The biology of human hematopoietic stem and progenitor cells in acute myeloid leukemia, aging and autologous transplantation

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CHAPTER 1

General introduction

The hematopoietic system

General introduction to the hematopoietic system

The adult bone marrow is capable of replenishing all mature blood cell types during the complete lifespan of an individual and is therefore a unique and intriguing tissue. Because of the short life span of most mature blood cells, the hematopoietic cell production is an ever ongoing process with an estimated output of 1.5 million blood cells every second.[1;2] The hematopoietic system, responsible for this process, is considered a hierarchical system with hematopoietic stem cells (HSCs) on top of the hierarchy. The experimental concept of HSCs can be traced back to the early fifties of the last century, when it was observed that the injection of spleen or bone marrow cells could prevent mice from the lethal effects of irradiation-induced bone marrow failure.[3;4] In 1961 Till and McCulloch reported their breakthrough experiments indicating the existence of cells in the marrow that could give rise to mixed myeloerythroid progeny and the ability of some of these cells to make more of themselves, i.e. to self-renew.[5] Since these first experiments that suggested the presence of multipotent HSCs, the field of HSC research has made major progress in identifying, characterizing and isolating HSCs and new insights in HSC biology are reported frequently.

The hematopoietic hierarchy

HSCs are defined as single cells with the lifelong ability to self-renew as well as to generate progeny that can differentiate in order to produce all blood cell lineages.[6;7] It is becoming more and more clear, at least for the mouse system, that HSCs can be subdivided into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs).[6;8] LT-HSCs are considered the slowest cycling HSCs and in a steady state adult hematopoietic system they reside in a quiescent cell cycle state. Maintenance of quiescence is crucial for HSC preservation and ensures the lifelong replenishment of mature blood cells.[9-11] ST-HSCs are as multipotent as LT-HSCs and are predominantly quiescent in steady-state conditions, but will cycle rarely yet consistently in order to maintain hematopoiesis and will be triggered more easily by injury or loss when enhanced blood cell production is needed. Being active contributors to steady-state hematopoiesis, ST-HSCs will ultimately exhaust and be replenished by LT-HSCs. Recent studies have shown that within the HSC compartment different cell subtypes can be distinguished. Based on a measurement of the predominant lineages within their total output HSC subsets were characterized as myeloid-biased, lymphoid-biased or balanced.[12;13] Other studies distinguished HSC subtypes as lymphoid-deficient (α), balanced (β), or myeloid-deficient (γ and δ). Both α and β HSCs are found to contribute similarly to the circulating pool of myeloid cells.[14] Serial transplantation experiments have shown that only α and β HSCs can be serially transplanted, and their unique differentiation behaviors are usually maintained over periods of years in primary transplant recipients.[14;15] In addition, by using a genetic model in which a tetracycline responsive fusion protein histone 2B green fluorescent protein (H2B-GFP) was used to pulse label hematopoietic

stem and progenitor cells, evidence was provided for the existence of two HSC populations with distinct division kinetics, namely a dormant subset and a more activated population.[16]

Following the branches of the hematopoietic tree, the immediate progeny of HSCs are multipotent progenitors (MPPs) that retain full lineage potential but have a very limited self-renewal capacity and are mainly cycling.[17] MPPs are followed in hierarchy by committed progenitors which are simply named after their lineage commitment, i.e. the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP).[18;19] The latter will further differentiate in the megakaryocyte erythrocyte progenitor (MEP) and granulocyte macrophage progenitor (GMP).[18;20;21] At the end of the hierarchy, the mature effector blood components are preceded by their lineage restricted precursors (Figure 1). Of note, recent studies conducted in the murine system suggest that MPPs initially differentiate into lymphoid-primed multipotent progenitors (LMPPs) with lymphoid and granulocyte macrophage but no megakaryocyte erythrocyte potential and a separate MEP population.[22-25]

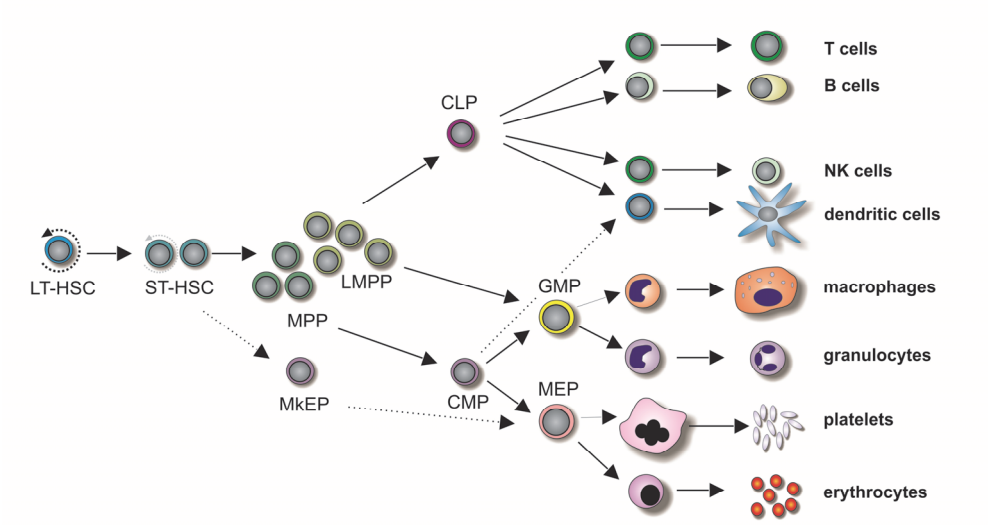

Figure 1 Schematic representation of the hematopoietic hierarchy

Abbreviations: LT-HSC: long-term hematopoietic stem cell, ST-HSC: short-term hematopoietic stem cell, MPP: multipotent progenitor, LMPP: lymphoid-primed multipotent progenitor, MkEP: early megakaryocyte erythrocyte progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, GMP: granulocyte macrophage progenitor, and MEP: megakaryocyte erythrocyte progenitor.

Identification of distinct hematopoietic populations by cell surface markers

Since studies in the human hematopoietic system are hampered by both ethical and experimental issues, most of our knowledge comes from the murine system. Although major progress has been made, the identification of the human counterparts of the murine hematopoietic stem and progenitor cells is still lagging behind and is subject to continuing discussion and investigation.[26] Already early on in the search for human HSCs, the cell surface protein CD34 was identified as an important marker for immature hematopoietic cells. CD34-enriched populations were demonstrated to be able to form colonies *in vitro* and reconstitute the hematopoietic system upon *in vivo* transplantation.[27-30] With the development of severe immunocompromised mice (NOD-SCID and SCID-humanized mice) a murine model became available for the analysis of human HSCs, making further characterization and purification possible.[31;32] To date, human HSCs are thought to reside in the $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ compartment, which give rise to $Lin^-CD34^+CD38^-CD90^-CD45RA^-$ MPPs.[33;34] However, the complexity of the human HSC compartment and its identification was evidenced by the demonstration of multipotent SCID repopulating cells negative for CD34 expression.[35] Moreover, recent studies identified CD49f (ITGA6) as a specific HSC marker in human cord blood.[36] In the same study HSCs negative for CD90 expression were identified. Human hematopoietic progenitors have also been characterized. The more committed $Lin^-CD34^+CD38^+$ can be separated into the myeloid branched CMP, GMP and MEP based on differential expression of CD123 (IL3RA), CD110 and CD45RA.[37;38] Less straight-forward is the characterization of the human CLP. Whereas marker definitions of the myeloid progenitors are consistent in bone marrow and cord blood, different marker expression profiles were shown for lymphoid progenitors. For bone marrow a $Lin^-CD34^+CD38^+CD10^+CD45RA^+$ CLP was identified,[39] while the human cord blood CLP was characterized as $CD34^+CD38^-CD7^+$, a population not existing in adult bone marrow.[40;41] To add to the complexity, the CD10 expression of the bone marrow CLP is still debated and an IL7RA expressing bone marrow CLP has been suggested.[7] In general, the transition and lineage restriction steps between early $CD34^+CD38^-$ human HSCs, MPPs and their more committed $CD34^+CD38^+$ progeny are poorly defined and a human LMPP population has not yet been identified.

The hematopoietic stem cell niche

HSCs do not function alone, but are highly dependent on signals from surrounding cells that are found within a specialized micro-environment within the bone marrow. This micro-environment is termed the HSC niche. For a long time, niches were a theoretical concept strongly supported by the observation that transplanted HSCs survive and grow only in particular tissue conditions. In recent years progress has been made in our understanding of cellular components that comprise the niche and mechanisms that underlie the interaction between hematopoietic stem and progenitor cells and their niche.[42-44] The HSC niche is thought to consist of osteolineage cells, sinusoidal endothelial cells, mesenchymal stromal and stem cells, sympathetic neurons, and the extracellular matrix. HSCs were

originally detected in the endosteal regions of the bone marrow[42;45] and recent findings have suggested the existence of distinct perivascular niches.[46-49] Most probably, both endosteal and vascular or perivascular niches contribute to HSC function. Continuing experiments will further expand our knowledge of HSC niches and their relevance in the hematopoietic system. Moreover, a potential role for the niche in disease pathogenesis is an intriguing concept that deserves further research.

Malignant hematopoiesis and acute myeloid leukemia

Malignant hematopoiesis

To ensure adequate blood cell production, the hematopoietic system is strictly regulated by both cell intrinsic as well as extrinsic factors.[50-53] As with cancers of other tissues, malignant hematopoietic disorders are characterized by a disturbance of the normal hematopoietic biology and of these disorders leukemia is probably one of the most dramatic examples. In general, leukemia can be characterized by uncontrolled proliferation and a block in maturation. Immature hematopoietic cells, also known as blasts, lose the ability to differentiate normally and to respond to normal cell regulating mechanisms. The leukemic blasts can find their origin at any early stage in the hematopoietic hierarchy. Leukemia can be subdivided both clinically and pathologically in four major categories combining two broad classifications. One classification stratifies leukemias according to lineage into either myeloid or lymphoid.[54;55] The other classification tends to indicate the level of disease progression and distinguishes acute from chronic leukemia. This thesis focuses on acute myeloid leukemia, which will be discussed in further detail.

Acute myeloid leukemia

Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells in the marrow and an arrest in their maturation. The condition is frequently resulting in hematopoietic insufficiency, i.e. granulocytopenia, thrombocytopenia and anemia, and can present with or without leukocytosis.[54;56] Most of the clinical symptoms can be directly related to the pancytopenia and patients typically present with fatigue, abnormal bleeding, frequent and unusual infections and fever. AML progresses rapidly and is typically fatal within months or even weeks if left untreated.

Table 1 French-American-British classification of acute myeloid leukemia

Type	Name
M0	Myeloid leukemia with minimal differentiation
M1	Myeloid leukemia without maturation
M2	Myeloid leukemia with maturation
M3	Promyelocytic leukemia
M4	Myelomonocytic leukemia
M4eo	Myelomonocytic leukemia with abnormal eosinophils
M5	Monocytic leukemia
M6	Erythroid leukemia
M7	Megakaryocytic leukemia

Classification of acute myeloid leukemias

AML is a very heterogeneous disease, both clinically as well as biologically.[54;56] For decades, the French-American-British (FAB) classification was used to classify AML according to the morphological appearance of the blasts and their immunohistochemical features (Table 1).[57] However, with the discovery of a number of recurring genetic alterations that could be associated with defects in maturation and proliferation in AML subtypes, the traditional FAB classification became less adequate. Importantly, the discovered genetic abnormalities could predict clinical behavior and outcome better than morphology alone. In 2001 a more clinically useful classification system was launched by the World Health Organization (WHO). The WHO classification attempts to incorporate those disease characteristics that have proved to have clinical and biologic relevance into a useful, working nomenclature.[58] Inherent to this ambition is the necessitation to revise the classification based on new findings in the rapidly evolving scientific field of AML. Indeed, recently a revised WHO classification was published incorporating newly identified prognostic factors.[59] For the first time, AML subgroups with a specific gene mutation have been included as provisional entities, i.e. AML with a mutation in CCAAT/enhancer binding protein alpha (CEBPA) and AML with mutated nucleophosmin 1 (NPM1). Table 2 summarizes the latest WHO classification for AML (2008).

Table 2 WHO classification of acute myeloid leukemia and related neoplasms

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
APL with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

Prognostic factors in acute myeloid leukemia

Clinical heterogeneity in AML implies the need for prognostic markers that could help doctors and their patients choose the best suitable therapy. While highly toxic therapies including allogeneic stem cell transplantation might be the only chance of survival for patients diagnosed with a poor prognostic AML subtype, therapy related morbidity and mortality risks should not outweigh disease related mortality risk in good prognostic patients. An accumulating number of studies have shown that karyotype and mutation status of certain genes provide important prognostic information. Among the disease related prognostic factors, pre-treatment cytogenetics remain the most important and patients can be divided in three major prognostic categories based on their karyotype. Patients with acute promyelocytic leukemia (APL) with the t(15;17)(q22;q12-21) and those with the core binding factor (CBF) leukemias with t(8;21)(q22;q22) or inv(16)(p13;q22)/t(16;16)(p13;q22) are characterized by a relative favorable prognosis and form the favorable risk group.[60-63] In contrast, patients diagnosed with abnormalities of 3q, deletions of 5q or 7q, monosomies of chromosome 5 and/or 7, abnormalities of 11q23, t(6;9), t(9;22) or a complex karyotype (i.e. three or more unrelated chromosomal abnormalities) have a poor prognosis.[60-62] An important recent observation is the existence of a subgroup of patients within the poor risk group with an extremely poor outcome, characterized by the presence of an autosomal monosomy in conjunction with at least one other autosomal monosomy or structural abnormality (monosomal karyotype).[64] The intermediate risk group includes all patients with a normal karyotype AML and those who do not fit in the favorable or poor prognostic categories. The intermediate risk subgroup represents the largest (approximately 45%) and most heterogeneous group of patients. In the last decades, there have been great efforts in identifying molecular abnormalities that have significant prognostic impact and can help further classify patients in the intermediate risk group. The most prognostically important and prevalent gene mutations in AML will be discussed briefly. Mutations in FMS-like tyrosine kinase 3 (FLT3) are among the most common genetic changes in AML.[65] FLT3 is a member of the class III receptor tyrosine kinase family and plays a role in proliferation, survival and differentiation of hematopoietic progenitor cells. Mutations in FLT3 come in two forms of which the most common are internal tandem duplications (ITD). Less frequently mutations affect the tyrosine kinase domain (TKD). Several studies have shown that prognosis of AML with FLT3-ITD is significantly worse compared with AML without the mutation, but the allelic burden seems of importance.[66-69] The prognostic relevance of the FLT3-TKD mutation has remained controversial.[70;71] Mutation of NPM1 is the most frequently found molecular abnormality in AML. The presence of an NPM1 mutation has major prognostic implications, especially in the absence of FLT3-ITD. Part of this thesis focuses on NPM1 mutated AML and this topic will therefore be discussed in more detail in the next paragraph. Like NPM1-mutated AML, AML with a mutation in CEBPA has been included as a provisional entity in the WHO classification system. CEBPA encodes for CCAAT/enhancer binding protein α, a granulocytic differentiation factor important in the regulation of myeloid progenitors.[72;73] CEBPA mutations are particularly frequent in

normal karyotype AML.[74] Importantly, CEBPA mutations can be divided in two subgroups, namely mono-allelic and bi-allelic mutations with distinct impact on prognosis, presence of concurring gene mutations and gene expression.[75;76] Currently, only CEBPA bi-allelic mutations are associated with favorable outcome and as a consequence, not all CEBPA mutated AML but only AML with a CEBPA bi-allelic mutation should be considered a distinct prognostic category.[77] With the application of whole genome sequencing technologies, a number of novel gene mutations have been discovered. Mutations in the isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2) genes have been identified as recurrent molecular abnormalities in AML and result in a substrate shift to α-ketoglurate (α-KG) with accumulation of 2-hydroxyglutarate (2-HG) that is a putative oncogenic metabolite.[78-80] Recently it was suggested that 2-HG can lead to DNA methylation alterations.[81] IDH1 mutations were reported to be an adverse prognostic factor in cytogenetically normal AML, although their prognostic impact in molecular subgroups based on NPM1, FLT3 and CEBPA mutations has been controversial.[78;79;82;83] The prognostic significance of IDH2 mutations is also still under discussion. Mutations in DNMT3A, encoding for a methyltransferase catalyzing the addition of a methyl group to the cytosine residue of CpG dinucleotides, were also discovered by next-generation sequencing techniques.[84;85] Their presence has been associated with an unfavorable prognosis.[86] Recently, mutations in the TET oncogene family member 2 (TET2) were observed in patients with AML and in other myeloid disorders.[87] TET2 was identified as a candidate tumor suppressor gene and the TET2 proteins are involved in epigenetic regulation. Like with the other novel gene mutations, the prognostic impact of TET2 mutations is still under debate.

To summarize, the prognostic classification of normal karyotype AML patients is rapidly progressing. Discoveries of disease related prognostic markers have led to new disease entities and revision of traditional classification systems within a decade. Testing for FLT3, NPM1 and CEBPA is now common in clinical practice and influences treatment decisions. Nonetheless, newly identified prognostic markers need to be carefully examined in independent and large cohorts of patients in order to evaluate their value as an independent prognostic factor.

Nucleophosmin in acute myeloid leukemia

Nucleophosmin as a multifunctional protein

Nucleophosmin is a highly expressed multifunctional phosphoprotein that mainly resides in the nucleoli of the cell. The gene encoding for this protein (NPM1) is mapping to chromosome 5q35 and contains twelve exons. Nucleophosmin is a member of the nucleoplasmin family of proteins and acts as a nuclear chaperone protein. Various functional domains are identified through which nucleophosmin can bind to many partner proteins, thereby participating in important cellular processes. Nucleophosmin is mainly localized in the nucleoli, but it can shuttle between the nucleus and cytoplasm. This shuttling activity plays an important role in ribosome biogenesis, since

nucleophosmin takes part in the transport of pre-ribosomal particles.[88] In addition to a nuclear localization signal (NLS), nucleophosmin also has a nuclear export signal (NES). With this NES nucleophosmin can provide the necessary export signal required to transport components of the ribosome to the cytosol. By direct interaction with the ribosomal L5 protein (rpL5), an integral component of the 60S ribosomal subunit containing 5S rRNA, nucleophosmin contributes to the nuclear export of rpL5-5S rRNA complexes. In the cytoplasm nucleophosmin was shown to be present in the ribosomal subunits 40S, 60S, 80S, and polysomes, implying a role in the ribosome maturation in the cytosol and also in the process of translation.[89] Involvement of nucleophosmin in the apoptotic response to stress and oncogenic stimuli is revealed by its ability to modulate ARF and p53. Nucleophosmin associates with ARF in the nucleolus and stabilizes ARF by retarding its turnover.[90] Since NPM has a role in ribosome transport, it has been suggested that NPM facilitates the interaction between ARF and the ribosomal machinery. Besides its activity in ARF pathways, nucleophosmin has also been shown to interact directly with the tumor suppressor p53 and increase its stability and transcriptional activation.[91] Although more research is needed to reveal the precise interaction between NPM and ARF, and NPM and p53, it seems that nucleophosmin plays an important role in modulating ARF and p53 and thereby regulating the oncogenic stress response.[88;92] Further contributing to the idea of nucleophosmin as an important protein in the balance between normal function and possible oncogenic transformation is its role in the maintenance of genomic stability. Functional studies show that nucleophosmin can act as a substrate of Ran-Crm1, a complex involved in regulating centrosome duplication.[93] For the role of nucleophosmin in centrosome duplication a model has been suggested in which nucleophosmin is located in the nucleolus during cell progression from S to G2 phase. Then, at the beginning of mitosis, the nucleolar membrane breaks down and nucleophosmin is thought to associate with the mitotic centrosomes, thereby ensuring the coordination of centrosome and DNA duplication, and restricting centrosome duplication to occur once within a cell cycle.[93;94] In addition to the above described functions of nucleophosmin, research in *Npm1* knockout and *Npm1* heterozygous mice demonstrated an essential role of nucleophosmin in embryonic development. Embryos of *Npm1* knockout mice show deficient anterior brain organogenesis. Interestingly, these embryos show distinct defects in primitive hematopoiesis and die at mid-gestational age. Inactivation of the *Npm1* gene leads to unrestricted centrosome duplication and genomic instability.[95] In summary, the diversity of cellular activities makes nucleophosmin a potential tumor suppressor protein. However, by alternating the NPM1 gene or disturbing the important influencing factors it can become an oncogenic factor.[88;95] The NPM1 gene can therefore function both as an oncogene and as a tumor suppressor, depending on gene dosage, expression levels, interacting partners and cellular localization.

NPM1 mutations in acute myeloid leukemia

In 2005 Falini and his colleagues discovered that approximately one third of all cases of AML harbor a heterozygous mutation in the NPM1 gene, leading to aberrant cytoplasmic localization of nucleophosmin in the leukemic blasts.[96] In normal karyotype AML the incidence of NPM1 mutations is even higher and with a frequency of approximately 60% in this subgroup, NPM1 mutations are the most frequently found mutations in AML.[96-100] Currently up to 50 different mutations in the NPM1 gene have been identified.[101;102] The great majority of these mutations involve exon 12, although rare mutations in exon 9 and 11 have been identified.[103-105] All NPM1 mutations result in a shift of the reading frame leading to common changes at the very end of the NPM1 protein C-terminus. The changes induced by the mutation lead to the generation of a new NES which reinforces the Crm1-dependent nuclear export of nucleophosmin protein and loss of tryptophan residues 288 and 290 (or residue 290 alone) which cause the three-helix structure of the nucleophosmin C-terminal domain to unfold, thus preventing or decreasing binding to the nucleolus.[106] The aberrant cytoplasmic localization of nucleophosmin in immunohistochemical stainings led to the discovery of the mutation and is still used for diagnostic purposes. Indeed, immunohistochemistry was described to be fully predictive for the detection of NPM1 mutations in AML and as a consequence NPM1 mutated AML is often indicated by the term NPMc$^+$ AML.[96;107-109] However, there has been controversy about the sensitivity of immunohistochemistry for the detection of NPM1 mutations.[110] This will be further discussed in Chapter 4 of this thesis.

NPM1-mutated AML has been recognized as a provisional entity in the WHO classification of AML. Indeed, distinct biological and clinical features can be observed in NPM1-mutated AML. Presenting clinical features associated with NPM1 mutated AML are the predominance of female sex and a high bone marrow blast percentage and peripheral blood white blood cell count.[96;101] NPM1-mutated AML typically presents with high CD33 but very low CD34 expression and multilineage involvement is often observed.[96;111;112] Moreover, a specific gene expression signature, characterized by high expression of several HOX genes, and distinct microRNA profile were observed.[100;113;114] NPM1 mutations have been shown to be associated with FLT3-ITD and more recently also with IDH and DNMT3A mutations.[78;79;83;84;86] The prognostic value of NPM1 mutations has been well recognized. The presence of an NPM1 mutation is associated with a good response to induction chemotherapy.[96;98;99] Several studies in normal karyotype AML have consistently shown a favorable prognosis for patients with an NPM1 mutation in the absence of a FLT3-ITD.[98-100;115;116] In patients younger than 60 years, outcome is even similar to those patients with CBF leukemias.[116] After conventional induction and consolidation chemotherapy, younger adult patients with mutated NPM1 without FLT3-ITD have an overall five year survival probability of approximately 60%. Recently it was shown that the favorable prognostic impact of NPM1 mutations also applies to older patients.[117-120]

Leukemic stem cells

The leukemic stem cell concept

Of all AML patients that are eligible for intensive treatment, the great majority achieves complete remission upon induction therapy. In approximately 70-80% of all patients under 60 years of age, conventional chemotherapy will initially result in normal bone marrow morphology, undetectable levels of cytogenetic abnormalities and bone marrow blast counts of less than 5%.[119;121;122] However, the disease typically relapses within months or years after initial diagnosis and overall survival rates do not exceed 40% at five year. It has therefore been postulated that, although current treatment regimens might be capable of eliminating the tumor burden, there must be a rare subset of malignant cells that are not effectively eliminated. Indeed, as is true for the normal hematopoietic system, it has been recognized that AML comprises of a heterogeneous group of cells with distinct differentiation status and proliferation potential.[123;124] These observations have ultimately led to the current model in which AML is organized as a hierarchical system with the so called leukemic stem cells (LSCs) on top of the leukemic hierarchy. LSCs share characteristics with normal hematopoetic stem cells and are defined by their ability to proliferate and renew themselves *in vivo* while at the same time generating clonogenic and more differentiated leukemic progeny. Historically characterized by their capability of transferring leukemia to severe combined immunodeficient (SCID) mice, the terms SCID leukemia-initiating cell (SL-IC) or simply leukemia-initiating cell (LIC) are also often used as synonyms for LSC. The initial evidence for the hierarchy model in AML was provided by landmark studies of the John Dick laboratory.[125;126] Since then, the LSC concept in AML has been subject to intensive investigation and will also be further discussed in this thesis.

Acute myeloid leukemia and hematopoietic aging

Acute myeloid leukemia at older age

AML can present at all ages, but is mainly a disease of the elderly. The incidence of AML increases steeply with age (Figure 2) and approximately 75% of all patients is aged 60 years or more.[127;128] The prognosis of patients with AML is generally considered poor, but is worse in elderly patients. Approximately 70-80% of patients younger than 60 years of age will achieve complete remission[121;122] and overall survival rates are 40-45% at five years after diagnosis. Among patients aged above 60 years with a good performance status complete remission will be achieved in 40-50%. However, cure rates in these elderly patients are less than 10% and median survival time is less than one year.[119;128] Prognosis for older patients with unfavorable cytogenetics or poor performance status is even worse. Cure rates for elderly AML patients have not significantly improved with improvements in supportive care and poor prognosis of these patients can only be partly related to diminished performance status or co-morbidity in older individuals. Other important factors should therefore be involved. Indeed,

several observations strongly suggest that the biological nature of AML changes as the age of the patient increases. Compared to AML in younger patients, AML in older patients is more often preceded by a hematological disorder like myelodysplasia,[129] is less proliferative and presents with lower white blood cell counts and lower percentages of marrow blasts.[130] Moreover, the spectrum of cytogenetic abnormalities changes with age and is characterized by a much higher incidence of abnormalities involving chromosomes 5 and 7, and a lower incidence of the translocations associated with favorable treatment outcomes in AML of the elderly.[129-132] Contributing to the difficulties of treating AML at older age might also be the observation that AML of older age more commonly expresses proteins involved in multidrug resistance.[133;134] Recently, we showed that AML of the elderly exhibits a distinct gene expression profile compared to AML of younger patients, including decreased expression of $p16^{INK4a}$.[135] Based on these different clinical and biological characteristics AML of older age is increasingly considered as a separate entity.

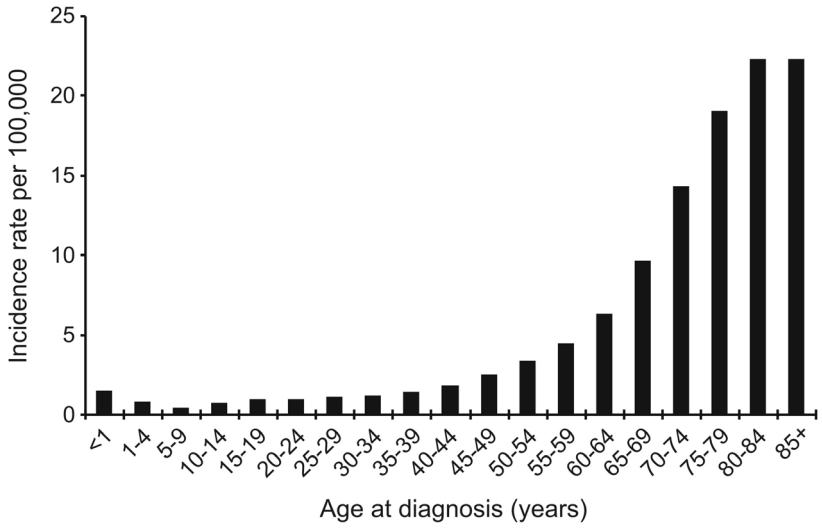

Figure 2 Age-specific incidence of acute myeloid leukemia
Data include all races and both sexes and have been derived from the National Cancer Institute (Surveillance Epidemiology and End Results, 2005-2009, http://seer.cancer.gov/).

Normal hematopoietic aging in relation to malignant transformation

It has been postulated that the phenotype of the leukemic clone(s) might be a reflection of the cell in which (epi)genetic lesions first arose. In this perspective it could be hypothesized that the distinct characteristics of AML at older age are a reflection of age-related changes in normal hematopoietic stem and progenitor cells. Moreover, it has been hypothesized that the aging process is largely the consequence of accumulated genetic damage to stem cells. Therefore, older hematopoietic stem cells might be more vulnerable to malignant transformation. In order to get insight in the impact of age on the development of AML, a thorough understanding of the aging process of the normal hematopoietic system is required. In Chapter 5 we review the effects of aging on normal hematopoietic stem and progenitor cells and speculate how the observations in murine models might be of clinical relevance.

Stem cell transplantation

Hematopoietic stem cell transplantation

HSCs are an appropriate source for stem cell transplantation since they can both self-renew as well as produce progeny that can differentiate into mature cells of all hematopoietic lineages. After the discovery of the regenerative effects of transplanted bone marrow in irradiated animals,[4;136] one of the first human bone marrow transplantations was performed between identical twins in 1959.[137] With the identification and typing of human leukocyte antigen (HLA), the human variant of the major histocompatibility complex,[138] allogeneic transplantation became feasible. In the seventies it was shown that some patients with end stage leukemia could be cured by transplanting marrow from their HLA-identical siblings after ablating the patient's marrow with total body irradiation combined with cyclophosphamide.[139;140] Nowadays, hematopoietic stem cell transplantation is a widely used treatment modality for a variety of hematologic malignancies and can be lifesaving. It is also increasingly used in the treatment of patients with defined congenital disorders of the hematopoietic system or severe systemic auto-immune diseases.

Mobilization of stem cells into the peripheral blood

Historically, bone marrow stem cells for transplantation were obtained by repeated aspiration of the posterior iliac crests while the donor is under general or local anesthesia. This procedure is associated with a very low risk of serious complications and is therefore generally considered save for healthy donors as well as patients.[141-143] Nevertheless, there has been an interest in the use of peripheral blood as a source for hematopoietic stem cells for a long time. Already in the early sixties, the presence of stem cells in the circulation was experimentally proven by transplantation experiments using whole blood or leukocytes in lethally irradiated recipients.[144-146] Under normal physiological conditions, undifferentiated stem cells are maintained within the bone marrow

throughout life. Only a small pool of stem cells within the bone marrow continuously produces high levels of immature and maturing myeloid and lymphoid blood cells with a limited lifespan, which are released into the circulation. However, very low levels of noncycling quiescent progenitor cells, including primitive stem cells, are also released into the peripheral blood.[147;148] Still, the physiological numbers of circulating stem cells are too low to collect enough stem cells for transplantation within a reasonable period of time. With the observations of increased numbers of circulating stem cells in patients presenting with chronic myeloid leukemia[149;150] and high numbers of circulating stem cells as a rebound phenomenon after intensive chemotherapy in patients with lymphoma or solid tumors,[151] peripheral blood became an available stem cell source. Since then important steps have been made with the discovery of many hematopoietic cytokines that induce the release of hematopoietic stem cells into the peripheral blood. This process of mobilization can be initiated by a wide number of molecules, including cytokines such as granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 7 (IL 7), stem cell factor, flt-3 ligand, chemokines like IL 8 or SDF-1, and chemotherapeutic agents including cyclophosphamide.[152] In current clinical practice, a variety of mobilization strategies are used, including growth factors alone or in combination with chemotherapy. Of the available growth factors, the most commonly used is the bacterially synthesized recombinant G-CSF analog filgrastim, which was shown to be well-tolerated, save and highly effective in mobilizing stem cells in most patients and healthy donors.[153-156] Whereas the molecular cloning and clinical development of G-CSF initially focused on the treatment of chemotherapy induced cytopenia,[157-161] it soon became clear that G-CSF also expanded the circulating hematopoietic stem cell pool by mobilizing $CD34^+$ hematopoietic stem and progenitor cells into the peripheral blood. More recently, the CXC chemokine receptor 4 (CXCR4) agonist plerixafor (AMD3100) was introduced as a mobilizing agent.[162;163] A synergistic effect of G-CSF and plerixafor on the mobilization of $CD34^+$ cells was shown.[164] In poor mobilizers, the combination of G-CSF and plerixafor has become the standard treatment regimen.

With the application of mobilized peripheral blood stem cells (PBSCs) a more convenient and less painful collection of stem cells for transplantation was introduced in which there is no need for anesthesia and a lower risk of bleeding, infection and nerve damage.[165;166] Not only are mobilized PBSCs a save alternative for bone marrow as a source for transplantation, filgrastim-stimulated peripheral blood also has a much higher content of blood progenitor cells than bone marrow. Importantly, time to neutrophil and platelet reconstitution is uniformly reported to be shorter with PBSCs than bone marrow as stem cell source.[165] With the documentation of this rapid recovery together with a lower need for transfusions and antibiotics and a shorter hospital stay, an almost complete shift from bone marrow to peripheral blood as stem cell source for autologous stem cell transplantation was observed between 1992 and 1996.[167] However, in allogeneic transplantation the use of PBSCs increases the incidence and prolongs the treatment of chronic graft versus host disease (GVHD), since more T cells are present in the PBSC graft compared to bone marrow.[168;169]

Therefore, the advantages and disadvantages of peripheral blood compared to bone marrow are less clear in the allogeneic setting. Although PBSCs are used in more than 70% of all allogeneic stem cell transplantations at the present time,[170] concerns about the relative benefits of this source of stem cells compared to bone marrow are still legitimate. It is clear that engraftment is more rapid with PBSCs than bone marrow and that this can be associated with a reduced transplant-related mortality. Chronic GVHD can be expected to be 15-20% higher with PBSCs than bone marrow, but is associated with significant antileukemic effects, which in turn provides a major survival benefit to patients with more advanced hematologic malignancies. It is therefore suggested that patients with less-advanced hematologic malignancies might not benefit from the use of PBSCs.[171;172] Possibly, hematopoietic cells collected from bone marrow after donor treatment with growth factors may facilitate engraftment without increasing the risk of GVHD.[173] Alternatively, the development of transplantation approaches that decrease the risk of acute and extensive chronic GVHD when either stem cell source is used can be of great benefit in the future.

Biology of stem cell mobilization

Although hematopoietic stem cell mobilization has been in clinical use for many years now, the biological mechanisms underlying the egression of stem cells out of the bone marrow into the peripheral blood are not yet fully understood. The homing and retention of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow are controlled by adhesive interactions between these cells and the bone marrow micro-environment. An important mechanism in this process is the interaction between vascular cell adhesion molecule 1 (VCAM-1) which is expressed by bone marrow stromal cells, and its counter-receptor integrin α4β1 or very late antigen 4 (VLA-4) expressed at the surface of HSPCs.[174-176] Homozygous targeted deletion of the integrin α4 gene results in decreased hematopoiesis in mouse embryos and decreased homing of myeloid and B lymphoid precursors in the spleen and bone marrow.[174;177] In adult mice, pretreatment of HSPCs with blocking anti-VLA-4 antibodies results in a profound reduction of donor HSPCs homing to the bone marrow of lethally irradiated recipients.[178] Moreover, administration of VLA-4 or VCAM-1 blocking antibodies induces mobilization in animal models, indicating important roles for these two molecules in this process.[178;179] It was also demonstrated that increased release of the proteolytic enzymes elastase and cathepsin G from murine bone marrow neutrophils during cyclophosphamide and G-CSF (and IL-8) mobilization correlated with stem cell egression. These enzymes cleave VCAM-1 from bone marrow stromal cells, preventing VLA-4 antigens expressed on the surface of hematopoietic progenitors to bind to their ligand VCAM-1 expressed by stromal cells.[180;181] However, the role of neutrophils and proteases in mobilization remains controversial since more recent studies demonstrate that mice lacking these proteases do not show impaired mobilization.[182-184] Clearly, also other mechanisms are involved.

Considerable evidence strongly suggests that the dominant mechanism by which G-CSF induces HSPC mobilization is through suppression of the CXCL12/CXCR4 axis.[185] The chemokine CXCL12 is

also known as stromal-derived growth factor-1 (SDF-1) and has a crucial role in regulating HSPC trafficking, homing and maintenance.[186-188] In mice lacking CXCL12[189] or CXCR4[190] there is a failure of the migration of HSPC from the fetal liver to the bone marrow, and $CXCR4^{-/-}$ bone marrow chimeras exhibit constitutive mobilization.[191] Treatment of humans or mice with a CXCR4 antagonist results in rapid HSPC mobilization.[162;192]

In summary, three main processes have been implicated in G-CSF mediated mobilization, i.e. activation of proteases, inhibition of adhesion molecules and attenuation of CXCR4/CXCL12 signaling. A recent study investigated the importance of the individual mobilization processes by attempting to mobilize $CXCR4^{-/-}$ bone marrow chimeras with G-CSF and other agents.[193] These mice failed to mobilize in response to G-CSF. Interestingly, the levels of activated metalloproteinases in their bone marrow were upregulated at levels similar to wild type controls, indicating that proteolytic activity is not an independent contributor to HSPC mobilization. In contrast, these chimeric animals successfully mobilized in response to a novel small molecule antagonist to the VCAM-1/VLA-4 niche interaction termed BIO5192.[194] This indicates that disruption of HSPC adhesion by antagonizing VCAM-1/VLA-4 signaling is both independent of CXCR4/CXCL12 signaling and essential for the process of HSPC mobilization.

Importantly, the above described mechanisms cover only part of the story and a number of additional factors are involved in the process of mobilization. Besides the involvement of other molecular pathways and interactions, there are data suggesting the involvement of the nervous system via peripheral β2-adrenergic signals[195] and of a circadian rhythm[196;197]. The relevance of these proposed mechanisms still has to be elucidated. Moreover, continued investigation of the molecular mechanisms underlying the action of G-CSF and other HSPC mobilizing agents may reveal new insights and define novel molecular targets that can be used to further develop the quality of HSPC collection for hematopoietic transplantation.

Clinical applications of autologous stem cell transplantation

More than twenty-five years ago, it was shown that high dose chemotherapy with autologous transplantation could cure some lymphomas after conventional chemotherapy had failed.[198] In multiple myeloma autologous stem cell transplantation was found to decrease the effects of toxicity of high dose melphalan, which was efficient in achieving remission in relapsed and refractory disease, but induced profound myelosuppresion.[199-201] These results led to the exploration of high dose therapy with autologous stem cell support as consolidation therapy in patients with newly diagnosed multiple myeloma.[202-204]

Autologous transplantation is now applied more often than allogeneic transplantation.[170] For autologous transplantation, hematopoietic stem cells are usually frozen at temperatures below -120°C and used within a few weeks. However, when frozen, the PBSC graft is viable for years, providing the possibility to use the initially mobilized and frozen cells in case of poor regeneration or

relapsing disease. Because autologous transplantation does not induce GVHD, it can be used in older patients. Indeed, mortality is considerably lower with autologous transplantation compared to allogeneic transplantation, but the absence of graft-versus-tumor activity reduces its effectiveness in diseases sensitive to the immunological effects of donor T cells. Autologous stem cell transplantation (ASCT) has become the mainstay of treatment for multiple myeloma patients younger than 65 years of age based on improved median overall survival of approximately 12-18 months compared with chemotherapy alone.[202;203;205] Accordingly, multiple myeloma is the number one indication for ASCT. Nevertheless, there is continuous debate whether ASCT should be included as the standard initial therapy for all eligible patients with multiple myeloma, especially after the introduction of induction therapy with the immunomodulatory drugs (lenalidomide or thalidomide) with or without the proteosome inhibitor bortezomib in combination with steroids.[206] High dose therapy followed by ASCT has also become the treatment of choice for patients with relapsed aggressive non-Hodgkin lymphoma.[207] For patients with Hodgkin lymphoma high dose therapy followed by ASCT can be used if no complete remission can be achieved with initial therapy regimens or in case of relapse.[208] Recently, results of a randomized trial in acute myeloid leukemia showed an advantage for ASCT as postremission therapy in terms of relapse rate.[209] Although clear survival benefits could not be detected in this study, ASCT might indeed be beneficial in particular subgroups of acute myeloid leukemia patients. Finally, ASCT is increasingly used in the treatment of severe auto-immune diseases.[170]

Scope of this thesis

Like the normal hematopoietic system, AML is thought to be hierarchically organized with a small subset of leukemia-initiating cells that initiate and maintain the leukemic population. These leukemia-initiating cells have been found to reside in the $CD34^+$ compartment in the majority of AML cases. Nevertheless, most gene expression profiling studies in AML have been performed with the total mononuclear cell fraction. In **Chapter 2** of this thesis we performed genome-wide gene expression studies on sorted $CD34^+$ and $CD34^-$ AML subfractions and compared these with a large group of normal $CD34^+$ bone marrow cells in order to identify AML $CD34^+$-specific gene expression profiles. This approach was chosen to obtain information about the transcriptome of the leukemic stem cell-enriched AML fraction.

We continued on studying the $CD34^+$ and $CD34^-$ subfractions of AML, but now focused on the subgroup of NPM1-mutated AML. As described, mutations in the NPM1 gene are frequently found in AML and are of significant prognostic relevance. However, it is currently unclear how mutant NPM1 contributes to leukemic transformation and which molecular mechanisms are involved in the maintenance of the leukemia. In **Chapter 3** we examined the long-term expansion and serial replating capacities of $CD34^+$ and $CD34^-$ NPM1-mutated cells in *ex vivo* stromal co-cultures, identified a $CD34^+$ NPM1-mutated AML gene expression profile and investigated the role of MEIS1 in the long-term expansion of $CD34^+$ NPM1-mutated cells.

Mutations in the NPM1 gene lead to aberrant accumulation of nucleophosmin protein in the cytoplasm of the leukemic cells. Immunohistochemical staining of nucleophosmin on bone marrow biopsies has therefore been widely used for the detection of NPM1 mutations. Moreover, the term cytoplasmic nucleophosmin positive ($NPMc^+$) AML is often used as a synonym for NPM1-mutated AML. In **Chapter 4** we assessed the sensitivity and specificity of immunohistochemistry compared with gold standard molecular analysis to predict NPM1 mutation status in a large cohort of AML patients from our institution. The cases that were found to have a discrepancy between the two methods were evaluated.

The relation between aging and AML is indicated by the strong increase in the incidence of myelodysplastic syndrome and AML with increasing age. Moreover, AML of older age shows distinct clinical and biological characteristics. Although major progress has been made by recent studies, data on the effects of normal aging on the human hematopoietic system are still limited. **Chapter 5** of this thesis provides a review on the effects of aging on the normal hematopoietic system with a focus on recent developments.

As is indicated in our review, aging of the hematopoietic system is especially studied in animal models. In **Chapter 6** we investigated $CD34^+$ cells derived from cord blood, young adult bone marrow and old adult bone marrow with respect to phenotype, *in vitro* function and gene expression profiles to reveal the potential effects of ontogeny and aging on human hematopoietic stem and progenitor cells. Furthermore, we considered ASCT as an experimental opportunity to study the impact of aging and stress response on human HSPCs *in vivo* and analysed the impact of age on mobilisation of HSPCs and on the short and long-term regeneration after autologous stem cell transplantation.

The setting of ASCT was also subject to our studies in the next chapter. Based on the clinical observation that ASCT induces a reduced tolerance to chemotherapy, we hypothesized that the ASCT procedure results in long-term effects on the bone marrow capacity. In **Chapter 7** $CD34^+$ bone marrow cells from patients at 6-9 months after ASCT were studied with respect to the myeloid progenitor composition, *in vitro* colony forming frequency and cell cycle status. In addition, the bone marrow compartments post-ASCT were studied *in vivo* using ^{18}F-FLT PET scans. The thymidine analogue ^{18}F-FLT can be used as a proliferation tracer, since it is phosphorylated by thymidine kinase 1 into ^{18}F-FLT-monophosphate after which it is trapped in the cell.

To follow-up on the observations in post-ASCT bone marrow, a more detailed examination of the hematopoietic stem and progenitor cell compartment post-ASCT was performed in **Chapter 8**. In this study, stem cell quiescence and stem cell frequency together with reactive oxygen species (ROS) production of $CD34^+$ cells from post-ASCT bone marrow (one year following transplantation) were studied and compared to normal bone marrow cells and mobilized peripheral blood stem cells. In addition, gene expression profiling was performed to obtain more insight in underlying molecular mechanisms.

The results of the studies outlined above are summarized in **Chapter 9**. This chapter also provides a general discussion and future perspectives.

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