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AUTOPHAGY IN HEMATOPOETIC STEM CELLS

The role of autophagy in hematopoietic stem cells, aging, acute myeloid leukaemia and acute myeloid leukaemia treatment

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Experimental Haematology | UMCG

Autophagy in hematopoietic stem cells

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Word count:
6359 words

Date:
25-11-2019

Abstract

Autophagy is a multistep intracellular degradation and recycling mechanism that is mainly used during cellular stress. Autophagy is used for the specific or non-specific degradation of dysfunctional organelles, like mitochondria, and protein aggregates. There are three different forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. These three forms differ in their target specificity, function and mechanisms but are all based on lysosomal degradation.

Autophagy seems to be implicated in many different ways, in different cell types and different diseases. One important implication of autophagy is its role in hematopoietic stem cells where it serves as a pro-survival mechanism by maintaining quiescence, allowing hematopoietic stem cells to preserve their quality and self-renewing capability. During the aging process hematopoietic stem cells seem to lose their quiescence, which is due to a reduced autophagic-flux. The loss of this degradation process often results in the accumulation of damage in a cell which could contribute to the development of age-related diseases like acute myeloid leukaemia. The role of autophagy in this specific disease is very complex, but it often seems to be increased in leukemic cells compared to hematopoietic stem cells. Additionally, autophagy seems to be induced by chemotherapy and provides a survival mechanism for cancer cells. Inhibition of autophagy during anti-cancer therapy therefore might be beneficial to reduce AML relapse.

Most studies regarding the role of autophagy have not focussed on specific subtypes of AML, therefore the results could be confounded by differences in genetic and transcriptomic profiles. Given the large amount of heterogeneity observed in tumours, it might be beneficial to study autophagy in a more single cell based context.

The purpose of this review was to provide an overview of recent discoveries regarding the role of autophagy in HSCs, aging, AML and AML treatment.

Abbreviations

Acute Myeloid Leukaemia	- AML
Autophagy related	- ATG
Bafilomycin A	- BafA
Bone marrow	- BM
Chaperone Mediated Autophagy	- CMA
Hydroxychloroquine	- HCQ
Hematopoietic Stem Cells	- HSCs
Hematopoietic Stem/Progenitor Cells	- HSPCs
Internal Tandem Duplication	- ITD
Knock-out	- KO
Leukemic Stem Cells	- LSCs
Micro RNA	- miRNA
Oxidative phosphorylation	- OXPHOS
Quantitative Polymerase Chain Reaction	- qPCR
Reactive Oxygen Species	- ROS
Short hairpin RNA	- shRNA

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Introduction

Autophagy is a multistep intracellular process for the degradation of cellular components, providing a survival mechanism for cells during cellular stress[1,2]. Additionally, autophagy serves as a recycling mechanism since it can reuse the degraded products as building blocks for cellular renovations and helps to keep cellular homeostasis[1].

Eukaryotes have two mechanisms for the degradation of cellular components: proteasomes and lysosomes[1]. Proteasomes are used for the selective degradation of ubiquitinylated proteins whereas lysosomes are used for the selective and unselective degradation of extracellular materials and plasma membrane proteins[1]. During autophagy the materials and organelles residing in the cytoplasm are degraded by lysosomes[1]. There are three different forms of autophagy; macroautophagy, microautophagy and Chaperon-Mediated Autophagy (CMA)[1,3]. These three pathways differ in their mechanism and function, as outlined by Figure 1[3].

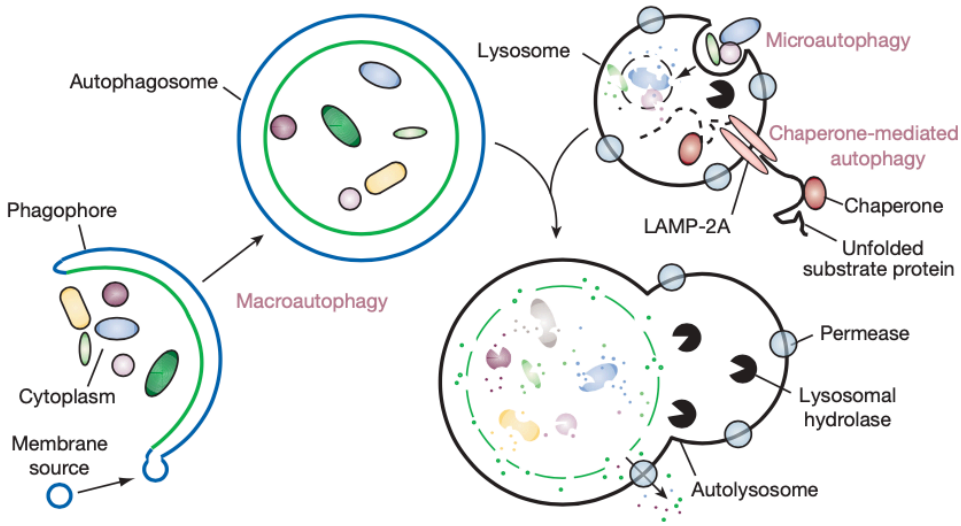

Figure 1 |Types of autophagy; macroautophagy, microautophagy and chaperone-mediated autophagy[3]

In macroautophagy, here after referred to as autophagy, a piece of intracellular membrane is isolated, referred to as a phagophore[1,3,4]. The phagophore can be used to form an autophagosome containing intracellular soluble materials and organelles[1,3,4]. The autophagosome then fuses with a lysosome, thereby becoming an autolysosome resulting in the degradation of the internal components[1,3,4]. During microautophagy a lysosome engulfs materials of the cytoplasm by endocytosis[1,3,4]. Autophagy and microautophagy can be used for the selective and unselective degradation of large structures[3]. CMA on the other hand is a more selective form of autophagy in which soluble proteins are not engulfed by the lysosome but are instead translocated directly across the lysosomal membrane by using chaperone proteins[1,3].

To date, around 40 proteins are identified to be involved in the autophagy pathways. These Autophagy-related (ATG) proteins can be divided in core proteins and additional proteins[2]. The core proteins are highly conserved and often involved in the formation of the autophagosome. Several studies, including multiple mouse models, have extensively studied the role of ATG5, ATG7 and ATG12 particularly in the hematopoietic system and aging[5]. These studies have implicated that besides providing a pro-survival mechanism for cells during

stress, autophagy also seems to be implicated in many other cellular processes like homeostasis, aging, disease onset, and the maintenance and quality control of stem cells[2,5].

One important application of autophagy seems to be in the maintenance of hematopoietic stem cells (HSCs)[2,5]. HSCs reside in the bone marrow (BM) where they preserve their self-renewal capacity through maintaining a quiescent state[6]. Cell divisions often result in the accumulation of DNA damage and dysfunctional mitochondria, eventually resulting in cell death or disease onset[6,7]. The slow cycling rate of HSCs therefore is thought to contribute to the self-renewal capacity, since this quiescent state helps to preserve the stem cell quality[6,7]. Deletion of genes involved in quiescence is shown to result in stem cell exhaustion due to the uncontrolled proliferation of HSCs[8]. During the cell division of HSCs one, both or neither of the daughter cells will maintain their stem cell properties[6,7]. The differentiated daughter cells, the multipotent stem cell, will form the starting point for a hierarchical process called haematopoiesis, in which HSCs give rise to all the mature blood cells in the human body during hematopoietic recovery[6,7]. HSCs that differentiate into precursor blood cells seem to lose their quiescence and switch their metabolism from aerobic glycolysis to mitochondrial oxidative phosphorylation (OXPHOS), due to the increase in energy demand[6,9].

The multipotent progenitor cells can give rise to two different lineage branches, the lymphoid and myeloid branch (Figure 2)[7,10]. The lymphoid progenitor cell can differentiate into natural killer cells, T-lymphocytes, B-lymphocytes and plasma cells[7,10]. These cells carry out the adaptive and innate immune responses in the human body[7]. The myeloid progenitor cells can give rise to myeloblasts that will differentiate into either monocytes, neutrophils, basophils or eosinophils that are all short-lived cell types[7,10]. Additionally, they can differentiate into erythroblasts that will give rise to reticulocytes and eventually to red blood cells[7,10]. Lastly, myeloid progenitor cells differentiate into megakaryocytes that will mature into platelets[7,10].

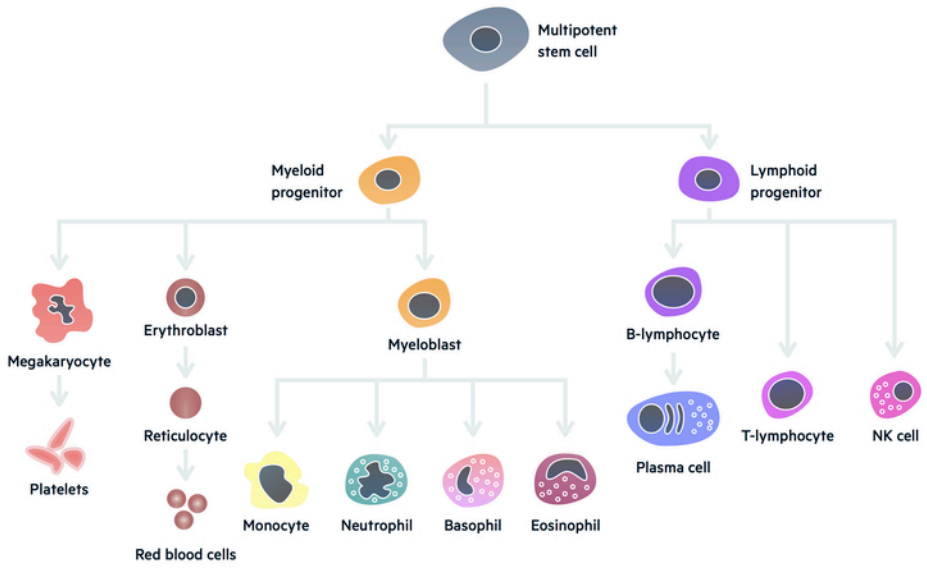

Figure 2 | Haematopoiesis: lineage branches[8]

Malignancies during haematopoiesis, like uncontrolled cell proliferation resulting in an accumulation of immature myeloid cells in the bone marrow, could initiate the onset of Acute Myeloid Leukaemia (AML)[11,12]. With AML there is an infiltration of poorly differentiated cells in the hematopoietic system in the BM of patients[11,12]. AML is mainly diagnosed in children

and elderly above the age of 60. Over the last few decades a lot of advances have been made regarding the treatment of AML, the prognosis however for most patients is still very poor due to treatment resistance and high relapse rates[12].

Studies have shown that AML is most likely initiated by HSCs that contain so called founder mutations in genes involved in epigenetics, like DNMT3A, ASCL1, IDH2 and TET2, forming Leukemic Stem Cells (LSCs)[12,13]. Over time these pre-leukemic cells will likely obtain additional somatic mutations providing a growth and survival advantage[12]. Cells containing these pre-leukemic mutations are often resistant to chemotherapy and are likely involved in cancer relapse[12]. Studies regarding the mechanisms involved in AML relapse are needed in order to increase the progress-free survival rate[12]. One mechanism that seems to be implicated in AML relapse is autophagy, indicating that this degradation pathway might not only be a pro-survival mechanism for cells during stress[1].

Since autophagy seems to be involved in many different biological processes, protective on one hand and tumour initiating on the other, it is important to get a clear view of the role of autophagy in health and disease[1]. If autophagy is involved for instance in the initiation of diseases like AML, then perhaps it can also be targeted to prevent of treat diseases. This review will provide an overview of recent discoveries regarding the role of autophagy in HSCs, aging, AML and AML treatment.

The role of autophagy

Role of autophagy in HSCs

Over the last two decades a lot of studies, especially in animal models, have suggested that autophagy might be involved in hematopoietic cell function[14]. One of these studies is the 2013 study conducted by Warr et al. in which they provided evidence that autophagy plays an essential role in the life-long protection and maintenance of HSCs during metabolic stress[14]. Additionally, they identified FoxO3a as a critical gene involved in this protective mechanism[14]. Warr et al. isolated HSCs from transgenic mice and confirmed autophagy induction upon ex vivo cytokine withdrawal by GFP-LC3 puncta[14]. The decrease in LC3 puncta after cytokine withdrawal indicates a role of autophagy in metabolic stress[14]. These experiments were also conducted in vivo in transgenic Gfp-Lc3 mice and again autophagy was shown to be induced in HSCs upon caloric restriction[14]. The protective function of autophagy was assessed by monitoring the levels of apoptosis in HSCs with and without cytokines [14]. The inhibition of autophagy with BafA resulted in a significant increase in apoptosis during starvation[14]. ATG12 knock-out (KO) mice were used to demonstrate the direct role of autophagy in this protective mechanism[14]. Similar to BafA, the deletion of ATG12 resulted in a significant decrease of HSCs due to apoptosis[14]. Warr et al. identified FoxO3a as transcriptional regulator capable of maintaining the expression of pro-autophagy genes in HSCs[14]. FoxO3a$^{-/-}$::Gfp-Lc3 mice confirmed lower levels of autophagy in HSCs and less autophagy induction, demonstrating the role of FoxO3a in maintaining the pro-autophagy gene expression[14]. With this study, Warr et al. proved that autophagy is essential for HSCs under metabolic as a survival mechanism[14]. This pro-survival pathway seems to be regulated through FoxO3a expression, which acts as a transcription regulator of autophagy related genes[14]. Additionally, Cao et al. demonstrated in 2014 that the loss of autophagy during metabolic stress decreased the number of cells going into G1/S phase of the cell cycle[15]. This study provided evidence that autophagy might be involved in regulating the cell cycle entry of HSCs during metabolic stress, thereby maintaining the HSCs population and haematopoiesis[15].

Numerous other studies demonstrated the importance of autophagy in the maintenance of HSCs, not only during metabolic stress[16–19]. Core autophagy protein ATG7 was shown to be important in maintaining HSCs[16–18]. The deletion of this gene in mice in multiple studies demonstrated that a loss of autophagy resulted in a loss of HSCs function and divergences in the hematopoietic system caused by dysregulated differentiation[16–18].

Studies, like the 2015 study of Folkerts et al., added to this growing body of evidence. They demonstrated with a knockdown of autophagy proteins ATG5 and ATG7 that these proteins are essential for the maintenance of Hematopoietic Stem/Progenitor Cells (HSPCs)[19]. During this study they first provided evidence that the autophagic flux of HSPCs is higher compared to more differentiated cells like myeloid and lymphoid progenitor cells similar to results of Warr et al[14,19]. To demonstrate the necessity of this process they used short hairpin RNAs (shRNA) to create a knockdown of ATG5 and ATG7[19]. They tested the effect of the lentiviral knockdown in vitro in CD34^{+} HSPCs as well as in vivo by transplantation of CD34^{+} cells in immunodeficient NSG mice[19]. The knockdown of either ATG5 or ATG7 in vivo and in vitro resulted in a reduced HSPC frequency, indicating an essential role for autophagy in the maintenance of these cells[19]. The reduced HSPC frequency could be caused by either a reduction in cell differentiation or cell cycle progression[19]. Folkerts et al. performed a cell cycle analysis which indicated that less cells entered the S-phase after the knockdown of ATG5 or ATG7[19]. This finding coincided with an increase in expression of p21, which is a cell-cycle

dependent kinase inhibitor[19]. These results are in concordance with the findings from Yan Cao et al.[15]. Additionally, Folkerts et al. demonstrated an increase in apoptosis of HSPCs upon knockdown, together with an increase in cleaved caspase-3, p53, BAX and PUMA which are all proteins involved in the promotion of apoptosis[19]. Lastly, measurements with H2DCFDA, a Reactive Oxygen Species (ROS) detection assay kit, showed an increase in intracellular ROS levels[19]. With this study, Folkerts et al. provided insight into the role of autophagy in HSPC maintenance and the molecular mechanisms underlying this phenomenon[19].

A recently published article focussing on ATG5 confirmed all these previously discussed findings[20]. With the conditional deletion of ATG5 in mice Jung et al. showed that lack of autophagy resulted in survival deficits and impairment of haematopoiesis[20]. These findings are similar to the results found with the deletion of ATG7 in mice and the findings of Folkerts et al.[16–20]. Autophagy seems to be involved in the regulation of HSPC proliferation, differentiation and cell survival[19,21]. Autophagy preserves stemness by regulating the homeostasis of organelles, preserving energy levels by recycling metabolites and the removal of specific proteins to protect the cell from pathogenic aggregates[21]. Autophagy seems to regulate numerous signalling pathways, thereby maintaining the HSCs function[14,21,22].

These studies all add to the evidence that autophagy is essential in preserving the homeostasis of HSCs[2,21]. Autophagy maintains stemness by maintaining the quiescence, eliminating dysfunctional mitochondria, regulating ROS production, preserving the glycolytic function and limiting the proliferation of HSCs[2,21]. Figure 3 provides an overview of the role of autophagy in HSCs[2,21]. If HSCs start to differentiate into progenitor cells the autophagic flux decreases, whereas it increases again during the differentiation of more specified blood cell types like red blood cells or macrophages[2,21].

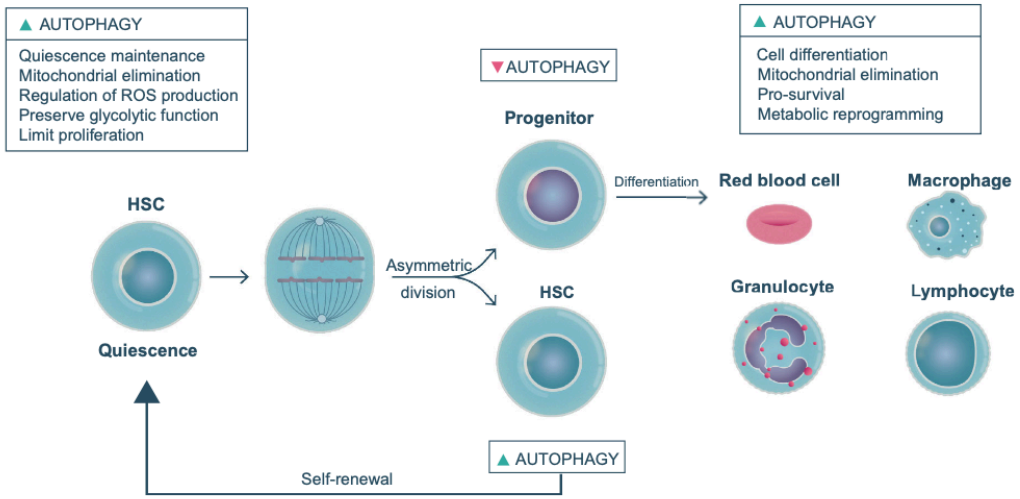

Figure 3 | Overview of the role of autophagy in hematopoietic stem cells and haematopoiesis[2]

Role of autophagy in aging and metabolism

Aging seems to be associated with decreased autophagic flux and functional decline in HSCs, underlying blood pathologies like AML[14]. In the 2013 study of Warr et al., which was discussed in the previous chapter, the authors already looked at the autophagy levels in HSCs from young and old mice[14]. With electron microscopy analysis and GFP-LC3 puncta they observed that HSCs isolated from old mice (24 months old) had higher basal autophagy levels compared to younger HSCs which were gathered from mice between the ages of 6 and 14

weeks[14]. There were no significant differences observed in FoxO3a induced pro-autophagic gene expression between the old and young HSCs[14]. Warr et al. then inhibited autophagy by plating HSCs with BafA demonstrating that autophagy is needed for the survival of older HSCs, indicated by a specific loss of cloning efficiency in old HSCs which was not observed in young HSCs[14]. With H2DCFDA they showed that old and young HSCs had similar levels of ROS and an equal increase in ROS levels upon autophagy inhibition[14]. Old HSCs however revealed a significant reduction in glucose-uptake, potentially driving the increase in autophagy[14]. Culturing of old HSCs combined with BafA and methylpyruvate, which alleviates metabolic stress, rescued the old HSCs and increased the plating efficiency[14]. With these experiments Warr et al. provided evidence that autophagy is essential for the survival of HSCs[14].

Theodore Ho published an article in 2017 regarding the role of autophagy in the maintenance and functioning of young and old stem cells[9]. With 4 weeks old ATG12^{KO} mice, they demonstrated that young HSCs with a loss of autophagy resemble the phenotype of old HSCs[9]. The young cells were characterized by a reduced engraftment capacity, reduced chimerism, myeloid bias and a reduction in HSCs[9]. After the second transplantation these characteristics became even more prominent, indicated by the loss of self-renewal capacity[9]. Ho et al. showed with electron microscopy analysis that the loss of autophagy was accompanied by an increase in elongated, healthy, mitochondria[9]. These findings were confirmed with immunofluorescence staining and indicate that autophagy might be involved in the removal and degradation of mitochondria in HSCs[9]. Gfp-Lc3 HSCs were isolated from mice and grown in cytokine rich conditions, to stimulate the cellular metabolism and to identify the relation between autophagy and the regulation of mitochondria[9]. The stimulated HSCs revealed an increase in OXPHOS levels to accommodate the increasing energy demand[9]. ATG12^{KO} HSCs and old HSCs revealed the same phenotype compared to these activated HSCs[9]. The only difference is that old HSCs also seemed to contain dysfunctional mitochondria[9]. Together, all these results indicate that autophagy is largely involved in the regulation of the metabolism of HSCs[9]. Additionally, during the aging process HSCs seemed to become more metabolically active[9]. Transcription analysis revealed for ATG12^{KO} HSCs that, apart from ATG12, there were no significant differences in gene expression for any other genes[9]. The differences in metabolism therefore would have to be regulated by epigenetics rather than gene transcription[9]. The KO of ATG12 in HSCs revealed significant changes in DNA methylation, indicating that epigenetic programming may be the driver of changes in HSCs[9].

Additional studies, performed by other research groups, focussing on ATG12 showed that this specific protein is needed for conjugation reactions between ATG12 and ATG3 or ATG5[23–27]. This conjugation is needed for the formation of autophagosomes in basal, not metabolic-stress, induced autophagy[23–27]. A knockout of ATG12 therefore results in the loss of autophagic capabilities of HSCs, reducing autophagic flux[23–27]. These studies provide evidence that ATG12 downregulation might not affect the transcription levels of other proteins resulting in higher metabolic rate, but rather changes the conjugation reactions[9,23–27]. Besides the epigenetic changes the metabolism of HSCs therefore might also change due to significant differences in conjugation[9,23–27].

Ho et al. revealed similar characteristics between ATG12^{KO} and old HSCs, while the 2013 study that was discussed before, stated that autophagy was increased during aging[9,14]. Additional experiment showed that old HSCs can have either high or low levels of autophagy, indicating possible subsets of cells in this older population[9]. Whereas most HSCs do not display a difference in autophagy levels during aging, a fraction of cells displays a higher basal level of autophagy[9]. The characteristics between the old HSCs were similar, apart from mTOR

expression levels that were significantly lower in HSCs resembling higher autophagy levels[9]. It seems likely that autophagy is used to preserve a subset of older HSCs to maintain healthy cells that can contribute to the production of blood during hematopoietic stress[9].

To date evidence implies that autophagic flux decreases with aging, coinciding with the loss of self-renewal capacity[28]. Autophagy seems to be mainly implicated in maintaining metabolism, since high autophagy levels in stem cells are accompanied by active proteostasis, low levels of mitochondrial activity, low accumulation of oxidative stress and a low metabolic state[28]. The decline in autophagy during aging results in a more metabolically active state which is resembled by an increase in OXPHOS levels in older HSCs compared to young cells[28]. Old HSCs are also characterized by a loss of proteostasis, an increase in mitochondrial activity and an increase in oxidative stress[28]. Figure 4 provides an overview of the role in autophagy in aging[28].
During the aging process autophagy levels decrease which could result in the development of blood malignancies or the onset of age-related diseases like AML due to an increase in oxidative stress and accumulations of DNA damage and dysfunctional mitochondria. Ho et al. showed that autophagy is used to preserve a part of the HSCs during aging which might be beneficial for healthy aging and might slightly reduce the risk of disease development[9].

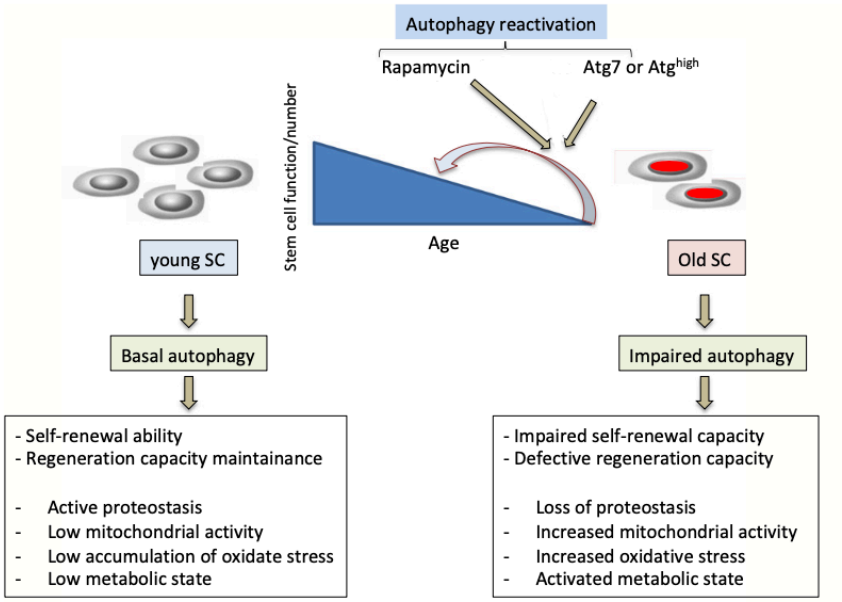

Figure 4 | Overview on the role of autophagy in aging and *metabolism* [28]

Role of autophagy in AML

Autophagy seems to have different applications in different diseases[4]. In AML for instance autophagy inhibition seems to accelerate disease development, whereas it slows down progression of chronic myeloid leukaemia[4,29]. During this review we will focus on the function of autophagy in AML, and recent publications regarding this subject.

Porter et al. published an article in 2017 about the implications of autophagy in AML[29]. During the study Porter et al. created three different mouse models; VavCre:Atg7$^{fl/fl}$ mice with a deletion of ATG7 in all hematopoietic cells, LysMCre:ATG7$^{fl/fl}$ mice with a deletion of ATG7 in all myeloid cells and Cre^{-}:Atg7$^{fl/fl}$ mice as a control[29]. In these mice MLL-AF9-induced AML was provoked with retroviral transduction[29]. After transduction the MLL-AF9 cells were

isolated form the BM and plated on methylcellulose[29]. The deletion of ATG7 in all hematopoietic cells resulted in reduced leukemic colony formation while there were equal amounts of colonies in the other two conditions[29]. Additionally, a higher rate of apoptosis was observed in the VavCre:Atg7fl/fl cells[29]. These findings might indicate that HSCs need autophagy for the initiation of leukaemia[29]. The authors then performed transplantation assays to determine the in vivo requirement of Atg7 in leukaemia initiation[29]. The VavCre:Atg7$^{fl/fl}$ cells demonstrated a delayed AML onset combined with a lower AML cell count compared to the controls[29]. There was still residual Atg7 protein expression and autophagic flux observed in the LysMCre:Atg7$^{fl/fl}$ cells, which was completely absent in the VavCre:Atg7$^{fl/fl}$ condition[29]. Together these findings suggest that autophagy is required to efficiently initiate AML in vivo and in vitro[29]. Without the ability to induce autophagy however cells were still able to achieve a, delayed, 100% AML penetrance, indicating that leukemic cells might be able to overcome the need for autophagy[29]. Piya et al. in 2017 also demonstrated, with the knockdown of Atg7 with shRNAs, that autophagy was not required in human AML cells to initiate the disease[29,30]. The Atg7 depletion however did increase the chemosensitivity of AML cells, indicating that an increase in autophagy during AML and AML treatment might be involved in chemoresistance and relapse[29,30].

Contradictory to statements made by Porter et al., Heydt et al. published an article in 2018 in which they state that autophagy is increased in AML cells and that it is even required for the proliferation of these cells[31]. In this specific article however they focused their attention on FLT3-ITD AML[31]. Given the complexity of autophagy this mechanism might be involved in different disease subtypes in different ways[31]. With this specific subtype of AML, which accounts for 25% of AML cases, there are internal tandem duplication (ITD) mutations in the FLT3 gene[31]. Heydt et al. observed a decrease in autophagy levels of FLT3-AML cells upon FLT3 inhibition, suggesting that FLT3-mediated AML cells exhibit a higher autophagic flux[31]. This data was gathered from both pharmacological inhibition as well as genetic downregulation of FTL3 in two individual FLT3-IDT positive cell lines and with pharmacological inhibition in primary AML samples[31]. The authors then identified a signalling pathway involved in the induction of autophagy in FLT3-IDT AML[31]. FLT3 inhibition was shown to be accompanied by the phosphorylation of eIF2α and a reduction in ATF4 protein levels, suggesting that ATF4 expression might be regulated by FLT3-ITD independent of eIF2α[31]. FLT3-ITD was proven to be important in the synthesis of ATF4 only in FLT3-ITD cells, and not in FLT3-wild type cells[31]. Other research groups had already published that ATF4 might be an important regulator of autophagy[31]. Heydt et al. added to these results by providing evidence that autophagic flux is significantly impaired upon ATF4 depletion, implicating a role of ATF4 in FLT3-ITD-mediated autophagy[31]. Autophagy was inhibited in the two FLT3-ITD positive cell lines to assess the role of autophagy in the proliferation of AML cells[31]. Cell counts performed with Trypan Blue revealed reduced proliferation rates after pharmacological inhibition and shRNA silencing of ATG12 and ATG5 specifically in the FLT3-ITD positive cell lines[31]. Additionally, ATF4 depletion also resulted in a reduced proliferation rate indicating that ATF4 is required for FLT3-ITD-mediated autophagy to support proliferation[31]. Lastly, Heydt et al. provided evidence for this mechanism in vivo in a NOD scid gamma mouse model[31]. In these mice that were transduced with FLT3-ITD cells, ATF4 and either ATG5 or ATG12 were silenced with shRNAs which significantly reduced the AML tumour burden and increased life-span[31].

With this publication Heydt et al. suggested that different AML subtypes might exhibit different levels of basal autophagy, depending on either the expression of certain oncogenes or mutations in autophagy-related genes[31]. Adding to this suggestion Folkerts et al. found

that a higher VMP1 expression in a subset of AML patient samples increased the basal autophagy rate[32]. VMP1 is a protein involved in activating autophagy[32]. To assess the function of VMP1, a knockdown was created with shRNAs to silence the gene in vitro and in vivo in HSPCs[32]. The depletion of VMP1 revealed a reduced autophagic-flux together with a decrease in expansion capacity[32]. The authors also assessed the consequence of VMP1 knockdown in AML cells by transducing these cells with shRNAs against VMP1 which were tagged with mCherry[32]. The knockdown in these cells, similar to leukemic cell lines, revealed that VMP1 is required for the survival and proliferation of cells as indicated by a reduced expansion capacity[32]. The overexpression of VMP1 in leukemic cells, initiated with a lentiviral overexpression vector, increased the autophagic-flux which was reflected by an increase in mCherry/GFP ratio in mCherry-GFP-LC3 autophagy reporter cells[32]. Given that VMP1 is often associated with genes regulating mitochondria, the functioning of mitochondria was determined with the amount of mitochondrial DNA and TOM20[32]. These experiments revealed that upon VMP1 overexpression the amount of mitochondrial DNA and mitochondrial mass were reduced[32]. Additionally, a reduction was observed in the amount of mitochondrial structures together with an increase in mitochondrial membrane potential in leukemic cells as determined with electron microscopy and tetramethylrhodamine[32]. The overexpression of VMP1 therefore in AML patients seems to be involved in an increased turnover rate of mitochondria[32]. Lastly, Folkerts et al. demonstrate that VMP1 overexpression in AML cells might be involved in therapy resistance[32].

Several research groups have published articles regarding the role of autophagy in AML. Many of these show reduced autophagic-flux in AML patients, like Porter et al. and Piya et al.[29,30]. There are however several studies that prove that in some AML subtypes but also in subsets of AML patients the autophagic-flux might be higher[31,32]. Given that tumours often exhibit large amounts of heterogeneity it is likely that some cells have higher autophagy levels compared to others, even cells within the same tumour could use autophagy in a different manner[33,34]. The complexity of the role of autophagy in AML therefore would have to be studied in a more subtype and cell specific way, for instance with more single-cell based methods, to gain more insight into its tumour-suppressive or tumour-promoting role in AML.

Autophagy as potential therapeutic target

The previous chapter already touched some light upon a possible role of autophagy in cancer treatment and chemoresistance[29–32]. Chemotherapy often seems to induce autophagy in cancer cells thereby preventing cell death and inducing chemoresistance[29–32]. Multiple research groups therefore have focused their work on identifying autophagy as a potential therapeutic target to treat cancer or to enhance currently used treatment methods. The studies that were discussed above by Piya et al., Heydt et al. and Folkerts et al. are examples of studies that, besides identifying a role for autophagy in AML also identified possible targets for treating the disease[30–32]. Piya et al. showed that depletion of Atg7 enhanced the sensitivity to chemotherapy whereas Heydt et al. demonstrated that resistance for FLT3 inhibitors could also be overcome by the inhibition of autophagy[30,31]. Additionally, Folkerts et al. showed that the overexpression of VMP1 coincided with an increase in autophagy and a reduction in responsiveness to venetoclax in AML cells[32]. Perhaps reduction of VMP1 expression or inhibition of autophagy might also enhance the therapeutic effect of venetoclax[32].

One way to inhibit autophagy is by using micro RNAs (miRNA), like MiR-34a, as demonstrated by Liu et al. in their 2017 publication[35]. Liu at all observed with quantitative Polymerase Chain Reaction (qPCR) and Western blots that the expression of MiR-34a was significantly lower in AML cells compared to stromal cells while the expression of the HMGB1 gene was much higher[35]. HMGB1 is involved in DNA mismatch repair and the regulation of gene transcription and is often found to be overexpressed in tumours[35]. The authors assessed the role of MiR-34a and HMGB1 in AML by transfecting AML and stromal cells with this miRNA molecule or with shRNAs targeting HMGB1[35]. The percentages of cells undergoing apoptosis were determined with flowcytometry which revealed that a higher expression of MiR-34a and a lower expression of HMGB1 specifically promoted apoptosis in AML cells[35]. Western blot analysis of both conditions revealed an increase in the expression of pro-apoptotic proteins[35]. Liu et al. also demonstrated that the increase in apoptosis coincided with the inhibition of autophagy by performing western blot analysis for autophagy-related proteins[35]. Upon either overexpression of MiR-34a or silencing of HMGB1 the Atg5 and LC3 protein levels were decreased, indicating a lower autophagic flux and a potential interaction between MiR-34a and HMGB1[35]. Co-transfection of MiR-34a with cDNA molecules of HMGB1 showed that overexpression of both molecules reduced the inhibition of autophagy by MiR-34a, suggesting that MiR-34a inhibits autophagy through targeting HMGB1[35]. Adding to these findings Liu et al. demonstrated that this co-transfection also reduced the percentage of cells undergoing apoptosis[35]. Liu et al. co-transfected AML cells with MiR-34a and shRNAs against HMGB1 and treated them with chemotherapy[35]. This co-transfection revealed that the higher expression of MiR-34a and the lower expression of HMGB1 reduced the autophagic flux and promoted cell death[35]. Given that AML cells normally have a lower expression of MiR-34a and a higher expression of HMGB1 Liu et al. demonstrated that this mechanism reduced apoptosis and promoted autophagy in AML cells, thereby protecting cells from chemotherapy induced apoptosis[35]. A potential therapeutic approach therefore could be to administer MiR-34a during chemotherapy[35]. This administration will result in a lower expression of HMGB1 thereby inhibiting autophagy and promoting apoptosis which reduces chemotherapy-induced autophagy[35]. This therapeutic approach might enhance the effectiveness of chemotherapy in certain AML patients with a high expression of HMGB1[35].

Hu et al. also identified autophagy as a mechanism for drug resistance in AML[11]. With their 2018 article regarding CXCR4-mediated signalling of autophagy they demonstrated that this signalling pathway regulates autophagy and that this is involved in the survival and drug resistance of AML cells[11]. CXCR4 is a cell surface molecule which presence might indicate the potential for AML relapse[11]. In the BM environment stromal cells secrete SDF-1α, known as CXCL12 which will bind to the CXCR4 receptor present on hematopoietic cells[11]. Research groups have already identified SDF-1α-CXCR4 interactions to be potentially involved in disease relapse[11]. This study adds to this hypothesis. Hu et al. demonstrated that culturing of AML cells with SDF-1α in the presence of chemotherapy drug Ara-C resulted in a reduced apoptotic rate and an increase in viable cells compared to treated cells without SDF-1α[11]. With JC-10 staining, which is used to determine the mitochondrial depolarization, Hu et al. showed that treating cells with SDF-1α increased the mitochondrial membrane potential[11]. These findings suggest that the interaction of SDF-1α with CXCR4 maintains the mitochondrial membrane potential during chemotherapy thereby preventing cell death[11]. To identify a possible role for autophagy in this mechanism Hu et al. performed different experiments like qPCR, electron microscopy, western blots and immunocytochemical staining[11]. Together these experiments indicated that SDF-1α-CXCR4 interactions led to an increase in autophagic flux, as indicated

by an upregulation of autophagy-related proteins, increase in autophagic vacuoles and an increase in LC3 puncta in AML cells upon co-treatment of SDF-1α with Ara-C[11]. Hu et al. then identified the signalling pathway involved in this chemoresistance, by creating a knockdown of CXCR4 which coincided with a downregulation of SIRT1, ATG5 and SQSTM1 in AML cells[11]. Additionally, this knockdown sensitized AML cells to Ara-C treatment resulting in a higher apoptotic rate, as indicated by an increase in Annexin V positive cells[11]. Similar results were observed with a knockdown of SIRT1 or ATG5 and ATG7, verifying the role of autophagy and this signalling mechanism in chemoresistance[11]. Upon inhibition of autophagy with Spautin-1, the Ara-C sensitivity of AML cells increased significantly[11]. The authors proved the role of autophagy in AML by inhibiting autophagy in vivo in a human AML mouse model[11]. Co-treating these mice with Ara-C and autophagy inhibitor Spautin-1 reduced the tumour burden and increased the overall survival[11]. Similar to Liu et al. the authors demonstrated here with this experiment that the inhibition of autophagy enhanced the sensitivity of leukemic cells to chemotherapy in vitro and in vivo[11].

Both studies demonstrated that an increase in autophagic flux induced chemoresistance in AML cells. Additionally, these studies therefore implicated autophagy as a potential therapeutic target for AML, since inhibition of autophagy enhanced the chemosensitivity. Figure 5 provides an overview of the role of autophagy in the chemoresistance of AML cells, summarizing both studies. Chemoresistance in both models can be overcome by inhibiting autophagy, or by increasing MiR-34a expression or decreasing SDF-1α-CXCR4.

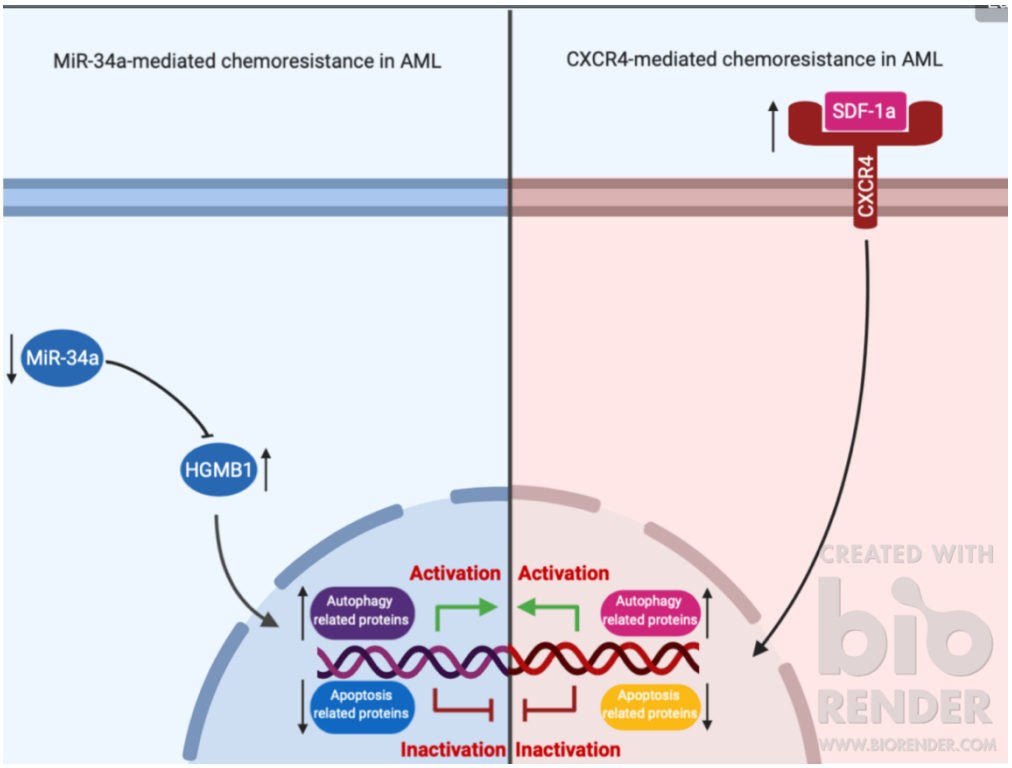

Figure 5 | Overview of chemoresistance in AML mediated by either MiR-34a (left) or CXCR4 (right)

Autophagy can be used as therapeutic target in AML by means of enhancing the effectiveness of current anticancer drugs which often induce autophagy, resulting in drug resistance[36–39]. The inhibition of autophagy as combinational therapy with currently used treatment methods might be beneficial for the patient's prognosis[36–38]. Two examples of autophagy inhibiting

drugs that can already be used for this purpose are chloroquine and hydroxychloroquine (HCQ), which are FDA approved drugs for malaria[36,37]. Results of preclinical trials and phase I and phase II trials regarding these drugs proved that these medicines alone or in combination with several other anticancer drugs significantly decrease tumour burden[36,37]. Chloroquine and HCQ however are not a very specific autophagy inhibitors which could result in several off-target effects, reflecting the need for more specific drugs[36–38]. Numerous specific autophagy inhibitor drugs, like ROC-325, histon deacetylase inhibitors, and combinational therapies are being tested in (pre-)clinical trials[36–38,40,41]. Highly specific and potent drugs and novel combination therapies that target autophagy might alleviate the poor prognosis of AML patients.

Discussion

The purpose of this review was to discuss recently published articles regarding the role of autophagy in HSCs, aging and AML and the therapeutic potential for targeting autophagy in AML patient. The role of autophagy seems to be very complex with different functions in different cells types and conditions. Autophagy is even implicated in different ways in different subtypes of a single disease.

The role of autophagy has been studied in various stem cell types, for instance in neural stem cells, however it seems to be highly indispensable specifically in HSCs[2,19]. The necessity for autophagy in HSCs functioning implies that this degradation process is of great importance. Multiple research groups therefore have focused their attention on the role of autophagy in HSPCs. HSPCs are important for the life-long supply of blood cells, especially during hematopoietic stress[7]. In order to preserve their quality and self-renewing capacities HSCs maintain a high autophagic flux. A reduced autophagic flux in HSCs is shown to increase the proliferation rate resulting in a loss of quiescence and eventually stem cell exhaustion, reflecting the importance of this specific degradation process[14,19,28]. Autophagy seems to maintain quiescence by regulating energy levels through the elimination of healthy mitochondria, thereby reducing ATP production, and preserving the glycolytic function[14,19,28]. Autophagy also provides a pro-survival mechanism and maintains the ROS production in HSCs[14,19,28].

During the aging process HSCs seem to become more and more active, losing their quiescence[9]. Studies, like the one from Ho et al., have shown that this loss of quiescence is induced by a reduced basal autophagic flux[9]. While in some HSCs the high autophagic flux is maintained, around two third of the stem cells loses their autophagy capabilities which coincides with their loss of quiescence[9]. The higher proliferation rate of the older HSCs is often accompanied by an accumulation of DNA damage, misfolded proteins and dysfunctional mitochondria or other organelles[9]. These accumulations could eventually result in the onset of age-related diseases like AML[9].

Indeed, differences in autophagic flux have been observed in primary AML patient samples[11,29–32,35]. There are however contradictory results and publications regarding the role of autophagy in leukaemia. First of all, the role seems to be entirely different between chronic myeloid leukaemia and AML. Second of all, when focusing on subtypes of AML there also seem to be differences. Like mentioned before, Porter et al. and Piya et al. demonstrated clearly that autophagy is a necessity for the efficient initiation of AML development, but afterwards this process seems dispensable[29,30]. Without autophagy the HSCs however were still able to develop into LSCs, but the disease onset was delayed[29]. Studies from Heydt et al. and Folkerts et al. on the other hand showed that a high autophagic-flux is needed for the development and progression of AML and that autophagy provides a pro-survival mechanism for AML cells[31,32]. Autophagy in AML cells even provides a mechanism for chemoresistance. Inhibition of autophagy during anticancer therapy therefore could significantly enhance the effectiveness of existing treatments[11,35]. Further improvements in AML treatment are necessary and the use of autophagy inhibitors might contribute to reducing the high relapse rate of the disease[11,35]. Currently numerous autophagy inhibitors are tested in (pre-)clinical trials to, these drugs might have the potential to alleviate the poor prognosis of AML patient[36–38].

Cancer stem cells often adapt their molecular pathways and genetic profiles in order to survive[33,34]. This adaptiveness combined with the complexity of autophagy makes it difficult to fully understand the role of autophagy in AML[31]. Besides the complexity, tumours

are often very heterogeneous with different mutations in different cells[33,34]. The role of autophagy in AML development and progression therefore has to be assessed in more detail, for instance with single cells studies[31]. Single cells studies enable the identification of more signalling pathways or specific genes or proteins involved in autophagy. Additionally, these studies could help to gain insight into the specific role of autophagy in specific cell types but also in subset of diseases.

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