Knock Knock Knock,
Who is there?
-Cell Crosstalk within the Bone Marrow

Jenny Stjernberg
"Aurora musis amica"

“Morgenstund har guld i mund”
Supervisor
Mikael Sigvardsson, Professor
Department of Clinical and Experimental Medicine
Faculty of Health Sciences
Linköping University, Sweden

Co-Supervisor
Jan-Ingvar Jönsson, Professor
Department of Clinical and Experimental Medicine
Faculty of Health Sciences
Linköping University, Sweden

Opponent
Hanna Mikkola, Professor
Department of Molecular, Cell and Developmental Biology
University of California
Los Angeles, USA

Committee Board
Fredrik Öberg, Docent
Department of Immunology, Genetics and Pathology
Uppsala University, Sweden

Mikael Benson, Professor
Department of Clinical and Experimental Medicine
Faculty of Health Sciences
Linköping University, Sweden

Stefan Thor, Professor
Department of Clinical and Experimental Medicine
Faculty of Health Sciences
Linköping University, Sweden
This thesis is focused on the subject of cell-cell interaction. Our body is composed of cells, most of them are integrated in a network with other cells that together forms tissues and organs. Every cell type in these complex organs has its special task and location. This is true whether we are doing research on humans or, as we have been, investigating mice. Mice are excellent models for studies of blood cell development since this process in mice resembles human blood cell generation in many regards.

Cells communicate with each other by sending out small molecules or by directly binding to surrounding cells; to cells of the same kind as well as to cells with different origins and tasks. A cell is surrounded by hundreds of different signals from its environment; soluble, bound to the extra cellular matrix or bound to its surface. Every cell has to distinguish and respond to the environment according to its own specific nature. We can look upon it as a big orchestra, where every person is responsible to play and stop playing its instrument at the exact right point in time set by the conductor for the tones to become music and not only sounds or even worse, noise. Cells also have their intrinsic cues to follow just as every member of the orchestra has its own notes. When a cell is playing its notes and listening to its environment accordingly, it will be told to survive, divide, differentiate and finally die. However when a cell stops listening, gets the wrong signal from its conductor or mess up the notes, it is at high risk of becoming a cancer cell. Therefore we need to learn more about intrinsic and extrinsic cell signals - to know who is knocking and why - in order to understand the normal events and eventually also understand what can go wrong, causing malignancies such as leukemia.
Articles and manuscripts included in this thesis


IV. Stjernberg, J., Qian, H. and Sigvardsson, M. Dynamic crosstalk between developing blood cells and mesenchymal stroma compartments. Manuscript for e-Blood
# Content

**Preface** ................................................................................................................................................................................... 5  
**Articles and manuscripts included in this thesis** ........................................................................................................ 6  
**Abbreviations** ..................................................................................................................................................................... 8  
**Background** ...................................................................................................................................................................... 11  
**Hematopoiesis** ............................................................................................................................................................ 11  
**Embryogenesis and the origin of hematopoietic stem cells** ............................................................................................ 12  
**Cell differentiation** ........................................................................................................................................................14  
**From hematopoietic stem cell to B-cell** ............................................................................................................................ 14  
**Transcriptional regulation of B-cell differentiation** ........................................................................................................... 18  
**Cytokine regulation in B-cell development** .................................................................................................................... 23  
**Bone and bone marrow** .................................................................................................................................................... 25  
**Hematopoietic stem cell niches** ............................................................................................................................................ 27  
**The endosteal niche** ...................................................................................................................................................... 27  
**The sinusoidal niche** ..................................................................................................................................................... 29  
**The intermediate niche** ................................................................................................................................................ 29  
**Cancer and stem cell niches** .......................................................................................................................................... 32  
**Homing and maintenance** ............................................................................................................................................... 34  
**Methodological considerations** ....................................................................................................................................... 37  
**Flow cytometry and cell sorting** ........................................................................................................................................ 37  
**Microarray analysis** ...................................................................................................................................................... 39  
**Aim of thesis** ..................................................................................................................................................................... 42  
**Conclusion** ........................................................................................................................................................................ 42  
**Results and discussion of papers in the thesis** .................................................................................................................. 43  
**Article I** ........................................................................................................................................................................... 43  
**Article II** ......................................................................................................................................................................... 45  
**Article III** ........................................................................................................................................................................ 46  
**Article IV** ........................................................................................................................................................................ 48  
**Populärvetenskaplig sammanfattning** .......................................................................................................................... 49  
**Acknowledgments** ......................................................................................................................................................... 50  
**References** ....................................................................................................................................................................... 52
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>ARC</td>
<td>adventitia reticular cell</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta, gonad, mesonephros</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic proteins</td>
</tr>
<tr>
<td>C1P</td>
<td>Ceramide-1 Phosphate</td>
</tr>
<tr>
<td>CAR-cell</td>
<td>Cxcl-12 abundant reticular cell</td>
</tr>
<tr>
<td>CD</td>
<td>cluster differentiation</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>Cx</td>
<td>connexin</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>Dpc</td>
<td>day post coitum</td>
</tr>
<tr>
<td>Ebf</td>
<td>early B-cell factor</td>
</tr>
<tr>
<td>Epo</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ETP</td>
<td>early thymic progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescent-activated cell sorting</td>
</tr>
<tr>
<td>FL</td>
<td>fetal liver</td>
</tr>
<tr>
<td>Flt-3</td>
<td>FMS-like tyrosin kinase 3</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescent protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GMP</td>
<td>granulocyte macrophage precursor</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HLH</td>
<td>helix loop helix</td>
</tr>
<tr>
<td>HPC</td>
<td>hematopoietic progenitor cell</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Lin-</td>
<td>lineage marker negative</td>
</tr>
<tr>
<td>LMPP</td>
<td>lymphoid primed multipotent progenitor</td>
</tr>
<tr>
<td>LSK</td>
<td>lineage 'sca' 'kit'</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEP</td>
<td>megakaryocyte erythroid precursor</td>
</tr>
<tr>
<td>MPP</td>
<td>multi potent progenitor</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>Pax-5</td>
<td>paired box gene 5</td>
</tr>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>platelet derived growth factor receptor α</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>Rag-1</td>
<td>recombination activated gene 1</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1 phosphate</td>
</tr>
<tr>
<td>Sca-1</td>
<td>stem cell antigen-1</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal cell derived factor 1 (Cxcl-12)</td>
</tr>
<tr>
<td>SLAM</td>
<td>signaling lymphocyte activation molecule</td>
</tr>
<tr>
<td>SNO-cell</td>
<td>spindle shaped, N-Cadherin positive osteoblastic cell</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Thpo</td>
<td>thrombopoietin (in the community,Tpo is sometimes used as well)</td>
</tr>
<tr>
<td>Tie2</td>
<td>endothelial-specific receptor tyrosine kinase</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stroma lymphopoietin</td>
</tr>
<tr>
<td>YS</td>
<td>yolk sac</td>
</tr>
<tr>
<td>VLA-4</td>
<td>integrin α4β1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Background

Hematopoiesis

Our blood system is one of the biggest organs in the human body; the circulating blood volume is about 8% of our body weight (5.6 l in a 70kg man). Blood cells have a rapid turnover, one trillion ($10^{12}$) blood cells are calculated to be produced every day, just to maintain steady state (Ogawa, 1993). This number can increase up to 10-fold in case of trauma resulting in large blood loss revealing a high degree of adaptability to physiological demands (Kaushansky, 2006). Blood cells are vital for our survival, cycling in our blood stream they are responsible for carrying oxygen and metabolites to our whole body and at the same time being our defense system towards surrounding viruses, bacteria and parasites as well as forming blood-clot after injury. There are specialized cells for each of these tasks. However, all of the blood cells originates from the same hematopoietic stem cell in the bone marrow (BM). This has been shown by single cell transplantation of HSCs to lethally irradiated mice, leading to reconstitution of the entire blood system (Smith et al., 1991; Osawa et al., 1996). Even though there exist an enormous demand for production of blood cells, the HSC itself resides mostly in a quiescent cell during homeostasis. It has been estimated that HSCs divide approximately every 145 days creating a need for a complex regulation of blood cell differentiation in order to fulfill the biological demands (Wilson et al., 2008).
Embryogenesis and the origin of hematopoietic stem cells

It is fascinating when you start thinking of how one single fertilized egg can give rise to a complete new organism. How the cell itself, upon intrinsic signals start dividing, together with extrinsic signals starts to differentiate and after numerous cell divisions a new baby is born. This new human, or mouse as we use as a model system for humans, will throughout its entire life need new blood cells and in health they will be provided by the bone marrow hematopoietic stem cells. The fertilized egg will first divide into two cells, that will become 4 and at next division 8, the morula. In the morula, all cells are identical but at the following division the cells will start differentiation towards external and internal embryonic organs. In mouse embryogenesis there are three germ layers, mesoderm, ectoderm and endoderm all responsible for the generation of specific tissues in the adult mouse. These layers are present from 6.5 day post coitum (dpc) (Tam and Behringer, 1997).

The hematopoietic cells are of mesodermal origin and are initially generated from at least two independent sites, the yolk sac (YS) in the extra-embryonic region, and the intra-embryonic region known as the para-aortic splanchnopleura, later to be the aorta-gonad-mesonephron (AGM) region. However, YS and AGM are no longer considered as the only potential sites for de novo hematopoiesis since the placenta and umbilical vein are also considered as a site both for early hematopoiesis and expansion of HSCs (Gekas et al., 2005; Rhodes et al., 2008; Van Handel et al., 2010). For a long period of time, the YS was seen as the main site of hematopoiesis in the early embryo and also the origin of hematopoietic stem cells (Moore and Metcalf, 1970). This was based on the observation that hematopoietic cells can be found in the YS from around 7.5 dpc thereby constituting the first site of hematopoiesis. The blood cells found in the YS is primarily erythrocytes expressing fetal hemoglobin (Palis et al., 1995). It has, however, been difficult to identify transplantable hematopoietic stem cells in the early YS and more recent studies indicates that the early hematopoietic stem cells (pre-HSC) origin from the AGM region where pre-HSCs can be found in low numbers around 10 dpc (Muller et al., 1994; Medvinsky and Dzierzak, 1996; Cumano et al., 2001; Taoudi et al., 2008).

The migration of erythroblasts from the YS to the embryo starts at 8.5 dpc, however, the circulation is limited but increase gradually and at 10.5 dpc there are circulating cells inside the embryo (McGrath et al., 2003). This also allows for cells from the YS and AGM to circulate in the embryo to reach both liver and placenta where the transplantable hematopoietic stem cells (HSCs) expand. In the placenta, HSC expands until the third trimester whereafter numbers decline (Gekas et al., 2005). Hematopoietic cells in the fetal liver (FL) has been reported to
Background

expand as early as 9 dpc, peak at 16 dpc and then decline in numbers until birth (Cumano et al., 1993; Morrison et al., 1995; Ema and Nakauchi, 2000).

Within the FL and perhaps also other niches like the placenta, there is a transition of the pre-HSC to transplantable HSCs. The characterization of the pre-HSCs is complicated by the fact that they cannot functionally reconstitute an adult bone marrow (Matsumoto et al., 2009). Pre-HSCs can be found in the AGM, however, this is not considered as the site for expansion of these cells. Rather they seem to migrate to the FL where they mature to HSCs (Matsumoto et al., 2009). Large number of HSCs can be found in the FL from 12.5 dpc, as verified by transplantation of FL cells to an irradiated recipient (Muller et al., 1994; Ema and Nakauchi, 2000). HSCs will home to the bone marrow around 17 dpc (Christensen et al., 2004; Gekas et al., 2005) and after birth, the bone marrow will be the major site for normal hematopoiesis throughout life.
B-cell differentiation

Cell differentiation

From hematopoietic stem cell to B-cell

All blood cells in the body originate from the long-term hematopoietic stem cell (HSC) that resides in the bone marrow (BM). HSCs were first described in the 1940s, however it took until 1956 until the nature of stem cells was reviled by transplantation of diluted bone marrow to irradiated recipient mice (Ford. et al., 1956) The functional characterization of HSCs is greatly enhanced using transplantation between congenic C57 Black/6 mouse strains expressing different forms of the surface marker CD45 on all nucleated hematopoietic cells. Hence, mature cells generated from transplanted stem cells can be identified by flow cytometry using antibodies specific for the different forms of CD45 (CD45.1 and CD45.2). The initial characterization of HSC was done in the late 80's with aid of flow cytometry and transplantations, revealing that the Sca-1\textsuperscript{+}, Thy1.1\textsuperscript{lo} and lineage marker negative (Lin\textsuperscript{-}) population contained transplantable HSCs (Spangrude, Heimfeld, and Weissman, 1988a; Morrison and Weissman, 1994).

The sub-fractionation of this population to identify a homogenous HSC population has been proven difficult, in part because of the rarity of the HSCs constituting approximately 4-8 cells/10\textsuperscript{5} nucleated BM cells (Abkowitz et al., 2002). For a long time it has been known that the Lin\textsuperscript{-} Sca-1\textsuperscript{+} c-Kit\textsuperscript{+} (LSK) CD34\textsuperscript{-} fraction contains the stem cells (Osawa et al., 1996). Today we can purify this population further by using additional antibodies towards newly discovered surface markers that can be detected and sorted by more advanced flow cytometers. The HSC is today mainly characterized by a c-Kit\textsuperscript{+} Lineage\textsuperscript{-} CD150\textsuperscript{-} Sca-1\textsuperscript{-} Flt-3\textsuperscript{-} phenotype (Papathanasiou et al., 2009). Cells in this population can be serially transplanted i.e. one cell from a donor mouse can be transplanted to an irradiated recipient to reconstitute hematopoiesis. From the reconstituted mouse it is possible to purify HSCs based on the same criteria and with single cell transplantation reconstitute a secondary host. The same procedure can be used to reconstitute a tertiary host proving the longevity and selfrenewal capacity of this purified cell population (Morita et al., 2010). Expression of additional members of the Signaling lymphocyte activation molecule (SLAM) family receptors as well as lack of CD48 or
CD244 expression has also been proposed to allow for the enrichment of functional HSCs (Kiel et al., 2005; Bryder et al., 2006) A fraction of the HSCs can be visualized by adding Hoechst-33342 or rhodamine-123 since the stem cells will efflux the dye and can be visualized as a side population using flow cytometry (Wolf et al., 1993; Goodell et al., 1996). The HSC populations defined by these markers are however probably to some extent overlapping, decreasing the impact of using a combination of all markers in order to obtain a homogenous HSC population (Ema et al., 2006; Weksberg et al., 2008).

In vivo, HSCs are found in BM cavities, close to both bone and blood vessels. Even though the high production rate of blood cells, HSCs may reside quiescent in their BM niche for several weeks or even months (Wilson et al., 2008). This is made possible due to highly proliferating downstream progenitors reducing the need for HSC cell division to maintain homeostasis. Functionally for a cell to be identified as a hematopoietic stem cell it has to fulfill several criteria including.

1: be able to self renew
2: be able to give rise to all different hematopoietic lineages.
3: functionally replace a damaged BM of a recipient.

Self renewal is crucial to avoid that the stem cell pool gets depleted through the generation of differentiating progeny. This has been proposed to be achieved either by asymmetric cell division or by symmetric cell division followed by commitment to either remain as HSC or to enter a differentiation pathway as a multi potent progenitor (MPP) in the shape of a short term stem cell (Spangrude, Heimfeld, and Weissman, 1988b; Morrison and Weissman, 1994; Morrison and Kimble, 2006). The surface marker phenotype of MPPs resemble that of the HSC but with the loss of CD150 (Papathanasiou et al., 2009) and gain of CD34 expression (Adolfsson et al., 2005; Yang et al., 2005). MPP divide more frequently than the HSC, and they presumably retain capacity to give rise to all the different blood cell lineages, however as they display limited self renewal capacity they will be depleted in 4-6 weeks (Morrison and Weissman, 1994; Akashi et al., 2000; Christensen and Weissman, 2001). As a result, in contrast to the HSC, these cells fail to maintain blood cell production over time, a characteristic limiting the possibility to investigate their lineage potential at the single cell level (Akashi et al., 2000; Adolfsson et al., 2005; Yang et al., 2005). HSCs have been reported to express low levels of lineage associated genes already at the stem cell stage (Hu et al., 1997). This could be due to the cell already at this stage are undergoing commitmtment to a certain lineage fate, however, since single cells can express genes associated with several lineages, it is more likely that they are primed towards a specific fate even if they retain the ability to change path upon differentiation (Hu et al., 1997; Månsson, Hultquist, et al., 2007).

Cells committing to a lymphoid and eventually the B-cell path, will proceed in development into lymphoid primed multipotent progenitors (LMPPs) (Adolfsson et al., 2005). These cells can be identified based on the expression of Flt-3 on LSK cells. Even though disputed (Forsberg et al., 2006), it appears that these cells display reduced capacity to generate megakaryocyte
B-cell differentiation

and erythroid lineages, while they retain potential for both Granulocyte/Monocyte (GM) and lymphoid lineages. Further restriction in myeloid lineage potential is associated with the up regulation of the IL-7 receptor on common lymphoid progenitors (CLPs). CLPs were originally characterized by displaying a Lin^{-} Sca^{1+} Kit^{+} IL7-R_{high} phenotype (Kondo et al., 1997). These cells can give rise to B-cells, T-cells, NK-cells and to some extent Dendritic cells (DC) and has long been considered as a homogenous multipotent population. However, in the last years increasing evidence suggest that this cell population can be dissected into at least three different stages where specific differentiation potentials are gradually lost. These stages can be defined using a combination of transgenic and surface markers in such way that lack of expression of the surface marker Ly6D on the CLP identifies cells with reduced granulocyte/macrophage (GM), but preserved NK/B/T and DC potential. These cells can also be identified as negative for the expression of a Rag-1 promoter regulated GFP. Subsequent differentiation is reflected in an up regulation of Rag-1 and the surface marker Ly6D, associated with a reduction of NK and DC lineage potentials (Inlay et al., 2009; Mansson et al., 2010). The idea that Rag-1 expression is associated with loss of NK cell potential is also supported by the finding that lineage tracing experiments using a Rag-1 regulated Cre only resulted in that a small fraction of the NK cells expressed the Cre-induced fluorescent marker (Welner et al., 2009). The actual lineage potential of the Ly6D^{+} CLPs is less clear although it is obvious that these cells possess in vitro T-cell potential at the single cell level (Mansson et al., 2010). Intrathymic injection suggest that they display a limited capacity to generate T-cells in vivo (Inlay et al., 2009). This issue also relates to whether the origin of the Early Thymic Progenitors (ETPs) is LMPPs or CLPs that will migrate from the bone marrow, to the thymus where Notch-induced signaling will provide maturation factors driving them to T-cell destiny (Bell and Bhandoola, 2008). A third developmental stage within the CLP compartment has been identified by the expression of a λ5 reporter transgene. The expression of this transgene is restricted to 5-10% of the CLP population and single cell analysis suggests that they have lost most of their T-cell potential and thus represents the first B-cell committed progenitors. This is somewhat in contrast to the current dogma stating that before commitment, the cells progress through the Pre-ProB or Fraction A stage, defined as CD19^{−} B220^{+} CD43^{+} CD24^{lo} AA4.1^{+} (Hardy et al., 1991). However, this population has been suggested to retain some T-cell potential that is not lost until the expression of CD19 can be detected on the cell surface (Rumfelt et al., 2006). Hence, the identification of committed B-lineage progenitors in the CLP compartment may be helpful to unravel mechanisms involved in lymphoid lineage restriction.
Figure 1: A schematic drawing of identified progenitor stages in the path from stem cell to committed B-cells. Each stage can be defined by surface marker expression and to some extent transcription factor reporters as follows. HSC: LinCD150⁺C-Kit⁺Sca-1⁺. MPP: LinCD150 C-Kit⁺Sca-1⁻. LMPP: LinC-Kit⁺Sca-1⁺Flt-3⁻. CLP: LinC-Kit Sca-1⁻Flt-3⁻IL-7R+. In addition, CLP with NK/DC/B/T Ly6D⁻ Rag⁺. CLP with B/T potential is Rag⁺⁺⁺⁺ Ly6D⁺. CLP with B potential: Ly6D⁺ λ5⁺.
Transcriptional regulation of B-cell differentiation

B-cell commitment is a highly regulated, multistep process beginning with a multipotent HSC ending with a mature B-lymphocyte, potent of responding to foreign antigens by differentiating to a plasma cell capable of secreting massive amounts of antigen specific antibodies. This process is highly dependent both on external environmental cues as well as internal sensitivity to those cues. This section will focus on the intrinsic regulation from hematopoietic stem cell to mature B-lymphocyte.

The intrinsic regulation of cell fate options may be reflected already in the transcriptome of the HSCs. For maintaining the HSCs pool, Ldb-1 forms a transcription complex together with E2A, Scl/Tal and GATA-2. This complex is considered to regulate almost 70% of the known genes for HSC maintenance both in fetal liver and in adult bone marrow. This idea is supported by the notion that deletion of Ldb-1 results in depletion of HSCs (Li et al., 2011). IKAROS and Pu.1 are transcription factors important for HSC function as well as for early restriction between the lymphoid and myeloid pathways. Complete deficiency of Pu.1 results in late embryonic lethality, between 18.5 dpc and birth. CD19+ as well as GR1+ Mac-1+ cells are missing and the cells also lack expression of the hematopoietic marker CD45 (Polli et al., 2005). Pu.1 expression levels also appear to have a special function when it comes to the bifurcation of the myeloid and lymphoid lineages. High expression results in a myeloid fate while low levels promotes B-cell differentiation (DeKoter, 2000; Arinobu et al., 2007) The unique role of Pu.1 appears to be limited to early progenitors since conditional knock out (KO) of Pu.1 using a CD19 promoter regulated cre did not result in downstream B-cell failure, likely as a result of that other Ets family transcription factors like Spi-b is highly expressed in committed B-cells (Polli et al., 2005). Pu.1 appears to act in an interesting interplay with the transcriptional repressor Gfi-1 that displays an ability to bind regulatory elements in the Pu.1 gene. In the absence of Gfi-1, these elements binds to Pu.1 creating a positive feedback loop and Gfi-1 can therefore directly modulate transcription of the Pu.1 gene and in the extension the dose of Pu.1. Hence the decision of lymphoid versus myeloid cell fate (Spooner et al., 2009). This regulatory loop also involves the transcription factor IKAROS, modulating the expression of Gfi-1 (Thompson et al., 2007; Spooner et al., 2009). HSCs from IKAROS-deficient mice have impaired self renewal capacity and lymphocyte development (Georgopoulos et al., 1994; Nichogiannopoulou et al., 1999) This is reflected at several levels since IKAROS −/− LSK cells lack the ability to up-regulate
Flt-3, resulting in an impaired LMPP compartment that fails to give rise to CLPs and B-lineage cells (Yoshida et al., 2006). IKAROS also seems to play another role in B-cell development by the regulation of Rag recombinase prohibiting the V(D)J rearrangement and by maintaining a stable phenotype of the committed B-cell progenitor (Reynaud et al., 2008). Other transcription factors acting at several stages of B-cell development is the basic helix loop helix (b-HLH) family transcription factors E12 and E47, generated by alternative splicing of mRNA transcript encoded by the E2A gene (Murre et al., 1989). E2A has an important role for the HSC as well as for B-cells. Heterozygous mice display a decrease in the number of HSC as well as the LMPP (Dias et al., 2008; Yang et al., 2008). E2A have a positive action on B-cell development as it directly binds regulatory elements and up-regulates other transcription factors of importance for B-lineage commitment such as Ebf-1 and Pax-5 (Kee and Murre, 1998; Lin et al., 2010). There are also inhibitory factors participating in the regulation of E2A. Id proteins that may heterodimerize with b-HLH proteins inhibiting the ability to bind DNA and thereby prohibiting B-cell development (Benezra et al., 1990; Norton et al., 1998). E2A KO mice have a block in B-cell development before the immunoglobulin rearrangement occurs (Bain et al., 1994; Zhuang et al., 1994; Borghesi et al., 2005). E2A is also of importance for recombination of Immunoglobulin as shown by the finding that overexpression of E2A and Rag in a non-lymphoid cell line resulted in Ig rearrangement (Romanow et al, 2000).

Ebf-1 (Olf-1 or O/E) is a HLH transcription factor (Hagman et al., 1993) crucial for B-cell differentiation, initiating the B-cell program within the progenitor cells (Lin and Grosschedl, 1995; Zandi et al., 2008; Article I). Ebf-1 is initially up-regulated at the CLP stage (Dias et al., 2005), after exposure to IL-7 via the IL-7R on the cell surface (Article I). This may be as a direct result of that IL-7 induced STAT5 (Vermeulen et al., 1998) bind to the Ebf-1 promoter (Kikuchi et al., 2005; Roessler et al., 2007). Ebf-1 and E2A expression also regulate PU.1, although low expression of PU.1 is prerequisite for lymphoid development, PU.1 is required for B220/CD45 expression (Medina et al., 2004). The hierarchical activation of B-lineage transcription continues with essential down regulation of Id2 and Id3 by Ebf-1 (Pongubala et al., 2008; Thal et al., 2009). As Id proteins negatively regulates E2A, induction of EBF increases E2A expression (Amin and Schlissel, 2008). Ebf-1 regulates a network of genes in early B-cell progenitors including mb-1 (CD79a) (Hagman et al., 1991), B29 (CD79b) (Akerblad et al., 1999), CD19 (Gisler et al., 1999; Månsson et al., 2004), λ5, VpreB (Sigvardsson et al., 1997), CD53 (Månsson, Lagergren, et al., 2007), OcaB and Foxo1 (Zandi et al., 2008) as well as Pax-5 expression (O’Riordan and Grosschedl, 1999). The idea that Ebf-1 is directly involved in the regulation of all these genes has recently been confirmed by chromatin precipitation experiments (Lin et al., 2010; Treiber et al., 2010). Several of these genes encode components of the pre-B cell receptor, of large importance for the transition from the pro-B to pre-B cell stage (Kitamura et al., 1992). Additionally, Ebf-1 binds to regulatory elements to upregulate expression of the Pax-5 gene (O’Riordan and Grosschedl, 1999). Pax-5 will then increase Ebf-1 expression further by binding to an Ebf-1 promoter region (Roessler et al., 2007), creating a positive feedback loop. Pax-5 is a key factor when it comes to lineage restriction of B-cells. Its expression is regulated by Pu.1, Ebf-1 and E2A (Decker et al., 2009; Lin et al., 2010). Pax-5 is
Transcriptional regulation of B-cell differentiation

Transcribed within the B-cell lineage, from pro-B-cell to the mature B-cell stage (Fuxa et al., 2007). Lack of Pax-5 results in the development of progenitor cells expressing low levels of B-lineage genes (Nutt et al., 1997, 1998; Cobaleda et al., 2007) and shows a high extent of lineage plasticity to alternative cell fates (Nutt et al., 1999; Rolink et al., 1999). Furthermore, the Ebf-1 target Foxo1 has been shown to be involved in the regulation of Rag-1 and -2 essential for Immunoglobulin rearrangements (Amin and Schlissel, 2008) highlighting the central role for Ebf-1 in early B-cell development.

**Figure 2:** The figure displays a schematic drawing of the regulatory network around Ebf-1 active in early B-cell development. Regulation of Ebf-1 is dependent both on upstream transcription factors as well as signalling through the IL-7 receptor. Ebf-1 binds to several genomic sites inducing B-cell program, amongst those a key player in lineage restriction, Pax-5. Pax-5 together with Ebf-1 will restrict the cell to a B-lineage path.
Extrinsic regulation of progenitor cells

Not only intrinsic transcription factors solely regulate cell expansion, differentiation and maturation. HSCs are also dependent upon environmental factors affecting the cells in maintaining stemness as well as making lineage choices and differentiate.

Many cytokines surround the cells in the marrow, some of which drive the cells to a myeloid fate, others to lymphoid lineage development. Cytokine signalling is primarily paracrine, however, also endocrine signalling affects the development of blood cells. Two hormones with impact on HSCs are Erythropoietin (Epo) and Thrombopoietin (Thpo). Epo is produced by the kidneys and Thpo is mainly produced by the liver (Naets, 1960; Nomura et al., 1997) but also by the stroma cells in the BM (Yoshihara et al., 2007). HSCs have expression of several cytokine receptors (Taichman, 2005) possibly as a mean of preserving multi potentiality with the potential to respond to a large variety of exogenous signals. HSCs are primarily quiescent and in close contact with stroma cells, providing them with adhesion molecules and cytokines. Still HSCs must remain ready to divide and differentiate upon physiological demands.

One area of intense discussion concerns whether cytokines act by instructive or permissive actions, instructive meaning that cytokines bind to the HSCs or multipotent progenitors and thereby give a signal that drives development towards specific cell fates. In a scenario with permissively acting cytokines, the signals generated would only stimulate survival or proliferation of the progenitors without directly driving development towards specific cell fates. In order to investigate instructive versus permissive roles of cytokines, several approaches have been undertaken. Increase in receptor number (Pharr et al., 1994), changes in downstream receptor signalling (Semrad et al., 1999), also increased expression of cytokine receptors results in an increased population of interest (Pawlak et al., 2000). Loss of function of cytokines such as IL-7, Flt-3L, GM-CSF results in minor or severe reductions in progenitors and mature cells (Stanley et al., 1994; McKenna et al., 2000; Carvalho et al., 2001). Instructive roles of cytokines has also been suggested from experiments where GM-CSFR were expressed in CLPs since this resulted in the generation of GM cells after incubation with GM-CSF (Kondo et al., 2000). Instructive roles of extrinsic signals is also evident from data revealing that Delta/Notch signalling suppress myeloid conversion of pro-T cells by the constraint of Pu.1 and also E protein inhibition and regulation of Gfi-1 (Franco et al., 2006) also resulting in block in B-lymphoid development of CLPs (Schmitt and Zúñiga-Pflücker, 2002). Even though these data would support the idea of instructive roles of cytokines, other lines of evidence supports the
Extrinsic regulation

idea of permissive roles of cytokines. Expression of CSF1-R (Bourgin et al., 2002) or a constitutively active EpoR (Pharr et al., 1994) in multipotent progenitors did not cause any major changes in lineage choices. Furthermore, a G-CSFR-EpoR fusion receptor retain the ability of G-CSF to stimulate granulocyte development and a targeted knock-in of the G-CSFR intracellular domain into the ThpoR locus is sufficient to rescue the thrombocytopenic phenotype of ThpoR deficient mice (Stoffel et al., 1999).

Furthermore, mice lacking functional genes encoding cytokines associated with certain lineages such as Epo (Wu et al., 1995), G-CSF (Lieschke, Grail, et al., 1994) or GM-CSF (Stanley et al., 1994), display rather mild steady-state phenotypes. This could, to some degree, be contributed to by functional redundancy and overlapping receptor expression; however, the combined inactivation of GM-CSF and G-CSF (Seymour et al., 1997) or GM-CSF and M-CSF (Lieschke, Stanley, et al., 1994) does not result in an enhanced disturbance of hematopoiesis as compared to the single knock-out mice. Although these data argue that the action of cytokines in early hematopoiesis is permissive, the two theories are not necessarily mutually exclusive, and it can be hypothesized that cytokines possess both permissive and instructive activity.

Even though cytokines influence the outcome of cell differentiation processes in the hematopoietic system, the precise understanding of these activities is obscured by the huge number of combinatorial activities achieved by their concerted action as well as the action of other regulatory factors in the BM microenvironment. In order to test the impact of cytokines on differentiation of CD34+ HSCs, single cells were seeded and subsequent to division daughter cells were moved to individual conditions and analyzed. Daughter pair analysis revealed that cytokine composition in vitro had a major impact for the outcome on the daughter cells (Takano et al., 2004). This was also confirmed with a video-imaging study demonstrating that the stroma cell microenvironment strongly influences the number of HSCs, self renewing or differentiating (Mingfu, 2008). Paired model investigation of the granulocyte-macrophage progenitor (GMP), that can give rise to both granulocytes and macrophages, revealed that providing the GMP with either G-CSF or M-CSF resulting in generation of granulocytes and macrophages respectively, proposing an instructive role for cytokines in this experimental setting (Rieger et al., 2009). Hence, it would appear as if permissive and instructive actions of environmental signals are not mutually exclusive and it should not even be excluded that the same cytokines may have different functions depending on the developmental stage of the exposed cell.
Figure 3: Schematic drawing of models for instructive and permissive function of cytokines. 
A) Cytokine instructs HSC to division and commitment, increasing the production of specific committed progenitors. B) Permissive regulation of HSC where cytokine affect the daughter cell to expand hence supporting selective survival of the cells.

Cytokine regulation in B-cell development

While several cytokines present in the BM display crucial roles in the regulation of hematopoiesis, some factors appear to be of special importance for B-cell development. Among these are the cytokine IL-7. The crucial effect of IL-7 on normal lymphoid development was first suggested from injection of antibodies towards either IL-7 or IL-7Rα in mice revealing reduction in mature B-cells as well as a reduced cellularity of T-cells in thymus (Grabstein and Waldschmidt, 1993). IL-7 acts via a stage specific receptor expressed on early lymphoid progenitors (Kondo et al., 1997). This receptor is composed of the IL-7 restricted and...
Extrinsic regulation

developmentally regulated α-chain and the more broadly expressed common gamma chain (γc). The γc was originally discovered as part of the IL-2 receptor (also consisting of a α and β subunit) in patients suffering from severe immune deficient disease (SCID) (Noguchi et al., 1993). Later on it was evident that also IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα, IL-21Rα were dependent on the γc unit for receptor signaling (Kovanen and Leonard, 2004). In order to activate the receptor with following downstream signalling, a ligand needs to bind it, leading to dimerization and phosphorylation of the receptor. There are three ways for γc receptors to activate intra-cellular signaling systems, the janus activated kinase (JAK) - signal transducer and activator of transcription (STAT) pathway, phosphatidylinositol 3-kinases (PI3K)-Akt pathway and mitogen activated protein kinase (MAPK) pathway leading to a quick transcription of various genes. IL-7R signaling activates all three signaling systems (Kovanen and Leonard, 2004). Upon IL-7 stimulation of the IL-7R activates JAK1 and JAK3, phosphorylating the intracellular receptor domain, providing docking sites for STAT proteins, predominantly STAT5A and STAT5B, that upon phosphorylation can gain access to the nucleus and target promoters (Hennighausen and Robinson, 2008).

The critical role of IL-7 signaling in lymphocyte development was confirmed by the generation of IL-7Rα and IL-7 deficient mice resulting in an almost complete block of B-cell development and a decrease of the T-cell compartment in thymus (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). To understand more about the nature of the developmental block in absence of IL-7 signaling, investigations of progenitor cell compartments were initiated. By using IL-7 deficient mice instead of IL-7R deficient mice, the CLP compartment (Lin−, Sca1+, Kitlo, Flt-3+, IL-7R+) could be investigated, revealing a block in B-cell development within the CLP stage suggesting that IL-7R signaling is vital for B-lineage commitment (Dias et al., 2005; Kikuchi et al., 2005). In order to further analyze the role of IL-7 in lineage restriction, sub fractionations of both the LMPP and CLP compartments were conducted revealing that IL-7 signalling is crucial for normal maintenance of the IL-7R expressing progenitors, as well as for up regulation of transcription factors promoting B-cell development (Article I). To enhance proliferation of the CLP compartment IL-7R signal, through the STAT5 pathway act synergistically with Flt-3 (Flk2) signaling possibly through the Akt pathway increasing proliferation of progenitor cells (Åhsberg et al., 2010). Flt-3 is a tyrosine kinase receptor expressed by LMPPs and CLPs. A lack of functional Flt-3 signaling results in impaired development of lymphoid progenitors and B cells (Mackarehtschian et al., 1995), (Sitnicka et al., 2003) and combined with lack of IL-7 there is a complete block of lymphoid progenitor development (Sitnicka et al., 2007). Even though IL-7 deficiency results in a dramatic impairment of B-cell development in the adult, the production of B-cells in the FL appears rather normal. This has been explained by that thymic stroma lymphopoetin (TSLP) may compensate for the lack of IL-7 within the fetal liver (Ray et al., 1996). IL-7 has also been suggested to collaborate with hepatocyte growth factor (HGF) that through the interaction with cMet receptor creates a powerful synergy with regard to stimulation of proliferation (Vosshenrich et al., 2003).
Bone is a mineralized, hard but yet light stabiliser and organ protector in our body. Bone is not a homogenous solid structure; rather it contains cavities that host a vast number of cells and cell types. This is reflected in that there are two major types of bone, compact and trabecular bone. The compact bone is what can be seen as the white bone surface, however, even in a long bone such as the femur the cortex contains cavities and trabecular structures, both harbouring bone marrow cells. The bone marrow contains a complex mixture of cells of both hematopoietic and mesenchymal origin. These include bone-synthesizing osteoblasts as well as bone-degrading osteoclasts. Osteoblasts and osteoclasts are in an equilibrium, the numbers and relative frequencies can be altered for periods of time, if needed, after for example an injury or when the bone needs to be remodelled (Hauge et al., 2001).

The long bones are normally highly vascularised by major arteries named according to their location, diaphysial, periosteal, metaphysial and endosteal arteries. These vessels traverse the cortex and branch out to reach the bone in Harvers's and Volkmann's channels to enter the marrow and the sinusoidal microcirculation. Some of these sinusoidal capillaries have an open lumen, permitting slow blood flow increasing the possibility for blood cells to adhere to the endothelium and exit the circulation. Venous blood is drained to the venous central sinus, and leaves the bone at the entry site of the artery (Santos and Reis, 2010). Even though hematopoietic stem cells are localized within the red marrow of the bone, there are interspecies differences as to where in the bone the HSCs are located. In mouse the marrow of long tubular bone is hematopoietically active throughout life while in humans hematopoiesis becomes restricted to the axial skeleton and portions of the long bone metaphyses over time and is reversibly lost in the rest of the marrow (Bianco, 2011).
Figure 4: Schematic drawing illustrating some potential means of cell-cell interaction in the bone marrow. Cells in the bone marrow are often in close contact with each other however it is not uncommon that their location is close but not direct. The interaction between a hematopoietic cell and its surroundings can occur in four different ways.

A): Direct contact. (This is probably the most important contact inside the bone marrow).
B): Paracrine signaling. Cytokines out in the open, affecting surrounding cells.
C): Endocrine signalling. Hormones that comes from other parts of the body, i.e. after blood loss.
D): Function regulated by an intermediate cell.
Hematopoietic stem cell niches

It has been assumed that the microenvironment within the bone marrow creates specific niches harboring defined populations of hematopoietic cells. The HSC niche provides HSCs with signals regulating maintenance, differentiation and proliferation to maintain a steady state of mature cells within the bloodstream (Hirao et al., 2004). In 1970s it was first proposed that stromal cells within the marrow support HSCs and their maturation (Dexter et al., 1977). The exact location of adult HSCs is debated, however it is generally accepted that a majority of the HSCs are located within the trabecular bone marrow cavities in either endosteal or sinusoidal niches or perhaps both simultaneously. Both of the niches consist of the same type of mesenchymal cells, that can produce an array of growth factors, factors for maintenance as well as homing (Sacchetti et al., 2007).

The endosteal niche

The molecular regulation of niche formation and HSC homing appear to be controlled by a complex network of secreted factors, cell interactions as well as physical parameters. Among the physical parameters, there has been an extensive focus on the role of hypoxia in HSC maintenance and differentiation (Eliasson and Jönsson, 2010). Furthermore, it has been reported that the high concentration of calcium surrounding the osteoclasts, as a result of bone degradation, works as an attractant for HSCs potentially directing them to home close to the bone (Adams et al., 2006; Porter and Calvi, 2008). Calcium receptor expression is therefore thought to be important for homing, lodging and adhesion of transplanted HSCs (Lam et al., 2011). Other factors involved in bone remodeling such as Osteopontin is also thought of as an important factor for the localization of HSCs to the endosteal region of the BM (Nilsson et al., 2005). Lining the bone is an osteoblastic cell, spindle shaped N-Cadherin positive osteoblast (SNO). HSCs are proposed to be located close to the bone and the morphologically distinct SNO cells. An increase in the number of SNO cells directly impacts the number of hematopoietic cells expressing N-Cadherin and retaining BrdU 70 days after staining, possibly being HSCs (Zhang et al., 2003). In addition, it has been reported that parathyroid hormone induce an increase of osteoblasts as well as an increase of the same population of N-Cadherin+ cells in
vivo (Calvi et al., 2003). Since HSCs were not analyzed by surface markers as CD150+ Lin- Sca- Kit+, the possibility to link these data to other investigations with a focus on HSC function is somewhat limited. In order to verify the importance of osteoblasts for normal HSCs function, targeted depletion of osteoblasts was conducted using a mouse model displaying osteoblast specific expression of Thymidine Kinase making the cells sensitive to treatment with Gangcyclovir. This resulted in decreased numbers of BM cells, however not specifically HSCs, rather a procentual increase was observed and a decrease in HSCs were not observed until weeks later (Visnjic et al., 2004; Zhu et al., 2007). In an attempt to address whether the HSCs directly depend on the osteoblast or rather indirectly, the expression of N-cadherin on HSCs were evaluated, without finding any expression by PCR, RT-PCR or flow cytometry (Kiel et al., 2007). Also the localization of HSCs were investigated, no more than 20% of the HSC were found within 5 cell distances from the endosteum and almost all HSCs were found within 5 cell distance from the sinusoids, indicating that direct contact with osteoblasts might not be necessary (Kiel et al., 2005, 2007; Ellis et al., 2011) However, this does not exclude that osteoblasts have an important function, perhaps as an indirect mediator of HSC maintenance rather then as a direct regulator of HSC function.
The sinusoidal niche

Most of the endosteum is highly vascularized hence a sinusoidal niche has been proposed. Within the sinusoidal or vascular niche, the stroma cells are adjacent to sinusoidal cells, lining the vessels (Kiel et al., 2005). Stromal cells arise from adventitial reticular cells (ARCs), or the presumed mouse counterpart, Cxcl-12 abundant reticular cells, in short CAR (Sugiyama et al., 2006). CAR cells are essential for blood cell progenitor maintenance, they express SCF as well as Cxcl-12 and matrix proteins. Specific ablation of CAR cells resulted in a 50 % reduction of HSCs within the bone marrow suggesting a central role for these cells in the regulation of blood cell development (Omatsu et al., 2010). There are also other cells that may be involved in the regulation of hematopoiesis within the vascular niche. Developmentally, vasculature has a crucial role in hematopoiesis since hematopoietic progenitors arise in vacuolated regions in the embryo (YS, AGM and placenta). Investigating the zebra fish, where hematopoiesis never occurs in bone, could indicate that bone per se is not directly involved in the regulation of HSC maintenance (Murayama et al., 2006). Endothelial cells lining the vessels express soluble proteins including Angiopoietins (Ang), factors that have been indicated as important for HSC as well as vessel maintenances and quiescence (Arai et al., 2004). HSCs express the endothelial-specific receptor tyrosine kinase (Tie2); Tie2 is only expressed by early hematopoietic cells and endothelial cells lining blood vessels. Ang-1 binding to Tie2, however, does not induce cell growth, rather maintenance of HSCs (Davis et al., 1996). Tie2 expressing HSCs comprise a side population within the bone marrow and also mediates stimulation of Ang-induced integrin adhesion between HSCs and osteoblasts (Arai et al., 2004). The HSC residing in the vascular niche can easily respond to environmental changes, as well as benefiting from stromal factors, generating an opportunity for HSCs to remain quiescent also in the vascular area (Arai et al., 2009).

The intermediate niche

It would appear as if both osteoblastic and endothelial cells can promote maintenance of HSCs, and influence each other, making it difficult to predict whether a specific niche is more important than the other with regard to blood cell development and HSC maintenance. HSCs have recently been found to home in areas close to vessels in the endosteal region of the bone post transplantation (Ellis et al., 2011). This presents the possibility that the vascular niche and the endosteal niche co-exist and are not mutually exclusive. Another event that supports this
theory is that osteoblasts can act as an positive regulator of vessels, promoting angiogenesis by the secretion of vascular endothelial growth factor (VEGF) (Street et al., 2002; Tombran-Tink and Bamstable, 2004) making it possible for the HSC to benefit from every cell type within the BM. Also stromal cells with different origin can benefit from each other. Cxcl-12 is vital for other processes than only chemoattraction and maintaenance of HSCs, for instance angiogenesis, (Tachibana et al., 1998) and as stroma supporter (Kortesidis et al., 2005). Also other cell types located in the marrow are reported as modulators of HSC activity, including macrophages (Chow et al., 2011) and adipocytes (DiMascio et al., 2007).

Hence, there is much to learn about the identity of the HSC supporting cells within the bone marrow. One reason for our limited knowledge about these stromal cells has been the lack of characteristic surface markers, resulting in the inability to purify them by flow cytometry and further investigate them in vitro. This has improved by the development of sorting protocols based on the expression of platelet derived growth factor receptor α (PDGFRα) and stem cell antigen-1 (Sca-1) on mouse stroma cells (Koide et al., 2007; Tokunaga et al., 2008; Morikawa et al., 2009).

However, much of our current understanding of stroma cell-HSC communication has been generated through in vitro studies using hematopoietic-supportive stromal cell lines such as OP9 supporting B-cell development and NIH3T3 which is not able to support B-cell development (Vieira and Cumano, 2004). From these cell lines we have learned a lot about the supportive properties of stroma cells as well as how genetic networks are established in the stroma cells. Ectopic expression of Ebf-1 in fibroblastic NIH3T3 cells result in an increased expression of genes of importance for stroma cell communication and hematopoesis regulation including Cxcl-12, Perisotin, Ccl-9 and Igf-2 (Article II).

There are transgenic models that can be used to investigate the properties of BM stromal cells and their capacity to form a bone marrow niche, like Ebf-2-LacZ (Kieslinger et al., 2010) and Nestin-GFP mice (Méndez-Ferrer et al., 2010). Knowledge of the transcriptional regulation of the stromal compartment is limited; however we know that Ebf-2 is required for proper stromal support of hematopoietic progenitor cells within the bone marrow as well as in vitro. Ebf-2−/− mice have a 2-4 fold decrease in numbers of HSCs compared to WT and die at week 6-8 (Kieslinger et al., 2010). Even though the stromal cells are altered, the mice retain normal numbers of osteoblast and normal bone formation (Kieslinger et al., 2005). There is also no indication that the endothelial cells are the cells responsible for Ebf-2 expression (Kieslinger et al., 2010). Nestin+ stroma cells are important for HPC as they can provide a wide range of important molecules such as Cxcl-12, SCF and IL-7. Difteria toxin depletion of the Nestin+ population results in impaired homing of HPCs (Méndez-Ferrer et al., 2010) further supporting the idea that Nestin expression marks a population of large relevance for the regulation of hematopoiesis.
Hematopoietic niche supporting the stroma

To evaluate whether HSCs are vital for the mesenchymal cell development in AGM, Runx\(^{-/-}\) mesenchymal cells were investigated. Runx\(^{-/-}\) embryos display fetal anemia and lethality at embryonic day 12.5 (North et al., 1999). Despite the severe hematopoietic effect of Runx\(^{-/-}\) AGM, the mesenchymal cells harboring it (adipogenic, chondrogenic and osteogenic progenitors) were found in normal numbers (Mendes et al., 2005). This however does not necessarily mean that the hematopoietic cells are not of importance for the maturation of mesenchymal cells in the adult bone marrow. In (Article III) we show that hematopoietic cells affected a stroma cell line \textit{in vitro} by direct cell-cell contact hence it remains to unravel the impact of blood cell progenitors on the mesenchymal cells \textit{in vivo}.

\textbf{Figure 5: A drawing of cells located within the bone marrow niches.} There are several cell types within the endosteal niche, all in close vicinity to each other. Not yet do we know enough to exclude the importance of any of these or additional cells.
Cancer and stem cell niches

As stem cell niches are crucial for the stem cells, keeping them quiescent and providing the cells with factors of importance for survival, there is an obvious risk that in case there are mutations in the stem cell, making it malignant, this cell will also be supported by the niche. Another problem occurring in this scenario is the alteration of stroma cells by the malignant HSC resulting in differences in cell adhesion and the ability to respond to target drugs (Lwin et al., 2007; Colmone et al., 2008). It must not always be the hematopoietic progenitors that are the only target for mutations in leukemia; also alterations in the niche cells can give rise to, or enhance malignancies. An example is the deletion of Dicer-1, an enzyme important for siRNA gene silencing. This protein is found in osteoprogenitor cells and stroma cells, where deletion of Dicer-1 resulted in an increased development of acute myeloid leukemia (AML) (Shiozawa and Taichman, 2010). Dicer-1 deficient stroma cells interacting with HPC can induce oncogenesis, hence the microenvironment can be the trigger in a multistep process resulting in cancer (Raaijmakers et al., 2010). Also increased expression of p53 can enhance tumor growth for acute lymphoblastic leukemia (ALL) patients and results in increasing amount of VEGF hence an increase in vessel formation and nutrition supply to the tumour (Narendran et al., 2003).

Stroma-stroma cell interactions

It is not only HPCs that are interacting with stroma cells within the BM, also supportive cells are connected and dependent on signaling from the environment. Today we know very little about the interplay between stroma cells and how the interactions with different surface molecules and cytokines affect these cells. Stroma cells expose several receptors to their expressed ligands (Article IV). Whether these signals are solely for regulating the amount of cytokine present in the bone marrow compartment, or if the cells themselves also respond to the cytokines in regards to growth and differentiation still needs further investigation. Besides soluble signaling molecules, stroma cells also use gap-junctions for communication. Gap-junctons are intracellular channels between cells, connecting stroma cells together in a complex functional syncytial network through which their supportive capacity is coordinated. The junction consists of homo- and hetero-hexamers of connexin proteins (Cxs) that facilitates
Niches

the transport of secondary messengers such as cAMP and calcium. The pore size of the channels is quite small thus only allowing for molecules with a size less than \( \sim 1000 \text{Da} \) (Warner et al., 1984). There are three different Cxs present in the BM stroma cells, Cx-43, Cx-45 and Cx-31, whereas there are seven expressed in the fetal liver (Cancelas et al., 2000). Cx-43 and Cx-45 are directly involved in the maintenance of HPC by up-regulation and secretion of Cxcl-12 (Schajnovitz et al., 2011). Cx-43\(^{-/-}\) mice survive through embryonic development but die hours after birth (Montecino-Rodriguez and Dorshkind, 2001), Cx-43\(^{+/+}\) mice have an impaired B-cell development with a reduction in IgM\(^{+}\) immature B-cells (Machtaler et al., 2011). Whether this is due to impairment of stroma function or a direct effect on B-cell development due to loss of gap-junction, regulating spreading and adhesion of these cells is yet to be fully resolved.
Homing and maintenance

Homing of HSCs is a prerequisite for bone marrow transplantation to succeed. Little is known about the molecules that direct this process. However, there seems to be several pathways involved in homing and lodging of HSCs to the bone marrow niche. Homing occurs already 15 min after injection of HSCs to the blood stream, and continues for the next 15 hours (Ellis et al., 2011). The amount of cells homing with time seems to peak at 3 hours (Cerny et al., 2002) and is impaired after in vitro cultivation of HSC together with various cytokines (van der Loo and Ploemacher, 1995) probably due to loss of stem cell features. In order for the circulating cells to enter the bone marrow through adhesion, rolling and finally diapedesis, there exist a prerequisite of endothelial expression of VCAM-1 on the endothelial cells and P- and E-selectin as well as integrin α4β1 (VLA-4) expression on the HSC (Frenette et al., 1998; Mazo et al., 1998). Multiple adhesion molecules are found on the HSCs within the bone marrow, including cadherins, selectins and integrins. All are of importance for cell-cell and cell-extracellular matrix interactions, mediating binding both to extracellular matrices, as well as cellular receptors.

Integrins are a large family of proteins and even though VLA-4 is the most studied integrin on hematopoietic progenitor cells, there are more integrin family members that seem to be important for HSCs including α4β7 and its receptor MadCAM-1 (Katayama et al., 2004). Deletion of VLA-4 results in embryonic haemorrhage as well as cranial and facial malformations and embryos die at 12 dpc due to heart failure (Yang et al., 1995). VLA-4 binds to VCAM1 on the stroma and endothelial cells that constitutively express this protein (Schweitzer et al., 1996). The interaction between VLA-4 on the HPC is important for maintaining progenitor cells within the bone marrow, and interruption of this interaction will increase the number of cycling HPC in the periferal blood (PB) (Craddock et al., 1997; Vermeulen et al., 1998). Mobilization of HSCs is of clinical importance for patients suffering from disease that needs to be treated with stem cell transplantation. In order to retain sufficient amounts of stem cells from the patient before depleting the bone marrow, HSCs are initiated to migrate from the BM to the circulation. This is done by addition of cytokines (G-CSF, SCF or both in combination) changing the BM environment by causing down regulation of VCAM-1 expression on the stroma cells hence mobilization of cells and entry to the blood stream (Le et al., 2001).

Stroma cell derived factor 1 (SDF-1) also named Cxcl-12, signals from the stroma cell to HSC by the Cxcr-4 receptor. This chemokine is reported as the key factor for homing of circulating
Homing and maintenance

HSCs as well as for maintaining "stemness" of the HSCs (Sugiyama et al., 2006). Cxcr-4⁻/⁻ as well as Cxcl-12⁻/⁻ defects are embryonically lethal at around 15-18 dpc (Nagasawa et al., 1996; Zou et al., 1998; Ma et al., 1999). Cxcl-12 is a member of a large family of chemotactic cytokines (chemokines) and was initially characterized as an essential growth factor for B-cells (Nagasawa et al., 1996; Egawa et al., 2001). Later on Cxcl-12 was shown to be of importance for B-cell and to some extent T-cell development during embryogenesis (Ara et al., 2003). Initially only Cxcr-4 was thought to bind Cxcl-12 acting through a G-protein coupled receptor mediating intracellular structural changes to induce movement of the cell towards the gradient of chemokines (Ourne, 1997; Iijima et al., 2002). However, more recently two other receptors for Cxcl-12 has been discovered, Cxcr-7 and Cxcr-11, not mediating movement, but rather cell proliferation (Burns et al., 2006). Cxcl-12 is secreted by stroma cells inside the marrow, however, the secretion only occurs if the stroma cells are in cell-cell contact via connexin gap junctions (Schajnovitz et al., 2011). The cells expressing Cxcl-12 are thought to also express other HPC regulating molecules such as Flt-3L and VCAM-1 (Schaumann et al., 2007). The Cxcl-12/Cxcr-4 axis can also be targeted to cause migration of HSCs to the periphery. This can be done by adding G-CSF, which downregulates Cxcl-12. Increase in the number of circulating HSC can also be seen after treatment with AMD3100 a Cxcr-4 selective antagonist (Dar et al., 2011). Together these results imply that Cxcl-12 is of importance for homing as well as maintenance HSCs.

In recent years the simple explanation that Cxcl-12 is the only attractive agent for homing has been challenged. Cxcr-4 deficient mice have an altered homing capacity during embryogenesis resulting in virtually no Pre- or Pro-B-cells in fetal liver or bone marrow. Abnormal amounts of progenitor cells can thus be found in the circulating blood, however, there are still cells homing to the bone marrow (Ma et al., 1999). Homing of cells co-incubated/injected with the Cxcr-4 antagonist AMD3100, is only mildly reduced (Christopherson et al., 2004). The concentrations used when investigating chemoattractive responsiveness can also be questioned, as the concentrations used are 100-fold higher than presumed to correspond to the in vivo environment (Gazitt and Liu, 2001; Basu et al., 2007). The fact that impaired Cxcl-12 gradient still results in homing of cells implies that other factors can compensate for the decrease. Several agents being part of the extra-cellular matrix have been investigated including shingosine-1 phosphate (S1P) (Seitz et al., 2005; Ratajczak et al., 2010), hyaluronan (HA) (Nilsson et al., 2003) and Ceramide-1 Phosphate (C1P) (Granado et al., 2009; Kim et al., 2011). HA is a polysaccharide found in the extracellular matrix of the BM, synthesised and secreted by hematopoietic cells and of importance for lodging and differentiation of HPCs. Enzymatic decrease of HA slows homing after transplantation (Nilsson et al., 2003). Significant levels of C1P and S1P have been found in mice post irradiation, hence their chemo-attractive ability might support Cxcl-12 induced homing (Ratajczak et al., 2010; Kim et al., 2011).

Expression of several members of the TGF-β family can be found when investigating mRNA levels in BM stroma cells by microarray. The TGF-β family includes 33 members in mammals: TGF-β, activins, inhibins and BMPs. They are all structurally related and secreted as dimeric proteins, acting either as hormones or, as local mediators regulating biological functions, such
as proliferation, differentiation, extracellular matrix production, apoptosis, tissue repair and immune regulation (Miyazono et al., 2010). Message RNA coding for receptors for these factors is expressed on hematopoietic cells, indicating that this receptor-ligand interaction is important for maintenance and possibly differentiation (Article IV). BMPs signals through enzyme-linked receptors that are single pass membrane proteins with a serine/threonine kinase domain on the cytosolic side of the plasma membrane. There are two different kinds of receptors type 1 (a & b) and type 2 (Heldin et al., 1997). For signal transduction to occur, both type 1 and type 2 receptor needs to be bound to the ligand (Rosenzweig et al., 1995). There are eight different SMADs, however, there are specific SMADs activated by BMP receptors as well as by TGF-β and activin receptor signaling (Larsson and Karlsson, 2005). The SMADs are structurally related to each other and the functional differences are still to be uncovered. Various BMPs and their antagonists are expressed by hematopoietic cells within the bone marrow suggesting of an active role in hematopoiesis (Passa et al., 2011). BMP signalling is thought to induce Id proteins (Miyazono and Miyazawa, 2002). As Id proteins are negative regulators of E2A, this should prohibit B-cell differentiation, however, in (Article III) in vitro differentiation of B-cells were increased after addition of BMP-4. Also over-expression of BMP-4 during embryogenesis results in increased adipocyte development (Taha et al., 2006) indicating importance of BMP signalling both at early stage as well as in the mature hematopoiesis. Adipocytes may contribute to the proliferating pool of HSCs (having an increased number of adiponectin receptors expressed) in vivo by the secretion of Adiponectin (DiMascio et al., 2007). Hence, blood cell development is regulated by a complex interplay between the hematopoietic progenitors and the stroma cells in the microenvironment.
Methodological considerations

Detailed descriptions of methods used are described in the materials and method sections in the articles included in this thesis; however some issues will be further explained and discussed in the following section.

Flow cytometry and cell sorting

Several of the functionally defined stages of blood cell development, from HSC all the way to fully differentiated mature cells can be distinguished by extra-cellular and to some extent intracellular markers. The extra-cellular markers are mainly surface receptors, many of which are carrying a cluster differentiation (CD) number. These markers are differentially expressed during differentiation giving each stage in blood cell development a unique panel of surface molecules. These molecules can be visualized by different methods. However, the most common is to attach an antibody directed towards a specific surface epitope coupled with a fluorochrome.

Bone marrow cells can be extracted from femur, tibia and crista illea in euthanized mice. The bones are crushed and filtered to obtain a single cell suspension and the cells can then be enriched by magnetic beads and labeled with antibodies. This procedure is rather harsh to the cells with both magnetic-bead and antibody labeling and takes a couple of hours to conduct. This can affect the cells and also have impact on downstream analysis such as microarray or RT-PCR. Hence, even though the cells originate from an in vivo environment it is more reasonable to talk about ex vivo analysis. In order to minimize the differences between in vivo and ex vivo, the handling should be kept to a minimum and with the cells kept on ice, to reduce the numbers of intracellular events to taking place.

The cells are injected to a flow cytometer, in which a laser will excite electrons resulting in the release of photons from the antibody conjugated fluorochrome. The spectrum of light emitted when the atom goes back to their steady state of energy is then measured by the cytometer, hence the cell surface antigens will be visualized. One cell can be labeled with several antibodies with different excitation and emission spectras, the number is limited by the
Methods

number of lasers and the number of emission spectra filters for each laser as different lasers excite atoms to emit light at different wavelengths. A standard staining today is 6-8 colors on a 2-3 laser instrument, however more or less colors are used depending on the addressed question. At our facility we can stain and sort cells using up to 14 colors, using a BD Aria special order system, or 9 colors for analysis on a BD Canto.

FACS is used to sort cells that pass through the laser beam and full-fill our pre-set criteria for sorting, i.e. have the desired surface markers stained with antibodies. For sorting, cells need to be placed in droplets. Before passing through the laser beam the stream of cells injected to the cytometer is broken and droplets are formed. In each droplet a cell can be localized. By adding an electric tension to the droplet with the desired cell inside of it, the tension will force the droplet to move, left or right of the flow stream into a collection tube.

It is also common to trace gene expression by the use of using fluorescent proteins (FP). A common way of doing this is to insert a FP under after the promoter of a stage restricted gene, forcing the transcription of the FP at the same time as the gene is transcribed resulting in fluorescence. The first of FP's to be isolated was the GFP, originating from a jellyfish (Tsien, 1998). Using FP's is very convenient when it comes to investigating intracellular proteins or transcription factors located in specific populations of cells or as in (Article III), where cells need to be trypsinized before they can be sorted. Protease treatment of the cells has a side effect as trypsin/EDTA cuts most surface proteins, hence the treatment can potentially alter the surface epitopes on the cells in such way that antibodies cannot bind, and cells cannot be separated upon antibody expression. Due to differences in the spectral-width of emission spectra of the cytometers used, different FP's can be visualized, and it is currently possible to use a wide spectra of different FP's simultaneously (Shaner et al, 2005).

Aspects of analyzing flow data

While the acquisition of flow data is rather straight-forward, there are several aspects that should be considered when the data is analyzed. To start with the compensation of the samples investigated is critical which has to be done for the same antibodies used within a particular experiment in order to get well compensated and hence more easily analyzed results. When it comes to staining, there are a few things that are important. First, the usage of antibody cocktail: this should always be done to avoid unequal amounts of antibodies in between samples. Second, number of cells stained: in order to get a proper comparison in between samples the number of cells stained with the antibody cocktail should be the same, otherwise there is a risk for over- or under-staining of the cells. This can result in misinterpretation of the data, either as events that will be analyzed as positives due to unspecific staining, or cells being analyzed as negative due to too few antibodies bound to cell surface receptors. Unequal
amount of cells are often observed when comparing cells with different genetic properties, for example Ebf-1 KO versus WT. Also in post *in vitro* differentiation the clones will have different size and hence might stain unequally. Therefore, when ending up with the analysis part, all these things have to be considered in order to draw proper conclusions.

**Microarray analysis**

DNA microarray is a high throughput method for investigating the expression of thousands of genes simultaneously within a cell population. It was revolutionary when initially set up, as in previous analyzes the number of mRNA to be investigated were highly limited (Lockhart et al., 1996). The approach involves determination of relative concentrations of mRNA expression based on hybridization of the entire mRNA pool from a cell population to a chip with synthetic oligonucleotides at a length of 25 bases, analyzing 34000 genes at once (Affymetrix, 2004). While there are several platforms available for this technique, in this thesis we only used Affymetrix whole genome 430 2.0 mouse chips.

**Labeling of mRNA for microarray analysis**

The cell population of interest is sorted by FACS straight down to an RLT buffer used to lyse the cells and stabilize the RNA. The sample is immediately frozen using dry ice to decrease the handling time of the cells and diminish extra steps involving handling of the cells. The buffer is then used when mRNA is extracted and converted to cDNA, amplified, labeled and fragmented before hybridization. To decrease the risk for RNA to be degraded, there is no refreezing of the extracted RNA, and all samples not yet extracted are kept at -80°C. The hybridized chip is scanned by an Affymetrix chip scanner (Affymetrix, n.d.). Raw data from the chip is then normalized and analyzed.
Methods

Data handling

The initial action taken is normalization of all the arrays to be analyzed, in order to correct for the overall intensity levels in the analyzed arrays. This is important as it will reduce variability of probe sets (Bolstad et al., 2003). Normalization of arrays can be done in a variety of ways; there are two main ways though, to either use scalar or linear normalization. The scalar normalization is based on using baseline arrays, it is dependent on using the array with median expression as baseline (Boes and Neuhäuser, 2005). I have chosen to use the Robust Multichip average (RMA) platform as it performs quintile normalization and is good in detecting differential gene expression (Irizarry et al., 2003). Verification of the mRNA expression can be done by qPCR, where fold changes can be calculated comparing a housekeeping gene with a gene of interest in two samples, using $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001).

GCINT and choosing software to work with

In the very beginning GCINT was located in Microsoft Excel, using the filtering tools inside the software to analyze present connections in different populations. In order to get a better view at specific interactions it was moved to Microsoft Access, and more arrays were added. Having the database in Access gave a lot of advantages when it came to searching specific interactions between two cell types. However as it only allowed us to investigate interactions between two samples at a time, it had limitations. This is why I for the last manuscript chose to change software once more, and write a script in MATLAB investigating cell-cell interactions. The advantage of doing this in MATLAB is that high throughput analysis of several arrays in different groups can be conducted at the same time. Using this approach also has an advantage when it comes to answering new questions regarding cell-cell interaction both in populations already investigated as well as new populations, as the same scripts can be used and modified for every question.
Relying on a database

A database is like a good wine when properly kept, it only gets better with time. Using a database for measuring potential interactions between cells has a huge advantage compared to practically testing the interactions directly using trial and error methods. It will quickly tell you what interactions to look for within the population of interest. Also interactions not previously known for a specific compartment will automatically be tested, potentially giving the investigator a new pathway to study. There are of course not only good things coming out of a database. A drawback of using and relying too much on it, is that you need to have in mind that all possible connections are probably not in the database. If this is the case for a specific interaction, it will not be revealed by asking for its presence. However this does not mean that the interaction does not occur in between two cells. Also if there are interactions in the database that might not be true, these will show as present when in reality they are not there. These false-negative or false-positive interactions will be decreased with time, and as new discoveries will be entered into the database, it will improve. Today we do not know about the receptors for every cytokine present in the bone marrow, this means that there are more interactions occurring than the database will tell us, as unknown interactions cannot be presented by this methodology. Another error with investigating connections based on mRNA levels from a microarray is that it is just that. mRNA levels can easily be verified using RT-PCR, however this does only tell us about the mRNA level. Hereby the protein levels still remain unknown and while a high-throughput measurement of total protein content within a cell population, in low numbers would be desired, this is yet not possible. I still think this is a very potent approach in searching for new pathways of interest in cell-cell communication whether looking in the bone marrow or any other tissue where cells are in close contact and depending upon each other.
Aim and Conclusion

Aim of thesis

To use gene expression analysis to gain a molecular insight to the interplay between hematopoietic progenitors and stromal cells in the BM. This can potentially shed light to the complex cellular signalling mediating maintenance as well as proliferation and differentiation of hematopoietic progenitor cells in health and disease.

Conclusion

Our results suggest that bioinformatic processing of gene expression data can be used to unravel novel means of cell communication in a complex environment. Even though this RNA based approach does not directly take into regard actual protein levels, the method still appears to give a fair overall estimation of protein expression of many genes. The quality of the information obtained through this type of analysis is highly dependent on the quality of the input material. This is valid for the technical quality of the gene expression data but also of the purity of the cell populations used for generation of RNA. In addition to generating ideas for novel communication pathways, this large scale approach allows for the investigation of directions of information flow. Our analysis suggests that the environment in the BM is not created only by defined mesenchymal cell populations but rather of interplay between the stroma and the developing hematopoietic cells. Hence, I believe that the work presented in this thesis shows that gene expression based analysis of cell-cell communication may serve as a mean to take systems biology one step closer to physiology and in vivo biology.
Results and discussion of papers in the thesis

Article I

**IL-7 mediates Ebf-1-dependent lineage restriction in early lymphoid progenitors**

IL-7 is a factor produced by the mesenchymal stroma cells in the bone marrow. It is critical for the development of B-lymphocytes possibly by increasing the Ebf-1 expression levels in the B-cell progenitors (Dias et al., 2005), (Roessler et al., 2007), placing Ebf-1 downstream of IL-7R signaling in B-cell development.

IL-7R is mainly expressed on lymphoid-restricted progenitor cells. The receptor however, can be found also on non-committed progenitor cells. Without IL-7 expression there is no B-cell development. Although B220^+^CD43^+^ cells can be found in the BM of IL-7 deficient animals, there are no detectable levels of B-lineage gene activation i.e. there are no coordinate expressions of Pax-5, Pou2afl or CD79a. When investigating the LMPP population in an IL-7 deficient mouse, this population is only 1.3-fold reduced with no significant differences in lymphoid gene expression. This indicates that IL-7 is not of critical importance for LMPP maintenance in vivo or for lymphoid lineage priming. When analyzing the CLP population, expressing IL-7R even in IL-7 deficient mice, this population is reduced 5-fold. Hence, IL-7 is crucial for maintaining the IL-7R expressing B-lymphoid-progenitor populations.

*In vitro*, CLPs from an IL-7 deficient background can differentiate to B-cells upon cultivation on OP9 cells providing an environment that supports B-cell development. This indicates that even though the cells have not been exhibited for IL-7 they have not lost their potential of becoming B-cells. Also when culturing these cells on OP9DL providing T-cell conditions there were no significant differences between the cloning efficiency or their ability of becoming T-lineage cells. One difference was observed; cells from IL-7KO animals did not develop to B-cells on OP9DL, an event happening as there is a fraction within the CLP population already primed for B-cell development. Lack of IL-7 therefore seems to result in lack of B-lineage priming. Consistent with this idea, global gene expression analysis of the CLP compartment from WT and IL-7 KO mice revealed a decreased expression of B-lineage genes. Among these were Rag-1 and Ebf-1, both important for B-cell development and reported to be up-regulated with the transition of Ly6D^-^ to Ly6D^+^ cells within the classical CLP compartment (Inlay et al., 2009; Mansson et al., 2010).
Results

This opened the possibility that IL-7 is critical for the transition from the Ly6D⁻ B/T/NK potent progenitor stage to the lymphoid restricted Ly6D⁺ stage. To investigate this, the two “CLP” populations were analyzed and compared between WT and KO animals. In the Ly6D⁻ population a 2.3-fold reduction was observed in the IL-7KO mice, however when investigating the Ly6D⁺ compartment, there was a 23-fold difference supporting the idea that IL-7 is important for the B/T lineage restriction in the CLP compartment. To investigate the lineage potential, single cell assay for \textit{in vitro} differentiation in B/NK condition of the Ly6D⁻ population within the CLP mediated no functional difference in retaining B or NK potential between IL-7 KO and WT cells. This was also verified \textit{in vivo} when Ly6D⁻ cells from IL-7 WT and KO CD45.2 animals were transplanted into CD45.1 WT mice. Analysis of the Ly6D⁺ population in WT and KO mice revealed a lower cloning frequency using cells from the IL-7⁻/⁻ mouse. The content of the clones also differed. While using cells from WT mice, essentially all the clones generated contained only B-lineage cells, in contrast, using cells, from the IL-7 KO mouse, a large frequency of the clones generated contained multiple lineages. This shows that the cells retained B, NK and DC potential indicating that these cells retain B-lineage potential but are not properly lineage-restricted.

\textit{Ebf}⁻¹ expression is thought to be directly regulated by IL-7 signaling (Kikuchi et al., 2005; Roessler et al., 2007). To address whether \textit{Ebf}⁻¹ expression directly respond to functional IL-7R signalling, \textit{Ebf}⁻¹ expression was analyzed within the Ly6D⁻ and Ly6D⁺ populations revealing a reduced expression of \textit{Ebf}⁻¹ in IL-7 KO cells.

In order to investigate if the plasticity observed in Ly6D⁺ cells from IL-7 deficient mice could be due to the reduced expression of \textit{Ebf}⁻¹, \textit{Ebf}⁻¹ WT, heterozygote and KO CLPs were cultured as single cells upon a feeder layer of OP9 and with cytokine stimulation of IL-7, SCF, Flt-3L, IL-15 and IL-2 \textit{in vitro}. Cloning frequency was equal between groups; however lineage restriction faded with reduced amount of \textit{Ebf}⁻¹. No B-cell potential was detected in the \textit{Ebf}⁻¹ KO. Further investigation of the CLP compartment revealed no significant difference in the numbers of Ly6D⁻ or Ly6D⁺ between Ebf⁻¹ KO and WT animals. This indicates that Ebf⁻¹ is not essential for functional IL-7 signalling. However, the Ly6D⁺ cells generated in Ebf⁻¹ deficient mice retained DC- and NK-cell potential to a higher degree than WT mice. Hence, Ebf⁻¹ is located downstream of IL-7 signaling and crucial for mediating lineage restriction.

We now know that Ly6D is expressed regardless of Ebf⁻¹ expression, hence the regulation is upstream of Ebf⁻¹ and we know that without IL-7R signaling there are no Ly6D⁺ cells. Thus STAT5 or STAT5 targets might have an impact of the regulation of Ly6D and should be further investigated.
Stroma cells are of importance for maintenance of hematopoietic cells in vivo and in vitro. These cells produce cytokines of importance for maintaining the cells as well as inducing differentiation.

In order to learn more about gene regulation and potential transcription factors of importance for the ability of stroma cells to support hematopoiesis, this comparative study was done. Two cell lines were investigated: fibroblastic NIH3T3 cells, unable to support lymphoid differentiation to B-cells in vitro and the stroma cell line OP9, highly efficient in this regard. The initial step to take was to investigate gene expression in these two cell lines. This was done using microarray-based global gene expression analysis, revealing many differentially expressed genes in these two cell lines. One factor that aroused curiosity was Ebf, since both Ebf-1 and -3 were highly expressed in the OP9 cells but not in NIH3T3. Ebf-1 is a transcription factor well known to be expressed and essential for early stages of B-cell development. The finding from the microarray were confirmed using RT-PCR and EMSA. Supporting the idea that functional Ebf protein is produced in stroma cells primary stroma cells were then investigated for the expression of Ebf family genes. Not only did the OP9 express Ebf, also primary stroma cells expressed Ebf-1 and -2. Gain of function analysis using NIH3T3 cells by inducing a retrovirus overexpressing Ebf-1 resulted in increased expression of an array of genes including cytokines, chemokines and growth factors. Among those, Periostin, Cxcl-12 and Ccl-9, their expression was also confirmed by RT-PCR. As a control ectopic expression of Ebf-1 in the hematopoietic cell line Batf3 did not result in up-regulation of the same genes as in the stroma cell lines. This indicates that expression of Ebf-1 results in a tissue specific gene regulation. Also on protein level the induction of genes was detectable. Ectopic expression of Ebf-1 resulted in a marked increase of Ccl-9 as measured with ELISA. To verify the binding of EBF to promoter elements, DNA binding analysis were conducted, implying that Ebf have binding sites on the Ccl-9, Cxcl-12 and Periostin promoters. Loss-of-function study was done using a dominant negative form of Ebf, where the activation domain of Ebf-1 is replaced by the repressor domain of engrailed. Analysis of the presumed Ebf targets suggested that mRNA of target genes was reduced however only modestly in Ccl-9. To further verify this shRNA expressing retrovirus targeting Ebf-1 was transduced to OP-9 cells. Levels of Ebf-1 decreased compared to control, also reduction in mRNA levels of Cxcl-12, Ccl-9 and Periostin was observed. This supports the idea that that Ebf has a role in the regulation of genes in bone marrow stroma cells.
Results

Article III

Genomics based analysis of interactions between developing B-lymphocytes and stromal cells reveal complex interactions and two-way communication

This paper is focused on the development and evaluation of the database GCINT, Genomics based cell-cell Interaction analysis, where it is possible to match locuslinks (Gene ID) from 2 microarrays with the GCINT database unravelling theoretical interactions between two cells. Each Locuslink corresponds to several probe sets measuring the intensity of a certain mRNA expression, matching a coding region of the DNA. This gives information about a theoretical appearance level of a certain protein expressed by the cell. Microarrays from hematopoietic cells in different developmental stages as well as stroma cell lines were added to the Microsoft Access database together with over 400 receptor-ligand interactions. Each interaction can occur both from the tested stroma cell to the hematopoietic cell and vice versa.

The evaluation of GCINT gave some potentially interesting receptor-ligand pairs, both commonly expressed and stage-specific interactions. To further investigate the significance of these interactions an in vitro differentiation experiment was performed. Primary sorted LSK cells from mouse bone marrow were cultured with two different stroma cell lines, one with OP9 known to support B-cell development and one other with NIH3T3 that is unable to perform this task. To investigate whether we could identify B-lineage growth factors differentially expressed between the two stroma cell lines, we compared the theoretical abilities of OP9 and NIH3T3 cells to interact with defined hematopoetic progenitor cells, identifying Cxcl-12 and BMP-4 as differentially expressed by OP9 cells. Testing Flt-3 ligand and IL-7, cytokines known to support B-cell differentiation on primary sorted LSK cells cultured with NIH3T3, gave no impact on the B220+ fraction. No increase in the B220+ fraction were seen with the addition of Cxcl-12, however when adding BMP-4 together with Flt-3 ligand and IL-7, a significant fraction of the LSK population differentiated towards B-cells on the NIH3T3 stroma cells. LSK in co-cultivation with OP9 cells were used as control.

Visual inspection of the wells revealed that progenitor B-cells growing with OP9 attached strongly to their surface and also drastically changed the morphology of the stroma cells after one week of co-cultivation. To investigate if these changes were due to cell-cell contact, control OP9 cells were incubated with the media from the co-cultures. There was no apparent change after this cultivation suggesting that the changes in morphology demanded close contact between the blood cell progenitors. Microarray analysis was made on both FACS-sorted OP9 co-cultivated with the pre-B cell line 230-238 and OP9 cells incubated with conditioned media in parallel culture experiments. Microarray analyses showed significant differences in gene expression pattern between the co-cultured cells and the control. To verify the microarray
results, RT-PCR was used, and the genes chosen was SpiB, Nov and Cxcl-10. To investigate if there were any difference in expression pattern induced by cells of different origin both pro B cells and myeloid cell lines were used. The RT-PCR was performed using Taq-man probes and analysed in the aspect of $2^{\Delta\Delta CT}$ with cells in parallel culture as controls. Changes in gene expression occurred early and increased over a 7-day period. Differences in mRNA expression pattern were seen in the OP9 cells when co-cultivated with a myeloid cell line compared to a B-cell line.

These results indicate that theoretical investigation of cell-cell contact is valid and can be used to unravel new interaction pathways. Also the cell-cell interaction occurring between stroma cells and hematopoietic cells is a two way communication, with signals going in both directions.
Results

Article IV

Dynamic crosstalk between developing blood cells and mesenchymal stroma compartments.

For this work, gene expression data generated from primary FACS sorted cells from both the hematopoietic and stromal compartment were analyzed using a refined version of GCINT. Three populations of primary stroma cells were investigated. Their cytokine producing ability was first analyzed using RMA pre-processed data in dChip. An evident difference was seen between the three populations with fewer cytokine genes expressed by the CD44+ populations as compared to Ebf-2+ and Cd44- populations, suggesting that these cells have distinctive roles in hematopoiesis. To further analyze if the ability to produce cytokines also reflect their ability to interact with hematopoietic cells in the bone marrow, the arrays were analyzed to identify potential receptor-ligand interactions using a refined version of GCINT allowing for high throughput analysis of several microarrays from different stroma as well as hematopoietic population simultaneously. Investigating the number of possible interactions between stroma cells and hematopoietic cells revealed an overall reduction in possible interactions for CD44+ stroma cells compared to Ebf-2+ and CD44- populations. Investigating these populations and their ability to interact via cell-cell contact with primary hematopoietic cells within the bone marrow revealed stage specific connections between stroma and hematopoietic cells. This suggests a dynamic stromal environment adopting to stage specific needs. Signals were not only produced by the stromal cells, but there were also several cytokines produced by the hematopoietic cells. If these are aimed as auto or paracrine signals are unclear, however, as the stroma cells have expression of receptors for some of the cytokines produced, it is likely they will respond to such stimuli. Investigating the receptor-ligand interactions between stroma cells there were many interaction pathways open. To verify this, flow cytometric measurement of receptors located on the stroma cells revealed that these cells do express receptors for produced cytokines like Cxcl-12 and IL-7, hence there is a possibility for feed-back mechanisms for factors secreted by the stroma cells to its environment.

One can speculate why CD44+ cells does not have the same number of options for cell-cell communication with hematopoietic cells and produce less number of cytokines. As previously shown in (Article II), hematopoietic cells interact and affect the stroma cell by direct cell-cell interaction and so does other stromal cells. A speculation could be that the CD44+ marker is present as a result of lack of cell- cell interactions or specific interactions promoting differentiation to a less supportive cell.
Allt vårt cirkulerande blod härstammar från stamceller i vår benmärg. Stamceller har förmågan att ensamma ersätta alla typer av blodceller i vår kropp. För att utvecklingen från stamcell till mogen cell ska ske, krävs ett flertal delningar som leder till mellanliggande utvecklingsstadier vilket till slut resulterar i att cellerna lämnar benmärgen för att mogna och därmed bidra till den ständiga nyproduktionen av blodceller. I blodet finns celler specialiserade på en rad livsviktiga funktioner: röda blodkroppar transporterar syre, trombocyter ser till att vi får stopp på blödningar, B- och T-cellers ger immuniteten mot sjukdom som vi redan träffat på, antingen genom vaccin eller faktiskt bekämpade sjukdomar, samt makrofager och granulocyter har som främsta uppgift att bekämpa bakterier. För att en stamcell ska kunna dela sig och mogna krävs influenser av omgivande faktorer från andra celler som också är lokalerade i benmärgen, s.k. stromaceller.

Acknowledgments

First of all I would like to thank everyone who has supported me in my everyday life, both private and professional. To all the wonderful people I have met and come to know, both at floor 9 and 13, you have all contributed to the work resulting in this thesis; it has been pleasure getting to know you and working with all of you!

Special thanks go to:

First and foremost my supervisor Professor Mikael Sigvardsson for your never-ending and contagious enthusiasm over science, it has been an honour working with you. I am grateful for the always encouraging mentoring and the wonderful scientific environment you have provided.

Professor Jan-Ingvar Jönsson, for being my co-supervisor and for mentoring me both during my undergraduate studies and during my PhD studies, for always being there, open for questions and discussion.

Josefine, for being a great friend colleague and room mate, sharing science discussions as well as one or two things regarding life, universe and everything. It is a great pleasure working with you

Every now and then you find these special people around you, those you can share good and bad times with, no matter if it is personal or work related things. Thank you Beatrice, for always being there, both in the best and worst of times.

"Lotta", for always having a spare hand, I appreciate your willingness to help in the lab and your effort in making everyone enjoy.

To Nils that always had a helping hand when it came to the Access database

To present and passed co-workers in the Sigvardsson lab, Hong, Sasan, Panagiotis, Robert, Eva, for contributing to a great environment and making the days in the lab fly.
The Jönsson group, past and present members, especially **Pernilla, Amanda** and **Pia**, for being great co-workers, both at floor 9 and 13. I have learned so many things from you.

To all the co-workers at Lab1 floor 13, no one mentioned - no one forgotten, for good laughter and nice discussions over a cup of coffee.

To my dear friends sharing my passion for horses, especially **Cecilia** and **Johanna** for always having a helping hand, in the dressage course as well as in the stable – when having to spend late nights in the lab, what would I do without you?

Your family, you can not choose, I’m happy to love mine. Thank you for being supportive at all times.

To **Gunnar** and **Linus**, you are my everything, thank you for always being there, I love you.
References


References


References


References


Kee, B.L., Murre, C., 1998. Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. The Journal of experimental medicine 188, 699-713.


Kikuchi, K., Lai, A.Y., Hsu, C.-L., Kondo, M., 2005. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. The Journal of experimental medicine 201, 1197-203.


References


References


Medvinsky, a, Dzierzak, E., 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86, 897-906.


References


References


References


References


References


References


