07.

Summary, Discussion and Future Perspectives
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Summary

The research topics in this thesis have a common theme: improving our understanding of the role of autophagy in normal and malignant hematopoiesis. **Chapter 2** concerns the diverse functions of autophagy in leukemic cells, leukemic stem cells and non-malignant cells, such as hematopetic stem and progenitor cells (HSPCs), T cells and B cells and the non-hematopoietic cells that comprise the bone marrow micro-environment. Therapeutic targeting of autophagy is also addressed. **Chapter 3** focuses on the role of autophagy in human cord blood-derived CD34$^+$ hematopoietic stem cells and their progeny. The study presented in this chapter concerns the level of autophagy in HSPCs and during differentiation towards the myeloid and erythroid lineage. The autophagic-flux was determined by analyzing the relative accumulation of autophagosomes, which was higher in HSPCs and declined with myeloid differentiation. Autophagy appeared to be functionally relevant for HSPCs. Knockdown of the essential autophagy genes ATG5 and ATG7 resulted in reduced HSPC frequencies and impaired *in vivo* engraftment. The negative phenotype observed after autophagy inhibition was in part due to increased apoptosis and reduced cell cycle progression. Knockdown of ATG5 or ATG7 in HSPCs caused an increase in reactive oxygen species (ROS). This is in line with observations in ATG5 or ATG7 knockout mice models, which have accumulation of dysfunctional mitochondria in the affected cells coinciding with increased ROS levels. In conclusion, this study indicates that autophagy is an essential mechanism in human HSPC cells whereby autophagy promotes survival by limiting cellular stress.

Low-risk MDS is clinically characterized by peripheral blood cytopenias despite the presence of a hypercellular bone marrow. The cytopenias are in part caused by an increased tendency of myeloid and erythroid progenitors to undergo apoptosis. Although, genetic and molecular aberrations are clearly important factors in MDS, increasing evidence indicates that the bone marrow microenvironment is an additional factor in the pathogenesis of the disease. **Chapter 4** concerns the impact of the microenvironment on programmed cell death (PCD) in erythroblasts derived from patients with low-risk MDS. The study presented in this chapter investigated PCD in freshly isolated MDS bone marrow erythroblasts derived from the mononuclear cell fraction or from hematons. Bone marrow-derived hematons are compact hematopoietic complexes containing various cell lineages including mesenchymal cells, endothelial cells and hematopoietic progenitors.

In these hematons, a large number of erythroblasts are located within their own microenvironment. The study demonstrated that erythroid cells directly isolated from bone marrow hematons and MNC fractions exhibited reduced levels of apoptosis. The ultrastructure results were compatible with the enhanced levels of autophagy. However, when MNCs from MDS bone marrow were cultured for 24 hours *in vitro*, a relatively large proportion of apoptotic erythroid cells was observed, which was not observed in erythroid cells of cultured hematons. Comparable results were obtained from erythroid progenitors: BFU-Es could not be efficiently cultured from the MNC fraction, while high numbers of BFU-Es were obtained from the hematon fraction. To study whether increased autophagy could be linked to altered expression of key autophagy genes, the expression level of ATG5, ATG7, Beclin-1 and LC3 was determined by qPCR. However, no difference in the expression of these genes was observed in MDS erythroblasts. These results indicate that MDS-derived erythroid progenitor cells are strongly dependent on their own microenvironment for their survival and that increased numbers of autophagy structures occur, probably due to increased deposition of iron in the mitochondria.

Due to the relevance of autophagy for maintaining stem cell properties in normal HSPC, the study presented in **Chapter 5** addressed the question of whether AML HSPC are dependent in a similar manner. We observed variation in autophagy activity in a panel of leukemic cell lines and primary AML CD34$^+$ cells. The level of autophagy was higher in AMLs clinically classified as poor-risk compared to intermediate-risk and favourable-risk groups. In addition, autophagy was higher in AMLs with TP53 mutations. The AML patients in the poor-risk group had a higher frequency of mutations in the TP53 gene. The increased autophagic flux in TP53 mutated cells was not due to an altered p53 protein expression. Modulation of p53 expression by either overexpression or knockdown did not affect the autophagic-flux. Autophagy was an important survival mechanism of AML cells. Inhibition of autophagy by hydroxychloroquine (HCQ) or knockdown of the essential autophagy genes ATG5 and ATG7 triggered p53-medated apoptosis. However, the p53-mediated apoptosis was dysfunctional in AMLs with a TP53 mutation. Therefore, TP53 mutant AMLs are less sensitive for HCQ-dependent apoptosis.

To determine whether the AML stem cell-enriched CD34$^+$ fraction contains cells that differ in their autophagy level, we separated AML CD34$^+$ cells based on the

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amount of intracellular ROS. Compared to AML mononuclear cells with high ROS levels, AML cells with low ROS levels have been shown to be enriched for leukemic stem cells. Separating AML CD34$^+$ cells based on ROS levels revealed a higher basal-autophagy activity and lower mitochondrial content in the AML CD34$^+$ROS^{low} cells. ROS^{low} cells were also more sensitive for HCQ-mediated apoptosis, indicating that autophagy is important for survival of these cells. Finally, the study showed that AML CD34$^+$ cells with knockdown of ATG5 failed to maintain leukemia *in vivo* after transplantation in NSG mice. In summary, these findings suggest that targeting autophagy could provide new therapeutic options for treatment of leukemia, especially in AMLs with wildtype TP53.

The aim of the study presented in **Chapter 6** was to identify which autophagy genes are differentially expressed in AMLs compared to normal bone marrow (NBM) controls. Interestingly, the putative autophagy protein VMP1 was more highly expressed in a subset of AML CD34$^+$ compared to NBM CD34$^+$ cells, in particular in AMLs with a monocytic phenotype. VMP1 can bind to the master autophagy regulator Beclin-1, thereby shifting the balance to induction of autophagy. Because little information was available regarding the role of VMP1 in hematopoiesis, we studied the functional consequences of VMP1 down regulation or overexpression. Downregulation of VMP1 expression by shRNA in CB CD34$^+$ cells showed reduced HSPC frequencies as determined with colony-forming cell assays. In line with this observation, these CB-derived CD34$^+$ cells had a reduced expansion rate under myeloid and erythroid-permissive culture conditions, coinciding with a delay in differentiation. In addition, transplantation of CB CD34$^+$ cells in mice after knockdown of VMP1 showed reduced engraftment compared to control cells. Similarly, in leukemic cell lines and primary AML CD34$^+$ cells, knockdown of VMP1 also resulted in a strong negative phenotype, which was at least in part due to increased apoptosis and reduced cell cycle progression.

Gene ontology (GO) analysis was performed with a publicly available AML gene expression datasets to identify gene sets which correlate with VMP1 expression. GO analysis revealed that VMP1 correlated positively with genes enriched for the GO terms lysosome and vesicle transport, but correlated inversely with genes enriched for GO terms mitochondria. To study whether VMP1 affects mitochondrial turn-over in AML, leukemic OCIM3 cells with overexpression or knockdown of VMP1 and control cells were analyzed with electron microscopy. Overexpression of VMP1 resulted in a marked decrease in mitochondrial structures, while the

number of lysosomal structures increased. In addition, cells overexpressing VMP1 had a higher autophagic-flux compared to control cells. The remaining mitochondria in the VMP1 overexpressing cells also appeared to perform better in terms of mitochondrial membrane potential and ATP production.

Members of the BCL-2 protein family regulate apoptosis by controlling the threshold for mitochondrial outer membrane permeabilization (MOMP), which is an early step in the apoptotic programming. VMP1 contains a BH3-binding domain, which is a characteristic of the BCL-2 protein family. Therefore, we hypothesized that high VMP1 expression could affect the MOMP threshold, thereby favoring survival of leukemic cells. To test this hypothesis, VMP1 overexpressing cells were cultured with venetoclax, which is a BH3 mimetic that blocks the anti-apoptotic function of BCL-2, thus de-repressing apoptosis. Leukemic cells overexpressing VMP1 were indeed less sensitive for venetoclax-induced apoptosis. These results demonstrate that VMP1 is essential for normal HSPC and AML cells by regulating mitochondrial quality control. The VMP1-mediated turnover of mitochondria therefore results in improved mitochondrial function and is protective against loss of MOMP and apoptosis induced by venetoclax treatment.

In summary, the studies reported in this thesis show that autophagy is an essential survival mechanism for normal HSPC, AML cells and MDS erythroblasts. Altered expression of autophagy-associated genes may have consequences for drug resistance. Therefore, these findings suggest that modulation of autophagy could be a promising therapeutic strategy for the treatment of AML.

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Discussion and future perspectives

To develop new therapeutic strategies in AML, we must improve our understanding of the intrinsic and extrinsic cellular mechanisms that control HSC maintenance. To this end we studied the role of autophagy in both normal and leukemic hematopoiesis.

Autophagy is essential for homeostasis in HSPC

Autophagy is generally assumed to be a pro-survival pathway that can preserve organelle function and mitigate cellular stress. Most studies on autophagy in hematopoiesis have been performed in mice by using knockout models of essential autophagy genes [1-3]. However, its functions in human hematopoiesis have been remained largely unexplored. We observed that the autophagic flux was high in human HSPCs and declined with cellular differentiation. Cytokine concentrations did not affect the level of autophagy in human HSPCs, in contrast to murine HSCs in which autophagy induction was observed upon cytokine deprivation [3, 4]. The observed high autophagy flux in human HSPCs is likely an intrinsic property of the HSPC. In mice, it has been shown that aging is associated with myeloid skewing [5], which has also been observed in mice with mono-allelic knockout of ATG7 and ATG12 [3, 6]. However, an increased lifespan of mice was observed after autophagy induction, which can be accomplished by using the mTOR inhibitor rapamycin [7]. These findings suggest that autophagy regulates stem cell function during aging. Future studies focusing on HSCs in the context of aging, preferentially in a more humanized microenvironment, are crucial to improve our understanding of autophagy in human hematopoiesis.

Knockdown of ATG5 or ATG7 prevented *in vivo* engraftment of human HSPCs after intravenous injection, indicating that autophagy is essential for long-term engraftment of human HSPCs *in vivo*. Inhibition of autophagy in HSPCs caused a delayed cell cycle progression and increased apoptosis. Moreover, ATG5 or ATG7 knockdown in human HSPCs resulted in increased ROS levels, which probably reflects accumulation of dysfunctional mitochondria. A similar phenotype has been observed in a hematopoietic-specific ATG7 knockout mouse model [8]. ROS plays a central role in cell signalling, and excessive oxidative stress can initiate mitochondrial-mediated apoptosis [9]. In addition, elevated ROS levels can also cause activation of tumor suppressor p53, resulting in cell cycle arrest and apoptosis [10]. Indeed, apoptosis induced by knockdown of ATG5 or ATG7 could

partially be rescued by the additional knockdown of p53, indicating that inhibition of autophagy causes p53 activation. A recent study has also shown that inhibition of autophagy prevented autophagy-mediated turn-over of FOXO3A, resulting in FOXO3a-medited upregulation of PUMA and consequently in apoptosis [11]. This could be an alternative explanation for induction of apoptosis as a consequence of autophagy inhibition. Together, these results show that HSPCs are dependent on functional autophagy for their survival by limiting cellular stress.

Mitophagy controls mitochondrial function in HSPCs

Mitophagy, a specific type of autophagy, is involved in clearing redundant or damaged mitochondria, thereby limiting accumulation of ROS [6]. Following differentiation, HSCs become metabolically more active in order to support cell growth and differentiation [3, 12]. Mitochondria are important bioenergetic organelles that produce biosynthetic metabolites and ATP [13]. For example, mitochondrial metabolites can be used to synthesize phospholipids, which are required for the construction of cellular membranes. In mice, mitophagy activation was shown to actively supress myeloid proliferation by limiting mitochondrial metabolism in HSPCs [3]. However, in contrast to studies in mice [3, 6], inhibition of autophagy in human HSPCs did not result in increased myeloid proliferation.

The high autophagic-flux in HSC might also indicate that immature stem cell populations could be identified by measuring mitochondrial activity. For this approach the appropriate techniques for studying mitochondrial content and function should be used. Mitochondrial mass can be determined by quantifying mitochondrial DNA relative to nuclear DNA, by using electron microscopy, or by using dyes. Mitochondrial activity can be determined by measuring the mitochondrial membrane potential (MMP), measuring ROS levels (a by-product of oxidative metabolism) or by directly measuring ATP. Importantly, MitoTracker a commonly used dye for staining mitochondria mass was shown to be effluxed out of cells via Ca^{2+} sensitive xenobiotic efflux pumps, resulting in unreliable results [14]. Therefore, different complementary assays should be used for quantifying mitochondrial function in cells.

In mice, the most immature HSC population has been defined based on the mitochondrial membrane potential [15] and mitochondrial mass [3, 15, 16]. Similarly, cells with low ROS levels had a higher self-renewal potential compared to cells with high ROS levels [3, 17]. These results appear to be contradicted by a recent

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study demonstrating that HSCs have higher mitochondrial mass compared to more differentiated cells [14]. Despite the higher mitochondrial mass in HSCs, the respiratory capacity in these cells was low [14]. Although it remains unclear what caused this disparity, it might be related in part to differences in experimental setup.

Based on *in vivo* transplantation experiments of Jordan's group, mononuclear AML cells can be separated into AML ROS^{low} and ROS^{high} cell populations. The AML ROS^{low} mononuclear cells are metabolically less active and enriched for leukemic stem cells [12, 18]. Our results show that CD34$^+$ROS^{low} cells are smaller, have lower mitochondrial mass and mitochondrial activity, and higher autophagy activity compared to AML CD34$^+$ROS^{high} cells. These findings are in line with the recent study of Pei *et al.* demonstrating that AML ROS^{low} cells have constitutively active AMKP signalling, which is an upstream positive regulator of FIS1-mediated mitophagy [18]. These findings suggest that leukemic stem cells rely on functional mitophagy for limiting mitochondrial activity and ROS production in order to maintain a more quiescent cellular state.

Autophagy in MDS

In myelodysplastic syndrome (MDS), intrinsic defects in HSCs are believed to be the driving forces for clonal expansion of dysplastic cells. This is reflected by bone marrow hyper-cellularity, defective maturation of different cell lineages and peripheral blood cytopenias [19, 20]. This apparent conundrum can be explained by increased programmed cell death or apoptosis in developing hematopoietic cells. However, only limited apoptosis was observed in megakaryocytes and erythroblasts freshly isolated from MDS bone marrow [21, 22]. Instead, MDS erythroid precursor cells showed signs of increased autophagy [22, 23]. The increased autophagy activity observed in MDS cells did not coincide with increased expression of the essential autophagy genes ATG5, ATG7, Beclin-1 and LC3. The MDS patients in these studies belonged predominantly to the subcategory MDS with ring sideroblasts (MDS-RS), a subgroup that is associated with a high prevalence of SF3B1 mutations [24]. Importantly, knockdown of SF3B1 has been shown to cause accumulation of iron in mitochondria, resulting in the development of ring sideroblasts [25]. Therefore it is conceivable that the mitochondrial iron overload causes cellular stress, which could trigger an autophagy response [26]. In line with these results, basal-autophagy has been shown to be upregulated in SF3B1 mutant cells [27, 28]. In another study, this

process corresponded with deregulation of genes associated with mTOR and AMPK signalling pathways [29]. Both the mTOR and AMPK pathways play a central role in regulation of autophagy. Together, these findings suggest that SF3B1 mutant cells activate autophagy in order to remove damaged mitochondria and prevent cellular stress caused by the excessive accumulation of iron.

Genome-wide gene expression profiling of a large cohort of low-risk MDS patient samples show alterations in genes involved in apoptotic programming, but not in core autophagy genes. Interestingly, the anti-apoptotic BCL-2 gene was silenced [30]. This might have consequences for the autophagy process since BCL-2 can also interact with Beclin-1. thereby repressing autophagy [31]. Silencing of BCL-2 could therefore potentially result in de-repression of autophagy due to an altered balance between BCL-2 and Beclin-1 [31]. In addition, aberrant splicing of ATG7 is another mechanism that can alter the level of autophagy activity in MDS cells. In 10% of MDS patients the splicing factor U2AF35 is mutated, which results in reduced expression of ATG7 [32]. Studies in mice have shown that the absence of ATG7 expression ultimately induces a myeloproliferative disorder with signs of MDS [6, 33]. These findings indicate that an altered autophagy process can be observed in MDS, which might contribute to malignant transformation.

A role for the microenvironment in MDS

Recent studies have indicated that the bone marrow microenvironment plays a critical role in the pathophysiology of MDS [34, 35]. However, it is difficult to study MDS in the context of its "own" micro-environment. For example, traditional patient-derived mouse xenograft models were unsuccessful in sustaining the propagation of MDS CD34$^+$ cells. It is conceivable that the mice bone marrow micro-environment does not completely recapitulate the human bone marrow niche. Attempts have been made to improve the engraftment of MDS cells by co-injection with human MSCs. However, the results so far have been inconsistent [36, 37]. We therefore investigated whether low-risk MDS-derived erythroid precursor cells are less prone to undergo programmed cell death if they are lodged in their own micro-environment. To this end, we determined in MDS whether the survival of freshly isolated erythroblasts from the mononuclear cell fraction (MNC) differed from erythroblasts embedded in hematons [38, 39]. MDS patient-derived hematons contained, among other cell types, endothelial and mesenchymal cells, which could represent a hematopoietic niche structure. Importantly, MDS erythroblasts embedded in their own micro-environment had

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significantly better survival. In other tumour types a similar strong interaction with the microenvironment has been shown. For example, detachment of breast cancer cells resulted in mitophagy activation, coinciding with a decline in mitochondrial content, inadequate NADPH production, increased ROS levels and non-apoptotic cell death [40]. MDS cells could have a similar response when they are dislodged from their microenvironment. A study in Shwachman Diamond Syndrome, which is a pre-leukemic disorder, suggested that the micro-environment could be a dominant factor in the transformation process [41]. In contrast, the majority of human studies have indicated that the microenvironment is supportive for the malignant clone. Malignant cells can potentially induce changes to the microenvironment, making the microenvironment more supportive for malignant cells versus normal HSPCs.

Autophagy in AML
In the second part of this thesis, we studied the role of autophagy in acute myeloid leukemia. Autophagy-associated genes have been shown to be mutated in ~14% of the MDS and AML patients [42]. This suggests that the autophagy process could be relevant during leukemia initiation and/or maintenance. To test this hypothesis, we determined the level of autophagy and the dependency on functional autophagy in AML CD34$^+$ cells.

A variation in autophagy activity was observed between various leukemic cell lines and primary AML samples. In particular, patients with poor-risk features with a complex karyotype had a higher autophagy activity compared to AMLs with low-risk and intermediate-risk features. Importantly, autophagy activity has also been associated with increased drug resistance in leukemia [43-46]. This suggests that the increased autophagy activity in the AML cells could be one of mechanisms contributing to increased drug resistance in poor-risk AML. Moreover, patients in our cohort with poor-risk features had a high frequency of TP53 mutations. Mutant TP53 protein accumulates in cells due to increased protein stability [47]. Therefore, autophagy could be increased to limit cellular stress. However, modulation of p53 expression by itself did not affect autophagy activity, suggesting that the p53 status in the leukemic cells does not dictate autophagic-flux. Besides the complex karyotype and TP53 mutations, no additional recurrent mutations could be identified in our study that correlated with altered autophagy activity. This is in contrast with a study from Heydt *et al.*, in which FLT3-ITD mutations were associated with increased basal-autophagy

[46]. Interestingly, in that study FLT3-ITD presence was linked to increased ROS formation and DNA damage [46]. However, those results were obtained with cell lines with over-expression of FLT3-ITD, which might provide different results compared to primary AML CD34^{+} cells.

**Autophagy as a therapeutic target in AML**

Because autophagy is important for survival of AML cells and is frequently associated with drug resistance, we explored whether autophagy could be used as potential therapeutic target in AML. The anti-malaria drug hydroxychloroquine (HCQ) has been used to inhibit autophagy, especially in combination with anticancer agents ([48, 49] **Table 1, Chapter 2**). However, inconsistent results have been obtained for autophagy inhibition at the maximum tolerated dosage in several clinical trials (**Table 1, Chapter 2**). In a recent screening, based on testing synthetic derivatives of chloroquine, Lys05 was identified as a promising and more potent autophagy inhibitor [50]. Lys05 was also an effective autophagy inhibitor *in vivo* and potentially has improved anti-leukemic properties compared to HCQ [50]. Besides systemic administration of autophagy inhibitors, antibody-drug conjugates could also be designed to deliver autophagy inhibitors more specifically to AML cells [51]. For example, this could be done by targeting differentially expressed plasma membrane proteins relative to normal HSPCs [51, 52].

VMP1 and hypoxia

We observed that the putative autophagy protein VMP1 is more highly expressed in AML compared to control. It has been shown that VMP1 can be regulated by HIF1 in human colon cancer cell lines. HIF1 stabilization increases VMP1 expression by binding to the hypoxia-responsive element in the VMP1 promoter region [53]. We hypothesized that VMP1 might be actively transcribed in AML cells residing in the hypoxic bone marrow micro-environment. However, in contrast to experiments with colon cancer cell lines, chip-seq experiments did not show HIF1 or HIF2 binding sites near the VMP1 promoter region in CB CD34^{+} cells (Wierenga *et al.*, manuscript submitted). In addition we observed a decrease in VMP1 protein levels in leukemic cell lines after 48 hrs of culture under hypoxic conditions. (**Fig. 1**). These findings suggest that VMP1 is not dominant in controlling autophagy during hypoxia in AML cells [54]. It is more likely that BNIP3 and BNIP3L are the main proteins to induce autophagy in response to hypoxia [55]. In line with these findings, we observed HIF1 binding to the promoter of BNIP3 and BNIP3L in CB CB34^{+} cells (data not shown). Together, these findings suggest that during hypoxia

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Fig. 1 Western blot showing TOM20 and VMP1 levels in HL60, OCIM3, MOLM13 and THP1 cells cultured under hypoxia (H) or normoxia (N) for 48 hrs, B-actin was used as control.
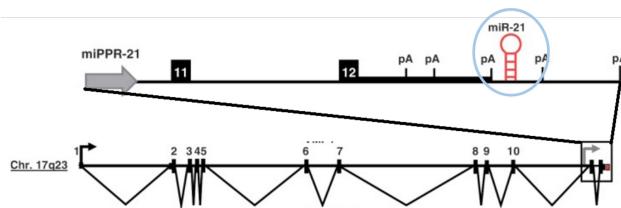
Fig. 2 Figure indicating the structure of the VMP1 and miR-21 loci at chromosome 17q23. The black and white numbers indicate exons of VMP1. The gray arrow indicates the miR-21 promotor and the red hairpin indicates the location of pre-miR-21. The black arrow represents the transcription start site of VMP1 [56].
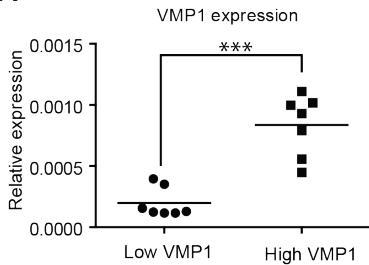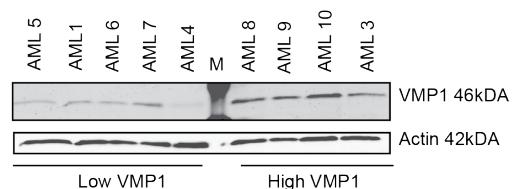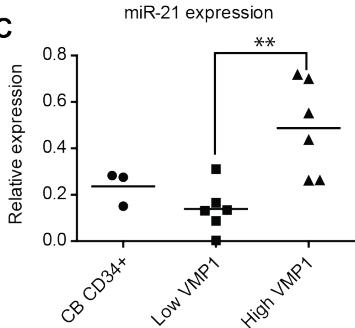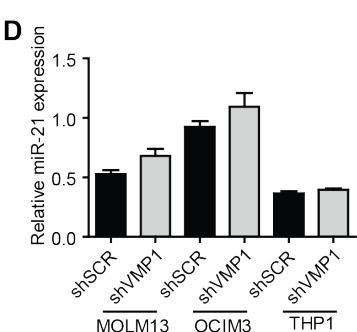
Fig. 3, A-B) VMP1 expression was confirmed at mRNA and protein level by qPCR and Western blotting in low- and high-VMP1 expressing AML CD34+ cells. **C)** miR-21 expression was determined by qPCR in cord blood (CB)-derived CD34+ cells and in low- and high-VMP1 expressing AML CD34+ cells, RNU48 was used as control. **D)** miR-21 levels in MOLM13, OCIM3 and THP1 cell lines with and without VMP1 knock down, RNU48 was used as control.

VMP1 does not facilitate increased mitochondrial turn-over; BNIP3 and BNIP3L are more likely candidates for regulation of mitophagy in response to hypoxia.

VMP1 as a source for onco-miR-21

Interestingly, the VMP1 gene is located upstream of onco-miR-21 [56]. Micro-RNAs are small non-coding RNAs that degrade mRNA thereby reducing translation. Although VMP1 and miR-21 both have their own promoter (**Fig. 2**) in human breast cancer cells, it has been shown that VMP1 transcripts can bypass polyadenylation signals, resulting in a VMP1-miR-21 transcript, thus providing an alternative source for miR-21 [56]. Importantly, miR-21 has also been linked to leukemia development [57]. Studies in mice demonstrated that elevated miR-21 expression caused pre-B-cell lymphoma-like phenotype [58]. In addition, miR-21 was associated with repression of SMAD7 signaling, which can be triggered by the cytokine TGF- [59, 60]. Therefore, we hypothesized that enhanced VMP1 expression in AML might be a source for onco-miR-21 expression, which could potentially contribute to malignant transformation. AMLs with low or high VMP1 expression were preselected based on available gene expression data [61]. Low or high VMP1 expression was confirmed on the mRNA and protein level in the selected AMLs (**Fig. 3A-B**). miR-21 expression levels positively correlated with VMP1 levels in primary AML CD34$^+$ cells (**Fig. 3C**). However, knockdown of VMP1 did not affect miR-21 levels in AML cell lines (**Fig. 3D**). CHIP-seq studies have demonstrated that the promoter regions of both VMP1 and miR-21 had active K27ac marks, while the repressive H3K27ME3 marks were absent (data not shown), suggesting that VMP1 and miR-21 are both actively transcribed. Together these findings suggest that it is more likely that miR-21 expression is not connected to VMP1 expression, but that VMP1 and miR-21 expression are regulated by overlapping upstream regulatory pathways in AML.

Conclusion

The results of my thesis research provide new insights into the involvement of autophagy as a central homeostatic process in both normal and leukemic hematopoiesis. One of the main functions of autophagy in hematopetic cells is controlling mitochondria, which play a central role in stem cell (and leukemic stem cell) function and differentiation. Subpopulations of leukemic cells often persist after chemotherapy treatment. Differences in metabolic activity could potentially be used as a tool to identify these persisting subpopulations. Subsequently, novel techniques, such as single cell sequencing or single cell CHIP, can be used

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to detect genetic and epigenetic alterations. Ultimately, this could provide new insights into the molecular mechanisms that enable leukemia-reinitiating cells to survive following chemotherapy treatment.

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Nederlandse samenvatting

In het menselijk lichaam worden constant nieuwe bloedcellen aangemaakt, dit proces wordt bloedcelvorming of hematopoëse genoemd. Er zijn verschillende soorten bloedcellen: rode bloedcellen, witte bloedcellen en bloedplaatjes. Rode bloedcellen, ook wel erytrocyten genoemd, verzorgen het transport van zuurstof en koolstofdioxide tussen de longen en verschillende weefsels. Witte bloedcellen, ofwel leukocyten, zijn een belangrijk onderdeel van het immuunsysteem van het lichaam. Bloedplaatjes of trombocyten zijn belangrijk voor de bloedstolling, bijvoorbeeld na verwonding. Bloedcellen hebben een beperkte levensduur waardoor er in het beenmerg constant nieuwe bloedcellen worden gevormd door hematopoietische stamcellen. De vorming van deze bloedcellen gebeurt stapsgewijs door middel van een proces wat differentiatie wordt genoemd. Daarnaast kunnen hematopoietische stamcellen zichzelf vernieuwen, waardoor de populatie aan stamcellen in het beenmerg in stand blijft. In de loop van het leven kunnen er afwijkingen (ofwel mutaties) ontstaan in het DNA (de genetische code) van de bloedvormende cellen. Hierdoor kan het proces van differentiatie en zelfvernieuwing verstoord raken wat kan leiden tot bloedkanker (leukemie). In die situatie wordt de normale bloedcelvorming verdreven. Er zijn verschillende soorten bloedkanker, waaronder het myelodysplastisch syndroom (MDS) en acute myeloïde leukemie (AML). MDS kan in sommige gevallen ontaarden in AML, een zeer agressieve vorm van leukemie. In beide situaties hebben de kwaadaardige stamcellen een groeivoordeel ten opzichte van de normale cellen waardoor er een tekort kan optreden van rode bloedcellen, witte bloedcellen, en bloedplaatjes.

Autofagie is een mechanisme binnen de cel waarmee overbodige organellen en eiwitten worden afgebroken. Deze vrijgekomen "bouwstenen" kunnen vervolgens weer worden hergebruikt. Autofagie wordt meestal geassocieerd met een betere overleving van cellen en is tevens een beschermingsmechanisme tegen de celdodende werking van chemotherapie. In dit proefschrift is het belang van autofagie bestudeerd in de context van normale hematopoëse en leukemie. Het is reeds bekend dat autofagie belangrijk is voor de differentiatie van bloedcellen. Wij hebben onderzocht hoe actief het autofagie proces is in onrijpe bloedvormende stam- en voorlopercellen en hoe dit verandert tijdens het uitrijpingsproces. Deze onderzoekingen tonen aan dat stamcellen een hogere autofagie activiteit hebben dan meer uitgerijpte bloedcellen. Daarnaast

is autofagie belangrijk voor de overleving van stamcellen wanneer deze cellen worden gebruikt voor een transplantatie.

Wanneer er sprake is van MDS, dan vormen de afwijkende stamcellen in het beenmerg misvormde (dysplastische) cellen. Deze dysplastische cellen zijn eerder geneigd om celdood te ondergaan. Hierdoor kan er een tekort aan functionele bloedcellen ontstaan. Daarnaast blijken ook afwijkingen in beenmerg ondersteunende cellen (ook wel het beenmergmicromilieu genoemd) een rol te spelen bij het ziekteproces. Daarom hebben wij de rol van het beenmergmicromilieu bestudeerd in de context van celdood van MDS cellen en met name de cellen die verantwoordelijk zijn voor de vorming van rode bloedcellen. Het blijkt dat MDS cellen omgeven door cellen uit het eigen beenmergmicromilieu minder gevoelig zijn voor celdood. Mogelijk speelt een versterkte autofagie hierbij een rol met name om afwijkende mitochondriën (ook wel bekend als de energiecentrale van de cel) in erytroïde cellen op te ruimen die beladen zijn met ijzer wat veelvuldig voorkomt bij dit type MDS.

Uit de bovengenoemde gegevens blijkt dat autofagie een belangrijk cellulair mechanisme voor gezonde hematopoietische cellen is. Op basis hiervan hebben wij de rol van autofagie onderzocht in humane AML cellen. Autofagie activiteit blijkt hoger in AMLs met een ongunstig risicoprofiel. Deze AMLs hebben ook vaker mutaties in het TP53 gen, wat een beschermende invloed kan uitoefenen op deze cellen wanneer het proces van autofagie wordt geblokkeerd. Echter uit aanvullende onderzoekingen blijkt dat het TP53 eiwit niet de directe prikkel is voor een hogere autofagie activiteit maar mogelijk samenhangt met metabole veranderingen in deze cellen.

In het vervolgonderzoek hebben we gekeken naar de functie van een nog niet goed bestudeerd autofagie gen in de hematopoëse, namelijk VMP1. Om de functie van VMP1 beter te begrijpen hebben wij VMP1 verminderd tot expressie gebracht in normale en leukemische hematopoietische cellen. Een verminderde aanwezigheid van VMP1 veroorzaakt een afname in autofagie. Deze afname in autofagie is geassocieerd met een sterke afname in het aantal onrijpe bloedcellen waarbij ook de uitrijping van deze cellen verstoord is. In AML cellen blijkt VMP1 hoger tot expressie te komen in vergelijking tot gezonde cellen. Een toename in VMP1 expressie resulteert in een toename in autofagie activiteit en daardoor in een afname van het aantal mitochondriën.

Ook blijken deze cellen minder gevoelig te zijn voor venetoclax, een medicijn welke celdood induceert door in te grijpen op mitochondriale functies.

In dit proefschrift hebben wij laten zien dat autofagie essentieel is voor de overleving van zowel gezonde als leukemische hematopoietische stam- en voorlopercellen. Vervolg onderzoek zal in de toekomst duidelijk maken in hoeverre de remming van autofagie de effecten van chemotherapie op AML cellen nog verder kan versterken.

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Publicaties

1. **Folkerts H**, Hilgendorf S, Vellenga E, Bremer E, Wiersma VR
   The multifaceted role of autophagy in cancer and the micro-environment.
   Med Res Rev. **2018**, 10.1002/med.21531

2. **Folkerts H**, Hilgendorf S, Wierenga ATJ, Jaques J, Mulder AB, Coffer PJ, Schuringa JJ, Vellenga E
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3. Gómez-Puerto MC, **Folkerts H,** Wierenga AT, Schepers K, Schuringa JJ, Coffer PJ Vellenga E
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4. **Folkerts H**, Hazenberg CL, Houwerzijl EJ, van den Heuvel FA, Mulder AB, van der Want JJ, Vellenga E
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5. Van Roermund, CWT, IJlst L, Majczak W, Waterham HR, **Folkerts H**, Wanders RJA and Hellingwerf KJ
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6. Hilgendorf S, **Folkerts H**, Schuringa JJ, Vellenga E
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7. **Folkerts H**, Pougovkina OA, Ofman R, Gloerich J, Wanders RJA, Houten SM, de Boer VCJ
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   Poster presentation, Bioenergetics meeting, April 13 - April 15, **2011**, Wageningen, The Netherlands

8. Van Beilen J, Blohmke CJ, **Folkerts H**, de Boer R, Zakrzewska A, Kulik W, Vaz FM, Brul S, Ter Beek A
   RodZ and PgsA Play Intertwined Roles in Membrane Homeostasis of Bacillus subtilis and Resistance to Weak. Organic Acid Stress.
   Front Microbiol. 2016, 7:1633. eCollection

Prijzen

American Society of Hematology 2017   Abstract Achievement Award
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American Society of Hematology 2014   Abstract Achievement Award