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BLIMP-1 and STAT3 Counterregulate MicroRNA-21 during Plasma Cell Differentiation

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During cellular differentiation, mRNA transcription and translation require precise coordination. The mechanisms controlling this are not well defined. IL-21 is an important regulator of plasma cell differentiation, and it controls the master regulator of plasma cell differentiation, B lymphocyte-induced maturation protein-1 (BLIMP-1), via STAT3 and IRF4. Among the other targets of STAT3 is microRNA-21 (miR-21). miR-21 is the most frequently deregulated microRNA in malignancy, including B cell lymphomas, and it has oncogenic potential downstream of STAT3. However, the regulation and function of miR-21 during plasma cell differentiation are not characterized. In contrast to the induction of miR-21 observed in response to STAT3 activation in other systems, we demonstrate that miR-21 is repressed during IL-21-driven plasma cell differentiation. We explored the molecular basis for this repression and identify primary miR-21 transcription as a direct target of BLIMP-1-dependent repression, despite continued STAT3 activation and phospho-STAT3 binding to the primary miR-21 promoter. Thus, STAT3 and BLIMP-1 constitute an incoherent feed-forward loop downstream of IL-21 that can coordinate microRNA with mRNA expression during plasma cell differentiation. The Journal of Immunology, 2012, 189: 253–260.

During the humoral immune response, both short-lived and long-lived Ab-secreting cells are made, and both require B lymphocyte-induced maturation protein-1 (BLIMP-1) expression (1). In response to Ag and follicular T cell help, B cells form germinal centers that generate high-affinity long-lived plasma cells. IL-21, which is produced by T follicular helper cells, is a potent inducer of class-switch recombination and plasma cell differentiation (2, 3). The effect of IL-21 on responsive B cells is mediated by the activation of STAT3, which, in conjunction with IRF4, results in the upregulation of BLIMP-1 (4, 5). The importance of STAT3 during plasma cell differentiation is further demonstrated by the selective loss of T-dependent IgG responses in mice that have Stat3-deficient B cells (6).

Beyond its role in lymphocyte differentiation, STAT3 has been identified as part of a regulatory circuit linking inflammatory responses to cellular transformation. In this context, STAT3 was shown to directly activate microRNA-21 (miR-21) (7). Aberrant expression of microRNAs is a feature of malignancies, and microRNA profiles can define disease groups (8). Expression of miR-21 is part of the characteristic profile in diffuse large B cell lymphoma (DLBCL) (9–11). miR-21 is also overexpressed in malignant plasma cells in which expression of miR-21 is dependent on IL-6 (12, 13).

Mature microRNAs are derived from processed primary transcripts, whose expression is driven predominantly from polymerase-II promoters (14). This implicates the prevailing transcription factor circuitry in the control of microRNA expression. BLIMP-1 is a key element of this circuitry in differentiating B cells, where it directly represses gene expression (15). A tumor suppressor function for BLIMP-1 was demonstrated based on genetic inactivation in DLBCL; this occurs preferentially in a subset of DLBCL that also shows evidence of constitutive STAT3 activation (16–18). We demonstrate in this study that, during an IL-21–driven cellular response, BLIMP-1 can override STAT3-dependent promoter activation by exerting direct repressive control over a shared target promoter. Expression of primary miR-21 (pri-miR-21) is repressed by BLIMP-1 during IL-21–driven in vitro human plasmablast differentiation, despite continued STAT3 activation and pri-miR-21 promoter occupancy by phospho-STAT3. As such, the relationship by which STAT3 and BLIMP-1 counterregulate the expression of miR-21 specifically conforms to a type 1 incoherent feed-forward loop in which the two arms of the feed-forward loop oppose the action of each other (19). Thus, we define the first connection, to our knowledge, between BLIMP-1 and the regulation of microRNA expression, as well as identify an incoherent feed-forward loop that contributes to coordinated microRNA and mRNA expression.

Materials and Methods

Cell lines and Abs

U266, H929, HepG2, HeLa, and CD40L-L cells (20) were cultured under standard conditions. Use of human samples was approved by the Leeds East Research Ethics Committee (REC reference 07/Q1206/47). The Abs used for flow cytometry included PE-conjugated anti-CD19 (Miltenyi Biotec) and PE-Cy7–conjugated anti-CD38 (BD Biosciences). The Abs used for chromatin immunoprecipitation (ChIP), EMSA, and immunoblotting were control rabbit IgG (Upstate Biotechnology), phospho-STAT3 (Tyr-705) (9131; New England BioLabs), total STAT3 (137000; Invitrogen), monoclonal anti–β-actin (Sigma-Aldrich), and rabbit anti–
BLIMP-1 (21, 22). Densitometry was performed using Quantity One analysis software (Bio-Rad).

ChIP, EMSA, and luciferase assays

ChIP and EMSA were performed as described (23). Nuclear extracts for EMSA were prepared from transfected COS cells, the H929 myeloma cell line, and in vitro-generated primary human plasmablasts at day 6 of culture. The primers used for amplification of ChIP material included: miR-21-5526946F (5′-GAAGATTTCTGTCCTAGATGCCC-3′), miR-21-5526946F (5′-TCTCCTTTGATACAGCTCC-3′), miR-21-55269836F (5′-GAGTGAGAGCTG-3′), mirVana miRNA inhibitor negative control #1 (4464076), mirVana mir-21-55269836R (5′-AGTTGACGACGAGGCTG-3′), RHOBF (5′-GGCTAAGGTAAGCTGGTGTAAGCACTGCCAAGACACTCTGCA-3′), and PDCD4R (5′-GGCGTGGTCGGCAGAGGCT-3′). Mature miR-21 was quantified using TaqMan hsa-miR-21 assay (4395549) and normalized to TaqMan U18-RNA assay (4380904) (Applied Biosystems).

Cell culture and differentiation

Total peripheral blood B cells, as well as naive and memory B cell populations, were purified using MACS CD19+ B cell isolation and memory B cell isolation kits (Miltenyi Biotec), according to the manufacturer’s instructions. Flow cytometry was performed with an LSR II cytometer (BD Biosciences), and cell sorting was performed with a MoFlo (Beckman Coulter). In vitro differentiation was performed using IL-2, IL-21 (Sigma-Aldrich), and irradiated CD40L-expressing fibroblasts, as described (26).

Results

miR-21 levels decrease during plasma cell differentiation

Given the role that miR-21 plays in tumor development, and in particular its association with hematological malignancies arising from late-stage B cells, we asked whether expression of miR-21 is reprogrammed during human B cell terminal differentiation. Total peripheral blood B cells were isolated and subjected to in vitro differentiation using IL-2, IL-21, and CD40L-expressing fibroblasts. Differentiation was assessed by flow cytometry (Fig. 1A). At day 6 of differentiation, B cells were sorted by CD38 expression, which identifies the majority of Ab-secreting plasmablasts in this system (27, 28). As expected, expression of the master regulator of plasma cell differentiation, BLIMP-1, increased with time and was substantially greater in the CD38+ve plasmablast population (Fig. 1B). The initial determinant of microRNA expression is the long primary transcript from which the functional mature microRNA is subsequently processed. As the B cells become activated and differentiate toward plasmablasts, transcription of the primary miR-21 transcript is rapidly extinguished. In fact, the levels of pri-miR-21 were inversely correlated with BLIMP-1, with significantly lower expression in CD38+ve plasmablasts relative to either the originating B cell population at day 0 or activated B cells at day 3 of differentiation (Fig. 1C).

To assess whether a similar pattern of miR-21 expression would be observed in plasmablasts derived from naive or memory B cells, these populations were independently differentiated. In freshly isolated cells, we observed high levels of pri-miR-21 but not the processed mature form. As the cells became activated, the mature form of miR-21 increased (Supplemental Fig. 1, data not shown). Expression of BLIMP-1 and miR-21 was then evaluated in CD38+ve and CD38−ve populations from each differentiation series at day 6. Expression of pri-miR-21 and mature miR-21 was repressed in CD38+ve plasmablasts derived from either naive or memory B cell populations and correlated with the induced expression of BLIMP-1 in these cells (Fig. 2). BLIMP-1 expression levels were consistently higher in the CD38−ve population derived from memory B cells than in that derived from naive B cells, and this also correlated with lower levels of pri-miR-21 expression in these cells (Fig. 2, Supplemental Fig. 1).

STAT3 binds to regulatory regions in both BLIMP-1 and pri-miR-21

IL-2 is a powerful inducer of plasma cell differentiation, with the ability to drive differentiation of various subsets of human B cells that is greater than other T cell-derived cytokines (27–31). The predominant biochemical mediator of IL-21 signaling was found to be activated STAT3 (23, 33). Indeed, STAT3 has proven essential for the induction of BLIMP-1 and the generation of plasma cells from both human and murine B cells (5, 34). In a prior as-

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Data shown are averages and standard deviations of triplicate samples.

The Journal of Immunology 255

This site was also identified as a region of STAT3 binding in the study of Kwon et al. (4). The binding of phospho-STAT3 to intron 3 of BLIMP-1 was ~8-fold by day 3 of culture and had increased to 11-fold by day 5.

STAT3 is also an important regulator of miR-21 expression and specifically acts downstream of IL-21 to induce the microRNA in CD4+ cells from patients with Sézary syndrome (35). In this instance, both the activation of STAT3 and the expression of miR-21 are transient. We performed ChIP to determine whether the activated STAT3 in differentiating B cells bound to the previously identified sites in the promoter of miR-21 (Fig. 3B). In agreement with previous reports, we detected phospho-STAT3 in a region proximal to the transcriptional start site. Somewhat surprisingly, the 4-fold binding of phospho-STAT3 to the miR-21 promoter was present throughout the time course of the experiment.

**BLIMP-1 binds to the promoter of pri-miR-21**

The continued binding of activated STAT3 to the miR-21 promoter in day-5 plasmablasts is at odds with the reduction in detectable transcript and suggests that a dominant inhibitory mechanism is operating to prevent transcription. BLIMP-1 is a transcriptional repressor that regulates many of its direct targets via binding sites within promoter elements (15). Therefore, we asked whether potential BLIMP-1 binding sites are present within the promoter region of pri-miR-21. Indeed, the defined promoter region of human pri-miR-21 contains two sequences that resemble the BLIMP-1 consensus (Supplemental Fig. 2B, data not shown) (13, 36–38). Using ChIP, we identified a distinct single peak of BLIMP-1 binding at the promoter of pri-miR-21 in two human B cells. Naive and memory B cells were selected and subject to in vitro differentiation for 6 d. CD38+ve or CD38−ve differentiating B cells were sorted. Expression of pri-miR-21 and mature miR-21 was quantified by RT-PCR. Expression of pri-miR-21 and mature miR-21 was normalized to GAPDH mRNA and mature miR-21 to U18 RNA. The relative expression was then normalized to the average of the CD38−ve samples. Data are averages and SD of independent differentiations from three donors. BLIMP-1 protein levels were determined by Western blot relative to β-actin in the same populations (bottom panels). Numbers above the blots indicate densitometry values for BLIMP-1 normalized against β-actin expression. *p < 0.05, CD38+ve versus CD38− , unpaired t test.
myeloma cell lines that constitutively express BLIMP-1 at levels analogous to plasma cells (Fig. 4A). This peak corresponds with the position of the more conserved consensus binding site. In a comparable experiment, we performed BLIMP-1 ChIP from day-6 plasmablasts generated from human B cells and observed the same pattern of binding to the region of conservation, and the degree of BLIMP-1 association was similar to that of another BLIMP-1 target, LMO2 (Fig. 4A) (38). To further evaluate the sequence bound by BLIMP-1, we performed EMSA with an oligonucleotide corresponding to the proposed BLIMP-1 binding site at this peak region. This oligonucleotide substantially bound BLIMP-1 protein (Fig. 4B).

BLIMP-1 represses pri-miR-21

To assess whether BLIMP-1 could regulate endogenous promoter activity, we transfected HeLa cells, which constitutively express miR-21, with BLIMP-1 or control expression plasmids (37). Levels of pri-miR-21 were substantially repressed by enforced BLIMP-1 expression (Fig. 5A). To examine whether the binding of BLIMP-1 to the pri-miR-21 promoter corresponded with regulation in myeloma cell lines, we examined the effects of BLIMP-1 knockdown in U266 cells. A consistent increase in pri-miR-21 expression was observed in BLIMP-1 siRNA-treated cells relative to control siRNA-treated cells (Fig. 5B).

To assess the ability of BLIMP-1 to modulate an alternate STAT3-mediated response at the pri-miR-21 promoter, the effect of BLIMP-1 on IL-6–dependent pri-miR-21 promoter activation was evaluated in the IL-6–responsive HepG2 cell line. As expected, the pri-miR-21 promoter was responsive to IL-6; however IL-6–induced promoter activity was significantly repressed by BLIMP-1 (Fig. 5C) (13). Thus, the ability of BLIMP-1 to repress the promoter is not cytokine specific but corresponds with the shared usage of STAT3.

miR-21 targets are upregulated in differentiating B cells

Our data showed that BLIMP-1 can bind to and control expression from the promoter of pri-miR-21. We next evaluated whether BLIMP-1 is capable of altering the expression of mRNA containing an miR-21 seed sequence in its 3’UTR. To this end, we cotransfected a luciferase reporter with the Pdcd4 3’9 UTR, which contains an miR-21 seed sequence, together with a control vector...
or BLIMP-1 expression construct, into HeLa cells (25). In these cells, introduction of BLIMP-1 represses expression of pri-miR-21 (Fig. 5A). Consistent with this, addition of BLIMP-1 led to derepression of the Pdcdd4 3’UTR luciferase reporter gene (Fig. 6A).

There are two potential models for the consequences of this repression in plasma cell differentiation. First, that genes regulated by miR-21 are not expressed in plasma cells and so miR-21 is switched off because its function is no longer required. Second, BLIMP-1 may control expression of miR-21 to ensure more efficient expression of miR-21 targets.

To distinguish between these possibilities, we looked at the expression of a variety of genes that are directly targeted by the mature miR-21 transcript in other cellular contexts. If the former model is correct, then these genes should be uniformly repressed; however, they should show stable, or even increased expression, if the latter model holds. In parallel with the downregulation of miR-21 in plasmablasts derived from both naive and memory B cells, we observed an increase in gene expression of a subset of miR-21 targets, including PDCD4, BTG2, and RHOB, in CD38+ve plasmablasts compared with nondifferentiated cells (Fig. 6B, 6C). This evidence favors a model in which BLIMP-1 coordinates the expression of miR-21 targets by directly modulating levels of miR-21.

**FIGURE 5.** BLIMP-1 regulates the miR-21 promoter. (A) BLIMP-1 represses pri-miR-21 expression. Expression of pri-miR-21 was quantified by real-time PCR in enhanced GFP (EGFP)-expressing HeLa cells, 24 h after transfection with IRES2-EGFP vector (Control) or BLIMP-1–IRES2-EGFP vector (BLIMP-1). Data shown are average and SD of three independent transfections, normalized to GAPDH and to the average value of the three control-transfected samples. Relative expression is plotted on the y-axis. *p < 0.05, vector versus BLIMP-1 transfectants, unpaired t test. (B) pri-miR-21 is responsive to BLIMP-1 knockdown in myeloma cells. Expression of pri-miR-21 was quantified by real-time RT-PCR in U266 cells 24 h after electroporation with BLIMP-1–specific (BLIMP-1) or scramble control (Scramble) siRNA. Relative expression, shown on the y-axis, is the average and SD of four independent transfections, normalized to GAPDH and to the average value of the control-transfected samples. Western blots of BLIMP-1 and β-actin in representative samples are shown (lower panels). Numbers above the blot indicate densitometry values normalized against β-actin expression. *p < 0.05, control versus BLIMP-1 siRNA, unpaired t test. (C) BLIMP-1 represses IL-6 response of pri-miR-21 promoter. HepG2 cells transfected with pri-miR-21 promoter luciferase reporter, TK229-pRL and pIRES2-EGFP (Control), or BLIMP-1–pIRES2-EGFP expression vector (BLIMP-1) were left untreated or treated with IL-6 for 6 h. Plots show relative luciferase activity (firefly/Renilla) normalized to pri-miR-21 basal promoter activity on the y-axis. Data shown are average and SD of three replicates and are representative of three independent experiments. *p < 0.05, IL-6–treated control versus IL-6–treated BLIMP-1 transfectants, unpaired t test.

**FIGURE 6.** miR-21 targets are responsive to BLIMP-1 levels and increase in CD38+ve plasmablast populations. (A) HeLa cells were transfected with a wild-type sequence Pdcdd4 3’UTR luciferase reporter, SV40-pRL and pIRES2-EGFP (Control), or BLIMP-1–pIRES2-EGFP expression vector (BLIMP-1). Bar graph shows relative luciferase activity (firefly/Renilla). Data shown are average and SD of three replicates and are representative of three independent experiments. *p < 0.05, vector versus BLIMP-1, unpaired t test. mRNA obtained from sorted CD38+ve or CD38−ve differentiating B cells derived from naive (B) or memory (C) populations, as in Fig. 2, were assessed for levels of PDCD4, BTG2, and RHOB by RT-PCR. Expression of each gene was normalized to GAPDH. Data are from independent differentiations from three or four different donors and are normalized to the average levels in naive B cells at day 3. *p < 0.05, CD38− versus CD38+, unpaired t test.
Enforced miR-21 expression does not prevent phenotypic or functional maturation of plasmablasts in vitro

The documented overexpression of miR-21 in a number of tumor settings suggests that its deregulation is an important factor in oncogenesis. Indeed, in the case of lymphoid-derived malignancies, such as multiple myeloma and Sézary syndrome, inhibition of miR-21 expression resulted in modest increases in the degree of apoptosis (13, 35). The function of miR-21 during normal cellular processes is less well known; at least two studies using miR-21-deficient mice showed that this microRNA is not required for normal tissue homeostasis (39, 40). To determine whether continued high-level expression of miR-21 or inhibition of miR-21 could influence the generation of human plasmablasts in vitro, we introduced an miR-21 mimic or inhibitor into activated B cells at day 3, the time point at which we observed the initial decrease in pri-miR-21. The transfected cells were subjected to the differentiation protocol, and parameters that distinguish the phenotypic and functional maturation of plasmablasts were assessed. Differentiating B cells were monitored for continued expression of the transfected microRNA through detection of a fluorescent label and by quantitative PCR of the processed transcript, which demonstrated ~10-fold greater expression of miR-21 over control transfectants during the course of the experiment (Supplemental Fig. 3A, 3B). Although there was a trend toward slightly increased numbers of cells in the presence of continued miR-21 expression, this did not reach statistical significance (Supplemental Fig. 3C). Analysis of other parameters, such as apoptosis, phenotypic markers, and Ab secretion, showed no significant differences (Supplemental Fig. 4, data not shown). We conclude that miR-21 repression is not essential to the core program of in vitro differentiation, as assessed in this model, but instead may contribute to more subtle aspects of plasma cell biology.

Discussion

The demonstration that STAT3 and BLIMP-1 counterregulate pri-miR-21 is of importance for two main reasons. To our knowledge, this provides the first evidence for the transcriptional regulation of a microRNA by BLIMP-1 and identifies a microRNA that is deregulated in B cell neoplasms as a BLIMP-1 target. Second, it establishes that, as well as being an IL-21 and STAT3 target, BLIMP-1 can act in an incoherent feed-forward loop to modulate IL-21– and STAT3-dependent gene regulation.

BLIMP-1 acts in the immune system and during development to control numerous gene-expression programs. Previous work demonstrated that it does so both directly, through target gene regulation, and indirectly, through the control of key transcription factors (15). Repression of miR-21 is distinct from the known regulatory mechanisms controlled by BLIMP-1 during B cell differentiation, because miR-21 itself acts as a repressor of gene expression at a posttranscriptional level. The reprogramming of microRNA expression can be inferred as an essential component of cellular differentiation because microRNAs impose broad control over mRNA translation and stability. Our data indicate that BLIMP-1 contributes to this process during plasma cell differentiation and may coordinate expression of both protein coding and microRNA genes, thus ensuring precision and stability in the expression program.

Plasma cells represent the ultimate outcome of B cell differentiation, but they may differ substantially in terms of lifespan and localization, which are determined by the nature of the responding B cell and input from the initiating stimuli (41). In the context of Ag and T cell help, IL-21 is a potent driver of plasma cell differentiation. Although IL-21 is capable of activating STAT1, STAT3, and STAT5, STAT3 appears to be the predominant downstream effector responsible for the B cell response to this cytokine (32, 33). The activation of STAT3 was shown to potentiate BLIMP-1 expression, and the molecular requirement for STAT3/IRF4 cooperation at an RE 3′ of the BLIMP-1 gene was recently described (4). Although STAT3 undoubtedly contributes to the regulation of BLIMP-1 during the differentiation of B cells to plasma cells, the extent to which individual STAT3 and/or IRF4 sites play a role has not been addressed. The identified 3′ element is not conserved in humans; therefore, additional sites are likely to be operative. ChIP-seq data suggest that STAT3 has the potential to bind multiple areas within the Blimp-1 locus, and our ChIP results confirm that at least one of these sites is occupied in differentiating human B cells (4).

STAT3 was also shown to regulate the expression of miR-21 in a number of cell types. Notably, direct regulation of miR-21 by STAT3 was documented in CD4+ T cells stimulated with IL-21 from patients with Sézary syndrome (35). Similarly, we provide evidence that STAT3 binds to a regulatory site in the miR-21 promoter following IL-21 treatment of human B cells. Although the level of pri-miR-21 in differentiating B cells diminished rapidly, the extent of STAT3 binding to the promoter did not. Instead, we observed a simultaneous recruitment of BLIMP-1 to the miR-21 promoter. This is likely to account for the loss of expression, given that expression of BLIMP-1 is sufficient to repress the miR-21 promoter in transient-transfection assays, and endogenous miR-21 expression is responsive to BLIMP-1 levels in two distinct cell line models. In conjunction with the established role for STAT3 as an inducer of BLIMP-1, the relationship between STAT3 and BLIMP-1 at the miR-21 promoter conforms to a network motif known as a type 1 incoherent feed-forward loop (19).

In such a feed-forward loop, the two arms oppose each other: transcription factor X activates target gene Z, but it also induces another transcription factor Y, which acts as a repressor of gene Z. In this case, STAT3 (X) activates miR-21 (Z), but it also represses miR-21 (Z) by activating the repressor BLIMP-1 (Y). This type of regulatory circuit is among the most commonly observed network motif in species such as Escherichia coli and yeast. Thus, the arrangement of BLIMP-1 both as a target of STAT3 and a repressor of target promoters induced by STAT3 identifies a recurring regulatory structure used to coordinate gene expression during plasma cell differentiation.

miR-21 has been implicated in the pathogenesis of non-hematopoietic tumors acting through repression of proapoptotic genes and the potentiation of oncogenic RAS signaling. In lung tumors, miR-21 is an essential component of an autoregulatory loop whereby repression of miR-21 targets, such as BTG2 and PDCD4, relieves inhibition of the RAS/MEK/ERK pathway, whereas downmodulation of other miR-21–regulated genes prevents cell death (42). We showed that these same targets (i.e., BTG2, PDCD4, and RHOB) are increased in differentiating plasmablasts in contrast to the disappearance of miR-21 (25, 43, 44). The role of these genes in the generation of plasma cells has not been characterized. However, one may speculate that higher levels of miR-21 target genes, such as BTG2 and RHOB, might contribute to features that typify plasma cells, such as exit from cell cycle and migration to survival niches.

In the context of the in vitro-differentiation system, we were unable to show a definitive effect of altered miR-21 expression, suggesting that downregulation of miR-21 is not an absolute requirement for the generation of Ab-secreting cells. We used an established in vitro system that ensures a robust level of B cell differentiation. Alternative strategies involving different combinations of Ag, cytokines, and/or innate signals also generate
plasmablasts and reflect the heterogeneous nature of this population. Akin to the finding that loss of miR-21 had no effect on normal kidney development or function, but rather manifested its importance in response to tissue injury, our results suggest that miR-21 targets may be operative only in particular situations that generate plasma cells (40). Moreover, the long-term survival of plasma cells in vivo is dependent on successful entry into bone marrow stromal niches where a number of factors act in concert to promote longevity. It is conceivable that miR-21 targets are required during this stage of the plasma cell life cycle, as suggested above. Furthermore, although we demonstrated elevation of certain miR-21 targets in plasmablasts, we cannot rule out the possibility that the key targets that may impact differentiation are themselves silenced and are no longer functioning.

Beyond its potential role in normal B cell physiology, miR-21 contributes to characteristic microRNA signatures in DLBCL, myeloma, and Hodgkin’s lymphoma (9–12, 45–47). Failure of BLIMP-1 to control miR-21 expression is likely to contribute to the effects of BLIMP-1 inactivation in B cell malignancies. In myeloma, loss of balance between the repressive effects of miR-21 targets in plasmablasts, we cannot rule out the possibility that the key targets that may impact differentiation are themselves silenced and are no longer functioning.

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Disclosures

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