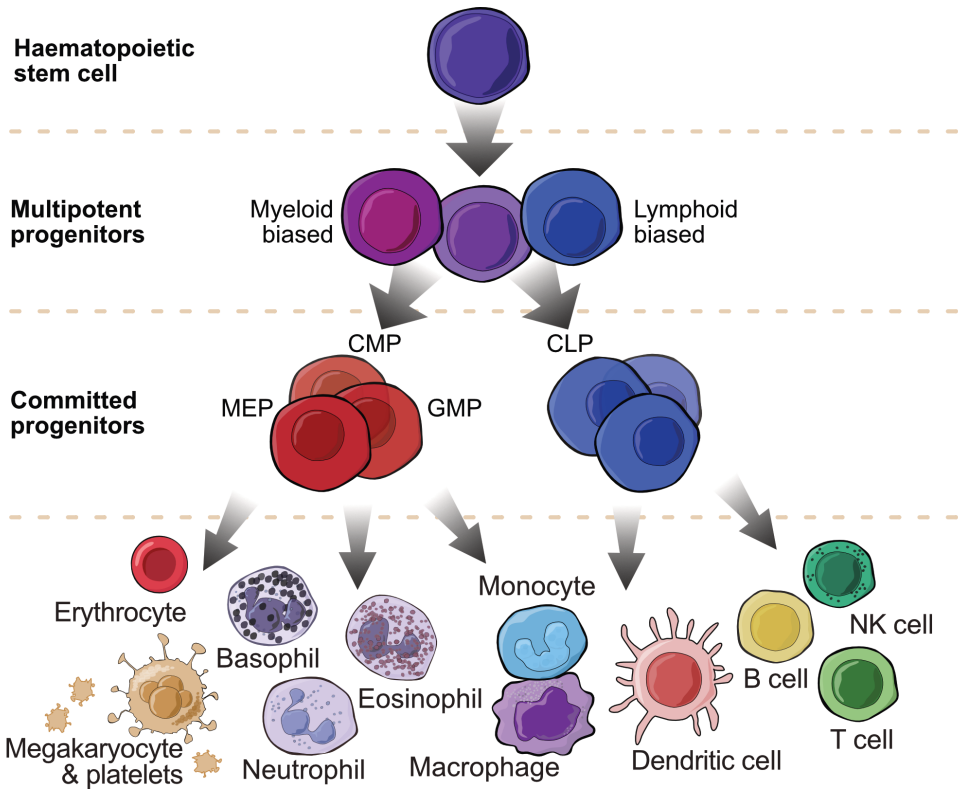

Figure 1. The haematopoietic hierarchy. The classical view of the haematopoietic hierarchy consists of specific cell types in a stepwise sequential differentiation. At the top of the hierarchy we find the HSCs, here represented by one cell but in reality consisting of a pool of long-term, intermediate-term and short-term HSCs. In the classical theory of cell differentiation, HSCs give rise to multipotent progenitors, which can be biased towards a myeloid or lymphoid differentiation path. It has been suggested that HSCs themselves can also be biased towards a myeloid or lymphoid differentiation path and that myeloid biased HSCs can directly differentiate to MEPs and GMPs, without having to differentiate via the more primitive CMP (not depicted). Ultimately, HSCs give rise to all of the haematopoietic cell types in the circulatory system. HSC: haematopoietic stem cell, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, MEP: megakaryocytic-erythroid progenitor, GMP: granulocytic-monocytic progenitor.
Illustration by Laura Tarnawski.

Haematopoietic stem cells

At the top of the haematopoietic hierarchy we find the HSC, with its capacity of both self-renewal and multipotential differentiation (Figure 1). In the 1960's Till and McCulloch published a series of studies showing clonal colony formation in the spleen of lethally irradiated mice receiving bone marrow transplants, as well as reconstitution of multiple blood lineages (Becker *et al.*, 1963; Siminovitch *et al.*,

1963; Till and McCulloch, 1961). They thereby provided evidence for the existence of the putative multipotent haematopoietic stem cell, a concept which had previously been discussed in pioneering work on blood and bone marrow by Florence Sabin (Sabin *et al.*, 1932; Sabin *et al.*, 1936) and E. Donnall Thomas (Thomas *et al.*, 1957). Till and McCulloch's work was followed by several studies that utilised retroviral DNA integration for cell tracking to prove a common origin for all haematopoietic lineages (Dick *et al.*, 1985; Jordan and Lemischka, 1990; Keller *et al.*, 1985; Keller and Snodgrass, 1990) and in the 1990's successful single cell transplantations provided unequivocal proof of a self-renewing HSC with the capacity for long term multi-lineage reconstitution of irradiated recipients (Osawa *et al.*, 1996).

Table 1. Haematopoietic cell functions.
List of different haematopoietic cells found in the blood and their functions.

Cell type	Main function
Erythrocytes	Transport oxygen and carbon dioxide
Leukocytes:	
Granulocytes:	
Neutrophil	Phagocytose bacteria
Eosinophil	Destroy parasitic worms; modulate allergic and asthmatic responses
Basophil	Release histamine in certain immune reactions; contain heparin (anticoagulant)
Agranulocytes:	
Lymphocytes:	
B cell	Produce and release antibodies
T cell	Kill virus-infected cells and regulate other leukocytes
NK cell	Kill virus-infected cells
Monocyte	Become tissue macrophages that phagocytose microrganisms, foreign bodies, and dying cells
Platelets	Cell fragments from megakaryocytes in bone marrow that intitate blood clotting and seal tears in blood vessels

More recent cell tracking studies have investigated the heterogeneity within the HSC pool, for example with regards to individual clones' contribution to steady state haematopoiesis, their cycling and self-renewal characteristics, or lineage bias. It is thought that different clones within the HSC population can be biased towards a lymphoid or myeloid fate (Muller-Sieburg *et al.*, 2004; Muller-Sieburg *et al.*, 2002) and that there are age-dependent changes in lineage bias, with an increased number of aged HSCs preferentially forming cells of the myeloid lineage (Sudo *et al.*, 2000). Historically there has also been contrasting views on the kinetics of clonal contribution to steady state haematopoiesis (Bystrykh *et al.*, 2012), though the hypotheses have been based largely on data from transplantation assays, which may not properly reflect the native state. In two recent studies, novel models of genetic labelling *in situ* were used to address the question of the dynamics of haematopoiesis at steady state. The results of these studies would argue for a model of successive recruitment of multi- and unipotent clones that continuously drive native haematopoiesis. In this model, a large number of ST-HSCs and other progenitor cells

are specified at an early age, after which LT-HSCs have limited further contribution to this pool of progenitors (Busch *et al.*, 2015; Sun *et al.*, 2014). This model moreover fits with the concept of quiescence. This is a cardinal feature of HSCs, which have been shown to exist in a quiescent, i.e. inactive, cell cycle state (Cheshier *et al.*, 1999; Goodell *et al.*, 1996). Cells progress through the cell cycle states known as G1 (interphase I), S (DNA synthesis phase), G2 (interphase II) and M (mitotic phase) (Alberts *et al.*, 2002), which was first discovered by Alma Howard and Stephen Pelc in 1953. Instead of proceeding from G1 to S, cells can remain undivided and withdraw from the cycle to enter G0, in which they are considered quiescent (Pardee, 1974). Quiescence is thought to protect HSCs from DNA damage, which could result in malignant transformation or malfunction (Moore and Lyle, 2011). Precise regulation of quiescence is additionally required to ensure sufficient blood production and to avoid stem cell exhaustion. Regulation of quiescence, and its balance vis-a-vis self-renewal and differentiation, also comes into play in the transplantation setting, where transplanted HSCs need to proliferate to rebuild a functional haematopoietic system in the recipient. Studies have also shown that there are negative effects of quiescence, such as error-prone DNA repair (Mohrin *et al.*, 2010). Quiescence may thus be a double-edged sword and its function in HSCs is not yet entirely understood. Though quiescence has generally been equated with self-renewal capacity and repopulation potential, some studies challenge this dogma, claiming no correlation between G0 phase and reconstitution capacity (Takizawa *et al.*, 2011). Other important features of HSCs include the ability of transplanted HSCs to find their way back to the bone marrow, so called homing (Tavassoli and Hardy, 1990), and their capacity to engraft in the bone marrow to reconstitute the haematopoietic system and sustain life-long haematopoiesis.

In contrast to multipotent tissue-specific adult stem cells, the stem cells in early development are totipotent or pluripotent. Totipotency refers to the ability to form all tissues of a developing organism, including extra-embryonic tissues, and is used in describing the zygote, i.e. the fertilized egg. Pluripotency is the ability to differentiate into all cells of the embryo proper, as is the case for embryonic stem cells (Lu and Zhang, 2015). Though both embryonic stem cells and certain tissue-specific stem cells such as neural stem cells can be grown *in vitro*, it has proven a difficult task to do the same with HSCs. These cells appear to have a higher propensity to lose their stem cell properties and differentiate into progenitors and mature blood cells when in culture (Verfaillie, 2002). It is thus a sought-after goal to be able to maintain and expand HSCs *in vitro*, for which at the current stage a deeper understanding is needed regarding their cellular regulation. The ability to maintain and grow HSCs in culture would not only revolutionize the research on haematopoiesis and HSCs itself, but also be of immense importance in the clinical use of HSCs, as described below.

Ontogeny of the haematopoietic system

Around embryonic day (E) 7.5, the first blood cells start emerging in the murine embryo (Haar and Ackerman, 1971; Silver and Palis, 1997). Haematopoiesis then takes place at several anatomical sites before establishment in the bone marrow (Figure 2) (Dzierzak and Speck, 2008). The haematopoietic system develops during two distinct waves. The first wave, primitive haematopoiesis, generates only erythrocytes and certain myeloid populations. This wave is thought to originate in the yolk sac from so-called haemangioblasts, i.e. primitive cells that give rise to both blood and endothelium (Choi *et al.*, 1998). During the second wave, definitive haematopoiesis, HSCs are generated that can give rise to all mature haematopoietic lineages (Medvinsky and Dzierzak, 1996). Though dogma states that the site of this second wave of haematopoiesis is the aorta-gonad-mesonephros region, definitive HSCs have been shown to co-exist in the aorta-gonad-mesonephros, yolk sac, and the placenta, and the true origin of HSCs is therefore a matter of discussion (Medvinsky *et al.*, 2011). As the circulatory system is established, HSCs migrate to the foetal liver, which remains a major site of haematopoiesis until a final relocation to the thymus, spleen and bone marrow takes place prior to birth (Ema and Nakauchi, 2000; Medvinsky *et al.*, 2011). The bone marrow later remains the primary site of adult haematopoiesis.

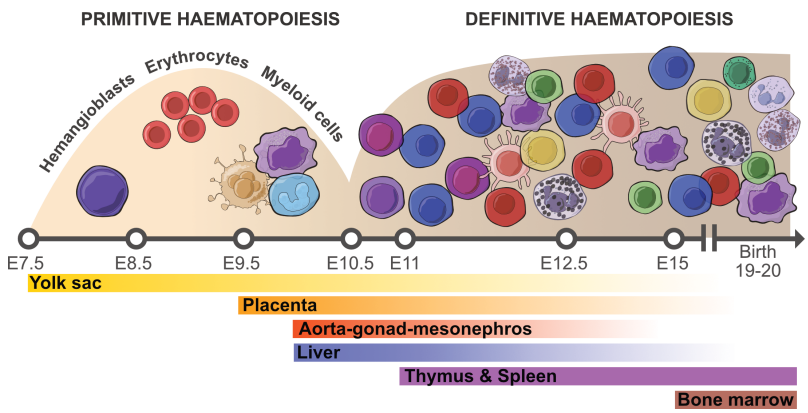

Figure 2. Development of haematopoiesis. In the mouse embryo, haematopoiesis develops in two waves, originating in different anatomic locations. The first wave, primitive haematopoiesis, produces limited types of haematopoietic cells, mainly erythrocytes and certain myeloid cells. Definitive haematopoiesis, the second wave, establishes the haematopoietic stem cell pool and generates all types of progenitors and differentiated haematopoietic cells. Timeline indicates embryonic day (E), from E7.5 to birth. Illustration by Laura Tarnawski.

Haematopoietic stem cell regulation

Due to their great regenerative potential, and thus also potential for malignancy, HSCs are kept under tight regulation in the bone marrow microenvironment, also referred to as their niche (Figure 3). The concept of an HSC niche was first proposed by Schofield in the late 1970's (Schofield, 1978) and the niche model has since been continuously developed and refined. HSC fate options such as quiescence, apoptosis, proliferation, differentiation, and self-renewal (Figure 4) (Wagers *et al.*, 2002), is regulated by both extrinsic signals (molecular feedback) from surrounding cells in the niche, as well as intrinsic signals from within the HSCs themselves. Through symmetric or asymmetric self-renewal, i.e. cell division producing two daughter stem cells or one stem cell and one differentiated progenitor cell (Morrison and Kimble, 2006) the HSC pool can either be expanded or maintained.

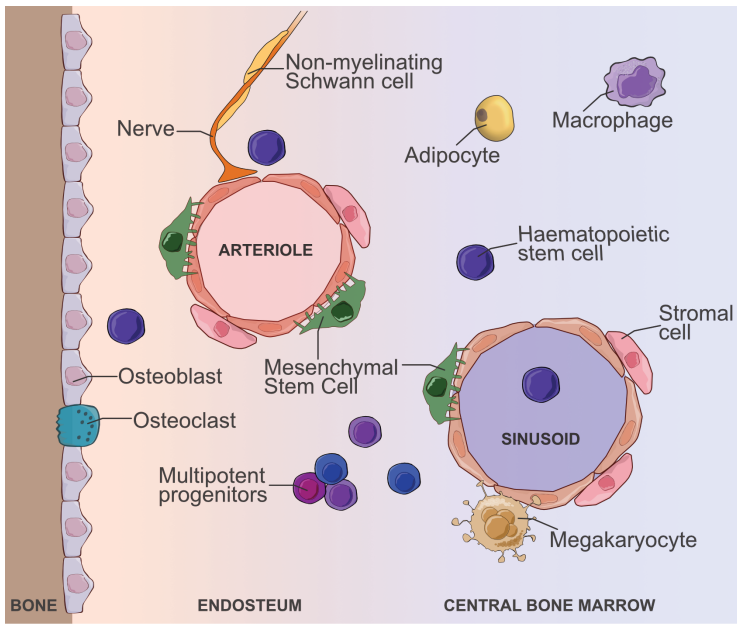

Figure 3. The haematopoietic stem cell niche. The HSC niche is a complex microenvironment where haematopoietic stem cells reside and proliferate. Various extrinsic factors within the niche help determine HSC fate. HSCs reside in the entirety of the bone marrow, but depending on their state they are likely to be found in specific sub-localizations. It is thought that quiescent HSCs are localized in the endosteum and adjacent to arterioles, while activated cycling HSCs reside closer to sinusoids and can also migrate into the peripheral circulation via entry into the bone marrow vasculature. Due to their localization HSCs are in close contact with mesenchymal stromal cells and endothelial cells, both of which provide factors required for HSC maintenance and affecting HSC fate options. Other cell types, such as osteoblasts, osteoclasts, Schwann cells, megakaryocytes, and macrophages, also regulate HSCs directly or indirectly through several different mechanisms. Illustration by Laura Tarnawski.

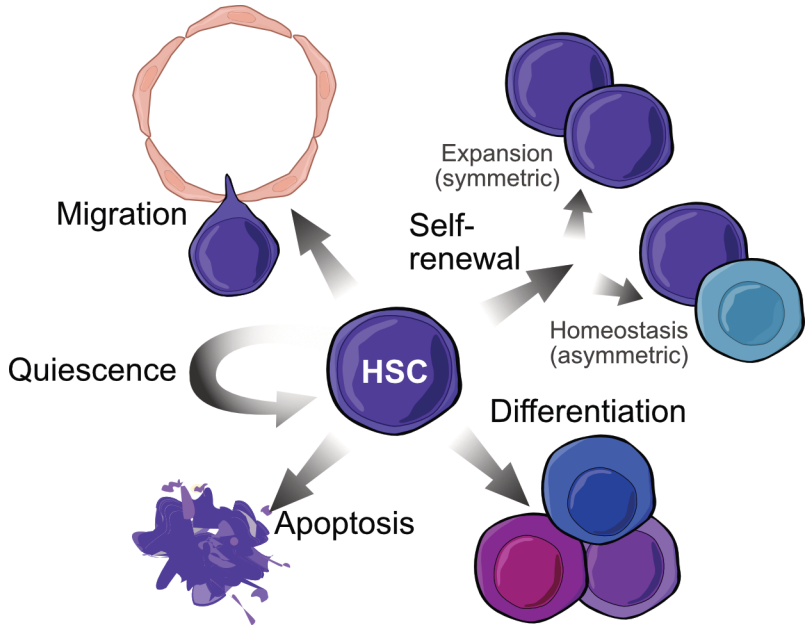

Figure 4. Haematopoietic stem cell fate options. Depending on intrinsic and extrinsic cues, HSCs can stay in a quiescent state or become active to either self-renew, migrate or differentiate. They can also undergo apoptosis or, in certain environmentally dependent situations, undergo necrosis (not depicted, as it is not defined as a fate option). Illustration by Laura Tarnawski.

An important concept in stem cell biology is the demarcation between dormant and active HSCs. It has long been thought that HSCs in these two different states are preferentially found in anatomically distinct regions within the bone marrow; in the classical model the endosteal and the vascular niche (Boulais and Frenette, 2015; Kiel *et al.*, 2005; Nilsson *et al.*, 2001; Sugiyama *et al.*, 2006). In this model, the endosteal niche is viewed as hypoxic, i.e. low in oxygen, and home to dormant HSCs (Cipolleschi *et al.*, 1993; Parmar *et al.*, 2007; Wilson *et al.*, 2007). This view on hypoxia in bone marrow can, however, be debated since oxygen measurements in bones of live mice have showed relatively higher oxygen levels in the endosteum compared to the central bone marrow (Spencer *et al.*, 2014). Nevertheless, it is proposed that the hypoxic environment protects the sensitive HSCs from reactive oxygen species, which could induce cell cycling and thereby exhaust the stem cell pool (Ito *et al.*, 2006). Additionally, the preference of quiescent HSCs to produce energy via glycolysis rather than oxidative phosphorylation (Simsek *et al.*, 2010; Takubo *et al.*, 2013) may also be a mechanism for protection against reactive oxygen species. Studies also show that specialised vascular niches regulate the quiescence and proliferation of HSCs (Asada *et al.*, 2017; Kunisaki *et al.*, 2013) and many cells and molecules partake in this regulation. For example, Schwann cells adjacent to arterioles in the bone marrow promote HSC quiescence via activation of TGFβ (Yamazaki *et*

al., 2011), as does netrin-1 produced by arteriolar endothelial cells and perivascular stromal cells (Renders *et al.*, 2021). This suggests that the vascular niche is not exclusively home to active HSCs as in the classical model, but also quiescent ones, despite this being a less hypoxic niche due to oxygen rich arterial blood supply. Updated models of the HSC niche accordingly uses terms such as the proliferative sinusoidal niche and the quiescent arteriolar niche and the hypoxic state of HSCs may actually be regulated by cues other than hypoxia in their environment (Boulais and Frenette, 2015).

In the endosteal niche, bone-remodelling cells such as osteoblasts play an important role in HSC regulation. Osteoblasts produce collagen to which HSCs can adhere, a function that is dependent on the calcium receptor. Consequently it is also dependent on the niche's calcium ion level, which in turn is determined by osteoblast- and osteoclast-mediated remodelling of bone (Adams *et al.*, 2006). Additionally, osteopontin, which is produced by osteoblasts, is needed for proper adherence and localization within the niche and it is also a suppressor of proliferation (Nilsson *et al.*, 2005). Several other extrinsic factors produced by cells in the niche also partake in HSC regulation, for example SCF, VEGF, TGFβ, and cripto (Goey *et al.*, 1989; Li and Johnson, 1994; Miharada *et al.*, 2011; Wang *et al.*, 2013; Yamazaki *et al.*, 2011). Additionally, several intrinsic HSC regulatory factors have been identified: Gfi1, Cebpa, p57, and the transcription factor PU.1 to name a few (Hock *et al.*, 2004; Matsumoto *et al.*, 2011; Staber *et al.*, 2013; Ye *et al.*, 2013). The field of HSC regulation is continuously evolving and new regulatory factors are identified and added to the vast list of extrinsic and intrinsic HSC regulators. In the past two decades, several studies have proposed a role for inflammation in HSC regulation. It has been shown that inflammation and interferon signalling has an important role in inhibition of HSC proliferation as well as in activation of quiescent HSCs (Baldridge *et al.*, 2010; de Bruin *et al.*, 2013; Essers *et al.*, 2009; Feng *et al.*, 2008; Pietras *et al.*, 2014; Sato *et al.*, 2009). Interferon exposure has not only been demonstrated to activate HSCs, but it also leads to alteration of their physical location within the bone marrow. Following interferon exposure, fewer HSCs are found in arteriolar proximity, a niche that is known for promoting quiescence (Kunisaki *et al.*, 2013). Other recent studies highlight the importance of metabolic pathways in HSC regulation, concerning both quiescence and stem cell survival. Recent studies demonstrate e.g. the role of endoplasmic reticulum stress in HSC apoptosis (Miharada *et al.*, 2014) and the role of glycolysis in regulation of HSC quiescence (Takubo *et al.*, 2013). The study by Miharada and colleagues investigated Dppa5, which is an RNA binding protein highly expressed in pluripotent stem cells. They found that Dppa5, by suppression of endoplasmic reticulum stress and apoptosis, increased HSC reconstitution levels when HSCs were transplanted following *ex vivo* culture. Conversely, when Dppa5 was knocked down endoplasmic reticulum stress levels were elevated and long-term reconstitution was impaired. Prior to this study

Takubo and colleagues had published data showing that pyruvate dehydrogenase kinases (PDKs) were essential for the function of glycolysis in HSCs. Loss of PDKs inhibited glycolysis and decreased quiescence and transplantation capacity. These two studies were among a number of early studies that showed the importance of metabolic pathways in HSC regulation. Though HSC regulation is extensively studied (Gottgens, 2015), much is still unknown regarding how various factors interact to form signalling networks and how these networks precisely regulate HSC fates depending on the shifting requirements of the haematopoietic system.

Clinical use of HSCs

HSCs have an enormous therapeutic potential and HSC transplantation has become a model system within stem cell based regenerative medicine. HSCs were first used in clinical applications in the late 1950's, when E. Donnall Thomas proved that leukemia could be cured by giving patients an infusion of bone marrow from their identical twin (Thomas *et al.*, 1959). In the clinic today, HSC transplantation is used as the only curative approach for numerous blood disorders and severe forms of leukaemia. Using chemotherapy or radiation, the patient's own haematopoietic system is first eradicated. The patient is then infused with new HSCs which can re-build a new, healthy haematopoietic system (Figure 5) (Thomas *et al.*, 1957). Transplantations can be autologous, i.e. from the patient themselves, or allogeneic when HSCs are taken from a matched donor. HSC transplantation comes with many risks, some of which are associated with the conditioning treatment needed prior to transplantation. Using milder conditioning regimens clinicians could potentially reduce morbidity and mortality associated with HSC transplantation. This will be discussed further in the coming sections of this dissertation.

Previously all HSC transplantations were done through infusion of bone marrow. Today HSCs can also be harvested for transplantation by stimulating them to leave the bone marrow. Donors are treated with e.g. the hormone granulocyte colony stimulating factor (G-CSF) to mobilize HSCs to the circulation, after which they can be extracted from peripheral blood (Antman, 1990; Duhrsen *et al.*, 1988). Another source for transplant material is cord blood from the umbilical cord of newly born infants (Broxmeyer *et al.*, 1989) which contains HSCs, but often not in high enough numbers for reconstitution of adult patients. In cord blood transplantations to adults it is therefore often necessary to pool two different cord blood samples (Ballen *et al.*, 2011) and this entails an increased risk of an immune response from the graft in the form of graft vs. host disease (GVHD) (Copelan, 2006). GVHD arises due to insufficient matching between donor and recipient human leukocyte antigen (HLA), which are present on leukocytes to distinguish between "self" and "non-self". It was

only in the late 1960's that the importance of HLA matching was fully appreciated (Storb *et al.*, 1968; Thomas *et al.*, 1971) and bone marrow transplantation could become a widespread clinical success. However, there is still limited donor availability; only 30% of patients in need of an HSC transplant have an HLA-matched sibling, and for about a third of all patients no matched donor will be found. The fraction of patients without a matched donor is further increased in non-caucasian populations (Ballen *et al.*, 2011). There is hope that current limitations in HSC transplantation will be overcome by, for example, the development of methods for *ex vivo* culture that allows expansion of HSCs. Another possible option would be to reduce the number of cells needed in a graft by enhancing the cells' reconstitution capacity through pharmacological pre-treatment of graft or recipient. Pharmacological pre-treatments could also serve as a potential method to decrease the so-called time to neutrophil recovery, which is the initial period of time following a transplant when HSCs have not yet engrafted or initiated haematopoiesis, leaving patients highly susceptible to infections and even bearing a risk of death (Centers for Disease Control and Prevention *et al.*, 2000).

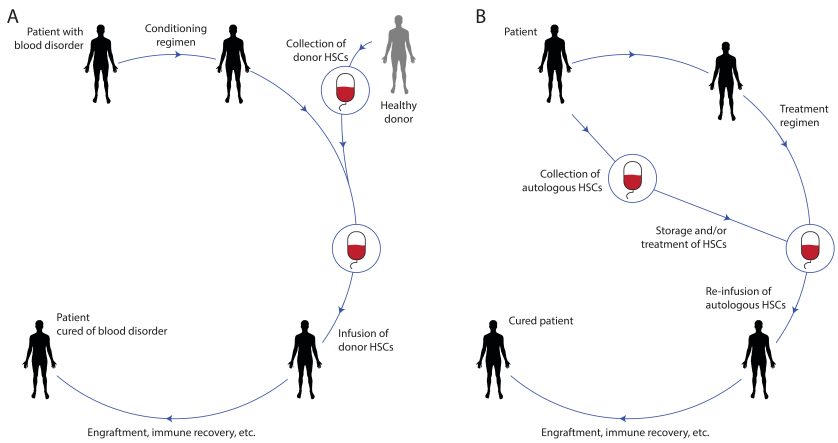

Figure 5. HSC transplantation in the clinic. Allogeneic (A) and autologous (B) stem cell transplantation can be used as a cure for a number of haematopoietic disorders. Haematopoietic stem cells (HSCs) are obtained via bone marrow aspiration or peripheral blood filtration after mobilization of HSCs to the circulation using the growth factor G-CSF (granulocyte-colony stimulating factor).

In the future there is also hope for transplantation of HSCs originating from induced pluripotent stem cells (Takahashi and Yamanaka, 2006) and for autologous transplantations in conjunction with gene therapy for monogenetic haematopoietic disorders, where the patient's own HSCs would be genetically corrected *ex vivo* by viral vector mediated gene insertion and thereafter transplanted back to the patient following chemotherapy or irradiation. Gene therapy has been used successfully to treat the haematopoietic disorder X-SCID (Cavazzana-Calvo *et al.*, 2000), but there is

still a need to thoroughly address the risk of insertional mutagenesis and leukemic transformation (Hacein-Bey-Abina *et al.*, 2003) before this would become a standard therapy. In our lab we have instead used lentiviral vectors for treatment of Diamond-Blackfan anaemia (DBA) and Gaucher disease in mouse models (Dahl *et al.*, 2021; Liu *et al.*, 2022). Lentiviral vectors as well the CRISPR-Cas system (discussed below) may be used more commonly in the future for gene therapy as they do not pose the same risk for insertional mutagenesis as in retroviral vector mediated gene correction. Also in the context of gene therapy, efficient HSC expansion protocols would be of use, as a large number of self-renewing HSCs could be more efficiently transduced.

Methods to study haematopoietic stem cells

Flow cytometry and cell surface markers

Flow cytometry is one of the established and most used methods to study cells in the haematopoietic system, a golden standard in haematopoiesis research today. When studying the haematopoietic system, peripheral blood can be easily obtained via venous sampling while murine bone marrow can be extracted post mortem from the mouse femur and other bones. HSCs, which represent only about 0.01% of total murine bone marrow, and other haematopoietic cells can be identified through flow cytometry analysis and isolated by fluorescence activated cell sorting (FACS) (Challen *et al.*, 2009; Cheng *et al.*, 2020; Mayle *et al.*, 2013; Rundberg Nilsson *et al.*, 2013).

Flow cytometry utilises the binding of fluorochrome-conjugated monoclonal antibodies to epitopes for the identification of cells. The labelled cells are arranged into a single file by a liquid vortex and inspected one by one using lasers of different wavelengths. This causes the fluorochromes attached to the cell surface to emit light, which is then recorded by detectors. Flow cytometry thereby enables researchers to identify specific marker proteins on the cell surface and accordingly determine the cell type (Rundberg Nilsson *et al.*, 2013). FACS combines this identification of cells with a technique to separate single cells from each other based on the desired antibody/epitope combination, enabling the isolation of live cell populations of specific phenotypes.

Table 2. Cell surface markers. Table on next page.

List of cell surface markers that can be used for flow cytometry analysis (or sorting) of murine haematopoietic stem and progenitor cells (HSPCs) and differentiated haematopoietic cells. Included markers are those most relevant for this dissertation, though more markers are known for the various existing haematopoietic cell populations. For example, LT-HSCs can also be defined as Hoechst low, Rhodamine low, EPCR+ and ESAM1+. Included data has been reviewed and originally published in Cheng *et al*. 2020, Karlsson *et al*. 2013, Mayle *et al*. 2013, and Pronk *et al*. 2007. LT/ST-HSC: long-term/short-term haematopoietic stem cell, (L)MPP: (lymphoid-primed) multipotent progenitor, LRP: lineage restricted progenitor, CLP: common lymphoid progenitor, CMP: common myeloid progenitor, GMP: granulocytic-monocytic progenitor, PreGM: pre-granulocyte-monocyte progenitor, MEP: megakaryocytic-erythroid progenitor, PreMegE: pre-megakaryocyte-erythrocyte progenitor, MkP: megakaryocytic progenitor, (Pre)CFU-E: (pre) colony forming unit erythroid, ProEry: proerythroblast.

	Lineage markers															
	Mac1/Gr1	B220	CD3/4/8	Ter119	Sca1	c-kit	CD9	CD150	CD48	CD34	Flt3	CD105	CD41	CD32/16	CD71	IL7rα
Primitive HSPCs:																
LT-HSC	–	–	–	–	+	+	high	+	–	–	–	+	–			
ST-HSC	–	–	–	–	+	+	mix			–	+					
MPP/LMPP	–	–	–	–	+	+	–/low	–	–	+	+	–				
LRP	–	–	–	–	+	+	–	–	+							
Committed progenitors:																
CLP	–	–	–	–	low	low	–				+					+
CMP	–	–	–	–	–	+				+				–/low		–
GMP	–	–	–	–	–	+	–/low	–		+			–	+		–
PreGM	–	–	–	–	–	+	–	–				–	–	–		
MEP	–	–	–	–	–	+				–				–		–
PreMegE	–	–	–	–	–	+	–/low	+				–	–	–		
MkP	–	–	–	–	–	+	high	+					+			
PreCFU-E	–	–	–	–	–	+	–	+				+	–	–		
CFU-E	–	–	–	–	–	+	–	–					–	–		
ProEry	–	–	–	+	–	+		–					–	–	+	
Differentiated cells:																
T cells	–	–	+													
B cells	–	+	–													
Myeloid cells	+	–	–													

Over the past decades many haematopoietic cell surface markers have been identified and utilised for flow cytometry analysis and cell isolation (Table 2). By using unique combinations of these markers, it is now possible to isolate distinct populations of haematopoietic stem and progenitor cells (HSPCs) and mature blood cells from both mouse and human blood, bone marrow and other tissues. It is well established that in the bone marrow the so-called LSK (lineage– Sca1+ c-kit+) cell population contains HSCs and multipotent progenitors (MPPs). Lineage negativity specifies that HSPCs do not express several lineage markers that are found on mature haematopoietic cells (e.g. Mac1, Gr1, B220, CD3, and Ter119). HSCs are furthermore positive for stem cell antigen 1 (Sca1) (Spangrude *et al.*, 1988). The other commonly used marker is c-kit, the receptor for stem cell factor (SCF) (Ikuta and Weissman, 1992). Differing levels of c-kit expression has recently been shown to affect stem cell potential; HSCs with lower levels of c-kit were found to be more quiescent and c-kit^{high} HSCs showed reduced self-renewal and bias towards megakaryocytic differentiation (Grinenko *et al.*, 2014; Shin *et al.*, 2014). The heterogeneous LSK population can be further divided into subpopulations using markers such as CD34 and Flt3, as well as the signalling lymphocyte activating molecule (SLAM) family members CD150 and CD48 (Adolfsson *et al.*, 2001; Christensen and Weissman, 2001; Kiel *et al.*, 2005; Osawa *et al.*, 1996). LT-HSCs are recognised as negative for CD34, Flt3, and CD48, but positive for CD150. Though the function for many of these markers remains unknown, CD34 is known to be associated with HSC quiescence and activated CD34+ HSCs can return to a quiescent CD34- state after transplantation (Sato *et al.*, 1999). Other LSK subpopulations are the ST-HSCs (CD34- Flt3+), lymphoid-primed MPPs (LMPPs; CD34+ Flt3+) (Adolfsson *et al.*, 2001; Osawa *et al.*, 1996; Yang *et al.*, 2005), MPPs (CD48- CD150-), and lineage restricted progenitors (LRPs; CD48+ CD150-) (Kiel *et al.*, 2005) (Figure 6). New markers for HSCs are continuously being identified, such as EPCR, CD9, and ESAM1 (Balazs *et al.*, 2006; Karlsson *et al.*, 2013; Ooi *et al.*, 2009), as well as the SLAM family members CD229 and CD244 that can be used to sub-fractionate HSC and MPP compartments (Oguro *et al.*, 2013; Tian and Zhang, 2016). CD9 has been shown to allow for high purity sorting of HSCs from murine bone marrow and the marker is advantageously used in separation schemes in conjunction with above markers. A study by Karlsson and colleagues showed that CD9, when used as a positive selection marker, captures all long-term engrafting HSCs. This held true even when sorting cells that were otherwise immunophenotypically not defined as LT-HSCs, such as CD150- or CD34+ cells (Karlsson *et al.*, 2013). A recent follow-up study on CD9 showed that within the LMPP population (LSK CD34+ Flt3+), a subpopulation of cells with intermediate expression of Flt3 and high CD9 expression (Flt3^{int}CD9^{high}) concurrently display stem-like and lineage-associated chromatin signatures. Though these cells could be shown to possess multi-lineage capacity, the study also demonstrated a lack of self-renewal ability as no long-term reconstitution of the

LSK compartment could be found (Safi *et al.*, 2022). This study complements the view of CD9 as not only an HSC marker, but also a marker of multi-potency in a heterogeneous progenitor population.

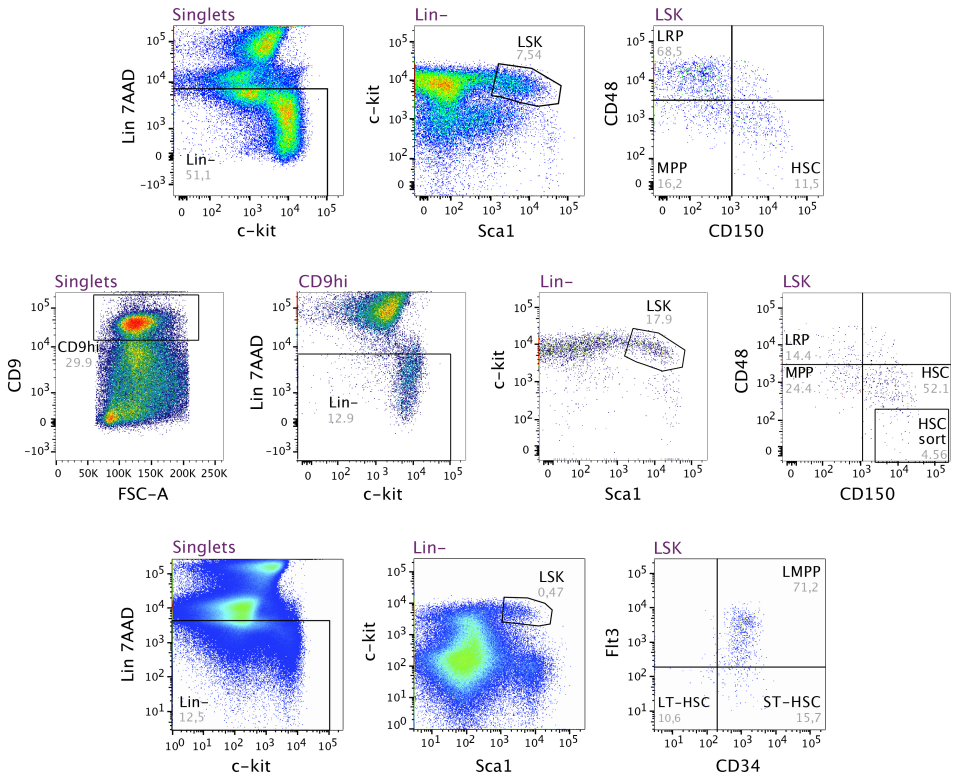

Figure 6. Flow cytometry analysis of HSPCs. Representative plots showing flow cytometry gating of c-kit enriched bone marrow (upper and middle rows) and whole bone marrow (bottom row). Lineage negative and Sca1 and c-kit positive (LSK) cells were analysed for CD150 and CD48 (top row plots) or CD34 and Flt3 (bottom row plots), subdividing the LSK population into different haematopoietic stem and progenitor cells (HSPCs). 7-AAD was used for exclusion of dead cells. Initial selection for CD9 high expressing cells (middle row plots) results in populations enriched for more primitive cells (Karlsson *et al*., 2013). This leads to a higher proportion of haematopoietic stem cells (HSCs) seen in the final plot (middle row), in comparison to without initial CD9 high selection (top row). When sorting HSCs for e.g. transplantation experiments a stricter gate is set to avoid contamination with unwanted cell types. Lin: lineage, LSK: lineage negative sca1 positive c-kit positive, LT/ST-HSC: long-term/short-term haematopoietic stem cell, (L)MPP: (lymphoid-primed) multipotent progenitor, LRP: lineage restricted progenitor.
Adaptation of illustration from Warsi *et al*. 2022.

Though there are many markers for HSCs, it is important to keep in mind that cell surface expression can be altered in response to environmental stimuli, e.g. up-regulation of Sca1 by inflammation (Snapper *et al.*, 1991), which can be a limitation in the phenotypic identification of HSCs. As an alternative to cell surface marker identification, HSCs can also be identified by using their dye efflux properties. HSCs contain, in comparison to more mature haematopoietic progenitor populations,

a large number of transmembrane pumps that expel dyes such as Rhodamine-123 and Hoechst-33342 (Bertoncello *et al.*, 1985; Goodell *et al.*, 1996; Wolf *et al.*, 1993). Using Hoechst 3342, Goodell and colleagues identified the so-called side population, which is now a recognized method for HSC isolation.

Development of fluorochrome-conjugated antibodies for intracellular epitopes have also made it possible to, for example, investigate intracellular protein phosphorylation levels and to categorise cells into different cell cycle phases or apoptotic states. Early and late apoptosis can be evaluated by using staining for AnnexinV (detected on cells in both early and late apoptosis) and the DNA-binding fluorescent chemical 7-aminoactinomycin D (7-AAD) (in late apoptosis and necrosis, as the cell membrane is compromised allowing for dye passage into the cell). 7-AAD is also routinely used for the exclusion of dead cells in flow cytometry and FACS. For cell cycle analysis, permeabilised cells may be stained for Ki67, which marks proliferating cells (Schluter *et al.*, 1993), alongside a DNA marker such as DAPI, making it possible to discern whether cells are in G0, G1, or S/G2/M (Figure 7). This method has previously been utilised to show that about 70% of mouse HSCs reside in G0 (Wilson *et al.*, 2008). Interestingly, over 90% of CD9^{high} cells lack expression of Ki67, suggesting that a high proportion of these cells reside in G_0 phase (Karlsson *et al.*, 2013). This characteristic lends further support for CD9 as a positive selection marker for HSCs.

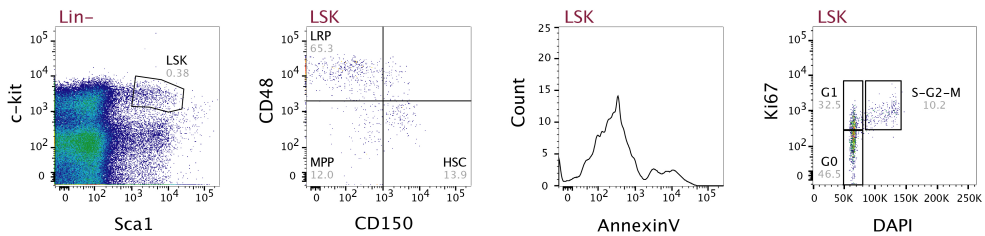

Figure 7. Analysis of cell cycle status and apoptosis. Representative plots depicting flow cytometry analysis strategy for cell cycle and apoptosis analyses. Haematopoietic stem and progenitor cell populations are visualised in whole bone marrow using LSK, CD48 and CD150 markers. Subsequently analysis of Ki67 and DAPI is used to determine cell fractions at different stages of the cell cycle (G0, G1, and S/G2/M), while AnnexinV is used to determine frequency of apoptotic cells. Lin: lineage, LSK: lineage negative, sca1 positive, c-kit positive.
Illustration from Warsi *et al*. 2022.

Genetic modelling

One of the most potent and versatile *in vivo* methods to study the effect of specific genes in a biological system is by using mouse models. The mouse is used as a standard model system for transplantation assays within haematopoiesis research and one of the most common techniques to study gene function is by using so-called knockout (KO) mice, where removal of a single gene of choice enables investigations of the role of that gene in a biological system. In a conventional KO model, a specific

genetic region is removed entirely from genome of the mouse. As an alternative, the conditional KO model allows for genomic deletion in one specific tissue system or after a specific time point or event. This is particularly useful when conventional deletion of a gene has been known to cause lethality during development. Conditional gene loss is often achieved by using the Cre-lox system, where specific gene segments flanked by loxP sites can be excised by the Cre recombinase (Sauer, 1998; Sauer and Henderson, 1989). The Mx-Cre system enables induction of KO through injection of substances that activate interferon signalling (Kuhn *et al.*, 1995), while other Cre systems can be triggered alongside activation of a gene during development. An example of the latter is the Vav-Cre system, where Cre transcription is initiated by Vav1, a gene activated at E10.5 in the haematopoietic system specifically (Stadtfeld and Graf, 2005). Similarly, reporter mouse models induce expression of e.g. GFP in tissues where a gene of interest is actively transcribed. Gene function can also be evaluated through over-expression studies, where a viral vector is used to transduce haematopoietic cells, which then express high levels of the gene of interest. Where KO is not feasible, knockdown (KD) through e.g. viral vector induced expression of shRNA can be used. New techniques can also induce single nucleotide mutations in the genome, making it possible to study gene function at an additional level of complexity. One such new and promising technique is CRISPR-Cas, which has emerged in recent years as a method for specific and targeted genetic alterations. CRISPR-Cas is a very powerful tool; it is a site-specific nuclease that causes double-stranded DNA breaks after which homology-directed repair can be promoted by providing a suitable DNA repair template (Cong *et al.*, 2013; Hochheiser *et al.*, 2018; Kühn, 2022; Tschaharganeh *et al.*, 2016). This technology allows for rapid generation of novel KOs, point mutations, specific site gene insertions and much more.

Though the above-described model systems are commonly used, they come with several caveats. The Mx-Cre system has recognized drawbacks such as spontaneous deletion (Velasco-Hernandez *et al.*, 2016) as well as known (and unknown) effects of interferons on HSC regulation. KO and over-expression are also criticised, as they are most often not representative of a physiological situation. It is questionable whether a gene's true function can be properly assessed in non-physiological conditions, but not many other techniques exist today that are equally powerful and could take their place as standard methods.

Stem cell transplantation

Though analysis of cell surface markers is fundamental in identification of HSCs, true stem cell properties of phenotypic HSCs are properly assessed only through competitive transplantation and serial transplantations. Only functional LT-HSCs

possess the capability of long-term reconstitution through primary, secondary, and tertiary recipient mice (Harrison *et al.*, 1993; Hellman *et al.*, 1978). Failure to reconstitute can be indicative of several problems, including homing, engraftment, survival, self-renewal, or a lack of *bona fide* HSCs in the graft. In competitive transplantation, the investigated bone marrow is transplanted together with a bone marrow sample of equal size from a normal mouse (a so called wild type, WT, mouse) into an irradiated recipient mouse. Irradiation is used to eliminate the bone marrow cells of the recipient, making space for the transplanted graft. If the cells of the investigated bone marrow graft have normal functionality they will have equal engraftment to the WT graft, and if not they might engraft better or worse in comparison. Engrafted cells from different sources can be distinguished through the CD45.1/45.2 system (Figure 8). CD45 is a pan-haematopoietic marker, i.e. it is expressed on the surface of all haematopoietic cells. Transplantation using donor cells from mice that exclusively express different isoforms (CD45.1 or CD45.2) allows for flow cytometric analysis of chimerism within the haematopoietic system of the recipient. 16-20 weeks post-transplantation, long-term engraftment can be evaluated and cells from the primary recipients are transplanted to secondary mice. This is then repeated once more, as a true HSC should be capable of long-term engraftment also of a tertiary recipient. Syngeneic, or congenic, bone marrow transplantation with the CD45.1/45.2 model has been widely used to characterise engraftment, as CD45 isoforms were shown to not provoke an immune reaction (Sykes *et al.*, 1989). Congenic strains have, however, been a debated topic as there has later been evidence of some immunogenicity (van Os *et al.*, 2001; Xu *et al.*, 2004), but for lack of other better model systems it is still the golden standard today. Following a publication from Mercier and colleagues, truly congenic transplantations with the so-called STEM mouse could become the new standard in the future (Mercier *et al.*, 2016). Additionally, owing to the development of humanized mouse models it is similarly possible to study human haematopoiesis *in vivo* through transplantation of human HSCs into immunodeficient mouse strains (Shultz *et al.*, 2007).

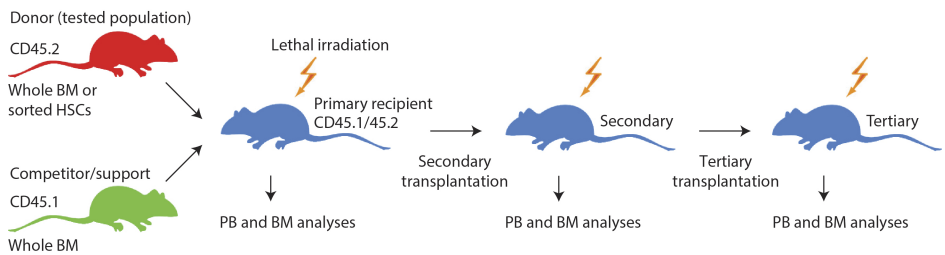

Figure 8. Murine HSC transplantation setup. Schematic overview of transplantation and analysis time points in serial transplantation experiments. CD45.1 and CD45.2 were used to discriminate between donor, competitor, and recipient populations in the bone marrow (BM) and peripheral blood (PB). HSCs: haematopoietic stem cells. Adaptation of illustration from Warsi *et al.*, 2022.

Transplantation assays can also be used for quantification of HSCs in a so-called limiting dilution assay. The test population is increasingly diluted and the different doses of cells are transplanted together with a fixed amount of competitor cells (Szilvassy *et al.*, 1990). The number of long-term reconstituted recipients from each dilution can be used to calculate the frequency of HSCs in the donor test population; these HSCs are termed competitive repopulating units (CRU). It is however important to take into account that not only the frequency of HSCs determine reconstitution. If a KO mouse model is assessed in a limiting dilution assay the resulting CRU is also affected by e.g. homing capacity, which can be altered in loss-of-function mouse models. It is therefore important to complement this type of assay with others such as the homing assay.

Ex vivo and *in vitro* assays of HSPCs and their function

Definitive proof of a fully functional HSC can only be obtained in transplantation experiments, but HSPCs and their functions can also be evaluated in numerous other assays. *Ex vivo* analysis of primary cells can be done using a multitude of techniques, one of which (flow cytometry) has been described above. Aside from HSC phenotype it is often also of interest to investigate gene expression patterns, which often change in response to e.g. KO of a gene in a mouse model. This is regularly done by using either microarray or RNA-sequencing analyses. In these analyses purified cell populations or single cells (Kalisky *et al.*, 2011) (e.g. sorted by FACS) can be compared for gene expression differences. Microarrays investigate a large set of RNAs using specific probes (Schena *et al.*, 1995) while RNA-sequencing, as the name suggests, sequences the whole transcriptome of a cell population (Wang *et al.*, 2009). Similarly, proteomics analyses differences in cells' protein expression (Blackstock and Weir, 1999). Apart from the aforementioned global analysis methods, expression levels of a smaller number of selected genes and proteins can be measured using classical techniques such as quantitative PCR and western blotting.

A commonly used method to evaluate haematopoietic function *ex vivo* is the colony forming unit (CFU) assay, providing information about progenitor cell frequency, proliferation, and differentiation capacity. In CFU assays a gel medium supplemented with cytokines is used to grow primary cells from murine bone marrow; cytokines are selected to promote growth and differentiation of specific haematopoietic lineages. Whole bone marrow, or an enriched or sorted cell population, is seeded and within 7-14 days a colony of cells forms around each primary cell capable of proliferation and differentiation in the specified medium. Colonies are counted to evaluate the sample for frequency of specific progenitor populations. Microscopic evaluation regarding colony size and cell type provides information on the progenitors' capacity for proliferation and differentiation respectively, the latter also indicating the progenitor's

primitiveness. Current protocols allow for evaluation of granulocyte/macro-phage progenitors (CFU-GM, CFU-G, and CFU-M) and erythroid progenitors (CFU-E and burst forming unit erythroid, BFU-E), as well as mixed potential progenitors (CFU-GEMM). The CFU assays have been criticised for their variability between laboratories due to the subjective nature of the analysis, however introduction of automated counting and microscopic evaluation holds promise for increased reliability of the method in the future (Khojasteh *et al.*, 2015; Pamphilon *et al.*, 2013).

Another method to evaluate haematopoietic progenitors *ex vivo* is the long-term culture initiating cell (LTCIC) assay, which can be used to quantify more primitive haematopoietic progenitors (upstream of CFU progenitors in the haematopoietic hierarchy). The method is based on that committed progenitors have a life span of only about 3-5 weeks, i.e. shorter than that of primitive progenitors that can survive longer in specific supportive cultures and continuously generate new committed progenitors. After 5-8 weeks of culture, the resulting cell population is assessed for CFU capacity, from which the number of primitive haematopoietic progenitors in the tested cell population can be quantified (Sutherland *et al.*, 1990).

Finally, there are currently a few immortalized cell lines with primitive haematopoietic cell properties, the HPC and FDCP-mix cell lines. HPC cell lines were generated by expression of Lhx2 in either haematopoietic cells derived from embryonic stem cells *in vitro* or in HSPCs derived from adult bone marrow. These cells are multipotent and the HPC cell line derived from primary HSPCs has also been shown to be capable of long-term reconstitution following transplantation in mice (Pinto do *et al.*, 1998; Pinto do *et al.*, 2002; Pinto do *et al.*, 2001). FDCP-mix is also a self-renewing haematopoietic cell line that can differentiate into all haematopoietic lineages upon appropriate cytokine stimulation *in vitro*, but cannot rescue lethally irradiated recipients upon transplantation (Greenberger *et al.*, 1983; Spooncer *et al.*, 1986). Though none of these cell lines can be considered true HSCs, due to their immortalized nature, the availability of cell lines with multi-lineage differentiation capacity, and even long-term engraftment, provides a valuable tool for *in vitro* analyses. This can be useful in e.g. gene expression analyses and in pilot studies of HSPC function, before moving on to experiments using animal models.

The Schlafen family of proteins

The murine Schlafen (SLFN) family of proteins are made up of ten known or predicted proteins and the SLFNs are widely distributed in mammalian species, especially among primates and rodents, but are also found in e.g. amphibians and orthopoxviruses (Bustos *et al.*, 2009; Mavrommatis *et al.*, 2013). Since their original

discovery in mice (Schwarz *et al.*, 1998), the *Slfns* have been candidate genes for meiotic drive and embryonic lethality, due to their connection to the mouse DDK syndrome (de la Casa-Esperon, 2011). They are all clustered within a 350 kb region on chromosome 11 (Bustos *et al.*, 2009). The role for each *Slfn* gene is, however, still unclear. The SLFN family can be subdivided into three groups. The group I SLFNs consist of SLFN1, SLFN1L, and SLFN2, and are the shortest of the SLFN proteins. SLFN3 and SLFN4 make up group II, which are slightly larger proteins, and group III (SLFN5, 8, 9, 10, and 14) are the largest SLFNs because of a C-terminal extension with predicted RNA helicase activity (Geserick *et al.*, 2004; Mavrommatis *et al.*, 2013; Neumann *et al.*, 2008). All SLFN proteins contain a conserved so-called Schlafen box, which is part of a larger region termed "ATP binding associated with cellular activities" (AAA). AAA domains are known to be present in other proteins involved in e.g. protein folding and transcription (Hanson and Whiteheart, 2005). Due to their differing protein structure, the SLFNs have varying intracellular expression patterns; subgroups I and II localize preferentially to the cytoplasm while subgroup III proteins are found exclusively in the nucleus (Neumann *et al.*, 2008). Interestingly, SLFN dysregulation is reported in several disorders, in particular carcinomas, and the SLFN family was also implicated in haematopoietic disease in a recent case study reporting T-cell dysfunction and T-cell lymphoma, which lead to the death of a 65-year old patient (Recher *et al.*, 2014). It was found that the patient had mutations across a genomic region containing several *Slfn* genes and many of the symptoms were reported to be recapitulations of the elektra mouse model phenotype, which is discussed more extensively below. This suggests that SLFN proteins may have a role in haematopoiesis warranting further study.

The most studied murine SLFN proteins are SLFN1, SFLN2, and SFLN3, but the full range of their biological functions is still unclear. Though several of the *Slfn* genes are expressed in HSCs (Bustos *et al.*, 2009), there are as of yet no studies of the effect of SLFNs in HSC regulation. A majority of studies on SLFN proteins has been carried out in cell lines, and within the haematopoietic system studies on SLFNs are limited only to specific cell lineages. It has been reported that over-expression of *Slfn1* in T-cell progenitors has a negative effect on growth and development of cells in the T lineage due to induction of cell cycle arrest (Schwarz *et al.*, 1998; Zhang *et al.*, 2008), and similar effects have been found for *Slfn8* (Geserick *et al.*, 2004). *Slfn3* is also expressed in a certain subset of T-cells, but contrary to *Slfn1*, the expression of *Slfn3* is up-regulated upon T cell activation (Condamine *et al.*, 2010). In 2005 Brady and colleagues reported that SLFN1-induced cell cycle arrest was caused by Cyclin D1 inhibition (Brady *et al.*, 2005). This mechanism has later been both confirmed (Zhang *et al.*, 2008) and contested (Zhao *et al.*, 2008). The SLFN of interest in this thesis, SLFN2, has been suggested as a positive regulator of osteoclastogenesis downstream of RANKL (Lee *et al.*, 2008) and it also has a role in macrophages, where microbe induced stimulation triggers *Slfn2* expression via activation of

the NF-κB and AP-1 pathways (Sohn *et al.*, 2007). The SLFNs are thus highly implicated but not yet well studied in haematopoietic regulation. Interestingly, *Slfn* genes are also reported to be up-regulated in response to interferon, an effect likely mediated by STAT and p38 MAPK (Katsoulidis *et al.*, 2009). As discussed above, it is known that interferons play a role in activation of quiescent HSCs (Essers *et al.*, 2009; Sato *et al.*, 2009), and it is possible that the SLFNs are somehow involved in this or are up-regulated in response to reduced quiescence. Katsoulidis and colleagues also showed that KD of *Slfn2* rescues an interferon-induced decrease in colony formation in CFU assays using isolated primary cells. They therefore described *Slfn2* as a key regulator of interferon-induced growth suppression. However, as this assay was done using the highly interferon-sensitive Sca1+ cell population the results may need to be tested for reproducibility in other cell subsets. Additionally, a clear though non-significant trend towards an increase in haematopoietic colony formation following *Slfn2* KD was seen also in CFU assays without interferon treatment (Katsoulidis *et al.*, 2009), suggesting a role for SLFN2 in regulation of haematopoietic progenitor proliferation. Another study found that loss of SLFN2 function caused increased susceptibility to infections due to immune system dysfunction; specifically an observed lack of T-cell quiescence and increased T-cell apoptosis (Berger *et al.*, 2010). In the same paper, Berger and colleagues also reported the development of a new mouse model, which they named the elektra mouse. This strain contains a point mutation in the *Slfn2* gene causing a single amino acid substitution mid-protein. Based on both the observed phenotype in the paper by Berger *et al.* and polymorphism phenotype analysis using PolyPhen-2 (Adzhubei *et al.*, 2010), the *Elektra* allele of the *Slfn2* gene is commonly considered a loss-of-function allele. This mouse model therefore provides an excellent means for studying the function of SLFN2 in, for example, haematopoiesis.

In 2004, Bruno and colleagues detected *Slfn2* expression in an HSC like haematopoietic self-renewing cell line and, importantly, showed that *Slfn2* expression was decreased upon differentiation of these cells to four separate haematopoietic lineages (Bruno *et al.*, 2004). This finding, along with SLFN2 being implicated in cell cycle regulation, cell quiescence, proliferation, and interferon signalling, makes it an excellent candidate gene to study in research on HSC regulation.

The BMP signalling pathway

The bone morphogenetic proteins (BMPs) have been studied for many decades and were first known for their roles in bone and cartilage formation as well as neurogenesis in the early embryo (Huang and Saint-Jeannet, 2004; Katagiri and Watabe, 2016). BMP signalling is also known to have an important role in

embryogenic mesoderm formation and developmental specification of haematopoiesis (Sadlon *et al.*, 2004; Snyder *et al.*, 2004). KO of the ligand genes *Bmp2* or *Bmp4*, or the receptors *Alk3* or *Bmpr2* cause embryonic lethality and mesoderm malformation (Beppu *et al.*, 2000; Mishina *et al.*, 1995; Winnier *et al.*, 1995; Zhang and Bradley, 1996), reflecting the importance of these proteins in embryogenesis. The BMPs are part of the larger TGFβ superfamily, a group of proteins that beyond development also have well-established roles in cell regulation and various malignancies and diseases (Massague *et al.*, 2000). Just like other members of the TGFβ family, canonical BMP signalling is driven by the SMAD proteins. The BMPs bind to and activate complexes of type I (ALK2, ALK3, ALK6) and type II (BMPR2, ActRIIA, ActRIIB) receptors. Following this, the canonical BMP pathway preferentially signals through the receptor-activated SMAD1, SMAD5, and SMAD8 (Derynck and Zhang, 2003). These receptor-activated SMADs form a complex with the shared co-SMAD (SMAD4) to translocate into the nucleus and act as transcription factors for BMP target genes such as *Id1* (Katagiri and Watabe, 2016; ten Dijke *et al.*, 2003). It was long assumed that the importance of BMP signalling during embryonic haematopoiesis was simply carried over into adult haematopoiesis. In accordance with this hypothesis, in 2003 Zhang and colleagues showed that conditional KO of *Alk3* (encoding a type I receptor) in adult mice lead to an expansion of the number of HSCs by increasing the size of the osteoblastic niche (Zhang *et al.*, 2003). Recently it was also demonstrated that BMP4 stimulation *in vitro* protects HSCs from culture-induced loss of homing capacity (Khurana *et al.*, 2013). In contrast to these findings, reduced levels of BMP4 in the bone marrow has been shown to reduce HSC numbers (Goldman *et al.*, 2009) and *in vitro* it has been reported that BMP4 stimulation has no effect on the proliferation of cultured HSCs (Utsugisawa *et al.*, 2006). Previous studies from our lab have also proved the dispensability of SMAD1 and SMAD5 (Singbrant *et al.*, 2010; Singbrant *et al.*, 2006). As these are the only BMP-specific receptor-activated SMADs that are expressed at detectable levels in haematopoietic cells (Utsugisawa *et al.*, 2006), endogenous canonical BMP signalling is most likely not required in HSCs. These results strongly questioned the importance of the BMP pathway in adult murine haematopoiesis and there has not been consensus on the role of BMPs in adult HSC regulation *in vivo*. However, though disruption of SMAD1 and SMAD5 does block canonical BMP signalling, the BMPs are also known to signal through several non-canonical mediators e.g. TAK1 and elements of the mitogen-activated protein kinase (MAPK) pathway such as p38 and JNK (Blank *et al.*, 2009; Derynck and Zhang, 2003; Shim *et al.*, 2009). Blocking of all BMP mediated signalling would provide a means for investigating the complete role of the BMP pathway in HSCs *in vivo*. This is readily achieved by KO on the receptor level, for which the type II receptor BMPR2 is a prime candidate, as it is expressed at much higher levels than other BMP-targeted type II receptors in primitive haematopoietic cells (Utsugisawa *et al.*, 2006).

Diamond-Blackfan Anaemia

DBA is a rare inherited blood disorder that often presents at 2-3 months of age and is therefore classically diagnosed very early in life (Campagnoli *et al.*, 2004; Lipton *et al.*, 2006; Orfali *et al.*, 2004; Willig *et al.*, 1999). Clinically, patients exhibit macrocytic anaemia, reticulocytopenia and selective erythroblastopenia in the bone marrow (Flygare and Karlsson, 2007). DBA is also associated with physical malformations and a predisposition to develop malignancies later in life (Lipton *et al.*, 2006; Willig *et al.*, 1999). In about 60% of DBA patients, mutations in ribosomal proteins have been discovered (Farrar *et al.*, 2011), the most frequent being *RPS19,* which comprises about a quarter of cases (Draptchinskaia *et al.*, 1999).

First-line therapy for DBA has long been treatment with systemic corticosteroids (Vlachos *et al.*, 2008). About four out of five patients respond to this treatment initially, but unfortunately half of these cannot continue corticosteroid treatment due to side effects or loss of response (Lipton *et al.*, 2006). Other pharmaceutical treatments have been or are being tested, but none are currently in regular use in the clinic. Surprisingly, and by unknown mechanisms, about 20% of DBA patients go into spontaneous remission, though not all of them stay in remission (Lipton *et al.*, 2006). For relapsed patients and for those where corticosteroids cannot be used, continuous blood transfusions are used to battle anaemia (Vlachos *et al.*, 2008). This comes with risks such as iron overload and immune reactions. The final and only definitive cure for the haematological symptoms of DBA is allogeneic HSC transplantation (Vlachos *et al.*, 2008). In the future, autologous HSC transplantation with gene therapy correction can become an important option for patients where monogenic mutation of e.g. *RPS19* is the cause of their disease. For this to be a feasible option, human HSC expansion protocols would be highly useful.

Present investigation

HSCs are to date the most studied stem cell in the human body. However, there are still many gaps in knowledge, leading to poor in-depth understanding of the underlying molecular mechanisms governing self-renewal and quiescence, two fundamental properties of stem cells. Accordingly, robust, reproducible and efficient *in vitro* expansion of functional HSCs has been difficult to achieve. Studying the regulation of HSC function during normal haematopoiesis could provide key insights for further development of expansion protocols and could also lead to other clinically relevant therapies. The study of normal HSC regulation has therefore been the focus of Paper I and Paper II.

In current clinical practice, patients with blood disorders need to undergo tough conditioning regimens prior to HSC transplantation to ensure proper engraftment of donor HSCs. However, fit and healthy transplanted HSCs may be able to out-compete diseased HSCs without the need for conditioning in transplantation for certain blood disorders. Reduced intensity conditioning protocols would be valuable in the clinic as they could reduce the risk of morbidity and mortality. This has been the focus of Paper III.

Specific aims of this thesis

Paper I

To investigate the role of SLFN2 in HSC regulation by using a mouse model with a point mutation in the *Slfn2* gene.

Paper II

To investigate the role of BMP signalling in HSC regulation by using a mouse model with conditional KO of the *Bmpr2* gene.

Paper III

To evaluate reduced intensity conditioning regimens in HSC transplantation using a DBA mouse model.

Summary of results

Paper I

Our interest in SLFN2 as a potential regulator of HSC function emerged from an *in vitro* study showing that *Slfn2* expression was down-regulated when a primitive hematopoietic cell line underwent differentiation (Bruno *et al.*, 2004). We analysed *Slfn2* expression in murine HSPC populations isolated by FACS and found that *Slfn2* is highly expressed in LT-HSCs, in comparison to ST-HSCs and LMPPs, which gave us further incentive to study the role of SLFN2 in HSCs. In other cell types SLFN2 has been shown to be involved in quiescence, differentiation, and interferon response. It is also a known regulator of immune response where studies have shown that T-cell quiescence and activation are dependent on SLFN2 (Berger *et al.*, 2010). Berger and colleagues developed the elektra mouse model for the study of SLFN2; a mouse where the *Slfn2* gene contains a point mutation (resulting in the so-called *Elektra* allele), which causes it to lose its function. In our paper we have used the elektra mouse model to study the role of SLFN2 in HSCs. We could show that though *Elektra* homozygous mice have essentially normal steady state haematopoiesis, with only a reduction in T-cells at younger ages, they show a decrease in primitive haematopoietic cells and importantly a decrease in LT-HSCs. When elektra bone marrow was tested functionally in serial transplantation we found a drastic reduction in regenerative capacity, with a significantly reduced donor engraftment already in primary recipients (mean engraftment 14% in PB of recipients of elektra bone marrow vs. 51% in recipients of WT bone marrow; $p<0.01$). In recipients of purified elektra HSCs we saw an even greater reduction in donor engraftment (3% in PB of elektra HSC recipients vs. 44% in WT HSC recipients; $p<0.01$). To investigate the reason for this defect in HSC function we performed microarray analysis of elektra HSCs and found clusters of deregulated genes involved in cell cycling and apoptosis. Further analysis of elektra HSPC populations showed that the HSCs did indeed show increased cycling (22% of elektra HSCs in G1 phase vs. 12% of WT HSCs; $p<0.05$). We also found an increase in apoptotic cells in elektra HSPC populations (40% apoptotic elektra HSCs vs. 20% of WT HSCs; $p<0.05$). We additionally showed a defect in stress response where 2/3 elektra mice treated with 5FU died, while all WT mice survived. We could show that elektra mice treated with 5FU could be rescued by bone marrow transplantation. We also found that *Slfn2* is up-regulated 1.7-fold in HSCs two days after 5FU treatment, lending further support to its role in stress response. Taken together, we report for the first time that SLFN2 is a critical regulator of HSC quiescence and stress response. Our data contributes to the accumulating evidence of SLFN2 as a general regulator of cell quiescence and proves it has an important role in stem cell regulation.

The main conclusions of this paper were:

- Loss of SLFN2 function causes a reduction in HSCs in the bone marrow.
- SLFN2-deficient HSCs have impaired regenerative capacity following transplantation.
- SLFN2 deficiency deregulates expression of genes involved in cell cycle and apoptosis. This consequently causes an increase in cycling and apoptotic HSCs and leads to impaired stress response following 5FU treatment.

Paper II

BMPs are well known and extensively studied, and though they play an important role in embryonic haematopoiesis, previous studies from our research group have shown that canonical SMAD-mediated BMP signalling is dispensable in adult HSCs (Singbrant *et al.*, 2010). Despite this, *Bmpr2* is highly expressed in HSCs leaving the possibility that BMPs may yet play role in these cells but via alternative circuitries. In this paper we used a conditional KO model for *Bmpr2* with KO induced at E10.5 by the *Vav-Cre* driver. This enabled complete shutdown of all downstream BMP signalling specifically in haematopoietic cells from E10.5 and onwards. Though this had no major effect on steady state haematopoiesis, aside from a reduction in LSK cells in the bone marrow, we found a reduced regenerative capacity of BMPR2 deficient HSCs in transplantation experiments (mean engraftment 37% in PB in recipients of *Bmpr2*-KO bone marrow vs. 51% in recipients of WT bone marrow; 8% in PB of recipients of FACS purified *Bmpr2*-KO HSCs vs. 27% in WT HSC recipients; $p<0.05$). In serial transplantations engraftment was further decreased in secondary and thereafter tertiary recipients. In line with previous studies, our study found it unlikely that this phenotype was caused by loss of canonical BMP signalling, as bone marrow cells stimulated with BMP did not respond transcriptionally via the SMAD pathway. Though no one clear mechanism behind our observed phenotype could be established, we found that (1) p38 was reduced in c-kit enriched bone marrow from *Bmpr2*-KO mice, (2) there was a trend towards increased cycling of HSPCs following *Bmpr2*-KO, and (3) gene expression of *Tjp1* was up-regulated 2.4-fold in *Bmpr2*-KO HSCs. When using shRNA to knock down *Tjp1* in BMPR2 deficient bone marrow cells we saw a partial rescue in transplantation experiments (mean engrafted LSK cells 0.0075% in bone marrow of recipients of WT scramble-transduced cells vs. 0.0018% in recipients of *Bmpr2*-KO scramble-transduced cells – $p<0.05$ – and 0.0029% in recipients of *Bmpr2*-KO *Tjp1*-KD-transduced cells – non-significant compared to WT). Taken together, we report that non-canonical BMP signalling is necessary for proper function of adult HSCs. We further show that this is in part mediated by TJP1, which is a known regulator of self-renewal in other stem cells. This would also suggest that TJP1 might be a universal stem cell regulator.

The main conclusions of this paper were:

- Loss of BMP signalling through *Bmpr2* KO causes a deficiency in regenerative capacity of HSCs in serial transplantations, suggesting a self-renewal defect.
- *Bmpr2* KO causes a reduction in p38 and up-regulated gene expression of *Tjp1*.
- *Tjp1* KD in BMPR2 deficient cells causes a partial rescue of engraftment of LSK cells following transplantation.

Paper III

DBA is a blood disorder, or more specifically a bone marrow failure disorder, which primarily causes erythroblastopenia and anaemia. It presents in early infancy and patients often also have developmental defects as well as an increased risk of leukaemia and cancers later in life. Patients undergo blood transfusions to alleviate symptoms, but HSC transplantation is the only curative treatment available today. Both the transplantation itself and the preceding conditioning regimen come with risks to the patient's health and well-being. As DBA-diseased HSCs are less fit in comparison to WT HSCs (Jaako *et al.*, 2011) we reasoned that it might be possible for healthy HSCs to out-compete innate diseased HSCs without extensive conditioning prior to transplantation, which is the standard today. A majority of patients show mutations in ribosomal proteins and a conditional RPS19-deficient mouse model has previously been developed to mimic the DBA haematopoietic disease phenotype and lethal bone marrow failure (Jaako *et al.*, 2011). In this model doxycycline treatment is used to induce disease phenotype. We used this mouse model to investigate whether transplanted HSCs can engraft with either low dose irradiation conditioning or no conditioning at all. Kusabira orange mice (Hamanaka *et al.*, 2013) were used as bone marrow donors to track engraftment in all haematopoietic lineages. Sixteen weeks after transplantation we found robust engraftment of donor cells in RPS19-deficient mice even with no prior irradiation, while there was no engraftment in WT recipients with no conditioning (mean engraftment 85% in bone marrow of RPS19-deficient mice; $p<0.001$). With low dose irradiation (200 cGy) we found on average 92% engrafted donor cells in bone marrow of RPS19-deficient mice, compared to 16% engraftment in WT recipient bone marrow ($p<0.001$). Unsurprisingly, there was complete reconstitution of donor cells in both RPS19-deficient and WT recipients given full myeloablative conditioning (900 cGy). In both low-dose irradiated and non-conditioned RPS19-deficient mice we found a correction of disease phenotype following transplantation, with significant contribution of donor cells to erythroid populations 16 weeks post-transplantation. Taken together, we report for the first time that HSCs can engraft in non-conditioned or low-dose irradiated RPS19-deficient recipients and correct the

disease phenotype seen in these mice. DBA patients with *RPS19* mutations may greatly benefit from future implementation of reduced intensity condition protocols prior to curative bone marrow transplantation, which has also been demonstrated in a recent retrospective study of 27 young DBA patients (Koyamaishi *et al.*, 2021).

The main conclusions of this paper were:

- Even with no conditioning or low dose irradiation prior to bone marrow transplantation there is significant engraftment of donor cells in RPS19-deficient DBA mice.
- In both the non-conditioned and low-dose irradiated groups there is significant contribution of donor cells to erythroid populations in RPS19-deficient mice.
- Non-conditioned and low-dose irradiated RPS19-deficient mice exhibit full correction of disease phenotype after bone marrow transplantation.

General discussion and future perspectives

Investigating HSC regulation and function

It has been a long-standing goal in haematopoiesis research to establish culture protocols in which fully functional HSCs can be expanded and maintained *ex vivo*. This would not only revolutionize the research itself, by providing ample material for continued studies, but could also have great clinical benefits, as it holds promise to enable greater availability of HSC therapies. The papers in this dissertation have identified novel intrinsic regulators of HSC fate options. The ultimate goal has been to expand current knowledge on the regulation of HSCs in their natural environment, which could contribute to improved techniques for efficient HSC culture. Additionally, in-depth mechanistic knowledge of normal HSC regulation is crucial to better understand leukaemogenesis and, in the future, aim to find new treatment options for these malignant transformations. Our studies have also provided novel insight into HSC function by showing that diseased HSCs can be out-competed by healthy transplanted HSCs with no or little conditioning. This discovery could be greatly beneficial for patients about to undergo HSC transplantation, as reduced chemotherapy or radiation would lead to improved outcome regarding morbidity and mortality.

HSC expansion

Currently, HSC transplantation is limited by donor obtainability, in the case of allogeneic transplantation, and by the limited number of cells that can be enriched from a donor or patient, in the case of both allogeneic and autologous transplantation. With efficient HSC expansion, these limitations could be overcome.

The HSC in its natural environment is tightly regulated; fate options are controlled by intrinsic factors within the HSC and extrinsic factors, both from within the niche and via whole system effects due to e.g. environmental factors. Accordingly, mapping how various factors affect HSC function both *in vivo* and *in vitro* is of key importance for the development of effective and biologically relevant protocols for HSC expansion. Importantly in this context, studies have observed discrepancies between

in vivo and *in vitro* findings regarding HSC function. For example, Notch signals seemingly are required for *in vitro* but not *in vivo* maintenance of human HSCs (Benveniste *et al.*, 2014). Certain cell culture effects are, once initiated, also difficult to reverse; for example cell cycle entry *in vitro,* which causes a decline in HSC engraftment ability (Foudi *et al.*, 2009; Glimm *et al.*, 2000; Passegué *et al.*, 2005). A precise balance is needed between proliferation, self-renewal, differentiation and apoptosis to enable HSC survival, maintenance and expansion *ex vivo*. Specifically for expansion we need approaches that promote symmetric division and self-renewal without differentiation, as well as promotion of quiescence for maintenance of engraftability. Interestingly, a recent study showed that engraftable functional murine HSCs could be maintained and expanded up to 899-fold during one month in defined culture conditions. The protocol in question replaced serum albumin with polyvinyl alcohol in the culture media, which was also supplemented with TPO, SCF, and fibronectin (Wilkinson *et al.*, 2019; Wilkinson *et al.*, 2020). A later study also identified that functional HSCs in this culture system were CD244 negative and showed that this population was better maintained *in vitro* when endoplasmic reticulum stress was suppressed using a molecular chaperone, as this correlated with improved reconstitution capacity (Koide *et al.*, 2022). Similar culture systems as the one reported by Wilkinson and colleagues are also being developed for human HSCs and several studies have had partial success in promoting expansion (Bai *et al.*, 2019; Dahlberg *et al.*, 2011; Hofmeister *et al.*, 2007; Sauvageau *et al.*, 2004). Importantly, in recent years, studies have shown successful expansion of human HSCs using the small molecules UM171 and SR1, which are also being evaluated in on-going phase I-II clinical trials (Fares *et al.*, 2022). Additionally, so-called HDAC inhibitors such as valproic acid have showed promising results for human HSC expansion in both pre-clinical studies and initial data from an on-going phase I clinical trial (Fares *et al.*, 2022). In the future, many such molecules will need to be tested to develop both short-term and long-term expansion protocols. To find novel regulatory factors that could be of use in these culture protocols, HSCs need to be further studied in their natural environment, which is best done in animal models.

The work presented in this thesis has identified two novel *in vivo* regulators of murine HSC quiescence and self-renewal respectively, SLFN2 (paper I) and the BMP-TJP1 pathway (paper II), which are discussed in more detail below. Future studies could investigate the possibility of activation or inhibition of SLFN2 or the BMP-TJP1 pathway using for example small molecules. These small molecules could further on be assessed *in vitro* in HSC culture, to investigate their potential effects on HSC expansion.

Mouse models in HSC research

The ultimate proof of stem cell function is the reconstitution potential in serial transplantations. Low reconstitution can indicate an HSC defect, for example impaired self-renewal or excessive proliferation and differentiation, which both lead to a depletion of the HSC pool. A gold standard in haematopoiesis research has long been experimental mouse models, providing a powerful method for studying HSC biology and disease modelling.

There are many approaches in working with mouse models, including gene KO, knock-in, silencing, and overexpression. In our research (paper II) we have used *Bmpr2* KO mice to study the role of BMP signalling in HSCs. As opposed to using a conventional mouse model, where a gene is knocked out in its entirety, we used a conditional model in which the receptor is knocked out upon expression of *Vav*, which occurs around day E10.5 exclusively in all haematopoietic cells (Stadtfeld and Graf, 2005). By doing so, we can study the signalling pathway following receptor loss specifically in definitive haematopoiesis and we avoid the detrimental effects linked to loss of BMP signalling during embryogenesis and early foetal development (Larsson and Karlsson, 2005; Massagué, 2012; Sadlon *et al.*, 2004; Snyder *et al.*, 2004). Loss of function can also be obtained by deleterious genetic mutations, as is the case for the elektra mouse model, which we used in our study of SLFN2 (paper I). This model is a conventional model and it is therefore important to keep in mind that any observed changes could also be due to effects on other organ systems in either adulthood or during development. In conventional models the HSC niche is also affected as well as other cell types that may affect HSC regulation. However, viable conventional models also show that the gene in question is not essential during development. Compared to KO, point mutations can lead to either a loss of function, a gain of function, or both. For this reason it can be valuable to complement point mutation experimental models with a KO or KD model.

To observe immediate effects of reduced expression of a protein, shRNA KD can be used. In these models, cells are transduced *in vitro* with viral vectors, which induce expression of shRNA strands that silence specific gene expression by RNA interference. Knock-in of a gene coding for a specific shRNA enables its use *in vivo* as well. An shRNA-based conditional system was used to create the DBA mouse model, which we used in paper III. Exposure of these mice to doxycycline induces expression of a knocked-in shRNA targeting *Rps19,* leading to a reduction of *Rps19* mRNA levels by approximately 50%. This in turn results in a DBA-like disease phenotype (Jaako *et al.*, 2011). Importantly, shRNA and other gene-silencing models only decrease gene expression as opposed to a KO, which completely removes it. Depending on the specific research question, one or the other may be more desirable.

Finally, viral vectors or knock-in can also be used to overexpress a gene of interest in order to investigate its effect on HSC functionality. However, integration of the promoter and gene at an undesirable site can cause oncogene overexpression and result in off target effects not connected to the gene of interest.

In more recent years CRISPR-Cas has emerged as a method in generation of various types of transgenic cells and mouse models. It is much faster than conventional methods for KO and knock-in, and is also considerably safer due to the high specificity with which one can insert or remove genetic material, lowering the risk of e.g. oncogene overexpression. Future studies of SLFN2 or other members of the Schlafen family could focus on utilising this system to knock out *Slfn2* or increase its expression. KO using CRISPR-Cas would be a more efficient and powerful method compared to e.g. shRNA to reduce gene expression levels.

Finally, it is important to remember that *in vivo* alterations of gene and protein expression can have extensive unknown effects. Cell signalling systems are often very intricate and display redundancy, which is to say if something were decreased, something else would increase in an effort to replace the lost function. For example, in our microarray analysis we observed a slight, but non-significant, up-regulation of *Bmpr1a* and *Smad3* in *Bmpr2*-KO HSCs. Similarly, in HSCs derived from elektra mice, microarray data showed a slight non-significant up-regulation of *Slfn3*. Even if these changes may counteract effects of the loss of a regulatory mechanism, the up-regulation evidently was not enough, since we saw a clear phenotype in both the *Bmpr2*-KO and elektra mouse models.

Heterogeneity among HSCs

When utilising genetic alterations to study HSC function it is important to consider that HSCs are a heterogenic population of cells, consisting of active and quiescent HSCs, located in the bone marrow and in the peripheral circulation, as well as contributing to long-term, intermediate-term, and short-term haematopoiesis. The many down-stream multipotent progenitor populations add additional heterogeneity to this system. It would be erroneous to assume that a KO, or any other genetic change, would affect all these cells equally. As there is redundancy within signalling networks, there may also be redundancy between cell populations. If a certain HSC subpopulation is severely affected by a genetic change, it may be possible for another subpopulation, which is less affected, to adapt in an attempt to fill its place. It could then be difficult to distinguish if an observed phenotype is due to the loss of one population or the gain of another.

Additional heterogeneity can be discussed within the population of quiescent HSCs. Though the terms quiescence and dormancy are often used interchangeably in the field of haematopoiesis research, it may be important that a distinction is made between them. By definition these two terms are not synonymous, which has been more extensively discussed in the scientific field of plant biology (Considine and Considine, 2016) and is occasionally mentioned in studies on cancer stem cells (Damen et al., 2021). Previous research has, however, also identified two different subpopulations of HSCs with characteristics that suggest the presence of distinctly different quiescent and dormant HSCs (Raaijmakers and Scadden, 2008; Wilson *et al.*, 2008). It would be of value in future studies to further investigate these putative HSC populations and to investigate what is needed to maintain these possibly different cell states.

Heterogeneity within the HSC pool also leads to limitations in HSC assays. In conventional bone marrow transplantation, the whole pool of heterogeneous HSCs and progenitors are transplanted together. The readout is thus affected by all the transplanted cells. Similarly, differences in HSC subpopulations and in HSC numbers can affect readout in other assays such CFU. This limitation can be addressed by using a purified HSC population for these assays.

By expanding our knowledge on HSC regulation and function we also learn more about the heterogeneity within the HSC pool. In paper I, we saw that engraftment seemingly increases in secondary and tertiary recipients following initial transplantation of purified HSCs from elektra mice. This could indicate the existence of a subpopulation of HSCs with limited dependence on SLFN2; because this population is potentially not affected by or can adapt to loss of SLFN2, it is able to increase in numbers in long-term serial transplantations. Investigation of possible subpopulations with varying dependence on SLFN2 could be a subject for future studies. The potential untangling of HSC heterogeneity, if linked to function and phenotypic markers, could be of considerable importance for the research field and for the clinical use of HSCs.

New insight into HSC quiescence and self-renewal

SLFN2 in health and disease

The SLFN family of proteins were discovered about twenty years ago and in the context of haematopoiesis they are sparsely studied. Knowledge on SLFN2 is limited to its role in T-cell quiescence and apoptosis, as well as in interferon response, and there are thus several gaps in knowledge regarding its role in the haematopoietic

system, especially in stem cell regulation. The novel data presented in our study (paper I) establishes SLFN2 as a critical regulator of adult murine HSC quiescence and apoptosis and shows that SLFN2 is essential for proper haematopoietic regeneration.

Due to the known role of SLFN2 in regulation of quiescence and apoptosis in T-cells, as well as the high expression of *Slfn2* in HSCs, we early on hypothesized that SLFN2 may be important in HSC regulation. Using the so-called elektra mouse model containing a point mutation in the *Slfn2* gene, we showed that loss of SLFN2 function led to smaller fractions of HSPCs in the bone marrow, in comparison to WT mice, including a significantly smaller fraction of LSK CD34- Flt3- LT-HSCs. Functionally, we could also show that SLFN2 deficient HSCs have lower reconstitution of the haematopoietic system following transplantation into irradiated recipient mice. This was seen using both unfractionated bone marrow and sorted LT-HSCs and was especially pronounced in the latter setting, suggesting a specific defect in HSC function following loss of SLFN2. To explore the mechanism through which SLFN2 acts we performed a microarray of LT-HSCs and found significantly higher expression of genes involved in cell cycle and cell division in SLFN2 deficient HSCs compared to WT HSCs. We also found lower expression of genes involved in cell quiescence in SLFN2 deficient HSCs. A putative effect on cell cycle regulation was further investigated in flow cytometry analysis using Ki67 and DAPI. We found that a lower fraction of SLFN2 deficient HSPCs were in a G_0 state and correspondingly found a higher fraction of HSPCs in G_1. We also found more apoptotic HSCs and MPPs in the bone marrow of SLFN2 deficient mice. The observed higher fraction of cycling HSCs can, at least in part, explain the lower engraftment following transplantation of SLFN2 deficient cells. We could also show how this affected HSC stress response by treating mice with 5FU. Two days after treatment we found higher expression of *Slfn2* in WT HSCs. Within 10-11 days two out of three SLFN2 deficient mice died whereas all WT mice survived, and SLFN2 deficient mice could be rescued by bone marrow transplantation following 5FU treatment.

In future studies it would be of interest to investigate the effect of *Slfn2* KO. In our studies we showed that cells with KD of Slfn2 have lower engraftment following transplantation, similar to what was seen using bone marrow from *Slfn2* mutated mice. It would, however, be valuable to also investigate this using a complete KO. Additionally, the effect of SLFN2 in the haematopoietic niche could be further investigated using either the elektra loss-of-function model or a future *Slfn2* KO.

Apart from up-regulation of cell cycle associated genes, our microarray analysis of HSCs also showed down-regulation of spliceosome components in SLFN2 deficient LT-HSCs compared to WT. This would also be an interesting subject to investigate further. The spliceosome is part of the cellular mRNA processing machinery and studies have shown that its proper function is vital for HSC development in both

zebrafish (Yu *et al.*, 2019; Zhao *et al.*, 2021) and mice (Wang *et al.*, 2022). Additionally, mutations in RNA-binding proteins involved in splicing have been reported in both myeloid and lymphoid leukaemias (Hodson *et al.*, 2019) and impaired minor intron excision via the minor spliceosome was recently shown to both drive cancer development and enhance HSC self-renewal (Inoue *et al.*, 2021). A putative connection between the Schlafen family of proteins and the spliceosome is yet to be investigated.

Finally, there is also a known association of the Schlafens with interferon response. Previous work has shown that *Slfn* gene expression is up-regulated in cell lines following interferon treatment (Katsoulidis *et al.*, 2009; Mavrommatis *et al.*, 2013). It is also known that interferon treatment causes HSCs to exit G_0 and progress to an active cell cycle state (Essers *et al.*, 2009; Sato *et al.*, 2009). The connection between interferon, SLFN2, and HSC quiescence would be interesting to explore in future studies. Our data shows that SLFN2 is a mediator of quiescence in HSCs and that *Slfn2* gene expression is up-regulated in HSCs two days following 5FU treatment. We have hypothesized that SLFN2 may be important for a negative feedback inhibition system to enable re-entry into quiescence following interferon or stress induced cycling of HSCs and thus prevent HSC exhaustion following a physiological interferon or stress response. Previous studies have presented data in support of this hypothesis using mouse embryonic fibroblasts and cell lines (Fischietti *et al.*, 2018), but it is yet to be investigated in HSCs or *in vivo*, which could be done using either the elektra mouse model or by generating and using a novel *Slfn2* KO mouse model.

BMP signalling in HSCs

The TGFβ superfamily of growth factors, including the BMPs, has been extensively studied over many decades. While it was previously thought that BMP signalling was dispensable for adult HSCs *in vivo,* our study (paper II) has provided novel mechanistic insight into the role of BMP signalling in HSC self-renewal.

Earlier studies on the role of BMP in HSC regulation have focused on the well-characterized SMAD pathway (the so-called canonical pathway), which operates downstream of BMP receptors, and found that this pathway is dispensable in adult HSCs (Singbrant *et al.*, 2010). However, non-canonical BMP signalling through non-SMAD pathways can also be activated by BMPs. Interestingly, the BMP receptor *Bmpr2* is highly expressed in HSCs, suggesting existence of a potential yet unknown role for BMPs in HSC regulation. In our study we sought to elucidate the role of non-canonical BMP signalling in HSCs. By conditional deletion of *Bmpr2* we effectively shut down all (both canonical and non-canonical) BMP signalling in haematopoietic cells and showed that signalling via BMPR2 is essential for self-renewal of adult HSCs. Specifically we show that in the absence of BMPR2, HSC

grafts fail to efficiently generate new HSCs in serial transplantations, resulting in significantly reduced engraftment. Our findings showed only small and non-significant differences in cell cycle status of BMPR2 null HSCs compared to WT and therefore an effect on cell cycle progression likely only plays a small part in HSC regulation by the BMP pathway. We further found that homing was not affected and therefore not a likely cause behind the observed reduction in engraftment and, accordingly, compromised self-renewal ability is likely.

In accordance with previous studies showing dispensability of BMP-activated SMADs in HSCs, we showed that a majority of haematopoietic cells do not engage a SMAD-dependent transcriptional response. This may be due to cross-regulation by other pathways and could be an interesting focus of future studies. While our data further showed that many of the known non-canonical pathways did not seem to be differentially activated following loss of BMPR2, both phosphorylated p38 and total p38 were reduced in primitive haematopoietic cells from *Bmpr2*-KO mice. This is in agreement with a previous study showing increased p38 phosphorylation in mouse HSPCs cultured in the presence of the ligand BMP4 (Khurana *et al.*, 2013). Though in our paper we observed decreased self-renewal following deletion of *Bmpr2,* which also coincided with a downstream reduction in p38, previous work has conversely indicated an association between high p38 activity and loss of HSC self-renewal (Hinge *et al.*, 2017). Inactivation of p38 has further been shown to protect against this loss of self-renewal (Ito *et al.*, 2006). Additionally, it has been shown that p38 inhibition promotes murine HSC expansion *in vitro* (Wang *et al.*, 2011), which also indicates a negative effect of p38 on self-renewal, in contrast to our findings. Accordingly, we reasoned that reduction in p38 was likely not a mediator of the loss of self-renewal seen in *Bmpr2*-KO HSCs, which might instead be attributed to a potential novel mechanism. To analyse this, we performed a microarray of LT-HSCs and found a significant up-regulation of *Tjp1* in *Bmpr2*-KO HSCs as compared to WT HSCs. TJP1 is a known negative regulator of self-renewal in embryonic stem cells (Xu *et al.*, 2012) and expression of *Tjp1* is also shared between many stem cell types (Ramalho-Santos *et al.*, 2002). Following KD of *Tjp1* in *Bmpr2*-KO cells we found a partial rescue of the *Bmpr2* null phenotype, as transplanted *Bmpr2*-KO *Tjp*-KD cells showed increased engraftment in the LSK cell compartment of the bone marrow. Therefore, the up-regulation of *Tjp1* is likely at least in part one of the underlying mechanisms of the *Bmpr2* null phenotype. Future studies should focus on further characterization of the BMPR2-TJP1 pathway in HSC self-renewal. It could be of value to investigate KO, instead of KD, of *Tjp1* in haematopoietic cells and possibly also to cross such a model with the *Bmpr2*-KO. Furthermore, there may be other systems contributing to the *Bmpr2* null phenotype, which could also be a focus of future studies.

Therapeutic relevance

The work comprised in this dissertation presents two novel pathways important for HSC regulation (papers I and II). Investigation of small molecule activation or inhibition of the SLFN2 or BMP-TJP1 pathways could be relevant for future HSC expansion protocols, which in turn can be useful for treatment of haematopoietic disorders through increased availability of donor graft material or by facilitating *ex vivo* gene therapy of autologous cells. Additionally, small molecules may have potential for direct therapeutic benefit in patients. Hypothetically, a pharmaceutical therapy that e.g. up-regulates *Slfn2* may lead to better engraftment, if a graft was pre-treated with such a drug or if the drug was administered in conjunction with HSC transplantation. A research topic such as this could be an interesting future project.

This thesis also presents findings that are closer to regular clinical application. In paper III we used a mouse model with conditional *Rps19* deficiency, which causes a DBA-like disease phenotype. The haematological symptoms seen in these mice can be cured with HSC transplantation, as is done for DBA patients in the clinic. To assess the need for conditioning prior to transplantation we evaluated HSC transplantation in groups of mice given no conditioning treatment or low dose irradiation (200 cGy). We found that mice were successfully long-term engrafted with transplanted healthy HSCs following no prior conditioning treatment and when conditioning consisted of only low dose irradiation. The transplantation also corrected the haematological symptoms seen in the mice. These results show how healthy HSCs are able to out-compete less fit diseased HSCs in *Rps19*-deficient DBA mice. This allows for milder conditioning regimens as a standard for patients with *RPS19*-mutated DBA, though further pre-clinical and clinical studies are needed. Case reports and a recent retrospective study of paediatric DBA patients undergoing HSC transplantation have shown that reduced intensity conditioning regimens can be used successfully in this patient group (Koyamaishi *et al.*, 2021). Additionally, our findings show that diseased HSCs have decreased fitness when in competition with HSCs from healthy individuals and it is possible that this is a universal trait for certain types of haematological disorders. It could therefore be of value to study additional haematological disorders in this manner, especially bone marrow failure disorders where bone marrow cellularity is reduced. It is, however, unlikely that this is also a trait of leukaemia, considering that leukemic disorders typically exhibit increased proliferation among other traits. Leukemic disorders would therefore likely still require full dose conditioning prior to curative HSC transplantation. We anticipate that milder conditioning regimens for patients with DBA and other similar disorders can become reality in the near future, which would undoubtedly reduce morbidity and mortality for this patient group.

Ethical considerations

When working with animals there are certain ethical aspects to consider. In society, animal experiments are a highly debated topic with widely varying opinions. Though a future where animal models are no longer needed is far-fetched, there is much that can be done in our everyday work to reduce, replace and refine animal work (the so-called three R's).

In certain areas of medical research, animal models cannot be avoided to be able to obtain robust and reliable data. Haematopoietic stem cell research is one of these fields, as the only way to prove actual stem cell function is by transplantation. In every aspect of animal work one must consider minimization and optimization. An important part of this is to only use as few mice as necessary, as well as to make sure animals are treated well and suffer as little as possible. When it comes to replacement of animal models, *in vitro* HSC expansion holds promise. By performing haematopoietic assays using cultured cells we could reduce the need for primary bone marrow cells in today's quantities or possibly even replace this completely in the future. The definitive functional test of HSCs will most probably still be transplantation assays for the foreseeable future, especially when working towards clinically applicable therapies.

In human HSC expansion it will be important to consider the source of cells and possibly also genetic ownership. The cell source for various human HSC cultures, if attained in the future, will be healthy volunteers and patients. The integrity of these individuals must be considered in this process.

Popular science summary

Our blood consists of multiple cell types with various functions like transporting oxygen and combating infections. On a daily basis, the body needs millions of blood cells to maintain these functions. Blood stem cells (also called haematopoietic stem cells; HSCs) reside in the bone marrow and are responsible for continuously supplying the blood with new cells. Due to their unique capabilities, HSCs have the capacity to re-establish a whole new blood system. This feature of HSCs enables the use of these cells in bone marrow transplantation (also called stem cell transplantation; SCT), which is currently used as a cure for many blood disorders, including severe leukaemias. However, SCT is dependent on a large amount of cells to rebuild the blood system of an adult patient. Additionally, many patients never find a bone marrow donor that is biologically compatible. To be able to overcome some of these issues in SCT, it is of utmost importance that we understand how HSCs are regulated on a molecular level. One of the goals of my research has been to investigate how different molecules or proteins regulate so-called fate options of HSCs, for example whether HSCs are in a resting state or actively proliferating to expand their numbers or make new blood cells. Such knowledge could contribute to improved methods to keep and even grow HSCs outside of the body, or improve their capacity to rebuild a blood system following transplantation.

In my first article we investigated a molecule called SLFN2. We saw that mice without normal SLFN2 had few HSCs in comparison to normal mice and that these HSCs were worse at rebuilding a blood system following transplantation. We observed that a possible reason for this was that the SLFN2 molecule affects cell division, which is of immense importance to stem cells so that the "pool" of stem cells is not diminished and for them to function properly after transplantation.

In my second article we investigated a signalling pathway, i.e. a cascade of molecule or protein interactions that together affect a cell. We used genetically modified mice in which one molecule was missing, the so-called BMP receptor in the BMP signalling pathway. This effectively shut down the signals that the molecule BMP would have generated in normal blood cells. We saw that HSCs without BMP signalling were worse at rebuilding a blood system following transplantation. We discovered that this was because another molecule, TJP1, was affected by the lack of BMP signalling. Both of the above-described studies contribute new observations and valuable knowledge on how these molecules regulate HSCs, especially in the context of SCT.

SCT is a procedure associated with many risks. Patients need to undergo tough treatments, often using chemotherapy or irradiation, to remove their own diseased blood cells, including their HSCs. After this, new HSCs are transplanted to rebuild a healthy blood system. Another goal of my research has been to evaluate if milder irradiation treatment would be sufficient before SCT in certain diseases. To investigate this, in my third article we used genetically altered mice with symptoms similar to what is seen in patients with the blood disorder Diamond-Blackfan anaemia, where production of red blood cells is severely decreased. We observed that SCT could be successful even without pre-treatment with irradiation in these mice. This shows, in principle, that healthy HSCs in a transplant can out-compete and replace diseased HSCs in the bone marrow. The knowledge generated through this project could be clinically valuable, as milder SCT pre-treatments could decrease the severity of side effects and lead to improved outcomes for patients.

To summarize, my thesis contributes with new insights into how blood stem cells are regulated in their natural environment as well as the possibility of milder pre-treatments for SCT. These discoveries could provide a stepping-stone for improved treatments for blood disorders and improved methods for SCT in the future.

Populärvetenskaplig sammanfattning

Vårt blod består av flera olika celltyper som är nödvändiga för att bland annat transportera syre och bekämpa infektioner. Varje dag behöver kroppen miljontals blodceller för att upprätthålla dessa funktioner. Blodstamceller finns i benmärgen och ansvarar för att kontinuerligt förse blodet med nya blodceller. Tack vare deras unika egenskaper har blodstamceller förmågan att återuppbygga ett helt nytt blodsystem. Detta möjliggör användning av blodstamceller för benmärgstransplantation (även kallat stamcellstransplantation), vilket för närvarande används för att bota många blodsjukdomar, inklusive svåra leukemier. Dock behövs det ett stort antal celler för att återuppbygga blodsystemet hos en vuxen patient. Många patienter hittar dessutom aldrig en benmärgsdonator som är biologiskt kompatibel. För att övervinna några av dessa problem med stamcellstransplantation är det av yttersta vikt att förstå hur blodstamceller regleras på molekylär nivå. Ett av målen med min forskning har varit att undersöka hur vissa molekyler eller proteiner reglerar blodstamcellers olika tillstånd, till exempel huruvida blodstamceller befinner sig i ett vilande läge eller aktivt förökar sig genom celldelning för att öka i antal eller bilda nya blodceller. Sådan kunskap skulle kunna bidra till förbättrade metoder för att upprätthålla och även tillväxa blodstamceller utanför kroppen, eller förbättra deras förmåga att återuppbygga ett blodsystem efter transplantation.

I min första artikel undersökte vi en molekyl som kallas SLFN2. Vi såg att möss som saknar normalt SLFN2 har färre blodstamceller än normala möss och att dessa blodstamceller var sämre på att återuppbygga ett blodsystem efter transplantation. Vi såg även att en möjlig orsak till detta var att SLFN2 har en påverkan på celldelning, vilket är av enorm betydelse för stamceller så att antalet stamceller inte minskar och för att de ska kunna fungera korrekt efter transplantation.

I min andra artikel undersökte vi en signalväg, det vill säga en kaskad av molekyl- eller protein-interaktioner som tillsammans påverkar en cell. Vi använde genetiskt modifierade möss där en molekyl saknades, den så kallade BMP-receptorn i BMP-signalvägen. Detta stängde av de signaler BMP skulle ha genererat i normala blodceller. Vi såg att blodstamceller som saknar BMP-signalering var sämre på att återuppbygga ett blodsystem efter transplantation. Vi upptäckte att detta berodde på att en annan molekyl, TJP1, påverkades vid avsaknad av BMP-signaler. Båda de ovan beskrivna studierna bidrar med nya observationer och värdefull kunskap om hur dessa molekyler reglerar blodstamceller, särskilt vid transplantation.

Stamcellstransplantation är en procedur som innebär många risker. Patienter behöver genomgå påfrestande behandlingar, ofta med cellgifter eller strålning, för att avlägsna deras egna sjuka blodceller, inklusive deras blodstamceller. Efter detta transplanteras nya blodstamceller för att återuppbygga ett friskt blodsystem. Ett annat mål med min forskning har varit att utvärdera om mildare strålbehandling skulle vara tillräckligt inför stamcellstransplantation vid särskilda blodsjukdomar. För att undersöka detta använde vi, i min tredje artikel, genetiskt förändrade möss med symptom liknande de man kan se hos patienter med blodsjukdomen Diamond-Blackfan-anemi, där produktionen av röda blodkroppar är kraftigt minskad. Vi såg att stamcellstransplantation kunde vara framgångsrik även utan förbehandling med strålning. Detta visar principiellt att friska blodstamceller vid transplantation kan utkonkurrera och ersätta sjuka blodstamceller i benmärgen. De resultat som presenterats i studien kan vara av kliniskt värde, eftersom mildare förbehandlingar inför stamcellstransplantation kan minska svårighetsgraden av biverkningar och leda till förbättrade utfall för patienter.

Sammanfattningsvis bidrar min avhandling med ny förståelse om hur blodstamceller regleras i sin naturliga omgivning och om möjligheten till mildare förbehandling innan stamcellstransplantation. Dessa upptäckter skulle kunna utgöra en språngbräda för framtida förbättrade behandlingar mot blodsjukdomar och förbättrade metoder vid stamcellstransplantation.

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"Do… or do not. There is no try."

Yoda (Star Wars Episode V)

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Paper II

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On the cover

The cover art for this dissertation is made by Ben Soper and Laura Tarnawski and shows an abstract interpretation of haematopoiesis. What is your view on it?

I see several aspects of haematopoiesis; red blood splatter representing mature blood cells in the circulation, the golden drop a rare haematopoietic stem cell, red droplets the process of haematopoiesis in-between stem cell and mature cells, and the black splatter apoptosis or haematopoiesis gone wrong as in leukaemia. It is also just very cool artwork and I am happy to be able to show it on my book.

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Novel Insights into Haematopoietic Stem Cell Regulation and Function

The work in this dissertation focuses on haematopoietic stem cells, i.e. the rare cells in the bone marrow responsible for the life-long production of blood cells. It summarizes research regarding two novel regulators of haematopoietic stem cell self-renewal and quiescence, as well as research investigating novel conditioning regimens prior to curative HSC transplantation for blood disorders.

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