THE HYPOXIC HEMATOPOIETIC STEM CELL NICHE Consequences of Hypoxia-induced Transcription on Stem Cell Fate

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THE HYPOXIC HEMATOPOIETIC STEM CELL NICHE

Consequences of Hypoxia-induced Transcription on Stem Cell Fate

Matilda Rehn
Abstract

Hematopoiesis is the process of blood formation that originates with the hematopoietic stem cell (HSC), a cell type that is responsible for the life-long supply of mature blood cells. HSCs are defined by their ability to self-renew as well as giving rise to differentiated cells of all blood lineages. Because of these features, HSCs are also the basis for bone marrow transplantation regimens for treatment of leukemia and various other hematopoietic disorders. HSCs reside in the bone marrow of adult mammals, in the so called niche. The HSC niche refers to the specific anatomical region where the stem cells reside, but also to the regulatory microenvironment consisting of adjacent cells and factors produced by these. An important feature of the HSC niche seems to be a relatively low level of oxygen: hypoxia. Hypoxia leads to activation of Hypoxia-Inducible Factors (HIF). We have investigated the role of hypoxia and HIFs in HSC biology. By in vitro hypoxic treatment, we could show that hypoxia leads to less proliferation of HSCs, with up-regulation of cell-cycle inhibitory genes, while full reconstitution potential of irradiated recipient mice is preserved. Ectopic activation of HIF, mediated by retroviral overexpression in hematopoietic stem and progenitor cells (HSPC), leads to even less proliferation and a disability to sustain hematopoiesis in vivo. The role of the HIF target gene Vegfa was studied in a mouse model where hypoxia-induced transcription of Vegfa is abrogated (Vegfa$^{−/−}$). HSCs could be maintained under steady-state conditions without hypoxic induction of Vegfa. In transplantation assays however, we show that when Vegfa upregulation upon hypoxia is lacking, adult HSC function is clearly impaired. On the contrary, HSCs isolated from the fetal liver of developing Vegfa$^{−/−}$ mice had a normal function. Furthermore, we show that erythropoiesis during development and to a lesser extent in the adult involves hypoxic Vegfa expression. In summary, this thesis contributes new findings to the role of the hypoxic HSC niche. We show that hypoxia and HIFs are involved in regulating HSC proliferation. Furthermore, hypoxia-induced Vegfa is identified as an important player in HSCs.

Keywords: Hematopoietic Stem Cells, Stem Cell Niche, Hypoxia, Hypoxia-Inducible Factor, Vegfa.
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Abbreviations

AGM          Aorta Gonad Mesonephros
AML          Acute Myeloid Leukemia
PBSC         Peripheral Blood Stem Cell
BFU-E        Erythroid Burst Forming Unit
BM           Bone Marrow
BrdU         5-Bromo-2-deoxyuridine
CAFC         Cobblestone Area Forming Cell
CDK          Cyclin Dependent Kinase
CDKI         Cyclin Dependent Kinase Inhibitor
CFU          Colony Forming Unit
CFU-GM       Granulocyte/Macrophage Colony Forming Unit
CFU-S        Spleen Colony Forming Unit
CLP          Common Lymphoid Progenitor
CMP          Common Myeloid Progenitor
CRA          Competitive Repopulation Assay
CRU          Competitive Repopulation Units
EPO          Erythropoietin
ESC          Embryonic Stem Cell
FACS         Fluorescence Activated Cell Sorting
FL           Fetal Liver
G-CSF        Granulocyte Colony Stimulating Factor
G-CSF-R       Granulocyte Colony Stimulating Factor Receptor
GFP          Green Fluorescence Protein
GVHD         Graft vs. Host Disease
HIF          Hypoxia Inducible Factor
HRE          Hypoxia Response Element
HSC          Hematopoietic Stem Cell
HSCT         Hematopoietic Stem Cell Transplantation
HSPC         Hematopoietic Stem and Progenitor Cells
KO           Knock Out
LMPP         Lymphoid Primed Multipotent Progenitor
LSC          Leukemic Stem Cell
LSK          Lineage^-SCA-1^-C-KIT^+
LTC-IC        Long Term Culture Initiating Cell
<table>
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<th>Abbreviation</th>
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<tr>
<td>LT-HSC</td>
<td>Long Term Hematopoietic Stem Cell</td>
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<tr>
<td>MPP</td>
<td>Multipotent Progenitor</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non Obese Diabetic SCID</td>
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<tr>
<td>ODD</td>
<td>Oxygen Dependent Degradation Domain</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short Hairpin RNA</td>
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<tr>
<td>SLAM</td>
<td>Signalling Lymphocyte Activation Molecule</td>
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<td>SRC</td>
<td>SCID Repopulating Cell</td>
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<td>ST-HSC</td>
<td>Short Term Hematopoietic Stem Cell</td>
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<tr>
<td>VEGFA</td>
<td>Vascular Endothelial Growth Factor A</td>
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<tr>
<td>wt</td>
<td>Wild Type</td>
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Papers Included in the Thesis


Although only a fraction of it actually ends up on paper, this thesis will summarize the work carried out during my PhD studies for the last five or so years at Lund Stem Cell Center and Molecular Medicine and Gene Therapy at Lund University. The first part of the thesis consists of a literature review of research in the field providing a background for the second part where the present investigation is summarized, followed by the three papers.

Hematopoietic stem cell biology has been an intense field of research for more than 50 years but there are still many things that we do not understand. In fact, more questions are generated the deeper we dig into the complex world of gene regulation and cellular interaction. Hematopoietic stem cells are the basis for several treatment strategies involving transplantation of hematopoietic cells to treat e.g. leukemia. Yet, the hematopoietic stem cell itself may be the cause of several hematopoietic diseases. To better understand hematopoietic disorders aiming at better treatment, we need to comprehend the underlying biology. At the same time, the knowledge we can generate in research is an important driving force in itself because we want to know how it works. We look deep into the nucleus of a cell and know that the truth is there for us to find.

In order to learn about gene and cell function in a context relevant to the human body, the mouse is commonly used as a model system. Mouse and human have very similar physiology and genetics. Indeed, approximately 95% of the murine genome is similar to ours and many findings in mouse models can be extrapolated to human biology. Research in mouse models has in many cases proven invaluable to understand human disease. In this thesis we have studied how low-oxygen concentrations affect hematopoietic stem cells in the mouse.
Background

Stem Cells
Stem cells are long-lived cells that are able to both self-renew and give rise to various types of mature cells. The ultimate stem cell is the fertilized egg, which during development of the embryo will give rise to all the different kinds of cells present in the organism. It is therefore called totipotent. Embryonic stem cells (ESC) are pluripotent cells derived from a developing embryo that are propagated in culture. The research involving human ESCs holds great promise because of its potential for creating new tissues, thereby giving hope for treatment of many different diseases or injuries where tissues or organs are damaged. At the same time, the use of human ESCs is somewhat controversial due to the fact that the cells are derived from fertilized embryos. Recently, the stem cell field experienced a major breakthrough when scientists managed to reprogram mature cells to revert to an ESC-like state. These cells are called induced pluripotent stem cells and are currently under intense investigation. Since they can be produced from almost any mature tissue cell and still have the properties of ESCs, they are an attractive alternative to ESCs for regenerative therapies.

Adult, or tissue stem cells are also self-renewing, but they can only give rise to mature cell types within a certain tissue. They are termed multipotent stem cells. Studies of tissue-specific stem cells are important to understand regeneration of a specific organ or tissue. Examples of well-studied adult stem cells are the neural stem cells and the epithelial stem cells of the gut. The focus of this thesis is the stem cells of the blood.

Hematopoiesis
Hematopoiesis is the process of blood formation where millions of blood cells are produced each hour in healthy humans. In cases of stress, such as a bleeding or an infection, the production increases even further. The blood consists of a mixture of many different cell types as well as blood plasma—a liquid containing nutrients, proteins and growth factors. The blood cells are generally divided into red and white blood cells. The red blood cells (erythrocytes) have the important function of oxygen delivery from the lungs to all parts of the body. The white blood cells,
myeloid or lymphoid, comprise the cellular part of the immune system with the
function to fight infectious or other harmful agents but also to clear dead cells
from the body. Blood platelets (thrombocytes) are formed from megakaryocytes
and are crucial in preventing bleedings from damaged blood vessels. Most mature
cells in the blood system are relatively short lived. Apart from some types of
lymphocytes, like memory B-cells which can survive for years, most blood cells
have a life-span ranging from a few days to a few months. This requires constant
replenishment of mature cells, a process where the hematopoietic stem cell (HSC)
is fundamental.

Hematopoietic Stem Cells

HSCs are rare cells present in the bone marrow (BM) of adult mammals and are
defined by their ability to self-renew as well as giving rise to differentiated cells of
all blood lineages. The long-term HSC (LT-HSC) is at the top of the hierarchy in
the stem cell model of the hematopoietic system and is defined by its ability to
provide life-long hematopoiesis in the host. LT-HSCs give rise to progeny cells
that sequentially lose self-renewal capacity while gaining the capacity to
proliferate extensively. Short-term HSCs (ST-HSC) have limited self-renewal
capacity but are still multipotent, and the multipotent progenitor (MPP) has lost
self-renewal capacity while still able to produce all lineages. Progenitors in the
hierarchy then become stepwise more restricted towards a specific lineage in the
hematopoietic system (Figure 1). Mixed populations consisting of both HSCs and
progenitor populations can be referred to as hematopoietic stem and progenitor
cells (HSPC). According to the classical model of hematopoiesis, the
hematopoietic differentiation hierarchy is divided in two parts at the level of the
common lymphoid progenitor (CLP), precursor of all lymphoid cells, and the
common myeloid progenitor (CMP), giving rise to myeloid and erythroid cells. However, this model has been challenged, and it is now thought that
megakaryocytic/erythroid progenitors deviate already at the level of ST-HSCs
while a lymphoid primed multipotent progenitor (LMPP) gives rise to lymphoid
and myeloid cells (Figure 1). Still, hematopoiesis is a highly complex system,
and any model trying to describe it is likely to be somewhat simplified.

The starting point for HSC research is considered to be a series of experiments
in the 1960s, where Till and McCulloch showed for the first time that self-
renewing cells were present in the BM of mice, and that these cells produced
mature blood cells in a clonal manner. The readout used at the time was the spleen
colony forming unit (CFU-S), and cells isolated from BM of mice were shown to
give rise to colonies in the spleen in a dose-dependent manner. These colonies
contained myeloid and erythroid cells, and some of the colonies could also give
rise to lymphoid cells if re-transplanted. Importantly, CFU-S could give rise to
secondary colonies in a new mouse as well as rescue irradiated mice. By cytological marking, it was also shown that each colony had one and the same ancestor since all the mature cells in the colony contained the same unique mark. It was concluded that the HSCs were located to the BM and that it was these cells that gave rise to the CFU-S.\textsuperscript{4,5} It is now known that both HSCs and more committed progenitor cells can form CFU-S, but the principle that self-renewing, blood-forming cells reside in the BM was clearly demonstrated and has since then been confirmed with more refined techniques and stricter assays.

\textit{Figure 1.} Schematic overview of the hematopoietic system
**HSC Fate Options**

In order to try to understand HSC biology, the different theoretical fate options of HSCs are usually discussed. Self-renewal or differentiation are the two fate options that can occur upon cell division. A single HSC that divides can do this symmetrically or asymmetrically. Symmetric division results in two identical cells, while asymmetric division leads to two distinct cells with different potential. Symmetric division can result in two new HSCs or two cells already committed towards differentiation. Asymmetric division results in one new HSC and one committed cell. Self-renewal requires that at least one of the daughter cells remains a stem cell (Figure 2). A symmetric division ending in two HSCs is usually regarded as an expanding division since the number of HSCs is doubled. On the other hand, an asymmetric division with one HSC and one committed cell as the endpoint is a maintenance division since the number of HSCs is the same as before. HSCs can also go into apoptosis or leave their residency in the BM and migrate to the periphery. It seems that a fraction of HSCs is mainly non-dividing (quiescent) or even dormant, meaning that they do not actively contribute to hematopoietic output during steady-state conditions but rather serve as a back-up pool. Thus, quiescence can be added to the list of fate options for HSCs. 

The different HSC fate decisions need to be strictly controlled but still flexible in order to meet the various demands imposed on the hematopoietic system at different times. At homeostasis, the number of HSCs is relatively constant, while any stress imposed to the hematopoietic system, such as acute blood loss, infection or myeloablative therapy, requires temporal expansion of the HSC pool. Once the insult has been cleared, the HSC pool can decrease again. A central goal in HSC research is to be able to achieve expansion of HSCs in vitro for clinical purposes. Furthermore, in hematopoietic malignancies, aberrant expansion usually takes place. This explains why the knowledge about how these processes are regulated is important.
Intrinsic Regulation of HSCs
Control over HSC fate options is regulated by a complex network of several intrinsic molecules that in turn integrate extrinsic signals from the surroundings (see section “The HSC Niche”). Transcription factors are the final targets of many signalling pathways and several transcription factors have been shown to have important HSC regulatory functions. C-MYC and N-MYC are both involved in HSC self-renewal and genetic ablation of these genes leads to reduced HSC function. The hox family of transcription factors also includes several members
with known HSC regulatory activity, most notably HOXA9 and HOXB4. HOXB4 has been shown to promote ex vivo expansion when overexpressed. Modulation of transcription factor activity naturally also has implications for HSC function and examples of proteins with such modulatory functions are GFI-1, BMI-1 and PRDM16.

Control over the cell cycle is another main point of intrinsic HSC regulation. Proliferating cells sequentially go through the different phases of the cell cycle: growth and preparation of the chromosomes for replication (G1), DNA synthesis (S), additional growth and preparation for cell division (G2) and mitosis (M). In addition, cells can exit the cell cycle and stay quiescent in the G0 phase.

Studies using the DNA-incorporating compound 5-Bromo-2-deoxyuridine (BrdU) in mice have shown that ~50% of HSCs enter the cell cycle every 6 days and by 6 months, 99% of LT-HSCs have cycled at least once. However, treatment with BrdU for extended periods might initiate proliferation in itself why these numbers may be somewhat overestimated. By instead using a short pulse of BrdU incorporation followed by a long chase period, it was shown that some LT-HSCs are able to retain the BrdU label for more than one year indicating that they have cycled very few times. Similarly, by labelling HSCs with a histone-GFP tag, label-retaining HSCs were detected after 72 weeks. Label-retaining LT-HSCs showed higher engraftment in competitive repopulation assays (CRA) and serial transplantation compared to phenotypically similar LT-HSCs that had lost the label. These studies suggest that there is a subpopulation of HSCs that is dormant and does not contribute to hematopoiesis at steady state but rather serves as a backup pool, reserved for stress situations. In contrast, using Biotin labelling, no evidence for a dormant HSC-population was found. Nonetheless, HSCs with slower proliferation kinetics, measured by Biotin retention, were shown to have increased function. This is also consistent with the finding that HSCs have a prolonged cell-cycle transit time. In summary, even though controversies still exist regarding the proliferative status of HSCs, it is commonly accepted that HSCs are relatively quiescent in the BM with a large fraction of LT-HSCs in a non-cycling state. It is estimated that 70–75% of LT-HSCs are in the G0 phase of the cell cycle at any one time.

Progression through the cell cycle is controlled by cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (CDKI) which in turn receive signals from growth-factor signalling. Several CDKIs seem to have essential functions in control over HSPC proliferation. Genetic ablation of the gene for p21, Cdkn1a, in mice leads to abnormal cycling of HSCs, with increased HSC numbers but this is followed by exhaustion of stem cells after serial transplantations. However, contradictory results exist, indicating that the outcome of the analysis of Cdkn1a deficient mice might depend on mouse strain background. Cdkn1b (p27) deleted mice have normal HSC numbers while proliferation of progenitors is affected. Cdkn1c, the gene coding for p57 has been shown to be highly expressed in
purified HSC populations. Finally, deletion of Cdkn2c (p18) in HSCs leads to increased proliferation but at the same time increased self-renewal.  

**HSCs During Development**

Around day 7 of development in the mouse, hematopoietic activity occurs in the extra-embryonic yolk sac. Yolk sac hematopoiesis provides the developing embryo with a transient wave of erythrocytes and macrophages and is termed primitive hematopoiesis because true HSCs, providing persistent hematopoiesis in the adult, are not believed to be present here. Later, HSCs appear in the aorta-gonad-mesonephros (AGM) region of the embryo proper around day 10 and in the placenta. These HSCs are termed definitive because they are able to establish hematopoiesis if transferred to an adult mouse. AGM and placental HSCs subsequently seed the fetal liver (FL) which is the main site for hematopoiesis during the later stages of development until birth. In the FL, the hematopoietic organ grows and there is substantial HSC expansion. Just before birth, HSCs start to migrate towards the BM which then remains the major site for hematopoiesis for life. HSCs in the FL are highly proliferative and continue to be so until around 3 weeks after birth in the mouse, when they transition into a less proliferative state.

**Methods to Study HSPC Biology**

**In Vivo Transplantation Assays**

HSCs are functionally defined by their dual ability to self-renew and give rise to all lineages of blood in vivo. In the mouse, this ability is tested by injecting populations of interest into lethally irradiated mice and thereafter measuring contribution to hematopoiesis in the blood and BM. Irradiation causes irreversible damage to DNA and therefore mainly harms dividing cells. Because of the high proliferative rate of many cells in the BM, the hematopoietic organ is one of the most sensitive ones to this treatment. Thus, irradiation efficiently wipes out the hematopoietic system, and without re-injection of hematopoietic cells, mice will die from BM failure. The exact mechanism as to why this happens is not fully understood; even though a fraction of HSCs are not rapidly cycling and therefore should not be affected by irradiation, lethality is an obvious effect. The major cause of lethality is the acute lack of immune cells, making combating of normally benign infections difficult. Re-engraftment of residual host HSCs is possible if transient mature support cells are injected, which shows that irradiation cannot completely abolish HSCs. Nevertheless, irradiation is an efficient method to clear the BM to prepare for transplantation of test populations containing HSCs, and it is thought that the procedure disturbs the HSCs enough to provide space for new ones.
The definitive proof for the existence of a HSC in an injected test population is that the irradiated animal survives long-term (usually more than 12 weeks) since this means that a new hematopoietic system has been established and is called **long-term repopulation**. The more immediate effect of injecting hematopoietic cells for survival of the lethally irradiated recipient is termed **radioprotection** and may not require a HSC. A variation on this theme is the **competitive repopulation assay (CRA)** considered as the gold standard test for HSC function. In this assay, a test population containing putative HSCs is transplanted to lethally irradiated mice together with a competitor population, usually unfractionated normal BM. Thereafter, relative reconstitution of the two populations in the transplanted animal is measured in the peripheral blood. Early on, to distinguish between test and competitor cells in this type of assay, researchers used cells from different strains of mice carrying different hemoglobin types and analyzed the contribution of each strain by electrophoresis. Alternatively, mice of different sex were used and the relative contribution of the Y-chromosome by PCR or southern blot was analyzed. Later, this type of analysis became much easier with the innovation of Ly5/CD45-distinguished mouse strains. CD45 is expressed on the surface of all hematopoietic cells and exists in two isoforms. Congenic strains are genetically identical apart from one locus, which enables transplantation without immunological rejection. By using congenic strains that express either the CD45.1 or CD45.2 isoform, it is possible to distinguish between donor, competitor and recipient contribution, by analyzing cell surface expression using **flow cytometry**. Another possibility is to use cells from transgenic mice expressing fluorescent proteins, e.g. green fluorescent protein (GFP), also readily detected by FACS. The CRA is a strict functional assay and measures the ability of HSCs to migrate to and engraft the irradiated host BM under competitive pressure. A positive readout requires that mature cells of all lineages are present in the blood, demonstrating multipotentiality. LT-HSCs are confirmed when donor contribution can be measured in the blood 12–16 weeks post transplantation or longer. The readout for ST-HSCs is somewhere around 8–10 weeks since the ST-HSC is multipotential yet with limited self-renewal. The frequency of functional HSCs, so-called **competitive repopulation units (CRU)** can be measured by applying limiting-dilution to the CRA. Different doses of the test population are administered to groups of irradiated mice, together with a fixed dose of competitor cells. Positively engrafted mice are usually defined with a cutoff level of more than 1% lympho-myeloid reconstitution. At or below the limiting dilutions, not all mice in the group will have a positive readout, and the frequency of CRUs can be calculated using Poisson statistics. At a dose where ~63% of the recipients are reconstituted, 1 CRU is present.

To provide long-term engraftment, the HSC needs to self-renew. Although 12–16 weeks is usually regarded enough to demonstrate this, more stringent assays are typically employed to show significant self-renewal. This can be done with
serial transplantations into secondary or tertiary recipients. In this case, a sample of the BM from the initially transplanted mouse is transferred to new groups of lethally irradiated recipients. 39

In vivo assays also exist for some progenitor populations. As discussed above, the CFU-S is an assay for hematopoietic progenitors, even though some HSCs can also read out in this assay. 35,40 The CFU-S also relies on the transplantation of cells into lethally irradiated mice and detection of colonies that appear on the spleen during the first 2 weeks after injection.

For obvious reasons, human HSPCs cannot be defined using the same criterion as mouse HSCs. However, in vivo xenograft assays have been developed that at least aim for a similar functional definition. To avoid cross-species immune rejection, putative human HSPCs can be transplanted to immune-compromised mice. Severe combined immunodeficient (SCID) 41 or non-obese diabetic (NOD/SCID) mice 42 accept human grafts, but differentiation of human hematopoietic cells is skewed towards B-cells, while lacking T- and NK-development, probably due to the existence of NK-cells in the hosts. By depleting NK-cells in NOD/SCID/B2m null 43 and NOD/SCID/IL2rg null mice 44, this assay has been improved. Two weeks after transplantation of human HSPC populations, short-term repopulating cells can be evaluated in the blood of recipients and at 16–20 weeks, SCID repopulating cells (SRC) are defined by the presence of human cells in the blood and BM. 45

**In Vitro Assays for HSPCs**

The frequency of different types of progenitors in hematopoietic cell populations can be measured using variants of the in vitro colony forming unit (CFU) assay. Hematopoietic cells are cultured in a semi-solid medium (usually methylcellulose) in the presence of growth-factors and cytokines directing their differentiation. After a defined culture period, colonies of mature cells appear and can be quantified. Because the cells grow in a semi-solid medium, a colony arises from one progenitor cell initially seeded in the culture. By applying different cytokines, different progenitor types can be assessed. For example, erythroid burst forming units (BFU-E) are assayed in the presence of Erythropoietin (EPO), while granulocyte/monocyte colony forming units (CFU-GM) are detected when myeloid promoting cytokines are added. In addition, the morphology and size of the colonies formed give some information regarding the differentiation and proliferative potential of the seeded progenitor. Colonies of mixed cells can also appear and is then the result of a multipotent progenitor. B-cells and T-cells are not as easily formed using only the addition of soluble factors, but they can be assayed using co-culture systems with stromal cell lines expressing vital cues for lymphoid development. 46–47

The long-term culture initiating cell assay (LTC-IC) is perhaps the best assay for human HSCs but it can also be used for mouse cells. 48–49 This assay
relies on the ability of adherent stromal cells to support HSPC growth over several weeks. Primary stromal cells, isolated from BM, or cell lines can be used. Examples of such cell lines are OP9, AFT024, or MS5. Putative HSC populations are cultured on the stromal cells for 5–6 weeks whereafter the CFU potential is measured. A cell that after several weeks of culture still maintains the progenitor capacity is regarded to have stem cell properties. Classically, the LTC-IC assay is performed using limiting dilutions, whereby quantification can be performed in a similar way as described for the CRU assay (see above). Variants of the LTC-IC assay instead takes into account the ability of HSPCs to form so called cobble-stone areas (cobblestone area forming cell; CAFC), where cells grow underneath the stromal cell layer.

**Phenotypical Identification and FACS Purification of HSCs**

Early on, researchers tried to subfractionate BM to achieve populations enriched for HSC activity based on different physical properties. By using for example density gradients or wheat-germ glutinin binding, some level of HSC enrichment could be achieved. However, it was not until researchers started to use fluorescence activated cell sorting (FACS) that the more detailed subfractionation of the hematopoietic hierarchy could start. The FACS methodology includes antibody recognition of specific cell-surface antigens with fluorochromes and detection of these on a single-cell basis. The fact that cell surface molecules are expressed differently depending on maturation stage makes it possible to prospectively isolate populations and thereafter test the function of these to narrow down populations containing the HSCs.

Weissman and colleagues pioneered the cell sorting of hematopoietic cells and showed that by selecting cells that did not express markers for a number of mature cells (lineage−, lin'), they could enrich for HSC activity. It was then found that all HSC activity was contained within the lin' population expressing the stem cell antigen 1 (SCA-1) and the receptor for Stem cell factor (SCF), C-KIT; the so-called LSK population. By further subdivision of the LSK compartment, a number of markers can be used to purify LT-HSCs to near purity. CD34 is a marker expressed on maturing and cycling HSCs, but adult LT-HSCs are CD34−. The signalling lymphocyte activation molecule (SLAM)-family of receptors can also be used to successfully isolate HSC, and LT-HSCs are CD150+ CD48−. The LSK SLAM cell surface phenotype is stable during development and can be used for FL HSC isolation as well as in aged mice where other markers have shown to be unstable. Furthermore, LT-HSCs are positive for the Endothelial protein C receptor (EPCR) and negative for the Fms-like tyrosine kinase 3 (FLT-3). HSCs express membrane pumps involved in export of toxic compounds taken up by cells. The presence of these pumps on HSCs enables isolation of HSCs based on their ability to exclude dyes, e.g. Hoescht33342 or Rhodamine.
Human HSPCs can be enriched by selecting for Lin− CD34+ or CD133+ compartments. Within these populations, the SRCs are found in the CD38−CD90−CD45RA− population. \textsuperscript{68-71} Dye efflux properties can also be used for human HSC enrichment as well as activity of the enzyme Aldehyde dehydrogenase. \textsuperscript{72} Recently it was shown that the CD90+ population also contains some SRCs and Integrin α6/CD49f is a novel marker that further enriches for SRCs both within the CD90+ and CD90− populations. \textsuperscript{73}

**Gain and Loss-of-function Studies**

In order to learn about the function of specific genes or their gene products in HSPC biology, gain-of-function or loss-of-function studies can be performed. Gain-of-function is expected to enhance the effect of the normal function of a gene, while loss-of-function should demonstrate what functions are lost when the gene is not expressed, giving information regarding the normal function of the gene.

A common way to achieve loss-of-function is to use knock-out (KO) mouse technology. By targeting a specific gene in ESC using homologous recombination and thereafter injecting those cells into blastocysts (day 3.5 developing embryo), mice with a non-functional gene can be produced. \textsuperscript{74} The phenotype of the KO mice with regards to hematopoiesis can be studied and the HSCs tested in various HSC assays to gain knowledge about whether the gene is needed for HSC function. Many genes are essential during embryogenesis, and KO animals for such genes will die in utero. Embryonic lethality is important information regarding the gene function in itself, but prevents the possibility to study tissue specific gene function in the adult. In such cases one can use inducible KO strategies. A common method involves the Cre-LoxP system. Cre recombinase is an enzyme that recognizes specific DNA-sequences (LoxP sites) inserted around the gene that is to be targeted. The Cre activity can be activated in adult animals in different ways. The Mx1-Cre mouse expresses Cre under the influence of an interferon-inducible promoter and is commonly used to inactivate genes in hematopoietic cells. By crossing a LoxP-targeted strain to the Mx1-Cre strain, deletion of the gene can be achieved by injecting the interferon-inducing substance pIpC. \textsuperscript{75} Another variant on this theme is the modified Estrogen Receptor (ER)-Cre, where Cre expression is induced by Tamoxifen. The Cre-LoxP system can also be used with tissue-specific promoters or promoters that are active during a specific phase of development, thereby restricting the loss-of-function in time and space. \textsuperscript{76} This may be especially important when the investigated gene is widely expressed, causing unspecific secondary effects on the tissue of interest when the gene is deleted globally.

Mice can also be genetically engineered to overexpress certain genes or express genes not normally expressed, for example human fusion genes involved
in malignancies. Here, injection of a piece of DNA into a fertilized oocyte leads to random integration in the chromosomal DNA, resulting in a transgenic mouse. Generation of KO or transgenic mice is quite time consuming. A faster way to study gene function in hematopoiesis may be to use viral overexpression. The most commonly used systems are the onco-retroviral or lentiviral vectors. These viruses both belong to the retroviral family of integrating RNA-viruses. Retroviral vectors have been modified so that they can be used to deliver a gene of interest to cells in an experimental setting, while not maintaining the ability to replicate as a wild type (wt) virus. The big advantage with the retroviral system in hematopoietic research is the ability of these vectors to integrate in chromosomal DNA. This feature certifies that the transgene is passed over to progeny cells upon cell division, especially important when studying gene-function in the HSC context. Typically, HSPCs are isolated from wt mice followed by in vitro transduction with the vector. Thereafter, transduced cells can be assessed in various in vitro or in vivo assays to study the effect of the overexpressed gene. Vectors usually contain some type of selectable marker, e.g. GFP that is co-expressed with the transgene to allow for tracking of overexpressing cells. Retroviral overexpression can be modulated in various ways, for example, by using different promoters driving the expression, or inducible transgene expression. Retroviral vectors can not only be used for overexpression but also to express short hairpin RNA (shRNA) molecules for RNA-interference. RNA-interference affects both mRNA stability and translation, in this way reducing gene function. However, the efficiency of knock-down varies, and residual gene expression might limit the conclusions that can be made from results obtained using this type of vectors. Even though the genomic integration of retroviral vectors is an asset, this feature also has drawbacks. Chromosomal integration is random and occasionally takes place in such a way that normal gene function is affected. Insertional mutagenesis is the phenomenon where the integrating vector disturbs a tumor suppressor or an oncogene leading to transformation. To control for this, empty vector controls can be used. Another limitation of retroviral vectors is that the in vitro transduction step likely affects HSCs, which might influence the results.

**Mechanistic Studies in HSPCs**

To study proliferation and cell-cycle kinetics in HSC biology, a number of different approaches can be used. DNA-staining can be combined with cell surface HSC phenotype and analysis by FACS. This type of experiment gives information about the distribution of cells at the different stages of the cell cycle since DNA-replication yields cells with double the amount of DNA compared to cells that have just divided. However, many HSCs are not actively cycling, but instead resting in G0, and this phase cannot be discriminated from G1 without using a marker for proliferation, for example Ki67. Many DNA staining protocols requires
fixation and permeabilization of cells and cannot be combined with functional analyses after sorting. An alternative is therefore to use PyroninY, an RNA stain that, combined with a vital DNA stain, can also separate the different cell cycle stages.  

FACS staining for cell cycle only gives information about the distribution of cells in different phases of the cell cycle at the very moment when measured. This type of analysis does not tell anything about the proliferating history of cells or the rate at which cells proceed through the cell cycle. In order to get this kind of information, cells can be labeled in vitro using Carboxyfluorescein ester (CFSE) or in vivo with the DNA-incorporating compound BrdU. These labels are diluted when cells divide, and in this way, one can measure the proliferative history of HSCs and their label-retention capacity. However, the initial labelling procedure using BrdU requires cell division for the dye to incorporate into DNA, and it is also possible that BrdU in itself stimulates cell division. Moreover, detection of BrdU requires fixation of cells why functional tests cannot be performed, but one has to rely solely on cell surface phenotype. Recently, alternative methods have been developed to study proliferation of HSCs in vivo. By tagging cells using a Doxycycline inducible H2b-GFP transgenic mouse or Biotin, it is possible to track the proliferative history of cells and combine the readout with functional studies of live cells.

Apoptosis is one of the fate options that can be affected when overexpressing or deleting genes in HSCs. Analysis of apoptotic cells can be done by FACS, where HSC phenotypical staining is combined with a staining for apoptotic markers. One such marker is AnnexinV that detects flipping of phosphatidylserine groups to the outer side of the cell membrane, a sign of early apoptosis. Another way to measure apoptosis is by intracellular TUNEL-staining that detects DNA-strand breaks, which is a sign of late apoptosis.

When HSPCs are assessed in transplantation assays, a positive readout requires that HSCs are able to first migrate to the BM (homing), thereafter engraft, proliferate, and differentiate. To specifically assess the initial homing step, transplantation assays can be performed, where the amount of cells that reach the BM is measured shortly after injection. A commonly used method is labelling of sorted HSPCs with a fluorescent dye, e.g. PKH or CFSE, prior to injection into irradiated mice, followed by measurement of labeled cells in the BM after 3–24 hours. However, the frequency of cells that can usually be detected using this method is low, which is why a high number of cells need to be injected and a large amount of BM cells need to be analyzed to obtain reliable data. Therefore, it is common to use impure or only partly HSC-enriched populations. Both HSCs and more committed cells can home to the BM, which is why this type of assay may be quite unspecific. A more stringent way to measure homing is to measure true HSPC function in the recipient mice directly after injection. This is done by first injecting the test population into lethally irradiated mice, harvest the BM shortly
after, and subsequently inject into secondary recipients. The secondary mice are then analyzed for short-term or long-term reconstitution after the appropriate time.

**Therapeutic Potential of HSCs**

**Leukemia**

Leukemia is the collective term for various cancers that arise in the hematopoietic system. Leukemia is characterized by the accumulation of malignant cells at a specific stage of differentiation, with aberrant self-renewal and lost ability to mature. The malignant cells expand at the expense of differentiated cells, and normal blood functions like oxygen transport, blood clotting and immune response are disrupted. It is believed that leukemia is hierarchically organized in a similar way as the normal hematopoietic system, and that there are **leukemic stem cells (LSC)**. This means that a small number of cells with self-renewal capacity are able to maintain the leukemic bulk. The first evidence for LSCs came from studies where CD34+ CD38- cells from primary acute myeloid leukemia (AML) could give rise to new leukemia in immunodeficient mice, showing that LSC have similar properties as normal HSCs.  

**Hematopoietic Stem Cell Transplantation**

HSCs are the basis for treatment strategies involving transplantation of HSCs (HSCT) to treat a variety of disorders of the hematopoietic system as well as non-hematopoietic malignancies. Autologous HSCT is the type of transplantation that can be carried out using cells from the same person being treated, while allogeneic HSCT instead uses cells from a donor.

Autologous HSCT can be used for patients suffering from hematologic or non-hematologic malignancies that need especially high doses of chemotherapy to be treated. High-dose chemotherapy or irradiation causes severe side-effects on the hematopoietic system. HSCs can therefore be isolated from the patients before therapy, and re-introduced after therapy to establish a new hematopoietic system, in this way counteracting the side-effects.

Allogeneic HSCT is used to treat leukemia, using cells from a donor. Allogeneic HSCT can not only rescue the patient from the initial treatment aimed at eradicating the leukemia, but also has some therapeutic effect in itself. This effect is termed the **graft-versus leukemia (GVL)** effect and is caused by T-cells present in the donor population causing immunological reactions towards the non-self tissue. However, such immunological activity also often leads to a severe side effect called **graft-versus host disease (GVHD)** where tissues in the receiving patient are attacked. To avoid GVHD, donor cells need to be isolated from genetically matched donors (HLA), and treated patients are given immunosuppressants.  

The source of cells for HSCT can be aspirated BM or mobilized peripheral blood stem cells (PBSC). For collection of PBSCs, patients are first treated with
Granulocyte stimulating factor (G-CSF) and/or Cyclophosphamide, which leads to release of HSPCs from the BM into the blood that can be readily harvested. Apart from BM or PBSCs, another source for allogeneic HSCT is cord blood, taken from umbilical cords of newborns. A major advantage with this source is that cord blood has shown to be less immunologically active than conventional sources for transplantation. This means that a higher degree of HLA-mismatch can be tolerated without developing severe GVHD.

**Ex Vivo Expansion**

A limitation of using cord blood, and to a lesser extent other sources, for HSCT, is that the numbers of cells that can be isolated is usually not enough for transplantation. Therefore, a major goal in HSC research is to achieve *ex vivo* expansion. *Ex vivo* expansion is when HSPC populations are grown in culture, allowed to proliferate, while still maintaining self-renewal, to achieve increased numbers at the end of the culture. Recently, a number of different molecules (Notch-ligand, StemRegenin, Pleiotrophin) were shown to have the capacity to induce *ex vivo* expansion as measured by SCID-repopulation, but the clinical use of these molecules remains to be tested.

**Gene Therapy**

HSCs are crucial in *gene therapy* of various monogenic disorders of the hematopoietic system. Such diseases are caused by inherited mutations in specific genes that ultimately disrupt blood-cell function, typically in a specific lineage. Because of the nature of the hematopoietic system, correction of the mutation in the HSC population will provide life-long cure, even though the affected gene is mostly active in a differentiated cell type. Gene therapy can be performed by isolating cells from the patient and subsequently transducing the cells *ex vivo* with a vector that carries a corrected version of the gene in question. Corrected cells are thereafter re-injected to the patient. Retroviral vectors are the most widely used vectors in gene therapy protocols because of their ability to stably integrate in the host cell chromosome, thereby providing persistent expression. X-linked SCID is an example of a monogenic hematopoietic disorder and is caused by a mutation in the gene for the common γ chain of cytokine receptors, leading to a deficiency of a number of immunological cells. Patients suffer from numerous infections and if left untreated usually do not survive past the first year of life. In clinical trials, several X-linked SCID patients have been cured using gene therapy protocols that involved retroviral introduction of the therapeutic gene. However, insertional mutagenesis caused by the retroviral vector that inserted into the LMO2 gene, lead to development of acute leukemia in some patients. Development of safer vectors has improved gene therapy protocols, and in a recent clinical trial a patient was cured from Thalassemia by the introduction of a corrected gene.
The HSC Niche

The term **stem cell niche** is used to describe the anatomical location of stem cells as well as the regulatory microenvironment in this location that can affect stem cell fate. The concept of the niche was initially introduced by Schofield in 1978. The proposed model suggested that the niche is a specified anatomical space, limited in size and number, where stem cells can be maintained and replicate, and where differentiation is inhibited. The hypothetical model was proposed for HSCs but conceptually apply to other stem cell types as well. The exact identity of the HSC niche in BM is not clear, but it seems that several cell types and factors are involved (Figure 3).

![Figure 3. Schematic overview of the HSC niche in the BM.](image-url)
Osteoblasts

It is clear that the endosteal surface covering trabecular and compact bone, at the inside of the marrow space, is one location where HSCs preferentially reside. Early studies showed that by subdividing mouse femurs along the central-peripheral axis, primitive cells (CFU-S) were enriched in the peripheral region. By labelling HSCCs with a fluorescent dye, followed by injection into recipient mice, it was shown that the labeled cells tended to localize to the endosteal surfaces. Additionally, HSCs isolated from the endosteal fraction of BM show higher stem cell function compared to phenotypically identical HSCs isolated from central BM. The endosteal surfaces are covered by osteoblasts—specialized bone-forming cells that produce and deposit bone matrix. An indication that these cells are essential niche cells came from studies where it was shown that osteoblasts can maintain HSCs in ex vivo co-cultures. Later it was demonstrated that osteoblasts indeed play an important role in HSC support in vivo. Two studies, where different genetic approaches in mice increased the volume of bone and thereby the numbers of osteoblasts, could show that HSC numbers increased as a consequence of this. In one of the studies, the gene for bone morphogenetic protein 1 receptor, Bmp1r, was conditionally deleted, leading to increased trabecular bone areas and osteoblast numbers, followed by increased HSC numbers. N-cadherin, expressed on both osteoblast and HSC surfaces was identified as one of the molecular players in the osteoblast-HSC interaction. The other study achieved increased osteoblast numbers by overexpression of a constitutively active Parathyroid hormone receptor, Pthr, specifically in the osteoblast lineage. In this study, Jagged-1 was shown to be expressed in osteoblasts, thereby identifying the Notch-pathway to be an important regulator of HSCs in the niche. Likewise, wt mice that were treated with Parathyroid hormone had increased trabecular bone, as well as HSC numbers and function. This latter finding is highly clinically relevant, since a pharmacologic agent could affect HSC numbers, thereby opening up possibilities for use in HSCT applications. Furthermore, it has been shown that depletion of osteoblasts in vivo can result in decreased hematopoietic activity in the BM, but in other models of osteoblast-perturbation, the same effect was not achieved. It seems that cells of the osteoblastic lineage at different maturation stages can affect HSCs and hematopoiesis to different extents. Scadden and colleges have shown that osteoprogenitors rather than mature osteoblasts are the crucial HSC niche cells. Along this line, Nakamura et al subdivided endosteal-associated cells based on ALCAM-1 and SCA-1 expression and tested the ability of the different fractions to support HSCs in co-cultures. They found that the ALCAM-1+ SCA-1+ population was most efficient in supporting HSCs in vitro as shown by competitive repopulation experiments of cultured LSK cells. However, the ALCAM-1+ SCA-1+ and ALCAM-1- SCA-1- populations were also able to support HSCs, although not to the same extent as the ALCAM-1+ SCA-1+ cells.
There are several ways in which osteoblasts may regulate HSCs. As mentioned above, N-cadherin and components of the Notch pathway are some of the molecular cues identified. Furthermore, osteoblasts express high levels of CXCL12, a chemotactic agent interacting with CXCR4 on the surface of HSCs and other hematopoietic cells. The CXCL12-CXCR4 interaction has been shown to affect not only chemotaxis and homing to the BM but also HSC proliferation and survival. Thrombopoietin (TPO) is expressed by osteoblasts and interacts with TPO-receptor on the surface of HSCs, thereby inducing quiescence. A similar situation is true for Angiopoietin-1 secreted by osteoblasts, binding to TIE-2 on HSCs; and membrane-bound SCF-C-KIT interactions. Osteopontin (OPN) is an extracellular-matrix molecule produced by osteoblasts that is involved in bone remodeling. Nilsson et al have shown that OPN is enriched in the endosteal areas of BM. Furthermore, it was demonstrated that the role of OPN in the HSC niche is to negatively regulate proliferation. Opn KO mice have increased numbers of HSPCs that are more sensitive to 5-FU compared to wt. When wt HSPCs were transferred to Opn KO mice, the distribution within the marrow space was random, suggesting that OPN is needed for proper localization of HSPCs to the endosteal surfaces.

**Osteoclasts**

Another player at the endosteal niche is the bone-resorbing osteoclast. In a constantly ongoing process of bone remodeling, the osteoclasts degrade bone, while new is formed by the osteoblasts. In the resorption process, Ca$^{2+}$ ions are released. The concentration of Ca$^{2+}$ ions can locally be very high in the vicinity of actively resorbing osteoclasts. Ca$^{2+}$ ions bind to the Calcium-sensing receptor, which is expressed on HSCs and has shown to be crucial for localization of HSCs to the niche, likely mediated through binding to Collagen I deposited by osteoblasts and the CXCR4-CXCL12 pathway. Osteoclasts have also been shown to regulate HSC movement out of the BM through a mechanism that involves Cathepsin K mediated cleavage of CXCL12. Moreover, actively resorbing osteoclasts are needed to maintain HSC function, shown in experiments where mice were treated with bisphophonates—inhibitors of osteoclast function. Treated mice had fewer HSCs, and the bisphophonates could even override the effect of a Parathyroid hormone-induced HSC increase. These results demonstrate that active osteoclasts are important niche cells, both in themselves and by affecting other niche cells such as osteoblasts.

**Endothelial Cells**

Apart from endosteal areas, another possible location for the HSC niche is the perivascular area. Using the SLAM-markers in immunohistochemical stainings of BM sections, a majority of putative HSCs were shown to reside close to BM vasculature. Furthermore, sinusoidal endothelial cells are crucial for engraftment of transplanted donor HSCs in irradiated hosts. In a genetically modified
mouse, where endothelial cells are activated by constitutively active Akt-signalling. HSC numbers and function are increased, demonstrating an essential role of endothelial cells as HSC niche constituents. Activated endothelial cells also support in vitro growth of HSCs through activation of the Notch-pathway. 

However, the endosteal and perivascular niches might not be separated from each other. By intra-vital imaging of fluorescently labeled, purified HSCs in the BM cavity of Calvaria (skull bone), it was demonstrated that endosteally located HSCs also was in contact with vascular cells. Yet, conflicting data exists arguing for HSC localization rather to areas of the BM that are not well vascularized. It is also possible that HSCs are present in perivascular sites, simply because they are migrating in and out of BM and therefore occasionally found there. In this case, the perivascular areas may not be true niches in the sense that they control stem cell fate, but this is a question of big debate, and more research is needed to clarify this issue.

**Mesenchymal Cells**

Adherent cells from BM have long been known to have HSPC-supportive function in vitro. Fibroblast-like reticular cells with high expression of CXCL12 localize both to the endosteum and close to sinusoidal walls together with putative HSCs in BM. This was shown in a Cxcl12-GFP knock-in mouse, where CXCL12 expression is visualized by GFP in sections of the BM. The CXCL12-expressing cell population identified here is likely to be overlapping with the Nestin+ mesenchymal stem cells (MSC) recently identified in murine BM. Nestin+ MSCs are mostly perivascular and express high levels of many HSC regulatory factors like CXCL12, SCF and OPN. In addition, specific depletion of this cell type in vivo leads to reduced retention of HSCs in the BM, providing evidence that the Nestin+ MSCs are another important part of the HSC niche.

**Monocytes/Macrophages**

Cells of the monocyte/macrophage lineage also seem to be critical components in the BM HSC niche. In two different studies, it was recently shown that by depleting monocytes from the BM, CXCL12 levels are reduced and HSPCs migrate out of the BM. In one of the studies, it was demonstrated that loss of monocytes affected mainly the osteoblasts, while another group reported that Nestin+ MSCs were the most affected. The exact role of monocytes in the HSC niche therefore remains to be resolved, but it is clear that these cells are central for retention of HSCs in the BM.

**Adipocytes**

With age, the marrow space starts to fill up with fat (adipose tissue). Adipose tissue is also a feature of chemotherapy-treated BM, where adipocytes replace blood-forming marrow. It seems that adipocytic cells take part in HSC regulation in the niche in a negative manner. Naveiras et al compared HSC numbers and
function in different bones in mice, containing more or less fat (tail vertebrae and thoracic vertebrae respectively). The authors found that in bones with more adipose tissue, the hematopoietic activity was the lowest. By depleting adipocytes using a transgenic mouse or by a chemical method, it was shown that HSCs engraft better in recipients with absence of fat in the BM. \textsuperscript{124} It is also known that HSCs, although increasing in numbers, have less functional activity in old mice compared to young. It is possible that the reason for this is the increasing presence of adipose tissue with age.

**Dormant vs. Activated Niches**

At homeostasis, a fraction of HSCs is mostly non-dividing, quiescent or even dormant, while others are in a more proliferative state. It is therefore possible that there could be two separate niches, supporting either of these two states. It has been proposed that the osteoblastic endosteal niche constitutes the dormant niche, since several “retention factors” that also mediate quiescence of HSCs (e.g. TIE-2, TPO, SCF, CXCL12, OPN, see above) are expressed by osteoblasts. \textsuperscript{18} In normal hematopoietic turnover and in situations when the hematopoietic system is under challenge, such as bleedings, infections, or transplantation, some of these HSCs are activated and proliferate to give rise to progeny that can generate mature hematopoietic cells. Co-culture of HSCs with immortalized endothelial cells from BM leads to HSC-expansion, indicating that perhaps the vascular niche is the preferred activated niche. \textsuperscript{114} A perivascular location also provides faster access to the circulation, which may be crucial for a fast response to an external damage.

**Migration in and out of Niches**

HSCs are mostly resident within the BM during adult life, but occasionally enter the circulation. This was shown in experiments, where the circulation of two mice with different CD45 isoforms were joined surgically, a procedure called parabiosis. In the parabiotic mice it was shown that stable hematopoiesis was established in the opposite parabiont, demonstrating that HSCs had migrated out of their initial BM microenvironment, circulated and repopulated the other BM. \textsuperscript{125} However, these observations could theoretically be artifacts caused by surgery-induced inflammation. What is for certain is that HSCs can be experimentally induced to move out of their niche (mobilization) and back again (homing). Mobilization can be induced by administrating G-CSF or Cyclophosphamide, and these procedures are used clinically in order to harvest HSPCs for HSCT. Homing of HSCs takes place in transplantation settings but also during development when FL HSCs repopulate the BM. Key to these processes is the CXCL12-CXCR4 axis. Conventional KO animals for either Cxcl12 or Cxcr4 are embryonically lethal due to the inability to establish BM hematopoiesis as well as cardiovascular and neural defects. The migration of Cxcr4-null FL cells towards a CXCL12 gradient was reduced, suggesting that the migration towards the BM CXCL12 gradient during establishment of BM hematopoiesis is also regulated by this pathway. \textsuperscript{126-127}
Transplantation of Cxcr4null FL cells showed that reconstitution in irradiated adult recipients was indeed reduced but not completely abrogated. 128-129 Studying the steady-state roles of this pathway in HSC biology using conditional KO models for Cxcl12 or Cxcr4 has shown that the role for these molecules seems to be regulation of HSC proliferation as well as retention of HSCs in the BM. Conditional deletion of Cxcr4 or Cxcl12 leads to increased cycling, expansion of the HSC pool and increased frequencies of HSPCs in the circulation. 120,130-131 By treating human HSPCs with blocking antibodies against CXCR4, Peled et al demonstrated that homing in NOD/SCID mice was reduced. 132 Furthermore, using a CXCR4 antagonist, HSPCs are mobilized, confirming that the CXCR4-CXCL12 interaction is needed to keep HSCs in place. Pharmacological inhibition of CXCR4 can now be used clinically as a mobilization regimen. 133 Roundabout-4 was recently discovered to be a co-receptor for CXCR4 and genetic deletion of the gene Robo4 in mice leads to reduced HSC function and BM localization. 134 G-CSF-mediated mobilization is partly mediated via modulation of the CXCR4-CXCL12 interaction. But the mechanism by which HSCs are mobilized seems to be indirect, since the G-CSF receptor (G-CSF-R) is not required on HSPCs in order for them to be mobilized. 135 Recently it has been shown that a possible cellular target for G-CSF-induced mobilization is the macrophage. In a mouse model where only CD68+ macrophages expressed G-CSF-R, mobilization upon G-CSF treatment was intact, but disrupted when macrophages did not express the G-CSF-R. 136 In addition, G-CSF treatment reduces the abundance of monocytic cells in the BM, which in turn negatively affects osteoblasts and MSCs, leading to less CXCL12 secretion, thereby releasing stem cells to the circulation. 122,123 G-CSF-induced mobilization probably also involves the expansion of neutrophils and activation of proteases that cleaves several of the niche retention molecules, releasing HSCs. 137

G-CSF-induced mobilization is now also known to work via involvement of the sympathetic nervous system. Both osteoblasts and MSCs are innervated by sympathetic nerves that negatively regulate the activity of these cells. When G-CSF is administered, the sympathetic tone increases, leading to reduced CXCL12 secretion and HSC retention. 138

When HSCs circulate under physiologic conditions or in a transplantation setting, they need to move from the blood stream and enter the BM space. This process is dependent on expression of the adhesion molecules P-Selectin, E-selectin and VCAM-1 on BM sinusoidal endothelial cells. HSPCs express the receptors to interact with these adhesion molecules. CD162 and CD44 interact with the selectins, and various integrins, e.g. Integrin α4β1 binds to VCAM-1. P- and E-selectin mediate the initial rolling step where HSPCs are caught on the BM endothelium. Next, HSPCs adhere more firmly to the endothelium via integrins and can thereafter transmigrate through the endothelium and into the BM space.
Blocking the function of the adhesion receptors on HSPCs leads to reduced homing to the BM. 139-141

**A Niche for Malignant Hematopoiesis?**

LSCs are likely to share several properties with normal HSCs. It is therefore also possible that the LSCs can benefit from being located in the normal HSC niches. Alternatively, there are separate leukemic niches that may be induced by malignant cells, or the leukemic niches may appear as part of the transformation process. Supporting the existence of leukemic niches is the fact that donor-derived leukemia in allogeneically transplanted leukemia patients has been observed, although rarely. 142 The leukemic cells in the patients are eradicated and replaced by healthy HSCs from a donor. When a new leukemia develops, in donor-derived cells, this could be due to signals from the new niche that supports leukemic development. In mice with deficiency in the genes for Retinoic acid receptor γ (Rarg) or Retinoblastoma (Rb1), a myeloproliferative-like disease appears. The disease is recapitulated when normal BM is transplanted into recipients with the gene deletions, clearly demonstrating the dependence of the microenvironment for development of the disease. 143-144 A similar situation occurs in genetically engineered mice with an osteoprogenitor dysfunction or deficiency in *Sbds* in osteoprogenitors, leading to development of myelodysplasia and leukemia. 101 Moreover, CXCR4 can be elevated in AML indicating that LSCs may use pathways that are overlapping with normal HSCs. 145 It has also been shown that LSCs, sorted from primary AML samples, home to endosteal areas of NOD/SCID/IL2rgnull recipients, further demonstrating that LSCs likely use similar niches as normal stem cells. 146

**Other Niches Than the BM HSC Niche**

The properties of HSC niches in the BM are beginning to be unraveled, but less is known about the characteristics of niches other than that in the BM where HSCs can be maintained. In the mouse, there is a small pool of HSCs present in the spleen. Morita et al showed that transplantable HSCs are present in the spleen, although at a lower frequency compared to BM. Spleen HSCs are interchanged in a parabiosis model to similar levels as HSCs in the BM, arguing that the spleen HSC pool is a permanent one and not merely an observation of circulating HSCs detected while passing by. Another prominent HSC residency is the FL. In the FL, HSCs are proliferating extensively in order to expand the hematopoietic organ. Moore and colleagues created stromal cell lines derived from FL that are able to maintain HSCs in vitro. 52 The gene expression profile of the most efficient cell line was determined and it is interesting to note that it expresses high levels of Pleiotrophin and Prostaglandin-associated genes 147, factors that have recently been shown to mediate HSC in vitro expansion. 85,148 Along the same line, Zhang et al isolated cells from FL that were also HSC-supportive in culture. These cells had a CD3+ Ter119+ cell-surface phenotype and expressed high levels of
Angiopoietin-like proteins. Both the spleen and liver are highly vascularized organs, and it is therefore probable that perivascular areas are part of the HSC niches in these organs. However, the more exact cellular identity of non-BM HSC niches remains to be shown.

**Hypoxia**

All multicellular animals are dependent on a continuous oxygen supply to survive. Efficient energy production is maintained by oxidative phosphorylation in the mitochondria, where glucose is the substrate and O₂ acts as the terminal electron acceptor. A reduced oxygen supply can lead to hypoxia. The oxygen level in ambient air is approximately 21%, but few parts of the body are exposed to such high oxygen concentrations. The blood is oxygenated in the lung alveoli, where the level of oxygen reaches 13–16%. As the blood is transported through the body, oxygen levels decrease. Venous blood contains around 5% oxygen and in certain peripheral tissues, oxygen levels are probably substantially lower than that. It is difficult to define hypoxia with a specific oxygen concentration threshold value. According to some definitions, hypoxia occurs when oxygen levels are reaching levels below which biologic functions are altered. This level can vary between different cell types, but a commonly used cutoff value used in experimental settings is oxygen concentrations below 8–10 mmHg (~1%). Hypoxia is a normal physiologic state that can occur during embryogenesis. Before the onset of circulation, the only means of oxygen transport within the developing embryo is through diffusion, which is limited to about 150 μm. Hypoxia can also occur in a number of pathological settings, such as anemia, cardiovascular disease, and cancer.

**Hypoxia Inducible Factors**

Tissues and cells exposed to hypoxia respond by activation of Hypoxia-inducible factors (HIF). HIFs are heterodimeric transcription factors composed of two helix-loop-helix proteins, an α-subunit and a β-subunit. There are three different oxygen-sensitive α-subunits known, HIF-1α, HIF-2α, and HIF-3α. HIF-1α and HIF-2α form heterodimers with the same β-subunit, the Aryl hydrocarbon nuclear translocator (ARNT, HIF-1β). Much less is known about HIF-3α, but it is thought that it is not highly transcriptionally active but rather functions as a negative regulator of the other α-subunits. HIF-1β is constitutively expressed, whereas the HIF-α subunits are regulated by oxygen levels at the post-transcriptional level. The N-terminal domain in the α-subunits contains the oxygen degradation domain (ODD). Whenever oxygen is available, proline residues in the ODD are hydroxylated by help of prolyl-hydroxylase containing enzymes (PHD). Hydroxylation leads to recognition by the von Hippel-Lindau (VHL) E3 ligase.
complex, resulting in ubiquitinylation followed by proteasomal degradation. In addition, hydroxylation occurs at an asparagine residue in the transactivation domain by Factor inhibiting HIF 1 (FIH1), thereby interfering with the transcriptional function. In this way, HIF activity is efficiently inhibited as long as oxygen is available. However, as the oxygen concentration decreases, hydroxylation is inhibited which leads to accumulation of HIF-α subunits that can dimerize with HIF-1β, recruit co-activators, and activate transcription by binding to hypoxia response elements (HRE) in the promoter regions of target genes (Figure 4). Apart from being regulated by oxygen availability, other pathways of HIF activation are known, e.g. SCF or activation of PI3K and MAPK signalling.

HIFs regulate a number of genes involved in adaptation of cells and tissues to the low-oxygen environment both systemically and locally. A response to systemic hypoxia caused by e.g. blood loss or high altitude is HIF-induced activation of EPO in the kidney. Increased EPO levels boost red cell production to increase the efficiency of oxygen delivery. At the local level, hypoxia can be counteracted by inducing vascularization/angiogenesis. HIFs drive this response by inducing genes like Vascular endothelial growth factor A (VEGFA) and Angiopoietin-1 and 2 (ANGPT1, ANGPT2) that mediate recruitment, growth and differentiation of vascular endothelial and smooth muscle cells to increase vascular supply. Cells in hypoxia also need to adapt to the low oxygen levels by switching to oxygen-independent metabolism. In this regard, HIFs activate transcription of genes involved in glycolysis, e.g. PDK1.

To identify unique or redundant genes for HIF-1α and HIF-2α, several studies have been performed where gene expression in response to hypoxia is measured in absence of one of the HIF-α subunits. Furthermore, studies have been performed in mice and ESCs where the Hif2a gene was knocked in to the locus of Hif1a. It appears that HIF-1α is the subunit that plays the bigger role in inducing genes in the hypoxic response. Several target genes overlap between the two subunits but HIF-1α mediates a greater deal of induction of e.g. glycolytic genes, while for example Pou5f1 (Oct3/4) is a HIF-2α unique target gene.

HIF-1α is abundantly expressed during development, while HIF-2α has been shown to have a more restricted expression pattern. Hif1a KO mice are embryonic lethal at day 11 of development due to a lack of vascularization, neural tube defects, and cardiovascular malformations. Most Hif2a KO strains are also embryonic lethal, but depending on strain background, some Hif2aKO mice are born. Defects reported in various Hif2a KO strains include placental defects, impaired heart rate regulation, vascular remodeling and lung development due to a lack of VEGFA. KO mice for Arnt is also embryonic lethal at day E10.5, and embryos have defective angiogenesis in the yolk sac and neural tube closure defects. The results from all these loss-of-function studies clearly show that hypoxia and HIF activity are crucial for normal development to take place.
The Hypoxic HSC Niche

In 1987, it was first postulated that the HSC niche in the BM is hypoxic, and that there is an oxygen gradient within the BM that correlates with the degree of immaturity, with HSCs at the lowest end of this gradient.\textsuperscript{180} This suggestion remained speculative for a long time, but lately, several indications that support this idea have emerged. Chow \textit{et al} used mathematical modeling to suggest that most hematopoietic progenitors experience very low oxygen levels in the BM.\textsuperscript{181} Furthermore, by measuring the oxygen concentration in BM aspirates of healthy volunteers, it was shown that the partial pressure of oxygen was significantly lower in BM compared to blood.\textsuperscript{182} However, this type of measurement only roughly estimates the oxygen level in the BM as a whole and is not taking into account that different subregions of the BM can differ in oxygen concentrations. Further information regarding this was provided by Levesque \textit{et al} that used \textit{in situ} staining with Pimonidazole within BM sections from mouse. Pimonidazole is
injected in mice before sacrifice, and at sites where the oxygen concentration is below 10 mmHg, this compound will react and form stable epitopes that can later be detected by antibodies. This study demonstrated that the areas within the BM that were the most Pimonidazole positive were the endosteal areas. After G-CSF treatment, Pimonidazole positive areas expanded towards the central BM, indicating that hypoxia is involved in regulation of the mobilization response. 183

Another study using Pimonidazole instead showed that long-label retaining cells that were highly Pimonidazole positive localized to sinusoidal areas. 184 By FACS analysis, Parmar et al showed that Pimonidazole staining correlated with the side-population phenotype of HSCs. In the same study, it was also demonstrated that subpopulations of BM that were not well perfused contained the highest HSC activity measured by transplantation assays. This was shown by injecting mice with the dye Hoescht33342 prior to BM harvest and functional assessment. Theoretically, the dye will distribute in tissues depending on the level of blood perfusion and therefore simulates oxygen distribution. 185 The same approach was used in a study that combined phenotypic HSC staining and Hoescht33342 perfusion followed by functional assessment. Here, it was demonstrated that LT-HSCs defined by the LSK CD150^+ CD41^- CD48^- cell surface phenotype divided into two distinct functional groups based on Hoescht33342 staining. Notably, purified HSCs with a lower Hoescht uptake showed higher reconstitution potential in serial transplantations, compared to phenotypically identical HSCs with slightly higher Hoescht uptake. 118 In a similar manner, by fractionating BM cells based on the level of reactive oxygen species (ROS), it was shown that populations containing lower levels of ROS had higher in vivo reconstitution potential. 186 This study assumed that oxygen concentrations and ROS levels correlate, since ROS are formed as a consequence of oxygen-dependent reactions. The hypoxic location of HSCs in the BM is also reflected by the propensity of HSCs to use cytosolic glycolysis rather than mitochondrial oxidative phosphorylation for energy production. Simsek et al measured the relative amount of mitochondria in different BM subpopulations and could demonstrate a clear relationship between primitiveness and decreasing amounts of mitochondria. 187

The Role of HIFs in Hematopoiesis and HSCs

As mentioned above, several developmental defects are detected in HIF KO mice. Therefore it has been difficult to investigate the role of HIFs specifically in embryonic hematopoiesis, but especially the Arnt KO mice display clear hematopoietic defects during development. 188 Using Hif1a KO ESC blastocyst complementation or conditional deletion of Hif1a, it has been shown that HIF-1α is important in B-cell development and differentiation, and function of myeloid cells recruited to hypoxic sites of inflammation. 189,190 A recent investigation using a conditional KO mouse for Hif1a with Mx1-Cre-mediated deletion demonstrated a major role for this gene in adult HSC function in vivo. The deletion was induced
in adult mice, and shortly after deletion, hematopoiesis seemed unaffected. In CRA however, contribution of Hif1a-deleted cells first increased while after extended time periods, the engraftment levels dropped. In secondary transplantations, the contribution of Hif1a-deficient cells was severely reduced. The authors showed that absence of Hif1a expression leads to increased cycling and HSC exhaustion that could be rescued by suppression of Ink4a/Arf cell cycle inhibitory genes through Bmi1 overexpression. Hif1a-deleted mice also showed a decrease in numbers of phenotypically defined HSCs with age. By deletion of Vhl in BM previously transferred to wt recipients, the frequency of LT-HSCs increased while their function was severely impaired. At the same time, Vhlnull HSCs had substantially lower numbers of HSCs in active cell cycle as measured by DNA/Ki67 staining. This shows that overactivation of HIF-1α impairs HSC proliferation and reconstitution ability. By co-deletion of Hif1a and Vhl however, HSC function and proliferation was restored to normal.

By keeping Hif2a KO mice on a mixed background (129 and B6), embryonic lethality can partially be avoided, and it was therefore possible to analyze the role of HIF-2α in adult hematopoiesis. It was shown that Hif2anull mice were cytopenic with defectve hematopoiesis. Although only ST-HSCs were analyzed in transplantation assays, it was clear that the hematopoietic defects were related to the microenvironment. By transferring Hif2a KO BM to wt mice, the hematopoietic defect was normalized. On the contrary, by performing the reverse transplantation with wt BM transfer to Hif2a KO recipients, the hematopoietic defects were recapitulated.

**What Is the Function of the Hypoxic HSC Niche?**

The study by Takubo et al clearly shows that control over proliferation via HIF-1α is a critical aspect of the hypoxic HSC niche. A relative quiescence is thought to protect HSCs from replication-associated mutations. Furthermore, many studies have shown that disrupting quiescence leads to decreased HSC function and exhaustion of the HSC compartment over time. That HSCs reside in hypoxic niches, with a low metabolic activity, fits well in this picture. It has been suggested that hypoxic niches are the preferential niches for dormant HSCs. In this regard, it is interesting to note that both low perfusion level and dormancy selects for LT-HSCs with superior self-renewal in highly purified HSC populations previously thought to be virtually heterogeneous.

Protection from ROS might be another central function of hypoxia. Low ROS levels correlate with HSC function and it is clear that elevated levels of ROS have detrimental effects on cell survival. Disruption of the Atm gene in mice results in elevated ROS levels with reduced HSC function as a consequence. These defects can be reversed by the antioxidant N-acetylcyestein. Furthermore, the FOXO proteins are responsible for counteracting ROS-production in cells and when the genes for all three murine FOXO genes, Foxo1, Foxo3 and Foxo4 are deleted,
HSCs are compromised. Nevertheless, the relationship between oxygen concentration and levels of ROS may not be as simple as it seems. Although counterintuitive, it appears that ROS can form even when oxygen is limited, perhaps due to the fact that the electron transport chain in mitochondria requires oxygen for the reactions to be completed. Since oxygen is the terminal electron acceptor, a lack of oxygen might leave reactive compounds behind. Besides, it has been shown that ROS produced in mitochondria is even required for HIF activity. It is therefore possible that moderate levels of ROS may be needed for HIF signalling in HSCs but that elevated levels are detrimental.

Given the large number of genes known to be hypoxia regulated, it is highly probable that hypoxia and HIF-activity play additional roles in HSCs, via induction of other genes. Even though direct evidence for HIF regulation in HSCs might not exist, several genes expressed in HSCs are known to be hypoxia induced in other cell types. One example is the ATP binding cassette transporter ABCG2, expressed on HSCs and used in the side population HSC phenotype, shown to be induced by both HIF-1 and HIF-2. Furthermore, hypoxia can induce Cxcl12 and Cxcr4 involved in HSPC migration as well as other stem-cell associated genes like NOTCH1 and KIT. A classical hypoxia-controlled gene is VEGFA with known implications in HSC biology (discussed further below, and Papers II and III).

**HSPCs and In Vitro Hypoxia**

Several studies have shown that various hematopoietic progenitors, both murine and human, can be increased by incubation under reduced oxygen concentrations. These studies typically measured CFU formation or short-term engraftment after different time periods of hypoxic treatment of unfractionated hematopoietic populations. Danet et al have demonstrated that human Lin− CD34+ CD38− BM cells expanded after a 4 day culture period in 1.5% oxygen as measured by SCID repopulation. This was accompanied by HIF-1α stabilization and upregulation of VEGFA protein and the cell surface receptors for VEGFA, VEGFR1 and VEGFR2, as well as angiopoietin receptors TIE-1 and TIE-2. Notably, the fraction of CD34+ cells in the G0 stage of the cell cycle decreased in hypoxia, although the G1 and G0 phases together increased. A similar study used cord blood CD34+ cells incubated in hypoxia for 6 days, followed by transplantation into NOD/SCID/IL2rgnull mice. With this setup, engraftment levels were not different from normoxia-treated cells if the whole fraction of cultured cells was transplanted. However, by transplanting equal numbers of cells, counted after the culture period, the hypoxia-treated cells showed higher engraftment. In essence, these results are correlating with the results from the study by Danet et al, since equal cell numbers after the culture were transplanted in that study. However, Shima et al observed increased fractions of Ki67+ cells accompanied by increased expression of the cell cycle inhibitory genes CDKN1A and CDKN1C.
We have achieved similar results using purified murine HSPCs (Paper I), discussed in a later section of this thesis.

**Hypoxia in Other Stem Cell Systems**

Hypoxia may be key to maintain the undifferentiated state of stem cells in general. Human ESCs cultured under low oxygen conditions were shown to proliferate at similar rates as cells grown in normoxia but with significantly fewer areas of differentiation.\(^{211}\) Similarly, differentiation of both myogenic and neural stem cell lines is inhibited in hypoxia, via a mechanism involving HIF1-Notch interaction.\(^{212}\) Hypoxia-induced \textit{NOTCH1} also promotes de-differentiation in neuroblastoma cells.\(^{201}\) Mazumdar \textit{et al} recently showed that the subgranular zone of hippocampus, a neural stem cell niche, is positive for Pimonidazole staining, indicating hypoxia. In this study, HIF-1 activity was shown to control neural stem cells via Wnt/β-catenin under hypoxia.\(^{213}\) Interestingly, \textit{Oct3/4} seems to be a HIF-2α specific target\(^{171}\) gene and is one of the 3–4 factors used to reprogram somatic cells to induced pluripotent stem cells. Other reprogramming factors have also been shown to be induced in hypoxia and are suggested to promote a stem-like state in cancer cell lines.\(^{214}\)

**Vascular Endothelial Growth Factor**

Vascular endothelial growth factor A (VEGFA) is a major inducer of angiogenesis, and it can bind to the receptors VEGF receptor 1 (VEGFR1, FLT1) and VEGF receptor 2 (VEGFR2, KDR). By binding to VEGFR2 on endothelial cells, it mediates recruitment, survival, growth, and differentiation of vascular endothelial cells. This leads to formation of new blood vessels (angiogenesis) or branching of already existing ones (vasculogenesis), but VEGFA can also promote vascular permeability and vasodilation.\(^{215}\) \textit{Vegfa} deficiency results in embryonic lethality at midgestation in mice. Notably, even when one \textit{Vegfa} allele is present in \textit{Vegfa}\(^{−/−}\) embryos, this cannot rescue the lethal phenotype. \textit{Vegfa} KO embryos have severe defects in angiogenesis and yolk sac blood island formation.\(^{216,217}\) Hypoxia occurring as the embryo grows past diffusion distances, is believed to drive these processes, and \textit{Vegfa} is a well established hypoxia/HIF-regulated gene. In reporter assays using co-transfection of a 5-prime sequence from the human VEGFA promoter together with HIF-1α expressing vectors, direct evidence for HIF-regulation of VEGFA expression has been provided.\(^{166}\) Interestingly, hypoxic induction of \textit{Vegfa} can also take place in a HIF-independent manner as shown in muscle cells and is in this case mediated by PGC-1α.\(^{218}\) Non-hypoxic inducers of VEGFA mRNA or protein release include Estrogen\(^{219}\) and various cytokines e.g. Interleukin-6.\(^{220}\) VEGFA not only regulates blood vessel development but has also been shown to be involved in endochondral ossification and ovarian development.\(^{215}\)
HIF-induced $VEGFA$ expression is a major event in tumor growth. As tumors grow beyond oxygen supply they become dependent on outgrowth of new blood vessels. Oncogenic signalling may also induce HIF and VEGFA independent of hypoxia, which is usually a sign of especially malignant tumors. As a means to inhibit tumor vascularization, VEGFA-inhibitors are now used clinically as anti-cancer therapy. 221

*Vegfa and HSCs*

Impaired formation of blood islands were among the defects discovered in $Vegfa$ deficient mice, indicating a role for VEGFA in developmental hematopoiesis. VEGFA has a clear role also in adult hematopoiesis as shown by conditional deletion of the $Vegfa$ gene in adult mice. $Vegfa$-deficient BM could not provide radioprotection when transplanted to lethally irradiated wt recipients. Nor did $Vegfa$-deleted cells contribute to engraftment in CRA. The fact that HSC engraftment of $Vegfa$ deficient BM cells was not possible, despite the presence of VEGFA in the microenvironment of wt recipients, shows that the requirement for VEGFA in hematopoietic cells is intrinsic. In line with this, engraftment could be restored when VEGFA was overexpressed in $Vegfa$-deficient BM cells by a retroviral vector. Rescue was provided also with overexpression of Placental derived growth factor (PLGF), a VEGF-related ligand, binding selectively to VEGFR1, suggesting that both VEGFR1 and VEGFR2 signalling is active in hematopoietic cells. CFU formation was also abrogated in $Vegfa$ deleted cells and the CFU inhibitory effect could be mimicked in wt cells by adding soluble small-molecule blockers of VEGFA signalling. Remarkably, the same inhibitory effect was not achieved by using an antibody that inhibits VEGFR only at the cell surface. The authors concluded that VEGFA impacts HSCs survival via a private, autocrine loop mechanism. 222

Even though VEGFA seems to be most important as an intrinsic factor in HSC survival, VEGFA may also play a role in both the osteoblastic and vascular niches. VEGFA is a regulator of endochondral ossification during development since conditional, tissue specific KO of $Vegfa$ in chondrocytes impaired bone growth. 223 In young mice, VEGFA antibody-blocking experiments also lead to impairment of this process. 224 Furthermore, the formation of ectopic niches via endochondral ossification has been shown to be dependent on VEGFA. Chan *et al* isolated skeletal progenitors from developing fetal bones and transplanted these under the kidney capsule of adult mice. The skeletal progenitors formed ectopic bone through a cartilage intermediate (endochondral ossification), that was able to recruit host BM cells, including phenotypic HSCs. By suppressing VEGFA activity, the ectopic niche formation was inhibited. In addition, ectopic niches did not form when skeletal progenitors were isolated from bones that do not develop via cartilage. 225 Finally, HSC engraftment after irradiation has been shown to be
dependent on VEGFR2-expressing sinusoidal endothelial cells in the BM, arguing that VEGFA signalling is important also in the vascular niche. 113
Present Investigation

The BM HSC niche is characterized by low oxygen levels, and hypoxia may regulate HSC quiescence, metabolism, and ROS status in vivo. Additional roles of hypoxia and HIF-induced gene expression in HSC biology remain elusive. To further delineate the function of the hypoxic HSC niche, the general aim of these studies has been to investigate the effect of hypoxia and HIF-induced gene expression in HSC biology. Specifically, we wanted to investigate the consequences of in vitro hypoxic treatment of HSPCs, HIF-gain-of-function, and the role of hypoxia-induced Vegfa expression in HSCs in vivo.

Paper I

Aims
Hypoxic in vitro treatment of HSPC populations affects their proliferation and differentiation. Studies on human BM or cord blood cells showed that HSC-enriched populations were maintained and/or expanded in hypoxia. However, similar studies previously performed on murine cells used unfractionated or only partly HSC-purified populations and mainly assessed progenitor function. To further investigate the role of hypoxia in HSC biology, our aim was to investigate how hypoxic culture conditions affect murine HSPC proliferation and HSC function measured by in vivo engraftment. Furthermore, we wanted to investigate the molecular mechanisms behind the hypoxic effect with specific focus on HIF-1.

Summary
Culture of LSK cells under hypoxic conditions (1% O₂) for 4 days resulted in significantly lower cell numbers at the end of the culture period compared to normoxia (21% O₂). This was accompanied by reduced proliferation of HSCs since purified LT-HSCs, with the LSK CD34⁺ FLT3⁺ cell surface phenotype, displayed increased label retention and an increased fraction of cells in the non-cycling, G₀ phase of the cell cycle. These results show that low-oxygen conditions favor quiescence of HSCs. When HSC function of the complete fraction of cultured cells was assessed in CRA, the reconstitution capacity of hypoxia-cultured HSCs were shown to be similar to that of cells cultured in normoxia. This means that hypoxic conditions did not result in increased HSC numbers compared
to initially seeded numbers. However, since the hypoxia-treated cultures resulted in fewer cells, engraftment on a per-cell basis was increased compared to normoxia. Hypoxic treatment resulted in increased expression of the genes coding for the CDKIs p21 (Cdkn1a), p27 (Cdkn1b), and p57 (Cdkn1c) in LSK cells. However, in LSK CD34+ FLT3− cells, only Cdkn1a was increased in hypoxia. To investigate whether the hypoxia-generated CDKI gene-expression changes were mediated through HIF-1, we carried out knock-down experiments utilizing shRNA against HIF-1α in LSK cells. We observed that Cdkn1b and Cdkn1c expression was reduced both in normoxia and hypoxia, in HIF-1α shRNA expressing cells, while Cdkn1a was not affected. To investigate whether HIF gain-of-function could mimic the effect of hypoxia, we carried out experiments where HSPCs were treated with a HIF-stabilizing agent or transduced with a retroviral vector overexpressing a constitutively active form of HIF-1α (ca HIF-1α). These experiments revealed that HIF gain-of-function, similarly to hypoxic culture conditions, reduced growth and proliferation of HSPC populations. Consistent with hypoxic pretreatment, transient HIF-activation resulted in preserved engraftment capacity in CRA. However, persistent HIF-activation in caHIF-1α-transduced cells resulted in reduced reconstitution ability in vivo. This result is likely due to the negative effect on proliferation and not caused by apoptosis, since caHIF-1α overexpression in the anti-apoptotic BCL2 background did not rescue the engraftment deficiency.

Conclusions and Points of Discussion

In conclusion, we show that hypoxic culture conditions maintain HSC function and favor quiescence in HSPCs via both HIF-1α dependent and independent mechanisms.

Our results are in agreement with the findings by Takubo et al.191 showing that HIF-1α monitors HSC quiescence and function in vivo as shown in the Hif1a conditional KO mouse. Nonetheless, even though hypoxia/HIF-mediated quiescence and control over proliferation is crucial in HSCs, normoxic/constitutive activation of HIF-1α has negative effects on HSC function. This conclusion can be drawn from the experiments using caHIF-1α overexpressing HSPCs in our study, consistent with the findings by Takubo et al where Vhl KO HSCs showed impaired reconstitution. The reason for these observations is probably that when the HIF-mediated proliferation block is not released in cells further down the hematopoietic hierarchy, multipotent differentiation is not possible since extensive cell division must be allowed as maturation proceeds. This further argues for a HSC-specific role of hypoxia since control over proliferation is important to maintain HSC quiescence, while committed cells must be allowed to divide in order to generate mature blood cells.
Previous studies using hypoxic culture of human HSPCs have concordantly to our results shown reduced proliferation and maintenance of engraftment capacity over the culture period. Although Danet et al claimed expansion of SRCs in hypoxia, this conclusion was drawn based on transplantation of equal numbers of cells from normoxia and hypoxia, counted after the culture period. Shima et al showed that the outcome differed whether the complete fraction of cultured cells or an equal number of cells were transplanted. Similarly, we show that engraftment is preserved compared to normoxia when transplanting all cells evolving in the hypoxic culture, while on a per-cell basis, hypoxia actually increases reconstitution compared to normoxia-treated cells. Another point of controversy is that Danet et al observed decreased fractions of cells in the cell cycle G0 phase in hypoxia, while we and others rather see that hypoxic HSPCs increase the relative amount of cells in G0.

**Future Directions**

Hypoxia may not be an efficient method to *ex vivo* expand HSCs over the input numbers. On the other hand, hypoxic culture conditions resembles the *in vivo* conditions better than culture at ambient air and may therefore be an alternative to be explored in combination with other factors used for HSC expansion. Recently, the compound StemRegenin was shown to be able to induce *ex vivo* expansion of cord blood SRCs. Interestingly, StemRegenin is an antagonist of HIF-1β. The exact mechanism behind the HSC-promoting effect of this compound is not known, but it is of course highly interesting in the context of hypoxia, and future research should provide insight into this.

We have shown that HSCs are affected by hypoxia *in vitro* and by ectopically activated HIF. *In vivo* however, the hypoxic environment is not isolated to hematopoietic cells but likely also affects surrounding cells. Given the many different cell types involved in HSC regulation in the niche, it will be important to delineate how hypoxia and HIF activity affect also these cells. Hypoxic regulation of niche cells is likely to have important indirect implications for HSC support.

The hypoxic HSC niche regulates normal HSC function. It is therefore plausible that also LSCs can be affected by low oxygen concentrations. Since hypoxia and HIF induce quiescence in normal HSCs, LSCs may be affected in the same way. If this is true, this has major implications for therapy, since non-cycling cells may escape chemotherapy. A recent paper suggests that HIF is indeed involved in LSC maintenance. Wang et al show that the HIF-inhibitor Echinomycin is able to eliminate LSCs in a mouse model of lymphoma and human AML. Research is needed to further investigate the relevance of hypoxia and HIF-induced gene expression in LSCs and leukemogenesis.
Papers II and III

Aims
The hypoxic HSC niche monitors quiescence through induction of cell-cycle inhibitory genes in HSCs. Hypoxia may also promote low metabolic activity and protect HSCs from damage caused by ROS to preserve HSCs throughout life. We hypothesized that an additional function of the hypoxic niche is to provide HSCs with VEGFA, a previously identified HSC-survival factor. Our aim was to investigate whether hypoxia and HIF activity control expression of Vegfa in HSCs, and whether this has any importance to HSC outcome.

Summary
To investigate the role of hypoxia-regulated Vegfa expression in HSCs, we characterized the hematopoietic phenotype in the genetically modified mouse Vegfaδ/δ, where the HRE in the Vegfa promoter is lacking. Vegfa expression in LT-HSCs from Vegfaδ/δ mice was significantly lower compared to wt LT-HSCs. This finding demonstrates that LT-HSCs reside in hypoxic niches in vivo where Vegfa expression is upregulated. In adult Vegfaδ/δ mice we observed that erythropoiesis was slightly impaired, resulting in anemia. In Vegfaδ/δ fetuses, erythropoiesis was more severely affected signified by pale embryos and reduced numbers of developing erythrocytes in the FL. Defined by cell surface phenotype, HSPC frequencies were increased in Vegfaδ/δ FL and BM, but when comparing total numbers of HSPCs, no significant differences were observed. When HSC function was assessed in CRA, we observed that BM HSCs lacking hypoxia-induced Vegfa had impaired engraftment. In a limiting-dilution assay, the numbers of CRUs was determined to be approximately 3 times lower in Vegfaδ/δ BM compared to wt. However, HSCs from FL showed normal reconstitution capacity in CRA. Furthermore, we observed a defect in endochondral bone formation in Vegfaδ/δ mice. Finally, the occurrence of embryonic lethality caused by an inability to induce Vegfa in hypoxia was determined to take place mainly between day 15.5 of development and birth.

Conclusions and Points of Discussion
In conclusion, we show that LT-HSCs induce Vegfa expression as a consequence of hypoxia, supporting the existence of hypoxic HSC niches in the BM. Hypoxic Vegfa expression regulates adult, but not fetal HSC function. Finally, hypoxic Vegfa expression is involved in both FL and adult erythropoiesis as well as in endochondral ossification of growing bones.

Although an important role of Vegfa in HSC survival has already been established, we demonstrate for the first time that specifically hypoxia is a major upstream
inducer of this factor in HSCs. Moreover, we confirmed that Vegfa is mainly acting intrinsically to HSCs since the HSC functional defect was evident despite the presence of VEGFA in the BM of wt recipients in our experiments.

The approach to delete a regulatory element in a promoter and not the complete gene in an experimental mouse model is rather uncommon. It is interesting that this “minor” modification has such big impact on survival and phenotype of surviving mice. It illustrates the major impact of the hypoxia-Vegfa axis in physiology in general since many different systems apparently are affected by disrupting it (HSCs, erythropoiesis, bone formation, sympathetic nervous system etc.). Still, the global presence of this mutation during development in the Vegfa$^{δ/δ}$ mouse may blur its effect in specific tissues since selection for individuals with a milder phenotype can take place. It is therefore intriguing to speculate that if the Vegfa$^{δ/δ}$ mutation would have been restricted in space and time in a conditional setup, the effect on hematopoiesis would have been much stronger.

**Future Directions**

The mechanism behind the erythropoietic defect in Vegfa$^{δ/δ}$ mice was not deeply investigated. It would be informative to determine whether hypoxic Vegfa has an intrinsic or extrinsic effect on erythropoiesis. To investigate this, FACS analysis for erythropoietic development could be performed in the hematopoietic chimeras, where donor and competitor derived erythrocytes can be measured separately. If erythrocytes in the chimeras are mainly from the competitor wt cells, this would argue for an intrinsic role of hypoxic VEGFA. Conversely, if Vegfa$^{δ/δ}$ erythropoietic development is rescued when in a wt environment, this would argue for an extrinsic effect. Furthermore, since the macrophage is a cell type that constitutes the erythroblastic islands, it would be interesting to investigate whether macrophage numbers are decreased in FL and BM of Vegfa$^{δ/δ}$ mice. If hypoxic VEGFA regulates erythoblastic island macrophages, a decreased abundance of these cells should be observed. Furthermore, measurements of Vegfa mRNA levels within developing erythrocytes and macrophages should be informative.

Processen där stamceller självförnyas och blir till mogna celler styrs av gener och proteiner inuti cellerna, som i sin tur kan påverkas av andra celler eller faktorer i närheten av stamcellerna i benmärgen. Syrehalten inuti benmärgen varierar och blodstamceller sitter troligen på de ställen där syrehalten är relativt låg (hypoxia) och påverkas därför av Hypoxia Inducible Factor (HIF). Vi har undersökt hur hypoxi och gener som aktiveras av HIF kan påverka blodstamceller på olika sätt. Eftersom människans biologi är mycket lik den i andra däggdjur, har vi använt stamceller från möss som en modell för att studera detta.

För att undersöka hur hypoxi påverkar blodbildande stamceller har vi isolerat stamceller från benmärgen i möss och därefter odlat dem i en syrefattig miljö. Vi har funnit att dessa celler delar sig långsammare. Detsamma sker när aktiviteten av HIF ökar utöver det vanliga vilket vi åstadkommit genom att överföra HIF-genen till blodceller med hjälp av ett virus. Viruset är modifierat på så sätt att det inte kan orsaka en infektion som sprider sig utan endast kan gå in i celler och leverera HIF-genen och sedan inte föröka sig ytterligare. En ökad HIF-aktivitet i blodstamceller
sätter igång uttryck av gener som förhindrar celldelning. I benmärgen kan hypoxi därför vara ett sätt att minska celldelning hos blodstamceller, något som anses viktigt för att upprätthålla en kontrollerad blodbildning, utan att cancer uppstår. Vi har också studerat VEGF, en annan gen som aktiveras av HIF. I en muterad musstam som inte uttrycker VEGF till följd av hypoxi fungerar stamcellerna sämre. Detta betyder att VEGF i normala fall är viktigt i den hypoxiska benmärgen för att stamcellerna ska kunna fungera. Sammantaget verkar det som att syrefattiga delar av benmärgen påverkar blodbildande stamceller på flera sätt. Olika gener som förhindrar celldelning samt påverkar cellens överlevnad och funktion aktiveras.
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