microRNA 28 controls cell proliferation and is down-regulated in B-cell lymphomas

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Burkitt lymphoma (BL) is a highly aggressive B-cell non-Hodgkin lymphoma (B-NHL), which originates from germinal center (GC) B cells and harbors translocations deregulating v-myc avian myelocytomatosis viral oncogene homolog (MYC). A comparative analysis of microRNAs expressed in normal and malignant GC B cells identified microRNA 28 (miR-28) as significantly down-regulated in BL, as well as in other GC-derived B-NHL. We show that reexpression of miR-28 impairs cell proliferation and clonogenic properties of BL cells by modulating several targets including MAD2 mitotic arrest deficient-like 1, *MAD2L1*, a component of the spindle checkpoint whose down-regulation is essential in mediating miR-28–induced proliferation arrest, and BCL2-associated athanogene, *BAG1*, an activator of the ERK pathway. We identify the oncogene *MYC* as a negative regulator of miR-28 expression, suggesting that its deregulation by chromosomal translocation in BL leads to miR-28 suppression. In addition, we show that miR-28 can inhibit MYC-induced transformation by directly targeting genes up-regulated by MYC. Overall, our data suggest that miR-28 acts as a tumor suppressor in BL and that its repression by MYC contributes to B-cell lymphomagenesis.

Germinal centers (GCs) form when mature naïve B cells start proliferating at high rates upon T-cell–dependent antigen stimulation. Within the GC, B cells undergo somatic hypermutation of their Ig-variable regions and class switch recombination, two processes that allow the generation of high-affinity antibodies of diverse isotype (1). Although the GCs are essential for humoral immunity, their importance in the immune system is counterbalanced by the fact that the majority of B-cell non-Hodgkin lymphomas (B-NHLs) arise from GC B cells.

Recently, the development of new technologies for comprehensive genomic analysis has led to the identification of a large number of genetic alterations with potential implications in the pathogenesis of B-NHL. Although, the focus of these studies has initially been restricted to protein-coding genes, it has been shown that noncoding RNA and in particular microRNAs (miRNAs) are implicated in a wide variety of biological processes (2). The role of miRNAs in normal B-cell development, as well as in lymphomagenesis, is to date largely unknown. In hematological malignancies, a role has been established for some miRNAs including the miR-17-92 cluster, which has been shown to accelerate v-myc avian myelocytomatosis viral oncogene homolog (MYC)-induced lymphoma development (3); miR-155, whose overexpression can cause immature B-cell malignancies (4); and the miR-15a/16-1 cluster, which has been implicated in the pathogenesis of B-cell chronic lymphocytic leukemia (5, 6).

To expand our understanding of the role of miRNAs in B-cell development and lymphomagenesis, we and others have investigated the miRNA expression profiles in the human mature B-cell compartment (7–9). Based on these studies, microRNA 28 (miR-28) emerged as a miRNA that is specifically induced during the GC reaction. miR-28 is an intragenic miRNA encoded by intron 6 of the LIM domain-containing preferred translocation partner in lipoma (*LPP*) gene, located on chromosome 3q28. Both mature miRNAs—miR-28-5p and miR-28-3p—that are produced by the miR-28 precursor are expressed in GC B cells, with miR-28-5p being the most abundant. miR-28 has been shown to be deregulated in several cancer types. Although increased levels of miR-28 expression have been observed in esophageal, cervical, renal cancer, as well as in the platelets of a fraction of myeloproliferative neoplasm patients (10–13), miR-28 is down-regulated in colorectal cancer cells and nasal-type natural killer/T-cell lymphoma. In both these latter cell types, reexpression of miR-28 leads to reduced proliferation in cell lines (14–16).

In this study, we investigate the role of miR-28 in B-cell lymphomagenesis and identify direct target genes that are involved in apoptosis and cell cycle control. Furthermore, we show that the oncogene *MYC* negatively regulates miR-28 expression, suggesting that miR-28 silencing contributes to lymphomagenesis.

Results

miR-28 Is Expressed in GC B Cells and Down-Regulated in GC-Derived Lymphomas. To investigate the miRNA expression profiles of mature B cells and GC-derived lymphomas, we performed small RNA sequencing of GC B cells isolated from human tonsils (four donors) and primary diffuse large B-cell lymphoma (DLBCL) biopsies (n = 25). Among miRNAs that displayed high levels of expression specifically in GC B cells, but were aberrantly down-regulated in DLBCL, we identified miR-28 as the top candidate

Significance

The majority of non-Hodgkin B-cell lymphomas arise from the malignant transformation of germinal center B cells. The molecular pathogenesis of these malignancies is not fully understood. Although a number of oncogenes and tumor suppressors have been identified among protein-coding genes, the role of microRNAs during lymphomagenesis remains largely unexplored. Our results identify a role for microRNA 28 (miR-28) in normal and malignant germinal center B cells. These data provide new insights on the microRNA-mediated posttranscriptional regulation occurring in normal germinal center B cells as well as during lymphomagenesis. In addition, the identification of a cross talk between miR-28 and v-myc avian myelocytomatosis viral oncogene homolog extends the relevance of our observations to a wide variety of malignancies.

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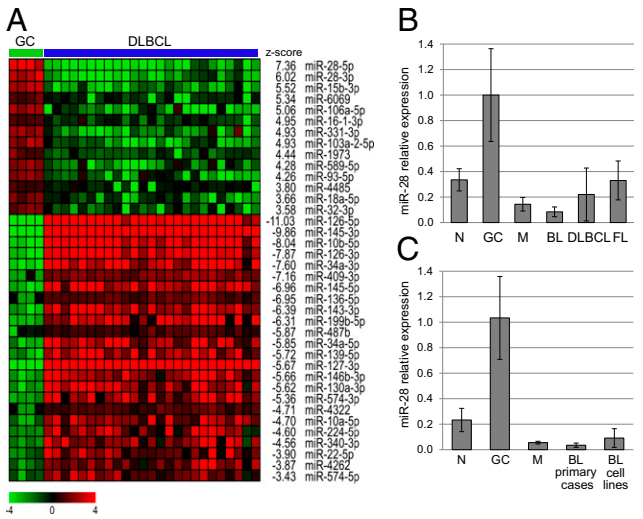

Fig. 1. miR-28 is highly expressed in germinal center (GC) B cells and is down-regulated in GC-derived lymphomas. (*A*) Heat map displaying miRNAs differentially expressed between normal GC B cells ($n = 4$) and diffuse large B-cell lymphomas (DLBCLs) ($n = 25$), as detected by small RNA sequencing. The *z* score values are displayed. (*B*) Microarray-based miR-28-5p expression in normal B cells (naïve, N, $n = 4$; GC, $n = 4$; memory, M, $n = 4$) and GC-derived lymphomas (Burkitt lymphoma, BL, $n = 12$; DLBCL, $n = 46$; follicular lymphoma, FL, $n = 15$). (*C*) Expression of miR-28-5p measured by quantitative RT-PCR (qRT-PCR) in GC ($n = 4$), primary BL ($n = 6$), and cell lines ($n = 6$). Data are shown as average ± SD.

(Fig. 1*A*). These results were confirmed in a larger set of DLBCL ($n = 48$) and extended to follicular lymphoma (FL) ($n = 16$) and Burkitt lymphoma (BL) ($n = 12$) that were analyzed by an array-based miRNA expression profiling approach (Fig. 1*B*). BL appeared to show the most dramatic and homogenous down-regulation of miR-28, both in primary tumor biopsies and cell lines, as further confirmed by quantitative RT-PCR (Fig. 1*C*).

Overall, these results indicate that miR-28 expression is specifically induced in GC B cells but is abnormally repressed in transformed GC B cells.

Reexpression of miR-28 in BL Cell Lines Impairs Proliferation and Clonogenicity. To investigate the possible role of miR-28 down-regulation in B-cell lymphomagenesis, we studied the effects of miR-28 on proliferation and clonogenicity. We engineered two BL cell lines (P3HR1 and RAJI) to inducibly express miR-28 and GFP from a doxycycline-responsive bidirectional promoter (Fig. S1*A*) (17). Expression of both mature miRNAs (miR-28-5p and miR-28-3p) in P3HR1-miR-28 and RAJI-miR-28 cells was confirmed 48 h upon induction by Northern blot analysis (Fig. S1*B*). After 48 h, more than 95% of the cells expressed GFP (Fig. S1*C*) and miR-28-5p expression was about 60- to 80-fold higher compared with cells expressing the empty vector (EV) and slightly higher than normal GC B cells (2.5- to 4.5-fold; Fig. S1*D*). We then monitored proliferation for 96 h and observed that miR-28–expressing cells displayed reduced or impaired proliferation in both cell lines (Fig. 2*A*). Cell cycle analysis on the GFP$^+$ cells showed that miR-28–expressing cells were arrested at the G_1 phase, whereas control cells displayed a normal cell cycle profile (Fig. 2*B*). Additionally, both cell lines showed an increased number of apoptotic cells upon induction of miR-28 (Fig. 2*C*).

We then tested the clonogenic properties of these cell lines by measuring their ability to form colonies in soft agar. Interestingly, miR-28 expression was sufficient to significantly reduce (RAJI) or completely abrogate (P3HR1) the formation of colonies in soft agar (Fig. 2*D*).

To investigate the overall transcriptional program associated with the reexpression of miR-28 in BL, we performed gene expression profiling on P3HR1-EV and -miR-28 cells at 12 and 24 h upon induction. Toward the identification of the biological programs that are affected by miR-28 expression, we performed gene set enrichment analyses (GSEAs) (18) using the KEGG, Biocarta, and Reactome databases. The results showed that several gene sets involved in cell cycle progression and DNA replication were significantly ($P \leq 0.05$) enriched for genes down-regulated in miR-28–expressing cells (Dataset S1). These results are consistent with the phenotype observed in BL cell lines after reexpression of miR-28.

The host gene of miR-28, *LPP*, displays a pattern of expression resembling that of miR-28 both in normal and malignant B cells (Fig. S2 *A* and *B*). This observation prompted us to characterize the effects of LPP reexpression in BL cells (Fig. S2*C*). Induction of LPP in both P3HR1 and RAJI cell lines did not interfere with proliferation (Fig. S2*D*), confirming that miR-28 is the genetic element negatively regulating proliferation in GC-derived lymphomas.

The cell phenotype induced by miR-28 is not a phenomenon observed by reexpressing any miRNA. Indeed, no effect on proliferation was observed when P3HR1 were engineered to express miR-151 that, similarly to miR-28, is significantly down-regulated in BL (Fig. S2 *E*–*G*).

In summary, the reexpression of miR-28 in BL cells specifically led to retarded proliferation due to a combination of apoptosis and cell cycle arrest. Together with the impaired transformed phenotype upon miR-28 reexpression, these results suggest a tumor-suppressor role of miR-28 in B cells.

miR-28 Directly Targets Genes Involved in Proliferation and Apoptosis. To identify miR-28 direct targets, we first computationally pre-

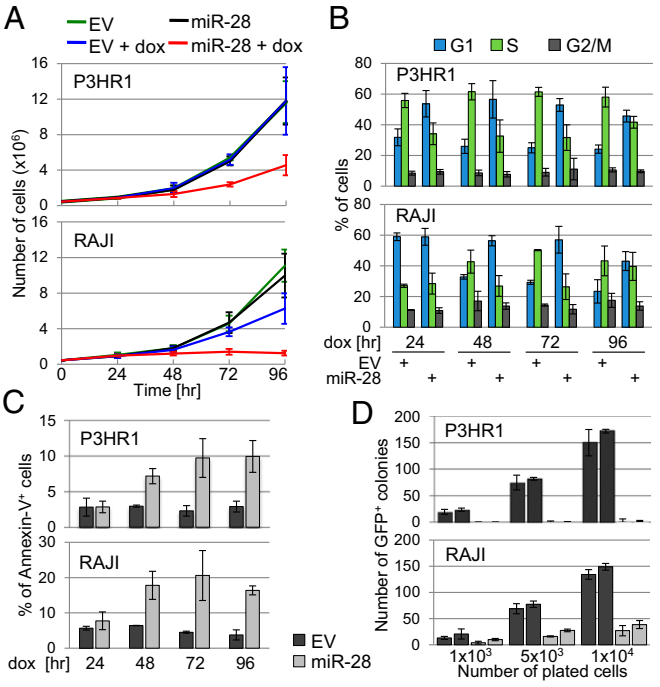

Fig. 2. miR-28 reexpression in BL affects proliferation and clonogenicity. (*A*) Cell proliferation curve of P3HR1 and RAJI cell lines engineered to inducibly express miR-28 or empty vector (EV) in a doxycycline-dependent fashion. Cells were cultured with or without doxycycline (dox) and counted at the indicated time points. (*B*) Cell cycle profile and percentage of cells synthesizing DNA were determined by 7-AAD assay and BrdU incorporation. (*C*) Percentage of apoptotic cells as determined by Annexin-V staining. (*D*) Clonogenicity in soft agar. The bar plots report the number (average ± SD) of GFP$^+$ colonies counted in two independent experiments, each including two bulk populations and performed in triplicates.

dicted genes with miR-28 binding sites in their 3′-UTRs using the mirSVR algorithm (19). mirSVR predicted 1,623 target genes for miR-28-5p and 1,562 for miR-28-3p (Dataset S2). To investigate the pattern of expression of miR-28 predicted targets in B cells, GSEA was performed on the gene expression profiles generated in P3HR1-miR-28 cells. This analysis detected a significant enrichment of miR-28-5p, but not miR-28-3p, predicted targets among the genes down-regulated upon induction of miR-28 (Fig. S3 and Dataset S3). Based on this observation and on the fact that miR-28-5p is more abundant than miR-28-3p in B cells (7), we identified genes that were predicted to have miR-28-5p binding sites in their 3′-UTR and were down-regulated in P3HR1 cells upon miR-28 induction. Among the 95 predicted targets that were significantly ($P < 0.05$) down-regulated in the miR-28–expressing cells, four genes, involved in spindle checkpoint control (*MAD2 mitotic arrest deficient-like 1*, *MAD2L1*), apoptosis (*BCL2-associated athanogene*, *BAG1*), and GTPase-mediated signal transduction (*RAP1B member of RAS oncogene family*, *RAP1B*; *RAB23 member of RAS oncogene family*, *RAB23*), were experimentally tested for the response of their 3′-UTR to miR-28. Cotransfection in 293T cells of the reporter constructs carrying the full-length 3′-UTRs along with a plasmid expressing miR-28, led to a dose-dependent reduction of the reporter activity. This negative regulation was dependent on the ability of miR-28-5p to directly bind to its predicted binding sites in all four tested target 3′-UTRs, because mutations affecting these sites fully abrogated the miR-28–mediated repression (Fig. 3*A*). Although both *BAG1* and *RAB23* displayed multiple predicted miR-28-5p binding sites in their 3′-UTRs, only one site in each of the 3′-UTRs was demonstrated to be functional (Fig. S4).

The effects of miR-28 on the protein levels of its target genes were tested based on antibody availability, for *MAD2L1*, *BAG1*, and *RAP1B* in P3HR1- and RAJI-miR-28 cells. As expected for miRNA targets, all three genes showed a reduction in protein expression upon induction of miR-28 (Fig. 3*B* and Fig. S5*A*). Based on these results, *MAD2L1*, *BAG1*, and *RAP1B* are bona fide direct targets of miR-28-5p in B cells.

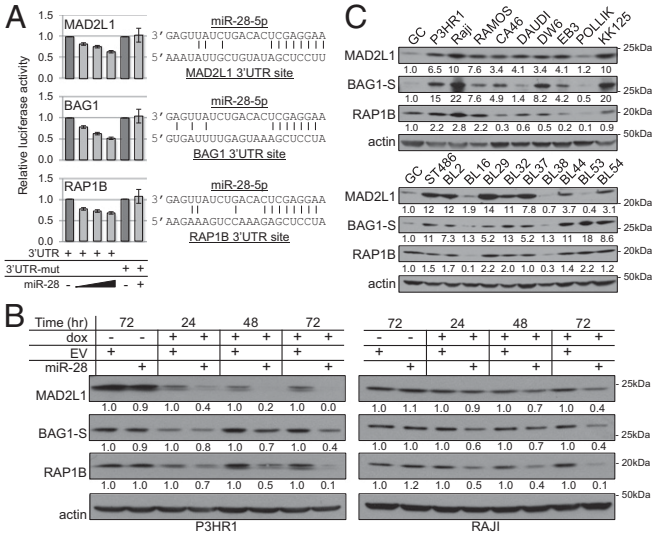

Fig. 3. miR-28 directly targets genes involved in proliferation and apoptosis. (*A*) Relative luciferase activity of the 3′ UTR reporter constructs upon normalization by *Renilla* activity. The bar plots represent the average ± SD of at least two independent experiments including three technical replicates/each. (*Right*) Schematic representation of miR-28-5p sites in the MAD2L1, BAG1, and RAP1B 3′-UTRs. (*B*) Immunoblotting for MAD2L1, BAG1, and RAP1B in P3HR1 and RAJI upon miR-28 reexpression. (*C*) Immunoblot analyses for MAD2L1, BAG1, and RAP1B in normal GC B cells and 19 BL lines. Normalized relative fold change values are reported below each lane.

Considering the different expression pattern of miR-28 in normal GC B cells and BL, we investigated whether the MAD2L1, BAG1, and RAP1B proteins are expressed at higher level in cells lacking miR-28 expression. Consistent with this hypothesis, the large majority of the 19 tested BL cell lines displayed higher levels of MAD2L1, BAG1, and RAP1B expression compared with normal GC B cells (Fig. 3*C*).

Taken together, these results are consistent with a role of miR-28 in the regulation of MAD2L1, BAG1, and RAP1B and their consequent deregulated expression in BL cells.

MAD2L1 Mediates miR-28–Induced Reduction of Proliferation. MAD2L1 is an essential component of the spindle checkpoint and its overexpression in mice leads to chromosomal instability and development of neoplasia, including B-cell lymphomas (20). Furthermore, silencing of *MAD2L1* in normal human fibroblasts and cell lines leads to chromosome missegregation and cell death (21). Based on these observations, we hypothesized that *MAD2L1* could represent a critical miR-28 target whose deregulated expression could be required for proper proliferation of lymphoma cells. To explore this hypothesis, we generated stable BL cell lines inducibly expressing GFP and shRNAs targeting *MAD2L1*. In both P3HR1 and RAJI cells, three different shRNAs against MAD2L1 impaired proliferation, in direct correlation with their ability to decrease MAD2L1 protein levels (Fig. 4 *A* and *B*). Similarly to what was observed upon miR-28 expression (Fig. 2*B*), MAD2L1 silencing led to an accumulation of cells in the G_1 phase of the cell cycle (Fig. 4*C*).

To investigate whether the reduced proliferation induced by miR-28 was mainly mediated by targeting *MAD2L1*, we generated inducible vectors simultaneously expressing miR-28 and *MAD2L1* coding sequence and 3′-UTR. The *MAD2L1* 3′-UTR was used in its wild-type form or upon introduction of mutations affecting miR-28-5p binding site. We aimed to establish cell lines where MAD2L1 levels would be restored, but not overexpressed, as it has been shown that overexpression of MAD2L1 leads to reduced proliferation (20). As expected, 48 h after induction of miR-28 in P3HR1 or RAJI cells, MAD2L1 protein levels were reduced compared with control cells. Although the cells expressing MAD2L1 with the wild-type 3′-UTR still showed slightly reduced MAD2L1 levels, mutating the miR-28-5p binding site in the 3′-UTR restored the protein expression completely (Fig. 4*D*). Indeed, in both cell lines reexpression of MAD2L1 increased proliferation significantly compared with cells expressing miR-28 only (Fig. 4*E*). Furthermore, expression of MAD2L1, carrying the mutated 3′-UTR resistant to miR-28 regulation, was sufficient to restore proliferation (Fig. 4*E*). These data confirm MAD2L1 as a main mediator of miR-28–induced proliferation impairment in BL cells.

miR-28 Affects ERK1/2 Phosphorylation. One of the validated direct targets of miR-28-5p, namely BAG1, is known for its anti-apoptotic function (22). We confirmed that shRNA-mediated reduction of BAG1 increases apoptosis in Burkitt lymphoma cells (Fig. S5 *B* and *C*), suggesting that BAG1 negative modulation contributes to the apoptotic phenotype induced by miR-28. In addition BAG1 has been reported to activate v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1) in a RAS-independent manner (23). Because RAF1 is involved in the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), we hypothesized that miR-28 may affect ERK1/2 phosphorylation through modulation of BAG1. In both BL cell lines, P3HR1 and RAJI, induction of miR-28 led to reduced ERK1/2 phosphorylation, without affecting total ERK1/2 protein levels (Fig. 5). These results suggest that miR-28, through modulation of BAG1 and potentially other targets, is involved in the regulation of ERK1/2, affecting an essential component of the MAP-kinase pathway.

MYC Negatively Regulates the Expression of LPP and miR-28. To dissect the mechanisms responsible for the lack of miR-28

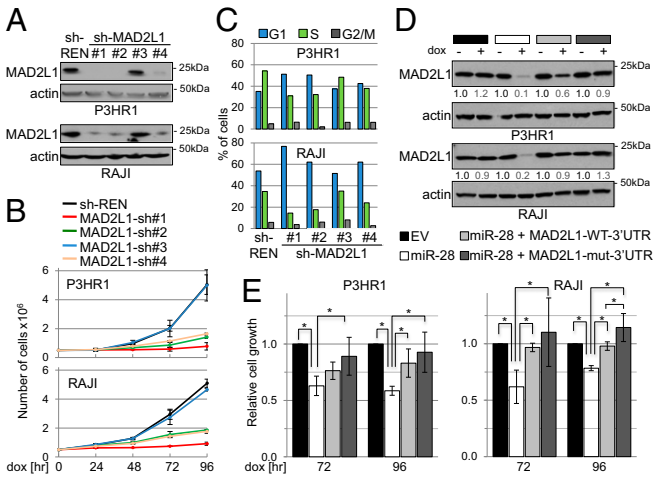

Fig. 4. miR-28 affects proliferation by targeting MAD2L1. (*A*) Immunoblot analysis of MAD2L1 in P3HR1 and RAJI cells 48 h upon induction of shRNAs against *Renilla* or MAD2L1. (*B*) Cell proliferation curve in P3HR1 and RAJI cells upon shRNA induction. Data are displayed as average ± SD of two independent experiments, each including two bulk populations and performed in triplicates. (*C*) Cell cycle profile and percentage of cells synthesizing DNA were determined by 7-AAD assay and BrdU incorporation. (*D*) MAD2L1 immunoblotting 48 h upon induction in P3HR1 and RAJI cells engineered to express the empty vector (EV), miR-28, or miR-28 with MAD2L1 including its 3′-UTR with (mut) or without (WT) mutations in the miR-28 binding site. Actin is a loading control. Relative fold change values normalized by actin are displayed. (*E*) Cell proliferation of P3HR1 and RAJI cells described in *D*. Cell counts (average ± SD) are from two independent experiments each including two bulk populations and performed in triplicates. *One-way ANOVA: Tukey's multiple-comparison test (value of $P < 0.05$).

expression in GC-derived lymphomas, we investigated both genetic and epigenetic factors. In FL, deletions affecting the host gene of miR-28 (*LPP*) have been reported (24). As expression of miR-28 and LPP correlates in normal and malignant B cells (Fig. S2 *A* and *B*), we hypothesized that deletions affecting LPP expression may also affect miR-28. FISH and Southern blot analyses were performed in cell lines lacking miR-28 expression (28 BL and 12 DLBCL), but failed to identify chromosomal losses in the LPP locus. Furthermore, no evidence of methylation-mediated silencing of the LPP/miR-28 locus was found following treatment of three BL lines with 5-azacytidine.

We then aimed to identify transcription factors that may be involved in the regulation of LPP and miR-28. Toward this goal, we used gene expression data obtained from normal mature B cells and GC-derived lymphomas and correlated the expression of transcription factors with the expression of miR-28/LPP. Based on correlative expression, we identified 39 transcription factors potentially involved in miR-28/LPP transcriptional regulation (Dataset S4). Because of its primacy in the pathogenesis of BL we focused on the oncogene *MYC*, which displayed a significant inverse correlation with miR-28/LPP (Fig. 6*A*). To test whether MYC is involved in the regulation of LPP and therefore miR-28, we silenced MYC expression in P3HR1 cells. Depletion of MYC by four independent shRNAs induced an increase in miR-28 and LPP levels that correlated with the efficiency of MYC silencing (Fig. S6). In addition, MYC was silenced in seven BL cell lines using one shRNA (sh-MYC #3). In six of seven cell lines tested, MYC silencing led to a significant ($P < 0.05$) increase in LPP and miR-28 expression (Fig. 6*B*). Conversely, enforced expression of MYC in four lymphoblastoid cell lines led to a significant down-regulation of LPP, as well as miR-28, expression (Fig. 6*C*). Taken together, these data show that MYC is involved in the negative regulation of miR-28 and suggest that it may play a critical role in miR-28 repression in GC-derived lymphomas.

miR-28 Inhibits MYC-Induced Transformation. Based on the observation that miR-28 inhibits proliferation and clonogenicity in BL cells, we tested whether miR-28 reexpression would affect MYC-induced transformation. Because immortalized normal GC B cells are not available, we used the nontumorigenic breast epithelial cell line MCF10A, a well-established system in which introduction of MYC (MCF10A-MYC) promotes anchorage-independent growth (25). These cells were transduced with an inducible vector encoding the red fluorescent protein "turboRFP" (tRFP) and the miR-28 precursor (26). Although expression of MYC in MCF10A cells led to efficient colony formation in soft agar, this phenotype was completely abrogated by expression of miR-28 (Fig. 7*A*).

Interestingly, both *MAD2L1* and *BAG1* have been shown to be induced by MYC (27, 28), and we confirmed that both genes were up-regulated in MCF10A-MYC cells. Consistent with the results obtained in B cells, induction of miR-28 led to a reduction of MAD2L1 and BAG1 (Fig. 7*B*). Taken together, these results suggest that miR-28 is able to inhibit MYC-driven transformation in MCF10A cells through a mechanism that includes the direct targeting of genes that are induced by MYC.

Discussion

Our investigation into the role of miR-28 in lymphomagenesis was prompted by the initial observation that mir-28 is specifically induced in normal GC B cells, whereas it is repressed in GC-derived lymphomas. The inhibitory effects of miR-28 expression on B-cell proliferation and the fact that the MYC oncogene requires the suppression of miR-28 to transform cells have provided supporting evidence for a role of miR-28 in interfering with B-cell malignant transformation. These results have implications for the role of miR-28 as tumor suppressor in GC-derived lymphomas as well as for the mechanism of MYC-induced malignant transformation.

miR-28 as a Candidate Tumor Suppressor in GC-Derived Lymphomas. Our results extend to GC-derived lymphomas and provide a mechanistic scenario to previous observations that have linked miR-28 to tumorigenesis in various tissues. Deregulated miR-28 expression has been observed in several types of cancer, including up-regulation in esophageal, cervical, and renal cancer, and in myeloproliferative malignancies and down-regulation in colorectal cancer and nasal-type natural killer/T-cell lymphoma (10–16). These apparently inconsistent observations are not surprising because it is well established that miRNAs can act both as tumor suppressors and oncogenes depending on the cell context (29). Analogously to colorectal cancer and nasal-type natural killer/T-cell lymphoma (14, 16), we found that miR-28 is significantly down-regulated in the majority of B-NHL and appears to be consistently silenced in all BLs. In addition, the notion that miR-28 may act as a tumor suppressor is greatly supported by the

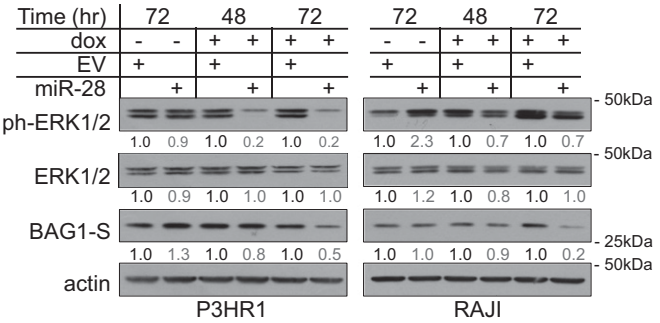

Fig. 5. miR-28 affects ERK1/2 phosphorylation in BL cells. Immunoblotting of BAG1 (BAG1-S, short isoform), phosphorylated ERK1/2 (ph-ERK1/2), and ERK1/2 in P3HR1 and RAJI cells upon miR-28 reexpression at 48 and 72 h. Relative fold change values normalized by actin are displayed.

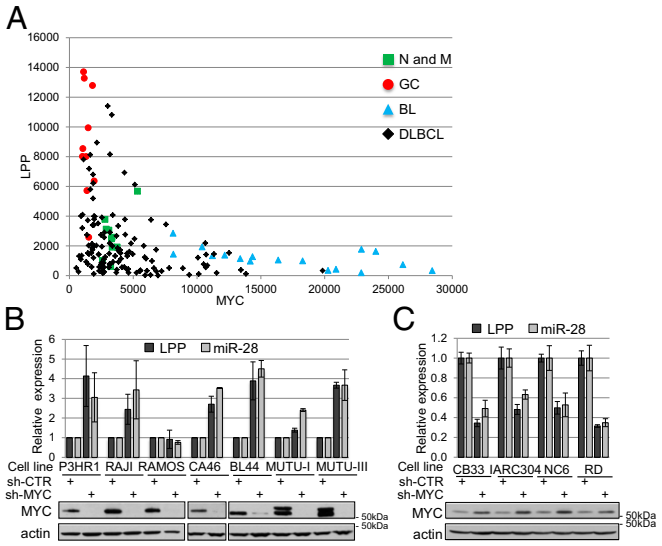

Fig. 6. MYC negatively regulates the expression of LPP and miR-28. (*A*) Correlation plot for MYC and LPP mRNA expression data (Affymetrix), in naïve (N, $n = 5$), memory (M, $n = 5$), and GC B cells ($n = 10$), BL ($n = 17$), DLBCL (112 primary cases and 18 cell lines). (*B*) (*Upper*) miR-28-5p and LPP qRT-PCR in BL cell lines expressing control (sh-CTR) or MYC (sh-MYC) shRNAs. (*Lower*) MYC immunoblotting in the same samples. (*C*) (*Upper*) miR-28-5p and LPP qRT-PCR in lymphoblastoid cell lines stably expressing MYC. (*Lower*) MYC immunoblotting in the same samples. Actin is a loading control.

observation that reexpression of miR-28 leads to impaired proliferation and loss of clonogenic properties in vitro and that its repression is mediated by MYC and specifically required for MYC-induced malignant transformation. The final proof that miR-28 may act as a tumor suppressor would require testing the effects of its down-regulation in an experimental mouse model. To implement this approach, both the miRNA and its binding sites in the target 3′-UTRs must be conserved between human and mouse. Unfortunately, although miR-28-5p is fully conserved, less than 15% of its targets, as predicated by mirSVR, are shared between human and mouse, suggesting that miR-28 may have divergent mechanisms of action in the two species.

MAD2L1 and BAG1 as Main Mediators of miR-28–Induced Phenotypes. The proliferation impairment and the loss of clonogenic potential that we observed in BL upon miR-28 reexpression are consistent with previous observations in colorectal cancer and in nasal-type natural killer/T-cell lymphoma, in which several miRNAs, including miR-28, have been reported to affect proliferation (14, 16). Nonetheless, the mechanisms and the genes that are involved in these phenotypes remained unknown. Only a few direct targets of miR-28 had been identified in different cell types (13, 14, 30) and most of them did not appear to contribute to the miR-28 program in mature B cells, because they have not been identified among the predicted targets that are responsive to miR-28 in our cell system (Dataset S3). Our results identify a significant core of direct miR-28 targets that are relevant in B cells and are involved in the proliferation-suppressing activity of miR-28. In particular MAD2L1 may be a main contributor of the miR-28–induced cellular phenotype consistent with the observation that MAD2L1 overexpression in mice leads to chromosomal instability and the development of several cancers, including B-cell lymphomas (20). Our results imply that decreased miR-28 expression in B-NHL contribute to lymphomagenesis by increasing the levels of MAD2L1 protein.

A second functionally validated target of miR-28, BAG1, may also contribute to the effects of miR-28 on proliferation by promoting apoptosis and by interfering with the ERK signaling pathway, a major growth-promoting signal transduction pathway. Deregulation of ERK signaling is found in many different cancers, including B-NHL, and its inhibition is also associated with an increase in apoptosis (31, 32), including MYC-induced apoptosis (28). Thus, elevated BAG1 levels can protect the cells from apoptosis and, by activating ERK1/2, may favor survival and proliferation in lymphomas lacking miR-28 expression.

The observation that miR-28 has proliferation-modulating properties raises the issue of its role in normal GC, a structure characterized by its highly proliferative rate. It is notable, however, that cellular proliferation is modulated in the transition between the dark-zone centroblasts and the light-zone centrocytes, which are entering the plasmacytic differentiation pathway. In addition, signal transduction pathways, including B-cell receptor signaling that induces ERK signaling, are analogously modulated during the GC reaction. Thus, it is conceivable that miR-28 expression may contribute to the fine-tuning of proliferation and signaling pathways during the complex biology of B cells transitioning and recirculating in the GC.

MYC and miR-28 in GC B Cells. The involvement of MYC in the regulation of miR-28 is an intriguing observation given that ectopic/deregulated MYC expression represents the hallmark of BL and is also frequently found in other GC-derived lymphomas (33, 34). MYC is known to directly induce the expression of the prooncogenic miR-17-92 cluster (3), but it is also involved in a widespread down-regulation of miRNA expression (35). In the specific context of the GC reaction, it is important to note that MYC is not expressed in most B cells because its transcription is directly repressed by BCL6 (36). Thus, the expression of miR-28 in normal GC B cells is consistent with the absence of MYC in the same cells, whereas the ectopic, deregulated MYC expression in BL is consistent with miR-28 repression in these tumor cells. Although our data demonstrate a role for MYC in the regulation of miR-28, we cannot exclude that other transcription factors as well as epigenetic and genetic mechanisms are involved in modulating miR-28 expression.

The precise mechanisms by which MYC represses LPP/miR-28 transcription could not be elucidated. We can exclude a direct or MIZ1-mediated repression (37) of *LPP* and miR-28 by MYC, as we failed to identify a region in the *LPP* promoter bound and/or regulated by MYC or MIZ1. These negative findings suggest that the regulation of miR-28 by MYC may not be direct but involves intermediate molecules that are currently unknown.

Regardless of the precise mechanism involved, it is notable that induction of MYC does lead to LPP/miR-28 repression in native B cells, and, most notably, that miR-28 reexpression in MYC-expressing cells prevents MYC-induced cell transformation. Interestingly, some of the miR-28 direct targets genes, such as *MAD2L1* and *BAG1*, have been shown to be transactivated by

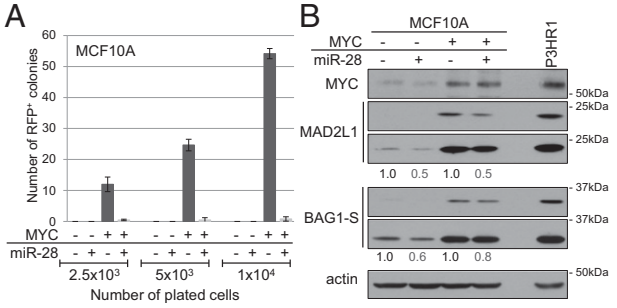

Fig. 7. miR-28 inhibits MYC-induced transformation. (*A*) Transforming ability of MCF10A and MCF10A-MYC cells upon miR-28 determined in soft agar as the number of RFP^+ colonies. The bar plots represent the average ± SD of two independent experiments, each including two bulk populations and performed in triplicates. (*B*) Immunoblot analysis for MYC, MAD2L1, and BAG1-S in MCF10A and MCF10A-MYC cells upon 48-h induction of miR-28. Two time exposures are displayed for MAD2L1 and BAG1-S.

MYC (27, 28), whereas ERK1/2 signaling is involved in the stabilization of MYC protein (38). These observations, together with their mutually exclusive expression in GC B cells, suggest the presence of multiple feedback loops between MYC and miR-28. These circuits may be variably regulated during the GC reaction but become unbalanced in the favor of MYC, at the expense of miR-28, in BL. Finally, the observation that the reexpression of miR-28 is sufficient to block MYC-dependent cell transformation and proliferation, suggests that reexpression of miR-28 represents a potentially useful therapeutic strategy, once technologies for efficient miRNA delivery will be optimized.

Materials and Methods

Details regarding the expression constructs, lentiviral transduction, gene and miRNA expression profiling, Northern blotting, and bioinformatics analysis are available in *SI Materials and Methods*.

Primary Cells and Cell Lines. Primary tumor tissue collection was approved by each institutional ethics committee. Purification of GC B cells was performed as previously reported (39). HEK293T cells and B-cell lines were grown in DMEM (Gibco) and Iscove's modified Dulbecco's medium (Gibco), respectively supplemented with 10% (vol/vol) FCS and 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37 °C in 5% CO_2. Cell numbers were determined using the Countess automated cell counter (Invitrogen), which performs cell count and viability measurements using trypan blue staining.

Cellular Assays. BrdU incorporation and DNA content were analyzed with the APC BrdU flow kit (BD Pharmingen). Annexin-V–PE (BD Pharmingen) and 7-aminoactinomycin D (7-AAD) (BioLegend) stainings were performed as recommended. Cells were analyzed on a FACSCalibur flow cytometer using the CellQuest software (BD Pharmingen). FlowJo 9.5.2 software (TriStar) was used for data analysis and plot rendering. Clonogenicity assays in soft agar were performed as previously described (22). Agar plates were incubated at 37 °C for 7–14 d. GFP^+ colonies were counted using a fluorescence microscope (Nikon Eclipse TS100). 3′-UTR–reporter assays were performed in HEK293T cells upon transient transfections by calcium phosphate precipitation method (40) or by polyethylenimine (Polysciences) (41). Firefly and *Renilla* luciferase activities were measured 48 h after transfection using the Dual-Luciferase Reporter Assay Kit (Promega), according to the manufacturer's instructions.

Quantitative RT-PCR. Total RNA was isolated by TRIzol Reagent (Invitrogen) and reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Absolute Blue QPCR SYBR Green Fluorescein Mix (Thermo) was used for quantitative PCR using the primers reported in Dataset S5. miRNA detection was performed using the human miR-28-5p, miR-151-3p, and RNU6B Mature Taqman MicroRNA Assays (Applied Biosystems).

Immunoblotting. Whole-cell lysates were prepared as previously described (42) and analyzed by SDS/PAGE and immunoblotting using the antibodies in Dataset S5.

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