Chapter 7
DISCUSSION AND FUTURE PERSPECTIVES
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

When microRNAs (miRNAs) were first discovered two decades ago it opened a whole new chapter in molecular biology.¹ Their implications in cancer development boosted research and the number of publications on miRNAs increased exponentially. Today, miRNAs are known to function in all kind of organisms and are implicated in a wide array of physiological as well as pathological cellular and developmental processes. The research described in this thesis is focused on the role of miRNAs in acute leukemia and more specifically in acute myeloid leukemia stem cells. The chapters of this thesis cover multiple aspects of miRNA expression and function that are important for diagnosis, classification and therapy development.

Our findings that miRNA expression profiles can be used to identify the underlying lineage of acute leukemias of ambiguous lineage (ALAL) are described in Chapter 3. The consequences of miRNA-based classification of these patients for therapy decision making are further discussed there.

Despite the fact that current risk scores for acute myeloid leukemia (AML) can effectively be used as predictors for prognosis much improvement is required. Further improving risk classification in AML can add to therapy decision making and thus consequently improve outcome. In Chapter 5 we report the identification of miR-551b as an independent marker for AML with stem cell characteristics associated with poor prognosis. It forms the basis to further discuss the inclusion and value of prognostic miRNAs in AML scoring systems and to hypothesize on the functional contribution of miR-551b expression in AML therapy sensitivity in light of the recently published literature.

To really improve outcome of AML new treatment options are needed. As relapse is thought to be initiated by the survival of chemotherapy resistant leukemic stem cells (LSC), therapies specifically targeting LSC are a promising strategy. The efforts to identify potential miRNA therapeutic targets directed against LSC are described in Chapter 6. There we showed that miR-126 can be regarded as a promising new target for LSC specific therapy with the capacity to spare normal HSC. The importance of accurate profiling and current status of miRNA based therapy, as well as the recent reports on miR-126 targeting in AML are discussed here.

MicroRNAs as diagnostic markers

The characteristics of cells of the hematopoietic system are well defined. On top of this hierarchical organized system is the HSC which gives rise to all mature blood cells and has the ability to self-renew.² Differentiation from HSC to all the mature lymphoid or myeloid hematopoietic cells is strictly regulated by various factors including miRNAs. MiRNAs are expressed at specific hematopoietic developmental stages and important for the lineage differentiation of these cells.³ A similar hierarchical system exists in acute myeloid leukemia.⁴ The malignant counterpart of the HSC, the leukemic stem cell, also has self-renewal capacity and can differentiate into more differentiated progenitors that
lack this ability. MiRNAs also play an important role in the hierarchical organization of leukemia. Upon malignant transformation the expression of miRNAs is deregulated and the resulting aberrant expression contributes to the character of the disease. miRNA expression profiles are associated with different types of leukemia but also with particular AML subgroups defined by cytogenetics and molecular aberrations. To accurately discriminate the lineage of origin of undefined acute leukemias we used the specificity of these miRNA expression profiles. MiRNA expression profile analysis can be used for defining whether acute leukemia is of lymphoid (ALL) or myeloid (AML) origin. However, until now this miRNA profiling is not used in clinical practice like well-established methods as cytomorphology and immunophenotypic characterization by flow cytometry. These methods are, in general, highly specific, fast and cheap which makes classifying acute leukemias using miRNA expression profiles only of interest when turnaround time and costs are reduced. One exception forms the diagnosis of ALAL. Immunophenotypical characterization to determine whether this subgroup of acute leukemias originates from a lymphoid or a myeloid cell precursor (or both) is difficult and forms a major hurdle in treatment decision making for hematologists. In Chapter 3 we show evidence that miRNA based classification of ALAL adds significant value to the traditional methods like cytomorphology and immunophenotyping in defining a lymphoid or myeloid lineage origin. In addition, we show that mixed phenotype acute leukemias (MPAL) are not a separate entity, unlike the current WHO classification suggests, and our results therefore challenge the currently used immunophenotypic classification of these leukemias. Thus, despite the fact that these leukemias show a mixed immune-phenotype they do not have a mixed genotype. The term mixed phenotype acute leukemia should therefore only be reserved for an immune-phenotypic classification but preferably not for the final diagnosis on which treatment choice is based. Our results indicate that miRNA expression based classification of ALAL is of additional value for the decision to apply either ALL or AML therapy and therefore deserves a place in the diagnostic workup of these leukemias. As we show that discrimination between lymphoid and myeloid origin can be made by using the expression of only 5 miRNAs, implementation of this technique could be relatively easy and at low costs. Turnaround time for the expression analysis of a small subset of miRNAs is short (two days) and the results will be available before the start of treatment. Notably, in all ALAL cases with a B-ALL phenotype and expression of MPO as sole myeloid marker, that are diagnosed as MPAL by the WHO 2008 criteria, we found a lymphoid miRNA expression profile. These cases are therefore expected to respond better to ALL treatment schemes. This is in line with recent reports that ALL based chemotherapeutic regimens seem to have better response rate in the total group of MPAL cases. Although clinical studies in ALAL will be difficult due to the low incidence of these leukemias, they will be needed to find out whether patients with immune-phenotypically defined ALAL have better disease outcome when treatment choice is made based on miRNA expression profiling. Their low incidence makes it crucial that these trials are conducted in international multicenter consortiums.
In our analysis we did not include bilineal (consisting of two distinct populations) or biclonal (arise from a separate transforming event) cases. However, also in these cases miRNA expression profiling, after flowcytometric isolation of the individual populations, might provide more information to whether there is a shared lineage or a true bilinear disease.

We suggest that in the future the WHO classification of acute leukemias should be revised. The entity MPAL should be removed and additional diagnostic testing using miRNA expression profiling in case of leukemia of ambiguous lineage should be advised to unravel the lineage of origin and direct therapy.

**MicroRNAs differentially expressed in LSC, HSC and leukemic progenitors (LP)**

Despite high remission rates the survival of AML patients is still poor which is, at least in part, due to the survival of LSC after chemotherapy. In order to improve outcome alternative therapies targeting LSC to reduce relapse, are needed. As LSC and normal HSC have many common characteristics and co-exist in the same microenvironment specific targeting of LSC, while sparing HSC, is crucial. Since LSC utilize a variety of mechanisms to resist therapy the major challenge in targeting LSC is their heterogeneity.

As miRNAs target multiple genes simultaneously and manipulation of their expression can affect multiple pathways, modulation of miRNA expression may hold the key to successful elimination of therapy resistant leukemic (stem) cells. In Chapter 6 of this thesis we purified LSC, HSC and leukemic progenitors from individual human AML bone marrows and reported for the first time the comparison of miRNA expression profiles between the normal and malignant stem cell populations and the two leukemic fractions. Isolation of leukemic and normal cell fractions from the AML bone marrow takes into consideration the influence of the leukemic microenvironment on miRNA expression in these cells. We hypothesized that comparing miRNA expression of LSC with that of HSC derived from the same AML bone marrow sample increases the chance of finding genuine LSC specific targets as compared to studies that compare LSC with normal HSC isolated from healthy bone marrow or cord blood. Since there is still heterogeneity in these small cell populations and the identity of the real relapse initiating cell herein is not known, future research should be directed to genetically and epigenetically characterize single cells. In the past, profiling of cancer stem cells have been performed with populations of cells wherein the percentage of stem cell-like cells have been very small. Stem cell associated miRNAs might have been overshadowed by miRNAs expressed in the bulk of cells that could have led to the missing of potential important targets.

As miRNAs can have different functions in different cell types and developmental stages and their effect is also dependent on available mRNA targets and other mechanisms influencing expression of these targets, it seems important to investigate the function of miRNAs in the appropriate cell within a heterogeneous tumor. Therefore, next to single
cell profiling, also functional experiments with very small cell populations and single cells have to be optimized and performed.

**MicroRNAs expressed in stem cells and their influence on prognoses**

In AML, cytogenetic and molecular aberrancies correlate strongly with outcome and are used to predict survival and direct treatment choices.\(^\text{11}\) When it became apparent that miRNA expression profiles were associated with specific AML subtypes and cytogenetic abnormalities, their expression was also found to be associated with prognosis\(^\text{12}\). To date, multiple profiles and individual miRNAs have shown to be associated with AML survival, relapse and/or complete remission rates.\(^\text{13-15}\) However, despite the fact that the first publication linking miRNAs to AML prognosis was published almost eight years ago\(^\text{16}\), still no prognostic classification model includes the expression of miRNAs. This probably has several reasons. One is that the well-known and widely accepted cytogenetic and molecular factors that are associated with AML prognosis have a strong effect on disease outcome. Prognostic differences between good, intermediate and poor cytogenetic AML risk groups are large and highly significant and justify the application of different treatment strategies. The association of miRNA expression and AML outcome is mostly reported in cytogenetically normal AML. This is likely because this cytogenetically normal intermediate prognostic risk group lacks the strong prognostic influence of cytogenetic aberrancies which gives room to the influence of miRNAs. Another reason why miRNA expression is not yet used in prognostication models is that karyotyping and molecular diagnostic technology is already routinely used and of low cost while a novel technique like miRNA-based diagnostics requires implementation and validation.

In Chapter 5 and 6 of this thesis we describe the prognostic value of miR-126 and miR-551b. Both miRNAs are highly expressed in cells with a stem cell phenotype and associated with “stemness” which could explain a direct link with chemotherapy resistance and disease outcome. MiR-126 expression levels are associated with poor prognosis in the subgroup of non-CBF AML patients (Chapter 6). In this group high miR-126 expression is associated with shorter relapse free survival and a trend towards poorer overall survival compared to patients with low miR-126 expression. Interestingly, the good prognostic subgroup of CBF AML also shows high expression of miR-126 but differential expression within this group was not associated with disease outcome. The lack of finding a prognostic association in CBF AML could be caused the small size of our cohort as Li et. al. recently reported poorer prognosis of patients with high miR-126 expression compared to patients with low expression in AML-ETO.\(^\text{18}\) The fact that miR-126 is highly expressed in HSC and associates with poor prognosis in various AML risk groups suggests a functional role in “stemness” possibly imposing therapy resistance. Indeed we also found higher expression of miR-126 in CD34+CD38- LSC compared to differentiated LP and show that knockdown of miR-126 reduces clonogenic capacity of leukemic stem cells by inducing apoptosis and possibly differentiation (chapter 6). The hypothesis that miR-126 might induce a stem cell genotype is strengthened by the recent observations of
Li et al., who demonstrated activation of genes highly expressed in more differentiated hematopoietic cells upon knockdown of miR-126 and activation of genes highly expressed in LSC and primitive HSPCs after miR-126 overexpression. Altogether it seems therefore possible that high expression of miR-126 in a subpopulation of leukemic cells within the total AML could induce “stemness” and decreased therapy sensitivity eventually leading to relapse after chemotherapy.

In Chapter 5 we show that high expression of miR-551b is significantly associated with poor survival and high relapse rates in AML. Importantly, miR-551b expression is an independent prognostic factor even when combined with the most important currently used prognostic indicators like white blood cell count and the presence of cytogenetic abnormalities and molecular aberrations. We observed that miR-551b is highly expressed in HSC, higher expressed in undifferentiated AML as compared to the more differentiated subtypes and correlates with the expression of genes that are present in a HSC-gene signature. Together this suggests that AML with high miR-551b expression has stem cell-like characteristics. This stem cell-like phenotype might be due to miR-551b-induced expression of stem cell genes however might also be the result of an initiating founder mutation in a normal HSC. In this later case, the intrinsic resistance of the HSC that is inherited by the leukemia is probably responsible for the adverse clinical outcome seen in patients with high expression of miR-551b. As this HSC inherited resistance is independent of the identity of the acquired molecular and cytogenetic aberrancies it could add additional value to risk stratification of AML patients in each risk group. However, the validation of miR-551b as a poor prognostic marker in larger patient cohorts is needed in order to find a validated cut-off value.

Currently there are only a few reports on the mechanisms whereby miR-551b regulates stem cell characteristics and therapy resistance. It has been shown that miR-551b is involved in acquired apoptosis resistance, chemotherapy resistance and promotion of tumor growth and metastasis, possibly by targeting genes like STAT3, c-Kit and catalase. More research to elucidate the functional role of miR-551b in AML (stem) cells and chemotherapy sensitivity is needed. Currently we are investigating whether miR-551b is an inducer of a stem-cell like phenotype and/or whether its high expression is associated with the existence of pre-LSC, indicative for a leukemia that has been derived from a HSC. To shed more light on this we are analyzing the presence of founder mutations, like DNMT3a, IDH1/2 and ASXL1, in residual HSC in primary AML. When AML with high miR-551b expression is associated with the presence of such mutations in HSC (pre-LSC) the leukemia is likely HSC-derived which might partly explain its inherited intrinsic (chemo) resistance and poor outcome.

**MicroRNA-based therapy**

The identification of miRNAs differentially expressed between HSC, LSC and leukemic progenitors generates new potential therapeutic targets. Various techniques to modulate miRNA expression are now available and have shown tremendous potential *in vitro*
and more recently also in vivo.\textsuperscript{23–25} In Chapter 6 we identified and explored miR-126 as a possible target for future anti-miRNA based therapy in order to target AML LSC. We show for the first time that targeting of miR-126, which is highly expressed in HSC and differentially expressed between LSC and leukemic progenitors, induces apoptosis of leukemic cells while sparing and even expanding HSC. The exact mechanism explaining this differential effect between LSC and HSC remains to be elucidated but differential expression of miR-126 target(s) in these populations or presence of unknown survival mechanisms in HSC that are lacking in LSC might be the cause for this. The potential of miR-126 as a therapeutic target preventing leukemia relapse was recently confirmed by the group of Dorrance et al., who showed that in vivo targeting of miR-126 using nanoparticles can lead to a reduction of LSC burden, a delay in AML development and an increased survival of secondary recipient transplanted mice without clear evidence of toxicity.\textsuperscript{23} These findings further strengthen the idea that miR-126 could be used as a therapeutic target for successful AML therapy.

That miRNA based treatment in humans is possible and safe has been demonstrated already for a few miRNAs; anti-miR-122 therapy in the treatment of hepatitis C virus and miR-34a therapy in cancer treatment.\textsuperscript{26,27} Depending on the expression and function of the identified miRNA targets, there are two approaches to develop miRNA-based therapies: antagonists and mimics. Mimics, double stranded oligonucleotides, are used to restore the expression and function of downregulated or deleted miRNAs mostly with tumor suppressor activity. Antagonists, made to inhibit miRNAs that have acquired a gain of function, will be needed for therapeutic targeting of miR-126. Often antagonists are single-stranded oligonucleotides that can efficiently silence miRNA activity. For in vivo usage these oligonucleotides require chemical modification to improve their binding affinity, biostability and pharmacokinetic properties. The most common modifications to improve the duplex melting temperature, their resistance to nucleases and reduce their clearance by glomerular filtration and urinary excretion include 2’-O-methyl (2’-\textsuperscript{O-Me}), 2’-Methoxyethyl (2’-MOE) 2’fluoro and the bicyclic locked nucleic acid (LNA) modifications. Among these modifications, LNA exhibits the highest affinity toward complementary RNA.

The major challenge remains the successful delivery of therapeutic miRNA(s) to the site of the tumor, in case of AML the leukemic bone marrow, without inducing toxicity. Accurate targeting will depend greatly on the delivery system that is used. There are several in vivo delivery methods available; i.e. conjugated-based (usually cholesterol), liposomal-based, nanoparticle-based and antibody-based methods. For the first two methods mentioned the liver, spleen and kidney are the main sites of localization. This also is mainly true for nanoparticle-based methods, although they can be conjugated with for example transferrin, to direct them. The high affinity and binding specificity of antibodies makes the antibody-conjugated method likely the most relevant method for leukemic (stem) cell targeting. Markers known to be specifically expressed on LSC, like CLL1, CD123 and TIM3 could be potential targets to direct miRNA-based therapy.\textsuperscript{28}
The fact that miRNA-based therapy is already under investigation in human trials without the occurrence of severe side effects is promising and encouraging. The results of these trials will hopefully be positive and pave the way for the development of more clinical therapeutic miRNAs like miR-126.

CONCLUSION
Research from the last two decades have provided us with an enormous amount of knowledge on miRNA biology and shed light on their role in hematopoiesis and leukemogenesis. While early research initially focused on discovery of new miRNAs and their expression it gradually shifted towards more functional analysis and more recently miRNA-based therapeutics. The studies performed in this thesis contribute to a better understanding of miRNA regulation in leukemogenesis and stem cells, offer a new tool for clinical practice and build the biological basis for miRNA-based therapy against AML LSC. Additional research by us and other groups extending the findings from this thesis hopefully will lead to more (therapeutic) miRNA applications further improving diagnosis, classification and prognosis of acute myeloid leukemia patients in the future.
REFERENCES


