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The Role of Hox Transcription Factors in the Regulation of Hematopoiesis

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With the approval of the Lund University Faculty of Medicine,
this thesis will be defended on February 23, 2007, at 13.00 in the Segerfalk lecture hall,
Wallenberg Neurocentrum, Sölvegatan 17, Lund

Faculty opponent
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LUND UNIVERSITY
Faculty of Medicine

To my parents

On the cover:
Scanning electron microscope image from normal circulating human blood.
Photographers: Bruce Wetzel and Harry Schafer,
Source: National Cancer Institute.
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Abstract Hematopoiesis is a lifelong, dynamic process, originating from a low number of hematopoietic stem cells (HSCs) residing in the adult bone marrow with the ability to self-renew and generate all blood lineages throughout life. Recent findings have demonstrated that the Homeobox (Hox) transcription factors are important regulators of both normal and malignant hematopoiesis, controlling proliferation, differentiation and self-renewal of hematopoietic cells at different levels of the hematopoietic hierarchy. This thesis focuses on the role of HOXA10 and Hoxb4, and its interaction with Hoxb3, Hoxa9 and the cell cycle regulator p21 in hematopoiesis. These Hox-genes are all expressed in the HSC compartment, and are then downregulated upon differentiation. In support of this, overexpression of HOXB4 is known to strongly increase HSCs self-renewal in vivo and in vitro. To investigate the physiological role of Hoxb4 we generated a Hoxb4 and a Hoxb3/Hoxb4 knockout mouse model and found that HSCs lacking Hoxb4 exhibit a reduction in their proliferative response to hematopoietic stress. This phenotype was slightly enhanced by the additional deletion of the neighboring gene Hoxb3, demonstrating redundancy of function between these genes. We further explored the complex interactions within the Hox clusters by generating a triple knockout lacking Hoxa9/b3/b4. However, the reconstitution ability of Hoxa9/b3/b4 knockout HSCs did not decline beyond the repopulating defect seen in Hoxa9 knockout HSCs. Using retroviral gene transfer we found that overexpression of HOXB4 in p21 deficient HSC further enhanced the HSCs self-renewal effect in vitro, demonstrating that temporary overexpression of HOXB4 and suppression of p21 could be useful for future HSC expansion protocols. The expression level of HOXB4 is crucial for the HSC fate decisions and to delineate the role of HOXA10 in hematopoiesis, we generated an inducible mouse model for HOXA10. Our findings from this study demonstrate that HOXA10 acts as a master regulator of hematopoiesis governing both proliferation and differentiation of hematopoietic progenitor and stem cells, through direct regulation of the genes Gfi-1, Dkk-1, HLF and Gata-1, where distinct fate outcomes depend on the HOXA10 concentration. In summary, our findings unravel new interactions and molecular pathways acting within, or downstream of Hox genes to regulate hematopoietic progenitor and stem cells.		
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List of abbreviation

5-FU	5-fluorouracil
ABC	ATP binding cassette
AGM	aorta-gonad-mesonephros region
AML	acute myeloid leukemia
AML1	acute myeloid leukemia 1
ANG1	angiopoetin1
Ant-C	antennapedia
Bmi1	B lymphoma Mo-MLV insertion region 1
BMP	bone morphogenic proteins
Bx-C	bithorax genes
C/EBP α	CCAAT/enhancer binding protein
CAFC	cobblestone area forming cell
CaR	calcium-sensing receptor
CB	cord blood
CFU-S	colony forming unit of the spleen
CDKI	cyclin-dependent kinas inhibitors
c-kit	receptor for c-kit ligand/SCF
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
c-Myb	myeloblastosis viral oncogene homolog
c-Myc	myelocytomatosis oncogene
CRU	competitive repopulating units
Cybb	phagocyte burst oxidase protein gp91phox
DBA	diamond blackfan anemia
Dkk-1	dickkopf homolog 1
DNA	deoxyribonucleic acid
E	embryonic day
EBF	early B-cell factor 1
ECM	extracellular matrix
Ezh2	enhancer of zeste homolog 2
FACS	Fluorescence Activated Cell Sorter
FG	phenylalanine glycine
FLT3	fms-related tyrosine kinase 3
FOG-1	friend of gata-1
Gfi-1	growth factor independent-1
GMP	granulocyte macrophage progenitors
HDAC	histone deacetylase
HES-1	hairly and Enhancer of Split homolog-1
HLA	human leukocyte antigens
HLF	hepatic leukemia factor
Hox	Homeobox genes
HSC	hematopoietic stem cells
HxRE	Hox response element
Lin	lineage
LMO2	LIM domain only 2
LoxP	locus of crossover of P1
LPM	lateral plate mesoderm

LSK	lin ⁻ , c-kit ⁺ , Sca ⁺
LTC-IC	long-term culture-initiating cell
LT-HSC	long-term HSC
Meis	myeloid ecotropic viral integration site
Mel18	polycomb group ring finger 6
MEP	megakaryocyte erythroid progenitor
MDR	multidrug resistance
miRNA	microRNA
Mll	mixed leukemia factor
MPP	multipotent progenitors
NF-Y	nuclear factor Y
NK-cell	natural killer cell
nls	nuclear localization sequence
NOD	non-obese diabetic
NUP98	nucleoporin gene 98
OPN	osteopontin
Pax-5	paired box protein Pax-5
Pbx	pre-B cell leukemia transformation related gene
PcG	polycomb
PPR	parathyroid hormone receptor
RA	retinoic acid
Rae28	polyhomeotic homolog 1
RNA	ribonucleic acid
rtTA	reverse tetracycline transactivator
Sca-1	stem cell antigen 1
SCF	stem cell factor
SCID	severe combined immunodeficiency
SCL	stem cell leukemia gene
Shh	sonic hedgehog
SLAM	signaling lymphocyte-activating molecule
SP	side population
Stat5	signal transducer and activator of transcription 5
ST-HSC	short-term HSC
TALE	three amino acid loop extension
tetO	tet operator sequence
TF	transcription factor
TGF- β	transforming growth factor- β
Tie2	tyrosin kinase receptor 2
TPO	thrombopoietin
TrxG	trithorax
tTA	tetracycline transactivator
USF	stimulating factor

List of articles

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

- I **Reduced proliferative capacity of hematopoietic stem cells deficient in Hoxb3 and Hoxb4.**
Bjornsson JM, Larsson N, Brun AC, Magnusson M, Andersson E, Lundstrom P, Larsson J, Repetowska E, Ehinger M, Humphries RK, Karlsson S. Mol Cell Biol. 2003 Jun;23(11):3872-83.
- II **Hoxb4 deficient mice have normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells.**
Brun AC *, Bjornsson JM *, Magnusson M *, Larsson N, Leveen P, Ehinger M, Nilsson E, Karlsson S. Blood. 2004 Jun 1; 103(11): 4126-33.
* contributed equally to this work.
- III **HOXB4-induced self-renewal of hematopoietic stem cells is significantly enhanced by p21 deficiency.**
Miyake N, Brun AC, Magnusson M, Miyake K, Scadden DT, Karlsson S. Scadden, Stem Cells. 2006 Mar; 24(3):653-61.
- IV **Hoxa9/hoxb3/hoxb4 compound null mice display severe hematopoietic defects.**
Magnusson M, Brun, AC, B, Lawrence, HJ, Karlsson, S. Manuscript.
- V **HOXA10 is a critical regulator for hematopoietic stem cells and erythroid/megakaryocyte development.**
Magnusson M, Brun, AC, B, Miyake, N, Larsson, J, Ehinger, M, Björnsson, J, Wutz, A, Sigvardsson, M, Karlsson, S. Blood in press.

Introduction

Stem cell biology has become a loaded topic for many reasons e.g. political, financial, health care and most importantly ethical concerns. For the scientific community it has opened doors of opportunities to unwind the mechanisms controlling the developmental process from a single stem cell to a mature organism. The discovery of adult tissue specific stem cells carrying the capacity to repair and/or reconstruct an organ, such as blood (hematopoiesis), liver, brain and muscle, has led to the concept of regenerative medicine, meaning replacing an injured organ or cells with stem cells that repair the damaged area (Lagasse et al., 2001). Even though these ideas sound like science fiction, regenerative medicine has been used in the clinic successfully for more than three decades. Through the use of bone marrow transplantation, patients with leukemia and genetic blood disorders such as sickle cell anemia and diamond blackfan anemia have been successfully treated (Roy et al., 2005; Shizuru et al., 2005; Vermynen, 2003). The availability of the blood stem cells (hematopoietic stem cells (HSCs)) in the bone marrow has made these cells the most characterized adult stem cell in the field. However, our current knowledge concerning the regulatory circuits governing HSCs is still limited, restricting the therapeutic potential of these cells.

Hematopoiesis is highly conserved throughout evolution and is largely similar between lower vertebrates and mammals (Zon, 1995). The focus of this thesis is on the HSCs, and in particular the role of Hox transcription factors in the regulation of HSCs using the mouse as the model system.

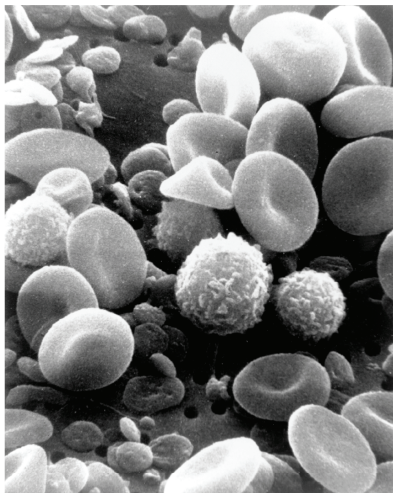


Figure 1. Scanning electron microscope image from normal circulating human blood. One can see red blood cells (donut shaped), several white blood cells including lymphocytes, a monocyte, a neutrophil, and many small disc-shaped platelets. Source: National Cancer Institute, Photographers Bruce Wetzel and Harry Schaefer.

Hematopoiesis

Hematopoiesis, from the ancient Greek haima (blood) and poiesis (to make), is the formation and development of blood cells. Most of the mature blood cells are short lived, and in an adult human the astonishing amount of approximately 1000 billion new blood cells are produced every day (Ogawa, 1993). Even though the ancient Greeks, lead by Hippocrates, Aristotle and Galen, knew the lifegiving properties of blood, it was not until the beginning of the 17th century when the father of modern

physiology, William Harvey, discovered and demonstrated that the blood cells were in a circulating system (Bardell, 1978). However, it took another 300 years from his discovery until the true origin of the blood cells was demonstrated. In the blood there are at least nine defined cell types, and most of them are vital for the organism (Figure 1 and Figure 2). Red blood cells constitute the majority of the blood cells. They contain hemoglobin for transport of oxygen from the lungs to the tissues. Platelets or thrombocytes circulate in the blood stream, and upon damage to the vessels, they form blood clots to prevent blood loss and initiate wound healing. White blood cells are responsible for the defense against infections and microorganisms, and consist of monocytes, granulocytes (neutrophils, basophils and eosinophils), B-cells, T-cells and NK-cells (Bryder et al., 2006; Orkin, 2000).

Hematopoietic stem cells

Hematopoiesis is dependent on a few rare cells, one in 30 000 bone marrow cells or in total about 11 000 per mouse (Adams and Scadden, 2006; Ema et al., 2005), which are called the hematopoietic stem cells (HSCs). These cells reside within the bone marrow in the adult individual. The definition of a true HSC is based on three unique properties (Weissman, 2000). (1) They can divide into two new HSCs both carrying the same properties as the original HSC, a process referred to as self-renewal. (2) They can differentiate into all the mature effector cells in the blood. (3) They can reconstitute the hematopoietic system in an ablated recipient and sustain the production of blood cells throughout its lifespan.

In 1909, Maximov first postulated the existence of HSCs (Maximov, 1909). However, it was not until a half century later that the first experimental evidence showed that blood cells were derived from one common cell. In 1961, Till and McCulloch published their ingenious assay that demonstrated that a subset of cells in the bone marrow were capable of generating macroscopic colonies in the spleen. These colonies were called colony-forming units of the spleen (CFU-S), and could be observed after bone marrow transplantation into lethally irradiated recipient mice (Till and McCulloch, 1961). In addition, these colonies were shown to originate from a single cell that had the capacity to generate new CFU-S colonies in a secondary host, indicating self-renewal capacity (Becker et al., 1963; Siminovitch et al., 1963). However, it was later demonstrated that the majority of the CFU-S content is of myeloid-erythroid origin, demonstrating that the colonies are derived from a more committed progenitor and not from the HSCs (Magli et al., 1982; Na Nakorn et al., 2002; Schofield, 1978). Nonetheless, the pioneering work by Till and McCulloch started the field of adult stem cell biology by demonstrating the concept of the HSCs.

In the mid 1980s a couple of groups used retroviral gene transfer into hematopoietic bone marrow cells and demonstrated common insertion sites in myeloid as well as lymphoid cells after transplantation into recipient mice (Dick et al., 1985; Keller et al., 1985; Williams et al., 1984). These observations were possible since retroviruses integrate randomly into the host genome enabling for tracking of individual cells. Furthermore, when the transduced bone marrow cells from the primary recipients were harvested and transplanted into secondary recipients, identical clones were found to contribute to hemtopoiesis in different secondary recipient mice (Keller and Snodgrass, 1990). Taken together these findings demonstrated the existence of a cell in the bone marrow that fulfilled the criterion of a true HSC.

The Hematopoietic hierarchy

The development of *in vitro* assays in the 1960s allowed for the discovery of several hematopoietic progenitor cells showing that there are different intermediate stages between the HSC to the mature blood cells (Bradley and Metcalf, 1966; Ichikawa et al., 1966; Pluznik and Sachs, 1965). At present, the hematopoietic development is described as a hierarchical irreversible process under normal physiological conditions. Hence, the long-term HSCs (LT-HSCs) are situated on top of the hierarchy, fulfilling the criterion of a true HSC, and is followed by several stages of progenitors down to the mature blood cells (Figure 2) (Weissman, 2000).

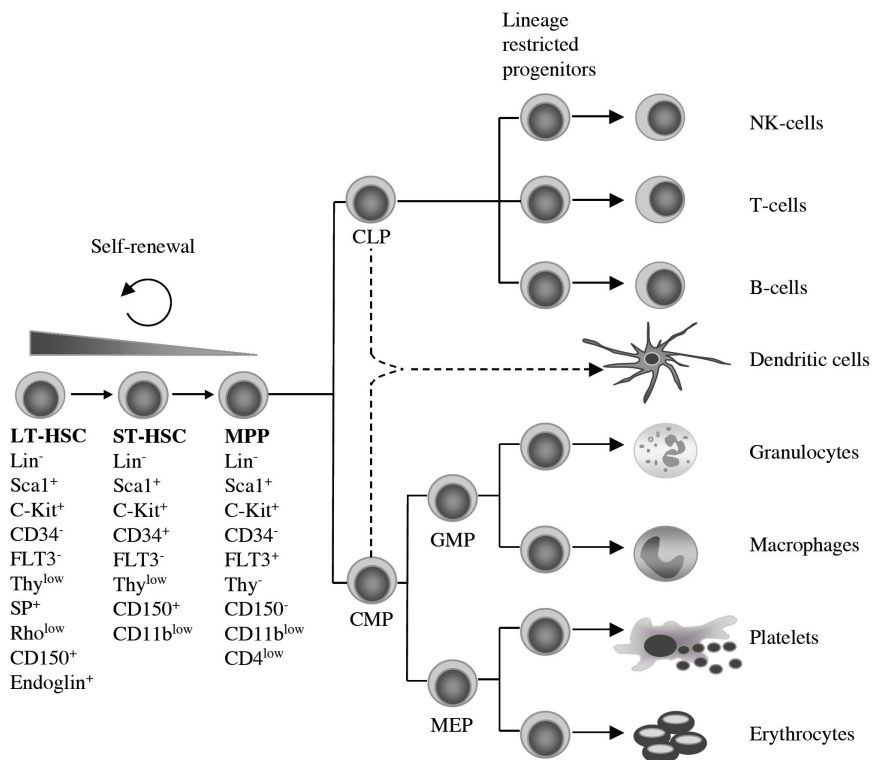


Figure 2. The hematopoietic hierarchy. The LT-HSCs, ST-HSCs, and MPP cells are identified by the delineated phenotypic markers.

The advances in Fluorescence Activated Cell Sorting (FACS) analysis made it possible to dissect the hematopoietic hierarchy and identify the intermediate stages. LT-HSCs, in mice, differentiate into short-term HSCs (ST-HSCs) (Morrison and Weissman, 1994) with limited self-renewal capacity for 6-8 weeks. These cells advance into multipotent progenitors (MPPs) having only 2 weeks of self-renewal potential (Morrison et al., 1997b). Still, these progenitors carry full potential to generate all mature effector cells. More importantly, they may reconstitute an irradiated recipient faster than the LT-HSCs, thus showing very important therapeutic potential for transplantation purposes. Next, the MPPs commit either to the myeloid or the lymphoid pathway by differentiating into common myeloid progenitor (CMP)

cells or common lymphoid progenitor (CLP) cells (Akashi et al., 2000; Kondo et al., 1997). The CMPs are restricted to the myeloid lineages differentiating into granulocyte macrophage progenitors (GMP) that give rise to granulocytes and macrophages, or differentiate into megakaryocyte erythroid progenitors (MEPs) that advance into platelets or erythrocytes (Akashi et al., 2000). The CLPs are restricted to the lymphoid lineages and differentiate into oligolineage restricted progenitors giving rise to T-cells, B-cells, and NK-cells (Kondo et al., 1997). Both CMPs and CLPs have been suggested to give rise to dendritic cells (Manz et al., 2001; Traver et al., 2000). However, the classification of the hematopoietic progenitors are not absolute, and alternative pathways have been suggested (Adolfsson et al., 2005) showing that further research is needed to understand the developmental stages from the LT-HSCs to the mature effector cells.

Origin and development of HSCs during ontogeny

The development of HSCs during embryogenesis is a very complex process involving several different anatomical sites including the yolk sac, the aorta-gonad-mesonephros region (AGM), the placenta and the fetal liver (Figure 3A). Subsequently, as the fetus develops the HSCs finally seed the bone marrow at birth. The shift from one anatomic site to another during embryogenesis is crucial due to the anatomy change during organogenesis. In addition, shifting anatomic site may also have instructive functions controlling the expansion of HSCs, and the generation of mature blood cells during ontogeny (Mikkola and Orkin, 2006).

The embryonic hematopoiesis in mice starts after gastrulation when specialized mesodermal precursor cells give rise to blood islands, which contain primitive erythrocytes in the yolk sac already at E7.0-8.25 (Ferkowicz et al., 2003; McGrath and Palis, 2005; Palis et al., 1999). These precursor cells are thought to be the hemangioblasts and have been hypothesized to be the common ancestor of both blood and endothelial cells (Jaffredo et al., 2005; Mikkola and Orkin, 2006). The hemangioblasts were first identified *in vitro* when ES cells were differentiated into blast like colonies containing cells with the capacity to generate both blood and endothelial cells (Choi et al., 1998; Kennedy et al., 1997). More importantly, the hemangioblasts were also identified *in vivo* in the gastrulating mouse embryo, and interestingly they were found in the primitive streak, indicating that the first hematopoietic cells migrate from the primitive streak to the yolk sac (Huber et al., 2004). Still, it is not clear whether the hemangioblasts do contribute to all hematopoietic cells in the yolk sac or even to the development of HSCs.

The primitive erythrocytes generated in the yolk sac are characterized by their large size and the retention of the nucleus while entering the circulation at day E8.5 to supply the embryo with oxygen (Ferkowicz et al., 2003; Palis et al., 1999). Shortly after the appearance of the primitive erythrocytes, definitive myeloerythroid progenitors develop in the yolk sac (Cumano et al., 1996; Palis et al., 1999). Since the yolk sac does not possess the capacity to differentiate the progenitors into mature blood cells, HSCs migrate to the fetal liver, whose microenvironment support the maturation of functional definitive blood cells (Jaffredo et al., 2005). Though HSC activity is detectable in the yolk sac at E11.5 (Figure 3B), it is still controversial whether these HSCs are generated *in situ*.

According to the current dogma, the first HSCs originate from the AGM region (Cumano et al., 1996; Godin and Cumano, 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994), developing from the lateral plate mesoderm (LPM). The

first “definitive” HSC, characterized by long-term multilineage reconstitution of the hematopoietic system in adult recipients, emerge at day E10.5–11. The HSCs at this stage have very poor engraftment capacity in adult mice, probably due to the lack of major histocompatibility complex (MHC) as well as deficient homing capabilities (Cumano et al., 2001; Matsubara et al., 2005). At day E11.5–12.5 a rapid expansion of the HSCs occur in the placenta (Gekas et al., 2005). It is not clear whether the HSCs that expand in the placenta are generated *in situ*, even though HSC activity has been detected as early as E10.5 (Figure 3B) (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), or if they are arriving via the circulation from the AGM region.

The first HSCs in the fetal liver, most likely originating from the AGM and/or the placenta, appear at day E11.5 (Figure 3B) (Gekas et al., 2005). At day E12.5 the fetal liver becomes the main hematopoietic organ during embryonic development both for HSC expansion and production of definitive blood cells. The HSCs expand dramatically in the fetal liver microenvironment until day E15.5–16.5 (Figure 3B) (Ema and Nakauchi, 2000; Gekas et al., 2005; Morrison et al., 1995) when they finally start seeding the bone marrow niche. However, it is still debated whether HSCs are generated from more than one site during ontogeny (AGM, yolk sac and placenta), which is why further studies are needed to unravel the complex journey of the developing HSCs.

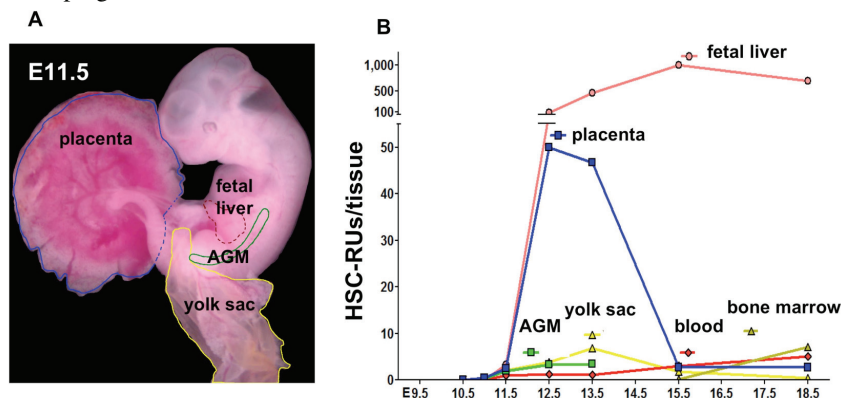


Figure 3. (A) Dissection of fetal hematopoietic organs E11.5. (B) Kinetics of HSC-Repopulating Units (RU) at each hematopoietic site during mouse development. HSC-RUs are calculated from the frequency of reconstituted recipients at 10–12 weeks after transplantation, divided by the transplanted cell dose (in embryo equivalents). The figure has been adapted from (Mikkola et al., 2005), with permission from Dr Hanna Mikkola.

Isolation of HSCs

During the last decades of the twentieth century intensive studies were performed in order to isolate and identify single HSCs. The major breakthrough came with the development of clonal assays of the hematopoietic progenitors and the discovery of monoclonal antibodies specific for cell surface molecules on the hematopoietic cells. Today, using combinations of monoclonal antibodies, single HSCs can be isolated by FACS with very high purity (Goodell et al., 1996; Osawa et al., 1996; Spangrude et al., 1988a).

The FACS machine was invented in the late 1960s to allow for flow cytometry and cell sorting of viable cells (Bonner et al., 1972) and in the early 1970s the commercial machines were available by Becton Dickinson Immunocytometry Systems. Over the years, the number of measured FACS parameters and sorting speed

have advanced considerably, and today at least 12 fluorescent colors plus 2 scatter parameters can be used.

Using the above approaches, the murine adult HSCs were discovered to be negative for markers that are expressed on lineage committed blood cells. These are referred to as the lineage specific markers and include Ter119, B220, CD4, CD8, Mac1 and Gr1 (Muller-Sieburg et al., 1986). Thus, the HSCs are lineage negative (Lin⁻) and removal of all lineage positive cells leads to an enrichment of HSCs. However, the Lin⁻ hematopoietic cells consist mainly of progenitor cells, which was why development of additional markers was needed. Further purification of the HSCs was done by adding the cell surface markers c-kit and Sca-1 (Morrison and Weissman, 1994; Spangrude et al., 1988b). These cells are referred to as Lin⁻, Sca-1⁺, c-kit⁺ (LSK) cells. The LSK cells have for long been referred to as HSCs but this population is still very heterogeneous and only one in 30 LSK cells are a true HSC (Bryder et al., 2006). Several studies have shown that additional markers, such as CD34, Thy1.1, endoglin, FLT3, and SLAM receptors, together with LSK can further enrich the HSC population (Bryder et al., 2006). Combinations of these markers can also be used to further distinguish the LT-HSCs from the ST-HSCs and MPPs (See Figure 2). The HSCs were found negative for CD34, FLT3 and low for Thy1.1, and approximately 20 % of single LSKCD34⁻ cells or LSKThy1.1^{low} cells gave rise to long term repopulation (Christensen and Weissman, 2001; Osawa et al., 1996; Wagers et al., 2002). Interestingly, HSCs were recently shown to be positive for CD150 (a member of the SLAM receptor family) and in combination with other SLAM family members (CD48 and CD244) or LSK, high purity of HSCs was obtained (Kiel et al., 2005). It was also shown that these markers could be used to obtain high purity of HSCs from the fetal liver (Kim et al., 2006).

Zhou et al demonstrated that the HSCs have high expression levels of ATP binding cassette/multidrug resistance (ABC/MDR) transporter genes (Zhou et al., 2001). These gene products result in a unique protection against foreign cytotoxic substances. Using these properties, a more functional approach to enrich for HSCs has been developed, which measures the ability to efflux dyes such as rhodamin-123 or Hoechst 33342. This capacity results in a very distinct FACS profile called the side population (SP). The SP cells are enriched at least a 1000-fold for HSCs (Goodell et al., 1996) and in combination with surface markers like LSKCD34⁻ highly purified HSCs can be obtained. Intriguingly, it has been shown that a single SP LSKCD34⁻ cell can give rise to long-term repopulation with astonishing 96% efficiency (Matsuzaki et al., 2004). However, these findings have so far not been reproduced (Camargo et al., 2006; Morita et al., 2006). Nonetheless, the ability to efflux dye is an important mechanism that is highly conserved between species e.g. pigs, monkeys and humans, which make HSC enrichment possible for species where no antibodies exist (Goodell et al., 1997). Furthermore, the Lodish laboratory has recently shown that endoglin, a co-receptor for certain members of the TGF- β super family, was highly expressed on HSCs and could be used for HSC enrichment in combination with other cell surface markers or the SP (Chen et al., 2003). The pursuit for obtaining absolute HSC purity will continue. However, it is possible that the HSC purity achieved today is higher than shown by the transplantation assay, since a single HSC has several undetectable fate options such as differentiation without self-renewal, apoptosis or quiescence, suggesting that additional assays to assess the true purity of HSC are needed. The most commonly used phenotypic markers for HSC isolation are shown in Figure 2.

The isolation of human HSCs using immunophenotype staining has not been as successful as for murine HSCs. The common combination of cell surface markers that has been used is Lin⁻CD34⁺CD38⁻. However the possibility to test the true properties of human HSCs is limited, and the model system used today is transplantation into NOD/SCID mice or other immunodeficient mice. Using the xenograft transplantation model it was estimated that one in 121 Lin⁻CD34⁺CD38⁻ was a SCID repopulating cell with multilineage reconstitution ability (McKenzie et al., 2006).

Regulation of HSCs

The adult HSC pool is relatively constant in size under physiological conditions (Weissman, 2000). Most of the HSCs *in vivo* are quiescent (resting phase) and reside in the bone marrow niche (Bradford et al., 1997). However, all murine HSCs do enter the cell cycle within one to three months and parabiosis studies using two different congenic mouse strains have demonstrated that the HSCs continuously enter the circulation to re-engraft into a new niche (Bradford et al., 1997; Cheshier et al., 1999; Wright et al., 2001). The advantage of having the majority of the HSCs in a quiescent state is that it protects them from metabolic side products such as oxidative stress, chemotherapeutic agents, and random mutations occurring during DNA-replication. This feature may well contribute to the long life span of these cells (Rossi et al., 2005) (Lerner and Harrison, 1990).

In order to sustain the immense production of mature blood cells generated every day, and to keep the HSC pool intact, several different HSC fate options exist. These include self-renewal (symmetric or asymmetric cell division), apoptosis and migration (Domen and Weissman, 1999). During symmetric self-renewal, the HSCs divide into two identical daughter cells both retaining HSC properties and thus resulting in HSC expansion (Lansdorp, 1997). During asymmetric self-renewal, one daughter cell retains HSC properties whereas the other initiates differentiation ultimately leading to HSC maintenance (Brummendorf et al., 1998; Ema and Nakauchi, 2000; Suda et al., 1984; Takano et al., 2004). The symmetric cell division, which results in a net expansion of HSCs, has important clinical applications. However, despite several attempts to mimic this mechanism, no major breakthroughs in the field have emerged. The fate options regulate the size of the HSC pool (Domen and Weissman, 1999). It is still debated though, whether the HSC pool is regulated in a spontaneous “stochastic” way or in a more instructive “deterministic” manner (Enver et al., 1998; Metcalf, 1998; Ogawa, 1999; Till et al., 1964). Nonetheless, several intrinsic (intracellular) and extrinsic (extracellular) signals have been identified in regulating HSC fate options.

Intrinsic regulators

The internal signals are represented by the gene expression pattern of transcription factors (will be discussed separately), cell cycle and apoptosis regulators (Kondo et al., 2003; Teitell and Mikkola, 2006). Given that HSCs divide, several cell cycle regulators, such as the cyclin-dependent kinase inhibitors (CDKI), have been identified to regulate HSC maintenance. The p21 (KIP family) and p18 (INK4 family), both members of the CDKIs, inhibit CDK2 and CDK4-6 respectively. In knockout studies, p21 null HSCs displayed increased cycling resulting in exhaustion

of the HSC pool (Cheng et al., 2000b). Similarly, lack of p27, another member of the KIP family, resulted in an increased proliferation on the progenitor cell level in p27 deficient mice (Cheng et al., 2000a). Strikingly, p18 deficient mice displayed increased numbers of HSCs in the bone marrow, which in contrast to p21 deficiency, did not result in exhaustion (Yuan et al., 2004). Also, p16 (INK4 family), has recently been shown to regulate HSC aging (Janzen et al., 2006). Taken together, these data clearly show that cell cycle regulators are involved in regulating HSC self-renewal.

Two different signal transducers (Pten and Lnk) were recently identified as regulators of HSC self-renewal. Pten deficient HSCs, defined by immunophenotype, showed increased proliferation but were unable to give long-term reconstitution when transplanted into myeloablated recipients (Yilmaz et al., 2006; Zhang et al., 2006b). In contrast, Lnk deficiency resulted in enhanced self-renewal and an increased number of HSC, suggesting that Lnk negatively regulate self-renewal signaling (Ema et al., 2005).

Maintenance of the HSC pool in the bone marrow is dependent on several signals inhibiting differentiation, exhaustion and apoptosis. The latter has best been demonstrated by overexpression of the anti-apoptotic Bcl2 gene in mice, resulting in an increased HSC pool (Domen et al., 2000; Domen et al., 1998). More recently, Mcl1, a member of the Bcl2 family, was found to be important for HSC survival (Opferman et al., 2005). In summary, the intrinsic factors governing HSC regulation may be potential targets for efficient HSC expansion protocols. However, several of the regulators are involved in leukemic transformations, emphasizing the importance of tightly regulated systems.

Extrinsic regulators

Several hematopoietic growth factors have been identified and characterized using mouse models and *in vitro* culture systems. Most of them act on progenitor cells and control differentiation, proliferation and survival such as IL-3, IL-6, IL-7, GM-CSF and G-SCF (Kaushansky, 2006). However, stem cell factor/steel factor (SCF), which binds to the c-kit receptor, and thrombopoietin (TPO), a ligand for the c-mpl receptor, are proven important regulators of the HSCs. Accordingly, c-kit and c-mpl mutant mice displayed reduced HSC function (Miller et al., 1996; Murone et al., 1998).

Different combinations of growth factors have been used in order to expand HSCs *in vitro*, but at best a very modest expansion has been detected during a limited time frame (Bryder and Jacobsen, 2000; Miller and Eaves, 1997). Encouragingly, fibroblast growth factors (FGFs) have recently been shown to maintain LT-HSC function for up to 5 weeks *in vitro* (de Haan et al., 2003; Yeoh et al., 2006). Concomitantly, FGF receptor knockout mice displayed impaired hematopoietic development supporting FGFs as important regulators of hematopoiesis (Faloon et al., 2000). More impressively, Zhang et al identified angiopoietin-like proteins (Angptl) as positive regulators of symmetric self-renewal *in vitro* since up to a 30 fold net increase of HSCs was obtained after 10 days of culture with Angptl2 and 3 (Zhang et al., 2006a).

The Notch and Wnt signaling transduction pathways, which are greatly conserved throughout evolution, regulate a wide range of developmental processes as well as hematopoiesis. The Notch receptor is activated upon binding of Jagged1 (a transmembrane receptor) and enhanced activation of Notch1 has been shown to increase HSC self-renewal (Stier et al., 2002). Correspondingly, experiments inhibiting the Notch pathway resulted in accelerated differentiation and a subsequent depletion of the HSCs (Duncan et al., 2005).

Wnt proteins bind to the frizzled receptors, which in turn leads to internalization of β -catenin into the nucleus. *In vitro* cultures with Wnt3A have been shown to increase HSC self-renewal and similar findings have been obtained by overexpressing activated β -catenin (Reya et al., 2003; Willert et al., 2003). Contradictory to the overexpression studies, conditional β -catenin deficiency had no effect on hematopoiesis (Cobas et al., 2004), indicating redundancy from other pathways. Also, Duncan et al demonstrated that Notch1 signaling was required for Wnt mediated self-renewal (Duncan et al., 2005), which further demonstrated the complex interactions in HSC regulation.

Transforming growth factor β (TGF β) and bone morphogenic proteins (BMPs), members of the TGF β superfamily of ligands, have been described as regulators of hematopoiesis by activating the canonical Smad signaling pathway (Larsson and Karlsson, 2005). Stimulation with BMP4 was shown to preserve human HSC function *in vitro* (Bhatia et al., 1999) and interestingly, Bhardwaj et al demonstrated that sonic hedgehog (Shh) regulates proliferation of human HSCs in a BMP dependent manner (Bhardwaj et al., 2001). Furthermore, by terminating Smad signaling at the receptor level through overexpression of Smad7 in mouse bone marrow, Blank et al demonstrated an increase in HSC self-renewal *in vivo* (Blank et al., 2006). In contrast, the same group showed that deletion of the common Smad4 resulted in an opposite effect to that of Smad7 overexpression (Karlsson, unpublished data). These results imply differences at the molecular level, possibly through crosstalk with other pathways.

In summary, present knowledge suggest that there is no molecule that is solely dominant in regulating HSC self-renewal, and that crosstalk between the different pathways is a common event. Therefore, to achieve efficient HSC expansion protocols in the future using growth factors, it will be necessary to understand these complex networks controlling the HSCs.

Regulation within the stem cell niche

In 1978, Schofield postulated the idea that HSCs reside in a specialized compartment in the bone marrow, called the HSC niche (Schofield, 1978). Today, several studies have shown that the HSC niche is creating a microenvironment controlling the HSC fate, and several important players have been identified (summarized in Figure 4).

The HSCs are thought to reside in close proximity to the endosteal surface of the bone and in close contact to the osteoblasts (Gong, 1978; Nilsson et al., 1997; Zhang et al., 2003). The osteoblasts are responsible for the regeneration of bone and additionally, constitute a crucial component in the HSC niche. In two studies, where increased numbers of osteoblasts were detected either by constitutively expressing the active form of parathyroid hormone receptor (PPR), or through deletion of the BMP receptor 1a, an increased number of functional HSCs was detected (Calvi et al., 2003; Zhang et al., 2003). Interestingly, the increased HSC pool caused by PPR was mechanistically explained by increased Notch signaling, since active signaling of the PPR in the osteoblasts resulted in upregulation of Jagged1 (Calvi et al., 2003). The deletion of BMP signaling indicated that N-cadherin is an important component for anchoring the HSCs to the osteoblast in the niche (Zhang et al., 2003). The important role of the osteoblasts in the HSC niche was further demonstrated by their secretion of angiopoietin1 (ANG1), which resulted in activation of the Tie2 receptor on LT-HSCs. More importantly, activation of the ANG1/Tie2 signaling pathway was shown to

preserve LT-HSCs in a quiescent state, possibly through upregulation of p21 (Arai et al., 2004; Wilson and Trumpp, 2006).

Besides the direct contact with the osteoblasts, two groups recently showed that osteopontin (OPN), a matrix glycoprotein secreted by osteoblasts, is a negative regulator of the HSCs by preventing cell division (Nilsson et al., 2005; Stier et al., 2005). In addition, Adam et al demonstrated that loss of Calcium-sensing Receptor (CaR) resulted in a defect in homing to the endosteal niche, which correlated with diminished binding to collagen type 1 (Adams et al., 2006). This suggests that the calcium gradient within the remodeling bone is important for engraftment of HSCs into the endosteal niche.

Furthermore, it has been postulated that the HSCs are distributed along an oxygen gradient with the most hypoxic area closest to the endosteal niche. In support to this theory, human SCID repopulating cells have been shown to expand during hypoxic conditions *in vitro* (Danet et al., 2003). The impact of different oxygen levels *in vivo* is still unknown but during ontogeny, HSCs do expand in the hypoxic placenta (Mikkola and Orkin, 2006).

Understanding and subsequently mimicking the HSC niche would have major clinical applications for stem cell expansion. Intriguingly, new data have challenged the exclusiveness of the endosteal niche in the bone marrow (Kiel et al., 2005). Thus, future studies need to determine whether different niches in the bone marrow retain functional differences or not.

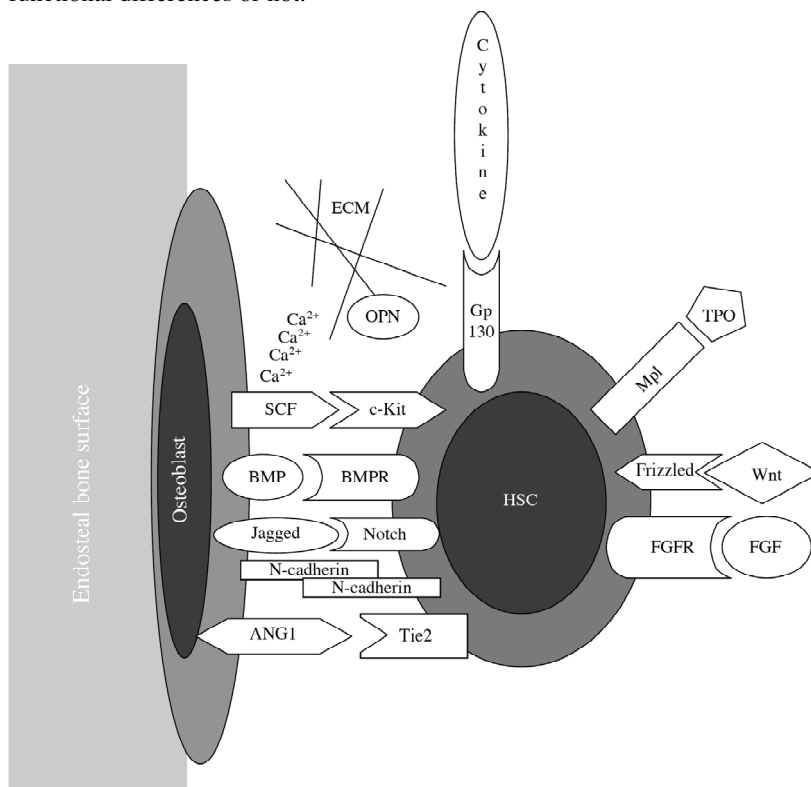


Figure 4. The HSC niche. A simplistic figure over the HSC bone marrow niche showing various signals regulating HSC fate within the bone marrow microenvironment. ECM (extra cellular matrix).

Transcription factors

A transcription factor (TF) is often defined as a protein that mediates binding of RNA polymerase II to DNA, initiating transcription of a gene. However, this is a very simplified model and the regulation of transcription is very complex involving the interaction of several proteins that may either stimulate or repress transcriptional activity. This depends on co-factors, the cellular context, and chromatin structure (epigenetics). The TFs usually bind directly to the DNA motifs upstream or in close proximity to the transcriptional start site either alone or with co-factors resulting in recruitment and activation of the general transcription complex leading to RNA transcripts. TF repression works by inhibiting the binding of TF activators. The regulatory mechanisms are largely unknown as well as the TF target genes. Still, a number of TFs have been shown to act as critical intrinsic factors for specification of HSCs during both ontogeny and/or postnatal hematopoiesis (Orkin, 2000; Teitell and Mikkola, 2006). Interestingly, many of these TFs have been identified as major regulators of hematopoiesis by the use of gain- (mainly overexpression by retroviral vectors) or loss of function assays (mainly knockouts). TFs have been shown to control self-renewal, proliferation and differentiation, and importantly, have been associated with chromosomal translocations causing leukemias (Orkin, 2000).

Transcription factors regulating HSCs

The TF stem cell leukemia gene (**SCL/Tal1**) was originally found to be crucial for the initiation of hematopoiesis during ontogeny (Robertson et al., 2000; Shivdasani et al., 1995). More recent experiments using conditional knockout mice demonstrated that SCL was dispensable for the development and function of adult HSCs, although the gene was still crucial for the lineage specification of erythroid and megakaryocyte cells. These findings indicated that SCL might be under epigenetic regulation (Mikkola et al., 2003; Schlaeger et al., 2005).

Mice deficient in **LMO2** or **Gata-2** exhibited major defects in primitive and definitive hematopoiesis (Tsai et al., 1994; Yamada et al., 1998). Furthermore, haploinsufficient Gata-2 adult HSCs resulted in decreased competitive repopulating ability, implying Gata-2 as an important regulator of both pre- and postnatal HSCs (Ling et al., 2004). However, the effect of LMO2 on adult HSCs is still not known.

Also **Runx1/AML1** has been shown to be essential for the development of definitive hematopoiesis during ontogeny (Okuda et al., 1996). More recent data using a conditional AML1 knockout model demonstrated that AML1 was not crucial for maintenance of adult HSCs (Growney et al., 2005; Ichikawa et al., 2004), even though a reduced repopulating capacity was detected in a competitive transplantation assay. This was mainly explained by blocked development of lymphoid cells and megakaryocytes.

Interestingly, mixed leukemia factor (**Mll**) deficient mice developed definitive hematopoietic cells but no HSCs, indicating that Mll is critical for maturation of definitive HSCs (Ernst et al., 2004). This might be explained by the loss of Hox gene expression since Mll is a critical regulator of Hox genes (discussed below).

The loss of the TF **Ikaros** displayed a severe reduction in competitive repopulating ability demonstrating a defective transition to definitive HSCs and reduced HSC self-renewal capacity (Nichogiannopoulou et al., 1999). In addition, Ikaros null mice displayed reduced numbers of hematopoietic progenitors and a block in B, T and NK-cell development.

In contrast, when **c-Myc** was deleted in adult HSCs an accumulation of HSCs was detected. Also an increased level of adhesion molecules, which are important for the interactions in the HSC niche, was found. This resulted in a blocked differentiation of all lineages and the c-Myc deficient HSCs were unable to reconstitute hematopoiesis in a myeloablated recipient. Conversely, enforced expression of c-myc had the opposite effect, suggesting that c-Myc is needed to allow the HSCs to exit the niche and initiate differentiation (Wilson et al., 2004).

Using a conditional knockout, **PU.1** deficient HSCs were found unable to compete with wildtype HSCs in a steady state or in a competitive transplantation assay (Fisher et al., 1999; Iwasaki et al., 2005). The deficient HSCs were able to home to the bone marrow but incapable of inducing maintenance. Furthermore, PU.1 deficiency inhibited the transition into CMPs and CLPs as well as myeloid maturation. Recently, a mutation in **c-Myb** disrupting its binding to the cofactor p300 resulted in an increased HSC pool and distinct effects on several lineages such as T-cells, B-cells and megakaryocytes (Sandberg et al., 2005). Similarly, **C/EBP α** deficiency, led to increased repopulating ability as well as a block in the transition from the CMP to GMP stage resulting in an accumulation of blast cells in the bone marrow (Zhang et al., 2004). This indicated that C/EBP α serves to limit HSC self-renewal. Conversely, **Stat5** knockout bone marrow showed a severe defect in competitive repopulating ability while overexpression of Stat5 promoted self-renewal of HSCs *in vitro* (Bunting et al., 2002; Kato et al., 2005). In addition, overexpression of **HLF** or **HES-1** in human bone marrow cells led to an increased repopulating ability implying that these TFs act as important regulators of HSCs (Shojaei et al., 2005).

Current studies have demonstrated that several transcriptional repressors are important factors in HSC regulation, particularly members of the Polycomb group (PcG) proteins (regulators of HOX proteins) like **Bmi1**, **Mel18**, **Rae28** and **Ezh2**. **Bmi1** was identified as an important regulator of symmetric self-renewal since overexpression of Bmi1 protein led to increased *ex vivo* expansion of HSCs (Iwama et al., 2004). Conversely, Bmi1 deficiency resulted in HSC exhaustion, possibly through upregulation of the cell cycle inhibitors p16 and p19, and simultaneous downregulation of the apoptosis inhibitor AI-6 (Lessard and Sauvageau, 2003; Park et al., 2003). **Mel18** deficiency had a reverse effect on HSC fate, resulting in increased HSC self-renewal, possibly by increased expression of Hoxb4 (see below) (Kajiume et al., 2004). HSCs deficient in **Rae28** displayed impaired repopulating ability in serial transplantation assays (Ohta et al., 2002) and overexpression of **Ezh2** prevented HSCs from exhaustion in a serial transplantation assay (Kamminga et al., 2006). Also, Hock et al and Zeng et al demonstrated that **Gfi-1**, a Zinc finger transcriptional repressor, retained HSC integrity by inhibiting HSC proliferation and exhaustion, possibly by regulating the cell cycle inhibitor p21 (Hock et al., 2004; Zeng et al., 2004). These results further support the important role of transcriptional repressors in HSC regulation.

In summary, even though several of the TFs may act as master regulators of HSCs and hematopoiesis, they most often operate in collaboration with each other. Nonetheless, a number of TFs such as PU.1, C/EBP α and Gata-1 have the ability to switch lineage specification when overexpressed in cells with different lineage fates (see below) (Orkin, 2000). Moreover, recent findings demonstrate that different expression levels of the TFs can result in different fate options, adding an additional dimension to the regulatory mechanisms governing HSCs (see general discussion). Figure 5 summarizes the impact of different TFs on hematopoiesis.

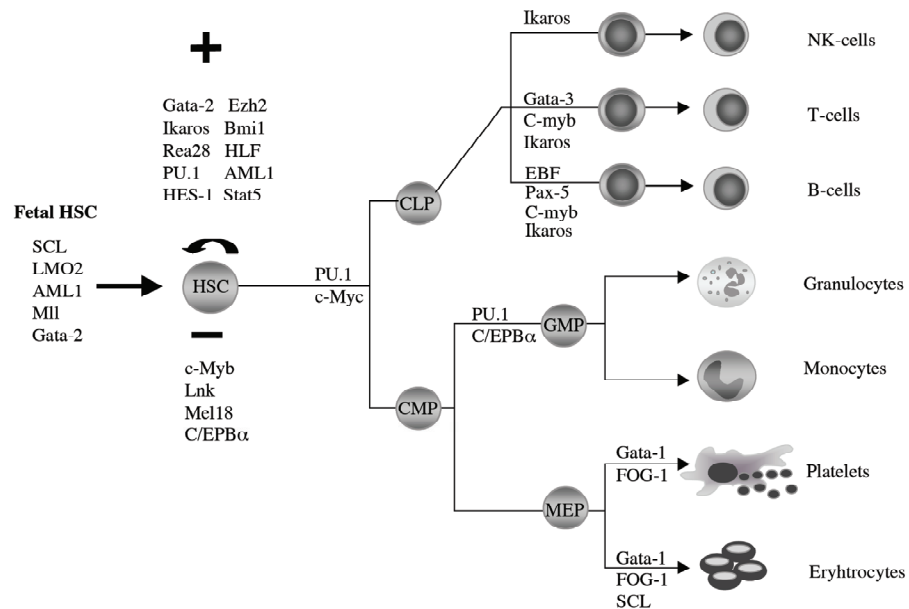


Figure 5. Transcription factors regulating HSC fate and lineage commitment. The picture shows TFs crucial for fetal hematopoiesis and TF that positively (+) and negatively (-) regulate adult HSC self-renewal. Furthermore, TFs important for different stages in the hematopoietic development are shown throughout the hierarchy.

Transcription factors regulating lineage commitment

Gain- or loss of function studies has demonstrated a crucial role for several TFs in lineage specification. **Gata-1** was found essential for the development of both primitive and definitive erythropoiesis, for maturation beyond the proerythroblast stage, as well as for megakaryopoiesis (Fujiwara et al., 1996; Pevny et al., 1995; Pevny et al., 1991). Interestingly, enforced expression of Gata-1 converted CLPs into progenitors with erythropoietic and megakaryopoietic potential (Iwasaki et al., 2003). Friend of gata-1 (**FOG-1**), a co-factor of Gata-1, demonstrated similar roles as Gata-1 in hematopoiesis (Fox et al., 1999; Tsang et al., 1997). Another member of the Gata family, **Gata-3**, is normally expressed in the CLP cells. The Gata-3 deficient mice do not develop T-cells, thereby suggesting that Gata-3 also play an important role in the initiation of T-cells.

A TF implied in the commitment and regulation of early B-cell development is **EBF** (Hagman et al., 1993; Hagman et al., 1995). EBF null mice failed to develop B-cells and lacked the expression of **Pax-5**, known to be crucial for B-cell maturation (Nutt et al., 1999). The Pax-5 null mice displayed a developmental arrest in B-cell development later than the EBF deficient mice. Interestingly, EBF was shown to directly bind and regulate the promoter activity of Pax-5, proposing a direct role for EBF in the regulation of Pax-5 (O'Riordan and Grosschedl, 1999).

Finally, much of the regulatory mechanisms in transcription known today arise from studies done in lower organisms and from these studies a large group of TFs have been identified as the homeobox genes (Hox). Initially, these genes were found to play critical roles in embryonic development but are now also known as major regulators of hematopoiesis.

Homeobox transcription factors

Introduction

Homeotic transformations, from the Greek word homeosis, are developmental mutations that direct one body part into another during ontogeny. This phenomenon was first described and named by Bateson in 1894 (Bateson, 1894). However, it was not until 1978, when Lewis presented his Nobel Prize winning work that the first genes regulating growth in *Drosophila melanogaster* were revealed (Lewis, 1978). He called these genes the bithorax genes (Bx-C) and demonstrated that they gave rise to distinct body segments. Dysregulation of these genes could result in abnormalities such as extra wing pairs or antennae. It was later found that the Bx-C genes all shared a common sequence called the homeobox, which led to the discovery of an additional cluster of homeobox genes in *Drosophila* named the antennapedia (Ant-C) (McGinnis et al., 1984; Scott and Weiner, 1984). At present, a large number of different homeobox genes have been identified with members present in the genome of all known animal species (Holland and Takahashi, 2005; Krumlauf, 1994).

The biggest and most studied group of homeobox genes is the Hox gene family. These genes are the mammalian homologs to the *Drosophila* Ant-C/Bx-C cluster (Holland and Takahashi, 2005). The Hox gene family consists of 39 members divided into the four clusters A, B, C and D, located on chromosomes 6, 11, 15 and 2 respectively in mice and 7, 17, 12 and 2 respectively in human (Boncinelli et al., 1989; Lappin et al., 2006). Each cluster is composed of 9-11 genes divided into 13 paralogs based on the homology among the individual genes in the clusters (Boncinelli et al., 1989; Scott, 1992). They are thought to arise from a common ancestor by amplification and duplication events that started about one billion years ago, hence the great homology between species (Holland et al., 1994; Kappen et al., 1989; Lappin et al., 2006). The Hox TFs can act as both transcriptional activators and repressors and a schematic overview of the genomic organization of the mammalian Hox clusters and their homolog genes in *Drosophila* is presented in Figure 6.

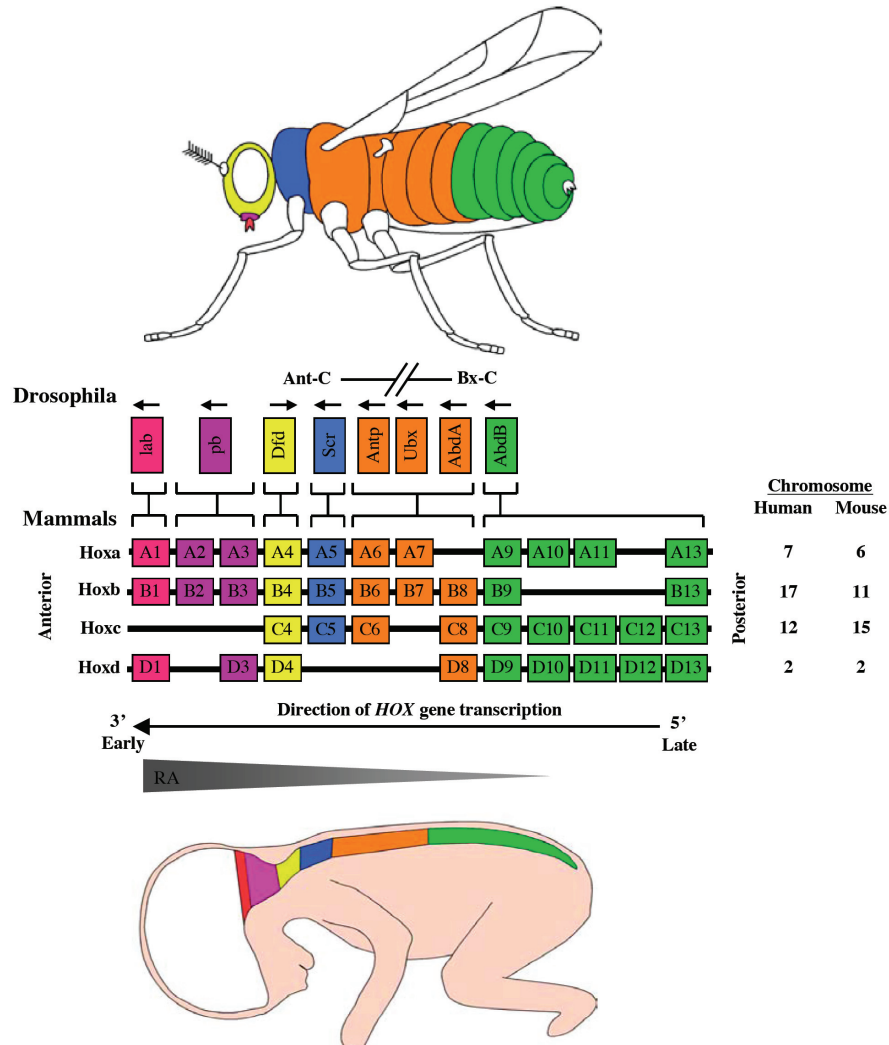


Figure 6. A schematic overview of the genomic organization in the mammalian Hox clusters showing their homologs in *Drosophila*. During development, the Hox genes are expressed in a pattern correlating with their chromosomal positions, as demonstrated by the different colors. The arrow indicates the direction of gene expression, and the grey scale demonstrates the response to retinoic acid, to which the anterior genes are more sensitive than the posterior genes (modified from (Lappin et al., 2006; van Oostveen et al., 1999)).

Hox structure

The mammalian Hox genes consist, in most cases, of two exons and one intron with the homeobox positioned in the second exon. The homeobox is a highly conserved sequence of 180 basepairs that transcribes into a helix turn helix motif serving as the DNA binding domain (see Figure 7) (Gehring et al., 1994; van Oostveen et al., 1999). The homeodomain can be divided into three helical regions (see Figure 7). Helix 3

contacts the major groove of DNA and recognizes a core sequence, which is TAAT or TGAT for the anterior Hox proteins and TTAT or TTAC for the posterior Hox proteins (Desplan et al., 1988; Phelan et al., 1995). Further contact of the homeobox domain to DNA is made by a sequence proceeding from helix 1 called the N-terminal arm, which binds to the minor groove (Wolberger et al., 1991). In addition, the variable sequence provides binding specificity to the individual Hox proteins. However, the Hox proteins have weak DNA binding activity and are therefore dependent on dimerization with co-factors that increase the stability of Hox-DNA binding (see below). These co-factors belong to the three amino acid loop extension (TALE) family and bind to a pentamer upstream of the homeodomain (see Figure 7).

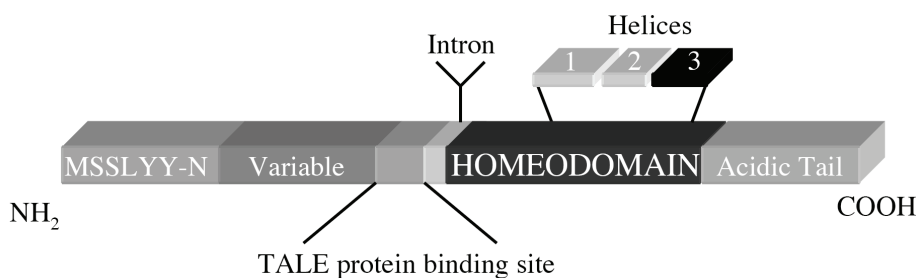


Figure 7. The general structure of Hox proteins. The homeodomain encodes for 60 aminoacids consisting of three helices in a helix-turn-helix motif, out of which helix three (black) binds to DNA. The variable domain is thought to give specificity to the protein and the carboxyl tail is often acidic, indicating that it might act as a transcriptional domain.

Regulation of Hox gene expression

Hox genes are organized in an order that is parallel with the expression pattern along the anterior-posterior body axis during development (see Figure 6). This means that the low number 3' genes are expressed earlier and more anteriorly during embryogenesis than the higher numbers at the 5' end (Duboule, 1994). This pattern is known as colinearity and enables the Hox genes to convey body patterning during the development. Similar patterns are also seen in adult organ systems such as hematopoiesis. All Hox genes are thought to have their own promoter. Interestingly, certain Hox genes share a master promoter that controls the expression of more than one Hox gene (Sharpe et al., 1998; van Oostveen et al., 1999). Moreover, regulatory elements have been identified in the introns of the Hox genes. For example, binding motifs for the TFs nuclear factor Y (NF-Y) and NF-YY1 were found in the Hoxb4 intron, regulating the expression pattern of the gene (Gilthorpe et al., 2002). In addition, by trimerization with stimulating factor 1 and 2 (USF1/2), NF-Y may regulate the expression of Hoxb4 by binding to the Hox response element 1 (HxRE-1) and HxRE-2/E-box, two critical DNA-binding sites in the Hoxb4 promoter (Giannola et al., 2000; Zhu et al., 2003).

The mechanisms regulating the expression of Hox genes are still not well characterized. However, it is known that Hox genes are regulated within the clusters of neighboring genes and by Hox genes from other clusters. For example, among the Hox paralog group 1 gene, Hoxa1 is required for the normal onset of Hoxb1. Hoxb1 is required for its own maintenance showing both cross- and auto regulation among the

Hox genes (Popperl et al., 1995; Studer et al., 1998). Furthermore, during embryogenesis, retinoic acid (RA) is known to regulate the expression of the 3' located Hox genes (Arcioni et al., 1992; Gould et al., 1997; Morrison et al., 1997a; Popperl and Featherstone, 1993). It has been shown that the regulatory relationship between Hox and RA is very dynamic and involves a feedback regulation system, which is crucial for axial development (Serpente et al., 2005). Moreover, it has recently been described that estrogen, progesterone and vitamin D regulate Hox gene expression during embryogenesis as well as in several adult organ systems where tissue regeneration is an ongoing process such as the reproductive tract, and hematopoiesis (Daftary and Taylor, 2006). For example, vitamin D was found to regulate differentiation of myeloid cells by activating HOXA10 as well as HOXB4 in human hematopoietic cell-lines (Du et al., 2005; Pan and Simpson, 2001a; Rots et al., 1998).

Two major groups of Hox regulators have emerged from studies performed in *Drosophila*, the Polycomb (PcG) and the Trithorax (TrxG) genes. The PcG genes function as transcriptional repressors maintaining the silencing of Hox gene expression. The PcG proteins repress transcription of Hox genes by modifying the chromatin structure and are central players in epigenetic regulation of X chromosome inactivation and imprinting. Several PcG genes have been identified in mammals. Bmi1, Me118, Ezh2, Rae28 and M33 have all been demonstrated to be important regulators of hematopoiesis (see above). The only TrxG gene identified in mammals is the mixed-lineage leukemia (Mll) gene, which act in an antagonistic manner to the PcG genes by activating the Hox genes. Mll is crucial for the development of HSCs (see above) and Mll deficiency results in complete absence of Hox genes (Hess et al., 1997).

Not many extrinsic factors that regulate Hox gene expression have been identified. However, TPO was found to both increase the expression levels of Hoxb4 as well as regulating the nuclear transport of Hoxa9, demonstrating that TPO modulates Hox gene function by several mechanisms (Kirito et al., 2003; Kirito et al., 2004). Hox protein activity has also been shown to be regulated by BMP stimuli and it was recently shown that Smad4 interacts with Hoxa9, resulting in disruption of the DNA binding capability of Hoxa9 (Li et al., 2006; Wang et al., 2006).

To further demonstrate the complexity in Hox gene regulation it was recently found that microRNA (miRNA) targets the disruption of Hox mRNA in mice. Yekta et al discovered that the miRNA, miR-196, targets the mRNA of Hoxb8 for degradation, demonstrating post-transcriptional regulation of Hox genes (Yekta et al., 2004). Intriguingly, several miRNAs regulating homeobox genes have been discovered in *Drosophila* (Ronshaugen et al., 2005; Stark et al., 2003) indicating that additional miRNA will be identified in the near future as regulators of Hox genes in mammals.

Hox co-factors

The high homology within the homeodomain of the Hox proteins results in poor sequence specificity and since most Hox proteins bind DNA weakly *in vitro* the arising question is how Hox protein specificity is obtained *in vivo* (Hoey and Levine, 1988). The specificity is established by the interaction of additional DNA binding proteins acting as Hox co-factors (Mann, 1995; Mann and Affolter, 1998; Mann and Chan, 1996). The first Hox co-factor, the Homeobox Extradenticle (*Exd*), was identified in *Drosophila*, as most of the homeobox genes (Peifer and Wieschaus,

1990). The mammalian homologs were found to belong to the TALE homeodomain proteins that are divided into two classes, Meis and PBC. The PBC proteins consist mainly of pre-B cell leukemia transformation related genes (Pbx) (Burglin, 1997; Burglin, 1998).

Pbx

The first Pbx gene, Pbx1, was identified by a chromosomal translocation, Pbx-E2a that causes B-cell leukemia (Kamps et al., 1990; Nourse et al., 1990). The Pbx homeodomain, a three amino acid loop, interacts with all Hox genes from paralogs 1-10 by binding to the TALE binding site containing a tryptophan-hexapeptide motif (N-Y/F-P/D-W-M-K/R) (See Figure 7) (Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom and Murre, 1997; Passner et al., 1999). The dimerization of Pbx and Hox has been demonstrated to bind DNA with high specificity *in vitro* (Chan et al., 1994; Chang et al., 1995). However, the binding effect of co-factors *in vivo* is still not understood and the Pbx/Hox complex has been shown to act as both activator and repressor, which may be dependent on additional factors (Moens and Selleri, 2006).

There are four known Pbx members in vertebrates (Pbx 1-4) and targeted mutations have demonstrated redundancy among the genes. In fact, experiments in zebrafish have suggested that the functional differences between them are more related to their expression than to their mechanism, and embryonic Pbx4 mutants were indeed rescued with any of the zebrafish Pbx genes (Popperl et al., 2000). Interestingly, Pbx1 knockout mouse embryos displayed several phenotypes related to organ systems where Hox genes are known to regulate the developmental program including a severe anemia and a 5-fold decrease in CMP cells (DiMartino et al., 2001). A number of *in vitro* studies have shown that Hox genes such as Hoxb3, Hoxb4, Hoxb8, and Hoxa9 (Knoepfler et al., 2001; Krosl et al., 1998; Schnabel et al., 2000) prevented differentiation and/or caused transformation of cells in culture in a Pbx dependent manner. This indicates that Pbx/Hox complexes might promote proliferation.

However, not all Hox functions are dependent on the Pbx genes. Hoxb6 was found to immortalize myeloid cells *in vitro* independent of its TALE binding site (Fischbach et al., 2005). Furthermore, two studies by the Sauvageau laboratory showed that Pbx interaction had a repressive effect on Hox function by inhibiting Hoxb4 stem cell expansion *in vitro* (Beslu et al., 2004; Krosl et al., 2003b). These findings suggest that Pbx/Hox interactions result in several different fate options and most likely are dependent on additional factors like Meis1.

Meis

The myeloid ecotropic viral integration site (Meis) gene was first identified as a common site for viral integration causing myeloid leukemia in BXH-2 mice (Moskow et al., 1995). In contrast to the Pbx proteins, Meis interacts with the posterior Hox gene paralogs 9-13 and the binding between Hox and Meis does not require the conserved hexapeptide (Shen et al., 1997). Meis1 deficient mice were recently found to be embryonic lethal, displaying hemorrhages and anemia (Azcoitia et al., 2005). Furthermore, the expression of Runx1 was almost absent showing that Meis1 was crucial for definitive hematopoiesis. In addition to Hox binding, Meis1 can heterodimerize with Pbx and bind to DNA (Chang et al., 1997). It has also been shown that Meis1 interacts with Pbx in the absence of DNA. The formed heterodimer has been shown to induce nuclear localization as well as rendering stability to the Meis-Pbx complex (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Waskiewicz et al.,

2001). Concomitantly, it has been shown that Meis, Pbx and Hox form a trimeric complex that regulates transcriptional activation. Both Meis and Pbx could act as DNA binding and non-DNA binding partner respectively to the Hox gene, where the non-binding partner increased the stability of the complex (see Figure 8) (Shanmugam et al., 1999; Shen et al., 1999). Moreover, Meis1 overexpression together with Hox genes such as HOXA9 and HOXA10 dramatically accelerated the onset of myeloid leukemia, demonstrating Meis as an important factor in leukemogenesis (Pineault et al., 2004; Thorsteinsdottir et al., 2001). The interactions between Pbx, Meis and Hox have largely been established from *in vitro* studies and how these interactions regulate individual Hox gene specificity *in vivo* have yet to emerge.

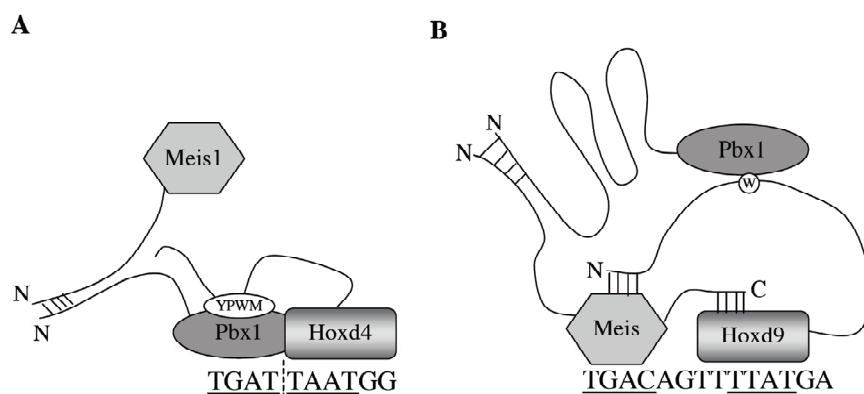


Figure 8. Schematic model illustrating the trimeric complexes including Hox, Meis and Pbx. (A) Meis1 as the non-DNA binding partner interacting to Pbx via their N terminus while Hox and pbx binds to a consensus binding site. (B) Pbx as the non-DNA binding partner. The Pbx connects to the Hox gene via tryptophan motif while Meis and Pbx interacts via their N termini. The core binding sites is underlined in both A and B (Shanmugam et al., 1999).

Hox genes in normal hematopoiesis

Hematopoiesis is a tightly regulated process and several Hox transcription factors have been identified as major regulators controlling proliferation, differentiation and self-renewal during different stages of the hierarchy. The first evidence of Hox gene expression during hematopoiesis came from *in vitro* studies performed on cell lines in the late 80s (Kongsuwan et al., 1988; Shen et al., 1989). It has now been established that Hox genes from the A, B and C clusters are expressed during normal hematopoiesis and genes from all four clusters have been detected in leukemia (Giampaolo et al., 1994; Moretti et al., 1994; Sauvageau et al., 1994). The expression pattern of the Hox clusters has been described as co-linear as well as lineage restricted e.g. HOXA genes are expressed primarily in myeloid cells, HOXB genes in erythroid cells and HOXC in lymphoid cells (Bijl et al., 1996; Care et al., 1994; Lawrence et al., 1993; Lowney et al., 1991; Mathews et al., 1991; Quaranta et al., 1996; Shen et al., 1989). However, such compartmentalization is in reality a simplistic model and the true Hox expression pattern in hematopoiesis is far more complex. Studies using murine HSCs demonstrate the presence of Hox genes from both the A and B clusters as well as low and high paralogous genes in the HSC compartment e.g. Hoxb3, Hoxb4, Hoxa9, Hoxa10, Hoxa5 (Ivanova et al., 2002). Interestingly, these genes were

found to be downregulated upon differentiation demonstrating the importance of Hox genes during early hematopoiesis.

In order to investigate the role of individual Hox genes, several Hox knockout mice have been generated. Still, not much is known about their effect on hematopoiesis since most of the studies have focused on embryogenesis and developmental effects (Barrow and Capecchi, 1996; Boulet and Capecchi, 1996; Capecchi, 1991; Condie and Capecchi, 1993; Goddard et al., 1996; Manley and Capecchi, 1995; Ramirez-Solis et al., 1993). Nevertheless, these studies have demonstrated an important biological event, the occurrence of redundancy among the Hox genes. Also, compound knockout mice missing more than one Hox gene have illustrated a dose dependent effect on the phenotypes (Chen and Capecchi, 1999; Maconochie et al., 1996; Manley and Capecchi, 1997). This phenomenon was also found to be true for hematopoiesis as shown in articles I and II. However, most information about the role of Hox transcription factors in the regulation of hematopoiesis have come from overexpression studies. These studies have provided evidence for their capacity to regulate HSCs as well as lineage commitment and for their leukemogenic potential, summarized in Table 1 (Abramovich and Humphries, 2005; Eklund, 2006).

HOXA genes in hematopoiesis

Expression analysis performed on human hematopoietic progenitor cells revealed that genes from the HOXA cluster are the most frequently expressed Hox genes in hematopoiesis (Moretti et al., 1994; Sauvageau et al., 1994). **HOXA9** is the most studied gene within the A cluster due to its common involvement in leukemia, but lately also for its role in normal hematopoiesis. Interestingly, Hoxa9 knockout mice displayed a 7-fold reduction in competitive repopulating units (CRU), although the frequency of immunophenotypically defined stem cells (by Lin⁻, c-kit⁺, flk-2, Sca-1⁺ (KLFS)) and homing of progenitor cells to the bone marrow were normal (Lawrence et al., 2005). In addition, the Hoxa9 null bone marrow cells showed impaired recovery following hematopoietic stress, and single cell cultures using KLFS cells revealed a reduction in high proliferating colonies. Furthermore, the Hoxa9 null mice displayed a reduction in spleen size as well as in white blood cell counts (Lawrence et al., 1997). Conversely, overexpression of HOXA9 resulted in an *in vivo* expansion of the HSC pool as well as an enhanced myelopoiesis and a partially blocked B lymphopoiesis (Thorsteinsdottir et al., 2002). In addition, our findings in article V further support Hoxa9 as an important proliferation regulator of HSCs and its progeny during adult as well as fetal hematopoiesis.

Hoxa10, the closely related neighbor to Hoxa9, is expressed in the HSC compartment as well as in most acute myeloid leukemias (Lawrence et al., 1995). (Lawrence et al., 1997). However, mice deficient in Hoxa10 display no HSC phenotypes (Lawrence et al., 2005). In contrast, gain of function studies by retroviral mediated gene transfer, have established HOXA10 as an important regulator of hematopoiesis. Early studies by Thorsteinsdottir et al, demonstrated that enforced expression of HOXA10 resulted in an increased number of myeloid progenitor cells and impaired B-cell development as well as the formation of large colonies containing megakaryocyte-blast cells (Thorsteinsdottir et al., 1997). The same laboratory found that overexpression of HOXA10 in human hematopoietic cells had similar effects including a reduction in T-cells and erythrocytes *in vitro* indicating species related differences (Buske et al., 2001), further discussed in article V. In addition, HOXA10 was suggested to be an important TF for monocyte development since enforced

expression in human hematopoietic cell progenitors resulted in a dramatic increase in CD14⁺ cells *in vitro* (Taghon et al., 2002). Moreover, our group have demonstrated a direct role of HOXA10 in proliferation of myeloid progenitors since, by using an inducible system, the increased number of CFU-S colonies was reversed on withdrawal of HOXA10 (Bjornsson et al., 2001). This model system was further explored in article V, demonstrating that HOXA10 is a critical regulator of HSCs and erythroid/megakaryocyte development in a dose dependent manner. Taken together, these data emphasize the importance of down regulation of Hoxa9 and Hoxa10 for normal differentiation of hematopoietic cells to occur.

The analysis of **Hoxa7** deficient mice resulted in a more stage specific effect than Hoxa9 and Hoxa10, as illustrated by the decreased number of MEP cells (So et al., 2004). Interestingly, enforced expression of HOXA10 resulted in increased levels of MEPs (article V), suggesting that correct Hox gene regulation is important for erythrocyte/megakaryocyte differentiation.

Finally, the expression of **Hoxa5** has been reported in the HSC compartment as well as in myeloid progenitor cells (Crooks et al., 1999; Ivanova et al., 2002). No effects have been reported on the HSC level, but increased levels of HOXA5 inhibited erythropoiesis and promoted myelopoiesis (Crooks et al., 1999). Concomitantly, antisense against HOXA5 resulted in an increased number of erythroid progenitors and an impaired myelopoiesis implying HOXA5 as an important regulator of lineage commitment and maturation (Fuller et al., 1999).

HOXB genes in hematopoiesis

HOXB4 is the most “famous” and studied Hox gene in hematopoiesis due to its dramatic effect on HSC self-renewal. Enforced expression of HOXB4 using retroviral vectors demonstrated an impressive *in vivo* expansion without affecting lineage commitment (Antonchuk et al., 2001; Sauvageau et al., 1995). Furthermore, the *in vivo* expansion led to a faster and more complete restoration of the HSC pool in the transplanted animal, which normally only reaches 10% of the non-irradiated recipients (Thorsteinsdottir et al., 1999). Interestingly, this expansion never exceeded normal HSC levels, indicating that HOXB4 overexpressing cells are controlled by the stem cell niche *in vivo* and so far no leukemia has been detected. Antonchuk et al further demonstrated that *in vitro* culture for 10 days after HOXB4 mediated gene transfer resulted in an impressive 40 fold net increase in HSCs, which is the most efficient expansion of HSCs known to the field of stem cell biology (Antonchuk et al., 2002). However, for clinical purposes retroviral vector integration is not favorable due to the risk of insertional mutagenesis. Subsequently, two attempts using the purified HOXB4 protein, thus circumventing the use of viral vectors and the risk of cancer development, were shown to successfully expand both murine and human HSCs *in vitro*, 4 and 2.5 fold respectively (Amsellem et al., 2003; Krosi et al., 2003a). The molecular mechanism causing the HOXB4 mediated HSC expansion is not well characterized but the interaction with Pbx has been studied. Surprisingly, downregulation of Pbx using antisense together with overexpression of HOXB4 had a favorable effect on the HSC expansion. In addition, it was shown that mutating the DNA binding domain of HOXB4 abrogated the HSC expansion, but mutations in the TALE binding sequence did not alter the HSC expansion indicating that Pbx and HOXB4 work in independent pathways in HSC regulation (Beslu et al., 2004). Recently, it has also been demonstrated that the obtained HSC expansion is dependent on the level of HOXB4 expression with high levels promoting differentiation and

impairing lineage commitment (discussed below) (Brun et al., 2003; Schiedlmeier et al., 2003).

Even though **HOXB3** is expressed in a similar pattern as HOXB4, overexpression of HOXB3 has a totally different effect on hematopoiesis. Enhanced expression of HOXB3 resulted in a myeloproliferative disorder and a perturbed lymphopoiesis (Sauvageau et al., 1997). In order to study the physiological role of Hoxb4 and Hoxb3/Hoxb4 we generated two knockout mouse models deficient in Hoxb4 or Hoxb3 and Hoxb4, presented in articles I and II. Our findings support a role for Hoxb4 in HSC regulation since loss of Hoxb4 resulted in reduced proliferation of HSCs during hematopoietic stress. In contrast, Bijl et al showed that fetal liver HSCs deficient in the whole Hoxb cluster, except hoxb13, retained full repopulating capacity, demonstrating a complex network of genetic interactions within the Hox genes (Bijl et al., 2006).

The expression pattern of **Hoxb6** appeared to be somewhat unique since it is not expressed until late in myeloid progenitor cells (Giampaolo et al., 2002; Kawagoe et al., 1999). However, a recent study overexpressing HOXB6 resulted in HSC expansion *in vivo* as well as in myeloid progenitors while inhibiting erythropoiesis and lymphopoiesis (Fischbach et al., 2005). Interestingly, the HSC expansion was, as for HOXB4, independent of the interaction with Pbx. Still, it is unclear whether the HOXB6 effect is physiologically relevant, since deficiency in Hoxb6 only resulted in an increased number of erythroid progenitor cells (Kappen, 2000; Shen et al., 1992).

HOXC genes in hematopoiesis

Most of the HOXC genes in hematopoiesis are expressed in the lymphoid compartment, such as HOXC4, HOXC5 and HOXC6 (Bijl et al., 1996). However, not many functional studies have been performed on HOXC genes and their involvement in hematopoiesis. Even so, **HOXC4** was found to be expressed in the human HSC pool and overexpression analysis of HOXC4 resulted in an expansion of early progenitor cells *in vitro* (Daga et al., 2000). This phenotype resembles the effect of the paralog gene Hoxb4, but the role of Hoxc4 has not been characterized *in vivo*. Furthermore, **Hoxc8** was found to be important for the myeloid differentiation since Hoxc8 knockout mice displayed reduced numbers of erythroid and myeloid progenitor cells. Yet, the effect on HSCs and the lymphoid development have not been characterized (Shimamoto et al., 1999).

Table 1. A summary the hematopoietic phenotypes caused by dysregulation of Hox genes

Hox gene	Phenotypes in transgenic mice		Association with human leukemia
	Gain-of-function	Loss-of-function	
Hoxa9	Increase in HSC self-renewal and myelopoiesis <i>in vivo</i> , induction of AML (Kroon et al., 1998; Thorsteinsdottir et al., 2002)	Severe reduction in proliferation and repopulating ability, normal homing. (Lawrence et al., 2005)	AML, chronic myeloid leukemia, acute lymphocytic leukemia and infant leukemia (Drabkin et al., 2002; Ferrando et al., 2003; Golub et al., 1999; So et al., 2004; Tedeschi and Zalazar, 2006)
HOXA10	Increase in proliferation of HSCs and myeloid progenitors, block in the differentiation of erythrocytes, megakaryocytes and T-cells, induction of AML. (article V)(Bjornsson et al., 2001; Buske et al., 2001; Thorsteinsdottir et al., 1997)	No HSC phenotype (Lawrence et al., 2005)	Acute myeloid leukemia and acute lymphocytic leukemia (Ferrando et al., 2003; Lawrence et al., 1995)
Hoxa7	Not investigated	Decrease in MEP cells (So et al., 2004)	Acute myeloid leukemia and infant leukemia (Afonja et al., 2000; So et al., 2004)
Hoxa5	Increase in myelopoiesis and impaired erythropoiesis (Crooks et al., 1999)	Increase in erythropoiesis and impaired myelopoiesis (Fuller et al., 1999)	Acute myeloid leukemia (Drabkin et al., 2002)
Hoxb4	Increase in HSC self-renewal in vitro and in vivo, high levels promotes differentiation and impairs lineage commitment. (Antonchuk et al., 2001; Brun et al., 2003; Schiedlmeier et al., 2003; Thorsteinsdottir et al., 1999)	Decrease in HSC proliferation Article I and II	No
Hoxb3	Perturbed lymphopoiesis, induction of a myeloproliferative disorders (Sauvageau et al., 1997)	Not investigated	Acute myeloid leukemia (Roche et al., 2004)
Hoxb6	Increase in HSCs and myeloid progenitors in vivo, impair erythropoiesis and lymphopoiesis, induction of AML (Fischbach et al., 2005)	Increase in erythropoietic progenitors (Kappen, 2000; Shen et al., 1992)	Acute myeloid leukemia (Giampaolo et al., 2002)
Hoxc4	Expansion of early progenitors in vitro (Daga et al., 2000)	Not investigated	Acute myeloid leukemia (Bijl et al., 1998)
Hoxc8	Not investigated	Decrease in erythroid and myeloid progenitors (Shimamoto et al., 1999)	No

Hox genes in malignant hematopoiesis

Given that Hox genes regulate proliferation and differentiation of hematopoietic cells it was not surprising that dysregulation of these genes contribute to malignant transformation of cells. For example, elevated expression levels of HOXA7–HOXA11, HOXB6 and HOXB8 have been reported in human leukemia (Buske and Humphries, 2000; Eklund, 2006). HOXA9 and HOXA10 are both expressed in all types of human acute myeloid leukemias (AML) (except promyelocytic leukemia), and HOXA9 is the single most correlated factor for poor prognosis in human AML (Buske and Humphries, 2000; Drabkin et al., 2002; Golub et al., 1999; Lawrence et al., 1995). In mice, overexpression studies with retroviral vectors expressing HOXA9 or HOXA10 in HSCs also generate AML, however with a latency period of several months up to one year (Kroon et al., 1998; Thorsteinsdottir et al., 1997). This implies that additional mutations are required for the onset of disease and overexpression of HOXA9 and HOXA10 together with Meis1 dramatically reduced the latency period of the AML (Pineault et al., 2004; Thorsteinsdottir et al., 2001).

A more direct involvement of Hox genes in leukemia is their frequent fusion with the nucleoporin gene NUP98 (Slape and Aplan, 2004), a component of the nuclear pore complex involved in transport of RNA and proteins across the nuclear membrane (Fontoura et al., 1999; Powers et al., 1997). The most common NUP98-HOX fusion protein is the NUP98-HOXA9, which results from the translocation t(7:11). The fusion product contains the HOXA9 homeodomain and FG repeats from the NUP98, known to have transcriptional activation capabilities. This fusion product has been associated with AML M2 and M4 (Barrow and Capecchi, 1996; Kasper et al., 1999; Nakamura et al., 1996). Indeed, overexpression of the fusion protein using retroviral vectors in HSCs resulted in the development of AML with a latency period of at least 4 months *in vivo* (Kroon et al., 2001). Interestingly, as for enforced expression of normal HOXA9, co-expression of NUP98-HOXA9 and Meis1 accelerated the onset of AML suggesting that additional mutations are required to fully transform the cells (Kroon et al., 2001). Six other Hox genes have been identified in NUP98 fusions (HOXA11, HOXC11, HOXD11, HOXA13, HOXC13, HOXD13) (Slape and Aplan, 2004). Recently, the NUP98-HOXA9 fusion protein were shown to cooperate with other translocations e.g. BCR-ABL and TEL-PDGFR in the development of leukemia (Dash et al., 2002; Slape and Aplan, 2004). Intriguingly, NUP98-Hox fusion proteins have recently been shown to enhance HSC proliferation *in vivo* and *in vitro* (Chung et al., 2006; S. Sekulovic and Humphries, 2005). These findings may open new avenues for these fusion products as new candidates in HSC expansion.

The Hoxb8 gene has been implied in leukemic transformation in collaboration with Il-3 (Perkins et al., 1990; Perkins and Cory, 1993). Furthermore, overexpression of HOXB6 was recently shown to induce AML with the same time lag as HOXA9, demonstrating the involvement of all Hox clusters in leukemic transformation (Fischbach et al., 2005).

Hox target genes

The mechanisms by which Hox genes regulate hematopoietic cell fates are still an open question and not many genuine target genes have been identified. It is clear from the studies performed during embryogenesis that Hox genes are transcriptionally activated by each other in a complex pattern. This suggested that auto- and cross regulation within the Hox clusters are an important mechanism controlling

organogenesis (Arcioni et al., 1992; Gould et al., 1997; Morrison et al., 1997a; Sharpe et al., 1998). Most of the Hox target genes in hematopoiesis arise from studies performed on cell lines revealing myeloid specific target genes and cell cycle regulators. HOXA10 was found to transcriptionally activate p21 in a myelomonocytic cell line resulting in differentiation and cell cycle arrest (Bromleigh and Freedman, 2000). Interestingly, the same group reported an additional binding site for HOXA10 that had a negative effect on p21 transcription, indicating that HOXA10 can activate or repress transcription of p21 depending on the cellular context. Furthermore, HOXA10 was shown to repress transcription of *Cybb* (phagocyte burst oxidase protein gp91phox) by forming a complex with histone deacetylase (HDAC2) and thereby blocking the access for transcriptional activators (Eklund et al., 2000; Lu et al., 2003). In contrast to HOXA10, HOXA9 activated transcription of *Cybb* by binding to the same binding site as HOXA10 together with Pbx (Bei et al., 2005). Furthermore, Shi et al demonstrated that HOXA9 is a transcriptional repressor of OPN, which may explain why overexpression of HOXA9 *in vivo* resulted in HSC expansion since OPN is known to inhibit HSC division (Shi et al., 2001).

Two studies by Raman et al demonstrated HOXA5 as a transcriptional activator of p53 and progesterone in breast cancer cell lines suggesting that loss of HOXA5 is the primary reason why breast tumors often lack p53 expression (Raman et al., 2000a; Raman et al., 2000b).

Even though the role of Hoxb4 is carefully studied in hematopoiesis, not many target genes have been identified. However, HOXB4 was found to block transcription of c-Myc in the HL-60 cell line resulting in differentiation (Pan and Simpson, 1999; Pan and Simpson, 2001b). Indeed, we found that the c-Myc was upregulated in fetal liver cells deficient in Hoxb4, which further supports c-Myc as a downstream target of Hoxb4 (article II). However, Satoh et al demonstrated that Hoxb4 activated the transcription of c-Myc in adult bone marrow cells and proposed that c-Myc supported self-renewal of HSCs as a downstream mediator of Hoxb4 (Satoh et al., 2004). This shows that Hoxb4 functions as a transcriptional activator as well as a repressor depending on the cellular context.

Recently, numerous potential Hox target genes have emerged from global gene expression profiling, which have provided valuable insight into the mechanisms underlying the role of Hox genes in hematopoiesis (Dorsam et al., 2004; Ferrell et al., 2005; Ghannam et al., 2004). However, these genes need to be validated as true targets before any real conclusions can be drawn. Intriguingly, in article V we identified four new putative target genes for HOXA10 in primary hematopoietic bone marrow cells, which may explain how HOXA10 mechanistically regulates HSC proliferation and erythrocyte/megakaryocytic development.

Finally, better understanding of the target genes, and how they are regulated together with co-factors, will be necessary in order to improve future treatments for HOX induced leukemias as well as for the use of Hox genes in HSC expansion protocols.

Applications in clinical medicine: HSC expansion

HSC transplantation is today a common approach in the field of hematology and oncology to treat multiple malignant diseases and non-malignant disorders (Sorrentino, 2004). The field developed from research to treat the sequelae of irradiation exposure, which became a great concern after the development of the nuclear bomb during world war two. Lorenz et al discovered that mice receiving bone marrow transfusion after lethal irradiation rescued them from radiation sickness and in 1956 Barnes et al described the first treatment of murine leukemia by HSC transplantation (Barnes et al., 1956; Lorenz et al., 1951). HSC transplantation in humans was pioneered by Dr E. Thomas and the first successful transplant into a leukemic patient was reported in 1959 (Thomas et al., 1959). However, these transplants were only successful when grafts came from identical twins and it was not until the late 60s and early 70s that allogenic bone marrow transplantations were possible thanks to the increasing knowledge about human leukocyte antigens (HLA). Taking HLA into account allowed for the selection of compatible donors and thereby reducing the risk for graft versus host disease (Thomas, 2000). The first transplantations used bone marrow as the source for HSCs, but today the most common source is peripheral blood after treating the donor with G-SCF that mobilizes the HSCs into the periphery (Schmitz et al., 1995). In addition, HSCs can be obtained from umbilical cord blood (CB) collected from newborn children during delivery (Broxmeyer et al., 1989). Interestingly, the CB HSCs are less immunogenic, however the number of HSCs from these grafts is small and so far only suitable for pediatric patients (Harris et al., 1992). Studies have shown that engraftment efficiency correlates with the number of HSCs transplanted (Shizuru et al., 1996).

To further explore the use HSCs from UB as well as reducing the risk and cost obtaining HSCs from adult donors, efficient HSC *ex vivo* expansion protocols would be extremely valuable. Indeed, several attempts have been performed in order to expand human HSCs *in vitro*, but so far only a very limited expansion have been reported and in most cases HSC activity is declining (Amsellem et al., 2003; Bhatia et al., 1997). HSC expansion for clinical use needs to be tightly controlled for safety reasons, since self-renewal is a property of leukemic stem cells. One approach that has been described is use of the HOXB4 protein in the culture medium (Amsellem et al., 2003; Krosil et al., 2003a). However, only a mild expansion was detected in comparison with the impressive expansion seen using retroviral mediated gene transfer in mice. The differences can be related to the protein levels (discussed below) and a major obstacle is the short half-life of the HOXB4 protein. Encouragingly, NUP98-Hox fusion proteins have recently been shown to be even more efficient in expanding HSCs than HOXB4 (S. Sekulovic and Humphries, 2005). Again, since these proteins are known oncogenes, safety needs to be established before clinical use.

The dissection of the HSC niche is opening new fundamental approaches for HSC expansion (Calvi et al., 2003). The idea of mimicking the HSC bone marrow niche *in vitro* is a very appealing strategy for safety concerns. However, it may be very challenging since the HSC niche during steady state does not support HSC expansion (Martin and Bhatia, 2005). The HSC niches during development are the fetal liver and the placenta and they contain microenvironments that naturally support HSC expansion (Martin and Bhatia, 2005; Mikkola et al., 2005). Therefore, these HSC niches may be more suitable to mimic than the bone marrow niche.

If HSC expansion becomes clinically feasible, it would have tremendous applications not only in hematological malignancies but also for treatment of genetic

disorders such as β -thalassemia, sickle-cell anemia, DBA and for the gene therapy field (Karlsson, 1991; Roy et al., 2005; Sorrentino, 2004). The properties of the HSCs make them suitable targets for gene therapy by introducing the therapeutic gene into the patients own HSCs *in vitro* using retroviral mediated gene transfer. However, this approach demands many HSCs and would greatly benefit from efficient HSC expansion protocols. Still, gene therapy has been successfully used in treating patients with severe combined immunodeficiency (SCID) (Aiuti et al., 2002; Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002). Unfortunately, three out of nine patients in this pioneering study developed a T cell lymphoproliferative disease as a consequence of insertional mutagenesis (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). Even so, this trial is a breakthrough for these patients since no other treatments are available, and if a small number of transduced HSCs could be efficiently expanded *in vitro*, the risk of insertional mutagenesis would be less significant.

Aims of the present studies

The studies presented in this thesis aim at resolving the role of Hox transcription factors in hematopoiesis using mouse models with altered Hox gene expression generated in our laboratory. In particular, the role of HOXA10 and Hoxb4, and its interaction with Hoxb3, Hoxa9 and the cell cycle regulator p21 in hematopoiesis. The mouse models used for this purposes are listed below.

- A conventional Hoxb4 knockout, generated by complete deletion of the entire Hoxb4 gene using the Cre-LoxP technique
- A conventional Hoxb3 and Hoxb4 knockout, generated by complete deletion of the entire Hoxb3 and Hoxb4 genes using the Cre-LoxP technique
- Enforced expression of HOXB4 in p21 knockout HSCs using retroviral mediated gene transfer
- A conventional Hoxb3, Hoxb4 and Hoxa9 knockout by mating our Hoxb3/Hoxb4 knockout with the Hoxa9 knockout, kindly provided by Dr H. Jeffery Lawrence
- An inducible HOXA10 mouse model by mating our tetO-HOXA10 mouse with the Rosa26rtTA-nls mouse, kindly provided by Dr Rudolf Jeanisch.

Specific aims

- To study the effects of Hoxb4 deletion in pre- and postnatal hematopoiesis
- To study the combined effects of Hoxb4 and Hoxb3 deletion in hematopoiesis and to what extent these genes collaborate in hematopoiesis
- To study whether the increase in HSC self-renewal generated by overexpression of HOXB4 is enhanced in p21-deficient HSCs
- To analyze what the combined effects of Hoxb3, Hoxb4 and Hoxa9 deficiency in hematopoiesis are, and to what extent these genes collaborate in HSC regulation
- To study whether tight regulation of HOXA10 is essential for the control of normal fate options in hematopoietic progenitors and stem cells.

Summary of results

(I) Reduced proliferative capacity of hematopoietic stem cells deficient in Hoxb3 and Hoxb4.

(II) Hoxb4 deficient mice have normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells

Several overexpression studies have shown that Hoxb4 and Hoxb3 are important regulators of hematopoiesis and that enforced expression of HOXB4 dramatically enhances the stem cell pool. Furthermore, both genes have been shown to have similar expression patterns in normal hematopoiesis indicating a possible redundancy among these genes. In order to study the physiological role of Hoxb4 alone or in concert with Hoxb3 in hematopoiesis, we generated two knockout mouse models. These knockout strains were generated using the Cre-LoxP technique where the whole gene sequence of Hoxb4, or Hoxb3 and Hoxb4, were flanked by LoxP sites. Upon excision at the LoxP sites with Cre-recombinase, conventional knockouts lacking either Hoxb4 or Hoxb3 and Hoxb4 were generated. Since previous Hoxb4 knockout models using the classical NEO cassette targeting strategy displayed lethality due to skeletal abnormalities upon loss of Hoxb4, we were surprised to find that both our knockout mouse strains were viable and born at normal Mendelian ratios. Only minor skeletal abnormalities, such as loss of the lowest rib pair, were found in 40% of the knockout mice. A mild hypocellularity in the bone marrow and spleen was also detected but this did not affect lineage distribution. Mice of both knockout strains displayed normal homing of HSC, but with a mild proliferation defect in the primitive hematopoietic progenitors (Lin⁻, Sca-1⁺, c-kit⁺ cells). More importantly, a significant reduction in repopulating ability *in vivo*, and a reduced proliferative response of repopulating HSCs to hematopoietic stress was detected when the knockout mice were treated with repeated 5-FU injections.

Overall, the phenotypes were mild but more pronounced in the double knockout when compared to the single knockout, suggesting a redundancy of function for these genes in hematopoiesis. Furthermore, our data demonstrates that the Hoxb4 effect is restricted to HSC function, supporting a role for Hoxb4 in HSC proliferation.

(III) HOXB4-induced self-renewal of hematopoietic stem cells is significantly enhanced by p21 deficiency

Overexpression of HOXB4 and downregulation of p21 can independently increase proliferation of murine HSCs. We therefore asked if simultaneous modulation of these pathways, by overexpression of HOXB4 using retroviral mediated gene transfer into p21 knockout cells, would further enhance the HSC proliferation *in vitro*. The HOXB4 transduced p21 knockout bone marrow cells were kept in liquid culture for up to 11 days revealing that modulation of both pathways resulted in increased expansion of primitive cells. The proliferation effect on the p21 knockout HSCs transduced with HOXB4 after 5 days of *in vitro* culture was enumerated by CRU assay, demonstrating a clear increase in CRU numbers compared to controls. These findings suggest that temporary overexpression of HOXB4 and suppression of p21 could be useful for future HSC expansion protocols.

(IV) Hoxa9/Hoxb3/Hoxb4 compound null mice display severe hematopoietic defects

Hoxb3, Hoxb4 and Hoxa9 are all expressed in the HSC compartment and deficiency of Hoxb3 and Hoxb4 or Hoxa9 display comparable HSC phenotypes suggesting that these genes have similar roles in HSC regulation. Hence, we asked whether deletion of Hoxa9, Hoxb3 and Hoxb4 together would further increase the hematopoietic phenotypes, and in particularly the HSC phenotype. Breeding our Hoxb3/hoxb4 double mutants with a Hoxa9 deficient mouse generated a triple (Hoxa9/b3/b4) knockout mouse model, which was viable but displayed a significant reduction in body weight, spleen size and spleen cellularity. Furthermore, Hoxa9/b3/b4 knockout mice demonstrated increased numbers of LSKCD150⁺ immunophenotypic HSCs, but when assessed in a competitive repopulating assay the repopulating capacity of Hoxa9/b3/b4 knockout HSCs did not decline beyond the repopulating defect seen in Hoxa9 knockout HSCs.

(V) HOXA10 is a critical regulator for hematopoietic stem cells and erythroid/megakaryocyte development

In order to delineate the role of HOXA10 in hematopoiesis, we generated an inducible HOXA10 mouse model by mating our previously published tetO-HOXA10 mouse model with the Rosa26-rtTA-nls mice that express the reverse tetracycline transactivator in all hematopoietic tissues. The expression level of HOXA10 was tightly regulated by the administration of doxycycline. High levels of HOXA10 blocked the development of erythropoiesis and megakaryopoiesis and lead to an accumulation of MEP cells, possibly due to a direct downregulation of GATA-1. Furthermore, intermediate expression levels of HOXA10 induced a 15-fold increase in the repopulating capacity of HSC after 13 days of in vitro culture, compared to uninduced control cells. Interestingly, the proliferation capacity was dependent on the expression level of HOXA10 since high levels of HOXA10 had no positive effect on HSC proliferation. The effect on HSC proliferation was associated with the alteration of several genes known to regulate stem cell self-renewal e.g. Dkk1, HLF and Gfi-1. Molecular studies revealed binding sites for HOXA10 in the promoter region of Dkk1 and HLF as well as transcriptional activation of Dkk1 and Gfi-1 upon enforced HOXA10 expression. Additionally, in vivo induction of HOXA10 recapitulated previous findings demonstrating impaired T-Cell development and increased myelopoiesis. These findings demonstrate that HOXA10 is acting as a master regulator of hematopoiesis, governing both proliferation and differentiation of hematopoietic progenitors and stem cells, where distinct fate options depend on graded expression of HOXA10.

General discussion

Mouse targeting strategies

It is now more than 20 years since the first transgenic mouse model was developed, starting an era for the analysis of gene function. Since then, several thousands of transgenic mice have been generated and analyzed. The targeting strategies have been modified over the years resulting in advanced genetic modifications such as conditional knockout strains, (Baubonis and Sauer, 1993; Sauer, 1994) and inducible transgene expression models (Gossen et al., 1995; Hochedlinger et al., 2005; Huettnner et al., 2000). Even though the use of transgenic mice have been extremely valuable, it is important to emphasize the significance of careful analysis and interpretation of the results. Some important aspects that we have encountered are addressed below.

Loss of function

The first common genetic targeting strategy for terminating the expression of a gene was performed by insertion of a selection cassette (e.g. neomycin) in a functionally defined exon followed by a stop codon to permanently disable expression of the gene of interest. These transgenic mice are called conventional knockout mice and several of the early Hox knockout strains were generated using this technique in order to study the role of the gene of interest during embryogenesis (Barrow and Capecchi, 1996; Boulet and Capecchi, 1996; Capecchi, 1991; Ramirez-Solis et al., 1993). Three major setbacks exist with this technique: (1) Embryonic lethality caused by the termination of the gene of interest preventing postnatal studies. (2) Tissue specific effects may be hidden or affected by phenotypes from other organ systems, since the gene is terminated in all tissues. (3) Due to strong promoters used in these knock out models, flanking downstream genes could also be affected by the insertion of the selection cassette, resulting in miss-interpretation of the resulting phenotype.

These problems were circumvented by the development of the Cre-LoxP technique, which allowed for the termination of the gene in a tissue and time specific manner, called conditional knockouts (Baubonis and Sauer, 1993; Sauer, 1994). Importantly, this technique allows for the removal of the selection cassette combined with its own promoter that otherwise may interfere with normal transcriptional activity and result in a non-specific gene knockout (Shivdasani et al., 1997). Hox genes may be very sensitive to this effect due to their co-linearity and sharing of regulatory elements (Boulet and Capecchi, 1996). Therefore, insertion of a selection cassette in a Hox gene may result in a strong phenotype due to the alterations of neighboring genes.

The first Hoxb4 knockout described by Ramirez-Solis et al (Ramirez-Solis et al., 1993), generated by insertion of a selection cassette in exon one and a stop codon in exon two, was lethal in the perinatal period due to a split sternum. In contrast, our Hoxb4 and Hoxb3/Hoxb4 knockouts, generated by deletion of the entire gene/s by the Cre-LoxP technique on the embryonic stem cell level, were both viable. They displayed a mild phenotype indicating that the previous conventional knockout was influenced by the transcription of other genes. However, when we removed the whole Hoxb4 gene we also removed regulatory elements that may interfere with the expression of other Hox genes. Indeed, gene expression analysis revealed that Hoxb2, Hoxb3 and Hoxb5 were downregulated in our Hoxb4 knockout model (article II). Still, our phenotypes for both knockout models are mild in contrast to the previously described Hoxb4 knockout strain. In line with the previous conventional Hoxb4

knockout model, the *Hoxa9* knockout mice that were also generated by insertion of a selection cassette, displayed a severe hematopoietic phenotype (Lawrence et al., 2005). Possibly, these phenotypes may be related to the promoter in the selection cassette, however this remains to be explored. Thus, for future generations of Hox knockout mice, it may be most optimal to mutate only the amino acids responsible for the DNA/co-factor binding. This would solely disrupt the function of the HOX gene of interest and thus circumvent the interference of additional genes.

Gain of function

Several different approaches can be used to study gain of function, such as transgenic mice where the expression is regulated by a constitutively active tissue specific promoter or an inducible promoter as well as by retroviral mediated gene transfer. Transgenic mice can be generated by introducing the transgene by random integration or by inserting the gene of interest into a specific locus using homologous recombination “knock in” technique (Bjornsson et al., 2001; Brinster et al., 1981; Wutz and Jaenisch, 2000). It is important to make sure that the site/s of integration does not interfere with the expected findings, thus several mouse lines need to be screened before further use. Furthermore, to avoid that the transgene eliminates endogenous gene/s at the integration site, the mice are kept haploinsufficient. In general, the generation of these transgenic mice are both time consuming and costly.

In contrast, retroviral mediated gene transfer is a fast and inexpensive way to study gene expressions. This technique has been very popular in the hematopoietic system, since stable integration in the HSC genome results in the expression of the transgene in all subsequent hematopoietic cells. In addition, high transduction efficiency is feasible in murine bone marrow cells and the effect on HSCs and its progeny can be assessed *in vivo* through bone marrow transplantation. The random numbers of integrations and integration sites have both advantages and disadvantages. Individual clones can easily be traced throughout the hematopoietic hierarchy due to the random integrations. However, the integration site and copy numbers of the retroviral vector can affect the expression level of the transgene (discussed below), and could cause insertional mutagenesis. Retroviral mediated gene transfer was used in article III to integrate a HOXB4 expression vector into the genome of p21 deficient HSCs. This vector was the same as the one used in the impressive HSC expansion studies performed by Antochuk et al (Antonchuk et al., 2002). Correspondingly, our data confirmed the positive effect of HOXB4 in HSC proliferation with no indications of leukemia detected one year after transplantation of gene modified cells (unpublished data).

In article V we explored the use of an inducible tetracycline transactivator system to analyze the role of HOXA10 in hematopoiesis by crossing the tetO-HOXA10 mouse and the Rosa26-rtTA-nls mouse. The tetracycline transactivator system enables tight regulation of the gene of interest through administration or withdrawal of doxycycline both *in vitro* and *in vivo* (Bohl and Heard, 1997; Furth et al., 1994; Hennighausen et al., 1995). A schematic overview over the tetracycline transactivator system is illustrated in Figure 9. The characterization of the tetO-HOXA10 mice generated in our laboratory is described in Björnsson et al (Bjornsson et al., 2001). As addressed above, several tetO-HOXA10 lines were screened before suitable lines were found, demonstrating no leakiness and a high inducibility. Moreover, the tetO-HOXA10 mice have been kept heterozygous for the HOXA10 transgene constructs for more than 5 years with no abnormalities detected. A limitation using this system has been the difficulties in producing transgenic mice or

viral vectors expressing the tetracycline transactivator (tTA)(tet-OFF) for activation of the transgene (Bjornsson et al., 2001). Most likely, this is due to toxicity of the herpes simplex virion protein 16 activation domain in the tTA sequence. We have previously used a complex vector generating low titers as well as low expression levels of tTA. However, recent advances in the tetracycline transactivator systems, such as the Rosa26-rtTA system, allowed us to optimize our HOXA10 mouse model further, excluding difficult transduction protocols as well as reducing variability in expression levels resulting from vector integration. The Rosa26-rtTA mouse model was generated by knocking in the reverse tTA (rtTA)(tet-ON) (Gossen and Bujard, 1992) into the *rosa26* locus (Hochedlinger et al., 2005; Wutz and Jaenisch, 2000). This locus has been shown to be transcriptionally active in all hematopoietic tissues (Hochedlinger et al., 2005; Soriano, 1999). By mating our HOXA10 transgenic mouse strain with the Rosa-rtTA mouse strain, we found that the expression level of HOXA10 could be tightly regulated in a dose dependent manner using varying levels of doxycycline *in vitro* and *in vivo* (article V).

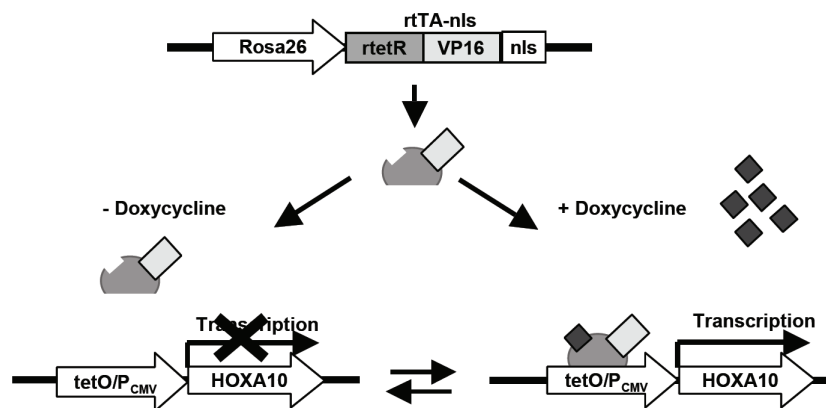


Figure 9. A schematic illustration of the reverse (tet-ON) tetracycline transactivator system. The rtTA is a fusion protein, composed of the rtetR repressor protein fused to the VP16 activation domain and the nls, driven by the Rosa26 locus. In the absence of doxycycline, rtTA will not recognize the tetO sequence and no expression of HOXA10 will occur. Addition of doxycycline results in binding of the rtTA to the tetO sequence and transcriptional activation of HOXA10. The inducible expression system is reversible and withdrawal of doxycycline terminates the expression.

Effects of transcription factor concentrations on hematopoietic fate options

It is clear that the molecular mechanism by which TFs regulate transcription is diverse and complex. Recent findings using transgenic mice have implied that hematopoietic cell fate can be regulated by graded expression of TFs.

PU.1 was one of the first TFs to demonstrate distinct hematopoietic cell fates due to different expression levels. It was found that high levels of PU.1 promoted macrophage development while lower levels turned differentiation towards granulocytes and B-cells (Dahl et al., 2003; DeKoter and Singh, 2000). Another TF that demonstrated dosage sensitivity in hematopoiesis is c-Myb. The c-Myb knockout mice displayed a complete block in progenitor cell development, whereas low levels of c-Myb, corresponding to 5-10 % of wild type, allowed for initial HSC

differentiation even though being insufficient in terminal differentiation (Emambokus et al., 2003). In addition, Gata-1 has been shown to regulate erythroid, megakaryocytic, mast cell and eosinophilic development in a concentration dependent manner (Migliaccio et al., 2003; Shivdasani et al., 1997; Yu et al., 2002).

New evidence suggests that Hox genes also regulate hematopoiesis in a concentration dependent manner. Enforced expression of HOXB4 using retroviral mediated gene transfer has been shown to increase HSC proliferation *in vitro* and *in vivo* as described above (Antonchuk et al., 2001; Antonchuk et al., 2002). In attempts to further explore the possibility to use HOXB4 in HSC expansion, we and others have demonstrated that higher levels of HOXB4, using different viral vectors than the previous studies, resulted in perturbed lineage commitment and decreased proliferation of primitive progenitor cells (Brun et al. 2003; Schiedlmeier et al. 2003). These findings indicate that the level of HOXB4 expression is crucial for HSC fate and may explain why the use of purified HOXB4 protein did not result in a substantial HSC expansion (Amsellem et al., 2003; Krosel et al., 2003a).

Our findings in article V, using the tetracycline transactivator system, further demonstrated the importance of Hox gene expression levels in the regulation of hematopoietic cell fate options. We showed that intermediate levels of HOXA10 resulted in increased proliferation of HSCs *in vitro*, while higher doses had no such effect. Furthermore, we found that high levels of HOXA10 blocked the development of erythropoiesis and megakaryopoiesis. However, in contrast to previous reports where enforced expression of HOXA10 using a retroviral vector resulted in AML (Thorsteinsdottir et al. 1997), we did not detect any signs of disease. This may again be related to the expression levels, and indeed, graded expression of both PU.1 and C/EPB α were found to regulate the development of AML in mice (Rosenbauer et al., 2005). The mechanisms governing the regulation of this phenomenon is still an open question. Knockout studies as well as vector-mediated overexpression may result in extreme experimental situations instead of effects correlating to the normal gene function. Thus, tightly regulated gene expression such as the tetracycline transactivator system may be needed for studying the true function of a TF.

HSC assays

Several different *in vitro* methods have been developed to study HSC function such as the Long-Term Culture-Initiating Cell (LTC-IC) assay and the Cobblestone Area Forming Cell (CAFC) assay (Gartner and Kaplan, 1980; Ploemacher and Brons, 1989; Sutherland et al., 1989). However, none of these *in vitro* assays are able to recapitulate the true properties of HSCs. Thus, the most reliable way to evaluate HSC function is to examine their capacity to reconstitute the hematopoiesis *in vivo* after transplantation into lethally irradiated recipients. The irradiation disrupts the recipients' own hematopoiesis, allowing the donor cells to seed the bone marrow and rebuild the hematopoietic system. During the years, several transplantation assays in mouse have been developed, for example the competitive repopulating assay where donor/test HSCs compete against a set number of wild type bone marrow cells (Domen and Weissman, 1999; Harrison, 1980; Jones et al., 1996; Spangrude et al., 1988b). Furthermore, the competitive repopulating unit (CRU) assay, which allows for the enumeration of functional HSCs *in vivo* by transplanting limited dilutions of donor cells together with a set number of unfractionated bone marrow cells (Szilvassy et al., 1990). To distinguish donor cells from test cells, different congenic mouse

strains have been developed. These strains are genetically identical except for one cell surface marker. We are using mice expressing different isoforms of CD45, CD45.1 or CD45.2, which are expressed on all nucleated hematopoietic cells (Loken et al., 1987; Thomas, 1989). The level of reconstitution and lineage distribution is measured by analyzing blood samples by FACS.

Transplanting donor cells in a non-competitive setting may not detect defects in HSC activity as shown by several mutant mice including ours (Lawrence et al., 1997; Nichogiannopoulou et al., 1999)(article I, II and IV). However, using competitive transplantations as well as serial transplantations, the HSCs are exposed to more challenging conditions that may reveal differences in HSC function. In this way, we detected reduced repopulating capacity in all our knockout models indicating that these mice exhibited decreased HSC proliferation capacity. In articles I and II, we explored the use of the antimetabolic drug 5-Fluorouracil (5-FU) to further verify whether the Hoxb4 and Hoxb3/Hoxb4 deficiency negatively affect the HSC proliferation. The drug was administered twice prior to harvest and transplantation. 5-FU induces hematopoietic stress by selectively kill cycling cells, which result in loss of all rapidly expanding progenitor cells, subsequently forcing the quiescent HSCs to divide. We hypothesized that the knockout stem cells needed more time to enter the cell cycle after the first hit of 5-FU than wild type HSC. Hence, the second hit would kill all the HSCs that responded to the previous hit, sparing more knockout than wild type HSCs. Transplantation after the second hit revealed that our hypothesis was correct, showing that antimetabolic drugs like 5-FU can be used for delineating HSC function *in vivo*.

Traditionally, enumeration of the HSCs is done by calculating the number of CRUs *in vivo*. However, it is important to know that this method is a functional assessment of HSCs, and that only proliferating HSCs are detected. Still, this is the only assay presently known to enumerate the HSCs. We used this method in article III in order to assess the HSC expansion *in vitro* after HOXB4 transduction into p21 deficient mice. However, using vector integration in *in vitro* HSC expansion does not exclude continuous effects from the transgene *in vivo*. Thus, the tetracycline system is more accurate in assessing HSC expansion *in vitro*, since it allows for the termination of the transgene before transplantation.

The genetic background in the mice can have major impact on the reconstitution ability, and may therefore affect the phenotype studied. In our studies we observed that bone marrow cells from the 129Sv had an increased repopulating capacity compared to B6.SJL derived cells (unpublished data). Our Hoxb4 and Hoxb3/Hoxb4 mice were primarily studied in a mixed genetic background (B6.SJLx129Sv) and to minimize the effect from the genetic background, littermate mice were used as controls (article I and II). Concomitantly, later experiments using backcrossed mice showed similar results.

Even though *in vivo* transplantation is so far the only way to study HSC activity, it is important to emphasize that HSC self-renewal is measured by its capacity to contribute to the mature blood cell production in the recipients. This means that defects in the reconstitution capacity may be related to defects in HSC differentiation or homing as they are in HSC self-renewal. Therefore, it is crucial to carefully design and interpret the experiments before any conclusions can be drawn.

Future directions

HOXA10 in HSC expansion

It is clear that different concentrations of HOXB4 result in different HSC fate and our data using the HOXA10 inducible mouse model demonstrate similar findings. To further explore this system, we are currently enumerating the number of HSCs after *in vitro* culture in different concentrations of HOXA10. Preliminary findings show that intermediate concentrations of HOXA10 following 13 days of culture lead to a 10-fold increase in CRUs compared to fresh input cells, assessed at 8 weeks after transplantation.

Concomitantly, long-term culture for up to 50 days with inducible HOXA10 LSK cells expressing intermediate levels of HOXA10 revealed a dramatic proliferative capability compared to uninduced cells. More impressively, FACS analysis demonstrated that approximately half of the cells were still of LSK phenotype (Figure 10), indicating an impressive expansion of phenotypically primitive cells. However, further evaluation *in vivo* will reveal whether HSCs are expanded or preserved in the *in vitro* culture. Intriguingly, preliminary data using 200 LSK cells cultured for 28 days with intermediate concentrations of HOXA10 displayed high multilineage reconstitution ability *in vivo*.

Taken together, these data suggest that HOXA10 is a potent protein for HSC expansion, and further adjustments of the expression levels and the *in vitro* conditions may be very valuable. Furthermore, long-term culture with HOXA10 may be used as an experimental tool for studying HSC function otherwise impossible due to the low number of primary HSCs obtained *in vivo* as well as the lack of good HSC lines.

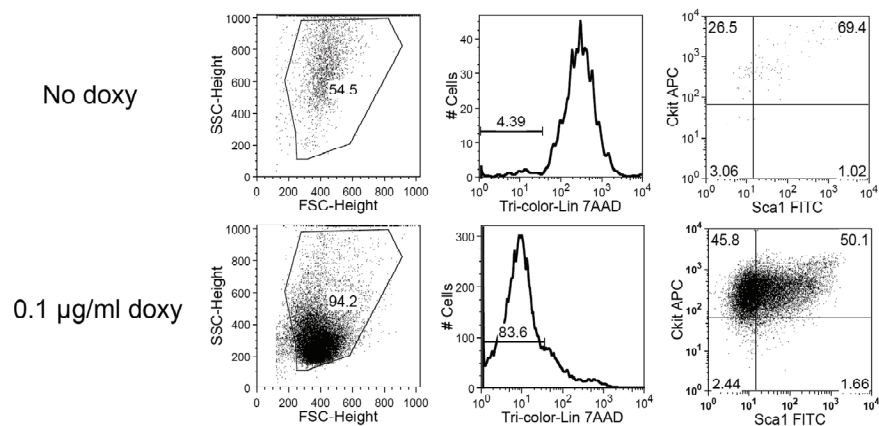


Figure 10. Intermediate levels of HOXA10 demonstrate strong expansion of LSK cells after 50 days of *in vitro* culture. Upper panel represents inducible HOXA10 LSK cells cultured without doxycycline (no HOXA10) for 50 days in culture. As expected, most cells have terminally differentiated and stopped their proliferation. Lower panel represents inducible HOXA10 LSK cells cultured with 0.1 $\mu\text{g/ml}$ doxycycline (intermediate levels of HOXA10) for 50 days which demonstrates high proliferation of LSK cells. The cells were culture in serum free medium containing SCF, FLT3 and TPO.

Identification of target genes

Recent data by the laboratory of Dr Humphries support a role for HOXA10 in HSC expansion since expression of the NUP98-A10 fusion protein, consisting of NUP98 fused with the HOXA10 homeodomain, resulted in an impressive HSC expansion *in vitro* (S. Sekulovic and Humphries, 2005). In order to further delineate the mechanisms by which HOXA10 regulates HSC self-renewal, our gene expression array data were compared with their microarrays. Interestingly, more than 50 genes were overlapping between the two studies, and the probability for genes to be randomly activated was less than 1 in 1×10^{49} , indicating a strong correlation between the arrays. Future analyses will reveal whether these genes are important regulators in HSC self-renewal.

Our mechanistic studies suggest that the putative HOXA10 binding site TTAT within the HLF, Dkk-1, Gfi-1 and Gata-1 gene, is dependent on additional base pairs or unknown co-factors, since HOXA10 displayed different binding efficiency to the different sites of the target genes. It would be of great interest to further analyze these data to gain new insights into HOX gene specificity. In addition, the target genes identified in article V can be further evaluated for their involvement in the phenotype caused by overexpression of HOXA10, especially the interaction with Gata-1, since Gata-1 is crucial for erythropoiesis and megakaryopoiesis.

Development of inducible leukemia

Overexpression of HOXA10 has been shown to generate myeloid leukemia in a fraction of mice that constitutively express HOXA10. The fact that only a fraction of the mice acquired leukemia, and that the latency period is long (20-50 weeks) indicate that secondary mutations are required for leukemia transformation to occur. Indeed, overexpression of Meis1 and HOXA10 simultaneously reduced the latency period dramatically (Pineault et al., 2004). By transducing the inducible HOXA10 HSCs with a retroviral Meis1 vector, an animal model for leukemia that is reversible may be feasible. We expect the leukemia generated with HOXA10 alone not to be reversible upon withdrawal of HOXA10 expression due to several secondary mutations that will be able to maintain the malignant phenotype. If a successful inducible model of leukemia requiring the interaction of two molecules can be generated, it will serve as an important model to investigate further molecular interactions in leukemic cells, and these investigations will be facilitated by the on-off inducibility to verify the relationship between molecular interactions and biological phenotype.

Furthermore, Smad4, a downstream signaling protein of the tumor suppressor TGF- β can directly interact with Hoxa9 and block its binding to DNA, which inhibits the transformation capability of Hoxa9 (Wang et al., 2006). Since HOXA9 and HOXA10 have very similar structure and are functionally similar, and that both genes induces AML in mice, we can exploit our inducible HOXA10 mice to define the Smad4-Hox interaction in leukemia by mating our inducible HOXA10 mouse with the conditional Smad4 knockout (already existing in our laboratory). By overexpression of HOXA10 in the Smad4 deficient HSCs we could study whether the transforming function of HOX genes is increased in Smad4 deficient hematopoietic progenitors.

In summary, these models will allow us to reveal the molecular pathogenesis of leukemia and allow for testing of new therapeutic strategies.

Conclusions

- Endogenous expression of Hoxb3 and Hoxb4 is not essential for development of definitive hematopoiesis.
- HSCs deficient in Hoxb3 and Hoxb4 demonstrate slower proliferation kinetics *in vivo* in response to hematopoietic stress.
- The additional deletion of Hoxb3 enhances the Hoxb4 knockout phenotype in a quantitative manner suggesting a cooperative or similar function for these two Hox proteins in HSCs.
- Overexpression of HOXB4 in p21 deficient HSCs leads to enhanced HSC proliferation *in vitro* compared to overexpression of HOXB4 alone.
- Hoxa9/b3/b4 knockout mice demonstrate reduced spleen weight and spleen cellularity.
- Hoxa9/b3/b4 knockout mice display increased frequency of HSCs defined by immunophenotype (LSKCD150), whereas the repopulating capacity of the Hoxa9/b3/b4 knockout HSCs is similar to hoxa9 knockout HSCs.
- Intermediate concentrations of HOXA10 induce a 15-fold increase in repopulating capacity of HSCs after 13 days of *in vitro* culture.
- The HOXA10-mediated effects on hematopoietic cells are associated with altered expression of genes that govern stem cell self-renewal e.g. Dkk1, HLF and Gfi-1, here identified as direct downstream targets of HOXA10.
- High levels of HOXA10 block erythroid and megakaryocyte development which is accompanied by downregulation of Gata-1, suggested here to be a direct downstream target of HOXA10.
- HOXA10 acts as a major regulator of hematopoiesis governing both proliferation and differentiation of hematopoietic progenitor and stem cells, where distinct fate outcomes depend on the HOXA10 expression level.

Svensk sammanfattning

Cellerna i blodet har till uppgift att dels förse kroppen med syre och transportera bort restprodukter, men också att skydda oss mot bakterier och virus. Dessa celler är kortlivade och behöver nybildas hela tiden, en process som kallas hematopoies och som härrör från ett litet antal celler i benmärgen, de s.k. hematopoetiska stamcellerna (HSC). HSC har förmågan att ge upphov till alla typer av blodceller, och har även möjlighet att dela sig i två identiska dotterceller med samma förmåga som modercellen, sk självförnyelse. Detta innebär att de kan förse kroppen med nästan 1000 miljarder nya blodceller varje dag genom hela livet. Dessa egenskaper gör att transplantation av blodstamceller är det enda alternativet och botemedlet för patienter med blod cancer (leukemi) eller vissa genetiska sjukdomar. I mer än 30 år har man genomfört lyckade benmärgstransplantationer men ett mycket centralt problem är att antalet HSC är ytterst få och behovet av donatorer är stort. Transplantationerna medför också i sig en stor risk pga den strålningsdos som måste ges till patienten för att göra plats åt de nya cellerna. Med bättre kunskap om hur HSC reglerar "självförnyelse" skulle man kunna kontrollera denna unika process och på detta sätt få fram fler HSC celler att transplantera. Detta skulle kunna leda till säkrare och effektivare transplantationer, och att öka möjligheter till genterapi eller cellterapi av andra organsystem.

För att hematopoiesen skall fungera krävs ett väloljat maskineri som involverar många olika gener. Trots mycket forskning vet man inte så mycket om hur denna process regleras. Dock har ny forskning visat att Hox generna är involverade i regleringen av hematopoiesen och att många av dessa Hox gener orsakar leukemi om de är aktiva på fel sätt, t ex HOXA9 och HOXA10.

Min doktorsavhandling har gått ut på att studera ett antal Homeobox (Hox) gener och deras roll i regleringen av hematopoiesen. Hox familjen består av 39 gener indelade i fyra grupper (A, B, C och D) som är utspridda på 4 olika kromosomer. Alla generna är mycket lika vilket man tror beror på att de multiplicerats under evolutionens gång. Varje gen är tilldelad en siffra mellan 1-13 beroende på hur de sitter i gruppen t ex Hoxb2, Hoxb3 Hoxb4 osv. Hox gener är transkriptionsfaktorer vilket innebär att de binder till specifika ställen på DNA molekylen vilket i sin tur leder till aktivering eller inaktivering av målgenerna. Hox generna har visat sig vara mycket viktiga under embryonal utveckling där de reglerar formationen av kroppens alla delar, men under de senaste åren har forskning också visat att dessa gener spelar en mycket viktig roll i hematopoiesen. Vidare har man visat att övertryck av HOXB4 via retroviral genöverföring är den hittills enda kända gen som riktigt leder till expansion (40 ggr) av HSC hos möss utanför kroppen. För att kunna studera HOXB4s naturliga roll tillverkade vårt laboratorium två sk knockout mus modeller. Dels en som helt saknar Hoxb4 och dels en som saknar både Hoxb3 och Hoxb4 eftersom det är känt att närliggande Hox gener kan ta över varandras funktion vid behov. Genom att studera dessa mus modeller fann vi att HSC som saknar en eller båda generna reagerar långsammare på stress t ex vid benmärgstransplantation. För att ytterligare förstå hur dessa Hox gener samverkar parade vi vår dubbel knockout mus med en knockout mus som saknar Hoxa9. Dessa trippel knockout möss var små till växten jämfört med normala möss, men vi kunde ej påvisa någon ökad effekt på de HSC jämfört med Hoxa9 knockout modellen. Vi fann dock att dessa gener tillsammans är viktiga för andra organs utveckling, t ex mjälten. Vi undersökte också möjligheten att ytterligare förstärka HOXB4s positiva effekt på de HSC genom att övertycka HOXB4 i HSC som saknar en negativ feedback mekanism mot cell delning (p21 knockout).

Resultatet blev att vi fick ännu fler HSC än med bara HOXB4 vilket visar att man kan påverka både en positiv och en negativ regulator av HSC för att expandera dem utanför kroppen.

De senaste årens forskning har visat att nivån av HOXB4-proteinet spelar stor roll för hur effekten blir på HSC. Därför tillverkade vi en inducerbar HOXA10 mus modell för att kunna studera hur olika nivåer av HOXA10 påverkar HSC och bildandet av de olika blodcellerna. Modellen innebar att vi kunde sätta på och stänga av genen efter behov men också reglera aktivitetsgraden av genen. Vi fann att intermediära nivåer av HOXA10 resulterade i ökad HSC funktion efter två veckors odling, medan höga nivåer av HOXA10 visade sig blockera utvecklingen av röda blodkroppar och blodplättar, vilket resulterade i anemi. Denna studie visade att nivån av en Hox gen kan ha stor betydelse för hur den påverkar regleringen av HSC och de olika blodcellernas mognad. Detta har i sin tur stor betydelse när man skall designa säkra och effektiva HSC expansionsprotokoll med hjälp av Hox gener i framtiden.

Papers not included in the thesis

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