miR-155 targets histone deacetylase 4 (HDAC4) and impairs transcriptional activity of B-cell lymphoma 6 (BCL6) in the Eμ-miR-155 transgenic mouse model

Sukhinder K. Sandhu*, Stefano Volinia*a,b, Stefan Costinean, Marco Galasso, Reid Neinast, Ramasamy Santhanam, Mark R. Parthun, Danilo Perrotti, Guido Marcuccio, Ramiro Garzon, and Carlo M. Croce*a,1,2

*aDepartment of Molecular Virology, Immunology, and Medical Genetics, Ohio State University Medical Center, Columbus, OH 43210; bData Mining for Analysis of Microarrays, Department of Morphology and Embryology, Università degli Studi, Ferrara 44100, Italy; and cDepartment of Molecular and Cellular Biochemistry and cDivision of Hematology, Department of Internal Medicine, Ohio State University Medical Center, Columbus, OH 43210

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Multiple studies have established that microRNAs (miRNAs) are involved in the initiation and progression of cancer. Notably, miR-155 is one of the most overexpressed miRNAs in several solid and hematological malignancies. Ectopic miR-155 expression in mouse B cells (Eμ-miR-155 transgenic mice) has been shown to induce pre-B-cell proliferation followed by high-grade lymphoma/leukemia. Loss of miR-155 in mice resulted in impaired immunity due to defective T-cell-mediated immune response. Here we provide a mechanistic insight into miR-155-induced leukemogenesis in the Eμ-miR-155 mouse model through genome-wide transcriptome analysis of naïve B cells and target studies. We found that a key transcriptional repressor and proto-oncogene, Bcl6 is significantly down-regulated in Eμ-miR-155 mice. The reduction of Bcl6 subsequently leads to de-repression of some of the known Bcl6 targets like inhibitor of differentiation (Id2), interleukin-6 (IL6), cMyC, Cyclin D1, and Mip1α/ccl3, all of which promote cell survival and proliferation. We show that Bcl6 is indirectly regulated by miR-155 through Mad1/Max1/Mad1 up-regulation. Interestingly, we found that miR-155 directly targets HDAC4, a corepressor partner of Bcl6. Furthermore, ectopic expression of HDAC4 in human-activated B-cell–type diffuse large B-cell lymphoma (DLBCL) cells results in reduced miR-155–induced proliferation, clonogenic potential, and increased apoptosis. Meta-analysis of the diffuse large B-cell lymphoma patient microarray data showed that miR-155 expression is inversely correlated with Bcl6 and Hdac4. Hence this study provides a better understanding of how miR-155 causes disruption of the BCL6 transcriptional machinery that leads to up-regulation of the survival and proliferation genes in miR-155–induced leukemias.

Results and Discussion

Signaling Pathways Modulated by miR-155. We have previously shown that miR-155 overexpression in mouse B cells induces pre–B-cell leukemia/lymphoma (6), but the exact mechanism of pathogenesis needs further investigation. To identify potential miR-155 targets involved in the pathogenesis of B-cell leukemia/lymphoma in the Eμ-miR-155 mice, we performed miRNA expression profiling of purified (naïve resting) B cells from transgenic and wild-type mice spleens. We found that 268 genes were down- and 1,077 were up-regulated in the Eμ-miR-155 transgenic mice B cells compared with wild-type controls (Dataset S1). We performed a comprehensive pathway analysis of the differentially expressed genes to obtain the systems biology overview of the miR-155–mediated gene regulation using the extensive knowledgebase at the Ingenuity Pathway Analysis (IPA).

MicroRNA analysis of human and mouse miRNAs reveals that miR-155 is one of the most frequently overexpressed miRNAs in various cellular processes including proliferation, differentiation, and development. Recent studies have established that expression of miRNAs is widely altered in a variety of cancers and miR-155 is one of the most frequently overexpressed miRNAs in various solid and hematological malignancies (1). miR-155 is highly up-regulated in Hodgkin, primary mediastinal, and diffuse large B-cell lymphomas (DLBCL) (2, 3) and is almost absent or significantly down-regulated in primary cases of Burkitt lymphoma (4). Overexpression of bic, host mRNA of miR-155, caused increased incidence of leukemia and a decrease in latency of lymphoma development in chickens with elevated levels of MYC (5). Overexpression of miR-155 in mice B cells (Eμ-miR-155) has been shown to cause pre–B-cell leukemia/high-grade lymphoma (6), whereas deletion of bic/miR-155 in mice has been attributed to immunodeficiency and impaired T-cell–dependent antibody response (7, 8). Additionally, sustained miR-155 expression in stem cell progenitors induced a myeloproliferative disease in transplanted mice (9). Despite the availability of multiple animal models and a plethora of target studies, the precise mechanism of miR-155–induced leukemogenesis remains elusive.

NfκB | Ingenuity Pathway Analysis

The proto-oncogene BCL6 belongs to the POK (Poxviruses and Zinc-finger and Kruppel) family of transcription repressors. It has a role in germinal center development, Th2 response, and regulation of lymphocyte function, survival, and differentiation (10). It is frequently dysregulated in various non-Hodgkin lymphomas (NHLs) due to translocations, deletions, or point mutations, which juxtapose its regulatory region to heterologous promoters. However, its down-regulation in other cancers is relatively less defined (11). HDACs are a class of chromatin modifiers that act by deacetylation the lysine tails of histones and are often recruited by corepressors to regulate target gene expression by deacetylation. POK family transcription factors like BCL6 and PLZF (promyelocytic leukemia zinc-finger) have been shown to mediate transcriptional repression by recruiting HDACs like HDAC4 in hematopoietic cell differentiation, leukemogenesis, and inflammation (12, 13).

To investigate additional targets and understand mechanisms of miR-155–induced leukemogenesis, we undertook this study of profiling naïve B cells from a miR-155 transgenic mouse model. We show that miR-155 directly targets HDAC4 and indirectly regulates BCL6 expression and activity and leads to deregulation of a BCL6 transcriptional program, both of which play an important role in B-cell leukemias.
Ingenuity Systems Inc.). Among the top five pathways represented by the up-regulated genes was Aryl Hydrocarbon Receptor (AHR) Signaling (Table 1, up-regulated pathways), a stress responsive pathway linked to B-cell differentiation by modulating B-cell development gene networks (14). Interestingly, AHR mediates signaling by transactivating MYC on interaction with the RelA subunit of NfkB, both of which are also up-regulated in Eq-miR-155 mice B cells (Dataset S1).

The canonical pathways represented by the down-regulated genes can be unified by processes involved in impaired hematopoietic progenitor cell signaling mediated by kinases like MAPK (Table 1, down-regulated pathways). Interestingly, the B-cell receptor signaling pathway, which is required for maturation of pre-B cells to mature B, was also significantly down-regulated ($P < 0.05$) in these mice. Among the molecules of this pathway were Bcl6, p38 Mapk, IkKkb, and Atf2, which may contribute to the disruption of normal B-cell development in Eq-miR-155 mice. Collectively, the gene signature represented in the miR-155 overexpressing mouse B cells was representative of increased proliferation and dysregulated B-cell-development-related pathways.

We first confirmed the down-regulation of Bcl6 mRNA in Eq-miR-155 mice spleen cells using quantitative real time PCR (qRT-PCR) (Fig. 1A, Left). Further analysis of Bcl6 mRNA from purified spleen pre-B (B220$^+$CD34$^+$ IgM$^-$) and naive-B (B220$^+$CD34$^+$ IgM$^+$) cells showed the most significant down-regulation in naive B cells (Fig. 1A, Left). However, pre-B cells from Eq-miR-155 transgenic mice also consistently showed lower Bcl6 expression compared with their wild-type counterparts (Fig. 1A, Right). We also found decreased Bcl6 protein levels in total splenocytes (Fig. 1B, Left) and purified naive B cells (Fig. 1B, Middle) of Eq-miR-155 mice compared with wild type. To verify this regulation in vitro, we cotransfected Bcl6 cDNA in HEK-293T cells when cotransfected with premiR-155 and also found significant down-regulation of endogenous Bcl6 mRNA compared with scrambled control (Fig. 1C). Further we also found significant down-regulation of ectopically expressed Bcl6 cDNA in HEK-293T cells when cotransfected with premiR-155 oligos versus the scrambled control (Fig. 1B, Bottom). Additionally, miR-155–deleted B cells from bic knock-out mice spleens (CD19$^+$ total B cells) expressed higher levels of Bcl6 as compared with those from wild-type mice (Fig. 1D).

Altogether, these results confirm that Bcl6 is down-regulated in the Eq-miR-155 model of B-cell leukemia, which was perplexing because it is often up-regulated in a subset of human B-cell lymphomas. However, interestingly, between the two subsets of human DLBCL, the germinal center B-cell DBLCL (GCB-DLBCL), which overexpresses Bcl6 also has lower levels of miR-155, and the activated B-cell DBLCL (ABC-DLBCL), which overexpresses miR-155, has low levels of Bcl6 (15, 16). Higher Bcl6 levels in GCB-DLBCL are also associated with better prognosis. This correlation substantiates our findings and provides an insight into the Eq-miR-155 mouse model, which mostly develops pre-B-cell leukemia but has a B-cell gene expression signature resembling the miR-155 overexpressing human ABC-DLBCL. This is also in line with the role of miR-155 in B-cell activation and its induction with BCR cross-linking (17). Altogether this implies the overlapping molecular signatures from multiple malignancies, which can take different courses. Next, we analyzed the Eq-miR-155 mice B cells’ transcriptome to study the fate of Bcl6-regulated gene.

### Downstream Targets of BCL6 Are Actuated in Eq-miR-155 Mice

We reasoned that BCL6 suppression by miR-155 might unblock its oncogenic transcriptional targets, which may play a role in leukemogenesis. BCL6 is known to act as a transcriptional repressor of various genes involved in B-cell activation, survival, cell cycle arrest, cytokine signaling, and differentiation (18). Several published studies have reported on BCL6 targets and its interacting partners using multiple biochemical techniques like Tandem Mass Spectrometry (19), ChIP-on-chip (20), and integrating biochemical and computational approaches (21). Hence, we compiled the dataset of published BCL6 targets from these studies and used it to investigate the up-regulated gene set from Eq-miR-155 mice B cells using hierarchical clustering analysis. We found a significant overlap among the known BCL6 targets in the up-regulated dataset from Eq-miR-155 mice B cells (Fig. 1F). Next, to assess the functions represented by these genes, we performed a Gene Set Enrichment Analysis (www.broadinstitute.org/gsea) to assess the functions represented by these genes, we performed a Gene Set Enrichment Analysis (www.broadinstitute.org/gsea) to assess the functions represented by these genes, we performed a Gene Set Enrichment Analysis (www.broadinstitute.org/gsea) to assess the functions represented by these genes, we performed a Gene Set Enrichment Analysis (www.broadinstitute.org/gsea) to assess the functions represented by these genes, we performed a Gene Set Enrichment Analysis (www.broadinstitute.org/gsea) to assess the functions represented by these genes, we performed a Gene Set Enrichment Analysis (www.broadinstitute.org/gsea) to assess the functions represented by these genes.

### Table 1. Categorization of top canonical pathways represented by the genes up-regulated and down-regulated in Eq-miR-155 mice naive B cells using IPA

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Molecules</th>
<th>–log(P value)</th>
<th>Ratio</th>
</tr>
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<tbody>
<tr>
<td>Up-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHR signaling</td>
<td>MGST1, CCNE2, NFIX, TFDP1, GSTM5, NQO2, POLA1, CD6, CCND1, GSTO1, CHEK1, MYC, TMG2, CCNA2, CCND3, ALDH1A1, MGST2, E2F1, DHFR, ESR1, AHR</td>
<td>4.74E+00</td>
<td>1.67E-01</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>GSR, MGST1, MGST2, GSTM5, G6PD, IDH2, GCLM, GLRX, ANPEP, GSTO1, RNPEP, IDH1</td>
<td>4.69E+00</td>
<td>2.45E-01</td>
</tr>
<tr>
<td>Mitotic roles of polo-like kinase</td>
<td>KIF23, PLK4, ESPL1, CDC20, PRC1, CCNB2, PLK1, CDK1, KIF11</td>
<td>3.01E+00</td>
<td>1.89E-01</td>
</tr>
<tr>
<td>Communication between innate and adaptive immune cells</td>
<td>TNFSF13, IL15, TL7R, FCER1G, TL13, IGHG1, CCL5, TL33, CDBA, Ccl9</td>
<td>2.58E+00</td>
<td>1.54E-01</td>
</tr>
<tr>
<td>Role of pattern recognition receptors in recognition of bacteria and viruses</td>
<td>IFIH1, CLEC7A, OAS1, C3, PIK3R6, TL7R, CCL5, EIF2AK2, TL33, RNASEl</td>
<td>2.26E+00</td>
<td>1.47E-01</td>
</tr>
<tr>
<td>Down-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAPK/JNK signaling</td>
<td>GADD45A, DUSP4, MAPK4K4, MAPK12, ATF2</td>
<td>2.98E+00</td>
<td>5.75E-02</td>
</tr>
<tr>
<td>Activation of IRF by cytokelic pattern recognition receptors</td>
<td>IKBKB, STAT2, MAPK12, ATF2</td>
<td>2.98E+00</td>
<td>8.16E-02</td>
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<tr>
<td>Toll-like receptor signaling</td>
<td>IKBKB, MAPK4K4, MAPK12</td>
<td>2.03E+00</td>
<td>6.25E-02</td>
</tr>
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<td>ATM signaling</td>
<td>GADD45A, MAPK12, ATF2</td>
<td>1.98E+00</td>
<td>6.12E-02</td>
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<tr>
<td>ERK/MAPK signaling</td>
<td>ETS2, DUSP4, RPS6K5A5, RAPGEF4, ATF2</td>
<td>1.82E+00</td>
<td>3.05E-02</td>
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<tr>
<td>B-cell receptor signaling</td>
<td>IKBKB, MAPK12, BCL6, ATF2</td>
<td>1.46E+00</td>
<td>2.90E-02</td>
</tr>
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</table>
regulation of a significant number of BCL6 targets in Eμ-miR-155 mice. B cells from bic/miR-155 knock-out (KO) mice consequently leads to de-repression of its targets, which may contribute to differentiation inhibition (Id2), increased proliferation (IL6, Mip1α, CMyC), and impaired apoptosis (Cnd1) of B cells. These genes can collaborate with MYC and multiple MYC targets to contribute to the miR-155-induced block in B-cell differentiation and increased proliferation observed in these mice.

**Bcl6 Transcription Is Indirectly Modulated by miR-155 Through Mxd1 Induction.** Next, we determined if the down-regulation of Bcl6 mRNA occurs through the canonical miRNA targeting through 3′ UTR interactions. Only one algorithm (27) predicted Bcl6 to be a miR-155 target, but luciferase assays did not confirm this in silico prediction (Fig. S1). Therefore, we searched for other genes that regulate Bcl6. Few known regulators of Bcl6 transcription include inducers like IFN regulatory factor 8 (IRF8) (28) or repressors like IRF4 (29) and the E-box factor Mxd1 (30). We found that Mxd1 is significantly up-regulated (2.5-fold) in Eμ-miR-155 mice B cells as revealed by the microarray analysis and confirmed by real-time PCR (Fig. S4A). MAD or MAX dimerization protein 1 (Mxd1) is one of the key spindle checkpoint proteins, and a MYC antagonist often co-pressed with MYC (31). To confirm that miR-155 can lead to Mxd1 up-regulation, we performed an ectopic overexpression of miR-155 in mouse RAW 264.7 cells. Results showed up-regulation of Mxd1 and subsequent down-regulation of Bcl6 with miR-155 in comparison with scrambled control over time (Fig. 2B). Further, we found that ectopically expressed full-length Mxd1 cDNA into Mxd1-low OCI-Ly10 cells resulted in significant down-regulation of BCL6 at both the RNA and protein level (Fig. S2, Left and Right, respectively). Next, to determine if miR-155-mediated Bcl6 down-regulation is dependent on Mxd1, we

Fig. 1. Bcl6 is down-regulated in Eμ-miR-155 mice. (A) qRT-PCR showing down-regulation of Bcl6 mRNA in Eμ-miR-155 transgenic mice (TG) total splenocytes (TS), spleen preB (preB), and naive B cells compared with wild-type mice (WT) (Left). qRT-PCR showing miR-155 levels in TG Sp preB and naive B cells (normalized to miR-155 expression in WT Sp preB and naive B) (Right). (B) Immunoblot analysis of total cell lysates from splenocytes (Top) and naive B cells (Middle) from TG and WT mice. Immunoblot of HEK-293a cells cotransfected with pCMV6-Bcl6 with premiR-155 or scrambled control (Bottom). β-actin is used as a loading control. (C) qRT-PCR showing significant down-regulation of Bcl6 mRNA by ectopic miR-155 expression in DLBCL cells OCI-Ly1 (Ly1). (D) qRT-PCR showing down-regulation of Bcl6 in miR-155–deleted B cells from bic/miR-155 knock-out (KO) mice compared with wild-type B cells. (E) qRT-PCR showing selected BCL6 targets that are up in TG mice B cells, normalized to Actin. Bars represent mean ± SEM of respective relative amounts from three independent experiments. *P < 0.05, two-tailed Student t test. (F) Heatmap showing cluster analysis of known BCL6 targets up-regulated in TG mice B cells.
Bcl6

tain the scale. (48 h posttransfection. miR-155 expression shown is log-transformed to main-
regulation of 
Mxd1
that miR-155
recovers
Bcl6
and found that cotransfection of siMxd1 along with miR-155

by P300-mediated acetylation (11). Because HDACs are im-
with HDAC2, -4, -5, and -7 by various studies (19), and HDACs
gene promoters (32). BCL6 has been shown to directly interact
SMRT/Ncor, Bcor, and selected class I and II HDACs to target
requires recruitment of various corepressor complexes containing

used small interfering RNAs (siRNA) against Mxd1 (siMxd1) and found that cotransfection of siMxd1 along with miR-155
reverses Bcl6 compared with miR-155 alone (Fig. 2C). This shows that miR-155–caused induction of Bcl6 is partially dependent on
Mxd1. Overall, these results indicate that miR-155–induced Bcl6 transcriptional repression is not direct but could be at least in part
mediated through Mxd1 up-regulation by miR-155. How miR-155
overexpression induces Mxd1 is unknown and needs further investigation.

We then investigated the BCL6 transcriptional machinery to
find out possible miR-155–regulated targets that may collabor-
ate with Bcl6 in leukemogenesis. Gene suppression by BCL6
requires recruitment of various corepressor complexes containing
SMRT/Neor, Bcor, and selected class I and II HDACs to target
gene promoters (32). BCL6 has been shown to directly interact with HDAC2, -4, -5, and -7 by various studies (19), and HDACs
are also important in maintaining its stability as it is inactivated by P300-mediated acetylation (11). Because HDACs are im-
portant chromatin modifiers and targets of various anticancer
therapies, we focused our attention on those HDACs that may be targeted by miR-155.

miR-155 Directly Targets HDAC4, the Corepressor Partner of BCL6.
Although Bcl6 is not a direct target of miR-155 and its expres-
sion is indirectly regulated through Mxd1, we wanted to in-
vestigate additional mechanisms by which miR-155 can lead to
up-regulation of oncogenic BCL6 targets. Among the HDACs
known to be BCL6 partners in mediating gene repression, we
found that HDAC4 was a predicted miR-155 target. Sequence
alignment of HDAC4 3′UTR across phyla showed two miR-
155 binding sites, a 7 mer (AGCATTA) and a bonafide 8 mer (AGCATTAA) (Fig. 3A). Luciferase reporter assay confirmed a
significant down-regulation of HDAC4 3′ UTR by miR-155
mimic compared with scrambled control in multiple cell lines
(Fig. 3B, Left). This interaction was abrogated when the miR-155
binding site in HDAC4-3UTR was deleted or mutated (Fig. 3B,
Right), which confirmed that HDAC4 is a direct target of miR-
155. Further, HDAC4 protein levels in Eµ-miR-155 spleens and
B cells were also significantly down-regulated (Fig. 3C, Upper
and Lower, respectively), and ectopic overexpression of miR-155
in HEK-293T cells resulted in significant reduction of endogenous HDAC4 protein (Fig. 3A) and mRNA in OCI-Ly1 cells (Fig.
3D, Left). Inhibition of miR-155 using anti-miRs in OCI-Ly3 cells
resulted in significant de-repression of HDAC4 transcript levels
(Fig. 3D, Right). Interestingly, inhibition of miR-155 in purified
naïve B cells from Eµ-miR-155 mice using anti-miR-155 showed
significant recovery of HDAC4 expression over time compared with negative control inhibitor (Fig. 3E). Higher HDAC4 expres-
sion in miR-155–deficient bic−/− mice B cells further substantiated
our findings (Fig. S3B). Altogether these results confirm that
HDAC4 is a bona fide target of miR-155.

Hence we believe that part of the BCL6 target genes that are
up-regulated in Eµ-miR-155 mice B cells may be those for which
HDAC4 is required. As multiple biochemical studies have shown
that the two directly interact with each other, we sought to look
for the de-repressed targets that may require both BCL6 and
HDAC4 in the up-regulated dataset from Eµ-miR-155 mice. Interestingly, one of the key BCL6 target genes, Id2, up-regulation

Fig. 2. Transcriptional regulation of BCL6 by miR-155. (A) qRT-PCR showing Bcl6 and Mxd1 mRNA levels in TG mice B cells. (B) qRT-PCR showing temporal regulation of Bcl6 and Mxd1 by miR-155 in mouse Raw 264.7 cells 24 and 48 h posttransfection. miR-155 expression shown is log-transformed to main-

Fig. 3. HDAC4 is a direct target of miR-155. (A) Sequence alignment of 3′ UTR of mouse (Mmu), rat (Rno), human (Hsa), and rhesus (Mml) HDAC4 highlighting two miR-155 binding sites, 8-mer and a more conserved 7-mer. (B) Luciferase reporter assay showing up to 50% reduction in reporter ac-
tivity with miR-155 in five cell lines compared with scrambled (scr) control (Left) and Luciferase re-
porter assay with wild-type (WT) or deleted (Del) or mutated (Mut) miR-155 binding site in 3′UTR of HDAC4 (Right) in HEK-293T cells. Values in graphs represent mean ± SEM from three independent experiments. *P < 0.05. (C) Immunoblot analysis of HDAC4 expression in TG mice splenocytes (Upper) and B cells (Lower) compared with WT mice. (D) qRT-PCR analysis of miR-155 mimic or scrambled (Scr) transfected OCI-Ly1 cells showing down-regu-
lation of HDAC4 (Left) and recovery of its expression by anti-miR-155 in OCI-Ly3 cells (Right). (E) Immuno-
 blot analysis of TG mice B cells treated with anti-
miR-155 showing recovery of HDAC4 expression with time. All experiments were repeated at least three times.
of which inhibits B-cell differentiation, has been proposed to require HDAC4 as one of the repressors (33). Therefore, we propose that BCL6 may recruit HDAC4 to the promoter of Id2 to suppress its transcription. However, down-regulation of Bcl6 and HDAC4 may be the cause of de-repression and up-regulation of Id2 in Eq-miR-155 mice B cells. This provides the connecting link between miR-155-mediated direct regulation of HDAC4 and indirect regulation of BCL6 that contributes to de-repression of some of the BCL6 transcriptional targets.

**Restoration of HDAC4 in B-Lymphoma Cells Have Anti-Tumor Effects.**

To evaluate the functional relevance of down-regulation of HDAC4/BCL6 expression in miR-155–induced leukemogenesis, we used a high miR-155 expressing human ABC-DLBCL–derived OCI-Ly3 cells (15), which expresses low HDAC4 and BCL6. We observed that exogenous HDAC4 (without Id2) in E10′BCL6. We observed that exogenous HDAC4 (without 3′ UTR) and BCL6 expression in OCI-Ly3 cells resulted in a significant reduction in their clonogenic potential and proliferation (Fig. 4 A and B). Ectopic HDAC4 expression also resulted in increased apoptosis in three cell lines (Bjab, Wehi-231, and OCI-Ly3) (Fig. 4C) and reduced Bjab proliferation (Fig. 4D, Right). Further, HDAC4 inhibited the miR-155–induced proliferation of OCI-Ly1 cells when cotransfected with miR-155 (Fig. 4D, Left). Collectively, these findings suggest that HDAC4 has the ability to dampen miR-155–induced proliferative signals in high-miR-155–associated DBLCLs.

HDAC4 has been linked to both proliferative and anti-proliferative pathways. Our study shows that HDAC4 has anti-tumor potential when overexpressed in B-lymphoma cells. HDAC4 has many hallmarks of a tumor suppressor, considering it was identified as a mediator of p53 and p16-RB-dependent proliferation arrest and senescence and is required for repair of ionizing radiation-induced DNA damage (34). In addition, HDAC4 is frequently mutated in certain human cancers (35). In light of these and our current findings, HDAC4 may play an important role in suppressing cancers in conjunction with corepressors like BCL6 that recruit HDAC4 for repressing oncogenes. Lately, HDAC inhibitors (HDIs) have been in use in trials for multiple cancers, but their efficacy varies widely. Our study raises an important concern in identifying roles of individual HDACs and designing specific HDIs for the most useful outcomes, along with patient and disease stratification based on miR-155 levels as one of the criteria. Hence it is important to study the role of HDAC4-mediated gene expression in B-cell development to design the most beneficial therapies.

**Bcl6 and Hdac4 Levels Are Negatively Correlated with miR-155/bic Expression in DBLCL Patients.** Finally, to establish the relevance of our findings to human disease, we investigated the correlation between miR-155, Bcl6, and Hdac4 expression in the DLBCL subtype of NHL patients. We obtained two microarray GEO datasets and analyzed 84 DLBCL patient samples from GEO accession nos. GSE12195 (36) and GSE12453 (37), respectively. We found that miR-155/bic expression level correlated negatively with Bcl6 (R = 0.51, P < 0.001) and Hdac4 (R = 0.33, P < 0.001) levels (Fig. 5). These findings independently support our results of negative regulation of Hdac4 and Bcl6 by miR-155. A recent study also showed that HDAC4 is methylated in B-cell CLL (B-CLL), alluding to its putative beneficial role (38). Such findings and ours further warrant the significance of these genes in stratifying patients for HDI-based therapies for the most beneficial outcomes. HDIs have anti-tumor effects in a wide range of tumors, but a phase I clinical trial with SAHA in relapsed DLBCL patients showed limited benefits (39). As recently shown by Pasqualucci et al. (36), tumors with mutations in acetyltransferase genes are not very likely to benefit from the HDI therapies. Hence, it is important to consider multiple factors to design efficient treatment therapies. We believe that miR-155, Hdac4, and Bcl6 are such candidates especially because BCL6 is a transcription factor and has the ability to act as a hub controlling multiple cellular functions and processes.

In summary we have shown that miR-155 suppresses Hdad4 and Bcl6 expression during miR-155–induced leukemogenesis, resulting in the up-regulation of gene products that may block B-cell development at an immature B-cell stage of differentiation and induce uncontrolled cell proliferation. Here we provide evidence linking miR-155 overexpression to Bcl6 down-regulation indirectly through HDAC4. Together these findings may have a significant clinical impact on the treatment of miR-155–induced tumors, especially with HDAC or BCL6 inhibitors.
Materials and Methods

Mice, Mice Lines, and Retrovirus. Eμ-miR-155 (B-cell miR-155 transgenic) mice have been described before (6). BiC/miR-155–deleted mice (B6.Cg-Mirem155tm1(Rsky)/J) were obtained from the Jackson Laboratories (B). Splenocytes from wild-type and three 6–9-wk-old BALB/c and wild-type mice as pre-B: B220+ IgM+ CD43- and naive B as B220+ IgM+ CD43+. B cells from biC–transduced mice were isolated by biotinylating CD19 microbeads. All mice experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University.

Human DLBCL cell lines, OCI-Ly1, -Ly3, and -Ly10 (gift from Dr. Ricardo C. T. Aguiar, University of Texas Health Science Center, San Antonio, TX) were maintained in IMDM, 20% (vol/vol) FBS and 1% Penicillin/Streptomycin. Full-length human Mxd1 cDNA clone was HEK-293T and Raw 264.7 cells were maintained in RPMI with 10% (vol/vol) FBS and 1% Penicillin/Streptomycin. Full-length human Mxd1 cDNA clone was a gift from Dr. Ricardo C. T. Aguiar, University of Texas Health Science Center, San Antonio, TX) were maintained in IMDM, 20% (vol/vol) FBS and 1% Penicillin/Streptomycin. All mice experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University.

Luciferase Reporter Assay. To confirm the miR-155 and target gene regulation, a luciferase reporter assay was performed using the Dual Luciferase Reporter Assay system from Promega. See Materials and Methods.

Microarray, Quantitative RT-PCR, and Immunoblotting. Total mRNA was isolated using TRIzol (Invitrogen) from splenic naive B cells isolated from Eμ-miR-155 transgenic and wild-type mice using a B-cell isolation kit (Miltenyi Bio-tech) and analyzed on Mouse Genome 430_2 arrays. The data were analyzed from the GSE12195 series were RMA normalized, managed, and analyzed by BRB-ArrayTools Version 3.8.1 (Affymetrix HG U133 Plus2.0). Genes whose expression differed by at least 1.5-fold from the median in at least 20% of the arrays were used. Using Spearman correlation, which measures the correlation of rank ordering between two variables, at P < 0.01 stringency, expression of BC and HADC4 negatively correlated with BiC (miR-155).

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Cell Proliferation, Apoptosis, and Colony Formation. For apoptosis analysis, cells were stained with Annexin (BD Pharmingen) and 7-AAD (Biolegend). The cells were stained on wet transfer (Biorad). Membranes were blocked for 1 h in 5% nonfat dry milk and incubated in primary antibodies overnight at 4 °C. The following primary antibodies were used: rabbit HADC4 (sc-11418), rabbit BC and HADC4 (sc-368 or sc-888), goat ACTIN (sc-1616), and Gapdh and ECL reagent for detection (GE Amersham).

For qRT-PCR, see Materials and Methods.

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For qRT-PCR, see Materials and Methods.

DLBCL Patient Sample Analysis. A total of 84 DLBCL patient samples from two Gene Expression Omnibus (GEO, National Center for Biotechnology Information (NCBI)) datasets were analyzed for HADC4, BC6, and biC/miR-155 mRNA expression correlation studies. Eleven samples from the GSE12453 and 73 from the GSE12195 series were RMA normalized, managed, and analyzed by BRB-ArrayTools Version 3.8.1 (Affymetrix HG U133 Plus2.0). Genes whose expression differed by at least 1.5-fold in at least 20% of the arrays were used. Using Spearman correlation, which measures the correlation of rank ordering between two variables, at P < 0.01 stringency, expression of BC and HADC4 negatively correlated with BiC (miR-155).

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Supporting Information

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SI Materials and Methods

Retrovirus. Full-length cDNA of HDAC4 and BCL6 was cloned into Murine Stem Cell Virus (MSCV) containing IRES and GFP (pMIG). Retrovirus was produced in Phoenix cells maintained in DMEM with 10% (vol/vol) FBS. DLBCL cell lines were transduced with 2 mL of virus per 1 × 10⁶ by spin-infection with 10 µg/mL polybrene (SCBT). Briefly, the cells containing virus were centrifuged at 37°C at 930 × g for 90 min, and incubated with virus for additional 4–5 h, after which it was replaced with fresh media for recovery overnight. After 3–4 d, GFP positive cells were sorted using BD FACS Aria and cultured for colony formation, apoptosis and proliferation assays.

Luciferase Reporter Assay. miR-155 target prediction was according to the Targetscan [Release 3.0 (1)] and RNA22 algorithms (http://cbcrsv.watson.ibm.com/rna22.html) (2). The 3’UTR of the human and mouse HDAC4 and BCL6 containing the two miR-155 binding sites was PCR amplified with XbaI flanked primers. The PCR products were purified, digested, and cloned downstream of the luciferase coding region in the pGL3 control vector (Promega). Human HEK295T, HeLa, Jurkat, and mouse NIH 3T3 cells were cotransfected with respective luciferase constructs and miR-155 or scrambled (Scr) oligos along with Renilla luciferase expression plasmid (pRL-TK) as a transfection control using Lipofectamine (Invitrogen). Deletion and mutant HDAC4 luciferase constructs were prepared by deleting the miR-155 binding sites in the original 3’UTR construct, respectively. After 48 h, cells were lysed and analyzed for relative luciferase activity using the Dual Luciferase Assay Kit (Promega). Results are representative of three independent experiments.

The primer sequences for luciferase constructs are as follows:

hsHdac4_WT3utr_L: ACCTCTAgAAgATTTCTATTTCTACgTGAAGACTgAGAg; hsHdac4_WT3utr_R: ACCTCTAgAAgTTTCTATTTCTACgTAGCTTCAgAGAGAGA; mmHdac4_WT3utr_L: ACCTCTAgAAgATTTCTATTTCTACgTAGCTTCAgAGAGAGA; mmHdac4_WT3utr_R: ACCTCTAgAAgATTTCTATTTCTACgTAGCTTCAgAGAGAGA; mmHdac4_m155Mut7mer_L: CACAAATgTgTACgAAACATACgTgTAgCCTTT; and mmHdac4_m155Mut7mer_R: gAAgAAgCTACACgTATggTgTgTAgCCTTT.

qRT-PCR. For qRT-PCR total mRNA from sorted cells was isolated using Phenol-free total RNA isolation kit (Amresco) and reverse transcribed using iScript first strand cDNA synthesis kit. About 1/20th of diluted cDNA was used for gene taqman analysis using respective taqman assays for mouse or human  Hdac4, Bcl6, Myc, and Mad1 (ActinB was used as normalizer). For miR expression analysis, total RNA was reverse transcribed using TaqMan miRNA reverse transcription kit using the manufacturer’s protocol, followed by miRNA RT-PCR for miR-155 and sno-135 (normalizer). BCL6 target genes (IL6, Id2, Ccnd1, and Ccl3/ Mip1α) were detected using sybr-green-based RT-PCR using primers picked from the RTPRIMER database (http://medgen. ugent.be/rtpRIMERdb/). The list of primers are as follows: mAc tin_ForSybr: ATG CTC CCC GGG CTG TAT; mActin_RevSybr: CAT AGG AGT CCT TCT GAC CCA TCT; mId2_ForSybr: AAG ACT TTT GGT ATG ACT TTG GAT CAT TT; mId2_RevSybr: GAC GAT CAT CCT TAG TTI CCC GC TTI CT; mId2_RevSybr: TCC AGT TTC CTT GGG AC; mIL6_RevSybr: GAT GAC TAA ATA GCC CTC CTA CTG; mCCL3_ForSybr: CTG TCC TAC CCC ATC; and mCCL3_RevSybr: TGT CAG TTC ATG CTT GCA TCA T. All RT-PCR assays were performed on the Biorad CFX96. Data were analyzed using 2–ΔΔCt method to calculate the relative amounts or 2–ΔΔCt for fold change. The overlap between up-regulated genes in mice and known BCL6 targets (3) in humans was calculated using Gene Cluster 3.0. The clustered genes were mean centered, and average linkage was performed using Euclidean distance, after filtering the genes with SD > 1.

Fig. S1. Sequence alignment of 3′ UTR of human and mouse Bcl6 with respect to miR-155 mature sequence (1), showing possible interaction through a less conserved binding site (Upper). Luciferase reporter assay showing no difference in pGL3-Bcl6-3′ UTR (mouse or human) reporter activity by miR-155 or empty vector (Lower).


Fig. S2. qRT-PCR (Left) and immunoblot (Right) analysis of full-length Mxd1 cDNA-transfected OCI-Ly10 cells.
Fig. S3.  (A) Ectopic miR-155 expression into HEK293T cells significantly down-regulates endogenous HDAC4. (UTC, Untransfected Control). (B) Immunoblot analysis of HDAC4 levels in miR-155-deficient CD19 cells from bic/miR-155 knock-out mice showing increased expression. jactin was used as a loading control in all immunoblots unless indicated otherwise. (KO-B, miR-155 knock out/bic−/− mice CD19+ B cells; KO-nonB, miR-155 knock out/bic−/− mice non-B-cells, CD19−; WT, wild type).

Other Supporting Information Files

Dataset S1 (XLSX)