microRNA-dependent modulation of histone acetylation
in Waldenstrom macroglobulinemia

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Abstract

Waldenstrom Macroglobulinemia (WM) cells present with increased expression of miRNA-206 and reduced expression of miRNA-9*. Predicted miRNA-206 and -9*-targeted-genes include HDACs and HATs, indicating that these microRNA (miRNA) may play a role in regulating histone acetylation. We were able to demonstrate that primary WM cells are characterized by unbalanced expression of histone deacetylases (HDACs) and histone acetyl transferases (HATs), responsible for decreased acetylated-histone-H3 and -H4, and increased HDAC-activity. We next examined whether miRNA-206 and -9* modulate the aberrant expression of HDAC and HATs in WM cells leading to increased transcriptional activity. We found that restoring miRNA-9* levels, by transfecting WM cells with precursor-miRNA-9*, induced toxicity in WM cells, supported by down-modulation of HDAC4 and HDAC5 and up-regulation of acetyl-histone-H3 and -H4. These, together with inhibited HDAC-activity, led to induction of apoptosis and autophagy in WM cells. To further confirm that miRNA-9*-dependent modulation of histone acetylation is responsible for induction of WM cytotoxicity, a novel class of HDAC inhibitor (LBH589) was used; we confirmed that inhibition of HDAC-activity leads to toxicity in this disease. These findings confirm that histone-modifying genes and HDAC-activity are de-regulated in WM cells, partially driven by the aberrant expression of miRNA-206 and -9* in the tumor clone.
INTRODUCTION
Waldenstrom Macroglobulinemia (WM) is a B cell low-grade lymphoma characterized by an arrest of B cells after somatic hypermutation and before isotype class switching.\textsuperscript{1,2} Characterization of the cytogenetic and genetic abnormalities in WM has led to the identification of the long arm deletion on chromosome 6 (6q-) in about 35\% of the patients with this disease through the use of fluorescent in situ hybridization (FISH).\textsuperscript{3} However, other cytogenetic and chromosomal abnormalities that are common in plasma cell dyscrasias, including multiple myeloma or in other low-grade B cell malignancies, are not present in WM. Gene expression profiling in these patients has also been able to show that there are minimal genetic changes, with an expression profile similar to chronic lymphocytic leukemia myeloma.\textsuperscript{4} Therefore, multi-level characterization of this disease at the epigenetic level is necessary to better identify molecular abnormalities that lead to tumor progression and survival in this disease. Epigenetic alterations include methylation, histone acetylation, and microRNA (miRNA) regulation.\textsuperscript{5} Histone acetylation is commonly deregulated in many cancers. The balance of nucleosomal histone acetylation leads to the transcriptional regulation of many genes: hypoacetylation is associated with a condensed chromatin structure leading to the repression of gene transcription, and acetylation is associated with a more open chromatin structure and activation of transcription.\textsuperscript{6, 7} This balance is maintained by a tight regulation of the level of histone deacetylase (HDAC) and histone acetyl transferases (HATs). In many malignancies, this balance is deregulated, with an increased expression of HDACs leading to enhanced gene transcription.\textsuperscript{8, 9} HDACs are enzymes that catalyze the removal of the acetyl modification on lysine residues of proteins, including the nucleosomal histones H2A, H2B, H3, and H4. In addition, other induced genes include the cell cycle kinase inhibitor p21\textsuperscript{WAF1}, p16\textsuperscript{ink4a}, and p27\textsuperscript{Kip}, p53, NF-YA, and GATA-1, leading to enhanced cellular functions such as proliferation, cell-cycle, and survival.\textsuperscript{10} Recent report has demonstrated that WM cells present with a specific miRNA signature characterized by increased expression of miRNA-206 and reduced expression of miRNA-9*.\textsuperscript{11} Predicted miRNA-206 and -9*-targeted-genes include HDACs and HATs, leading us to hypothesize that miRNA-206 and -9* play a role in regulating
histone acetylation in WM. To date, the histone acetylation status in WM patients has not been investigated.

In this study, we investigate the role of miRNA-206 and -9* in the regulation of histone modification in WM. We first demonstrated that primary WM cells are characterized by unbalanced expression of HDACs and HATs at the mRNA level. We found that restoring miRNA-9* levels, by transfecting WM cells with precursor-miRNA-9*, resulted in induction of toxicity in WM cells, supported by down-modulation of HDAC4 and HDAC5 and up-regulation of acetyl-histone-H3 and –H4. These, together with inhibited HDAC activity, led to induction of apoptosis and autophagy in WM cells.

MATERIALS AND METHODS

Cells
WM cell line (BCWM.1) and IgM secreting low grade lymphoma cell lines (WM-WSU, MEC-1 and RL) were used in this study. The BCWM.1 is a recently described WM cell line that has been developed from a patient with untreated WM. MEC-1 was a gift from Dr. Kay (Mayo Clinic, Rochester, MN). RL was purchased from the American Tissue Culture Collection (Manassas, VA). Primary WM cells were obtained from bone marrow (BM) samples using CD19+ microbead selection (Miltenyi Biotec, Auburn, CA) with over 90% purity, as confirmed by flow cytometric analysis with monoclonal antibody reactive to human CD20-PE (BD-Bioscience, San Jose, CA), as described. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy subjects by Ficoll-Hipaque density sedimentation, and subsequently CD19+ selection was performed as described above. All cell lines and primary cells were cultured at 37°C in RPMI-1640 containing 10% fetal bovine serum (FBS; Sigma Chemical, St Louis, MO), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (GIBCO, Grand Island, NY). Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki protocol.
**Reagents**

LBH589 was provided by Novartis Pharmaceuticals (Cambridge, MA). The maximum final concentration of DMSO (< 0.1%) did not affect cell proliferation and did not induce cytotoxicity on all the cell lines and primary cells tested (data not shown).

**HDAC activity assay**

HDAC activity was determined by using Colorimetric HDAC Activity Assay Kit. (BioVision, Mountain View, CA), as described.\(^{14}\)

**Gene expression profiling**

Total RNA has been isolated from primary CD19+ cells isolated from bone marrow of patients with Waldenstrom Macroglobulinemia and from peripheral blood mononuclear cells of healthy donors, using RNeasy kit (Qiagen), as described by the manufacturer. Purified cRNA (15 \(\mu\)g) was hybridized to HG-U133Plus2.0 GeneChip (Affimetrix). RNA integrity was verified with the Agilent 2100 Bioanalyzer (Agilent, CA, USA).\(^{15}\)

**microRNA expression profiling**

RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA), as reported in previous miRNA studies.\(^{16-18}\) The expression of 318 miRNAs was investigated using liquid phase Luminex microbead miRNA profiling (Luminex, Austin, TX), as described.\(^{19}\) Briefly, 500 ng of total RNA was labeled with biotin using the FlexmiR MicroRNA labeling kit (Luminex, Austin, TX), which labeled all RNA molecules including small RNA, by first using Calf Intestinal Phosphatase (CIP) for removal of 5’-phosphatases from the terminal end of the miRNAs. In the second step, a biotin label was then attached enzymatically to the 3’-end of the miRNAs in the total RNA sample. After an enzyme inactivation step, the sample was hybridized with beads containing one of 100 different fluorophores and coated with oligonucleotides complementary to each known miRNA. Addition of streptavidin-phycoerythnn (Molecular Probes) then yielded fluorescence with wavelengths and amplitudes characteristic of the identity and quantity of miRNAs,
respectively. Normalization of arrays and calculation of median fluorescence intensity was performed according to the manufacturer’s instructions.

**Quantitative reverse transcription-PCR**

Stem-loop quantitative reverse transcription-PCR (qRT-PCR) for mature miRNAs (TaqMan microRNA Assays, Applied Biosystems, Foster City, CA) was performed as described on an Applied Biosystems AB7500 Real Time PCR system. All PCR reactions were run in triplicate and miRNA expression, relative to RNU6B, was calculated using the $2^{-\Delta\Delta Ct}$ method.

**miRNA transfection**

BCWM.1, MEC.1, RL cell lines were transfected with either pre-miRNA-9* (Ambion, Austin, TX), anti-miRNA-206 (Exiqon, Vedbaek, Denmark), or scramble probe at a final concentration of 40 nM, using Lipofectamine 2000 following manufacturer’s instructions (Invitrogen, Carlsbad, CA), as described. Culture medium was changed after transfection and replaced with RPMI 10% FBS. Cells were then used for functional assays at different time points (24 hours, 48 hours, and 72 hours). Both untransfected and scramble probe-transfected BCWM.1, MEC.1 and RL cell lines were used as controls. Efficiency of transfection was validated by qRT-PCR and microRNA assay.

**Growth inhibition assay**

The inhibitory effect of LBH589 on WM cell growth was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) (Promega, Madison, WI) dye absorbance of cells, as described.

**DNA synthesis and cytotoxicity assay**

DNA synthesis was measured by [3H] - thymidine ([3H]-TdR; Perkin Elmer, Boston, MA) uptake, as previously described. All experiments were performed in triplicate. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International, Temecula, CA) dye absorbance, as previously described.
Effect on paracrine WM cells growth in the BM
To evaluate the role of miRNA-9* and HDAC inhibition in regulating growth in WM cells in the context of primary bone marrow stromal cells (BMSCs), untransfected, scramble probe- and pre-miRNA-9*-transfected BCWM.1 cells were cultured in presence or absence of BMSCs for 48 hours. DNA synthesis was measured as previously described. Similarly, BCWM.1 cells were cultured in BMSC-coated 96-well plates for 48 hours in the presence or absence of LBH589. DNA synthesis was measured as previously described.

Flow cytometric analysis
Cell-cycle analysis was profiled by flow cytometry using propidium iodide (PI) staining (5 μg/mL, Sigma Chemical) after 24 hours culture with LBH589. Apoptosis was quantitated using Apo2.7 flow cytometric analysis (Beckman Coulter Inc., CA), as described.

Immunoblotting
BCWM.1, RL and MEC-1 cells were harvested and lysed using lysis buffer (Cell Signaling Technology, Beverly, MA) reconstituted with 5 mM NaF, 2 mM Na3VO4, 1 mM PMSF(polymethilsulfonyl fluoride), 5 μg/mL leupeptine, and 5 μg/mL aprotinin. Whole-cell lysates (50 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The antibodies used for immunoblotting included: anti-acetyl-tubulin (clone 6-11B-1) (Sigma, St Louis, MO); -HDAC4, -HDAC5, -acetylated histone H3, -acetylated histone H4, -acetylated lysine, -α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); -LC3B, -Rab7, -CDK2, -CDK4, -p21waf1, -p27kip1, -p53, -Bcl-XL, -Mcl-1, -caspase-3, -caspase-8, -caspase-9, -PARP, -p-IkB (Cell Signaling Technology, Beverly, MA); -Myst3 (Novus Biologicals, Littleton, CO). Nuclear extracts of the cells were prepared using the Nuclear extraction kit (Panomics Inc., Redwood City, CA, USA) and subjected to immunoblotting with anti-
HDAC4, -HDAC5, Myst3, and (Cell Signaling Technology, Beverly, MA). -nucleolin (Santa Cruz biotechnology, Santa Cruz, CA).

Statistical analysis
Statistical significance of differences in drug-treated versus control cultures was determined using Student’s t-test. The minimal level of significance was $P<0.05$. Gene-expression profiling supervised clustering analysis has been performed using the dChip software (www.dchip.org) ($P<0.05$). miRNA expression data was analyzed according to manufacturer’s instructions (Luminex, Houston, TX). The expression patterns of unfiltered data were performed using unsupervised hierarchical clustering of samples based on centroid linkage and 1-correlation distance metric, using dChip (www.dchip.org). To further define those miRNAs differentially expressed between groups (patients vs normal), the data were filtered on significance of differences using ANOVA test, ($P < 0.01$). To identify specific predicted miRNAs-targeted mRNAs, TargetScan, PicTar, and miRanda algorithms were used.22,23 To reduce the numbers of false positives, only putative target genes predicted by the three algorithms were considered.
RESULTS

Increased expression of miRNA-206 and decreased expression of miRNA-9* modulate histone acetylation in WM patients

We have previously demonstrated that bone marrow (BM)-derived WM CD19+ selected cells present with a specific miRNA signature which differentiates tumor cells from their normal cellular counterpart. Among the deregulated miRNAs, miRNA-206 and -9* are respectively increased and decreased compared to normal cells (Fig. 1A; \( P < 0.01 \)). We next analyzed the predicted gene targets for miRNA-206 and -9*, using miRanda, TargetScan, andPicTar, algorithms commonly used to predict human miRNA gene targets and specifically identified genes predicted by all three methods. Predicted targets for the increased miRNA-206 and decreased miRNA-9* included histone-acetyltransferases (HATs) and histone-deacetylases (HDACs), respectively, leading us to hypothesize that miRNA-206 and -9* play a role in modulating the histone-acetylation status in primary WM cells. To determine whether an imbalance of HDAC/HAT activity occurs in WM, we first examined HDACs and HATs expression at the mRNA level in primary CD19+ cells isolated from WM patients compared to their cellular counterpart. We found that primary WM cells were characterized by significant increased expression of HDAC-2, -4, -5, -6, -8, -9, and significant decreased expression of HAT-1, -2, and -3 (Fig. 1B).

We next examined how this imbalance could affect acetylated-histone status and HDAC activity level. Therefore, acetylated histone-H3 and -H4, as well as HDAC activity have been evaluated in bone marrow derived CD19+ WM cells compared to their normal counterpart. HDAC activity in WM and other IgM-secreting low-grade lymphoma cell lines was also investigated. We found a lower level of acetylated histone H3 and H4 in primary CD19+ WM cells compared to control (Fig. 2A-2B). Conversely, the level of HDAC activity was significantly higher in all WM patient samples and cell lines compared to the average of 3 normal controls, indicating that indeed HDAC activity is elevated in WM cells (Fig. 2C).

To further define the role of miRNA-206 and -9* in targeting histone-acetylation in WM cells as well as in other IgM-secreting low-grade lymphoma cell lines, acetylated-
histones expression and HDAC activity were examined in miRNA-206 knockdown and miRNA-9*-precursor (pre-miRNA-9*)-transfected cells, compared to controls (control-probe transfected- and untransfected cells). Efficiency of transfection was evaluated by qRT-PCR at 48 hours after transfection (Suppl. Fig. 1A-B-C). We first investigated whether predicted HDAC4/HDAC5 and HAT (Myst3) mRNAs were targeted at the protein level by miRNA-9* and miRNA-206, respectively. We first demonstrated that miRNA-9* induces down-modulation of HDAC4 and HDAC5 without affecting Myst3 protein expression, as shown by western blot in precursor (pre)-miRNA-9*-transfected cells (Fig. 3A), compared to either untransfected or scramble probe-transfected cells, used as controls. We also validated that miRNA-206 targets HAT (Myst3), as shown by up-regulation of Myst3 protein level in anti-miRNA-206-transfected cells compared to controls (Fig. 3B). pre-miRNA-9*- and anti-miRNA-206-dependent modulation of the relative targets was also confirmed at mRNA level (Suppl. Fig. 1D). We also confirmed that acetyl histone-H3 and -H4 were upregulated in pre-miRNA-9*- and –anti-miRNA-206-transfected cells, with a higher acetyl histone-H3 and –H4 up-regulation upon miRNA-9* modulation (Fig. 3C). We next investigated whether miRNA-9*- and -206-dependent regulation of acetyl histone-H3 and –H4 depends on changes in HDAC activity in cell lines; and found that HDAC activity was down-regulated in pre-miRNA-9*-transfected cells compared to either untransfected or scramble probe-transfected cells (Fig. 3D). HDAC activity was not modified in anti-miRNA-206-transfected cells compared to controls (Fig. 3D). Further validation of HDAC inhibition inducing up-regulation of acetyl histone-H3 and -H4 in WM, as well in other IgM secreting low grade lymphoma cell lines, was demonstrated by Western blot in cells treated with the pan-HDAC inhibitor LBH589 in a dose-dependent manner (Fig. 3E). Similarly, modulation of HDAC activity was observed in WM and other IgM secreting low grade lymphoma cell lines upon LBH-589 treatment in a dose-dependent manner (Fig. 3F).

**miRNA-9*-dependent regulation of proliferation and survival in WM cells**

Based on the ability of miRNA-9* to modulate HDAC activity in WM cells, we next sought to determine the effect of miRNA-9* in regulating cell proliferation and survival. We showed that DNA synthesis was significantly reduced in pre-miRNA-9*-transfected
cells compared to control at both 48 hours and 72 hours ($P < 0.05$; Fig. 4A). In addition, induction of toxicity was observed in pre-miRNA-9*-transfected BCWM.1 cells compared to controls ($P < 0.05$; Fig. 4A). Similar results were confirmed in other IgM secreting low grade lymphoma cell lines, MEC.1 (Fig. 4B) and RL (Fig. 4C). In contrast, DNA synthesis and toxicity were not demonstrated in anti-miRNA-206-transfected cells (Suppl. Fig. 2A-B-C). To better define whether miRNA-9*-induced cytotoxicity is functionally related to HDAC inhibition, BCWM.1 cells were cultured in presence or absence of LBH589 50nM for 8 hours, washed, and subsequently transfected using pre-miRNA-9* probe, and harvested after 48 hours. Untransfected-untreated cells were used as control. We observed that pre-miRNA-9*-transfected cells did not present with significant increased toxicity as compared to LBH589-pretreated cells, suggesting that miRNA-9*-dependent induction of cell death is related to HDAC inhibition (Suppl. Fig 2D). We next evaluated cell cycle profiling in pre-miRNA-9* transfected cells: increased number of cells in G1 phase was observed upon pre-miRNA-9* transfection (65% vs 53%), together with a decreased number of cells in S phase (20% vs 34%). It has been previously demonstrated that HDAC4 promotes the repression of cyclin-dependent kinase inhibitors p21.24 We demonstrated that p21 expression was up-regulated in pre-miRNA-9*-transfected cells compared to either untransfected or scramble probe-transfected cells (Fig. 4E). Since p21 does not represent a miRNA-9*-predicted mRNA, this could possibly be driven by miRNA-9*-dependent HDAC4 modulation in WM cells. To further validate the role of HDAC inhibition in reducing WM cell proliferation, the effect of the HDAC inhibitor LBH589 was next tested in primary WM cells. LBH589 induced cytotoxicity in primary CD19+ cells isolated from the bone marrow of three patients with WM (IC$_{50}$ 30-40 nM; Fig. 4F). In contrast, LBH589 had no cytotoxic effect on CD19+ cells isolated from normal PBMCs (Suppl. Fig. 3A). Induction of cytotoxicity was also observed in WM and other IgM secreting low grade lymphoma cell lines exposed to increasing concentrations of LBH589. LBH589 inhibited BCWM.1 proliferation, as measured by MTS assay, with an IC$_{50}$ between 20 and 40 nM. LBH589 demonstrated similar activity on all cell lines tested, with IC$_{50}$ between 20 and 40 nM at 48 hours (Suppl. Fig. 3B-3C). We next confirmed that cell cycle progression was modulated upon HDAC inhibitor treatment. LBH589 induced subG1 arrest (1.5%, 5.9%,
13.2% and 27.7% at LBH589 0 nM, 20 nM, 40 nM, and 60 nM, respectively) with an associated decrease in the percentage of cells in proliferative phases S-G2/M (36.5%, 24.1%, 20.7% and 12.4% at LBH589 0 nM, 20 nM, 40 nM and 60 nM, respectively) (Suppl. Fig. 3D). To determine the mechanism of LBH589-induced cell cycle arrest, we investigated the effect of LBH589 on BCWM.1 cells using immunoblotting. BCWM.1 cells were treated with LBH589 (0-60 nM) for 16 hours. LBH589 induced the up-regulation of cyclin kinase inhibitor proteins p21Cip1 and p27kip1, as well as the down-regulation of cyclin D2, and cyclin-dependent kinase (cdk2, cdk4) protein levels (Suppl. Fig. 3E).

**Mechanisms of miRNA-9*-dependent toxicity in WM cells**

Cell cycle profiling was also able to show an up-regulation of sub-G1 phase in pre-miRNA-9*-transfected cells compared to controls (Fig. 4D), indicating presence of apoptotic cells; indeed, induction of apoptosis was observed in pre-miRNA-9*-transfected cells, as shown by Apo2.7 staining and flow cytometry analysis (Fig. 5A). miRNA-9*-dependent modulation of apoptosis in WM cells was supported by PARP-, caspase-8- and caspase-9-cleavage as shown by Western blot in pre-miRNA-9*-transfected cells compared to either untransfected or scramble probe-transfected cells (Fig. 5B).

To further confirm that miRNA-9*-dependent modulation of HDAC activity leads to toxicity in WM cells, the effect of LBH589 has been tested in WM cells. Histone acetylation regulates the function of many genes involved in cell survival.10,26 We therefore sought to examine the effect of LBH589 on the expression of proteins known to be regulated by histone acetylation; we observed an up-regulation of p53, together with down-regulation of BCL-XL, Mcl-1 and c-myc (Fig. 5C). In addition, LBH589-dependent inhibition of HDACs resulted in the activation of both intrinsic and extrinsic apoptotic pathways with caspase-9, caspase-8, caspase-3, and PARP cleavage in a dose-dependent manner (Fig. 5D). We next examined the functional effect of HDAC inhibition on apoptosis of WM cells; we demonstrated that LBH589 induced significant apoptosis in a dose-dependent manner, as evidenced by Apo2.7 staining and flow cytometry analysis. The percentage of apoptotic BCWM.1 cells increased from 3.2% (untreated) to
27.8% and 65.6% after 48 hours of treatment with LBH589 20 nM and 60 nM, respectively (Fig. 5E). Similar data were obtained with other IgM secreting cell lines (data not shown).

It has been demonstrated that HDAC inhibition leads to apoptosis though caspase-independent mechanisms, such as inducing autophagy. We therefore evaluated the efficacy of miRNA-9* in modulating autophagy in WM cells; and found upregulation of Rab7 and LC3B in pre-miRNA-9*-transfected cells compared to either untransfected or scramble probe-transfected cells (Fig. 6A). The effect of LBH589 in inducing autophagy was next evaluated. BCWM.1 cells were incubated with LBH589 (40 nM for 3, 6, 12, and 24 hours), and whole-cell extracts were then analyzed using immunoblotting. We found that LBH589 increased LC3B and Rab7 expression in a time-dependent manner (Fig. 6B). We further confirmed that LC3B and Rab7 protein levels were upregulated by LBH589 in BCWM.1 cells using immunofluorescence (Fig. 6C; Fig. 6D). These data confirm that HDAC inhibition, due to either miRNA9*-dependent modulation or LBH589 treatment, results in induction of autophagy in WM cells.

Neither adherence to BMSCs nor growth factors protect against miRNA-9*-dependent effect on WM cells
Since the BM microenvironment confers growth and induces drug resistance in malignant cells, we next investigated whether miRNA-9*-dependent modulation of WM cell proliferation and survival could inhibit WM cell growth even in the context of the BM milieu. Untransfected, scramble probe-transfected and pre-miRNA-9*-transfected WM cells were cultured in the presence or absence of BMSCs for 48 hours; we found that the adherence of untransfected, scramble probe, or pre-miRNA-9*-transfected BCWM.1 cells to BMSCs triggered an increase of 70%, 68%, and 47% in proliferation, respectively, as compared to untransfected, scramble probe-, and pre-miRNA-9* transfected cells cultured alone (Fig. 7A), indicating the ability of miRNA-9* to inhibit WM cells proliferation, even in the presence of bone marrow milieu.

Previous studies using gene expression analysis in WM have demonstrated an upregulation in IL-6 signaling. IL-6 also promotes plasmacytoid lymphocyte growth in WM, and serum IL-6 levels reflect tumor burden and disease severity. Similarly, it has
been previously shown that IGF-1 induces the proliferation of WM cells.\textsuperscript{16} We therefore next tested whether the addition of recombinant human IL-6 (25 ng/mL) or IGF-1 (50 ng/mL) can overcome miRNA-9*-dependent WM cytotoxicity. Both IL-6 and IGF-1 induced proliferation of either untransfected or scramble probe-transfected cells, in contrast with pre-miRNA-9*-transfected cells where IL-6 and IGF-1 did not exert any pro-proliferative effect (Fig. 7B).

To further confirm the role of HDAC inhibition in reducing WM cell proliferation, even in the context of BM milieu and in presence of IL-6 and IGF-1, the effect of the HDAC inhibitor LBH589 was next tested in WM cells in presence or absence of BMSCs and cytokines. BCWM.1 cells were cultured with LBH589 (10-40 nM) in the presence or absence of BMSCs for 48 hours. The viability of BMSCs, assessed by MTS, was not affected by LBH589 treatment (data not shown). Using \textsuperscript{[3]H}-TdR uptake assay, adherence of BCWM.1 cells to BMSCs triggered an increase of 77\% in proliferation, which was inhibited by LBH589 in a dose-dependent manner (Fig. 7C), even in the presence of the bone marrow milieu. In addition, both IL-6 and IGF-1 induced proliferation of BCWM.1 cells and the addition of LBH589 (10-40 nM) inhibited IL-6- and IGF-1-induced proliferation of BCWM.1 cells, indicating that LBH589 can overcome resistance induced by growth factors such as IL-6 and IGF-1 (Fig. 7D).
DISCUSSION
Alterations in the balance between HAT and HDAC activity in many cancers will lead to deregulated gene expression and the induction of proliferation and survival in tumor cells.\textsuperscript{5-10} HDACs mediate the function of oncogenic translocations in many malignancies including PML-RAR in acute promyelocytic leukemia.\textsuperscript{32} Most of the aberrant HAT and HDAC activity has been due to translocation, amplification, overexpression or mutation in many malignancies, including hematological malignancies.\textsuperscript{9,25,26} However mechanisms responsible for the modulation of histone acetylation and HDAC activity in WM have not been fully elucidated.
Recent studies have demonstrated that miRNAs may exert their activity by interfering with the epigenetic machinery, such as modulating the expression of enzymes regulating DNA methylation or histone modification.\textsuperscript{33-35} Indeed, miRNA-449a regulates histone acetylation status in prostate cancer cells by targeting HDAC1. It has been demonstrated that up-regulation of miRNA-449a in prostate cancer cells exerts an anti-proliferative effect on the tumor clone, supported by cell cycle arrest and induction of a senescence-like phenotype and apoptosis.\textsuperscript{35} In addition, other miRNAs are responsible for targeting histone methyltransferases. For example, it has been recently reported that miRNA-101 tags the enhancer of Zeste homolog 2 (EZH2); the low expression level in several tumor types could lead to up-regulation of EZH2 in aggressive tumors with an invasive phenotype.\textsuperscript{36,37} These findings confirm the role of miRNAs in regulating histone acetylation status in clonal tumor cells.
We have been able to demonstrate that primary WM cells present with decreased expression of miRNA-9* and increased expression of miRNA-206 compared to their normal cellular counterpart. Predicted targets for miRNA-206 and -9* include histone-modifying genes, such as HDACs and HATs. We first confirmed the efficacy of miRNA-9* and miRNA-206 in targeting HDACs and HAT, respectively. We therefore hypothesized that altered miRNA signature in WM cells could modulate histone acetylation and HDAC activity in the tumor clone. We demonstrated that primary WM cells are characterized by unbalanced expression of HDACs and HATs at the mRNA level, responsible for decreased acetylated-histone-H3 and -H4, and increased HDAC activity in primary WM cells compared to normal cells, suggesting that histone
modification may play a role in the pathogenesis of WM.

We next showed that miRNA-9* and –206 play a functional role in regulating histone-acetylation and HDAC activity in WM cells, leading to induction of toxicity in WM cells, as shown by decreased DNA synthesis, cell cycle arrest, and induction of apoptosis in pre-miRNA-9*-transfected cells. We established that reduced expression of miRNA-9* and increased expression of miRNA-206 in primary WM cells resulted in higher HDAC activity together with lower acetylated status of H3 and H4. This was accomplished through the ability of these miRNA to target HDAC4/HDAC5 and Myst3, respectively. The aberrantly upregulated HDAC activity in WM cells was inhibited by over-expressing miRNA9* in WM cells, as well in other IgM secreting low grade lymphoma cell lines, which led to cytotoxicity and induction of apoptosis in WM cells. Similar results were obtained when tumor cells were exposed to the HDAC inhibitor LBH589, indicating that targeting HDAC is critical in this disease.

In summary, our data indicate that loss of miRNA-9* may be responsible for up-regulation of HDAC4 and HDAC5 in primary WM cells, contributing to the pathogenesis of this disease; this also indicates the potential therapeutic value of synthetic miRNA oligonucleotides as epigenetic modulators with a mechanism of action similar to chemical HDAC inhibitors.
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AUTHOR CONTRIBUTION
AMR, AS, IMG: designed research, analyzed data, wrote the paper
AS, XJ, AKA, HTN, PM, PQ, FA, JR: performed research

CONFLICT OF INTEREST
Aldo M. Roccaro: No Conflict of Interest.
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REFERENCES


FIGURE LEGEND

Figure 1. Primary WM cells present with higher level of miRNA-206, lower level of miRNA-9*, together with increased expression of histone-deacetylases and reduced expression of histone-acetyl transferases, as compared to healthy donors.

(A) miRNA analysis has been performed on total RNA isolated from BM CD19+ WM cells, normal bone marrow (NBM)- and peripheral blood (PBMC)-derived CD19+ counterparts, and WM cell line (BCWM.1). Heatmap was generated after supervised hierarchical clustering analysis was performed using ANOVA test. Differential expression of miRNA patterns is shown by the intensity of red (up-regulation) versus blue (down-regulation). (B) Purified cRNA (15 μg) isolated from primary CD19+ cells isolated from bone marrow of 6 WM patients, and from CD19+ cells isolated from peripheral blood mononuclear cells of 2 healthy donors were hybridized to HG-U133Plus2.0 GeneChip (Affimetrix). Fold change is shown by the intensity of induction (red) or suppression (blue).

Figure 2. Lower expression of acetylated histone-3/histone-4 and higher HDAC activity characterize primary WM cells as compared to healthy donors.

Immunocytochemical analysis of primary CD19+ cells isolated from bone marrow of 4 WM patients (A), and CD19+ cells isolated from peripheral blood mononuclear cells of 4 healthy donors (B), was performed using anti-acetyl-histone- H3 (Lys18), -H3 (lys9/14), -H4 (lys12) antibodies. DAPI was used to stain nuclei. (C) HDAC activity was assessed using nuclear extracts using a Colorimetric HDAC Activity Assay Kit, on primary CD19+ cells isolated from bone marrow of 6 WM patients (WM1, WM2, WM3, WM4, WM5, WM6); primary CD19+ cells isolated from peripheral blood mononuclear cells of 3 healthy donors (average HD); and BCWM.1, and low grade lymphomas IgM secreting cell lines (P all ≤ .05).
Figure 3. miRNA-9* and miRNA-206 target HDAC4/HDAC5 and Myst3 respectively.

(A) BCWM.1 cells (scramble probe-, pre-miRNA-9*-transfected; and untransfected) were harvested at 12 hours after transfection. Nuclear lysates were subjected to Western blot using anti-HDAC4, -HDAC5, -Myst3, and -nucleolin antibodies. (B) BCWM.1 cells (scramble probe-, anti-miRNA-206-transfected; and untransfected) were harvested at 12 hours after transfection. Nuclear lysates were subjected to Western blot using anti-Myst3, and -nucleolin antibodies. (C) BCWM.1 cells (scramble probe-, pre-miRNA-9*-, anti-miRNA-206-transfected; and untransfected) were harvested at 12 hours after transfection. Whole cell lysates were subjected to Western blotting using anti-acetyl-histone H3, -acetyl-histone H4, -acetylated-tubulin, -acetylated-lysine and -actin antibodies. (D) BCWM.1 cells (scramble probe-, pre-miRNA-9*-, anti-miRNA-206-transfected; and untransfected) were harvested at 12 hours after transfection. HDAC activity was assessed in vitro using nuclear extracts by Colorimetric HDAC Activity Assay Kit. (* P <0.05). (E) BCWM.1, RL and MEC-1 cells were cultured with LBH589 (20-60 nM) for 16 hours or control medium. Whole cell lysates were subjected to Western blotting using anti-acetyl-histone H3, -acetyl-histone H4, -acetylated-tubulin, -acetylated-lysine and -α-tubulin antibodies. (F) BCWM.1 cells were cultured in presence or absence of LBH589 (0-80nM; 8 hours). HDAC activity was assessed in vitro using nuclear extracts by Colorimetric HDAC Activity Assay Kit. (P all ≤ 0.05).

Figure 4. miRNA-9* regulates proliferation and survival in WM cells as well as in low grade lymphoma IgM secreting cells.

(A) WM cells (A: BCWM.1), low grade lymphoma IgM secreting cell lines (B: MEC1; C: RL) (scramble probe-, pre-miRNA-9*-transfected and untransfected) were harvested at 24-48-72 hours after transfection; DNA synthesis and cytotoxicity were assessed by thymidine uptake and MTT assays, respectively. (* P<0.05). (D) Cell cycle analysis was performed by propidium iodide staining using BCWM.1 cells (untransfected, scramble probe- and pre-miRNA-9*-transfected). (E) BCWM.1 cells (untransfected, scramble probe- and pre-miRNA-9*-transfected) were harvested at 12 hours after transfection. Whole cell lysates were subjected to Western blotting using anti-p21 and -actin
antibodies. (F) Freshly isolated normal CD19+ cells from normal PBMCs were cultured with LBH589 (0-80 nM) for 48 hours. Cytotoxicity was assessed by MTS assay.

**Figure 5. miRNA-9*-dependent HDAC inhibition exerts a pro-apoptotic effect on WM cells.**

(A) Percentage of cells undergoing apoptosis was studied by Apo2.7 staining and flow cytometry in WM and low grade lymphoma IgM secreting cell lines (untransfected, scramble probe-, pre-miRNA-9*-transfected were harvested 48 hours after transfection). (B) BCWM.1 cells (untransfected, scramble probe- and pre-miRNA-9*-transfected) were harvested at 12 hours after transfection. Whole cell lysates were subjected to Western blot using anti-PARP, -caspase-8, -caspase-9, and -actin antibodies. (C, D) BCWM.1 cells were cultured with LBH589 (0-60 nM) for 16 hours. Whole cell lysates were subjected to Western blot using anti-p53, -BCL-XL, -Mcl-1, -c-myc, -β-actin, -PARP, -caspase 9, -caspase 8, -caspase 3, and -α-tubulin antibodies. (E) BCWM.1 cells were cultured with LBH589 for 48 hours at doses that range from 0 to 60 nM, and the percentage of cells undergoing apoptosis was studied by Apo2.7 staining by flow cytometry.

**Figure 6. miRNA-9*-dependent-HDAC inhibition and modulation of autophagy in WM cells.**

(A) BCWM.1 cells (untransfected, scramble probe- and pre-miRNA-9*-transfected) were harvested at 24 hours after transfection. Whole cell lysates were subjected to Western blot using anti-Rab7, -LC3 and -actin antibodies. (B) BCWM.1 cells were cultured with LBH589 (40 nM) for 0 to 24 hours. Whole cell lysates were subjected to Western blotting using anti-Rab7 and -LC3B antibodies. (C; D) BCWM.1 cells were cultured in presence or absence of LBH589 (40 nM) for 16 hours. Immunocytochemical analysis was assessed using anti-Rab7 (C) or –LC3B (D) antibodies.

**Figure 7. miRNA-9*-dependent modulation of WM cell proliferation in the context of bone marrow milieu.**
(A) BCWM.1 cells (untransfected; scramble probe-, pre-miRNA-9*-transfected) were cultured for 48 hours in presence or absence of primary bone marrow stromal cells (BMSCs). Cell proliferation was assessed using the [³H]-thymidine uptake assay. (B) BCWM.1 cells (untransfected; scramble probe-, pre-miRNA-9*-transfected) were cultured in presence or absence of IL-6 (25 ng/mL) or IGF-1 (50 ng/mL) for 48 hours. DNA synthesis was assessed using [³H]-thymidine uptake assay. (C) BCWM.1 cells were cultured with LBH589 (0- 40 nM) for 48 hours in the presence or absence of WM patient-derived BMSCs. Cell proliferation was assessed using the [3H]-thymidine uptake assay. (D) BCWM.1 cells were cultured with LBH589 (0-40 nM) in the absence and presence of IL-6 (25 ng/mL) or IGF-1 (50 ng/mL) for 48 hours. DNA synthesis was assessed using the [3H]-thymidine uptake assay.
Figure 2

(A) Acetyl-H3 (Lys18) and Acetyl-H3 (Lys9/14) staining in WM CD19⁺ cells.

(B) Acetyl-H3 (Lys18), Acetyl-H3 (Lys9/14), and Acetyl-H4 (Lys12) staining in Normal CD19⁺ cells.

(C) Bar graph showing HDAC activity (% of control) for various cell lines: average HD, WM1, WM2, WM3, WM4, WM5, WM6, BCWM1, RL, MEC.1.
Figure 4

A

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B

C

D

E

F

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Figure 5

(A) Apoptotic cells (% of control) for BCWM.1, MEC.1, and RL treated with untransfected, scramble-probe, and pre-miRNA-9*.

(B) Western blot showing PARP, Caspase-8, Caspase-9, and actin levels in untransfected, scramble-probe, and pre-miRNA-9* treated cells.

(C) Western blot showing p53, β-actin, BCL-XL, Mcl-1, c-myc, and α-tubulin levels at different LBH589 concentrations (0, 20, 40, 60 nM).

(D) Western blot showing PARP, Caspase-9, Caspase-8, Caspase-3, and actin levels at different LBH589 concentrations (0, 20, 40, 60 nM).

(E) Graph showing the % of apoptotic cells in response to LBH589 concentrations (0, 5, 10, 20, 40, 60 nM).
Figure 7

A

DNA synthesis (cpm x 10^3)

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B

DNA synthesis (% of control)

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C

DNA synthesis (cpm x 10^3)

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D

DNA synthesis (% of control)

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<tr>
<td>Control</td>
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<td>IGF-1 (50ng/ml)</td>
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LBH589 [nM]
microRNA-dependent modulation of histone acetylation in Waldenstrom macroglobulinemia

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