MicroRNA-130a targets \textit{ATG2B} and \textit{DICER1} to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells.

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Abstract

Toxicity and relapses from the immunochemotherapy used to treat chronic lymphocytic leukemia (CLL) prompt continued interest in gentle but effective targeted treatment options for the mainly elderly population suffering from this disease. Here we report the definition of critical CLL cell survival pathways that can be targeted by ectopic re-expression of the microRNA (miRNA) genes miR-130a and miR-143 which are widely downregulated in CLL. Notably, miR-130a inhibited autophagy by reducing autophagosome formation, an effect mediated by downregulation of the genes ATG2B and DICER1, the latter of which is a major component of the miRNA silencing machinery. In support of the concept of a fundamental connection between miRNA disregulation and altered autophagic flux in this cancer we demonstrated that RNAi-mediated knockdown of DICER1 expression was sufficient to reduce autophagy in primary or established cultures of CLL cells. Together, our findings demonstrate that miR-130a modulates cell survival programs by regulating autophagic flux, and they define roles for miR-130a and Dicer1 in a regulatory feedback loop that mediates CLL cell survival.

Introduction

B-cell chronic lymphocytic leukemia (CLL) is characterized by the expansion of CD5+/CD19+/CD23+ B-lymphocytes and shows diverse pathogenic mechanisms and a heterogeneous clinical course (1, 2). Inherent defects in cell death of CLL B-lymphocytes that are nourished by a small proliferative pool in lymph nodes, spleen and bone marrow are responsible for the massive accumulation of malignant cells. Prolonged CLL cell survival is induced by autocrine signaling pathways and survival stimuli from the microenvironment (3, 4). MicroRNAs (miRNAs) are endogenously expressed small RNA molecules that mediate post-transcriptional gene silencing and have the capacity to simultaneously regulate tens to hundreds
of target genes (5). Thereby, these non-coding RNAs are involved in the regulation of multiple cellular processes including proliferation, apoptosis, development and differentiation (6), and have been also associated with cancer. Specific miRNAs were shown to be differentially expressed between normal and tumor cells, including CLL (7, 8) and are therefore potential targets for anti-cancer therapy.

Autophagy is one of the key protective cellular pathways that mediate stress-induced adaptation and damage control. Macroautophagy (hereafter referred to as autophagy) is a major type of autophagy, which sequesters organelles and long-lived proteins in membrane-coated vesicles, so-called autophagosomes. This survival mechanism, induced by various types of stress, can block intrinsic and extrinsic apoptotic pathways (9) and cancer cells exploit autophagy to overcome starvation and hypoxia. Interestingly, anti-cancer therapy such as DNA-damaging drugs or hormone antagonists induce autophagy as pro-survival response (10, 11) and the combination of standard therapies with chloroquine, an inhibitor of autophagy, has been shown to enhance treatment efficacy (12). Therefore, the ability to modulate autophagy is extensively studied in cancer research. However, only few studies have investigated autophagy regulation on the miRNA level (13, 14). The involvement of autophagy in CLL has been reported in the literature, where induction of autophagy in CLL cells upon dasatinib or dexamethasone treatment was described (15, 16). In addition, it was shown that chloroquine induces apoptosis in CLL cells in vitro (17) and might therefore enhance the efficacy of drugs currently used for CLL treatment.

In this study we investigate the effects of five miRNAs that are down-regulated in CLL on the regulation of cell death/cell survival programs. We show that enforced expression of miR-130a impairs cell viability and inhibits autophagic flux in CLL cells, suggesting a new miRNA-autophagy regulatory axis for modulating survival mechanisms in CLL cells.
Material and Methods

Cell lines. HEK293T and HS-5 cells were purchased from ATCC (Manassas, VA, USA) and retested and authenticated in 03.2011 and 05.2008, respectively. MEC-1 cells were obtained from DSMZ (Braunschweig, Germany) in 2009 and not passaged longer than six months continuously. Cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Gibco BRL, Invitrogen) (referred to as complete medium) at 37°C and 10% CO2.

Primary cells. Peripheral blood (PB) samples were collected from CLL patients (Supplementary Table 1) and healthy donors after informed consent. All cases matched standard diagnostic criteria (18). Mononuclear cells were isolated and cultured as reported before (19).

RNA and DNA isolation. Total RNA with or without miRNA fraction was isolated using miRNeasy or RNeasy Mini Kit, respectively (Qiagen, Hilden, Germany). Genomic DNA was isolated using Blood & Cell Culture DNA Midi Kit (Qiagen).

miRNA expression analysis. 700 ng total RNA was employed for miRNA expression analysis using Bead-based miRNA expression assay (Illumina, San Diego, USA). The array data were analyzed using BeadStudio v3.2 software (Illumina) and are available at the NCBI Gene Expression Omnibus (GEO) database, accession no. GSE31599. For further validation of miRNA expression, quantitative reverse transcription (qRT)–PCR analysis was carried out using TaqMan MicroRNA Assays (Applied Biosystems, Forster City, USA).

miRNA precursor molecules, miRNA inhibitors and siRNAs. All synthetic miRs and anti-miRs including Negative Control pre-miRNA (AM17110) and Negative Control anti-miR Inhibitor (AM17010) were purchased from Applied Biosystems. Silencer Select Negative Control siRNA (4390843) and DICER1 siRNA (s23755) were obtained from Applied Biosystems.
**Nucleofection of cell lines and primary CLL cells.** 1x10^7 primary B-cells were transfected using the human B-cell Nucleofector Kit and program U-015 with 1 µM miRNA or siRNA according to the manufacturer’s instructions (Lonza, Cologne, Germany). After nucleofection, primary cells were cultured in 4 mL of sterile filtered conditioned medium obtained from HS-5 cell cultures. Transfection of MEC-1 cells was performed by nucleofection (solution V, program X-001) using 5x10^6 cells and 0.5 µM miRNA or siRNA and subsequent culture in complete medium.

**Construction of DICER1 and ATG2B 3′UTR luciferase plasmids and reporter assay.** The 3′UTR-fragments of DICER1 and ATG2B containing the miRNA target sites were amplified from MEC-1 genomic DNA by PCR using FastStart Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany) and the primers listed in Supplementary Table 2. PCR products were cloned into pMIR-REPORT luciferase plasmid (Applied Biosystems) using HindIII and SpeI restriction sites. For reporter assays, 1x10^5 HEK293T cells were co-transfected with 5 ng of 3′UTR reporter plasmid, 50 ng pRL-TK renilla luciferase reporter vector (Promega, Karlsruhe, Germany), and 30 nM miR-130a or Negative Control miRNA using TransIT transfection reagent, according to the manufacturer’s instructions (Mirus BioLLC, Madison, WI, USA). Transfected cells were lysed 36 hours after transfection and luciferase activities were assayed using a Dual-Luciferase Reporter System (Promega).

**Cell viability analysis.** Cell viability was assessed by flow cytometry after annexin V-phycoerythrin (PE) and 7-amino-actinomycin (7-AAD)staining (BD Biosciences, Heidelberg, Germany) as described before (20). Double-negative cells were counted as viable cells. FACS analyses were carried out using a FACSCanto II flow cytometer equipped with FACSDiva V6.1.2 software (BD Biosciences).
Quantitative reverse transcription polymerase chain reaction (qRT-PCR). For mRNA quantification, total RNA was isolated from cells 48 hours after transfection and transcribed using SuperScript II and anchored oligo-d(T)\textsubscript{20} primer (Invitrogen). Amplification and quantification of cDNA was performed using SYBR Green ROX Mix (Abgene, Epsome, UK) according to the manufacturer’s protocol and as described before (20). QRT-PCR was performed in 7900 HT Fast Real-Time PCR System (Applied Biosystems). Relative quantification was performed in relation to two housekeeping genes (\textit{LAMINB1}, \textit{PGK1}). The qRT-PCR primer sequences are listed in Supplementary Table 2.

Western blotting. Cells were harvested 48 hours after transfection and lysed in 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP-40, containing Complete Mini protease inhibitor mixture (Roche Diagnostics). Protein extracts were electrophoresed on 4–12% linear gradient Bis-Tris ready gels (Invitrogen) and transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany). Membranes were probed with antibodies specific for Dicer1 (1:1000, ab14601, Abcam, Cambridge, UK), LC3-B (1:1000, 2775 S, Cell Signaling, Inc., Danvers, USA), α-tubulin (1:2500, Sigma Aldrich, Munich, Germany), and GAPDH (1:2500, CB1001, Calbiochem, Darmstadt, Germany), and subsequently with horseradish peroxidase-coupled anti-rabbit or anti-mouse antibodies (both 1:2500, Cell Signaling Inc.). Specific bands were visualized using ECL Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, England) and quantified with Image J software.

Generation of MEC-1/GFP-LC3 cells. To generate a stable cell line expressing GFP-LC3 fusion protein, MEC-1 cells were transduced with the pWPI lentiviral vector containing the GFP-LC3 sequence, using a titer of 2.6x10\textsuperscript{6} for 5x10\textsuperscript{5} cells and selected in the presence of puromycin (Invitrogen) for 2 weeks.
**Imaging of autophagic flux.** Autophagic flux was quantified by using an imaging flow cytometer (Image Stream, Amnis, Seattle, USA) employing 2×10⁶ MEC-1/GFP-LC3 cells per analyzed sample. For starvation, cells were transferred to Krebs-Henseleit buffer (Sigma-Aldrich) 24 hours after transfection and incubated for further 8 hours in the presence or absence of 100 nM lysosomal H1-ATPase inhibitor bafilomycin A1 (Merck KGaA, Darmstadt, Germany). Subsequently, cells were harvested and resuspended in 100 μl of cold PBS/2%BSA. To exclude dead cells from the analysis, TO-PRO3 (Quinolinium, 4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl], diiodide, Invitrogen) was added directly before acquisition at a final concentration of 1 μM. For each sample, images of 5000 cells were acquired in the bright field channel and the fluorescence emission channels at 660 nm (TO-PRO-3) and 520 nm (GFP). The acquired images were analyzed with IDEAS software (Amnis) as described in the supplement. The statistical significance of the differences between the autophagic flux distributions was assessed using a Kolmogorov-Smirnov test. In addition, the histogram channels of the autophagic flux distribution presenting a significant difference in frequencies were evidenced by a Student’s *t*-test (see supplementary material for details).

**Results**

**A set of miRNAs is down-regulated in chronic lymphocytic leukemia.**

Previous studies have reported that the majority of miRNAs are down-regulated in CLL when compared to normal B-cells (21). To identify deregulated miRNAs we performed miRNA expression profiling on RNA samples of 18 CLL patients and on pooled RNA of peripheral blood B-lymphocytes of five healthy donors by miRNA arrays. MiR-126, miR-130a, miR-143, miR-181a and miR-326 were among the top 20 down-regulated miRNAs in CLL patients (GEO...
no. GSE31599). To confirm this finding, we quantified the respective miRNAs by qRT-PCR in samples of six CLL patients. Both methods showed lower expression levels of these five miRNAs in CLL cells compared to normal B-cells and qRT-PCR values ranged from 1.5- to approximately 20-fold reduction (Figure 1).

To elucidate whether the lower expression levels of these miRNAs in CLL is due to epigenetic transcriptional silencing, we assessed the methylation status of putative miRNA promoter regions by MassArray. Out of the tested miRNAs, only miR-126 showed a significant gain in methylation within its predicted transcriptional start site in CLL cells compared to healthy B-cell control (p=0.007; Supplementary Figure 1). Small subsets of patients showed hypermethylation of miR-326 and miR-181a, but the average methylation values were not significantly different compared to the controls. This result indicates that the majority of the tested sequences are not affected by epigenetic regulation and aberrant methylation is not the main reason for the deregulated expression of the studied miRNAs in our CLL patient cohort. However, we cannot rule out that other, not yet identified miRNA regulating sequences are under the influence of epigenetic regulation.

**miR-130a and miR-143 induce cell death in CLL cells.**

To investigate the function of the underrepresented miRNAs in CLL, we first assessed their impact on cell viability, since accumulation of quiescent CLL cells is primarily due to defects in apoptosis induction, rather than increased cell proliferation as reflected in gene expression (22, 23). Freshly isolated CLL cells were transiently transfected with one of the following synthetic miRNAs: miR-126, miR-130a, miR-143, miR-181a, miR-326, or with an unspecific miRNA used as negative control (NC). Cell viability was measured 48 hours after transfection by flow cytometry after annexin V–PE/7-AAD staining. Introduction of miR-130a, miR-143 and miR-
181a led to an average reduction in the number of viable cells of 21%, 15%, and 23% respectively, when compared to the negative control set as 100% viability (p_{miR-130a}=0.01; p_{miR-143}=0.02; p_{miR-181a}=0.03; Figure 2A). Furthermore, we tested whether the above-mentioned five miRNAs have an impact on the viability of the CLL cell line MEC-1. Twenty-four hours after transfection of miR-130a or miR-143 the number of viable MEC-1 cells was decreased by 57% or 50%, respectively, when compared to unspecific miRNA control (p_{miR-130a}=0.001; p_{miR-143}=0.001; Figure 2B). MiR-181a slightly, though not significantly, reduced cell viability of MEC-1 cells, whereas miR-126 and miR-326 did not affect viability of either primary or cell line CLL cells. These results indicate that miR-130a and miR-143 induce cell death both in primary CLL and MEC-1 cells.

**Autophagy is induced in CLL cells under starvation.**

We next sought to investigate the mechanism of how miR-130a and miR-143 impact survival-related pathways in CLL. Autophagy is one possible mechanism of how cancer cells overcome cellular stress and maintain cell survival and resistance to apoptosis (11). To investigate the relevance of autophagy in CLL cell survival we used chloroquine, a late stage inhibitor of autophagy, in primary CLL cultures and observed decreased cell viability in a dose-dependent manner (Supplementary Figure 2), which confirms results published earlier (17). In addition, chloroquine treatment resulted in enhanced fludarabine-induced cell death in primary CLL cells (data not shown). To evaluate the role of autophagy in CLL cells in more detail we followed the specific autophagosome marker LC3, a microtubule-associated protein 1 light chain 3, which is involved in the formation of autophagosomes and translocates from a soluble form, LC3-I, to the autophagosomal membrane-bound form LC3-II (24). For this means, we generated a stable MEC-1 cell line expressing GFP-tagged LC3 (MEC-1/GFP-LC3). Accumulation of fluorescent
dots, representing autophagosomes, was evaluated in these cells by imaging flow cytometry as described in the methods section. Since autophagic flux can be measured as the difference in autophagosome formation between samples in the presence or absence of inhibitors of autophagosome degradation, (25) we quantified GFP-positive autophagosomes in cells treated for eight hours with or without the lysosomal H1-ATPase inhibitor bafilomycin A1 (BaF).

In non-stress conditions, the basal level of autophagy can be very low, but might be induced upon starvation. Therefore, we determined autophagic flux in MEC-1/GFP-LC3 cells cultured in complete medium or under starvation conditions. Representative images of analyzed cells are presented in figure 3A, showing increased GFP-LC3 clustering in bafilomycin-treated cells under starvation conditions. The level of GFP-LC3 clustering was extracted from the individual cellular images and represented by the bright detail intensity feature (Figure 3A lower panel). A non-parametric description derived from the Kolmogorov-Smirnov (K-S) statistics was applied for the quantification and representation of these data, as described in the supplement. Briefly, we calculated the autophagic flux of MEC-1/GFP-LC3 cells as the difference in bright detail intensities between bafilomycin-treated and untreated states (Figure 3B, left and middle panel). The obtained data indicate that MEC-1 cells have a constitutively active autophagy in complete medium conditions, which is extensively up-regulated upon starvation as indicated by the right-shift of the curve (Figure 3B, right panel).

Autophagy might therefore be a survival mechanism of CLL cells. All subsequent analyses of autophagic flux in MEC-1/GFP-LC3 cells were performed under starvation conditions to obtain high assay sensitivity.

miR-130a inhibits autophagic flux in CLL.
To investigate the impact of miR-130a and miR-143 on autophagy in CLL cells, MEC-1/GFP-LC3 cells were transfected with these two synthetic miRNAs or a scrambled miRNA used as negative control, and autophagic flux was analyzed. Introduction of miR-130a resulted in a robust change in the autophagic flux distributions showing lower frequencies when compared to the negative control. A summary of four independent experiments demonstrated statistically significant reduction of autophagosome formation (Kolmogorov-Smirnov test for each individual experiment, p<0.05; mean histogram channels of 4 experiments with statistical difference are labeled with dots, Student’s t-test, p<0.05), indicating that miR-130a inhibits autophagic flux in MEC-1 cells (Figure 3C). Transfection of cells with miR-143 did not reveal statistically significant changes in the autophagic flux distribution (data not shown).

To confirm our observations by an independent assay, we performed Western blot analysis for endogenous LC3 protein by using MEC-1 cells treated under the same conditions as described above. Autophagic flux was assessed by calculating the difference of LC3-II protein of samples in the presence or absence of bafilomycin (ΔLC3-II) as reported (26). These experiments revealed decreased ΔLC3-II in miR-130a transfected samples (Figure 3D left), which confirms reduced autophagic flux in MEC-1 cells upon introduction of miR-130a. We further investigated the role of miR-130a in autophagy in primary CLL cells by Western blot analysis. Similar as in MEC-1 cells, transfection of primary CLL cells with miR-130a resulted in a decrease in ΔLC3-II (Figure 3D right). These results show for the first time that autophagy is an active process in primary CLL cells and is regulated by miR-130a.

**DICER1 and ATG2B are direct targets of miR-130a.**

To identify genes that are regulated by miR-130a we analyzed previously reported transcriptome data and searched for transcripts with higher expression levels in primary CLL samples...
compared to healthy B-cells (27). These differentially expressed genes were analyzed for miR-130a binding sites by Targetscan software. By this means, DICER1 was selected as a novel potential miR-130a target gene, since it was up-regulated 2.3-fold in CLL cells relative to healthy donor control, and has a highly conserved binding site for miR-130a. In addition, autophagy-related genes were screened for miR-130a binding sequences and thereby ATG2B, a mediator of autophagy, was identified and selected for further studies. The miR-130a binding site within the 3’ untranslated region (3’UTR) of DICER1 is conserved between human, mouse, rat, dog and chicken; and of ATG2B between human, dog and chicken (Figure 4A).

To examine miR-130a-mediated regulation of the selected genes, we transfected primary CLL cells with synthetic miR-130a and assessed DICER1 and ATG2B mRNA levels by qRT-PCR 48 hours after transfection. This resulted in a 1.4-fold reduction of DICER1 (p=0.002) and a 1.8-fold reduction of ATG2B (p>0.05) mRNA levels when compared to transfection with unspecific control miRNA (Figure 4B).

Furthermore, to confirm that the down-regulation of the tested mRNA is due to direct interaction between miR-130a and its predicted binding site, we co-transfected HEK293T cells with miR-130a and pMiR luciferase reporter constructs containing 3’UTR fragments of DICER1 and ATG2B. In the presence of miR-130a, relative luciferase activity assessed 36 hours after transfection for the DICER1 and ATG2B constructs was reduced by 25% (p=0.09) and 37% (p=0.03), respectively (Figure 4C). Importantly, inhibition of endogenous miR-130a by transfection of anti-miR-130a (inhibitor of miR-130a) in HEK293T cells increased the luciferase activities of reporter constructs for DICER1 and ATG2B by 10% (p=0.05) and 33% (p=0.03), respectively (Figure 4C).
These results demonstrate that miR-130a down-regulates DICER1 and ATG2B mRNAs by direct interaction with their 3’UTRs, and therefore identifies these genes as novel targets for miR-130a in CLL cells.

**miR-130a down-regulates Dicer1 protein expression in primary CLL and MEC-1 cells.**

We next assessed whether the observed regulation of DICER1 and ATG2B by miR-130a is reflected by changes on protein level. Due to the lack of ATG2B-specific antibodies this could not be achieved for this protein so far. For Dicer1, Western blot analyses were performed using lysates of primary CLL cells transiently transfected with miR-130a or control miRNA. Dicer1 protein levels were analyzed by quantifying the bands corresponding to full-length Dicer1 protein of 218.7 kDa, which is the upper band in the blots shown in Figure 5A. In three analyzed samples of primary CLL cells introduction of miR-130a resulted in reduced Dicer1 levels when compared to unspecific miRNA transfection (Figure 5A). A respective experiment using B-lymphocytes of a healthy donor showed reduced Dicer1 protein after miR-130a transfection in these cells as well (Figure 5A). Furthermore, introduction of miR-130a in MEC-1 cells resulted in a down-regulation of Dicer1 by 2.3-fold relative to unspecific miRNA control (Figure 5B).

**Knock-down of Dicer1 leads to reduced autophagy in primary CLL and MEC-1 cells.**

To investigate whether Dicer1 is involved in autophagy in CLL cells we transfected DICER1-specific siRNA in MEC-1 and primary CLL cells, which resulted in a reduction of Dicer1 protein 48 hours after transfection of around 75% and 60%, respectively. Autophagic flux was evaluated in the transfected cells in the presence and absence of bafilomycin by Western blotting of LC3 protein and revealed a considerable reduction of ΔLC3-II after knock-down of Dicer1 both in
MEC-1 and primary CLL cells (Figure 6). This suggests that inhibition of autophagy in CLL cells by miR-130a is mediated at least partly by its target gene *DICER1*.

Since Dicer1 is a key component of the miRNA biogenesis machinery, it was likely that down-regulation of Dicer1 by miR-130a results in reduced levels of miRNAs in general. Therefore, we measured expression levels of seven different miRNAs (miR-21, miR-26a, miR-28, miR-146a, miR-148b, miR-193b, let-7c) after transfection of miR-130a in MEC-1 cells. This resulted in reduced expression levels of all seven tested miRNAs (between 0.4- and 0.8-fold) compared to transfections of unspecific miRNA (data not shown). Therefore, the observed effect of miR-130a on cell survival and autophagy in CLL cells might be due to major changes in the miRNA profile of the cells.

Taken together, our data show that miR-130a regulates autophagic flux and cell viability in both MEC-1 and primary CLL cells and suggest that its target gene *DICER1* is involved in this process.

**Discussion**

Deregulated expression of miRNAs is a common feature of many tumor entities, including CLL. Here, specific miRNA signatures were shown to differentiate patients with short and long intervals to therapy (28, 29). The biological role of most of these miRNAs in tumor development and progression is however still unclear. The aim of this study was therefore a functional analysis of five deregulated miRNAs in CLL, miR-126, miR-130a, miR-143, miR-181a and miR-326, which were shown to be underrepresented in CLL cells compared to normal B-cells by us and others (21). For some of these deregulated miRNAs implications in CLL biology have been suggested: miR-181a has been reported to regulate the CLL-relevant oncogenes *TCL1* and
PLAG (21, 30), and miR-143 which is down-regulated in several B-cell malignancies, was shown to target ERK5, a MAPK protein (31).

One of the reasons for deregulated miRNA expression in cancer is promoter silencing due to aberrant DNA methylation. Therefore, we examined the promoter methylation status of the down-regulated miRNAs in CLL patients and observed a significant increase in promoter methylation of miR-126 in comparison to normal B-cells. This is again in accordance with data published by Pallasch et al. (21) who additionally showed a tendency for hypermethylation of the promoter regions of miR-130a, miR-181a and miR-326.

Our primary interest was to elucidate a possible role of the down-regulated miRNAs in the persistent survival of CLL cells. By miRNA transfection studies we showed that miR-130a and miR-143 reduce viability of both primary CLL and MEC-1 cells. To investigate the molecular mechanism of how these miRNAs regulate survival of CLL cells, we proceeded with studying the role of autophagy in CLL cell survival and a possible involvement of miR-130a or miR-143 in its regulation.

Autophagy is a highly conserved pro-survival mechanism to maintain cellular homeostasis upon different types of stress and plays a crucial role in cancer. In tumor cells with defects in apoptosis, autophagy was shown to prolong cell survival (32), but the interplay between autophagy and apoptosis and its role in cancer are still poorly understood. Of crucial importance is to increase our knowledge on the regulation of autophagy in cancer with the aim to modulate autophagy in tumor cells and thereby inhibit their survival. Inhibitors of autophagy that sensitize apoptosis-resistant tumor cells to anti-cancer therapy are under current investigation in clinical trials (33). The potential regulation of autophagy by miRNAs may offer an efficient multi-target strategy to accomplish a robust autophagy inhibition unaffected by single target gene mutations, cell-to-cell heterogeneity or other hallmarks of cancer cell resistance. Some miRNAs have been
reported to regulate autophagy-related genes, such as miR-30a, regulating \textit{BECN1} and miR-196, regulating \textit{IRGM} (13, 14).

In both primary CLL and MEC-1 cells, we monitored autophagic flux and showed for the first time that autophagy is an active process in these cells. In addition, we demonstrated that MEC-1 cells were able to respond to starvation by an increased autophagic flux. These data suggest that autophagy is a constitutive mechanism in CLL and is part of the stress response of these cells to the lack of nutrients. Since introduction of miR-130a and miR-143 reduced viability of both primary CLL and MEC-1 cells, we investigated their impact on autophagy. Indeed, miR-130a reduced autophagic flux in primary CLL cells and MEC-1 cells, which for the first time identified miR-130a as a regulator of autophagy in CLL. In accordance with our results, a recent systems biology-based computational analysis study reported that miR-130a is one of five putative miRNAs that regulate the autophagy-lysosomal pathway (34). Although, the role of miR-130a in CLL biology was unclear, in ovarian cancer miR-130a was associated with anti-cancer drug-resistance, since it was found to be down-regulated in cell lines resistant to paclitaxel and cisplatin (35).

To further elucidate the molecular mechanism of miR-130a-mediated CLL cell survival and autophagy, we aimed at identifying putative target genes of this miRNA. By luciferase reporter assay we identified the autophagy-related gene \textit{ATG2B} as a direct target of miR-130a and showed down-regulation of \textit{ATG2B} expression in CLL cells transfected with miR-130a. \textit{ATG2B} interacts with \textit{ATG2A} and WDR45 and thus is possibly involved in vesicle nucleation and the initial steps of autophagosome formation (36). Interestingly, \textit{ATG2A} was found to be 1.5-fold up-regulated in CLL samples relative to healthy donor controls (27). Association of \textit{ATG2B} and cancer has been reported: frameshift mutations were found in the mononucleotide repeats in the coding sequence of \textit{ATG2B} gene in gastric and colorectal carcinomas (37). Our data imply that
ATG2B might be involved in miR-130a-mediated autophagy in CLL cells. Its functional role in this process has to be further investigated.

In addition, we identified DICER1 as a direct target gene of miR-130a and showed that knock-down of Dicer1 results in reduced autophagic flux in CLL cells. Dicer1 is a highly conserved protein, with endonuclease RNase III activity, required for siRNA- and miRNA-mediated silencing. Therefore, miR-130a is most likely involved in autophagy and cell survival in CLL cells via regulating the maturation and activity of many miRNAs, multiplying the amount of indirect effective target genes. Indeed, our results showed that reduction of Dicer1 levels by overexpression of miR-130a lead to reduced levels of several miRNAs in CLL cells. The effects of miR-130a in CLL cells might therefore be mediated by a more global change in the miRNA profile of the cells and the presence of feedback loops involving Dicer1 and several miRNAs are very likely.

As a key enzyme in RNA-induced silencing, Dicer1 is essential for maintaining many crucial cellular processes including B-cell development. Koralov et al. showed that in the absence of Dicer1, B-cell development is almost completely blocked at the transition from pro-B- to pre-B-cells due to failure in response to survival signals at this early stage of development (38). Increased expression of DICER1 has been demonstrated in several tumor types including ovarian and prostate cancer (39, 40), and was often associated with tumor progression and poor prognosis. In addition, deletion of Dicer1 in B-cells in a Myc-induced lymphoma model significantly inhibited lymphomagenesis (41).

Since the DICER1 gene harbors a long 3’UTR (>4000bp) with multiple sites for potential miRNA binding, it is likely to be targeted by several miRNAs. In addition to our data showing regulation of DICER1 by miR-130a, the miRNA let-7 and the miR-103/107 cluster have been reported to regulate Dicer1 protein (42, 43).
Taken together, our results demonstrate that miR-130a plays a role in CLL cell death/cell survival pathways including autophagy. We identified ATG2B, as an autophagy-related target gene of miR-130a, which might be involved directly in CLL cell resistance to apoptosis. The regulatory network of miR-130a is presumably highly complex since it also targets Dicer1, an essential component of the miRNA biogenesis machinery, suggesting the presence of feedback loops and the involvement of other miRNAs. Which miRNAs and respective target genes are essentially involved in this regulation will be of major interest on the way to identify novel drug targets. Our results also point towards a cancer-supporting role of Dicer1 in CLL since its down-regulation by miR-130a leads to a decrease in autophagy and induces cell death. The understanding of these regulatory networks offers a great potential for the intelligent multi-targeted design of new cancer therapies.

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Conflict of Interest Disclosures

The authors declare no competing financial interests.
References


Figure legends

Figure 1: miR-126, miR-130a, miR-143, miR-181a and miR-326 are down-regulated in CLL cells. miRNA expression was assessed by array and qRT-PCR. Array data were normalized to the median of all miRNA expression values within an individual sample. qRT-PCR data were normalized to the average of RNU-6B and RNU-66. Mean log2 fold changes and standard deviations (array: n=18; pRT-PCR: n=6) compared to a pool of 5 healthy donor B-lymphocyte samples (HD) are depicted.

Figure 2: miR-130a and miR-143 induce cell death in primary CLL and MEC-1 cells. (A) Primary CLL cells were transfected with miRNAs and cell viability was quantified after 48 hours by annexin V-PE/7-AAD staining. One representative FACS data and a summary of 4 independent experiments are depicted. Mean values and standard deviations of relative cell viability are shown (NC miRNA set to 100%). Paired t-test was applied to calculate statistical significance. * p≤0.05, ** p≤0.01. (B) Respective experiments were performed with MEC-1 cells, where cell viability was assessed 24 hours after transfection.

Figure 3: miRNA-130a reduces autophagic flux in CLL cells. (A) Autophagy was assessed in MEC-1/GFP-LC3 cells using an imaging flow cytometer. Upper panel: Representative images of analyzed cells cultured in complete medium or under starvation conditions in the presence or absence of bafilomycin. Lower panel: Bright detail intensity feature distribution representing GFP clustering. (B) Cumulative distribution function of bright detail intensity of MEC-1/GFP-LC3 cells incubated with or without bafilomycin in complete medium (Left) or under starvation (Middle). Autophagic flux of MEC-1/GFP-LC3 cells cultured in complete medium (black), or under starvation conditions (red), calculated as the difference between the cumulative
Figure 4: DICER1 and ATG2B are direct targets of miR-130a. (A) Schematic representation of the 3’UTRs of DICER1 and ATG2B indicating the fragments which were cloned into the luciferase reporter construct pMIR-REPORT. Evolutionary conserved regions containing the miR-130a binding sites are highlighted by frames in the sequence alignments of different species. (B) Expression level of DICER1 and ATG2B mRNA quantified by qRT-PCR 48h after miR-130a transfection in primary CLL cells. mRNA levels were normalized to two housekeeping genes (LAMINB1 and PGK1) and are presented as mean values ± SEM relative to negative control (NC) results (DICER1: n=6; ATG2B: n=3). Paired t-test was applied to assess statistical significance. p_{DICER1}=0.002; p_{ATG2B}=0.2. (C) 3’UTR luciferase reporter assay for DICER1 and ATG2B. HEK293T cells were co-transfected with pMIR-3’UTR luciferase reporter construct and miR-130a or anti-miR-130a and respective controls (NC or anti-NC). Relative
luciferase intensity was assessed as described in the methods section and is represented as mean ± SEM of 4 independent experiments. * p≤0.05.

**Figure 5: Western blot analysis confirms down-regulation of Dicer1 protein by miR-130a.**

(A) Western blot for Dicer1 using primary CLL samples as well as B-cells from a healthy donor (HD) transfected with miR-130a or NC miRNA. Non-transfected B-cells were used as control (NT). Dicer1- and GAPDH-specific bands were quantified by ImageJ software and Dicer1 protein expression levels were normalized to GAPDH values. (B) Respective experiments were performed with MEC-1 cells, of which one representative result is shown. Mean values ± standard deviation of 3 independent experiments are shown.

**Figure 6: Knock-down of Dicer1 results in reduced autophagic flux in MEC-1 and primary CLL cells.** MEC-1 (A) or primary CLL cells (B) were transfected with DICER1-specific siRNA (siDicer) or Negative Control siRNA (siCo) and cultured for 40 hours in complete medium. MEC-1 cells were then transferred to Krebs-Henseleit buffer and bafilomycin was added where indicated (BaF) for further 8 hours. Results of non-transfected MEC-1 cells either in complete medium (CM) or under starvation (KHS) are shown in addition. Western blot results for Dicer1 and LC3-II were normalized to α-tubulin and differences of LC3-II in BaF-treated and untreated samples were calculated (ΔLC3-II) and set to siCo=1. Mean values ± standard deviations of relative ΔLC3-II of 2 independent experiments each are depicted on the right.
Figure 1
Figure 2

A

CLL109 (48h after transfection)

- NC
- miR-130a

Summary of primary CLL transfections

% of viable cells relative to NC

miR- 126 130a 143 181a 326

B

MEC-1 (24h after transfection)

- NC
- miR-130a

Summary of MEC-1 transfections

% of viable cells relative to NC

miR- 126 130a 143 181a 326

7AAD:

- 72%
- 51.7%
- 72.1%
- 41.8%
Figure 3

(A) Complete medium vs. Starvation

- Steady state
- + Bafilomycin

(B) Complete medium vs. Starvation

- Steady state
- + Bafilomycin

(C) MEC-1 in starvation conditions

- Negative control
- miR-130a

(D) CLL primary cells in complete medium

- BaF NC
- BaF + miR-130a

Normalized Bright Detail Intensity

Change in Cumulative frequency

Relative LC3-II

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Figure 4

DicER13 UTR

sequence with mR-130a target site

Luciferase

ATG2B 3'UTR

sequence with mR-130a target site

Luciferase

Relative mRNA expression

Relative luciferase intensity

A  B  C

NC  mR-130a  NC  mR-130a  antiNC  antiNC

Human: 1494 tGGGtGAGGCAAGGCAAGGGAAGG
Mouse: 1438 tGGGtGAGGCAAGGCAAGGGAAGG
Rat: 1438 tGGGtGAGGCAAGGCAAGGGAAGG
Chicken: 1438 tGGGtGAGGCAAGGCAAGGGAAGG

Human: 1494 tGGGtGAGGCAAGGCAAGGGAAGG
Mouse: 1438 tGGGtGAGGCAAGGCAAGGGAAGG
Rat: 1438 tGGGtGAGGCAAGGCAAGGGAAGG
Chicken: 1438 tGGGtGAGGCAAGGCAAGGGAAGG

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Mouse: 1438 tGGGtGAGGCAAGGCAAGGGAAGG
Rat: 1438 tGGGtGAGGCAAGGCAAGGGAAGG
Chicken: 1438 tGGGtGAGGCAAGGCAAGGGAAGG
Figure 5

(A) Western blot analysis showing Dicer and GAPDH expression in HD, CLL135, CLL24, and CLL139 samples. The expression levels are compared between NC and miR-130a groups.

(B) Bar graph illustrating relative Dicer expression levels in NC and miR-130a groups for HD, CLL135, CLL24, and CLL139 samples. The graph shows a significant increase in Dicer expression in the miR-130a group compared to the NC group.
Figure 6

A

BaF
CM
KHS
siCo
siDicer

Dicer
α-tubulin
LC3-I
LC3-II

0.14 0.39 0.21 0.73 0.17 1.13 0.13 0.75

1
0.8
0.6
0.4
0.2
0

relΔLC3-II

siControl
siDICER

MEC-1

B

BaF
siCo
siDicer

Dicer
α-tubulin
LC3-I
LC3-II

1
0.8
0.6
0.4
0.2
0

relΔLC3-II

siControl
siDICER

CLE
MicroRNA-130a targets ATG2B and DICER1 to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells.

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