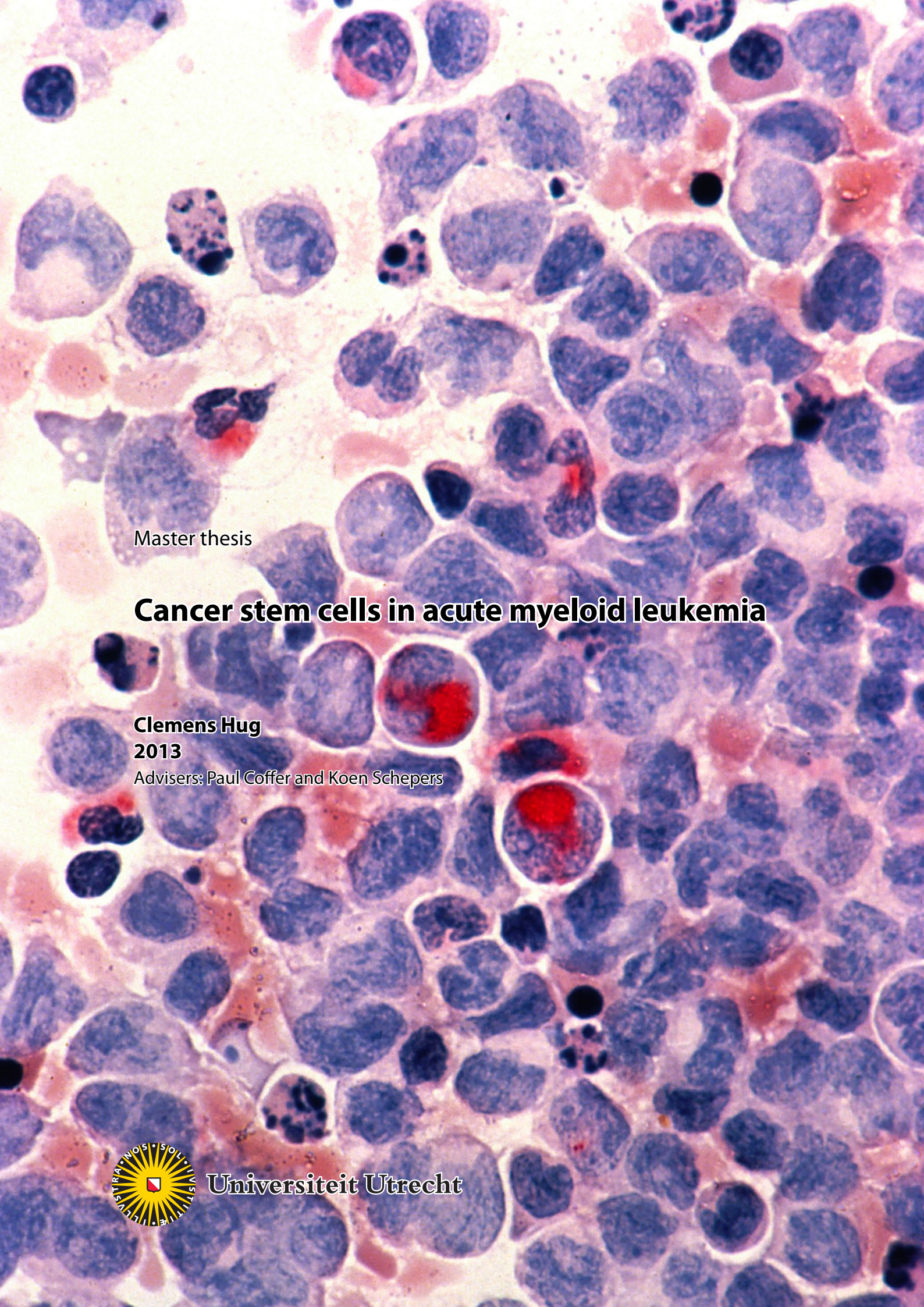

Master thesis

Cancer stem cells in acute myeloid leukemia

Clemens Hug
2013
Advisers: Paul Coffer and Koen Schepers

Universiteit Utrecht

Abstract

Since the original description of leukemic stem cells (LSC) in acute myeloid leukemia (AML) as a rare subpopulation of leukemic cells located in the CD34+/CD38- compartment much has changed. Supported by continuously improving immunodeficient mouse models, LSC were found in cells which were previously thought not to contain any LSC activity. Furthermore, the phenotype of LSCs as well as the phenotype of the offspring they form, were found to be astonishingly variable and dynamic over time, challenging the perception of LSCs as classical stem cells in a static developmental hierarchy. This thesis focusses on the change in perception of LSC in AML regarding their phenotype, development and differentiation and also touches on the subject of clonal evolution and development of AML LSCs.

Contents

1 Introduction **2**
- 1.1 Cancer stem cells . . . 2
- 1.2 Scope of this thesis . . . 4

2 LSC in acute myeloid leukemia **4**
- 2.1 Methods for studying LSCs . . . 4
- 2.2 Proof for LSCs in AML . . . 4
- 2.3 Implications of the proof of the cancer stem cell concept for AML . . . 6

3 LSC in AML: A moving target? **6**
- 3.1 Advances in xenograft models to study AML LSC . . . 8
- 3.2 AML LSCs without HSC phenotype . . . 9
  - 3.2.1 CD38+ AML LSC . . . 9
  - 3.2.2 CD34- AML LSC . . . 11
  - 3.2.3 Additional markers expressed on AML LSCs . . . 12
- 3.3 Hierarchical orders in AML . . . 14
- 3.4 LSC as a cellular state . . . 15

4 Implications for future research **16**
- 4.1 LSC phenotype . . . 17
- 4.2 Cancer stem cells vs. clonal evolution . . . 18
- 4.3 Future directions . . . 19

1 Introduction

The hematopoietic system is the developmental system by which all blood cells are formed. Multipotent hematopoietic stem cells (HSCs) are at the apex of the hematopoietic lineage tree and ensure sustained production of the different blood cell lineages through production of a hierarchy of developmentally restricted progenitor populations [1]. Genetic alterations in hematopoietic cells can cause changes in hematopoiesis that lead to leukemia. There are different forms of leukemia which affect different parts of the hematopoietic system and show different clinical progression. Essentially, there is distinction between myeloid and lymphoid, acute and chronic leukemias. Acute myeloid leukemia (AML) is a cancerous disease of the white blood cells, particularly the myeloid lineage. During AML, normal blood cells are rapidly replaced by abnormal, nonfunctional leukemic blasts which interfere with the regular function of the blood. AML is the most common acute leukemia in adults and is responsible for about 1.8% of all cancer related deaths in the United States in 2012 [2]. It is highly correlated with a number of abnormal karyotypes and genetic lesions. While 41% of AML patients have normal karyotype, t(15;17)(q22;q21) (13% of patients), t(8;21)(q22;q22) (7%) and inv(16)(p13q22)/t(16;16)(p13;q22) (5%) are especially common cytogenetic abnormalities in AML [3]. Some of these chromosomal alterations lead to the expression of an abnormal fusion protein with oncogenic potential, others lead to the deactivation of tumor suppressors [4]. AML with normal cytogenetics is very heterogeneous in its clinical and molecular features and its pathogenesis is much less clear than for AML with chromosomal rearrangements. A study by Ley et al. [5] comparing the whole genome of leukemic blasts from a patient with cytogenetically normal AML with healthy cells revealed eight alterations in the coding regions of known genes. Two of which occurred in already well known genes, namely the *NPM1* and the *FLT3* gene, whose mutational status serve as a prognostic marker in the clinical assessment of both cytogenetically normal and abnormal AML [4]. Several more genetic alterations in cytogenetically normal AML have been characterized since. However, the exact pathogenetic mechanisms by which the identified mutations lead to AML are not well understood [6].

1.1 Cancer stem cells

It has early been noted that most cancers are not homogeneous, but instead consist of cells with marked differences in their phenotype and function [7]. Two models are considered to explain these observations. The clonal evolution theory (Fig. 1a) proposes that there are several competing clonal populations in a tumor that differ in their proliferative and tumorigenic potential but are homogenous within themselves. There is no interconversion or differentiation between the different populations, but there is Darwinian competition among cells. The cancer stem cell model (Fig. 1b) views tumors as organ-like organized entities similar to other organs in the body. It proposes that the cancer is sustained and can only be propagated by a relatively small pool of cells called cancer stem cells. They are viewed as the origin of a hierarchy of tumor cells, of which the bulk is predominant in number but cannot propagate and recapitulate the disease on its own. Based on this model, cancer stem cells are considered to be 1) a subpopulation of the bulk tumor cells 2) able to recreate all different phenotypic and morphologic subtypes of the original tumor and 3) able to self-renew and thereby sustain the tumor.

In addition to these two conflicting models there is also a synthetic model that combines elements from both clonal evolution and cancer stem cell theory (Fig. 1c).

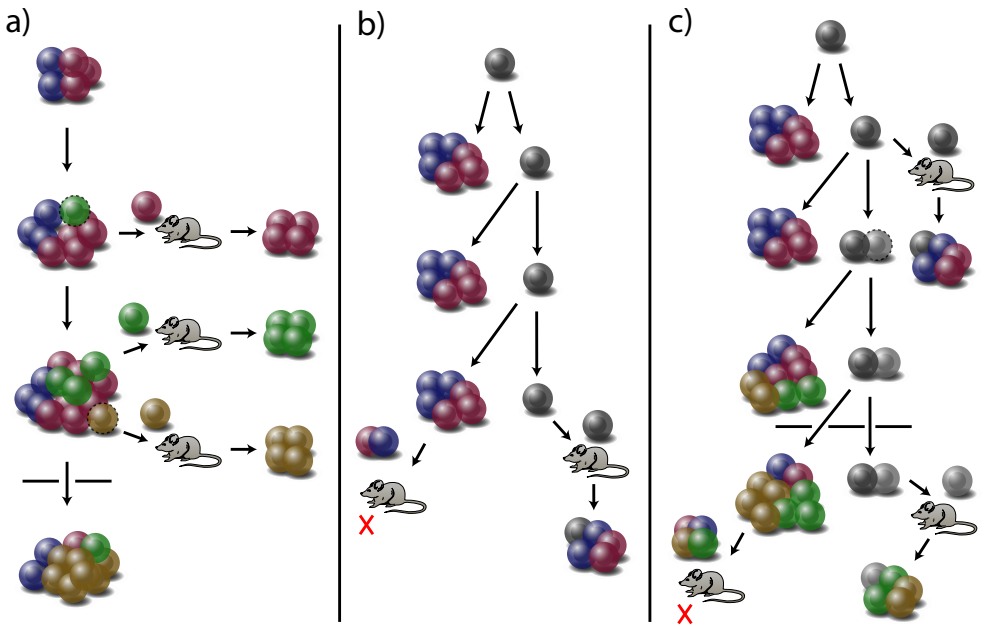

Figure 1: Illustration of the different models that are considered to explain tumor heterogeneity in AML. **a)** Clonal evolution model **b)** Cancer stem cell model **c)** Combined theory incorporating clonal evolution into the cancer stem cell model.
Dashed lines denote novel cellular clones which aquired a distinguishing new mutation. The black line with a gap represents a selection pressure, favouring specific clones within the cell population. Also the outcomes of xenotransplantation experiments of single clonal cell populations into mouse models are shown, illustrating the differentiative capacity of these cells.

1.2 Scope of this thesis

AML was the first disease for which it was shown that it is sustained by a small population of so called leukemic stem cells (LSCs) [8, 9] and it is now widely accepted that growth of several types of leukemia are initiated and/or sustained by a small population of LSCs. Since then much effort has been put into further identifying and characterizing LSCs in AML. This has led to the publication of a wide array of literature, which often contains conflicting reports on phenotype, features and origin of AML LSCs. Here, I will summarize studies concentrating on the ongoing effort to pinpoint the phenotype of LSCs in AML, by presenting the current state of research, as well as mentioning and explaining different concepts that have been put forward to interpret the different phenotypes that have been observed. The literature contains many contradicting and confusing observations that I will try to present in order to create a consistent general picture of our current understanding of LSCs and their phenotypes in AML.

2 LSC in acute myeloid leukemia

2.1 Methods for studying LSCs

To determine whether a certain population of AML cells contains LSCs, similar approaches are used as for the study of human HSCs. Cells are evaluated on their ability to engraft into and sustain their development in immunodeficient mice. The immunodeficiency is an important property of the xenograft mouse model because certain parts of the mouse immune system will recognize and destroy transplanted human cells, thereby prohibiting engraftment.

The relationship between leukemia initiating cells (LICs) and LSCs is not resolved definitively. LICs are cells that initiate leukemia in xenograft models, making them model-specific, therefore a LIC for one model is not necessarily a LIC in another model. LSCs are self-renewing cells which can regenerate the full phenotypic and functional diversity of the leukemic cells by itself, therefore in an adequately permissive xenograft model LSCs are always LICs. The opposite is not necessarily true, because there may be short- and medium-term repopulating cells which can transiently initiate but not sustain leukemia, similar to the short-/medium-term repopulating cells that have been described for the normal hematopoietic system [10].

Successful engraftment can be assessed by a number of methods: Most commonly, peripheral blood or bone marrow is aspirated from the mouse and the percentage of human cells is measured by southern blots probing for human-specific DNA or, more recently, by flow cytometric analysis with antibodies specific for human surface markers, e.g. hCD45. Using antibodies against lineage markers, for example of the myeloid, lymphoid and erythroid populations, the engrafted cells can also be tested for their ability to differentiate into various hematopoietic lineages.

2.2 Proof for LSCs in AML

The idea of stem cells in leukemia has been around for a long time. Already in the early 80s it was discovered that not all leukemic blasts have unlimited proliferative capacity. This was based on experiments that showed that only a small fraction of leukemic cells formed leukemic colonies in *in-vitro* assays as measured by the colony forming ability of individual cells. [11]. The relevance of these findings was still questionable, as it could not be excluded that certain cell types simply did not grow under the *in-vitro* culture conditions employed.

In 1994, the group of John Dick tested the possibility that the heterogeneity reflects a developmental hierarchy within the tumor similar to the normal developmental hierarchy of hematopoietic

cells, with immature LSCs at the apex of the hierarchy [8]. They found that indeed only a minor fraction of leukemic cells were able to engraft in specially engineered mice that lack certain parts of the adapted and innate immune system. This small population of cells, termed leukemia initiating cells (LIC) was able to recreate leukemia in the mice including the patient specific phenotypes of leukemic blasts. These findings [8, 9] can be considered first definitive proof of the existence of cancer stem cells.

However, it was only in 1994 that Lapidot et al. [8] for the first time managed to demonstrate, in a stringent and convincing manner, that a small and defined subpopulation of leukemic cells is able to regenerate AML *in-vivo*. Their experimental approach employed injecting the leukemic cells intravenously into immunodeficient Scid mice. To achieve proper engraftment they treated their mice with certain human growth factors (PIXY321, a fusion protein of human granulocyte-macrophage colony stimulating factor and human IL-3, as well as human mast-cell growth factor). This treatment improved engraftment efficiency by a factor of 10-100 compared to untreated mice. Upon injection of unfractionated AML cells, the human leukemic cells expanded, homed towards and colonized the bone marrow of the host mice. Depending on the specific AML sample and number of cells injected up to 95% of the bone marrow of transplanted mice was determined to be of human origin after 17-45 days *in-vivo*, indicating solid and stable engraftment of the leukemic cells. Importantly, they could show by morphological and cytogenetic analysis that the leukemic blasts in the recipient mice showed the same aberrant features as the patient's blasts from which they were derived.

It was also found that the pool of AML-colony forming units was expanding *in-vivo* in the bone marrow of mice transplanted with unfractionated patient peripheral blood, indicating the presence of a LIC with self-renewal capacity. Together with the observation that LIC were more than 1000-fold less frequent than AML CFU this indicates that a leukemia initiating cell, more immature than blasts was present and expanding in the xenograft. To identify these cells phenotypically Lapidot et al. [8] used flow cytometry to sort the leukemic cells according to their expression of the surface markers CD34 and CD38 (for an overview of the heterogeneity of AML samples regarding the expression of these markers see Fig. 3 on page 11). During normal hematopoiesis, these markers can distinguish between cells with hematopoietic stem cell activity (CD34+/CD38-) and more mature progenitor cells (CD34+/CD38+, see Fig. 2 on page 7). Transplantation of these purified AML fractions revealed engraftment capacity exclusively in the CD34+/CD38- fraction, which in normal individuals marks the hematopoietic stem cell containing population. Although this result had to be taken with care, since the experiment was performed with only one patient sample, this result implicated that the CD34+/CD38- AML cells contain the LICs.

Proof of self-renewal of the LIC While Lapidot et al. [8] were successful in showing the disease recapitulating capacity of the CD34+/CD38- population of leukemic cells, they failed to establish definitive proof for the self-renewal capacity of the LICs they found. In xenograft models an elegant and convincing way to show self-renewal is secondary transplantation of cells from a primary to a secondary recipient mouse. By assessing the frequency of the LICs in the original sample and in the primary recipient using limited dilution analysis it is possible to determine whether the population of LICs was expanded *in-vivo* in the primary recipient. Expansion of the LIC population implies self-renewal capability in-vivo of these cells.

While secondary transplantation of the putative LSCs failed in the Scid mouse model [8] the same research group of John Dick in 1997 showed that CD34+/CD38- AML LICs were able to engraft and differentiate into leukemic blasts of the same phenotype observed in the original patient sample in both primary and secondary recipients [9]. Together these results confirm that

CD34+/CD38- AML cells contain true LSCs with self-renewal capacity.

2.3 Implications of the proof of the cancer stem cell concept for AML

Implications for prognosis and therapy The proof of cancer stem cells in AML has implications with respect to prognosis and therapy of AML. Treatment of AML patients with chemotherapeutics regularly leads to quick improvements up to complete remission, where no residual disease is detectable anymore. However, remission is unfortunately very often short-lived and commonly results in relapse. The cancer stem cell theory offers an elegant explanation for this clinical observation. While the rapidly dividing leukemic blasts which represent the majority of the leukemic population are killed efficiently by chemotherapy, the long-lived and quiescent LSCs are spared and can re-initate the disease. It is believed that leukemic stem cells, compared to the bulk of leukemic cells, possess extraordinary resistance to chemo- and radiotherapy, the mechanisms of which are still under investigation but probably depend on the interaction with the bone marrow microenvironment [12]. Therefore it is vital to also target LSCs for therapy to completely eradicate the disease. This is one reason for the importance of the LSC phenotype, which has been the subject of intensive research in the last decade. If the phenotype and properties of LSCs are precisely known it might be possible to use this insight to create targeted therapies which specifically eliminate LSCs.

Implications for the cell of origin Around the time of the original discovery of AML LSCs, two possible models had been suggested to explain the heterogeneity of AML blast phenotypes and clinical outcomes [9]: First, the leukemic transforming event can take place on all levels of the hematopoietic hierarchy from the HSC to more committed progenitors and the nature of the transformed cell determines the phenotype of the blasts. Second, the transforming event always occurs in the HSC and the blastic phenotype is controlled by the nature of the specific genetic lesions involved. From these models certain predictions about the phenotype of the LSCs can be devised. The first model makes the prediction that the phenotype of the LSC mimics the phenotype of their respective blasts. In the second model, the LSC always mimics the immature HSC phenotype, regardless of the blast phenotype.

A key feature of the AML LSCs as described in the early studies by the group of John Dick [8, 9] was that they were exclusively found in AML cells with HSC (CD34+/CD38-) phenotype, completely independent of the phenotype of the total AML sample, AML cell morphology or FAB-classification of the AML samples. Since LSCs were not detected in the CD38+ fraction, which is highly enriched for committed myeloid progenitors (see Fig. 2), Bonnet and Dick [9] therefore concluded that in AML, LSCs originate from HSCs and not from committed progenitors.

3 LSC in AML: A moving target?

Soon after the initial report of Lapidot et al. [8] that leukemia initiating cells reside in the immature fraction of leukemic cells with a CD34+/CD38- phenotype conflicting data was published by Terpstra et al. [19]. They found leukemia initiating activity in both CD34+ and CD34- cells as assayed by xenotransplantation in Scid mice and through *in-vitro* culturing assays. However, they showed this for a single patient only and thus LSC phenotypes aberrant from CD34+/CD38- were long seen as a rare and untypical occurrence. Recently evidence accumulated indicating that indeed AML LSCs are not restricted to the immature CD34+/CD38- phenotype but, depending on the AML sample, can be found in a wide variety of phenotypic compartments. These findings were

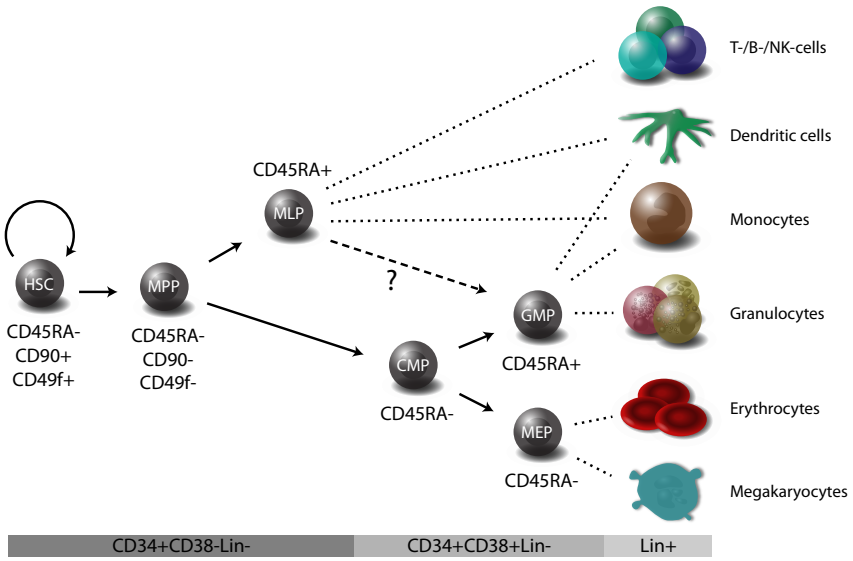

Figure 2: Simplified diagram depicting the human hematopoietic lineage tree.
HSC, hematopoietic stem cell. MPP, multipotent progenitor. MLP, multilymphoid progenitor. GMP, granulocyte-macrophage progenitor. CMP, common myeloid progenitor.
Nomenclature and parts of the figure are adapted from Doulatov et al. [10].

Phenotype	**Remarks**	**Model**	**Reference**
Lin-/CD34+/CD38-		NOD	[8]
		NOD-Scid	[9, 13]
	CD90-/CD45RA+ MLP-like	NSG/αCD122/IVIG	[14]
	ALDH^{int} only	NSG	[15]
	Also in Lin^{dim} [16]	NSG or NOD-Scid β2m-/- IVIG	[17, 16]
Lin-/CD34+/CD38+		NOD-Scid/αCD122	[13]
	CD90-/CD45RA+ GMP-like	NSG/αCD122/IVIG	[14]
		NSG/IVIG	[18]
	Also in Lin^{dim} [16]	NSG or NOD-Scid β2m-/- IVIG	[17, 16]
Lin-/CD34-/CD38+		Nod-Scid/αCD122	[13]
		NSG or NOD-Scid β2m-/- IVIG	[17, 16]
Lin-/CD34-/CD38-		Nod-Scid/αCD122	[13]
		NSG or NOD-Scid β2m-/- IVIG	[17, 16]

Table 1: Overview of published phenotypes for AML LSCs.
αCD122, antibody against CD122. IVIG, intraveneous immunoglobulin. See chapter 3.1 and table 2.

Model	NK-cell level	Macrophage level	Myeloid engraftment	Reference
Scid	++	++	-	Bosma et al. [20]
NOD-Scid	++	-	+	Shultz et al. [21]
NOD-Scid β2m-/-	+	-	+	Glimm et al. [22]
NOD-Scid anti-CD122	+	-	+	McKenzie et al. [23]
NOD/ShiJic-scid/IL2Rγ-/- (NOG)	-	-	+	Ito et al. [24]
NOD/ShiLtSz-scid/IL2Rγ-/- (NSG)	-	-	+	Shultz et al. [25]
BALB/Rag2-/-/IL2Rγ-/-	-	+	+	Traggiai et al. [26]

Table 2: Overview of immunodeficient mouse models which are generally used in studies of AML leukemic stem cells [10, 27].

made possible primarily by many recent advances in the field of mouse xenograft models, whose improved sensitivity enabled reasearches to detect engraftment for cells that did not engraft in Scid mice.

3.1 Advances in xenograft models to study AML LSC

Not all AML LSC engraft in all xenograft models with the same efficiency and the choice of the model system has decisive influence on the outcome of transplantation experiments [10]. This availability of advanced mouse models was an important prerequisite for the recent discoveries of new phenotypes of AML LSCs. Here, I briefly describe the most important advances in these xenograft models since the NOD-Scid mice and will indicate throughout this thesis how they contributed to improving our understanding of AML LSCs.

The shortcomings of the Scid mice (no serial transplantation, poor multilineage engraftment, high cell doses required for transplantation) were partially alleviated with the introduction of the NOD-Scid model, which was generated by Shultz et al. [21] backcrossing Scid mice into non-obese diabetic (NOD) mouse strains. Transplantation studies showed that these mice showed increased human hematopoietic engraftment as compared to the Scid mice. The mutation responsible for this favorable behavior was later identified to be in the *SIRPA* gene, which encodes for the transmembrane receptor SIRPα on phagocytic cells such as macrophages and dendritic cells. SIRPα binds to CD47 expressed on hematopoietic cells and thus infers macrophage phagocytosis resistance [28]. The NOD mutated SIRPα, not the wild-type version, is able to recognize human CD47 and therefore prevents phagocytic clearance of the human xenograft. However, also the NOD-Scid mice were found to possess some major limitations. The reactivity of the innate immune system, especially of NK-cells towards the human graft is uncompromised. Furthermore, it was noticed that NOD-Scid mice have a strong tendency to develop spontaneous thymic lymphomas, preventing long-term studies of human engraftment. In addition, NOD-Scid mice do not support the full differentiation of human hematopoietic xenografts. Especially T-cells are virtually absent, which also leads to arrested proliferation of T helper cell-dependent B-cells [29].

A major breakthrough was the generation of mouse models with reduced or nearly absent NK-cell activity. Several approaches were taken to achieve this goal. Most commonly used today are mice with deletions or truncations in the interleukin-2 receptor gamma chain in a NOD-Scid background [24, 25]. The deletion or truncation of *IL2Rγ* leads to a complete loss of interleukin signaling, which is important for the development of B-, T- and NK-cells. NOD/ShiLtSz-scid/IL2Rγ-/- (NSG) mice are a popular example of a mouse model employing this strategy to eliminate NK-cell activity. NOD/ShiJic-scid/IL2Rγ-/- (NOG) mice, which only have a truncation in the extracellular signalling

domain of the *IL2R*γ gene behave generally very similar to the NSG mice in most applications, but NSG mice were found to be slightly more permissive to normal hematopoietic engraftment than NOG mice [30]. Alternatively, mutations in the gene encoding for β2-microglobulin, which is necessary for the expression of MHC molecules and therefore NK-cell development, are another commonly used aproach to repress NK-cells in mice with a NOD-Scid background [22].

According to Vargaftig et al. [31] NSG mice are significantly more permissive to leukemic engraftment than the previously used NOD-Scid mice. When assayed in the NSG model the apparent number of AML LIC increases on average 31-fold compared to the NOD-Scid model. However, it is important to note that AML samples that did not engraft in NOD-Scid mice at all were also not engraftable in NSG mice. This is consistent with earlier findings of McDermott et al. [30], who compared the engraftment efficiency of normal human hematopoietic cells in different mouse models. They found an 3.6-fold increase of the apparent hematopoietic stem cell frequency when using NSG mice as compared to NOD-SCID mice. Also the human engraftment level was higher in NSG mice than in NOD-Scid mice, as assayed by the percentage of human CD45-positive cells in the peripheral blood of transplanted mice.

Immunosuppressive antibodies Another important way to improve the immunodeficiency of mouse models is to infuse immunosuppresive antibodies into their bloodstream. Primary choices are antibodies against CD122 and pooled purified human IgG immunoglobulins (IVIG, Intravenous immunoglobulin). CD122 is an antigen found on many mature hematopoietic cells including macrophages and NK cells, which are depleted efficiently by administering antibodies directed against CD122. The mechanism by which IVIG suppresses the immune system is less clear, but it is widely used in xenotransplantation experiments and also in the treatment of human autoimmune diseases [32]. McKenzie et al. [23] compared the engraftment of normal human hematopoietic cells in NOD-Scid treated with anti-CD122 and the newer NOD-Scid β2m-/- mice. The engraftment level was highest in the anti-CD122 treated NOD-Scid mice, followed by untreated β2m-/- mice, while untreateted NOD-Scid mice showed the lowest human engraftment. Also the effect of direct intrafemural injection of the transplant versus intraveneos infusion was assessed. Intrafemural injection generally lead to higher engraftment levels than intraveneous infusion, indicating the presence of residual immunoreactive cells even in NOD-Scid β2m-/- mice, which can prevent the transplanted cells from reaching and engrafting the bone marrow of the host. This is also supported by the fact that the differences in engraftment between the three models (NOD-Scid, NOD-Scid + anti-CD122, NOD-Scid β2m-/-) was not as pronounced when performing intrafemural injections, presumably because the transplanted cells were less exposed to host immune cells with this method as compared to intraveneous infusions. The significantly higher engraftment in anti-CD122 treated NOD-Scid than in NOD-Scid β2m-/- mice indicates that even in the more immunodeficient β2m-/- background there is still significant immunoreactivity towards transplanted human cells. Therefore also these mice most likely benefit from anti-CD122 treatment, although this was not directly shown by McKenzie et al. [23]. Today, IVIG and anti-CD122 administration is a routine method to improve immunodeficiency and is widely used even in the most recent mouse models.

3.2 AML LSCs without HSC phenotype

3.2.1 CD38+ AML LSC

Largely owing to improved mouse models compared to the NOD-Scid mice used in the first AML LSC studies recently more and more evidence has been published that LSCs can be found

in phenotypic subpopulations of AML cells that initially were not believed to contain any LSC activity. An overview of the most commonly observed phenotypes of LSC in AML is given in Table 1. As Taussig et al. [18] show, the leukemia initiating capacity of CD38+ cells has been largely underestimated. They discovered that several antibodies commonly used in the enrichment of hematopoietic cells have inhibitory effects on the engraftment of both normal and leukemic cells in mice. Especially the anti-CD38 antibody clones HIT2 and AT13/5, widely used in flow cytometry of hematopoietic cells, inhibit engraftment of leukemic cells in both NSG and NOD-Scid β2m-/- mice. Depending on the sample, the percentage of engraftment was reduced 3-500 fold in the presence of these antibodies and in some instances lost altogether.

This finding offers a convincing explanation why people did not find significant quantities of CD38+ LSC before. Apparently cells coated with anti-CD38 antibody are cleared from the circulation by a yet undetermined mechanism which leads to the observed reduction in engraftment efficiency. Several methods have been found effective to overcome this issue. Immunosuppresive antibodies, especially anti-CD122 and IVIG, negate the negative effect of CD38 antibodies on engraftment efficiency and can even enhance engraftment above the base level (see chapter 3.1).

Using IVIG to alleviate the effect of the anti-CD38 antibody Taussig et al. showed the presence of LIC with CD34+/CD38+ phenotype in six AML samples upon engraftment in NSG mice. The self-renewal potential of these cells was demonstrated later by the same group so they can be considered true LSC [17]. In the samples where Taussig et al. [18] detected CD38+ LSC, only one case of leukemic engraftment following the transplantation of CD34+/CD38- cells was demonstrated. However, since typically only $>1 \cdot 10^3$ CD38- AML cells per mouse were transplanted and the frequency of LICs in CD34+/CD38- cells is generally estimated to be $4.5 \cdot 10^2 - 100 \cdot 10^6$, their low engraftment might have been caused by the low amounts of cells transplanted. All in all this report as well as others clearly show that the CD38+ subpopulation of cells in AML can contain LSCs [13, 14, 17, 16].

Importantly, even the group of John Dick, which published the first paper on AML LSCs, recently reported the notion that AML LSCs are not necessarily confined to the CD34+/CD38- population of AML cells. Using NOD-Scid mice treated with anti-CD122 antibody they assessed the engraftment potential of sorted peripheral blood samples from subjects with AML via intrafemural injection [13]. In 13 out of 14 cases LSCs were found in the CD34+/CD38- compartment, but in 8 out of these 14 they were also identified in the CD34+/CD38+ subpopulation. The relative distribution of LSC among the subpopulations was highly variable from sample to sample, without obvious correlation with absolute cell numbers in each subpopulation, clinical parameters, mutational status or FAB subtypes.

Goardon et al. [14] have provided additional data on the phenotype of CD34+ LSCs. They used a rigorous immunophenotyping approach followed by xenotransplantation to identify different cellular populations and phenotypes of LSCs in CD34+ AML samples. In comparison to normal blood samples they found that in AML patients certain cell populations were characteristically expanded. In the vast majority (87.8%) of AML samples they found that >90% of the CD34+ cell population had the immunophenotype Lin-/CD34+/CD38-/CD90-/CD45RA+, which corresponds to multilymphoid progenitors (MLPs) by the nomenclature of Doulatov et al. [10], but in contrast to MLPs do not express the early lymphoid markers CD7/10. In the same samples most CD38+ cells are CD34+/CD38+/CD45RA+, which closely corresponds to Granulocyte-macrophage progenitor cells (GMPs).

In contrast, in 13.8% of the examined samples the predominant cell population was Lin-/CD34+/CD38-/CD90-/CD45RA-, mimicking the phenotype of multipotent progenitor cells (MPPs). In these samples there is also expansion of the common myeloid progenitor-like (CMP) population. On the basis of this definition AML samples can be classified either into a MLP/GMP-

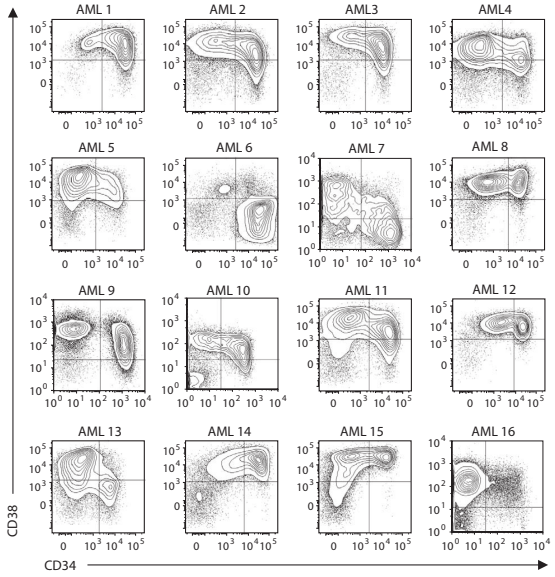

Figure 3: Representative flow cytometry plots showing the expression of CD34 and CD38 on cells from the peripheral blood of 16 different AML patients, thereby illustrating the wide variety of phenotype distributions observed in AML cells. Adapted from Eppert et al. [13].

expanded or MPP/CMP-expanded group.

Transplantation of purified GMP- and MLP-like cells into NSG mice treated with CD122 antibody revealed that in most MLP/GMP-expanded patients both populations have LSC activity. The self-renewal capacity of those LSC was confirmed by secondary and tertiary transplantations. Although both populations contain LSCs the frequency is much higher in the MLP-like than in the GMP-like population. This data indicates that CD34+ AML (only samples with >5% CD38+ cells were analyzed) is a disease in which certain hematopoietic progenitors gain self-renewal capacity.

All this indicates that there is much more heterogeneity in the LSC phenotype in AML as previously described, especially the finding that multiple populations of LSC coexist within one patient is surprising but very well documented in recent literature. This finding raises the question how different LSC populations are clonally related to each other and what the distinguishing molecular features are. These issues will be addressed later in this thesis.

3.2.2 CD34- AML LSC

AML with mutated *NPM1* accounts for approximately one third of all AML cases and has some specific clinical, molecular and immunological features that distinguishes it from other types of AML and justify its categorization as a distinct diagnostic criterion [33]. Recently, two studies concerned with the phenotype of the leukemic blasts and LSC in AML with mutated *NPM1* have been published [17, 34]. Studying *NPM1*-mutated AML is interesting because the blood from such patients generally contains on average only 0.66% CD34+ cells compared to 24.5% in AML with WT *NPM1* [17]. The question then was whether LSC are predominantly present CD34+ fraction although this population is comparatively small. Taussig et al. [17] report that they found LIC activity in the CD34- fraction of all 15 examined *NPM1*-mutated AML samples, assayed by transplanting in the order of $1 \cdot 10^6$ cells into NSG or NOD-Scid β2m-/- mice. Specifically, the LIC were located in the CD34-/CD38+ fraction in all 12 samples tested. They were also found in the

CD34-/CD38- fraction in 6 out of 12 samples.

The CD34+/CD38- fraction, which represents the classical immunophenotype for LSC, gave rise to leukemic engraftment only in a subclass of *NPM1*-mutated AML, referred to as class C samples by Taussig et al. [17], which have a relatively large CD34+ population but the CD34+/CD38- population does not appear as a clearly distinct and separated entity in flow cytometry analysis. In samples with a clearly separated CD34+/CD38- population or a total CD34+ population below 0.5% (subtype B and A, respectively) the CD34+/CD38- cells either did not engraft or lead to normal multilineage engraftment.

For a subset of the *NPM1*-mutated AML samples serial transplantations were performed, both CD34+ and CD34- cells were able to engraft secondary recipients and established stable populations within the host for up to 34 weeks. In two instances also tertiary engraftment with CD34-/CD38+ cells was attempted and successfully established. This clearly indicates that both CD34+ and CD34- from these samples cells can initiate leukemia and possess self-renewal capacity, meeting the strict definitions for LSCs. Martelli et al. [34] mostly came to similar conclusions, but also clearly showed that CD34+ LSC do exist in some *NPM1* mutated AML samples when more than 1% of leukemic cells are CD34+. All in all this data indicates that in AML with mutated *NPM1* where CD34+ cells make up less than 1% of the total leukemic population, the LSCs are usually CD34-.

Similarly also John Dick's lab found LSC in the CD34- compartment in three out of ten examined samples, of which one contained ITD in the *NPM1* gene, one was *NPM1* wild-type and for one the *NPM1* status was not determined [13]. Interestingly, none of the published studies show any correlation between the distribution of LSC phenotypes in a sample and the distribution of phenotypes among all cells. Although the phenotype of AML LSCs is therefore proven to be variable, LSCs in AML are still rare and highly enriched in specific immunophenotypic fractions, which vary between different AML cases.

3.2.3 Additional markers expressed on AML LSCs

Additionally to the traditional markers associated with the normal hematopoietic system a wide array of markers specific for leukemic cells have been discovered. They can be used to distinguish between normal hematopoietic cells and leukemic cells and also to enrich for LSCs.

ALDH Aldehyde dehydrogenase (ALDH) is a metabolic enzyme that oxidizes aldehydes to carboxylic acids. It represents a rather new marker for AML LSC and was recently put forward by Gerber et al. [15]. They found that three distinct cell populations with low, intermediate and high ALDH staining can be detected in the CD34+/CD38- fraction of peripheral blood from AML patients while the population with intermediate staining is absent in non-leukemic blood samples. Xenograft experiments in NSG mice revealed that only transplantation of the $ALDH^{int}$ population lead to leukemic engraftment. By contrast, transplantation of $ALDH^{high}$ cells leads to normal hematopoietic engraftment while $ALDH^{low}$ cells show no engraftment potential whatsoever even at very high doses. Even more interesting, when comparing the ALDH staining of patients during morphologically defined complete remission (CR) they found two distinct patterns which strikingly correlated with the clinical outcome for the patient. The "normal" pattern resembles the pattern found in non-leukemic samples and contains no discernible $ALDH^{int}$ population. It is highly correlated to a positive clinical outcome, since none of the seven patients possessing this pattern had relapsed over an average of 509 days of observation post diagnosis.

Patients with "MRD" pattern during CR show a strong $ALDH^{int}$ population representing minimal residual disease (MRD) not detectable with traditional morphological methods. All four patients

with this pattern had relapsed on average 53 days after MRD detection. One of the relapsed patients achieved CR again after repeated chemotherapy and converted to a "normal" pattern and remained in CR since then. Two retained the MRD pattern in their second CR after repeated chemotherapy and subsequently relapsed again.

Of interest, this is one of only a few studies that followed patients during the progress and therapy of the disease. Valuable information about relevant properties of AML LSC is gained by this approach. For example, it appears that while the total load of leukemic cells is reduced by two orders of magnitude between diagnosis and CR for MRD patients, the number of CD34+/CD38-ALDH^{int} cells is reduced by only one order of magnitude. As these cells represent a population which is highly enriched for LSC and as they are the major cell fraction left in MRD it is not surprising that relapse was observed in all cases which exhibited MRD pattern.

This study shows that ALDH is potentially useful as a marker for LSCs in AML as well as a diagnostic tool to track the progression of the disease.

CD47 CD47, which was already mentioned earlier as being important for the evasion of the host immune system in xenotransplantation experiments, has been shown to be expressed highly on leukemic cells [35]. The same mechanism that leads to the immune evasion in NOD-Scid mice, the interaction of CD47 on the leukemic cells with SIRPα on phagocytic cells, apparently helps leukemic cells to counter the immune system of the patient. Majeti et al. [35] show that in many AML patients CD47 is significantly higher expressed on leukemic than on normal hematopoietic cells. At the same time high CD47 expression also correlated significantly with unfavorable clinical prognosis and was found to be positively correlated with ITD in the *FLT3* gene. the phagocytic clearance of human LSC injected into NSG mice is significantly enhanced by the addition of anti-CD47 antibody, while normal human HSC are unaffected. Existing leukemic engraftment was efficiently eliminated by the administration of anti-CD47 antibody. This effect was indeed at least party due to phagocytic clearance by macrophages, as demonstrated by the inhibiting effect of the macrophage toxin chlodronate.

However, while selection for CD34+/CD38-/CD47^{low} cells efficiently depletes any leukemic cells from the sample its usefulness as marker for LSC is still questionable. For their comparison of CD47 expression among bulk leukemic cells and LSC the LSC fraction was not functionally validated but only defined by their Lin-/CD34+/CD38-/CD90- phenotype (as opposed to Lin-/CD34+/CD38-/CD90+ which is the phenotype defining normal HSC). Still it seems that LSC are more susceptible to anti-CD47 treatment, as secondary transplantation from anti-CD47 treated mice failed despite leukemic human cells still being present in the transplanted whole bone marrow.

Consequences for our view of the LSC All in all, LSC can no longer be considered confined to a distinct phenotypic compartment. However, in many cases there is still significant enrichment of LSC in otherwise infrequent cell populations, especially CD34+/CD38-. This remains an important issue and an important question is now whether LSCs in AML preferentially originate from the phenotypic compartment that they are enriched in or that genetic lesions conferring LSC-activity somehow lead to the expression of specific phenotypic markers.

A completely reliable and unambiguous marker set for LSCs has not been found yet and depends strongly on the specific AML sample. Even with the most sophisticated purification techniques usually thousands of cells need to be transplanted to get engraftment. This will hopefully change in the future, when the specifics of the different AML subtypes are understood better. However, I do not expect that a universal set of markers will be found which marks LSCs in all kinds of AML.

The fundamentally variable genetic background of AML LSCs and their phenotypic plasticity make it unlikely that a unifying marker set present on all AML LSCs can be found. However, it should be possible to devise a strategy to purify LSC from any AML sample based on the knowledge of its underlying molecular changes and pathogenesis.

3.3 Hierarchical orders in AML

Complementary to the idea of LSC initiating the disease there is the idea that LSC can differentiate into phenotypically distinct leukemic blasts and form a hierarchy of cells with distinct behaviors, especially regarding their proliferative potential and divison frequency. A powerful method to study these hierarchichal structures is to mark primary AML or transduced healthy hematopoietic cells with virally delivered markers. Upon xenotransplantation these cells can be efficiently tracked by fluorescence markers and clonal relationships between cells can be established by analyzing the integration site of the viral vector, which is unique for each clone. Hope et al. [36] retrovirally transduced primary AML cells with a GFP construct to track the leukemic cells *in-vivo* after transplantation into NOD-Scid mice. Clonal analysis revealed that the clonal population of cells initially populating host mice was disappearing after approximately 12 weeks and being replaced by a population not detected earlier. This finding can be explained by the existence of multiple distinct LSC populations with varying self-renewal capacity, termed short-term and long-term LSCs, the former one having limited self-renewal capacity. Analysis of the clonal markup in secondary and tertiary recipients revealed that often clones detectable in the primary mouse were also present in the secondary mouse, indicating self-renewal of this clonal population in the primary mouse. Interestingly, some clones were contributing persistently for >12 weeks in primary mice but consistently failing to engraft more than 12 weeks in secondary and tertiary recipients. This can be considered evidence that short-term LSC arise from long-term LSCs, which are believed to be quiescent and persistent below the detection limit in the primary recipient.

In a related study Barabé et al. [37] investigated the clonal relationship between different LSC populations and how it evolves during disease progression. They retrovirally tranduced lineage depleted human cord blood cells with with GFP and the *MLL-AF9* fusion gene, which is sometimes found in AML patients. These tranduced cells were transplanted into NOD-Scid mice, which subsequently developed symptoms of B-precursor ALL, AML or mixed leukemia. It was found that the graft was derived from two to five clones, of which usually either one or two were numerically dominant. Upon serial transplantation into a second mouse the graft was usually monoclonal and derived from one of the predominant clones from the primary recipient.

They found that leukemic blasts are clonally derived from a rather small subpopulation of cells. However, the hierarchy does not necessarily adhere to lineage constraints of the normal hematopoietic system. Indeed it was observed that during *in-vitro* culturing of B-ALL complete cells lineage switches occurred from lymphoblastic to myeloblastic populations and in one instance this switch was later reversed. Examination of the IgH gene arrangement revealed that this switch must have occurred in progenitor cells with rearranged IgH allele, which are normally strictly committed to the lymphoid B-cell lineage. Generally, a progression of the IgH gene from germline state to rearranged state during disease progression was observed, as the proportion of germline configured alleles decreased consistently after the switch from primary to secondary recipients.

More data on the cellular hierarchy in AML is presented in a paper by Goardon et al. [14]. They have shown that in most AML samples two LSC populations with MLP- and GMP-like phenotype are present. There was abnormal enrichment of self-renewal genes normally only found in stem-cells, which may allow those LSCs to self-renew while retaining the proliferative activity innate to progenitor cells. By xenotransplantation of both these populations into CD122-treated

NOD-Scid mice they show that either can recapitulate the disease and both possess self-renewal capacity when serially transplanted. However, while MLP-like cells consistently gave rise to the GMP-like population upon transplantation, the reverse transition from GMP-like to MLP-like was not observed, neither *in-vivo* nor *in-vitro*. This might be related to the fact that the global gene expression profile of these two populations was most similar to their normal hematopoietic counterparts, which would probably restrict their capability to give rise to cells of different lineages. While the MLP- and GMP-like LSC population was distinct in both immunophenotype and gene-expression profile, there still exists the possibility that they are actually derived from one homogenous population. This is owing to the rather arbitraty selection of CD38+ and CD38- cells from the continuous distribution of CD38-expression among hematopoietic cells.

All this clearly indicates that leukemic cells in AML follow a hierarchical pattern of development, which does not necessarily reflect the natural hematopoietic hierarchy. Unorthodox lineage switches of cells with commited progenitor features have been observed on multiple occassions and might be rule rather than exception.

3.4 LSC as a cellular state

Investigating AML in all kinds of different subtypes and for many cytogenetic and mutational malignancies revealed the presence of LSC in more and more immunophenotypic compartments. This questions the validity of studies relying on phenotypically defined, not functionally validated LSCs in AML. The variability of LSCs makes it likely that sometimes cell populations labeled as LSCs are not actually LSCs unless some kind of functional validation, ideally quantitative by limiting dilution analysis in xenograft models, is given.

Phenotypical descriptions are far from useless, as there are certainly clear correlations between specific genetic lesions and the phenotype of leukemic blasts [38, 33]. The clinical relevance of certain subpopulations, namely cells with CD34+/CD38- $ALDH^{Int}$ immunophenotype, has recently been shown by Gerber et al. [15] as discussed earlier. Also the relevance of LSC as such for progression and prognosis of the disease is rarely disputed, as convincing data exists that links certain LSC parameters to clinical outcome and prognosis Pearce et al. [39], Majeti et al. [35], Eppert et al. [13].

However, in light of results that convincingly show that the phenotype of LSC is not restricted to a primitive phenotypic compartment and that it can shift and evolve during disease progress or upon transplantation into mice it becomes clear that new approaches on how to define LSCs are required.

Some reports indicate that there is similarity between LSC and HSC expression patterns in gene expression microarrays, [13], while others found the expression profiles of LSCs to resemble commited progenitors (MLPs and GMPs) [14]. Therefore it is more beneficial to define AML LSCs primarily in a functional and genetic way instead of staying fixed solely on immunophenotypes. Instead, immunophenotypic markers can be seen as a representation of the cellular state, which can be useful to distinguish AML LSCs from the bulk of leukemic cells once they have been defined functionally.

A recent study by Gibbs Jr. et al. [40] of HoxA9-Meis1 driven AML in mice gave valuable insight about the phenotype of LSC at disease initiation and during its progression. In this study, mouse bone marrow cells were retrovirally transduced with HoxA9 and Meis1 and transplanted into mice, reliably generating AML by this single oncogenic lesion. The presence of tumor initiating activity in several immunophenotypic compartments was assessed by injecting 100 sorted cells into mice and evaluating the number of mice falling sick. Consistent with previous findings presented here, most LSCs were found in the immature hematopoietic compartment (Lin- c-kit+) and also in the

compartment containing immature myeloid cells (Gr1+/c-kit+). Somewhat unexpectedly also the compartment analogous to lymphoid progenitors (Lym+/c-kit+) contained tumor initiating activity. The clonality of this initiation was confirmed by plating single sorted cells and expanding them *in-vitro*. Subsequently transplanted single colonies of Lym+/c-kit+ as well as Gr1+/c-kit+ cells initiated AML and lead to the same phenotypic spectrum of leukemic blasts as in the original full population they were derived from, including the immature Lin-/c-kit+ subpopulation and terminal Gr1+/c-kit- blasts.

This already provides some evidence that leukemic cells do not necessarily adhere to the classical cellular hierarchy of the hematopoietic system, making immunophenotypic description of the tumor initiating population difficult. This notion was confirmed by transplanting cells with LSC activity from different immunophenotypic compartments into cohorts of mice. The phenotype transplanted GFP positive leukemic cells was analyzed periodically by means of generating SPADE trees, which visualize the interrelatedness of cellular populations from flow cytometric data. Seven to eight days after transplantation first evidence of engraftment of GFP positive cells appeared, at which stage the phenotypes of the leukemic cells was confined to a few compartments with strong tumor initiating capacity. However, already after 14 days the phenotypic variation between cohorts almost disappeared and were indistinguishable in terms of their phenotypic distribution, regardless of the phenotype of the cells originally transplanted. Taken together these findings demostrate that LSC activity can be seen as a cellular state independent of the immunophenotype.

Clonal evolution in AML Some clues on how the diversity of LSC phenotypes and its apparent dynamic and plastic nature is generated can be drawn from the work of Anderson et al. [41]. They provide convincing data about the clonal architecture of leukemic cells in childhood acute lymphoblastic leukemia with *ETV6-RUNX1* gene fusion. By analyzing copy number alterations of a limited number of relevant genes on single cell level by FISH they generate a map of the different subclones present in each case of ALL and are able to generate a hypothetical evolutionary tree representing the ancestral relationship between different subclones. The data shows that even at the early stages of disease, in this case at diagnosis, leukemic cells are composed of several distinct subclones with greatly varying frequency. Interestingly, the clones with the most genetic alterations were not necessarily the ones with highest frequency, suggesting that there might be specific niches of differentiation with divergent genetic requirements.

4 Implications for future research

Challenges One of the major challenges in AML cancer stem cell research is the relative scarcity of LIC. This imposes some major limitations on experimental design, especially for working with primary cells derived from patient blood samples. The current gold standard for determining the LSC frequency are limiting dilution assays, for which specific amounts of cells are injected into immunodeficient mice and the engraftment is measured after usually about 10 weeks. If this is done for a series of different dilutions in multiple mice it is possible to calculate an estimate for the LSC frequency in the injected sample using Poisson statistics. In AML the LSC frequency in the unfractionated peripheral blood of patients varies greatly but is generally reported to be very rare, with about one LSC per $4.5 \cdot 10^2 - 100 \cdot 10^6$ mononuclear cells. [9, 16, 31] However, current methods to measure the frequency of stem cells are believed suffer from several biases, resulting in systematical over- or underestimation of the number of stem cells [42]. In case the putative stem cell can be enriched to high purity it is also possible to do single cell transplantation experiments. This methods provides evidence of the stemness of clones on a single-cell level. It was used by

Quintana et al. [43] to show that cancer stem cells in human melanoma are rather frequent and make up approximately 27% of the unsorted primary tumor cells in single cell transplantation experiments into highly immunocompromised NOD-Scid-IL2RγNull mice. The downside to this method is the high purity demands imposed on the stem cell sample which makes it unfeasible for situations in which the stem cells are rare and their markers are not well known. However, in the future higher purity of AML LSC samples might be obtained using new or better combinations of LSC markers so that single cell transplantation experiments with AML LSC become possible.

It is an open question whether AML LSC are truly as rare as current data suggests or whether the xenotransplantation experiments systematically underestimate their frequency, for example because some LSC might be particularly susceptible to clearance by the host immune system or because they need supportive human tissue like bone marrow, spleen or liver to engraft. Parts of this question will probably be answered when chimeric mouse models containing these human tissues become available, as discussed later. In support of the rarity of AML LSC it is worth to note that the current immunodeficient mice are already very supportive of normal hematopoietic engraftment. Notta et al. [44] were able to establish long-term multilineage engraftment following the transplantation of a single CD34+/CD38−/CD45RA−/CD90+/Rho^{low}/CD49f + HSC into a NSG mouse.Taking this into consideration, it is not immediately clear why AML LSC, which have been shown to possess similar gene expression profiles as normal human HSCs or multipotent progenitors [13, 14], would be less able to engraft in these mice than normal human HSCs.

Non-engrafting AML Another challenge to AML LSC research is that certain samples do not engraft. This might pose a problem if generailized conclusions are drawn from research done solely on the subset of engrafting AML samples, which may have special features distinguishing them from non-engrafting AML. The reason why certain patient samples fail to engraft are not yet known but several possible explanations have been put forward. Most prominently, the CD47 expression levels of the transplant has been correlated to the ability to engraft in xenotransplantation models. It is expected that higher CD47 levels lead to more successful engraftment because of the increased immune-evasive potential towards macrophages, but Vargaftig et al. [31] found no significant difference in CD47 expression between engrafting and non-engrafting samples. Pearce et al. [39] and Rombouts et al. [45] were able to correlate engraftment of AML samples to a series of clinical variables. They observed that engraftment is highly increased for secondary AML as well as for certain FAB subtypes. However, the single most predictive parameter was the white blood cell count at diagnosis, suggesting that the pure proliferative potential of the AML cells might be more important for engraftment than LSC frequency.

4.1 LSC phenotype

The fact that LSCs in AML are found in more and more phenotypic compartments is astonishing. In the early days of AML research, when LSC were evident mostly in the CD34+/CD38- subset of cells, it seemed logical that LSC are basically transformed HSC. Based on the strong phenotypic similarity and stem-cell like function of the LSC, such as the capacity to self-renew upon transplantation into immunodeficient NOD-Scid mice over a period of 8 weeks and the ability to differentiate into typical leukemic blast cells [9], the conclusion that LSCs are probably transformed hematopoietic stem cells was valid. However, as early as 1996 doubts where raised whether LSC were always and exclusively CD34+ and thus share an immunological phenotype with the HSC. With improved mouse models and refined transplantation techniques LSCs were discovered in various immunologically distinct subpopulations of leukemic cells. This challenged the original model of Lapidot et al. [8] and led to a confusing diversity of described LSC phenotypes.

Several models have been proposed that are able to explain the LSC phenotype in AML as we understand it today [16, 41, 36, 46]:

The missing marker model proposes that LSC are a homogenous and phenotypically well-defined subpopulation of leukemic cells and can be unambiguously defined by a specific combination of absent or present surface markers. Such unique of marker set that identifies LSCs in each individual AML patient is not yet known and there are good reasons to believe they may not exist. AML research has revealed an enormous variety of mutations, cytogenetical alterations and epigenetic changes in leukemic cells that lead to a astonishing and still growing multitude of leukemic phenotypes [38, 47]. Moreover, recent studies on AML and ALL have shown that even within one patient various subclones of LSCs with distinct genetic changes exist that are subject to clonal evolution and selection [37, 41]. Even within one individual the leukemic cells are in a constant change, driven by decreasing DNA stability, selection pressures imposed by chemotherapeutical treatment and necessary evasion of the host immune system lead to rapid evolution of aberrant cellular clones. It seems very unlikely that among all this changing conditions the LSC is maintaining one distinct immunological phenotype. Considering current literature such possibility cannot be excluded, but it seems highly unlikely that the LSC of such a heterogeneous and multifactorial disease like AML can be defined by a single combination of markers. More likely it will be possible that LSCs can be functionally defined by a specific set of genetic alterations which give rise to a certain specific phenotype.

Another model is a tumor plasticity and clonal evolution model. In this model the LSC is not defined by a specific immunophenotype but the LSC property is more seen as a cellular state that can be induced by a multitude of cellular changes. The classical clonal evolution model proposes several clonal subpopulations in a tumor, of which some possess tumor initiation capacity and some do not. However, there is no developmental hierarchy like the development of LSC into non-self renewing leukemic blasts in this model. Subpopulations may only differ in their tumorigenicity but all subclones of one population may only differ by stochastic variation. These stochastic differences together with newly acquired genetic traits lead to competition between clones, where those with favorable changes are selected.

4.2 Cancer stem cells vs. clonal evolution

The hierarchical nature of AML has been shown convincingly in numerous studies [9, 36, 37, 14, 40] so that the classical pure clonal evolution can be regarded as disproven. However, our current knowledge of LSC might be convincingly represented by a combination of cancer stem cell and the clonal evolution model. The clonal evolution model applied to different clonal LSC populations which differ in their self-renewal, repopulation and differentiation capacity and compete for clonal dominance with these properties. Some subclones of a population might loose tumor initiating capacity others might gain genetic lesions that increase their tumorigenicity. However, the tumor as a whole is maintained by a limited subset of LSCs with capacity to self-renew. These reside at the apex of a developmental hierarchy with terminally differentiated blasts at its end, which cannot recapitulate the disease.

A major point which makes validation of these findings so complex is the large number of potential contributing factors that make a cell a leukemic cell. Almost every case of AML is different in its origin, genetic changes and history, making generalized statements about the causal links between its different features very difficult. The changes from normal cell to leukemic cell can be of intracellular as well as extracellular origin, for example changes in the microenvironment or the supply with growth and differentiation factors that the LSC is exposed to [48, 12]. For AML alone more than 200 genetic alterations have been described that seem to be contribute

to the disease [38]. The emergence of high-throughput genetic screening technologies enabled researches to study the genetics of cancer at unprecedented detail and gave insight into the clonal architecture of cancer cells versus normal cells. However, progress has been hampered by the fact that most of technologies employed for these studies require the analysis of a whole ensemble of cells derived from the tumor. This approach conceals the genetic diversity of different cancer subclones expected to be present in a single patient, contributing to the confusion of what constitutes the phenotype of AML LSCs.

Recently, more and more technologies to study genetics at a single cell level have been applied to cancer cells. Using these methods enables researchers to identify and describe single subclones from the whole tumor mass, which is highly interesting given the heterogeneity of clones even among cells that share one immunocompartment.

All this highlights how problematic it is to treat cells and their features as a static entity, as it classically done when discussing the cancer stem cell model disregarding the mechanisms of clonal evolution. Anderson et al. [41] show that there is great variety in the clonal composition of ALL tumors both within a patient and in between patients, as well as a temporal development when the disease is treated and eventually relapses. Specific subclones that only make up a small amount of the total population can suddenly become dominant when circumstances change. This is especially interesting in conjunction with the results from Sarry et al. [16] and others [17, 15, 14, 13]. They show that LSCs are highly enriched in the relatively rare CD34+/CD38- population, but but when considering absolute numbers are also present in large numbers in the other subpopulations of cells. Keeping the concept of clonal evolution in mind it is possible that the LSC originated from this naive phenotype but during disease progression was able to acquire additional mutations that lead to differentiation or expression of more mature phenotypic markers.

It seems logical that some genetic lesions only lead to transformation into a LSC only in the context of a cell that already possesses self-renewal capacities, for example the HSC. Other genetic lesions may directly or indirectly lead to the expression of stem-cell specific genes relatively independent of the cell in which they occur in, as it seems to be the case in the *Hoxa9/Meis1* model of AML [40]. In many cases clonal evolution apparently leads to the accumulation of additional mutations [49] which may alter the appearance of LSC in a way that they longer retain the native immunophenotype of the original but need to be defined functionally.

On this background it would be highly interesting to know how the genomic status of leukemic stem cells in the various immunophenotypic subpopulations compare and whether the apparent lineage infidelity of LSCs and their ability to spontaneously switch lineage commitment is a result of changing genetics due to clonal evolution or true dedifferentiation to a more naive cell type and redifferentiation.

4.3 Future directions

I predict that with the advent of efficient tools to study the genomics of single cancer cells fresh light can be shed on the relationship between the immunological phenotype of cancer stem cells and their specific genomic lesions. At the moment most studies are tainted by the intermixing of heterogeneous cells and the fact that diseases at different stage of progress are evaluated. In my opinion these factors are major reasons for the current confusion in literature about the phenotype and identity of the LSC in AML.

It is necessary to view LSCs as dynamic, constantly changing entities with a lot of diversity even within one patient. For this reason, I believe that further research must concentrate on two things: First, the stage and progression of each patient sample needs to be determined and taken into account individually, it is usually not helpful to pool results from patients in widely varying

stages of leukemia, for example patients with relapsed and primary AML. Considerable evidence has been accumulated that LSC-characteristics can change considerably during these events [49, 50, 41, 51, 15]. Furthermore, all results obtained with patient blood are to be considered only snapshots at a fixed timepoint from a very dynamic system, which is likely to considerably change over time. It would therefore be highly interesting to conduct more timecourse studies, following LSCs during disease progression. Second, there is a high degree of heterogeneity between leukemic cells isolated from on patient and often there are multiple LSC populations. Therefore it would be highly preferable to obtain more data on a single cell level instead of from a whole of bulk cells. This is increasingly made possible by newly developed single cell sequencing and tracking techniques as pioneered by Navin et al. [52] for the study of breast tumor evolution, which allowed for much higher resolution compared to previous studies where FISH staining of a limited number of loci was used to track the clonal relationship between cells.

Deep-sequencing of single cells with next-generation sequencing techniques, enabling researchers to define clonal relationships between cells with unprecedented precision. Using this data relationship trees can be inferred from single nucleotide polymorphism and copy number alteration patterns found in multiple cells. In this way it may be possible to follow in detail how leukemic cells develop during disease progression and treatment and which genetic features determine certain properties like the lineage commitment of blasts, the LSC phenotype and mechanism of chemotherapy resistance.

In the end it will hopefully be possible to formulate a contingent theory of LSCs, that encompasses the whole spectrum of observed phenotypes. Included will probably be a theory of origin, explaining which hematopoietic cells, especially HSCs and multipotent progenitors, are susceptible to which genetic lesions. In the early days of AML LSC research the LSC immunophenotype was often used to infer the cell of origin, an approach we now know is very unreliable given the apparent flexibility and instability of the LSC phenotype [40, 14, 17, 16]. A more promising approach is the backtracing of the origin by expression analysis. Already a lot is known on this subject from gene expression microarrays, generating profiles of functionally validated LSCs which can be compared to different normal hematopoietic cells [13]. It was found that HSC signature genes were also highly enriched in LSC. There are basically two different explanations for this observation: On the one hand, the similarity of expression profiles can be interpreted as a hint that LSCs are transformed hematopoietic stem cells, on the other hand it is possible that the transforming tumorigenic hits lead to a hematopoietic stem cell like expression of proteins in the target cell, regardless of its original identity. Also here single cell techniques like transcriptome sequencing could give more precise information on the active genes of LSCs. At some point it may even be possible to sequence functionally validated single clones.

New mouse models Further improvement of xenograft mouse models can be achieved by humanizing mice with human tissues, which can support engraftment by providing transplanted human cells with their natural environment. This includes cellular niches to which xenografts can home to and the production of growth factors.

Several groups are currently working on the development of mouse models with a functional human bone marrow niche supporting hematopoietic engraftment. The interaction between the bone marrow microenvironment and hematopoietic stem cells is essential for their maintenance. Also malignant hematopoietic cells often are dependent on a functional bone marrow. Their interaction is of high clinical importance, contributing to chemotherapy resistance and eventually relapse of leukemia in remission [53, 54]. All this shows the deficiencies present in current immunocompromised mice and highlights the need for humanized mouse models to properly

study hematopoiesis and its malignancies.

Most of the humanized mouse models currently in development are based on the ectopic implantation of an artificial human bone marrow graft supported by some kind of scaffold. This includes scaffolds made from polymeric material [55], biphasic calcium phosphate (BCF) [56] and matrigel [57], a commercially available, complex mixture of proteins which somewhat mimics the extracellular environment in human tissues. These scaffolds can be seeded with multipotent human stromal cells, which differentiate and form the basis of the artificial human bone marrow. Several groups have shown that human hematopoietic stem cells can successfully engraft and differentiate in this environment, even mature human B- and T-cells have been detected, whose differentiation is still a problem in conventional models [56, 57]. Also primary human multiple myeloma cells engrafted successfully in the artificial bone marrow and were maintained *in-vivo* for up to four months [55, 56], which has not successfully been done before without the support of fetal human bone material.

As to how these models compare it can be concluded that all of them seem to provide an adequate human bone marrow environment including the production of human growth factors and cytokines necesary to suport the growth of certain human cells, such as primary multiple myeloma cells. Further studies that use additional materials that are difficult to engraft in current xenograft models are necessary to determine the supportive potential of these new models. However, using BCF as scaffold material has some clear advantages over artificial polymeric material. BCF consists of the natural building material of bones and can be considered bioactive. It can be biodegraded by osteoclasts and new material can be deposited by osteoblasts, which brings mimicking real bones a step further compared to polymeric scaffolds.

To date, there are very few published reports where these new humanized mouse models have been used to engraft AML. However, humanized mouse with functional human bone marrow show great promise for AML research, as the interactions between leukemic cells and their environment in these models are much more representative for what happens in the patients than what can be achieved in classical immunodeficient mice. The huge potentials of this technology to study leukemia-bone marrow interactions was demonstrated by Chen et al. [57], who knocked down hypoxia inducing factor 1α in the supporting mesenchymal stem cells, which lead to a significant reduction in the engraftment level of the human MOLM13 AML cell line. Future studies will hopefully use primary human AML cells, whose capability to engraft into these new mouse models was already shown very briefly by Groen et al. [56].

Another interesting direction would be to study the response of AML to certain external cues like chemotherapy. The mechanisms by which relapses after complete remission occur are not really clear yet. In new mouse models with a functional human bone marrow microenvironment it is much more likely that the mechanisms of therapy resistance work than in the conventional non-humanized mice, because LSC resistance to chemotherapy is often conferred by the microenvironment [58, 53, 12]. Knowing the specific property that allows LSC to withstand chemotherapy could have far-reaching consequences for therapy and could lead to the development of drugs that specifically target processes LSCs are dependent on to function.

References

[1] Orkin, S. H., and Zon, L. I. (2008) Hematopoiesis: an evolving paradigm for stem cell biology. *Cell 132*, 631–644.

[2] Siegel, R., Naishadham, D., and Jemal, A. (2012) Cancer statistics, 2012. *CA Cancer J Clin 62*, 10–29.

[3] Grimwade, D., Hills, R. K., Moorman, A. V., Walker, H., Chatters, S., Goldstone, A. H., Wheatley, K., Harrison, C. J., Burnett, A. K., and National Cancer Research Institute Adult Leukaemia Working Group, (2010) Refinement of

cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood 116*, 354–365.

[4] Estey, E. H. (2012) Acute myeloid leukemia: 2012 update on diagnosis, risk stratification, and management. *Am J Hematol 87*, 89–99.

[5] Ley, T. J. et al. (2008) DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature 456*, 66–72.

[6] Welch, J. S. et al. (2012) The origin and evolution of mutations in acute myeloid leukemia. *Cell 150*, 264–278.

[7] Nowell, P. C. (1976) The clonal evolution of tumor cell populations. *Science 194*, 23–28.

[8] Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M. A., and Dick, J. E. (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature 367*, 645–648.

[9] Bonnet, D., and Dick, J. E. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med 3*, 730–737.

[10] Doulatov, S., Notta, F., Laurenti, E., and Dick, J. E. (2012) Hematopoiesis: a human perspective. *Cell Stem Cell 10*, 120–136.

[11] Griffin, J. D., and Löwenberg, B. (1986) Clonogenic cells in acute myeloblastic leukemia. *Blood 68*, 1185–1195.

[12] Konopleva, M. Y., and Jordan, C. T. (2011) Leukemia stem cells and microenvironment: biology and therapeutic targeting. *J Clin Oncol 29*, 591–599.

[13] Eppert, K. et al. (2011) Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med 17*, 1086–1093.

[14] Goardon, N. et al. (2011) Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. *Cancer Cell 19*, 138–152.

[15] Gerber, J. M., Smith, B. D., Ngwang, B., Zhang, H., Vala, M. S., Morsberger, L., Galkin, S., Collector, M. I., Perkins, B., Levis, M. J., Griffin, C. A., Sharkis, S. J., Borowitz, M. J., Karp, J. E., and Jones, R. J. (2012) A clinically relevant population of leukemic CD34(+)CD38(-) cells in acute myeloid leukemia. *Blood 119*, 3571–3577.

[16] Sarry, J.-E., Murphy, K., Perry, R., Sanchez, P. V., Secreto, A., Keefer, C., Swider, C. R., Strzelecki, A.-C., Cavelier, C., Récher, C., Mansat-De Mas, V., Delabesse, E., Danet-Desnoyers, G., and Carroll, M. (2011) Human acute myelogenous leukemia stem cells are rare and heterogeneous when assayed in NOD/SCID/IL2Rγc-deficient mice. *J Clin Invest 121*, 384–395.

[17] Taussig, D. C., Vargaftig, J., Miraki-Moud, F., Griessinger, E., Sharrock, K., Luke, T., Lillington, D., Oakervee, H., Cavenagh, J., Agrawal, S. G., Lister, T. A., Gribben, J. G., and Bonnet, D. (2010) Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood 115*, 1976–1984.

[18] Taussig, D. C., Miraki-Moud, F., Anjos-Afonso, F., Pearce, D. J., Allen, K., Ridler, C., Lillington, D., Oakervee, H., Cavenagh, J., Agrawal, S. G., Lister, T. A., Gribben, J. G., and Bonnet, D. (2008) Anti-CD38 antibody-mediated clearance of human repopulating cells masks the heterogeneity of leukemia-initiating cells. *Blood 112*, 568–575.

[19] Terpstra, W., Prins, A., Ploemacher, R. E., Wognum, B. W., Wagemaker, G., Löwenberg, B., and Wielenga, J. J. (1996) Long-term leukemia-initiating capacity of a CD34-subpopulation of acute myeloid leukemia. *Blood 87*, 2187–2194.

[20] Bosma, G. C., Custer, R. P., and Bosma, M. J. (1983) A severe combined immunodeficiency mutation in the mouse. *Nature 301*, 527–530.

[21] Shultz, L. D., Schweitzer, P. A., Christianson, S. W., Gott, B., Schweitzer, I. B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T. V., and Greiner, D. L. (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol 154*, 180–191.

[22] Glimm, H., Eisterer, W., Lee, K., Cashman, J., Holyoake, T. L., Nicolini, F., Shultz, L. D., von Kalle, C., and Eaves, C. J. (2001) Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID-beta2 microglobulin-null mice. *J Clin Invest 107*, 199–206.

[23] McKenzie, J. L., Gan, O. I., Doedens, M., and Dick, J. E. (2005) Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of CD122+ cells. *Blood 106*, 1259–1261.

[24] Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., Ueyama, Y., Koyanagi, Y., Sugamura, K., Tsuji, K., Heike, T., and Nakahata, T. (2002) NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood 100*, 3175–3182.

[25] Shultz, L. D., Lyons, B. L., Burzenski, L. M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S. D., King, M., Mangada, J., Greiner, D. L., and Handgretinger, R. (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol 174*, 6477–6489.

[26] Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J.-C., Lanzavecchia, A., and Manz, M. G. (2004) Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science 304*, 104–107.

[27] Shultz, L. D., Ishikawa, F., and Greiner, D. L. (2007) Humanized mice in translational biomedical research. *Nat Rev Immunol 7*, 118–130.

[28] Ide, K., Wang, H., Tahara, H., Liu, J., Wang, X., Asahara, T., Sykes, M., Yang, Y.-G., and Ohdan, H. (2007) Role for CD47-SIRPalpha signaling in xenograft rejection by macrophages. *Proc Natl Acad Sci U S A 104*, 5062–5066.

[29] Melkus, M. W., Estes, J. D., Padgett-Thomas, A., Gatlin, J., Denton, P. W., Othieno, F. A., Wege, A. K., Haase, A. T., and Garcia, J. V. (2006) Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat Med 12*, 1316–1322.

[30] McDermott, S. P., Eppert, K., Lechman, E. R., Doedens, M., and Dick, J. E. (2010) Comparison of human cord blood engraftment between immunocompromised mouse strains. *Blood 116*, 193–200.

[31] Vargaftig, J., Taussig, D. C., Griessinger, E., Anjos-Afonso, F., Lister, T. A., Cavenagh, J., Oakervee, H., Gribben, J., and Bonnet, D. (2012) Frequency of leukemic initiating cells does not depend on the xenotransplantation model used. *Leukemia 26*, 858–860.

[32] Bayry, J., Thirion, M., Misra, N., Thorenoor, N., Delignat, S., Lacroix-Desmazes, S., Bellon, B., Kaveri, S., and Kazatchkine, M. D. (2003) Mechanisms of action of intravenous immunoglobulin in autoimmune and inflammatory diseases. *Neurol Sci 24 Suppl 4*, S217–S221.

[33] Falini, B. et al. (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med 352*, 254–266.

[34] Martelli, M. P. et al. (2010) CD34+ cells from AML with mutated NPM1 harbor cytoplasmic mutated nucleophosmin and generate leukemia in immunocompromised mice. *Blood 116*, 3907–3922.

[35] Majeti, R., Chao, M. P., Alizadeh, A. A., Pang, W. W., Jaiswal, S., Gibbs, K. D., Jr, van Rooijen, N., and Weissman, I. L. (2009) CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell 138*, 286–299.

[36] Hope, K. J., Jin, L., and Dick, J. E. (2004) Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat Immunol 5*, 738–743.

[37] Barabé, F., Kennedy, J. A., Hope, K. J., and Dick, J. E. (2007) Modeling the initiation and progression of human acute leukemia in mice. *Science 316*, 600–604.

[38] Bagg, A. (2007) Lineage ambiguity, infidelity, and promiscuity in immunophenotypically complex acute leukemias: genetic and morphologic correlates. *Am J Clin Pathol 128*, 545–548.

[39] Pearce, D. J., Taussig, D., Zibara, K., Smith, L.-L., Ridler, C. M., Preudhomme, C., Young, B. D., Rohatiner, A. Z., Lister, T. A., and Bonnet, D. (2006) AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML. *Blood 107*, 1166–1173.

[40] Gibbs Jr., K. D., Jager, A., Crespo, O., Goltsev, Y., Trejo, A., Richard, C. E., and Nolan, G. P. (2012) Decoupling of tumor-initiating activity from stable immunophenotype in HoxA9-Meis1-driven AML. *Cell Stem Cell 10*, 210–217.

[41] Anderson, K., Lutz, C., van Delft, F. W., Bateman, C. M., Guo, Y., Colman, S. M., Kempski, H., Moorman, A. V., Titley, I., Swansbury, J., Kearney, L., Enver, T., and Greaves, M. (2011) Genetic variegation of clonal architecture and propagating cells in leukaemia. *Nature 469*, 356–361.

[42] Bystrykh, L. V., Verovskaya, E., Zwart, E., Broekhuis, M., and de Haan, G. (2012) Counting stem cells: methodological constraints. *Nat Methods 9*, 567–574.

[43] Quintana, E., Shackleton, M., Sabel, M. S., Fullen, D. R., Johnson, T. M., and Morrison, S. J. (2008) Efficient tumour formation by single human melanoma cells. *Nature 456*, 593–598.

[44] Notta, F., Doulatov, S., Laurenti, E., Poeppl, A., Jurisica, I., and Dick, J. E. (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science 333*, 218–221.

[45] Rombouts, W. J., Martens, A. C., and Ploemacher, R. E. (2000) Identification of variables determining the engraftment potential of human acute myeloid leukemia in the immunodeficient NOD/SCID human chimera model. *Leukemia 14*, 889–897.

[46] Kelly, P. N., Dakic, A., Adams, J. M., Nutt, S. L., and Strasser, A. (2007) Tumor growth need not be driven by rare cancer stem cells. *Science 317*, 337.

[47] Deneberg, S. (2012) Epigenetics in myeloid malignancies. *Methods Mol Biol 863*, 119–137.

[48] Heidel, F. H., Mar, B. G., and Armstrong, S. A. (2011) Self-renewal related signaling in myeloid leukemia stem cells. *Int J Hematol 94*, 109–117.

[49] Ding, L. et al. (2012) Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature 481*, 506–510.

[50] van Rhenen, A., van Dongen, G. A. M. S., Kelder, A., Rombouts, E. J., Feller, N., Moshaver, B., Stigter-van Walsum, M., Zweegman, S., Ossenkoppele, G. J., and Jan Schuurhuis, G. (2007) The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood 110*, 2659–2666.

[51] Dorantes-Acosta, E., and Pelayo, R. (2012) Lineage Switching in Acute Leukemias: A Consequence of Stem Cell Plasticity? *Bone Marrow Res 2012*, 406796.

[52] Navin, N., Kendall, J., Troge, J., Andrews, P., Rodgers, L., McIndoo, J., Cook, K., Stepansky, A., Levy, D., Esposito, D., Muthuswamy, L., Krasnitz, A., McCombie, W. R., Hicks, J., and Wigler, M. (2011) Tumour evolution inferred by single-cell sequencing. *Nature 472*, 90–94.

[53] Mony, U., Jawad, M., Seedhouse, C., Russell, N., and Pallis, M. (2008) Resistance to FLT3 inhibition in an in vitro model of primary AML cells with a stem cell phenotype in a defined microenvironment. *Leukemia 22*, 1395–1401.

[54] Chan, W.-I., and Huntly, B. J. P. (2008) Leukemia stem cells in acute myeloid leukemia. *Semin Oncol 35*, 326–335.

[55] Calimeri, T. et al. (2011) A unique three-dimensional SCID-polymeric scaffold (SCID-synth-hu) model for in vivo expansion of human primary multiple myeloma cells. *Leukemia 25*, 707–711.

[56] Groen, R. W. J. et al. (2012) Reconstructing the human hematopoietic niche in immune deficient mice, opportunities for studying primary multiple myeloma. *Blood*

[57] Chen, Y., Jacamo, R., Shi, Y.-X., Wang, R.-Y., Battula, V. L., Konoplev, S., Strunk, D., Hofmann, N. A., Reinisch, A., Konopleva, M., and Andreeff, M. (2012) Human extramedullary bone marrow in mice: a novel in vivo model of genetically controlled hematopoietic microenvironment. *Blood 119*, 4971–4980.

[58] Ishikawa, F. et al. (2007) Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol 25*, 1315–1321.