SUMMARY

Acute myeloid leukemia (AML) is a haematological malignancy caused by the accumulation of immature myeloid cells in the patient’s bone marrow and peripheral blood which becomes rapidly fatal when patients are not immediately treated after diagnosis. AML is classified based on cell morphology, the presence of molecular aberrancies and immune phenotypic features, which finally leads to distinguished patient groups with a predicted outcome ranging from favorable to poor. Most AML patients are treated with intensive chemotherapy resulting in complete remission (CR) rates exceeding 80%. Despite this initial good response, a small subpopulation of AML cells survives the treatment and can ultimately reinitiate the leukemia. AML recurrence is very difficult to treat and rarely results in a new complete remission.

AML cells that contain stem cell features, often referred to as leukemic stem cells (LSCs), are thought to be the cause of relapse and targeting these LSCs is therefore needed to improve survival. LSCs have many properties in common with normal hematopoietic stem cells (HSCs), including the CD34^+CD38^- immune phenotype and a shared core transcriptional program that partly confers a stem cell like state supporting self-renewal and therapy resistance. Since LSCs and HSCs are similar in many ways, specific therapies targeting LSCs need to be designed in a manner that prevents the elimination of HSCs, aiming at restoring normal haematopoiesis after chemotherapy. Therefore, these anti-LSC therapies should be developed against features differentially expressed or active between LSCs and HSCs. Several attempts have been made to compare genes differently expressed between HSCs from healthy donors and LSCs from AML patients, however, since AML cells enforce changes on the bone marrow microenvironment and suppresses HSC functions, we hypothesized that this comparison is most relevant between normal and malignant stem cell fractions derived from the same patient. To discriminate HSCs from LSCs in an AML bone marrow, several groups, including ours, have identified leukemia associated immune phenotypic markers. These include the lineage markers CLL-1, CD7, CD56, CD45RA and CD123, as well as functionally defined aldehyde dehydrogenase (ALDH) activity. Using these markers, we purified HSCs, LSCs and leukemic progenitors from AML bone marrows and determined their gene expression profiles. This resulted in the identification of genes differentially expressed between LSCs and HSCs and between LSCs and leukemic progenitors. These genes might be potentially involved in sensitivity to chemotherapy and self-renewal, however, these genes might also be targets for therapeutic strategies.

Increased chemotherapy resistance can be facilitated in multiple ways, including increased DNA repair, enhanced drug efflux, quiescence, drug target modification, a block in apoptosis and enhanced survival signalling routes. A signalling route that is frequently linked to AML cell survival and therapy resistance is the insulin-like growth factor receptor 1 (IGF1R) pathway. The activation of the IGF1R pathway can be regulated in multiple manners, including IGF1R expression, IGF-1 ligand availability and the expression of insulin-like growth factor binding proteins (IGFBPs). In chapter 2 of this thesis we have studied the role of insulin-like growth factor binding protein-7 (IGFBP7), a member of the IGF1R signalling route, in the context of AML cell survival and chemotherapy sensitivity. IGF1R inhibition, IGFBP7 overexpression and incubation with human recombinant
(rhIGFBP7) all resulted in decreased AML cell survival and sensitization to chemotherapy. Furthermore we showed that IGFBP7 overexpression induced a G2/M cell cycle block and apoptosis in AML cell lines, which was mainly independent of IGF1R activation. Importantly, patients that have low IGFBP7 expression at diagnosis show a trend towards decreased overall survival, event free survival and relapse free survival, indicating that AML cells with reduced IGFBP7 levels are associated with decreased sensitivity for chemotherapy-induced cell death.

In chapter 3 of this thesis, the differential gene expression between LSCs and HSCs derived from the same bone marrow was compared to the difference in gene expression between LSCs and leukemic progenitors. This revealed IGFBP7 as one of the top candidate genes differently expressed between HSCs, LSCs and leukemic progenitors. Lower expression of IGFBP7 in LSCs was validated in an AML patient cohort of 23 AML cases that compared LSCs versus non-LSC fractions. Moreover, this same AML patient cohort showed the upregulation of the IGF1R on LSC relative to progenitors which we have validated on plasma membrane cell surface expression levels in a subset of AML patients. Together these data suggest that LSCs might use the IGF1R pathway for survival, proliferation and chemotherapy resistance. This hypothesis is supported by our findings since we show that the addition of recombinant IGF-1 stimulates colony formation of primary AML cells. Importantly, inhibition of the IGF1R pathway, using a tyrosine kinase inhibitor, selectively kills AML cells in contrast to normal bone marrow cells derived from healthy volunteers. Based on these observations we hypothesized that the persistence of LSCs might be partly due to the high expression of the IGF1R and the low expression of IGFBP7. We showed that lowering expression of IGFBP7 reduces chemotherapy sensitivity of AML cells. Furthermore, IGFBP7 expression is frequently downregulated in cells derived from relapsed AML patients relative to their diagnosis counterparts, indicating that in several AML cases IGFBP7low expressing leukemia cells have a survival advantage over IGFBP7high expressing cells. To study whether enforced levels might influence stem cell-like features we overexpressed IGFBP7 in primary AML and observed reduced clonogenic capacity in vitro, as well as sensitization of LSCs to chemotherapy in vivo. Next, we studied the inhibitory effect of rhIGFBP7 on primary AML cells and observed that rhIGFBP7 reduces AML cell survival, which is at least partly explained by the induction of cell death. In vivo application of rhIGFBP7 results in reduced AML engraftment in immunodeficient mice. Moreover, rhIGFBP7 induces differentiation in primary AML and eradicates leukemic progenitors and long term culture initiating cells (LT-LICs). Importantly, the treatment with rhIGFBP7 eliminates malignant CD34+CD38- cells in vitro and hampers the engraftment of AML cells in secondary recipients indicating the elimination of LSCs. These findings are supported by gene expression profiles made from rhIGFBP7 treated AML versus untreated cells, which showed the reversal of a stem cell-like signature upon rhIGFBP7 treatment. We have also explored the effect of rhIGFBP7 in addition to currently used cytostatic regimens and show that rhIGFBP7 can add to reduced LSC survival in vivo. Finally, we did not observe an inhibitory effect of rhIGFBP7 on the self-renewal capacity of healthy normal bone marrow cells, or on the engraftment potential of normal hematopoietic cells. Together, these data indicate that application of rhIGFBP7, in addition to the current used chemotherapy regimens, might improve therapy outcome for AML patients.
In chapter 4 we used a leukemia cell line model to study dynamic resistance to anthracyclines, one of the components of the chemotherapy treatment for AML patients. This study revealed that minor subpopulations of leukemia cells are able to acquire a reversible anthracycline-tolerant state that can be maintained by culturing in presence of doxorubicin. These anthracycline-tolerant clones (ATCs) show increased expression of the LSC markers CD44, MDR1 and GRP56, likely indicating their stem cell-like phenotype. MDR1 is probably upregulated on the ATCs by instruction, since its expression is upregulated during incubation with doxorubicin and again decreased after doxorubicin removal. High CD44 expression in ATCs likely develops due to the selection of CD44\(^\text{high}\) cells during treatment, since incubation with doxorubicin does not instruct CD44 upregulation and CD44\(^\text{high}\) parental cells are less sensitive to doxorubicin than CD44\(^\text{low}\) cells. Although MDR1 inhibition re-sensitized partly for doxorubicin, we sought to find additional manners to target these ATCs since MDR1 inhibitors have failed in multiple clinical trials. We observed that doxorubicin had a stimulatory effect on H3K4 and H3K27 methylation, however, the ATCs showed reduced H3K27m3 modifications, likely indicating enhanced activity of a histone demethylase in the ATCs. By studying gene expression profiles of ATCs compared to parental cells we found that KDM6A and KDM6B are upregulated in the ATCs. Moreover, the inhibition of KDM6 activity using GSK-J4 selectively kills the ATCs, while the parental cells remained unaffected. Together, these data imply that KDM6 inhibition might be an attractive novel therapeutic strategy to overcome anthracycline tolerance of myeloid leukemia cells.

Part of the AML patients is treated with all-trans retinoic acid (ATRA) instead of the conventional chemotherapy. So far, ATRA has been proven to be very effective to treat acute promyelocytic leukemia (APL) and turned this poor prognostic malignancy into a curable disease. This patient group, accounts for approximately 10% of the AML patients and form a separate entity within AML, characterized by a PML-RARA translocation t(15;17). In APL, ATRA drives cells into differentiation which leads to decreased proliferation and self-renewal capacity. Despite the success for this particular subgroup of AML patients, the effect of ATRA is limited for other AML patient groups. In chapter 5 of this thesis we have studied the response to ATRA for AML patients with high expression of the Ecotropic Viral Integration site-1 (EVI-1) oncogene. EVI-1-positive AML cases have a very poor risk profile, since the two year survival of those patients is around 5%. This indicates the critical urgency to identify novel therapeutic strategies for this group of AML patients. For this reason we evaluated the effect of ATRA on differentiation and cell survival in a panel of 13 primary EVI-1 positive AML cases. We found that 9/13 AML samples show an induction of differentiation and 5/13 a reduction of myeloid cell survival upon treatment with ATRA, this in contrast to non-EVI-1 positive AML. The reduced cell survival of EVI-1 positive AML cases upon ATRA treatment can at least be partly explained by the induction of apoptosis. Moreover, ATRA inhibited clonogenic capacity of leukemic progenitors in most EVI-1 positive samples that we tested. Importantly, we showed that ATRA significantly inhibited human myeloid engraftment of EVI-1 positive AML samples in vivo. Together, these data suggest that EVI-1 positive AML patients might benefit from ATRA treatment. Currently, it is still unclear why EVI-1 positive patients have an increased response to ATRA and why a subset of EVI-1 positive patients is unresponsive.
Chapter 6 covers a review that addresses the most recent insights with respect to reprogramming of non-APL AML cells towards ATRA sensitivity. APL patients express the chimeric gene fusion PML-RARA and are successfully treated with ATRA, in combination with arsenic trioxide (ATO). ATRA drives APL cells into differentiation towards a neutrophilic lineage, and ATO subsequently induces apoptosis in the differentiated cells. Although non-APL AML cells are in general considered to be unresponsive to ATRA, there is increasing evidence that specific molecular aberrations or expression levels of epigenetic modifiers like the lysine-specific demethylase 1A (KDM1A/LSD1), can determine susceptibility to ATRA. The same might hold true for high EVI-1 expressing cells since a large subset of EVI-1 positive AML patients that we have tested showed high susceptibility for ATRA compared to patients that lack EVI-1 expression. This hypothesis is supported by functional data from another group, showing that enforced EVI-1 expression in an EVI-1 negative cell line increased cell cycle arrest, differentiation and apoptosis upon treatment with ATRA. Together, these data suggest that EVI-1 expression might be needed to force differentiation into AML cells.

EVI-1 has been shown to interact with several transcription factors and epigenetic regulators among others, P/CAF, CBP, GATA2, PBX1, PML, EZH2, SUV39H1, G9a, DNMT3A and PU1, suggesting that EVI-1 expression might induce a distinct transcriptional or epigenetic cell state, creating susceptibility to ATRA. Since we also find non-responders to ATRA among the EVI-1 positive cases it is possible that other genes that are mutated or differently expressed abolish this susceptible state. Because more ATRA sensitive AML cases were reported in subsets of AML patients with a MLL translocation, an IDH-1 mutation and a NPM1 mutation, we suggest that the identification of novel biomarkers that can predict a response to ATRA therapy might be of critical importance to improve therapy outcome. Despite the urge to identify novel biomarkers that predict response, more research is needed to elucidate whether ATRA can be given to patients as monotherapy, or much more likely in combination with other therapeutic agents like conventional chemotherapy, arsenic trioxide or epigenetic inhibitors.

In chapter 7, the most important findings are discussed with a focus on the potential mechanisms for the proposed novel therapeutic strategies that are identified in this thesis and the possible implications for the clinic to improve AML outcome.