Chapter
GENERAL INTRODUCTION
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Normal hematopoiesis

Hematopoiesis, derived from the Greek words “haima” and “poiein” meaning respectively “blood” and “to make”, is the process by which all cell components of the blood are formed. In the adult human this takes place in the bone marrow. The formation of blood cells is a hierarchical arranged process with at the origin the hematopoietic stem cell (HSC) of which all blood cells are derived. HSCs have the ability to self-renew and give rise to more differentiated progenitor cells that lack self-renewal capacity and are more restricted towards a specific hematopoietic lineage. The hematopoietic system can be divided in two distinct lineages. The myeloid lineage gives rise to erythrocytes, megakaryocytes, monocytes and granulocytes that are responsible for transportation of oxygen, defense against pathogens and hemostasis. The lymphoid lineage gives rise to B-, T- and NK-cells that together form the adaptive immune system. The process of lineage differentiation is tightly regulated by various genes and microRNAs that are expressed at particular stages of hematopoietic cell development and are influenced by signals from the microenvironment.

Acute leukemia

Acute leukemia is malignant clonal disease that is characterized by an uncontrolled proliferation and accumulation of immature hematopoietic progenitor cells in the bone marrow. Due to this uncontrolled proliferation of aberrant cells normal hematopoiesis is hampered resulting in anemia, thrombocytopenia and leukopenia. Clinically this is reflected by symptoms of pallor, tiredness, easy bruising or bleeding and recurrent opportunistic infections. Grossly there are two types of acute leukemia; acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). AML occurs when HSC or more restricted myeloid progenitor cells acquire molecular mutations or chromosomal aberrations that result in a block in differentiation and an induction in proliferation. In case of ALL these aberrations occur restricted lymphoid progenitor cells.

Diagnosis of acute leukemia is based on the combined examination of peripheral blood and/or bone marrow by morphology, flow cytometry, cytogenetics and molecular analysis. Treatment consist of high dose chemotherapy sometimes followed by autologous or allogeneic hematopoietic stem cell transplantation. AML and ALL require different chemotherapeutic treatment strategies and therefore the discrimination between the two, before start of treatment, is important. As AML and ALL in general have different morphology, phenotype and genetic abnormalities this distinction is usually straightforward. Occasionally leukemias with an undifferentiated or mixed phenotype are seen. These mixed phenotype acute leukemias (MPAL) comprise less than 3% of the total acute leukemias diagnosed. They are associated with a worse prognosis as compared to AML and ALL and pose a problem for hematologists as to decide what treatment scheme should be chosen due to the fact that the underlying lineage of MPAL is unknown.
Acute myeloid leukemia

AML is the most common form of acute leukemia in adults. The yearly incidence of AML in Western Europe is estimated to be around 2-3 per 100,000. In the Netherlands around 700 patients per year are diagnosed. The incidence increases with age which makes AML a disease of the elderly (approximately 65% of AML patients is over 60 years). Due to the aging overall population, the incidence of AML in The Netherlands is increasing. The prognosis of AML is highly dependent on age and overall performance status of the patient but also on intrinsic cell properties like cytogenetic and molecular aberrations. The standard treatment is a combination of cytostatic drugs consisting of cytarabine and an anthracycline such as daunorubicine. This combination treatment results in a complete remission (CR) in the majority of patients. However despite these high remission rates the overall survival (OS) for AML patients under 60 years of age is around 30-40% and only 10-20% for patients over 60 years.

AML is thought to arise via a process of clonal evolution in which healthy HSCs or restricted progenitor cells acquire somatic mutations, often in epigenetic regulators, leading to pre-leukemic stem cells. Subsequent mutations lead to a diversity of pre-leukemic clones with a different molecular makeup. These clones remain functionally normal but can progress into frank leukemia after the acquisition of additional mutations in genes that promote proliferation. Alternatively AML could arise from more differentiated myeloid progenitor cells that acquire self-renewing properties. The degree of lineage commitment of the cell of origin is thought to influence the characteristics of the leukemia. Moreover, AML can arise from myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPNs), or can develop after exposure to genotoxic agents that have been used to treat other (un)related malignancies.

AML is a very heterogeneous disease and the response to treatment depends greatly on the intrinsic cell properties. Cytogenetics is one of the most important determinants for treatment response and thus plays an important role in classification of AML. Recurrent cytogenetic abnormalities like; t(8;21)(q22;q22), inv(16) or t(16;16)(p13;q22), t(15;17) (q22;q12), or 11q23 (MLL) rearrangements are strongly associated with clinical outcome and form the basis for risk stratification upon which the intensity of the additional treatment scheme is based. In general, favorable prognostic cytogenetic abnormalities include t(8;21)(q22;q22) and inv(16) or t(16;16)(p13;q22). Complex cytogenetic abnormalities, monosomal karyotype, 11q23 (MLL) rearrangements, inv(3)(q21q26.2) or t(3;3)(q21;q26.2) and t(6;9)(p23;q34) are associated with a poor prognosis. Acute promyelocytic leukemia (APL), characterized by a t(15;17), the PML-RARA fusion protein, is seen as a separate entity within AML due to the characteristic clinical presentation and the good response to all-trans retinoic acid (ATRA) in combination with arsenic trioxide. APL patients have an overall survival of around 95% and are often excluded from clinical trials and risk scores that involve AML patients of all other subgroups. The largest cytogenetic subgroup of AML patients, about 55%, has a normal karyotype with an intermediate prognosis. Although they are cytogenetically identical there is still a large degree of
heterogeneity and a broad variety in clinical outcome between these patients. Partly, this difference is the result of molecular aberrations that each contribute to the character of the disease and affect therapy sensitivity. Over the last decades a large number of gene mutations as well as deregulated expression of genes have been identified. Recurrent somatically acquired gene mutations include amongst many other; fms-related tyrosine kinase 3 (FLT3), CCAAT/enhancer binding protein alpha (CEBPA), nucleophosmin 1 (NPM1), DNA (cytosine-5)-methyltransferase 3A (DNMT3A), Tumor Protein P53 (TP53), Tet Methylcytosine Dioxygenase 2 (TET2), runt-related transcription factor 1 (RUNX1). These mutations frequently occur in cytogenetically normal AML but are also seen in AML with cytogenetic abnormalities. As with the cytogenetic abnormalities, also these mutations are associated with clinical outcome. In general, internal tandem duplications in FLT3 (FLT3-ITD) and mutations in TET2, RUNX1, TP53 and DNMT3A are associated with adverse prognosis. Favorable prognostic gene mutations include NPM1 and CEBPA. Next to gene mutations, also deregulated expression of genes affect outcome. For example, patients with overexpression of the Ecotropic Virus Integration Site 1 (EVI-1), Brain And Acute Leukemia, Cytoplasmic (BAALC), ETS-related gene (ERG) or meningioma (disrupted in balanced translocation) 1 (MN1) have an adverse prognosis. The large number of mutated or dysregulated genes combined with the many recurrent cytogenetic abnormalities illustrates the enormous heterogeneity of AML. Prognostic risk scores, for example the classification according to the European LeukemiaNET (ELN), is helpful to guide treatment decision. Currently patients with a good prognostic risk score are treated with an additional course of chemotherapy whereas patients with an intermediate or unfavorable risk are treated with autologous or allogeneic stem cell transplantation respectively provided that they have a good performance score and have achieved a CR.

Another important determinant influencing the development of relapse is the frequency of residual leukemic cells after chemotherapy treatment. The survival of these chemotherapy resistant cells are responsible for the re-initiation of AML. The presence of high levels of minimal residual disease (MRD) is associated with a poor outcome in all cytogenetic and molecular risk groups and is therefore recently added as a factor to inform post remission therapy. Patients with a favorable cytogenetic and/or molecular risk but with high levels of MRD are now eligible for a stem cell transplantation. MRD can be detected by flow cytometry but also by measuring leukemia specific molecular aberrancies that are present. The sequential measuring of MRD can also be used as a monitor to alert for beginning relapse.

Leukemic stem cells

AML is a heterogeneous disease but also within individual AML cases there is diverse clonal and cellular heterogeneity. Like normal hematopoiesis, AML has a hierarchical organization. Only a small fraction of the leukemic cell population has the ability to self-renew. These leukemic stem cells (LSC) can give rise to more differentiated leukemic progenitor cells that do not have this ability. LSC are thought to be responsible
for relapse and high LSC load after therapy and at diagnosis is associated with a worse prognosis. AML LSC share many characteristics with normal HSC. LSC were first described to be only present in the CD34+CD38- fraction of the leukemic population. Later, with the advent of improved immune-deficient mouse models, also other phenotypically defined cell populations were shown to contain LSC. However, the CD34+CD38- leukemic cell population still remains the most potent leukemia initiating fraction in most AML cases. LSC show great resemblance to their normal counterparts. For example, gene expression profiles of LSC and HSC show a shared core transcriptional program with enrichment of genes that are implicated in stem cell regulation. The fact that LSC, like HSC, are quiescent and resistant to apoptosis make them insensitive to conventional chemotherapeutic treatment. To achieve long-term cures, the elimination of LSC is necessary. Development of new anti-leukemic therapies specifically targeting LSC, while sparing normal HSC is therefore needed. To find new therapeutic targets that specifically deplete LSCs it is important to distinguish between normal and malignant stem cells within the AML bone marrow. LSC can express cell surface markers showing lineage infidelity or can express aberrant leukemia associated markers which can discriminate them from HSC. These markers include myeloid antigens CD13, CD33, CD123, and CLL-1 and other lineage defining markers like CD7, CD11b, CD22, and CD56. CD44, CD96, and TIM-3 are often overexpressed on LSC compared to HSC. The enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) is another way to discriminate LSC and HSC. ALDH1 is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes and has been shown to be increased in HSC compared to LSC in a large part of AML cases. These differential expressed markers potentially form an attractive target for LSC specific (antibody-mediated) therapy. Currently various monoclonal antibodies target specific LSC markers like CD44, CD123, and CD47 that have demonstrated efficacy against LSC in xenotransplantation models. Clinical trials investigating the safety and efficiency of these new drugs are currently underway.

MicroRNAs
MicroRNAs (miRNAs) are small single stranded RNA molecules of about 22 nucleotides that post-transcriptionally regulate protein expression. MiRNAs are located in introns or in exons of protein coding genes and are transcribed from their own promoters by RNA polymerase II as long primary transcripts called pri-miRNAs or are formed as byproducts from transcription of the genes in which they are genomically localized. Pri-miRNAs are large hairpin loop structures that can contain multiple miRNA precursors. The endonuclease Drosha together with the double-stranded RNA (dsRNA)-binding protein DGCR8 cleave the stem–loop structure of the pri-miRNA into a precursor miRNA (pre-miRNA). The RanGTP-dependent dsRNA-binding protein Exportin 5 (XPO5) transports this pre-miRNA from the nucleus to the cytoplasm where the pre-miRNA is cleaved by the endonuclease Dicer. The resulting miRNA–miRNA* duplex is then loaded
into the Argonaut (AGO) protein after which the miRNA* strand is expelled. The AGO protein together with the single stranded miRNA forms the mature RNA-induced-silencing complex (RISC) which is able to bind target mRNA. The subsequent binding of the RISC complex to complementary mRNA leads to mRNA degradation or prevention of translation. Complete complementary binding of the miRNA and the target mRNA sequence leads to mRNA degradation through cleavage of Ago2. Partial complementary binding leads to reduced protein expression by multiple mechanisms including the inhibition of translation initiation, the induction of premature termination of translation (ribosomal drop-off) or the induction of mRNA deadenylation leading to mRNA destabilization. The most important region of miRNAs that recognizes the target mRNA are the first 6-8 nucleotides at the 5' end of the miRNA, the so called seed sequence. Through this seed sequence one miRNA can potentially target hundreds of mRNAs and one mRNA can be regulated by multiple miRNAs.

In normal hematopoiesis, miRNAs regulate important steps in lineage differentiation. For example in granulocytic differentiation miR-223 is involved in the negative-feedback loop that promotes differentiation together with the transcription factors NFI-A and C/EBPalpha. Another example is miR-451, which is strongly upregulated during erythropoiesis by GATA-1 and has been shown to target GATA-2, a repressor of erythroid maturation. MiRNAs also regulate quiescence, self-renewal and apoptosis of HSCs and important stem cell regulatory miRNAs include miR-126, miR-196b and miR-125a. MiR-126 has shown to target the AKT-pathway which controls the balance between HSC quiescence and expansion. In early hematopoiesis three important regulatory proteins, HOXA9, MEIS1 and FAS are under the control of miR-196b that thereby fine tunes proliferation, survival and differentiation. Another miRNA regulator of HSC function is miR-125a which can repress the pro-apoptotic protein BAK1 protecting cells from apoptosis and promoting expansion of the HSC pool.

Next to their role in normal biological processes miRNAs have been shown to play a role in cancer. Cancer is characterized by aberrant expression of miRNAs and miRNA profiles can accurately distinguish between different types of cancer and their expression changes at different developmental stages. Also for acute leukemia, miRNA expression profiles can classify AML, B-ALL and T-ALL. AML, like other cancers, is characterized by aberrant miRNA expression profiles which can recognize cytogenetic subgroups of AML. For example, core binding factor AML is associated with high expression of miR-126 and AML with MLL-translocation has increased expression of miR-196b and the miR-17–92 cluster. Also molecular aberrations display specific miRNA expression profiles. Cytogenetically normal AML with CEBPA mutation has lower expression of miR-34 whereas FLT3-ITD positive AML is associated with higher expression of miR-155. MiR-9 is downregulated when EVI-1 is overexpressed.

Interestingly, multiple miRNAs that have been identified to function in normal stem cell regulation are often deregulated in AML. Functional experiment in mice with
miR-125b, miR-22, miR-29a and miR-155 showed that overexpression of these miRNAs in normal HSC can lead to a myeloproliferative disorder that, in case of miR-125b, miR-22 and miR-29a, could progress to an aggressive form of AML.\(^\text{81–84}\)

One of the first studies that showed an association between miRNA expression and overall survival of patients was performed by Takamizawa et al. who investigated the expression of let-7 in lung cancer.\(^\text{85}\) Patients who had undergone a potentially curative resection and of which the tumors showed reduced let-7 expression had significantly shorter survival than patients with high expression. Similar associations are found in AML. For example, high expression levels of miR-181a in cytogenetically normal AML were associated with increased CR rates and with longer disease free and OS.\(^\text{86}\) In a more heterogeneous cohort, including normal karyotype AML and AML with cytogenetic and molecular abnormalities, miR-212 was found to be significantly associated with prolonged overall and relapse-free survival.\(^\text{87}\) However, other miRNAs are related to adverse outcome. High miR-155 expression is significantly associated with WBC count, blast percentage, less \(\text{CEBPA}\) mutations, more \(\text{FLT3-ITD, RUNX1}\) and \(\text{WT1}\) mutations and higher expression of \(\text{ERG}\) and \(\text{BAALC}\). Despite all these correlations high miR-155 expression remained an independent prognostic factor for CR rate and OS in a large AML cohort with patients under 60 years of age.\(^\text{88}\) Moreover, miR-3151, located in intron 1 of \(\text{BAALC}\), has independent prognostic value predicting OS and disease free survival in older patients with cytogenetically normal AM, while high expression of \(\text{BAALC}\) is an independent adverse prognostic factor by itself.\(^\text{83,89}\)

The aberrant expression of miRNAs and their function in cancer associated processes like therapy resistance make them interesting therapeutic targets. MiRNA-based therapy could potentially restore deleted or mutated tumor suppressor miRNAs or downregulate oncogenic miRNAs. The multifactorial nature of tumor therapy resistance makes therapies that target a single gene often ineffective. As miRNAs can regulate multiple mRNA targets and thus affect different cellular mechanisms at the same time, these therapies might be more effective. Many miRNAs showed anti-leukemic effects after modulation in cell lines or primary AML cells but only a few studies showed promising results in AML xenograft mouse models.\(^\text{90–93}\) One of these is the tumor suppressor miR-29b which is decreased in AML.\(^\text{90}\) Restoration of miR-29b expression in AML may be a potential therapeutic strategy. When overexpressed, miR-29b induces DNA hypomethylation and re-expression of hypermethylated and silenced genes in AML cell lines through the targeting of \(\text{DNMT3}\) and \(\text{DNMT3B}\).\(^\text{94}\) Functionally restoration of miR-29b in AML cell lines and primary AML induces apoptosis and a reduction of leukemic burden in a xenograft leukemia model.\(^\text{95}\) Moreover, the delivery of miR-29b transferrin-conjugated nanoparticles showed downregulation of its targets in leukemic cells in a xenograft mouse model and intravenous injection of miR-29b resulted a reduction of splenomegaly and leukemic cells of miR-29b treated mice.\(^\text{90}\)

MiR-126 regulates the balance between quiescence and expansion of HSC.\(^\text{66}\) In normal HSC knockdown of miR-126 increases AKT signaling resulting in expansion of long-term...
repopulating HSC \(^{66}\), whereas in LSC increased AKT signaling induces differentiation and proliferation leading to chemo-sensitivity \(^{97}\) or apoptosis \(^{98}\). Due to its opposing role in HSC and LSC, miR-126 could be the ideal candidate for miRNA-based therapy in AML without inducing toxicity for HSC. The feasibility and efficacy of anti-miR126 therapy was recently demonstrated by Dorrance et. al. who showed that targeting of miR-126 using nanoparticles led to depletion of LSC and improved survival of secondary transplanted mice, with minimum toxicity \(^{91}\).

To date only a few miRNAs have been tested in a clinical trial. The antagonim of miR-122 (Miravirsen) has been tested in patients with a chronic hepatitis C infection and showed a dose dependent effect without adverse events \(^{99}\). Currently, the safety for MRX34, a miRNA mimic of the tumorsupressor miR-34, is tested in a phase 1 trial including patients with hematological malignancies.

**Scope of the thesis**

Although the characterization of acute leukemias is well defined and the underlying genetic factors that contribute to leukemogenesis are now being unraveled by new large scale sequencing techniques, leukemias of ambiguous lineage remain. To date, difficult to classify acute leukemias with undefined, undifferentiated or mixed phenotypes still pose a therapeutic dilemma as the underlying lineage of these leukemias is unknown and no prospective, controlled trials exist to guide therapy. In Chapter 2 we present such a case that demonstrates the challenges in diagnosing an acute leukemia with ambiguous morphologic, immunohistochemical and flow cytometric characteristics. This case, as well as similar other cases, demonstrate the limitations of conventional diagnostic tools. Therefore, we hypothesized that miRNA expression profiling could contribute to a better characterization of these leukemias. The results of this profiling study, it’s relation with previous and current immunophenotypical scorings systems and the potential therapeutic consequences are described in Chapter 3.

Over the last years the central role for LSC in AML has become increasingly clear. LSC are thought to be responsible for relapse and poor outcome of AML patients. This is mainly due to their intrinsic properties like self-renewal and resistance to chemotherapy. MiRNAs can target hundreds of genes, are playing key roles in all developmental biological processes and have shown to control HSC function. It is therefore not surprising that they also regulate important functions in LSC and that aberrant expression of stem cell related miRNAs in AML can influence the course of disease. Targeting of these miRNAs might be an new therapeutic strategy in the treatment of AML. The current knowledge on miRNA expression and function in hematopoietic and leukemic stem cells as well as the therapeutic approaches for miRNA-based specific elimination of leukemic stem cells are reviewed in Chapter 4.

In order to find miRNAs that could be targeted to specifically eliminate AML LSC, we conducted a miRNA profiling study on normal residual HSC and leukemic stem and progenitor cells from AML bone marrows. Despite the limitations of deriving low HSC
numbers from AML samples, we were able to obtain and compare the expression profiles of HSC, LSC and leukemic progenitors and identified multiple differential expressed miRNAs. This profiling revealed that miR-551b is highly expressed in residual HSC. MiRNAs that are expressed and functional in HSC are often dysregulated in AML. In Chapter 5 we describe the expression of miR-551b in various hematopoietic stem and progenitor cell populations from the normal bone marrow as well as in a cohort of 154 AML patients, where we correlate its expression with clinical characteristics, cytogenetic and molecular abnormalities and prognosis.

Our profiling revealed differential expression of miR-126 between stem cells and differentiated progenitors in normal hematopoiesis and AML. Moreover, miR-126 is higher expressed in HSC as compared to LSC and modulation of its expression in both cells showed divergent outcomes. The results of our profiling as well as the functional role of miR-126 and it’s potential as a target for LSC specific therapy is further explored in Chapter 6.

Finally, the findings described in this thesis and the implications they may have for future miRNA research and AML are discussed in Chapter 7.
REFERENCES


