Aus der Medizinischen Klinik mit Schwerpunkt Hämatologie, Onkologie und Tumorimmunologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

# Overexpression of HoxA9 in CD150- hematopoietic stem cells leads to rapid progression of acute myeloid leukemia

zur Erlangung des akademischen Grades
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von

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# 1. Abbreviations

| | |
|---|---|
| µg | micrograms |
| µL | microliters |
| µm | micrometer |
| AML | acute myeloid leukemia |
| BM | bone marrow |
| BMCM | bone marrow culture medium |
| BSA | bovine serum albumin |
| Bala | balanced |
| $CD150^{neg}$ | HCS that were sorted for CD150- |
| $CD150^{neg}$/HoxA9 | HCS that were sorted for CD150- and transduced with a retroviral vector that overexpresses HoxA9 |
| $CD150^{pos}$ | HCS that were sorted for CD150+ |
| $CD150^{pos}$/HoxA9 | HCS that were sorted for CD150+ and transduced with a retroviral vector that overexpresses HoxA9 |
| HSC | hematopoetic stem cell |
| FAB | French-American-British |
| Fig | Figure |
| FACS | Fluorescence activated flow cytometry |
| HOX | homeobox gene |
| dH2O | double distilled water |
| DMEM | Dulbecco's modified Eagle's medium |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| GFP | green fluorescent protein |
| *et al.*(*lat.*) | *et alii*; and others |
| g | gram |
| LB medium | lysogeny broth medium |
| Ly-bi | lymphoid-biased |
| mAb | monoclonal antibody |
| mg | milligram |
| min | minute |

| | |
|---|---|
| mL | milliliter |
| mM | millimolar |
| My-bi | myeloid-biased |
| ng | nanogram |
| PBS | phosphate buffered saline |
| pH | negative decimal logarithm of the hydrogen ion activity in a solution |
| PI | propidium iodide |
| RCF(*g*) | relative centrifugal force or g-force |
| SD | standard deviation |
| vs. | versus |
| WHO | World Health Organization |

# 2. Abstract

## 2.1 Abstract in English

**Introduction:** The hematopoietic stem cell (HCS) compartment does not represent a homogenous group of cells but instead consists of 3 subsets termed myeloid-biased (My-bi), lymphoid-biased (Ly-bi), and balanced (Bala) HSCs. Each of these possess a distinct pre-programmed proliferation and differentiation behavior, which helps us to determine if only 1 of these subsets contains cells that induce acute myeloid leukemia (AML).

**Method:** Highly purified HSCs were separated into 2 different subsets Ly-bi (CD150-) and My-bi (CD150+) using fluorescence-activated cell sorting. The sorted cells were transduced with a retroviral vector expressing HoxA9, a gene known to induce AML, and injected into mice (primary hosts). Blood samples were taken regularly and analyzed in terms of leukemic behavior. After euthanizing the mice, spleen and bone marrow were analyzed, and whole bone marrow cells from these mice were serially transplanted into naive mice (secondary hosts). In line with the previous experiments, the leukemic behavior was again examined using blood analysis. Finally, whole bone marrow and spleen from these secondary hosts was transplanted into new tertiary hosts, and the time of survival of these mice was determined.

**Results:** Mice injected with Ly-bi HSCs expressing HoxA9 displayed a shift of differentiation towards the myeloid lineage and a high percentage of Mac-1 positive cells, which is typical for AML. Additionally, typical signs of leukemia were observed in 70% of the primary hosts, which was characterized by an enlarged spleen. After serial transplantation, the development of leukemia became more aggressive as indicated by increased spleen weight and the loss of blood cell markers. The tertiary host mice died within 4 weeks of transplantation. In contrast, mice injected with My-bi HSCs expressing HoxA9 showed no signs of leukemia in primary host mice and lower levels of HoxA9 expression in bone marrow and spleen. Transduced cells did not differentiate towards the myeloid lineage and did not behave like those of AML. Only after bone marrow serial transplantation were differentiation shifts towards the

myeloid lineage and enlarged spleens observed in these mice. Strikingly, the tertiary host mice survived up to 5 months.

**Conclusion:** Important variations in the onset of primary AML and survival were observed between mice that received Ly-bi and My-bi HSCs when overexpressing HoxA9. Mice that received the transduced subset Ly-bi showed an early onset and a fulminant progress of AML. In contrast to this, mice injected with My-bi HSCs showed a more gradual onset of disease and a longer time of survival.

## 2.2 Abstract in German

**Einleitung:** Das hämatopoetische Stammzellenkompartiment besteht nicht, wie lange angenommen, aus einer homogenen Masse, sondern aus drei verschieden Stammzelluntergruppen: myeloid-biased (My-bi), lymphoid-biased (Ly-bi) und balanced (Bala). Aus dieser Erkenntnis ergeben sich neue Fragestellungen und Antwortmöglichkeiten. In dieser Arbeit wird die Frage behandelt, ob nur eine dieser Untergruppen die Zellen enthält, die eine Akute Myeloische Leukämie (AML) auslösen.

**Methodik:** Zunächst wurden hochreine hämatopoetische Stammzellen in My-bi Stammzellen (CD150+) und Ly-bi Stammzellen (CD150-) unterteilt. Die sortierten Zellen wurden anschließend mit einem HoxA9-exprimierenden retroviralen Vektor (einem AML auslösenden Gen) transduziert und in Mäuse (primäres Wirtstier) injiziert. Bei regelmäßigen Blutkontrollen wurden diese auf leukämisches Verhalten überprüft. Nach dem Töten der Mäuse wurden die Milz und das Knochenmark untersucht, zusätzlich wurden Knochenmarkszellen in neue Mäuse (sekundäres Wirtstier) transplantiert. Bei diesen Mäusen wurde, wie beim ersten Experiment, das leukämische Verhalten mit Hilfe von Bluttests kontrolliert. Schließlich wurden Milz- und Knochenmarkszellen dieser Mäuse in tertiäre Wirtstiere transplantiert und es wurde die Überlebenszeit bestimmt.

**Ergebnisse:** Mäuse, die HoxA9-überproduzierende Ly-bi Stammzellen erhalten haben, zeigten eine Verschiebung der Differenzierung hin zur myeloischen Reihe und eine große Anzahl Mac-1-positiver Zellen in Blut, Milz und Knochenmark. Dieses

Verhalten ist typisch für die AML. Des Weiteren zeigten 70 % der primären Wirtsmäuse weitere typische Anzeichen von Leukämie, unter anderem eine deutlich vergrößerte Milz. Nach der seriellen Transplantation von Knochenmarkszellen in die sekundären Wirtsmäuse wurde die Leukämie aggressiver, was durch stark vergrößerte Milzen und den Verlust von Blutzellmarkern erkennbar wurde. Die tertiären Mäuse starben alle innerhalb von 4 Wochen. Im Gegensatz dazu traten bei den primären Mäusen, die My-bi-Stammzellen mit HoxA9-Expression erhalten hatten, keinerlei Anzeichen für Leukämie auf. Zudem waren bei diesen eine niedrigere Expression von HoxA9 in Milz und Knochenmark festzustellen, dazu keine Verschiebung der Differenzierung und auch keine anderen Anzeichen für Leukämie. Erst nach der seriellen Transplantation von Knochenmarkszellen kam es zu einer Verschiebung der Differenzierung hin zur myeloischen Reihe und zu vergrößerten Milzen in den sekundären Wirtsmäusen. Als auffällig hat sich erwiesen, dass die tertiären Mäuse bis zu 5 Monate überlebten.

**Schlussfolgerung:** Mäuse, die transduzierte Ly-bi-hämatopoetische Stammzellen erhalten haben, zeigten ein frühes Auftreten und einen deutlicheren Verlauf einer der AML beim Menschen vergleichbaren Leukämie. Auf der anderen Seite ist die Leukämie bei Mäusen, die transduzierte My-bi-Stammzellen erhalten haben, langsamer vorangeschritten und sie haben länger überlebt.

# 3. Introduction

## 3.1. Stem cells

Stem cells are defined by their ability for self-renewal and for differentiation into different cell types. They play an essential role during embryonic development as well as tissue replenishment and repair. One way to classify stem cells is by their cell potency. Totipotent stem cells have the capability to give rise to virtually all types of cells in an organism, such as the zygote, which is a single cell formed from the fusion of an egg cell and a sperm cell. Pluripotent stem cells, like embryogenic stem cells, have the ability to form all cell types of the 3 germ layers - endoderm, mesoderm, and ectoderm. Multipotent stem cells possess the potential to differentiate into different cells of 1 lineage. The best-described multipotent stem cell population is the HSC population. Unipotent stem cells can only give rise to 1 cell type. (1-3)

### 3.1.1. Hematopoietic stem cells

Alexander A. Maximow developed one of the first theories of hematopoiesis and HSCs at the beginning of the 20th century (4, 5). HSCs are defined by their ability to self-renew and to generate all cells from the myeloid and lymphoid lineage through cell differentiation, and they can be found in different locations during development. Early hematopoiesis takes place in the placenta, yolk sac, the aorta-gonad mesonephros region, the fetal liver, and bone marrow. In adult mammals, the subset of HSCs is highly limited. HSCs are mostly found in bone marrow and less frequently in the circulating bloodstream, and they represent the top of the hierarchical process of hematopoiesis. Moreover, HSCs are capable of differentiating into progenitors that become progressively restricted to several or single lineages (Fig. 1) (6).

A major step in the research of HSCs was achieved in 1951, by the research teams of Lorenz and Jacobson, demonstrating that lethally irradiated mice managed to survive after receiving bone marrow tissue from donor animals (7, 8). Similar results were achieved by Ford *et al.* (9) suggesting that single bone marrow cells are capable of replenishing the whole blood compartment. In 1988, Spangrude *et al.* (10) managed to isolate HSCs using phenotypic markers. Since then, great effort has been made to improve the protocols for isolating and purifying HSCs (11, 12).

Obtaining highly purified HSCs is one crucial aspect of improving stem cell research. One of these protocols was introduced by Osawa *et al*. (13). They used lineage markers negative (Lin-), c-Kit+, Sca-1+ (LSK+), CD34- mouse bone marrow cells that resulted in a cell population highly enriched for HSCs. These early findings led to, among other things, HSCs being the first adult stem cells used successfully to treat and cure patients (14).

Despite extensive research and wide application in modern medicine, the biology of the HSC compartment is still not fully understood and requires intense investigation to unravel the features of the HSC compartment.

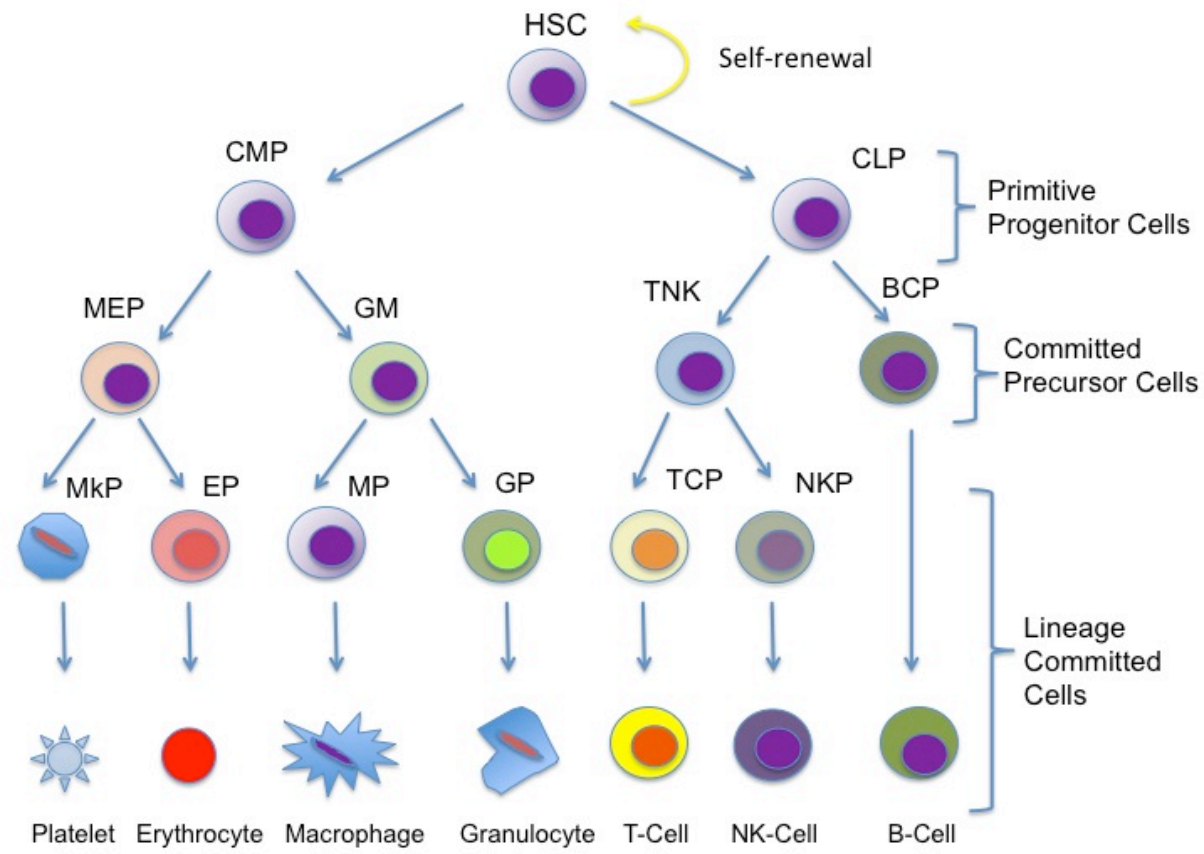


**Figure 1: Schematic representation of hematopoietic development.** This scheme illustrates the complex hematopoietic system, starting with the HSC and its differentiation into various lineages. HSCs sit at the top of the hematopoietic tree as they differentiate into progenitors that become progressively restricted to multiple or single lineages. The results of this cell differentiation are mature cells with several functions (6). CMP - Common Myeloid Progenitor; CLP - Common Lymphoid Progenitor; MEP - Megakaryocyte and Erythroid Progenitor; GM - Granulocyte and Macrophage Progenitor; TNK - T-Cell and Natural Killer Cell Progenitor; BCP - B-Cell Progenitor; MkP - Megakaryocyte; EP - Erythrocyte Progenitor; MP - Monocyte Progenitor; GP - Granulocyte Progenitor; TCP - T-Cell progenitor; NKP - Natural Killer Cell Progenitor; Partially adapted from reference (15).

#### 3.1.1.1. Heterogeneity of the hematopoietic stem cell compartment

Data from recent studies showed that the HSC compartment is not a homogenous population. It consists of several subpopulations of HSCs, each with distinct, pre-programmed proliferation and differentiation behaviors (16). The theory of multi-subpopulations is in contrast to the established opinion that the hematopoietic stem cell compartment is a homogenous population. However, it provides a better explanation for the complex behavior of the hematopoietic system. The widely accepted view was that 1 HSC gave rise to 1 myeloid and 1 lymphoid progenitor. However, this simplified theory could not explain the different ratios of myeloid to lymphoid cells in the blood. Additionally, this could not provide an explanation for the changes in blood cell composition during aging (17). These observations were first questioned by Jordan and Lemischka (18) using analysis of retrovirally marked HSCs, who suggested that there might be lineage-biased HSCs (16). The laboratory of Christa Müller-Sieburg was the first to discover that the normal HSC compartment consists of 3 different types of HSCs, which differ in their ability to differentiate into the different blood cell types (19, 20). Other research groups have made similar discoveries in recent years, resulting in a revolution in how the hematopoietic system is viewed today (21-24). The subsets can be classified by their differentiation potential, each with an epigenetic fixed differentiation and self-renewal capacity. Each subset consists of true HSCs, which give rise to all types of mature blood cell. However, the various types of HSCs induce different ratios of lymphoid and myeloid cells. These 3 subsets are named My-bi, Ly-bi, and Bala HSCs. Bala HSCs generate about 80% lymphocytes and 20% myeloid cells. Furthermore, My-bi HSCs produce few lymphocytes but normal numbers of myeloid cells, and thus have a low lymphoid to myeloid ratio. Ly-bi HSCs differentiate partially to myeloid cells, but produce normal numbers of lymphoid cells, resulting in a high ratio of lymphoid to myeloid cells (Fig. 2). In addition, these different subsets can be discriminated by their altered finite lifespans. In experiments using *in vivo* mice models and computer algorithms, it was shown that every HSC possesses an intrinsically limited life span (25). Notably, HSC clones differ markedly; some only live a few months, others survive several years. It was further demonstrated that My-bi HSCs have a higher self-renewal capacity than Ly-bi HSCs, resulting in the accumulation of My-bi HSCs in the aged, while Ly-bi HSCs dominate in young animals (17). Moreover, these subsets express different cell surface markers, which make it possible to enrich for the different HSC

compartments. One of these markers is CD150, which is expressed at higher levels on My-bi HSCs than on Ly-bi HSCs (21, 23, 24). Unpublished data, provided courtesy of Dr. Christa Müller-Sieburg and Dr. Hans Sieburg, show that with the aid of the marker CD150 it is possible to use fluorescence-activated cell sorting (FACS) for My-bi HSCs and Ly-bi HSCs. Sorting results in stem cell mixtures, but it can be assumed that the subset that is overrepresented after sorting is the one that is affected by the experiments performed in this thesis (Fig. 3).

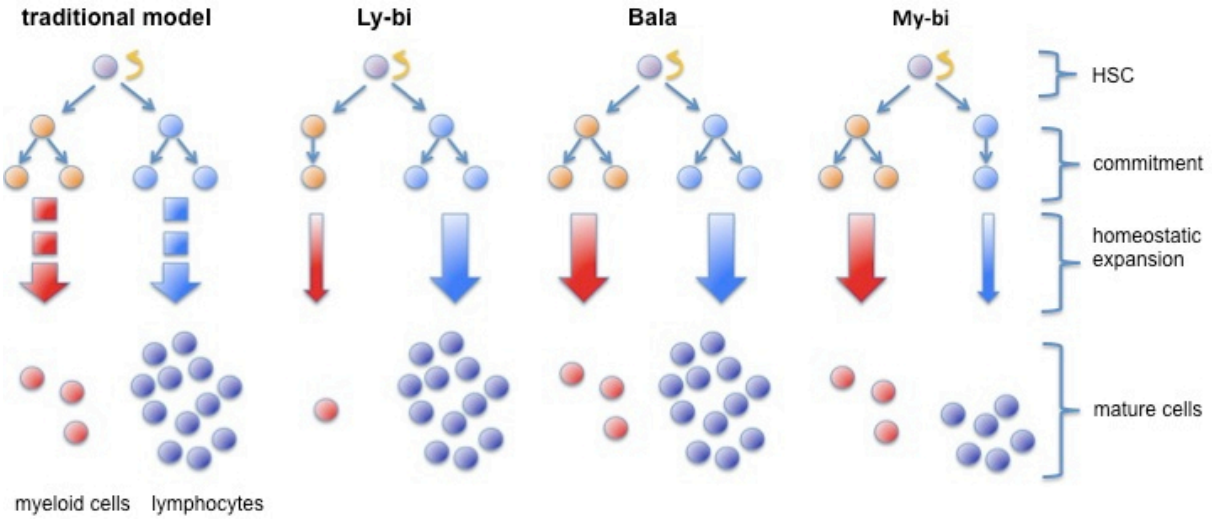


**Figure 2: Hematopoietic stem cell heterogeneity.** This diagram illustrates the novel view of the HSC compartment. The traditional model suggested only 1 type of HSC. However, more recent studies indicate that the HSC compartment consists of 3 distinct subsets with a certain epigenetically fixed differentiation (blue and red arrows) and self-renewal capacity (yellow arrow). The various types of HSCs give rise to different ratios of lymphoid and myeloid cells. Bala HSCs generate about 80% lymphocytes and 20% myeloid cells. My-bi HSCs generate few lymphocytes but normal amounts of myeloid cells and thus have a low lymphoid to myeloid ratio, while Ly-bi HSCs generate few myeloid cells but normal amounts of lymphoid cells, resulting in a high ratio of lymphoid to myeloid cells. Adapted from (16).

**Table 1:** Summary of similarities and disparities between My-bi HSCs and Ly-bi HSCs

| | Ly-bi HSC | My-bi HSC |
|---|---|---|
| Stem cell classification | multi-potent | multi-potent |
| Ratio lymphoid to myeloid cells | normal lymphoid cells, few myeloid cells | few lymphoid cells, normal myeloid cells |
| Self-renewal capacity | low self-renewal capacity | high self-renewal capacity |
| Pattern | dominate in young animals | accumulate in aged animals |
| FACS markers | c-Kit+; Sca-1+; lineage markers negative (Lin-); CD34-; CD150- (**CD150$^{neg}$**) | c-Kit+; Sca-1+; lineage markers negative (Lin-); CD34-; CD150+ (**CD150$^{pos}$**) |

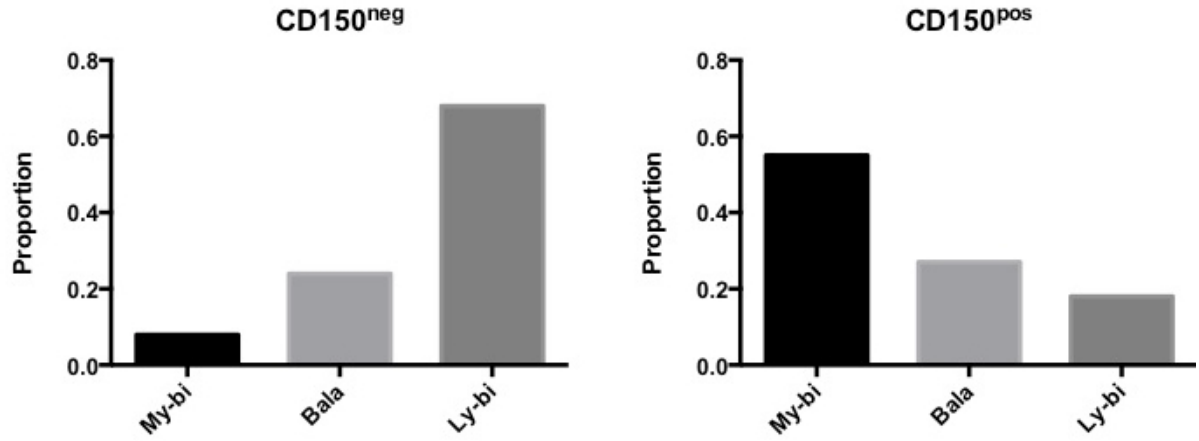


**Figure 3: Clonal analysis showed that CD150$^{neg}$ and CD150$^{pos}$ preparations contain long-term repopulating HSCs, which differ significantly by HSC lineage potential distribution.** Bar charts show the distribution of lineage potential CD150$^{neg}$ or CD150$^{pos}$ sorted HSCs. This data and graph were provided courtesy of Dr. Christa Müller-Sieburg and Dr. Hans Sieburg and contain unpublished data.

## 3.2 Leukemia

Normal hematopoiesis malfunction may result in proliferation of certain blood components and a loss of normal functions of the blood. Transformation of the blood system was first described by John Bennett (1845) (26). He examined a patient post-mortem, who had died rapidly after displaying signs of a “splenic tumor”. Microscopic examination of the blood revealed changes that were independent of inflammation and suggested a transformation affecting the whole blood. Bennett´s paper was the first published clinical case of a patient with leukemia. In line with Bennett´s

observations, Rudolf Virchow described a patient who died of a similar disease (27). Virchow introduced the name “leukhemia” (white blood) for an uncontrolled cell proliferation of white blood cells (28, 29). Interestingly, neither researcher could explain the cause or mechanism of this new disease. In the year 1869, Ernst Neumann drew a link between blood and bone marrow (30) and finally suggested that leukemia was a disease of the bone marrow (31).

Today the diagnosis of leukemia is accompanied with a severe negative impact on the quality of peoples’ lives. About 13,000 people are diagnosed annually with some form of leukemia, resulting in leukemia being the 11th most common newly diagnosed tumor in Germany. Despite intensive research into treatment, 7,300 people died from the consequences of leukemia in Germany in 2008 (32). Leukemia is a heterogeneous group of diseases. Each form of leukemia is described by a variety of symptoms, age of occurrence, therapies, and outcomes. In humans, leukemic cells can be classified by their morphological and immunological characteristics (i.e. acute and chronic forms). The acute forms are characterized by uncontrolled proliferation of immature cells referred to as blasts, whereas chronic forms display proliferation of mature but pathologically modified cells. Moreover, leukemic cells can be distinguished based on which types of blood cell they are derived from. Hence, it is possible to discriminate between a myeloid and a lymphoid form. If these 2 classifications are combined, they result in 4 different forms of leukemia, and this is the most common classification today (33). In particular, each form is determined by different age patterns as depicted in Figure 4.

Despite the devastating numbers of patients and the great efforts that have been made in research, the causes of leukemia are still widely unknown, though some risk factors have been identified, such as exposure to ionizing radiation and aromatic hydrocarbons, particularly benzene. Furthermore, genetic factors have been implicated in the mediation of leukemia. Chromosome aberrations (trisomy 21) and chromosome translocation (Philadelphia translocation: t(9;22)(q34;q11)) have been suggested to increase the risk of leukemia. Nevertheless, most patients do not have any known risk factors (33, 34).

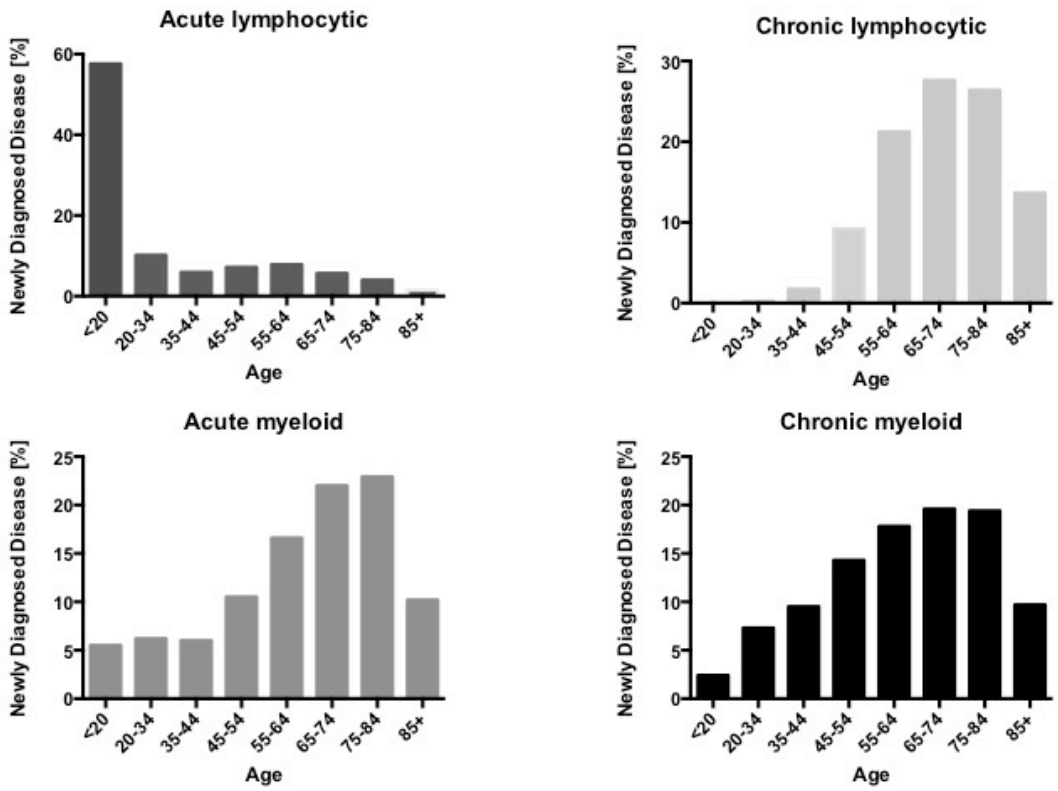


**Figure 4: Age patterns of different forms of leukemia.** The graphs show the age at diagnosis for leukemia in the United States between 2008 and 2012 for both genders. About 62,000 people are newly diagnosed with some form of leukemia every year in the United States. Occurrence of acute lymphoid leukemia peaks during childhood, whereas acute myeloid leukemia is more prevalent in older adults. Data from (35).

### 3.2.1. Acute myeloid leukemia (AML)

The initial symptoms of AML are considered as unspecific. They are caused by an infiltration of blast cells in the bone marrow and a suppression of healthy bone marrow cells, which cause a failure of normal hematopoiesis. The results are fatigue and exhaustion caused by a lack of red blood cells. Furthermore, the lack of platelets may lead eventually to petechiae and mucous membrane bleeding. In addition, malfunction of the immune system can enable serious infections like pneumonia. It is possible to detect alterations caused by AML in blood tests, and in most cases the blood count shows a leukocytosis. Other variations are an increase in blast cells, anemia, and thrombocytopenia as well as enhanced levels of lactate dehydrogenase, blood sedimentation rate, and uric acid levels. Furthermore, to diagnose AML,

distinct parameters such as clinical symptoms, blood count, and a bone marrow biopsy are required. Crucial parameters for diagnosis are the specific numbers of blast cells in the bone marrow and their allotment to the myeloid lineage. Nowadays, 2 classifications are commonly used. A precise classification is necessary to better predict the patients´ prognosis and to choose the appropriate therapy. The FAB-classification (French-American-British) requires at least 30% of blast cells in the bone marrow. It is based on morphological and cytochemical characteristics of the cells, resulting in 8 subclasses (M0-M7). Depending on the subclass, the patients receive different prognoses and treatments (36-38). In addition, the World Health Organization (WHO) classification uses a clinical approach for its classification and divides the subtypes according to cytogenetic features. This approach was developed considering the FAB system and also considers underlying chromosomal abnormalities. It requires at least 20% of blasts cells in the bone marrow (39, 40).

The current therapy for leukemia has 2 mainstays: chemotherapeutic agents and stem cell therapy. Chemotherapeutic agents are designed to specifically target rapidly growing cells; however, their action does not distinguish between cancer cells and healthy cells. Hence, treatment results in severe side effects including hair loss, nausea, and susceptibility to infection. Besides this, long-term effects lead to damage to nerves and the cardiovascular system. Thus, intense research is required for a better understanding of molecular changes and to find target cells of leukemic transformation, which might provide a more suitable therapy (36, 37). Stem cell transplantation is often the treatment of last resort for patients. There are 2 different methods used in stem cell transplantation - autologous and allogeneic transplantation. In an autologous transplantation, the patient's own stem cells are returned after they have undergone high-dose chemotherapy. This method is mostly used to treat solid tumors. In an allogeneic transplantation, the patient receives stem cells from a healthy donor who has been matched for the HLA type. This type of transplantation is limited by the availability of a volunteering donor. It can take up to a month to find a suitable donor, and this delay can be critical for patients, especially with an acute type of leukemia (41).

### 3.2.2 Leukemic stem cell

In the last few years, the theory has been proposed that a small number of normal cells are transformed, which drives tumorigenesis and metastasis, resulting in the formation of cancer cells. Nevertheless, the initial transformed cell remains unknown in most cancer types. Cancer cells are characterized by an unlimited self-renewal potential, resulting in extensive proliferation (42).

The hematopoietic system is one of the best-studied organ systems in terms of cancer cell theory. However, to date it is not known which cells are the target cells of leukemic transformation. This question is of major importance for clinical questions and patient outcome.

It is assumed that leukemia arises from a transformation which takes place in a few or even single cells, which then leads to uncontrolled self-renewal and differentiation. It has been proposed that these transforming events take place in HSCs. Alternatively, leukemia may also arise from more committed progenitors (15). There is evidence suggesting that leukemia rises from an HSC. HSCs already have the ability to self-renew, which is an important aspect of a leukemic stem cell, and it seems more likely that this mechanism is turned into uncontrolled self-renewal than that self-renewal capacity is created in a cell which is already more differentiated. Another explanation why stem cells might be the target is that they are long-lived cells, and it also seems more likely that during their longer lifetime mutations could occur which transform these cells. On the other hand, there is some evidence that the leukemic transformation may take place in a progenitor cell. In mouse models using fusion genes like BCR-ABL and PML/RARα, it has been shown that myeloid leukemia also arises from more restricted progenitor cells (15). Another argument for the restricted progenitor is that HSCs are capable of differentiating into all lineages of the blood. However, in most cases leukemia only produces pathological cells from a single lineage. In this case a leukemic transformed progenitor, which is already committed to one lineage, could be a good explanation.

## 3.3. HoxA9

HoxA9 is part of the homeobox gene family, which was first described in *Drosophila*.

In mammals 39 different homeobox genes are known, which are arranged into 4 clusters and labeled A, B, C, and D (43, 44).

These genes play an important role during embryonic development, where they are essential for specifying cell identity and positioning. Mutations in these genes can result in morphologic abnormalities involving many body structures (45).

During development HoxA9 plays an important role in many parts of the body, for example in skeletal tissue or urogenital tract development. HoxA9 is also known to play a role in cancer, for example, it is downregulated in breast cancer (46).

HoxA9 is best known for its crucial role during hematopoiesis. It is the most highly expressed gene of the homeobox family in HSCs and plays an essential role during normal hematopoiesis. However, abnormal expression can lead to abnormal stem cell behavior and cell expansion, and eventually to leukemia. HoxA9 is highly expressed in early hematopoietic progenitors whereas it is downregulated during differentiation (47-49). On the other hand, loss of HoxA9 expression during early stages of hematopoiesis results in a hematopoietic phenotype with reduced numbers of peripheral blood granulocytes and lymphocytes (50). In patients with AML the expression of HoxA9 is associated with a poor prognosis, and about 50% of patients with AML express HoxA9. The overexpression of HoxA9 leads to AML in mice, which makes this gene a valuable tool for leukemia studies (51). Nevertheless, it remains unknown by which molecular mechanisms HoxA9 promotes leukemogenesis (48, 52).

## 3.4. Aim and outline of the thesis

### 3.4.1. Motivation and goal

Despite extensive research and great progress in treatment, the prognosis for patients with acute leukemia is still poor. New insights, specifically the heterogeneity of the HSC compartment, offer many novel opportunities to address questions that could not be investigated before. Nevertheless, it still remains enigmatic which cells are the preferred target cells for leukemic transformation. Identifying the preferred target cell population would be an important step towards the development of more efficient therapeutic approaches.

This thesis was designed to analyze whether My-bi HSCs are a target of transformation in myeloid leukemia. If the hypothesis is true, this could explain why a transformed HSC has progeny skewed toward the myeloid lineage. Furthermore, My-

bi HSCs accumulate in the aged animal. This fits well with the clinical observation that acute myeloid leukemia occurs more frequently in the elderly. The aim of this thesis is to show whether My-bi HSCs and Ly-bi HSCs behave differently when transduced with a retroviral vetctor overexpressing a gene that induces AML. Another question we want to answer is whether My-bi HSCs are the target cells for leukemic transformation induced by HoxA9.

### 3.4.2. Outline

**1. How do My-bi and Ly-bi cells transduced with HoxA9 repopulate in the blood?**

To answer this question, whole bone marrow cells were sorted for LSK+ CD34+ to collect highly purified HSCs. These cells were further sorted to select a CD150+ or CD150- population. The acronym $CD150^{pos}$/HSCs is used to refer to the cell population that is enriched for My-bi HSCs. On the other hand, the acronym $CD150^{neg}$/HSCs is used for the cell population that is highly enriched for Ly-bi HSCs. The sorted cells were then transduced with a retroviral vector overexpressing HoxA9. For these transduced cells the acronym $CD150^{neg}$/HoxA9 HSCs or $CD150^{pos}$/HoxA9 HSCs is used. Transduced cells were then injected into lethally irradiated mice. These mice are called primary host mice. The repopulation levels of donor cells and transduced donor cells that express the HoxA9 gene were monitored in primary host mice using the cell surface marker CD45.1 and GFP (green fluorescent protein). The blood levels were monitored for a period of 5 months.

**2. Is HoxA9 capable of inducing leukemia in My-bi and Ly-bi HSCs?**

If a primary or secondary host mouse showed signs of leukemia, the mouse was sacrificed and the spleen weight was used as an indicator for leukemia. Signs of leukemia were lethargy, lack of motion, and refusal to eat.

**3. What cell marker changes can be seen in the blood?**

Control samples of blood were drawn after 1, 3, and 5 months. The blood was then analyzed for the composition of B-cells, T-cells, and myeloid cells. To visualize the HoxA9 activity, the overexpressing gene was linked to GFP.

**4. Which roles do the bone marrow and spleen play in leukemic behavior?**

If a primary host mouse showed signs of leukemia, the mouse was sacrificed and the bone marrow and spleen were analyzed further. An age-matched mouse from the other group (either $CD150^{pos}$/HoxA9 or $CD150^{neg}$/HoxA9) was euthanized at the same time and was used to compare the progress of leukemia.

**5. Does the leukemic behavior stay consistent after serial transplantation of bone marrow cells into new host mice**?

To observe if the type of leukemia stayed consistent and to further observe leukemic behavior, several serial transplantation experiments were performed. Therefore, whole bone marrow from the primary host mice was injected into lethally irradiated mice. These mice are called secondary host mice. Blood from these mice was drawn at the same intervals, and the same analyses were used as for the primary mice.

**6. Does the leukemic behavior differ if spleen cells are serially transplanted compared to bone marrow cells?**

To provide deeper insights in the composition of bone marrow and spleen and to address the question of whether the spleen could be a place of extra-medullary hematopoiesis, serial transplantations with spleen and bone marrow from leukemic mice were performed. Therefore, bone marrow cells or spleen cells from secondary host mice of both groups were injected into lethally radiated mice. These mice are called tertiary host mice. These mice were observed for the length of their survival time.

**7. Does the number of transplanted cells impact the leukemic behavior?**

To demonstrate that the number of transplanted cells has no impact of the onset, behavior, and type of leukemia, different numbers of whole bone marrow cells were transplanted from the primary into the secondary host mice for both $CD150^{pos}$/HoxA9 and $CD150^{neg}$/HoxA9 groups.

# 4. Material and Methods

## 4.1. Buffers and solutions

**Table 2:** The composition of buffers and solutions

| Medium/Solution | Composition |
|---|---|
| Bone Marrow Culture Medium (BMCM) | 500 mL RPMI-1640, 10 mL GluPest, 25 mL Fetal Calf Serum, 0.5 mL Beta Mercapato-Ethanol |
| GluPest | 200 mM L-Glutamine, 5000 Units/mL Penicillin/Streptomycin |
| Staining Medium | 500 mL Newborn Calf Serum, 33.3 mL of 10% Sodium Azide, 2170 mL dH20, 250 mL of 10X dPBS |
| Propidium Iodide (PI) Medium | staining medium with 10 µg/mL PI |
| Sterile PI Medium | staining medium with 10 µg/mL PI, sterile filtered |
| Basal Medium | 400 mL Iscove's Modified Dulbecco's Medium (IMDM), 75 mL Fetal Calf Serum, 25 mL GluPest |
| FACS Medium | 400 mL IMDM, 100 mL Fetal Calf Serum |
| Cytokine Medium | 5 mL Basal Medium, 25 µL mSCF(100ng/mL), 25 µL hIL6(10 ng/mL), 37 µL Thrombopoietin TPO(75 ng/mL), 5 µL bFGF (10 ng/mL), 5 µL Flt3-1(10 ng/mL), 5 µL FGF (10 ng/mL) |
| 293T Medium | 25 mL Fetal Calf Serum, 217.5 mL Isocoves IMDM, 2.5 mL MEM Non-Essential Amino Acids Solution (100X), 5 mL GluPest |
| S17 Medium | 250 mL Alpha-MEM, 50 mL Fetal Calf Serum |
| Dexter Medium | 50 mL IMDM, 75 mL horse serum (HS), 75 mL FBS, 5 mL 200 mM L-glutamine, 2.5 mL 10,000 U/mL penicillin/10,000 µg/mL streptomycin, 500 µL 0.1 M 2-mercaptoethanol, 500 µL 1 mM hydrocortisone |
| 10 mM PBS/EDTA | 5.13 mL 0.5M EDTA, bring volume to 200 mL with 1xPBS, add HCl until pH is 7.0 |
| Lysogeny Broth (LB) | 10 g tryptone, 5 g yeast extract, 10 g NaCl in 950 mL DI H2O, adjust pH to 7.0 using 1N NaOH and bring volume up to 1 liter, autoclave on liquid cycle for 20 min at 15 psi, add ampicillin 50 µg/mL |
| LB agar-plates | Prepare LB medium, but add 15 g/L agar before autoclaving, after autoclaving add ampicillin 50 µg/mL and pour into Petri dishes |
| Gey Solution Stock A | 35 g NH4Cl, 1.85 g KCl, 1.5 g Na2HPO4*12H2O, 0.12 g KH2PO4, 5 g Glucose, bring volume to 10 mL with DI H2O, sterile filtered |
| Gey Solution Stock B | 0.42 g MgCl12*6H2O, 0.14 g MgSO4*7H2O, 0.34 g CaCl2, bring volume to 100 mL with DI H2O, sterile filtered |
| Gey Solution Stock C | 2.25 g NaHCO3, bring volume to 100 mL with DI H2O, sterile filtered |

**Table 2:** Continued

| Medium/ Solution | Content |
|---|---|
| 2% Dextran | 10 g of Dextran T500, bring volume to 500 mL with PBS |
| 2% Bovine Serum Albumin | 2 g Bovine serum albumin (BSA) (100 mg/mL), 100 mL DI H2O |

## 4.2. Antibodies

**Table 3:** Antibodies used for these experiments

| Characterization | From | Antibody | Company |
|---|---|---|---|
| Purified anti-mouse/human CD45R/B220 Antibody (**6B2**) | rat | primary Ab | BioLegend, San Diego CA, USA |
| GR-1 Antibody (RB6-**8C5**) | rat | primary Ab | Life Technologies, Carlsbad CA, USA |
| Anti-Mouse CD11b Purified (**Mac-1**) | rat | primary Ab | Biosciences, Franklin Lakes NJ, USA |
| Anti-Mouse Thy-1 Antibody (**31-11**) | rat | primary Ab | Santa Cruz Biotechnology, Santa Cruz CA, USA |
| Mouse Anti-Mouse **CD45.1**-APC (Ly-5.1) | mouse | primary Ab | SouthernBiotech, Birmingham, AL, USA |
| FITC anti-mouse Ly-6A/E (**Sca-1**) Antibody | rat | primary Ab | BioLegend, San Diego CA, USA |
| PE anti-mouse **CD34** Antibody | Armenian hamster | primary Ab | BioLegend, San Diego CA, USA |
| APC anti-mouse CD117 (**c-Kit**) Antibody | rat | primary Ab | BioLegend, San Diego CA, USA |
| Biotin anti-mouse **Lineage** Panel | rat/Armenian hamster | primary Ab | BioLegend, San Diego CA, USA |
| PE/Cy7 anti-mouse **CD150** (SLAM) Antibody | rat | primary Ab | BioLegend, San Diego CA, USA |
| PE Goat anti-rat IgG (minimal x-reactivity) Antibody(**G@RPE**) | goat | secondary Ab | BioLegend, San Diego CA, USA |
| **APC**/Cy7 Streptavidin | *Streptomyces avidinii* | secondary Ab | BioLegend, San Diego CA, USA |

## 4.3. Mice

C57BL/6 (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All other mice were bred at the Sanford-Burnham facility. These included B6 mice congenic for the CD45.1 allele (B6-CD45.1) and F1 mice. F1 mice are generated by mating a B6-CD45.1 mouse with a B6-CD45.2 mouse. The blood cells express both a CD45.1 and a CD45.2 gene. David Harrison (Jackson Laboratories, Bar Harbor, ME, USA) generously provided the breeder pairs of B6-W41W41 mice; these mice have a mild mutation in the *c*-Kit gene and served as sublethally irradiated hosts for transplantation experiments (17). The work reported in this dissertation was supported by the United States National Health Grants DDK48015 and AG 023197 to Prof. Dr. Christa E. Muller-Sieburg. Experiments, if any, were performed in Prof. Dr. Muller-Sieburg's laboratory at the Sanford-Burnham Institute in La Jolla, California. All experiments were approved by the Institutional Animal Care and Use Committee at the Sanford-Burnham Institute in La Jolla, California.

## 4.4. Plasmid MigR1- HoxA9

The MigR1-HoxA9 construct contains the GFP reporter under control of the HoxA9 promoter and an ampicillin resistance cassette (Fig. 5). This plasmid was transfected into a retrovirus (see 4.7.) and the resulting retroviral vector was later used to transduce the target cells (see 4.8.).

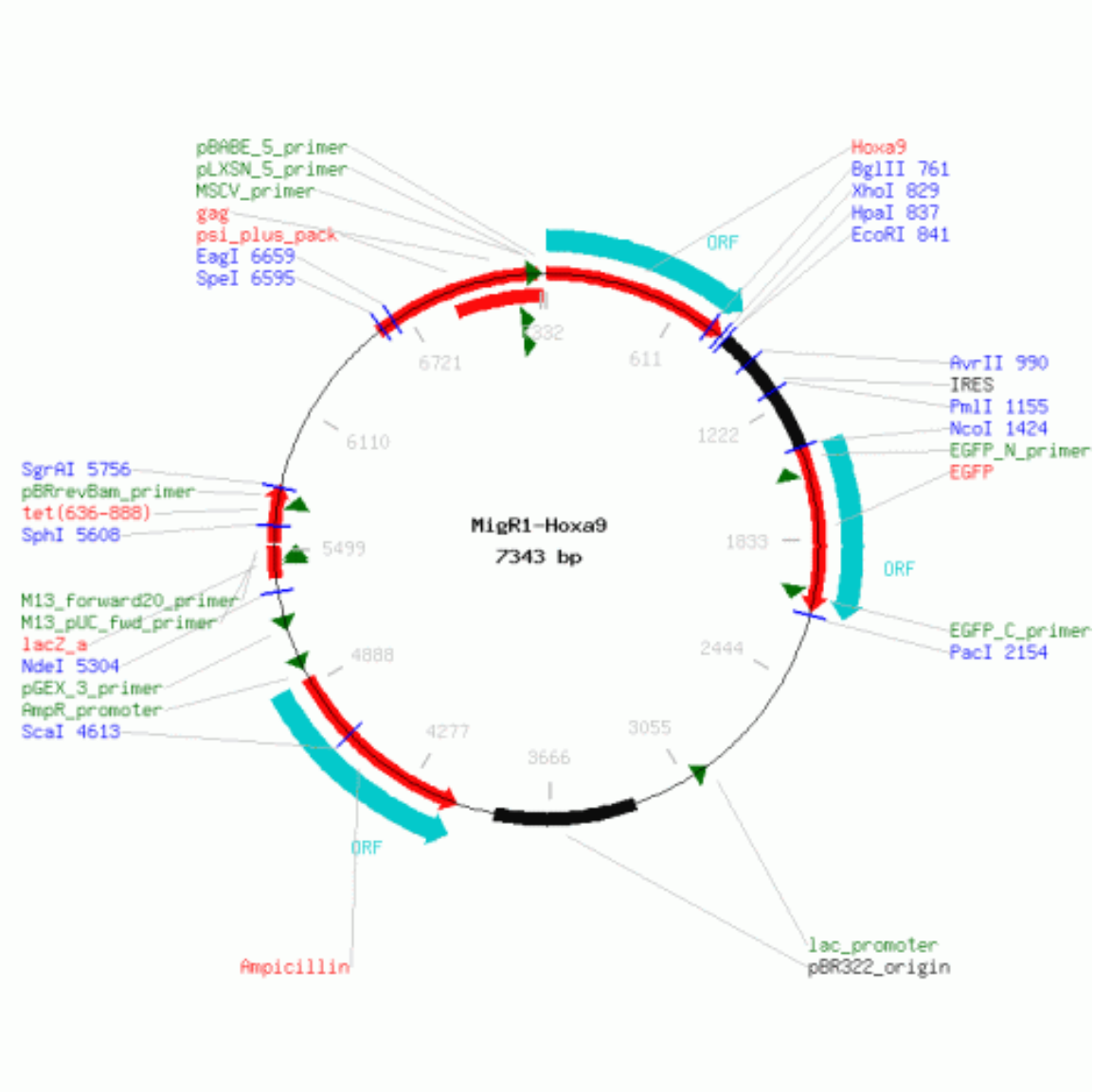


**Figure 5: MigR1-HoxA9 plasmid.** The plasmid consists of a total of 7,343 base pairs (bp). It contains the GFP reporter under control of the HoxA9 promoter. This means that when HoxA9 is expressed GFP will also be expressed. The ampicillin resistance cassette helps to select the bacterial colonies that have incorporated the plasmid during transformation (see 4.5 Transformation). The possible restriction enzyme cutting locations are marked in blue. Thanks are due to Dr. Jeffrey Bernitz for providing the plasmid construct.

## 4.5. Transformation

We mixed 1 µL of MigR1-HoxA9 DNA (see 4.4.) with competent *Escherichia coli* (*E.coli*) cells and incubated for 30 minutes on ice. After incubation, the tube was removed from the ice and put in a 42°C heated block for 90 seconds. Then, the tube was returned to ice for 5 minutes, and 500 µL of lysogeny broth (LB) was added to

the cells. They were then transferred to a sterile falcon tube and incubated for 1 hour with shaking at 37°C. Afterwards, the cells were plated on LB plates supplemented with 50 µg/mL ampicillin and incubated overnight at 37°C. Only bacteria that had incorporated the plasmid had the resistance gene for ampicillin and were able to grow on the plates. The following day single colonies were picked up with sterile tips and grown in 5 mL LB medium supplemented with 50 µg/mL ampicillin for 8 hours with shaking at 20°C.

## 4.6. Isolation of plasmid DNA

Bacteria from at least 5 cultures were pelleted via centrifugation for 7 minutes at 312 RCF(*g*) in a tabletop centrifuge. Isolation of plasmid DNA was then performed with a QIAGEN Plasmid Midi Kit (Qiagen N.V., Venlo, Netherlands) according to the manufacturer's instructions. Finally, the plasmid DNA pellet was air-dried for 5-10 minutes and redissolved in 100 µL TE buffer, pH 8.0. The concentration of DNA was determined by measuring the absorbance at 260 nm ($A_{260}$) in a spectrophotometer.

## 4.7. Producing the retroviral vector; transfection

On the first day, $2x10^7$ 293T cells were plated in a 15 cm dish with 30 mL of medium. The next day, 24 µg of Plasmid Migr1-HoxA9 DNA (see 4.4.) was mixed with 8 µg ECOpack DNA (Clonetech, Mountain View, CA, USA). Additionally, 144 µL of FuGENE (Roche Applied Science, Basel, Switzerland) was added dropwise to 2 mL of Opti-MEM medium (Life Technologies, Carlsbad, CA, USA) and then rested for 5 minutes at room temperature. Then, the DNA mix was added dropwise to the solution with FuGENE, and everything was gently shaken and rested for 20 minutes at room temperature. In the meantime, the medium from the 293T cells was aspirated and replaced with 10 mL of fresh medium. The DNA and FuGENE mix was added dropwise to the 293T cells, and everything was incubated at 37°C overnight. The next day 10 mL of medium was added to the 293T cells, and the plate was incubated for another 2 days at 37°C. After the incubation time, the media containing the retroviral vector was collected and centrifuged for 10 minutes at 312 RCF(*g*). At the next step, the supernatant was filtered through a 40 µm filter (Falcon™ Cell Strainers, Fischer Scientific, Waltham, MA, USA), aliquoted, and immediately stored at -70°C.

## 4.8. Retroviral transduction of HSCs

After sorting, the cells were centrifuged and then seeded into a Falcon 24-well Tissue Culture Plate (Corning Life Sciences, Corning, NY, USA) in cytokine medium for 2 days. The following day, 4 wells of another 24-well plate were coated with RetroNectin (RN) (Clontech Mountain View, CA, USA) solution and then rested overnight at 4°C. The next day, the virus-loaded plates were prepared. For this, the RN solution was removed, 2% BSA was added, and the plates rested for 30 minutes at room temperature. Afterwards, the wells were washed with Dulbecco's Phosphate-Buffered Saline (dPBS) (lonza Group, Basel, Switzerland). Then, 0.5 mL of the virus supernatant containing the retroviral vectors (see 4.7.) overexpressing HoxA9 was added to 2 of the 4 wells. The other 2 were kept as control wells, and everything was incubated for 5 hours at 32°C. After incubation, the wells were washed once again with dPBS, and the transduction was performed. The sorted cells that were in pre-stimulation conditions were transferred to the Hoxa9 retrovirus-loaded wells. The following day all cells were collected and washed with Iscove's Medium (IMDM) (Lonza Group, Basel, Switzerland). The cells were centrifuged and resuspended in IMDM. They were then ready for injection into sublethally irradiated B6-W41W41 mice.

## 4.9. Harvesting of bone marrow

The mice were anesthetized using isoflurane (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anesthesia vaporizer (Parkland Scientific, Inc., Coral Springs, FL, USA). Subsequently, mice were sacrificed by cervical dislocation. Next, 97% ethanol was applied to clean the fur. For the extraction of bone marrow, skin and muscles were separated from the tibia and femur using forceps and scissors. In the next step, these bones were separated from the rest of the body and stored on ice in bone marrow culture medium (BMCM). Separation of bone marrow was performed using a 10 mL syringe containing BMCM, which was injected into the bone in order to flush out the bone marrow. The obtained bone marrow was centrifuged for 7 minutes at 312 RCF(*g*) in a tabletop centrifuge (Hettich Rotina420R, Hettich Instruments LP, Beverly, MA, USA). Finally, the supernatant was discarded and 1 mL of BMCM was added to the pellet and the cells resuspended. In order to determine the number of extracted cells, a cell count was performed (see 4.14.).

## 4.10. Sorting of HSCs

In order to sort the bone marrow cells into fractions of $CD150^{pos}$ and $CD150^{neg}$ HSCs, cells were labeled with fluorescent antibodies and sorted using a FACSaria (BD Biosciences, Franklin Lakes, NJ, USA). Cells isolated from bone marrow (B6-CD45.1 mice) were counted (see 4.14.); $1.0x10^7$ cells were used for sorting and $1.0x10^6$ cells were used for each of the compensation tubes. The cells for sorting were put into a Falcon 15 mL Conical Centrifuge Tube, and $10^6$ cells for compensation were put into Falcon FACS-tubes (both BD Biosciences, Franklin Lakes, NJ, USA). All tubes were centrifuged for 7 minutes at 312 RCF(*g*) in a tabletop centrifuge. The supernatant was aspirated and discarded, and the pellet was dispersed. Then primary antibodies were added. The combinations of antibodies used are described in Table 4, and 6 µL of each antibody was added per $10^6$ cells. All tubes were incubated at 4°C for 15 minutes. After incubation, the cells for sorting were washed with 10 mL of staining medium, and the cells for compensation with 1 mL of staining medium. All tubes were centrifuged for 7 minutes at 312 RCF(*g*) in the tabletop centrifuge. The supernatant was aspirated and discarded, and the pellet was dispersed again. Then, the secondary antibody was added to the comp APC-Cy7 tube and to the cells for sorting. The tubes were incubated at 4°C for 15 minutes. After incubation the cells for sorting were washed with 10 mL of staining medium, and the cells for compensation with 1 mL of staining medium. All tubes were centrifuged as before, and the supernatant was aspirated and discarded. The pellets of the compensation tubes were resuspended with 200 µL of staining media. For the single PI compensation, 200 µL of PI medium was used for resuspension. Then 2 mL of staining medium was added to the cells for sorting and the cells were filtered through a nylon mesh (Falcon™ Cell Strainers, Fischer Scientific, Waltham, MA, USA). The mesh was washed with 5 mL of PI staining media to collect all remaining cells. The cells for sorting were centrifuged again, and the supernatant was aspirated and discarded. The pellet was resuspended in 2 mL of sterile PI staining medium. The cells were now ready for sorting. Two tubes with 5 mL sorting media were prepared for collecting the sorted cells. The cells were sorted with a FACSaria and the gates were set for white blood cells, living cells, lin-, Sca+, c-Kit+, CD34- and CD150+/- (Fig. 6), with the help of the FACS-facility supervisor.

**Table 4:** Antibody combinations for sorting CD150$^{pos}$ HSCs and CD150$^{neg}$ HSCs

| Tubes | SCA-FITC | CD34-PE | c-kit-APC | Lin-bio | AvAPC-Cy7 | CD150-PEcy7 | PI |
|---|---|---|---|---|---|---|---|
| unstained | - | - | - | - | - | - | - |
| comp FITC | + | - | - | - | - | - | - |
| comp PE | - | + | - | - | - | - | - |
| comp PI | - | - | - | - | - | - | + |
| comp APC | - | - | + | - | - | - | - |
| comp APC-Cy7 | - | - | - | + | + | - | - |
| comp PE-cy7 | - | - | - | - | - | + | - |
| sample to sort | + | + | + | + | + | + | + |

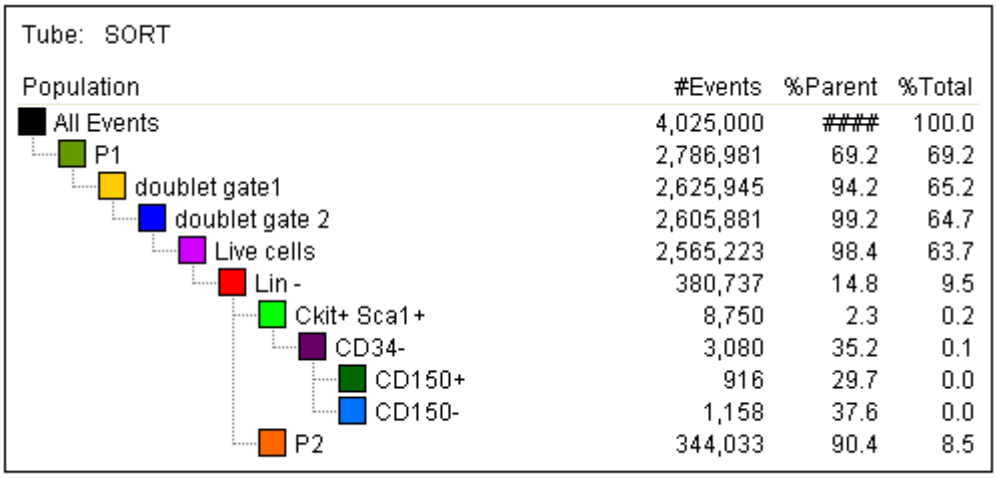


**Figure 6: Protocol for sorting HSCs.** 4,025,000 whole bone marrow cells were sorted and the gates were set for white blood cells (P1, doublet gate 1, doublet gate 2), Live cells, Lin-, c-Kit+ and Sca1+, CD34- and CD150+ or CD150-. The beginning population of 4,025,000 bone marrow cells was sorted into 916 CD150$^{pos}$ HSCs and 1158 CD150$^{neg}$ HSCs.

## 4.11 Performing a blood test

Repopulation levels were monitored by staining white blood cells with monoclonal antibodies (mAb) specific for the CD45.1 allele. The HoxA9 activity was measured with GFP at the same time. The white blood cells were stained with mAb specific for Thy-1 (31-11/T-lymphocytes), B220 (6B2/B-lymphocytes), and Gr-1 (8C5/granulocytes) and Mac-1 (macrophages). The final samples were run on a

FACSCalibur (BD Biosciences Franklin Lakes, NJ, USA) and analyzed using FlowJo software (FLOWJO, LLC Ashland, OR, USA) (Fig. 7).

A preparation of 500 µL of 10mM PBS/EDTA was added to Falcon FACS-tubes (BD Biosciences Franklin Lakes, NJ, USA) to prevent coagulation of the blood and the tubes were kept on ice. The mice were warmed for about 5 minutes under a heating lamp. A single mouse was put in a mouse restrainer, and the tail was slightly cut with a razorblade; then about 4-6 drops of blood were collected in the FACS-tubes. The tubes were gently shaken to mix the blood completely with the PBS/EDTA and prevent coagulation. After bleeding all the mice, 1 mL of 2% Dextran (T-500 Dextran Pharmacosmos Holbaek, Denmark) was added to each tube and the tubes were incubated at 37°C for about 40 minutes. After the incubation time, the red blood cells had settled on the bottom of the tubes. The clear supernatant, which contained the white blood cells, was carefully transferred with a disposable plastic transfer pipette to new FACS-tubes. The supernatant was washed by adding 1 mL of staining media to each tube and was centrifuged for 7 minutes at 312 RCF(*g*) in a tabletop centrifuge. After spinning, the supernatant was decanted into the sink and the pellet, which contained a few remaining red blood cells and enriched white blood cells, was dispersed by shaking the tube holder vigorously back and forth 3-4 times. The remaining red blood cells were lysed with Gey solution by adding 1 mL of Gey solution and incubating the mixture for 90 seconds. The Gey solution was neutralized with 1 mL of staining media. Afterwards, the contents were centrifuged for 7 minutes at 312 RCF(*g*) in a tabletop centrifuge. The clear supernatant was decanted into the sink and the pellet was dispersed by shaking the tube holder vigorously back and forth 3-4 times. Then,100 µL of staining media was added, the solution was split into 3 new FACS-tubes, and 30 µL of 1x solution of the primary antibody Ly-APC was added to every tube. Also, 30 µL of 31-11, 6B2, and 8C5/Mac-1 antibodies were added to the corresponding tubes as shown in Table 5. The cells were incubated for 15 minutes at 4°C, protected from light, and after incubation 1 mL staining medium was added to remove unbound antibodies. After another centrifugation, the supernatant was decanted. Subsequently, the secondary antibody G@RPE was added to the tubes and incubated for 15 minutes at 4°C while protected from light. After a final washing step with 1mL staining media, 200 µL of PI medium was added to each tube except the non-PI single color control tubes (usually control tube 1).

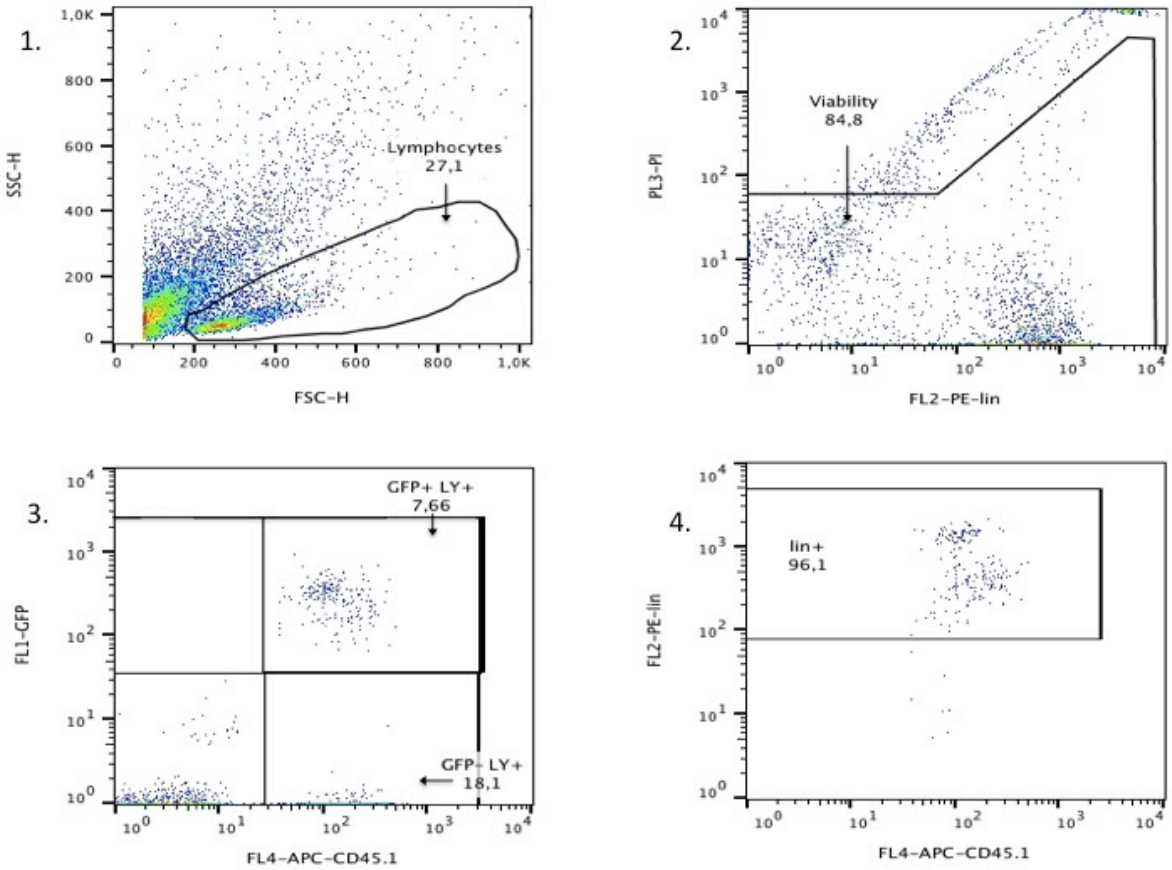


**Figure 7: Gating of blood samples.** The samples were analyzed with a FACSCalibur flow cytometer and FlowJo software. First (1.), the cells were gated for SSC (indicator for cell size) against FSC (indicator for granularity). This allowed the identification of the lymphocytes. Then (2.), the lymphocytes were gated for PI, which helped to distinguish between live and dead cells. The next step (3.) was to gate the live lymphocytes for GFP+ and CD45.1 (Ly+). This only identified transplanted lymphocytes (CD45.1) that were transduced with the retroviral vector (GFP). The final step (4.) was to gate these cells for the linage marker, which could be 31-11, 6B2, or 8C5/Mac-1 to distinguish between T-cells, B-cells, and myeloid cells.

**Table 5:** Antibody combinations used for blood sample analysis

| Tubes | Antibodies | | |
|---|---|---|---|
| Control tube 1 | - | - | G@RPE |
| Control tube 2 | - | 31-11 | G@RPE |
| Control tube 3 | Ly-APC | - | G@RPE |
| Control tube 4 | Ly-APC | 31-11 | G@RPE |
| Control tube 5 | Ly-APC | 6B2 | G@RPE |
| Control tube 6 | Ly-APC | 8C5/Mac-1 | G@RPE |
| Test tube 1 | Ly-APC | 31-11 | G@RPE |
| Test tube 2 | Ly-APC | 6B2 | G@RPE |
| Test tube 3 | Ly-APC | 8C5/Mac-1 | G@RPE |

## 4.12. Harvest of spleen

The mice were narcotized with isoflurane (Santa Cruz Biotechnology, Santa Cruz CA, USA) and the help of an anesthesia vaporizer (Parkland Scientific Inc., Coral Springs, FL, USA). After narcotizing, the mice were sacrificed by cervical dislocation and the fur was cleaned with 97% ethanol. The abdomen was carefully opened with scissors and the organs were observed. Photographs were taken, and then the spleen was carefully removed and freed from fat. After that, the weight and length of the spleen was measured. A BD Falcon Cell Strainer (40 µm, blue) was placed on a BD Falcon 50 mL conical tube (both BD Biosciences Franklin Lakes, NJ, USA). The spleen was cut into multiple pieces and squeezed through the filter. Then, 1 mL of BMCM was added to the top of the filter and the spleen was squeezed through again. Afterwards, another 1 mL of BMCM was added, and with the help of a pipette the last drops were pulled through the filter. The contents were resuspended with a 1 mL pipette tip, and the spleen cells were centrifuged for 7 minutes at 312 RCF(*g*) in the tabletop centrifuge. Then the supernatant was aspirated and 1 mL of BMCM was added. A cell count was taken (see 4.14).

## 4.13. Staining of bone marrow and spleen cells

For staining experiments, $1x10^6$ cells were utilized, and 30 µL of 1x solution of the primary antibody Ly-APC was added to every tube. Also 30 µL of 31-11, 6B2, 8C5, and Mac-1 were added to the corresponding tubes (Table 6). Further washing and staining was performed as described in 4.11.

**Table 6:** Antibody combinations used for bone marrow and spleen samples

| Tubes | Tissue | Antibodies | | |
|---|---|---|---|---|
| Tube 1 BM | BM | | | |
| Tube 2 BM | BM | 6B2 | GaR-PE | Ly-APC |
| Tube 3 BM | BM | 8C5 | GaR-PE | Ly-APC |
| Tube 4 BM | BM | Mac-1 | GaR-PE | Ly-APC |
| | | | | |
| Tube 1 Spleen | Spleen | | | |
| Tube 2 Spleen | Spleen | 31-11 | GaR-PE | Ly-APC |
| Tube 3 Spleen | Spleen | 6B2 | GaR-PE | Ly-APC |
| Tube 4 Spleen | Spleen | 8C5 | GaR-PE | Ly-APC |
| Tube 5 Spleen | Spleen | Mac-1 | GaR-PE | Ly-APC |

## 4.14. Counting cells

For counting cells, a new dilution was made with 190 µL Turk's solution and 10 µL of the corresponding cell suspension. Turk's solution stains the nuclei of leukocytes and hemolyzes erythrocytes which makes it easy to visualize and count leukocytes. The cell count was made using a hemocytometer.

## 4.15. Producing and staining slides

A solution of $10^6$ bone marrow cells in 1 mL staining media was prepared, and 100 µL of this cell solution was put in a CytoSep™ funnel and centrifuged in a Shandon Cytospin 4® (Thermo Scientific, Waltham, MA, USA) for 10 minutes at 412 RCF(*g*). The centrifuge chambers were prepared with microscope slides and filters so that the cells were centrifuged as a drop onto the microscope slides. After air-drying, 2 drops of Wright-Geimsa stain were added to the slides for 2 minutes. Afterward, 2 drops of distilled water (DI H2O) were added and the slides were incubated for another 2 minutes, followed by a final washing step with DI H2O.

# 5. Results

The aim of this thesis was to study the leukemic behavior of transduced $CD150^{pos}$ HSCs and $CD150^{neg}$ HSCs *in vivo*. Therefore, these cells were transduced with a retroviriral vector overexpressing HoxA9 and injected into host mice. Blood samples were drawn and analyzed with fluorescent antibodies and FACS analysis. After symptoms of leukemia developed, mice were sacrificed and the spleen and bone marrow were analyzed. Both $CD150^{pos}$ and $CD150^{neg}$ are a heterogenic mixture of different HSCs (Fig. 3). This means that $CD150^{pos}$ HSCs contain mostly My-bi HSCs, but also some Ly-bi HSCs and Bala HSCs, and *vice versa* for the $CD150^{neg}$ HSCs that mostly contain Ly-bi HSCs. However, it can be assumed that the overrepresented HSC subtype is the one, which gets transduced by the retroviral vector. This means that for the $CD150^{pos}$ HSCs, My-bi HSCs are the ones that become transduce, and vice versa for the $CD150^{neg}$ where the Ly-bi HSCs are transduced. Seventeen primary mice received $CD150^{neg}$/HoxA9 HSCs and also 17 received $CD150^{pos}$/HoxA9 HSCs (see 5.1.). If the percentage of GFP+ cells was below 2% at time of sacrifice in any of the 3 tested tissues (blood, spleen or bone marrow) a reasonable analysis was not possible and these mice were excluded from further analysis. Details for further exclusion of mice and possible reasons for this are discussed under 6.6. Limitations. This resulted in 10 primary host mice that received $CD150^{neg}$/HoxA9 HSCs and 6 primary host mice that received $CD150^{neg}$/HoxA9 HSCs that were used for detailed analysis (5.2. and onward). For the secondary hosts, 4 mice each were analyzed for the $CD150^{neg}$/HoxA9 respectively $CD150^{neg}$/HoxA9 group.

## 5.1. How do My-bi and Ly-bi HSCs transduced with HoxA9 repopulate in the blood? HoxA9 slowed down the growth of $CD150^{pos}$ HSCs

The blood levels of transplanted cells were analyzed in the mice after 1, 3, and 5 months. Repopulation levels were monitored by staining white blood cells with monoclonal antibodies specific for the CD45.1 allele. CD45.1(Ly5.1) is a marker that is expressed on all donor cells that were transplanted. A total of 17 mice were transplanted with cells from each group, one group received $CD150^{pos}$/HoxA9 HSCs,

and the other CD150$^{neg}$/HoxA9 HSCs. For 12 mice of each group, data points at 1, 3, and 5 months were available. For the remaining 5 mice, 1 or 2 data points were missing. This was for different reasons - for example because some mice were euthanized earlier than 5 months because they showed signs of leukemia.

The mice transplanted with CD150$^{pos}$/HoxA9 HSCs have a higher percentage of donor-type cells compared to the CD150$^{neg}$/HoxA9 group. All mice that received CD150$^{pos}$/HoxA9 HSCs showed an increase of donor cells over the 5-month experiment duration. In mice that received CD150$^{neg}$/HoxA9 HSCs the mean percentage of donor-type cells stayed almost the same after 5 months.

The next step was to analyze how many of the donor cells were expressing the HoxA9 gene. Therefore, the GFP levels of donor cells were measured in the blood. Mice that received CD150$^{pos}$/HoxA9 HSCs showed a decline in the number of cells expressing the HoxA9 gene, in contrast to the CD150$^{neg}$/HoxA9 mice that showed an increase of cells expressing the HoxA9 gene (Fig. 8).

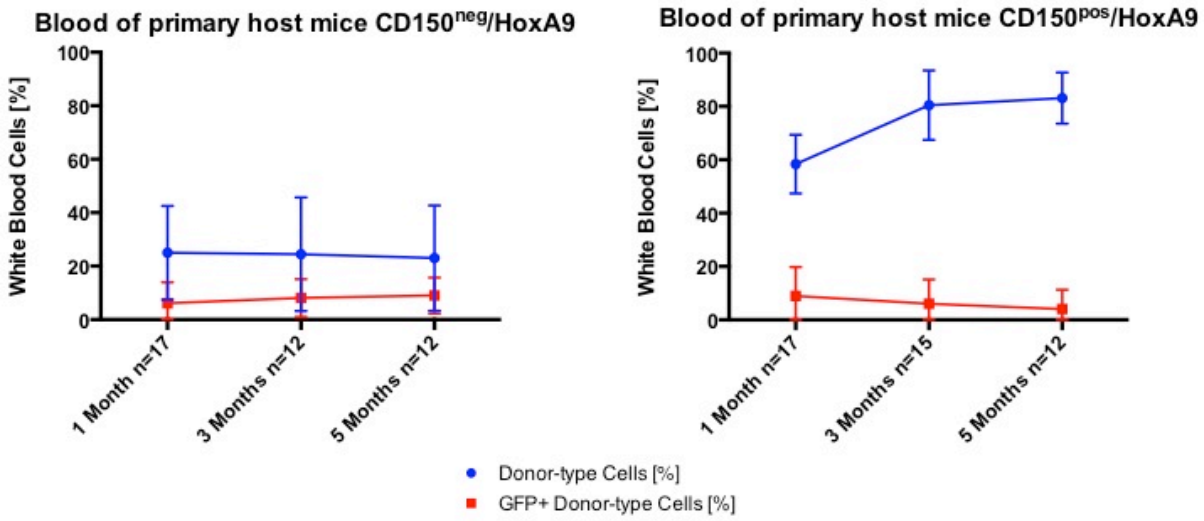


**Figure 8: Comparison of the kinetics of donor-type cells in primary host mice.** Donor-type cells were measured at 1, 3, and 5 months (horizontal axis) after transplantation. The graphs show the mean and the standard deviation (SD) of donor-type cells (blue) or GFP+ donor-type cells (red). The mice that received CD150$^{pos}$/HoxA9 HSCs (3 months n=12) had higher numbers of donor-type cells and showed an increase over 5 months compared to the mice that received CD150$^{neg}$/HoxA9 HSCs (3 months n=12). A 2-sided t-test between the 2 groups was significant ($P$<0.05) for all months. Interestingly, the donor-type cells expressing the HoxA9 gene (GFP+) behaved differently. The mice that received CD150$^{pos}$/HoxA9 HSCs showed a decrease in GFP+ donor cells from 8.9% at 1 month (n=17) to 4% at 5 months (n=12). However, the CD150$^{neg}$/HoxA9 mice showed an increase of cells that express HoxA9, from 6.1% at 1 month (n=17) to 9% at 5 months (n=12).

## 5.2. Is HoxA9 capable of inducing leukemia in My-bi and Ly-bi HSCs? Mice injected with CD150$^{neg}$/HoxA9 HSCs showed an enlarged spleen

As AML causes splenomegaly, the spleen weight was used as an indicator for the progress of leukemia. Differences in spleen size were already seen macroscopically (Fig. 9). For the CD150$^{neg}$/HoxA9 HSC population (mean=0.17 g; SD=0.07 g; n=10) 7 out of 10 mice showed an enlarged spleen $\geq$0.15 g. This was in contrast to the CD150$^{pos}$/HoxA9 HSCs group (mean=0.09 g; SD=0.02 g; n=6) where none of the 6 analyzed mice showed an enlarged spleen at time of death. An unpaired *t*-test was significant with a *P* value of 0.006 for the spleen weights of both groups.

No signs of leukemia were observed in primary host mice that received CD150$^{pos}$/HoxA9 HSCs. To further examine the leukemic behavior, whole bone marrow cells from primary host mice were serially transplanted into secondary host mice. Interestingly, 2 to 2.5 months post-transplantation, spleens of secondary host mice displayed enlarged morphology (Fig. 10). Moreover, in all secondary host mice treated with CD150$^{neg}$/HoxA9, we observed spleens markedly increased in size. In contrast to the primary host, all 4 secondary host mice of the CD150$^{pos}$/HoxA9 group (mean=0.25 g; SD=0.09 g; n=4) also showed an enlargement (spleen weight $\geq$0.15 g). Nevertheless, the spleens of the CD150$^{neg}$/HoxA9 mice (mean=0.36 g; SD=0.08 g; n=4) were slightly bigger.

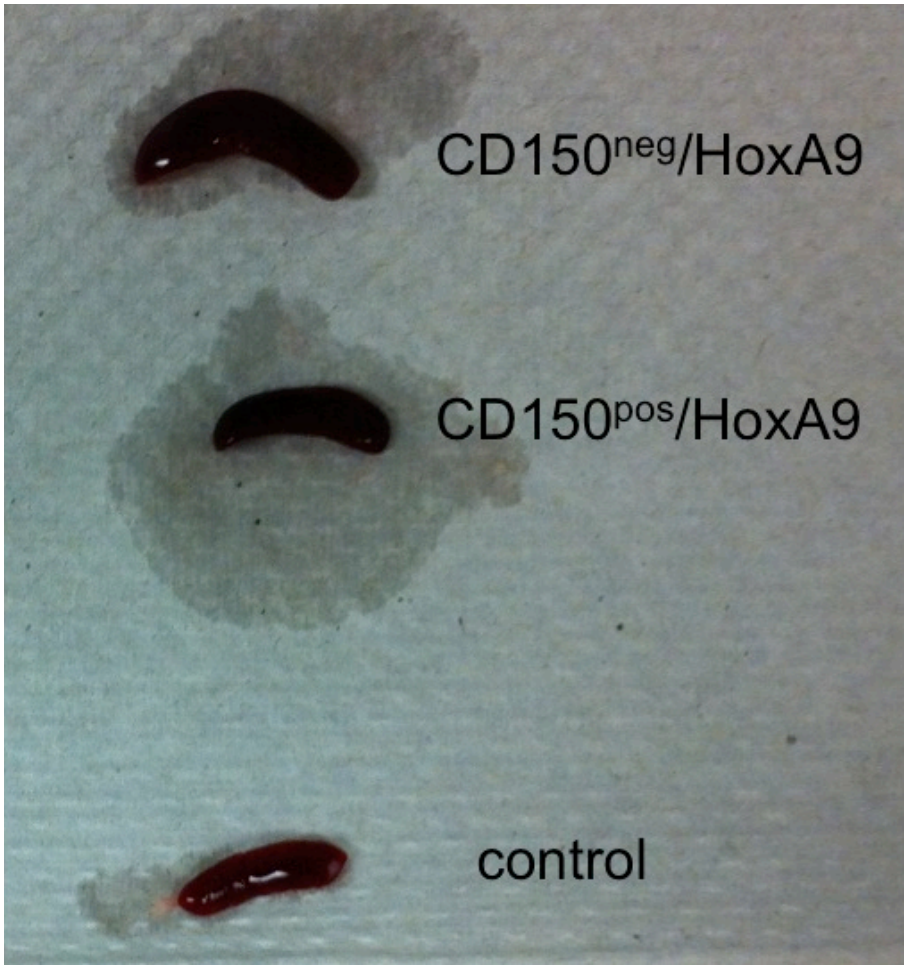


**Figure 9: Comparison of spleen size of primary host mice**. Primary host mice were injected with HSC subsets and sacrificed at 208 days post-transplantation. As a control mouse an age-matched untreated animal was used. The spleens were freshly harvested, and the surrounding fat tissue was removed. Notably, the size between the spleens differed markedly.

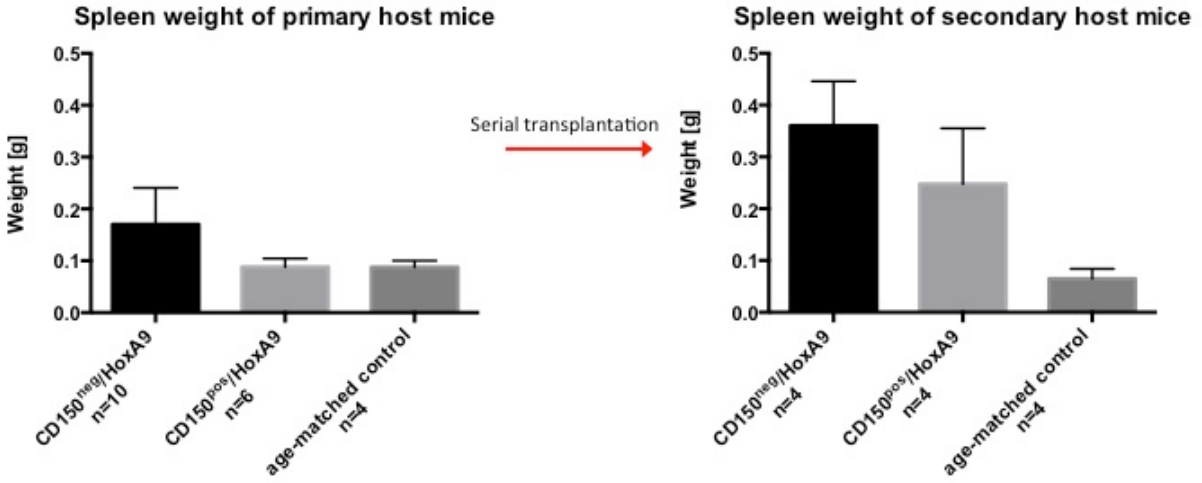


**Figure 10: Spleen weights of primary and secondary host mice (mean and SD).** Spleen weight (vertical axis), at time of death, of primary and secondary host mice injected with $CD150^{pos}$ HoxA9 transduced HSCs or $CD150^{neg}$ HoxA9 transduced HSCs. For $CD150^{neg}$/Hoxa9 HSCs (mean=0.17g; SD=0.07g; n=10), 7 out of 10 primary mice showed an enlarged spleen $\geq$0.15g in contrast to $CD150^{pos}$/Hoxa9 HSCs (mean=0.09g; SD=0.015; n=6), where none of the 6 analyzed primary mice showed an enlarged spleen at time of death. All secondary host mice showed an enlargement of the spleen (spleen weight $\geq$0.15g). Nevertheless, the spleens of the $CD150^{neg}$/HoxA9 mice (n=4) were slightly bigger than the $CD150^{pos}$/HoxA9 mice (n=4). The control mice (n=4) were age-matched animals that were not injected with any cells.

## 5.3. Mice injected with $CD150^{neg}$/HoxA9 HSCs showed higher HoxA9 activity

The HoxA9 activity is linked to GFP expression, which makes it possible to measure the activity of HoxA9. At time of euthanasia, the mouse cells were stained for CD45.1, and then the percentages of GFP+ cells for blood, spleen, and bone marrow were checked with a FACSCalibur cytometer (Fig. 11). The data showed that in both groups the activity of GFP in the blood was about 16%, and was significantly lower than in the bone marrow for both groups together $P< 0.0001$ in a paired *t*-test.
Interestingly, the GFP activity in spleen ($P$=0.0076 unpaired *t*-test) and bone marrow ($P$=0.014 unpaired *t*-test) in $CD150^{neg}$/HoxA9 HSCs was significantly higher than in the $CD150^{pos}$/HoxA9 group. At time of euthanasia, the bone marrow consisted of about 84% of HoxA9-expressing cells and the spleen consisted of 33.5%. This extensive growth was not observed in the $CD150^{pos}$/HoxA9 population, where the percentage of HoxA9-expressing cells was about 43% in bone marrow and 16% in spleen. The data suggest some correlation between the GFP expression levels in

blood and spleen (Pearson product-moment correlation coefficient of r=0.5148). By contrast, the GFP expression levels in blood and bone marrow were uncorrelated (r=0.0199).

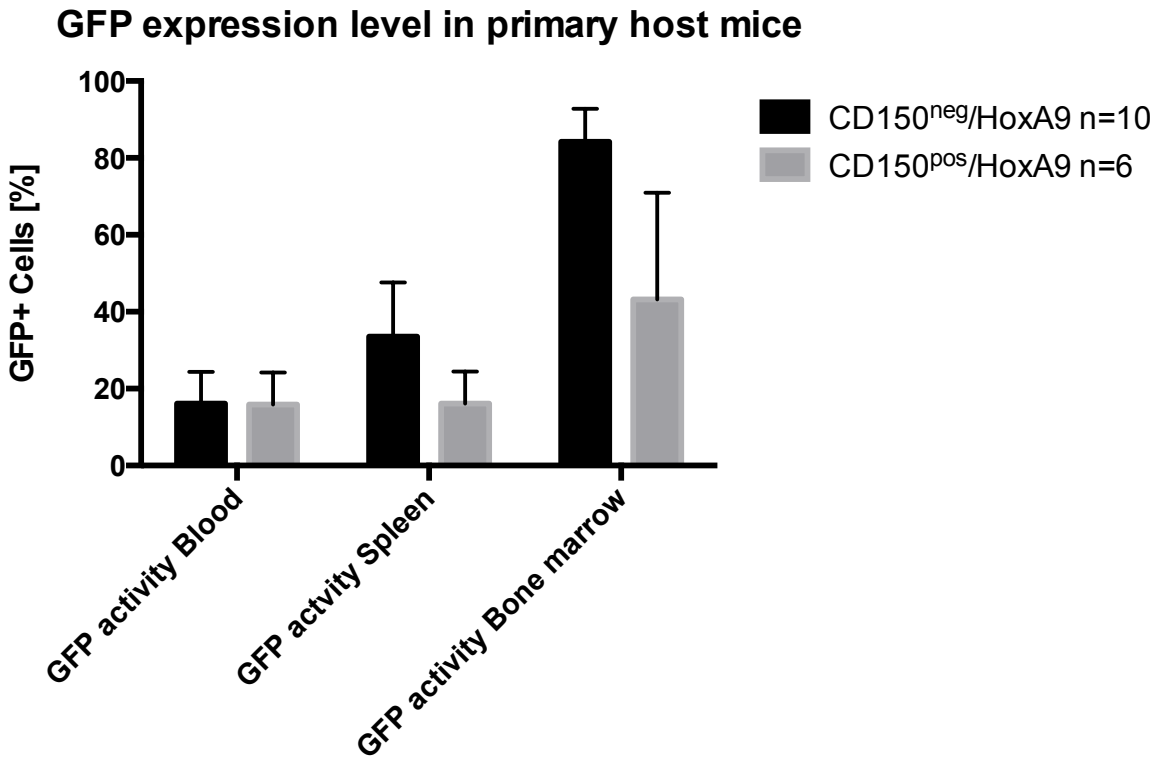


**Figure 11**: **GFP expression level in primary host mice (mean and SD).** GFP is an indicator for HoxA9 activity. Both CD150$^{neg}$/HoxA9 (n=10) and CD150$^{pos}$/HoxA9 (n=6) mice showed low levels of GFP activity in the blood compared with bone marrow. The bone marrow of the CD150$^{neg}$/HoxA9 mice consisted almost completely of cells positive for GFP. In the mice that received CD150$^{pos}$/HoxA9, the bone marrow is composed of less than half of transduced cells. For both groups together a correlation analysis showed a Pearson product-moment correlation coefficient of r=0.5148 between the GFP activity in the blood and spleen. The correlation between GFP activity in the blood and bone marrow was negligible (r=0.0199).

## 5.4. What blood marker changes can be seen? CD150$^{neg}$/HoxA9 primary host mice showed high numbers of Mac-1 positive cells in the blood

Blood samples of injected mice were taken after 1, 3, and 5 months and were analyzed for B-cells (6B2), T-cells (31-11), and myeloid cells (Mac-1/8C5) using fluorescent antibodies, and the emissions were measured with a FACSCalibur cytometer. A total of 17 mice were injected with CD150$^{neg}$/HoxA9 HSCs and 17 mice

received CD150$^{pos}$/HoxA9 HSCs. Mice which showed at least 2% of GFP activity in the blood were considered for analysis. For the CD150$^{neg}$/HoxA9 samples, a total of 10 mice were analyzed. However, not every mouse had blood samples taken every month. There were different reasons for this, for example, because of a failure of the transplanted HSCs or because some mice were euthanized earlier than 5 months as they showed signs of leukemia. In addition, if the GFP levels were below 2% a reasonable evaluation was not possible. For CD150$^{pos}$/HoxA9 HSCs a total of 6 mice were analyzed, and they had various numbers of samples for every month. The behaviors of CD150$^{neg}$ and CD150$^{pos}$ HSCs, which remained uninfected, have been published before (16, 19, 20, 53). The data for Figure 12 were acquired by gating for Ly45.1+ and GFP- cells. Therefore, the data represent HSCs sorted for CD150$^{pos}$ and CD150$^{neg}$ that have not been transduced with the HoxA9-overexpressing retrovirus vector. The non-infected CD150$^{neg}$ HSCs showed a noticeable bias towards differentiation into lymphoid cells. However, the blood samples of the HoxA9 transduced CD150$^{neg}$ HSCs behaved differently. These cells showed high numbers of myeloid cells from the first month onward, whereas CD150$^{pos}$ HSCs transduced cells showed a similar behavior to non-infected CD150$^{pos}$ cells (Fig. 12 and Fig. 13).

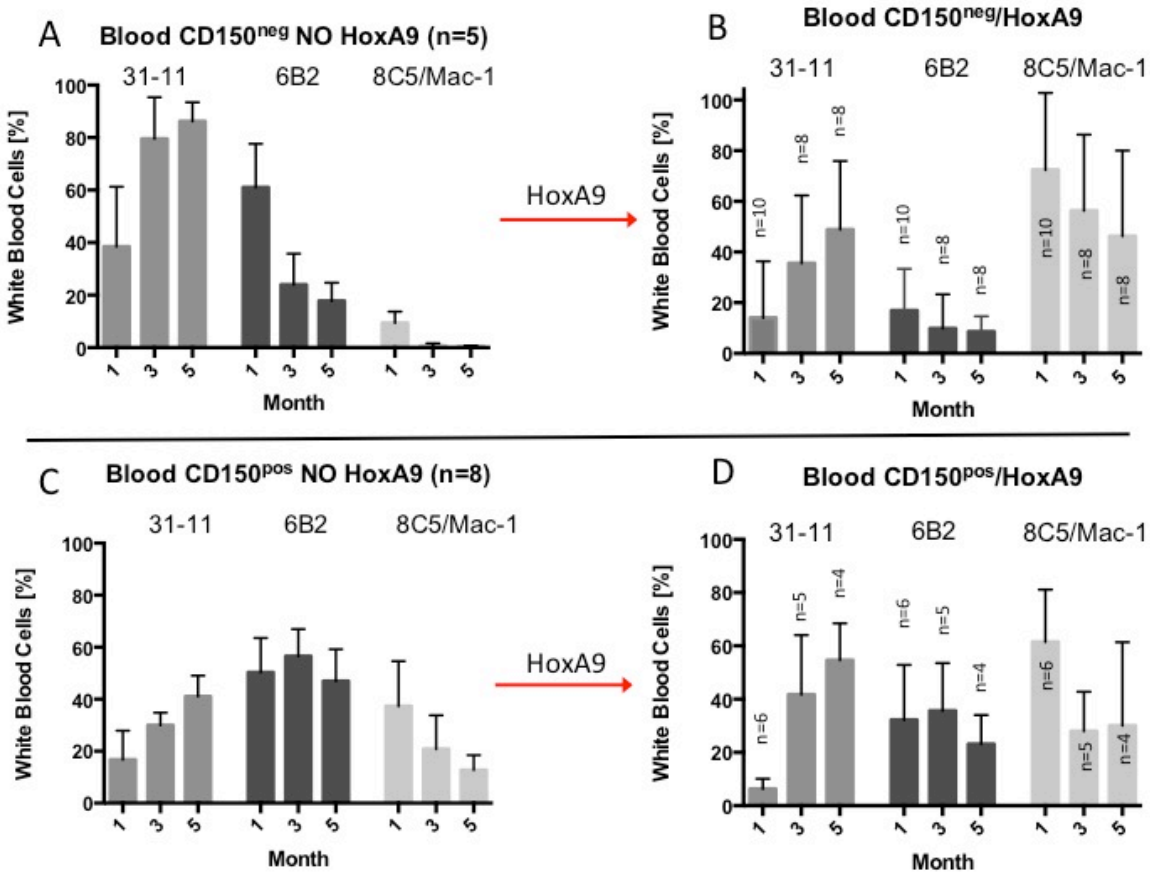


**Figure 12**: **Comparison of blood marker profiles of non-infected and transduced CD150$^{pos}$ and CD150$^{neg}$ HSCs.** The blood samples were taken after 1, 3, and 5 months (horizontal axis). The non-infected CD150$^{neg}$ HSCs (A) (n=5) showed a noticeable bias towards differentiation into lymphoid cells (31-11 and 6B2). However, HoxA9-transduced CD150$^{neg}$ HSCs (B) showed high numbers of myeloid cells (8C5/Mac-1) starting with the first month (n=10). At the third and fifth month blood samples were drawn from 8 mice (n=8).

Interestingly, CD150$^{pos}$ HoxA9-transduced cells (D) showed an almost similar behavior to non-infected CD150$^{pos}$ cells (C) (n=8). From CD150$^{pos}$/HoxA9 mice (D), at the first monst blood was drawn from 6 mice (n=6) at the third month from 5 mice (n=5) and at the fifth month from 4 mice (n=4).

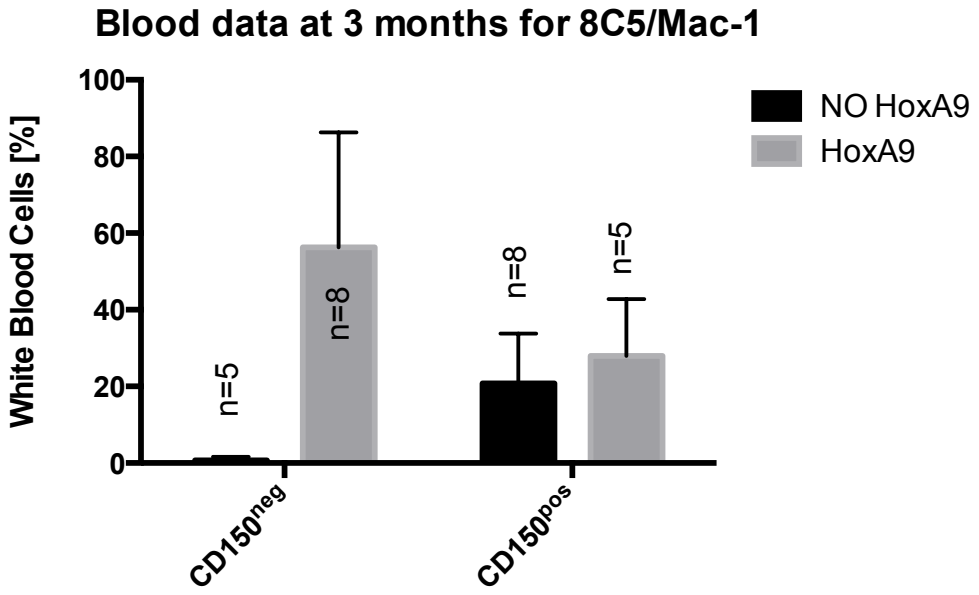


**Figure 13**: **Blood data at 3 months for 8C5/Mac-1.** This graph highlights the data from Figure 12 for the blood marker 8C5/Mac-1. There was a significant difference *(P*<0.002) for the CD150$^{neg}$ HSCs between the transduced (n=8) vs. non-infected mice (n=5). However, there was no significant (*P*=0.3756) difference between the transduced (n=5) vs. non-infected CD150$^{pos}$ HSCs (n=8).

## 5.5. Which roles do the bone marrow and spleen play in the leukemic behavior? CD150$^{pos}$/HoxA9 primary host mice showed high numbers of Mac-1 cells in the bone marrow but not in the spleen

The white blood cells were stained with monoclonal antibodies specific for Thy-1 (T-lymphocytes), B220 (B-lymphocytes), and Gr-1 (granulocytes) and Mac-1 (macrophages). For the CD150$^{neg}$/HoxA9 and CD150$^{pos}$/HoxA9 groups, 10 mice and six mice were analyzed, respectively. In bone marrow no significant difference between the 2 groups was detected. The HoxA9-expressing cells in the bone marrow were almost completely Mac-1 positive cells. In CD150$^{neg}$/HoxA9 mice, the mean was 85%. In the CD150$^{pos}$/HoxA9 group the mean was 77%. A non-parametric Mann-Whitney test had a *P* value of 0.0714 for this marker. A macroscopic picture of bone marrow cells is shown in Figure 15, and micrographs of the bone marrow are shown in Figure 21.

In the spleen, significant differences between the CD150$^{neg}$/HoxA9 and CD150$^{pos}$/HoxA9 group were detectable. The CD150$^{neg}$/HoxA9 consisted mostly of Mac-1 positive cells (mean 68.7%). An unpaired *t*-test showed a *P* value of 0.0365 for this marker. In the spleens of the CD150$^{neg}$/HoxA9 mice there were also cells

positive for 31-11 (20.8%) and 8C5 (15.4%). Again, the number for 6B2 positive cells was low at 7.8%. The HoxA9 positive cells of CD150$^{pos}$/HoxA9 primary hosts showed a completely different picture; here the cells that were positive for 31-11 represented the largest group (46.1%). An unpaired *t*-test for this marker was not significant (*P*=0.0866). However, a non-parametric Mann-Whitney test showed significance with a value *P*=0.042. About 36.2% of the cells were positive for 6B2 (unpaired *t*-test *P*=0.0267).

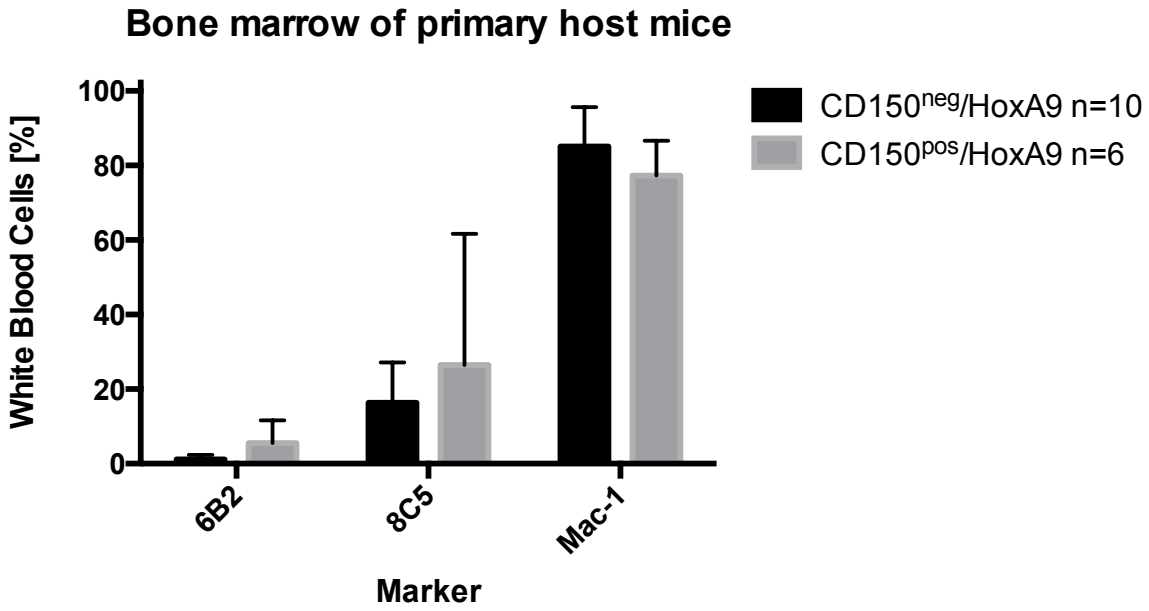


**Figure 14: Marker profile of bone marrow cells of primary host mice.** CD150$^{neg}$/HoxA9 mice (n=10) and CD150$^{pos}$/HoxA9 mice (n=6) showed a similar marker profile in the bone marrow with high numbers of Mac-1 positive cells.

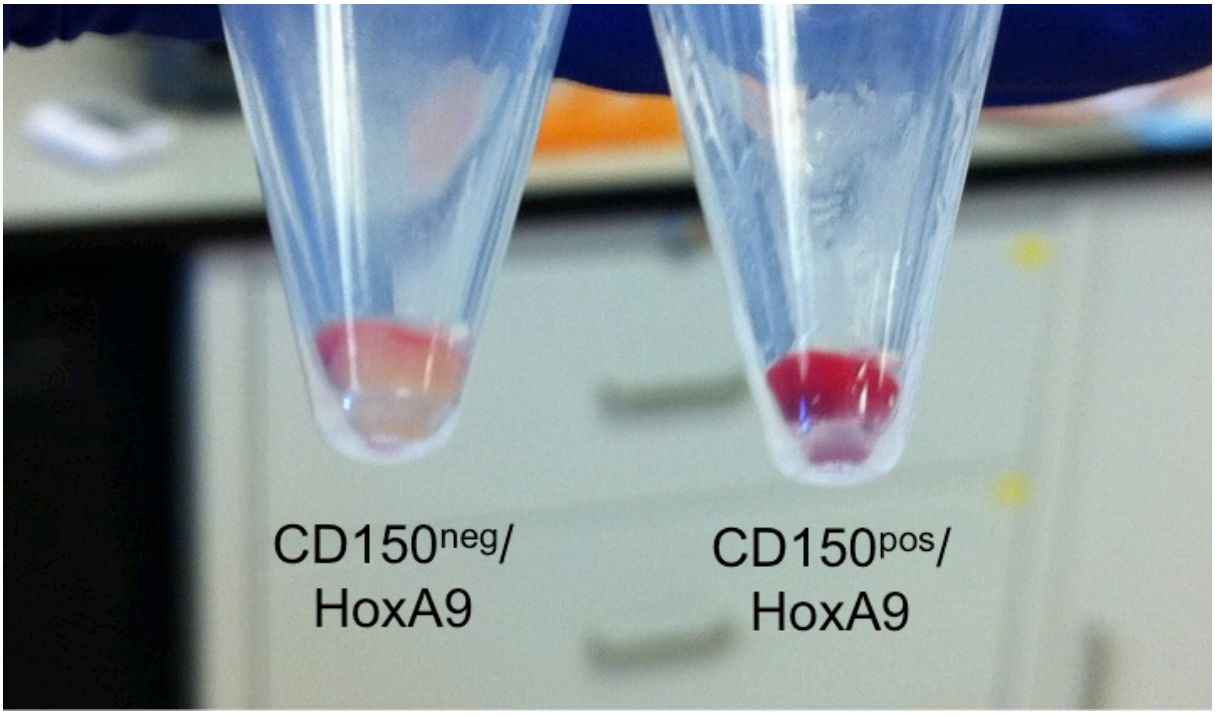


**Figure 15: Bone marrow cells of 2 primary host mice.** One mouse received CD150$^{neg}$/HoxA9 HSCs and the other mouse received CD150$^{pos}$/HoxA9 HSCs. The bone marrow was harvested, filtered, and then centrifuged. We can clearly see a difference in the color of these cell pellets. Although the marker profile is not different, the number of transduced cells is much higher in the bone marrow of the mice which were injected with CD150$^{neg}$/HoxA9 HSCs, and the bone marrow consists almost completely of cells that have been transduced with HoxA9. The left test tube shows the typical white color of a leukemic bone marrow sample due to the enrichment of leucocytes characteristic of leukemia. Hence, we can easily understand why Virchow coined the name leukemia for this disease.

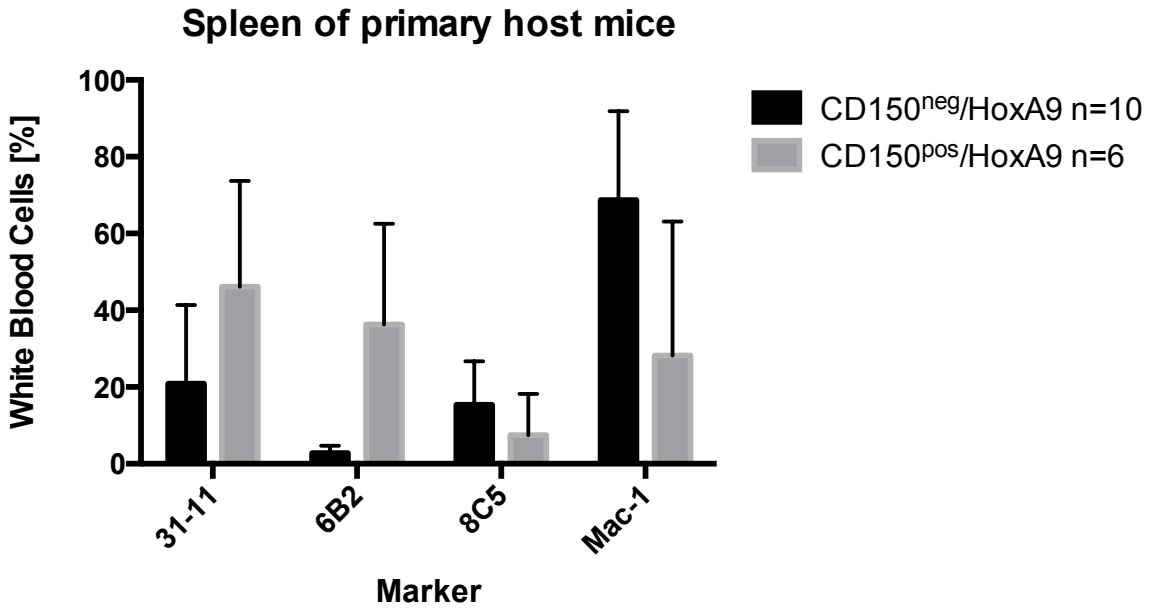


**Figure 16: Marker profile of spleen cells of primary host mice.** The spleen cells of mice (n=10) which received CD150$^{neg}$/HoxA9 HSCs showed a similar marker profile as in the bone marrow with a high number of Mac-1 positive cells. Spleen cells of CD150$^{pos}$/HoxA9 HSCs mice (n=6) showed a completely different picture; here the cells that were positive for 31-11 were the largest group (46.1%), and about 36.2% of the cells were positive for 8C5. In contrast to the bone marrow, the number of Mac-1 positive (28.2%) cells is relatively low.

## 5.6. Does the leukemic behavior stay consistent after serial transplantation of bone marrow cells into new host mice?

For further investigations, 1 mouse of the primary CD150$^{neg/}$HoxA9 group and 1 of the CD150$^{pos}$/HoxA9 group were chosen at random, and the unseparated bone marrow was transplanted into 4 new host mice for each donor. For analysis and further serial transplantations these mice were all sacraficed between 2 and 2 1/2 months after the start of the experiment.

### 5.6.1. Secondary host mice showed an increased HoxA9 activity

After euthanasia, the blood, spleen and bone marrow of the secondary host mice were analyzed using the same antibodies and procedures as in the primary hosts. In comparison to the GFP levels of the primary host mice, the levels of GFP+ cells had increased dramatically (Fig. 17). In particular, the number of GFP+ cells in the blood and spleen were increased. The bone marrow consisted almost exclusively of GFP+

cells in both groups with no significant difference between the groups. The GFP activity in the spleen (*P*=0.0254 unpaired *t*-test) was significantly higher in the CD150$^{neg}$/HoxA9 group. For the GFP levels in the blood a non-parametric Mann-Whitney test was not significant (*P*=0.0571).

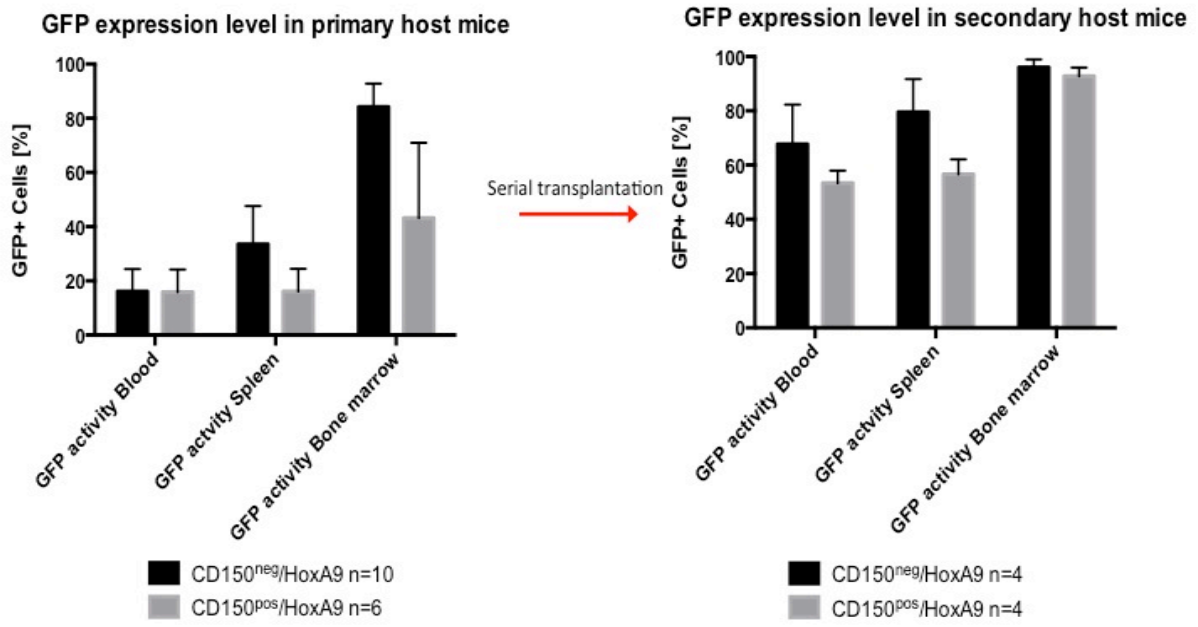


**Figure 17: GFP expression level of primary and secondary host mice.** GFP is an indicator for the HoxA9 activity. The data for the primary CD150$^{pos}$/HoxA9 mice (n=6) and primary CD150$^{neg}$/HoxA9 mice (n=10) were already shown in Figure 11. Both CD150$^{pos}$/HoxA9 (n=4) and CD150$^{neg}$/HoxA9 (n=4) showed an increase of GFP+ cells in all 3 examined tissues in the secondary host mice. The bone marrow of both groups consisted almost completely of GFP+ cells; in addition, the increase of GFP activity in blood and spleen compared to the primary group was striking.

### 5.6.2. Secondary host mice showed high numbers of Mac-1 positive cells in bone marrow, spleen, and blood

The analysis of the GFP+ cells in blood, spleen and bone marrow showed a dramatic increase of Mac-1 positive cells in the secondary host mice of the CD150$^{pos}$/HoxA9 group. In the secondary hosts no significant difference in the behavior of the transduced cells between the 2 groups was detected. Interestingly, the cells of the CD150$^{neg}$/HoxA9 group began to show a loss of cell surface markers in spleen and bone marrow, which is indicated by a low percentage when all markers are added together. The beginning of the loss of markers and function, especially in the bone marrow, is also visible under the microscope (Fig. 21).

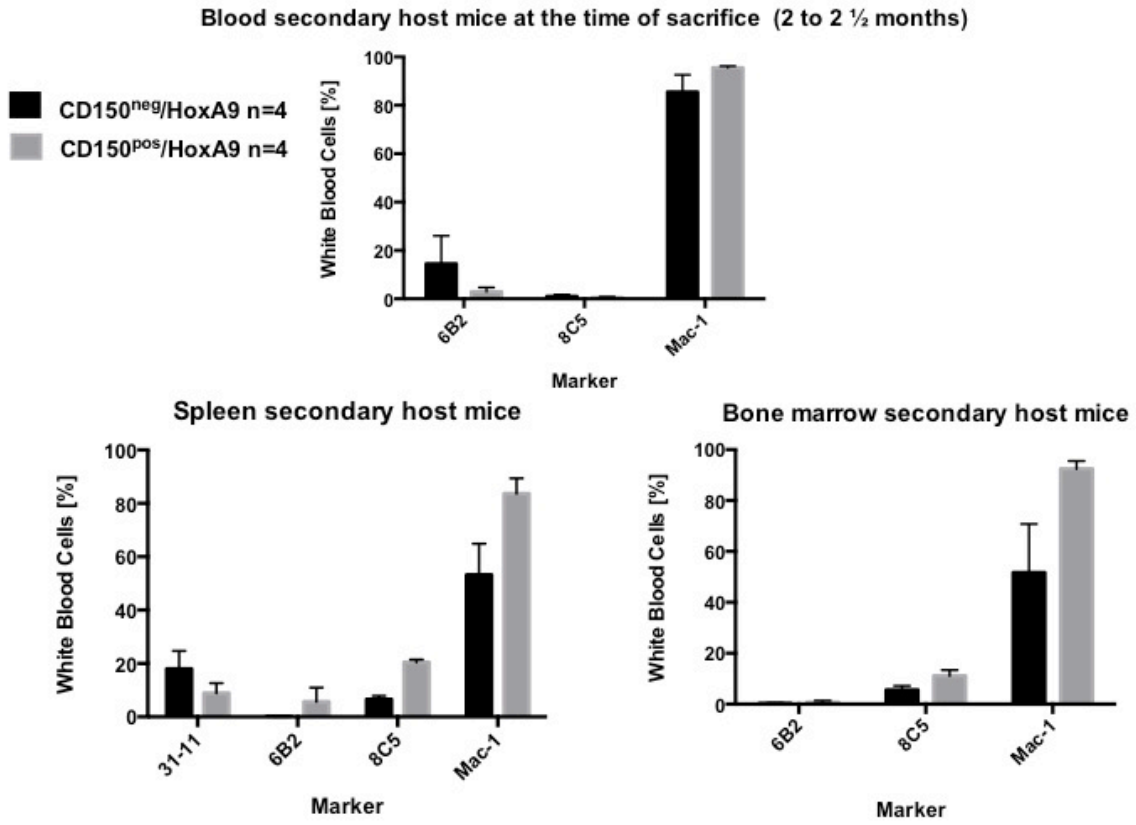


**Figure 18: Marker profile in blood, spleen, and bone marrow cells of secondary host mice.** The analysis of GFP+ cells showed a marked increase of Mac-1 positive cells in the secondary host mice of the CD150$^{pos}$/HoxA9 (n=4) group. The secondary host mice of both groups now show a similar marker profile. Furthermore, the marker profile in the 3 tissues looks very similar with a high number of Mac-1 positive cells. The spleen and bone marrow cells of the CD150$^{neg}$/HoxA9 (n=4) secondary host mice began to show a loss of cell surface markers with a decrease in Mac-1 positive cells.

## 5.7. Spleen cells and bone marrow cells showed no difference in leukemic behavior when serially transplanted

The primary host mice showed low numbers of HoxA9 activity (GFP+) in the blood compared to the high numbers in the bone marrow. This could suggest that extra-medullary hematopoiesis takes place and keeps the organism alive. One possible place for this extra-medullary hematopoiesis is the spleen. To investigate this further, spleen cells and bone marrow cells from secondary host mice were transplanted into new hosts to see if different behavior could be observed. Two secondary host mice for both the CD150$^{neg}$/HoxA9 group and for the CD150$^{pos}$/HoxA9 group were used. Staining of bone marrow and spleen cells with mAb was performed. The remaining

bone marrow cells were mixed and transplanted into the 2 new hosts for each group. In the same way, the remaining spleen cells were utilized. The main observation made was that the leukemia became more aggressive and resulted in the sudden death of all 4 mice within 1 month that received either bone marrow or spleen cells from the CD150$^{neg}$/HoxA9. No obvious differences were visible between the mice that received bone marrow and the ones that received spleen cells. Because of the sudden death of those mice, no statistically valid staining was possible. The tertiary host mice of the CD150$^{pos}$/HoxA9 group lived up to five months, and showed no difference between the mice which received spleen cells and the mice which received bone marrow cells.

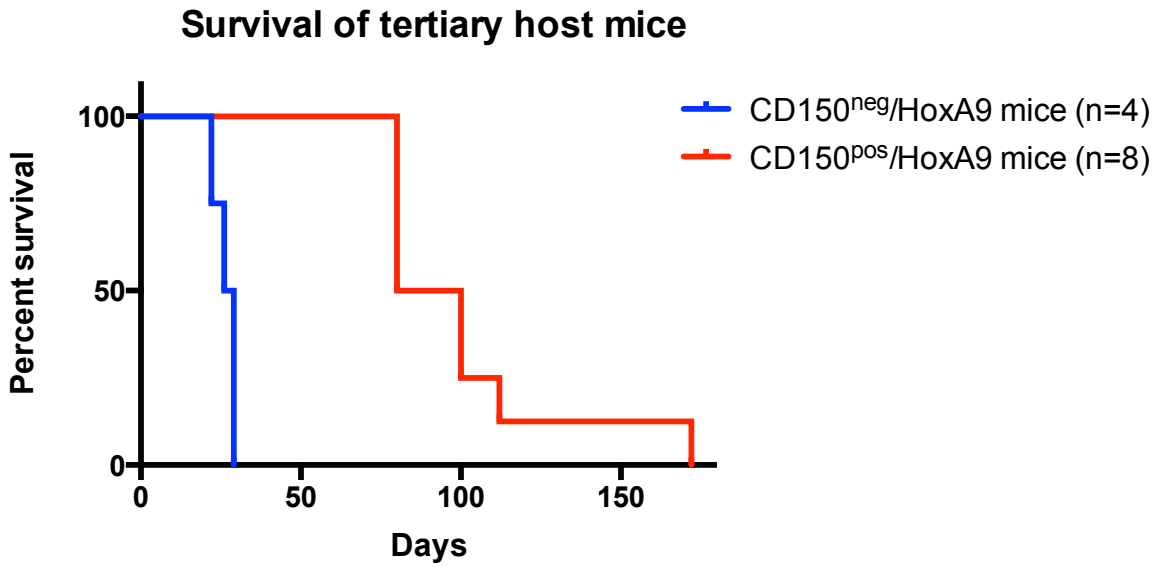


**Figure 19: Time of survival of tertiary host mice.** The mice were either euthanized when they showed signs of leukemic disease or they were found dead. All mice that received CD150$^{neg}$/HoxA9 cells (n=4) died within 1 month and the mean survival was 27,5 days. However, the mice that received CD150$^{pos}$/HoxA9 cells (n=8) lived up to 5 months and the mean survival was 90 days. The Mantel-Cox test shows a significant difference between the two groups (*P*=0.0004)

## 5.8. The number of transplanted cells had no impact on the leukemic behavior

To demonstrate that the number of transplanted cells had no influence on the leukemic behavior, a serial dilution serial transplantation experiment was performed. Consequently, a primary host mouse of the CD150$^{pos}$/HoxA9 and the CD150$^{neg}$/HoxA9 group was euthanized, and unseparated whole bone marrow was

serially transplanted into new hosts. Different amounts of bone marrow cells were serially transplanted $10^6$, $3x10^6$, and $10^7$. The behavior in the blood was monitored with monoclonal antibodies specific for Thy-1 (T-lymphocytes), B220 (B-lymphocytes), and Gr-1 (granulocytes) and Mac-1 (macrophages) after 1 month. The primary hosts showed typical behavior as described in section 5.4. The blood of the primary $CD150^{neg}$/HoxA9 host consisted almost exclusively of 8C5/Mac-1 positive cells (90%). The blood of the $CD150^{pos}$/HoxA9 mouse was more diverse with 48.1% cells positive for 6B2 and only 23.4% positive for 8C5/Mac-1. The number of 31-11 positive cells was 6.73% lower than in the $CD150^{neg}$/HoxA9 host (20.5%). After serial transplantation of different numbers of unseparated whole bone marrow cells into new secondary host mice, the blood was evaluated after 1 month. A multivariate analysis of variance (MANOVA) showed that the number of transplanted cells had no influence on the leukemic behavior. The $CD150^{neg}$/HoxA9 cells again consisted for the most part of 8C5/Mac-1 positive cells with all figures greater than 95%. In the new hosts of the $CD150^{pos}$/HoxA9 the MANOVA also showed that the number of transplanted cells had no influence. Interestingly, the blood of the $CD150^{pos}$/HoxA9 mice shifted towards the myeloid lineage, with 8C5/Mac-1 positive cells being the biggest component (between 46% and 59%). This is consistent with the observations from 5.6.2.

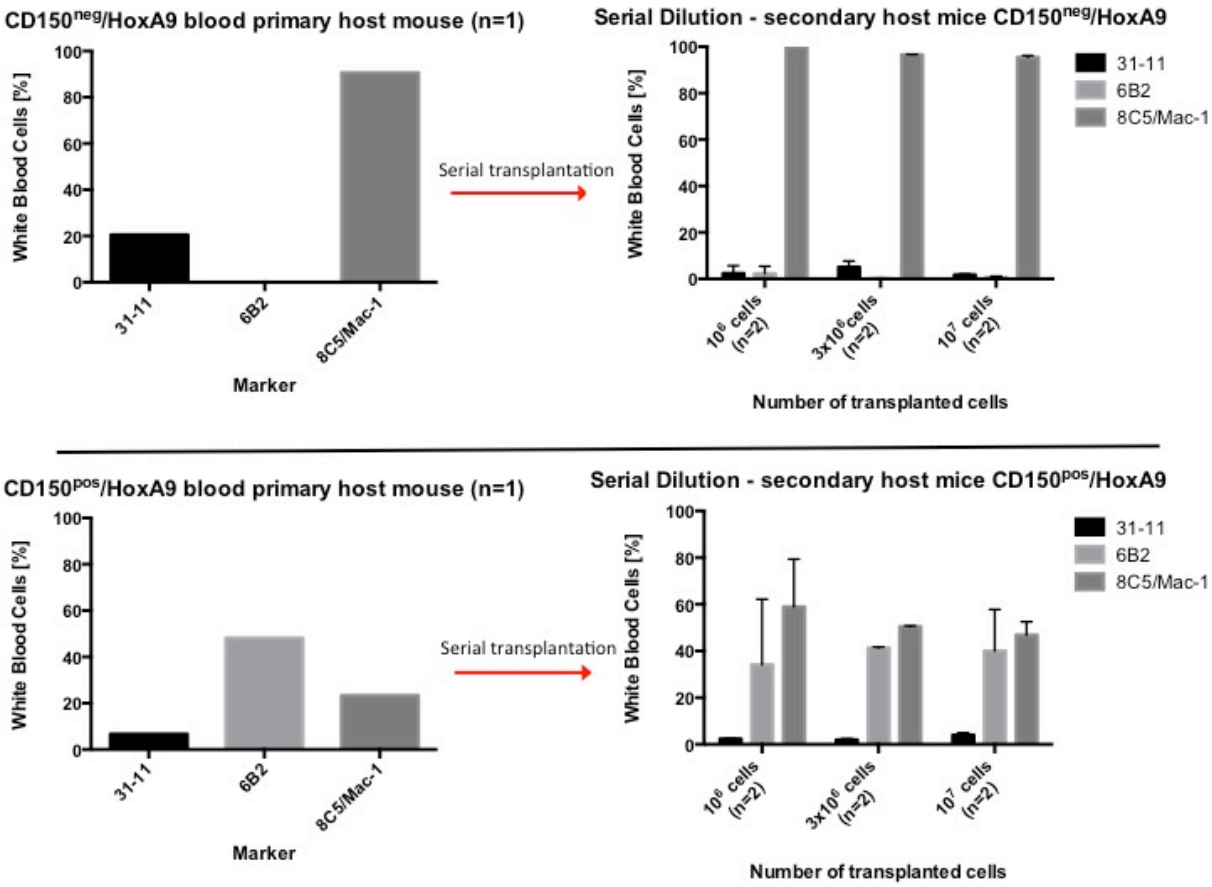


**Figure 20**: **Serial transplantation of different cell numbers.** The left side represents the primary host of a CD150$^{neg}$/HoxA9 (n=1) and a CD150$^{pos}$/HoxA9 (n=1) mouse. The red arrow represents the transplantation of different numbers ($10^6$, $3x10^6$, $10^7$) of whole unseparated bone marrow cells from the primary host into new secondary hosts. Every bone marrow dilution was transplanted int 2 secondary host mice (n=2 for every secondary host mice). The right sides are the data from the first month blood sample of the secondary host mice. A MANOVA showed that the number of transplanted cells had no influence on the leukemic behavior. However, the CD150$^{pos}$/HoxA9 cells shifted towards the myeloid lineage.

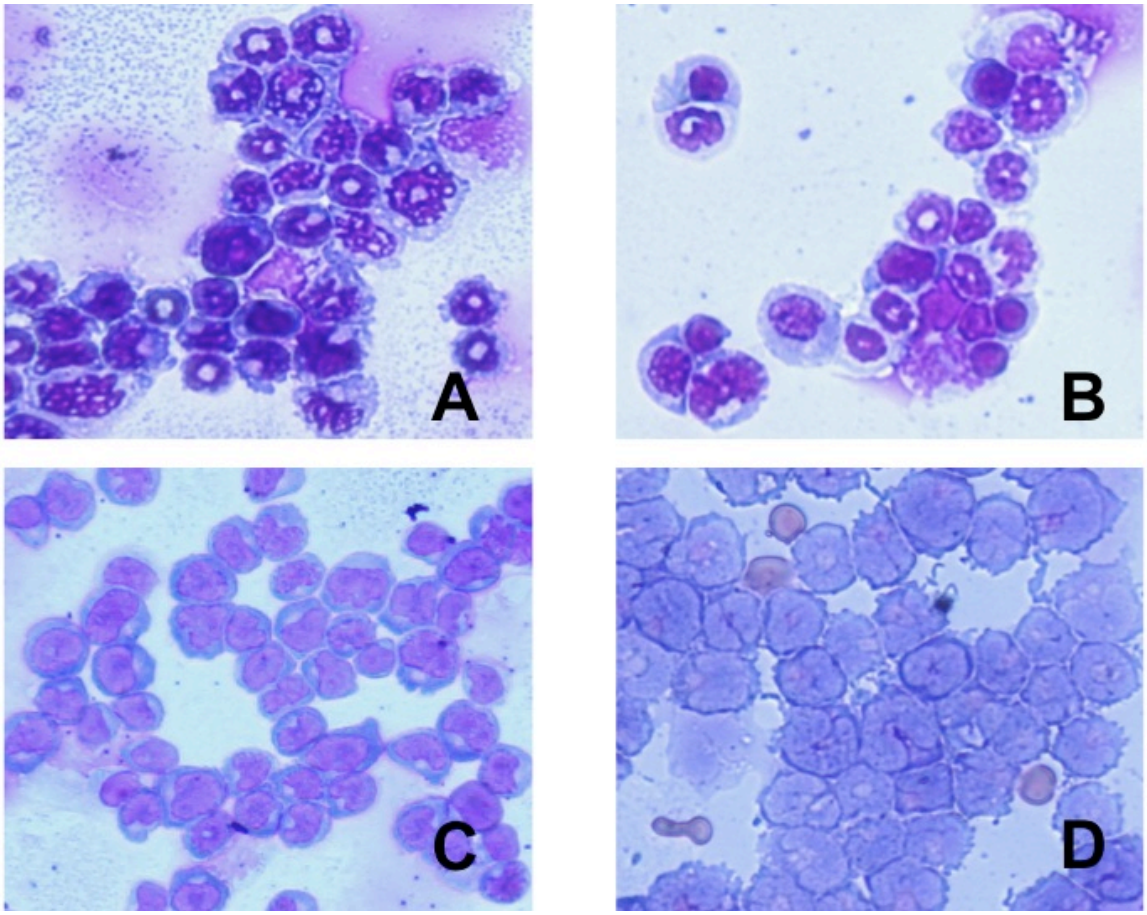


**Figure 21**: **Photomicrographs of bone marrow cells.** The micrographs are from different host mice (original magnification x200). Note that the difference in cell density could be due to the production process of the slides when the cells were diluted (See 4.15.) and does not represent the actual cell density. Picture A shows bone marrow from a primary host mouse that received $CD150^{neg}$/HoxA9 HSCs. Picture B shows bone marrow of a primary host mouse that received $CD150^{pos}$/HoxA9 HSCs. Picture C shows bone marrow of a secondary host mouse. The cells appear to be uniform and to be myeloid blast cells, which is typical for the acute state of myeloid leukemia. Picture D shows bone marrow of a tertiary host ($CD150^{pos}$/HoxA9) with uniform cells that have a totally altered form and appearance.

# 6. Discussion

In these experiments the properties of several groups of HSCs that were modified in the same way were observed. Both $CD150^{pos}$/HSCs and $CD150^{neg}$/HSCs are a heterogenic mixture of various cell types. However, it can be assumed that the most prominent type of HSCs become transduced by the retroviral vector overexpressing HoxA9. This means that although $CD150^{pos}$ HSCs contain My-bi, Ly-bi and Bala HSCs, the My-bi are the ones who become transduced, and vice versa for the $CD150^{neg}$, where the Ly-bi HSCs become transduced. Therefore, we observed the behavior of different stem cell mixtures, which become transduced with a HoxA9 overexpressing retroviral vector. It can be assumed that the HoxA9 mutation not only alters the HSCs but also affects all daughter cells along the differentiation tree.

The laboratory of Christa Müller-Sieburg was the first to investigate the different leukemic behavior of My-bi and Ly-bi HSCs transduced with a retroviral vector overexpressing HoxA9. Rather than confirming the original hypothesis that only My-bi (CD150+) HSCs can give rise to myeloid leukemia, different leukemic behavior was seen in the 2 groups. Eventually, mice injected with transduced My-bi or Ly-bi (CD150-) developed myeloid leukemia. However, the mice that received Ly-bi HSCs showed an early and more aggressive onset of leukemia with an acute stage. The mice that received My-bi HSCs developed leukemia with a prolonged latency, and with no enlargement of spleens in the primary host.

## 6.1. Mice injected with $CD150^{neg}$/HoxA9 show early signs of leukemia

The spleen weight was used as an indicator for the progression of leukemia. The enlarged spleens of primary host mice injected with $CD150^{neg}$/HoxA9 HSCs indicate a rapid progressive leukemia, whereas in the $CD150^{pos}$/HoxA9-injected mice normal spleen weights and no macroscopic signs of leukemia were detectable. This was consistent with previous data, where transduction of unseparated HSCs with HoxA9 did not result in leukemia-specific phenotypes such as enlarged spleens (54). In line with these findings, we observed a similar phenotype within the $CD150^{pos}$/HoxA9 group. Notably, 70% of primary mice injected with $CD150^{neg}$/HoxA9 HSCs exhibited

enlarged spleens and signs of leukemia. Thus, the results indicate that Ly-bi HSCs mediate an early onset of leukemia in primary mice.

Strikingly, both groups of secondary host mice revealed a marked increase in spleen sizes. Although the mice were euthanized at an early age (2.5 months), their spleen size almost doubled compared to that of the primary hosts. This indicated a more aggressive progression of the leukemic disease. Nevertheless, the spleen sizes of the mice of the $CD150^{neg}$/HoxA9 group are larger than the mice of the $CD150^{pos}$/HoxA9. This fulminate progression of disease continued into the tertiary host mice, where all 4 recipients of the $CD150^{neg}$/HoxA9 cells died within 1 month of transplantation. Tertiary host mice of $CD150^{pos}$/HoxA9 HSCs survived up to 5 months, but then also showed signs of leukemia. This means mice that received $CD150^{pos}$/HoxA9 HSCs showed a survival benefit.

A possible explanation for the different behavior is that My-bi possess a high self-renewal capacity and a high repair rate for mutations, which protects them from “additional hits”; these are necessary to transform normal HSCs into leukemic stem cells when transduced with a retrovirus vector overexpressing HoxA9 (54). On the other hand, Ly-bi HSCs, which are short-lived, might not have these efficient repair mechanisms, more mutations could occur, and the cells could be transformed. This explanation would agree well with the observations of Thorsteinsdottir *et al.* (51) that HoxA9 overexpression alone is not enough to induce myeloid leukemia, and additional genetic events are necessary to trigger this leukemia. Beachy *et al.* suggest that isolated HoxA9 overexpression leads to a form of lymphoid leukemia (55). They established a transgenic mouse model to achieve the overexpression of HoxA9. However, the disease presentation of their mice was varied and some showed the typical signs of AML with enlarged spleens. From the long latency period of the disease onset, up to 18 months, they also concluded that other genetic factors were needed for disease development. The onset of leukemia in the secondary host mice of the $CD150^{pos}$/HoxA9 group could be due to the additional stress of the serial transplantation, which could induce mutations and finally transform the My-bi HSCs into leukemic stem cells. A better understanding of the molecular processes which protect My-bi HSCs could lead to an improvement in leukemia research. Another explanation could be supplied by the studies of Thorsteinsdottir *et al*. (51). They showed that overexpression of HoxA9 leads to greatly enhanced HSC regeneration.

Similar results have been observed with the human fusion gene NUP98-HoxA9. Overexpression of this fusion gene leads to a greater proliferation rate of HSCs and a longer period of time over which HSCs can self-renew (56, 57). As My-bi HSCs are already characterized by high self-renewal rates and long periods of self-renewal, it is possible that they can cope better with the additional stress of the HoxA9 overexpression. Ly-bi HSCs which have a low self-renewal capacity might be "overwhelmed" with the new conditions and more mutations might occur that transform Ly-bi HSCs into leukemic cells.

One limitation to this finding could be due to the serial transplantation experimental procedure. Although the results were significant, the bone marrow from 1 primary mouse was serial transplanted into 4 new secondary host mice. This means that although every secondary host mice was analyzed separately, the cells all originated from 1 primary host mouse.

## 6.2. HoxA9 slows down the repopulation of My-bi HSCs in the blood

In the primary hosts the blood repopulation behavior showed some interesting differences. We observed an increase of GFP+ cells in the mice that received $CD150^{neg}$/HoxA9 HSCs cells over 5 months. This could be a sign of a selection advantage and also of extensive growth, which is typical for leukemia cells. HoxA9 seems to increase the rate of growth of Ly-bi HSCs, whereas the $CD150^{pos}$/HoxA9 primary host mice showed a decrease of GFP+ cells over 5 months. This is especially noteworthy as My-bi HSCs are rapidly cycling cells and an overall increase of donor-type cells was seen in these mice. This might suggest that HoxA9 can slow down the growth of My-bi HSCs.

To further investigate the repopulation of $CD150^{neg}$/HoxA9 HSCs and $CD150^{pos/}$HoxA9 HSCs, a competitive *in vitro* experiment was designed (data not shown). Consequently, HoxA9 transduced HSCs were seeded together with F1 bone marrow cells in 1 cell flask. The hypothesis was that the $CD150^{neg}$/HoxA9 would suppress the healthy bone marrow cells. However, this was not observed, and although $CD150^{neg}$/HoxA9 HSCs possess a higher survival capacity in a competitive environment than $CD150^{pos/}$HoxA9 HSCs, both groups showed a decline in the number of transduced cells (GFP+) over a period of 2 weeks. However, the decline in the number of $CD150^{pos}$/HoxA9 cells was greater. There are multiple reasons why

the experiment did not show the predicted results. It is always difficult to grow leukemic cells *in vitro* and to find adequate culture conditions. Kroon *et al.* suggest that leukemic cells need 10% fetal calf serum and IL-3 to grow *ex vivo* (54). For these experiments, Dexter's medium was used that did not contain IL-3.

## 6.3. CD150$^{neg}$/HoxA9 mice show the typical marker profile of AML

The leukemic cells showed a noticeable bias towards the myeloid lineage. The CD150$^{neg}$/HoxA9 primary host mice showed high levels of Mac-1 positive cells in the blood from the first month onward. Mac-1 positive cells also dominated in the bone marrow and spleen of these mice. These findings are similar to the results of Kroon *et al.* (54). They induced leukemia by transducing HSCs with a virus overexpressing HoxA9/Meis-1, and the leukemic cells were 100% Mac-1 positive. The same research team transduced mice with a retrovirus vector overexpressing only HoxA9. In these mice, after 4 and 8 weeks post translation, these researchers saw normal bone marrow and thymus cellularity and only mildly enlarged spleens, which is consistent with the findings in the CD150$^{pos}$/HoxA9 primary host mice of these experiments (51). Another research group with similar results induced AML by overexpressing MLL-AF9 and also observed that almost all leukemic cells expressed Mac-1 or Gr-1 (58). However, Mac-1 positive cells seem to play an important role not only in mice but also in humans, where this kind of cell is observed in AML. The research group of Graf *et al.* (59) investigated the expression of Mac-1 (CD11b) in human AML. They found high expression of this marker especially in patients with the FAB subtypes M5, M4, and M1 and also in patients with an adverse cytogenetic risk. Therefore, they concluded that the high expression of Mac-1 is associated with a worse prognosis in humans with AML. The high levels of Mac-1 positive cells shown early by CD150$^{neg}$/Hoxa9 primary host mice are further evidence for the more acute state of this type of leukemia. High levels of Mac-1 positive cells in the CD150$^{pos}$/HoxA9 mice were only observed after serial transplantation into secondary host mice.

## 6.4. The spleen could be a place of extra-medullary hematopoiesis

The blood from the primary hosts of both groups showed low levels of GFP/HoxA9 activity. However, the GFP activity in the bone marrow was much higher. The difference between peripheral circulating blast cells and the blast levels in the bone marrow is not well understood. Amin *et al.* performed a monocentric study where they compared these 2 blast levels in patients with myelodysplastic syndrome (MDS), acute lymphocytic leukemia (ALL), or AML. They showed that in patients with MDS and ALL, higher peripheral blast cell levels were associated with a more aggressive disease and a shorter survival. In patients with AML this was not true, as the circulating blast levels seemed to have no clinical relevance. They hypothesized that adhesion molecules, chemokine, angiogenetic factors, and homing play a role in mobilizing blast cells in the bone marrow. However, they had no explanation why some AML patients have “massive infiltration of bone marrow by blasts but lack circulating blasts” (60). This lack of circulating blast cells, while the bone marrow is almost completely infiltrated, could be an indicator for extra-medullary hematopoiesis. When the bone marrow begins to fail, healthy hematopoiesis and also some parts of the leukemic hematopoiesis are relocated to organs like the spleen and liver to keep the organism alive. However, the origin of these hematopoietic precursor cells and the molecular mechanisms underlying their development in the spleen is uncertain. Hsieh *et al.* suggested that in chronic myeloproliferative disorders these cells originate from transformed bone marrow cells (61). In our experiment the correlation analysis suggests that the cells that are circulation in the blood correlate more with the cells in the spleen than in the bone marrow. Which suggests that the hematopoiesis in the bone marrow fails, and then the cells are not mobilized from the bone marrow and do not circulate in the blood. The spleen and probably other organs take over hematopoiesis and their cells circulate in the blood. The $CD150^{neg}$/HoxA9 HSCs mice showed higher levels of GFP+ cells in the bone marrow and spleen in primary hosts compared to the $CD150^{pos}$/HoxA9 cells. From these results it can be assumed that $CD150^{neg}$/HoxA9 cells grow extensively and suppress normal cells, until the bone marrow consists exclusively of HoxA9-transduced cells. This is typical for an acute stage of leukemia. In the secondary host mice, the main observation regarding the GFP levels is the markedly increased number of GFP+ cells in the blood and spleen, which indicates that the healthy extra-medullary hematopoiesis is

not sufficient or that it is dominated by leukemia cells. The organism fails to produce healthy cells, and we can observe the outcome to be fully developed leukemia. To investigate the behavior of extra-medullary hematopoiesis further, bone marrow and spleen cells from secondary host mice were transplanted into new host mice to observe their leukemic behavior. There was, however, no significant difference between mice that received spleen cells and mice that received bone marrow cells. This might be due to our use of secondary host mice. In this population the percentage of HoxA9 expressing cells was already very high in both tissues, and both tissues showed a similar marker profile with high numbers of Mac-1 positive cells, which is typical for AML. Transplantation of spleen and bone marrow from primary host mice, when the extra-medullary hematopoiesis is still intact, might reveal different results.

## 6.5. Through serial transplantation leukemia becomes more aggressive

Beginning in the secondary, and continuing in the tertiary hosts, we saw a loss of markers in the $CD150^{neg}$/HoxA9 mice. This is another indicator that the AML is more aggressive and progresses faster in these mice. Loss or shifts of cell surface markers are widely observed in cases of leukemia. Tomová *et al.* saw a frequent loss of CD34 and CD10 in their patients with ALL. They also observed a loss of CD1 and CD2 in patients with T-ALL (62). Yoo *et al*. even suggested that the expression of CD7 should be included in the diagnosis of natural killer-cell neoplasms, because they observed a frequent loss of CD7 in patients with aggressive natural killer-cell leukemia (63). These observations also led to the following conclusion - that not only was the potential to initiate leukemia maintained upon serial transplantation, but also that the progression of the leukemic disease became more and more aggressive from host to host. Serial transplantations of leukemia cells have been performed before. The one that is most similar to these experiments was performed by Kroon *et al.* (64). They used the fusion gene NUP98-HoxA9 to trigger leukemia, and they also observed that the outbreak of leukemia was much faster in the secondary host. In addition, this led them to the conclusion that NUP98-HoxA9 alone was not sufficient to induce leukemia, and that additional genetic events were required. Schulze *et al.* (65) induced AML by overexpressing MLL-AF9. These researchers were also able to

show that the potential to initiate leukemia was maintained following serial transplantation. However, they did not see an increase of aggressiveness in the leukemia. These different findings are probably due to the different genes that were overexpressed. Overexpression of MLL-AF9 alone is already sufficient to induce AML. For our experiments HoxA9 was used, which is not capable alone of causing AML in HSCs. Use of serial transplantation puts additional stress on the cells and more mutations occur, which can then lead to leukemia. Other research groups have used different species or studied different types of leukemia (66-68). However, all the work had a common feature in that there was no observation of an increase in the aggressiveness of the disease. A serial dilution experiment was performed to rule out the possibility that the number of transplanted cells has an effect on the type of leukemic behavior. The analysis showed that the number of transplanted cells had no influence on the leukemic behavior. Although the experiment showed a valid result statistically, it was only performed once, which could be a point of limitation.

## 6.6. Limitations

One of the limitations was transplant failure respectively the decrease of HoxA9 transduced cells in the blood, spleen and bone marrow. Out of the 17 primary host mice that received $CD150^{pos}$/HoxA9 HSCs, 9 mice showed a transplant failure with not enough transduced/GFP+ cells at the time of sacrifice in any tissue. From the 17 primary host mice that were injected with $CD150^{neg}$/HoxA9 HSCs, only 3 mice showed a transplant failure. One might assume that this could be simply due to problems with venipuncture injection. However, this can be ruled out by the fact that the mice were lethally irradiated and would not have survived without a successful injection of cells. The data of all 17 mice for each group is shown in Figure 8. This figure shows the decrease of HoxA9 expressing cells in the primary host mice that received $CD150^{pos}$/HoxA9 HSCs. This is a main finding of this thesis. However, this means that after 5 months there were not enough HoxA9 expressings cells left for a reasonable analysis. To explain why HoxA9 slows down the growth of these cells, further research is needed. Four primary host mice that were injected with $CD150^{neg}$/HoxA9 HSCs, died suddenly (e.g on a weekend) and were found dead, so that an analysis of bone marrow and spleen was not possible.

The lack of circulating GFP+ cells in the blood was another challenge. Even when the bone marrow showed a massive infiltration of transduced cells (above 80%), the number of circulating transduced cells in the blood stream was often below 10%. Only mice that had more than 2% of transduced cells in all 3 tissues (bone marrow, spleen, and blood) were included for analysis. This excluded 2 primary mice from analysis that received $CD150^{pos}$/HoxA9 HSCs. Although the 2 mice had sufficient transduced cells in their bone marrow (41% and 28.5%), the number of GFP cexpressing cells in their spleens and blood was below 2%. This could be a compensation by the organism to keep it alive or simple due to the effect that HoxA9 slows down the growth of My-bi cells. Nevertheless, the low numbers of transduced cells make a reasonable analysis impossible. As mentioned in 6.4., the difference between the infiltration of bone marrow and circulating blast cells is not well understood in AML, and further research of adhesion molecules, chemokine, angiogenetic factors, and homing is needed (60).

Only about 1 of every 10,000 to 100,000 bone marrow cells is an HSC - this makes HSCs very rare cells in mammalian bone marrow (69, 70). For these experiments, highly purified HSCs that were sorted with antibodies and a FACSaria cytometer were used. The marker profile of c-Kit+; Sca-1+; lineage markers negative (Lin-); CD34- gives one of the highest yields when sorting for HSCs. It has been shown that small numbers of these cells are enough for the long-term repopulation of an irradiated host (13, 71). However, these populations are still not completely pure, and it is possible that instead of an HSC, the target for the leukemic transformation is a more committed progenitor. This could be a restricted myeloid progenitor or a short-lived multi-potent progenitor. A great effort has been made to improve the sorting protocols, and many different protocols and cell surface markers have been used to identify and isolate HSCs from mouse bone marrow. However, a consensus regarding which markers are consistently expressed on HSCs has not yet been reached (72). Recently, a new way to isolate HSCs was introduced (72). Bone marrow cells are stained with nontoxic dye and transplanted into new host animals. Some of the injected cells migrate to the bone marrow of the recipients within 48 hours. When these cells are harvested and serially transplanted into a new host, the cells are highly enriched for stem cells.

Another way to investigate if stem cells are the target of leukemic transformation is to improve the virus used for transfection. The retrovirus vector used for these

experiments was not selective, and therefore it could transduce all cells it came in contact with. To advance the method, it would be necessary to develop a retrovirus vector that only transduces HSCs. Our laboratory has been working on improving the plasmid construct by using promoter genes only expressed in HSCs, but the new plasmid was not completed in time to be used in these experiments. Improving the purity of the sorted HSC population and developing a retrovirus that only transduces HSCs could help to solve the question of which cells are the target cells of leukemic transformation.

## 6.7. Prospects – My-bi HSCs might improve HSCs transplantation

The results show that mouse HSCs sorted for $CD150^{pos}$ are more resistant to leukemia caused by an overexpression of HoxA9 than HSCs sorted for $CD150^{neg}$. As HoxA9 is expressed in about 50% of patients with AML, these findings could have an enormous impact on the clinical treatment for patients with leukemia. One of the cornerstones of leukemic therapy in humans is the transplantation of HSCs. To achieve this, all remaining cells in the bone marrow of a patient are killed by chemotherapy, radiotherapy, or other modalities (e.g. tyrosine kinase inhibitors [TKIs] and antibodies) to reach a state of remission. Then the HSCs are transplanted, either from the patient (autologous), or from a healthy donor (allogeneic). This therapy is often the treatment of last resort, as it results in high failure rates. Depending on the type of leukemia and the time point of the treatment, the relapse rates are 20% to 80% for allogeneic stem cell transplantation (73). The management of failure of transplantation is a major challenge, and the therapies range from simple support and palliation to second attempts to cure with second transplants. At present, unsorted HSCs are used for transplantation to cure patients with various forms of leukemia. However, these experiments could show that mice injected with $CD150^{pos}$/HoxA9 HSCs survive significantly longer than mice injected with $CD150^{neg}$/HoxA9 HSCs. A sorting of the HSCs which are transplanted into patients for CD150 could be a major benefit for patients. Nevertheless, at this early stage of research, all prospects are mainly speculative.

## 6.8. Conclusion

Rather than confirming the hypothesis that only one HSC compartment is responsible for inducing AML, the results showed convincingly that mice injected with $CD150^{neg}$/HoxA9 HSCs showed an early onset of AML, a more rapid progression of the disease, and eventually a shorter lifespan. Conversely, mice that received $CD150^{pos}$/HoxA9 HSCs showed signs of AML only after serial transplantation into secondary host mice. Furthermore, the experiments showed that the number of transplanted cells had no effect of the type of leukemia. There was also no difference in the leukemic behavior whether it was spleen cells or bone marrow cells that were serially transplanted. This suggests that the clonal heterogeneity in the normal blood system is causative for heterogeneous manifestations of acute myeloid leukemia derived from the same initial transformation. The experiments presented in this thesis suggest that, in fact, disease pathways diverge and are manifest in typical clinical identifiers such as enlarged spleens. These findings could have important implications for the treatment of acute myeloid leukemia and, potentially, other forms of leukemia.

# 7. References


1. Wagers AJ, Weissman IL. Plasticity of adult stem cells. Cell. 2004;116(5):639-48.

2. Slack JM. Origin of stem cells in organogenesis. Science. 2008;322(5907):1498-501.

3. Jaenisch R, Young R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell. 2008;132(4):567-82.

4. Konstantinov IE. In search of Alexander A. Maximow: the man behind the unitarian theory of hematopoiesis. Perspect Biol Med. 2000;43(2):269-76.

5. Maximov. Über experimentelle Erzeugung von Knochenmarks-Gewebe. Anat Anz. 1906;28:24-38.

6. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008;132(4):631-44.

7. Lorenz E, Uphoff D, Reid TR, Shelton E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J Natl Cancer Inst. 1951;12(1):197-201.

8. Jacobson LO, Simmons EL, Marks EK, Eldredge JH. Recovery from radiation injury. Science. 1951;113(2940):510-11.

9. Ford CE, Hamerton JL, Barnes DW, Loutit JF. Cytological identification of radiation-chimaeras. Nature. 1956;177(4506):452-4.

10. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241(4861):58-62.

11. Thomas TE, Miller CL, Eaves CJ. Purification of hematopoietic stem cells for further biological study. Methods. 1999;17(3):202-18.

12. Wognum AW, Eaves AC, Thomas TE. Identification and isolation of hematopoietic stem cells. Arch Med Res. 2003;34(6):461-75.

13. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. 1996;273(5272):242-5.

14. Little MT, Storb R. History of haematopoietic stem-cell transplantation. Nat Rev Cancer. 2002;2(3):231-8.

15. Passegue E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci U S A. 2003;100 Suppl 1:11842-9.

16. Muller-Sieburg CE, Sieburg HB, Bernitz JM, Cattarossi G. Stem cell heterogeneity: implications for aging and regenerative medicine. Blood. 2012;119(17):3900-7.

17. Cho RH, Sieburg HB, Muller-Sieburg CE. A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. Blood. 2008;111(12):5553-61.

18. Jordan CT, Lemischka IR. Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev. 1990;4(2):220-32.

19. Muller-Sieburg CE, Sieburg HB. Clonal diversity of the stem cell compartment. Curr Opin Hematol. 2006;13(4):243-8.

20. Sieburg HB, Cho RH, Dykstra B, Uchida N, Eaves CJ, Muller-Sieburg CE. The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. Blood. 2006;107(6):2311-6.

21. Challen GA, Boles NC, Chambers SM, Goodell MA. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell. 2010;6(3):265-78.

22. Dykstra B, Kent D, Bowie M, McCaffrey L, Hamilton M, Lyons K, Lee SJ, Brinkman R, Eaves C. Long-term propagation of distinct hematopoietic differentiation programs in vivo. Cell Stem Cell. 2007;1(2):218-29.

23. Beerman I, Bhattacharya D, Zandi S, Sigvardsson M, Weissman IL, Bryder D, Rossi DJ. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. Proc Natl Acad Sci U S A. 2010;107(12):5465-70.

24. Morita Y, Ema H, Nakauchi H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. J Exp Med. 2010;207(6):1173-82.

25. Sieburg HB, Rezner BD, Muller-Sieburg CE. Predicting clonal self-renewal and extinction of hematopoietic stem cells. Proc Natl Acad Sci U S A. 2011;108(11):4370-5.

26. Bennett JH. Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. Edinburgh Medical and Surgical Journal. 1845;64:413-23.

27. Virchow R. Weisses Blut. Notiz a d Geb d Nat- u Heilk. 1845;33:151.

28. Piller G. Leukaemia - a brief historical review from ancient times to 1950. Br J Haematol. 2001;112(2):282-92.

29. Virchow R. Zur pathologischen Physiologie des Blutes. II. Weisses Blut. Archives of Pathology, Anatomy and Physiology. 1847;I:563 - 72.

30. Neumann E. Ueber die Bedeutung des Knockenmarkes fur die Blutbildung. Ein Beitrag zur Entwicklungsgeschichte der Blutkorperchen. Archives Heilkunde. 1869;10:68 - 102.

31. Neumann E. Ein neuer Fall von Leukamie mit Erkrankung des Knochenmarks. Archives Heilkunde. 1872;13:502 - 8.

32. Kaatsch P, Spix C, Katalinic A, Hentschel S. Krebs in Deutschland 2007/2008. published on edoc: 2012-02-22T14:00:00Z
access: 2013-11-09T09:59:57Z: Robert Koch-Institut; 2012.

33. Davis AS, Viera AJ, Mead MD. Leukemia: An overview for primary care. Am Fam Physician. 2014;89(9):731-8.

34. Gilliland DG, Jordan CT, Felix CA. The molecular basis of leukemia. Hematology Am Soc Hematol Educ Program. 2004:80-97.

35. SEER Cancer Statistics Review, 1975-2012, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2012/, based on November 2014 SEER data [Internet]. April 2015.

36. Creutzig U, van den Heuvel-Eibrink MM, Gibson B, Dworzak MN, Adachi S, de Bont E, Harbott J, Hasle H, Johnston D, Kinoshita A, Lehrnbecher T, Leverger G, Mejstrikova E, Meshinchi S, Pession A, Raimondi SC, Sung L, Stary J, Zwaan CM, Kaspers GJL, Reinhardt D. Diagnosis and management of acute myeloid leukemia in children and adolescents: recommendations from an international expert panel. Blood. 2012;120(16):3187-205.

37. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;341(14):1051-62.

38. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol. 1976;33(4):451-8.

39. Rulina AV, Spirin PV, Prassolov VS. Activated leukemic oncogenes AML1-ETO and c-kit: role in development of acute myeloid leukemia and current approaches for their inhibition. Biochemistry (Mosc). 2010;75(13):1650-66.

40. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100(7):2292-302.

41. Lennard AL, Jackson GH. Stem cell transplantation. BMJ. 2000;321(7258):433-7.

42. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105-11.

43. Duboule D, Dolle P. The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes. EMBO J. 1989;8(5):1497-505.

44. Krumlauf R. Hox genes in vertebrate development. Cell. 1994;78(2):191-201.

45. Duverger O, Morasso MI. Role of homeobox genes in the patterning, specification and differentiation of ectodermal appendages in mammals. J Cell Physiol. 2008;216(2):337-46.

46. Gilbert PM, Mouw JK, Unger MA, Lakins JN, Gbegnon MK, Clemmer VB, Benezra M, Licht JD, Boudreau NJ, Tsai KK, Welm AL, Feldman MD, Weber BL, Weaver VM. HOXA9 regulates BRCA1 expression to modulate human breast tumor phenotype. J Clin Invest. 2010;120(5):1535-50.

47. Alharbi RA, Pettengell R, Pandha HS, Morgan R. The role of HOX genes in normal hematopoiesis and acute leukemia. Leukemia. 2013;27(5):1000-8.

48. Collins CT, Hess JL. Role of HOXA9 in leukemia: dysregulation, cofactors and essential targets. Oncogene. 2015.

49. Sitwala KV, Dandekar MN, Hess JL. HOX Proteins and Leukemia. Int J Clin Exp Pathol. 2008;1(6):461-74.

50. Lawrence HJ, Helgason CD, Sauvageau G, Fong S, Izon DJ, Humphries RK, Largman C. Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. Blood. 1997;89(6):1922-30.

51. Thorsteinsdottir U, Mamo A, Kroon E, Jerome L, Bijl J, Lawrence HJ, Humphries K, Sauvageau G. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood. 2002;99(1):121-9.

52. Li DP, Li ZY, Sang W, Cheng H, Pan XY, Xu KL. HOXA9 gene expression in acute myeloid leukemia. Cell Biochem Biophys. 2013;67(3):935-8.

53. Muller-Sieburg CE, Sieburg HB. The GOD of hematopoietic stem cells: a clonal diversity model of the stem cell compartment. Cell Cycle. 2006;5(4):394-8.

54. Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J. 1998;17(13):3714-25.

55. Beachy SH, Onozawa M, Silverman D, Chung YJ, Rivera MM, Aplan PD. Isolated Hoxa9 overexpression predisposes to the development of lymphoid but not myeloid leukemia. Exp Hematol. 2013;41(6):518-29.e5.

56. Chung KY, Morrone G, Schuringa JJ, Plasilova M, Shieh JH, Zhang Y, Zhou P, Moore MA. Enforced expression of NUP98-HOXA9 in human CD34(+) cells enhances stem cell proliferation. Cancer Res. 2006;66(24):11781-91.

57. Takeda A, Goolsby C, Yaseen NR. NUP98-HOXA9 induces long-term proliferation and blocks differentiation of primary human CD34+ hematopoietic cells. Cancer Res. 2006;66(13):6628-37.

58. Somervaille TC, Cleary ML. Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. Cancer Cell. 2006;10(4):257-68.

59. Graf M, Reif S, Kroll T, Hecht K, Nuessler V, Schmetzer H. Expression of MAC-1 (CD11b) in acute myeloid leukemia (AML) is associated with an unfavorable prognosis. Am J Hematol. 2006;81(4):227-35.

60. Amin HM, Yang Y, Shen Y, Estey EH, Giles FJ, Pierce SA, Kantarjian HM, O'Brien SM, Jilani I, Albitar M. Having a higher blast percentage in circulation than bone marrow: clinical implications in myelodysplastic syndrome and acute lymphoid and myeloid leukemias. Leukemia. 2005;19(9):1567-72.

61. Hsieh PP, Olsen RJ, O'Malley DP, Konoplev SN, Hussong JW, Dunphy CH, Perkins SL, Cheng L, Lin P, Chang CC. The role of Janus Kinase 2 V617F mutation in extramedullary hematopoiesis of the spleen in neoplastic myeloid disorders. Mod Pathol. 2007;20(9):929-35.

62. Tomová A, Babusíková O. Shifts in expression of immunological cell markers in relapsed acute leukemia. Neoplasma. 2001;48(3):164-8.

63. Yoo EH, Kim HJ, Lee ST, Kim WS, Kim SH. Frequent CD7 antigen loss in aggressive natural killer-cell leukemia: a useful diagnostic marker. Korean J Lab Med. 2009;29(6):491-6.

64. Kroon E, Thorsteinsdottir U, Mayotte N, Nakamura T, Sauvageau G. NUP98–HOXA9 expression in hemopoietic stem cells induces chronic and acute myeloid leukemias in mice. EMBO J. 2001;20(3):350-61.

65. Schulze I, Tschanter P, Rohde C, Krause A, Linhart H, Hebestreit K, Dugas M, Rosenbauer F, Berdel WE, Müller-Tidow C. Identification of leukemia suppressive genes by inducible overexpression of the DNA methyltransferase DNMT3B during leukemogenesis in mice. Blood. 2013;122(21):2493-.

66. Adams RA, Foley GE, Uzman BG, Farber S, Lazarus S, Kleinman L. Leukemia: serial transplantation of human leukemic lymphoblasts in the newborn Syrian hamster. Cancer Res. 1967;27(4):772-83.

67. Imada K, Takaori-Kondo A, Sawada H, Imura A, Kawamata S, Okuma M, Uchiyama T. Serial transplantation of adult T cell leukemia cells into severe combined immunodeficient mice. Jpn J Cancer Res. 1996;87(9):887-92.

68. Miyagi T, Kubonishi I, Ohtsuki Y, Ohyashiki JH, Toyama K, Miyoshi I. Direct and serial transplantation of Ph1-positive acute lymphoblastic leukemia into nude mice. Int J Cancer. 1989;43(6):1149-54.

69. Harrison DE, Jordan CT, Zhong RK, Astle CM. Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. Exp Hematol. 1993;21(2):206-19.

70. Hawley RG, Ramezani A, Hawley TS. Hematopoietic stem cells. Methods Enzymol. 2006;419:149-79.

71. Challen GA, Boles N, Lin KK, Goodell MA. Mouse hematopoietic stem cell identification and analysis. Cytometry A. 2009;75(1):14-24.

72. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell. 2001;105(3):369-77.

73. Barrett AJ, Battiwalla M. Relapse after allogeneic stem cell transplantation. Expert Rev Hematol. 2010;3(4):429-41.

# 8. Appendix

## 8.1 Eidesstattliche Versicherung

„Ich, Michael Ziller, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Overexpression of HoxA9 in CD150- hematopoietic stem cells leads to rapid progression of acute myeloid leukemia“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.
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## 8.2 Resume

Mein Lebenslauf wird aus wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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