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

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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 mice 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 *Mxd1/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.

NfκB | Ingenuity Pathway Analysis

MicroRNAs (miRNAs) are 18–24-nucleotide-long noncoding RNA molecules that regulate gene expression in many 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.

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 deacetylating 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.

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 mRNA 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,

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo [accession no. GSE30248 (Eμ-miR-155 transgenic mice B cells)].

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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 NfκB, both of which are also up-regulated in Eμ-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*, *IKbKb*, and *Atf2*, which may contribute to the disruption of normal B-cell development in Eμ-miR-155 mice. Collectively, the gene signature represented in the miR-155 overexpressing mice B cells was representative of increased proliferation and dysregulated B-cell-development-related pathways.

We first confirmed the down-regulation of *Bcl6* mRNA in Eμ-miR-155 mice spleen cells using quantitative real time PCR (qRT-PCR) (Fig. 1*A*, *Left*). Further analysis of *Bcl6* mRNA from purified spleen pre-B ($B220^+$ $CD43^-$ IgM^-) and naïve-B ($B220^+$ $CD43^-$ IgM^+) cells showed the most significant down-regulation in naïve B cells (Fig. 1*A*, *Left*). However, pre–B-cells from Eμ-miR-155 transgenic mice also consistently showed lower *Bcl6* expression compared with their wild-type counterparts (Fig. 1*A*, *Left*). Analysis of miR-155 expression in these B-cell subsets showed that naïve B cells from Eμ-miR-155 transgenic mice had the highest levels of miR-155 (Fig. 1*A*, *Right*). We also found decreased BCL6 protein levels in total splenocytes (Fig. 1B, *Top*) and purified naïve B cells (Fig. 1*B*, *Middle*) of Eμ-miR-155 mice compared with wild type. To verify this regulation in vitro, we show that transfection of premiR-155 oligos into the OCI-Ly1 cells resulted in significant down-regulation of endogenous *Bcl6* mRNA compared with scrambled control (Fig. 1*C*). 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. 1*B*, *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. 1*D*).

Altogether, these results confirm that BCL6 is down-regulated in the Eμ-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 DLBCL (GCB-DLBCL), which overexpresses *Bcl6*, also has lower levels of miR-155, and the activated B-cell DLBCL (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 Eμ-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 Eμ-miR-155 mice B cells' transcriptome to study the fate of *Bcl6*-regulated geneset.

Downstream Targets of *BCL6* Are Activated in Eμ-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 geneset from Eμ-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 Eμ-miR-155 mice B cells (Fig. 1*F*). Next, to assess the functions represented by these genes, we performed a Gene Set Enrichment Analysis (www.broadinstitute.org/gsea) of the up-regulated BCL6 target geneset. Interestingly, we found a significant enrichment for MYC-MAX targets ($P = 4.96e\text{-}10$) in this set of genes, as *cMyc* is also a BCL6 target. Hence the up-

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

Pathways	Molecules	–log(*P* value)	Ratio
Up-regulated			
AHR signaling	MGST1, CCNE2, NFIX, TFDP1, GSTM5, NQO2, POLA1, CDK6, CCND1, GSTO1, CHEK1, MYC, TGM2, CCNA2, CCND3, ALDH1A1, MGST2, E2F1, DHFR, ESR1, AHR	4.74E+00	1.67E–01
Glutathione metabolism	GSR, MGST1, MGST2, GSTM5, G6PD, IDH2, GCLM, GLRX, ANPEP, GSTO1, RNPEP, IDH1	4.69E+00	2.45E–01
Mitotic roles of polo-like kinase	KIF23, PLK4, ESPL1, CDC20, PRC1, CCNB2, PLK1, CDK1, KIF11	3.01E+00	1.89E–01
Communication between innate and adaptive immune cells	TNFSF13, IL15, TLR7, FCER1G, Tlr13, IGHG1, CCL5, TLR3, CD8A, Ccl9	2.58E+00	1.54E–01
Role of pattern recognition receptors in recognition of bacteria and viruses	IFIH1, CLEC7A, OAS1, C3, PIK3R6, TLR7, CCL5, EIF2AK2, TLR3, RNASEL	2.26E+00	1.47E–01
Down-regulated			
SAPK/JNK signaling	GADD45A, DUSP4, MAP4K4, MAPK12, ATF2	2.98E+00	5.75E–02
Activation of IRF by cytosolic pattern recognition receptors	IKBKB, STAT2, MAPK12, ATF2	2.98E+00	8.16E–02
Toll-like receptor signaling	IKBKB, MAP4K4, MAPK12	2.03E+00	6.25E–02
ATM signaling	GADD45A, MAPK12, ATF2	1.98E+00	6.12E–02
ERK/MAPK signaling	ETS2, DUSP4, RPS6KA5, RAPGEF4, ATF2	1.82E+00	3.05E–02
B-cell receptor signaling	IKBKB, MAPK12, BCL6, ATF2	1.46E+00	2.90E–02

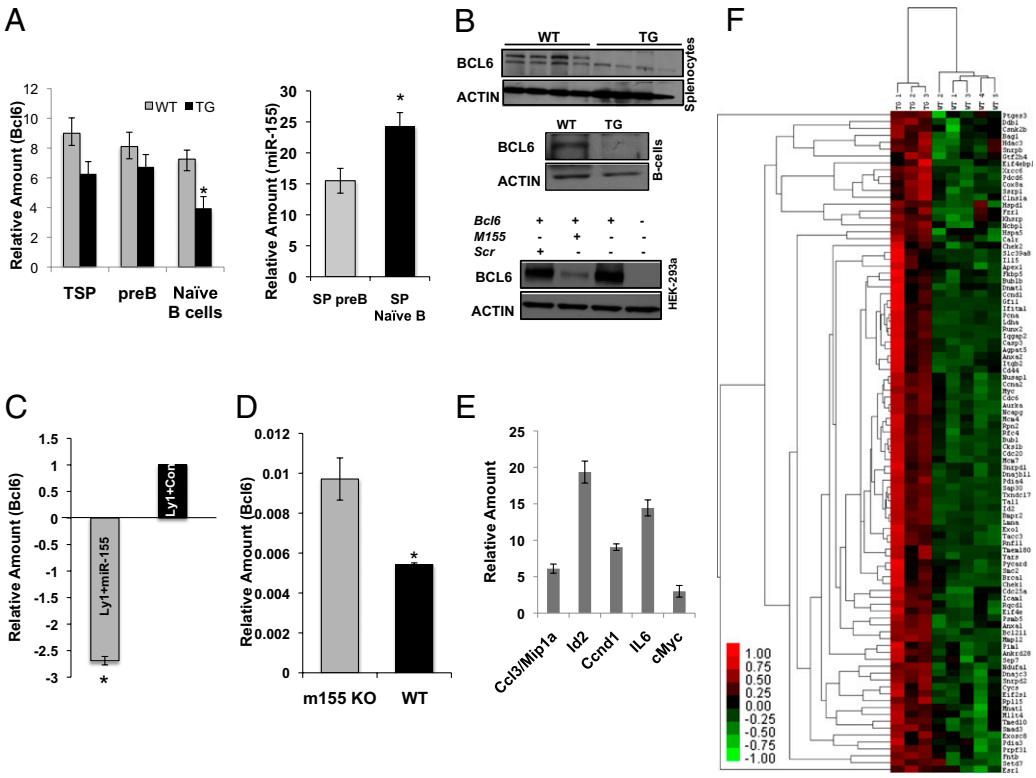

Fig. 1. BCL6 is down-regulated in Eμ-miR-155 mice. (*A*) qRT-PCR showing down-regulation of *Bcl6* mRNA in Eμ-miR-155 transgenic mice (TG) total splenocytes (TSP), spleen preB (preB), and naïve B cells compared with wild-type mice (WT) (*Left*). qRT-PCR showing miR-155 levels in TG Sp preB and naïve B cells (normalized to miR-155 expression in WT Sp preB and naïve B) (*Right*). (*B*) Immunoblot analysis of total cell lysates from splenocytes (*Top*) and naïve 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.

regulation of a significant number of BCL6 targets in Eμ-miR-155 mice B cells corroborated the significance of BCL6 down-regulation, as it represented the de-repressed genes that may be contributing to leukemia in the miR-155 mouse model.

We then focused our attention on some of those genes that may be relaying their oncogenic potential in the absence of BCL6. These include inhibitor of differentiation (*Id2*), Chemokine Ligand 3 or macrophage inflammatory protein 1α (*Ccl3*/*Mip1α*), Interleukin 6 (*Il6*), Cyclin D1 (*Ccnd1*), and *cMyc*, which were significantly up-regulated in the transgenic mice (Fig. 1*E*). It has been previously shown that down-regulation of *Id2* is essential for B-cell commitment and its up-regulation in mouse bone marrow blocks B cells at the prepro-B stage (22). Another target of *Bcl6*, *Ccl3*, or *Mip-1α* is involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes. Increased *Mip-1α* activity has been associated with various lymphomas in a recent report on the significance of this chemokine as a prognostic marker in chronic lymphocytic leukemia (CLL) (23). IL-6 is a proinflammatory cytokine that promotes lymphoma and leukemia proliferation, and its suppression by BCL6 has been shown to negatively regulate macrophage proliferation (24). Further $Bcl6^{-/-}$ macrophages were shown to hyperproliferate due to accelerated G1/S transition accompanied by increased *Cyclin D2* and *c-myc* and decreased expression of p27 (24). Likewise, *Ccnd1*, an anti-apoptotic cyclin with a role in G1/S transition, increases proliferation and is overexpressed in many tumors (25). The miR-155 encoding *bic* locus has been long known to cooperate with the cMyc oncogene that controls cellular proliferation, growth, and cell death and induces B-cell lymphomas (26). Therefore, it is possible that significant loss of *Bcl6* in naïve/mature B cells of Eμ-miR-155 mice consequently leads to de-repression of its targets, which may contribute to differentiation inhibition (*Id2*), increased proliferation (*IL6*, *Mip1α*, *cMyc*), and impaired apoptosis (*Ccnd1*) 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*/*Mad1* (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. 2*A*). MAD or MAX dimerization protein 1 (MXD1) is one of the key spindle checkpoint proteins, and a MYC antagonist often coexpressed 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. 2*B*). 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

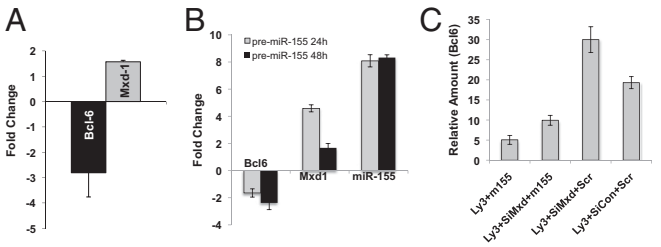

Fig. 2. Transcriptional regulation of BCL6 by miR-155. (*A*) qRT-PCR showing *Bcl6* and *Mxd1* mRNA levels in TG mice B cells. (*B*) qRT-PCR showing temporal regulation of *Bcl6* and *Mxd1* by miR-155 in mouse Raw 264.7 cells 24 and 48 h posttransfection. miR-155 expression shown is log-transformed to maintain the scale. (*C*) Effect of silencing *Mxd1* and miR-155 overexpression on *Bcl6* mRNA (*Right*). siMxd1, siRNA against Mxd1; Scr, scrambled miR control; siCon, siRNA control. Bars represent mean ± SEM from three independent experiments. $*P < 0.05$, two-tailed Student *t* test.

used small interfering RNAs (siRNA) against *Mxd1* (siMxd1) and found that cotransfection of siMxd1 along with miR-155 recovers *Bcl6* compared with miR-155 alone (Fig. 2*C*). 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 other possible miR-155–regulated targets that may collaborate with *Bcl6* in leukemogenesis. Gene suppression by BCL6 requires recruitment of various corepressor complexes containing SMRT/Ncor, 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 important 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 expression is indirectly regulated through *Mxd1*, we wanted to investigate 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. 3*A*). 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. 3*B*, *Left*). This interaction was abrogated when the miR-155 binding site in HDAC4-3UTR was deleted or mutated (Fig. 3*B*, *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. 3*C*, *Upper* and *Lower*, respectively), and ectopic overexpression of miR-155 in HEK-293T cells resulted in significant reduction of endogenous HDAC4 protein (Fig. S3*A*) and mRNA in OCI-Ly1 cells (Fig. 3*D*, *Left*). Inhibition of miR-155 using anti-miRs in OCI-Ly3 cells resulted in significant de-repression of *Hdac4* transcript levels (Fig. 3*D*, *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. 3*E*). Higher HDAC4 expression in miR-155–deficient bic$^{-/-}$ mice B cells further substantiated our findings (Fig. S3*B*). 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

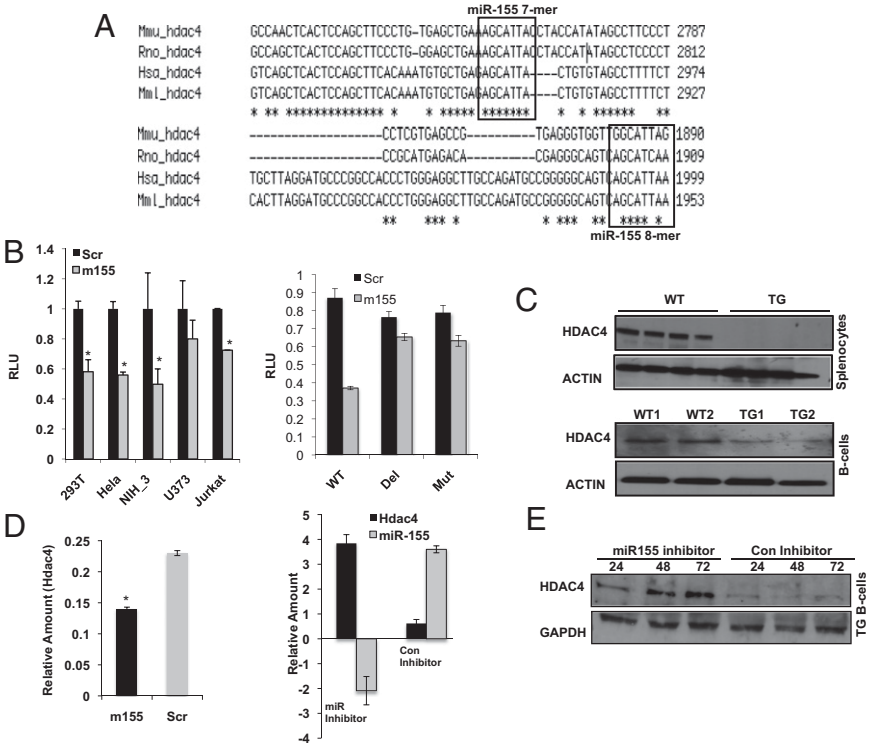

Fig. 3. HDAC4 is a direct target of miR-155. (*A*) Sequence alignment of 3′ UTR of mouse (Mmu), rat (Rno), human (Hsa), and rhesus (Mml) HDAC4 highlighting two miR-155 binding sites, 8-mer and a more conserved 7-mer. (*B*) Luciferase reporter assay showing up to 50% reduction in reporter activity with miR-155 in five cell lines compared with scrambled (scr) control (*Left*) and Luciferase reporter 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-regulation of *Hdac4* (*Left*) and recovery of its expression by anti-miR-155 in OCI-Ly3 cells (*Right*). (*E*) Immunoblot 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-rgeulation of *Id2* in Eμ-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 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. 4*C*) and reduced Bjab proliferation (Fig. 4*D*, *Right*). Further, HDAC4 inhibited the miR-155–induced proliferation of OCI-Ly1 cells when cotransfected with miR-155 (Fig. 4*D*, *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 $p19^{ARF}$-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.

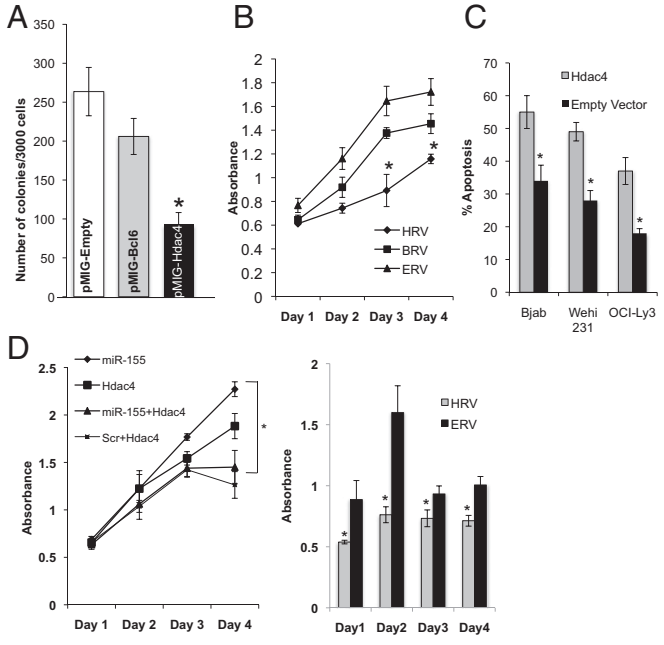

Fig. 4. HDAC4 reduces clonogenic potential and proliferation and increases apoptosis of B cells. (*A*) Quantitation of methylcellulose colony formation of OCI-Ly3 cells transduced with empty retrovirus (pMIG-Empty or ERV), pMIG-Bcl6 (or BRV), and pMIG-HDAC4 (or HRV). (*B*) MTS proliferation assay of HRV-, BRV-, or ERV-transduced OCI-Ly3 cells. (*C*) Apoptosis of human Bjab, OCI-Ly3, and mouse Wehi-231 (preB) cells transfected with pCMV6-Hdac4 or Empty vector alone. (*D*) Proliferation assay of OCI-Ly1 cells cotransfected with miR-155+Hdac4, Scr+Hdac4, miR-155 alone, or Hdac4 alone (*Left*) and Bjab cells transduced with HRV or ERV (*Right*). All experiments were repeated three or more times. *$P < 0.05$.

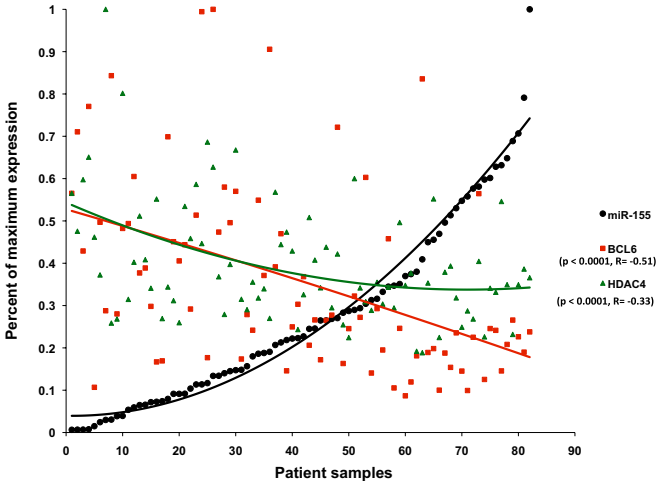

Fig. 5. *Hdac4*, *Bcl6*, and *bic*/*miR-155* mRNA correlation analysis in 84 DLBCL patients showing negative correlation between *bic*/*miR-155* with *Hdac4* and *Bcl6*.

***Bcl6* and *Hdac4* Levels Are Negatively Correlated with miR-155/bic Expression in DLBCL 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 II 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 HDAC4 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.

CELL BIOLOGY

Materials and Methods

Mice, Cell Lines, and Retrovirus. Eμ-miR-155 (B-cell miR-155 transgenic) mice have been described before (6). Bic/miR-155–deleted mice (B6.Cg-Mirn155tm1.1Rsky/J) were obtained from the Jackson Laboratories (8). Spleen B-cell stages were flow sorted from three 6–9-wk-old transgenics and wild-type mice as pre-B: $B220^+$ IgM^- IgD^- $CD43^-$ and naïve B as $B220^+$ IgM^+ IgD^+ $CD43^-$. B cells from *bic*$^{-/-}$ mice were isolated using biotinylated 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. 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 obtained from Origene and nucleofected into OCI-Ly10 cells using Lonza Sol V (Program A-020). siRNAs against Mxd1 (siMxd1) (Santa Cruz, Biotechnology) and controls were nucleofected at a concentration of 100 nM using Lonza Sol V. For retroviral constructs and infection protocol, see *SI Materials and Methods*.

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 *SI Materials and Methods*.

Microarray, Quantitative RT-PCR, and Immunoblotting. Total mRNA was isolated using TRIzol (Invitrogen) from splenic naïve B cells isolated from Eμ-miR-155 transgenic and wild-type mice using a B-cell isolation kit (Miltenyi Biotech) and analyzed on Mouse Genome 430_2 arrays. The data were analyzed using BRB array tools. GC-RMA normalized data were used to identify "canonical pathways" affected by miR-155 using IPA.

For immunoblotting, cells were lysed in RIPA, and lysates were run on 4–20% SDS/PAGE (Lonza) gel and transferred to nitrocellulose membrane using 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 HDAC4 (sc-11418), rabbit BCL6 (sc-368 or sc-858), goat ACTIN (sc-1616), and Gapdh and ECL reagent for detection (GE Amersham).

For qRT-PCR, see *SI Materials and Methods*.

Cell Proliferation, Apoptosis, and Colony Formation. For apoptosis analysis, cells were stained with Annexin (BD Pharmingen) and 7-AAD (Biolegend). The cells were analyzed on BD FACS LSR-II using the FACS DiVa software (BD Biosciences). Cell proliferation was assayed using CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega). Colony formation was assayed using Methocult media (H4100, Stem Cell Biotechnologies). Briefly, FACs-sorted GFP+-transduced cells were diluted to 10,000 cells/mL, and about 3,000 cells (0.3 mL) mixed with 2.7 mL of Methocult were plated on six Petri-dishes (10 cm). Colonies were scored at 7 and 15 d. Experiment was repeated three times.

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 *Hdac4*, *Bcl6*, 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 from the median in at least 20% of the arrays were used. Using Spearman correlation, which measures the correlation of rank ordering between two values, at $P < 0.01$ stringency, expression of BCL6 and HDAC4 negatively correlated with BIC (miR-155).

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