Bromodomain and extra-terminal domain inhibition modulates the expression of pathologically relevant microRNAs in diffuse large B-cell lymphoma

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ABSTRACT

Aberrant changes in microRNA expression contribute to lymphomagenesis. Bromodomain and extra-terminal domain inhibitors such as OTX015 (MK-8628, birabresib) have demonstrated preclinical and clinical activity in hematologic tumors. MicroRNA profiling of diffuse large B-cell lymphoma cells treated with OTX015 revealed changes in the expression levels of a limited number of microRNAs, including miR-92a-1-5p, miR-21-3p, miR-155-5p and miR-96-5p. Analysis of publicly available chromatin immunoprecipitation sequencing data of diffuse large B-cell lymphoma cells treated with bromodomain and extra-terminal domain (BET) inhibitors showed that the BET family member BRD4 bound to the upstream regulatory regions of multiple microRNA genes and that this binding decreased following BET inhibition. Alignment of our microRNA profiling data with the BRD4 chromatin immunoprecipitation sequencing data revealed that microRNAs downregulated by OTX015 also exhibited reduced BRD4 binding in their promoter regions following treatment with another bromodomain and extra-terminal domain inhibitor, JQ1, indicating that BRD4 contributes directly to microRNA expression in lymphoma. Treatment with bromodomain and extra-terminal domain inhibitors also decreased the expression of the arginine methyltransferase PRMT5, which plays a crucial role in B-cell transformation and negatively modulates the transcription of miR-96-5p. The data presented here indicate that in addition to previously observed effects on the expression of coding genes, bromodomain and extra-terminal domain inhibitors also modulate the expression of microRNAs involved in lymphomagenesis.

Introduction

The important role of non-coding elements of the genome, specifically microRNAs (miRNAs), in mediating cellular transformation was first demonstrated in chronic lymphocytic leukemia.[1] Since then, numerous miRNAs have been shown to function as tumor suppressors or oncogenes in both hematologic and solid tumors.[2-8] miRNAs are short sequences of 19 – 25 nucleotides that function as part of an RNA-induced silencing complex (RISC).[9] In humans, they function primarily by destabilizing messenger RNA (mRNA) and inhibiting the translation of mRNA into protein. This is achieved through binding of the 5' seed region of a

miRNA to its recognition sequence in the 3' untranslated region of its target gene.[10] A single miRNA can recognize multiple target genes and, conversely, different miRNAs can target an individual gene.[11] Thus, in the context of cancer, miRNAs can intricately and markedly influence individual driver genes and entire signaling pathways crucial to the survival of cancer cells. Furthermore, a number of miRNAs have been shown to participate in a feedback loop with the protein product of their target gene.[11]

Diffuse large B-cell lymphoma (DLBCL) is an aggressive lymphoma that accounts for approximately 35-40% of all lymphoma cases.[12] DLBCL frequently harbors mutations in chromatin-modifying enzymes indicating that perturbation of epigenetic regulation is an important trigger for B-cell transformation.[13,14] A class of epigenetic drugs that has recently shown promising results in pre-clinical and clinical settings, and particularly in DLBCL, inhibits members of the bromodomain and extra-terminal domain (BET) protein family.[15-25] In mammals, the BET family comprises four proteins, BRD2, BRD3, BRD4 and BRDT, which all share two highly conserved N-terminal bromodomains (BRD) and a C-terminal extra-terminal (ET) domain. BET proteins specifically bind to acetylated lysine residues via their dual BRD motifs, acting as epigenetic readers of acetyl-lysine marks. They therefore constitute an important component of the write-read-erase model via which epigenetic information is interpreted by cells.[17] BET inhibitors act by preventing the interaction of BRD4 with acetylated histones.[26] Here we show direct and indirect regulation of miRNA expression in DLBCL by a BET inhibitor.

Methods

Cell lines and molecules

Established human cell lines derived from DLBCL were cultured according to recommended conditions. Two germinal-center B-cell type DLBCL (GCB-DLBCL) cell lines, DOHH-2 and OCI-LY-1, were cultured in Roswell Park Memorial Institute medium and Iscove's Modified Dulbecco's Medium, respectively. The activated B-cell–like DLBCL (ABC-DLBCL) cell lines SU-DHL-2 and HBL-1 were cultured in Roswell Park Memorial Institute medium. Cell lines were obtained as previously described,[27] and their identity was authenticated by short tandem repeat DNA profiling (IDEXX BioResearch, Ludwigsburg, Germany). All media were supplemented with fetal bovine serum (10%; DOHH-2 and OCI-LY-1 or 20%; SU-DHL-2 and HBL-1), penicillin-streptomycin-neomycin (5,000 units penicillin, 5 mg streptomycin and 10 mg neomycin/mL, Sigma) and L-glutamine (1%). OTX015 (MK-8628, birabresib) was provided by Oncoethix (Lausanne, Switzerland).

In vivo xenograft model

The xenograft model used here has been described elsewhere.[15] Total RNA, previously extracted from these tumors, was used to analyze OTX015-mediated modulation of miRNA expression *in vivo*.

Western blotting analysis

Protein extractions, sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblotting were performed as previously described.[15] The antibodies used were anti-PRMT5 (A1520; NeoBiolab), anti-GAPDH (9131; Cell Signaling) and anti-BRD4 (A301-985A; Bethyl).

One-step quantitative reverse transcription - polymerase chain reaction

Total RNA was extracted from cells treated with dimethyl sulfoxide (DMSO) or OTX015 using TRIzol (Thermo Scientific, Lausanne, Switzerland). One-step quantitative reverse transcription - polymerase chain reaction (qRT-PCR) was performed as previously described[15] using 20 ng of RNA for each reaction. Forward and reverse primers used for quantification of *PRMT5* mRNA were, respectively, 5'-TCTCATGGTTTCCCATCCTC-3' and 5'-ACACAGATGGTTTGGCCTTC-3'. Quantification of GAPDH expression served as an endogenous control. *GAPDH* primer sequences were, 5'-CGACCACTTTGTCAAGCTCA-3' (forward) and 5'-CCCTGTTGCTGTAGCCAAAT-3' (reverse). Expression of *GAPDH* was verified to be stable between the analyzed groups.

MicroRNA expression profiling

Total RNA was extracted as previously described.[15] miRNA expression profiling was performed on RNA from DLBCL cell lines treated with DMSO or OTX015 using the Agilent Human microRNA microarray v. 3 or Nanostring nCounter Human V3A miRNA Expression Assay Kits. Profiling was done on RNA extracted from untreated lymphoma cell lines[27,28] using the Nanostring nCounter Human V2. All samples were processed as previously described.[29,30] Profiling data are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (*http://www.ncbi.nlm.nih.gov/geo*) database under the GEO project number GSE99208.

MicroRNA quantification with TaqMan microRNA assays

Profiling results for selected miRNAs were validated using the following TaqMan MicroRNA Assays (Applied Biosystems): hsa-miR-96-5p, assay ID: 000186; hsa-miR-92a-1-5p, assay ID: 002137, hsa-miR-21-3p, assay ID: 002438; hsa-miR-155-5p, assay ID: 002623; RNU6B, assay ID: 001093. Reverse transcription and quantitative PCR were performed using the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan Universal PCR Master Mix according to the manufacturer's instructions. Briefly, for each sample, 10 ng of total RNA was used for reverse transcription and 1.33 μL of the reverse transcription product was used in triplicate wells for the quantitative PCR (qPCR). All qPCR reactions were performed on an Applied Biosystems StepOnePlus System. Amplification of RNU6B served as a normalizing control for RNA quantity. Data were analyzed using the ΔΔCt method to obtain relative quantities. Expression of *RNU6B* was verified as stable between the analyzed groups.

Data mining

miRNA expression data obtained from each profiling platform were analyzed independently. For Agilent arrays, the hybridization signal values for the multiple probes were obtained using the Agilent Feature Extraction Software 10.7.3 (Agilent Technologies). For the Nanostring nCounter, raw expression data were log-transformed and normalized by the quantile method after application of manufacturer-supplied correction factors. For both platforms, differentially expressed miRNAs were defined using R/Bioconductor with the linear model for microarray data analysis (limma) with a contrast matrix for the comparisons of interest on the datasets filtered to exclude features below the detection threshold (defined for each sample by a cut-off corresponding to twice the standard deviation of negative control probes plus the means) in at least half of the samples. The transcripts bearing an absolute log-fold change greater than 0.2 and a *P*-value less than 0.05 at any experimental time point were defined as differentially expressed. Overlapping among lists was performed using the VENNY on-line tool.[31] Experimentally validated transcript targets

of the miRNAs were obtained using the MicroRNA Target Filter in Ingenuity Pathway Analysis (Qiagen). Functional annotation of the targets was performed with the Gene Set Enrichment Analysis tool for overlap analysis using the hallmarks and the c2.cp of the Molecular Signatures Database 5.2[32] and hypergeometric P-values after correction for multiple hypothesis testing according to Benjamini and Hochberg.

Publicly available chromatin immunoprecipitation (ChIP) sequencing datasets obtained by ChIP followed by high-through-put DNA sequencing were downloaded and re-analyzed. They comprised datasets obtained in the ABC-DLBCL cell line HBL-1 (SRP043524)[23] and in the GCB-DLBCL cell line OCI-LY-1 (SRP022129),[22] both treated with the BET inhibitor JQ1 or DMSO alone. Sequence reads obtained from ChIP fragments were aligned to the human reference genome hg19 using Bowtie, allowing up to one mismatch per fragment length. Redundant reads were removed and only reads uniquely mapping to the reference genome were used for further analysis. The peaks that were genomic regions enriched by ChIP, relative to the background reads, were detected using HOMER (v2.6), a suite of tools for Motif Discovery and next-generation sequencing analysis, with a default option (false discovery rate = 0.001 and Poisson P-value cut-off = 1e-04). Differential peaks were defined as having at least a four-fold difference in enrichment within a 200 bp region between the two conditions (DMSO versus JQ1) and a Poisson enrichment P-value less than 1e-04. All discovered putative peaks were ranked by their normalized tag counts (number of tags found at the peak, normalized to $10x10^6$ total mapped tags) and annotated with the annotatePeaks.pl subroutine. We defined miRNA promoters using FANTOM5[33] and the precursors of microRNAs downloaded from miRBase (v20)[34] to annotate the BRD4 ChIP sequencing datasets. We defined enriched regions located within 5 kb regions of predicted promoters and pre-miRNAs as candidate BRD4 binding sites. For global ChIP sequencing visualization, we used ngs.plot (https://code.google.com/p/ngsplot/) for inspection of both average and 'laid out' coverages as curves or heatmaps.

Chromatin immunoprecipitation

Cells (SU-DHL-2 and HBL-1) were cross-linked with 1% formaldehyde. Crosslinking was quenched with 125 mM glycine. Cells were washed with ice-cold phosphate-buffered saline containing 1 x HALT protease inhibitor (Thermo Scientific, Lausanne, Switzerland) and resuspended in sodium dodecylsulfate lysis buffer (ChIP Assay Kit, Millipore, Schaffhausen, Switzerland) before sonication using the Bioruptor Plus. For each immunoprecipitation reaction, chromatin from $1x10^6$ cells was incubated overnight with anti-PRMT5 (A1520; NeoBiolab), anti-BRD4 (A301-985A; Bethyl) or 3 μg of the negative control antibody, anti-IgG (Millipore). Immune complexes were collected by incubation with 20 μL magnetic protein G beads at 4°C for 1.5 h. Protein G-bound complexes were sequentially washed with Low Salt Wash Buffer, High Salt Wash Buffer, LiCl Wash Buffer and twice with TE Buffer (ChIP Assay Kit, Millipore). Protein/DNA complexes were eluted using 1% sodium dodecylsulfate and 0.1 M NaHCO₃. Following reversal of crosslinks (65°C overnight), samples were treated with RNAse A and then Proteinase K. DNA samples were purified using the QIAquick PCR purification kit (Qiagen, Hombrechtikon, Switzerland). Chromatin samples to which no antibody had been added were processed in parallel as input references. For qPCR analysis of ChIP samples, triplicate wells containing 1 μL of purified ChIP DNA plus PCR master mix were prepared. Reactions were performed on a StepOnePlus Real-Time PCR system (Applied Biosystems). Standard curves were constructed using sonicated and purified chromatin. ChIP-qPCR was performed using primers specific for the upstream regulatory regions of PRMT5 and miR-96-5p. Primer sequences were as follows: PRMT5 forward; 5'-AGCGCGAGGAGAAAGATG-3', PRMT5 reverse; 5'-CTATTTCGGGGACGCAATTC-3', miR-96 forward; 5'-AGCTGGGAGACCTTGCTTC-3', miR-96 reverse; 5'-TCACCCCTCCTAACCCAAAT-3'.

Results

BET inhibition modulates the expression of a subset of microRNAs

We have previously shown that the BET inhibitor OTX015 modulates the expression of multiple coding transcripts in DLBCL cells.[15] Here we assessed the effect of OTX015 on global miRNA expression. GCB-DLBCL OCI-LY-1 and ABC-DLBCL HBL-1 cells were treated with 500 nM OTX015 for 4 and 24 h. Total RNA isolated from vehicle- and OTX015-treated cells was interrogated with the Nanostring nCounter. Fourteen miRNAs were modulated (5 downregulated, 9 upregulated) by the BET inhibitor in OCI-LY-1 cells and 11 (5 downregulated, 6 upregulated) in HBL-1 cells (Table 1).

Additionally, we used the Agilent Human miRNA microarray v.3. platform to perform miRNA profiling on two more DLBCL cell lines, DOHH-2 (GCB-DLBCL) and SU-DHL-2 (ABC-DLBCL), the same cell lines we had previously used for mRNA profiling of OTX015-treated cells.[15] In this case, seven miRNAs (3 downregulated, 4 upregulated) were affected by OTX015 in the GCB-DLBCL, and five (2 downregulated, 3 upregulated) in the ABC-DLBCL cell line (Table 1).

A few miRNAs were affected in more than one cell line, although we could not determine clear subtype-specific differences in miRNA modulation since only one GCB- and one ABC-DLBCL cell line were interrogated on each profiling platform. The oncogenic miR-92a-1-5p,[35] belonging to the miR-17-92 cluster, was downregulated in three of four cell lines (2 ABC-DLBCL, 1 GCB-DLBCL). miR-204-5p, involved in BRAF resistance in melanoma,[36] was downregulated and miR-487b-3p, expressed at lower levels in DLBCL versus follicular lymphoma,[37] was upregulated in both cell lines analyzed with the Nanostring platform. The tumor suppressor miR-96-5p[38,39] was upregulated in HBL-1 and DOHH-2 cells. Besides these, among the miRNAs modulated by the BET inhibitor there were others known to be involved in lymphomagenesis. The oncogenic miRNAs hsa-miR-21-3p[40-44] and miR-155[45,46] were downregulated, while, besides miR-96-5p, another miRNA with a tumor suppressor function, miR-16-5p,[47] was also upregulated by the BET inhibitor. qRT-PCR was used to validate the expression of two lymphoma oncomiRNAs modulated by BET inhibition: miR-155-5p and miR-92a-1-5p (Online Supplementary Figure S1). The latter also appeared significantly downregulated after in vivo treatment of SU-DHL-2 xenografts (Online Supplementary Figure S2).

microRNAs modulated by BET inhibition control important pathways in diffuse large B-cell lymphoma

Functional annotation analysis identified the p53 pathway, apoptosis, MYC-targets, cell cycle regulation, B-cell receptor signaling, interleukin-6 signaling, the STAT3 pathway, PI3K and nuclear factor-κB signaling among the biological processes significantly associated with the miRNAs that exhibited expression changes in HBL-1 and

OCI-LY-1 DLBCL cells treated with OTX015 (*Online Supplementary Table S1*). The same pathways were predicted to be affected based on the modulated miRNAs in DOHH-2 and SU-DHL-2 cells treated with OTX015 (*Online Supplementary Table S1*). These signaling pathways and processes were similar to those we previously observed when analyzing the gene expression profiles of OTX015-treated DLBCL cells.[15]

BRD4 binds to the upstream regulatory regions of multiple miRNAs

To further study the role of BET proteins in miRNA regulation, we took advantage of two publicly available ChIP-sequencing datasets obtained in the ABC-DLBCL cell line HBL-1 (SRP043524)[23] and in the GCB-DLBCL cell line OCI-LY-1 (SRP022129),[22] both treated with the BET inhibitor JQ1 or DMSO alone. Analysis of these datasets revealed that half of the regions bound by BRD4 were in intronic and intergenic regions where miRNAs are often located.[48] We detected 794 miRNAs with at least one BRD4-binding event within their regulatory regions in ABC-DLBCL HBL-1 cells and 757 in the GCB-DLBCL OCI-LY-1 cell line (*Online Supplementary Table S2*).

To determine whether BRD4 binding was associated with the expression of miRNA genes we profiled miRNA expression levels in a panel of 35 lymphoma cell lines using the Nanostring nCounter (*Online Supplementary Table S3*). Comparison of miRNA expression levels and BRD4 binding sites demonstrated that BRD4 peaks were more prevalent in the proximity of expressed miRNAs than non-expressed miRNAs ($P<0.001$) and were positively correlated with miRNA expression levels (Figure 1A). When we compared BRD4 binding in the presence or absence of the BET inhibitor JQ1, we identified 707 miRNAs with decreased BET bromodomain binding after exposure to the BET inhibitor in ABC-DLBCL HBL-1 cells and 348 in GCB-DLBCL OCI-LY-1 cells (*Online Supplementary Table S2*). Downregulation of miR-92a-1-5p and miR-155p expression following BET inhibitor-mediated reduction of BRD4 binding was also confirmed by qRT-PCR analysis (Figure 1B).

BET inhibition mediates upregulation of miR-96-5p by downregulating PRMT5 expression

The observed upregulation of the tumor suppressor miR-96-5p after exposure of DLBCL cell lines to OTX015 could not be explained by a direct BRD4-mediated effect of the BET inhibitor on the miRNA promoter. Thus, the miRNA profiling results were further validated by qRT-PCR in two GCB-DLBCL cell lines (DOHH-2, OCI-LY-1) and two ABC-DLBCL cell lines (SU-DHL-2, HBL-1) treated with OTX015 for 4, 24 and 48 h. For DOHH-2, SU-DHL-2 and HBL-1 cells, there was a time-dependent upregulation of miR-96-5p. For OCI-LY-1 cells, miR-96-5p was similarly upregulated at all three time points (Figure 2A).

In lymphomas, miR-96-5p expression is regulated as part of a negative feedback loop with the protein arginine methyltransferase, PRMT5.[39] Overexpression of PRMT5 mediates transcriptional repression of this miRNA via symmetric dimethylation of histones H3 and H4 in the promoter of miR-96-5p. Conversely, binding of miR-96-5p to the 3' untranslated region of *PRMT5* inhibits its translation.[39,49] We hypothesized that the upregulation of miR-96-5p observed in OTX015-treated DLBCL cells could be due

Table 1. miRNAs modulated by the BET inhibitor OTX015 in four DLBCL cell lines.

| microRNA_ID | OCI-LY-1 (GCB-DLBCL) | | | |
| | 4 hours | | 24 hours | |
	log$_2$FC	*P* value	log$_2$FC	*P* value
hsa-miR-639	-1.38	0.005	-0.29	n.s.
hsa-miR-204-5p ^	-0.46	n.s.	-1.61	0.048
hsa-miR-6511a-5p	-0.43	n.s.	-1.38	0.034
hsa-miR-106a-5p+hsa-miR-17-5p	-0.33	n.s.	-0.95	0.035
hsa-miR-3613-5p	0.30	n.s.	-3.30	<0.001
hsa-miR-1254	0.96	0.031	0.73	n.s.
hsa-miR-760	1.07	0.012	0.84	n.s.
hsa-miR-221-5p	1.46	0.004	-0.07	n.s.
hsa-miR-498	1.84	0.013	-0.48	n.s.
hsa-miR-487b-3p ^	-0.02	n.s.	1.58	0.016
hsa-miR-16-5p	0.07	n.s.	1.22	0.012
hsa-miR-182-5p	0.24	n.s.	0.95	0.025
hsa-miR-3136-5p	0.43	n.s.	1.51	0.013
hsa-miR-3605-3p	0.48	n.s.	1.47	0.014

| microRNA_ID | HBL-1 (ABC-DLBCL) | | | |
| | 4 hours | | 24 hours | |
	log$_2$FC	*P* value	log$_2$FC	*P* value
hsa-miR-204-5p ^	-1.59	0.011	-0.58	n.s.
hsa-miR-133a-5p	-1.13	0.020	0.26	n.s.
hsa-miR-155-5p	-0.67	n.s.	-1.11	0.017
hsa-miR-580-3p	-0.22	n.s.	-1.11	0.017
hsa-miR-92a-1-5p ^	-0.45	n.s.	-1.93	0.009
hsa-miR-487b-3p ^	1.59	0.007	-0.32	n.s.
hsa-miR-652-5p	2.08	<0.001	0.56	n.s.
hsa-miR-191-5p	0.16	n.s.	1.17	0.007
hsa-miR-96-5p ^	0.41	n.s.	0.99	0.023
hsa-miR-433-3p	0.49	n.s.	1.75	0.014
hsa-miR-582-3p	0.72	0.059	1.88	0.001

| microRNA_ID | DOHH2 (GCB-DLBCL) | | | |
| | 4 hours | | 8 hours | |
	log$_2$FC	*P* value	log$_2$FC	*P* value
hsa-miR-196a-3p	-0.44	0.039	0.29	n.s.
hsa-miR-21-3p	-0.37	0.045	-0.48	n.s.
hsa-miR-92a-1-5p ^	-0.32	n.s.	-0.52	0.0177
hsa-miR-630	0.63	0.036	-0.33	n.s.
hsa-miR-935	0.36	0.049	1.45	n.s.
hsa-miR-1181	0.07	n.s.	0.61	0.0086
hsa-miR-96-5p ^	0.01	n.s.	0.39	0.0415

| microRNA_ID | SU-DHL-2 (ABC-DLBCL) | | | |
| | 4 hours | | 8 hours | |
	log$_2$FC	*P* value	log$_2$FC	*P* value
hsa-miR-92a-1-5p ^	-0.78	0.004	-2.01	0.004
hsa-miR-29b-1-5p	-0.25	n.s.	-0.46	0.043
hsa-miR-765	0.39	0.102	0.48	0.011
hsa-miR-1246	0.23	n.s.	0.64	0.025
hsa-miR-345-5p	0.13	n.s.	0.58	0.015

^:modulated in more than one cell line; n.s., not significant. GCB: gerinal center B-cell; ABC: activated B-cell; DLBCL: diffuse B-cell lymphoma; FC: fold change.

to a perturbation of its downregulation by PRMT5. To assess this, we performed qRT-PCR analysis of *PRMT5* in DLBCL cells treated with OTX015 for 4, 24 and 48 h. *PRMT5* mRNA was markedly downregulated at 4 and 24 h. At 48 h *PRMT5* levels were similar in DMSO- and OTX015-treated cells for all four cell lines (Figure 2B). At the protein level (Figure 2C), moderate downregulation of PRMT5 was evident at 24 h in DOHH-2 cells treated with OTX015. In SU-DHL-2 cells, PRMT5 was moderately downregulated at 24 h and was negligible at 48 h. These results indicate that OTX015 could downregulate the levels of expression of both PRMT5 RNA and protein in DLBCL cells. Hence upregulation of miR-96-5p following OTX015 treatment was associated with downregulation of PRMT5 protein, particularly in SU-DHL-2 cells.

BRD4 binds to the 5' regulatory region of *PRMT5* in diffuse large B-cell lymphoma cells and treatment with a BET inhibitor reduces BRD4 binding

As OTX015 mediates transcriptional repression by displacing BRD4 from chromatin,[15,21] we hypothesized that *PRMT5* was transcriptionally regulated by BRD4. To assess this, we re-analyzed the two public ChIP sequencing datasets of DLBCL cells treated with the BET inhibitor JQ1,[22,23] which has a similar mechanism of action to OTX015 and exhibits an overlapping profile of targeted genes.[15,22] This revealed that BRD4 bound to the 5' region of PRMT5 and that this binding was reduced following treatment with the BET inhibitor (Figure 3A). In agreement with the public ChIP sequencing data of JQ1-treated DLBCL cells, when we performed ChIP-
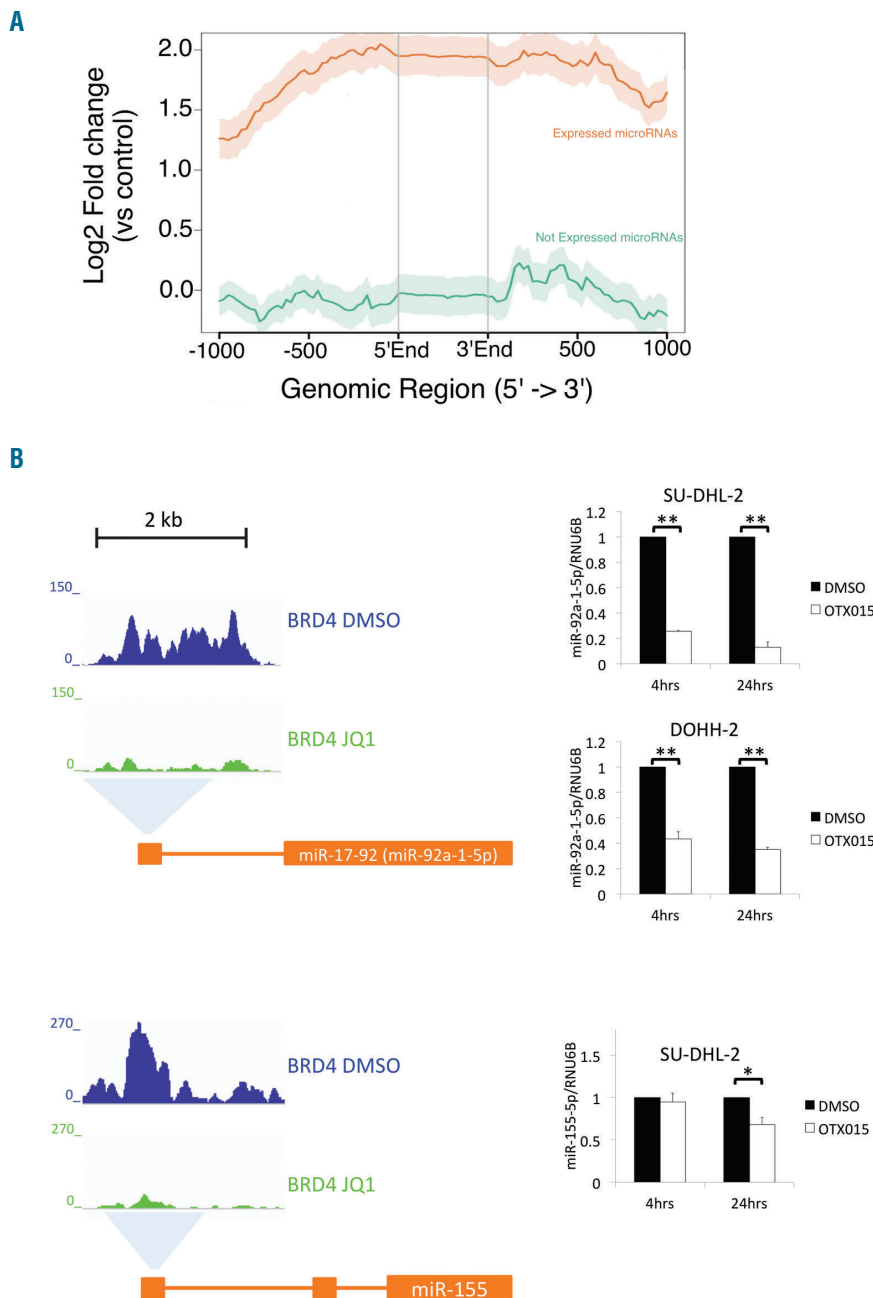

Figure 1. BRD4 binds to the regulatory regions of microRNAs. (A) The genomic regions within ± 1 kb of miRNA promoters that are bound by BRD4 obtained using ngs.plot. Lines represent the average expression profiles of "expressed" (red line) and "not expressed" (green line) miRNA. (B) Analysis of publicly available chromatin immunoprecipitation sequencing data of diffuse large B-cell lymphoma (DLBCL) cells showed that BET inhibitor treatment reduces BRD4 binding at the 5' regulatory regions of the miR-17-92 cluster, which contains miR-92a-1-5p, and the miR-155 host gene (left panel). An activated B-cell (ABC)-DLBCL cell line (SU-DHL-2) and a germinal center B-cell (GCB)-DLBCL cell line (DOHH-2) were treated with OTX015 for 4 and 24 h before TaqMan quantitative reverse transcription polymerase chain reaction analysis of miR-92a-1-5p and miR-155-5p expression (right panel). miR-155-5p expression is only shown for SU-DHL-2 as it is an ABC-DLBCL specific oncomiR. Expression of RNU6B was used for normalization. For each timepoint, the mean fold-change relative to the dimethyl sulfoxide (DMSO) control is shown. Charts show the mean of at least three independent experiments. *$P<0.05$; **$P<0.01$. Error bars denote the standard error.

qPCR analysis of SU-DHL-2 cells treated with OTX015, we observed decreased binding of BRD4 to the 5' region of *PRMT5* (Figure 3B). The decrease in *PRMT5* expression following OTX015 treatment was, therefore, likely due to a reduced association of BRD4 to the 5' regulatory region of *PRMT5*. Additionally, DNAse hypersensitivity sites and H3K27 acetylation, both marks of active transcription, were enriched at the BRD4 binding site in *PRMT5* (*data not shown*). As *PRMT5* and miR-96-5p regulate each other in a negative feedback loop, we tested for binding of PRMT5 to the promoter of miR-96-5p. OTX015 treatment led to reduced recruitment of PRMT5 to the miR-96-5p promoter (Figure 3C) indicating that upregulation of miR-96-5p in BET inhibitor-treated cells was mediated through downregulation of PRMT5 (Figure 3D).
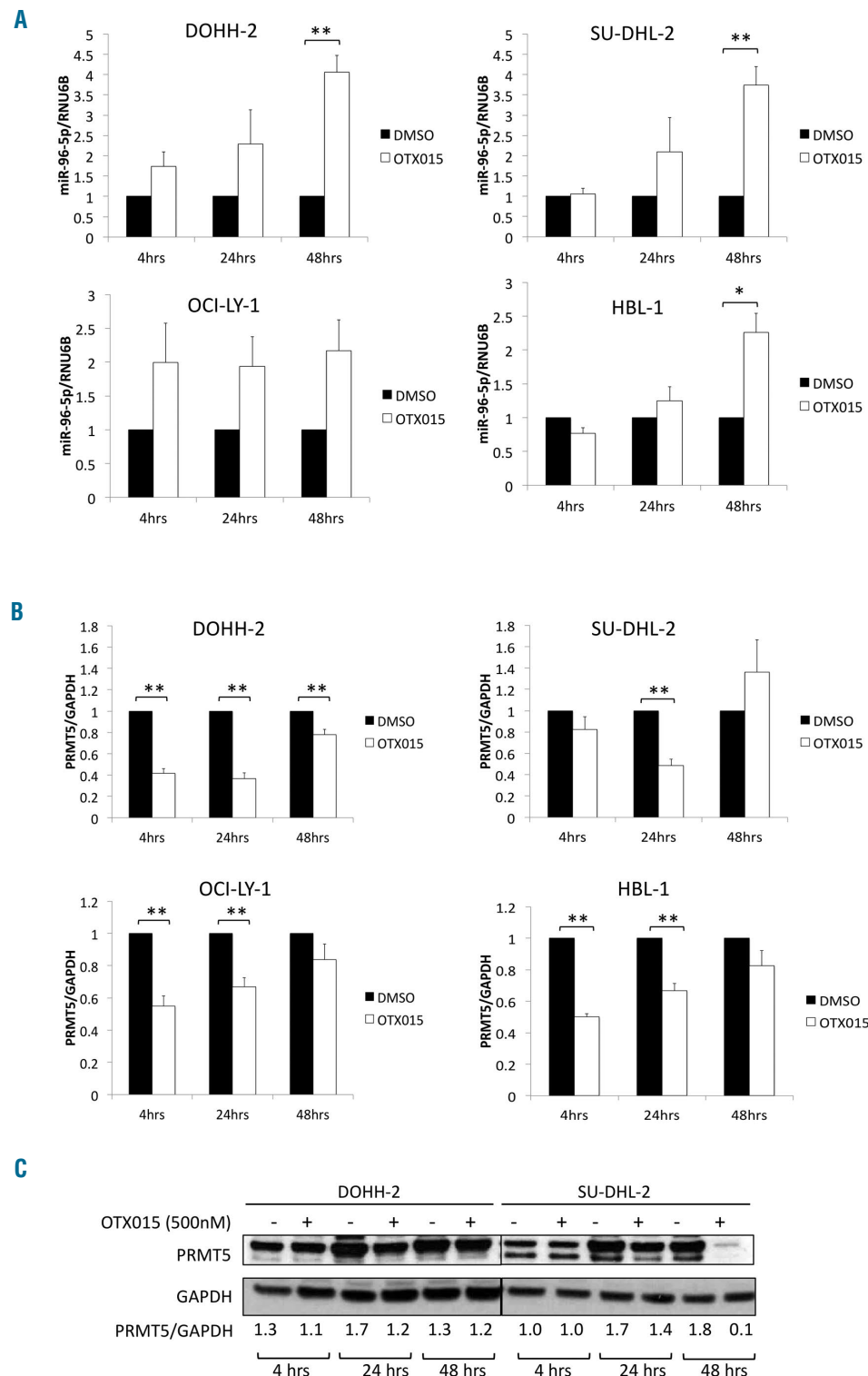
Figure 2. OTX015 modulates microRNA-96-5p expression in diffuse large B-cell lymphoma models. (A) OTX015 upregulates miR-96-5p in a time-dependent manner. Two germinal center B-cell (GCB)-diffuse large B-cell lymphoma (DLBCL) cell lines (DOHH-2, OCI-LY-1) and two activated B-cell (ABC)-DLBCL cell lines (SU-DHL-2, HBL-1) were treated with dimethyl sulfoxide (DMSO) or 500 nM OTX015 for 4, 24, and 48 h. Expression of miR-96-5p was determined by TaqMan quantitative reverse transcription polymerase chain reaction (qRT-PCR). Expression of *RNU6B* was used for normalization. For each time-point, the mean fold-change relative to the DMSO control is shown. (B) OTX015 treatment of DLBCL cells downregulates PRMT5. Two GCB-DLBCL (DOHH-2, OCI-LY-1) and two ABC-DLBCL (SU-DHL-2, HBL-1) cell lines were treated with DMSO or 500 nM OTX015 for 4, 24, and 48 h. Expression of *PRMT5* was determined by qRT-PCR. *GAPDH* expression was used for normalization. For each time-point, the mean fold-change relative to the DMSO control is shown. (C) OTX015 reduces *PRMT5* protein levels in DOHH-2 and SU-DHL-2 cells treated with DMSO or 500 nM OTX015. GAPDH was used as a loading control. PRMT5 signals were quantified using ImageJ (http://rsbweb.nih.gov/ij/) and normalized to GAPDH signals. Representative images of two independent Western blot analyses are shown. The graphs show the mean of three independent experiments. **$P<0.01$. Error bars denote the standard error.
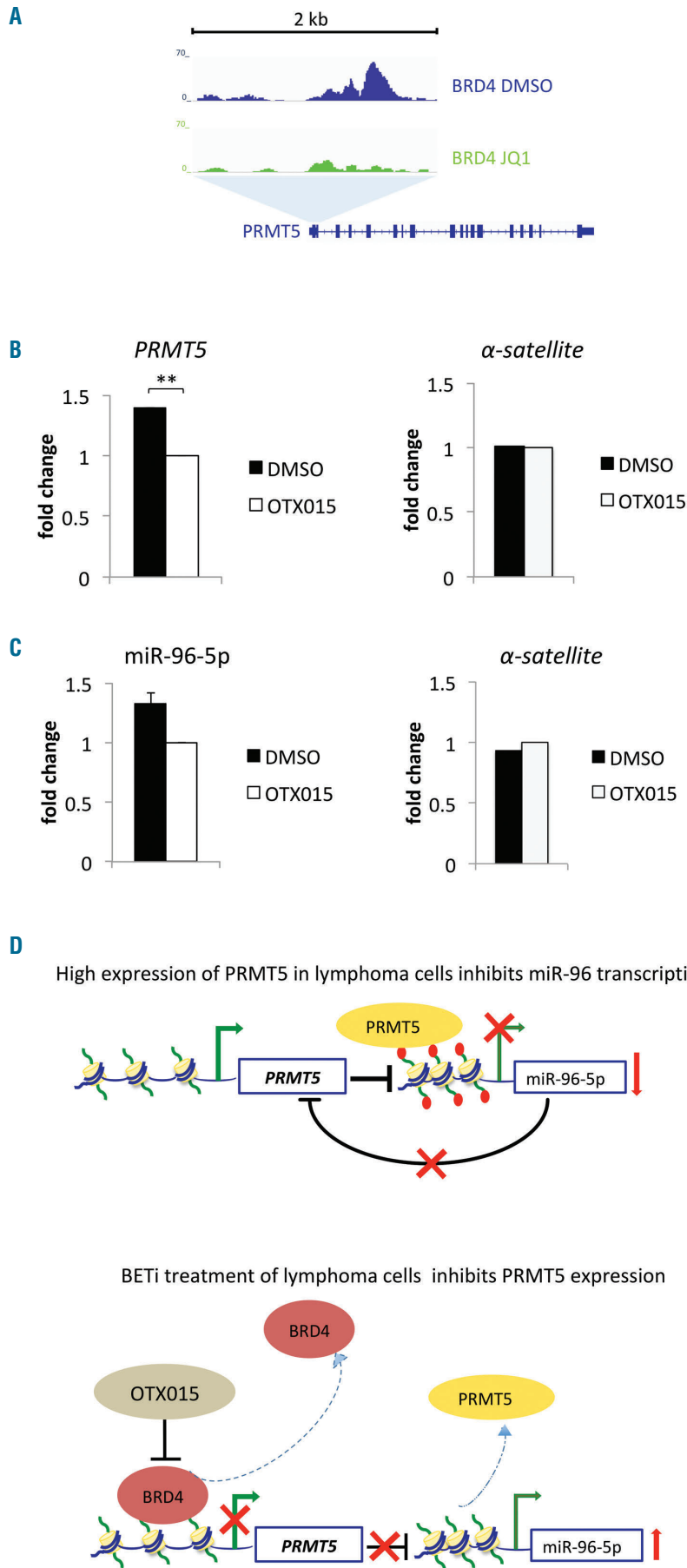
Figure 3. OTX015 reduces binding of BRD4 to *PRMT5* and diminishes recruitment of PRMT5 to the microRNA-96-5p promoter. (A) Analysis of publicly available chromatin immunoprecipitation (ChIP) sequencing data of diffuse large B-cell lymphoma (DLBCL) cells treated with the BET inhibitor JQ1 showed that BRD4 binds to the 5' regulatory region of *PRMT5* and that BET inhibitor treatment reduces BRD4 recruitment to *PRMT5*. (B,C) ChIP was performed for DLBCL cells treated with dimethyl sulfoxide (DMSO) or 500 nM OTX015 for 48 h. Anti-BRD4, anti-PRMT5 and anti-IgG (negative control) antibodies were used for immunoprecipitations. (B) Chromatin pulled down with anti-BRD4 and anti-IgG in DMSO- and OTX015-treated SU-DHL-2 cells was amplified with primers specific for the 5' regulatory region of *PRMT5* identified by the analysis in (A). (C) Chromatin pulled down with anti-PRMT5 and anti-IgG antibodies in DMSO- and OTX015-treated HBL-1 cells was amplified with primers specific for the promoter of miR-96-5p. Amplification of the same immunoprecipitated chromatin samples was performed with primers specific for the chromosome 4 human alpha satellite sequence as an additional negative control (representative results from one of two biological replicates are shown). The graphs show the mean fold-difference between DMSO- and OTX015-treated cells after normalization to input and IgG background subtraction. ChIP-quantitative polymerase chain reaction experiments were repeated twice in triplicate. (D) Proposed model for the upregulation of miR-96-5p expression following treatment of DLBCL cells with OTX015. Upper panel; previous work by others has shown that *PRMT5* is overexpressed in lymphoma cells in which it mediates transcriptional repression of miR-96 and that overexpression of miR-96 negatively regulates *PRMT5* translation.[39,49] Lower panel; here we showed that BRD4 binds to the upstream regulatory region of *PRMT5*. Treatment of DLBCL cells with a BET inhibitor reduced BRD4 occupancy at the *PRMT5* locus and also reduced the expression of PRMT5 mRNA and protein. Additionally, the BET inhibitor diminished the occupancy of PRMT5 at the miR-96 promoter and increased miR-96 expression. **$P<0.01$. Error bars denote the standard error.

Discussion

The present study shows that the BET inhibitor OTX015 modulates the expression of miRNAs in DLBCL cells. The regulation may occur directly, due to the binding of BRD4 to the regulatory regions of specific miRNAs, or indirectly as demonstrated for miR-96-5p, a miRNA with important functions in the proliferation and survival of B-cell malignancies.[49] The ability of OTX015 to alter miRNA expression demonstrates that the effects of BET inhibition on the transcriptome extend beyond coding genes to comprise also non-coding regions of the genome. miRNAs function by regulating the expression of genes at the transcript level, where they can mediate mRNA degradation, reduce mRNA stability or prevent translation of mRNA into protein.

By analyzing publicly available ChIP sequencing data[22,23] in combination with our miRNA profiling data of baseline and BET inhibitor-treated lymphoma cells, we determined that a subset of miRNAs were bound by BRD4 and that this binding decreased after BET inhibitor treatment. For a number of these miRNAs reduced binding of BRD4 after BET inhibition was associated with reduced expression. Our finding that BRD4 directly binds to the regulatory regions of miRNA genes to regulate their expression in lymphomas complements the recent report describing that components of the miRNA processing machinery, namely DGCR8 and Drosha, are localized to super-enhancers of miRNAs in a tissue-specific manner and that association of these proteins with super-enhancers is reduced following treatment with the BET inhibitor JQ1.[50] Indeed, these observations indicate that the targeted effects of BET inhibition on specific genes in different cellular contexts also likely comprises non-coding transcripts that are specifically modulated in different transformed cell types. With respect to this, we observed that the promoters of two established lymphoma oncomiRNAs, miR-155-5p and miR-92a-1-5p, were bound by BRD4. When lymphoma cells were treated with a BET inhibitor, BRD4 was diminished at these sites and this was associated with downregulation of miRNA expression. miR-155-5p is upregulated in activated B cells and ABC-DLBCL,[45,46,51] often because of amplification of its locus.[45] Its high expression is associated with poor outcome and resistance to R-CHOP therapy and its knockdown compromises the viability of ABC-DLBCL cells.[43,45] Of interest, BET inhibition decreased miR-155-5p expression only in the two ABC-DLBCL cell lines, while the GCB-DLBCL cell line showed upregulation of miR-155-5p suggesting that different mechanisms may regulate the transcription of this miRNA gene in different DLBCL subtypes.

miR-92a-1-5p is one of the six members of the miR-17-92 cluster located on chromosome 13q31.3 and is overexpressed in different lymphoma subtypes including DLBCL.[35] It is a transcriptional target of the MYC oncoprotein,[35] which is itself rapidly and robustly downregulated by treatment with a BET inhibitor.[15] Downregulation of miR-92a-1-5p was already very pronounced after 4 h of BET inhibitor treatment. Additionally, miR-92a-1-5p was the only miRNA commonly identified by the two different platforms that we utilized for miRNA profiling. The miR-17-92 cluster is involved in activation of the PI3K/AKT/mTOR pathway, lymphoma pathogenesis and chemoresistance.[52,53] The miR-17-92 cluster can acquire super-enhancers during neoplastic transformation[50,54] and BRD4 exhibits a preference for binding at super-enhancers.[22] This provides further support for our observation of direct regulation of the miR-17-92 cluster by BRD4.

miR-21-3p was also downregulated by BET inhibitor treatment. This miRNA is overexpressed in B-cell lymphomas.[40-42] It inhibits translation of the tumor suppressor *PTEN*[43] and knockdown of miR-21 increases the sensitivity of lymphoma cells to CHOP treatment.[44]

When we performed functional annotation analysis of the OTX015-modulated miRNAs, we identified the same signaling pathways and processes that we had previously identified from gene expression profiles of OTX015-treated DLBCL cells,[15] indicating that changes in miRNA expression likely contribute to modulating some of the transcripts and pathways that have been previously identified in cells treated with a BET inhibitor.[15,22,23]

In lymphoma cells, which overexpress PRMT5, the negative feedback loop comprising miR-96-5p and PRMT5 is usually poised in favor of PRMT5.[39] PRMT5 catalyzes the symmetric methylation of arginine residues on histones H3 and H4, giving rise to the repressive epigenetic marks H3R8me2S and H4R3me2S. Inhibition of PRMT5 expression, either pharmacologically or by the use of antisense oligonucleotides, severely compromises lymphoma cell viability and induces apoptosis.[39,49] In the DLBCL cells we treated with OTX015, PRMT5 protein was negligible after 48 h of treatment in the ABC-DLBCL cell line SU-DHL-2, which we previously showed undergoes pronounced apoptosis in response to OTX015.[15] DOHH-2 cells, in which we did not observe apoptosis following OTX015 treatment,[15] exhibited moderate PRMT5 downregulation. The less marked downregulation of PRMT5 protein in DOHH-2 cells treated with OTX015 was in contrast to its pronounced downregulation at the transcript level. It was nevertheless associated with a pronounced increase in miR-96-5p levels indicating that, for this cell line, factors other than PRMT5 downregulation may contribute to releasing miR-96-5p from transcriptional repression. Inhibition of PRMT5 has been shown to release miR-96-5p from transcriptional repression in lymphoma cells.[39] We observed that BET inhibitor-mediated inhibition of PRMT5 was associated with decreased binding of BRD4 to the 5' regulatory region of *PRMT5* and that this resulted in reduced occupancy of PRMT5 at the miR-96-5p promoter. The disruption of the negative feedback loop comprising PRMT5 and miR-96-5p by OTX015 shows how the anti-tumor effects of BET inhibition are further amplified by modulation of secondary targets, which might themselves also contribute to further suppress direct targets of BET inhibitors. With respect to this, overexpression of miR-96-5p downregulates phosphorylated STAT3 (p-STAT3) without affecting levels of total STAT3 in T-cell anaplastic large-cell lymphoma cells.[38] We have previously shown that OTX015 treatment decreases p-STAT3 in ABC-DLBCL cells.[15] It is therefore possible that in addition to the direct effects of OTX015 on the expression of genes involved in JAK/STAT signaling,[15] the overexpression of miR-96-5p contributes to maintaining p-STAT3 repressed.

Our study provides the first evidence of BET inhibitor-mediated modulation of miRNAs in lymphomas. This modulation can occur by inhibiting the interaction of

BRD4 with genes whose products regulate miRNA expression, or through the direct inhibition of BRD4 at miRNA regulatory regions, or, as recently suggested, by interfering with the processing of pri-miRNA to pre-miRNAs.[50] Unlike coding transcripts, miRNAs are highly stable in blood and as such, levels of circulating miRNAs have been used for diagnosis and screening in a number of diseases. In lymphomas, the overexpression of specific miRNAs in plasma and serum samples has been shown to be an accurate biomarker for diagnosis, prognosis and response to therapy.[45]

The circulating miRNAs that have been identified as biomarkers in lymphoma are among those that we have identified as regulated by BET inhibition (miR-92, miR-21, miR-155). The assessment of circulating miRNAs could, therefore, be used as a robust and non-invasive way to monitor response to BET inhibitor treatment.

In conclusion, our observations contribute to a better understanding of the targeted effects of BET inhibitors, revealing a novel aspect of the activity of this class of compounds in lymphomas.

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