Live and Let Die

Critical regulation of survival in normal and malignant hematopoietic stem and progenitor cells

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Cover picture is an illustration made by the author of a hematopoietic stem cell in the trabecular bone of the bone marrow

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During the course of the research underlying this thesis, Pernilla Eliasson was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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“The universe is full of magical things patiently waiting for our wits to grow sharper.”

Eden Phillpotts
ABSTRACT

The hematopoietic stem cell (HSC) is characterized by its ability to self-renew and produce all mature blood cells throughout the life of an organism. This is tightly regulated to maintain a balance between survival, proliferation, and differentiation. The HSCs are located in specialized niches in the bone marrow thought to be low in oxygen, which is suggested to be involved in the regulation of HSC maintenance, proliferation, and migration. However, the importance of hypoxia in the stem cell niche and the molecular mechanisms involved remain fairly undefined. Another important regulator of human HSCs maintenance is the tyrosine kinase receptor FLT3, which triggers survival of HSCs and progenitor cells. Mutations in FLT3 cause constitutively active signaling. This leads to uncontrolled survival and proliferation, which can result in development of acute myeloid leukemia (AML). One of the purposes with this thesis is to investigate how survival, proliferation and self-renewal in normal HSCs are affected by hypoxia. To study this, we used both in vitro and in vivo models with isolated Lineage^Sca-1^Kit^+^ (LSK) and CD34^Flt3^-LSK cells from mouse bone marrow. We found that hypoxia maintained an immature phenotype. In addition, hypoxia decreased proliferation and induced cell cycle arrest, which is the signature of HSCs with long term multipotential capacity. A dormant state of HSCs is suggested to be critical for protecting and preventing depletion of the stem cell pool. Furthermore, we observed that hypoxia rescues HSCs from oxidative stress-induced cell death, implicating that hypoxia is important in the bone marrow niche to limit reactive oxidative species (ROS) production and give life-long protection of HSCs. Another focus in this thesis is to investigate downstream pathways involved in tyrosine kinase inhibitor-induced death of primary AML cells and cell lines expressing mutated FLT3. Our results demonstrate an important role of the PI3K/AKT pathway to mediate survival signals from FLT3. We found FoxO3a and its target gene Bim to be key players of apoptosis in cells carrying oncogenic FLT3 after treatment with tyrosine kinase inhibitors. In conclusion, this thesis highlights hypoxic-mediated regulation of normal HSCs maintenance and critical effectors of apoptosis in leukemic cells expressing mutated FLT3.
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## TABLE OF CONTENTS

List of papers included in the thesis ................................................................. 7  
Abbreviations ...................................................................................................... 8  
Hematopoiesis .................................................................................................... 9  
The hematopoietic stem cell ............................................................................. 10  
  Identification of HSCs ................................................................................... 11  
  *In vitro* and *in vivo* assays to detect HSCs ................................................. 13  
  Clinical use of HSCs ...................................................................................... 15  
  Can HSCs be expanded *in vitro*? ................................................................. 16  
  Regulation of the cell cycle in HSCs ............................................................... 18  
  The hematopoietic stem cell niche ................................................................. 21  
  The hypoxic stem cell niche ......................................................................... 31  
**Hypoxia inducible factor-1** ........................................................................ 27  
  HIF-1 and regulation of important niche molecules ...................................... 29  
  Glucose metabolism in hypoxic cells ............................................................ 30  
  HIF-1 and Cell cycle ...................................................................................... 32  
**Apoptosis** .................................................................................................... 33  
  Pro- and anti-apoptotic Bcl-2 members .......................................................... 33  
**Oxidative stress and aging of HSCs** ............................................................ 36  
  FoxO proteins are essential in the resistance to oxidative stress ............... 37  
**The role of FLT3 in normal and malignant HSCs and progenitors** .......... 39  
  Tyrosine kinase receptors ............................................................................ 39  
  The FLT3 receptor ....................................................................................... 39  
    Stimulation of the FLT3 receptor ............................................................... 40  
    Mutations in the FLT3 receptor ................................................................. 41  
    FLT3 signaling in normal and mutated receptor ........................................ 42  
  Other mutations implicated in AML ............................................................. 44  
**Therapeutic strategies for AML** ................................................................. 46  
  FLT3 inhibitors .............................................................................................. 46  
  Therapies that targets the leukemic stem cell niche ................................... 47  
  BH3 mimicking drugs ................................................................................... 48  
**Aims of the present investigation** ............................................................... 50  
**Methodological considerations** ................................................................. 51  
  Isolation of HSCs using FACS based cell sorting ...................................... 51  
  RNA interference .......................................................................................... 52  
  Real time PCR ............................................................................................... 54
# Table of Contents

Results and discussion of the papers in this thesis .......................................................... 57
Conclusions ........................................................................................................................... 65
Future aspects ...................................................................................................................... 67
En livsviktig balans mellan liv och död – ................................................................. 69
  en populärvetenskaplig sammanfattning ......................................................................
Acknowledgements ........................................................................................................... 72
References .......................................................................................................................... 74
Paper I-IV ............................................................................................................................ 97
LIST OF PAPERS INCLUDED IN THE THESIS

I  Hypoxia expands primitive hematopoietic progenitor cells from mouse bone marrow during in vitro culture and preserves the colony-forming ability.

II Hypoxia, via hypoxia-inducible factor (HIF)-1α, mediates low cell cycle activity and preserves the engraftment potential of mouse hematopoietic stem cells
   Manuscript revised submitted

III Hypoxia rescues hematopoietic stem cells from oxidative stress-induced cell death and preserves the long-term repopulation ability.
   P. Eliasson, E. Widegren, and J-I. Jönsson
   Manuscript

IV BH3-only protein Bim more critical than Puma in tyrosine kinase inhibitor-induced apoptosis of human leukemic cells and transduced hematopoietic progenitors carrying oncogenic FLT3.
   Blood. 2009. 113 (10): 2302-2311
   *Contributed equally to this work
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma-2</td>
</tr>
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<td>Bim</td>
<td>Bcl-2-interacting modulator of cell death</td>
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<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>BSO</td>
<td>L-buthionine sulfoximine</td>
</tr>
<tr>
<td>CAFC</td>
<td>Cobblestone-area-forming cells</td>
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<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinases</td>
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<tr>
<td>CFC</td>
<td>Colony-forming cell</td>
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<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
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<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CX chemokine receptor 4</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activating cell sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FL</td>
<td>FLT3 ligand</td>
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<tr>
<td>FLT3</td>
<td>human c-fms-like tyrosine kinase 3 (Flt3 in mouse)</td>
</tr>
<tr>
<td>FoxO</td>
<td>Forkhead box transcription factor</td>
</tr>
<tr>
<td>GMP</td>
<td>granulocyte/monocytes progenitors</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor-1</td>
</tr>
<tr>
<td>HPP</td>
<td>High proliferative potential</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HSCT</td>
<td>hematopoietic stem cell transplantation</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IGF-2</td>
<td>insulin-like growth factor-2</td>
</tr>
<tr>
<td>ITD</td>
<td>Internal tandem repeats</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus-activated kinases</td>
</tr>
<tr>
<td>JM</td>
<td>Juxtamembrane</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemic stem cells</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage Sca-1/c-Kit+</td>
</tr>
<tr>
<td>LT</td>
<td>Long term</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>Long term cell-initiating cell</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>MPP</td>
<td>Multi-potent progenitors</td>
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<tr>
<td>NPM1</td>
<td>Nucleophosmin-1</td>
</tr>
<tr>
<td>PDK1</td>
<td>Pyruvate dehydrogenase kinase-1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PIM</td>
<td>Pimonidazole</td>
</tr>
<tr>
<td>Puma</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen-1</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal derived factor-1</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SOD</td>
<td>superoxidedismutase</td>
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<tr>
<td>SP</td>
<td>Side population</td>
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<tr>
<td>ST</td>
<td>Short term</td>
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<tr>
<td>TKD</td>
<td>Tyrosine kinase domain</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial factor</td>
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Background

HEMATOPOIESIS

In order to maintain a steady level of mature and functional cells in the blood system, 1 trillion \(10^{12}\) new cells are produced every day in an adult man (Ogawa, 1993). The hematopoietic cells have several distinct functions, such as delivering oxygen to all tissue cells, protecting the organism against infectious agents, as well as regulating the blood coagulation. Early in life, hematopoiesis takes place in the yolk sac, the aorta-gonad-mesonephos (AGM)-region, spleen, and the fetal liver. The bone marrow becomes the primary site for hematopoiesis from the time of birth (Muller et al., 1994). Extramedullary hematopoiesis occurs if the bone marrow is damaged or stressed due to irradiation or chemotherapy. A common extramedullary place for hematopoiesis is the spleen. All blood cells, including erythrocytes, myeloid and lymphoid leukocytes, and platelets (derives from megakaryocytes), arise from one common stem cell, the hematopoietic stem cell (HSC). Once lineage commitment has occurred, the choice is taken and cannot be reversed.

![The classical hematopoietic tree](Figure 1. The classical hematopoietic tree. The HSCs are divided in long term (LT) HSCs, short term (ST) HSCs, and multipotent progenitor cells (MPP) according to their self-renewal potential. They give rise to common myeloid progenitors (CMPs) and lymphoid progenitor cells (CLPs) which give rise to all myeloid and lymphoid cells, respectively. Mouse HSCs can be isolated by their distinct expression pattern of the surface markers c-Kit, Sca-1, Flt3 and CD34 (Modified from Reya et al., 2001).)
The differentiation to mature blood cells is a multistep process starting with the HSC giving rise to multi-potent progenitors (MPP) followed by production and commitment to lymphoid (common lymphoid progenitors, CLP) and myeloid restricted progenitors (common myeloid progenitors, CMP) which divide frequently and differentiate into distinct hematopoietic lineages (Figure 1) (Reviewed in (Reya et al., 2001). Identification of the lymphoid primed multi-potent progenitors (LMPP), which lack erythrocyte and megakaryocyte potential but possess the potential to undergo lineage segregation into lymphoid and myeloid lineages, has questioned this classical model of hematopoiesis (Adolfsson et al., 2005). How the hierarchy and the lineage commitment for the hematopoietic three is ordered remains to be investigated and the debate continues (Forsberg et al., 2006; Månsson et al., 2007). Differentiation to specialized lineage cells is regulated by both intrinsic (transcription factors) and extrinsic (cytokine receptor signaling) factors (Laiosa et al., 2006).

THE HEMATOPOIETIC STEM CELL

The true HSC is a rare cell type, constituting 1 per $10^5$ bone marrow cells (Harrison 1988) and can maintain the turnover of new blood cells throughout a lifespan. The first revolutionary experiments on, perhaps the best characterized stem cell today, the HSC, was done nearly a half century ago by Till, McCulloch and Becker (Becker et al., 1963; Till and Mc, 1961). They found a clone of murine marrow cells capable of repopulating all lineages in an irradiated mouse host.

The definition of HSCs is that they are unspecialized cells capable of producing all hematopoietic lineage cells as well as renewing themselves (Till and Mc, 1961). The HSC is divided in long term (LT) stem cells, which have a lifelong reconstitution ability, and short term (ST) stem cells, with a time-limit reconstitution of around 8 weeks (Morrison and Weissman, 1994). Early studies in the stem cell research area suggested that some HSCs remain outside the cell cycle in a dormant state (Kay, 1965). However, a more recent study has shown that 8% of the LT-HSCs are in the cell cycle at any given time and that nearly all (99%) divide within a two month time frame (Cheshier et al., 1999). The number of HSCs in young mice is relatively constant (Harrison et al., 1988), and to be able to divide and differentiate into progenitor cells they must have a self-renewal capacity in order not to decimate themselves. The HSC must undergo asymmetric cell division to be able to self-renew itself and at the same time produce progeny cells which divide further and mature into different cell fates.
Hematopoietic stem cells

The determination of the HSC to self-renew or to differentiate is thought to be regulated by intrinsic factors via a stochastic process (Abkowitz et al., 1996; Mayani et al., 1993; Suda et al., 1984), but deterministic regulation by extrinsic signals from the microenvironment is also of importance (Ho, 2005; Metcalf, 1998). The regulation between asymmetric and symmetric cell division needs to be well balanced in order to maintain homeostasis in the hematopoietic system.

Identification of HSCs

HSCs are mainly located in the bone marrow, but can also be found in the spleen, umbilical blood, and the placenta. The use of HSCs in the clinic requires an efficient and highly purified isolation procedure. Two techniques which are frequently used to isolate stem cells are enrichment with an immunomagnetic method and fluorescence-activating cell sorting (FACS) selection, both based on labeling cell surface markers with specific antibodies. Human HSCs and progenitors can be isolated by a positive selection of CD34 expressing cells (Civin et al., 1984). However, this population is very heterogeneous with only 1% being HSCs (Larochelle et al., 1996). A way to achieve a purer stem cell population is to remove mature cells by negative selection (Civin et al., 1984; Spangrude et al., 1988). As mentioned above, the HSCs are unspecialized hematopoietic cells and do not express lineage specific surface markers such as B220 (B cells), Mac-1 (myelomonocytic cells), Gr-1 (granulocytes), Ter-119 (erythrocytes), CD4 and CD8 (T cells). CD38 is another marker that can be used to distinguish human progenitor cells from CD38− HSCs, as well as a positive selection for CD133 (reviewed in (Wognum et al., 2003)).

The mouse HSC is better characterized than the human HSC and enables a purer isolation of stem cells. The lineage negative (Lin−) selection method for mouse HSCs is routinely used together with a positive selection for the stem cell antigen (Sca-1, also referred to as Ly-6A/E) and c-KIT receptor (also called CD117) (Ikuta and Weissman, 1992; Li and Johnson, 1995), usually called the LSK compartment, which contains all cells with repopulating activity (Spangrude et al., 1988; Uchida and Weissman, 1992). Thy-1 can be used to further enrich the murine HSCs within the LSK population because murine LT-HSCs, similar to human LT-HSCs, are found in the Thy-1low population (Spangrude and Brooks, 1992). Mouse HSCs are CD34low, which are all found in the LSK compartment (Osawa et al., 1996). Recently, expression of FMS-like tyrosine kinase 3, Flt3 (also called Flk2 or CD135) has shown to be accompanied by a loss of self-renewal capacity (Adolfsson et al., 2001; Christensen and Weissman, 2001). In addition, the LSKthy-1low compartment, containing the LT-HSC, completely
overlaps the LSK phenotype lacking expression of Flt3. Staining the LSK phenotype with antibodies against CD34 and Flt3, it is possible to distinguish ST-HSCs, expressing CD34 but not Flt3, from LT-HSC negative for both markers (Yang et al., 2005). However, FLT3 is ubiquitously expressed on hematopoietic cells in the entire human bone marrow as well as in cord blood, including CLPs, granulocyte/monocytes progenitors (GMPs), some CMP and HSCs with long term reconstitution capacity (Figure 2) (Kikushige et al., 2008). This shows that the expression patterns for mouse Flt3 and human FLT3 is different. Recently, Morrison and his group defined an alternative way to identify and isolate mouse HSC by using the SLAM family markers, in particular CD150 and CD48 (Kiel et al., 2005; Yilmaz et al., 2006). By isolating CD150+CD48−Sca-1−Lineage−c-Kit+ bone marrow cells, they were able to nearly double the fraction of cells capable of long-term reconstitution.

In addition to surface markers, isolation based on functional markers is also used to identify HSCs. HSCs have the ability to efflux certain fluorescent dyes. This led to the characterization of a Hoechst-low flow cytometric profile of HSCs, called the “side population” (SP) because of its location in the lower left corner of a dot-plot for Hoechst fluorescence (Goodell et al., 1996). The SP phenotype is associated with long-term reconstitution potential and expression of high levels of a multidrug resistant pump, the ABC transporter Bcrp1/ABCG2, which is responsible for mediating the efflux of the Hoechst dye (Zhou et al., 2001). However, the use of SP to isolate human HSCs has recently been questioned due to findings that the majority of cells in the SP population were mature cells expressing lineage markers. Instead, another characteristic for LT-HSCs, the high aldehyde dehydrogenase (ALDH) activity, is a

Figure 2. Proposed expression of FLT3 on human and mouse hematopoietic stem and progenitor cells. FLT3 in the human hematopoietic system is more widely expressed compared to a more restricted Flt3 expression in mouse cells. Human LT-HSCs express low levels of FLT3 and its expression increases during commitment to primitive lymphoid (CLP), myeloid (CMP) and granulocyte/monocytes (GMPs) progenitors. CMPs primed to develop megakaryocytes and erythrocytes do not express FLT3. In contrast, mouse Flt3 expression is limited to multipotent progenitor cells (MPP) and early lymphoid progenitors (CLP), although a fraction of CMPs express Flt3 at a low level (modified from (Kikushige et al., 2008)).
better marker for isolation of human HSCs (Jones et al., 1996). In contrast, the most suitable functional method to isolate murine HSC is suggested to be the Hoeschst exclusion and not the ALDH activity (Pearce and Bonnet, 2007). Although the purity of HSC in the SP has been questioned, the most primitive quiescent human HSC is suggested to be Lin CD34\(^{-}\)CD38\(^{-}\)ALDH\(^{\text{bright}}\)SP\(^{+}\) (Pierre-Louis et al., 2009).

**In vitro and in vivo assays to detect HSCs**

There are several *in vitro* assays for detection of hematopoietic stem and progenitor cells (Figure 3). A common way to quantify lineage-committed progenitor cells is the colony-forming cell (CFC) assay where cells are seeded in semi-viscous media dishes. In this way, colonies from one single cell can be analyzed and scored in an inverse light microscopy. To quantify more primitive human and mouse hematopoietic cells a long-term culture (LTC) assay was established 20 years ago (Sutherland et al., 1989). A layer of adherent feeder cells, primary bone marrow stromal cells or a stromal cell line, are first established and irradiated. Then test cells, unseparated or purified hematopoietic cells, are added and cultured for 4-5 weeks. During this time, more committed progenitors differentiate, die and disappear and only very primitive cells remain in the culture. The culture is then transferred to new dishes containing methylcellulose, a semi-viscous media. After 12-14 days *de novo* CFCs, formed from a LTC-initiating cell (LTC-IC) can be detected and scored. LTC-IC assays can detect some but not all HSCs (Larochelle et al., 1996). Another variant to score primitive cells is to analyze the test culture 28-45 days after seeding *in situ* on the feeder layer, where they form very distinguished flat colonies of cells tightly adhered to the feeder cells resembling cobblestones, hence called “cobblestone-area-forming-cells” (CAFC) (Ploemacher et al., 1991). The CAFC assay can easily overestimate the number of primitive hematopoietic cells and a validation of the method needs to be done to get reliable results (Denning-Kendall et al., 2003).

Detection of colony forming cells with high proliferative potential (HPP-CFC) have been frequently used for detecting, in particular human, hematopoietic stem cells. HPP-CFCs have been defined as dark colonies greater than 0.5 mm and containing more than approximately 50,000 cells (McNiece et al., 1990).
Although in vitro assays are needed to further study and characterize stem cells, it is important to state that the existence of a true HSC can only be proved by using in vivo long-term reconstitution assays. A HSC has the capacity to recover the endogenous hematopoietic system when transplanted into an irradiated, i.e. myeloablated, mouse. A common in vivo assay used is the competitive repopulation assay, which is a relative measurement of the repopulation ability of the test cells compared to a reference standard of normal non-fractioned bone marrow cells. The competitive bone marrow fraction is also necessary to support the hematopoietic tissue initially before primitive stem and progenitor cells have produced mature and functional hematopoietic cells. Myeloablated mice are transplanted with genetically marked test cells from donor mice (Szilvassy et al., 1990). Using CD45 congenic (genetically different in one locus) mouse strains whose leukocytes can be distinguish by their expression of CD45.1 or CD45.2 forms of the alloantigen enables selective tracking of test cells and competitor cells in congenic mice. If normal B6 (C57BL/6)
mice, expressing CD45.2, are used as recipient mice, then isolated HSCs from CD45.1 expressing B6.SJL mice can be used as donor cells. Competitor cells are syngenic to the recipient strain (Figure 4).

To evaluate the self-renewal of human HSCs, immunodeficient xenogeneic nonobese diabetic–scid/scid (NOD/SCID) mice are used as recipients (Conneally et al., 1997). Engraftment potential for the test cells are evaluated for multilineage differentiation in the peripheral blood of the recipient for various endpoints.

Figure 4. Schematic representation of the competitive repopulation assay. Donor cells from C57B6/J mice (CD45.2) are, together with competitor bone marrow (BM) cells (CD45.1), injected in the lateral tail vein of lethally irradiated recipient B6.SJL mice (CD45.1). At different time points after transplantation, engraftment potential is analyzed using flow cytometry in samples taken from peripheral blood.

Clinical use of HSCs
The pioneer for hematopoietic stem cell transplantation (HSCT) was E. Donnall Thomas who developed bone marrow transplantation as a treatment for leukemia more than 50 years ago (Thomas et al., 1957). Dr. Thomas, together with Dr. Joseph E Murray, was awarded with the Nobel Prize in medicine in 1990. HSCT is used in the treatment of multiple myeloma (Blade and Kyle, 1998) or severe leukemia (Michallet et al., 1996), where the patient has become resistant to chemotherapy. For other inherited blood diseases, such as severe combined immunodeficiency (SCID) and sickle cell anemia, BMT is the only curative treatment (Pinto and Roberts, 2008). The donor can either be the patient him-/herself (autologous bone marrow transplant) or a genetically matched donor (allogenic bone marrow transplant). For the latter, graft rejection is a major problem, which partly can be overcome with immunosuppressive medicine. Most allogenic stem cell transplantations use mobilized peripheral blood as a stem cell source instead of bone marrow. Granulocyte colony-stimulating factor (G-
CSF) is routinely used to mobilize bone marrow stem cells into the peripheral blood. A recent study reported that usage of mobilized peripheral HSCs instead of using bone marrow stem cells in BMT increases the risk of developing graft-versus-host disease although survival rate is not affected (Gallardo et al., 2009). To establish engraftment of hematopoiesis, a large amount of donor bone marrow cells are needed. A shortage of donor cells is often a limiting factor. Ex vivo expansion of hematopoietic stem and progenitor cells could increase both the usage of BMT in clinics and also enhance hematopoietic recovery.

Can HSCs be expanded in vitro?

Basically, although clearly oversimplified, the proliferation and maturation of mature hematopoietic lineage cells are regulated by specific growth factors such as erythropoietin (Epo) for erythrocytes, macrophage colony-stimulating factor (M-CSF) for macrophages and granulocyte colony-stimulating factor (G-CSF) for granulocytes. In contrast, proliferation and survival of multipotent progenitors are stimulated with overlapping cytokines such as interleukin (IL)-3, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4. Our knowledge about extrinsic factors regulating self-renewal (asymmetric division) in long-term HSC, which natural state in vivo is mainly dormant, is limited. A balanced hematopoiesis in vivo at steady-state is dependent on the asymmetric cell division of HSCs, whereas expansion of stem cells requires symmetric cell division. An interaction of early acting cytokines including IL-6, IL-11, fibroblast growth factor (FGF), stem cell factor (SCF, or c-KIT ligand, or steel factor), thrombopoietin (Tpo), and FLT3-ligand (FL) is thought to play a significant role in survival and maintenance of HSCs (Borge et al., 1997; de Haan et al., 2003; Ogawa, 1993). However, a successful expansion of human HSCs in vitro by using cytokines has shown to be difficult. Culture of stem cells with growth cytokines often results in massive proliferation, which is thought to be coupled with the loss of self-renewal (Ogawa, 1993; van der Loo and Ploemacher, 1995). Long-term culture of whole mouse bone marrow was pioneered by Dexter and colleagues in 1984 (Dexter, 1984), where bone marrow cells were cultured on stromal cells together with the addition of growth factors.

Finding the optimal conditions to expand stem cells without affecting the “stemness” would be of great importance for clinical use. Since the initial use of HSC in BMT therapy in the late fifties, several improvements in the field have been made, although clinical significance for in vitro expansion of human HSCs has not been achieved today. Conflicting results for which combination of cytokines that is optimal
for maintenance and expansion of HSCs indicate that more reliable and reproducible studies need to be done before expansion of human HSCs can be used in clinics. Some early acting cytokines, such as FL, affect murine and human HSCs differently, which makes the interpretation of data even harder.

Growth conditions for mouse HSCs are better established. The first known HSC self-renewal division that occurred in *in vitro* cultures was reported in 1992 (Fraser et al., 1992). Mouse marrow cells enriched for slow-dividing stem cells using 5-fluorouracil (5-FU) were cultured for four weeks on an irradiated layer of marrow feeder cells. By injecting cultured stem cells into myeloablated recipient mice, they demonstrated a maintained ability for long-term repopulation. Several studies with similar results followed showing maintenance of self-renewal but not expansion of stem cells. At this time, attention focused mainly on finding soluble growth factors, cytokines, to achieve a reproducible and controlled regulation of stem cell division. Five years later, a combination of FL, SCF, and IL-11 in serum-free media showed to be a successful combination for the expansion of HSCs with long-term self-renewal (Miller and Eaves, 1997). Sca-1^+^Lin^-^ marrow cells were cultured for 10 days and *in vivo* studies showed a 3-fold net increase in multilineage repopulation ability.

FGF receptor (FGFR), involved in the maintenance and developing of a wide range of tissues, is expressed on mouse LT-HSCs and stimulation with FGF-1 alone in serum-free media is capable of their expansion (de Haan et al., 2003). It is, however, possible that these results could be caused by indirect effects due to culture of unfractionated cells instead of purified HSCs.

A combination of SCF and FL together with Tpo (Ramsfjell et al., 1996) or IL-6, FL, and Tpo (Matsunaga et al., 1998) support survival of murine long-term HSCs. A body of evidence implicates that Tpo is important for *in vivo* maintenance of HSC and capable of generating *de novo* HSCs in culture (Borge et al., 1997; Kirito et al., 2003; Qian et al., 2007; Yagi et al., 1999). While SCF and FL appear to be important for survival and proliferation of HSCs, retention of stem cell activity needs stimulation with IL-11 or IL-6 (Borge et al., 1997; Sauvageau et al., 2004). Recently, Dr Lodish and his group developed a culture system for murine HSCs using SCF, Tpo, FGF-1 and the insulin-like growth factor (IGF)-2 in serum-free media, and were able to expand repopulating HSCs eight-fold compared to freshly isolated HSCs (Zhang and Lodish, 2005). A more remarkable expansion of HSCs was achieved when any of the members of the angiopoietin-like family of proteins were added to the culture showing a 30-fold increase of LT-HSCs (Zhang et al., 2006a).
It has also been shown that members of the Wnt growth factors are important for regulation of HSCs. Overexpression of β-catenin, a downstream effector in the Wnt pathway, results in an expansion of HSCs (Reya et al., 2003). Wnt induces expression of HOXB4 and Notch1, which are both suggested to be important for self-renewal (Reya et al., 2003; Stier et al., 2002; Varnum-Finney et al., 2000).

There is an increasing interest in culturing HSC on feeder layer of stromal cells or other supporter cells, referred to as co-culture. The use of feeder cells for in vitro culturing of stem cells would mimic the in vivo condition found in the bone marrow (see section; The hematopoietic stem cell niche?). A wide range of soluble cytokines, known and unknown, are secreted from the stromal feeder cells regulating self-renewal, survival and expansion. In addition, the feeder cells provide important cell-cell contact with the HSCs including ligand-receptor and interactions between integrins and the extracellular matrix. Special interest in Notch signaling, because of its role in supporting the HSCs, has lead to the finding that its ligands Delta1 and Jagged1 had no or little effect on the increase of ST-HSCs expansion, but significantly increased numbers of LT-HSCs (Kertesz et al., 2006; Suzuki et al., 2006).

Regulation of the cell cycle in HSCs
The fact that all cells produced during hematopoiesis are derived from HSCs, which are mostly dormant, implicates that a balanced regulation of proliferation and maintenance of a quiescent state is fundamental to enable formation of new cells without causing exhaustion of the HSC pool. Investigation of in vivo 5-bromo-2-deoxyuridine (BrdU) labeling of dividing cells has shown that 50% of all LT-HSCs divide every 6 days, whereas 99% of all LT-HSCs have entered the cell cycle after 57 days (Cheshier et al., 1999). Furthermore, at any given time point, 75% of all LT-HSCs are in a quiescent state. The cell cycle consists of four phases; G1, S, G2 and M with the addition of G0, which is a quiescent state outside the cell cycle. Growth factors stimulate cells to enter the G1 phase, where they grow and make preparations for the DNA replication, which takes place in the S phase. The G2 phase is a second growing phase needed to enable cell division, or mitosis, in the M phase. The cell cycle is controlled by cyclins, cyclin-dependent kinases (CDK), and CDK-inhibitors. In mammalian cells CDK4/6 and CDK2, which are involved in the activation of the cell cycle, are inhibited by CDK-inhibitors of the INK4 family (p16INK4A, p15INK4B, p18INK4C and p19INK4D) and of the Cip/Kip family (p21Cip1/Waf1, p27Kip1, and p57Kip2), respectively (Sherr and Roberts, 1999). I will highlight some of the CDK-inhibitors that have
received special attention in their role of regulating cell cycle activity in hematopoietic stem cells.

A slow cell cycle and maintenance of quiescence is important to protect stem cells and prevent exhaustion of HSC activity (Cheng et al., 2000b; Nygren and Bryder, 2008). Mice lacking the gene coding for p21"Cip1/Waf1" (p21 hereafter) show increased cycling of primitive HSCs with an impaired self-renewal capacity (Cheng et al., 2000b). Cell cycle inhibition in the late G1 phase by p21 seems to be stem cell restricted, due to the fact that progenitor cells from p21"−/−" mice show a decreased proliferation rate (Braun et al., 1998; Mantel et al., 1996). In contrast, deletion of the early G1 phase CDK inhibitor, p18"INK4C" (p18), increased the number of primitive HSCs with self-renewal capacity (Yuan et al., 2004). Together this shows that different CDK inhibitors can have distinct effects on the cell cycle control of HSCs. Furthermore, deletion of p18 counteracted the exhaustion of stem cells caused by p21 deficiency (Yu et al., 2006).

The CDK inhibitor p27"Kip1" (p27) is, in contrast to p21, not regulated by the tumor suppressor p53 (Polyak et al., 1994) and is controlled both by translational and post-translational mechanisms (Hengst and Reed, 1996; Pagano et al., 1995). Deletion of p27 does not affect the number of stem cells and they show a normal cell cycle, whereas the number of progenitors in the hematopoietic system is increased (Cheng...
et al., 2000a). The finding that there is dominance of p21 in stem cell kinetic regulation and of p27 in progenitor cells implicates a divergent function of different CDK inhibitors in distinct cell stages depending on intrinsic mechanisms. This difference in the CDK inhibitor function might contribute to the explanation why stem cells have a slower cell cycle compared to highly proliferative progenitor cells.

Bmi-1 is necessary for the maintenance of HSCs and is involved in the regulation of cell cycle by repressing the expression of p16$^{\text{INK4A}}$ (p16) and p19$^{\text{INK4D}}$ (p19) (Park et al., 2003), which are both involved in cell aging.

Until recently, the significance for p57$^{\text{Kip2}}$ (p57) in HSC kinetic was unknown. Expression analysis of different CDK inhibitors in the quiescent bone marrow SP cells and non-SP cells revealed novel data showing p57 specific expression in SP cells (Umemoto et al., 2005). This suggests a critical role of p57 in maintaining HSCs in a quiescent state. Similarly, data from Nakauchi and his group showed abundant expression of p57 in freshly isolated primitive CD34$^-$LSK cells, whereas p57 was downregulated in CD34$^+$LSK cells, HSCs with less self-renewal capacity (Yamazaki et al., 2006). The predominance of p57 in HSC, in contrast to p21 and p27, might imply a major role of p57 as a specific CDK inhibitor within the HSC cell cycle. It has also been shown that quiescence of HSCs by two growth factors, transforming growth factor (TGF)-β (Scandura et al., 2004) and Tpo (Qian et al., 2007), is mediated by p57 upregulation.

Another regulator of retaining HSC in the G0 phase is the phosphatase and tensin analog (PTEN). Abrogation of PTEN increases the cycling of HSCs and causes exhaustion of the stem cell pool (Zhang et al., 2006b). PTEN controls the cell cycle through inhibition of the phosphatidylinositol-3 kinase/AKT pathway and is commonly mutated in malignant tumor cells (Vivanco and Sawyers, 2002). Similar to the p21$^{-/-}$ mouse, depletion of PTEN, as well as the growth factor independent 1 (Gfi1) or Forkhead box transcription factors (FoxO) 1,3 and 4, show a HSC phenotype with increased cycling leading to HSCs exhaustion (Orford and Scadden, 2008).

The transcription factor MEF/ELF4 regulates both self-renewal and quiescence of HSCs (Lacorazza et al., 2006). Mef null mice have increased number of HSCs, which are more quiescent than wild type HSCs. Recently, it was found that this increase in HSCs quiescence in Mef null mice was abrogated in the absence of p53, suggesting an important role of p53 in the regulation of stem cell quiescence (Liu et al., 2009). Furthermore, maintenance of quiescence by p53 is thought to be mediated by the
p53 target genes \textit{Gfi1} and \textit{Necdin}. Gfi-1 has an essential role in restricting the proliferation of HSCs and retaining their self-renewal capacity (Hock et al., 2004). Contradictory data for the role that p53 plays in HSCs engraftment have been reported (Akala et al., 2008; Chen et al., 2008; Hock et al., 2004; TeKippe et al., 2003). Therefore, p53 seems to be important for quiescence of HSCs, whereas its significance for increasing functional HSCs is controversial (Figure 5).

The interaction of the tyrosine kinase receptor Tie-2, expressed on HSCs, and its ligand angiopoietin-1 (Ang-1) is another important way to maintain the HSC quiescence (Arai et al., 2004). This is one, of many, receptor-ligand pairs involved in the maintenance of HSCs in the stem cell niche, which will be explained in more detail in the next chapter.

The hematopoietic stem cell niche

Although the HSC is the best characterized adult stem cell today, its precise location in the bone marrow where it self-renews and differentiates is not fully defined. In contrast, the place for stem cells in other tissues, such as the skin and brain, is well identified and described. The microenvironment in the bone marrow provides the HSCs with important signals for their maintenance (self-renewal and survival), migration and differentiation. These signals are mediated by secreted- and membrane-bound factors from bone marrow cells. The concept of a microenvironment with a special architecture housing the stem cells was first proposed by Schofield more than 30 years ago when he introduced the term “stem cell niche” (Schofield, 1978). The criteria for a stem cell niche are: first, the number of stem cells is well regulated; second, an interaction with a heterogeneous population of other cells is necessary for the stem cell maintenance; third, a balanced regulation of stimulatory and inhibitory signals from membrane-bound and secreted molecules; and fourth, non-stem cells can acquire stem cells-like properties when located in the niche (Adams and Scadden, 2006). Although the bone marrow is the common place for HSCs, they undergo regular trafficking into the peripheral blood where they reside for shorter periods and then return to the bone marrow (Wright et al., 2001). The function for the stem cells in the peripheral blood is unknown, but their mobilization into the peripheral blood following various stresses such as chemotherapy or administration of G-CSF has been utilized in BMT therapy. In the niche, HSCs reside in fragments of spongious bone, called the trabecular bone, in close contact to the osteoblasts (Figure 6). This part of the bone contains a variety of cells such as
Hematopoietic stem cells

osteoblasts, adipocytes, reticular stromal cells, vascular endothelial cells, and small blood vessels called sinusoids.

The crucial role of the niche has been recognized for a long time, starting with the finding that mutation in the SCF gene, expressed on niche cells, in Sl/Sld mice, had a dramatic effect on the bone marrow HSCs (McCulloch et al., 1965). The first evidence that osteoblasts were important for the HSC niche came when a conditional (tissue specific) inactivation of the bone morphogenic protein (BMP)-1 receptor showed a bone defect causing an increase of the number of osteoblasts coupled to an increase of HSCs (Zhang et al., 2003). They found that LT-HSCs appeared to be attached to early spindle-shaped N-cadherin expressing osteoblastic (SNO) cells. Another study showed that the deletion of the oncogene c-Myc in HSCs resulted in severe cytopenia and an accumulation of HSCs in the bone marrow, whereas overexpression of c-Myc was shown to decrease the expression of N-cadherin on HSCs with lost self-renewal (Wilson et al., 2004). This indicates that c-Myc controls the balance between self-

Figure 6. The hematopoietic stem cell niche. In the bone marrow, HSCs reside in the spongyous bone called trabecular bone. HSCs receive important survival and maintenance signals from other cells in the niche (e.g. osteoblasts, reticulocytes, and endothelial cells). The nutrient and blood supply in the niche is rather limited and carried out via small blood vessels called sinusoids.
renewal and differentiation. Lately, the role of N-cadherin in the HSC niche has been questioned. Two recent studies have shown that conditionally deletion of N-cadherin in HSCs did not affect the bone marrow cellularity or the maintenance of the HSCs (Kiel et al., 2009; Kiel et al., 2007). Another evidence for the importance of osteoblasts in the niche was reported in a study showing that constitutively activation of parathyroid hormone receptors, expressed on osteoblasts, led to an increase of trabecular osteoblasts supporting the expansion of HSCs (Calvi et al., 2003). Furthermore, the expansion of HSCs caused by increased number of osteoblastic cells showed to be dependent on Notch signaling, although the role of Notch in the niche has been challenged (Mancini et al., 2005). The mineral environment in the bone marrow with a high quantity of calcium has shown to play an important role in the niche. Ca2+ ion concentration is recognized by the seven-transmembrane calcium-sensing receptor (CaR) which is expressed on HSCs (Adams et al., 2006). Mice deficient for CaR show a distinguished decrease in HSCs in the trabecular bone whereas the numbers of more mature progenitors were intact. Further analysis revealed that HSCs had entered the circulation and the spleen, however, no differences in the cell cycle profile were detected. It is likely that the calcium gradient in the niche has a major impact on homing and retaining HSCs in the bone marrow niche. Quiescence is a signature for HSCs located in the stem cell niche. The interaction of angiopoietin (Ang)–1 expressed on osteoblasts with the tyrosine kinase receptor Tie-2 on HSCs plays a critical role in maintaining the quiescent HSC state and strengthening of the adhesion to the endosteal surface (Figure 7) (Arai et al., 2004).

Lately, the role of the osteoblasts as the single niche cell important for HSC maintenance has been questioned in a study reporting that depletion of osteoblasts in the trabecular zone did not affect the frequency of HSCs (Kiel et al., 2009; Kiel et al., 2007). It is likely that there is interplay between several cell types in the niche, and that not only direct signaling with cell-cell contact is importance. One possibility is that soluble factors secreted by endosteal, perivascular or other cells create a gradient of secreted factors which contribute to the regulation of HSCs in the niche. Ang-1, Tpo, as well as the CXC chemokine ligand (CXCL) 12, also known as stromal derived factor (SDF)-1, are secreted by niche cells and known to regulate HSC maintenance (Arai et al., 2004; Sacchetti et al., 2007; Yoshihara et al., 2007). Several findings reveal that quiescent HSCs with long-term potential are associated with osteoblasts in the “osteoblastic stem cell niche” (Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003), while other findings point towards the importance of vascular cells to maintain HSCs in the “vascular stem cell niche” (Kiel et al., 2005).
It has been suggested that the vascular niche resides more mitotically active HSCs and play a role in the mobilization, while quiescent HSCs are located in the osteoblastic niche (Avecilla et al., 2004; Heissig et al., 2002). This indicates that the osteoblastic niche is the primary niche for maintenance of LT-HSC and the vascular niche is a secondary niche which function is to produce progenitor cells and maintain the homeostasis in the hematopoietic system (Wilson and Trumpp, 2006). A body of evidence shows the importance of osteoblasts, but it remains to be investigated if they are actually required for HSC maintenance (reviewed in (Kiel and Morrison, 2008)). Recent evidence support the earlier findings that quiescent HSCs are closer located to the osteoblasts, whereas more mitotically active HSCs are found more distant to osteoblasts (Lo Celso et al., 2009). However, sinusoids are also present in the endosteal niche (Kubota et al., 2008; Lo Celso et al., 2009; Xie et al., 2009) and it is therefore likely that the HSC niche is created through a combined influence of several specialized niche cells. Like adipocytes and osteoblasts, endothelial cells

Figure 7. A simplified picture of some of the signal transduction pathways in the HSC niche. Both soluble and membrane-bound interactions between osteoblasts and HSCs in the niche are thought to regulate the balance between quiescence, self-renewal, migration, and adhesion (Rizo et al., 2006).
surrounding sinusoids, are derived from mesenchymal stem cells. Recent data has revealed that endothelial cells express the HSC regulators CXCL12 and Ang-1 (Sacchetti et al., 2007). Experiments have shown that deletion of the receptor for CXCL12, CXCR4, had a severe effect on the number of HSCs, indicating that CXCL12-CXCR4 signaling plays an essential role in maintaining quiescent HSCs. In addition, bone marrow HSCs seem to be co-localized to special reticular cells expressing high amounts of CXCL12, called CXCL12-abundent reticular (CAR) cells, which are found near endothelial cells or the endosteum (Sugiyama et al., 2006). However, another study has reported that CXCL12 and CXCR4 have a key role in the G-CSF-induced mobilization of HSCs (Petit et al., 2002). Endothelial cells are thought to regulate HSC functions in the niche and maintaining stem cells properties in vitro (Kiel et al., 2007; Kiel et al., 2005; Li et al., 2004; Ohneda et al., 1998). These data together raise the possibility that HSCs can reside at different locations in the bone marrow and that several different cells are involved in the regulation of HSCs. So far, it is known that the maintenance of HSCs in the niche is dependent on a variety of membrane-bound and soluble factors secreted from osteoblasts, endothelial cells and other known or unknown cells. The anatomic microenvironment in the bone marrow is dynamic and the question of whether there is one or more niches remains.

The hypoxic stem cell niche
To better understand the stem cell niche in the bone marrow and how the regulation of HSCs is controlled, many studies have focused on the interplay between niche cells and HSCs. The oxygen level in the bone marrow microenvironment is another factor that lately has been given special attention in the hematopoietic stem cell research. It is suggested that the bone marrow is low in oxygen (hypoxic) (Ceradini et al., 2004; Harrison et al., 2002). The hypoxic region is thought to be caused by lower blood perfusion. Due to difficulties in measuring the partial pressure of oxygen (pO$_2$) in the bone marrow, the exact physiological concentration of oxygen in the endosteal area has not been specified. A provocative, but sensational, study used a mathematical model to estimate the oxygen concentration in bone marrow and speculated that HSCs were located in areas of low oxygen, whereas more proliferative blood cells were located in areas of higher oxygen concentration (Chow et al., 2001). Physiological measurements with a blood gas syringe in the bone marrow of a healthy human adult revealed a pO$_2$ average of 54.9 mmHg (Harrison et al., 2002). This could be compared to a pO$_2$ in capillaries of 95 mmHg and 40 mmHg in the interstitial fluids that surround tissue cells (Guyton and Hall, 1996), whereas in ischemic tissues the O$_2$ tension can decrease to 4 mmHg (Ceradini et al., 2004).
recent study by Parmar et al tested the hypothesis that HSCs were located in areas of low oxygen by utilizing that hypoxic regions have a lower blood perfusion which was measured by injecting mice with the fluorescent dye Hoechst 33342 (Ho). Bone marrow cells were isolated and divided according to a Ho dye diffusion gradient which simulates the in vivo level of oxygen. To investigate the stem cell character of cells with low and high Hoechst fluorescence, transplantations into lethally irradiated mice were done. The results showed that the bone marrow fraction with the lowest Ho uptake, inferred to be hypoxic, had the highest amount of long-term repopulating cells. Furthermore, HSCs in the SP showed high staining for the hypoxic probe pimonidazole (PIM), which covalently binds to protein thiol groups when pO$_2$ is below 10 mmHg (corresponding to less than 1.3% oxygen) (Parmar et al., 2007). Moreover, another study shows that PIM stains hypoxic areas in vivo in the bone marrow of mice. PIM staining was intense in endosteal areas and decreased rapidly within a short distance of 50 µm (Levesque et al., 2007), postulating that the osteoblastic niche is hypoxic. Recently it was shown that quiescent cells, label-retaining cells stained with BrdU, are found in hypoxic sinusoids containing areas distant from the “vascular niche” (Kubota et al., 2008). This finding, together with Lo Celso’s data that dormant stem cells are closer to osteoblasts compared to cells with a more active cell cycle (Lo Celso et al., 2009), indicates that areas containing sinusoids, close to the endosteum, distant from capillaries and likely hypoxic, maintain HSCs in a quiescent state.

Consistently, it has been shown that cultivation of bone marrow cells in hypoxia in vitro increases the number of primitive colony forming cells, and sustains their repopulating ability better compared to normoxic conditions and inhibited differentiation (Cipolleschi et al., 1993; Eliasson, 2006; Ivanovic et al., 2000; Ivanovic et al., 2002; Ivanovic et al., 2004). Moreover, cultivation of purified human HSCs (Lin$^{-}$CD34$^{+}$CD38$^{-}$) for 4 days in hypoxia revealed a 6-fold increase of repopulating cells compared to stem cells cultured in normoxia and also a nearly 4-fold increase compared to freshly isolated HSCs (Danet et al., 2003). This is consistent with findings in a recent study on human HSCs showing that hypoxia supports the maintenance of HSCs but reduces proliferation (Shima et al., 2008). Similarly, culture of CD34$^{+}$ cord blood cells in very low (0.1%) oxygen levels maintains survival and favors return of cycling stem cells to G0 (Hermitte et al., 2006).
HYPOXIA INDUCIBLE FACTOR-1

The main regulator in hypoxic cells is the transcription factor hypoxia-inducible factor (HIF)-1. HIF-1 regulates multiple genes involved in glucose metabolism, as well as stem cell mobilization, proliferation, survival, and differentiation. HIF-1 was discovered in 1988 when a hypoxia-response element (HRE) was found in the enhancer for erythropoietin, a protein involved in erythropoiesis (Goldberg et al., 1988; Semenza et al., 1991). HIF-1 is a heterodimeric transcription factor consisting of a 120 kDa subunit HIF-1α and a constitutively expressed β subunit, which is also called aryl hydrocarbon receptor nuclear translocator (ARNT). Three different HIF-α subunits have been characterized, HIF-1α, HIF-2α and HIF-3α. HIF-2α is predominately expressed in lung and endothelial tissues, whereas HIF-3α is more selectively expressed in neuronal cells and corneal epithelium (reviewed in (Ke and Costa, 2006)). The two subunits of HIFα and HIFβ are members of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) protein family, which are responsible for the heterodimerisation of the protein. In the domain structure of HIFα, two transactivation domains (TAD) have been identified; one N-terminal (N-TAD) and one C-terminal (C-TAD) (Figure 8). Regulation of HIF-1 activity is mediated by posttranslational modification of the oxygen-dependent degradation domain (ODD) on the HIF-1α subunit.

![Domain structure of HIF-1α](image)

Figure 8. **Domain structure of HIF-1α.** HIF-1α belongs to the bHLH and PAS protein family. The oxygen dependent degradation domain (ODDD) is important for oxygen regulated stability of HIF-1α. Oxygen levels higher than 5% cause hydroxylation of P402 and P564 and acetylation of K532. HIF-1α contains two transactivation domains (TAD) domains involved in activation of gene transcription.

At oxygen levels above 5%, hydroxylation of two conserved proline residues, Pro 402 and Pro 564 in the ODD, enables binding of the ubiquitin ligase von Hippel-Lindau (VHL) tumor suppressor protein, which leads to polyubiquitination and rapid degradation of HIF1-α by the proteosome (Maxwell et al., 1999). During hypoxic conditions, hydroxylation is inhibited, leading to the stabilization of HIF-1α and translocation to the nucleus where it dimerizes with HIF-1β and becomes transcriptionally active (Figure 9). The oxygen sensors in the oxygen dependent regulation of HIF-1α are prolyl hydroxylases (PHDs) which requires both oxygen and ferrous ions (Fe^{2+}) to be able to hydroxylate residues of the ODD. Diminished levels of Fe^{2+} caused by iron chelators or metal ions such as cobalt (Co^{2+}) are able to stabilize
HIF-1α (reviewed in (Ke and Costa, 2006)). Once HIF-1α is stabilized, a further decrease in oxygen inhibits the activity of factor inhibiting HIF (FIH), which modulates C-TAD of HIF-1α and enables recruitment of the transcriptional coactivator p300 and CREB binding protein (p300/CBP) (Mahon 2001).

Figure 9. **Oxygen-dependent regulation of HIF-1α stabilization.** In normal oxygen concentration, prolyl hydroxylases (PDHs) mediate hydroxylation of proline residues of HIF-1α. This enables binding of the ubiquitin ligase von Hippel Lindau (VHL) following ubiquitination and degradation by the proteosome. In low oxygen, however, HIF-1α proteins accumulate and form dimers with the partner HIF-1β. Cofactors such as p300/CBP bind to the HIF-1 dimer, which results in gene transcription of HIF-1 target genes.

HIF-1α activity is in addition to hydroxylation, regulated by several post-translational modifications such as ubiquitination, acetylation, phosphorylation, and SUMOylation (Agbor and Taylor, 2008; Ke and Costa, 2006). Phosphorylation of HIF-1α by the mitogen-activated protein kinase (MAPK) p42/44 has shown to enhance the transcription activity but not the stability of the protein (Richard et al., 1999). Lately, it has become evident that HIF activity can be induced in normal oxygen levels by specific cytokines, such as PDGF, FGF, IGF, SCF, and Tpo (Conte et al., 2008; Pedersen et al., 2008; Yoshida et al., 2008). The biological significance for induction of HIF-1 by cytokines, which is less intense, compared to hypoxic-induced expression, is under discussion. It is thought that the induction of HIF-1 by growth factors is a result of increased transcription of HIF-1, although stabilization of the protein is also suggested (Pedersen et al., 2008). The stabilization could be mediated by reactive oxidative species (ROS). The question whether ROS generated from the mitochondria in the electron transport chain is sufficient to stabilize HIF-1α under normoxic conditions is controversial (Brunelle et al., 2005; Gorlach et al., 2001; Yoshida et al.,
HYPOXIA INDUCIBLE FACTOR-1α

2008). Together, this shows that the regulation of HIF activity is a fine balance between HIF stabilization, promoted mainly by hypoxia, and PHD dependent degradation. It is possible that this regulation of HIF is cell type specific.

HIF-1 activates expression of several genes involved in angiogenesis and erythropoiesis. Abrogation of HIF via deletion of *Hif-1α* or *Arnt* genes have shown to defect the numbers of hematopoietic progenitors in the yolk sac (Adelman et al., 1999) and cause embryonic death before birth (reviewed in (Ke and Costa, 2006)), which makes it difficult to fully study the involvement of HIF-1 in adult hematopoietic progenitor and stem cells. HIF is known to activate 70 genes via binding to the HRE site, located in the promoter and enhancer of the target genes. More than 200 target genes are thought to exist, although not all are regulated by direct binding to a HRE region (Wenger et al., 2005). Furthermore, whether HIF-1 and HIF-2 have the same target genes is under discussion, but it is proposed that it is the cooperation with other transcription factors that distinguishes the expression pattern of specific genes.

As mentioned earlier, HIF-1 regulates the transcription of erythropoietin, which is required for the formation of erythrocytes. Another well known and important target gene for angiogenesis is the vascular endothelial factor (VEGF), which has the function to recruit and increase proliferation of endothelial cells (Josko et al., 2000; Neufeld et al., 1999). In addition, HIF-1 induces transcription of matrix metalloproteinases (MMP), which are involved in matrix metabolism and vessel formation (Ben-Yosef et al., 2002).

HIF-1 and regulation of important niche molecules

As discussed above, it is suggested that hematopoietic stem and progenitor cells are distributed along an oxygen gradient in the bone marrow, where the HSCs are sited in hypoxic areas and more proliferative progenitors are located in more oxygen-rich areas closer to large blood vessels (Levesque et al., 2007; Parmar et al., 2007). This implies that a hypoxic niche plays a fundamental role in the maintenance of HSCs. Many of the genes induced by HIF-1 are expressed in HSCs and the HSC niche (Figure 10). The HIF-1 target gene VEGF has been shown to have an important role for survival and *in vivo* repopulation of HSCs (Gerber et al., 2002). Moreover, perichondrial cells and chondrocytes, present in the osteoblastic niche, are known to express high levels of VEGF. A recent study showed that suppressing VEGF in mice inhibits niche formation (Chan et al., 2009), which indicates that VEGF has an essential role in the niche, both for maintenance of HSCs and non-hematopoietic niche cells. Hypoxia is known to increase growth factor signaling by inducing c-KIT,
Notch-1 (Jogi et al., 2002), IGF-2 (Feldser et al., 1999), and FGF (Conte et al., 2008), which are suggested to be regulated in a HIF-1 dependent way. However, it is not clear whether these regulatory mechanisms are transcriptional and if they are HIF-1 targets in HSCs. During hypoxia, Notch-1 is known to interact with HIF-1, which leads to expression of Notch-1 downstream target genes and a block of differentiation of neuronal and myogenic progenitor cells (Gustafsson et al., 2005). CXCL12, which binds to the chemokine receptor CXCR4 expressed on hematopoietic cells are important for homing to the bone marrow (Hattori et al., 2001), as well as maintaining quiescence of HSCs (Sugiyama et al., 2006). HIF-1 regulated CXCL12 expression in hypoxic regions in the bone marrow recruits CXCR4-expressing stem and progenitor cells, suggesting a fundamental role of HIF-1 in homing of HSCs (Ceradini et al., 2004).

HIF-1 has also been proposed to be involved in the mobilization of hematopoietic progenitor cells (Levesque et al., 2007). G-CSF, used in the clinic to elicit mobilization of transplantable HSCs, increases the number of granulocytes, which deplete the storage of oxygen in the bone marrow, leading to stabilization of HIF-1. This is accompanied by increased levels of VEGF that increase the permeability of the blood vessels. HIF-1 induced expression of MMP (Ben-Yosef et al., 2002), needed for
penetration of the endothelium, causes degradation and inactivation of CXCR4, CXCL12 and c-KIT, which are necessary to retain stem and progenitor cells in the bone marrow (Levesque et al., 2007). Although paradoxically, this implicates that HIF-1 can be involved both in mobilization and homing as well as maintenance of HSCs in the bone marrow niche.

Another target gene for HIF-1 and is the Bcrp1/ABCG2 gene (Krishnamurthy et al., 2004b), which is highly expressed on LT-HSCs. BCRP-1/ABCG2 is important for both the survival as well as protection of HSCs from toxic agents. This indicates that HIF-1 might protect primitive HSCs from severe damage caused by the accumulation of toxic compounds. HIF-1 has also been reported to control self-renewal by inducing the expression of telomerase (though hTERT) (Nishi et al., 2004), Oct4 (Covello et al., 2006) and Notch-1 (Gustafsson et al., 2005), although it remains to elucidate if these genes are increased by HIF-1 in hematopoietic cells.

Glucose metabolism in hypoxic cells
Under low oxygen supply, cells switch their glucose metabolism from the oxygen-dependent tricarboxylic acid cycle (TCA) to the oxygen-independent glycolysis, a process thought to be regulated by HIF-1 (Seagroves et al., 2001; Semenza et al., 1996). Glycolysis is a less energy effective pathway and hypoxic cells compensate this by increasing glucose uptake. This is achieved by upregulation of glucose transporters on the cell surface, which are transcriptionally regulated by HIF-1 (Wenger, 2002). In addition, by increasing expression of pyruvate dehydrogenase kinase-1 (PDK1) (Kim et al., 2006), lactate dehydrogenase A (LDH-A) (Semenza et al., 1996), and other enzymes involved in glycolysis, HIF-1 enables a higher production of adenosine triphosphate (ATP) through an anaerobic metabolism. PDK1 attenuates the pyruvate metabolism in TCA by inactivation of pyruvate dehydrogenase (PDH), the enzyme that converts pyruvate to acetyl-coenzyme A (Kim et al., 2006; Papandreou et al., 2006; Semenza et al., 1996). Instead, LDH-A favors the cytosolic lactate production from pyruvate (Figure 11). Recently, it was found that mice lacking prolyl hydroxylase 1 (Phd1), a negative regulator of HIF-1 and HIF-2 stabilization, showed signatures of oxygen-independent glucose metabolism (Aragones et al., 2008), indicating that HIF proteins play a key role in hypoxic metabolism. The ability of cells to adapt to conditions of limited oxygen supply and maintain ATP production is of particular importance in tumor development and stem cell maintenance.
HIF-1 and Cell cycle

Despite the crucial role of hypoxia in the regulation proliferation of normal and tumor cells, the mechanism behind this is poorly understood. Hypoxic conditions can cause cell cycle arrest in the G1 phase of primary cells and also in immortalized cell lines (Gardner et al., 2003). HIF-1α is suggested to induce cell cycle arrest by activating p21 (Goda et al., 2003; Koshiji et al., 2004) and p27 expression (Goda et al., 2003). Until recently, the HIF-1α activated p21 expression has been a mystery due to the fact that p21 lack a HRE sequence in the promoter. Koshiji and colleagues showed that neither the DNA binding domain, nor the transcriptional activity was needed for HIF-1α-induced p21 expression. Instead, HIF-1α binds to c-Myc and counteracts its transcriptional repression of p21 (Koshiji et al., 2004). Lack of HIF-1α increases proliferation in hypoxia and leads to decreased levels of p21, whereas p27 is unchanged (Carmeliet et al., 1998). However, another study claimed that p21 was not required for HIF-1α mediated cell cycle arrest, and that p27 was the key regulator of reduced proliferation in hypoxia independently of HIF-1 (Gardner et al., 2001). Whether HIF-1α regulates expression of p27 or not is under discussion and remains to be investigated. Low levels of oxygen decreases proliferation of human cord blood HSC and increases the number of quiescent cells without disturbing engraftment potential (Hermitte et al., 2006; Shima et al., 2008). This was associated with increased levels of p21 and p57 (Shima et al., 2008).
APOPTOSIS

Apoptosis is important in maintaining a balance between the formation of new blood cells and the depletion of aged and damaged cells in the hematopoietic system with a high turnover rate. Deregulation of apoptosis is involved in many diseases such as cancer, where tumor cells through mutations have evolved mechanisms to avoid apoptosis, whereas several neurodegenerative diseases, such as Alzheimer’s and Parkinson’s disease, are associated with an increase of apoptosis of neurons. Apoptosis is mediated by the activation of a cascade of asparatate specific cystein proteases (caspases), which through proteolytic activity cause degradation of proteins or activate DNAse which in turn cause degradation of DNA. The activation of caspases leading to apoptosis can be triggered by two pathways, the intrinsic and the extrinsic pathway. The intrinsic pathway is characterized by increased permeability of the outer mitochondrial membrane, which leads to the release of several pro-apoptotic mediators such as cytochrome c. The mechanism for permeabilization of the outer membrane is not fully understood, but it is likely that the Bcl-2 family members play a significant role (Green and Reed, 1998). Once released, cytochrome c forms, together with the adaptor protein Apaf-1 and procaspase-9, a complex called the apoptosome (Zou et al., 1997; Zou et al., 1999). The apoptosome activates procaspase-9, which in turn cleaves and activates the executioner caspases-3 leading to cell death. The extrinsic pathway is initiated by the activation of special cell surface death receptors, the tumor necrosis factor receptor 1 (TNFR1) and the Fas receptor, which are activated by the binding of TNF or Fas ligand (Locksley et al., 2001; Screaton and Xu, 2000). Both receptors contain death domains (DDs), which interact with specific adaptor proteins with trigger an intracellular cascade of cleavage and activation of executer caspases.

Pro- and anti-apoptotic Bcl-2 members

The first Bcl-2 (B cell lymphoma-2) member, provided the name to the family containing both pro- and anti-apoptotic members, was identified in 1985 for its involvement in human follicular B cell lymphoma (Tsujimura et al., 1999). The following years, studies in D. melanogaster and C. elegans led to much of the knowledge we know today concerning the Bcl-2 proteins as critical regulators in apoptosis. The different members of the Bcl-2 family are classified according to their function and the presence of one to four conserved Bcl-2 homology domains (BH1-4). The anti-apoptotic members, namely Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and A1/BFL-1, share three or four BH domains, which are necessary for their ability to bind and sequester...
POPTOSIS

pro-apoptotic members. The pro-apoptotic members are divided into two subgroups according to if they contain one or multiple BH domains. Bax, Bak, Bok, among others, belong to the later group, whereas Bad, Bid, Bik, Bim, and Puma are examples of members in the subgroup sharing the BH3 domain, and are therefore named BH3-only members. The BH3 domain is responsible for binding and inactivating the anti-apoptotic members. The release of cytochrome c is thought to be regulated by a complex interaction between pro- and anti-apoptotic Bcl-2 members forming homo- and heterodimers in the outer mitochondrial membrane. It is suggested that both Bax and Bak can form homodimers in the mitochondria upon an apoptotic stimuli, leading to increased permeabilization, possible through the formation of pores inducing mitochondrial protein release (Eskes et al., 2000; Wei et al., 2001). The pore formation is proposed to be inhibited by the anti-apoptotic members, Bcl-2 and Bcl-Xl (Cheng et al., 2001; Zong et al., 2001), whereas Bim and other BH3-only members facilitate the activity of Bax and Bak by binding and sequester Bcl-2 and Bcl-Xl (Figure 12) (Desagher et al., 1999). Precisely how the Bcl-2 proteins form monomers, homodimers or heterodimers which activate or inhibit their activity to regulate the permeability in the mitochondria is unclear and controversial. Regulation of Bcl-2 proteins is complex and involves both transcriptional and post-transcriptional mechanisms. Upon stress signals such as DNA damage or metabolic stress, p53 and FoxO3a, can induce expression of the Puma or Bim gene, respectively (Dijkers et al., 2000; Nakano and Vousden, 2001). Lately, it has become evident that p53 and FoxO signaling pathways interact to determine the choice between cell death and survival (You and Mak, 2005). Both transcription factors are regulated by posttranslational modification such as phosphorylation and acetylation, but the outcome might be different. Post-transcriptional modification, such as phosphorylation, regulates several pro-apoptotic proteins. For instance, growth factor stimulation of the PI3K/AKT pathway leads to the phosphorylation of Bad, which attract 14-3-3 proteins resulting in the sequestration of Bad due to transportation out of the nucleus (Datta et al., 1997; del Peso et al., 1997). Bid is instead regulated by caspases-8 cleavage into an active truncated form, resulting in Bax-dependent protein leakage from the mitochondria. In this way, the extrinsic and intrinsic pathways are integrated together to activate apoptosis (Li et al., 1998).

To study the individual role of Bcl-2 members in hematopoiesis and development, knockout technology in mice has been performed. Bcl-2−/− mice have an immunoedeficient phenotype due to apoptosis in peripheral lymphocytes (Veis et al., 1993). Studies have shown that HSCs ablated of Bcl-2 can perform long-term reconstitution of myeloid but not lymphoid cells, suggesting an important role of Bcl-
2 in lymphopoiesis (Matsuzaki et al., 1997). In contrast, Bim-deficient mice have increased numbers of lymphoid and myeloid cells, which are resistant to growth factor starvation but vulnerable to apoptosis induced by DNA damages (Bouillet et al., 1999). This is likely explained by different pathways for specific apoptotic stimuli mediated via the Forkhead and p53 pathways described above. Mcl-1−/− mice show peri-implantation lethality, which complicates the investigation regarding its role in adult hematopoiesis. However, a recent study that performed conditional deletion of Mcl-1 in mice, which were anemic, showed ablation of bone marrow cells (Opferman et al., 2005). The requirement of Mcl-1 for survival of hematopoietic cells was particular pronounced in HSCs and progenitor cells. Furthermore, Mcl-1 was highly expressed in HSCs and induced by early-acting cytokines, such as SCF.

![Figure 12. Regulation of apoptosis by Bcl-2 family members.](image)

In the absence of growth factors, apoptosis can be triggered through the intrinsic pathway. This inhibits important survival signaling pathways such as PI3K/AKT. A possible effect can be transcription of the BH3 only protein Bim. In the mitochondria, anti-apoptotic Bcl-2 proteins bind and sequester Bax proteins. However, in the presence of Bim, this survival promoting effect of Bcl-2 will be inhibited. Instead Bax proteins form pores in the mitochondria membrane resulting in cytochrome c release. Cytochrome c forms a complex with Apaf-1 and procaspase 9, called the apoptosome, which results in activation of caspases 9. Caspase 9 activates the executioner caspases-3 that starts a cascade leading to cell death. Apoptosis can also be induced by death receptors via the extrinsic pathway, which activate caspases without involving the mitochondria.
OXIDATIVE STRESS AND AGING OF HSCs

Several thousand DNA damages occur every day in every cell and the major part of these are caused by ROS (Conger and Fairchild, 1952). During production of energy, ATP, in the mitochondrial respiratory chain, water is formed when oxygen molecules accept electrons. Some O₂ molecules are, however, only partially reduced and form superoxide O₂⁻. The superoxide undergoes rapid dismutation by superoxide dismutase (SOD) to form hydrogen peroxide, H₂O₂, and O₂. H₂O₂ is more stable but can be extinguished by three different processes: 1) it is removed by catalase and glutathione (GSH) peroxidase; 2) it is spontaneously transformed into the highly reactive hydroxyl radical, OH, in the fenton reaction catalyzed by Fe²⁺; or in neutrophils 3), H₂O₂ is converted by myeloperoxidase to hypochlorous acid, a toxic product used in the defense against bacteria (reviewed in (Ghaffari, 2008)).

HSCs seem to be particular sensitive for oxidative stress and the accumulation of ROS causes defects in the regulation of hematopoiesis (Ito et al., 2004; Ito et al., 2006; Miyamoto et al., 2007; Tothova et al., 2007). It is therefore not surprising that HSCs are mostly in a dormant quiescent state with low metabolic activity, which spares them from DNA and lipid damages caused by ROS formed in the respiratory chain. The ataxia telangiectasia mutated (ATM) gene is essential for the regulation of genomic stability and studies by Suda and colleagues have showed that deletion of the Atm gene results in bone marrow failure associated with increased levels of ROS in HSCs, leading to a decrease in stem cell numbers and defects in their reconstitution ability. The accumulation of ROS in HSCs from Atm⁻/⁻ mice results in upregulation of the tumor suppressor genes p16 and p19 caused by the activation of the p38 mitogen-activated protein kinase (MAPK) (Ito et al., 2004). The inhibition of p38 MAPK or treatment with antioxidant reagent N-acetylcysteine (NAC) allowed HSCs from Atm⁻/⁻ mice to maintain a quiescent state and improve the self renewal of LT-HSCs (Ito et al., 2004; Ito et al., 2006). In addition to the dysfunction of cell cycle regulation caused by p38 MAPK, it is thought that adhesion molecules in the niche, such as N-cadherin, are affected by ROS, leading to the exit of HSCs from the niche (Hosokawa K, 2007). Increased levels of ROS and upregulation of p16 and p19 leads to a reduced repopulation ability and is associated with the aging of stem cells (Ito et al., 2004; Janzen et al., 2006; Krishnamurthy et al., 2004a). Primitive bone marrow cells from young mice deficient of p16 showed no difference in cell cycle or apoptosis compared to cells from wild type mice, whereas cells from older p16⁻/⁻ mice showed higher cycling activity and more dead cells compared to their wild type counterparts.
Oxidative stress and hematopoietic stem cells

Lately, the significance of p16 as a role in HSCs aging has been questioned (Attema et al., 2009). Another suggested signature for aging of HSCs is a myeloid skewing of differentiation (Janzen et al., 2006; Liang et al., 2005; Morrison et al., 1996), which has been suggested to depend on the increased levels of ROS in aging of HSCs (Janzen et al., 2006). The capacity to generate lymphoid differentiation is dramatically reduced in aging HSCs, whereas the myeloid potential is maintained and these changes are accompanied by upregulation of myeloid specific genes in LT-HSCs from older mice, indicating that aging of HSCs is regulated by intrinsic mechanisms (Rossi et al., 2007).

FoxO proteins are essential in the resistance to oxidative stress

During the last decade, several studies have underscored the importance of the FoxO family in the regulation of cell cycle arrest, stress resistance, apoptosis, glucose metabolism, and DNA repair. Particular in stem cell homeostasis, FoxO has attracted a lot of attention due to its role in the regulation of maintenance, survival, and quiescence. In mammals, the FoxO family consists of four members: FoxO1, FoxO3, FoxO4 and FoxO6. In hematopoietic cells the first three mentioned play a critical role. The FoxO protein was first characterized in C.elegans as the ortholog DAF-16 involved in the extension of lifespan (Kenyon et al., 1993). The FoxO family has diverse physiological functions and is well conserved from yeast to humans, which proclaims their importance in development. It seems like a paradox that FoxO proteins can regulate both cell death and resistance to oxidative stress. However, this is explained by a tight regulation of post-translational modifications controlling the diversity of functions achieved by FoxO factors. These include phosphorylation, acetylation and ubiquitylation (reviewed in (van der Horst and Burgering, 2007)). Insulin, insulin-like growth factors (IGF), as well as other growth factors including IL-3, SCF, FL, and Tpo activate the PI3K/AKT signaling pathway which leads to the inactivation of FoxO by phosphorylation of three specific serine and threonine residues (Thr32, Ser253 and Ser315 for FoxO3), subsequently leading to the interaction with the 14-3-3 and translocation out of the nucleus (Brunet et al., 1999; Engström et al., 2003; Jönsson et al., 2004; Tanaka et al., 2001). Instead, inactivation of the PI3K/AKT pathway or stress stimuli leads to activation of FoxOs (Greer and Brunet, 2005). In contrast to the phosphorylation of AKT, phosphorylation of FoxO caused by another kinase, JNK, results in nuclear translocation and activation (Essers et al., 2004). Oxidative stress activates FoxO proteins by mammalian sterile 20 like kinase 1 (MST-1) phosphorylation, monoubiquitination, as well as interaction with β-catenin, and SIRT1 deacetylase (reviewed in (Ghaffari, 2008)). Today, it is not known if JNK regulates
FoxO proteins in hematopoietic cells. The function of FoxO proteins to protect cells from oxidative stress by detoxification of ROS is thought to be mediated by transcriptional regulation of the scavenger enzymes SOD2, catalase (CAT), glutathione, and peroxidase-1 (Kops et al., 2002). Recently, studies have shown that FoxO proteins, in particular FoxO3, protect the maintenance of HSCs by regulating oxidative stress (Figure 13) (Miyamoto et al., 2007; Tothova et al., 2007). Although proliferation and differentiation of hematopoietic progenitor cells in Foxo3−/− mice were normal, serial transplantations showed an obvious failure of HSCs to maintain long term repopulation (Miyamoto et al., 2007). Due to suggested functional redundant mechanism for FoxO proteins in the hematopoietic system, mice with simultaneous conditional deletion of Foxo1, Foxo3 and Foxo4 were engineered. A marked decrease in HSCs as well as increased cell cycle and defects in long-term repopulation together with increased levels of apoptosis, which was not detected in mice deficient for a single FoxO member (Miyamoto et al., 2007; Tothova et al., 2007). This demonstrates that functional loss of one member can, at least partially, be compensated by other FoxO members.

Figure 13. FoxO proteins are critical mediators of HSC stress resistance. FoxO activity is regulated by several posttranslational modifications. Stimuli can regulate FoxO proteins differently. Activation of the PI3K/AKT pathway causes inhibition of FoxO, whereas oxidative stress activates FoxO by phosphorylation, monoubiquitination, and through interaction with β-catenin and the SIRT1 deacetylase. This leads to transcription of FoxO target genes involved in cell cycle arrest and oxidative stress resistance. FoxO proteins are thought to be important for the decrease of ROS levels in HSCs and maintenance of long term repopulation ability.
THE ROLE OF FLT3 IN NORMAL AND MALIGNANT HSCs AND PROGENITORS

As mentioned earlier, there are a number of cytokines important for stem cells and progenitors in the hematopoietic system for regulating survival, proliferation, differentiation, and self-renewal. While the knowledge which extrinsic factors are necessary for controlling self-renewal are fairly limited, much has been learned regarding cytokines important for survival and proliferation of HSCs. One cytokine with well-known capabilities to mediate survival and proliferation of HSCs is FL (Veiby et al., 1996). Although there are other cytokines important for survival-mediated pathways in HSCs, the focus in this thesis will be FL and its receptor FLT3.

Tyrosine kinase receptors
The majority of growth factors bind to receptors with intrinsic tyrosine kinase activity. Ligand binding causes the receptors to assemble into receptor dimers enabling cross-phosphorylation of the cytoplasmic part of the receptors. This initiates intracellular signaling cascades involved in controlling cellular processes such as growth, differentiation, migration, chemotaxis, and angiogenesis. There are 58 different human receptor tyrosine kinases (RTK); TIE2, FGFR, IGF, FLT3 and c-KIT just to name a few, which all have the ability to self-interact as stable dimers within the membrane (Finger 2009). FLT3 belongs to the subset of class III RTK family, which also includes FMS and the platelet-derived growth factor receptor (PDGFR). The RTK class III family is characterized by an extracellular domain compromised of five immunoglobulin-like domains, a transmembrane domain, a juxtamembrane (JM) domain and a cytoplasmic tyrosine kinase domain that are split into two motifs (reviewed in (Gilliland and Griffin, 2002)).

The FLT3 receptor
The murine Flt3 receptor was cloned independently by two groups in 1991. One group applied a hybridization technique using a probe from the FMS receptor to clone the novel receptor which was named FMS-like tyrosine kinase 3 (Flt3) (Rosnet et al., 1991). The other group cloned Flt3 from the fetal liver and named the receptor fetal liver kinase 2 (FLK-2) (Matthews et al., 1991). Today both names are used but the term FLT3 will be used hereafter in this text. Shortly after, the human FLT3 gene, also called the stem cell tyrosine kinase 1 (STK1), was cloned (Rosnet et al., 1993; Small et al., 1994), showing 85% similarity in the amino acid sequence with the murine Flt3. FLT3 ligand (FL), cloned two years later (Lyman et al., 1993), can activate
the tyrosine kinase receptor both in a soluble homodimeric form as well as a cell surface bound ligand. FLT3 is mainly expressed on human HSCs and other immature hematopoietic cells such as early lymphoid and myeloid progenitors, but not on more committed cells (Rasko et al., 1995; Rosnet et al., 1996). Bone marrow transplantations with HSCs lacking FLT3 has shown a reduction in both T cells and myeloid cells, whereas targeted disruption of FLT3 results in a decrease of primitive B cell progenitors, natural killer (NK) cells, and dendritic cells (reviewed in (Gilliland and Griffin, 2002; Small, 2006). Together this shows that FLT3 is essential for a multi-lineage differentiation. While the FLT3 expression is restricted to primitive hematopoietic stem and progenitor cells, FL is ubiquitously expressed on several cell types, including hematopoietic B-, T-, and myeloid cells, fibroblasts in the bone marrow microenvironment and also by non-hematopoietic tissues, including prostate, ovary, testis, heart, and placenta (Brasel et al., 1995; Lisovsky et al., 1996; Stirewalt and Radich, 2003). FLT3 signaling is important for the survival and proliferation in HSCs and progenitor cells. FL is by itself not a strong growth factor, but in synergizes with other cytokines to stimulate proliferation ((Gilliland and Griffin, 2002)).

**Stimulation of the FLT3 receptor**

Stimulation with FL results in receptor homodimerization and the activation of intracellular pathways involved in survival and proliferation. Inactive FLT3 receptors are found as monomeric proteins in the plasma membrane. Due to steric inhibition, the inactive state is unable to form receptor dimers. The JM domain stabilizes the inactive kinase conformation through an autoinhibitory mechanism. The JM domain is divided in three distinct topological components: the JM binding motif (JM-B), the JM-switch motif (JM-S) and the zipper segment (JM-Z). The JM-B stabilizes a closed conformation and buries the activation loop in the cleft between the N and C lobes of the FLT3 kinase, thereby blocking access to peptide substrate and ATP-binding sites. The JM-S is positioned next to the C-lobe, which enables the binding of the JM-B. The role of the JM-Z is simply to correctly align and maintain the JM-S in the right position during the inactive state of the kinase protein (Griffith et al., 2004). Activation of the FLT3 by FL leads to rapid conformational changes promoting dimerization of the receptor. This leads to transphosphorylation of specific tyrosine residues in the JM domain which allows it to move away from the active center of the kinase and enable the autophosphorylation of Tyr842 in the activation loop, which is the final step for activation of the kinase (Choudhary et al., 2005).
Mutations in the FLT3 receptor

In addition to the normal expression of FLT3 on primitive immature hematopoietic cells, FLT3 is also expressed in a wide range of hematopoietic malignancies, including B-lineage acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML). High expression of FLT3 has been reported on 70-100% of the cases of AML (Birg et al., 1992; Carow et al., 1996; Nakao et al., 1996; Stacchini et al., 1996). This implicates a significant role of FLT3 in leukemic malignancies. An aberrant form of the FLT3 has been found in about one third of the patients with AML. Two types of mutations leading to constitutively active receptor signaling have been found; internal tandem duplications (ITDs) in the JM domain and point mutations in the activation loop in the tyrosine kinase domain.

The ITD mutation was first reported by Nakao and colleagues in 1996 (Nakao et al., 1996). They investigated FLT3 expression in 30 patients with AML and in 50 patients with ALL and found 73% and 78% expression, in the respective groups. Interestingly, 5 of the AML patients had unexpectedly longer FLT3 transcripts. Sequence analyses revealed that this was caused by tandem repeats of certain regions in the JM domain, which were all in-frame enabling formation of non-truncated proteins. The length of the ITD varies from 3 to more than 400 base pairs (Stirewalt and Radich, 2003). The FLT3-ITD mutation, the second most common mutation in AML after mutations in the nucleophosmin (NPM1) gene (Falini et al., 2005), has been associated with increased relapse risks and poor overall survival rates (Kottaridis et al., 2001; Thiede et al., 2002). FLT3-ITD expression causes factor-independent growth and resistance to radio-induced apoptosis in factor-dependent cells lines (Mizuki et al., 2000). Mice injected with these cells developed leukemia-like syndromes. The frequency of FLT3-ITD mutation in adult AML ranges between 23-35%, whereas the incidence of FLT3-ITD in pediatric AML is strikingly lower ranging from 5-16%. However, pediatric AML with FLT3-ITD is associated with an inferior prognosis compared to adult AML with ITD (reviewed in (Parcells et al., 2006)). This implicates that age-related mechanisms are involved in the pathogenesis of AML. In addition, the incidence of AML increases with age (Appelbaum et al., 2006). The aging marker p16 increases in normal human CD34+ cells, which is suggested to be important in order to induce apoptosis to protect against accumulation of DNA damages that might lead to cancer. A low expression of p16 in older AML patients have been reported to be associated with decreased overall survival rates (de Jonge et al., 2009). Surprisingly, this pattern of p16 as a prognostic marker could only be seen in AML without FLT3-ITD.
The second most common mutation in the FLT3 receptor involved in AML is the point mutation of aspartate 835 to tyrosine in the activation loop in the tyrosine kinase domain (TKD) (Abu-Duhier et al., 2001). It is suggested that Asp835 provides a crucial stabilization of the closed loop configuration although this has been questioned (Griffith et al., 2004). This could explain how the TKD mutation causes the loop to transform into an open configuration allowing access to the kinase. Like the FLT3-ITD mutation, the FLT3-TKD mutation is associated with the constitutively activation of the receptor (Yamamoto et al., 2001). The prognostic impact of FLT3-TKD mutations in AML has been uncertain. A recent study reported that the 10-year overall survival rates for AML patients with FLT3-TKD was 51% compared to 23% for patients with ITD mutations, indicating that the former has a favorable prognostic outcome (Mead et al., 2007). The prevalence of AML patients with FLT3-TKD has been reported to be 7% (Thiede et al., 2002).

**FLT3 signaling in normal and mutated receptor**

The downstream signaling of FLT3 is today not fully elucidated. Several studies have demonstrated the involvement of different survival pathways in FLT3 expressing cells, both in primary cells and cell lines. Restriction of the interpretation has to be considering due to the fact that FLT3 signaling between cell types and organisms might differ. Although both FLT3-TKD and FLT3-ITD, like normal FLT3 stimulated with FL, leads to activation of the FLT3 receptor, the downstream signaling may differ (Choudhary et al., 2005). The ITD insertion occurs mainly in the JM-Z which offsets the position of JM-S and thereby disrupts the insertion of JM-B into its autoinhibitory binding site, which results in a configuration of the receptor that is continuously active (Griffith et al., 2004). Both normal and mutated FLT3 activate several downstream signaling pathways, including STAT5, PI3K/AKT and RAS/MAPK pathways (Figure 14) (Mizuki et al., 2000; Zhang and Broxmeyer, 1999; Zhang et al., 2000).

*The RAS/MAPK pathway*

Stimulation of FLT3 activates the RAS/MAPK pathway. FL induced phosphorylation of son of sevenless (SOS), which associates with the adaptor protein GRB2, which in turn, binds to the phosphorylated tyrosine residues in the activated receptor via its SH2 domain. This binding to the phosphorylated receptor causes translocation of SOS to membrane-bound RAS, which becomes activated through GTP-binding. RAS-GTP then activates RAF-1, which in turn, activates the two MAPK proteins, ERK-1 and ERK-2 (Figure 14). ERK-1 and ERK-2 are kinases primarily involved in regulation of cell growth and survival, whereas two other kinases, p38 and JNK are involved in
apoptosis caused by stress (Wada and Penninger, 2004). Activation of p38 MAPK is associated with dysfunction of hematopoietic stem cells (Ito et al., 2006). Similar to FLT3-ITD, the FLT3-TKD mutation leads to the constitutively activation of ERK1 and ERK2 (Mizuki et al., 2000).

The PI3K/AKT pathway
Similar to Ras-MAPK signaling, the activation of the PI3K and its downstream target protein kinase B (PKB or AKT) is important for cell proliferation, growth, survival, and metabolism in many cell types (reviewed in (Vanhaesebroeck and Alessi, 2000)). Type IA PI3K is a heterodimer consisting of a catalytic subunit (p110α) and a regulatory subunit (p85α). PI3K is a lipid kinase that catalyzes the phosphorylation of inositol phospholipids at the 3′position generating lipids called phosphatidylinositol-3,4-bisphosphate, PI(3,4)P₂, or phosphatidylinositol-3,4,5-triphosphate, PI(3,4,5)P₃. The lipids products, PI(3,4)P₂ and PI(3,4,5)P₃, recruit phosphatidylinositol-dependent kinase 1 and the serine/threonine kinase AKT to the plasma membrane, leading to phosphorylation and activation of AKT. The activation of murine Flt3 associates directly with p85, whereas human FLT3 lacks a binding site for p85. However, adaptor proteins, such as GRB2 binding protein (GAB) -1, GAB-2 and SHP-2, become phosphorylated and activated upon FL stimulation and associate to p85 (Zhang and Broxmeyer, 1999). Activation of AKT by FL stimulation leads to inactivation of the transcription factor FoxO3a through phosphorylation (Jönsson et al., 2004), which enables docking of the transporter protein 14-3-3 and nuclear export (Brunet et al., 2002). This prevents transcription of Bim and p27 promoting survival and cell cycle. Another downstream target of AKT is the pro-apoptotic protein Bad. AKT phosphorylates Bad, which results in sequestration and blocked apoptosis (Figure 14). In AML blasts and in cell lines with FLT3-ITD mutations increased activation of AKT has been observed, which negatively regulates FoxO3a and thereby promotes cell survival and proliferation (Brandts et al., 2005; Scheijen et al., 2004).

The JAK/STAT pathway
The Janus-activated kinases (JAKs) are cytoplasmic tyrosine kinases that mediate survival and proliferation signaling via activation of signal transducer and activator of transcription (STAT) proteins. Comparative analyses of normal FLT3 with FLT3-TKD and FLT3-ITD signaling has revealed differences in the activation of STAT5 (Choudhary et al., 2005; Mizuki et al., 2000). Preferential activation of STAT5 by FLT3-ITD, weak activation of FLT3-TKD and absence of STAT5-signaling by ligand-activated normal FLT3 have been described. Although the majority of STAT5 activation in AML is associated with autophosphorylation of FLT3 (Knapper et al., 2006), some cases of constitutively STAT5 phosphorylation have shown no activation of FLT3 (Birkenkamp
et al., 2001). This suggests that there might be a FLT3-independent mechanism for the activation of STAT5 in AML. An activation of the STAT5 leads to expression of its target genes PIM1 and PIM2. Increased expression of both PIM1 and PIM2 has been observed in AML patients with FLT3-ITD mutation (Kim et al., 2005; Mizuki et al., 2003). This upregulation of PIM is thought to contribute to proliferation and survival following FLT3-signaling.

Other mutations implicated in AML
FLT3 mutations block myeloid differentiation and ligand-dependent proliferation. However, this is not sufficient to induce acute leukemia. It has been suggested that two classes of mutations are required to develop AML. Another mutation often found expressed together with FLT3-ITD is the t(15:17). This is a balanced reciprocal translocation of the promyelocytic leukemia (PML) gene at chromosome 15 and the retinoic acid receptor-α (RARA) gene at chromosome 17 causing impaired differentiation of hematopoietic cells. The t(8;21) gene arrangement, resulting in

Figure 14. The FLT3 signaling pathways. The figure shows signaling cascades that are thought to be triggered through FLT3 activation. Binding of FL causes activation of the PI3K/AKT pathway involved in proliferation and survival. Association of GRB2 with the activated receptor results in activation of RAS, which in turn activates the RAF/MEK/ERK cascade. Another mediator of survival and proliferation activated by FLT3 is the STAT5 protein, although this activation likely is restricted to mutated FLT3 signaling (Stirewalt and Radich, 2003).
expression of the fusion gene *AML1/ETO*, is also listed in this class of mutations that frequently occur in conjugation with FLT3 mutations. This cooperation between mutations leading to inhibited differentiation with mutations associated with proliferation and survival is thought to be sufficient to cause leukemic malignancy. This “two-hit” model of leukemia proposes that only one mutation in each class is necessary for AML to develop. In support of this, mutation in the *RAS* gene, leading to increased proliferation and survival, never or very seldom coexist with FLT3 mutations (Gilliland and Griffin, 2002; Stirewalt and Radich, 2003). Ras mutations occur in 15-20% of AML patients, but have less prognostic significance compared to mutations in FLT3 (reviewed in (Levis and Small, 2005)). Another mutations associated with poor prognosis are c-KIT mutations, although the frequency is relatively rare (1-5%) in AML cases (Gilliland and Griffin, 2002).

Recently, a mutation in the *NPM1* gene was detected and described in 35% of AML cases (Falini et al., 2005). A high frequency of co-expression of NPM1 mutations and FLT3-ITD mutations in AML has been reported. Interestingly, NPM1 mutation alone is associated with a significantly better prognosis than absence of this mutation (Thiede et al., 2006). NPM1 protects cells from stress-induced cell death by inhibition of p53 (Li et al., 2005). Mutation and the loss of functional NPM1 are suggested to be explained by increased sensitivity to chemotherapy.
TREATMENT OF AML

AML is an aggressive malignancy with short survival rates if not treated. AML is thought to arise in leukemic stem cells (LSCs) and is characterized by aberrant proliferation of myeloid progenitor cells coupled to a partial block in differentiation. This in turn causes bleeding, anemia and immune suppression due to the decreased number of mature functional leukocytes, erythrocytes, and thrombocytes. The overall incidence is 3.4 cases per 100,000 inhabitants, but increases significantly with age (Tallman et al., 2005). Poor prognosis is often associated to AML due to therapy-induced mortality or resistance to chemotherapy (reviewed in (Weisberg et al., 2009)). As described above, deregulation of tyrosine kinases in particular FLT3, has for the last decade been implicated in the molecular pathology of AML.

The current treatment of AML is chemotherapy and BMT. Growing insight in specific pathogenic molecular mechanism has led to the development of novel therapeutic drugs, such as FLT3 inhibitors, inhibitors of bone marrow niche targets and BH3 mimetic drugs.

FLT3 inhibitors
The high frequency of FLT3 mutations associated with a poor prognosis has made FLT3 an interesting molecular target for treatment of AML. To date, many tyrosine kinase inhibitors have been developed where some are in clinical trials. Although they all interact with and inhibit FLT3 signaling, no FLT3 kinase-specific inhibitor has been developed. FLT3-inhibitors often recognize other RTKs such as PDGFR, VEGFR, and c-KIT. After the success of the tyrosine inhibitor imatinib (Glivec; Novartis) used in treatment of chronic myeloid leukemia (CML) by inhibiting the kinase activity in the fusion protein BCR/ABL, there has been a massive struggle to find an inhibitor as effective for treatment of AML. It must however be noticed that BCR/ABL might be the only molecular abnormality leading to the development of CML in contrast to AML, which is a multi-mutational leukemia, where constitutively active FLT3 is only one of many genetic alterations. The molecular mechanism for these small molecule kinase inhibitors is to mimic the adenosine structure and compete with ATP for binding to the ATP-binding pocket in the kinase domain. The first report that FLT3 is a potential therapeutic target for AML used the FLT3 inhibitor AG1295. AG1295 inhibits FLT3-ITD and wild type FLT3 and noticeably reduced the number of AML blasts in vitro (Levis et al., 2001). Because of its low bioavailability and low potency, AG1295
cannot be used clinically but is used in research to study cellular and molecular mechanisms influenced by FLT3-inhibition. Since then, several FLT3 inhibitors, such as SU11248, CEP701, PKC412, and MLN-518, have been discovered and investigated. PKC412 was originally developed as an inhibitor against protein kinase C, but was shown to be a more powerful inhibitor of FLT3 kinase activity. Through its inhibition of FLT3-ITD and FLT3-TKD it caused cell cycle arrest and apoptosis in leukemic cells (Weisberg et al., 2002). In addition, administration of PKC412 to mice with FLT3-ITD-induced leukemia showed prolonged survival. In a phase 2 study, PKC412 reduced peripheral blast count to 50% or less in 14 of 20 patients, whereas bone marrow blast counts were reduced to 50% in only 6 patients (Stone et al., 2005). This low response in reduction of bone marrow blasts is generally described for all FLT3 inhibitors used in clinical trials so far. In addition, other clinical problems, such as maintaining plasma concentrations of the drug and avoiding drug toxicity, need to be resolved before tyrosine inhibitors can be used in treatment of AML. The acquisition of additional mutations in the kinase domain inhibiting drug binding, increased expression of FLT3, and the activation of compensatory signaling pathways for survival are potential mechanisms by which the drug response can be reduced. The rapid development of resistance to tyrosine inhibitors as sole therapeutic agents has lead to clinical trials of FLT3 inhibitors in combination with conventional cytotoxic therapy. Hopefully, this would target the pathologic mechanism of AML while avoiding some toxicity from chemotherapy, as well as resistance to kinase inhibitors (reviewed in (Knapper, 2007)).

Therapies that targets the leukemic stem cell niche
The high incidence of relapse in AML is thought to be explained by leukemic stem cells (LSCs) escaping conventional chemotherapy. LSCs have self-renewal capacities and the ability to give rise to more mature hematopoietic cells (Somervaille and Cleary, 2006). A high percentage of leukemic stem cells at the time of diagnosis often reflect a number of remaining chemotherapy-resistant cells associated with poor survival (van Rhenen et al., 2005). If FLT3-ITD mutations occur in leukemic stem cells remains to be investigated. Data regarding the FLT3-ITD expression on AML blasts at the time for diagnosis and relapse provide useful information for this question. In addition, the mutant-to-wild type ratio might provide meaningful information to when the FLT3-ITD mutation occurs. Some studies have shown that patients diagnosed with FLT3-ITD all showed FLT3-ITD expressing blast cells at relapse, whereas others have shown the mutations were lost at relapse (reviewed in (Levis and Small, 2003)). A loss of FLT3-ITD indicates that this is a “late mutation” not
present in cancer stem cells. If instead, the mutation occurs early in stem cells with self-renewal capacity, the mutant-to-wild type ratio would be 1 or greater.

Leukemic cells can escape chemotherapy by “hiding” in special niches in the bone marrow referred to as “the leukemic stem cell niche”, which provides them with survival signals activating expression of anti-apoptotic proteins (Konopleva et al., 2002). Bone marrow stromal cells protect leukemic cells from chemotherapy-induced death (Konopleva et al., 2002; Panayiotidis et al., 1996; Tabe et al., 2004). CXCR4 has been shown to be important for migration and homing of AML cells to the bone marrow niche (Tavor et al., 2004). Interestingly, high expression of CXCR4 in AML cells with FLT-ITD has been reported to be associated with poor prognosis and an increased risk for relapse (Rombouts et al., 2004), indicating that FLT3-ITD expressing AML cells are favored by signals from the leukemic stem cell niche. One explanation to why peripheral blood blasts are more responsive to treatment than bone marrow blast might be that the bone marrow protects leukemic cells from toxicity. Therefore, disruption of protective niche-signals could be used to increasing responses to current therapies and reducing resistance to conventional chemotherapy and tyrosine kinase inhibitors. However, consideration is required before niche targeted therapies can be used.

BH3 mimicking drugs
Aberrant overexpression of Bcl-2 proteins in AML patients is associated with increased resistance to chemotherapy and FLT3 inhibitors. A deregulation of apoptosis caused by increased expression of anti-apoptotic proteins is common in the majority of malignancies which make them ideal targets for developing anti-cancer drugs. Overexpression of anti-apoptotic members inhibits Bax and Bak from forming homodimers in the mitochondria membrane and causing release of cytochrome c. Tumor cells with overexpression of Bcl-2 are therefore less sensitive to drug-induced apoptosis. In a normal cell, the Bcl-2 family proteins are controlled by BH3 only pro-apoptotic members. The docking of the BH3 domain to Bcl-2 annuls their protective effects against increased mitochondrial permeability. An attractive approach has been to mimic the BH3 domains and thereby reducing the anti-apoptotic ability for Bcl-2 protein family members. Several BH3 mimic peptides have been described where some are in clinical trials (reviewed in (Zhang et al., 2007)). The most promising Bcl-2/Bcl-X\textsubscript{i} inhibitor in the treatment of cancer is ABT-737. It was recently reported that treatment of AML cells, expressing high levels of Bcl-2, with ABT-737 neutralized achieved resistance to FLT3 inhibitors (Kohl et al., 2007; Konopleva et al.,
ABT-737 induced apoptosis in primary AML blast cells but showed no effect in normal lymphocytes. However, AML cells with a high expression of Mcl-1 showed significantly lower sensitivity to ABT-737. Together, this demonstrates that normal hematopoietic cells are tolerant to treatment with BH3 mimetics which might lower the dose of conventional chemotherapy required for response in combination with specific BH3 mimetics. Furthermore, the expression pattern of different Bcl-2 family proteins might indicate which treatment is the most proper to choose. For instance, treatment with ABT-737 should only be used in malignancies with low expressions of Mcl-1. Hopefully, new BH3 mimetics with specificity for Mcl-1 will be developed in the near future.
AIMS OF THE PRESENT INVESTIGATION

The overall goal of the present study was to gain an increased understanding in how maintenance is regulated in normal and leukemic hematopoietic stem and progenitor cells. Towards this the main objects were;

- To investigate the role hypoxia and HIF-1α play in survival and self-renewal of hematopoietic stem and progenitor cells.
- To study pro- and apoptotic signaling pathways activated by the tyrosine kinase receptor FLT3 in primitive AML cells.

The specific aims in the papers included in the thesis were the following;

Paper I: To evaluate the affect hypoxia has on survival and expansion of in vitro culturing of primitive hematopoietic progenitor cells from mouse bone marrow.

Paper II: To investigate the role of hypoxia in HSCs maintenance and expansion in vitro and in vivo, focusing on the importance of HIF-1α-dependent regulation of proliferation and cell cycle.

Paper III: To study the involvement of hypoxia and HIF-1α in HSCs exposed to oxidative stress.

Paper IV: To determine which apoptotic pathways are involved in FLT3 inhibitor-induced cell death of AML cells.
Detailed description of methods used is written in the material and methods section in each paper. Still, there are some issues that need to be clarified and explained more thoroughly.

Isolation of HSCs using FACS based cell sorting
Bone marrow is normally taken from femur and tibia from sacrificed mice. After crushing the bones in a mortar a single cell suspension is prepared by washing and filtering. Before sorting of HSCs using fluorescent activating cell sorting (FACS), primitive multipotent cells are enriched by depletion of mature lineage positive cells (express lineage specific markers e.g. Ter119 for erythrocytes) or concentrated for stem- and progenitor cells (express CD117/KIT ligand). Both ways generate a population of cells containing a heterogeneous population of stem- and progenitor cells. In brief, bone marrow cells are labeled with immunomagnetic beads conjugated with either antibodies specific for lineage surface antigens (lineage depletion) or antibodies specific for CD117 (CD117 positive selection). Labeled cells are then passed through a magnetic field, which enable separation of labeled and unlabeled cells. By using this pre-separation method, the time needed for sorting is reduced with a factor of 50 to 100 times, which is of advantage for the operator as for the viability of the cells.

The two most frequent populations used in this thesis are the lineage^-Sca-1^-c-Kit^- (LSK) cells and the CD34^-Flt3^-LSK cells, containing MPP, ST-HSCs, and LT-HSCs or LT-HSCs, respectively. The enriched stem- and progenitor cell sample is labeled with fluorochromes-conjugated antibodies specific for lineage markers, Sca-1, c-Kit, Flt3 and CD34 and further sorted on a FACS cell sorter. The cell sorter used in this study is the FACS Aria from BD Bioscience. It is a high-speed cell sorter (approximately 20 000 cells/second) connected to a FACS analyzing unit. The sample runs from the sample tube to the sample injection chamber to the cuvette flow cell and finally to the collection device. Sheet fluid is pumped from the fluid reservoirs into the cuvette flow cell where hydrodynamic forces focusing the sample into a single-file stream through the cuvette. Within the cuvette single cells are focused in the interrogation point by the laser beam and enter thereafter the nozzle (Figure 15).
Fluorochrome-conjugated antibodies are excited by different lasers. The FACSARia used here has four lasers; 488nm (blue), 633nm (red), 407nm (violet), and 355nm (ultra violet). The lasers can detect 2-6 different fluorochromes, which give the possibility to distinguish 14 colors in one sample at the same time. When the cells pass through the laser beam the fluorochromes absorb photons and become excited. Returning to their ground state results in release of energy as emitted light also referred to as fluorescence, which is transferred to specific detectors. The stream containing the cells is accelerated in the nozzle where the stream is broken into droplets. When a cell is detected that meets the criteria for the sorting, an electric charge is applied to the droplet at the time it leaves the nozzle tip. The charged droplets passes between two charged deflection plates and is attracted or repulsed towards the plates depending on its charge. This causes the droplet containing the cell to move to the right or the left collection tube. Uncharged droplets pass straight down in the waste container. By calibrating the instrument before use and select specific criteria for sorting, high purity of sorted population can be achieved.

RNA interference
In year 2006 Andrew Fire and Craig C. Mello were rewarded with the Nobel Prize in medicine for their work on RNA interference in *C. elegans*. They showed that anti-sense RNA is important for regulation of gene expression (Fire et al., 1991). The
discovery of RNA interference (RNAi) is one of the most important breakthroughs in modern time both for basic and for applied research. Exploring the mechanism for the RNAi machinery has resulted in a valuable research tool that can be utilized in therapeutic applications. RNAi is triggered by double-stranded (ds) short RNA exogenously transferred as small interfering RNAs (siRNAs) or endogenously produced as microRNAs (miRNAs). These short dsRNAs are around 21-23 nucleotides and is today commonly used in research to induce sequence-specific gene silencing. Exogenously supplied siRNAs induce gene-silencing by triggering RNA cleavage after binding to their target mRNA. The endogenous miRNAs are important regulators of genes involved in development, proliferation, hematopoiesis and apoptosis (reviewed in (Rana, 2007)). One important difference between miRNAs and siRNAs is the way of inhibiting the production of functional mRNA. Unlike siRNAs, miRNAs do not usually cause cleavage of RNA, but instead repress translation of mRNA. However, synthetic siRNAs can suppress mRNA translation by binding partially complementary to target sequences and simulate functions similar to endogenous miRNAs (Doench et al., 2003; Zeng et al., 2003). This less stringent mechanism to control RNA silencing can explain nonspecific effects sometimes observed in experiments using siRNAs.

RNA interference using siRNAs is transient and to be able to achieve a stable gene-silencing DNA vectors expressing short hairpin (sh) RNAs have been developed. Long dsRNAs, hairpin RNAs and siRNAs can all initiate the RNAi machinery. In the cytoplasm, both long dsRNAs and hairpin RNAs are first processed by the RNase III enzyme Dicer to shorter siRNAs with an overhang at the 3’ end and a phosphate group at the 5’ end. The anti-sense is known as the guide strand because it is responsible for complementary binding to the target RNA sequence. The siRNA oligo assemble into a complex with proteins called the RNA-induced silencing complex (RISC). This results in unwinding of the helix and the sense strand is rapidly degraded. The single stranded RNA-RISC complex then recognizes and binds the target RNA resulting in cleavage between nucleotide 10 and 11 upstream of the 5’ end of the guide strand (Figure 16) (reviewed in (Rana, 2007)).
In the present investigation, both siRNA (paper IV) and shRNA (paper II and III) are used as molecular tools to silencing specific genes. The choice to use shRNA was taken considering several aspects. First, a sustained long-term silencing of our target gene was desired. Second, our target cells are non-dividing primary stem cells that require lentiviral delivery of RNAi oligos to introduce gene-silencing. Third, using lentiviral vectors containing shRNA oligos encoding the selection marker enhanced green fluorescent protein (EGFP) enables sorting of cells expressing shRNA shortly after introduction of the viral vectors. The lentiviral vectors used in this study carry the human U6 polymerase III promoter for expression of the GFP reporter and the shRNA oligos. In addition to the lentiviral shRNA vector, vectors containing viral encoding gag, pol, and env, which are structural and enzymatic genes necessary for formation of viral particles and replication.

Real time PCR
To measure the expression of a gene of interest, quantification of transcribed mRNA molecules can be used. One common method to quantitate mRNA expression is real time polymerase chain reaction (PCR). Real time PCR is a sensitive method that
requires minimal amounts of starting material which makes it useful for determine
difference in gene expression when only a few number of primary cells are available.
Before running a real time PCR, mRNA is converted by reverse transcriptase to
complementary DNA (cDNA). The initial step for real time PCR is denaturation of the
two DNA strands which enables amplification of the target gene sequence using
specific primers. The PCR product accumulates for every cycle in an exponential way
and in contrast to traditional PCR, the amplification product is measured before the
final plateau phase, which increases the sensitivity and precision for quantification.
Detection of the PCR product in real time PCR can be monitored in two ways, non-
specific detection using DNA binding dyes or specific detection using target specific
probes. The latter includes a fluorescent labeled probe for detection, whereas the
former, used in this study, utilize double-strand DNA-specific dyes. The most widely
used fluorescent dye that bind double-stranded DNA is SYBR® green. Unbound SYBR®
green exhibits a very weak fluorescent signal, which increases extensively upon
binding to the minor groove in the DNA helix. Due to the fact that binding of SYBR®
green to DNA is non-specific it is important to optimize the settings to generate only
one gene product. Evaluation of the melting curve that monitors the kinetics of the
melting temperature for the DNA helix which depends on the products length and
the nucleotides composition is important to make sure the true target gene is
detected and not unspecific gene products or primer-dimer artifacts. To normalize
the total amount of RNA between the different samples, detection of an endogenous
constitutively expressed housekeeping gene is used. Mouse β-actin and human β-
A glucuronidase (GusB) are used in paper II and III, and paper IV, respectively. The
increase in SYBR® green fluorescence will follow the amplification of the gene
product. Measurement of the cycle number is where the increase of fluorescence
(and therefore amplification) is exponential. This measuring point is called C_T (cycle
threshold) value. The C_T value is inversely proportional to the amount of DNA
product, meaning the lower C_T value the higher amount of starting DNA copies in the
sample. In this way comprising in expression of a target gene between different
samples can be achieved. A common method to analyze the C_T values generated from
different samples is the relative quantitative method 2^ΔΔC_T (Livak and Schmittgen,
2001), which have been used in the present investigation. First, the C_T value for the
target gene (C_{T,X}) is normalized to the value for the endogenous housekeeping gene
(C_{T,R}), also referred to as reference gene. The equation that describes this is;

\[ C_{T,X} - C_{T,R} = \Delta C_T \]

The next step is to relate the ΔC_T for the sample (ΔC_{T,q}) to the untreated sample
(ΔC_{T,cb}), referred to as calibrator, given by;

\[ C_{T,X} - C_{T,R} = \Delta C_T \]
∆C<sub>T,q</sub> - ∆C<sub>T,cb</sub> = ∆∆C<sub>T</sub>

A simplified calculation of the fold change of target gene normalized to the endogenous reference gene and calibrated to the untreated sample is given by;

Fold change of target = 2<sup>-∆∆CT</sup>

Calibrators used in this study are sample cultured in normoxia (paper II and III) and sample treated with 0 nM PKC412 (paper IV).
RESULTS AND DISCUSSION OF THE PAPERS IN THIS THESIS

Hypoxia expands quiescent primitive progenitor *in vitro* and maintains functional HSCs *in vivo* (Paper I and II)

It has been suggested by several investigators that HSCs are located in a microenvironment in the bone marrow low in oxygen (Ceradini et al., 2004; Chow et al., 2001; Kubota et al., 2008; Levesque et al., 2007; Parmar et al., 2007). To simulate the oxygen conditions in the HSC niche, we cultured primary bone marrow cells in low oxygen levels (1% O₂). The key findings in paper I were an expansion of primitive hematopoietic multipotential colony forming cells and maintenance of a primitive stem cell phenotype in hypoxic *in vitro* cultures compared to cultures in ambient oxygen levels. The functional *in vitro* colony assays used to detect primitive progenitors were pre-CFCₚₘ₃, HPP-CFC, and CAFC. These colony forming cells resemble the true HSC with long term potential (Cheng et al., 2000b; Iscove and Yan, 1990), which make them suitable *in vitro* systems to detect stem cell activity. After hypoxic cultivation we could detect a preserved LSK phenotype, whereas more committed progenitors generating CFU-GM were decreased in number. Together, these observations indicate that hypoxia promotes expansion of cells with multipotent ability and inhibits differentiation of hematopoietic cells, which is in agreement with two previous studies using unfractionated murine bone marrow cells (Cipolleschi et al., 1993; Ivanovic et al., 2000). The significance of hypoxia in regulating differentiation of stem- and progenitor cells has been stated earlier by several groups and in various tissues, including neural crest stem cells and adipocytes (Morrison et al., 2000; Yun et al., 2002). Hypoxia-induced maintenance of an undifferentiated state is dependent on Notch-signaling (Gustafsson et al., 2005). A model where HIF-1α interacts with and stabilizes the cleaved Notch intracellular domain, promoting transcription of Notch downstream targets important for stem cell maintenance, has been proposed (Gustafsson et al., 2005). Similar to our observation of HSCs cultured in hypoxia, constitutively active Notch-signaling in HSCs generates a primitive phenotype and a morphology resembling immature blasts (Varnum-Finney et al., 2000). Together with our results from paper I it is possible that maintenance of HSCs in response to hypoxia could be elicited by activation of the Notch-pathway.

This study was followed by paper II where functional HSCs were detected using a competitive repopulation *in vivo* assay. In this study, a purer population of stem cells
RESULTS AND DISCUSSION

was isolated allowing a more direct evaluation of the stem cell response to hypoxia. In a heterogeneous cell population, the influence of non-stem cells on HSCs cannot be ignored. In paper II we focused on the role HIF-1α has in hypoxic culture of HSCs. One of the major findings from paper II was that hypoxia maintained quiescence of HSCs and reduced the proliferation rate which resembles the steady-state of HSCs. Furthermore, similar findings on proliferation and cell cycle arrest were observed by overexpression of HIF-1α in a multipotent progenitor cell line. Interestingly, despite a lower number of cells generated in the hypoxic culture, transplantation of these cells generated equal long-term reconstitution capacity compared to transplanted normoxic cell cultures, containing several fold higher total cell numbers. This indicates that hypoxia can maintain LT-HSCs whereas proliferation of more committed progenitor cells with lost long-term potential is reduced. In contrast, normoxia increased the proliferation of more mature progenitors in addition to decreased preservation of a primitive progenitor morphology and phenotype observed in paper I. An increase in cell cycle can cause an exhaustion of stem cells with long-term engraftment potential. It is therefore possible that the effects of hypoxia in maintaining stem cells capable of long-term engraftment can be explained by a reduced proliferation rate in addition to reduced differentiation. However, it cannot be excluded that hypoxia in vitro could regulate maintenance of functional stem cells by other mechanisms. Several niche molecules important for self-renewal of HSCs are regulated by hypoxia; VEGF, Notch-1, Bcrp1/ABCG2 just to name a few, demonstrating the importance of hypoxia in vivo. It has been shown that slowly cycling HSCs are most abundant in hypoxic regions of the bone marrow (Kubota et al., 2008; Parmar et al., 2007), which goes hand in hand with our observation of HSCs response to hypoxia in culture.

In paper II, we showed that hypoxia increased the expression of the CDK inhibitor p21 in CD34-FLT3-LSK cells, enriched for LT-HSCs. Two other CDK inhibitors, p27 and p57, were not affected by hypoxia in LT-HSCs. To our cultures we add the survival factor Tpo, which is suggested to increase the expression of p57 (Qian et al., 2007). Tpo-induced p57 expression and a predominance of p57 in CD34-FLT3-LSK cells (Yamazaki et al., 2006) might conceal possible increased levels caused by hypoxia. Earlier studies have shown that p21 is selective for regulating proliferation of HSCs, whereas p27 is more important for regulation of cell cycle in progenitors (Cheng et al., 2000a; Cheng et al., 2000b). Today, the interaction between hypoxia, p21, p27, and p57 is not fully understood. It has to be taken into consideration that p27 also can be regulated posttranscriptionally (Hengst and Reed, 1996; Pagano et al., 1995). In most cases mRNA expression is direct related to protein abundance (Greenbaum et al., 2002),
RESULTS AND DISCUSSION

but some proteins are regulated at the protein levels (e.g. HIF-1α through degradation by the ubiquitin-pathway). This might explain the conflicting results from earlier studies in how hypoxia regulates p27. It is known that the regulation of CDK inhibitors is multiple and dependent on the microenvironment and cell type. Our results showed that stable expression of HIF-1α in normoxic conditions could, similar to hypoxia, reduce proliferation and induce cell cycle arrest. Therefore, it is tempting to speculate that the effect hypoxia has on stem cell proliferation is HIF-1α mediated. However, cultivation of primitive progenitors in normoxic conditions in the presence of FG-4497, an oxygen-independent stabilizer of HIF-1α, reduced the initial engraftment potential, whereas the long-term engraftment was unaffected. Interestingly, we observed that HIF-1α induces expression of p27 and p57 in LSK cells, whereas p21 levels were stable. Those findings indicate that HIF-1α mainly decreases proliferation of progenitor cells through increased levels of p27, whereas p21 expression is stable and thereby maintain the number of stem cells with long-term potential. This could explain the decrease in initial engraftment, which is dependent on the activity and the number of progenitor cells, after treatment with the HIF-1α-stabilizer. In agreement with this, primary bone marrow cells with stable expression of constitutively active HIF-1α showed an initially reduction of engraftment potential. However, this decline in engraftment was maintained over time. A possible explanation could be that HIF-1α must be transiently expressed in order to enable proliferation and differentiation to more committed progenitors and further development of the hematopoietic lineages. According to our results, stable expression of HIF-1α would induce cell cycle arrest of all hematopoietic cells tested. Due to the fact that HIF-1α regulates a variety of signaling pathways involved in apoptosis, differentiation, glucose metabolism, in addition to cell cycle regulation, it is likely that overexpression of HIF-1α can cause a disturbance in regulation of other cell fates. Another explanation for the reduced repopulation ability could therefore be HIF-1α inducing cell death through transcriptional induction of the pro-apoptotic gene BNIP3 (reviewed in (Wenger et al., 2005)). However, overexpression of the anti-apoptotic protein Bcl-2 failed to rescue reconstitution of donor cells expressing constitutively active HIF-1α, indicating that the negative effects we see on repopulation cannot be explained by HIF-1α induced apoptosis. A third factor that can affect reconstitution is homing, which has not been investigated in the present study.

To determine how loss of HIF-1α affects HSCs we have used HIF-1α specific shRNA. We were able to knock down endogenous HIF-1α mRNA levels in primary HSCs by 75% in normoxia and 68% in hypoxia. We cannot rule out that remaining HIF-1α
mRNA transcripts can be translated into proteins which can activate HIF-1α target genes. Gene-silencing using shRNA does not induce a total knock-out of a gene, in contrast to genes in cells from knock-out mice. Studying the biological role of HIF-1α in bone marrow hematopoietic cells has been hampered due to the fact that HIF-1α−/− mice die before birth in the uterus (Adelman et al., 1999). A HIF-1α-conditional knockout mouse would be the optimal way to directly study the effects of HIF-1α, but until then, partial silencing with HIF-1α specific shRNAs is a comparatively good substitute.

Hypoxia protects HSCs from oxidative stress (paper III)
One essential role for the hypoxic stem cell niche could be to protect HSCs from exhaustion caused by oxidative stress. To investigate this, we induced oxidative stress in primary HSCs by adding L-buthionine sulfoximine (BSO) that disturbs the synthesis of the important ROS scavenger, glutathione peroxidase. The most important discovery in paper III was the ability of hypoxia to maintain survival and reconstitution capacity of primary HSCs even at high concentration of BSO. In contrast, HSCs exposed to oxidative stress in normal oxygen conditions showed a pronounced increase of cell death. The significance of hypoxia in HSC maintenance is further supported by the finding that HSCs with high self-renewal capacity and low ROS levels are associated with high expressions of niche molecules known to be induced by hypoxia (Jang and Sharkis, 2007).

As demonstrated in paper II, hypoxia decreases cell cycle and favors HSCs in a quiescent state. A slow cell cycle of HSCs is thought to prevent depletion of the stem cell pool (Cheng et al., 2000b; Nygren and Bryder, 2008). Likewise, a low metabolic activity can also spare HSCs from damages caused by ROS generated as by-products from the mitochondrial respiratory chain during aerobic glucose metabolism. Another possible mechanism of how hypoxia could save HSCs from oxidative stress is by lower the accumulation of ROS through HIF-1α-induced PDK1 expression. In normal oxygen conditions, energy is formed during mitochondrial respiration, which is the major source of ROS. However, in hypoxic cells, increased levels of PDK1 increase glycolysis and maintain a sufficient ATP production necessary for survival and growth at the same time as the ROS generation from the mitochondria is attenuated (Semenza, 2007). In line with this, our results showed a pronounced increase in intracellular ROS levels (H$_2$O$_2$) in cells cultured in normoxia, whereas cells cultured in hypoxia after addition of BSO showed continuous low levels of ROS. It has been shown by others that overexpression of PDK1 in cells deficient of HIF-1α, cultured in hypoxia, can rescue cells from cell death and decrease oxidative stress (Kim et al., 2006). Similar to
RESULTS AND DISCUSSION

this, we observed increased cell death when HSCs deficient for HIF-1α or PDK1 were cultured in hypoxia, which indicate that PDK1 as well as other HIF-1α targets are necessary for survival of hypoxic cells. Furthermore, we observed that bone marrow transplantations with HSCs silenced for PDK1 or HIF-1α revealed a significant decrease in engraftment capacity. A likely explanation could be that the transplanted cells with insufficient levels of HIF-1α and its target gene PDK1 die in a hypoxic in vivo microenvironment in the bone marrow. These findings might reflect the presence of a hypoxic stem cell niche.

We observed that hypoxia can depress accumulation of ROS and that HIF-1α and PDK1 are important for survival of cells in hypoxia. With these observations and the theoretical knowledge that PDK1 is important to reduce mitochondrial ROS production, it was therefore surprising that silencing of either HIF-1α or PDK1 did not abolish the resistance to oxidative stress generated by hypoxia. When we challenged the system by increasing the amounts of ROS through disturbance of the scavenger system in attenuating ROS, hypoxia was able to reduce apoptosis despite decreased levels of HIF-1α or PDK1. In this model, it is possible that increase of ROS might be generated in a mitochondrial independent way, which could explain why PDK1 expression is not important for hypoxic mediated resistance to oxidative stress. Noteworthy, mitochondria are not the only major source of ROS, they are also very sensitive to damages caused by ROS. Damages on mitochondrial DNA coding for proteins involved in the respiratory chain might result in cell death due to lack of ATP (reviewed in (Gogvadze et al., 2008)). The protection from oxidative stress-induced cell death we observed in hypoxic treatment might be explained by a decrease in metabolism. HSCs in hypoxia only consume a minimal amount of ATP, and therefore, lack of energy production caused by dysfunctional mitochondria or decrease in glycolysis due to decreased expression of HIF-1α-regulated glycolytic enzymes does not cause lethal effects in hypoxic conditions. Conversely, damaged mitochondrial ATP production caused by ROS would have detrimental effects on survival of normoxic cells with high metabolic activity. This might explain why HSCs survive better in hypoxia when exposed to oxidative stress.

Another possible explanation to hypoxia-mediated rescue of HSCs from ROS-induced cell death might be increased levels of FoxO proteins. As mentioned earlier, FoxO proteins, in particular FoxO3, are important for maintenance of HSCs by counteracting oxidative stress (Miyamoto et al., 2007; Tothova et al., 2007). In our study we observed an increase of FoxO3a followed by increased levels of SOD and CAT in HSCs cultured in hypoxia. The two ROS-scavenger enzymes SOD and CAT are
RESULTS AND DISCUSSION

responsible for dismutation of superoxide (\(O_2^-\)) to hydrogen peroxide (\(H_2O_2\)) and converting \(H_2O_2\) to water and oxygen, respectively. Hypoxia-induction of FoxO expression could therefore be involved in the process by how hypoxia maintains survival of HSCs exposed to oxidative stress. A cross-talk between FoxO3 and HIF-1 during hypoxia has been observed allowing fine-tuning of HIF-1 by a negative feedback mechanism of FoxO on HIF-1 (reviewed in (Hoogeboom and Burgering, 2009)). Furthermore, oxidative stress can also trigger interaction of both FoxO and HIF-1 with β-catenin leading to enhanced transcriptional activity (Essers et al., 2005). In addition to activation of scavenger enzymes, other FoxO3 targets can also induce cell cycle arrest (e.g. \(p27\)), which is important to allow more time for repair (e.g. \(GADD45\)) of ROS-damaged DNA (reviewed in (van der Horst and Burgering, 2007)). However, FoxO3 can also mediate apoptosis through increased levels of \(Bim\) and \(Fas ligand\) (Brunet et al., 1999; Dijkers et al., 2000). How can one protein both protect against cell death and induce apoptosis? One explanation is stimuli-specific post-translational regulation of FoxO proteins. In response to oxidative stress, the deacetylase SIRT1 associates with FoxO3 causing deacetylation of FoxO3 and evokes cell cycle arrest and DNA repair, whereas FoxO3-mediated apoptosis is attenuated (Brunet et al., 2004). In contrast, inhibition of many growth factor receptors, including FLT3, prevents PI3K/AKT mediated phosphorylation and inactivation of FoxO3, which then can translocate to the nucleus and activate transcription of \(Fas ligand\) and \(Bim\), resulting in apoptosis (See next section). In addition to hypoxia-mediated survival benefits of oxidative-stressed cells, we observed maintained reconstitution ability. The mechanism for how hypoxia restores HSC activity remains to be elucidated. However, possible key components might be hypoxia-induced quiescence and increased FoxO activity, strengthened by the fact that both a slow cell cycle and presence of FoxO proteins are closely coupled to maintenance of self-renewal capacity.

The PI3K/FoxO3/Bim pathway is critical in apoptosis induced by inhibition of FLT3 (Paper IV)

As mentioned earlier in the background, resistance to FLT3 inhibitors is a major problem in the treatment of AML. Therefore, it is important to investigate downstream signaling of FLT3 in order to find critical factors that could be possible new targets for anti-leukemic drugs. In paper IV, we report our discovery that FoxO3a and Bim play important roles in mediating apoptosis in AML cells after treatment with the FLT3 inhibitors AG1295 and PKC412. We found that inhibition of PI3K was essential to induce apoptosis of FLT3-ITD expressing leukemic cells through activation
of Bim due to inhibition of AKT and FoxO3a. In addition to Bim (Bcl-2-interacting modulator of cell death), another BH3-only member, Puma (p53 upregulated modulator of apoptosis), was increased following inhibition with tyrosine kinases and was closely associated with cell death. Bim is a well-known target gene for FoxO3a, whereas Puma is a classical p53-regulated mediator in response to genotoxic stress. Recent data has revealed that Puma can also be induced by FoxO3a when the PI3K/AKT pathway is inhibited (You et al., 2006). Although Puma was induced in a similar manner as Bim, we showed, by gene silencing, that Puma was not critical for mediating apoptosis upon tyrosine kinase inhibition. In contrast, FLT3-ITD expressing AML cells, silenced for either Bim or FoxO, were resistant to apoptosis after RTK inhibitor treatment. Both Puma and Bim are important effectors of apoptosis due to the fact that they bind and inhibit all anti-apoptotic Bcl-2 like family members (Chen et al., 2005). Recent evidence confers the importance of Bim in mediating apoptosis in chemotherapy-treated leukemic cells (Kuroda et al., 2006). Bim can be upregulated by other pathways in addition to PI3K/AKT signaling. A recent study has reported that Bim is critical in triggering apoptosis in AML cells after treatment with the RTK inhibitor Sorafenib (Zhang et al., 2008). Sorfenib induced Bim expression by inhibition of the RAF/MEK/ERK pathway. However, our study shows that blocking of MAP kinase activity with a more specific ERK inhibitor only had minor effect on FLT3-induced survival, in contrast to blocking of PI3K activity which resulted in a markedly increase in cell death through increased levels of Bim. This suggests that in AML cells, signaling via PI3K plays a significant role for survival, although signaling differences between patients do occur due to different molecular abnormalities. It is likely that other pathways in addition to PI3K/AKT signaling are involved in FLT3-induced transformation. This was demonstrated recently with microarray analysis of AML samples with FLT3-ITD showing induction of genes involved in multiple signaling pathways including PI3K/AKT, JAK/STAT, and RAS/MAPK, which likely together contribute to cell cycle progression, and decreased apoptosis and differentiation (Kim 2007). Constitutively expression of STAT5 has been associated with FLT3-ITD (Levis 2002; Weisberg 2002; Knapper 2007). This is dependent on phosphorylation of specific tyrosine residues in the juxtamembrane domain of FLT3-ITD, which is not phosphorylated in normal FLT3 (Rocnic 2006). Activation of the PI3K/AKT and RAS/MAPK are not always dependent on FLT3 signaling, which has been demonstrated in AML cells resistant to RTK although FLT3 is inhibited (Piloto et al., 2007). This justifies the development of new drugs targeting effector proteins (e.g. Bcl-2 proteins) in the PI3K/AKT and RAS/MAPK pathways in order to increase the sensitivity to RTK inhibitor treatment. This would result in a broader and more effective treatment with decreased risk of developing drug resistance.
Overexpression of Bcl-2 proteins is common in several leukemic malignancies which might lead to resistance to conventional chemotherapy and FLT3-inhibitors. Together with our results, this implicates that increased activity of Bim-mediating effects could trigger apoptosis in leukemic cells upon treatment with anti-cancer drugs and reduce the risk of developing resistance. In fact, BH3-only mimicking drugs are in clinical trials in combination with RTK inhibitors. Targeting downstream effectors can overcome FLT3 inhibitory-resistance by inhibiting their pathway regardless of the upstream protein activator. However, disturbing the balance between anti- and pro-apoptotic Bcl-2 members can also have negative side effects on survival of normal cells. Therefore, it is necessary to find a treatment that targets downstream signaling effectors of survival at the same time as sensitivity of tumor cells to apoptosis is increased. Using FLT3 inhibitors in a combination with drugs targeting effectors molecules in the survival signaling pathways would allow an effective and tumor specific treatment.
CONCLUSIONS

Maintenance of functional stem cells in the bone marrow is dependent on a balance between survival, proliferation, differentiation, and self-renewal. We show here that hypoxia regulates three of these; survival, proliferation, and differentiation.

Hypoxia maintains the primitive population of stem and progenitor cells and decreases the differentiation of more committed progenitor cells. In addition, hypoxia decreases cell cycle progression in progenitor cells possible through upregulation of the CDK inhibitor p27, which partly is dependent on HIF-1α. Furthermore, HSCs cultured in hypoxia maintain their slow cell cycle better compared to HSCs in normal oxygen levels. This is important to preserve the long-term engraftment capacity, which is coupled to expression of p21. In this study we show that hypoxia increases p21 expression in LT-HSCs and maintains functional HSCs. We suggest that HIF-1 activity declines the expansion of progenitor cells and decreases initial repopulation of committed progenitor cells in vivo.

Based on findings in this thesis, we proclaim that hypoxia protects HSCs from oxidative stress-induced cell death. The mechanism for this is not delineated, but FoxO3 might be involved. HIF-1 and PDK1 do not seem to be involved in the rescue of HSCs from oxidative stress. However, both are important for survival of HSCs in hypoxia. Although PDK1 might be involved in protecting cells from uncontrolled production of ROS from aerobic mitochondrial respiration, our findings implicate that other mechanism for attenuating ROS are induced by hypoxia. In addition, oxidative stress-exposed HSCs cultured in hypoxia maintain their engraftment potential, indicating that hypoxia can protect HSCs from cell death as well as preserve functional active HSCs.

To summarize, the major role of hypoxia in normal HSC biology is to protect them from damages caused by persistent cell cycle as well as endogenous and externally induced ROS accumulation. This enables HSCs to be maintained and functional throughout an individual’s lifetime.

The PI3K/AKT pathway is critical in tyrosine kinase inhibitor-induced cell death of AML cells expressing FLT3-ITD mutations. Inhibition of PI3K causes activation of FoxO3 and its downstream target Bim. Loss of FoxO3 or Bim results in preserved survival and resistance to RTK inhibitor, implicating that FoxO3 and Bim are essential to mediate apoptosis in AML cells with constitutively active FLT3 receptors. This
CONCLUSIONS

findings provide increased knowledge about survival pathways critical in FLT3-ITD signaling, which could have a clinical significance in the design of new effective drugs in the treatment of AML patients.
FUTURE ASPECTS

This thesis adds novel knowledge on the importance of a balance between survival and cell death in normal and malignant hematopoietic stem and progenitor cells. During my work, new questions have been raised just waiting to be answered. For instance, we have found that hypoxia can protect HSCs from cell death caused by oxidative stress, but the underlying molecular mechanisms are still unsolved. However, we observed that hypoxia can induce expression of the transcription factor FoxO3 that lately has caught a lot of attention due to its role in regulating maintenance of HSCs and scavenging ROS (Kops et al., 2002; Miyamoto et al., 2007; Tothova et al., 2007). We speculate that hypoxia might rescue HSCs from cell death by attenuating oxidative stress in a FoxO-dependent way. In addition to scavenging ROS, FoxO3, similar to HIF-1, regulates several cellular processes that include apoptosis, cell cycle arrest, differentiation, and metabolism. In some pathways FoxO3 and HIF-1 synergize, but in others they counteract each other. Just to make things more difficult to interpret, hypoxia can induce apoptosis in some cell types, whereas others appear to be resistance. This could possibly be explained by differences in intrinsic pathways. Maybe one of the roles of FoxO3 in HSCs is to fine-tune the effects mediated by hypoxia and HIF-1. It would be highly interesting to investigate if cross-talk between HIF-1 and FoxO3 exists and if so, is this of importance in stem cells? The regulation of FoxO proteins activity is multiple and complex, and involves transcriptional and post-transcriptional regulation as well as interaction with cofactors. Therefore, to be able to understand if FoxO proteins are important in hypoxic-mediated cell survival of HSCs exposed to oxidative stress a thorough investigation of FoxO proteins activity is necessary. Investigating the effect gene silencing of FoxO3 might have on hypoxic mediated survival would provide valuable information. Furthermore, it would be interesting to compare FoxO3 activity in purified primary stem cells and more mature progenitor cells exposed to oxidative stress while cultured in normoxia or hypoxia in order to see if FoxO proteins act differently depending on the cell stage. FoxO3 is known to activate different transcriptional programs depending on the stimuli. Inactivation of the PI3K/AKT pathway causes FoxO-mediated apoptosis, whereas stress-induced activation of FoxO by the deacetylase SIRT1 causes cell cycle arrest and stress resistance. How hypoxia interacts with these pathways in HSCs has not been investigated, but a favor of quiescence and protection from oxidative stress is likely. Notable, in the present study we show that hypoxia depresses accumulation of ROS and decreases the proliferation of HSCs, which resembles the effect mediated by FoxO proteins.
Whether hypoxia mediates these effects via FoxO proteins or through other effectors remains to be investigated. Increased knowledge of how FoxO proteins are regulated both in normal and malignant stem cells could provide important potential therapeutic applications.

In the present investigation we have also investigated the role of hypoxia on HSC maintenance, postulating that hypoxia in the HSC niche provides critical regulation of stem cell activity. However, as discussed earlier, signals from other niche cells are necessary for proper regulation. It is possible that hypoxia upregulates molecules on niche cells important for the interaction and maintenance of HSCs. Therefore, investigation of how niche cells are affected, both functionally and genetically, by cultivation in hypoxia would provide meaningful information to increase the understanding of the interplay between different factors in the stem cell niche. Moreover, co-culture of HSCs and niche cells in hypoxia could have a potential influence on the HSC potential. To investigate whether this could increase the expansion of functional HSCs would be intriguing. Increased knowledge of possible hypoxic targets in the stem cell niche will help to understand how self-renewal and survival of HSCs is regulated.

In this thesis, I discuss and highlight the importance of a hypoxic niche in maintaining and protect normal HSCs. Can this also be applied to the maintenance of leukemic stem cells (LSCs)? Dormant LSCs are located in the bone marrow where they are supported by survival signaling from niche cells (Konopleva et al., 2002). Many studies reveal evidence that this might retain LSCs and protect them from chemotherapy, at least in part explaining the high occurrence of relapse in AML. A possible way to get around this is by targeting niche molecules which would result in disrupting the interaction with LSCs. A recent study by Zheng and colleagues has shown that inhibition of CXCR4 increases sensitivity of leukemic bone marrow blasts to FLT3 inhibitors. Furthermore, decreased signaling of CXCR4 efficiently mobilizes FLT3-ITD expressing human AML cells from murine bone marrow into the peripheral blood, which causes enhanced sensitivity to anti-leukemic drugs (Zeng et al., 2009). Since hypoxia and HIF-1 regulate many interaction molecules between niche cells and HSCs (e.g. CXCR4/SDF-1 signaling and BCRP-1/ABCG2), which have proved to be involved in survival and maintenance of both normal HSCs and LSCs, it is tempting to speculate that hypoxia plays a key role in the leukemic stem cell niche. Maybe hypoxia is an important factor in promoting the escape of leukemic stem cells from chemotherapy.
EN LIVSVIKTIG BALANS MELLAN LIV OCH DÖD –
en populärvetenskaplig sammanfattning


Den här avhandlingen handlar om hur balansen mellan överlevnad och programmerad celldöd regleras och vad som händer vid en störd balans. Jag har här fokuserat på blodstamcellen och omogna förstadieceller. Valet mellan att självförnya sig eller att producera andra blodceller regleras av speciella signalmolekyler som kallas cytokiner vilka produceras av andra celler i omgivningen. Dessa binder till specifika mottagarmolekyler (receptorer) på stamcellens cellyta och skickar budskap som t.ex. ”Självförnya dig!” eller ”Bilda mer vita blodkroppar!”. Alla kroppens celler kan även få budskap om att ”begå självmord”. Kroppen kan på så vis göra sig av med gamla och skadade celler. Celler kan även få signaler från hormon och nerver samt från andra fysiologiska faktorer.

I delarbete I-III har jag studerat hur en låg syrehalt (hypoxi) påverkar blodstamceller. Andra forskargrupper har bl.a. visat att en låg syrehalt är viktigt för att bevara blodstamceller, men vilka mekanismer som sätts igång är till stor del oklart. Vi har studerat blodstamceller isolerade från benmärgen på möss. Mössens blodstamceller är bättre karaktäriserade än människans och det är på så vis lättare att rena fram
stamceller från andra celler. Dessutom underlättar användandet av en djurmodell genomförandet av transplantationsförsök, vilket krävs för att till fullo studera stamcellens unika egenskaper att förnya sig och bilda alla blodets celler under en organsims livstid. Här har vi undersökt hur odling av blodstemcells under 1-8 dagar i en syre- och miljö påverkar stamcellernas egenskaper i en cellkultur och blodstemcellens förmåga att nybilda sig själv och dela sig efter att de transplanterats till andra djur. Vidare har vi studerat om hypoxi skyddar blodstemceller mot fria syreradikaler. Tidigare forskning har visat att en ökning av mängden fria syreradikaler skadar stamcellens förnyelse förmåga. Vissa forskare anser även att fria syreradikaler leder till åldrande, men mer forskning krävs för att förtydliga och befästa dessa fynd.


Om ett fel uppstår i regleringen mellan överlevnad och celldöd kan blodcancer, leukemi, utvecklas. Akut myeloid leukemi (AML) är en speciell form av leukemi där en viss typ av förstadieceller delar sig ohämmat och dessutom är okänsliga för signaler att begå självmord. Ett vanligt fel som kan leda till AML är att det blir en DNA-skada, en mutation, på en speciell receptor som kallas FLT3. Denna sitter på cellytan och kan bli aktiverad att skicka signaler in i cellen som leder till överlevnad. En muterad FLT3 leder till en ständigt aktiv signal för överlevnad. AML tros ha sitt ursprung i blodstemceller eller omogna förstadieceller. En ny typ av behandling av leukemi är läkemedel som hämmar FLT3-signalen. Detta leder till att överlevnadssignalen i de leukemiska cellerna stängs av, vilket resulterar i celldöd. Leukemiska celler uttrycker ofta fler FLT3 mottagare och/eller muterade FLT3 mottagare, vilket gör att det främst
är de som drabbas och inte friska celler. Tyvärr har det visat sig att leukemiska cellerna utvecklar resistens mot dessa FLT3-hämmare. Därför måste nya behandlingsformer utvecklas. I arbete IV har vi studerat vilka signalmolekyler som är av betydelse för att inducera celldöd av AML-celler efter behandling med FLT3-hämmare.


Den här avhandlingen bidrar med nya forskningsfynd om hur överlevnad och celldöd regleras i normala och leukemiska stam- och förstadieceller.
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