Molecular and Cellular Analysis of Lhx2 Function in Hematopoietic Stem Cells

Karin Richter
Till pappa
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ABSTRACT

The formation of blood, hematopoiesis, is a dynamic process originating from a small number of hematopoietic stem cells (HSCs). To sustain hematopoiesis throughout life HSCs have the unique capacity to differentiate into all mature hematopoietic lineages as well as generating more HSCs by a mechanism referred to as self-renewal. However, the regulation of these processes is largely unknown. During embryonic development HSCs expand in the fetal liver, indicating that this environment supports HSC self-renewal. The LIM-homeobox gene Lhx2 is expressed in the fetal liver during this period and Lhx2 null mutant mice die in utero due to severe anemia caused by an environmental defect in the fetal liver. Embryonic stem cells differentiate in vitro, forming embryoid bodies (EBs) containing various tissues including hematopoietic progenitor cells. Introduction of Lhx2 into this system by retroviral transfer led to the generation of cytokine dependent HSC-like cell lines that were multipotent and expressed surface markers similar to embryonic HSCs. However, the specificity and efficiency of this event could not be elucidated.

To further evaluate the function of Lhx2 expression during hematopoietic development, Lhx2 was introduced into an ES cell system where expression could be efficiently turned on. This approach revealed that Lhx2 induce self-renewal of distinct multipotent hematopoietic progenitor/stem cells present in the EB, with the ability to form HSC-like cell lines. The Lhx2 induced self-renewal is growth factor specific since stem cell factor and interleukin-6 are necessary and sufficient for this process. However, Lhx2 expression blocked erythroid differentiation and interfered with early ES cell commitment, indicating that the effect of Lhx2 is cell type specific.

Since HSCs of early embryonic origin are inefficient in engrafting adult recipients upon transplantation, we wanted to address whether we could generate cell lines retaining this capacity by expression of Lhx2 in hematopoietic cells from adult bone marrow. This led to the generation of clonal and cytokine dependent HSC-like cell lines capable of generating erythroid, myeloid and lymphoid cells upon transplantation into lethally irradiated recipients. When transplanted into stem cell-deficient mice, they contributed to circulating erythrocytes for at least 18 months, revealing a remarkable potential for self-renewal and differentiation in vivo. However, expression of Lhx2 was maintained in vivo and most engrafted mice developed a transplantable myeloproliferative disorder resembling human chronic myeloid leukemia. Thus, elucidation of the mechanism for Lhx2 function
in HSC-like cell lines would give insights into both normal and pathological regulation of HSCs.

Down-regulation of Lhx2 expression in HSC-like cell lines with inducible Lhx2 expression led to rapid loss of stem cell characteristics and differentiation into various hematopoietic cell types. Thus, global gene expression analysis comparing Lhx2⁺ HSC-like cell lines to their Lhx2⁻ progeny would give insights into the molecular basis for Lhx2 function in stem cells. A number of differentially expressed genes overlapped with previously reported HSC enriched genes, further emphasizing the resemblance between HSCs and the HSC-like cell lines also at the molecular level. Moreover, a number of genes were identified with functions or expression patterns related to Lhx2 in other organs. Collectively, these data suggest that these HSC-like cell lines represent a relevant model system for normal HSCs on the molecular and the functional level as well as for evaluating Lhx2 function in the development of various tissues in the embryo as well as in disease.
PAPERS IN THIS THESIS

This thesis is based on the following papers which will be referred to in the text by their corresponding Roman numerals.


* The first two authors equally contributed to this work

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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta gonads mesonephros</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
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<tr>
<td>Anptl</td>
<td>angiopoietin-like</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>CAFC</td>
<td>cobblestone area forming cell</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin dependent kinase inhibitor</td>
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<tr>
<td>CFC</td>
<td>colony forming cell</td>
</tr>
<tr>
<td>CFC-M</td>
<td>CFC-macrophage</td>
</tr>
<tr>
<td>CFC-G</td>
<td>CFC-granulocyte</td>
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<tr>
<td>CFC-meg</td>
<td>CFC-megakaryocyte</td>
</tr>
<tr>
<td>CFC-GEMM</td>
<td>CFC-granulocyte/erythrocyte/macrophage/megakaryocyte</td>
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<tr>
<td>CFU-S</td>
<td>colony forming unit - spleen</td>
</tr>
<tr>
<td>CMD</td>
<td>chronic myeloproliferative disorder</td>
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<tr>
<td>CML</td>
<td>chronic myeloid leukemia</td>
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<tr>
<td>CRU</td>
<td>competitive repopulating unit</td>
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<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>dox</td>
<td>doxycycline</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
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<tr>
<td>EB</td>
<td>embryoid body</td>
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<tr>
<td>ECM</td>
<td>extra cellular matrix</td>
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<tr>
<td>epo</td>
<td>erythropoietin</td>
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<tr>
<td>eryp</td>
<td>primitive erythroid cell</td>
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<tr>
<td>eryd</td>
<td>definitive erythroid cell</td>
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<tr>
<td>ES</td>
<td>embryonic stem cell</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>Flt3L</td>
<td>fms-related tyrosine kinase 3 ligand</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte-CSF</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte/macrophage-CSF</td>
</tr>
<tr>
<td>Gpi</td>
<td>glucose phosphate isomerase</td>
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<tr>
<td>Hb</td>
<td>hemoglobin concentration</td>
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<tr>
<td>Hh</td>
<td>hedgehog</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>HPP-CFC</td>
<td>high proliferative potential-CFC</td>
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<tr>
<td>Hox</td>
<td>homeobox</td>
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<tr>
<td>HPC</td>
<td>hematopoietic progenitor cell</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>ICN</td>
<td>intracellular notch</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>LIM-HD</td>
<td>LIM-homeodomain</td>
</tr>
<tr>
<td>Lin</td>
<td>lineage specific markers</td>
</tr>
<tr>
<td>LSK</td>
<td>lin⁻ Sca⁺ c-Kit⁺</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>long term culture initiating cell</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>long term repopulating-HSC</td>
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<tr>
<td>MCHC</td>
<td>mean corpuscular hemoglobin concentration</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage-CSF</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
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<tr>
<td>rTAA</td>
<td>reverse tetracycline transactivator</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>short term repopulating-HSC</td>
</tr>
<tr>
<td>tpo</td>
<td>thrombopoietin</td>
</tr>
<tr>
<td>TRE</td>
<td>tetracycline response element</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>W</td>
<td>white spotted locus</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>YS</td>
<td>yolk sac</td>
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INTRODUCTION

The hematopoietic system

The mammalian hematopoietic system is comprised of the blood and the blood forming organs, such as the bone marrow (BM) in the adult and the fetal liver (FL) in the fetus. This system has many important functions, such as transportation of oxygen and nutrients around the body as well as providing protection against microorganisms. The blood is composed of a liquid part, plasma, and a cellular part, constituted of a variety of cells with diverse functions crucial for survival. The red blood cells, erythrocytes, transport oxygen from the lungs and comprise >99% of the circulating cells and 40-45% of the blood volume. The white blood cells, leukocytes, can be divided into platelets, lymphocytes and myeloid cells, and are involved in wound repair and protection against microorganisms. Circulating platelets are produced by megakaryocytes residing in the bone marrow (BM) and spleen and are implicated in blood clotting. Lymphocytes are involved in the adaptive immune system and are divided into two main cell types: B-cells (antibody producing) and T-cells (T helper cells and cytotoxic T cells). The myeloid cells comprise the innate immune system and are divided into granulocytes (neutrophilic, basophilic and eosinophilic), monocytes/macrophages and mast cells. Natural killer (NK) cells are sometimes also included in the lymphocyte group and are important for killing virus infected cells and developing tumour cells (Lee et al., 2007; Zamai et al., 2007) and dendritic cells are important for presenting antigens to the cells of the adaptive immune system. With the exception of some white blood cells, hematopoietic cells are relatively short lived (less than a day for neutrophilic granulocytes, 120 days for erythrocytes) and needs to be constantly replaced. An astonishing amount of cells, approximately 1000 billion, are produced daily in adult humans (Ogawa, 1993). The generation of blood, hematopoiesis, occurs in the hematopoietic organs and is sustained by a small number of specialized cells, the hematopoietic stem cells (HSC).
INTRODUCTION

The hematopoietic stem cell

The hematopoietic system is a hierarchal system with the hematopoietic stem cell (HSC) as the founder cell. HSCs have the unique potential to self renew (generate more stem cells) as well as to undergo differentiation into all mature cell types of the hematopoietic system (Figure 1). The differentiation process is successive and the cells gradually undergo differentiation into more committed progenitor cells. Since differentiation is coupled to expansion, it is possible for a few stem cells to sustain the enormous amount of mature hematopoietic cells required each day (Bryder et al., 2006). These characteristics of HSCs are the reasons why BM-transplantation is an efficient way to reboot the hematopoietic system after ablation caused by chemo- or radiotherapy and therefore makes HSCs of great clinical importance.

A historical perspective

The first experimental evidence that several hematopoietic lineages could be generated from a common progenitor was published in the early 1960s. Till and McCulloch demonstrated that a subset of cells in the BM had the capacity to generate macroscopic colonies on spleens when transplanted into lethally irradiated recipients (Till and McCulloch, 1961). It was shown that these colonies contained several hematopoietic lineages and were of clonal origin (Becker et al., 1963; Wu et al., 1968). The cell type capable of forming these colonies was designated colony forming unit spleen (CFU-S). If cells from these colonies were transplanted into secondary recipients they generated new spleen colonies, indicating that they contained new CFU-S and thereby proved the existence of multipotent cells with some self-renewal capacity in the BM (Siminovitch et al., 1963), although it was later shown that the CFU-S population represents more committed progenitor cells (Magli et al., 1982). This discovery launched the enormous field of adult stem cell biology by proving the existence of multipotent progenitors in the BM with some regenerative potential. In the mid 1980s, the existence of multipotent HSCs with self-renewal capacity and the ability to generate all hematopoietic lineages was finally demonstrated. The capacity of retroviruses to insert at random positions in the genome, causing unique insertion sites, was used to follow contribution to myeloid and lymphoid progeny of single BM cells in recipient mice upon transplantation (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986). This showed that single BM cells could contribute to myeloid and lymphoid cells and
also that the same clone could contribute to multilineage hematopoiesis in several secondary recipients. These experiments formally proved the existence of HSCs with the capacity for self-renewal as well as the ability to generate all hematopoietic lineages.

The hematopoietic hierarchy and functional assays for hematopoietic stem and progenitor cells

Since the basic principles of hematopoietic development are conserved among vertebrates, several model systems are available to explore the biology of HSCs and the development of the hematopoietic system. The mouse (M. musculus) model system is the most widely used, but also chicken (G. gallus), frog (X. laevis) and fish (D. Rerio) are important models to study hematopoiesis. A number of methods have been developed to analyze the HSC and the different stages of hematopoietic differentiation, however most of these methods study the read-out upon differentiation and thereby assign the potential of the cell studied retrospectively.

The generally accepted way to detect HSC activity is to perform transplantation assays and analyze for contribution to all hematopoietic lineages over a long period of time, e.g. long term (LT) repopulation or LT-HSC. LT-HSCs generates progeny of all lineages for >16 weeks and continue to do so upon serial transplantation into secondary recipients (Keller et al., 1985; Szilvassy, 2003). The frequency of LT-HSCs can be determined by a competitive assay/competitive repopulation unit (CRU) assay where BM derived competitor cells are cotransplanted with different number of test cells into irradiated recipients. Since the contribution to mature cells in the recipient will directly correlate with the number of HSCs in the test sample it is possible to calculate the number of CRUs, hence HSCs, in the test sample (Szilvassy et al., 1990).

Several steps in the hematopoietic hierarchy have been defined. The LT-HSCs first differentiate into short term repopulating HSCs (ST-HSC) that retain full lineage potential but have limited self-renewal potential and will eventually be exhausted, thus not contributing to mature cells for longer periods of time (Morrison and Weissman, 1994). The ST-HSCs in turn give rise to the common lymphoid progenitors (CLP) that lack potential to generate myelo-erythroid cells and the common myeloid progenitors (CMP) that lack lymphoid potential but are able to generate myelo-erythroid cells (Akashi et al., 2000; Kondo et al., 1997b). These progenitors differentiate into more lineage-restricted progenitors that eventually give rise to the mature cell types. However, exceptions to this strict
division into CMP and CLP progenitors have been reported in fetal as well as adult hematopoiesis, since progenitors with B-macrophage and B-T-macrophage potential have been identified (Cumano et al., 1992; Kawamoto et al., 1997; Lacaud et al., 1998; Montecino-Rodriguez et al., 2001). An overview of the hematopoietic hierarchy taking all these data in consideration is depicted in Figure 1.

The radioprotection assay detects cells that upon transplantation into lethally irradiated recipients will rescue the recipient from radiation induced death, since they are capable of restoring functional hematopoiesis during the initial critical period. Cells able to rescue lethally irradiated mice are referred to as radioprotective cells and have been shown to consist of a heterogenous population of multipotent cells, including the ST-HSC, CMP and myeloid-erythroid progenitors (Na Nakorn et al., 2002). LT-HSCs are considered to be too slow in generating hematopoietic progeny to confer radioprotection and must therefore be co-transplanted with sufficient numbers of supporting cells that give radioprotection (Jones et al., 1990). Moreover, a number of in vitro based assays have been developed to detect both immature progenitors/stem cells as well as more committed progenitors/precursors. Dexter and colleagues developed an in vitro culture system in which active hematopoiesis could be maintained in culture for several months (Dexter et al., 1977). This system (Dexter culture) is based on the presence of BM derived stroma cells that interact with hematopoietic cells in the culture and thereby presumably mimic the BM microenvironment. The cobblestone area forming cell (CAFC) assay measures the presence of cells capable of interacting with the stroma (e.g. migrate beneath) as indicated by formation of distinct colonies easily recognized as cell clusters resembling cobblestones under a light microscope with phase contrast (Gartner and Kaplan, 1980). CAFCs capable of maintaining cobblestones at day 28-35 of culture are highly enriched in immature hematopoietic progenitor activity while early CAFCs (at day 7) include more committed progenitors (Ploemacher et al., 1991; Ploemacher et al., 1989). In the long term culture initiating cell assay (LTC-IC) the culture is followed for 5-8 weeks studying the production of clonogenic cells into the media. This assay assesses the presence of immature cells with the capacity to interact with stroma and generate progeny for long periods of time and might include HSCs (Cho and Muller-Sieburg, 2000; Sutherland et al., 1989; Weilbaecher et al., 1991). Since many in vitro assays are strongly myeloid in their read out, specific assays have
Figure 1. The hematopoietic hierarchy. The hematopoietic stem cells self renew (marked by curved arrow) and differentiate into all hematopoietic lineages. Proliferation index for different populations is indicated. Functional assays and the populations they detect are depicted in the picture. Modified from (Keller, 1992)
been developed to investigate lymphoid potential. The most common in vitro assays for detecting these cell types are based on coculture with stromal cell lines known to support lymphoid differentiation, such as S17 or OP9 (Collins and Dorshkind, 1987; Hirayama et al., 1992; Kodama et al., 1994; Nakano et al., 1994; Whitlock and Witte, 1982). Another commonly used in vitro assay is the colony forming cell (CFC) assay that is based on the use of semisolid media, leading to the formation of colonies generated from single progenitor/precursor cells (Metcalf, 1969; Metcalf, 1970). By supplying the media with combinations of hematopoietic factors it is possible to assess the number and type of hematopoietic progenitors/precursors with ability to respond to that factor combination. The analysis of cell types present in the resulting colonies reveals the potential of the original progenitor/precursor cell, hence the term colony forming cell. The most committed precursors will form uni-lineage colonies (CFC-Erythrocyte, CFC-Granulocyte, CFC-Monocyte, CFC-Megakaryocyte), while more immature progenitors have the potential to form bi-lineage or multi-lineage colonies (CFC-GM, CFC-GEMMeg). The size of colonies can also be indicative of the potential of the CFC since high proliferative potential-CFC (HPP-CFC; generate colonies larger than 0.5 mm in diameter) is thought to include more immature progenitors and possibly HSCs. However, the read out of the CFC assay is biased towards myeloid cell types, although CFC assays have been developed that detect lymphoid cells (Hirayama et al., 1992). The assays and the approximate range of hematopoietic progenitors/precursors they detect are depicted in Figure 1.

The hematopoietic stem cell phenotype

Since HSCs are rare cells residing in hematopoietic organs, several methods to enrich or isolate these cells have been developed. HSCs are slow dividing and it was shown that they can be enriched in the BM by treatment with the cytostatic drug 5-fluorouracil (5-FU), since this drug kills rapidly dividing cells while sparing quiescent cells, including HSCs (Hodgson and Bradley, 1979; Van Zant, 1984). The development of fluorescence-activated cell sorting (FACS) and the usage of monoclonal antibodies have greatly increased the ability to isolate pure populations of hematopoietic cells, including stem cells, based on cell surface molecule expression patterns. In the mid 1980s, it was shown that a sub-population of hematopoietic cells lacking myeloid, B and T-lineage specific cell markers but expressing low levels of the marker Thy1 was enriched in cells capable of initiating long term B-cell cultures (Muller-Sieburg et al., 1986). These cells could also
rescue lethally irradiated mice and contribute to all hematopoietic lineages and it was suggested that this population included the HSCs (Berman and Basch, 1985; Muller-Sieburg et al., 1986). It was later shown that one can highly enrich for HSCs by the exclusion of cells expressing markers specific for to myeloid, lymphoid, and erythroid lineages (Mac-1, Gr-1, B220, CD4, CD5, CD8, Ter119), hence retaining cells not expressing lineage markers (lin⁻), and subsequently selecting cells expressing the stem cell markers c-Kit and Sca-1 (Ogawa et al., 1991; Okada et al., 1991; Spangrude et al., 1988). This lin⁻ Sca-1⁺ c-Kit⁺ (LSK) population, was enriched a 1000-fold (1/30 cells) for HSC activity (Osawa et al., 1996). The exclusion of lineage marker expressing cells has since become standard procedure in most purification protocols for HSCs. Combination of the LSK-population with the Thy1 marker further enhanced purification, although it should be mentioned that only the Thy1.1 and not the Thy1.2 allele is useful as a marker in this respect (Spangrude et al., 1988; Uchida and Weissman, 1992). Since HSCs express high levels of ABC/multidrug resistance (MDR) transporters, they can also be enriched based on the ability to exclude fluorescent dyes such as rhodamine 123 and Hoechst 33342 (Bertoncello et al., 1985; Chaudhary and Roninson, 1991; Wolf et al., 1993; Zhou et al., 2001). One example based on Hoechst 33342 staining and analysis by FACS at two wave lengths (red and blue), allows for the isolation of a HSC enriched population with low uptake of fluorescent dye, the so-called side population (SP) (Goodell et al., 1996). It was recently discovered that the expression of the SLAM receptors CD48, CD150 and CD244 can distinguish between stem- and progenitor cell populations and that the CD150⁺ CD48⁻ (all CD150⁺ CD48⁻ cells are CD244⁻) population was highly enriched in stem cell activity. One in five CD150⁺ CD48⁻ cells have long term repopulation potential upon transplantation into recipient mice, similar to the frequencies obtained from the LSK Thy1.1lo population. If the SLAM markers are combined with the LSK Thy1.1lo population the frequency of long term repopulating cells increases to 1 in 2 (Kiel et al., 2005), showing the tremendous improvement of purification procedures since the 1980s. The discovery of SLAM receptors might simplify the purification of HSCs in the future. Combinations of the previously mentioned markers and other surface markers specific for certain stages of differentiation have also been used to purify progenitor cells with different potential, such as the CMP, CLP, megakaryocyte/erythroid progenitor, granulocyte/macrophage progenitor etc. (Akashi et al., 2000; Bryder et al., 2006; Kiel et al., 2005; Kondo et al., 1997b; Morrison and Weissman, 1994). The quest for new and better techniques to isolate
HSCs is ongoing and the development of fast screening technologies (such as microarray) will presumably reveal new and more selective markers in the future.

**Development of the hematopoietic system**

The development of the hematopoietic system is a stepwise process occurring in different organs at different stages, involving both de novo formation and immigration of hematopoietic cells between organs. The first sign of hematopoiesis in the early mouse embryo is the formation of blood islands at embryonic day (E) 7.5 in the extra-embryonic yolk sac (YS) (Moore and Metcalf, 1970). At E11-12 hematopoiesis shifts to the fetal liver, that will become the major hematopoietic organ until around the time of birth when the BM takes over as the key organ for hematopoiesis (Cumano and Godin, 2007; Mikkola et al., 2005). The first hematopoietic cells to emerge in YS blood islands is a transient wave of large nucleated erythrocytes, called primitive erythrocytes (eryp) that express the embryonic form of hemoglobin in contrast to their adult counterparts, definitive erythrocytes (eryd) that are enucleated and express adult type of hemoglobin (Brotherton et al., 1979; Palis and Yoder, 2001). The generation of eryp is transient and the precursor cannot be detected after E9 (Palis et al., 1999); this stage of hematopoiesis is therefore referred to as primitive hematopoiesis. Although YS blood islands more or less exclusively contain primitive erythroid cells, it has been shown that precirculation YS derived progenitors have myeloid (CFC-M, -mast, -GM) but no lymphoid potential in in vitro assays (Cumano et al., 1996; Palis et al., 1999). Blood islands are surrounded by vascular endothelium and the close association between the endothelial cells and hematopoietic cells within these units led to the suggestion of a common precursor for both lineages, the hemangioblast, almost a century ago (Sabin, 1920). The existence of such a precursor was recently shown (Choi et al., 1998; Huber et al., 2004). However, contribution of individual cells to both endothelial and hematopoietic lineages in blood islands is infrequent (Ueno and Weissman, 2006), indicating that hemangioblasts rapidly differentiate into progenitors of the vascular or hematopoietic lineages prior to YS blood island formation. The extra-embryonic and embryonic vessels become linked around the time of onset of the heart function at E8.25-E8.5 and primitive erythrocytes start to appear in the embryo proper (Dzierzak et al., 1998; McGrath et al., 2003). The first appearance of multipotent progenitors within the embryo proper occurs within the mesodermally derived portions beginning at E8.5-9 (Godin et al., 1995; Medvinsky et al., 1993; Muller et al., 1994). The specific region is called para aortic
splanchnopleura that will form the aorta, gonad and mesonephros (AGM) region. The AGM region is the site where the first hematopoietic stem cells with adult engraftment potential appear around E10 (Figure 2) (Cumano et al., 1996; Medvinsky and Dzierzak, 1996; Muller et al., 1994). However, YS cells from E8-10 engraf embryonic or fetal mice and can contribute to multilineage repopulation of hematopoietic cells throughout adulthood (Toles et al., 1989; Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b). Moreover, in 2001, Matsuoka and colleagues showed that precirculation yolk sac cells from E8 embryos had the ability to long term engraft adult mice after coculture with AGM-S3, a stromal cell line derived from AGM of an E10.5 embryo (Matsuoka et al., 2001). This suggest that YS as early as E8 produce HSCs and that failure of these cells to engraft adult recipients could be due to failure of homing, engraftment or due to low expression levels of MHC class I and subsequently NK-cell mediated destruction in the adult recipient (Bix et al., 1991). It has been suggested that YS maintains progenitor production even after the fetal liver takes over as the main hematopoietic organ (McGrath et al., 2003). Recent lineage tracing studies to follow precirculation hematopoietic progenitors from YS suggest that YS-derived cells from as early as E7.5 contribute to adult hematopoiesis and therefore participate in establishing adult hematopoiesis (Samokhvalov et al., 2007). The first HSCs appear in the fetal

Figure 2. Development of the hematopoietic system. A. Overview of the E14 embryo and the hematopoietic organs. B. Schematic view of HSC development and major movements of cells between organs (arrows): Primitive streak mesoderm gives rise to the hemangioblasts, Yolk sac: the site for primitive hematopoiesis and generation of definitive progenitors/HSCs, AGM: de novo generation of HSCs, Placenta: a potential novel site for supporting expansion/de novo generation of HSCs, Fetal liver: the established site for expansion of HSCs during development, Bone marrow: the major site for adult HSCs. Modified from (Mikkola et al., 2005)
liver at E11.5 although the liver does not initiate hematopoiesis itself but becomes colonized by hematopoietic cells from other sites i.e. YS and the AGM-region (Houssaint, 1981; Johnson and Moore, 1975; Medvinsky et al., 1993). The fetal liver will become the main hematopoietic organ from E12.5 until birth when hematopoiesis shifts to the bone marrow (BM). HSCs expand in the fetal liver to generate the number of HSC that is needed to sustain hematopoiesis during adult life (Ema and Nakauchi, 2000), and a large proportion of fetal liver HSCs are actively cycling in contrast to their BM counterpart (Harrison et al., 1997; Morrison et al., 1995; Rebel et al., 1996). This indicates that the fetal liver microenvironment stimulates the HSCs to expand in numbers, in contrast to the BM where HSC numbers are held constant. The liver apparently provides the microenvironment required for the expansion of HSCs and might provide important clues to understanding the regulation of self-renewal vs differentiation. Another site that has lately emerged as a potential hematopoietic organ during development is the placenta. It is unclear however, whether placenta is a site for de novo generation of HSCs or a temporary supportive niche for expansion of HSCs generated elsewhere (Mikkola et al., 2005) (Figure 2).

The surface expression profile of HSCs changes during embryonic development. YS progenitors with definitive erythroid and lymphoid potential are c-Kit+, CD34+ and CD41+ but express only low levels of Sca-1 (Corbel and Salaun, 2002; Ferkowicz et al., 2003; Mikkola et al., 2003a; Mitjavila-Garcia et al., 2002; Yoder et al., 1997a). YS progenitors were found both in the CD45 negative and the CD45 positive cell fraction despite the fact that CD45 is considered to be a pan-hematopoietic marker in the adult (Mikkola et al., 2003a). Fetal liver and AGM derived HSCs can be enriched by sorting for the c-Kit+, Sca-1+, AA4.1+, Mac-1+ and CD34+ population while adult HSCs are Mac-1, AA4.1 and CD34 negative. (Ikuta and Weissman, 1992; Ito et al., 2000; Jordan et al., 1990; Morrison et al., 1995; Spangrude et al., 1988; Yoder et al., 1997a). These results further emphasize that HSC change their characteristics during hematopoietic development, not only in location but also in respect to their phenotype and function.

The embryonic stem cell system - a model to study early hematopoietic development

The inaccessibility of the early embryo and the limited amount of tissue available hamper the ability to study molecular and cellular events during early development. To overcome this problem, a model system has been established based on
embryonic stem (ES) cells. ES cells are derived from the inner cell mass of the blastocyst and are pluripotent as shown by their contribution to all tissues, including the germ line when reintroduced into blastocysts that are allowed to develop into embryos (Bradley et al., 1984; Evans and Kaufman, 1981; Martin, 1981). This potential of ES cells has been fundamental in developing methods to perform gene targeting studies in mice (knock out mice). ES cells were also shown to be able to spontaneously differentiate in culture (Doetschman et al., 1985). The factor responsible for keeping murine ES cells undifferentiated in vitro was shown to be leukemia inhibitory factor (LIF) (Williams et al., 1988). Upon removal of LIF, ES cells differentiate in a process mimicking the embryonic gastrulation process (Gadue et al., 2006). Differentiation leads to the generation of three dimensional structures, called embryoid bodies (EBs), that contain cells from all three germ layers; ectoderm, endoderm and mesoderm, where the latter subsequently differentiates into hematopoietic cells (Keller, 1995). Hematopoietic development within the EB recapitulates the earliest steps of hematopoiesis within the embryo, both in respect to gene expression and the emergence of progenitor cells. As in the embryo, the first hematopoietic cells to appear in the EB are the primitive erythrocytes followed by definitive precursors of the myeloid and erythroid lineages (Keller et al., 1993). In addition, a cell population with hemangioblast properties has been identified within the ES/EB system as well as in the embryo (Choi et al., 1998; Huber et al., 2004). Thus, the ES/EB system provides a reliable model system to analyze the earliest stages of hematopoietic development. However, generation of HSCs capable of adult hematopoietic reconstitution from the ES/EB system has been difficult to achieve. Only a few reports are published where hematopoietic cells with adult engrafting potential from unmanipulated ES cells have been detected (Muller and Dzierzak, 1993; Palacios et al., 1995; Potocnik et al., 1997). However, these reports either show poor reconstitution or remains to be verified. The difficulty in achieving adult reconstitution upon transplantation is probably due to the same reasons as the previously mentioned inability of YS derived hematopoietic progenitors to engraft the adult system. However, overexpression of HoxB4 or the fusion protein, BCR-ABL, leads to the generation of cells within the EB that have the ability to engraft adult recipients (Kyba et al., 2002; Perlingeiro et al., 2001; Peters et al., 2001).
Regulation of HSC development, self-renewal and differentiation

The quest for clues about the fate choices of HSCs has been going on for the last few decades. HSCs undergoing division are able to generate two daughter cells that adapt the same fate (symmetric division) or different fates (asymmetric division). Two theories have been proposed to explain commitment of HSCs. The stochastic theory suggests a random loss of differentiation potential and subsequent acquisition of lineage specific markers. The instructive theory on the other hand, suggests that environmental signals determine the outcome during differentiation (Douagi et al., 2002). Probably the answer could be a combination of these two theories. The search for genes important or defining for stem cells has been complicated and the key to stem cell self-renewal and differentiation is still unknown. However, a number of genes with important functions in HSC development, self-renewal, quiescence and differentiation have been identified through development of techniques such as gene targeting, global gene expression studies and analysis of translocations in leukemia and lymphomas. The array techniques in combination with more refined sorting have greatly increased the possibility of elucidating a “stem cell signature” in terms of pattern of mRNA expression (Ivanova et al., 2002; Ramalho-Santos et al., 2002). The future challenge will be to evaluate these enormous quantities of data and find the key molecules and signaling pathways involved in these important processes.

The hematopoietic stem cell microenvironment

HSCs are not autonomous entities but reside in hematopoietic organs surrounded by a complex network of supportive cells such as endothelial cells, fibroblasts and smooth muscle cells. Since the HSCs are self renewing cells with an enormous capacity to differentiate and produce immense amounts of progeny, these cells must be tightly regulated both in respect to total numbers and differentiation. Deregulation leading either to fewer cells (cytopenia), such as anemias, or to increased number of cells, such as myeloproliferative disorders or leukemias, are potential threats for the survival of the organism. The idea that the number of stem cells must be tightly controlled is supported by results indicating that the number of HSCs between animals of different sizes (mouse and cat) is held constant and approximately the same (1.1×10⁴ HSCs/animal) indicating that there is a predetermined limit that is strictly regulated (Abkowitz et al., 2002). The regulation
of HSCs is suggested to be achieved by reciprocal interactions between the stem cells and the specific microenvironment where they reside, called the niche. The stem cell niche is defined as a subset of tissue cells and extra-cellular substrates that can harbor one or more stem cells controlling their self-renewal and progeny production in vivo (Schofield, 1978). The BM niche was proposed to be located at the endosteal surface and to be composed of osteoblasts physically interacting with HSCs. This was confirmed in experiments where increased number of osteoblasts and trabecular bone led to an increase in total numbers of HSCs (Calvi et al., 2003; Zhang et al., 2003), while decreased number of osteoblasts led to a decreased number of HSCs (Visnjic et al., 2004). The tight connection between the HSC and the niche could be involved in important decisions such as regulation of asymmetric or symmetric cell divisions thereby regulating self-renewal vs. differentiation depending on the plane of division, as has been reported for the Drosophila male germ line stem cells (Yamashita et al., 2003; Yamashita et al., 2007). The niche also limits the space available for HSCs in the BM, but whether the number of niches is constant or can vary is not known. Several genes and pathways have been implicated in niche regulation such as osteopontin, calcium sensing receptor, Angiopoietin-1/Tie-2, the Wnt-, Notch-, Hedgehog and BMP-pathways and many more (Adams and Scadden, 2006) (Figure 3). These data have demonstrated the importance of osteoblasts in regulating the stem cell pool in the BM. However, HSCs are also released into the circulation, a process that can be greatly enhanced by treatment with cytokines. It has been shown that murine HSCs remain circulating for only a few minutes and that 100- 400 such cells are present

**Figure 3. Interactions between HSC and the BM-niche.** The HSC interacts with the osteoblast through a number of cell-cell interaction molecules. The niche is also regulated by morphogens such as Wnts, hedgehogs and BMPs as well as cytokines and other factors.
in the circulation at any time, indicating that a large number of stem cells pass through circulation each day and that HSCs are not by any means static within the BM (Wright et al., 2001). Moreover, during conditions where the BM microenvironment is disturbed, such as osteopetrosis (increase in bone matrix) or leukemias, HSCs are able to home to sites other than the BM, such as the spleen, were osteoblasts are not present (Adams and Scadden, 2006). However, the complicated nature of the stem cell niche in the BM might explain the difficulty in achieving expansion of HSCs in vitro. Removing the HSCs from their natural environment leads to proliferation, which in most cases is followed by differentiation and consequently loss of stem cell activity. The development of coculture systems with stromal cells derived from BM (Fraser et al., 1992) or fetal liver (Moore et al., 1997), has been shown to support HSC expansion in combination with cytokines, indicating that the microenvironment and cell-cell interactions might be important if not crucial for maintaining stem cell properties for longer periods of time in culture.

**Hematopoietic stem cells in disease - chronic myeloproliferative disorders**

A number of hematological diseases originate from the stem/progenitor cell compartment, some of which are classed as chronic myeloproliferative disorders (CMDs). The CMDs are pre-leukemic diseases that include chronic myeloid leukemia (CML), essential thrombocytopenia (ET), polycythemia vera (PV) and myelofibrosis with myeloid metaplasia (MMM). Rarer subtypes comprise chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), hyper-eosinophilic syndrome (HES), and unclassifiable CMDs (Haferlach et al., 2004). The CMDs are clonal stem cell disorders that are characterized by an initial chronic phase with increased production of one or more myelo-erythroid lineages in peripheral blood or BM (Adamson et al., 1976; Fialkow et al., 1981; Fialkow et al., 1967; Fialkow et al., 1977; Jacobson et al., 1978). CMDs have the potential to undergo transformation into blast crisis or to myelofibrosis leading to severe clinical complications (Haferlach et al., 2004).

CML is characterized by an initial chronic phase with high peripheral white blood cell counts (primarily neutrophilic granulocytes and myeloid precursors), extramedullary hematopoiesis and splenomegaly. This disorder usually accelerates into a terminal phase (acute phase or blast crisis) characterized by accumulation of transformed immature hematopoietic cells (Gordon and Goldman,
1996). CML is caused by a translocation between chromosome 9 and 22 t(9;22)(q34;q11), leading to a shortened chromosome 22, that is the cytogenetic hallmark of CML, referred to as the Philadelphia chromosome (Ph) (Nowell and Hungerford, 1960; Rowley, 1973). This translocation leads to the fusion between the genes Bcr and c-Abl (Deininger et al., 2000; Konopka et al., 1984). BCR is an ubiquitously expressed signaling molecule that appears to play a specific role in neutrophilic function (Deininger et al., 2000; Voncken et al., 1995). cABL on the other hand, is a protein tyrosine kinase with important functions in many cellular processes, such as cell cycle regulation, apoptosis, cytoskeletal rearrangements and cell migration (Deininger et al., 2000; Hernandez et al., 2004). The fusion between the Bcr and c-Abl genes leads to the expression of an abnormal fusion protein, BCR-ABL, with an increased tyrosine kinase activity (Konopka and Witte, 1985). The presence of the BCR-ABL fusion protein affects cell proliferation and cell survival, eventually leading to leukemogenesis (Chopra et al., 1999). Overexpression of BCR-ABL in murine HSCs leads to the development of a CML-like disorder and also to a number of other hematological malignancies (Daley et al., 1990; Elefanty et al., 1990; Kelliher et al., 1990; Pear et al., 1998; Zhang and Ren, 1998). Classical CML is 100% BCR-ABL positive, however there are cases of BCR-ABL CML-like disorders (Tefferi and Gilliland, 2007), indicating that BCR-ABL is not the sole mechanism for developing a CML-like disorder. The stem cell origin of CML suggests that further understanding of the molecular mechanisms involved in the regulation of HSCs might provide important information into the molecular basis for the development and progression of this disorder.

**Extrinsic regulators of hematopoiesis**

**Hematopoietic cytokines/growth factors**

Cytokines/growth factors are soluble or membrane bound proteins or glycoproteins. The action of cytokines varies from short range cell-cell signaling to effects seen throughout the organism. A number of cytokines/growth factors are involved in regulating survival, proliferation and differentiation of hematopoietic stem and progenitor/precursor cells.

Cytokines that act directly to induce proliferation or differentiation of lineage-committed hematopoietic precursor cells into mature cell lineages belong to the late acting cytokines. Many of the late acting cytokines exert their effect on
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precursors of a single lineage, such as Epo, G-CSF and IL-7, as is also indicated by the phenotypes of the respective mouse mutants (Socolovsky et al., 1998). EpoR⁻/⁻ embryos die due to severe anemia resulting from absence of erythrocytes, G-CSF knockout mice suffer from severe neutropenia while IL-7 mutants display abnormal B- and T-lymphocyte development (Lieschke et al., 1994; Lin et al., 1996; von Freeden-Jeffry et al., 1995).

Although single late acting cytokines are able to induce growth of hematopoietic precursors in CFC-assays, primitive progenitors require more than one cytokine to induce proliferation (Heimfeld et al., 1991; Okada et al., 1992). This is due to synergistic effects, i.e. that two cytokines added together elicit a response that is greater than the added effect of the two on their own. Cytokines that have this effect on immature progenitor cells are called early acting. This property has been described for a number of cytokines including stem cell factor (SCF, also called steel factor or c-Kit ligand), Fms-related tyrosine kinase 3 ligand (Flt3L), Thrombopoietin (Tpo), interleukin (IL)-3, IL-4, IL-6, IL-11, IL-12 and GM-CSF (Ogawa and Matsunaga, 1999). The most potent combinations of early acting factors shown to induce proliferation of primitive progenitors in vitro include SCF, Flt3L, IL-3, IL-6 and Tpo, although their capacity to induce self-renewal is limited (Audet et al., 2001; Matsunaga et al., 1998; Miller and Eaves, 1997; Ogawa and Matsunaga, 1999; Peters et al., 1997; Sitnicka et al., 1996; Sui et al., 1995; Yagi et al., 1999; Yonemura et al., 1997). In agreement with their activity, loss of any of these factors (with the exception of IL-3) or their receptors leads to severe hematological phenotypes in knockout models. SCF and its receptor c-Kit are encoded by the murine white spotting (W) and steel (Sl) loci, respectively. Null mutations for SCF or c-Kit are embryonic lethal due to severe anemia (Nocka et al., 1990; Russell, 1979). However, many independent strains with mutations in the W loci are known with varying severity of defects in hematopoiesis, pigmentation and fertility (Geissler et al., 1981). SCF/c-Kit signaling is not important for YS hematopoiesis but is critical for definitive hematopoiesis within the fetal liver (Ogawa et al., 1993). SCF has been shown to play an important role for both proliferation and differentiation of HSC and progenitor cells in vivo and as a synergistic factor in vitro (Broudy, 1997). IL-6, like SCF, is important for definitive hematopoiesis but not required for proper YS hematopoiesis (Takizawa et al., 2003; Yoshida et al., 1996). Null mutants for IL-6 show reduced numbers of primitive progenitors as well as decreased functionality of LT-HSCs (Bernad et al., 1994). The relatively mild phenotype of the IL-6⁻/⁻ mice could be explained by IL-6 sharing the signaling co-receptor glycoprotein 130
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(gp130) with other members of the IL-6 family (LIF, IL-11, oncostatin M, ciliary neutrophic factor and cardiotrophin) possibly leading to functional redundancy (Kishimoto et al., 1995). Activation of signaling from gp130 requires ligand binding to specific α-receptors controlling the cell specific responses from gp130. The null mutation of gp130 can lead to embryonic lethality between E12.5 and term due to multiple developmental defects (Yoshida et al., 1996). Mutant embryos suffer from hematological problems such as reduced numbers of HSCs and committed progenitors in the fetal liver as well as anemia. The role of gp130 in the adult system was investigated by generation of mice with inducible inactivation of gp130. Postnatal inactivation of gp130 led to multiple defects in several organ systems including hematopoietic defects, such as decreased number of progenitors in the BM and defects in thrombopoiesis (Betz et al., 1998), indicating an important role for gp130 signaling within the adult as well as the embryonic hematopoietic system. Null mutations in the Tpo receptor, c-Mpl, lead to a severe deficiency in megakaryopoiesis as well as a decrease in progenitors of multiple lineages (Alexander et al., 1996). HSCs from c-Mpl−/− mice are unable to compete with normal HSCs in CRU-assays, suggesting that self-renewal is compromised (Kimura et al., 1998; Murone et al., 1998). Tpo addition to long term BM cultures (Dexter culture) supported ex vivo expansion of HSCs, further indicating a role for Tpo in self-renewal of HSCs (Yagi et al., 1999). Flt3L, with its receptor Flt3 has turned out to be an important synergistic factor in many aspects of hematopoiesis, although alone it does not have any strong effect on proliferation or differentiation, thereby sharing many properties with SCF. The knockout for Flt3L is viable but displays hematopoietic defects, such as lowered cellularity of hematopoietic organs and a decrease in both lymphoid and myeloid progenitors (McKenna et al., 2000). The null mutant mice for the receptor, Flt3, are viable and display weak hematopoietic defects such as a decreased number of B-cells and less efficient repopulation upon transplantation (Mackarehtschian et al., 1995). The causes of the difference in phenotype between the receptor and the ligand mutants are not known. Interestingly, when Flt3−/− mice are crossed to a c-Kit mutant (W/Wv), which is viable but have severe defects in hematopoiesis, the resulting mice display reduced hematopoiesis and postnatal mortality indicating the importance of combined functional Flt3 and c-Kit signaling for normal hematopoiesis (Mackarehtschian et al., 1995). However, later it has been shown that the majority of LT-HSCs are Flt3− and that upregulation of Flt3 expression marks loss of self-renewal ability in stem cells (Adolfsson et al., 2001). Unexpectedly, knockouts of one of the early acting factors that is most potent, IL-3, are normal with respect to
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the HSC and the early hematopoietic progenitor compartment (Lantz et al., 1998; Nishinakamura et al., 1996). Recently, it was reported that members of the angiopoietin-like protein (angptl) family in combination with SCF, Tpo, insulin growth factor-2 (IGF-2) and fibroblast growth factor-1 (FGF) could stimulate in vitro expansion of HSCs (Zhang et al., 2006). Angptls are orphan ligands that do not bind the angiopoietin receptor tie-2 and thereby signal through an unknown mechanism (Morisada et al., 2006). This indicates that there are still unknown molecules to be discovered which could improve the ex vivo expansion of HSCs and give further insights into HSC self-renewal.

It has been debated whether cytokines acts as master regulators during hematopoietic differentiation (instructive theory) or if they simply allow cells already adapted to a specific fate to survive (permissive theory). The permissive action of cytokines is supported by data where the anti-apoptotic gene, Bcl-2, is overexpressed in cytokine dependent cell lines thereby rendering them cytokine independent (Fairbairn et al., 1993). Bcl-2 expression also rescues the phenotype of cytokine or cytokine-receptor deficient mice (Kondo et al., 1997a; Lagasse and Weissman, 1997; Maraskovsky et al., 1997). The permissive theory is also supported by experiments where a chimeric receptor was made from the extracellular domain of c-mpl combined with the intracellular domain of the G-CSFR (Stoffel et al., 1999). This chimeric receptor can functionally replace the c-Mpl receptor and restore megakaryopoiesis and thrombopoiesis in c-Mpl deficient mice while no increase in granulocytic progenitors was observed, indicating permissive roles of these receptors in hematopoiesis. However, there is support for the instructive model since the expression of cytokine receptors, such as the GM-CSFR in CLPs (restricted to lymphoid cells) could redirect their fate toward myeloid cells (Kondo et al., 2000). Collectively these data suggest that decisions during differentiation of hematopoietic cells are governed by permissive as well as instructive mechanisms.

**Hedgehog, BMP, Notch, Wnt and FGF Signaling pathways**

The Hedgehog (Hh), bone morphogenetic protein (BMP), Wingless (Wnt), Notch and fibroblast growth factor (FGF) signaling pathways are known to be important for embryonic patterning during development. The Notch ligands act via cell-cell interactions thereby regulating cell differentiation through a process called lateral inhibition (Radtke et al., 2004), while ligands of the Hh, BMP and Wnt pathways act as morphogens during development by the coordinated secretion from
organizing centers and the formation of gradients. These gradients elicit cellular responses depending on the concentration and combination of morphogens at a particular site (Capdevila and Belmonte, 1999). These pathways also play important roles in many processes in the adult and have been implicated in regulation of HSC fate decision and self-renewal.

Hedgehog proteins are a family of signaling molecules comprising three members in mammals; Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog. Shh is the most widely expressed and most extensively investigated of the three. Shh binds the primary receptor patched (Ptc) which leads to the release of another transmembrane protein, smoothened (Smo), which regulates transcription of target genes such as the Gli family of transcription factors (Riobo and Manning, 2007). Addition of anti-Hh antibodies to purified primitive blood cells inhibited cytokine induced proliferation indicating a role of the Hh pathway in HSC regulation in vitro (Bhardwaj et al., 2001). Addition of Shh on the other hand induced proliferation. The importance of the Hh pathway for hematopoiesis was further established by experiments in the zebrafish model system, where blocking Hh signaling led to deficient definitive HSC formation (Gering and Patient, 2005). In addition, Ihh has been reported to play a role in the induction of hematopoiesis and vasculogenesis from mesoderm in the early embryo (Dyer et al., 2001). The murine model system has had limited use for dissecting the roles of several Hh proteins since the knockouts for Shh, Ptc and Smo results in embryonic lethality with major developmental defects (Chiang et al., 1996; Zhang et al., 2001). The development of mice where genes can be specifically mutated in HSCs (conditional knockouts) would be important tools for understanding the significance of Hh signaling for the development of definitive HSCs in mammals. A recently generated conditional knockout of Ptc demonstrated an important function for the induction of lymphoid cell fate. However, transplantation of Ptc\(^{-/-}\) BM cells showed normal reconstitution of all lineages, indicating that constitutively active Hh signaling has no effect on hematopoietic cell directly but rather on the BM stroma compartment (Uhmann et al., 2007).

Interestingly, the Shh induced proliferation of human hematopoietic stem cells was suggested to be mediated by BMP4 (Bhardwaj et al., 2001). BMPs are important for defined ex vivo culture of human HSCs, increasing survival and maintaining repopulating blood cells, thereby indicating a role of BMP signaling in adult stem cells (Bhatia et al., 1999).

The Notch signaling pathway has been implicated in the regulation of hematopoiesis and HSC proliferation. In mammals there are four Notch receptors
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(Notch 1-4) and five known ligands (Jagged1 and 2, and Delta-like (Dll) 1, 3 and 4). Ligand binding to the extracellular part of Notch leads to proteolytic cleavage of the transmembrane part releasing the intracellular domain (ICN) of the receptor. ICN is translocated into the nucleus where it binds directly to the DNA binding protein CSL/RBPJκ. The formation of the ICN/CSL/RBPJκ complex leads to transactivation of target genes such as the HES family of transcription factors (Radtke et al., 2004). Notch-1 deficient embryos die in utero at E10.5 with many defects including deficient angiogenesis and reduced definitive hematopoiesis in the P-Sp/AGM region (Krebs et al., 2000; Kumano et al., 2003; Swiatek et al., 1994). The perturbed development of definitive HSCs was confirmed in chimeric mice consisting of normal and Notch-1 deficient cells. Although the Notch-1 mutant cells showed strong contribution to several organ systems, they did not contribute to the hematopoietic system after E15.5 (Hadland et al., 2004). This indicates that Notch-1 is required, in a cell-autonomous manner, for generating or expanding definitive HSCs during embryogenesis. However, conditional knockouts of Notch-1 in mouse BM display a block in T-cell development while the HSCs show normal engraftment and differentiation to lineages other than T-lymphocytes (Mancini et al., 2005; Radtke et al., 1999). Moreover, OP9 stromal cells (normally supporting B-cell development) genetically engineered to express Dll1 can support T-cell differentiation (Schmitt and Zuniga-Pflucker, 2002). These data suggest that Notch-1 signaling supports T-cell differentiation in the adult system. However, overexpression of active ICN from Notch-1 in KSL cells from adult BM led to the generation of multipotent cell lines capable of long term engraftment upon transplantation, indicating that constitutively active Notch signaling affects HSC self-renewal (Varnum-Finney et al., 2000). In other studies, using the 32D myeloid progenitor cell line, it has been suggested that Notch-1 inhibits differentiation of hematopoietic progenitor cells (Bigas et al., 1998; Kumano et al., 2001; Milner et al., 1996). However, activated Notch-1 has also been shown to induce differentiation in hematopoietic cell lines, indicating that the experimental setup determines the outcome of these experiments (Schroeder and Just, 2000; Schroeder et al., 2003).

The Wnt pathway has been implicated in many processes such as specification of cell fate, proliferation of progenitor cells and control of asymmetric cell division. There are 19 known Wnt-encoding genes in the human and mouse genome. These encode secreted lipid-modifying glycoproteins that bind receptors of the frizzled family (Staal and Clevers, 2005). Upon binding, a complex series of events leads to the stabilization and translocation of β-catenin into the nucleus.
where it replaces corepressors of the T-cell factor (TCF) thereby activating transcription of target genes (Staal and Clevers, 2005). β-catenin dependent Wnt signaling is referred to as the canonical pathway. However, Wnt signaling is also known to occur independent of β-catenin via so called non-canonical pathways (Gordon and Nusse, 2006). Several reports have shown that Wnt signaling could play a role in HSC self-renewal and/or differentiation. It has been reported that constitutively active β-catenin induces self-renewal of purified HSCs (Baba et al., 2006; Reya et al., 2003). However in contrary to this, two other studies have shown that conditional expression of active β-catenin leads to a block in differentiation and also proliferation of HSCs, resulting in their depletion (Kirstetter et al., 2006; Scheller et al., 2006). Moreover, conditional knockouts of β-catenin in the hematopoietic system do not impair self-renewal or differentiation of HSC, questioning an essential role in hematopoiesis (Cobas et al., 2004). This however, does not exclude the possibility that Wnts could exert their effect in β-catenin null mice through the non-canonical pathways or that other β-catenin related proteins transfer signals through the canonical pathway, such as γ-catenin (plakoglobin) (Reya and Clevers, 2005).

The FGF family comprises over twenty members in vertebrates (Itoh and Ornitz, 2004). Only four FGF receptor (FGFR) genes have been identified, although these undergo significant alternative splicing, giving rise to multiple isoforms of the receptors with varying ligand specificity (Givol and Yayon, 1992). FGF signaling is important for many developmental processes such as patterning of mesoderm and the nervous system (Vasiliauskas and Stern, 2001). Knockout studies have revealed a diverse range of abnormalities related to certain FGFs or FGFRs, such as in limb development and skeletal growth (Colvin et al., 1996; Martin, 1998). It has been reported that FGFR1-/- ES cells are deficient in hemangioblast generation in vitro, indicating a role in establishment of the hematopoietic system (Faloon et al., 2000). The FGFR1 gene has also been shown to be involved in translocations detected in patients with myeloproliferative disorders leading to constitutive activation of the receptor indicating a potential role in adult hematopoiesis (Cross and Reiter, 2002). Moreover, addition of FGF1 and 2 to ex vivo BM cultures can maintain and even lead to some expansion of stem cells capable of long term repopulation of recipient mice, although the mechanism is not clear (de Haan et al., 2003; Yeoh et al., 2006).
Intracellular regulators of hematopoiesis

Cell cycle regulators
The cell cycle machinery is a tightly regulated system with several checkpoints ultimately regulating the proliferation status of all cells. In mammalian cells, the entry into the cell cycle requires sequential activation of cycline-dependent kinases (CDKs) that can be inhibited by CDK inhibitors (CDKIs). CDKIs constitute an important class of cell cycle inhibitors composed of the INK4 and Cip/Kip families. Several CDKIs are expressed in early hematopoietic progenitors or their progeny, and have been shown to play a role in regulating HSC quiescence and self-renewal (Ezoe et al., 2004; Furukawa et al., 2000).

p21cip1/waf1 is a CDKI and G1 checkpoint regulator important for the maintenance of the quiescent state and prevention of HSC depletion, since p21-/- adult HSCs show increased cell cycling, and exhaustion upon serial transplantation (Cheng et al., 2000b). Another CDKI, p27kip1, is not preferentially expressed in HSCs but rather in more differentiated progeny (Furukawa et al., 2000). Therefore, it is not surprising that p27 does not affect stem cell number, cell cycling or self-renewal but rather causes increased proliferation of intermediate progenitors (Cheng et al., 2000a). Similar to p21, p18INK4c is an inhibitor of HSC self-renewal (Yuan et al., 2004). HSCs lacking p18 expression have increased self renewal but in contrast to p21 this does not lead to depletion. Null mutants for p18 suffer from gigantism, organomegaly and increased tumorogenicity indicating that the function of p18 is not restricted to hematopoiesis (Franklin et al., 1998). Another member of the INK4 family, p16INK4a, is highly expressed in human CD34+ hematopoietic cells and its expression is downregulated upon differentiation (Furukawa et al., 2000). However, p16 expression is virtually undetectable in tissues of young animals while expression increases with age (Krishnamurthy et al., 2004). Therefore it is not surprising that p16 null mice exhibit no difference in hematopoiesis at a young age compared to wild-type mice. On the other hand, with age, loss of p16 leads to increased number of HSCs and improved long term repopulation ability due to higher resistance to apoptosis and increased cycling, suggesting that inhibition of p16 could improve resistance to stress and injury repair in aging cells (Janzen et al., 2006). The importance of CDKIs, especially in HSCs and during the early stages of differentiation, makes it evident that proper cell cycle control is required for maintaining normal hematopoiesis and function of HSCs.
Apoptosis in the regulation of hematopoiesis

The anti-apoptotic gene Bcl-2 is expressed in erythroid-myeloid progenitor populations and in megakaryocytes (Hockenbery et al., 1991). Bcl-2 expression is downregulated upon differentiation into the myeloid lineage correlating with the short lifespan of neutrophils (Hockenbery et al., 1991). Overexpression of Bcl-2 within the hematopoietic system leads to an increased number of HSCs within the BM and growth advantage in competitive repopulation studies (Domen et al., 2000). When Bcl-2 was overexpressed during ontogeny under the control of the Ly-6E/A (Sca-1) promoter it caused an increase in number of HSCs found in E11 AGM and E11-12 fetal liver (Orelio et al., 2004), suggesting that apoptosis plays a role in HSC development. Mcl-1 is another member of the Bcl-2 group of anti-apoptotic genes. Mcl-1 has been shown to be a specific regulator essential for ensuring homeostasis of early hematopoietic progenitors. Mcl-1 deficiency leads to early embryonic death (Rinkenberger et al., 2000), however, inducible knockout of Mcl-1 in the BM of adult mice led to depletion of HSCs and progenitor populations in the BM through extensive apoptosis and leading to death of the mutant animal due to severe anemia (Opferman et al., 2005). Thus, apoptosis appears to be an important mechanism in regulating hematopoiesis and HSC numbers and might involve various anti-apoptotic and pro-apoptotic genes.

Transcription factors

Transcription factors are the executors of signals provided to the cell from its environment via cytokines, hormones, cell-cell contact etc. There are a number of important transcription factors known for regulating development, self-renewal and differentiation of HSCs. The importance of many of these genes has been uncovered through their involvement in leukemia and subsequently through gain- and loss-of-function studies.

A few transcription factors have been shown to be involved in the earliest stages of hematopoiesis. Scl/tal is known as a master regulator due to the complete failure of YS hematopoiesis and death of null mutants as early as E8.5 (Robb et al., 1995). Further studies showed that the Scl mutants are unable to contribute to any definitive lineages in mice chimeric for wild type (wt) and Scl-/- cells and that Scl is needed in mesodermal precursors for specification of both the primitive and definitive hematopoietic lineages (Endoh et al., 2002; Porcher et al., 1996; Robb et al., 1996). Scl is thereby one of the earliest regulators of HSC specification known to date. However, Scl has been shown to be dispensable for stem cell maintenance.
in the adult, although Scl expression is crucial for proper differentiation of the erythroid and megakaryocytic lineages (Mikkola et al., 2003b). Thus, Scl is essential for the generation of HSCs but its continued expression is not necessary for HSC maintenance. Rbtn2/LMO2 is a LIM-domain protein that forms a complex with Scl and other factors, such as the GATA family of zinc finger transcription factors (Wadman et al., 1997). The LMO2 knockout displays similar defects to the Scl knockout with embryonic lethality around E9.75 and complete block in primitive and definitive hematopoiesis, indicating the importance of LMO2/Scl interaction for development of the hematopoietic system (Warren et al., 1994; Yamada et al., 1998). One member of the GATA family of transcription factors, GATA2, is also involved in the earliest specification events during YS hematopoiesis and the knockout dies at E10-11 due to severe anemia. In vitro experiments using the ES system and analysis of mice chimeric for wt and GATA2−/− cells revealed a profound defect in generation of cells of all hematopoietic lineages, including primitive erythrocytes, from GATA2 deficient cells (Tsai et al., 1994a). It has also been shown that GATA2 is involved in generation of HSCs in the AGM region (Ling et al., 2004).

A number of transcription factors are implicated in establishment and/or expansion of definitive hematopoiesis but play no obvious role in primitive hematopoiesis. One of these genes, Runx1, is required for the establishment of definitive hematopoiesis and the knockout dies around E12.5 due a block in all definitive hematopoietic lineages (Okuda et al., 1996; Wang et al., 1996a). Similarly to Scl, Runx1 has been shown to be dispensable for maintenance of adult HSCs although essential for megakaryocytic and lymphocytic differentiation, further emphasizing the difference between generation and maintenance of HSCs (Ichikawa et al., 2004). CBFβ is a binding partner of Runx1, and disruption leads to the same phenotype as for the Runx1 knockout, showing that this interaction is important for the proper development of the definitive hematopoietic system (Wang et al., 1996b). Another important regulator in definitive hematopoiesis is c-myb, the founding member of the myb family of transcription factors. Null mutant mice for c-myb die from severe anemia at E15 due to the failure to initiate proper fetal liver hematopoiesis (Mucenski et al., 1991). The interaction between c-myb and its transcriptional coactivator p300 is important for controlling proliferation and differentiation of hematopoietic stem cells/progenitors in the adult since disruption of this interaction leads to increased proliferation of HSCs and perturbed differentiation of several lineages (Sandberg et al., 2005).
Several transcription factors have been identified as playing roles in proper differentiation into specific mature lineages. PU.1 homozygous mutant mice lack myeloid and lymphoid lineages and die at E17.5-18 due to anemia caused by a specific defect in erythroblast maturation (Scott et al., 1994). PU.1 is expressed in adult HSCs and is important for migration and homing to the BM and long term repopulation (Fisher et al., 1999; Iwasaki et al., 2005). The GATA-1 transcription factor on the other hand, is important for proper development of the erythropoietic lineage. GATA-1⁻/⁻ mice die in utero due to deficient primitive erythropoiesis (Fujiwara et al., 1996). It has been shown that GATA-1 interacts with the DNA binding domain of PU.1 and thereby represses myeloid gene expression and that PU.1 also represses GATA-1 transcriptional activation (Rekhtman et al., 1999) (Nerlov et al., 2000), suggesting that these factors act in an antagonistic fashion to determine the hematopoietic lineage choice.

Clustered homeobox genes
The homeobox superfamily of genes encodes proteins with a DNA-binding homeodomain. Many homeodomain containing proteins act as transcription factors and regulate gene expression during developmental patterning or cell differentiation (Holland and Takahashi, 2005). In mammals there are 39 homeobox genes specifically located in four clusters (A, B, C and D). These genes belong to the Hox family of clustered homeobox genes and are crucial for the proper development of the embryo (Iimura and Pourquie, 2007). Hox genes from all four
clusters have been implicated in the regulation of both normal and leukemic hematopoiesis (Abramovich et al., 2005). A number of Hox genes are expressed in the HSC compartment (such as HoxA2, HoxA5, HoxA9, HoxA10 and HoxB4) (Ivanova et al., 2002; Pineault et al., 2002; Sauvageau et al., 1994). Overexpression of HoxA10 in vivo leads to the development of AML and perturbed myeloid and lymphoid differentiation (Thorsteinsdottir et al., 1997). Moreover, ectopic expression of HoxA10 at low levels in LSK cells in vitro led to maintenance/proliferation of HSCs, suggesting that HoxA10 could be involved in regulating HSC self-renewal/proliferation (Magnusson et al., 2007). HoxA9 and HoxB4 have been implicated in regulation of HSC self-renewal and are able to expand HSCs if overexpressed (Antonchuk et al., 2002; Thorsteinsdottir et al., 2002). HoxA9 has been shown to be involved in translocations leading to the development of some cases of acute myeloid leukemia (AML) (Golub et al., 1999). Furthermore, overexpression of HoxA9 leads to HSC expansion and eventually the development of AML upon transplantation (Kroon et al., 1998; Thorsteinsdottir et al., 2002). The knockout for HoxA9 has normal numbers of HSCs although these are defective when tested in CRU assays, further indicating a role for HoxA9 in regulating HSCs (Lawrence et al., 2005).

HSCs overexpressing HoxB4 contribute to long term myeloid-lymphoid repopulation of adult recipients (Antonchuk et al., 2002), although mice with high expression levels of HoxB4 develop a chronic myeloproliferative disorder (CMD) over time (Milsom et al., 2005; Pilat et al., 2005; Schiedlmeier et al., 2003). The knockout for HoxB4 displays only a mild proliferative defect in HSCs indicating that there could be redundancy with other Hox genes (Brun et al., 2004). Interestingly, a knockout for all HoxB genes (HoxB1-9) was normal in respect to HSC self-renewal and differentiation (Bijl et al., 2006), indicating that all these genes are dispensable for hematopoiesis possibly due to a more complex cross-regulation between the Hox clusters.

Non-clustered homeobox genes

A large number of homeobox-containing genes are not located within clusters in the genome. These genes can be divided into six groups depending on their evolutionary relationships (LIM-, POU-, PAX-, PRD-type-, SIX- and NK-homeodomain proteins) (Hobert and Westphal, 2000). The group of most interest for this thesis is the LIM-homeodomain (LIM-HD) family. The characteristic feature of the LIM-homeodomain proteins are two specialized protein binding zinc fingers,
called LIM domains. The LIM domain derives its name from the initials of three members of the LIM-HD family, Lin-11, Islet-1 and Mec-3 (Freyd et al., 1990; Hobert and Westphal, 2000; Karlsson et al., 1990; Way and Chalfie, 1988). The LIM domains can interact with cofactors to modulate the specificity and DNA binding of the homeodomain (Curtiss and Heilig, 1998). This group of transcription factors is important in diverse developmental processes in many organ systems such as asymmetric cell division, tissue specification and differentiation (Hobert and Westphal, 2000). Due to the combination of LIM domains and the homedomain, LIM-HD proteins have a large capacity to interact with numerous cofactors thereby changing the specificity depending on the context of the cell, which may explain the multifunctionality of many LIM-HD factors (Curtiss and Heilig, 1998; Hobert and Westphal, 2000). The LIM-HD family is divided into six undergroups named by their founding members (Apterous, Lhx6/7, Islet, Lmx, Lim-3 and the Lin-11 group). LIM-HD transcription factors functions both during early pattern formation and later in regulating tissue specific gene expression in a range of organs throughout the body (Curtiss and Heilig, 1998; Hobert and Westphal, 2000).

**LIM-homeobox gene 2 (Lhx2)**

Lhx2 belong to the Apterous subfamily of the LIM-HD group of transcription factors (Nohno et al., 1997; Xu et al., 1993). Lhx2 plays an important role during the development of the nervous system and is expressed throughout the cerebral cortex in the developing forebrain, outer layers of the dorsal midbrain and the rostral hindbrain as well as in the spinal cord, retina of the developing eye and the olfactory epithelium (Porter et al., 1997; Xu et al., 1993). Lack of Lhx2 expression led to anophtalmia (lack of eyes), lack of mature olfactory sensory neurons (Hirota and Mombaerts, 2004; Kolterud et al., 2004a) and hypoplasia of the cerebral cortex and hippocampus caused by decreased proliferation of progenitor cells (Porter et al., 1997). The anophtalmia was caused by arrested development after formation of the optic vesicle but prior to the formation of the optic cup (Porter et al., 1997) (Figure 5). Lhx2 is also expressed during the hair cycle and was suggested to play a role in the regulation of hair follicle stem cell maintenance, since loss of Lhx2 expression led to a more rapid entry into the hair cycle and increased proliferation of stem cells in the hair follicle (Rhee et al., 2006). Lhx2-/- mice also displayed small, pale and severely fibrotic fetal livers as well as severe anemia leading to death around E16 (Figure 5). Transplantation of Lhx2-/- hematopoietic cells and the
analysis of mice chimeric for wt and Lhx2\(^{-/-}\) cells showed that mutant cells could contribute to all hematopoietic lineages in wild-type recipients, suggesting that the anemia in null mutants was caused by a cell nonautonomous mechanism (Porter et al., 1997). The fibrotic liver phenotype of Lhx2 mutant mice indicates that the hematopoietic phenotype is caused by a defective hematopoietic microenvironment and/or niche (Porter et al., 1997; Wandzioch et al., 2004). The cell type mainly contributing to the development of hepatic fibrosis is hepatic stellate cells. This cell type comprises a subset of vitamin A storing cells that are activated upon chronic injury to the liver, leading to the production of scar tissue and inflammation causing the fibrotic phenotype (Friedman, 2000). Lhx2 was shown to be expressed in hepatic stellate cells and proved to be an important inhibitor of their activation (Kolterud et al., 2004b; Wandzioch et al., 2004). This suggests that Lhx2 deficiency in hepatic stellate cells cause the fibrotic phenotype in Lhx2\(^{-/-}\) livers and that these cells contribute to the defective hematopoietic microenvironment (Kolterud et al., 2004b) (Wandzioch et al., 2004). Interestingly, expression of Lhx2 in hematopoietic progenitor cells derived from ES cells differentiated in vitro led to the generation of multipotent hematopoietic progenitor cell lines (HPC-lines) (Pinto do et al., 1998). The HPC-lines are dependent on SCF for survival and self renew through a cell nonautonomous mechanism in agreement with the cell nonautonomous phenotype in Lhx2\(^{-/-}\) mice (Pinto do et al., 2001), suggesting that Lhx2 controls the expression of soluble mediator/s involved in this process. However, since Lhx2 was introduced into the cells through retroviral transfer, it was not formally possible to exclude the possibility that inactivation of tumor suppressor genes or activation of oncogenes caused by retroviral insertions contributed to the observed phenotype. Thus, it is not clear whether Lhx2 is the sole cause for generation and the phenotype of HSC-like cell lines or if additional events are required.
AIMS

- To determine whether Lhx2 is solely responsible for self-renewal of HSC-like cell lines
- To elucidate if Lhx2 expression in adult BM can lead to the generation of HSC-like cell lines and if these cell lines retain their ability to engraft
- To elucidate the consequences of Lhx2 expression in hematopoietic cells in vivo
- To elucidate the molecular mechanism for Lhx2 mediated expansion of HSC-like cell lines
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Generation of ES cells with inducible Lhx2 expression (Paper I)

Previously, when we generated cell lines from ES cells differentiated in vitro we used ES cells transduced with retroviral vectors containing Lhx2. However, it was not possible to determine the specificity or efficiency of this event since the retroviral expression system was inefficient and gave unpredictable expression patterns upon differentiation into EBs (Laker et al., 1998; Pinto do et al., 1998). In addition, it was not clear whether Lhx2 expression was solely responsible for the expansion of hematopoietic progenitors or if mutagenesis due to retroviral insertion contributed to this process. Therefore, we introduced Lhx2 into ES cells (Ainv15) where expression is controlled by a Tet-on system (Kyba et al., 2002). Expression is induced by the addition of the tetracycline analogue doxycycline (dox), which binds the tet activator (rtTA) and enables induction of transcription from the tet response element (TRE) (Figure 6A). To ensure efficient expression upon differentiation, the rtTA and the TRE were inserted into the constitutively active ROSA26 and HPRT loci respectively (Gossen et al., 1995; Kyba et al., 2002; Wutz et al., 2002; Zambrowicz et al., 1997) (Figure 6B). Three ES cell lines with inducible expression were generated; a control cell line expressing GFP (Ainv15-GFP), a cell line expressing Lhx2 (Ainv15-Lhx2) and a third expressing Lhx2 together with GFP (Ainv15-Lhx2-GFP) (Figure 6B). Expression from the different cDNA constructs was efficiently turned on after adding dox to the culture media. The addition of dox to the control ES cells and hence inducing GFP expression did not interfere with any stage of differentiation. Thus, this system was well suited to elucidate the effects of Lhx2 expression during ES cell differentiation in vitro.

Lhx2 expression specifically induces self-renewal of a distinct hematopoietic progenitor/stem cell population in developing embryoid bodies (Paper I)

To further dissect the direct effect of Lhx2 on ES-derived hematopoietic progenitors, we allowed the ES cells to differentiate and induced Lhx2 expression in CFC-assays performed on EB cells at day 6 of differentiation when primitive and definitive hematopoietic progenitors are present in the EBs. Expression of Lhx2 led to a complete block of primitive erythroid colony formation as well as an approximately 3-fold increase in definitive colony formation (compare Figure 7A
and C). In addition, we observed little or no contribution of definitive erythroid cells in the definitive colonies that usually develops in the factor combination we used, indicating that Lhx2 expression could interfere with erythroid differentiation in general (Figure 8). However, the effect of Lhx2 was influenced by expression levels since only a marginal increase in responding definitive hematopoietic progenitors and a partial block in primitive erythroid colony formation was observed when the expression level of Lhx2 was lower (Ainv15-Lhx2-GFP cell line, expressing 50% less Lhx2). Thus, this shows that Lhx2 expression have different effects on hematopoietic progenitors within the EB depending on cell type and expression level.

Figure 6. Schematic view of the inducible ES system. A. Expression is induced by addition of doxycycline (dox) leading to binding of tet activator (rtTA) to the tet response element (TRE) and transcription of the inserted gene/s. B. Three cell lines were generated through CRE mediated insertion of cDNA for GFP, Lhx2 and Lhx2-GFP upstream of the HPRT locus. Upon insertion of the plox vector in the correct orientation, restored neo activity allowed for screening of correct clones.
The synergistic effect of Lhx2 on definitive colony formation was observed in most factor combinations including SCF. However, if SCF was excluded the effect was absent and the same was observed if the cells were placed in SCF alone (or together with epo), suggesting that activation of c-Kit is necessary but not sufficient for the synergistic effect of Lhx2 on colony formation. The least complex factor combination showing the most pronounced synergistic effect was SCF/IL-6, indicating that activation of the c-Kit and gp130 signaling pathways is necessary and sufficient to promote the synergistic effect of Lhx2 expression. Another member of the IL-6 family of growth factors that also promotes proliferation of immature hematopoietic progenitor cells is IL-11 (Musashi et al., 1991; Schibler et al., 1992). However, addition of IL-11 did not mimic the effect of IL-6, suggesting that only signaling via the IL6R through the gp130 signal transducer is efficient in these cells. We do not know whether this is an effect caused by specific signaling of gp130 and the IL6R or whether IL11R is not expressed in these progenitors. It has been suggested that gp130 signaling is independent of coreceptor type, since a combination of IL6 and soluble IL6R, that can activate gp130 signaling, substitutes the LIF receptor complex during ES cell self-renewal (Yoshida et al., 1994). These data suggest that lack of response to IL-11 would be due to lack of IL11R. Thus, Lhx2 acts specifically in synergy with c-Kit and gp130 signaling to promote definitive colony formation (Figure 7).

Due to the inefficient expression of Lhx2 from the retroviral expression system, it was not possible to determine whether the previously reported Lhx2-induced HSC-like cell lines originated from a progenitor normally responding to this factor combination or whether Lhx2 expression activates an additional progenitor (Pinto do, 2002). The synergistic effect on definitive hematopoietic progenitors in the inducible system suggested that Lhx2 activates an additional progenitor and that the increase in responding cells could correspond to cells able to generate HSC-like cell lines. We could confirm this since the efficiency of generating cell lines from randomly picked colonies in SCF/IL6/dox approximately corresponded to the increase in definitive colony formation (60-69%) (Figure 7C). However, none or very few cell lines could be established from CFC-assays with limited or no synergistic effect after dox addition (eg. SCF/epo/dox) or from cells expressing lower levels of Lhx2 (Ainv15-Lhx2-GFP). The cell lines were designated dox dependent HPC (doxHPC) lines and expressed cell surface markers (i.e. CD45+, C-Kit+, CD41+, lin-) similar to the HPC lines generated from retrovirally transduced ES cells (unpublished observation). Both SCF and IL-6 were required for generation of doxHPC-lines although only SCF was required for
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Figure 7. Lhx2 acts in synergy with SCF/IL-6 to induce proliferation of a distinct hematopoietic progenitor population. A. Definitive progenitors normally responding to SCF/IL-6 (light grey colonies) B. Lhx2 expression without addition of cytokines does not induce proliferation of hematopoietic progenitors C. SCF/IL-6 in combination with Lhx2 expression gives a 3.1 fold increase in responding definitive hematopoietic progenitors, corresponding to 65% of total CFCs (dark grey colonies). Expansion of randomly picked colonies led to generation of HSC-like cell lines from 60-69% of the colonies, corresponding to the increase in definitive CFCs.

survival after establishment. However, removal of IL-6 led to a temporary stall in growth for a few days and then resumed growth but at a lower growth rate. The resumed growth correlated to increased expression of endogenous IL-6 although the lower growth rate suggested that endogenous production of IL-6 could only partly compensate for the exogenously added IL-6. This shows that both SCF and IL-6 are required for generation and optimal maintenance of the doxHPCs. However, to elucidate whether also gp130 signaling is necessary for maintaining these cell lines, it would be needed to block gp130 signaling to overcome the problem of endogenous expression. Upon withdrawal of dox, the doxHPC lines lose their ability to form cobblestone areas and rapidly differentiate into various myeloid cells (macrophages, neutrophils, megakaryocytes and mast cells) (paper IV). Thus, Lhx2 expression induces self-renewal of a distinct SCF/IL-6 dependent multipotent definitive hematopoietic progenitor/stem cell leading to the generation and maintenance of HSC-like cell lines (Figure 7 and 8).

To try to exclude a possible effect of Lhx2 on earlier stages of hematopoietic development, such as the prehematopoietic mesoderm or the hemangioblast, CFC-assays were performed at different timepoints during EB development. However, the synergistic effect of Lhx2 was limited to timepoints when significant numbers of definitive hematopoietic progenitors are present in the
EB, starting at day 5 of differentiation and peaking at day 6 when the highest frequency of progenitors normally responding to this factor combination was present. This indicates that Lhx2 directly affects definitive multipotent hematopoietic progenitors rather than increasing hematopoietic commitment from the prehematopoietic mesoderm. Moreover, none of the ES-derived HSC-like cell lines (HPC-lines or doxHPCs, including those where Lhx2 was induced in day 3-4 EBs (see next section)), showed any primitive hematopoietic potential (i.e. commitment to primitive erythroid cells and/or responsiveness to VEGF) (Pinto do et al., 1998; Pinto do et al., 2001) (unpublished observations), lending further support to that Lhx2 expands hematopoietic progenitor cells that have lost their primitive hematopoietic potential.

The specificity of Lhx2 is unique so far compared to other factors tested in the ES system to date, such as HoxB4, Cdx4, Smad1, mMix1, BCR/ABL and Hox11, since the effects of these genes appear to be less specific and overexpression led to more general proliferation of hematopoietic progenitors of multiple lineages (Helgason et al., 1996; Keller et al., 1998; Kyba et al., 2002; Perlingeiro et al., 2001; Peters et al., 2001; Pilat et al., 2005; Wang et al., 2005; Willey et al., 2006; Zafonte et al., 2007). Moreover, of the above mentioned genes only Hox11 and BCR/ABL are capable of generating stable cell lines. However, the cell lines generated by Hox11 overexpression show various potential depending on the growth factor combinations used during their generation (Keller et al., 1998), while BCR/ABL expression generates cytokine independent hematopoietic progenitor cell lines that have limited differentiation potential due to transformation (McLaughlin et al., 1987; Peters et al., 2001). Therefore, the specificity and capacity for reproducible generation of HSC-like cell lines from ES cells is unique for Lhx2.

**Lhx2 affects the hematopoietic potential and development of embryoid bodies (Paper I)**

Self-renewal of hematopoietic progenitor cells during EB development is limited, since they rapidly decrease after day 6 of differentiation and are lost by day 8 in the absence of added cytokines. Thus, indicating that the increase in progenitors seen until day 6 is not due to self renewal of hematopoietic progenitor cells but rather due to generation of definitive hematopoietic progenitors from the prehematopoietic mesoderm. This suggests that the microenvironment within the EB is inefficient in expanding hematopoietic progenitors. Since Lhx2 is not
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expressed during normal EB development and has been associated with a cell nonautonomous microenvironmental defect in Lhx2-/- mutant fetal livers leading to severe anemia, we wanted to analyze whether Lhx2 expression in EBs could support expansion of hematopoietic progenitors within the EB. Addition of dox to day 3 or 4 EBs led to a 3-4 fold increase in CFCs responding to SCF/IL-6 at day 6. The continued expression of Lhx2 in CFC-assays led to a synergistic effect on colony formation as compared to if it was turned off in the CFC-assay, indicating that the Lhx2 responsive progenitor underwent self-renewal as well as differentiation within the EB. Moreover, expression of Lhx2 from day 4 of differentiation did not increase the frequency of generating cell lines from CFCs plated at day 6, since 65% of the colonies generated cell lines under these conditions, further supporting that these progenitors underwent self-renewal as well as differentiation in the EB.

To evaluate the capacity of Lhx2 to maintain progenitor activity in the EB after the time when they are normally exhausted, we also performed CFC-assays at day 7 and 8, revealing significant amounts of progenitors responding to SCF/IL-6/epo at these stages. This effect of Lhx2 expression, leading to expansion and maintenance of hematopoietic progenitors within the EB, could be caused by a cell autonomous effect of Lhx2 directly on hematopoietic progenitors. However, since Lhx2 mediates self-renewal of HSC-like cell lines through a cell nonautonomous mechanism and has been suggested to be involved in the expansion of the hematopoietic system in the fetal liver by controlling environmental factors (Porter et al., 1997), it is tempting to speculate that Lhx2 expression alters the microenvironment of the EB to permit expansion of hematopoietic progenitors. Since SCF is known to be expressed in EBs from an early stage while IL-6 is not (Schmitt et al., 1991), it is possible that Lhx2 expression leads to up-regulation of IL-6 thereby supporting expansion of hematopoietic progenitors. However, no increase in IL-6 expression or gp130 was detected by RT-PCR, neither was the expression of c-Kit or SCF affected, suggesting that this theory is unlikely. However, we can not exclude that other factors, including those signaling through gp130, could be involved.

To address the effect of Lhx2 on ES cells and in the earliest steps of EB development, we induced Lhx2 expression prior to onset of gastrulation (day 0-2), as determined by expression of Brachyury, a marker of primitive mesoderm (Keller et al., 1993; Wilkinson et al., 1990). Induction of Lhx2 expression during this time interfered with both EB formation and hematopoietic commitment (Figure 8), while Lhx2 expression from day 3 of differentiation (after onset of gastrulation) did
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not. The defective hematopoietic commitment could be caused by a specific effect of Lhx2 expression on the prehematopoietic mesoderm or due to interference with the induction of gastrulation, although this would require confirmation by studying development of endoderm and ectoderm in these cultures.

![Diagram of hematopoietic development](image)

**Figure 8. Model for the effects of Lhx2 expression during ES differentiation.** Lhx2 expression interferes with ES differentiation and hematopoietic mesoderm commitment if induced prior to onset of gastrulation, while expression during hematopoietic development blocks erythropoietic differentiation and leads to the generation of HSC-like cell lines (doxHPC lines) by inducing self renewal of a distinct definitive multipotent hematopoietic progenitor cell (DefHPC).


**Generation of HSC-like cell lines from adult BM (Paper II)**

The inability of HSCs of early embryonic origin (ES derived and early embryonic) to engraft in the adult system is well established (Toles et al., 1989; Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b). Therefore it is not surprising that HSC-like cell lines derived from ES cells are unable to engraft adult recipients (Pinto do et al., 1998) (unpublished observation). To investigate the effect of Lhx2 expression in adult hematopoietic progenitor/stem cells and to determine whether Lhx2 can generate cell lines with maintained engrafting potential, we introduced Lhx2 into 5-fluorouracil (5-FU) treated BM through retroviral transfer. In the presence of SCF or SCF/IL-6, clonal cell lines could be established denoted BM derived hematopoietic progenitor cell lines (BM-HPCs) (Figure 9). However, generation as well as growth of these cell lines was more efficient in SCF/IL-6 than
in SCF alone and BM-HPCs generated only in SCF differed slightly in respect to surface molecule expression pattern (Sca1\textsuperscript{low}) from those generated in SCF/IL-6. Furthermore, it was not possible to generate cell lines from ES cells with inducible Lhx2 expression in SCF alone (Paper I), indicating that additional events could be required for generation of HSC-like cell lines in SCF alone, such as mutations caused by the retroviral insertion.

The BM-HPCs expressed markers similar to adult HSCs (Sca-1\textsuperscript{+}, c-Kit\textsuperscript{+}, CD34\textsuperscript{-}, lin\textsuperscript{-/low}) (data not shown) and required high density cultures for survival, suggesting that the mechanism for Lhx2-induced expansion and self-renewal is similar for embryonic as well as adult hematopoietic progenitors/stem cells (Pinto do et al., 2001). Moreover, although the starting material, 5-FU treated BM, is a relatively heterogenous cell population, the different BM-HPC lines were homogeneous, indicating that Lhx2 targets a specific and rare hematopoietic progenitor/stem cell also in the BM, similar to that we have observed in EBs (Paper I). If Lhx2 had induced proliferation of any SCF-responsive progenitor/precursor we would have expected that individual cell lines would differ more from each other, representing a more diverse range of cell types, such as progenitors/precursors with erythroid, mast cell and megakaryocytic potential, since c-Kit is expressed by a wide range of hematopoietic progenitors/precursors (Broudy, 1997).

**BM-HPCs are multipotent and long term engraft stem cell deficient hosts (Paper II)**

The golden standard for elucidating the potential of hematopoietic cells in a sample is to analyze their function in vivo by transplantation studies. To be considered a true HSC the cell should be able to contribute to long term (i.e. > 16 weeks) multilineage repopulation after transplantation into irradiated recipients. However, a more efficient way to rapidly determine in vivo hematopoietic potential and the capability of generating functional hematopoietic cells is to analyze whether the cells can protect recipient mice from radiation induced death. Transplantation of high numbers of BM-HPCs (3×10\textsuperscript{6} cells) into lethally irradiated recipients provided some radioprotection (64%) while all mice receiving fewer cells died within 21 days. The high number of cells required to provide partial radioprotection could be due to lack of potential of the cells or that the cells are unable to produce mature progeny quickly enough to rescue the recipient. Analysis of the spleens of transplanted animals 10 days post transplantation revealed that BM-HPCs
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generated spleen colonies and that the frequency of CFU-S in these cell lines were between 1/150 and 1/250 cells, showing that a significant proportion of transplanted BM-HPCs enter hematopoietic organs and generate progeny shortly after transplantation. FACS analysis of the BM of surviving mice revealed contribution to the myeloid and lymphoid compartment, establishing that the BM-HPCs were truly multipotent i.e. capable of contributing to all lineages in vivo (Figure 9A). However, contribution to peripheral blood (PB) erythrocytes was lost approximately three months post transplantation and to BM shortly thereafter,

Figure 9. Generation and transplantation of BM-HPCs. C57BL/6-cast mice were treated with 5-FU. 5-FU treated bone marrow was cocultured with virus producing cells and expanded in SCF or SCF/IL-6. Lhx2 expressing cultures generated homogenous cell lines called BM-HPCs. No cell lines could be established by infection with control virus (MND-X-sn) A. BM-HPCs were transplanted into lethally irradiated C57BL/6-SJL and recipients were analyzed by FACS for multilineage contribution to hematopoietic organs. B. BM-HPCs were serially transplanted into sublethally irradiated HSC deficient recipients (C56BL/6-W41/W41) (1st primary, 2nd secondary and 3rd tertiary recipient), donor contribution (%) to peripheral blood was analyzed over time by glucose phosphate isomerase (gpi) analysis.
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indicating that the BM-HPCs were multipotent but unable to contribute to long term hematopoiesis in wt recipients, showing that BM-HPCs are multipotent and include cells with CFU-S and short term repopulation potential.

We wanted to analyze whether the loss of donor contribution was caused by lack of long term repopulation capacity or whether Lhx2 expression hampered the cells ability to compete with wt HSCs. To further address the long term repopulation potential of the BM-HPCs we therefore used a HSC deficient mouse model (W^{41}/W^{41}). These mice have a mutation in the c-Kit receptor leading to decreased signaling, thereby providing a less competitive environment for transplanted hematopoietic cells (Harrison and Astle, 1991). When transplanted into the W^{41}/W^{41} strain, the BM-HPCs were able contribute to hematopoiesis for >16 weeks as determined by contribution to PB (Figure 9B), suggesting that the lack of long term repopulation was caused by a competitive defect in our cells compared to normal HSCs. We followed contribution of BM-HPCs to PB for more than 18 months in serial transplantations, demonstrating a remarkable differentiation and self-renewal capacity of BM-HPCs in vivo (Figure 9B). Thus, BM-HPCs have the capacity for long term reconstitution of HSC deficient mice, but are unable to efficiently compete with the wt environment, indicating that these cells are HSC-like but less potent compared to unmanipulated HSCs. Nonetheless, BM-HPC lines are multipotent, have long term engraftment potential, generate CFU-S and confer some radioprotection upon transplantation.

It should be noted that high cell numbers (> 3×10^6 cells), the same as required to provide partial radioprotection, were needed to achieve long term engraftment in primary recipients, suggesting that 1 in 3×10^6 cells had long term repopulation potential. However, since HSC-like cell lines require high cell density for survival the reason could also be dilution effects post transplantation. Interestingly, upon transplantation into wt secondary recipients, BM-HPC derived cells contributed to hematopoiesis for more than three months, indicating that the cells had adapted in the primary recipients and increased their ability to compete with host cells. In agreement with this, the engrafting efficiency of donor cells was also higher in W^{41}/W^{41} secondary and tertiary recipients, since a lower number of transplanted cells could provide long term engraftment compared to primary transplantations of BM-HPCs. This indicates that cells capable of engrafting and generating mature progeny for long periods of time are selected during the first months post transplantation. This hypothesis could also explain the transient decrease in contribution seen after transplantation in most primary recipients (Figure 9B). Furthermore, the retroviral integration site remained the same in primary,
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secondary and tertiary recipients showing that the original cell transduced with Lhx2 and expanded for at least 10 weeks in culture, generated mature hematopoietic cells for over 18 months in vivo. However, despite maintenance of high Lhx2 expression in vivo (Paper III), we were unable to reestablish cell lines from BM of engrafted mice, suggesting that changes intrinsic to the BM-HPCs occur although the reason for these changes remains unknown.

**Lhx2 overexpression in vivo results in development of a chronic myeloproliferative disorder and anemia (Paper III)**

The mice engrafted with BM-HPC derived cells gave us an opportunity to further evaluate the consequences of Lhx2 expression in vivo, since expression was maintained at high levels after transplantation. Lhx2 is not expressed in normal hematopoietic cells but has been shown to be misexpressed in human CML cells (Wu and Minden, 1997). CML is caused by a translocation between chromosome 9 and 22 leading to the fusion of Bcr and c-Abl, causing the expression of a tyrosine kinase fusion protein (BCR-ABL) with increased kinase activity (Deininger et al., 2000). The Lhx2 gene is located close to c-Abl on chromosome 9 and it was therefore suggested that Lhx2 expression in CML could be a consequence of cis-acting effects caused by the reciprocal translocation site (Wu and Minden, 1997). Interestingly, animals engrafted with BM-HPCs developed a chronic myeloproliferative disorder characterized by splenomegaly with disrupted splenic architecture, myeloid cell accumulation and extramedullary hematopoiesis, fulfilling the criteria for myeloproliferation (genetic) in mouse (Kogan et al., 2002). Malignant transformation was seen in one group of secondary recipients (9/62 animals, 15%) transplanted with cells from the same primary donor, strongly suggesting that the malignant transformation occurred in the primary recipient and not as independent events in the respective secondary recipients. The relatively rare development of blast crisis and long chronic phase indicated that this event was caused by additional mutation(s) in that one primary recipient, rather than as a direct effect of Lhx2 expression. Therefore, we conclude that engrafted mice develop a CMD similar to the chronic phase of human CML. Interestingly, Lhx2 was found to be expressed in most pre-B and pre-T cell lines immortalized by v-Abl, an oncogenic form of c-Abl (Moyer and Graves, 1981; Wu and Minden, 1997; Xu et al., 1993; Yancopoulos et al., 1984). This suggests that Lhx2 expression in CML cells and v-Abl transformed lymphoid cells could be due to the activity of the truncated ABL protein encoded by BCR-ABL and v-Abl.
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All engrafted mice suffered from anemia due to low red blood cell (RBC) counts independent of the donor contribution level to PB (0-100%). Despite the highly variable contribution to PB, engrafted mice displayed similar contribution to BM, indicating that loss of PB contribution was not caused by depletion of progenitors/stem cells (Paper II). Mice with high donor contribution to circulating erythrocytes also displayed low hemoglobin concentration (Hb) (Paper III). However, the reduction in RBC counts did not account for the entire decrease in Hb. Further tests revealed that the Hb concentration per cell (mean corpuscular hemoglobin concentration, MCHC) was low compared to controls, indicating a defect on the single cell level. The reduced RBC counts together with the reduction in MCHC explained the low Hb detected in these mice. Although the specific cause for this defect is unknown it is reasonable to assume that Lhx2 overexpression interferes with both erythroid differentiation and hemoglobin expression. This is also confirmed by the block caused by Lhx2 expression in erythrocyte development that we observed upon differentiation of ES cells with inducible Lhx2 expression (Paper I). Since several LIM proteins and LIM binding proteins, such as LIM binding protein 1, (ldb1) and LIM domain only 2 (LMO2), are involved in erythropoiesis (Valge-Archer et al., 1994; Warren et al., 1994; Visvader et al., 1997), it is highly likely that Lhx2 could physically interact and compete with these proteins or binding partners and hence act in a dominant negative fashion. Certainly, since Lhx2 is a transcription factor, it may also influence the expression of genes important for erythropoiesis or disturb this process in other ways that could explain the cell autonomous defect in erythrocyte development. In addition, CML is often associated with anemia, although BCR-ABL has been shown to promote erythropoiesis and can functionally replace the Epo receptor (Eaves et al., 1998; Ghaffari et al., 2001; Ghaffari et al., 1999), suggesting that other genes that interfere with erythropoiesis in a cell autonomous manner could be expressed in CML and thereby cause the anemia, similar to Lhx2.

The potential gene therapy candidate, HoxB4, which is known for its remarkable potential to expand hematopoietic stem cells in vitro has been reported to induce a similar type of CMD as we have seen with Lhx2 (Milsom et al., 2005; Pilat et al., 2005; Schiedlmeyer et al., 2003). This undesirable effect has been suggested to be due to high expression levels of HoxB4 causing perturbed differentiation, suggesting a limited therapeutic window for this gene (Klump et al., 2005; Schiedlmeyer et al., 2003). The effect of Lhx2 was also shown to be determined by Lhx2 expression levels in the ES system (Paper I), suggesting that the expression level of Lhx2 could also be a factor in the development and severity
of disease in mice engrafted with HSC-like cell lines, as donor derived cells expressed high levels of Lhx2 in vivo.

**HSC-like cell lines represent a relevant tool for elucidation of molecular mechanisms involved in HSC and organ expansion (Paper I, II, III)**

Lhx2 expression in hematopoietic tissue derived from both adult and embryonic sources leads to the generation of HSC-like cell lines that resemble the normal HSC population from their respective ontogenic origin (Pinto do et al., 1998), Paper I, II, IV). In addition, Lhx2 expression does not prevent differentiation of these cell lines when subjected to conditions inducing differentiation. The putative target cell of Lhx2 seems to be quite specific, since the resulting cell lines are functionally and phenotypically homogeneous and share similar growth requirements despite the heterogeneous starting populations (EBs and BM). These features of HSC-like cell lines make them unique in comparison with other reported hematopoietic stem cell-like cell lines, since those cell lines either provided limited contribution in vivo (or not reported) or was generated through unknown or less specific events (Baba et al., 2006; Reya et al., 2003; Spooncer et al., 1984; Tsai et al., 1994b; Varnum-Finney et al., 2000; Wong et al., 1994; Yanai et al., 1999).

All HSC-like cell lines generated by Lhx2 expression independent of origin are dependent on SCF or SCF/IL-6 for generation and maintenance. It is well established that signaling via gp130 and c-kit is efficiently inducing proliferation of HSCs including those of human origin (Audet et al., 2001; Bernad et al., 1994; Kollet et al., 1999; Oostendorp et al., 2000; Peters et al., 1997; Sui et al., 1995). Moreover, several other hematopoietic stem cell-like cell lines established previously by the expression of constitutively active Notch1, β-catenin or a dominant negative retinoic acid receptor, are also dependent on SCF or SCF/IL-6 as a minimal growth and survival requirement (Baba et al., 2006; Tsai et al., 1994b; Varnum-Finney et al., 2000). This suggests that signaling from c-Kit and gp130 is important in combination with other signals to support HSC self-renewal in vitro.

The HSC-like cell lines appear to self renew by a cell nonautonomous mechanism (Pinto do et al., 2001) (Paper II). This is in agreement with the cell nonautonomous hematopoietic defect observed in Lhx2 knockout mice (Porter et al., 1997), and would suggest a role for Lhx2 in the expansion of the hematopoietic
system in fetal liver by controlling expression of secreted factors and/or other factors involved in cell-cell interactions. In addition, we also observed that severely enlarged spleens from mice engrafted with BM-HPCs could have relatively low donor contribution (30%) (Paper III), indicating that BM-HPC derived cells influenced the recipient hematopoietic system through cell non-autonomous mechanism/s leading to recruitment/expansion of recipient hematopoietic cells. This further indicates that HSC-like cell lines secrete molecules relevant in expansion of hematopoietic progenitor/stem cells. In addition to this, Lhx2 has been suggested to be involved in the expansion of several other organ systems during development such as the liver, the forebrain, olfactory epithelium and limb (Hirot a and Mombaerts, 2004; Kolterud et al., 2004a; Porter et al., 1997; Rodriguez-Esteban et al., 1998; Wandzioch et al., 2004), as well as being important for the regulation of hair follicle stem cell maintenance (Rhee et al., 2006). This suggests a more general role for Lhx2 in regulation of stem cell populations and in the expansion of organs during development. Elucidation of the molecular mechanism for Lhx2 induced self-renewal of HSC-like cell lines could give insight into the cell nonautonomous defect observed in Lhx2 null mutants and give further insights into the control of self-renewal of HSCs and the expansion of organs during development, thereby potentially providing important information for a variety of clinical applications.

**DoxHPCs provide a system for analyzing Lhx2 target genes (Paper IV)**

Lhx2 was the factor solely responsible for generation and self-renewal of doxHPC lines and expression was efficiently down-regulated after dox withdrawal, making this system suitable for global gene expression analysis. This gave us the opportunity to compare a very homogenous population of undifferentiated cells to their immediate progeny by only changing one parameter, and to identify genes indirectly and directly regulated by Lhx2 as well as genes potentially important for both HSC physiology and pathology. Global gene expression analysis was performed using cDNA arrays comprising 14 121 clones originating from cDNA libraries generated from mouse brain lateral ventricle wall, neurospheres and BM-HPC clone 5 (Paper II) (Williams et al., 2006). Two cell lines were chosen for comparison, a high Lhx2 expressing cell line (doxHPC1, Lhx2) and a low expressing cell line (doxHPC7, Lhx2-GFP). The expression level of Lhx2 in doxHPC1 was 2.7 fold higher (M value 1.43, comparison E) compared to
doxHPC7, although they were similar in terms self-renewal, surface phenotype and differentiation capacity. Since these cell lines differ from each other in Lhx2 expression but still share all the characteristics of HSC-like cell lines, we hypothesized that the comparison between these cell lines would detect the genes most important for Lhx2 function. cDNA from doxHPC1 and 7 isolated at 36, 72 and 96h after dox withdrawal, was compared to cDNA from Lhx2 expressing cells (0h), in order to analyze changes in gene expression early after Lhx2 down regulation and prior to the emergence of mature myeloid cells (Figure 10A). The data for doxHPC1 and doxHPC7 were combined to identify genes differentially expressed upon Lhx2 down regulation and consequently differentiation. 365 genes at 36h, 535 genes at 72h and 528 genes at 96h were differentially expressed, 267 of these genes were shared between all three time points (141 up-regulated and 126 down-regulated) (cutoff : p>0.01 and M<0.4) (Figure 10B). Among these, Lhx2 was the most down-regulated gene confirming the efficiency of this inducible system. Moreover, of the 50 most down-regulated genes (shared at all three time points) 39 were more highly expressed in doxHPC1 line compared to doxHPC7 (comparison E, Figure 10) (see additional files 1 and 2 in paper IV), showing a
Correlation between the level of Lhx2 expression and the expression levels of these 39 genes. However, if this correlation reveals genes directly regulated by Lhx2 or merely reflects differentiation status of the respective doxHPC lines remains to be elucidated.

**Global gene expression analysis of doxHPC gene expression reveals a stem cell like molecular signature (Paper IV)**

Differentially expressed genes obtained from the cDNA arrays were compared to other studies performed on purified hematopoietic stem cell populations and differentiated progeny. Ivanova et al. compared seven subsets of BM derived HSCs, progenitors and mature blood cell populations (i, LT-HSC, ii, LT-HSC and ST-HSC iii, LT-HSC, ST-HSC and early progenitors, iv, early progenitors, v, intermediate progenitors, vi, late progenitors, vii, mature blood cells) (Ivanova et al., 2002), while Ramalho-Santos et al. compared the SP⁺/c-Kit⁺/Sca-1⁻ HSC subset to total BM (Ramalho-Santos et al., 2002). The Ivanova as well as the Ramalho-Santos studies were performed using Affymetrix oligonucleotide arrays. When our differentially expressed genes were compared to the HSC specific genes obtained in these studies (Ivanova groups i-iv), 21% overlapped with the Ramalho-Santos study, 17% with the Ivanova study and 9% with both, indicating an overlap although limited. However, it should be noted that the overlap between the probes spotted on our array compared to the Affymetrix arrays used in the Ivanova and Ramalho-Santos studies was only 50% and 57% respectively. In addition, when the HSC enriched gene subsets from the Ivanova and Ramalho-Santos studies were compared to a third study using similar material and methods, only 13% of the HSC enriched genes were shared between all three studies and 34% were shared between at least two (Zhong et al., 2005), suggesting that array studies on BM derived HSCs show limited overlap between separate studies, despite similar platforms (Affymetrix), cell populations and the same mouse strain. Moreover, most of the genes that overlapped between our differentially expressed genes and the HSC enriched subsets were down-regulated upon Dox withdrawal (58% and 65%), as might be expected since the doxHPC lines are HSC-like and loss of Lhx2 expression leads to differentiation. In agreement with this, most genes overlapping with more differentiated cells (Ivanova group v-vii) were up-regulated (80%) after Lhx2 down-regulation. Collectively, these data indicate that there is an overlap between doxHPCs and HSCs also on the molecular level. Thus, suggesting that
these cell lines could serve as a relevant in vitro model system for HSCs on the molecular as well as cellular level.

**Genes and pathways identified in HSC-like cell lines with relevance for HSC physiology and pathology (Paper IV)**

Several studies have implicated the importance of the Notch, Wnt, Hedgehog and BMP signaling pathways for HSC maintenance and differentiation (Baba et al., 2006; Bhardwaj et al., 2001; Bhatia et al., 1999; Reya et al., 2003; Varnum-Finney et al., 2000). Some of these pathways are also affected by Lhx2 down regulation in doxHPCs, suggesting a role in the regulation of doxHPC maintenance and self-renewal.

Wnt signaling has been proposed to play a role in HSC self-renewal since Wnt3a and constitutively active β-catenin signaling have been reported to induce self-renewal of HSCs (Baba et al., 2006; Reya et al., 2003). However, the role of β-catenin signaling in HSCs is not entirely clear, since other studies have shown that expression of constitutively active β-catenin leads to a block in differentiation as well as compromised HSC maintenance (Kirstetter et al., 2006; Scheller et al., 2006). In our study the down-regulated β-catenin binding protein (catnbip, ICAT) provided a link to the Wnt signaling pathway, since ICAT is known to interfere with β-catenin and inhibit Wnt signaling (Tago et al., 2000). An earlier study in our lab showed that another Wnt inhibitor, dickkopf (Dkk1), also was down-regulated in doxHPCs as Lhx2 expression was turned off (Kolterud, 2004). In addition, Dkk1 was shown to be expressed in fetal liver and that expression was down-regulated in Lhx2 knockouts. In agreement with this, it has been shown that active Wnt/β-catenin signaling is almost completely absent in fetal liver (E9.5-14), based on analyses of mice with a reporter construct controlled by β-catenin/Tcf/Lef responsive elements (Kolterud, 2004). Furthermore, β-catenin expression was not detected in hematopoietic cells in the early fetal liver (Monga et al., 2003). Collectively, these data suggest that inhibition of the canonical Wnt signaling pathway through Dkk1 and other inhibitors could be involved in HSC expansion in the fetal liver as well as in maintenance/self renewal of HSC-like cell lines. However, the lack of β-catenin signaling in fetal liver does not exclude that non-canonical Wnt signaling pathways are active. This has indeed been indicated by global gene expression analysis of fetal liver vs. BM derived stromal cells where the expression of non-canonical signaling molecules, such as Wnt5A, was increased in fetal liver derived cells (Martin and Bhatia, 2005). It has also been
shown that Wnt5A has a proliferative effect on fetal liver derived HSCs (Austin et al., 1997). In addition, inhibition of the canonical Wnt signaling pathway via Dkk1 promoted self-renewal of BM derived mesenchymal stem cells in vitro, indicating a role in supporting proliferation of stem cells (Gregory et al., 2003). Furthermore, a member of the Sox family of transcription factors, Sox4, was down-regulated upon dox removal. The Sox family of transcription factors is a subfamily of the HMG (High Mobility Group) family also including Tcf/Lef which is a major player in the canonical Wnt signaling pathway (Laudet et al., 1993). Interestingly, some Sox proteins have been suggested to modulate β-catenin/Tcf/Lef induced transcriptional activation through interaction with β-catenin, suggesting a potential role for Sox4 in modulating the transcriptional response to Wnt signals (Zorn et al., 1999). Thus, Wnt regulators could be important for regulating self-renewal of HSC-like cell lines and the secreted factor, Dkk-1, could be a mediator of Lhx2 function in the fetal liver.

BMP signaling has been implicated in HSC maintenance in vitro, since BMP4 can act as a survival factor for human HSCs when added at high concentrations in liquid culture (Bhatia et al., 1999). Twisted gastrulation (Twsg), a gene that can act both as a BMP agonist and antagonist was down-regulated after dox withdrawal. Twsg has also been identified as a HSC-enriched gene in earlier studies (Ramalho-Santos et al., 2002). The role of Twsg in HSC-like cell lines is not clear although this is an indication that regulation of BMP signaling could be important for self-renewal and/or survival of these cell lines. The BMP effect on human HSCs was suggested to be mediated by Shh signaling since blocking Shh signaling interfered with the function of BMP4 on HSC maintenance (Bhardwaj et al., 2001). Smoothened (Smo), a transmembrane protein involved in sonic hedgehog (Shh) signaling and expressed in HSCs (Ivanova et al., 2002; Ramalho-Santos et al., 2002), was one of the transcripts down-regulated in doxHPCs after dox removal, suggesting that Shh mediated signaling could be important for the HSC-like cell lines. However, we could not detect any expression of the Hh ligands (Sonic, Indian or Desert hedgehog) in doxHPCs (data not shown), indicating that this pathway does not significantly influence these cell lines.

Thus, several molecules involved in signaling pathways known to control HSC self-renewal and survival are differentially expressed when Lhx2 expression is down-regulated, suggesting that these pathways might play important roles in self-renewal and maintenance of doxHPCs. Moreover, these pathways mediate signals from the environment by means of soluble factors and could therefore be directly involved in the cell nonautonomous mechanism controlling self-renewal of
HSC-like cell lines as well as being involved in Lhx2 regulation of the fetal liver microenvironment.

**Lhx2 target genes in HSC-like cell lines provide information about Lhx2 function in non-hematopoietic cells and disease (Paper IV)**

Lhx2 has been shown to be important for stem cell function and organ development of various tissues, such as in forebrain, olfactory epithelium, liver, and hair follicles (Hirota and Mombaerts, 2004; Kolterud et al., 2004a; Porter et al., 1997; Rhee et al., 2006; Wandzioch et al., 2004). We found that a number of genes putatively regulated by Lhx2 (or stem cell specific genes in our study) had partly overlapping expression patterns with Lhx2 in various organs (see Table 2, additional file 1, Fig. 5 in Paper IV). This suggests that the mechanism for Lhx2 in generation and maintenance of HSC-like cell lines might overlap with the function of Lhx2 in the development of a variety of organs. In agreement with this, a number of differentially expressed genes involved in processes related to Lhx2 function in other organs as well as in disease were identified and some examples are listed below.

**Liver:** Several differentially expressed genes were identified with functions related to the fibrotic liver phenotype in Lhx2-/- embryos. Loss of Lhx2 expression has been shown to lead to continuous activation of hepatic stellate cells (Wandzioch et al., 2004), that is the major cell type involved in the development of liver fibrosis (Friedman, 2000). CSRP2 that was down-regulated with loss of Lhx2 expression, encodes a LIM domain protein expressed in hepatic stellate cells and implicated in negative regulation of their activation. This suggests that CSRP2 could be downstream of Lhx2 in the negative regulation of hepatic stellate cell activation (Weiskirchen et al., 2001). Another down-regulated gene with an indirect effect on this process is Autotaxin (Enpp2), an extracellular enzyme that is involved in producing lysophosphatidic acid (LPA). LPA is an intercellular lipid growth factor/mediator with a wide variety of biological actions, such as induction of cell proliferation, migration and survival (van Meeteren and Moolenaar, 2007). Interestingly, LPA also plays a role in hepatic injury and the proliferation and migration of hepatic stellate cells, although the precise mechanism is not entirely clear (Svetlov et al., 2002). A gene that is not directly involved in the activation of hepatic stellate cells but might be involved in the fibrosis process by stabilizing
collagen fibrils is the proline-4-hydroxylase (P4ha1) which was up-regulated when Lhx2 expression was turned off. P4ha1 is critical for posttranslational modifications of collagen, leading to increased stability of collagen fibrils. P4ha1 is also up-regulated in fibrotic Lhx2\textsuperscript{-/-} fetal livers when compared to normal fetal liver (Wandzioch et al., 2004). The differential expression of these genes after Lhx2 down regulation suggests that this system also uncovers genes that might play a role for the development of liver fibrosis in Lhx2 null mice.

**Forebrain:** Lhx2 null mice have severely malformed forebrains as well as anophtalmia and several differentially expressed genes have been identified that relates to these defects. The knockouts for the Wnt and BMP regulators, ICAT and Twsg1, have been shown to display defects in neural development. ICAT\textsuperscript{-/-} embryos are anophthalmic and exhibit malformation of the forebrain as well as having additional problems, such as abnormal development of craniofacial bones (Satoh et al., 2004). In addition, it has also been shown that ICAT induce forebrain cell character by inhibiting Wnt signaling in differentiating ES cells (Satoh et al., 2004), suggesting a link between the inhibition of Wnt signaling and Lhx2 in the formation of forebrain and eye. Twsg has also been suggested to play a role in forebrain specification as indicated by experiments in *X. tropicalis* (Wills et al., 2006). However, the phenotypes of Twsg mutant mice are strain dependent, although some strains display defects in forebrain and cranio-facial development, indicating that there could be functional redundancy between Twsg and other factors in mice (Petryk et al., 2004). Tle6 is a groucho related protein that was up-regulated upon dox withdrawal from doxHPCs. Tle6 is involved in regulation of neurogenesis in the forebrain by inhibiting the activity of the transcriptional repressor FoxG1. The inhibition of FoxG1 led to decreased progenitor proliferation and increased differentiation into postmitotic neurons (Marcal et al., 2005). This suggests that Tle-6 could be deregulated in the Lhx2 knockout and thereby contribute to the proliferative defect in forebrain development.

**Dysregulation of hematopoiesis:** Since Lhx2 was shown to be expressed in human CML cells and mice engrafted with BM-HPCs developed a CMD-like disorder (Wu and Minden, 1997) (Paper III), this approach might also identify differentially expressed genes with functions related to the development of hematological disorders. The down-regulated gene encoding protein kinase D2 (Prkd2) has been shown to be expressed in HSCs as well as being a target of the BCR-ABL kinase activity in CML leading to activation of NFkappaB (Mihailovic
et al., 2004; Ramalho-Santos et al., 2002). Activated NFkappaB contributes to transformation and blockage of apoptosis in CML cells (Chopra et al., 1999), thereby indicating a role for Prkd2 in disease progression. Since Lhx2 has been shown to be expressed in human CML (Wu and Minden, 1997), and potentially regulates the expression of Prkd2 this could suggest a link between Lhx2 and BCR-ABL in the progression of CML. The previously mentioned transcriptional activator Sox4 is expressed in HSCs and has been shown to be important for B-cell development (Ivanova et al., 2002; Schilham et al., 1996). Sox4 has also been implicated in the development of malignancies, such as murine myeloid leukemias and splenic marginal zone lymphomas, as a consequence of retroviral insertional mutagenesis (Li et al., 1999; Shin et al., 2004). However, since Lhx2 is inserted at a specific location upstream of the HPRT locus, Sox4 expression in doxHPCs is likely to relate to Lhx2 and/or stem cell function in these cell lines. Both Sox4 and Prkd2 are expressed in normal stem cells (Ivanova et al., 2002; Ramalho-Santos et al., 2002), although aberrant expression leads to increased risk of developing hematological disorders. Sox4 and Prkd2 are expressed at higher levels in doxHPC1 (high Lhx2) compared doxHPC7 (low Lhx2) (comparison E, Figure 10) indicating that the expression of these genes could be correlated to Lhx2 expression level. This suggests that these genes could contribute to the CMD-like disorder in BM-HPC engrafted mice, since Lhx2 levels were maintained at a high level in vivo (Paper II).

In conclusion, expression analysis of doxHPCs has revealed a number of genes with interesting functions in a variety of processes related to Lhx2 and HSC function. This indicates that these cell lines could be a valuable tool for analyzing the mechanism for Lhx2 in generating and maintaining HSC-like cell lines, as well as for the study of HSC physiology and pathology. Since a number of differentially expressed genes involved in processes related to Lhx2 function in other organs were identified, it seems likely that the molecular mechanism and function of Lhx2 overlap between HSC-like cell lines and these processes. Therefore, the analysis of the molecular mechanism for Lhx2 in the doxHPC lines might generate information important for understanding the role of Lhx2 in other organs. In addition, the identification of the molecule/s responsible for self-renewal of HSC-like cell lines could potentially provide information about HSC expansion in the fetal liver and thereby provide clues about the regulation of HSC self-renewal. However, further characterization of potential Lhx2 effector genes will require extensive in vitro and in vivo studies of individual candidate genes.
CONCLUSIONS

- Lhx2 expression influence a specific subset of hematopoietic progenitor cells from both adult and embryonic sources and continued expression lead to the generation of HSC-like cell lines in the presence of specific cytokines

- Lhx2 in the presence of SCF/IL-6 (eg. c-Kit and gp130 signaling) is both necessary and sufficient for the generation of HSC-like cell lines

- BM-HPC lines are able to engraft and long term repopulate HSC deficient mice and contribute to all hematopoietic lineages in vivo

- Engrafted mice maintain high Lhx2 expression leading to the development of a chronic myeloid disorder similar to human CML, suggesting a connection between aberrant Lhx2 expression and this disease

- Molecular analysis of HSC-like cell lines with inducible Lhx2 expression suggest that these cell lines share gene expression patterns and hence regulation of important signaling pathways with normal HSCs

- Analysis of molecular mechanisms for Lhx2 in HSC-like cell lines has revealed putative connections to Lhx2 function in other organ systems and disease
ACKNOWLEDGEMENTS

First of all I wish to thank my supervisor Leif Carlsson for offering this opportunity to do science and to learn in your lab as well as for your patience and optimism when my own was not enough. Thank you for always keeping your door open and for many interesting and philosophical discussions around the field of hematology and other things, I will miss them. Thank you for believing in me!

The lab that has been changing over the years, but always felt like a second home. All the LC girls for being such great friends, discussion partners and most importantly for “pulka” days and strange dinners. I just can’t believe it was such a long time ago. Perpetua, I miss you still, maybe not the 18h shifts, but everything else, I will stand on your doorstep any day now! Ewa, for your young mind and for making the “old ladies” do crazy things! You are the true scientist, always keeping your focus on the money, I wait for the big breakthrough. Åsa, for being the calm one... (although “tokrolig”) in the lab, and for putting things in perspective. Anna-Carin, I love lunch time and fighting for the teabag. Your “it’s the way it is” mentality is to die for. Lina, my partner in colony counting and “modeguru”, although you haven’t managed to turn me around just yet! Good luck with everything!! Gunilla, your heading my way...it’s doable that’s all I can say! Shizuko, thank you for sushi recipies and baby walks. Good luck with your “new” carrier! We’ll meet again...! Jörgen, finally a true FACS MAN in the lab, it has been a shortage of both...!! Anna, for bringing new energy to the old lab. Christofer, Maria, Ludmilla, Artur, Markko, Anna, Lars, Maria N and all the other people who passed through the lab and made it such a great place to work in!

The department of molecular biology and all the people in “bunkern” in the good old days, it seems quite hazy at this time….wonder why!

The people in UCMM, grp HE, UA och TE for for the fantastic environment you all contribute to. Thank you for “fika” when it’s really needed and for the “friendly” harsch jokes that always keeps you on your toes. I don’t think there are many places like this!

My, thank you for going first!! It wouldn’t have been the same to do it alone! Kelly, for taking the time and energy to read my thesis and for sorting out the worst language mistakes. Anna R, for finding the energy to have those extra guests for dinner. The next dinner is on me...! UCMM book club, for off work meetings and exciting conversations. I hope I still possess chair no1 although I was let free...

PA for your patient, happy mood despite last minute orders and for being the central person in the fika corner. Kristina, my nextdoor neighbour these last months, thank you for all the help and hope my sighing did not disturb you too much! Elisabeth at the animal facility, for your help and for always finding that last cage that I forgot to order. Marita, Berit and Anita for helping me through the administrative work.
Gamla vänner: Maria, Lovisa och Elin och era familjer, för att ni är de bästa som finns. Ni är alltid med mig oavsett var i världen ni finns. Tack för allt stöd genom åren och för att ni delar det fantastiska mamma livet med mig!

Johanna och Emma, för att ni hjälpt mig hålla kontakten med det ”riktiga” livet som väntar där ute. Jag kommer verkligen sakna er och våra ”pluggkvällar” nu när jag skall tillbaka igen, önskar att vi kunde fått göra det tillsammans!

Sophie, Göran och Love för att ni finns där! Sophie, tack för att du stöttade mig och förstod när ingen annan fanns! Jenny, ser fram emot att få vara mamma tillsammans med dig igen. Tack för många mysiga eftermiddagar (...och förmiddagar för den delen) i ditt kök och för att hitta nya fantastiska vänner på ”gamla” dar.

Tack alla gamla och nya vänner som det inte finns plats för att nämna vid namn, ni vet vilka ni är och att ni har betytt så mycket för mig under alla dessa år!

Min familj för att ni alltid finns och för att vi håller ihop när det blåser hårt! Älskade Mamma, för tidiga telefonmorgnar och för ditt unga sinne...bli aldrig gammal! Lene, storasyster, som jag alltid ringer när jag behöver stöd. Elisabeth, roliga, galna lillasyster, tack för att du muntrar upp mig när det verkligen behövs! Andreas, för att du är du och för alla löften om att komma upp...vi väntar fortfarande! Gustav, för att du sticker ut och kommer med nya influenser, kämpa på! Pappa, jag önskar att du fanns med mig, livet är så kort....

Magnus, det finns inte ord för vad du betyder för mig, tack för att du har funnits med mig i alla dessa år. Det nya livet börjar nu! Jag ålskar dig Ylva, min älskade lilla dotter som jag har haft turen att få lära känna. Din ankomst gav mig nya perspektiv på livet, inget blir någonsin detsamma! Tänk att du finns och att det är en till på väg......jag långtar efter att spendera resten av mitt liv tillsammans med er!


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