Cytokine Regulation of Hematopoietic Stem Cells and Lymphopoiesis

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Cytokine regulation of hematopoietic stem cells and lymphopoiesis

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With the approval from the Faculty of Medicine, Lund University, the thesis will be defended on March 17, 2007, at 10.00, Conference Room D1539, BMC, Lund

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1. Original articles and manuscripts in this thesis

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II. Identification of Flt3+ lympho-myeloid stem cells lacking erythromegakaryocytic potential: a revised road map for adult blood lineage commitment.


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* These authors contributed equally to this paper.
2. Additional articles and manuscripts produced during Ph.D. training period

Key role of FLT3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool.

Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis.

Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express FLT3: distinct FLT3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells.

Critical role of the cytokine tyrosine kinase receptor FLT3 in regulation of T lymphopoiesis and lymphoid-primed multipotent progenitors.
Sitnicka E, Buza-Vidas N, Ahlenius H, Cilio CM, Gekas C, Svensson M, Agace W, Jacobsen SEW. *Manuscript submitted*

Biological and genetic evidence for a hierarchical organisation of lineage potentials conserved in fetal and adult hematopoietic stem cells.

Permissive roles of Flt3 ligand and IL7 but not TSLP in B-1 and B-2-lymphopoiesis.

Critical role of thrombopoietin in regulation of postnatal HSC quiescence and maintenance.
3. Svensk populärvetenskaplig sammanfattning (Swedish summary)

Blodbildande, s.k. **hematopoetiska** stamceller (HSC), har två viktiga egenskaper som utmärker dem från andra celler i blodsystemet; de kan genom självförnyelse i princip genomgå ett **obegränsat antal celldelningar** och de kan **differentiera till alla slags blodceller**. Hos en vuxen indivis återfinns de flesta av de hematopoetiska stamcellerna i benmärgen och i och med att de är ursprungsceller till alla blodcellstyper har benmärgstransplantation revolutionerat behandlingen av många blodsjuksommar, såsom leukemi. Även om kunskapen om normal blodbildning, s.k. hematopoes, har ökat de senaste 10-15 åren, vet vi fortfarande väldigt lite om hur de tidigaste celltyperna i hematopoesen bildas och hur normal blodbildning regleras. Vi vet ganska lite om hur tillväxtfaktorer och specifika gener kodar för olika val som resulterar i utmognaden av de olika blodcellstyperna vi har i kroppen, som fyller de livsnödvändiga funktionerna transport av syre, skydd mot olika slags infektioner samt blodkoagulering. Den kunskap vi erhåller om hur normal blodbildning sker kommer också förhopningsvis leda till att vi också bättre förstår uppkomsten av leukemier och hur dessa kan behandlas mer effektivt och specifikt.

Benmärgstransplantation och cellgiftsterapi som används vid behandling av flera blodsjuksommar är idag fortfarande förknippade med ett antal olika komplikationer som kan innebära ökad risk för infektioner eller i värsta fall döden. Vid en autolog transplantation används patientens egna stamceller, som återinförs efter cellgiftsbehandling. Denna behandling är dock förknippad med ökat återfall av sjukdomen. Ett alternativ är därför s.k. allogen transplantation, där man transplanterar stamceller från en annan frisk människa. Detta leder dock till ett annat slags problem, nämligen en Graft versus Host (GVH) reaktion, en immunologiskt betingad bortstötning av de främmande cellerna. En fördel med denna behandling är dock att de friska transplanterade cellerna också reagerar...
mot tumören hos patienten, en s.k. Graft versus Tumör (GVT) effekt uppstår. Det är således en stor utmaning att stimulera GVT effekten och eliminera GVH reaktionen. En annan utmaning inom stamcellsforskningen har varit att utveckla odlingssystem för att föröka (expander) stamceller utanför kroppen. Friska stamceller kan på så sätt ökas i antal, och samtidigt kan sjuka stamceller eventuellt genmodifieras och sedan transplanteras till patienten. Vidare kan stamcellsexpansion underlätta studier av de cellulära och molekylära mekanismerna som reglerar stamceller och deras självförnyelse och utveckling till olika typer av specialiserade blodceller.

I den första studien som ingår i mitt doktorsarbete har vi försökt förstå vilka mekanismer som reglerar självförnyelse och expansion av stamceller. Vi har använt oss av en musmodell, där genen som kodar för proteinet LNK tagits bort. LNK har tidigare visat sig vara en negativ regulator av ett flertal tillväxtfaktorer viktiga för blodcellsutvecklingen. Vi fann att LNK defekta möss hade up till 20 gånger fler hematopoetiska stamceller än normala möss och att dessa celler var överkänsliga för tillväxtfaktorn Thrombopoetin (THPO), som visat sig sedan tidigare stimulera expansion av stamceller. Vår hypotes blev därför att LNK negativt reglerar THPO stimulerad stamcellsexpansion. Genom att korsa LNK defekta möss med THPO defekta möss, fann vi 65 gånger färre stamceller i LNK-THPO dubblefekta möss jämfört med LNK defekta möss. Vi drar slutsatsen att LNK inhiberar THPO stimulerad HSC expansion.

I den andra studien försöker vi att bättre förstå hur stamcellerna i vuxen benmärg hos möss blir specialiserade mot specifika blodceller genom att karakterisera de tidigaste cellstadierna som utvecklas från de hematopoetiska stamcellerna. Den klassiska hierarkin av blodcellsutvecklingen postulerar en strikt separation mellan två olika sorters blodcellsförstadier, antingen myeloida förstadier som producerar blodplättar, röda blodceller, makrofager och
granulocyter eller lymfoida förstadier som bildar olika typer av s.k. vita lymfocytter (B, T och NK celler). I våra studier fann vi dock bevis för ett tidigt förstadie som i princip har förlorat förmågan att producera röda blodceller och blodplättar, men fortfarande kan producera andra typer av celler av både den myeloida och lymfoida linjen. Identifikationen av detta tidiga förstadie indikerar att processen för specifikation av olika typer av blodceller åtminstone delvis måste ske på ett annat vis än man tidigare trott.

**Den tredje och fjärde studien** syftar till att öka förståelsen för hur tillväxtfaktorn FLT3 ligand (FL) är viktig för återbildningen av stamceller och immunförsvaret efter transplantation och cellgiftsbehandling. Tidigare har man trott att de observerade förhöjda värdena av FL i blodserumet hos patienter efter cellgiftsterapi resulterar i förhöjd stamcellsregeneration och funktion som kompensation för den förlorade blodcellsbildningen. I våra försök där vi använder en musmodell som inte producerar FL finner vi däremot normala nivåer av stamceller. Efter transplantation av FL defekta benmärgsceller till FL defekta möss (på så sätt säkerställer en totalt FL fri miljö) är också stamcellsregenerationen normal medan B cells produktionen är kraftigt försämrad, ända från de tidigaste B cellsförstadierna till mogna B celler. Efter cellgiftsbehandling av FL defekta möss påvisar vi att även T cells produktionen är betydligt nedsatt. Vi drar slutsatsen att FL är av kritisk betydelse för återuppbbyggnaden av immunförsvarets B och T celler efter cellgiftsbehandling och benmärgstransplantation, men inte för antalet eller funktionen av stamceller.
4. List of abbreviations

AGM  aorta-gonads mesonephros
ALL  acute lymphocyte leukemia
AML  acute myeloid leukemia
Angptl2, 3 angiopoietin like 2, 3
BM  bone marrow
BMP  bone morphogenic protein
BMT  BM transplantation
CAMT congenital amegakaryocytic thrombocytopenia
CB  cord blood
CFU-C colony-forming unit cell
CFU-S  CFU-spleen
CLP  common lymphoid progenitor
CML  chronic myeloid leukemia
CMP  common myeloid progenitor
CSF  colony stimulating factor
E  embryonic day
EPO  erythropoietin
ET  essential thrombocytemia
FACS  fluorescence activated cell sorting
FGF-1 fibroblast growth factor 1
FLK2 fetal liver kinase 2
FLT3 fms like tyrosine kinase 3
FL  FLT3 ligand
FO follicular
5-FU  5-fluorouracil
GFP  green fluorescent protein
GIST gastrointestinal stromal tumors
GM  granulocyte/macrophage
GMP  GM progenitor
GVHD  growth versus host disease
G-CSF granulocyte CSF
GM-CSF granulocyte/macrophage CSF
HLA  human leukocyte antigen
HSC hematopoietic stem cell
HSCT  HSC transplantation
IGF-2 insulin like growth factor 2
IL  interleukin
ITD  internal tandem duplication
JAK  Janus kinase
KGF  keratinocyte growth factor
LMPP lymphoid primed multipotent progenitor
LSC  leukemic stem cell
LSK  Lin^-Sca-1^c-KIT^+
LT-HSC  long-term HSC
MAPK  mitogen-activated protein kinase
MCSF-R macrophage CSF receptor
MDS  myelodysplastic syndrome
MEP  megakaryocyte/erythroid progenitor
MPP  multipotent progenitor
MZ B  marginal zone B cells
PB  peripheral blood
PCV  polycythemia vera
PDGF  platelet-derived growth factor
PI3K  phosphatidylinositol-3 kinase
R  receptor
SCF  stem cell factor
SCID  severe combined immune deficiency
SCN  severe congenital neutropenia
STAT  signal transducers and activators of transcription
ST-HSC  short-term HSC
THPO  thrombopoietin
TSLP  thymic stromal-derived lymphopoietin
YFP  yellow fluorescent protein
5. Overall aims
The knowledge about the cellular and molecular aspects of hematopoietic development has increased considerably during the past 20 years. Although stem cells in general have received enhanced attention in media during the last decade, hematopoietic stem cells (HSCs) have in fact been used for more than four decades in bone marrow (BM) transplantations (BMTs) (Storb, 2003), and studies of the HSC properties initiated by the pioneers James E. Till and Ernest A. McCulloch in the 1960s have made HSCs the best characterized stem cells today. As a consequence of these developments, many patients with hematological malignancies, such as lymphoma, myeloma and leukemia, are today successfully treated with high dose chemotherapy and BM or HSC transplantations (HSCTs) (Devine et al., 2003; Little and Storb, 2002). However, this treatment modality is still associated with high mortality and morbidity, and consequently it remains a considerable challenge to better understand the cellular and molecular aspects of HSCs and hematopoiesis, as this hopefully will lead to improvements of the current therapy of hematopoietic diseases.

The focus of my thesis work has been in part to delineate the cellular pathways of early HSC lineage commitment. Furthermore, I have investigated the role of hematopoietic growth factors, so called cytokines, in regulation of HSCs and early lymphoid development, and how these factors regulate immune recovery after BM transplantation.

6. Hematopoiesis
Hematopoiesis is the process in which the hematopoietic system ensures the balanced production and replenishment of all the different types of blood cells that constitute the blood and the immune system. Billions of blood cells are being produced daily in man (Ogawa, 1993), and the pool of HSCs is ultimately
responsible for replenishment of all the blood cell types and their progenitors (Fig. 1). The hematopoietic system can be viewed as a hierarchical organisation with infrequent HSCs at the top and all the types of mature blood cells at the bottom (Fig. 1). When moving down in the hierarchy the progeny of HSCs gradually lose ability to self-renew as well as the ability to generate all the different kinds of mature blood cells. In general this process is accompanied by reduced proliferative capacity and most of the mature blood cells have little or no replicative potential. Mature blood cells consist of two main lineages, the lymphoid and myeloid blood cell lineages. The lymphoid cells include B cells, T cells, natural killer (NK) cells and the myeloid lineage contains granulocytes, macrophages, erythrocytes and blood platelets. The complexity of the blood system is further enhanced by several of these lineages consisting of different subtypes and by the fact that certain lineages have lymphoid as well as myeloid origins, such as dendritic cells (DCs) (Laiosa et al., 2006; Shortman and Liu, 2002). Furthermore, recently, it has been shown that NK cells, previously thought only to be derived from the BM, also are generated in the thymus, and interestingly have different characteristics than BM-derived NK cells (Vosshenrich et al., 2006).

Erythrocytes are responsible for transporting oxygen, whereas platelets are involved in blood clotting. The macrophages and granulocytes represent the innate immune defense, that react to infections or inflammation (Akashi et al., 2000). The myeloid cells are relatively short lived, compared to lymphoid cells, reflected by a short half-life that is a few hours (granulocytes) or approximately 100 days (erythrocytes) (Kawamoto and Minato, 2004) compared to up to several years for lymphoid cells (Zinkernagel, 2000).

B and T cells are part of the adaptive immunity, namely they are activated upon encounter of a cell with an antigen that is recognized by a B or T cell. B
Cells are when activated producing plasma cells that secrete antibodies, whereas T cells differentiate into either cytotoxic T cells, that kill cells infected with virus, or another class of T cells that activate other cells such as B cells and macrophages (for detailed review see (Blom and Spits, 2006)). NK cells are lymphoid cells that kill cells that have been infected by microbes as well as tumor cells and are activated in response to interferons or macrophage-derived cytokines (Blom and Spits, 2006).

Figure 1. The classical hematopoietic hierarchy. The HSCs pool can be divided into long-term self-renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSCs) and multipotent progenitors (MPPs). MPPs give rise to the CLPs and the CMPs. The CLPs give rise to lymphoid progenitors of the B, T, natural killer (NK) and dendritic cell (DC) lineages and the CMPs give rise to granulocyte/macrophage progenitors (GMP), megakaryocyte/erythroid progenitors (MEP) as well as DCs.
Mature blood cells are derived via intermediate stages or progenitors, exemplified by the common lymphoid progenitors (CLPs), the common myeloid progenitors (CMPs), and through increasingly restricted progenitors within these two main lineages (Fig. 1). The lineage commitment process is regulated by both intracellular (such as transcription factors) and extracellular (such as cytokines) cues (Laiosa et al., 2006). Whereas we know much about the regulation of the stages from committed progenitors to mature blood cells (reviewed in (Busslinger, 2004; Cantor and Orkin, 2001)), regulation of the earliest stages of HSC commitment is to a high degree unresolved.

The general principles of development, organisation and regulation of HSCs and the blood system are largely shared between species (Godin and Cumano, 2002; Godin and Cumano, 2005; Swiers et al., 2006), in particular within the mammalian system, but there are also distinct differences, and in the case of human hematopoiesis our knowledge remains limited due to the lack of optimal assays and tools (Bonnet, 2002; Guenechea et al., 2001). For this reason, most of our knowledge about human HSCs and hematopoiesis has emerged secondary to similar findings in the mouse. As the basic questions and goals addressed in my thesis appeared to be best addressed in mouse models, the general part of the thesis refers to the mouse system unless otherwise indicated (for detailed reviews of human hematopoiesis see (Blom and Spits, 2006; Bonnet, 2002)).

Primitive hematopoiesis begins with the first blood cells arising from the blood islands in the extra-embryonic yolk sac at embryonic day 7.5 (E7.5) (Godin and Cumano, 2005), consisting mainly of primitive erythroblasts and macrophages, and usually defined as the first wave of primitive hematopoiesis (Palis and Yoder, 2001). The primitive erythroid cells are enucleated and express embryonic and adult globins (Mikkola and Orkin, 2006). The second wave of
hematopoiesis consists of definitive cell types, thought to originate from different subsets within the mesoderm compared to primitive hematopoiesis, and takes place in the yolk sac and in the intra-embryonic sites para-aortic-splanchnopleura (PAS) and aorta-gonads-mesonephros (AGM) (Godin and Cumano, 2005; Mikkola and Orkin, 2006). Definitive erythroid cells express only adult globins and are enucleated before entering the circulation (Mikkola and Orkin, 2006). It is first in the second wave of hematopoiesis, where single lineage colony forming unit–cell (CFU-C), bi-potential CFU-Cs and later also progenitors with lymphoid potential can be found. However these latter cells could only be found in the PAS pre-circulation and not in the yolk sac (Cumano et al., 1996). The site of HSC origin in the embryo is still controversial (Godin and Cumano, 2005; Lengerke and Daley, 2005). There are major challenges related to resolving the site of HSC origin; the limited tools available for assessment of fetal HSC activity (Yoder, 2004), and the fact that the blood circulation connects the different embryonic sites complicates the interpretations of finding HSCs at different locations. Even though yolk sac was shown to harbour the first blood cells in the embryo (Palis and Yoder, 2001), it was rather in the intra-embryonic site, in the AGM, at day 8 of gestation, where the first detectable HSCs were observed upon transplantation into lethally irradiated adult recipients (Godin et al., 1999). However these AGM cells had to be cultured to become transplantable, and it was first at day E10 that functional, adult reconstituting HSCs could be found in the AGM (Medvinsky and Dzierzak, 1996; Muller et al., 1994) and vitelline and umbilical arteries (de Bruijn Embo J 2000). Explant cultures of AGM cells showed that HSCs can be generated from this site independently of the influx from other sites (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996). Further, the yolk sac hematopoietic progenitors isolated from day 8.0, i.e. before onset of circulation ~day E8.5, did not contribute to multilineage reconstitution after transplantation (Cumano and
Godin, 2001; Yoder, 2001). Interestingly, it has been shown that the day E8 yolk sac hematopoietic progenitors have repopulating capacity after co-culture with an AGM-derived stromal cell line (Matsuoka et al., 2001), and it has therefore been argued that the yolk sac would need a maturation step to contribute to definitive hematopoiesis. This has been argued to be a potential reason why yolk sac cells did not show any long-term multilineage reconstitution ability after transplantation into lethally irradiated adult recipients (Yoder, 2004). In addition, E8 or E9 blood islands injected in the yolk sac of fetuses support hematopoiesis for life (Moore and Owen, 1967; Weissman et al., 1978), and E9 and E10 yolk sac HSCs are capable of engrafting sublethally irradiated newborn mice (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b). However it is not until E11 HSCs from yolk sac have been shown to reconstitute adult recipients (Mikkola and Orkin, 2006). Although most available data support an AGM origin of HSCs, the HSCs in this site can only be found within a short period of time and the number of HSCs are few, and therefore it has been questioned whether this is the only site that can supply the rapidly expanding fetal liver with HSCs, or whether yolk sac HSCs could be another contributing independent site (Kumaravelu et al., 2002). Interestingly, recently there have been reports suggesting that the placenta could be yet another site containing HSCs during fetal hematopoiesis (Gekas et al., 2005; Ottersbach and Dzierzak, 2005) and it was demonstrated that the precursor tissue of the placenta (i.e. chorion and allantois) contained endogenous hematopoietic potential (Zeigler et al., 2006). However, due to similar limitations as mentioned above, it has not been ultimately proven that these HSCs originate from the placenta.

The fetal liver is the main site of hematopoiesis from day E12.0, and the fetal liver hematopoietic progenitor cells migrate to the spleen and BM, which make them the most active sites of hematopoiesis at day E18.0 (Yoder, 2004).
Interestingly, studies using in vivo fate tracing with cre-mediated conditional stem cell leukemia (SCL) marker (Gothert et al., 2005) and conditional β1-integrin null mice (Potocnik et al., 2000), strongly support that adult BM HSCs are indeed ancestors of fetal intraembryonic HSCs that have migrated from the fetal liver. The BM remains the primary site of HSCs in the adult, although low numbers of HSCs have been found in other sites such as peripheral blood (PB) (Fleming et al., 1993) and liver (Taniguchi et al., 1996).

7. Hematopoietic stem cells

7.1. The legacy of Till and McCulloch

Most, if not all, of the characteristic properties of HSCs, which we continue to study today, were in principle already established in the classical experiments performed by James E. Till and Ernest A. McCulloch in the 1960-1970s. As late as in 2005, they were awarded the prestigious Lasker Award for their contributions to the field (McCulloch and Till, 2005).

The groundbreaking experiments of Till and McCulloch were those showing that BM cells could give rise to macroscopic colonies in the spleen of irradiated recipient mice after transplantation, so called colony forming units of the spleen (CFU-S) (Till and McCulloch, 1961), consisting mainly of myeloid and erythroid cells. Methods for radiation-induced chromosomal translocations provided means for uniquely marking the progeny of individual stem cells, allowing Till and McCulloch to demonstrate that the CFU-S colonies generated were clonal, namely that they originated from single cells (Wu et al., 1968). Furthermore, by investigating different hematopoietic tissues, it was shown that a single stem cell could give rise to cells of both the myeloid and lymphoid lineages; thus the injected BM cells were multipotent (Abramson et al., 1977; Edwards et al., 1970; Wu et al., 1967; Wu et al., 1968).
Interestingly, *self-renewal* properties of the CFU-S were demonstrated by transplantation of cells isolated from the CFU-S colonies into secondary recipients (Siminovitch et al., 1963). The colony forming cells showed a decrease in proliferative capacity with serial transplantations and did not sustain multilineage reconstitution for the life of a lethally irradiated recipient (Magli et al., 1982; Siminovitch et al., 1964). Therefore these clonogenic BM cells were not considered as HSCs with long-term self-renewal potential, which is currently recognized as one of the hallmarks of HSCs. Even though the CFU-S assay developed by Till and McCulloch did not primarily reflect long-term HSC activity, it was the key discovery which introduced the concept of multilineage self-renewing HSCs to the HSC field.

In the 1980s, analysis of common integration sites in tissues of mice transplanted with retrovirally transduced BM cells, further confirmed that both myeloid and lymphoid cells can be derived from the same single stem cell (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986). The distribution of proviral markers in different blood cell lineages could identify distinct types of stem cell developmental behavior. In addition to cells with long-term repopulating capacity, cells with temporal and transient multilineage repopulating ability were detected, potentially reflecting distinct LT-HSC and ST-HSC cell populations, or alternatively that LT-HSCs can go in and out of cell cycle. The design of these experiments was however questioned due to the use of viruses that potentially could reinfect cells in vivo following a single cycle and reverse transcription (Jordan and Lemischka, 1990). Further it was not possible to in detail examine the clonal contribution of individual stem cells and it was also not possible by these methods to efficiently mark all the stem cells and thereby an underestimation of the number of HSCs that can contribute simultaneously to hematopoiesis could not be excluded (Morrison et al., 1997a). In addition,
concerns have later been raised regarding clonal selection after retroviral marking, by demonstrating that retroviral integrations themselves may trigger clonal expansion (Kustikova et al., 2005).

7.2. Hematopoietic stem cell self-renewal and expansion
HSCs can undergo alternative fates (Fig. 2), self-renewal being the most essential one. Self-renewal is both required and sufficient to sustain hematopoiesis in mouse and humans for life (Ogawa, 1993; Osawa et al., 1996). Thus it is essential to understand the mechanisms that regulate self-renewal. Although major advances have also been made in mammalian models, much knowledge about the self-renewing process has so far been gained from model organisms, such as Drosophila (Lin, 2002; Tulina and Matunis, 2001), and from stem cells in other tissues that can be studied more easily in their natural physical environment and niches, such as the hairpin and testis (Moore and Lemischka, 2006; Spradling et al., 2001).

Figure 2. HSC fates. HSCs can self-renew, undergo programmed cell-death (apoptosis), differentiate or migrate to other locations or tissues.

Based on different assessments, the HSCs in mouse BM have been found to represent approximately 1 in 10-20,000 BM cells (Szilvassy et al., 1990).
HSCs can undergo self-renewing and non-self-renewing divisions, and both of these can be symmetrical or asymmetrical (Fig. 3). In symmetrical self-renewing divisions, both of the daughter cells remain HSCs and it is only by this process HSC expansion can occur. In asymmetrical self-renewing divisions, one of the daughter cells remains a HSC whereas the other one adapts an alternative fate (Weissman, 2000), such as programmed cell death (apoptosis) or lineage commitment (Fig. 2). The mechanisms regulating the different alternative HSC fates are still poorly understood.

**Figure 3. HSC self-renewing divisions.** HSCs can divide symmetrically or asymmetrically in a self-renewing or non self-renewing manner. In HSC self-renewing divisions at least one of the daughter cells remains a HSC, while in non-self-renewing divisions both daughter cells have lost their self-renewing capacity. It is only by symmetrical self-renewing divisions HSCs can expand. White cells represent HSCs, black represents committed cells, and grey alternate committed cells or apoptotic cells.

HSCs expand massively during fetal development, as demonstrated by a 40-fold increase in the number of long-term repopulating units found in the fetal liver from day 12 to 16 of gestation (Ema and Nakauchi, 2000; Pawliuk et al., 1996). The expansion of the HSC population during fetal development must at least in part be the result of HSCs undergoing extensive symmetrical self-renewing divisions. Furthermore, it has been demonstrated that the fetal liver
HSCs are actively cycling in contrast to adult BM HSCs, that mostly reside in the quiescent G0 state (Bowie et al., 2006) (see below).

In contrast to the developing fetal liver, steady state adult BM has a rather stable number of HSCs (Harrison, 1980; Harrison et al., 1988). Recent reports show that HSCs are actively cycling until 3 weeks after birth, when most of the HSCs become quiescent (Bowie et al., 2006) and it has also been demonstrated that fetal liver HSCs have a higher proliferative capacity than adult BM cells (Morrison et al., 1995; Pawliuk et al., 1996; Rebel et al., 1996). Early models, such as the clonal succession model, proposed that HSCs remain out of cell cycle for much of the life of an animal and furthermore that there are only a few HSCs at a time that are recruited into cycle, and when reaching exhaustion of their proliferative capacity they are replaced by other HSCs (Kay, 1965; Lemischka et al., 1986). It later became clear that although being mostly resting, adult stem cells do cycle, although slowly, as demonstrated by all long-term HSCs dividing approximately every 60 days (Bradford et al., 1997; Cheshier et al., 1999).

HSC quiescence is considered to be an important stem cell feature, essential to minimize the susceptibility for mutations giving rise to leukemia and leukemic stem cells, and for avoiding premature stem cell exhaustion (Jordan et al., 2006). Furthermore, the quiescent stem cells in adult mice act as a reservoir and can be driven to self-renew and expand upon stress such as cyclophosphamide and granulocyte colony stimulating factor (G-CSF) (Morrison et al., 1997b), as well as upon irradiation and transplantation (Iscove and Nawa, 1997; Plett et al., 2002). The differences in cell cycle kinetics between fetal and adult HSCs could reflect mainly differences in cell intrinsic properties. However, the fact that adult HSCs expand rapidly and efficiently reconstitute all the hematopoietic lineages after transplantation (Iscove and Nawa, 1997; Pawliuk et al., 1996), would rather implicate an important role of extrinsic regulators in
governing HSC cell cycle progression. These extrinsic cues are today referred to as components of the stem cell niche (Moore and Lemischka, 2006). As our understanding of the composition of the HSC niche is increased (Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003), it is likely that we also will better understand the extrinsic factors controlling HSC fate decisions, such as self-renewal and lineage commitment.

7.3. Cytokine regulation of HSCs

Hematopoiesis and HSC fate decisions are regulated by extrinsic and intrinsic cues. The overwhelming evidence of an important role of transcription factors in hematopoietic lineage decisions (Cantor and Orkin, 2001; Georgopoulos, 2002; Lessard et al., 2004; Mikkola and Orkin, 2006; Zhu and Emerson, 2002), would suggest that intrinsic factors ultimately decide the fate of HSCs. In my thesis however, I have focused on the role of extracellular cues; in particular cytokines and their receptors. Although their role in HSC fate determination remains disputed, their important role in regulating lineage restricted progenitors and in maturation of different blood lineages is unequivocally established (Hofmann et al., 2002; Kaushansky, 2006). However, less is known about their role in HSC regulation. There are also other ligands and receptors not belonging to the classical hematopoietic growth factors that have been implicated to be important for HSC regulation, such as the Wnt and Notch pathways (for review see (Molofsky et al., 2004)).

7.3.1. Role of cytokine receptors and ligands in regulation of HSCs

Although the existence of HSCs has been known for half a century, the knowledge about the regulators of HSC fate decisions, including self-renewal and commitment, remain largely unknown. During the 1990s there were a wide
number of hematopoietic growth factors identified, so called cytokines, existing in soluble and membrane-bound forms. These bind to receptors expressed on the cell membrane and can promote the survival and proliferation of their target cells (Metcalf, 1993; Ogawa, 1993). For most of the hematopoietic lineages, one principal cytokine regulator has been identified, such as thrombopoietin (THPO) for platelets, erythropoietin (EPO) for erythroid cells, interleukin (IL) 15 for NK cells, IL-7 for B and T cells, and G-CSF for granulocytes (Kaushansky, 2006; Metcalf, 1993). However, an important role for these cytokines has primarily been implicated at the stage of committed progenitors and mature lineages, and not in regulation of HSCs and multipotent progenitors.

A number of studies have clearly demonstrated that cytokines, such as IL-6, IL-3, IL-11, fms like tyrosine kinase 3 (FLT3) ligand (FL), stem cell factor (SCF) and THPO can promote proliferation and perhaps to some extent also limited self-renewal and expansion of purified HSCs, and potent synergies between these cytokines have been demonstrated (Metcalf, 1993; Miller and Eaves, 1997; Ogawa, 1993; Sauvageau et al., 2004; Takano et al., 2004). On the basis of these findings it was suggested that cytokines might also play a physiologically important role in regulation of HSCs. However, most cytokine ligand or receptor knock-outs do not have a HSC phenotype, suggesting that the role of cytokines in HSC regulation is highly redundant (Enver et al., 1998; Laiosa et al., 2006; Metcalf, 1993).

It has been documented that in order to promote survival of HSCs, multiple signals may be required to prevent apoptosis. Interestingly, the prevention of apoptosis by enforced expression of the oncogene bcl-2 results in increased numbers of HSCs in vivo, suggesting that cell death plays a role in regulating the homeostasis of HSCs (Domen et al., 1998; Domen and Weissman, 2000).
In the early 1900s dominant white spotting (W) was observed in mice (Russell, 1979), later found to be encoded by c-KIT (Chabot et al., 1988; Geissler et al., 1988). c-KIT belongs to the type III receptor protein tyrosine kinases that all share the same topology: five immunoglobulin like domains in the extracellular-binding domain, a single transmembrane segment, and a cytoplasmic domain (Fig. 4). There are two different splice forms of the c-KIT ligand also called SCF; the transmembrane bound and the soluble form, and it has been demonstrated that stimulation with the transmembrane bound form, lacking a proteolytic cleavage site, leads to sustained activation of the c-KIT receptor, while the soluble form gives rise to rapid and more transient activation and auto-phosphorylation of the receptor (Lyman and Jacobsen, 1998; Miyazawa et al., 1995). Interestingly, Sl/Sl<sup>d</sup> mutant mice that only produce the soluble SCF form, have anemia, pigmentation defects and lack tissue mast cells, demonstrating that the soluble form of SCF can not replace the function of the membrane bound (Brannan et al., 1991; Flanagan et al., 1991; Russell, 1979). Although the mechanistic differences between the two isoforms are not completely understood, signaling differences have been reported, where the membrane bound form of SCF gives rise to a prolonged activation of the Erk1/2 and p38 mitogen-activated protein kinase (MAPK) in contrast to the soluble form (Kapur et al., 2002).
Fig. 4. Members of the tyrosine kinase receptor III family. Receptors for CSF-1, colony stimulating factor 1; PDGF, platelet derived growth factor, fms like tyrosine kinase 3, FLT3; c-KIT. Modified from

Signals through the c-KIT receptor are important for erythropoiesis, lymphopoiesis, mast cell development, megakaryopoiesis, gametogenesis and melanogenesis in mice (Lyman and Jacobsen, 1998; Ronnstrand, 2004). Loss of function mutations in humans lead to piebaldism (Spritz, 1994), characterized by abnormal pigmentation of the hair and skin, deafness and megacolon. Gain-of-function mutations have been found in various human malignancies, such as gastrointestinal stromal tumors, ovarian neoplasm and small lung cancer (Heinrich et al., 2002; Krystal et al., 1996). A role for c-KIT in HSC regulation has been implicated, due to the high levels of expression on HSCs (Ikuta and Weissman, 1992), the ability to act as a HSC survival factor (Keller et al., 1995; Li and Johnson, 1994), as well as being able to act in synergy with other cytokines to promote HSC proliferation (Lyman and Jacobsen, 1998). Interestingly, in steady state hematopoiesis c-KIT^{W41/W41} and c-KIT^{W42/+} (Miller et
mutant mice or viable c-KIT-deficient mice c-KIT\textsuperscript{w/w} (Vickid) (Waskow et al., 2002) have normal numbers of HSCs. Furthermore, studies of Sl/SI mice suggested that c-KIT is not essential for fetal HSCs (Ikuta and Weissman, 1992). Even though c-KIT-deficient fetal liver HSCs and adult BM HSCs are present at close to normal levels, the maintenance of HSCs after competitive transplantation was demonstrated to be severely impaired after transplantation of both fetal liver and adult BM cells (Geissler et al., 1981; Geissler and Russell, 1983; Miller et al., 1996). These studies suggest that c-KIT plays a redundant role in regulation of steady state maintenance of HSCs, but rather plays a crucial role in HSC self-renewal and/or regulation of committed progenitors post-transplantation. Interestingly, no hematopoietic defects have been found in humans with c-KIT deficiencies (Lyman and Jacobsen, 1998), suggesting that c-KIT might have a less significant role in human compared to mouse hematopoiesis. To exclude or establish a role of c-KIT in human hematopoiesis, more in-depth studies are however needed.

The type III class of receptors, includes in addition to c-KIT also the platelet derived growth factor receptor (PDGFR) (α- and β- chains), the macrophage colony stimulating factor (CSF-1) receptor c-fms, and the FLT3 receptor (also called fetal liver kinase 2, FLK2) (Fig. 4). The FLT3/FLK2 receptor was cloned and identified by two groups independently in the early 1990s (Matthews et al., 1991; Rosnet et al., 1991a; Rosnet et al., 1991b), and shortly after, the FLT3 ligand was cloned as well (Hannum et al., 1994; Lyman et al., 1993). Although initially cloned as a HSC specific receptor, recent studies have suggested that FLT3 is in fact not expressed on mouse long-term repopulating stem cells and that upregulation of the FLT3 receptor is rather associated with loss of self-renewal (Adolfsson et al., 2001; Christensen and Weissman, 2001). However, a role for FLT3 in the regulation of HSCs has been
suggested based on the reduced reconstitution ability of FLT3/FLK2 receptor deficient (FLK2<sup>-/-</sup>) BM cells (Mackarehtschian et al., 1995). In apparent contrast to these results, normal HSC levels in FL<sup>-/-</sup> mice have been observed, suggesting a redundant role of FL in regulation of HSCs (Sitnicka et al., 2002). Thus, it still remains to be resolved whether FLT3 signaling is involved in HSC regulation.

The THPO receptor c-MPL belongs to the type I hematopoietic growth factor receptor family (Kaushansky, 2005). Initially it was reported that THPO is an exclusive regulator of megakaryocyte and platelet production, just as EPO is for red blood cell production (de Sauvage et al., 1996; Gurney et al., 1994; Kaushansky, 2006; Krantz, 1991). An important role of THPO in regulation of HSCs was later implicated due to the high levels of expression of c-MPL on HSCs (Solar et al., 1998) as well as the THPO responsiveness of highly purified HSCs (Borge et al., 1996; Sitnicka et al., 1996). Furthermore, more recent and detailed studies have suggested, although not directly studying the HSC compartment itself, that mice deficient in THPO or c-MPL have reduced numbers and/or function of HSCs (Fox et al., 2002; Kimura et al., 1998; Solar et al., 1998), establishing a crucial role of THPO in regulation of HSCs. Interestingly, patients with congenital amegakaryocytic thrombocytopenia (CAMT) who have lack of function mutations in the c-MPL receptor, develop a multilineage deficiency and aplastic anemia within 2 years of birth, compatible with a stem cell deficiency (Ballmaier et al., 2003; Ballmaier et al., 2001).

### 7.3.2. Cytokine signaling pathways

Hematopoietic growth factor receptors can in general be divided into two main groups, those with ligand binding and tyrosine kinase domains within the same polypeptide chain, and those that lack intrinsic tyrosine kinase activity but instead
associate with and activate multiple families of nonreceptor protein-tyrosine kinases (Smithgall, 1998).

Receptor protein-tyrosine kinases of hematopoietic growth factor families including FLT3 and c-KIT, all share a similar structure (Figs. 4 and 5). Upon ligand binding, the receptors are dimerized, upon which the receptor dimers autophosphorylate on specific tyrosine residues. The phosphorylated tyrosines on the intracellular portion of the receptor create high affinity docking sites for Src homology (SH)-2 domain containing signal transduction molecules. As a consequence, different intracellular signaling molecules are recruited and thereby activate diverse intracellular signaling pathways, such as phosphatidylinositol-3 kinases (PI3K)/Akt, Janus kinases (JAK)/ signal transducers and activators of transcription (STAT) and Ras and MAPK pathways, leading to survival and proliferation of the cells (Linnekin, 1999; Ronnstrand, 2004) (Fig. 5).

Figure 5. Signal transduction pathways of c-KIT. Dimerization and autophosphorylation of c-KIT result in cell proliferation and inhibition of apoptosis through several pathways, such as PI3K/Akt system, Ras/MAP kinase cascade, Src family members and the JAK/STAT system. Modified from (Kitamura and Hirota, 2004).
The type I hematopoietic growth factor receptor family, including c-MPL, lack intrinsic tyrosine kinase activity, in contrast to the receptor tyrosine kinase family, and therefore depend on the association with multiple members of various cytoplasmic tyrosine kinase families (Kaushansky, 2005). The c-MPL receptor consists of an extracellular domain with cytokine receptor motifs, a transmembrane domain, and the intracellular domain containing domains that bind with intracellular proteins (Fig. 6). In the case of THPO signaling, binding of THPO to c-MPL results in receptor homodimerization and subsequent activation of JAK kinases that in turn activate molecules that promote cell survival and proliferation including the STATs, PI3K, and the MAPKs (Fig. 6) (Kaushansky, 2005). As indicated in figures 5 and 6, these two distinct families of hematopoietic growth factor receptors activate largely the same signaling pathways. It has therefore been questioned where the specificity of the cytokine signaling lies (Smithgall, 1998), other than in different expression patterns of the receptors themselves. It has been shown though that the phosphorylated receptors c-KIT, FLT3 and c-MPL bind to different downstream molecules (Kaushansky, 2005; Scheijen and Griffin, 2002). Furthermore, different cytokines might induce different gene transcription within a cell.
Figure 6. Signaling pathways activated by THPO. An activated (phosphorylated) form of the c-MPL receptor is shown. Once phosphorylated upon THPO binding to the receptor, several signaling cascades are initiated, such as MAPK, PIP3 and STATs. Modified from (Kaushansky, 2005).

Adaptor proteins are important mediators and regulators of cytokine signaling transduction. These proteins lack catalytic function but possess interaction domains, i.e. Src homology (SH) 2 domains which bind tightly to phosphotyrosine residues or SH3 domains that bind proline rich sequences (Birge et al., 1996). As illustrated in Fig. 6, the initial binding and phosphorylation of the Shc adaptor creates a binding site for a second adaptor, Grb2, that then directs Sos, which in turn acts on Ras (Kaushansky, 2005). The SH2-domain containing adaptor protein family includes APS, SH-2Bα, SH-2Bβ, SH-2Bγ and LNK (Fig. 7). APS and SH2-B have been shown to be phosphorylated after stimulation of growth factor, cytokine and immune receptors, in for instance mast cells (Kubo-Akashi et al., 2004; Nishi et al., 2005).
Figure 7. The SH2-domain containing adaptor proteins. The SH2B/APS/LNK adaptor protein family share two proline-rich (Pro) segments, as well as a conserved pleckstrin homology (PH) domain and a Src homology (SH) 2 domain. The related PH domains are of different sizes. Modified from (Rudd, 2001).

LNK in rat (Huang et al., 1995), mouse (Takaki et al., 1997) and human (Li et al., 2000), share a similar structure as APS and SH2-B, namely a SH-2 domain, an NH2-terminal homologous domain, a pleckstrin homology domain and a COOH-terminal conserved tyrosine phosphorylation site (Osborne et al., 1995; Takaki et al., 2000; Yokouchi et al., 1997). LNK has been demonstrated to inhibit signaling through a broad range of hematopoietic cytokine receptors including c-KIT, IL-3R, IL-7R and c-MPL (Tong and Lodish, 2004; Velazquez et al., 2002). The dominating phenotype of $Lnk^{-/-}$ mice, is an extensive expansion of cells of the B cell lineage, in agreement with the high expression of LNK in B cells (Takaki et al., 2000). Although not explored in detail there were also prior to our studies preliminary indications that primitive stem or progenitor cells might be expanded in $Lnk^{-/-}$ mice (Takaki et al., 2002).
7.3.3. Stochastic and deterministic models of hematopoiesis and instructive and permissive roles of cytokines

The balance between different HSC fate decisions, including self-renewal, lineage commitment and subsequent differentiation is regulated by extracellular cues, such as cytokines, as well as intrinsic mechanisms, including transcription factors. In both cases these regulators might act through instructive or permissive actions, in the first case actively imposing specific fate decisions on HSCs most compatible with a deterministic model for hematopoiesis, and in the second scenario allowing survival of specific cells and thereby their stochastic regulatory programs to be activated. A stochastic or probabilistic model for regulation of HSCs and hematopoiesis, was supported by elegant primary and secondary CFU-S experiments performed by Till and McCulloch, and their observation of random distributions of numbers and sizes of secondary CFU-S colonies derived from single primary CFU-S colonies (Till et al., 1964). Ogawa’s equally elegant blast colony experiments provided further support for hematopoiesis being predominantly a stochastic process (Nakahata et al., 1982).

Although HSC decisions might largely be based on stochastic control mechanisms, this does not preclude that regulatory cues can act in part to alter the probabilities for different cell fates. In that regard it appears as if many transcription factors act in an instructive manner to modify the probabilities for different cell fate outcomes. In contrast most available data would suggest that cytokines act primarily through permissive actions, promoting the selective survival of cells expressing the cytokine receptor involved. This is in part supported by studies in which overexpression of the anti-apoptotic regulator Bcl-2 promotes cytokine-independent growth and differentiation of otherwise cytokine-dependent cell lines (Fairbairn et al., 1993), as well as rescues the phenotype of cytokine ligand or receptor deficient mice (Kondo et al., 1997a;
Lagasse and Weissman, 1997; Maraskovsky et al., 1997). A permissive rather than instructive role of cytokines is further supported by experiments with chimeric cytokine receptors, consisting for instance of the extracellular domain of c-MPL receptor and the intracellular signaling domain of the G-CSFR, still expressed under the control of the c-MPL regulatory elements (Stoffel et al., 1999). Notably, the thrombocytopenic phenotype in c-MPL deficient mice is rescued by the expression of the chimeric receptor, while granulocytic differentiation was not promoted (Stoffel et al., 1999). However, in support of an instructive role of cytokines, ectopic expression of the human IL-2 receptor (IL-2Rβ) in CLPs resulted in granulocyte/monocyte (GM) differentiation after culture in IL-2, while normal CLPs formed exclusively B cells (Kondo et al., 2000), suggesting that introduction of specific cytokine receptors can indeed result in instruction of committed progenitors to differentiate along a route that they normally would not embark on.

8. Delineating the cellular pathways of HSC lineage commitment

8.1. Definition of lineage commitment

Lineage commitment is a process by which a multipotent cell becomes restricted in its lineage fate options to eventually become restricted to a progenitor of a single lineage. Although in the hematopoietic system largely pertaining to commitment of multipotent stem and progenitor cells towards increasingly restricted myeloid and lymphoid progenitors (Reya et al., 2001), the term commitment can also be used to describe how diversity can be obtained within a single blood cell lineage such as the T cell lineage (Starr et al., 2003). The HSC is the only cell that possesses the unique potential to produce all the different blood cell lineages, and is therefore the only truly uncommitted or pluripotent cell within the hematopoietic system. It might not be immediately evident, but
essential, to consider the process of lineage commitment as a process of loss rather than gain of potential, and thus this process might equally well be termed lineage restriction. As will be eluted to below, this fact represents a major challenge and potential problem when studying the molecular as well as cellular pathways of lineage commitment.

8.2. Importance and implications of a better understanding of HSC lineage commitment

Although it remains unclear and controversial how the lineage commitment process from pluripotent HSCs to uni-potent lineage-restricted progenitors occurs (Akashi et al., 2000; Allman et al., 2003; Katsura, 2002; Kawamoto et al., 2000; Kondo et al., 1997b; Laiosa et al., 2006; Lu et al., 2002; Singh, 1996), it is important to appreciate that even the unequivocal demonstration of one such pathway, does not exclude the existence of alternative paths. Identification of the cellular commitment pathways in hematopoietic development will be key towards a better understanding of how normal hematopoiesis is regulated. Lessons can be learned from the B and T cell fields, where far advanced identification and isolation of distinct stages of B and T cell development, have not only provided a highly detailed knowledge of cellular pathways from committed B and T cell progenitors to mature B and T cells, respectively, but also facilitated the identification and characterization of essential regulators of development within these lineages (Anderson, 2006; Bhandoola and Sambandam, 2006; Busslinger, 2004; Hardy and Hayakawa, 2001; Rothenberg and Anderson, 2002). Further, to better understand the cellular origins of leukemias and leukemic stem cells in particular, it is essential to know the identity of their normal counterparts (Bonnet and Dick, 1997; Jordan et al., 2006; Passegue et al., 2003).
8.3. HSC lineage commitment: State of the art

Whereas the existence of a truly adult pluripotent HSC has been unequivocally established (Osawa et al., 1996; Smith et al., 1991), the subsequent stages of commitment remain less well defined (Laiosa et al., 2006). The current prevailing hypothesis of HSC commitment is that the HSC gradually loses self-renewal potential while sustaining pluripotentiality with subsequent stepwise lineage restriction towards increasingly committed progenitors (Reya et al., 2001). The development of a number of improved assays for evaluation of different lineage potentials as well as tools for candidate progenitor purification (fluorescence activated cell sorting (FACS) and monoclonal antibodies) have enabled the identification, characterization and prospective purification of distinct progenitors, such as the CMPs (Akashi et al., 2000) and CLPs (Kondo et al., 1997b), lacking lymphoid and myeloid lineage potentials, respectively. These findings have been key to the establishment of the “classical” hematopoietic commitment model, proposing that the first lineage restriction or branching point from a HSC results in a strict separation between myeloid and lymphoid development (Fig. 1) (Reya et al., 2001). Importantly, the lineage potentials of the CLPs and the CMPs were analyzed and confirmed at the single cell level in vitro (Akashi et al., 2000; Kondo et al., 1997b). Furthermore, subsequent gene expression analysis demonstrated that the CMPs co-express GM and megakaryocyte/erythroid (Mk/E) but not lymphoid affiliated genes while the CLPs co-express B and T lymphoid but not myeloid associated genes, further supporting the lineage restriction of these two multipotent progenitors (Miyamoto et al., 2002). In the human system, a CMP has also been identified and characterized at the single cell level (Manz et al., 2002), while a human CLP (CD34+Lin−CD10+) population has been identified in adult human BM, giving
rise to B, NK and DC cells at a clonal level (Galy et al., 1995). A similar lymphoid progenitor has been identified in human cord blood (CB) (CD34⁺CD38⁺CD7⁺), giving rise to B, NK cells and DCs (Hao et al., 2001).

At variance with lineage-restricted progenitors, HSCs and MPPs have been demonstrated to express multiple lineage-affiliated programs, proposed to reflect their multilineage potentiality (Akashi et al., 2003; Delassus et al., 1999; Hu et al., 1997; Miyamoto et al., 2002). The low levels of gene expression prior to commitment is considered to be a consequence of changes in the chromatin structure of myeloid or lymphoid gene loci, which is a pre-stage to a full-scale transcriptional activation (Felsenfeld et al., 1996; Georgopoulos, 2002; Hu et al., 1997). However, the biological significance of this multilineage transcriptional priming remains to be established.

8.4. Alternative models for HSC lineage commitment

The classical model of HSC lineage commitment postulates that each of the lymphoid and myeloid lineages are generated from a common CLP and CMP, respectively (Dorshkind, 1994; Reya et al., 2001). However, alternative models have been proposed, primarily based on gene targeting studies. One such model is the “sequential model” described by Singh et al, suggesting that commitment could be a process of sequential loss of lineage potentials of a HSC (Fig. 8), mediated primarily by a hierarchy of transcription factors (Singh, 1996).
Figure 8. Alternative model of lineage commitment. The model was proposed based on gene targeting studies of the transcription factors indicated. Progenitor populations with thick ovals indicate unidentified cell populations. Transcription factors positioned at stages where they are required for the commitment or differentiation of the subsequent progenitor. HSC, hematopoietic stem cell; E, erythrocytes; G, granulocytes; M, monocyte; L, lymphocyte; Meg, megakaryocytes. Modified from (Singh, 1996).

c-Myb mutant mice have with the exception of intact megakaryocytopoiesis a severe defect in all hematopoietic lineages, including erythropoiesis (Mucenski et al., 1991). PU.1 deficient mice have, as c-Myb mutants, defective development of lymphoid and myeloid progenitors but normal megakaryocytopoiesis and erythropoiesis (Scott et al., 1994), whereas lack of Ikaros results in a selective defect in development of lymphoid progenitors (Georgopoulos et al., 1994). Further, E2A deficiency leads to virtual loss of B cell progenitors while the T cell lineage is intact (Bain et al., 1994; Zhuang et al., 1994). These data support the intriguing model shown in Fig. 8. However, the validity of this model is somewhat limited by the fact that it remains to be established whether each of these lineage deficiencies reflect a pre- or post-commitment role in lineage development of the investigated transcription factors.

Another alternative model for lineage commitment has been proposed based on studies primarily in the fetal liver, implicating that the first lineage restriction of a HSC results in the initial generation of either a monocyte/megakaryocyte/erythroid (M/Mk/E) progenitor or a M/B/T progenitor,
which subsequently commit into either M/B or M/T progenitors, respectively (Lu et al., 2002). In this model, multipotent lymphoid, B/T progenitors or CLPs are rare or non-existing, in contrast to the classical adult model for lineage commitment.

Although a model in which B and T cell potentials might follow different pathways prior to loss of myeloid potentials is supported by similar findings in other studies (Cumano et al., 1992; Kawamoto et al., 1997; Lacaud et al., 1998; Mebius et al., 2001), conclusions from these studies are limited in part by the use of mixed progenitor populations showing highly heterogeneous lineage outcomes in culture, and/or by the application of assays inefficient at promoting or detecting specific lineage outcomes. Combined, these two limitations make it difficult to conclude whether a specific combination of restricted lineage readouts of a fraction of investigated progenitors, reflects a true lack of the undetected lineage potentials, or whether the assays used to promote or detect specific lineages are not 100% efficient. The same limitations can be argued in the case of paired daughter cell experiments in which purified HSCs appear to occasionally generate Mk/E and GM restricted progenitor cells after a single HSC division, a finding which would implicate that HSCs can commit directly to Mk/E and GM restricted progenitors through asymmetrical cell divisions (Takano et al., 2004). A further limitation of these studies is that lymphoid potentials were not at all investigated.

In further support of alternative pathways of lineage commitment, is the fact that macrophages are the most ancient cells from an evolutionary point of view (Anastassova-Kristeva, 2003; Metchnikoff, 1884). Further, during development erythroid and myeloid cells are detectable at day 7.5 in yolk sac of the mouse, a time point that precedes detectable HSCs as well as lymphoid cells (Godin and Cumano, 2005).
Although it is established that long-term (LT)-HSCs are required and sufficient for the life-long reconstitution of all hematopoietic lineages (Reya et al., 2001; Spangrude et al., 1988), we know from clinical experience that the lack of LT-HSCs is probably not the most significant problem in clinical BM transplantation, but rather, the need for a rapid and efficient replenishment of short-term myeloerythroid cell lineages to overcome the cytopenia caused by the hematopoietic insults (Na Nakorn et al., 2002). The LT-HSCs, not only because they are so rare, but also because they typically are quiescent during steady state hematopoiesis, are not the most efficient stem-progenitor cells at ensuring rapid reconstitution of the entire hematopoietic system to rescue lethally irradiated recipient mice (Jones et al., 1990; Nakauchi et al., 1999; Osawa et al., 1996; Yang et al., 2005; Zhao et al., 2000). The rapid replenishment of myeloid progenitors has rather been suggested to be a key property of a short-term (ST) HSC population, that can provide erythrocytes, platelets and granulocytes to maintain the host viability until LT-HSCs can take over (Na Nakorn et al., 2002). Although several groups have identified populations of cells with potent ST-HSC activity (Osawa et al., 1996; Reya et al., 2001; Yang et al., 2005), it remains to be established whether truly pluripotent ST-HSCs exist at the single cell level, or whether the observed ST-HSC activity rather reflects the activity of a mixture of potent lineage restricted progenitors.

8.5 Methodological challenges and limitations

As eluted to above, to prove lineage commitment of a certain cell population, one has to face the daunting task of proving the absence of lineage potentials. Thus, to convincingly prove that a cell population is committed to a certain lineage is greatly dependent on the efficiency of the in vitro or in vivo assays used to detect the production of both myeloid and lymphoid lineages, as well as the
proliferative capacity of the cell population investigated. In vitro assays are well suited to evaluate the potential of low proliferative cell populations at the single cell level, in contrast to in vivo models in which a single cell is diluted amongst hundreds of millions of cells and therefore single lineage potentials could be very difficult to detect. On the other hand, in vitro conditions do not necessarily provide the same physiological conditions as the in vivo model, and therefore a lack of readout of a certain lineage, could simply reflect a suboptimal assay and not the properties of the analyzed cell population. When evaluating the lineage potential of a cell population, it is therefore crucial to include an upstream multipotent progenitor of which all the lineage potentials efficiently can be evaluated with the assay used, and if possible also a “downstream” lineage restricted progenitor that also efficiently would read out in the culture system used. In addition to optimizing in vitro assays, it is key to establish in vivo models that allow physiological evaluation of the potential from lineage restricted progenitors. An important aspect to consider is the different kinetics of production and survival of different cell lineages, in vitro as well as in vivo. Thus, the optimal time point for detecting a specific lineage could be restricted to a narrow window of time in which the lineage has had time to mature and at which time viable cells still must be present. As an example granulocytes are very short-lived (hours) in contrast to monocytes that accumulate with time.

The advantage of using in vivo models is the possibility to evaluate the behavior of a cell population in its proper environment. In addition, it is possible to investigate how the cells of interest behave under stress, for instance after irradiation which is highly clinically relevant. However, after transplantation the cells need to home to their proper niche, a feature that the cell perhaps normally is not expected to have.
Although clear improvements have been achieved in development of assays for different lineage potentials in vitro as well as in vivo, considerable challenges remain before we have assays in which multipotent progenitors other than HSCs, can read out all lineages with high efficiency at the single cell level.

8.6. Current and future directions and challenges
As outlined above, the main challenge with delineating the cellular pathways of lineage commitment is to identify and purify close to 100% pure stem/progenitor cells and to evaluate all their lineage potentials with high efficiency at the single cell level. With the current in vitro and in vivo models available, it is difficult to prove true multipotentiality at the single cell level. One of the challenges is therefore to improve the current culture conditions and thereby obtain optimal cloning frequencies. Furthermore, one of the major challenges is to evaluate multiple lineage potentials from single hematopoietic/progenitor cells in vitro, especially since it is well established that different lineages require different conditions in vitro. Although not yet developed, there is today great hope put into the established OP9/OP9-DL1 cultures, that enable efficient evaluation of B and T cell potentials from primitive and uncommitted progenitors (Schmitt and Zuniga-Pflucker, 2002; Vieira and Cumano, 2004). In addition, better in vivo mouse models for tracing lineage commitment should be developed. It is questionable, though, whether it at all will be possible to evaluate the lineage potentials of single non-HSCs using in vivo models. A more feasible approach appears to be to further develop multilineage assays for single progenitors in vitro, and to use the in vivo experimental setting to functionally evaluate the cells of interest at a population level.

Therefore, the combined efforts of prospective purification of distinct stages of lineage commitment, functional evaluation of their lineage potentials in
vitro and in vivo, as well as their molecular characterization, will provide complimentary tools to delineate the early cellular events of lineage commitment and thereby also uncover the regulatory mechanisms, and eventually provide specific molecular signatures of distinct lineage commitment fates.

9. B lymphopoiesis

9.1. Developmental aspects of B lymphopoiesis

9.1.1. From HSCs to mature B cells

The earliest commitment steps of HSCs to committed progenitors remain largely unresolved. Although the CLP (Lin⁻Sca-1⁻c-KIT⁺IL-7Rα⁺) is a candidate for the earliest restricted lymphoid progenitor in the adult BM, giving rise to B, T and NK cells but no myeloid cells (Kondo et al., 1997b), alternative lymphoid restricted progenitors have been identified during the past few years. Recently the CLP-2 (c-KIT⁺B220⁺CD19⁻pre-Tα⁺) was identified in the BM using a transgenic reporter mouse in which human CD25 is expressed under control of the pre-TCRα promoter (Martin et al., 2003). Cell culture experiments using single cells showed a bipotent B and T cell potential (Martin et al., 2003) and intravenous transplantations showed that the CLP-2 population was able to produce T, B, NK and dendritic cells (Gounari et al., 2002; Martin et al., 2003). Furthermore, it was proposed although not directly demonstrated that CLP-2s are progeny of conventional CLPs (Martin et al., 2003). A potential precursor to the CLP was recently identified, the early lymphocyte progenitor (ELP; Lin⁻Sca-1⁺c-KIT⁺CD27⁺), that initiate RAG1 and RAG2 expression and starts to undergo D_{HJ} rearrangements at the immunoglobulin heavy-chain (IgH) locus, and that have combined B, T and NK cell potential (Igarashi et al., 2002). Although reduced, the myeloid potentials of the ELP have yet to be investigated carefully at the single cell level. Based on these results and gene expression analysis showing a
lymphoid restricted gene expression pattern it was suggested that ELPs are precursors to CLPs, that express low levels of Sca-1 and c-KIT, and in addition express IL-7Rα. However, once again the direct relationship between these multipotent lymphoid progenitors has not been established. Interestingly, a recent study from Balciunaite et al identified an early B220+ B/T lymphoid precursor population (B220+c-KIT+CD19−) in the adult BM with residual macrophage potential (Balciunaite et al., 2005).

Whereas the earliest steps from HSCs to lineage restricted precursors remain to be determined, the understanding of the cellular pathways from the earliest committed B cell progenitors to mature B cells is well established. Restricted lymphoid progenitors, such as CLPs, develop stepwise into pre-pro-B (B220+CD43+AA4.1+CD19−Ly-6C−) (Allman et al., 1999; Hardy et al., 1991; Li et al., 1996; Tudor et al., 2000), pro-B (B220+CD43+CD19+AA4.1+) (Hardy et al., 1991; Li et al., 1993; Li et al., 1996) and pre-B cells (B220+CD43−IgM−) after functional rearrangement and expression of immunoglobulin heavy chain genes as part of the pre-B cell receptor (BCR) complex (Meffre et al., 2000; Osmond et al., 1998). Pre-B cells that rearrange immunoglobulin light-chain genes differentiate further into immature B (B220lo/intIgM+) and finally mature B cells (B220int/hiIgM+IgD+) (Meffre et al., 2000) (Fig. 9). The different stages of B cells have distinct rearrangement statuses, co-express different cell surface markers and key cytokine receptors such as FLT3 and IL-7 receptor α (IL-7Rα).
Figure 9. B cell development in adult bone marrow. Rearrangement status and expression pattern of cytokine receptors are indicated at specific stages of B cell development. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; Fr, fraction.

9.1.2. Fetal B-1 and conventional B-2 B lymphopoiesis

Mature B cells can be divided into B-1 and B-2 cells, demonstrated to differ in phenotype, developmental origin, distribution and function (Hardy and Hayakawa, 2001; Herzenberg, 2000). The conventional B-2 cells can be further divided into follicular (FO) B cells and marginal zone (MZ) B cells (Martin and Kearney, 2002; Oliver et al., 1997). FO B cells represent the major subset of B cells which recirculate through secondary lymphoid organs, and are found in the B-lymphoid follicles in the spleen and lymph nodes. MZ B cells are a minor subset localised to the marginal sinus of the spleen (Lopes-Carvalho and Kearney, 2004; Martin and Kearney, 2002). FO B cells are part of the adaptive immune responses, and mostly participate in antigen-stimulated antibody responses. MZ B cells on the other hand are part of the innate immunity and participate mostly in T-independent responses, even though some are stimulated by T dependent antigens (Herzenberg, 2000). MZ B cells are through their intimate contact with the blood and in conjunction with specialized macrophages, able to screen blood borne pathogens, upon which they divide and develop into IgM producing plasma cells within 3 days (Martin and Kearney, 2000). In this way MZ B cells provide fast antibody and/or T cell responses, until the adaptive antibody responses reach peak levels (Bendelac et al., 2001). Furthermore, MZ B...
cells appear to be mostly produced during a small window of time postnatally but subsequently sustained through peripheral mechanisms (Martin and Kearney, 2002).

B-1 B cells develop *de novo* from progenitors present in fetal/neonatal life and persist thereafter as a self-replenishing population, whereas B-2 cells develop during fetal life and continue to be *de novo* generated from B cell progenitors in the BM throughout life (Herzenberg, 2000). B-1 cells are considered to be independent of T cell help and thereby a part of the innate immunity and the major source of spontaneously produced antibodies. Most of the B cells found in the peritoneal and pleural cavities in adult mice are B1 cells and are IgM$^{hi}$IgD$^{lo}$CD11b$^{+}$ (Hayakawa et al., 1985; Lalor et al., 1989), and can be further subdivided into B-1a cells, expressing CD5, or B-1b cells, that are CD5 negative (Herzenberg, 2000; Herzenberg et al., 1986; Kantor and Herzenberg, 1993; Stall et al., 1992). B-1 cells can also be found in the spleen in adult mice and have a different phenotype (IgM$^{hi}$CD23$^{lo/-}$CD43$^{+}$) (Martin and Kearney, 2002; Wells et al., 1994). The functional properties of B-1a and B-1b cells are distinct as demonstrated by B-1a B cells secreting natural antibodies and are crucial in the rapid response to for instance bacteria, whereas antibodies produced by B-1b cells are induced after antigen exposure and are required for long-lasting protective immunity against pathogens (Alugupalli et al., 2004; Haas et al., 2005).

Early transplantation experiments demonstrated that fetal liver cells efficiently reconstituted B-1 B cells, whereas adult BM cells were better in generating B-2 cells (Hardy and Hayakawa, 1991; Herzenberg, 2000; Kantor and Herzenberg, 1993), suggesting that B-1 cells are primarily produced by distinguishable progenitors in fetal/neonatal life. It was long thought that B-1 cells derive exclusively from fetal/neonatal precursors, however it was recently
demonstrated that B-1a and B-1b cells, but not B-2 cells, can also be derived from rare Lin\(^{-}\)CD45R\(^{\text{neg/lo}}\)CD19\(^{+}\) progenitors in adult BM, although they should still be considered as primarily of fetal or early postnatal origin (Montecino-Rodriguez et al., 2006). Furthermore, it has been suggested that B-1a, B-1b and B-2 cell progenitors develop independently and can be subdivided based on the differential expression of syndecan I (CD138) and MHC class II (Montecino-Rodriguez and Dorshkind, 2006; Tung et al., 2006).

9.2. Cytokine regulation of B lymphopoiesis

B lymphopoiesis has been shown to be regulated both by cell intrinsic and extrinsic mechanisms (Busslinger, 2004). A multitude of lineage restricted transcription factors have been shown to control B cell development from HSCs to immunoglobulin secreting plasma cells (Busslinger, 2004). The commitment and development of lymphoid precursors to B cells involve three key transcription factors; E2A, early B cell factor (EBF) and Pax5 (BSAP). The E2A gene encodes the alternative splice products E12 and E47 (Murre et al., 1989). Although B cell development in E2A, EBF as well as Pax5 deficient mice is arrested at the pro-B cell stage (Bain et al., 1994; Lin and Grosschedl, 1995; Urbanek et al., 1994; Zhuang et al., 1994), a hierarchical relationship between these B cell specific transcription factors has been proposed, in which E2A acts upstream of EBF, in turn acting upstream of Pax5 (Busslinger, 2004).

Both fetal and adult B lymphopoiesis have also been demonstrated to be regulated by a range of cytokines, such as IL-7, thymic stromal-derived lymphopoietin (TSLP), FL and SCF (Carvalho et al., 2001; Kang and Der, 2004; McKenna et al., 2000; Sitnicka et al., 2003; Waskow et al., 2002; Vosshenrich et al., 2003; Vosshenrich et al., 2004). c-KIT is expressed on the earliest B cell progenitors and ceases to be expressed on pre-B cells (Lyman and Jacobsen,
A role for c-KIT in regulation of B cells was implicated from early studies using anti-c-KIT blocking antibodies in pro-B cell cultures (Rolink et al., 1991), findings recently corroborated by adult viable c-KIT null (c-KIT\textsuperscript{Vickid}) mice having reduced B cell progenitors (Waskow et al., 2002). FLT3 although not expressed on mouse HSCs (Adolfsson 2001) is expressed at very early hematopoietic stages, including MPPs, but ceases to be expressed around the pro-B cell stage (Fig. 9) (Busslinger, 2004; Karsunky et al., 2003; Wasserman et al., 1995). Mice deficient in either FLT3/FLK2 receptor (Mackarehtschian et al., 1995) or FLT3 ligand (McKenna et al., 2000; Sitnicka et al., 2002) have severely reduced CLPs, B cell progenitors, as well as NK cells and DCs, demonstrating an important role of FLT3 signaling in early B lymphopoiesis. Interestingly, FLT3 has been shown to act in potent synergy with IL-7 to promote the generation of committed B cell progenitors from MPPs (Hunte et al., 1996; Lyman and Jacobsen, 1998; Veiby et al., 1996).

IL-7R is a member of the common gamma (γc) chain receptor superfamily, a signaling receptor subunit shared by a number of cytokines, including IL-2, -4, -7, -9, -15 and -21 (Kang and Der, 2004). IL-7R is a heterodimer composed of a IL-7Rα ligand binding chain and a γc signaling chain which also confers high affinity ligand binding to the IL-7R. IL-7Rα starts and ceases to be expressed at somewhat later stages in B cell development than FLT3 (Fig. 9) and IL-7 is important for the in vitro survival, proliferation and differentiation of B cell progenitors (Milne and Paige, 2006). IL-7\textsuperscript{-/-} and IL7Rα\textsuperscript{-/-} mice have severely impaired B lymphopoiesis (Carvalho et al., 2001; Dias et al., 2005; Maki et al., 1996; Peschon et al., 1994; von Freeden-Jeffry et al., 1995), and interestingly IL-7Rα\textsuperscript{-/-} mice have been reported to have a more severe phenotype than IL-7\textsuperscript{-/-} mice (Vosshenrich et al., 2003; Vosshenrich et al., 2004). The signaling through IL-
7Rα and/or FLT3 has been demonstrated to be absolutely crucial for B cell development in both fetal and adult mice (Sitnicka et al., 2003).

The α chain of the IL7R is also a component of the high affinity receptor complex for TSLP, which heterodimerizes with the unique TSLP receptor γc-like chain (Park et al., 2000). The TSLPR is expressed on pro-B cells (Vosshenrich et al., 2003) as well as on mouse pre-B cell lines (Isaksen et al., 2002), and interestingly it was recently demonstrated that fetal but not adult pro-B cells were responsive to TSLP (Vosshenrich et al., 2003; Vosshenrich et al., 2004). Furthermore, IL-7 has been implicated to be important for adult B lymphopoiesis and to be more redundant in fetal or early post-natal B lymphopoiesis, since IL-7−/− mice show an age-progressive loss of conventional B cell production but sustained fetally derived B1 cells (Carvalho et al., 2001; Vosshenrich et al., 2003). Importantly though, it is difficult to state whether the decline of B cell progenitors in adult IL-7−/− mice reflects an important role of IL-7 exclusively in adult B lymphopoiesis or whether this is a secondary effect of impaired fetal B lymphopoiesis. Since TSLP is the other ligand of the IL-7Rα, and due to the redundant role of TSLP in adult B cell development observed in TSLPR deficient mice, it has been suggested that TSLP might be distinctly important for IL-7-independent fetal B lymphopoiesis (Al-Shami et al., 2004; Carpino et al., 2004; Montecino-Rodríguez and Dorshkind, 2006; Vosshenrich et al., 2003; Vosshenrich et al., 2004). However, as B cell development has not been investigated in TSLPR−/− fetal mice its role in fetal B lymphopoiesis remains to be established. As alternative explanations for the different B cell phenotypes of IL-7 and IL-7Rα deficient mice, it could be speculated that there could be other, not yet identified, ligands of the IL-7Rα that could play an important role in fetal B lymphopoiesis. Moreover, it has been proposed that c-KIT can transactivate IL-7Rα signaling in the absence of IL-7Rα ligands (Jahn, 2002). To ultimately
understand the exact roles of IL-7R, FLT3 and TSLPR in B cell development during different stages of ontogeny, it would be crucial to generate conditional cytokine receptor knock-out mice.

Interestingly, Pax5 has been demonstrated to activate the lineage specific genes CD19 and mb-1, while repressing the myeloid macrophage colony stimulating factor receptor (M-CSFR) and T cell specific Notch1 (Nutt et al., 1998; Souabni et al., 2002), and is often referred to as a “B cell lineage locker”. Recently, it was demonstrated that Pax5, regulates B cell maturation in part by repressing FLT3 expression on pro-B cells, and in the absence of FLT3 down-regulation B cell development is blocked at this stage (Holmes et al., 2006). The transcription factor PU.1 is also crucial for B cell development (McKercher et al., 1996; Scott et al., 1994) and interestingly IL-7Rα has been identified as a direct PU.1 target (DeKoter et al., 2002). Moreover, PU.1 has been shown to be important for early B cell development perhaps acting though regulation of FLT3 expression (Laslo et al., 2006). Ikaros is able to activate FLT3 expression in multipotent progenitors (Nichogiannopoulou et al., 1999) and interestingly Ikaros null MPPs lacking FLT3 expression sustain myeloid but virtually lack lymphoid potential (Yoshida et al., 2006). Taken together these data suggest that the upregulation of FLT3 on primitive stem/progenitor cells as well as its subsequent down-regulation at the pro-B cell stage are pre-requisites for normal B lymphopoiesis.

10. Clinical aspects of normal and malignant hematopoiesis

10.1. BM transplantation

10.1.1. Historical perspectives and current status

The concept of HSCT began with observations associated with the first atomic bomb explosion in 1949 demonstrating that mice could be rescued from the
ionizing radiation by protecting their spleens and femurs with lead (Jacobson, 1949). It was however not until 1955 that the pioneering studies were initiated with the human BM grafting program by E. Donall Thomas and his colleagues, and today HSCT is used to treat malignant and non-malignant hematological and immunological diseases, but also used as a supportive treatment of solid tumors (Little and Storb, 2002; Storb, 2003). HSCT after high dose chemotherapy has developed into an effective treatment for many patients with life-threatening diseases, and this breakthrough in modern medicine was recognized through the Nobel prize awarded to E. Donall Thomas in 1990 (Thomas, 1995).

Most patients that undergo HSCT have a malignancy resistant to standard chemotherapy and/or are considered to have a high risk of relapse (Shizuru et al., 2005). Allogeneic transplantations, i.e. transplantation of cells from a healthy donor, is usually used in patients that suffer from a disease thought to originate from stem cells or early progenitors and are frequently associated with the life threatening graft versus host disease (GVHD) due to mismatches in the histocompatibility between donor and host cells. However, a similar graft versus leukemia (GVL) or graft versus tumor (GVT) effect is equally essential to eliminate the residual tumor cells frequently remaining after aggressive chemotherapy (Auletta and Lazarus, 2005). Another serious side effect of the high dose chemotherapy used to condition the recipient BM prior to transplantation, is the life threatening cytopenia resulting from the chemotherapy-induced elimination of highly proliferative hematopoietic progenitor cells, rendering the patients highly susceptible to bleedings and infections until progeny of the transplanted HSCs have differentiated into fully functional platelets and white blood cells (granulocytes), respectively (Storb, 2003).

In autologous transplantations, in which the patients’ own stem cells are transplanted, obviously the problem with GVHD is eliminated, but at the same
time the benefit of GVL is lost, and this combined with the graft itself potentially containing tumor cells, results in considerably enhanced risk for relapse of the malignancy. Thus, in contrast to allogeneic transplantations, autologous HSCT rarely offers a curative potential.

In order to simplify, improve and increase the HSC harvest for transplantation, it is now routine to mobilize HSCs from the BM to the PB by treatment of the donor with G-CSF, sometimes together with low-dose chemotherapy (Little and Storb, 2002). PB stem cell transplants result in accelerated myeloid recovery (Devine et al., 2003) and eliminate the need for anesthetic treatment of the donor. HSCs can also be harvested from CB, which due to its primitive nature give rise to less GVHD, however the low amounts of HSCs that can be retrieved has largely limited its use to children (Wagner et al., 2002). Recently, promising results have been obtained when CB from two donors have been co-transplanted, perhaps resulting in improved engraftment, and without any obvious rejection between the samples (Barker et al., 2001; De Lima et al., 2002; Nauta et al., 2005). If confirmed in larger trials, this would suggest that a 2-fold ex vivo expansion of CB stem and progenitor cells prior to transplantation could be sufficient to improve the outcome of single CB transplants in adults.
10.1.2. Challenges in clinical BM transplantation

For many patients with hematological malignancies, HSCT represents the only curative option (Buckley et al., 1999; Haddad et al., 1998). However, as outlined above HSCT is still associated with high mortality and morbidity (Devine et al., 2003; Little and Storb, 2002). GVHD can be virtually eliminated if the donor graft is depleted of T cells (Shizuru et al., 2005), although such treatment is often associated with increased incidences of graft failure (Martin et al., 1985). This is probably due to the observed engraftment promoting effect of T cells (Dey and Spitzer, 2006; Gandy et al., 1999; Kaufman et al., 1994).

Due to the low amounts of HSCs found in CB, there have been attempts to ex vivo expand CB HSCs for transplantation. However, most efforts have rather resulted in reduced HSC numbers or the increase has been marginal or questionable (Bhatia et al., 1997; Fernandez et al., 2001; Glimm and Eaves, 1999; Sauvageau et al., 2004; Sorrentino, 2004). Thus, it is key to better understand the mechanisms that regulate HSC survival and self-renewal, and some recent studies suggest that regulators such as Hedgehog proteins, bone morphogenic proteins (BMPs), Notch and HOXB4 might eventually prove to be helpful (Sauvageau et al., 2004). In the mouse system recent reports have documented up to a 30-fold expansion of HSCs using a combination of growth factors including insulin like growth factor 2 (IGF-2), fibroblast growth factor 1 (FGF-1) and angiopoietin like 2 and 3 (Angptl2, 3) (Zhang et al., 2006), but it remains to be established whether this approach can be used for ex vivo expansion of human HSCs.

Whereas the myeloid cells recover relatively rapidly after chemotherapy and HSCT, the levels of B and T cells (key components of the adaptive immunity) frequently remain severely reduced and function abnormally for several months or even years after transplantation (Auletta and Lazarus, 2005),
increasing the risk for infections and relapse of the disease. The delayed recovery has been explained by several factors, such as the immunosuppressive effect of high dose chemotherapy, the effect of GVHD, treatment of GVHD by immunosuppressants and decreased thymic function in older patients (Auletta and Lazarus, 2005). Interestingly, FL, IL-7 and keratinocyte growth factor (KGF) have been shown to restore T lymphopoiesis through thymic preservation (Fry et al., 2004; Mackall et al., 2001; Rossi et al., 2002) and in addition both FL (Teshima et al., 2002; Yunusov et al., 2003) and KGF (Krijanovski et al., 1999; Panoskaltsis-Mortari et al., 2000) have been demonstrated to attenuate GVHD as well as preserving GVL effects. However, it has been demonstrated that administration of IL-7 and FL worsens the GVHD effect after BMT (Blazar et al., 2001; Sinha et al., 2002). Notably, FL and IL-7 treatment in humans has no significant effect on B cell recovery (Milne and Paige, 2006; Wodnar-Filipowicz, 2003). Even though no cytokine treatment has been established to accelerate B and T cell recovery post-transplantation (Auletta and Lazarus, 2005), recent studies show that IL-7 administration after BMT selectively can increase the non-T regulatory CD4+ T cells and CD8+ T cells (Rosenberg et al., 2006) that potentially could be used in the clinic.

10.2. Cytokines in clinical hematology

10.2.1. Activating mutations in cytokine receptors and signaling pathways

Activating mutations in the FLT3 receptor have been identified as the most common mutations in acute myeloid leukemia (AML) (Levis and Small, 2003), where the most common mutation is the FLT3 internal tandem duplication (ITD) found in approximately 30% of adult (Rombouts et al., 2000) and 5-15% of childhood (Iwai et al., 1999; Meshinchi et al., 2001) AMLs. ITD mutations result in ligand-independent dimerization and tyrosine phosphorylation (Kiyoi et al.,
1998) as well as activation of STAT5, RAS/MAPK and PI3K pathways (Hayakawa et al., 2000; Mizuki et al., 2000), by which the cytokine receptor is constitutively activated. FLT3 “activation loop” mutations also result in constitutive activation of the receptor, and can be found in 7% of AML patients (Gilliland and Griffin, 2002). Both the FLT3-ITD and activation loop mutations can also be found in other malignancies, such as myelodysplastic syndromes (MDS) and acute lymphocyte leukemias (ALLs), although much less frequently, and interestingly there are no reports showing that the FLT3-ITD nor activation loop mutations can on their own lead to leukemogenesis (Gilliland and Griffin, 2002; Kelly et al., 2002; Kiyoi et al., 1998). In addition to FLT3 mutations in AML, N-RAS and K-RAS mutations are observed in approximately 20% of adult AML cases and c-KIT mutations in an additional 5% (Gilliland and Griffin, 2002). There are several lines of evidence that support the hypothesis that a combination of at least two different types of mutations is required for leukemia development, where one class of mutations would induce a proliferative or survival advantage and where the other one would interfere with differentiation (Gilliland and Griffin, 2002; Kuchenbauer et al., 2005; Valk et al., 2004).

Although the mechanisms by which the mutations in cytokine receptors would confer leukemia transformation are not resolved (Gilliland and Griffin, 2002), there are several ongoing trials using potent FLT3 inhibitors (Brown and Small, 2004), as there are promising results from the mouse system (Levis et al., 2001; Naoe et al., 2001; Tse et al., 2001). Other tyrosine kinase inhibitors, in particular imatinib mesylate (Gleevec) inhibiting the BCR-ABL tyrosine kinase, has with considerable success been used in patients with Philadelphia positive chronic myeloid leukemia (CML) (Druker, 1996; Sawyers, 1999). Although these inhibitors might not efficiently target the leukemic stem cells in these patients
(see below), they efficiently eliminate downstream progenitors, resulting in extensive and prolonged clinical remissions.

Activating mutations of c-MPL exist but are rare, resulting in cytokine independent signaling. Such mutations have been observed in a family with essential thrombocythemia (ET) (Ding et al., 2004), resulting in a normocellular and normoplastic bone marrow except for increased megaryopoiesis.

The existence of leukemic stem cells have been clearly demonstrated in CML and AML and they have been implicated in ALL (Dalerba et al., 2006; Jordan et al., 2006). Even though drugs such as imatinib have been developed to target leukemia, this drug mainly targets the progeny of the leukemic stem cells and does not appear to efficiently kill the leukemic stem cells themselves (Graham et al., 2002). It is still not entirely clear what role cytokine receptor signaling and mutations play in the regulation of leukemic stem cells, however it has been demonstrated that AML stem cells have constitutive activation of the PI3K pathway promoting survival of malignant cells, something that is not seen in normal stem cells (Xu et al., 2003).

10.2.2. Cytokine lack-of-functions and clinical implications

Lack of function mutations of the c-MPL receptor have been found in children with CAMT (King et al., 2005). CAMT is a rare disease that usually presents at birth in the form of thrombocytopenia. However children with CAMT develop aplastic anemia within one to five years after birth, most likely due to a stem cell defect (Ballmaier et al., 2003).

Autosomal–dominant piebaldism is associated with loss-of-function mutations in c-KIT, a syndrome associated with deafness, megacolon, abnormalities in pigmentation of skin and hair (Lennartsson et al., 2005). Piebald trait represents the human homologue of the W mutation in the mice. No
hematopoietic defects have been reported as a result of human c-KIT deficiency (Lyman and Jacobsen, 1998), suggesting that c-KIT might play a less significant role in human hematopoiesis. However, more detailed studies would be required to more definitely establish the role (or lack thereof) of c-KIT in human hematopoiesis. In addition, no loss of function mutations of FLT3 or FL have been reported.

In patients with severe congenital neutropenia, mutations in the G-CSF receptor (G-CSFR) results in hypoproliferation but hampered maturation of myeloid cells and normal affinity to G-CSF (Dong et al., 1995; Dong et al., 1993). Interestingly, approximately half of the severe congenital neutropenia (SCN) patients with mutations in G-CSFR develop AML (Bernard et al., 1998). Despite of this, these mutations are rarely seen in de novo AML (Carapeti et al., 1997).

Children with X-linked severe combined immune deficiency (SCID), have a lack of function mutation in the γc cytokine receptor. Interestingly, when compared to γc-deficient mice which have a severe B and T cell deficiency (DiSanto et al., 1995), these patients have a restricted T cell deficiency but normal B cells (Milne and Paige, 2006). Similarly, patients with lack of function IL-7Rα mutations sustain normal B lymphopoiesis in contrast to IL-7Rα deficient mice (Peschon et al., 1994; Puel et al., 1998). Importantly, these findings unequivocally establish an important difference in cytokine regulation of mouse and human B lymphopoiesis, and raise the question as to which cytokine(s) might be critical for human B lymphopoiesis. One intriguing possibility would be that FL would play a more important role in human B cell development.
10.2.3. Applications of cytokines in clinical haematology

As mentioned above G-CSF has been used in order to mobilize HSCs from BM to PB in association with HSC transplantation, in treatment of congenital cytopenias, as well as in post-chemotherapy and post-transplantation granulocytopenia (Couban et al., 2000; Devine et al., 2003). EPO administration is often used in patients with anemia (Chang et al., 2005; Lu et al., 2005). THPO has been used in clinical trials in an attempt to accelerate platelet recovery after chemotherapy, however disappointingly THPO failed to significantly accelerate platelet recovery due to absence of sufficient progenitors responsive to THPO, and in addition several subjects started to produce antibodies against a truncated form of THPO, and these antibodies cross reacted with endogenous THPO, causing severe thrombocytopenia (Kaushansky, 2005). Therefore THPO has not yet been approved for use in the clinic. Attempts have been made to use IL-7 as well as FL for enhancement of immune recovery post transplantation, however so far these attempts have not been successful (Milne and Paige, 2006; Wodnar-Filipowicz, 2003).
11. Abstracts of articles and manuscripts in thesis

11.1. Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK


The role of cytokines as regulators of hematopoietic stem cell (HSC) expansion remains elusive. Herein, we identify thrombopoietin (THPO) and the cytokine signaling inhibitor LNK, as opposing physiological regulators of HSC expansion. Lnk^{-/-} HSCs continue to expand post-natally, up to 24-fold above normal by 6 mo of age. Within the stem cell compartment, this expansion is highly selective for self-renewing long-term HSCs (LT-HSCs), which show enhanced THPO responsiveness. Lnk^{-/-} HSC expansion is dependent on THPO, and 12-wk-old Lnk^{-/-}Thpo^{-/-} mice have 65-fold fewer LT-HSCs than Lnk^{-/-} mice. Expansions of multiple myeloid, but not lymphoid, progenitors in Lnk^{-/-} mice also proved THPO-dependent.
11.2. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: a revised road map for adult blood lineage commitment.


All blood cell lineages derive from a common hematopoietic stem cell (HSC). The current model implicates that the first lineage commitment step of adult pluripotent HSCs results in a strict separation into common lymphoid and common myeloid precursors. We present evidence for a population of cells which, although sustaining a high proliferative and combined lympho-myeloid differentiation potential, have lost the ability to adopt erythroid and megakaryocyte lineage fates. Cells in the Lin^Sca-1^c-kit^+^ HSC compartment co-expressing high levels of the tyrosine kinase receptor Flt3 sustain granulocyte, monocyte, and B and T cell potentials but in contrast to Lin^Sca-1^c-kit^-Flt3^- HSCs fail to produce significant erythroid and megakaryocytic progeny. This distinct lineage restriction site is accompanied by downregulation of genes for regulators of erythroid and megakaryocyte development. In agreement with representing a lymphoid primed progenitor, Lin^Sca-1^c-kit^CD34^Flt3^+^ cells display upregulated IL-7 receptor gene expression. Based on these observations, we propose a revised road map for adult blood lineage development.
11.3. Crucial role of FLT3 ligand in immune reconstitution following bone marrow transplantation and high dose chemotherapy


Almost five decades after the first clinical transplantations, delayed immune reconstitution remains a considerable hurdle in bone marrow (BM) transplantation, and the mechanisms regulating immune reconstitution post-transplantation remain to be established. Whereas adult fms-like tyrosine kinase 3 ligand deficient (FL−/−) mice have reduced numbers of early B and T cell progenitors, they sustain close to normal levels of mature B and T cells. Herein, we demonstrate that adult BM cells fail to reconstitute B cell progenitors and conventional B cells in lethally irradiated FL−/− recipients, which also display delayed kinetics of T cell reconstitution. Similarly, FL is essential for B cell regeneration following chemotherapy-induced myeloablation. In contrast, fetal progenitors reconstitute B lymphopoiesis in FL−/− mice albeit at reduced levels. A critical role of FL in adult B lymphopoiesis is further substantiated by an age-progressive decline in peripheral conventional B cells in FL−/− mice, whereas fetally and early postnatally derived B1 and marginal zone B cells are sustained in a FL-independent manner. Thus, FL plays a crucial role in sustaining conventional B lymphopoiesis in adult mice, and as a consequence, our finding implicate a critical role of FL in promoting immune reconstitution following myeloablation and BM transplantation.
11.4. FLT3 ligand is dispensable for optimal engraftment and expansion of fetal and adult hematopoietic stem cells


Originally cloned from hematopoietic stem cell (HSC) populations, and its ligand being extensively used to promote ex vivo HSC expansion, the cytokine receptor fms-like tyrosine kinase 3 (FLT3) and its ligand, was expected to emerge as a regulator of HSC maintenance and expansion. However, the role of FLT3 in HSC regulation remains unclear and controversial. Herein, FLT3 ligand (FL)-deficient mice were used to precisely assess the importance of FL in regulation of actively cycling and expanding HSCs during development and post-transplantation. Advanced phenotypic analysis established that HSC reconstitution of lymphopoiesis is highly FL-dependent, but that HSC expansion in fetal liver and maintenance in adult steady state hematopoiesis is FL-independent. Further, primary and secondary transplantations of bone marrow (BM) as well as fetal liver cells into FL-deficient mice confirmed a key role of FL in B cell reconstitution, but a dispensable role in post-transplantation HSC engraftment and expansion. In conclusion, these studies unequivocally establish that FL is dispensable for steady state HSC maintenance, and for HSC expansion during fetal development and post-transplantation.
12. Discussion of articles in thesis

12.1. Role of LNK and THPO in regulation of hematopoietic stem cells

12.1.1. Limitations and future directions

A) Implications of current findings

As ex vivo HSC expansion for clinical purposes would be of great benefit, for increasing the number of HSCs for transplantation and for gene therapy purposes, it is crucial to better understand by which mechanisms regulators of HSC numbers are controlling HSC survival, self-renewal and differentiation. Although not entirely clear, the limited success of extensive efforts to ex vivo expand HSCs is probably related to HSCs having a propensity towards commitment rather than self-renewal under current culture conditions.

In our studies we demonstrated that LNK is a negative physiological regulator of HSC expansion in steady state hematopoiesis, acting at least in part through inhibition of THPO-triggered c-MPL signaling, providing new insights into the dynamic positive and negative regulation of postnatal HSC expansion. Although the role of THPO as a key regulator of human HSCs is strongly implicated through identification of an apparent stem cell deficiency in CAMT patients (Ballmaier et al., 2003; Ballmaier et al., 2001), the role of LNK and its interaction with c-MPL-mediated signaling in human HSCs, has not yet been investigated. Obviously, it would be of considerable interest to aim to specifically target these pathways in order to promote HSC self-renewal and expansion ex vivo, in particular from CB. CB is an attractive source of HSC transplants, due to the higher toleration of HLA mismatch compared to adult BM, however due to the low HSC numbers in CB there is a considerable need to develop more efficient means for ex vivo HSC expansion (Sorrentino, 2004).
**B) How is LNK limiting postnatal THPO-promoted HSC expansion?**

Through analysis of phenotypically defined HSCs at different stages of development, we observed an age-progressive expansion of HSCs in *Lnk*−/− mice, resulting in as much as a 17-fold increase of LT-HSCs in adult *Lnk*−/− mice. An important role of LNK in HSCs was already observed in day 14.5 fetal liver and more clearly in 2wk old mice, in apparent discrepancy with the recent report by Ema et al. (Ema et al., 2005), in which it was suggested that LNK starts to be important as a negative regulator of HSC expansion first after 4wks postnatally. We believe this difference could be explained by Ema et al using LSKCD34− as a marker for LT-HSCs, even though it has been established that fetal and early postnatal LT-HSCs are mainly CD34 positive (Ito et al., 2000).

Interestingly, upon transplantation we found that *Lnk*−/− HSCs had a reconstitution and self-renewal potential comparable to that of WT HSCs, suggesting that LNK has little or no effect on the expansion of HSCs post transplantation. Ema et al. suggested in contrast to our findings, that at least a fraction of *Lnk*−/− HSCs possess a higher repopulating potential than WT cells (Ema et al., 2005), proposing that LNK is also important for negative regulation of HSC self-renewal after transplantation. One possible explanation to this apparent discrepancy, could be that whereas we mostly evaluated reconstitution in mice transplanted with multiple WT or *Lnk*−/− HSCs, Ema et al evaluated reconstitution with single HSCs (Osawa et al., 1996). Regardless, both studies implicate that LNK plays a more important role as a negative regulator of HSC expansion in steady state adult hematopoiesis than after transplantation or in fetal liver, conditions in which HSCs cycle and expand extensively (Pawliuk et al., 1996). Thus, we would propose that LNK acts as an important regulator of steady state HSC self-renewal, probably by increasing the probability for HSCs to undergo symmetrical rather than asymmetrical cell divisions.
The observed higher expression of *Lnk* and *c-Mpl*, and the selectively increased THPO responsiveness in LT-HSCs compared to ST-HSCs and LMPPs, suggested that the expansion of HSCs in *Lnk*<sup>-/-</sup> mice was likely to involve enhanced c-MPL signaling. This was confirmed by phenotypic analysis of HSCs in adult *Lnk*<sup>-/-Thpo</sup><sup>-/-</sup> mice, which had a similar fold reduction in LT-HSCs as *Thpo*<sup>-/-</sup> mice, suggesting that THPO is absolutely required for the HSC expansion in *Lnk*<sup>-/-</sup> mice. In addition, LNK also proved to be important for restricting expansion of myeloid progenitors in response to THPO. In contrast, the well established B lymphoid phenotype of *Lnk*<sup>-/-</sup> mice (Takaki et al., 2000) could not at all be explained by an action on the THPO pathway, as *Lnk*<sup>-/-Thpo</sup><sup>-/-</sup> and *Lnk*<sup>-/-</sup> mice had comparable increases in lymphocytes. This suggests that there must be other signaling pathways promoting lymphopoiesis that are subjected to negative regulation by LNK. We predict that this most likely includes IL-7R signaling but potentially also FLT3, c-KIT and TSLPR signaling. Taken together, our findings establish that LNK acts downstream of the c-MPL receptor to limit THPO-promoted HSC expansion.

To unequivocally prove that LNK is directly inhibiting c-MPL signaling in HSCs further experiments are needed. One approach would be to use a THPO responsive 32D cell line expressing both the c-MPL receptor and LNK. Co-immunoprecipitation studies could reveal whether LNK directly associates with the phosphorylated c-MPL receptor, and furthermore introduction of specific mutations within the c-MPL receptor could provide new insights to identify potential specific binding target sites of LNK. Since loss of LNK has been shown to enhance multiple THPO induced signaling pathways (such as STAT3, STAT5, MAPK and Akt) it is likely that LNK inhibits JAK2 activation and c-MPL phosphorylation. Due to the fact that THPO stimulates downstream signaling molecules that also are stimulated by other cytokines, it will be a challenge to in
vivo ultimately prove a direct inhibition of LNK on THPO driven proliferation of HSCs. One approach could however be to use c-MPL blocking antibodies and thereby block THPO signaling in single $Lnk^{-/-}$ HSCs, and subsequently investigate (by antibody staining against phosphorylated downstream signaling molecules) whether the enhanced activation of STATs and MAPKs in $Lnk^{-/-}$ HSCs is sustained or not in the absence of c-MPL signaling.

**C) Is c-MPL signaling the only pathway through which LNK is inhibiting postnatal HSC expansion?**

Our data clearly demonstrate that THPO is a main regulator of HSCs and that LNK is inhibiting its stimulatory action. However the results from the limiting dilution transplantation experiments, showing 7-fold higher competitive repopulating units in $Lnk^{-/-}Thpo^{-/-}$ mice than in $Thpo^{-/-}$ mice, would suggest that the observed expansion of HSCs in $Lnk^{-/-}$ mice could in part also be due to enhanced signaling triggered by other cytokines than THPO. This would implicate that LNK potentially inhibits other pathways regulating HSC expansion, and it will be of considerable interest to identify these. Several other cytokine receptors, such as c-KIT, have been implicated to play an important role in promoting HSC survival and proliferation (Miller et al., 1996). Since LNK has been shown to inhibit c-KIT mediated proliferation in vitro (Velazquez et al., 2002), it appears likely that LNK could potentially also inhibit c-KIT mediated HSC maintenance or expansion. In that regard, we have recently found that $Lnk^{-/-}$ LT-HSCs have slightly enhanced KL responsiveness compared to wild type LT-HSCs, although the enhanced responsiveness of $Lnk^{-/-}$ LT-HSCs to THPO is much more pronounced. Although not excluding other candidates, based on the knowledge about the known HSC phenotypes of cytokine deficient mice, we
would propose that the LNK inhibition of c-MPL and potentially c-KIT signaling might explain the whole HSC phenotype in *Lnk*−/− mice.

**D) Role of THPO in regulation of HSCs**

An important role of THPO signaling in maintenance of adult BM HSCs has been implicated in previous studies (Fox et al., 2002; Kimura et al., 1998; Solar et al., 1998). We extended these findings by phenotypically analysing the HSC subsets in THPO−/− mice, and interestingly we found as much as an 88% reduction of LT-HSC in 12 wk old THPO−/− mice. However, neither we or others, have shown exactly how THPO is regulating HSCs, i.e. whether THPO regulates self-renewal, promotes proliferation, provides anti-apoptotic signaling or regulates quiescence of HSCs. THPO has been shown to promote in vitro survival of highly purified HSCs (Borge et al., 1996; Sitnicka et al., 1996), and we found that *c-Mpl* gene expression levels are the highest in LT-HSCs, considered as mainly quiescent, compared to the more rapidly cycling ST-HSCs and MPPs. To investigate by which mechanism THPO is regulating HSC expansion, detailed cell cycle and apoptosis analysis would be required. Further, enforced expression of the anti-apoptotic regulator *Bcl-2* in *Thpo*−/− HSCs could unravel whether THPO serves as a major anti-apoptotic regulator. Furthermore, the action of LNK and THPO on the regulation of self-renewal, could be performed in paired daughter cell experiments (Takano et al., 2004), allowing analysis of the mode of cell division at the single cell level.

Whereas THPO so far has been implicated as an important regulator of adult HSCs, a role in the rapidly cycling fetal liver has not been investigated. This is of special interest in the light of LNK playing a very limited role in regulation of fetal HSCs in striking contrast to postnatal HSCs. Interestingly, CAMT children, born with a mutation in the c-MPL receptor, at birth have
thrombocytopenia as a result of deficient megakaryocytepoiesis, but otherwise normal hematopoiesis indicating a normal HSC compartment at birth, but subsequently develop a progressive multilineage BM failure (Ballmaier et al., 2001; King et al., 2005). This would suggest that THPO also plays an important role in regulation of postnatal but probably not fetal human HSCs. Interestingly, also the receptor tyrosine kinase angiopoietin has been shown to be redundant for fetal but essential for adult HSCs (Puri and Bernstein, 2003). Taken together, these findings would suggest that the requirement for cytokine regulated HSC maintenance increases with declined cycling, as fetal liver HSCs and BM HSCs post transplantation cycle more rapidly than steady state adult BM HSCs (Pawliuk et al., 1996).

**E) Potential involvement of LNK and c-MPL signaling in hematopoietic malignancies**

A role of LNK in hematological malignancies has so far not been investigated. Interestingly, we have not observed leukemia development in Lnk−/− mice, but this does by no means exclude that LNK lack of function mutations could enhance the propensity for leukemia development. Although eventually it would be important to screen leukemic patients for mutations in LNK, there are mouse models available in which one first can investigate the potential role of LNK deficiency, acting in collaboration with other mutations in leukemogenesis. As LNK acts as a broad inhibitor of various hematopoietic growth factor receptor signaling pathways, it will be of particular interest to cross Lnk−/− mice with mice carrying mutations that frequently are seen in leukemia along with other activating mutations in signaling pathways, such as PML-RARA (Schnittger et al., 2002) and AML1-ETO (Schessl et al., 2005; Valk et al., 2004).
c-MPL was originally cloned as a human homolog of the v-mpl oncogene from a cDNA library from a human erythroid leukemic cell line (Vigon et al., 1992). However, so far there have only been a few cases reported with activating mutations of the c-MPL receptor (Ding et al., 2004).

12.2. Defining the cellular pathways for HSC lineage commitment

12.2.1. Implications of current findings

Although there is substantial evidence in support of the classical model of lineage commitment in adult hematopoiesis, in which a strict separation of myelopoiesis and lymphopoiesis is the first lineage commitment/restriction step of HSCs (Fig. 1) (Reya et al., 2001), this does not necessarily imply this as an obligatory route for lineage development. On the contrary, several lines of recent studies imply alternative routes of lineage commitment. In our studies we identified lymphoid primed multipotent progenitors (LMPPs) as a fraction of the Lin−Sca-1+c-KIT+ (LSK) HSC compartment expressing high levels of FLT3, with combined B, T and GM lineage potentials, but little or no Mk/E potential (Adolfsson et al., 2005). Although our studies do not provide any answer as to what alternative route LSK cells with sustained Mk/E potential might have taken, there are several possibilities. One possible scenario is that HSCs at an early stage can commit directly to Mk/E progenitors, a possibility supported by recent paired daughter cell experiments (Takano et al., 2004). Alternative possibilities include the generation of CMPs or simply a parallel track to LMPPs in which pluripotent LSKCD34+FLT3− ST-HSCs sustain all lineage potentials to subsequently follow the classical strict CMP-CLP separation (Reya et al., 2001). Further studies will be required to distinguish between these and other possibilities.

Our studies also raise a number of additional important questions in need of further experiments. First, does the sustained GM potential of LMPPs
represent an alternative pathway for GM development, and if so to what degree does this pathway contribute towards replenishment of the GM lineage in steady state hematopoiesis and in situations in which enhanced demands are applied to the hematopoietic system. What are the next commitment steps of LMPPs? Do LMPPs generate GMPs and CLPs and/or B/M and T/M progenitors as suggested in fetal development (Lu et al., 2002)? Finally, are the LMPPs unique intermediates in the adult lineage commitment pathways or can we also identify and purify fetal LMPPs?

12.2.2 Limitations

A) Limitation of biological assays used to detect lineage potentials

In the short time since the publication of our identification of the LMPP, considerable controversy has emerged regarding the existence of adult LMPPs, and its implication of alternative pathways for lineage commitment. In agreement with our studies, recent studies by Yoshida et al., and Lai and Kondo used different approaches to identify adult BM populations corresponding closely to the LSKCD34^{+}FLT3^{hi} LMPPs, and as the LMPPs sustaining combined lymphoid and GM potentials but lacking MkE potential (Lai and Kondo, 2006; Yoshida et al., 2006).

However a recent study by Forsberg et al., casts serious doubts on the interpretation and implications of these studies, in part by demonstrating that large numbers of LSKFLT3^{hi} cells do in deed contribute towards reconstitution of the Mk and E lineages in vivo (Forsberg et al., 2006). The authors make a highly relevant and justified point that conclusions with regard to absence of lineage potentials of primitive progenitors require careful evaluations, of particular importance being detailed kinetics analysis of the emergence of cells of each lineage, as production of short-lived myeloid lineages might otherwise go
unnoticed. Another important point made by Forsberg et al was that in vitro assays might fail to efficiently detect lineage potentials, simply because we do not necessarily know the optimal conditions for generating each of the investigated cell lineages. In that regard, Forsberg et al clearly demonstrate that if transplanting as much as 500-1000 LSKFLT3\textsuperscript{hi} cells into irradiated mice they do contribute significantly also to platelet and erythrocyte production. Although we would agree that the Forsberg studies convincingly demonstrate that LSKFLT3\textsuperscript{hi} cells as a population can contribute towards the Mk/E lineages, we would actually argue that we in our studies had data fully compatible with this, as we in our in vitro studies found up to 2-3% of LSKFLT3\textsuperscript{hi} cells to have a residual Mk/E potential, in vitro as well as in vivo (Adolfsson et al., 2005). Thus, the low platelet and erythrocyte reconstitution from LSKFLT3\textsuperscript{hi} cells could be very compatible with our findings, although in the Forsberg studies no clonal (single cell) evaluation of the Mk/E potential of LSKFLT3\textsuperscript{hi} cells was performed in vitro or in vivo, with the exception of the CFU-S potential which was detected at a frequency as low as 1/230 LSKFLT3\textsuperscript{hi} cells (Forsberg et al., 2006).

As outlined above, a main challenge associated with studies of HSC lineage commitment is that one is facing the daunting task to prove a loss of lineage potentials, meaning that conclusions are relying to a large degree on the reliability of negative data. As there today do not exist any assays that efficiently can detect all the blood lineages from single cells, an alternative approach is to combine the current existing in vitro and in vivo assays with gene expression analysis of isolated progenitors (outlined below).

**B) Alternative approaches towards studies of HSC lineage commitment**

Studies of the lineage potentials of stem and progenitor cells require efficient single cell assays in which all lineage potentials can be evaluated simultaneously.
With the exception of self-renewing HSCs it is at the moment difficult to envision how one should be able to develop in vivo assays in which all lineage potentials (even reading out at different time points) of single progenitors can be evaluated with sufficiently high sensitivity. Thus, there must be devoted major efforts towards development of more efficient in vitro assays in which all lineage potentials can be assessed simultaneously at the single cell level. Even if successfully developed, a main limitation with this approach is that the evaluation of lineage potentials is done retrospectively, enhancing the risk that inefficient assays will underscore lineage potentials. Thus, the ultimate goal should be to establish specific molecular signatures for progenitors with different lineage potentials. Although this might seem like an impossible goal, our published studies of the LMPP (Adolfsson et al., 2005) and more recent extension of these studies, provide an indication of the usefulness of this approach. Through a detailed molecular profiling of LSKCD34^{+}FLT3^hi LMPPs, LSKCD34^{+}FLT3^- ST-HSCs and LSKCD34^{+}FLT3^- LT-HSCs with global gene profiling and multiplex single cell PCR, we can demonstrate that LMPPs have downregulated the transcriptional priming of Mk/E affiliated genes and upregulated lymphoid priming, with sustained GM priming. This is in striking contrast to ST-HSCs and LT-HSCs which have virtually no lymphoid transcriptional priming but extensive GM and Mk/E priming (Jacobsen et al, unpublished observations). Although the significance of these distinct patterns of multilineage transcriptional priming remains to be established, it helps to support the finding of downregulated Mk/E potential and enhanced propensity for lymphoid development in LMPPs.

12.3. Role of FL and other cytokines in normal B cell development

In our studies, we show that FL is important not only for the maintenance of early B cell progenitors, including the CLPs, but also for mature B cells. Previously a
crucial role of FL in B cell development had been reported, however the close to normal levels of mature B cells in adult FL\(^{-/-}\) mice suggested a more dispensable role of FL in production and maintenance of mature B cells (Sitnicka et al., 2002). Also, we found that fetally and post-natally derived B1 and MZ B cells are maintained at normal levels in FL\(^{-/-}\) mice, in agreement with previous findings (Sitnicka et al., 2003), suggesting that B1 and MZ B cells are sustained by mechanisms mediated by other pathways than FLT3 signaling. IL-7 has been implicated to play a similar role as FL in B cell development, namely a crucial role for adult B lymphopoiesis, and a more redundant role during fetal/postnatal stages of B cell development, as adult IL-7\(^{-/-}\) mice have normal levels of B1 cells (Carvalho et al., 2001). Furthermore, IL-7 has been implicated to play a compensatory role for mature B cells in the absence of FL, due to the total lack of B cells in FL\(^{-/-}\)IL-7R\(\alpha\)^{-/-} mice (Sitnicka et al., 2003). However, to ultimately establish the relative role of FL and IL-7 in adult B cell development, it would be crucial to generate conditional knock-out mouse models, since a B cell deficiency in adult FL\(^{-/-}\) and IL-7\(^{-/-}\) mice can not be ruled out to be a consequence of impaired fetal B lymphopoiesis. Interestingly, mice lacking both FL and IL-7R\(\alpha\) were totally devoid of conventional B-2 cells and fetally derived B-1 cells (Sitnicka et al., 2003). Since TSLP is a second ligand of the IL-7R\(\alpha\) and has previously been implicated as a regulator of IL-7 independent fetal B lymphopoiesis (Vosshenrich et al., 2003; Vosshenrich et al., 2004), the total absence of B-1 cells in FL\(^{-/-}\)IL-7R\(\alpha\)^{-/-} mice could be explained by the lack of TSLP signaling. However, since B-1 cells only have been investigated in IL-7R\(\alpha\)^{-/-} and IL-7\(^{-/-}\) mice, and not in TSLPR\(^{-/-}\) mice, this has not been ultimately proven. Furthermore, although unlikely, not yet identified ligands of the IL-7R\(\alpha\) could potentially be responsible for fetal B lymphopoiesis.
12.4. Role of FL in immune deficiency and reconstitution

In our studies, we clearly demonstrate, that FL is critical for the regeneration of B cells after chemotherapy and BMT, although playing a redundant role in generation of fetally and postnatally derived B1 and MZ B cells.

Patients have increased levels of FL following myeloablation and BMT, suggesting an important role of FL during immune reconstitution after transplantation (Blumenthal et al., 2000; Wodnar-Filipowicz et al., 1996). FL administration has however failed to demonstrate a positive effect on B cell regeneration (Brasel et al., 1996; Ceredig et al., 2006; Maraskovsky et al., 2000), but increases the generation of dendritic cells and has therefore been used in the treatment of patients with metastatic colon cancer to enhance immune response against tumor cells (Morse et al., 2000). So far there have been no reports showing patients with lack of FL. Interestingly, it has been demonstrated that FL levels in the plasma after chemotherapy or BMT could be used as an indicator to monitor immune recovery (Blumenthal et al., 2000). It would be of great interest to perform a detailed screening of patients having low plasma levels of FL, potentially reflecting deficient FL function. Such patients could potentially benefit from FL administration to accelerate their immune recovery post-chemotherapy or BMT. FL mRNA is highly expressed by a variety of hematopoietic and non-hematopoietic tissues, however the FL protein has only been found in stromal cells in the BM and T cells (Hannum et al., 1994; Lyman et al., 1993; Wodnar-Filipowicz, 2003). The lack of effect of FL administration on B cell recovery in mice and humans (Brasel et al., 1996; Ceredig et al., 2006) could potentially by explained by that it is the membrane bound and not soluble form of FL that is important for B cell generation. Notably, the predominant form of FL in human and mouse are membrane bound isoforms (Hannum et al., 1994; Lyman and Jacobsen, 1998; Lyman et al., 1994; Lyman et al., 1993; Lyman et
al., 1995), whereas the soluble form seems to be relatively rare (Lyman et al., 1995).

IL-7 administration to normal mice promotes B lymphopoiesis in BM, spleen and lymph nodes (Morrissey et al., 1991) and furthermore IL-7 treatment of mice after irradiation and cyclophosphamide treatment promotes B cell recovery (Faltynek et al., 1992).

IL-7 administration in patients leads to enhanced numbers of peripheral B cells in the recipients after BMT (Alpdogan and van den Brink, 2005). The IL-7 administration has not yet been established in clinical applications, since the safety has not been investigated in detail, and there are conflicting results regarding a potential augmentation of the GVHD effect upon IL-7 treatment (Alpdogan et al., 2003; Alpdogan et al., 2001; Sinha et al., 2002). Once this is settled, one could imagine IL-7 being used together with FL in an attempt to enhance B cell recovery upon BMT.
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14. References


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15. Appendices I-IV