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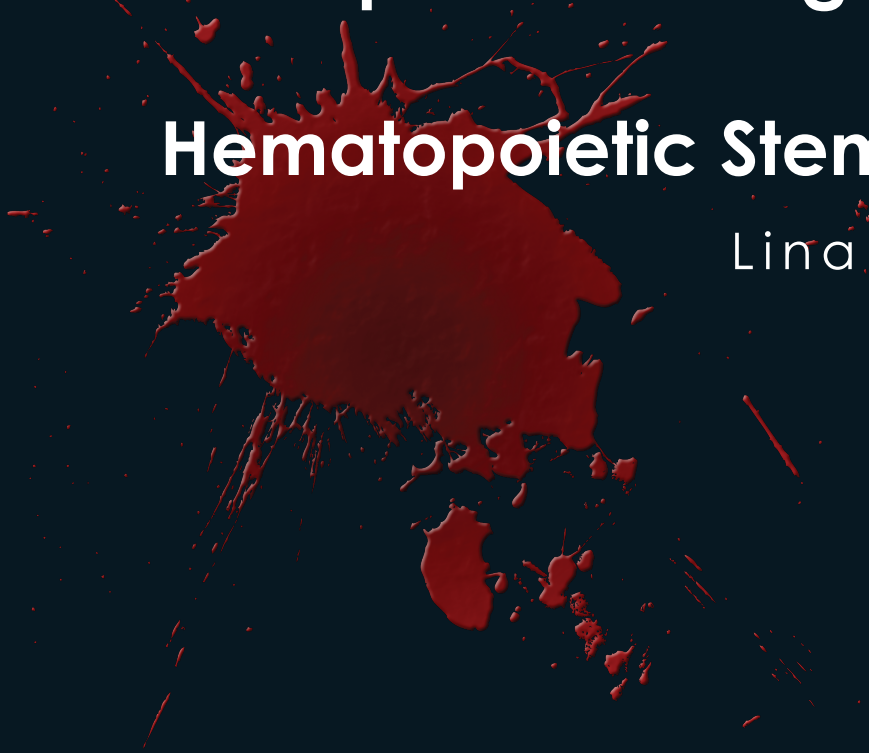

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Stem Cell Biology and Cell Therapy
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To my family

Articles and Manuscripts Included in this Thesis

Article I **Kit regulates maintenance of quiescent hematopoietic stem cells.**
L. Thoren, K. Liuba, D. Bryder, J. Nygren, C. Jensen, H. Qian, J. Antonchuk, SE Jacobsen.
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Article II **Important role of intact Kit signaling in thrombopoietin-independent maintenance of adult hematopoietic stem cells.**
L. Thoren*, H. Qian*, SE. Jacobsen
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* equal contribution

Article III **Critical role of Thrombopoietin in maintaining adult quiescent hematopoietic stem cells.**
H. Qian, N. Buza-Vidas, C. Hyland, C. Jensen, J. Antonchuk, R. Mansson, L. Thoren, M. Ekblom, W. Alexander, SE Jacobsen.
Cell Stem Cell. 2007. 1(6): 671-684

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List of Abbreviations

AGM	aorta-gonads-mesonephros
AML	acute myeloid leukemia
BM	bone marrow
BrdU	bromodeoxyuridin
CAMT	congenital amegakaryocytic thrombocytopenia
CFU-S	colony forming unit-spleen
CMP	common myeloid progenitor
CDKI	cyclin dependent kinase inhibitor
CRU	competitive repopulating unit
FL	fetal liver
Flt3L	flt3 ligand
FACS	fluorescence activated cell sorting
5-FU	5-fluorouracil
GMP	granulocyte macrophage progenitor
GVHD	graft versus host disease
γc	common gamma chain (mouse strain, same as SCID)
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IL	interleukin
IV	intra venous
KL	kit ligand
LSK	$lineage^{-} Sca1^{+} kit^{+}$
LMPP	lymphoid primed multipotent progenitor
MPP	multipotent progenitor
MEP	megakaryocyte erythroid progenitor
MHC	major histocompatibility complex
NK	natural killer
PB	peripheral blood
pSp	para-aortic splanchnopleura
RU	repopulating unit
SCID	severe combined immunodeficiency (mouse strain, same as γc)
SH2	scr homology 2
Sl	steel locus (mouse strain)
SLAM	signaling lymphocyte activation molecule
Thpo	thrombopoietin
WT	wild type
YS	yolk sac

Introduction

The zygote is the ultimate stem cell. It harbors the potential to create an entire organism through numerous differentiation steps that ultimately lead to the development of organs and tissues, each responsible for specific biological functions. The current belief implicates that several organs and tissues, like the hematopoietic system, are colonized by tissue specific stem cells that are able to provide a life-long supply of organ specific mature cells.

Hematopoietic stem cells (HSC)s have been characterized in more depth compared to other tissue specific stem cells, and the accumulated knowledge concerning the properties of these cells have made it possible to utilize them in treatment of several hematological diseases through bone marrow (BM) transplantation. Several circumstances have facilitated the characterization of the HSCs such as 1) the blood system being a soluble organ where the cells are in a single cell suspension, making them easy to isolate and handle in an experimental setting, 2) the development of transplantation assays for *in vivo* functional evaluation of the HSCs and 3) the development of fluorescence activated cell sorting (FACS) as an analytical tool for subfractionation and phenotypic analysis.

Despite the fact that much knowledge has been gathered concerning the biology of HSCs, many questions still remain. Gaining a deeper understanding for the identity of HSCs and the mechanisms governing their maintenance, expansion, survival and fate choices are of importance for improving therapies utilizing HSCs. For this reason we have chosen to study the role of two different cytokines, Kit ligand (KL, also referred to as Mast Cell Growth Factor or Stem Cell Factor) and Thrombopoietin (Thpo) and their respective receptors Kit and c-Mpl, and their effect on HSCs during steady-state maintenance as well as during regeneration of hematopoiesis post transplantation. Throughout this thesis I will describe the current understanding of *murine* HSCs. I have for simplicity entitled the enriched HSC populations "HSC", with the awareness that these are considered to be heterogeneous populations comprising early stem and progenitor cells.

Hematopoiesis and the Immune System

The Blood System

The hematopoietic system provides several important functions in the organism such as oxygen transportation, blood coagulation, and protection against bacterial, viral and parasitic infections. To fight infection the blood system affords two lines of defense; innate and adaptive immunity; processes mediated by distinct cellular subsets commonly referred to as white blood cells. Some mature blood cells can live for years whereas the majority is more short-lived, with some cell types persisting for only a few hours. This dictates a high demand on the precursors for continuous blood cell production. Current understanding poses that the ultimate precursor of blood cell production in an adult mouse or human is the HSC. In a healthy human of 70 kilograms it is estimated that around 1,000,000,000,000 new mature blood cells are produced per day. However, during infections the production of white blood cells can be increased by a factor of 10 or more to meet the increased needs (Kaushansky, 2006). The road from HSCs to a mature blood cell is characterized by numerous differentiation steps, involving commitment of progenitor cells thus leading to increasingly restricted lineage potential (illustrated in figure 1). The classical model of hematopoiesis strictly divides differentiation into one myeloid and one lymphoid pathway. Both myeloid (common myeloid progenitor, CMP) and lymphoid (common lymphoid progenitor, CLP) restricted progenitors and have been identified (Akashi et al., 2000; Kondo et al., 1997; Pronk et al., 2007), but also progenitors capable of lymphoid and myeloid lineage commitment but restricted to the erythroid and megakaryocytic lineages (lymphoid primed multipotent progenitors, LMPPs)(Adolfsson et al., 2005) arguing against the classical model being the only way that commitment can take place. Through further differentiation steps, these intermediate progenitors can generate the mature cells of the hematopoietic system and in the next section I will introduce these cell types and their functions.

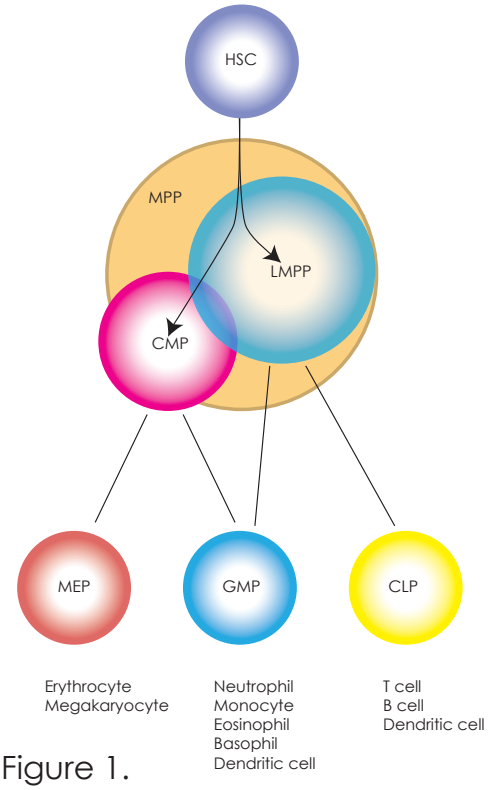

Figure 1.
The descendants of HSCs. *The HCS is thought to be the cell constantly replenishing the blood system with mature blood cells. Lineage commitment is accompanied by numerous differentiation steps bypassing several identified lineage restricted precursors like the CMPs, LMPPs, MEPs, GMPs and CLPs. The illustration is a speculative drawing of how the hematopoietic system might be organized. The overlapping circles represent the overlapping properties within the populations.*
(Adopted from: Iwasaki H. et al 2007)

Oxygen Transportation and Coagulation

Erythrocytes and thrombocytes are both believed to originate from a common downstream precursor, the megakaryocyte erythroid progenitor (MEP). However, the end products serve very different functions in the organism. The erythrocyte, or red blood cell, mediates the transportation of oxygen throughout the organism by binding of oxygen molecules to its iron containing heme group. By contrast, the thrombocytes, or platelets, arise from megakaryocytes and are vital for the blood coagulation process and wound repair.

Cells of the Innate Immunity

The cells of the innate immunity provide an instant defense against infections by elimination of cells through phagocytosis or destruction. They include:

- Macrophages, derived from monocytes, efficiently eliminate bacteria or infected cells by phagocytosis.
- Granulocytes, of which there exist several subtypes (neutrophils, basophils and eosinophils). The phagocytic neutrophils are the most abundant cell type of granulocytes and particularly involved in the defense against bacterial infections whereas eosinophils and basophils combat parasitic infections.
- Natural killer (NK) cells are cytotoxic lymphocytes and eliminate virus infected and tumor cells by release of cytoplasmic granules that initiates apoptosis of their target cells.
- Dendritic cells function as potent antigen presenting cells and serve as a link between the innate and the adaptive immune system.

The effects of the innate immunity are not long-lasting, but they serve an important function as they activate the adaptive immune system by presenting foreign antigens.

Cells of the Adaptive Immunity

The adaptive immunity is often considered a second line of defense, which in contrast to the innate immune system mounts a more specific defense against invading pathogens. In addition, it has the ability to confer long-lasting protection. A fundamental property of the adaptive immune system is the ability to recognize antigens belonging to self (host cells), non-self (bacteria or parasites) or missing-self (virus or tumor cells) to ensure proper selection of cells to eliminate. Lymphocytes, divided into B and T cells, are the effector cells of the adaptive immune system. Antigen presenting cells (macrophages, neutrophils or B cells) activates the adaptive immune response by antigen presentation on their cell surface mediated by major histocompatibility complex (MHC) molecules (Class I for invaded and Class II for engulfed pathogens). Viruses lack antigen specificity and tumor cells can re-modulate their normal MHC Class I and will therefore be recognized as missing-self.

Hematopoietic Stem Cells

The hematopoietic system is thought to be organized in a hierarchical manner, starting with HSCs that are able to generate downstream progeny of all hematopoietic lineages through numerous differentiation steps. Our current definition of HSCs proposes that HSCs can supply the organism with mature blood cells of all lineages (multilineage potential) for a lifetime (long-term) while at the same time maintaining the size of the HSC pool (self-renewal potential). Depending on the physiological demands, HSC can chose to stay quiescent, differentiate, self-renew or undergo apoptosis to maintain tissue homeostasis.

The Commencement of Stem Cell Research

Almost 50 years ago, it was shown that transplantation of BM cells could rescue lethally irradiated mice, suggesting that BM failure was the ultimate cause of the irradiation induced deaths seen after the atomic bombs (Little and Storb, 2002; Main and Prehn, 1955); however, the mechanism behind the successful transplantations was at that time unknown. Dr. James Till and Dr. Earnest McCulloch were the two pioneers of stem cell research. Through a series of ground breaking experiments during the 1960s, they discovered that by quantitatively transplanting BM cells of healthy mice into irradiated recipient mice (myeloablation) and assessing the number of survivors (McCulloch and Till, 1960), one could measure the number of cells that retained the capacity of forming macroscopic spleen colonies, so called Colony Forming Units of the Spleen (CFU-S) (Till and McCulloch, 1961), representing hematopoietic colonies found on the spleen 7-11 days post transplantation. By the use of irradiation induced chromosomal marking, they could prove that one CFU-S originated from one single cell, proving their clonality (Becker et al., 1963). In addition, they were serially transplantable into secondary recipients (Siminovitch et al., 1963) although at declining frequencies (Siminovitch et al., 1964) for the first time demonstrating the existence of a stem cell. The experiments tracing cells with irradiation induced chromosomal marking were subsequently criticized since the irradiation could have caused chromosomal translocations within the genomic DNA possibly generating abnormally high levels of cell proliferation within a clone that could be mistaken for a stem cell. To circumvent this, they used retroviral marking of genomic DNA and demonstrated for the first time that HSCs upon transplantation could give rise to cells of both myeloid and lymphoid lineages (Dick et al., 1985; Keller et al., 1985; Keller and Snodgrass, 1990). Furthermore, single HSC clones shown to be capable of multilineage engraftment in both primary and secondary recipients (Jordan and Lemischka, 1990).

Although later it has been shown that the cells colonizing the spleen and subsequently generating the CFU-Ss are *not* HSCs, but rather early myelo-erythroid progenitors (Jones et al., 1989; Magli et al., 1982; Na Nakorn et al., 2002), Dr. Till and Dr. McCulloch's work showed that one cell can multilineage differentiate, a hall mark property of the HSCs. Their findings initiated the efforts in stem cell biology of which we can see the clinical benefits today in terms of patients with hematological diseases being rescued by BM transplantation.

When and Where are the First HSCs Generated?

Hematopoietic cells are derived from the mesodermal layer, which is one of three germ layers that are formed during gastrulation. In the mouse, the first blood cells appear at day 7 in the yolk sac (YS) and are subsequently found in the para-aortic splanchnopleura (pSP) aorta-gonad-mesonephros (AGM), fetal liver (FL) and finally in spleen and BM. Both the pSp-AGM and the YS have been postulated to be the location of the earliest HSC in the embryo (Medvinsky and Dzierzak, 1996; Samokhvalov et al., 2007) creating an yet unsolved controversy within the field, and finding experimental evidence to resolve it has proven to be a challenge for two reasons. First, it is unknown if the *in vivo* repopulation assays used for evaluation of fetal HSCs of day 7-9, using new born or immunocompromised mice as recipients, are truly optimal conditions for these cells, as they appear unable to read out without pre-culturing (Yoder, 2004). Next, the establishment of circulation at day 8 makes it difficult to determine whether HSC were generated at the site or have colonized it through the circulation. Below, I will day by day describe the current understanding of the development of HSCs in the YS, pSP-AGM, FL, spleen and BM.

Day 7

The first hematopoietic cells arise around day 7-7.5 in the mesoderm of the YS, an extra embryonic site (Moore and Metcalf, 1970). YS mesodermal cells generate blood islands, aggregates of hematopoietic cells surrounded by layers of endothelial cells, probably through direct commitment to endothelial and hematopoietic cells, although an intermediate precursor called hemangioblast has been suggested (Ueno and Weissman, 2006). At day 7.0 only nucleated erythrocytes are present, sometimes entitled primitive erythrocytes because they retain their nucleus, and around day 7.5 myeloid progenitors appear as well (Cumano and Godin, 2007).

Day 8

During day 8 the blood circulation is established (McGrath et al., 2003) and intraembryonic hematopoietic precursors will appear at around day 8.5 for the first time (Godin et al., 1995). However, it has been difficult to demonstrate that these cells are HSCs. For instance, cells isolated from day 8 YS followed by 3 days of explant culture do not multilineage engraft *Rag2*$^{-/-}$ *x common gamma chain* (γc)$^{-/-}$ mice, even though these mice provide a NK cell free environment suitable for lymphoid engraftment (Cumano et al., 2001). However, some engraftment can be observed following pre-culture for two days on pSP-AGM stroma (Matsuoka et al., 2001). This could either reflect that the cells are too immature, or in need of extrinsic signals from the pSP-AGM stroma. Cells of the pSP-AGM can after explant culture short term engraft *Rag2*$^{-/-}\gamma c^{-/-}$ mice at this time point (Cumano et al., 2001). In addition, by inducible *in vivo* tracing of *Runx1*, induced day 7.5 in the YS, it has been suggested that cells migrate from the YS into the circulation and the embryo around day 8 (Samokhvalov et al., 2007).

Day 9

It is disputed whether the hematopoietic cells in the YS have the potential to produce lymphoid cells at day 9. Some studies have demonstrated the potential of the YS blood islands to long-term multilineage reconstitute new born mice after transplantation (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b), while others are unable to demonstrate B cell potential of these cells upon repopulation into severe combined immunodeficiency (SCID) mice. In contrast, cells from day 9 aorta (part of the pSP-AGM) can generate B cells upon transplantation into SCID mice (Godin et al., 1993; Mikkola and Orkin, 2006).

Day 10

At day 10, cells from the pSP-AGM will upon transplantation give rise to CFU-S, which is in contrast to cells from the YS at this timepoint (Medvinsky et al., 1993). Furthermore, cells of the pSP-AGM will upon transplantation long-term repopulate adult mice (Medvinsky and Dzierzak, 1996) providing the first evidence of multipotent HSCs in the embryo that read out in traditional BM transplantation assays into myeloablated adult recipients. Upon separation of the three pSP-AGM layers, the HSCs were predominantly found within the aorta (Godin et al., 1999). However, the numbers are quite small as the entire pSP-AGM has at day 10 only generated about 500 HSCs (Godin et al., 1999).

In addition, inducible *in vivo* lineage tracing of *Runx1*, labeled at day 7.5 in the YS, show high levels of YS contribution to cells within the dorsal aorta around day 10.5 (Samokhvalov et al., 2007), suggesting that some cells in the dorsal aorta are YS descendants.

Finally, during this day hematopoietic cells initiate the seeding of the FL. However, the site(s) that seeds the liver is/are still controversial (Cumano and Godin, 2007).

Day 11

The first HSCs appear in the FL during day 11. At the same time point, cells from the YS are able to repopulate adult mice (Kumaravelu et al., 2002); however, it is unclear whether the presence of HSCs in the YS is due to de novo generation of HSCs or due to colonization.

Day 12

The FL has by day 12 become the primary site for HSCs and their downstream progenitors. In addition, the placenta has also been indicated to be a site of HSCs around day 12.5; however, whether this is due to de novo HSC production or to transient colonization remains unclear (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). In addition, inducible *in vivo* lineage tracing studies using *Runx1*, labeled in the YS at day 7.5, has revealed contribution from YS descendants to myeloid, erythroid and lymphoid lineages in FL and thymus at day 12.5 (Samokhvalov et al., 2007).

Day 13-16

During day 13-16 the HSCs in the FL are expanding. It has been shown that from day 12-16 the absolute number of competitive repopulating unit(s) (CRU; an assay for quantifying HSCs, which will be described in more detail in the section "BM transplantation in mice") in FL expands almost 40-fold (Ema and Nakauchi, 2000) and at this stage the HSCs are rapidly dividing. In parallel, the homing to the fetal spleen starts at day 13, but at this site HSCs rather differentiate than expand (Bertrand et al., 2006; Godin et al., 1999).

Day 17- 18

At day 18 the seeding of the BM starts, and the BM remains the primary site of the HSCs throughout the adult life, except during situations of stress such as myeloablation that has been suggested to cause extramedullary hematopoiesis in adult mice including relocation of HSCs (Heissig et al., 2005; Kopp et al., 2005). In addition, inducible *in vivo* lineage tracing studies using *Runx1*, labeled in the YS at day 7.5, has revealed YS derived contribution to myeloid, erythroid and lymphoid lineages in FL and thymus of day 18.5 (Samokhvalov et al., 2007).

Taken together, most findings suggest, although not finally proven, that the first hematopoietic cells arise in the YS at day 7, and the first HSCs appear in the YS and/or pSP-AGM day 9-10, and it remains unclear whether the YS and placenta are capable of any de novo production of HSCs (Cumano and Godin, 2007; Mikkola and Orkin, 2006; Samokhvalov et al., 2007). In addition, it is unclear if the pSP-AGM is the major site ultimately seeding the FL, although many experiments points in that direction. Finally, the FL is the primary site of HSC expansion during day 13-16 resulting in massive expansion of HSCs that ultimately will seed the BM around day 18.

The Hematopoietic Stem Cell Niche

The concept of a niche was first proposed in 1978 by Dr. Robert Schofield (Schofield, 1978) suggesting that the stem cell activity would be preserved if the HSC remained in a BM microenvironment in close contact with other cells - the niche (illustrated in figure 2). Further, an asymmetric cell division would generate one daughter cell remaining in the niche and retaining stem cell properties, while the other would differentiate out to a mature cell unless finding a vacant niche.

The signals required for HSC maintenance and self-renewal are thought to, at least in part, come from the niche. The exact physical location of the niche has been difficult to establish due to technical difficulties originating in the number of antigens needed for visualization of HSCs in BM sections (Wagers, 2005). However, recent studies have opened up new venues in this area by identification of the three novel lymphocyte activation molecules (SLAM) markers (Kiel et al., 2005), allowing for examination of HSCs in their physiological location.

Figure 2.
Extrinsic signals provided by the niche. *The bone marrow in the trabecular bone area is the primary site of HSCs in the adult mouse or human, also referred to as the niche, and is thought to provide HSCs with extrinsic signals promoting self-renewal and quiescence. Two different niches have been proposed, the endosteal niche, made up of osteoblasts lining the bone, and the vascular niche made up of sinusoidal endothelial cells lining the sinusoids; however, the exact biological functions of these two niches remains unclear.*
(Adopted from: Yin et al., 2006)

HSCs seed numerous niches in several anatomically distinct places during ontogeny, from fetal development in YS, pSp-AGM and FL, followed by the seeding of the adult niches in spleen and BM. The spatial relocation of the niche seem to time wise correlate well with the organogenesis of these tissues, suggesting that the niche environment is created before HSCs arrive, and further, the formation of the niche is the rate-limiting factor during the HSC seeding of these tissues (Jones and Wagers, 2008).

HSCs have been postulated to be localized in at least two independent anatomical niches, the endosteal niche and the vascular niche, but the functions of these two niches remain controversial (Wagers, 2005; Wilson and Trumpp, 2006; Yin and Li, 2006). The endosteal niche has been proposed to physically be located at the endosteum of the bone in the trabecular bone area and to be composed of osteoblasts that through signaling via N-cadherin, Tie-2 and Ang-1 anchors HSCs to the niche, possibly keeping them in a quiescent state (Arai et al., 2004; Zhang et al., 2003). Studies have shown that an increase in the number of osteoblasts correlates with an increase in HSCs (Calvi et al., 2003; Zhang et al., 2003) Further, KL and in particular the membrane bound KL expressed

on osteoblasts, has also been suggested to play an important role in the endosteal niche (Driessen et al., 2003; Miyazawa et al., 1995).

Evidence for the existence of a vascular niche has only recently emerged due to robust visualization of HSCs *in situ* (Heissig et al., 2002; Kiel et al., 2005) and consequently our knowledge in this area is limited. The vascular niche has been suggested to be located near the center of the marrow, close to the vascularization network. Previously, it was thought that the vascular niche emerged temporarily due to myeloablative stress (Avecilla et al., 2004; Heissig et al., 2002; Kopp et al., 2005), but more recent studies have suggested that HSCs are attached to the endothelial cells lining the vascular niche during steady-state hematopoiesis, indicating a role of this niche during steady-state conditions as well (Kiel et al., 2005).

Further, endothelial cells have been indicated to play an essential role in the hematopoietic system as a study silencing the VE-cadherin, which is expressed on endothelial cells, by the use of blocking antibodies, resulted in hematopoietic failure (Avecilla et al., 2004; Yin and Li, 2006), which is similar to studies eliminating osteoblasts in the endosteal niche (Visnjic et al., 2004).

Self-renewal of Hematopoietic Stem Cells

Self-renewal is an essential mechanism of the HSC in order to maintain the HSC pool during homeostatic conditions and providing a lifelong continuous blood supply for the organism (Domen and Weissman, 1999; Wilson and Trumpp, 2006). A cell division producing at least one daughter cell that is a replica of the mother cell is a self-renewing cell division, and in the context of the HSC, it means that at least one of the two daughter cells maintains HSC identity. To maintain tissue homeostasis, a fine balance between symmetric (producing two identical daughter cells) and asymmetric (producing two different daughter cells) cell divisions have to be attained. Even though the knowledge concerning the mechanism of self-renewal is limited, it has been proposed that regulation of HSC self-renewal is dependent upon extrinsic signals, such as those provided by the niche environment, but also by intrinsic signals, such as anti-apoptotic proteins, polycombgroup proteins, transcription factors, adaptor proteins and cell cycle regulators that are expressed by the HSC itself (illustrated in figure 3).

The regulation of survival and death of HSCs needs to be tightly regulated to uphold homeostasis without development of hematological disease (Oguro and Iwama, 2007). Anti-apoptotic proteins, in particular members of the Bcl-2 family, have been implicated to be important for HSC self-renewal. For example, *Mcl-1* conditional knock-out mice show near complete BM failure, including loss of the hematopoietic stem and progenitor cells as well as severely impaired survival in steady-state. Overexpression of *BCL-2* under the H2K (MHC Class I) promoter, led to an increased number of phenotypic (Domen et al., 1998) and functional (Domen et al., 2000) HSCs as well as increased radioprotection (Domen et al., 1998).

Several transcription factors have been suggested to be important in the process of HSC self-renewal where the family of homeodomain containing Hox transcription factors have caught much attention since overexpression of *HoxB4* can expand HSCs *in vivo* and *ex vivo* without any signs of leukemia (Antonchuk et al., 2001; Antonchuk et al., 2002; Argiropoulos and Humphries, 2007; Sauvageau et

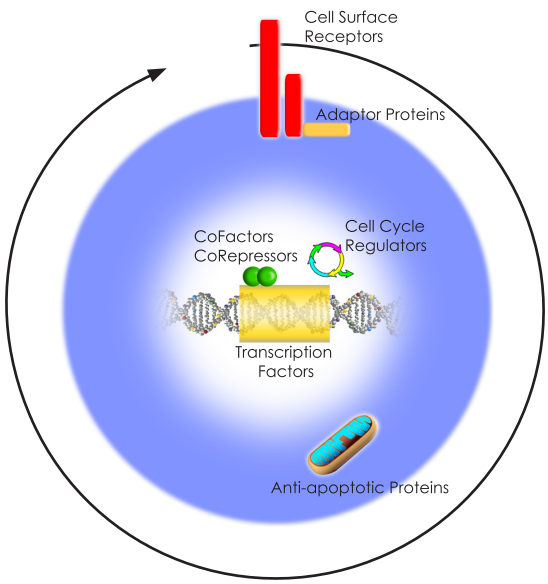

Figure 3.
Intrinsic signals provided by a range of cellular proteins. *HSCs are thought to retain intrinsic signals that promote self-renewal and quiescence by a wide range of cellular proteins. For example; cell surface receptors, adaptor proteins, cell cycle regulators, transcription factors and cofactors and anti-apoptotic proteins have been shown to be important for these processes.*

al., 1995; Thorsteinsdottir et al., 1999). Surprisingly, the *HoxB4*$^{-/-}$ mice only showed a marginally reduced HSC compartment (Bijl et al., 2006; Brun et al., 2004), suggesting a lot of redundancy among the Hox-genes, or that HoxB4 normally do not regulate self-renewal. HoxB4 mediated *ex vivo* expansion has mechanistically been proposed to be a consequence of direct DNA interaction with the Hox cofactor, Pbx1 and Meis (Beslu et al., 2004; Moskow et al., 1995). A recent study showed that simultaneous repression of *Pbx1* and overexpression of *HoxB4* resulted in a 100,000-fold *ex vivo* expansion of HSCs (Cellot et al., 2007). Another Hox-gene, HoxA9 has also been shown through repopulation studies to be central for HSC self-renewal (Lawrence et al., 2005).

The zinc finger repressor Gfi-1 is a transcription factor demonstrated to be an important regulator of self-renewal. *Gfi-1*$^{-/-}$ mice have reduced numbers of functional HSC (Hock et al., 2004a; Zeng et al., 2004), which is most likely explained by increased turnover in the HSC compartment, indicating that Gfi-1 plays an important role in restricting proliferation in the HSC compartment possibly by inhibition of the cyclin-dependent kinase inhibitor (CDKI) p21$^{cip1/waf1}$ (Hock et al., 2004a), although disputed (Zeng et al., 2004).

Further, the transcription factor c-Myc has been shown to play an important role in regulating the balance between self-renewal and differentiation of the HSC compartment by regulating proper release of HSCs from their BM niche, thereby allowing differentiation to take place (Wilson et al., 2004; Wilson and Trumpp, 2006).

During ontogeny, the requirement for factors regulating self-renewal changes and several transcription factors such as, Tal-1/Scl and Aml-1/Runx-1 have been shown to be important during fetal development, but dispensable during adult hematopoiesis (Ichikawa et al., 2004; Mikkola et al., 2003; Okuda et al., 1996; Shivdasani et al., 1995). Tel/Etv6, an Ets-related transcriptional repressor, has on the other hand been shown to be essential for both fetal hematopoiesis (Wang et al., 1997) and adult HSCs maintenance (Hock et al., 2004b).

The polycomb group proteins are epigenetic transcriptional repressors that have been indicated to play an active role in regulation of HSC self-renewal possibly through regulation of Hox gene clusters (Zeisig et al., 2004). Bmi-1, a polycomb group repressor, has through knock-out studies been demonstrated to play an important role in the maintenance of adult HSCs, although dispensable for fetal hematopoiesis (Iwama et al., 2004; Park et al., 2003). Interestingly, overexpression of *HoxB4* in *Bmi-1*$^{-/-}$ HSCs does not promote the HSC expansion that is usually seen, suggesting that HoxB4 mediated HSC expansion requires functional Bmi-1 (Iwama et al., 2004).

The function of adaptor proteins is to integrate different signaling pathways and one of these proteins, Lnk, has recently been shown to be involved in Thpo dependent HSC self-renewal. Lnk has by two independent groups been shown to regulate the size of the HSC pool in mice, as HSCs in young adult *Lnk*$^{-/-}$ mice were shown to be expanded both phenotypically and functionally (Buza-Vidas et al., 2006; Ema et al., 2005). Further, this expansion has been suggested to primarily be induced by Thpo (Buza-Vidas et al., 2006; Seita et al., 2007).

As all other cells, HSCs are thought to communicate and receive signals from the environment through different cell surface receptors. An example is the importance of HSC interaction with osteoblasts, as part of the niche, as has been previously described. The Wnt frizzled receptors, via the downstream signaling trough β-catenin, have been shown to be essential for proper HSC maintenance and differentiation (Kirstetter et al., 2006; Scheller et al., 2006) although disputed (Reya et al., 2003). In addition, the cell surface receptors Kit and c-Mpl, and their respective ligands, KL and Thpo, have been implicated to be important for self-renewal, and these will be discussed in much more detail under the heading "Cytokine regulation of HSCs".

Maintaining quiescence is a key property of the HSC (which is discussed in the section "Turnover and cell cycle kinetics in the HSC compartment"), and several cell cycle regulators have been implicated in HSC self-renewal. The late G_1 phase CDKI $p21^{cip1/waf1}$ of the Cip/Kip family has been suggested to maintain HSC quiescence, as demonstrated by increased turnover in the steady-state HSC compartment of $p21^{cip/waf1-/-}$ mice, and exhaustion of HSCs upon serial-transplantations (Cheng et al., 2000b), although, this was recently challenged (van Os et al., 2007). Further, the complete reverse phenotype was found in $p27^{kip1-/-}$ mice, a member of the same CDKI family (Cheng et al., 2000a).

Studies of CDKIs of the Ink4 family have demonstrated expansion of both the stem and progenitor cell compartments in mice lacking $p18^{Ink4c}$ (Yuan et al., 2004), while no apparent hematopoietic phenotype have been demonstrated in mice lacking the CDKIs $p16^{Ink4a}$ and $p19^{Arf}$ (Stepanova and Sorrentino, 2005) or the Retinoblastoma protein (Walkley and Orkin, 2006). Collectively, these data suggests that different CDKIs administrate very different, but distinct functions in the HSC compartment, some of which are essential for self-renewal.

Methods to Study Properties of HSCs

In order to study the factors governing the regulation of HSCs, such as cytokines, it is essential to purify the HSCs from the remaining cells of the BM. The HSCs are a rare population of cells and identification and enrichment continues to be problematic since *one* specific HSC phenotype has not yet been identified, and presumably even the best enrichment strategies available generate heterogeneous HSC populations. Below I will describe different approaches and enrichment strategies that have been undertaken in order to better purify HSCs – 1) cell intrinsic physical properties 2) monoclonal antibodies and 3) fluorescent vital dyes.

Enrichment of HSCs by Cell Intrinsic Physical Properties

Isolation of HSCs from samples of whole BM was initially done by investigation of their physical properties. A morphological description of the cells based on their light scattering properties was measured by FACS as described as forward scatter and side scatter, indicative of the size and granularity, respectively. HSC enriched populations had a diameter of 7.1-7.5 micron and their granularity increased with maturation (van den Engh and Visser, 1979; Visser et al., 1980). In addition, isolation by a density gradient

followed by labeling with fluorescent wheat germ lectin allowed for further purification (Visser and Bol, 1982).

Identification of HSCs by Cell Surface Antigens

Cell surface markers identified by monoclonal antibodies is a well developed method for enrichment of HSCs. The Stem Cell Antigen-1 (Sca-1, also referred to as Ly-6A.2) was early identified as a way of enriching for BM HSCs (Spangrude et al., 1988), as well as elimination of mature blood cells by negative selection for B220 (B cells), Gr-1 (granulocytes), Mac-1 (myelomonocytic cells) and CD4, CD8 (T cells) that are collectively referred to as lineage negative cells (Muller-Sieburg et al., 1986; Spangrude et al., 1988; Uchida and Weissman, 1992; Wineman et al., 1992). However, activated HSCs, such as in FL or after 5-fluorouracil (5-FU) treatment, have later been shown to express both Mac-1 and CD4 (Morrison et al., 1995; Morrison et al., 1997; Randall and Weissman, 1997; Szilvassy and Cory, 1993). In mice expressing the Thy-1 allele, but not the Thy1.2 counterpart, the antigen Thy1.1 can be used to enrich for HSCs which are found in the Thy-1^{low} fraction (Spangrude and Brooks, 1992). Another robust marker is the expression of Kit which is routinely used together with Lineage negative, Sca-1^{+} to enrich for HSCs (Ikuta and Weissman, 1992; Li and Johnson, 1995).

Human HSCs have been shown to express the CD34 antigen (Civin et al., 1984), while murine BM HSCs were found in the $CD34^{-/low}$ fraction of the Lineage negative, $Sca1^{+}$, Kit^{+} (LSK) compartment (Osawa et al., 1996). However, murine embryonic HSCs from FL (Sanchez et al., 1996) and AGM (Yoder et al., 1997a) have also been identified as $CD34^{+}$ fraction. The discrepancy in expression pattern of CD34 could be explained by the activation status of the HSC, due to the fact that CD34 has been shown to be upregulated on activated BM HSCs after 5-FU treatment (Sato et al., 1999), while steady-state BM HSCs downregulate CD34 around 7-8 weeks after birth (Ito et al., 2000). Taken together, these data indicate that CD34 is upregulated on activated HSCs and downregulated on quiescent HSCs.

Furthermore, Flt3 (or Flk2 or CD135) is expressed at low levels on HSCs and upregulated on more mature progenitor cells in both FL (Mansson et al., 2007), and BM (Adolfsson et al., 2001; Christensen and Weissman, 2001). In addition, endoglin (or CD105) was identified as a useful marker for purification of HSCs (Chen et al., 2002). More recently, as a result of gene expression profiling of purified HSCs, a trio of signaling SLAM markers, $CD150^{+}$, $CD244^{-}$ and $CD48^{-}$ were identified to enrich for HSCs within the BM and FL (Kiel et al., 2005; Kim et al., 2006).

Identification of HSCs through Vital Dyes

Vital dyes select for metabolic states and biological properties of the HSCs, rather than selecting for identified cell surface markers, with the advantage that biological properties of HSC are thought to be conserved over tissue and species boundaries (Bunting, 2002), which is not the case with cell surface markers and their matching monoclonal antibodies.

Two fluorescent vital dyes have been used to enrich for HSCs, namely Rhodamine 123 (Mulder and Visser, 1987; Spangrude and Johnson, 1990) and Hoechst 33342 (Baines and Visser, 1983; Goodell et al., 1996). Both dyes utilize the fact that HSCs are capable of eliminating entry of toxic substances to the cell, either due to expression of multidrug resistance pumps (Zhou et al., 2001), or to decreased mitochondrial activation (Spangrude and Johnson, 1990). HSC activity is enriched in the Rhodamine 123^{low} fraction of the BM (Spangrude and Johnson, 1990) as shown by transplantation of this population into myeloablated mice (Spangrude et al., 1995) The Hoechst 33342 staining reveals a so called side population of cells where the HSC activity is enriched for in the very tip of this population (Goodell et al., 1996), although this has recently been challenged as HSCs were found outside the tip as well (Morita et al., 2006).

Bone Marrow Transplantations in Mice

Bone Marrow Transplantation Assays

The most rigorous way of identifying HSCs is to evaluate their capacity to regenerate the hematopoietic system by transplantation into a new mouse, which has had its endogenous hematopoietic system eradicated i.e. myeloablated. Myeloablation is most commonly done by whole body irradiation of the mouse, which induces double stranded breaks in the DNA that leads to cell death once the cell enters cell cycle (van Gent et al., 2001), or by treatment with a chemotherapy drug, like 5–FU, a nucleotide analog, ultimately inhibiting cell division (Peters et al., 2002).

Qualitative or Quantitative Evaluation

Quantitative and/or qualitative biological evaluation of HSCs is assessed by experimental transplantation of BM cells into myeloablated mice. To evaluate HSC qualitatively, a long-term repopulating assay can be used, where HSCs are transplanted into myeloablated mice, usually together with a constant reference pool of cells that also serve a radio-protective function during the first 3-4 weeks post transplantation. Around 4 months post transplantation the mice are analyzed for donor derived multilineage engraftment in the peripheral blood (PB) by presence of lymphoid cells and the short lived myeloid cells, indicative of HSC activity.

Quantification of the number of HSCs is commonly performed by either measuring the repopulating activity within 100,000 whole BM cells, the repopulating unit (RU) (Harrison et al., 1993), or by determining the frequency of repopulating units within a whole BM sample based on Poisson statistics, the CRU (Szilvassy et al., 1990). To compare the repopulating potential between different sources of cells the Mean Activity of Stem Cells can be calculated by dividing RU over CRU (Ema and Nakauchi, 2000). The assays include transplantation of whole BM cells, either by competitive bulk transfers (RU) or by limiting dilutions (CRU) together with a constant reference pool of cells, into myeloablated mice, followed by examination of the PB 4 months post transplantation for donor derived long-term multilineage engraftment.

Originally it was believed that the ability to rescue myeloablated mice was a stem cell

property only. However, it was eventually proven that HSCs are not responsible for the initial wave of reconstitution post transplantation, but is rather the task of more mature progenitor cells, and the HSCs were the cells responsible for the long-term reconstitution securing the survival of the organism. The non-competitive repopulation assay was the original transplantation assay used (Jones et al., 1989) and is still used to measure long-term engraftment and repopulation capacity of whole BM cells (usually from genetically modified mice) without a reference population, thereby not competing with wild type (WT) HSCs for vacant niches.

Transplantation of Single HSCs

Transplantation of a single cell is a powerful assay and deserves extra attention as it allows a functional clonal read out of a HSC. However, transplantation of single cells is accompanied with difficulties in achieving high frequencies of transplanted mice generating long-term multilineage engraftment. Therefore, the assay is considered to be inefficient and resource demanding. The highest frequency of long-term engrafted mice reported is 96% (Matsuzaki et al., 2004), however, this has not been reproduced (Camargo et al., 2005) and on average most groups achieve around 20-30% (Osawa et al., 1996). The low reconstitution efficiency has been addressed and one report suggests that the HSC in principle always will produce initial engraftment, but fails to generate long-term output, probably related to loss of self-renewal capacity (Benveniste et al., 2003). The reason for this failure remains to be shown, but the use of heterogeneous cell populations containing multipotent progenitor (MPP) cells that have lost their long-term self-renewal capacity could be one explanation.

Serial Transplantation

Dr. Hayflick proposed, based on work done on fibroblasts, that a cell undergoes limited number of divisions before entering senescence, known as the "Hayflick limit", a process involving shortening of telomeres (Hayflick and Moorhead, 1961; Shay and Wright, 2000).

Early studies done on murine HSCs, tried to establish the number of sequential transplantations, referred to as serial transplantations, which the HSC could fulfill before going into senescence. Evaluation of regeneration capacity by measuring erythrocytes or CFU-S ability established that the HSC could be serially transplanted around 4-5 times (Cudkowicz et al., 1964; Siminovitch et al., 1964). However, the outcome in these experiments is probably affected by factors such as experimental set-up i.e. competitive vs. non-competitive transplantations, and time elapsing between each of the transplantations. Diminished HSC function, as measured by survival and lineage distribution, was demonstrated during the process of serial transplantations (Harrison and Astle, 1982; Spangrude et al., 1995).

Interpretation of Data

When evaluating the results of all transplantation assays a few assumptions have to be made, some of which especially pertain to the work with genetically modified mice. We assume that the test cells after intra venous (IV) injection into the periphery home to the BM with no further delay and find a vacant niche space. In addition, we assume that the test cells can differentiate to mature blood cells, as all assays are evaluated by the presence of mature cells in the PB. If one is trying to address self-renwal, these are all limitations of the transplantation assay. Because of these limitations, interpretation of negative data is more complicated than interpretation of positive data. To address these issues, assays specifically addressing the homing of the HSC are readily available (Katayama et al., 2003) and phenotypic analysis of the donor derived HSC compartment and further staging of progenitors can reveal if and where a differentiation block is located.

Regaining Steady-State Hematopoiesis after Transplantation

Myeloablation is a harsh treatment and it takes a significant period of time for the BM to revert back to steady-state hematopoiesis, if ever. Several parameters are taken into account when evaluating if the BM has regained its steady-state characteristics. For example, cell cycle activity and the telomere length have been evaluated within the HSC compartment four months post transplantation, at a time when the hematopoietic system has recovered in absolute numbers of blood cells. In the primary recipient mice four months post transplantation, Allsopp and co-workers found an enhanced portion of the HSCs to be actively dividing (in SG_2M phase) as compared with non-transplanted WT mice. Evaluation of the telomere length in HSCs at these time points revealed a shortening that increased as the transplantation cell dose was decreased, and was even further exacerbated upon serial transplantation (Allsopp et al., 2001). Shortening of telomeres can be considered an irreversible process, which may suggest that the BM is unable of reverting back to steady-state hematopoiesis post transplantation.

In the clinical setting, young human patients who had undergone allogeneic BM transplantation displayed shorter telomere length in PB cells than their BM donors when investigated 4-82 months post transplantation. The reduction was on average estimated to be equivalent to 15 years of aging, and may represent an irreversible intrinsic change in the HSC post transplantation (Wynn et al., 1998). This could at least in part explain that BM transplant recipients have enhanced propensity of developing leukemia.

Turn Over and Cell Cycle Kinetics in the HSC Compartment

The cell cycle has been divided into several phases where the G_0-phase denotes a stage of cellular quiescence. When signals induce the cell to start dividing, it enters into G_1 where the cell is growing and preparing for chromosomal duplication followed by DNA synthesis and duplication of chromosomes in the S-phase. In G_2M, a second growth phase takes place, followed by the actual cell division. The turnover in the HSC compartment is shifting throughout ontogeny and during stress. Gaining insight into the HSC cell cycle properties will help us understand HSCs in situations of steady-state or stress.

Proliferation Kinetics of HSCs in Steady-State Fetal Liver and Bone Marrow

During ontogeny the HSC cell cycle kinetics changes dramatically, adapting and adjusting to the diverse requirements (expansion or maintenance) of each site (FL or BM). Through Bromodeoxyuridin (BrdU) incorporation studies, it has been shown that nearly all (99%) FL HSCs have cycled at least once after 25 hours (Nygren et al., 2006), while it takes 3-6 months for the BM HSC compartment to achieve the same task (Bradford et al., 1997; Cheshier et al., 1999). The alteration in HSC proliferation kinetics has been suggested to take place between week 3 and 4 after birth, as it has been shown that the BM HSCs undergo a conversion from rapidly cycling HSCs to quiescent non-cycling HSCs during this week (Bowie et al., 2007; Bowie et al., 2006).

Importance of HSC Cell Cycle Status in Bone Marrow Transplantations

The ability of HSCs to regenerate hematopoiesis after transplantation into a myeloablated recipient has been postulated to be entirely dependent on the cell cycle status of the transplanted HSCs (Passegue et al., 2005). Transplantation of BM HSC from the G_0 fraction reconstitute myeloablated mice long-term, in contrast to BM HSCs from G_1 and SG_2M that only reconstitute the recipient transiently (Passegue et al., 2005). This could implicate that all reconstituting activity of adult BM HSCs is found within the G_0 population of adult BM HSCs, however, given the low number of cells transplanted (10 or 50) from heterogeneous populations of HSCs, it cannot be excluded that engraftment failure of HSCs in G_1 could be caused by lack of transplanted HSCs. In contrast, day 14.5 FL HSCs within G_0/G_1 as well as in SG_2M have been shown to long-term multilineage reconstitute ablated recipients, albeit with lower engraftment frequencies from the latter population (Nygren et al., 2006). In support, Bowie and colleagues found that all long-term reconstituting activity in day 14.5 FL and BM from 3 week old mice was found in the G_1 fraction, but BM from 4 week old mice was long-term reconstituted with HSCs from the G_0 fraction (Bowie et al., 2006). Further, a prolonged transition of HSCs through G_1 has been proposed to be a HSC defining property (Nygren et al., 2006).

Cytokine Regulation of Hematopoietic Stem Cells

Cytokine Families

Cyto means "cell", and Kinein means "to move". Cytokines are signaling molecules involved in hematopoiesis, adaptive immunity, and innate immunity and has been divided into five families:

- Immunoglobulin tyrosine kinase receptor family
- Hematopoietin receptor family
- Interferon receptor family
- Tumor necrosis factor receptor family
- Chemokine receptor family

This section will entirely focus on the role of the two cytokine receptor - ligand pairs, a) Kit - KL and b) c-Mpl - Thpo, belonging to the immunoglobulin tyrosine kinase receptor and hematopoietin receptor families, respectively.

Kit - KL

Kit Receptor and Kit ligand - The W and Steel locus

Both the *W* (de Aberle, 1927) and the *Steel (Sl)* (Sarvella and Russell, 1956) locus were well known before their respective gene products were identified and cloned. Mice harboring mutations in one or the other of the loci demonstrate overlapping phenotypes (defects in anemia, gametogenesis and pigmentation) which made researchers early on suggest an interaction between the two (Russell, 1979). However, because the two loci were located on different chromosomes in the mouse (*W* on chromosome 5, and *Sl* on chromosome 10) it was unlikely that they were the products of the same gene and it was suggested that the two loci might encode for a receptor ligand pair (Lyman and Jacobsen, 1998; Russell, 1979).

The Discovery and Cloning of Kit and Kit Ligand

Kit was mapped to the *W* locus by two independent groups (Chabot et al., 1988; Geissler et al., 1988) and identified as the cellular homologue of the *v-kit* gene (Besmer et al., 1986), and to be located on mouse chromosome 5. The Kit receptor exists in two isoforms differing in four amino acids ($GNNK^+$ or $GNNK^-$) (Reith et al., 1991), where both isoforms generally are expressed in all tissues, although the $GNNK^-$ being the predominant form (Ronnstrand, 2004). The $GNNK^-$ form has been shown to have low constitutive activity in the absence of the KL, in contrast to the $GNNK^+$ that is phosphorylated upon KL activation only (Reith et al., 1991).

Several independent groups purified the Kit ligand and provided the DNA sequence of the gene located on mouse chromosome 10 (Huang et al., 1990; Martin et al., 1990; Williams et al., 1990; Zsebo et al., 1990a; Zsebo et al., 1990b). KL exists in both a

membrane bound and a soluble form that are generated by alternative splice variants. One splice variant contains a proteolytic cleavage site in either exon 6 or 7 that can generate the soluble form of KL by proteolytic cleavage (Huang et al., 1990; Martin et al., 1990). The functions of the membrane bound vs. soluble KL will be discussed in the section "Kit and Kit ligand signaling".

Spontaneous Kit Receptor Mutations in the Mouse

Several spontaneous mutations exist within the *W* locus where some confer complete loss of tyrosine kinase activity resulting in embryonic lethality, whereas others lead to partial loss of function mutations generating viable mice. Just to mention a few, the $Kit^{W/W}$ (78 amino acid deletion), $Kit^{W19H/W19H}$ (complete deletion of *Kit*), $Kit^{W37/W37}$ (point mutation) and $Kit^{W42/W42}$ (point mutation) are all examples of tyrosine kinase dead mutations in the *W* locus and these mice exhibit severe anemia and die perinatally (Nocka et al., 1990; Tan et al., 1990). In contrast, the $Kit^{W41/W41}$ (point mutation) and $Kit^{Wv/Wv}$ (point mutation) mutations generate viable mice with only a mild form of anemia. All viable mice with a *W* mutation display in addition to various hematopoietic phenotypes also pigmentation defects of varying degrees as well as impaired gametogenesis (Nocka et al., 1990). Only one viable Kit null mutation has been reported, the Vickid mouse (Viable c-kit deficient), that arose from a backcrossing colony of $Kit^{W/+}x\gamma_c^{-/-}$ mice (γ_c is the common subunit of interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 receptors) and demonstrated defects in early lymphopoiesis (Waskow et al., 2002). The molecular reasons behind the survival of the Vickid mouse are still unclear. However, overexpression of *Erythropoietin* was shown to rescue the otherwise embryonic lethal $Kit^{W/W}$ mice, suggesting that erythropoietic thresholds could contribute to the phenotype and lethality of mice harboring Kit mutations (Waskow et al., 2004).

Spontaneous Kit Ligand Mutations in the Mouse

Spontaneous mutations of the *Sl* locus have also been identified and the homozygous *Sl/Sl* mutations are embryonic lethal (Sarvella and Russell, 1956). Steel-Dickie *(Sl^{d})* is a severe, but viable mutation in the *Sl* locus that has been shown to encode a truncated form of the soluble KL whereas the membrane bound form is lacking (Brannan et al., 1991). Several other mutations have been described as well and named according to the color coat appearance of the mouse including Sl^{sh} (Steel-Grizzle-Belly), Sl^{to} (Steel-Sooty), Sl^{cg} (Steel-Cloud-Gray), Sl^{con} (Steel-Contrasted) and Sl^{du} (Steel-Dusted) just to mention a few (Russell, 1979). Administration of KL into Sl/Sl^{d} mice have been shown to reverse their anemia (Zsebo et al., 1990a).

Kit and Kit Ligand Signaling

Most studies on intracellular signaling downstream of the Kit receptor are performed on different cell lines or on cultures of primary hematopoietic mast cell. Knowledge regarding the intracellular signaling pathways in primary HSCs remains limited due to experimental difficulties in studying signaling in such rare populations as HSCs.

KL molecules bind as a homodimer, to its receptor Kit, thereby inducing dimerization of two Kit receptors resulting in autophosphorylation of tyrosine residues serving as docking sites for signaling molecules containing Src homology-2 (SH2) sites (Ronnstrand, 2004), such as Phospholipase Cγ1 (Reith et al., 1991). Consequently, a signaling cascade is activated including the Ras/Erk, JAK/STAT and MAPK pathways, promoting mainly proliferation, and the PI3K/Akt pathway supporting survival and inhibition of apoptosis (Illustrated in figure 4) (Reber et al., 2006). Two of the genes that have been reported to be targets of downstream signaling from the Kit/KL complex, are the transcription factors Slug and Mitf (Lennartsson et al., 2005).

The $KitW^{41/W41}$ mutation is a point mutation in the conserved kinase domain

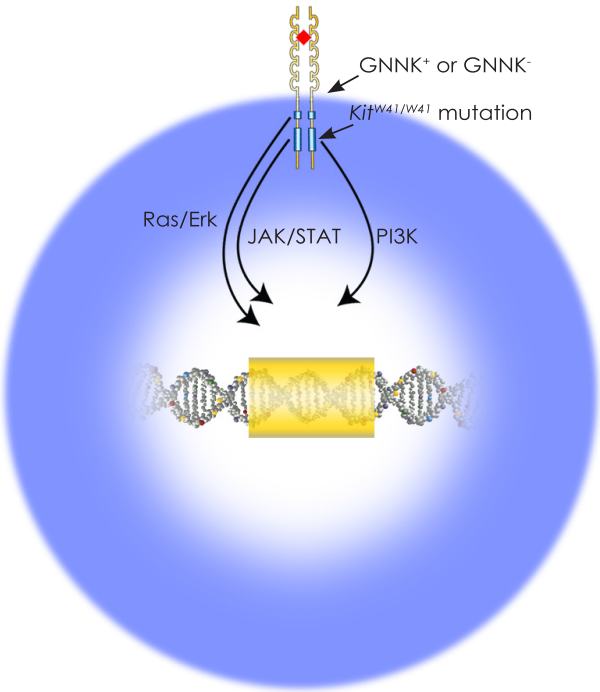

Figure 4.
The Kit receptor. *Binding of the KL to the Kit receptors results in dimerization and induction of downstream signals through the Ras/Erk, JAK/STAT and PI3K pathways. The Kit receptor is expressed in two forms, GNNK+ and GNNK-, encoded within the extracellular part of the receptor. The $Kit^{W41/W41}$ mutation is located within one of the kinasedomains.*

substituting a valine with a methionine at position 831 (Nocka et al., 1990; Reith et al., 1990). This results in a significantly weaker kinase autophosphorylation as well as decreased interaction with Phospholipase Cγ1, which is thought to dock with the receptor at tyrosine 730 (Reber et al., 2006; Reith et al., 1991).

The distinct role of membrane bound versus soluble KL is not entirely clear; however, studies on a stromal cell line established from FL cells, have shown a more rapid and transient signal downstream of the receptor followed by a quick degradation when activated by soluble KL, whereas the membrane bound form generates a more persistent activation (Ronnstrand, 2004). In addition, soluble KL cannot compensate for the lack of membrane bound KL as shown in Sl^{d}/Sl^{d} mice (Brannan et al., 1991).

Expression of Kit and Kit Ligand within the Hematopoietic System

The Kit receptor is highly expressed on HSCs both in FL and in BM and is a useful marker for the enrichment of these cells. In addition, Kit is also expressed on other cells throughout the blood system, for example MPPs (Morrison et al., 1997), LMPPs (Adolfsson et al., 2005), CMPs, MEPs, granulocyte macrophage progenitor (GMP)s (Akashi et al., 2000; Pronk et al., 2007), early T cell progenitors (Ceredig and Rolink, 2002; Godfrey et al., 1993; Porritt et al., 2004), and mast cells (Nocka et al., 1989).

Membrane bound KL is predominantly expressed in the BM, spleen, thymus and placenta whereas the soluble form is mostly produced in spleen, thymus and placenta (Huang et al., 1992).

Involvement of Kit and Kit Ligand in HSC Maintenance and Regeneration

Kit has long been thought to be involved in the regulation of the HSC pool, but due to Kit null mice being embryonic lethal (with the exception of the Vickid mouse (Waskow et al., 2002) this has been difficult to show. Instead, researchers have used the partial loss of function mutant mice of the *W* and *Sl* locus, as well as functional blocking antibodies.

By repeated IV injections of a Kit functional blocking antibody, ACK2, into mice investigators found decreased numbers of myeloid and erythroid progenitor cells in the BM, whereas the number of B cell stayed constant. In addition, the number of CFU-S were similarly decreased, indicating either a block in maturation of the blood cells, or a failure of the HSCs to produce new progeny (Ogawa et al., 1991).

Taking the complete opposite approach, it was demonstrated that continuous IV administration of KL could reverse the anemia in the Sl/Sl^{d} mice (Zsebo et al., 1990a). In addition, they observed radioprotection of lethally irradiate mice following a short term IV injection protocol (Zsebo et al., 1992), whereas prolonged administration of KL led to mobilization of HSCs to the PB and spleen (Bodine et al., 1993).

In addition, several groups have tried to address the role of Kit in steady-state hematopoiesis and have reported no phenotypic abnormalities in either FL or BM, suggesting that Kit does not play a role in HSC maintenance in either FL or BM (Miller

et al., 1997; Miller et al., 1996; Sharma et al., 2007). In addition, regeneration of the HSC compartment by transplantations of whole FL (Miller et al., 1997) or BM cells (Miller et al., 1996) from different *W* or *Sl* mutant mice into myeloablated hosts, have demonstrated reduced levels of donor derived mature blood lineages post transplantation. But as no phenotypic defects of HSCs were detected in steady-state (except for in the Vickid mouse that lacked all c-kit expression (Waskow et al., 2002)), it was concluded that the lack of mature blood cell production post transplantation was due to a differentiation block (Miller et al., 1996). However, until recently (Sharma et al., 2007), no one had performed an analysis of the donor derived HSCs in the BM of the transplanted recipients, to determine the HSC regeneration capacity. Sharma and colleagues provided evidence for reduced numbers of donor derived HSC post transplantation, for the first time showing that the HSCs from the Kit mutant mice are functionally distressed (Sharma et al., 2007). However, the mechanism behind the finding remains unknown.

c-Mpl - Thpo

The Discovery and Cloning of c-Mpl and Thpo

The Thpo receptor c-Mpl, located on the murine chromosome 4, was discovered through molecular cloning of a murine myeloproliferative leukemia virus identifying the proto-oncogene *v-mpl* (Souyri et al., 1990), with an amino acid sequence that was strikingly similar to previously identified cytokine receptors of the hematopoietin family (Souyri et al., 1990). Shortly thereafter, the murine (Vigon et al., 1993) and human (Vigon et al., 1993) homologues were cloned.

The ligand, Thpo which is located on murine chromosome 16, was identified by three independent groups that also linked the receptor ligand pair to megakaryopoiesis and platelet production (Bartley et al., 1994; de Sauvage et al., 1994; Lok et al., 1994).

Expression of c-Mpl and Thpo

c-Mpl is expressed on HSCs in both FL and BM (Solar et al., 1998). The *c-Mpl* expression pattern has been characterized in more detail in BM, showing high levels of expression in HSCs, and a gradual decrease during maturation to the LMPP (Buza-Vidas et al., 2006; Luc et al., 2008). In addition, c-Mpl is also expressed on cells of the megakaryocytic lineage including platelets (Debili et al., 1995).

Thpo is mainly produced by hepatocytes in the liver, but can also be detected in lower amounts in kidneys and on BM stroma cells (Nagahisa et al., 1996; Sungaran et al., 1997; Yoshihara et al., 2007).

Signaling upon c-Mpl and Thpo binding

The c-Mpl receptor is composed of two receptors with motifs that upon ligand activation phosphorylates tyrosine residues in the intracellular domain of the receptor. The phosphorylated tyrosine residues serve as a docking site for the kinase Jak2, leading to recruitment of STATs, PI3K and MAPK which regulate cell survival and proliferation

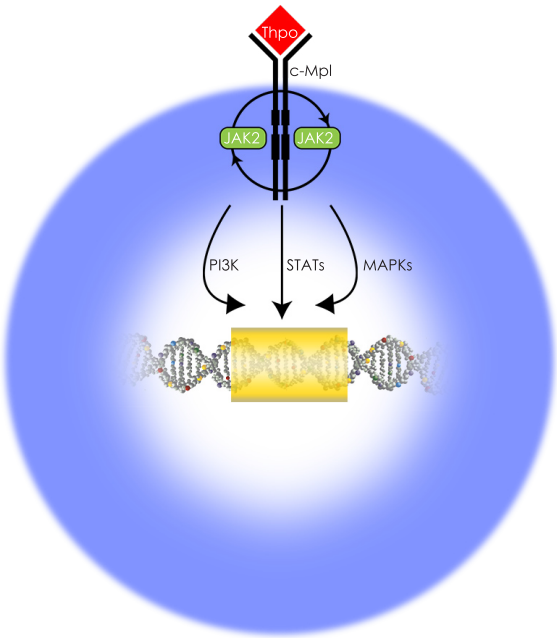

Figure 5.
The c-Mpl receptor. *Binding of Thpo to the c-Mpl receptor triggers downstream signaling, activating JAK2. This further triggers downstream pathways like PI3K, STATs and MAPKs supporting cell survival and proliferation.*

(illustrated in figure 5). The central role of Jak2 in c-Mpl and Thpo signaling is further verified in *Jak2*$^{-/-}$ mice that are unable to respond to Thpo and consequently fails to produce megakaryocytes (Kaushansky, 2005).

Further, Thpo has also been shown to activate HoxB4 transcription (Kirito et al., 2003) and trigger nuclear localization of HoxA9 (Kirito et al., 2004), suggesting a role of Thpo in regulation of the Hox-genes, that have been shown to be important in *ex vivo* expansion of HSCs (Antonchuk et al., 2002). Induction of Thpo expression in HSCs has also been suggested to activate and stabilize transcription of *Hif-1a* that subsequently activates expression of VEGF. This protein has been shown to enhance survival and proliferation of HSCs in culture, suggesting an autocrine regulation loop which might explain the favorable effects of Thpo on HSC survival and proliferation (Kirito et al., 2005). Importantly, these studies were only performed under *in vitro* conditions.

Thpo and c-Mpl Knockout Mice

Both *c-Mpl*$^{-/-}$ (Alexander et al., 1996) and *Thpo*$^{-/-}$ (de Sauvage et al., 1996) mice have been generated, and are born at Mendelian ratios with a normal life span. Both mice display similar phenotypes, including defective megakaryopoiesis and consequently reduced number of platelets, arguing that Thpo is the sole ligand of the c-Mpl receptor. Adequately, the platelet phenotype displayed in the *Thpo*$^{-/-}$ mice is reversible by IV administration of Thpo (de Sauvage et al., 1996; Fox et al., 2002).

Involvement of Thpo and c-Mpl in HSC Maintenance and Regeneration

Several studies on *Thpo*$^{-/-}$ and *c-Mpl*$^{-/-}$ mice have suggested cell intrinsic effects in steady-state maintenance and regeneration of the adult BM HSC compartment as well as reduced radioprotective ability of irradiated mice (Fox et al., 2002; Kimura et al., 1998; Solar et al., 1998). However, through these studies it has been impossible to distinguish between a block in differentiation, as recently suggested (Abkowitz and Chen, 2007), and a reduced function of the BM HSC compartment since all studies have utilized, as a sole identification of HSC activity, a measurement of mature blood cells in the PB to assess

multilineage engraftment (Fox et al., 2002; Kimura et al., 1998; Solar et al., 1998). No phenotypic reductions of HSCs in *c-Mpl*$^{-/-}$ FL have been reported, but transplantation of whole *c-Mpl*$^{-/-}$ FL or AGM cells, have suggested a role of c-Mpl and Thpo in HSC self-renewal during fetal stages (Petit-Cocault et al., 2007).

A mentioned above, Thpo has been implicated to act upstream of HoxB4 (Kirito et al., 2003) and HoxA9 (Kirito et al., 2004), suggesting that the *ex vivo* expansion of HSC seen upon overexpression of *HoxB4* could be Thpo induced (Antonchuk et al., 2001; Antonchuk et al., 2002), as well as the failure to regenerate hematopoiesis post transplantation in *HoxA9*$^{-/-}$ mice (Lawrence et al., 2005) could be explained by incomplete Thpo mediated nuclear localization of HoxA9 (Kirito et al., 2004). In addition, Thpo has also been shown to be the primary cytokine driving the BM HSC expansion seen in the *Lnk*$^{-/-}$ mice (Buza-Vidas et al., 2006; Ema et al., 2005; Seita et al., 2007).

Cytokine Redundancy

A combined effect of two cytokines that is greater than the additive effect of each two factors alone is commonly referred to as cytokine synergy, or in the context of knockout mice also referred to as cytokine redundancy. As cytokines exhorted strong effects on hematopoietic cells in *in vitro* cultures (Ramsfjell et al., 1996), researchers expected the cytokine specific knockout mice to show dramatic defects in lineage commitment (Robb, 2007). However, it turned out that many of the genetically engineered mice exhibited very marginal phenotypes, triggering the hypothesis of cytokine redundancy.

As an example, both IL-3 and GM-CSF promoted proliferation and formation of granulocyte macrophage colonies, respectively, *in vitro*, but the *IL3a-chain*$^{-/-}$, *IL3β-chain*$^{-/-}$ and *GM-CSFβ-chain*$^{-/-}$ mice showed very marginal reductions in mature blood cells (Ichihara et al., 1995; Nicola et al., 1996; Stanley et al., 1994). To try to overcome the issues of redundancy, researchers generated double knockout mice deficient in two cytokines or cytokine receptors. Numerous double knockout mice have been made, but most of them display additive effects only. Neither *c-Mpl*$^{-/-}$*xIL-6*$^{-/-}$, *c-Mpl*$^{-/-}$*xIL-11*$^{-/-}$ nor *c-Mpl*$^{-/-}$*xGM-CSF*$^{-/-}$ (Gainsford et al., 2000; Scott et al., 2000) revealed further reductions of platelet numbers as compared with *c-Mpl*$^{-/-}$ alone, but the *c-Mpl*$^{-/-}$*xKit*$^{Wv/Wv}$ mice displayed both reduced platelets and erythroid precursors arguing for a synergistic relationship between KL and Thpo (Antonchuk et al., 2004). The cytokines responsible for granulocyte and monocytes development were analyzed in the *Thpo*$^{-/-}$*xG-CSFReceptor*$^{-/-}$ and *G-CSF*$^{-/-}$*xGM-CSF*$^{-/-}$ mice (Kaushansky et al., 2002; Seymour et al., 1997), but revealed no further reductions.

In lymphopoiesis, studies on *Kit*$^{W/W}$*xγc*$^{-/-}$ mice showed that T cell lymphopoiesis was abrogated, in striking contrast to the two single mutant mice that showed only mild T cell phenotypes (Rodewald et al., 1997). Further, interaction between the IL-7 receptor and Flt3 ligand (Flt3L) have been demonstrated in *IL-7*$^{-/-}$*xFlt3L*$^{-/-}$ mice revealing a dramatic reduction in the number of both fetal and adult B cells (Sitnicka et al., 2003) and a somewhat milder effect on T cell lymphopoiesis in contrast to the two single mutant mice, suggesting a synergistic interaction between IL-7 and Flt3L on lymphopoiesis (Sitnicka

et al., 2007). These studies triggered the hypothesis that interactions of members of the immunoglobulin and hematopoietin receptor families might be working in synergy in development of blood lineages. However, so far none of these cytokine combinations have revealed any synergistic effects on the HSC pool.

Instructive vs. Permissive Role of Cytokines

Whether hematopoiesis is regulated in a deterministic or stochastic manner with cytokines playing an instructive or a permissive role in fate determination has been a long-standing controversy (Robb, 2007). Instructive action of cytokines conceptually suggests that cytokines are the main fate determinants of hematopoietic cells, and other regulators like transcription factors play a less dominant role. In contrast, the permissive hypothesis recognizes transcription factors as the definitive fate determinants, arguing that cytokines provide survival and proliferation signals rather than drive lineage commitment.

In support of the instructive hypothesis, researchers have overexpressed *IL2β-receptor* or *GM-CSF-receptor* in CLPs cultured on stromal cells, and found the lymphoid primed CLPs to produce cells of the myeloid lineages, suggesting that cytokines can modulate the lineage fate of an already committed cell (Kondo et al., 2000). This has been further supported *in vivo* by experiments showing that overexpression of human *GM-CSF-receptor* in *IL7*$^{-/-}$ mice, can upon IV administration of GM-CSF, instruct committed lymphoid cells to differentiate into myeloid cells (Iwasaki-Arai et al., 2003). Collectively, this would support that cytokine signals can modulate fates of already committed hematopoietic cells thereby arguing for an instructive role in hematopoiesis.

In contrast, the permissive model gained support when *Bcl-2* was overexpressed in a hematopoietic cell line, and demonstrated that upon suppression of apoptosis, the cells could survive and differentiate in the absence of cytokines, arguing that lineage determination is directed by intrinsic rather than extrinsic signals (Fairbairn et al., 1993). These *in vitro* findings have been further extended *in vivo*, providing evidence for a stochastic model, by overexpression of *BCL-2* in *IL7*$^{-/-}$ and *M-CSF*$^{-/-}$ mice (Akashi et al., 1997; Lagasse and Weissman, 1997; Maraskovsky et al., 1997) demonstrating a rescue of T cell and monocyte deficiencies respectively; however, not on B cells, suggesting cell type specific effects. In addition, experiments using mice expressing a chimeric receptor of extracellular Thpo and intracellular G-CSF-receptor did not display any signs of reductions of platelets or increases of granulocytic progenitors, suggesting that G-CSF could replace the role of Thpo, arguing for a permissive role of cytokines (Stoffel et al., 1999).

Clinical Aspects of Kit and Thpo

History of Bone Marrow Transplantation

In the 1950's researchers found that it was possible to rescue mice exposed to total body irradiation by transplantation of BM cells from non-irradiated mice (Ford et al., 1956; Lorenz et al., 1951). At that time, since the MHC and human leukocyte antigen (HLA) were yet undiscovered, one did not realize the importance of the genetic matching between recipient and donors used in the pre-clinical experiments. Therefore, the clinical trials following upon the pre-clinical experiments resulted in extensive mortality (Bortin, 1970) due to an immunological reaction that we today know as graft vs. host disease (GVHD); a complication caused by donor T cells recognizing host tissues as non-self and eliminate these by a cytotoxic response. The HLA was discovered in 1958 (Dausset, 1958; Van Rood et al., 1958) and studies in dogs further emphasized the importance of matching donors with recipients (Storb et al., 1971), thus making it clear that problems concerning the immunological barrier had to be solved for BM transplantations to be a useful treatment strategy. It was observed that cytotoxic agents reduced the propensity for mice to develop GVHD (Uphoff, 1958), and subsequently when using combinations of these drugs on patients advantageous effects on survival were observed (Storb et al., 1986). In addition, elimination of lymphocytes by antibody blocking (Boak et al., 1967; Brent et al., 1967) or cell depletion of the whole BM cell graft were tested to try to circumvent the GVHD (Prentice et al., 1984; Reisner et al., 1981). Today, clinicians are treating a wide variety of hematological diseases by BM transplantation, however, GVHD remains a problem and a future challenge.

Human Disease Associated with Mutations in Kit and Kit Ligand

Piebaldism is a syndrome associated with lack of function mutation in the Kit receptor and most closely resembles the *W* mutation in mice. It is characterized by deafness, megacolon and pigmentation abnormalities, but few hematopoietic defects have been observed (Lennartsson et al., 2005).

Acute Myeloid Leukemia (AML) is a disease associated with fast accumulation of myeloid progenitor cells within the BM, and if left untreated will result in a fatal outcome within a few months. The disease is associated with the elderly and the median age at diagnosis is around 60 years.

A constitutive activating mutation of Kit has been found in AML; however, the mutation is very rare. Since both the Kit receptor and its ligand are expressed on AML blasts an autocrine stimulation loop has been suggested in driving and sustain the growth of the tumor cells (Lennartsson et al., 2005). The exact role of Kit in AML is still unclear but is of interest, especially since the tyrosine kinase inhibitor Imatinib (also called Gleevec or STI-571) that among other tyrosine kinases also inhibits Kit, is readily available (Lennartsson et al., 2005).

Kit is also mutated in many other diseases, for example: gastrointestinal stromal tumors, mastocytosis, melanoma, small cell lung cancer (Lennartsson et al., 2005).

Human Disease Associated with Mutations in c-Mpl and Thpo

C-Mpl is expressed on fetal, neonatal and adult primitive human stem and progenitors cells (Ninos et al., 2006). Congenital Amegakaryocytic Thrombocytopenia (CAMT) is a stem cell disease that is caused by *c-Mpl* mutations, affecting young children (0-2 year). The symptoms include BM failure with low platelet and red blood cells counts and decreased levels of white blood cells, and in addition, age progressive reductions in the number of stem and progenitor cells in the BM. The CAMT phenotype has been shown to be caused by missense or nonsense mutations, or deletions in the *c-Mpl*, leading to partial or complete loss of signaling downstream of the receptor. Interestingly, no patients have been diagnosed with mutations in *Thpo*. The only cure to this otherwise lethal disease is BM transplantation, and most of the children are successfully treated (Ballmaier et al., 2003).

Polycythemia vera, essential thrombocythemia and chronic idiopathic myelofibrosis all have identified Jak2 mutations and are thought to be driven by Thpo, erythropoietin or G-CSF. Upon ligand binding, the HSCs are transformed and Jak-Stat signaling is initiated. A activating mutation of c-Mpl has also been reported in myelofibrosis with intact Jak2 activity (Levine and Gilliland, 2007).

Present Investigations

Aims of the Present Investigations

Understanding the steady-state physiology of a biological system is crucial to further comprehend what happens during disease. Animal models allow us to gain mechanistic insight into the biology of the organism, subsequently allowing us to build hypothesis that can be further extrapolated and tested in the human system.

The cytokine receptors Kit and c-Mpl and their respective ligands, KL and Thpo, have been implicated to play an important role in HSC biology. By the use of mice with a partial (*Kit$^{W41/W41}$*) and complete (*Thpo$^{-/-}$*) loss of function mutation we have aimed at specifically addressing the following question:

Article I — To further investigate the HSC defect in Kit partial loss of function mutant mice, and to investigate the potential mechanism behind the defective regeneration of the HSC compartment post transplantation seen in Kit mutated HSCs. Is it due to defective homing, differentiation, survival or self-renewal?

Article II — To investigate the interdependency between Kit and Thpo in steady-state maintenance of the HSC compartment as well as in the regeneration of hematopoiesis post transplantation.

Article III — To investigate the role of Thpo in HSC expansion and maintenance during ontogeny and post transplantation.

Discussion

Signaling through the Kit and c-Mpl pathways are thought to be crucial for sustaining HSC function and homeostasis of the hematopoietic system. Gaining a better understanding of the mechanisms that govern the activity of these two pathways and how/if they collaborate are important in furthering our understanding of HSC biology, and thereby increasing the knowledge about their associated diseases with the possibility of improving therapies.

Fluorescent Activated Cell Sorting

In all three papers, evaluation of HSC numbers and performance is investigated by FACS analysis of different hematopoietic tissues in steady-state conditions or post transplantation. Flow cytometry allows us to recognize subpopulations of cells within a tissue based on the presence or absence of cell surface receptors or intracellular proteins that are detected by fluorescently conjugated antibodies. By staining for combinations of cell surface markers or intracellular proteins known to identify HSCs, progenitors, mature blood cells, or recipient or host (for example CD45.1 or CD45.2 in a transplantation setting), one can evaluate the cells phenotypically and functionally and get clues about the ongoing intracellular signaling. However, the number of receptors expressed on the cell has to exceed a certain threshold in order to be detected, which means that low levels of expression can go unnoticed. Importantly, ensuring optimal detection of the surface antigens by flow cytometry involves finding the best antibody clone and the best conjugate combination. The antibody should bind with high specificity to the specific protein of interest, but at the same time provide low levels of unspecific binding (< 1%), and the conjugate selection depends on the lasers, filters and detectors that the FACS machine is equipped with. With multicolor FACS machines becoming increasingly available our possibilities in analyzing subpopulations based on combinations of more than ten antigens has become feasible.

Age Progressive Reductions in Hematopoietic Stem Cells in the Absence of Kit or Thpo

To investigate the effect on Kit and Thpo on HSC biology, we utilized mouse models with impaired ($Kit^{W41/W41}$) or deleted ($Thpo^{-/-}$) signaling of these cytokine pathways. Phenotypic analysis of HSC enriched cell populations can by no means be regarded as an absolute quantification of HSCs as these populations are assumed to be heterogeneous, but they provide us with general information about the size of the HSC compartment during steady-state hematopoiesis. Initially we studied the number of phenotypic HSCs in day 14.5 FL (defined by LSK $Flt3^-$) and BM of adult mice (defined by LSK $CD34^-Flt3^-$) through FACS analysis. In FL we found that neither $Kit^{W41/W41}$ nor $Thpo^{-/-}$ mice harbored any reductions in the phenotypic HSC compartment, in agreement with previously published data on Kit mutant mice (Miller et al., 1997). In fact, the FL in $Thpo^{-/-}$ displayed an increase in cellularity that resulted in a slight but significant increase in absolute

number of phenotypic HSCs. However, in the BM a 2-fold reduction of HSCs in the *Kit*$^{W41/W41}$ mice and a 9-fold reduction in the *Thpo*$^{-/-}$ mice were revealed and interestingly, the reductions in the BM in both mice were further exacerbated with age (analyzed in 6 months old *Kit*$^{W41/W41}$ and 2 years old *Thpo*$^{-/-}$ mice) suggesting an age progressive loss of HSCs during steady-state hematopoiesis, (Article I and III).

Interestingly, similar to the *Thpo*$^{-/-}$ mice, CAMT patients display normal numbers of platelets and erythroid cells at birth, indicative of a Thpo independent role during fetal development (Ballmaier et al., 2003). In addition, the CAMT patients are thought to develop an age progressive multilineage BM failure within their first two years of life. This has been further supported by studies showing that c-Mpl is expressed on human CD34^{+}CD38^{-} cells that are enriched for stem and progenitor cell activity, but is downregulated upon differentiation (Ninos et al., 2006). Further, the CD34^{+}CD38^{-} cell populations have also been reported to be progressively reduced in the BM of the CAMT patients (Ballmaier et al., 2003), suggesting a role of Thpo in steady-state maintenance of human stem and progenitor cells, similar to what we report in the *Thpo*$^{-/-}$ mice, (articel III).

Kit and Thpo Maintains Hematopoietic Stem Cell Quiescence

The majority of HSCs are suggested to be in cycle during expansion in the FL and to become predominantly quiescent when settled in the BM 3-4 weeks after birth (Bowie et al., 2006). Due to the reductions of phenotypic HSCs that we observed in BM of both *Kit*$^{W41/W41}$ and *Thpo*$^{-/-}$ mice, it was of interest to investigate if the turnover rate within the HSC compartment during steady-state conditions could explain these findings. As both Kit and Thpo have been suggested to promote proliferation *in vitro* (Ramsfjell et al., 1996), it was surprising to find elevated levels of cell cycle activity in both mice. This was measured by increased BrdU incorporation in the LSK CD34^{-}Flt3^{-}, enriched for HSC activity, as well as increased percentage of cells in G_1 and SG_2M phase of the cell cycle as measured by Ki67 and Dapi in both *Kit*$^{W41/W41}$ and *Thpo*$^{-/-}$ mice as compared with WT controls. These data suggested that the Kit and c-Mpl receptors play a role in keeping the HSC enriched cell population in the BM quiescent. A possible role of Kit and Thpo in keeping HSCs quiescent would also fit with the minor phenotype observed in the FL of *Kit*$^{W41/W41}$ and *Thpo*$^{-/-}$ mice where the HSCs are actively cycling, and a more pronounced phenotype in the BM of these mice where the HSCs are thought to stay more quiescent (Bowie et al., 2006). However, we were unable to show if the observed increase of cell cycle activity was a primary or a secondary event, caused by the mutation in Kit and Thpo. One could speculate that impaired Kit and/or c-Mpl signaling could primarily inhibit the anchoring of HSCs to osteoblasts in the niche, thereby as a secondary consequence enhance cell cycle activity. In support of this hypothesis, treatment of BM cells with anti-c-Mpl blocking antibody resulted in reduced beta 1 integrin expression, known to play a role in cell adhesion (Yoshihara et al., 2007). Investigations of BM sections of the *Kit*$^{W41/W41}$ and *Thpo*$^{-/-}$ mice stained with the SLAM markers (Kiel et al., 2005), could possibly reveal if HSCs are located further away from the osteoblasts in the niche than WT HSCs,

although would only be correlative. (Articles I and III). Several questions remain unclear: Is it possible that both Kit and c-Mpl signaling can provide survival and proliferation signals *in vitro* through for instance HoxB4 (Kirito et al., 2003) and HoxA9 (Kirito et al., 2004), and play a role in keeping the HSCs quiescent *in vivo*? If so, what signals does the BM microenvironment provide that alter the functions of the two signaling pathways? Maybe by Ang-1/Tie-2 or N-cadherin that are molecules expressed by osteoblasts in the niche (Arai et al., 2004; Yoshihara et al., 2007; Zhang et al., 2003)?

Critical Role of Kit and Thpo in Regeneration of the Hematopoietic Stem Cell Compartment Post Transplantation

To address the role of Kit and Thpo during situations of stress in the hematopoietic system we evaluated their capacity in regeneration of hematopoiesis following myeloablation. By transplanting BM cells from $Kit^{W41/W41}$ into WT recipients or WT cells into $Thpo^{-/-}$ recipients and evaluate the mulilineage engraftment in PB, we were able to confirm previous findings which reported reduced regeneration post transplantation (Kimura et al., 1998; Miller et al., 1996). Importantly, analysis of donor derived HSCs post transplantation (in both competitive and non-competitive BM transplantation settings) showed severely reduced numbers of these cells in the BM as previously shown (Sharma et al., 2007), suggesting that the HSCs had not been able to restore the HSC compartment. This observation made it unlikely, although not proven, that a differentiation block would be the only cause explaining the reduced levels of mature blood cells seen in PB, as one then would expect an intact HSC compartment (Articles I and III). Therefore, we chose to investigate other possible mechanisms, such as homing and survival that also could explain the reduced HSC activity in these mice.

To address homing, we transplanted BM cells from $Kit^{W41/W41}$ mice at 50:1 ratios with WT BM cells and evaluated the ratio $Kit^{W41/W41}$:WT in HSC compartment two weeks post transplantation. This is a rough evaluation of the homing efficiency and initial settlement in the BM, and this experiment will not detect minor homing defects or delays, as the majority of the HSCs home back to the BM within hours post transplantation. With these limitations, we could not find any evidence for a severe homing defect of the $Kit^{W41/W41}$ HSCs. (Article I)

The observed reduction of HSCs could also be explained by increased cell death, but finding evidence for reduced cell survival *in vivo* by the presence of apoptotic cells is difficult since these cells are rapidly cleared from the circulation. To initially screen for evidence of enhanced apoptosis we investigated HSCs from $Kit^{W41/W41}$ mice by FACS for presence of intracellular active Caspase-3, a protein expressed during late stages of apoptosis. As expected, we found no evidence for increased apoptotic activity within the steady-state HSC compartment of $Kit^{W41/W41}$ mice. However, due to the rapid clearance of apoptotic cells from the circulation as well as the limitated capacity of flow cytometry detection of antigens expressed at low levels, we decided to address survival by crossing both the $Kit^{W41/W41}$ and $Thpo^{-/-}$ mice to a transgenic mouse line overexpressing

BCL-2 from the H2K promoter. This mouse line ensures high levels of BCL-2 in the hematopoietic system, including the HSC compartment (Domen et al., 1998). By this model we investigated if suppression of apoptosis would rescue cells with deficient/absent Kit and Thpo signaling.

During steady-state conditions in $Kit^{W41/W41}$*xBCL-2* and $Thpo^{-/-}$*xBCL-2* mice, limited evidence of a reversed phenotype was found when examining LSK subpopulations, based on expression of CD34 and Flt3, that include long-term and short-term HSCs as well as MPPs. However, transplantation of whole BM cells from $Kit^{W41/W41}$*xBCL-2* mice into myeloablated WT recipients, revealed partial restoration of the levels of mature blood cells in the PB as well as HSC enriched populations (LSK) in the BM. These findings cannot fully rule out apoptosis as a possible mechanism for the reduced HSC compartment observed in $Kit^{W41/W41}$ and $Thpo^{-/-}$ mice, at the same time do we find very little evidence for apoptosis being a major and important mechanism during steady-state hematopoiesis and post transplantation. However, we cannot not rule out that a different anti-apoptotic protein could have a larger rescue effect than *BCL-2*. (Article I and III)

As of today, there is no *in vivo* assay that can **exclusively** evaluate self-renewal of HSCs as other mechanisms, like homing and survival, also play important roles during transplantation and therefore have to be carefully examined. Therefore, both homing and survival have to be ruled out, and even then one has to consider the problems with interpretations of negative data and the limitations of the assays, before effects on self-renewal can be put forward as possible explanations for changes in HSC activity and/or numbers.

Interdependance of Kit and Thpo in Maintenance of the Hematopoietic Stem Cell compartment

To investigate if the residual HSC activity seen in $Thpo^{-/-}$ mice was Kit dependent, we generated $Kit^{W41/W41}$*x*$Thpo^{-/-}$ mice. Steady-state BM analysis of 3-4 months old $Kit^{W41/W41}$*x*$Thpo^{-/-}$ revealed a 2-3 fold reduction in the stem and progenitor compartments as compared with $Thpo^{-/-}$ mice and a 15-fold reduction compared to Kit deficient mice. Although, the further reduction in the double mutant mice was rather limited, these findings did establish that Kit plays a role in Thpo-independent maintenance of HSCs in steady-state hematopoiesis. To further address this, we transplanted day 14.5 whole FL $Kit^{W41/W41}$ cells, that have normal HSC numbers, into a $Thpo^{-/-}$ environment, thereby analyzing the receptor deficient cells in the ligand deficient environment. About two weeks post transplantation we observed reduced survival of the transplanted mice; however, the early deaths are not necessarily an indication of reduced HSC activity, as previously suggested (Fox et al., 2002), but could rather reflect thrombocytopenia, as Thpo plays an important role in the development of the megakaryocytic lineage as well. More importantly, donor derived HSCs of $Kit^{W41/W41}$ mice were reduced 3-fold compared with transplantation of WT cells into $Thpo^{-/-}$ mice, and this effect was even more evident in secondary transplantations, again supporting a role of Kit in Thpo independent maintenance of HSCs also post transplantation. (Article II)

In Article I and II, the *Kit*$^{W41/W41}$ mouse has been used and one should keep in mind that the data we observe are based on a partial loss of function mutant mouse, and it is possible that the effects of Kit are much greater when using a null mutant mouse. One way to address this could be by the use of a conditional Kit knockout mouse which can be crossed to appropriate cre-mice, like Mx1-cre (Kuhn et al., 1995), that upon interferon-γ stimulation will induced the targeted deletion, allowing for investigations of a Kit null mutation in the adult mouse.

Taken together we have shown, through studies of the *Kit*$^{W41/W41}$ and *Thpo*$^{-/-}$ mice, that HSC maintenance during steady-state conditions as well as HSC regeneration post transplantation is highly dependent on intact signaling of the Kit and c-Mpl receptors. We find evidence supporting involvement of Kit and c-Mpl in keeping HSCs quiescent in the BM, possibly explaining the reductions of HSC numbers seen in the HSC compartment, but find little or no support of homing or apoptosis being involved in these reductions. In addition, we hypothesize that the Thpo independent HSC function is partially Kit dependent, although our findings are fully compatible with other cytokines also being involved in the HSC regulation.

Articles and Manuscripts not Included in the Thesis

Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitor cells.
R. Mansson, A. Hultquist, S. Luc, L. Yang, K. Andersson, S. Kharazi, S. Al-Hashmi, K. Liuba, L. Thoren, J. Adolfsson, N. Buza-Vidas, H. Qian, S. Soneji, T. Enver, M. Sigvardsson, SE. Jacobsen.
Immunity, 2007. **26**(4): 407-419.

Identification of Flt3$^+$ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: a revised road map for adult blood lineage commitment.
J. Adolfsson, R. Mansson, N. Buza-Vidas, A. Hultquist, K. Liuba, C. Jensen, D. Bryder, L. Yang, O. Borge, L. Thoren, K. Andersson, E. Sitnicka, Y. Sasaki, M. Sigvardsson, SE. Jacobsen.
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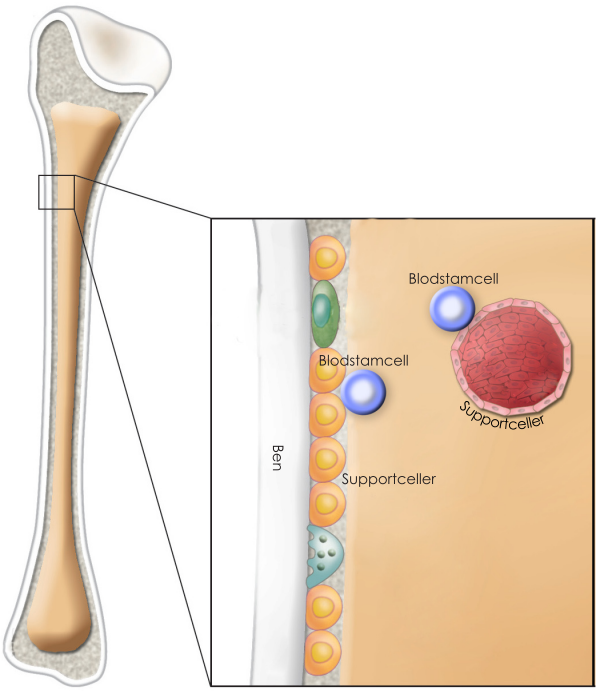
J. Nygren, K. Liuba, M. Breitbach, S. Stott, L. Thoren, W. Roell, C. Geisen, P. Sasse, D. Kirik, A. Björklund, C. Nerlov, B. Fleischmann, S. Jovinge, SE. Jacobsen.
Accepted into Nature Cell Biology

NMD is essential for immature hematopoietic cells and for eliminating programmed DNA rearrangement by-products.
J. Weischenfeldt, I. Damgaard, D. Bryder, K. Theilgaard-Mönch, L. Thoren, F. Cilius Nielsen, SE. Jacobsen, C. Nerlov, B. Porse.
Submitted manuscript

Populärvetenskaplig Sammanfattning



Figur 1
Blodstamcellernas hem. *Blodstamcellerna finner man i benmärgen hos både möss och människor där de tros de sitta i nära kontakt med olika typer av supportceller.*

Blodet och Blodstamcellen

Vårt blod består av olika celler som har till uppgift att transportera syre från lungorna ut till kroppen. Cellerna skall även stoppa blödningar när vi skär oss, samt skydda oss mot infektioner orsakade av bakterier, parasiter eller virus. Behovet att dagligen nyproducera blodceller är mycket stort. Uppskattningsvis omsätter en person, som väger 70 kg, ca 1 000 000 000 000 celler **per dag** under hela sitt liv. Blodcellernas livslängd varierar och en del kan bara leva i några dagar, medan andra kan leva i flera år. **Blodstamcellen** har till uppgift att producera den enorma mängd blodceller som behövs för att vi skall vara friska.
Blodstamcellerna finns huvudsakligen i benmärgen och är nära sammanbundna med benmärgens support celler (se figur 1). De är få till antalet, och endast en av 15 000 blodceller är en blodstamcell. Tre egenskaper definierar blodstamcellen:

- Den kan nyproducera alla typer av celler i blodsystemet.
- Den kan göra detta under ett helt liv.
- Den förnyar sig själv för att bibehålla ett konstant antal blodstamceller i kroppen.

Signalmolekyler hos Möss och Människor

I blodet hos både möss och människor finns signalmolekyler. De kan skicka följande fyra signaler eller order till blodstamcellerna:

- Håll dig still
- Förnya dig
- Mogna
- Dö

Leukemi och Benmärgstransplantation

När fel uppstår i blodets signalsystem sätts det ur balans. Detta kan leda till sjukdomar så som leukemi, vilket betyder ”vitt blod” och är ett samlingsbegrepp för olika typer av blodcancer. En vanlig behandlingsform för patienter som drabbats av leukemi är benmärgstransplantation. Detta innebär att man med hjälp av gift och strålning eliminerar patientens sjuka blodsystem, inklusive dess blodstamceller i benmärgen. Patientens sjuka blodsystem ersätts genom att transplantera nya blod- och blodstamceller från en frisk donator. Att ersätta patientens egna celler med celler från en donator är förknippat med vissa risker. Bland annat kan patientens kropp stöta bort de nya friska cellerna eftersom den inte känner igen dem som patientens egna. För att minimera riskerna vid benmärgstransplantation försöker man finna en donator med en liknande arvsmassa som patienten själv, t e x ett syskon eller en förälder. För att kunna förbättra behandlingen av de leukemidrabbade patienterna är det viktigt att vi lär oss att skilja blodstamcellerna från övriga blodceller, samt att förstå hur de fungerar. I forskningen används modeldjur, t e x möss, för att pröva olika teorier om blodstamcellernas signalsystem.

Genomförda Experiment

Under en fyraårs period ha vi genomfört ett stort antal experiment som huvudsakligen har varit inriktade på att kunna förstå hur blodstamcellernas signalsystem fungerar.

Frågeställningar och Slutsatser

I våra studier har vi undersökt två signalmolekyler som heter Kitligand och Thrombopoietin. För att bättre förstå vilken funktion dessa signalmolekyler har vid vanlig blodbildning eller efter benmärgstransplantation har vi undersökt två olika musstammar vilka har modifierats till att sakna antigen Kitligand- eller Thrombopoietinsignaler.
Denna avhandling behandlar följande frågeställningar:

Frågeställning 1

Vilka signaler skickar Kitligand och Thrombopoietin till blodstamcellerna?

Slutsats

Vi har med hjälp av olika experimentella metoder funnit att både Kitligand och Thrombopoietin skickar signaler till blodstamcellerna:
Håll dig still
Förnya dig
Detta medför att det uppstår båda kvantitativa och kvalitativa reduktioner av blodstamcellerna hos de möss, som är i avsaknad av dessa signaler. Vi har dock inte funnit några starka bevis som tyder på att Kitligand eller Thrombopoietin skickar signaler till blodstamcellerna om att mogna eller att dö.

Frågeställning 2

Är Kitligand och Thrombopoietins signaler summerbara **eller** synergistiska?

- Med summerbara avses om deras gemensamma signaleffekt på blodstamcellerna **är lika med** summan av de två.
- Med synergistiska avses om deras gemensamma signaleffekt på blodstamcellerna **är större än** summan av de två.

Slutsats

Vi har funnit att Kitligand och Thrombopoietins signaler är summerbara, dvs ej synergistiska. Vi har funnit bevis för att deras gemensamma signaleffekt på blodstamcellerna är lika med summan av de två. Dock kan vi inte utesluta att synergistiska effekter kan förekomma.

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