What's in a name?
Sub-fractionation of common lymphoid progenitors

Stem cell to B cell: Delineation of B cell commitment and heterogeneity in lymphoid progenitor compartments

by

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For Goli

Summary

The hematopoietic system is a highly dynamic organ developed in many multi-cellular organisms to provide oxygen, prevent bleeding and to protect against microorganisms. The blood consist of many different specialized cells that all derive from rare hematopoietic stem cells (HSCs) located in the bone marrow in mice and humans. Blood cell production from HSCs occurs in a stepwise manner through development of intermediate progenitors that gradually loose lineage potentials. This is a tightly regulated process with complex regulatory mechanisms and many checkpoints that ensure a high and balanced production of blood cells. One of the fundamental questions in hematopoiesis relates to how the maturation of the cells is controlled and driven towards defined cell fates. The understanding of these processes is largely facilitated by isolation of intermediate populations of cells at defined stages of development.

This thesis is focused on the regulatory mechanisms that regulate the maturation of B-lymphocytes constituting an important part of adaptive immunity by being responsible for the production of antibodies. It has been suggested that all the lymphoid cells have a common lineage restricted ancestor defined as a $Lin^{-}Kit^{lo}Sca1^{lo}Flt3^{+}IL7R^{+}$ common lymphoid progenitor (CLP). These cells are believed retain the combined potentials for B, T and NK cells and it has been presumed that commitment of CLPs to B lineage is associated with expression of CD19 and B220 on progenitor B-cells.

The aim of this thesis has been to identify the point of no return in B-cell development in order to allow for a better understanding of lineage restriction events in early lymphopoesis.

To this end, we have used reporter transgenic mice where marker gene expression has been controlled by the transcription regulatory elements from one early lymphoid marker (Rag1) and one B-lymphoid restricted gene (λ5, Igll1). This allowed us to identify three functionally distinct sub-populations within the conventional CLP compartment. The cells were identified as $CLPRag^{low}\lambda5^{-}$ cells retaining B, T, Nk and a limited myeloid potential while up-regulation of Rag1 to generate $CLPRag^{high}\lambda5^{-}$ cells, was associated with loss of Nk potential as well as of the residual myeloid potential. Ultimately expression of λ5 in the $CLPRag1^{high}\lambda5^{+}$ compartment identifies the first committed B cells. Hence, our data suggest that the point of no return in B-cell development can be found within the $CD19^{-}$ CLP compartment. Using this new model for B-cell development, we investigated the instructive vs. permissive role of IL7 signaling in B cell commitment. Our results show that in absence of IL7, CLP maturation is impaired and generation of the earliest

committed B-lineage cells is severely impaired. CLP maturation could not be rescued by ectopic expression of the anti-apoptotic Bcl2 protein even though the cells were able to generate normal B lineage cells after restoration of the IL7 signal. These findings suggest that Il7 is crucial for the maturation of lineage restricted CLPs and provide support for an instructive role of IL7 in early B-cell development.

This thesis highlights the importance of precise identification of the point of commitment in B cell development and provides insight to the hematopoietic hierarchical model with the potential to serve as a map to better understand the mechanisms of hematopoietic disorders.

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List of Abbreviations

AGM	aorta-gonad mesonephros
ALL	acute lymphoblastic leukemia
AML	acute myeloblastic leukemia
Bcl2	B-cell leukemia/Lymphoma 2
Blnk	B-cell linker
BM	bone marrow
CFU-S	colony forming unit spleen
ChIP	chromatin immunoprecipitation
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CML	chronic myelogenous leukemia
CMP	common myeloid progenitor
CSC	cancer stem cell
dpc	days post coitum
E	erythrocyte
E2A	transcription factor E2A (also known as Tcfe2a)
Ebf1	early B-cell factor1
ELP	early lymphoid progenitor
EMSA	electro mobility shift assay
Epo	erythropoietin
ETP	early thymic progenitor
FACS	fluorescence activated cell sorting
FL	fetal liver
Flt3	FMS-like tyrosine kinase 3
Foxo1	forkhead box O1
G	granulocyte
Gata1	GATA binding protein
G-CSF	colony stimulating factor 3 granulocyte (Csf3)
G-CSFR	colony stimulating factor 3 receptor granulocyte (Csf3r)
GFP	green fluorescent protein
Gr1	lymphocyte antigen 6 complex locus G (Ly6g)
HoxB4	homeobox 4
HSC	hematopoietic stem cell
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
Ikaros	IKAROS family zinc finger 1 (IKzf1)
IL7	interleukin 7
IL7r	interleukin 7 receptor
Kit	kit oncogene
KO	knockout
Lin-	lineage negative

LMPP	lymphoid primed multipotent progenitor
LSC	leukemic stem cell
LSK	Lin-Sca1+Kit+
LT-HSC	long term hematopoietic stem cell
M	macrophage
Mb1	CD79a, Immunoglobulin associated alpha
M-CSFR	colony stimulating factor 1 receptor (Csf1r)
MEP	megakaryocyte erythrocyte precursor
Mk	megakaryocyte
MPP	multipotent progenitor
NK	natural killer cell
NOD	non-obese diabetic
Notch1	notch1 gene
OCA-B	POU domain, class 2, associating factor 1 (Pou2af1)
Pax5	paired box gene 5
PB	peripheral blood
PU.1	SFFV proviral integration 1 (Sfpi1)
Rag1/2	recombination activating gene 1/2
RBC	red blood cell
Sca1	stem cell antigen-1 (Ly6a)
SCID	severe combined immunodeficiency disease
ST-HSC	short term hematopoietic stem cell
Tdt	deoxynucleotidyltransferase, terminal (Dntt)
Ter119	lymphocyte antigen 76 (Ly76)
Tpor	thrombopoietin receptor c-mpl
VpreB 1/2/3	Pre-B lymphocyte gene 1/2/3
YS	yolk sac
λ5	immunoglobulin lambda like polypeptide 1 (Igll1)

Articles included in this thesis

I. **Zandi, S**.*, Mansson, R.*, Tsapogas, P., Zetterblad, J., Bryder, D., and Sigvardsson, M. (2008). EBF1 is essential for B-lineage priming and establishment of a transcription factor network in common lymphoid progenitors. J Immunol 181, 3364-3372. published Sep 1, 2008; DOI 181/5/3364/JI Copyright 2008. The American Association of Immunologists, Inc.* Authors contributed equally

II. Mansson, R., **Zandi, S**., Anderson, K., Martensson, I.L., Jacobsen, S.E., Bryder, D., and Sigvardsson, M. (2008). B-lineage commitment prior to surface expression of B220 and CD19 on hematopoietic progenitor cells. Blood 112, 1048-1055. Prepublished Dec 8, 2009; DOI 10.1182/blood-2009-08-236398.

III. Mansson, R.*, **Zandi, S.***, Welinder, E., Tsapogas, P., Sakaguchi, N., Bryder, D., and Sigvardsson, M. (2010). Single cell analysis of the common lymphoid progenitor compartment reveals functional and molecular heterogeneity. Blood 115, 2601-2609. Prepublished Aug 15, 2008; DOI 10.1182/blood-2007-11-125385. * Authors contributed equally

IV. Tsapogas, P., **Zandi, S.**, Berntsen, J., Welinder, E., Månsson, R., Qian, H., and Sigvardsson, M. (2010). Interleukin-7 is critical for the development of lymphoid lineage restricted events in the common lymphoid progenitor compartment. (Submitted)

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Introduction

The hematopoietic system is one of the largest and most proliferative organs of the body. Blood and bone marrow make up about 12% of an adult's total body weight. There are more than 12 specialized cell types in the hematopoietic system being responsible for oxygen delivery, coagulation, healing and immunity. It has been estimated that the normal adult, the marrow produces about 2.5 billion red cells, 2.5 billion platelets, and 1.0 billion granulocytes per kilogram of body weight per day (Marshall A. Lichtman, 2006).

The hematopoietic system requires fast response mechanisms to cope with life threatening crises like infection, blood loss, hypoxia and hemolysis, which increase the demand for production and dispatch of one or several hematopoietic cell types. Hence the proliferation and differentiation of hematopoietic cells requires a well controlled regulatory mechanism to keep up with the high demands. Any disturbance in proliferation control, differentiation, trafficking of cells or even change in microenvironment can cause an array of blood related disorders.

Despite their vast diversity in terms of morphology and function, the blood's cellular components are tightly interconnected, and in some cases dependent on each other for their function. One beautiful examples of this is the process of wound healing which involves almost all sorts of blood cells as well as plasma proteins.

It was not until the beginning of the 20th century that Ernst Numann demonstrated that in fact blood cells are produced in bone marrow. In a normal healthy adult most of the marrow is filled with fat cells and only 10% of the marrow, referred to as the "red" or "active" bone marrow, is involved in blood cell production (Orkin et al., 2009). Red marrow is found mainly within the hip bone, sternum, ribs, vertebrae and the epiphyseal ends of long bones and, to a lesser extent within the skull bone. In stress conditions like chronic blood loss the fatty part of the marrow and even extra-medullary sites like liver and spleen can engage in blood cell production. Other organs referred to as peripheral lymphatic organs like the thymus, spleen and lymph nodes, are also participating in hematopoiesis. Each one of these organs has a specialized microenvironment that provides the right type of signal for proliferation and maturation of particular blood cell types.

Anemia, polycytemia and hemoglobin disorders are the result of pathologic erythropoiesis. Defective lymphocytes and myelocytes contribute in many immunodeficiency and autoimmune

diseases. Dysregulated proliferation and apoptosis at any stage of hematopoiesis can lead to different varieties of lymphomas and leukemias.

The concept of transplanting BM to correct blood abnormalities has been around ever since 1939, when Osgood injected BM cells intravenously to treat aplastic anemia for the first time. (Marshall A. Lichtman, 2006). However it took more than three decades for the scientists and clinicians to master the method and identify the suitable donors and recipients. Today both autologous and allogenic BM transplantation are widely practiced with very high success rates to treat a variety of blood disorders and brings hope to cure many blood diseases that was known as incurable in the past.

After more than a century of systematic research we know a great deal about different types of hematopoietic cells and their development as well as about the mechanisms and signaling pathways that govern their differentiation and maturation. However, there are still a lot to learn about the hematopoietic system and its normal and pathologic development before we can manipulate it in an effective and safe way and thereby take full advantage of its therapeutic potential for treatment of blood disorders.

The origin of blood cells

The integrity of the hematopoietic system hinges upon the presence of a cell or cells capable of both self renewal and differentiation; cells that we call hematopoietic stem cells. Unlike most organs, blood is a dynamic system and its appearance during embryogenesis coincides with endothelium and blood vessel formation. Tracing the exact origin of the blood cells is complicated due to the fact that the blood begins to flow shortly after the formation of the first cells. However, understanding the earliest stages of blood formation will allow us to better understand the mechanisms of differentiation and make better use of the therapeutic potentials of embryonic pluripotent stem cells.

It appears that the first hematopoietic cells form in the yolk sac which is an extra-embryonic site, at day 8 of gestation in mice (Moore and Metcalf, 1970; Palis and Yoder, 2001). However, the multipotency of these cells, particularly the ability to generate lymphoid lineages has been a matter of debate (Cumano et al., 1996; Cumano et al., 2001; Cumano et al., 1993; Yokota et al., 2006). In later stages of embryonic development, HSC's can be detected in the aorta-gonad-mesonephros (AGM) region around embryonic day 9 (Medvinsky and Dzierzak, 1996). Early HSC's begin to bud off from the ventral part of the AGM into aorta and soon after this, HSC's are found in both

placenta and Liver (Gekas et al., 2005). It has been suggested that yolk sac, placenta and AGM HSC's all contribute to the in colonization of the fetal liver which will remain the major source of definitive hematopoietic pluripotential stem cells preceding the shift of hematopoiesis to bone marrow shortly before birth (Medvinsky and Dzierzak, 1996; Muller et al., 1994). It is not known whether the cells from the yolk sac migrate to AGM regions and contribute in blood formation or if there is a de novo hematopoietic stem cell activity developing in the AGM independently of the yolk sac (Kinder et al., 1999; Samokhvalov et al., 2007). After birth bone marrow will be the major site of hematopoiesis for the rest of adult life (Mikkola and Orkin, 2006; Zanjani et al., 1992). Figure1 shows the hematopoiesis sites in human prenatal and post natal time. Although liver does not contribute to adult hematopoiesis in healthy person, but under extreme conditions such as chronic loss of blood, both liver and spleen can serve as extra-medullary sites of hematopoiesis.

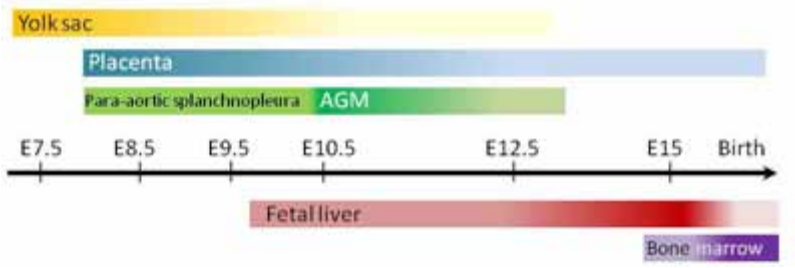


Figure 1. Kinetic of early hematopoietic development. E, embryonic day

Stem cell regulation and commitment

Discovery and identification of the hematopoietic stem cell

The concept postulating the existence of a pluripotential hematopoietic stem cell has been around since 1940s when Jacobson et al described the repopulation of the BM after lethal irradiation by shielding part of the body (Jacobson et al., 1950). Two decades later, Till and McCulloch eventually revealed the clonal nature of hematopoietic stem cells by transplanting serially diluted BM mononuclear cells to lethally irradiated mice (McCulloch and Till, 1960). They reported a linear relationship between the number of transplanted BM cells and the amount of radioprotection as well as the number of nodules visible in recipient's spleen which they referred to as CFU-S (colony

forming unit spleen). Examination of the nodules revealed that the CFU-S contained immature hematopoietic cells (Till and Mc, 1961). To show that each CFU-S was derived from a single cell and not from different types of progenitors, they induced random mutations in donor cells by in-vivo irradiating the transplanted cells and showed that all the cells in each clone carried the same karyotype abnormality and therefore should be derived from a single colony-initiating cell (Becker et al., 1963). Later on, Boggs and others proposed the presence of two different types of stem cells with high or low self renewal capacity by serially transplanting different number of BM cells to secondary recipients. (Boggs et al., 1982). To fulfill the prophecy of stemness it was required to show that a single cell can give rise to all hematopoietic lineages. Marking the progenitor cells with integrated retroviruses, assuming unique integration sites, and following their progeny after transplantation revealed that both myeloid and lymphoid cells are derived from the same initiating cell, suggesting the multipotency of single transplanted cell (Dick et al., 1985; Keller and Snodgrass, 1990).

The invention of Fluorescence Activated Cell Sorting technique in the late 1960s by Herzenberg and others, together with the availability of monoclonal antibodies, led to the identification and purification of HSCs from mouse and human BM. This was based on the absence of mature lineage markers associated with mature cells and the presence of primitive hematopoietic surface markers (Herzenberg and Sweet, 1976; Spangrude et al., 1988). Exclusion of cells with maturation surface markers, collectively referred to as lineage markers (Lin) is common in most HSC purification strategies. Population of cells identified as $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ or (LSK) contain about 10% true HSC's based on limiting dilution transplantation assays (Ikuta and Weissman, 1992). Later on the LSK cells were subdivided further based on absence or presence of CD34 and Flt3 (CD135/Flk2) into so called long-term HSCs ($LSKCD34^{-}Flt3^{-}$), short-term HSCs ($LSKCD34^{+}Flt3^{-}$) that despite preserved multipotency they have limited self renewal ability and therefore incapable of engrafting recipients beyond 16 weeks, and cells lacking Erythroid and megakaryocytic potentials ($LSKCD34^{+}Flt3^{high}$) denoted LMPP (lymphoid primed multipotent progenitors) (Adolfsson et al., 2005; Osawa et al., 1996; Yang et al., 2005). More recently Sean Morrison's group showed that $LSKCD150^{+}CD48^{-}$ cells are highly enriched for stem cells and that more than 50% of mice are reconstituted upon single cell transplantation (Kiel et al., 2005).

Lineage commitment

HSCs are generally dormant and divide very slowly, with about one division every 145 days (Wilson et al., 2008). Instead, they produce an enormous amount of cells per day through rapidly dividing intermediate progenitors. Stem cells should be able to divide in an asymmetric fashion to generate progenitors committed to differentiation as well as by symmetric division to expand the stem cell pool if necessary. The nature and mechanisms underlying symmetric versus asymmetric decision are not fully understood but there is evidence in favor of both intrinsic events, that could be stochastic, like asymmetric partition of cell components, and extrinsic or environmental cues to influence the decisions (Morrison and Kimble, 2006). After an asymmetric division one of the daughter cells may gradually lose lineage potentials and eventually reach the point of no return at which the decision has been made for a single cell fate. The precise time or the numbers of divisions possible or required for stem cell to produce committed daughter cells and the mechanisms governing this process for each hematopoietic cell lineage remain elusive (Giebel et al., 2006; Takano et al., 2004).

The question of whether stem cell commitment is a cell intrinsic (stochastic) or cell extrinsic (deterministic) process has been a matter of debate although the existing data shows that both mechanisms could contribute in cell fate decision. Among the environmental cues cell-cell interaction has been shown to be important both in asymmetric and symmetric division, as well as for maintaining the self renewal attribute of the stem cells. There is strong evidence that hematopoietic stem cells are in close contact with specific cellular niches in the BM microenvironment (Calvi et al., 2003; Kiel et al., 2007; Nilsson et al., 1997; Zhang et al., 2003a). These direct interactions and probably the chemokine gradient around the niche allow the stem cells to sustain their stemness and perform symmetric cell division. Stem cells are, however, not confined to their niches. 1 to 5% of the HSCs are estimated to leave the bone marrow and circulate in the blood every day without going through any cell division (Bhattacharya et al., 2009; Mendez-Ferrer et al., 2008).

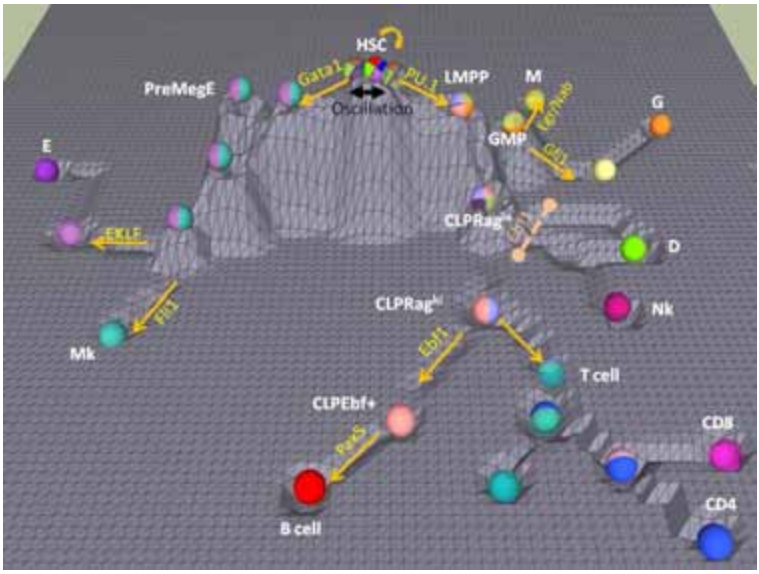
Interaction with BM stroma cells is not unique to stem cells. Other hematopoietic progenitors also need to get supporting signals from their niche either in bone marrow, liver, thymus or lymph nodes to commit to particular lineage, proceed with their development or perform other biological tasks.

The process of commitment is associated with a cascade of events in the cell, involving an array of transcription factors which consecutively establish the particular program associated with a certain cells lineage and at the same time suppress the genetic program of alternative cell lineages.

Reciprocal expression of transcription factors like GATA1 and PU.1, can direct HSCs to either erythroid or myeloid cell fate. Initial expression of GATA1 in HSCs can induce erythrocyte differentiation while expression of PU.1 before GATA1 can induce myeloid fate (Arinobu et al., 2007). Different levels of transcription factors have also been shown to play a role in fate decision and commitment of HSCs and progenitors. High levels of PU.1 can induce macrophage development while low levels of PU.1 expression promote B-cell development in BM (DeKoter and Singh, 2000; Spooner et al., 2009). Temporal and spatial expression of transcription factors is crucial for normal cell differentiation and commitment. Inappropriate expression of transcription factors in either the wrong cell type or at the wrong stage of development can induce variety of hematopoietic disorders. Thus, identifying the intermediate stages of development, and particularly the point of commitment, is important to understand the exact role and mechanisms of transcription factor in cell fate choice in health and disease.

Based on what we have learned from gain-of-function and loss-of-function studies, one can envisage that a certain pattern of gene expression represents the identity of a cell which in turn can be boiled down to the transcription factors status of a given cell. Even though there will be a huge number of possible combinations of transcription factors, there may be only a countable number of states in which they can be stably maintained. These are the states of transcription where cells, like intermediate progenitors, can resist mild environmental changes and keep their identities. Studies of global gene expression patterns in knock-out (KO) models suggest the presence of genetic networks highly dependent on one or a few transcription factors that maintain the integrity of the networks. Even though the transcription factors may differ during cellular development, these experiments show that a change in functional activity of a single transcription factor may tip the balance of the whole network and drive the cell into an unstable state which is undesirable for the cell (Gurdon and Melton, 2008). Therefore the cell proceeds by changing the combination of expressed genes to find the next possible stable genetic state. This new state of transcription normally represents a new developmental stage where the cell has lost some of its lineage potentials. The distinct stable state affiliated to each lineage can be envisioned as an attractor node in the basin of the lineage differentiation model, conceptualized from Waddington's landscape model (Figure 2) (Enver et al., 2009; Graf and Enver, 2009). In this model multipotent cells can oscillate in their stable basins by changing the values in their probability matrix without changing

their identity. If an environmental cue becomes strong enough it can destabilize the cell and dislocate it from its stable basin. The nature of the environmental cue combined with the matrix of probability for a cell at a given time and the type of environmental cue will then determine the path that the cell might take, to reach to the next stable state (Figure 2). In this model commitment is represented by a state with very low energy and high stability so the cells cannot easily regain multipotentiality (Enver et al., 2009). This has been supported by the observation that ablation of pax5 in committed B cells can induce infidelity in Pre-B cells allowing them to differentiate into T-, myeloid- or Nk- cells and even osteoblasts (Mikkola et al., 2002; Nutt et al., 1999; Rolink et al., 1999). The last few years have also seen the development of protocols using transcription factors that allows reprogramming of fully mature cells to pluripotential stem cells referred to as induce pluripotent stem cells (iPS) (Takahashi et al., 2007). The fact that cell fate can be changed experimentally raises the question as to whether this process happens in normal situations and perhaps contributes to pathogenesis of disease.



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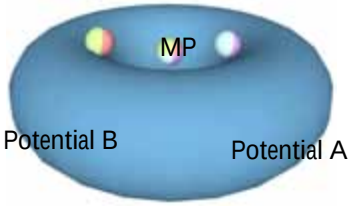

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Figure 2. Hematopoietic differentiation in a modified Waddingtonian epigenetic landscape. a, Transcriptionally stable states of development present as basins in the epigenetic landscape. Change in transcription factors expression can force the cell out of the basin and roll to the attractor basins bellow **b**, Multipotent cells fluctuate between different potentials so that at any given time they have a different probability of choosing a certain lineage. The probability for a lineage and expression of transcription factors collectively roll the cells out and down to one or the other possible lineages. MP, multipotent progenitor.

Instructive or permissive? Or something else?

Looking at the hierarchy of the hematopoietic system with the HSCs at the apex above the cascade of intermediate progenitors, it brings several fundamental questions are brought to mind: How can HSCs and progenitors sense the demand for certain type of cells at any given time and condition? In other words how do they regulate the differentiation? Does this regulated at the progenitor level or by direct action on HSCs? How stem cells divide and yet keep their stemness throughout whole life?

Serial and single cell transplantation assays are probably the most stringent ways to show that HSCs indeed self-renew and even expand. Transplanting a single HSC can reconstitute the full hematopoietic system for life-time (around 2 years) in mice, and yet the HSCs derived from this single HSC can fully reconstitute another recipient for an extended time (Iscove and Nawa, 1997; Osawa et al., 1996).

Upon any division HSCs have to make a decision whether to divide symmetrically or asymmetrically (Figure 3) and in order to explain the self-renewal and expansion ability of stem cells they also have to be able to alternate between these two options.

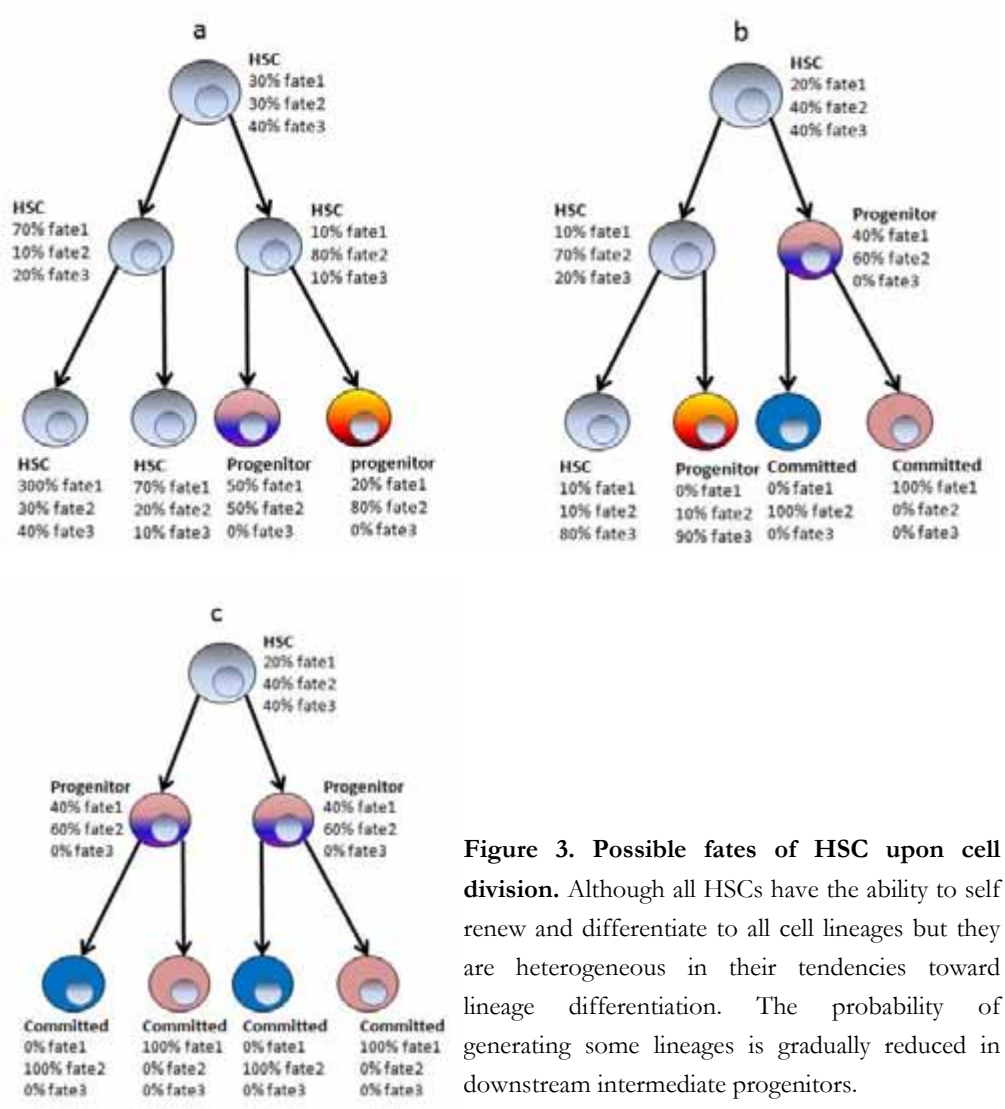

Figure 3. Possible fates of HSC upon cell division. Although all HSCs have the ability to self renew and differentiate to all cell lineages but they are heterogeneous in their tendencies toward lineage differentiation. The probability of generating some lineages is gradually reduced in downstream intermediate progenitors.

Looking at the outcome of hematopoiesis it is evident that environmental cues somehow feed back to hematopoietic stem and progenitor cells to regulate their development either by directly instructing them (Instructive signals) or simply by selecting the cells belonging to the specific

lineages, based on their temporal demand (permissive signals). Secretion of hormones and growth factors like Tpo, Epo, G-CSF, etc can promote production of a certain blood lineage over the others. Some of these factors are widely used clinically to treat blood disorders like anemia, thrombocytopenia, chemotherapy-induced leukopenia and to mobilize stem cells in a donors before transplantation (Metcalf, 2008). These practical applications can be equally explained by either instructive or permissive models and in fact evidence has been provided to argue for both (Fairbairn et al., 1993; Kondo et al., 2000; Stoffel et al., 1999). Recently Rieger et al. performed a live time-lapse microscopy to follow the progeny of a single bipotent cell (Granulocyte-Macrophage) in presence of either G-CSF or M-CSF. They used an elegant reporter system to mark the time points at which cells committed to G or M potential (Rieger et al., 2009). Although a relatively high number of cells died and the authenticity of true bipotency of original cells was hard to prove, they nevertheless demonstrated the importance and necessity of single cell assays and follow-up of all the progenies of a cell to address the question of instructive vs. permissive activity of extrinsic signals.

Manipulation of transcription factors can also cause stem cells to self renew or change their fates independently or dependently of growth factors and microenvironment. For example, over expression of the transcription factor HoxB4 or deletion of the signal modulator Lnk, lead to expansion of HSCs in-vivo and in-vitro (Antonchuk et al., 2002; Buza-Vidas et al., 2006). Ectopic expression of GATA1 in HSCs can induce erythro-megakaryocyte production at the expense of monocytes (Farina et al., 1995). Over-expression of mutant forms of CEBP/α have been shown to promote stem cell self-renewal and promote myeloid cell lineage while antagonizing lymphoid lineage development (Bereshchenko et al., 2009). Skewing of B cell production and block in non-B lineages have been reported after forced expression of EBF1 in HSCs (Zhang et al., 2003b). Apart from growth factors and cytokines the cross talk between the cellular microenvironment and matrix proteins can also deliver short-distance signals that contribute in HSC cell fate decision (Wineman et al., 1996; Wu et al., 2008).

When we talk about HSCs, the general assumption is that all the HSCs are similar and have similar potentials. However, it turns out that if the equivalent of a single HSC is expanded in vivo and serially transplanted to several hosts, despite the ability of all cells to self renew and fully reconstitute the recipient the relative contribution from each HSC to defined lineages would be different (Muller et al., 1994). In a related study, Dykstra et al. serially transplanted single purified HSCs to three consecutive recipients and identified four types of HSCs with distinct lineage bias and self-renewal potential (Dykstra et al., 2007). So it seems that HSCs are not under the

instructive rule of cytokines (they don't even have the receptors presumably needed for that!) however, at later stages, at the level of progenitors cytokines might have an instructive roles.

Based on these recent studies one can envisage that a single HSC has a certain distribution of probabilities to move toward every possible lineage but this probability is liable to change. In other words, the law of uncertainty is also applicable for stem cell lineage choice. The role of cytokines in this scenario is to give the final push to those stem or progenitors cells that have already have a high probability for a certain lineage.

Hematopoietic hierarchy

In recent years developments in multicolor cell sorting have allowed for purification of subsets of hematopoietic cells. Single-cell functional assays to determine cell potentials and transplantation assays have also made it possible to identify the intermediate cells that form on the way between hematopoietic stem cells and mature effector cells. The identification and isolation of clonogenic common lymphoid progenitors (CLPs) (Kondo et al., 1997) with B,T and Nk potential followed by the isolation of common myeloid progenitors (CMPs) (Akashi et al., 2000) with myeloid and erythomegakaryocytes potentials led to the proposition of a hierarchical model for hematopoietic development (Laiosa et al., 2006). In recent years the classic model of hematopoiesis based on strict divergence of lymphoid and myeloerythroid lineages has been challenged both by investigators increasingly identifying cell populations based on more surface markers and by improved single-cell functional and genomic assays. Adolfsson et al identified a population of cells with high expression of Flt3 within the LSK (Lin-Sca1+Kit+) compartment that contains both myeloid and lymphoid potential but lacks megakaryocytes or erythroid (MkE) potential, hence called lymphoid primed multi potent progenitors (LMPP). Other studies suggest that the Mk and E cells branch out directly from the HSC pool and that these cells do not share any common ancestor with the myeloid lineage (Adolfsson et al., 2005; Mansson et al., 2007; Yoshida et al., 2006). The classical hematopoietic tree has also been challenged by studies suggesting that the CLPs have a limited contribution to the T cell pool (Allman et al., 2003; Schwarz and Bhandoola, 2004) and that CLPs retain some myeloid potential (Balciunaite et al., 2005; Mansson et al., 2008; Rumfelt et al., 2006). The nature of CLPs in also the focus of paper II and III of this thesis. As will be discussed further in the discussion we revealed heterogeneity of CLPs at the single-cell level

which has prompted us to propose a revised developmental pathway for the lymphoid lineage differentiation (Figure 4).

Several studies have also showed heterogeneity among CMPs (Arinobu et al., 2007; Nutt et al., 2005; Pronk et al., 2008; Pronk et al., 2007; Takano et al., 2004). Using the Endoglin (CD105), CD41 and SLAMF1 (CD150) markers, Pronk et al subdivided early hematopoietic progenitors into cells biased toward either E, Mk or GM lineages, respectively (Pronk et al., 2007). This provides further supports for the notion that the hematopoietic tree is under continues reconstruction and that the resolution of the epigenetic map of hematopoiesis is gradually increasing.

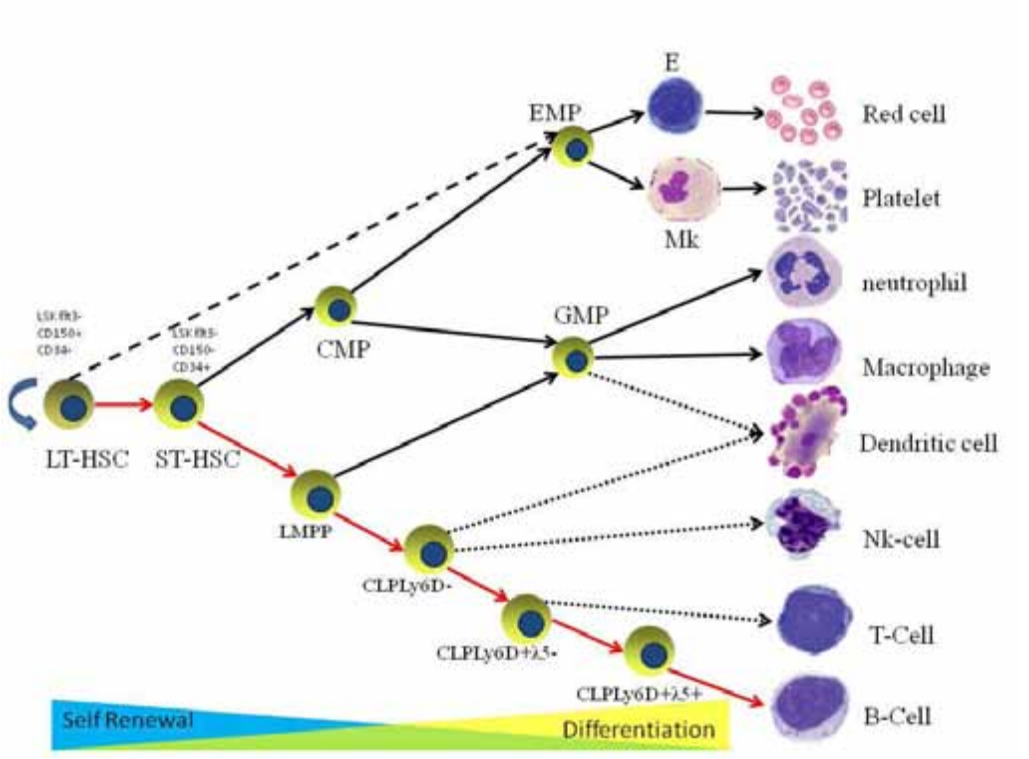

Figure 4. Alternative model for lymphoid development in hematopoietic hierarchy.

Lymphopoiesis

Lymphocytes are the class of blood cells that mediates all the adaptive immunity. This class of immune cells consists of B and T cells, which morphologically are very similar, even undistinguishable with normal Giemsa staining. Upon interaction with antigen, B and T lymphocytes form receptor complexes that mediate the antigen-specific recognition of pathogens through their immunoglobulin components. Lymphocytes ultimately derive from HSCs and diverge from erythomegakariocytes and myeloid lineages during the development into LMPPs, and CLPs (Adolfsson et al., 2005; Igarashi et al., 2002; Mansson et al., 2007; Medina et al., 2001; Welner et al., 2008). Initiation of lymphoid development begins with the expression of Flt3 and Il7-receptor on the surface and promiscuous expression of Tdt, Rag1, Rag2 and sterile IgH as early as at the LMPP stage. While development of B cells continues in the bone marrow, some lymphoid progenitors migrate to the thymus, which is the primary site for T-cell development. Reciprocal transplantation of BM and thymus between nude and NOD/SCID mice showed that thymic progenitors from bone marrow have to continually seed the thymus in order to sustain the T-cell production in the thymus. The exact nature of the cells that migrate from the BM to the thymus remain evasive. Some studies proposed the LMPP or $Lin^{-}Kit^{hi}CD25^{-}CD44^{+}$ populations as the early thymic progenitors rather than CLPs that rarely can be found in circulation (Figure 5) (Bell and Bhandoola, 2008; Schwarz and Bhandoola, 2006). T-cell development and maturation is largely dependent on the microenvironment of the thymus, which provides a high amount of Notch ligands (Pui et al., 1999; Sambandam et al., 2008; Sambandam et al., 2005). The question of whether T-cell commitment happens in bone marrow before early thymic progenitors (ETPs) seed the thymus or after seeding of the thymus remains to be answered, but Interplay of Notch1 with Ebf1, Pax5 and Lrf signal in the bone marrow has been proposed as a mechanism of T-cell versus B-cell fate determination in bone marrow microenvironment. (Maeda et al., 2007; Pui et al., 1999; Smith et al., 2005)

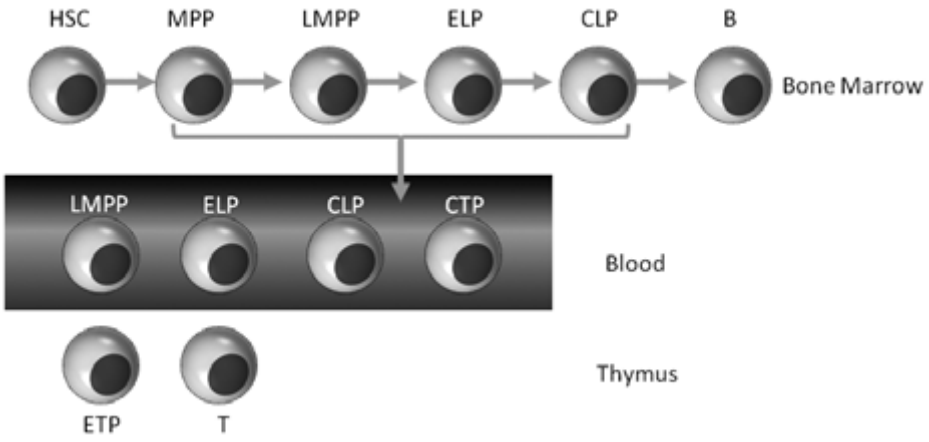

Figure 5. Constant migration of lymphoid progenitors from BM to thymus is required to sustain the T cell development in the thymus.

B cell development

Early B cell development

B cells are the source of antibody production; they can produce a huge repertoire of antigen-specific immunoglobulins (Igs) due to tremendous diversity of their immunoglobulin-coding genes, owing to rearrangement and recombination of the several variable (V), joining (J) and diversity (D) modules in the germline genomic DNA. Immunoglobulins are expressed both on the cell surface within the so called B-cell receptor (BCR) complex, and in their secreted form as antibodies. Alternative splicing of the Ig encoding mRNA in the terminally differentiated plasma cells govern the production of the soluble secreted form of Ig. While the primary Ig isoform generated is IgM, a second recombination event in the constant Ig genes, the so called class switch, allows for the production of IgG, IgE, IgD or IgA antibodies carrying the same antigen-binding amino-terminal domain.

B lymphocytes, like all other hematopoietic cells, derive from HSCs through a step-wise and complex process. During most of the fetal period, the B cells are generated and differentiated in the liver but shortly before birth, hematopoiesis shifts to the BM, where it normally remains for the rest of the life. Early stages of B cell development in adults take place in BM where stroma cells support their differentiation. The BM microenvironment provides the developing B cells with a number of cytokines and growth factors, such as kit ligand, IL7, Flt3 and SDF-1. The generation of B-lymphoid cells in mice has been extensively studied. Classification of different stages of B cell development is based on the presence of surface markers, expression of genes as well as Ig rearrangement status. Based on these characteristics two classification models have been developed for B cell staging: The Basel and Philadelphia (or Hardy) model (Hardy et al., 1991; Hardy et al., 2000; Li et al., 1996; Osmond et al., 1998).

The expression of Flt3 in early multipotent progenitors marks a population of cells that has lost MkE potential while retaining combined myeloid and lymphoid potentials (LMPP). These cells are believed to lose their myeloid potential to further develop toward lymphoid restricted cells. The first early lymphoid progenitor with combined B and T cell potential and significantly diminished myeloid potential was identified by Kondo et al. as Lin-$Kit^{lo}Sca1^{lo}CD127(IL\text{-}7R)^{+}$ cells and referred to as the common lymphoid progenitors (CLP) (Kondo et al., 1997). However, the use of mice carrying a GFP-encoding gene under the regulatory elements of the Rag-1 gene allowed for the isolation of lymphoid-restricted cells within the LSK compartment with a Lin-$Kit^{hi}Sca1^{hi}CD27^{+}Rag1^{+}$ phenotype (Igarashi et al., 2002). In the most recent studies in quest for the earliest lymphoid precursor, Irving Weismann's group combined Flt3 expression and CD27 with their old definition of CLP (Kondo et al., 1997) and showed by BM and intrathymic transplantations that these cells have strong B, T and perhaps Nk but limited myeloid potentials (Karsunky et al., 2008). Although in-vivo transplantation is probably the best method to show the potentials of cells in a physiologically relevant environment, investigation of populations with low proliferative capacity or of cells at the single cell level is not feasible. Additionally, factors that affect the homing and migration preference of cells are largely unknown and therefore difficult to control. Even though reduced as compared to the LMPP, in-vitro assays reveled that CLPs still retain a residual myeloid potential (Paper II). A low level of B cell associated genes like sterile IgH, TdT and Rag1 are detectable in early lymphoid progenitors (Mansson et al., 2007). Eventually an increased expression of Ebf1 followed by Pax5, activates the B cell programs and suppress the alternative lineage programs (Nutt et al., 1999; Zandi et al., 2008) (Paper I). Expression of CD19 activated by Pax5 has been suggested as the hallmark of B cell commitment (Nutt et al., 1999).

However, in paper I and II we show clearly that the early lymphoid progenitor population can be sub-fractionated into at least three distinct functional populations. Using Rag1 and λ5 reporters, we refer to these populations as CLPRag^{lo}λ5-, CLPRag^{hi}λ5-, CLPRag^{hi}λ5+ (Mansson et al., 2008; Mansson et al., 2010). CLPRag^{lo}λ5- cells showed B, T and Nk potentials and a residue of myeloid potential, CLPRag^{hi}λ5- on the other hand lost the Nk and Myeloid potentials and eventually expression of λ5 marks the first committed B cell. Identifying the first committed B cell and the makeup of its genetic network is a key to delineate B lymphocyte development and understand the mechanisms underlying B cell malignant transformations.

Regulation of B cell commitment

Expressions of Flt3 in the LMPP and IL7R in the CLP compartments are among the earliest events in lymphoid lineage restriction and the capacity to signal through these receptors is of crucial importance in B cell development. Disruption of IL7 or Flt3 signaling in mouse models result in a profound reduction in B cell production and mice lacking both Flt3 and IL7 display an even more dramatic phenotype and fail to produce any B lineage cells (Sitnicka et al., 2003). It appears that IL7, via Stat-5 and Flt3 perhaps through Akt pathways, deliver their synergistic effect on B cell development (Ahsberg et al., 2010). Mice with conditional deletion of PU.1 in early progenitors show a significant block in CLP formation, however, deletion of PU.1 at the ProB stage doesn't have any adverse effect on B cell generation and function (Dakic et al., 2005; Polli et al., 2005) Hence, it appears that even though crucial for early development, the importance of this protein is less pronounced, possibly due to high expression of related factors like SpiB, post commitment. PU.1 has also been suggested to act as a regulator of lymphoid/myeloid cell fate choices in a dose dependent manner since high expression results in the development of myeloid and lower levels in the development of lymphoid lineage cells (DeKoter and Singh, 2000). It has been proposed that in order to modulate the functional levels of PU.1 so to allow for lymphoid cell fate, the transcriptional repressor Gfi-1 can replace PU.1 on auto-regulatory elements in the PU.1 gene (Spooner et al., 2009) thereby modulating the levels of functional PU.1. Gfi-1 expression is regulated by the zinc finger transcription factor IKAROS (Ikzf1), known to be of crucial importance for development of the earliest B-lymphocyte progenitors (Wang et al., 1996 ; Yoshida et al., 2006). It has also been reported that PU.1 through interaction with regulatory elements creates an epigenetic landscape for the activity of downstream lineage restricted transcription factors (Heinz et al., 2010).

Another factor involved in the earliest stages of B-cell development is Ebf1, a helix-loop-helix protein that can be detected already in CLPs (Dias et al., 2005). Mice deficient for this protein were reported to develop $B220^{+}CD43^{+}$ cells leading to the initial conclusion that even though crucial for the development of $CD19^{+}$ B-lineage cells, this factor was not crucial for the development of proB cells (Lin and Grosschedl, 1995). However, as shown in paper I, mice deficient of Ebf1 display a complete block at the PrePro-B cell stage and the CLPs fail to express B cell associated genes like Pax5, CD79a,b, Igll1, and VpreB (Paper I). One of the factors involved in the regulation of the Ebf-1 gene is the basic Helix-loop-helix proteins E12 and E47 encoded by the E2A gene (Kee and Murre, 1998; Smith et al., 2002). These factors belong to the E-protein family and collaborate with Ebf1 to activate B cell programs (O'Riordan and Grosschedl, 1999; Sigvardsson et al., 1997). One of the two defined Ebf1 promoters also contains a Stat-5 binding site and therefore it has hypothesized that IL7 might act upstream of Ebf1 to activate transcription (Roessler et al., 2007). Mice lacking IL-7 have a reduced CLP compartment mainly due to a dramatic reduction in $Ly6D^{+}$ cells while the effect on the $Ly6D^{-}$ compartment is less pronounced (Manuscript IV). The deficiency in B-cell development can be partially rescued by ectopic expression of Ebf-1 (Dias et al., 2005) or constitutively active Stat-5 (Kikuchi et al., 2005). Thus, IL-7 may act via Stat-5 to instructively induce Ebf1 expression and cause lineage restriction in the $Ly6D^{+}$CLPs. Ebf1 also activates Pax5 (Decker et al., 2009; O'Riordan and Grosschedl, 1999), another crucial factor in B cell development and maintenance (Urbanek et al., 1994). One possible explanation for these apparently contradicting findings came from investigations of mice where the Pax-5 gene was inactivated in mature B-cells (Cobaleda et al., 2007). This resulted in de-differentiation of the mature B-cells presumably enabling them to adopt alternative cell fates (Cobaleda et al., 2007). Hence, even though Pax-5 may not be crucial for B-lineage specification and all the lineage restriction events, the protein is essential for the stable commitment of the B-lineage progenitors. Very recently Chromatin immunoprecipitation ChIP combined with high throughput sequencing revealed that Ebf1 interact with a large number of regulatory elements in Pro-B and Pre-B cells (Lin et al., 2010; Treiber et al., 2010). They also report that Ebf1 share many of these binding sites with Foxo1 and E2A.

Late B cell development

The Use of combination of surface markers that robustly correlate with developmental events such as rearrangement of heavy and light immunoglobulin chains, has made it possible to identify and isolate successive developmental stages of B cells (Figure 6). The earliest stage of B cell

development after the CLP stage is identified as $CD19^{-}B220^{+}CD43^{+}CD24^{lo}AA4.1^{+}$ and is variously called Pre-ProB or Fraction A (Hardy et al., 1991). The Pre-ProB stage is associated with high expression of Ebf1, CD79a, CD79b, λ5 as well as Rag1 and Rag2. Expression of Pax5 activates the CD19 expression and pre-proB cells develop into $CD19^{+}B220^{+}CD43^{+}CD24^{+}$ cells denoted ProB cells (equivalent of Fr.B) (Allman et al., 1999). This stage of development coincides with expression of VpreB and is followed by rearrangement of Ig heavy chain. Rearranged heavy chain then assembles with the surrogate light chain complex (SLC) composed of λ5 and VpreB that together with immunoglobulin-associated proteins Igα (Cd79a) and Igβ (CD79b) form the Pre-B cell receptor (pre-BCR) (Gong and Nussenzweig, 1996; Pelanda et al., 2002). Combined signaling through the Pre-BCR and IL7r will drive the cells to the highly proliferative large Pre-B stage (Fr.C′) that is characterized by a $CD19^{+}B220^{+}CD43^{-}IgM^{-}$ surface profile (Erlandsson et al., 2005). The highly proliferative Pre-B cells have to cease proliferation before they can rearrange the light chain. Attenuation of the IL7r signal and activation of the Ras-Mek-Erk pathway by Pre-BCR signaling is required at this stage to terminate proliferation and initiate light chain kappa rearrangement in small Pre-B cells (Fr.D) (Mandal et al., 2009). Naturally Rag1 and 2 expression are detectable again in small Pre-B cells, as they are required for light chain recombination. Heavy and light chain then assembled together and is express on the surface to make IgM^{+} immature B cells. Upon antigen encounter in the bone marrow, immature B-cells undergo clonal deletion, receptor editing before they develop to mature B cell with expression of IgD after leaving the bone marrow to reside in peripheral lymphoid organs. If the mature B cells get activated by antigen they can develop to memory B cell or antibody producing plasma cells in lymphoid organs.

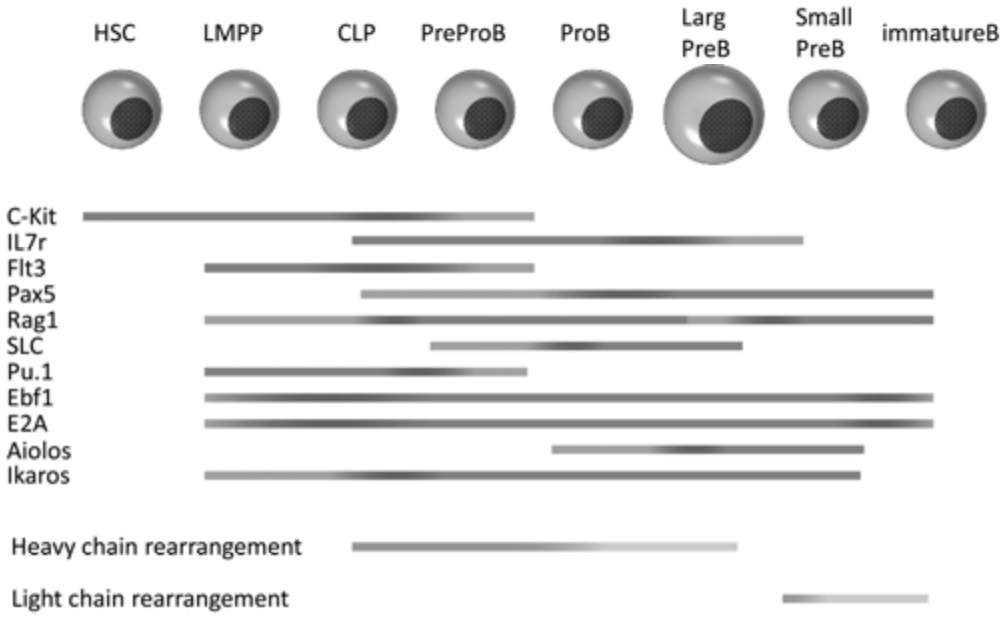

Figure 6. Transcription factor expression in at early developmental stages of B-cell development in mouse BM.

Aging of Blood cells

The cardinal sign of the aging process is that tissues lose their regenerative capacities. The hematopoietic system, like other organs, is also affected by aging. Most of mature blood cells have relatively short life span, ranging from minutes to months (Ogawa, 1993). So arguably HSCs and the supportive microenvironment are the only factors that keep the integrity of hematopoietic system throughout life. Although the frequency of HSCs increases by age, the ability of HSCs to engraft and regenerate the hematopoietic system in serial transplantation studies diminishes (Morrison et al., 1996; Rossi et al., 2005). Additionally, the composition of blood cells shifts from lymphopoiesis to more myelopoiesis (Morrison et al., 1996). The production of B-cells particularly, is reduced by advancing age, and interestingly the incidence of myeloid leukemia is also increases by age (Lichtman and Rowe, 2004; Rossi et al., 2005). Reduction in B-cell generation is noted already at early stages of development, partly due to 2.5 fold reduction in CLP and LMPP

frequency (Miller and Allman, 2003). The exact mechanism of aging is remain elusive, nevertheless, both intrinsic alterations such as accumulation of DNA damage due to oxidative stress, radiation, alkilating agents or telomere attrition can contribute in the process of aging (Rossi et al., 2008).

Alterations in the activation of pathways which are involved in protection of cells against neoplastic development, like P53 and retinoblastoma (Rb), are common with older age (Krishnamurthy et al., 2004). Loss of P53 leads to an array of neoplasms (Donehower et al., 1992), whereas over-expression of a short isoforms of P53 reduce the risk of cancers but at the same time accelerate the process of aging and impair the regenerative mechanisms (Maier et al., 2004). So it is plausible to think that by advancing age and accumulation of genetic damages, the risk of neoplasm increases so the cell activates anti-neoplastic mechanisms that adversely impair the regenerative capacities of the cells and as a result induce ageing. Hopefully by understanding the mechanisms of aging and keeping the balance between neoplasia protection and repair ability to repair, we can reverse some side effects of senescence.

B lymphoid malignancies: the other side of the coin

B cell neoplasias are a group of lymphoid abnormalities with diverse morphology, genetic and clinical pictures. B-cell neoplastic cells can be approximated to normal B-cell development stages based on morphology surface marker expression and recombination status of immunoglobulin gene (Figure 7). Neoplastic events are typically associated with alterations in the genome, such as chromosomal translocations, point mutations, and deletions as well as epigenetic and niche-related abnormalities. Natural events in B-cell development such as Ig recombination, somatic hypermutation and receptor editing, that can potentially induce instability in the genome and make B-cells more susceptible to neoplastic transformation (Kuppers and Dalla-Favera, 2001; Shaffer et al., 2002). This transient genetic instability could explain why 75% of all lymphoid leukemias and 90% of all lymphomas have B cell origin (Kuppers, 2005; Longo, 2009). As an example, the t(14,18) translocation occur in about one third of diffuse large B cell cases. Juxtapositioning of the anti-apoptotic Bcl-2 gene next to the immunoglobulin heavy chain gene could be the result of a mistake in Ig recombination machinery of B cells (Tsujimoto et al., 1985a; Tsujimoto et al., 1985b).

Differentiation of early lymphoid progenitors into B, T and NK lineages depends on the precise sequential expression of transcription factors that control the initiation and termination of cellular programs. Thus transcription factors are one of the most frequent targets that can be hijacked by

malignant transformation. Pax5, Ebf1, Tcf3 and IKAROS are indispensible transcription factors for the early B cell development. Mice lacking any of these factors show sever block in very early B cell development (Bain et al., 1994; Georgopoulos et al., 1994; Lin and Grosschedl, 1995; Urbanek et al., 1994). Disruption of Pax5 gene has been reported in 30% of acute lymphoblastic leukemia's (Mullighan et al., 2007; O'Neil and Look, 2007). Over-expression of Pax5 on the other hand, often as a result of translocation t(9,14) that juxtapose Igh and Pax5 is associated with lympho-plasmacytic lymphomas and diffuse large cell lymphomas happen in later stages of development in germinal center of lymphoid follicle (Busslinger et al., 1996; Iida et al., 1996; Kuppers, 2005). Using global high-throughput approaches like gene expression microarrays and single nucleotide polymorphism studies, revealed that the deletion of Ebf1, Ikzf1, Tcf3,Lef1 and Ikzf3 can also contribute to pathogenesis of ALL probably through blocking the differentiation (Mullighan et al., 2007). Higher prevalence of Ebf1 genetic lesion in 25% of pediatric ALL relapses suggests a role for Ebf1 in the etiology, drug resistance and relapse in children ALL (Yang et al., 2008).

E2A is a transcription factor known to collaborate with Ebf1 to initiate the B-cell program at the early stages of lymphopoiesis (Sigvardsson et al., 1997). Fusion of E2A-PBX1 is detectable in 25% of adult ALL cases with PreB phenotype; however over-expression of this fusion protein in the bone marrow of mice causes acute myeloid leukemia but not lymphoma (Dedera et al., 1993; Kamps et al., 1991). TEL-AML1 is another fusion gene as a result of translocation t(12,21) occurring in 20-25% of childhood ALL (Ferrando and Look, 2000). Although expression of this fusion gene in mice bone marrow doesn't initiate any malignancy (Andreasson et al., 2001), but both TEL and AML are required for self renewal of B-cell precursors (Morrow et al., 2007).

Classification of lymphoid malignancies and hence the treatment approaches are mainly based on morphology and few surface markers (Staudt, 2003). However in many of the categories the response of patients to therapy and behavior of disease is extremely heterogeneous, suggesting the possibility of molecularly distinct population of cells within the same category. Particularly in lymphoid associated cancers there is a clear need to develop a method to accurately predict the course of disease and response to available therapies for individual patients as well as the molecular pathogenesis of them.

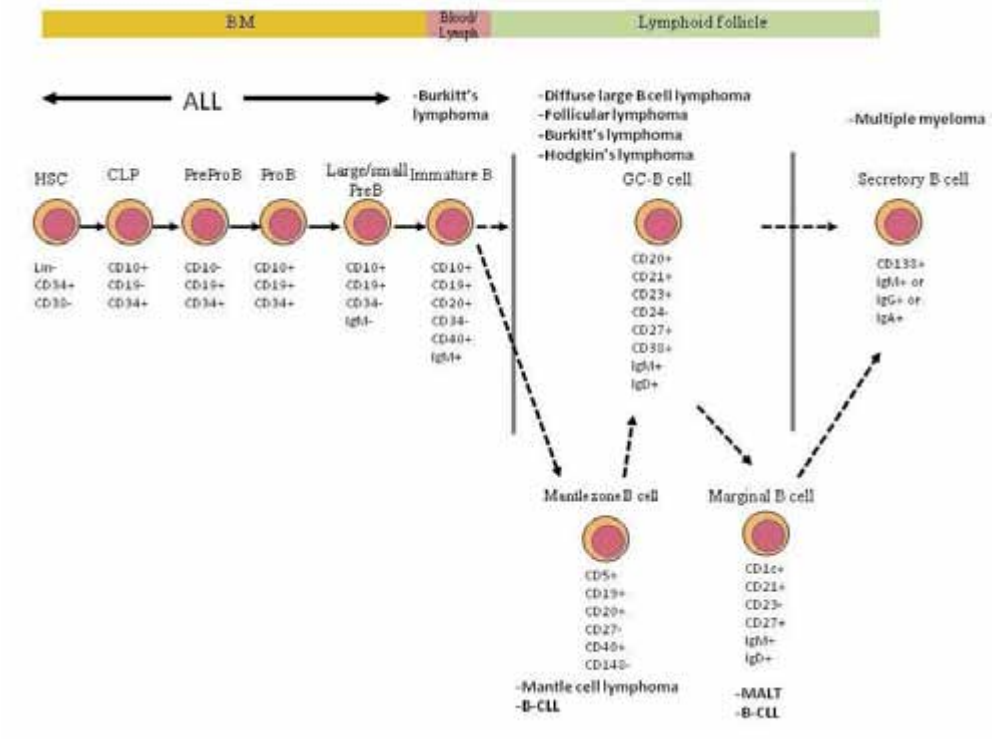

Figure 7. Normal human B-cell differentiation and its relationship to B-cell lymphomas.

Discussion of the articles

Specification and commitment of B lymphocytes is largely dependent on the appropriate expression of transcription factors to build up a network of genes that define the identity of B cells and repress the formation of alternative lineages. Unrevealing the elements of this genetic network and description of how they are connected in a temporal manner will be essential to understand B cell development and how perturbation in this network lead to abnormalities like autoimmune diseases, immune deficiency and lymphoid malignancies. Studies of animal models including knock outs, transgenic and reporter mice unveiled many of the cardinal regulators of B cell development and their complex interactions has began to resolve. Another key issue is to develop the understanding for the precise point of lineage restriction within the defined differentiation pathways.

The overall aim of this thesis has been to identify the point of no return in normal B-cell development.

Ebf1 is a helix-loop-helix (HLH) transcription factor with an amino-terminal domain containing a zinc-coordination motif (Hagman et al., 1993). The helix-loop-helix domain of Ebf1 is required for homo-dimerization of protein (Hagman and Lukin, 2005). Members of Ebf family are expressed in neurons, adipocytes, stroma cells and osteoblasts (Akerblad et al., 2002; Wang and Reed, 1993; Wang et al., 1997). Ebf1, however, is the only family member reported to be expressed in the hematopoietic system. Ebf1 expression is mostly detected in B lineage cells and it can be traced back to the CLP stage. Mice lacking Ebf1 display an absence of mature B-cells in bone marrow and periphery. The block in B cell development was reported to reside at the Pre-ProB (FrA) cell stage (Lin and Grosschedl, 1995). However studies of gene expression and in-vitro differentiation of Pre-ProB cells in Ebf1 deficient cells shows a perturbation of lineage identity in these cells (Lin and Grosschedl, 1995; Medina et al., 2004). In Paper I we investigated the earliest stages of B-cell differentiation in Ebf1 deficient mice. Although cells phenotypicaly similar to Pre-ProB cells ($B220^+CD43^+AA4.1^+CD19^-$) could be found in the bone marrow of mice transplanted with Ebf1 deficient fetal liver, global gene expression analysis and RT-PCR showed an absence of B cell restricted genes such as λ5, Pax5, CD79a,CD79b, Pou2af1, Foxo1 and VpreB. We could also detect reduced expression of these genes in Ebf1 deficient CLPs suggesting that Ebf1 play a crucial role already in these early progenitor cells. In accordance with this, our single cell RT-PCR data revealed that the CLP compartment contained a fraction of cells displaying coordinated

expression of B-lineage genes that were completely lost in the absence of Ebf1. Ectopic expression of Ebf1 in HSCs from E2A deficient mice was sufficient to rescue B cell development up to the Pro-B cell stage even though that E2A was required for normal proliferation of these cells (Seet et al., 2004). Ebf1 can also partially rescue IL7 knock out CLPs and restore B cell development in the absence of this crucial cytokine (Dias et al., 2005). Interestingly, the presumed B-lineage commitment factor Pax5 is unable to rescue neither the E2A nor IL7 knockout phenotype. Hence, it seems as if Ebf1 has a central and non redundant role in the formation of the B cell transcriptional regulatory network in early stages of B cell development. Therefore, characterizing the target genes and regulators of Ebf1 is essential to understand the B cell commitment and the genetic network that defines B-lineage cells. This far, the Ebf1 gene has been reported to harbor two promoters denoted α and β (Figure 8) (Roessler et al., 2007). The Ebf1α promoter has binding sites for Stat5, E2A and Ebf1 whereas Ebf1β has binding sites for PU.1, Pax5 and Ets1, suggesting that the expression of Ebf1 is regulated by a complex network of transcription factors acting both upstream and downstream of Ebf1 itself. The action of E2A and Il7 induced Stat-5 may be of crucial importance to initiate high levels of Ebf1 expression in the CLP compartment while regulation by Pax5 is crucial to establish a stable state in the committed B-cell progenitor.

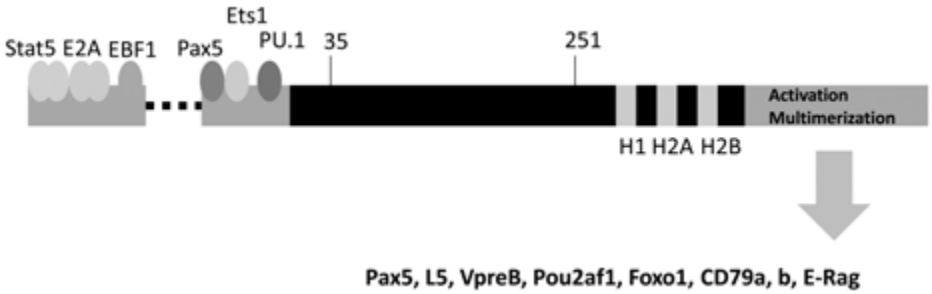

Figure 8. Schematic drawing of the Ebf1 promoters.

A relatively large number of target genes for Ebf1 has been identified including λ5, Pax5, CD79a, CD79b, and VpreB. Additionally, our global gene expression analysis comparing wild type and Ebf1 deficient CLPs, revealed dramatic reductions in the expression of the transcription factors Pou2af1 and Foxo1. Further investigations with electro mobility shift assay (EMSA) and luciferase assay revealed strong functional binding sites for Ebf1 on both the Pou2af1 and Foxo1 promoters. This suggested that Ebf1 is not only capable to induce the expression of Pax5, but also of other

transcription factors with important functions in B-cell development. Pou2af1 deficient mice display a rather mild phenotype in the early stages of B cell development while the formation of germinal centers and immunoglobulin class switching is dramatically impaired (Hess et al., 2001; Kim et al., 1996; Schubart et al., 1996). Pou2af1 is a co-factor for Oct proteins that are known to be involved in the regulation of immunoglobulin V-gene transcription. Hence, even if it would appear that Pou2af1 is not essential for early B cell development, the dramatic induction of transcription upon B-cell restriction may motivate a more careful investigation of the CLP compartment in Pou2af1 deficient mice. Foxo1 is expressed in early hematopoetic progenitors but high levels of expression in the CLP compartment depends on functional Ebf-1 (Paper I). Foxo1 has been shown to be important for early B-cell development (Dengler et al., 2008) where this factor has been suggested to directly regulate Rag1 transcription (Amin and Schlissel, 2008). Interestingly, Chromatin Immuno precipitation experiments revealed that Ebf1 and Foxo1 share a large number of target genes (Lin et al., 2010). Thus, the functional collaboration between these factors may participate in the co-ordination of the B-lineage program.

The finding that Ebf1 apparently regulated the expression of B-lineage genes in the presumably multipotent CLP compartment and the fact that single cell PCR revealed that a fraction of the CLPs from wildtype mice coordinately expressed a set of B-lymphoid lineage genes, suggested to us that the CLP compartment contained specific subpopulations of cells. It has been proposed that B cell commitment may not occur until after that the combined expression of B220 and CD19 can be detected at the Pro-B cell stage (Rumfelt et al. 2006). This would be well in line with the idea that Pax5 regulate CD19 expression as well as act as a B-lineage determination factor. However, the apparently coordinated regulation of most of B cell associated genes, including Pax5, by Ebf1 already in the CLP compartment prompted us to further investigate the temporal regulation of B-cell commitment. In order to investigate if the expression of B-lineage genes was a sign of that the CLPs are primed to B cell lineage development or if lineage commitment occur already at the CD19 negative stage of development, we utilized reporter mice that express hCD25 under the control of λ5 promoter (Paper II, III). Phenotypic characterization of CLPs in these mice revealed that 5 to 10% percent of the CLPs, expressed the transgene suggesting that the B-lineage restricted program had been initiated (Paper II and III). Investigating the lineage potentials of single $\lambda5^+$ and $\lambda5^-$ CLPs in vitro using OP9, OP9D stroma cell cultures as well as myeloid conditions, revealed that even on OP9D cells, that are strong promoters of T-cell development, the absolute majority of the λ5 expressing CLPs developed into B lineage cells. Hence, while almost all $\lambda5^-$CLPs cells have both B and T potential, the λ5 expressing cells are largely B-cell restricted. Culturing single

cells under myeloid promoting conditions revealed that all the residual myeloid potentials of the CLPs was retain in the $\lambda5^-$ fraction of cells. Furthermore global gene expression of $\lambda5^+$CLPs showed that the B cell associated program is already active in these cells and the pattern of expression is more similar to ProB cells than total CLP and LMPP populations. To ensure that the B cell program is active in every single cell of the $\lambda5^+$ CLP compartment, we performed single cell PCR in more than 100 cells and we could confirm the co-expression of B-cell restricted genes, including Ebf1 target genes, in more than 50% of these cells, a result similar to those obtained using $CD19^+$ ProB cells (Paper II).

The single cell PCR analysis did also suggest that the CLP compartment was heterogeneous with regard to the expression of Rag1. Expression of Rag genes in early progenitors like LMPPs and CLPs, have been considered as a sign of lymphoid specification. It has been suggested that high expression of Rag1 in progenitors mark the B and T potent cells while Rag1 negative cells retain Nk and dendritic cell potential (Igarashi et al., 2002; Luc et al., 2008; Welner et al., 2009). However, the usefulness of Rag expression in identifying lymphoid restricted cells has been a matter of debate. Thus, to follow up on our findings using single cell PCR and in an attempt to gain further insight into lymphoid development within the conventional CLP compartment, we crossed the λ5 reporter mice to Rag1-GFP reporter mice. This allowed us to sub-fractionate CLPs into three distinct populations: $CLPRag^{low}\lambda5^-$, $CLPRag^{high}\lambda5^-$ and $CLPRag^{high}\ \lambda5^+$. Upon investigation of the single cell lineage potential of these populations under B, T, Nk and myeloid promoting conditions (For B: OP9+IL7, FL, KIT for T: OP9D+IL7, FL, KIT, for Nk: OP9+IL7, FL, IL2, IL15, for myeloid: FL, KIT, GMCSF, MCSF) it became apparent that $CLPRag^{low}\lambda5^-$ cells retain Nk, T, B and residual myeloid potentials. $CLPRag^{high}\lambda5^-$ cells, on the other hand, show very little Nk but robust B and T potentials and virtually no capacity to form myeloid cells in vitro. Eventually almost all $CLPRag^{high}\lambda5^+$ cells display only B cell potential as suggested from Paper II (Figure 9). In vitro analysis of the kinetics of how these newly identified populations were generated suggests that they are developmentally related in such way that $CLPRag^{high}$ are generated from $CLPRag^{low}$ cells and that further maturation of these cells results in expression of λ5 that marks the restriction to B cell lineage. Global gene expression and single cell PCR also supported the idea of a gradual development of cells co-expressing B cell associated genes. Even though the Rag1 reporter provided us with an excellent tool to investigate lineage restriction events in the CLP compartment, we wanted to identify an endogenous marker that would allow us to investigate these events in mice models without first crossing them to reporter transgenes. To this end, we analyzed our microarray data collected from Rag positive and Rag negative CLPs, to find a marker

that would enable us to isolate the same populations in non-transgenic animals. Among the potential markers, we found that Ly6D to have the highest overlap with expression of Rag1 in CLPs. In an independent line of investigation, Inlay et al (Inlay et al., 2009) reported that the Ly6D$^+$CLP fraction retains B cell potential but display reduced T, Nk potentials. This is largely in line with our in vitro differentiation data with the exception that we detect robust T-cell potential up until expression of the λ5 reporter gene in a sub-fraction of the Ly6D$^+$ cells. This apparent discrepancy may be a result of reduced expression of CCR9 (Tsapogas and Zandi Unpublished observation), important for correct homing to the thymus micro-environment. This may disturb the ability of these cells to develop into T-lineage cells even after intrathymic injection. Sub-fractionation of CLP into more homogenous and functionally distinct population helps us to explain many unanswered questions about lymphoid commitment.

One of the immediate applications of sub-fractionation of CLPs and identification the commitment point within the CLP compartment is the investigation of the role and mechanisms of cytokines in B-cell commitment process. In paper IV we show that the defect in IL7 KO mice is actually within CLP compartment and precisely in transition from CLPLy6D$^-$ to CLPLy6D$^+$ cells. While CLPLy6D$^-$ cells from the Il7 deficient mice can develop normal B cells in-vitro upon IL7 substitution, the remaining CLPLy6D$^+$ cells cannot be rescued by addition of IL7 cytokine in-vitro. Crossing the IL7KO mice with Bcl2 transgenic mice didn't rescue the development of CLPLy6D$^+$ cells, arguing against a simple permissive role of IL7 at this stage of B cell development. We would suggest that IL7 is required for CLPLy6D$^-$ cells in an instructive manner to develop into lineage restricted CLPLy6D$^+$ cells. This clarifies the role of Il-7 in early lymphoid development and puts emphasis on the relevance of the recently defined lineage restricted progenitor cells in lymphoid differentiation.

In conclusion, our work reveals that the earliest stages of B-cell development, including commitment to B-lymphoid lineage, is dependent of Ebf1 and Il7 and occurs in CD19$^-$ progenitors. Furthermore, the CLP compartment is not a homogenous population but rather consists of at least three functionally different subpopulations. Our data would suggest that the most immature cells do not express either Rag1 or λ5 and retain lineage potentials for NK, B and T cells. Expression of Rag1 marks the loss of NK cell potential while further differentiation into λ5 expressing cells is associated with B-lineage commitment.

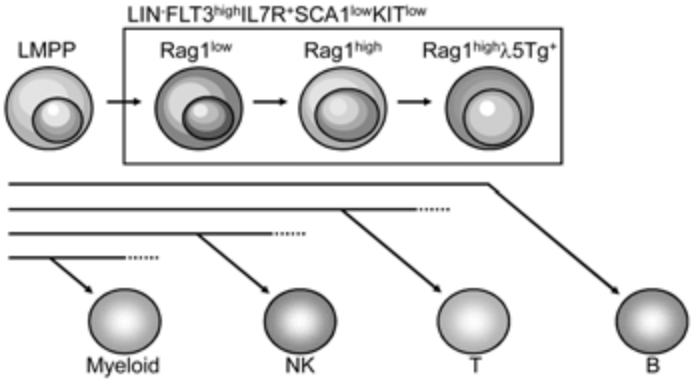

Figure 9. Alternative model for lymphoid lineage commitment events.

Our finding provide fresh a comprehensive insight to lymphoid restriction events that may help to explain several controversial and unknown phenomenons like the role of transcription factor networks in B cell commitment, instructive versus permissive roles of extrinsic factors like IL7, and open new questions like the origin of Nk cells and weather there is only one or several developmental pathways for B cell development. Additionally, the understanding of lineage relationships and transcription factor function can provide important clues to the roles of regulatory networks in malignant transformation.

Populärvetenskaplig beskrivning på Svenska:

En av de historiskt sett största vetenskapliga kontroverserna inom medicinen har cirkulerat kring frågan om hur ett befruktat ägg kan utvecklas till den oerhört komplicerade skapelse som utgör ett djur eller en människa. Denna debatt har engagerat forskare ända sedan Aristotoles tid men den moderna medicinska forskningen har tydligt visat att detta sker genom epigenes, det vill säga, skapande av organ genom utmognad från en stamcell. Denna förståelse har legat till grund för den medicinska användningen av stamceller vilken spås öka i takt med att kunskapen om dessa cellers beteende och mognad utvecklas. Även om stamcells terapi skulle kunna användas inom flera områden så har den till dags dato primärt använts vid benmärgs transplantation. Detta till stor del beroende på att det finns en bra förståelse för hur blodets celler mognar från stamceller i benmärgen. För att vi skall må bra behöver vår benmärg producera miljardtals nya blodceller varje dag. Dessa fyller en rad olika funktioner i kroppen såsom att transportera syre, hindra blödningar samt att försvara oss mot infektions sjukdomar. Dock kräver dessa uppgifter att blodcellerna specialiseras för en viss funktion. Exempelvis så sköts syretransporten av röda blodkroppar, kontroll av blödning av blodplättar och försvar mot infektion av vita blodkroppar. Trots att blodcellerna har olika funktioner och olika utseende så kommer de alla från en och samma blodstamcell i benmärgen. Denna cell delar sig och kan sedan ge upphov till alla de olika celltyperna beroende på vilket behov som kroppen har. Befinner vi oss på hög höjd med låg syrenivå tillverkas extra mycket röda blodkroppar och har vi en infektion produceras mer vita blodkroppar. Stamcellens förmåga att generera flera typer av blodceller kallas multipotens och borgar för både den stabilitet och flexibilitet i blodcells bildning som krävs för att vi skall vara friska. Störningar i blod cells bildning ligger även till grund för de flesta leukemier där tumör cellerna stannar i sin utveckling. Detta resulterar i en okontrollerad cell delning av dessa omogna celler vilket stör produktionen av normala blodceller. Denna avhandling är fokuserad på att öka förståelsen för de processer som ligger till grund för normala blodcellers mognad. Vi har genom detta arbete kunnat visa på nya typer av regler system som ger cellen möjlighet att utvecklas till en normal B-lymfocyt.

Jag hoppas att denna nya kunskap på sikt skall kunna hjälpa oss finna nya vägar för att förbättra förutsättningarna att kunna bota allvarliga blodsjukdomar som leukemi och bristande immunförsvar samtidigt som vi lär oss mer om stamcellers biologi och cell mognads processer. Denna kunskap tror vi kommer att kunna appliceras även i andra biologiska system och därmed ytterligare bredda användningen av cell baserade behandlings metoder.

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