

Original Article

The Epstein-Barr virus microRNA BART11-5p targets the early B-cell transcription factor EBF1

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Abstract: Epstein-Barr virus (EBV) is a ubiquitous B-cell trophic herpesvirus associated with a variety of histologically diverse B-cell lymphomas, each associated with specific viral-latency gene expression programs. Initial infection drives resting B-cells to differentiate via an atypical germinal centre reaction into memory B-cells, where the virus resides in a latent state. The mechanisms that underpin this process have yet to be fully elucidated. EBV expresses more than 40 microRNAs (miRNAs). The alternatively spliced BamHI A rightward transcripts (BARTs) are the template for two large miRNA clusters (BARTs A and B), that comprise the majority of all known EBV-miRNAs. Although BART-miRNAs are abundantly expressed in all latency programs, few BART-miRNA targets have been identified and their function is poorly understood. The early B-cell factor 1 (EBF1) was identified using bioinformatic analysis as a novel target of EBV-miRNA BART11-5p, encoded by BART cluster B. EBF1 is an important B-cell transcription factor that regulates many B-cell specific genes including Pax5, BCR and CD40 and is critical for germinal centre formation. Using luciferase reporter assays and a series of BART-constructs, we confirmed silencing via the EBF1 3' untranslated region (UTR) and identified the target site as 2137-2159 bp after the stop codon. Results were confirmed following transfection of a BART11-5p mimic, which was able to silence via the predicted target site. Our findings highlight a potential role of BART-miRNAs in the regulation of B-cell differentiation.

Keywords: EBV, microRNA, BamHI A rightward transcripts, early B-cell transcription factor, B-cell

Introduction

The B-cell lymphotropic gamma-herpesvirus Epstein-Barr virus (EBV) infects over 90% of the population [1]. Following primary infection, the virus establishes latency within host B-cells [2]. Through a series of viral gene expression programs, known as latency programs, the virus evades immune surveillance and drives infected cells through a B-cell terminal differentiation process, the mechanisms of which have yet to be fully elucidated [3]. This gives the virus access to the long lived memory B-cell compartment allowing persistence for the life of the host. Although infection is typically benign, EBV is strongly implicated in a range of histologically diverse B-cell lymphomas including Burkitt lymphoma (BL), Hodgkin lymphoma (HL), diffuse

large B-cell lymphoma (DLBCL) and post-transplant lymphoproliferative disease (PTLD) [4-8], the morphologies of which are identical to their EBV-negative malignant counterparts. Each is associated with distinct latency expression profiles [3, 9].

miRNAs are small RNA molecules (usually 20-22 nucleotides in length) that attenuate gene expression at a post-transcriptional level [10, 11]. They are important developmental regulators and have been associated in the development of many cancers. A number of viruses have now been identified that encode miRNAs, the first of which was EBV [12]. Currently, more than 40 miRNAs have been identified within the EBV genome [13-15]. EBV encodes these miRNAs within two genomic

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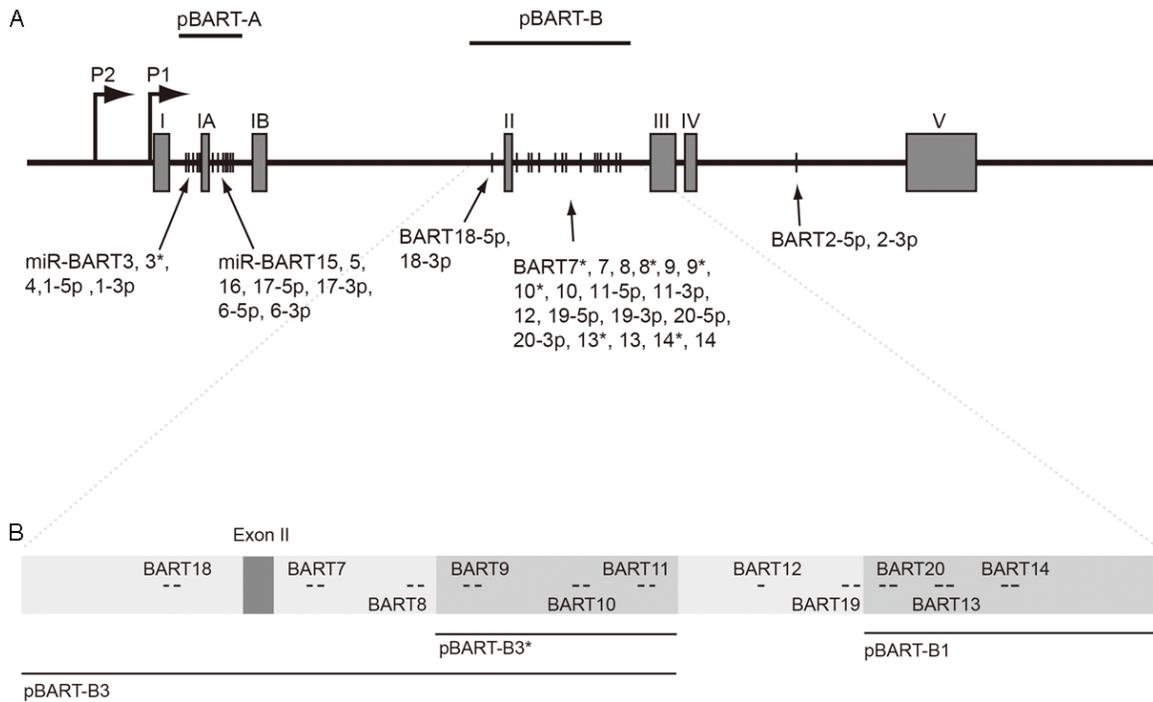


Figure 1. A: Location of the BART miRNAs within the EBV genome. Coding regions are indicated by shaded boxes with names above. Positions of miRNAs are indicated by vertical bars. Arrows represent promoters P1 and P2. Regions used for EBV-miRNA expression constructs are indicated by horizontal bars above. B: Location the restricted EBV-miRNA expression constructs. The entire region shown is expressed by the EBV-miRNA expression construct pBART-B and identifies the miRNAs expressed by each EBV-miRNA expression construct.

regions, termed BamHI-M rightward open reading frame 1 (BHRF1) and BamHI A rightward transcripts (BART). The miRNAs are found in the intronic regions with three miRNAs identified within the BHRF1 region and two large clusters found in the BART region (BART-A and BART-B, as outlined in **Figure 1A**), expressing many alternatively spliced transcripts and of unknown coding content.

EBV is also known to deregulate several host miRNAs, which may contribute to its oncogenic potential [16-20]. Furthermore, the expression of EBV-miRNAs has been demonstrated in several lymphoma types although their contribution to lymphomagenesis is not known [21, 22]. The expression of BHRF1 miRNAs is restricted to latency III expressing lymphomas such as PTLN whereas the expression of BART miRNAs has been detected in all tumor and latency types [12, 13, 21]. The expression of BART miRNAs can vary widely between tumor types [23]. Further, the expression of several EBV-miRNAs has been demonstrated to be deregulated within lymphoma and that tumor type can be distinguished by profiling of EBV-miRNAs [24]. This

suggests that EBV-miRNAs are indeed involved in the pathogenesis of lymphoma and may prove to be useful biomarkers for disease status or therapeutic targets.

Although studies have determined EBV-miRNAs play a role primarily in protecting infected cells from apoptosis and immune evasion [25], relatively few targets of EBV-miRNAs within the human genome have been identified and fully verified [22, 26-29]. As such their role in EBV pathogenesis and B-cell lymphomagenesis is not well understood. Using a combination of bioinformaticss analysis, luciferase reporter assays and a series of BART-constructs, we identify early B-cell factor 1 (EBF1), an important B-cell transcription factor that is critical for germinal centre formation, as a novel target of EBV-miRNA BART11-5p.

Methods

Bioinformaticss

An *in silico* analysis was performed to predict potential targets of EBV-miRNAs within the

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Table 1. Primer sequences

Primer	Primer Sequence (5'-3')
A. EBV miRNA expression constructs	
BART-A-Eco-F1	ATATGAATTCACTGATAAGGACCGCGGAGT
BART-A-Eco-R1	TTAAGAATTCCTCCAGAGGGCAGACGTTAG
BART-B-Eco-F1	TAATGAATTCCTCCAAATGTCACCACAGAA
BART-B-Eco-R1	ATATGAATTCCTCAACAACCCATAACGAT
B. Luciferase-3'UTR Expression Constructs	
LMP1-Xba1-F1	ATAATCTAGACCTTTCTTTACTTCTAGGCATTACCA
LMP1-Xba1-R1	AATTTCTAGACTGTACTTTTATTATTGCATCACAAAG
LY75XbaF1	TAATTTCTAGACCCTGCTAAATGCCATGTTT
LY75XbaR1	ATATTTCTAGACAGCTGTGCTTTTTCATCAAA
CTDSP2XbaF1	TAATTTCTAGACAGTAGGGGACTTTCCACACA
CTDSP2XbaR1	ATATTTCTAGACAGTCCAGTGGGAAGCAGAA
MLLXbaF1	TAATTTCTAGATGTTTGTGCCCTGTTGACAT
MLLXbaR1	ATATTTCTAGATGACCCATCACAAATTCCTCA
BCL2L11XbaF1	TAATTTCTAGAAAACCAACAAGACCCAGCAC
BCL2L11XbaR1	ATATTTCTAGAGTAAGCCAGGGAAACTGCAA
HIF1ANheF1	TAATGGCTAGCGGCACTCCACTCCTATTTGG
HIF1ANheR1	ATATGGCTAGCCAGATTTTGAAGGGTGGAA
BCL11ASpeF1	TAATACTAGTGGTTTGACAGAGGGGAACAG
BCL11ASpeR1	ATATACTAGTAAAGGGACAAAATGGGTGTATG
EBFSpeF1	TAATACTAGTAACAAAATCACCGACCTTGC
EBFSpeR1	ATATACTAGTAGGGCAATGTTATGCAATCC
CD59NheF1	TAATGGCTAGCCTGCGTAGTCCGCTTTCTCT
CD59NheR1	ATATGGCTAGCAGTTAGCAGGAGGCTGGATG
CD59NheF2	TAATGGCTAGCGCACAAACAACCTCCTGTCA
CD59NheR2	ATATGGCTAGCTTTTTCCCCCTTACTCCAAGA
HLADOASpeF1	TAATACTAGTAAATGACTTGTGGGAGACACC
HLADOASpeR1	ATATACTAGTGAAACACCTTTCCAATGATGC
CD207XbaF1	TAATTTCTAGATGCTTCAAAATGTCCACACA
CD207XbaR1	ATATTTCTAGATGACCCAGGCAGCATTTTAT
C. Real time primers	
RTQ-primer	CGAATTCTAGAGCTCGAGGCGAGCGACATGGCTGGCTAGTTAAGCTTG- GTACCGAGCTCGGATCCACTAGTCTTTTTTTTTTTTTTTTTTTTTTTTTTTT
miR-RTQ-Probe	AGTGGATCCGAGCTCGGTACCAAGC
RTQ-uniR2	CGAATTCTAGAGCTCGAGGCA
U6	CAAATTCGTGAAGCGTCCATA
miR-BART1-5p	TCTTAGTGGAAAGTGACGTGCTGT
miR-BART1-3p	TAGCACCGCTATCCACTATGTCT
miR-BART4	GACCTGATGCTGCTGGTGTGCT
miR-BART5	CAAGGTGAATATAGCTGCCATCG
miR-BART6-5p	GTTGGTCCAATCCATAGGCTTAAAA
miR-BART6-3p	CGGGGATCGGACTAGCCTTAGA
miR-BART8*	GTCACAATCTATGGGGTCGTAGAAAAA
miR-BART11-5p	GACAGTTTGGTGCCTAGTTGT
miR-BART11-3p	ACGCACACCAGGCTGACTGCC
miR-BART14	TAAATGCTGCAGTAGTAGGGATAAAAAA
miR-BART15	AGTGGTTTTGTTCTTGTATGAAAAAA
miR-BART20-3p	CATGAAGGCACAGCCTGTTACC

human genome. A library of human 3' untranslated region (UTR) sequences was compiled from the NCBI Reference Sequence (RefSeq) database using the UCSC Table Browser (<http://genome.ucsc.edu/cgi-bin/hgTables?org=human>) and scanned using the miRanda v1.0b software package, using a minimum score threshold of 130 and a minimum energy of -16 kcal/mol. The level of false positive results was estimated by first analysing the GC content distribution of the 3'UTR library using the CodonW package (<http://codonw.sourceforge.net/>) and then generating 1000 1 kb random sequences using the Fabox online toolbox (http://www.birc.au.dk/~biopv/php/fabox/random_sequence_generator.php) exhibiting the same GC distribution. The random library was scanned with miRanda using the same thresholds and cut off values determined based on false-positives observed in the random data set. The targets exceeding these cut off values were then scanned using a second miRNA prediction algorithm, TargetScan 4.1 and targets predicted by both algorithms were ranked.

Preparation of EBV-miRNA expression and luciferase reporter constructs

EBV-miRNA expression constructs were devel-

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Table 2. EBV miRNA targets

A. Potential targets of EBV-encoded miRNAs						
Accession #	Target Gene	miRanda score	miRanda energy	RNA hybrid	EBV miRNA	EBV-miR expressor
NM_002349	LY75	146	-29.34	-32.0	BART1-5p	BART-A
NM_024007	EBF1	145	-30.12	-33.1	BART11-5p	BART-B
NM_005730	CTDSP2	144	-32.75	-37.3	BART4	BART-A
NM_006538	BCL2-L11	143	-30.62	-35.7	BART4	BART-A
NM_203331	CD59	141	-23.25,	-28.5	BART10	BART-B
		139	-30.36	-33.8	BART13	
NM_017902	HIF1AN	139	-33.21,	-35.5	BART5	BART-A
		132	-26.39	-33.6	BART11-3p	BART-B
NM_005933	MLL	139	-32.09	-33.3	BART5	BART-A
NM_002119	HLA-DOA	139	-29.87	-31.6	BART8*	BART-B
NM_015717	CD207	136	-28.08	-30.7	BART20-3p	BART-B
NM_022893	BCL11A	136	-23.04	-24.5	BART15	BART-A

B. EBF1 BART11-5p Target Site	
EBF1	5' TAACTGTGCACCAGAACTGTCAGT 3'
	: : : : : : : : : :
BART11-5p	3' GUUGAUCGCGUGGUUUGACAGACU 5'

oped by high fidelity PCR amplification of the two primary BART miRNA clusters using template gDNA isolated from an established EBV-transformed lymphoblastoid cell line (LCL) infected with the QIMR-WIL EBV strain, using primers listed in **Table 1A**. Of note, the common laboratory strain B95-8 was not used as it includes a deletion in the BART region, removing most of the BART miRNAs. PCR was performed with the following PCR reaction, 1x HF Buffer, 0.2 μ M of forward and reverse primers, 0.2 mM dNTPs, 1 U Phusion Hot Start High-fidelity DNA polymerase (Finnzymes) and 30 ng of template DNA. The following PCR program was used: 98°C for 30 seconds, 35 cycles of amplification (98°C for 5 seconds, 64°C for 15 seconds and 72°C for 1 minute/kb) and 72°C for 5 minutes. Amplicons were cloned downstream of the CMV promoter of the pcDNA3.1 vector (Invitrogen) using EcoRI restriction sites at the 5' end of the miRNA sequences. The resulting two miRNA expression constructs were termed pBART-A, which encodes a cluster of BART miRNAs not including BART11-5p, and pBART-B which encodes BART11-5p (**Figure 1A**). Constructs expressing restricted subsets of BART miRNAs were developed from pBART-B, by the removal of miRNA sequences by digesting pBART-B with XhoI or HindIII, followed by re-ligation of the construct backbone, generating pBART-B1 and pBART-B3. A final EBV-miRNA expression construct was developed by

the digestion and subsequent re-ligation of pBART-B3 with NheI, generating pBART-B3* (**Figure 1B**). All constructs were verified by sequencing.

Luciferase reporter plasmids were constructed by cloning 3'UTR sequences of predicted target genes downstream of the luciferase ORF of the pGL3-Control vector (Promega). Primers were designed to encompass the miRNA target sites identified by the bioinformatics analysis (**Table 1B**). Either Xba I, Nhe I or Spe I sites were added to the 5' ends of primers dependent on the presence of restriction sites within the region to be amplified, enabling cloning into the Xba I site of the pGL3-Control vector. High fidelity PCR products were amplified as described above using template human gDNA (Roche) and constructs were verified by sequencing.

Transfections

HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) and 10% foetal calf serum (FCS) and incubated at 37°C, 6.5% CO₂. Cells were passaged every two to three days. Cells were seeded at 80,000 cells/well in 24 well plates 24 hours prior to transfection.

When using EBV-miRNA expression constructs, cells were transfected using Lipofectamine LTX (Invitrogen). 500 ng total plasmid DNA was

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Table 3. The number of predicted targets for each EBV miRNA over the course of the bioinformatics analysis. For each EBV miRNA examined the number of potential targets predicted by the miRanda software from the RefSeq 3'UTR library and from a library of randomly generated sequences is indicated. To obtain a measure of false positive target prediction the number obtained from the random library was subtracted from the number obtained from the RefSeq library. The number of potential targets from the RefSeq library exhibiting scores above those observed in the random library are shown. Potential targets identified by inputting the positive 3'UTRs identified by miRanda in a second program TargetScan. Final putative targets identified by both miRanda and TargetScan analyses are shown

miRNA Name	miRanda				TargetScan RefSeq hits	Hits common to miRanda and TargetScan
	RefSeq hits	Random hits	RefSeq minus Random hits	RefSeq hits above Random scores		
BART1-5p	779	999	-220	7	92	4
BART1-3p	160	185	-25	4	67	2
BART2	242	148	94	13	446	9
BART3*	208	370	-162	3	66	2
BART3	134	148	-14	16	20	3
BART4	240	370	-130	5	99	4
BART5	603	703	-100	7	135	5
BART6-5p	353	185	168	5	58	2
BART6-3p	66	222	-156	8	33	3
BART7	211	333	-122	5	108	1
BART8	197	518	-321	5	17	0
BART8*	128	333	-205	6	131	3
BART9	135	222	-87	5	98	2
BART10	364	222	142	13	123	7
BART11-5p	232	407	-175	4	109	4
BART11-3p	113	74	39	41	38	2
BART12	1401	1368	33	7	177	5
BART13	380	296	84	6	68	2
BART14*	42	111	-69	2	24	0
BART14	519	407	112	5	188	4
BART15	501	259	242	18	82	4
BART16	366	370	-4	3	78	1
BART17-5p	281	222	59	5	157	4
BART17-3p	122	148	-26	8	157	2
BART18	89	74	15	4	124	2
BART19	629	259	370	11	330	8
BART20-5p	333	74	259	66	104	19
BART20-3p	232	0	232	48	178	30

*Potential BART-miRNA targets.

transfected/well in a ratio of 450 ng EBV-miRNA expression construct or empty pcDNA3.1, 45 ng 3'UTR firefly luciferase reporter construct and 5 ng pRL-TK for the renilla luciferase normalising construct.

When co-transfecting miRNA mimics and luciferase reporters, cells were transfected using Lipofectamine 2000 (Invitrogen). Cells were transfected with 250 ng 3'UTR firefly luciferase

reporter construct, 50 ng pRL-TK as a renilla luciferase normalising construct and 10 pmol of either BART11-5p miRNA mimic or negative siRNA control (Qiagen).

Real time reverse-transcription polymerase chain reaction

In order to confirm the expression of BART miRNAs from the EBV-miRNA expression con-

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structs, HEK 293T cells were transfected with EBV-miRNA expression constructs. Cells were seeded at 240,000 cells/well in 6 well plates and transfected with 2 µg EBV-miRNA expression construct. Transfected cells were pelleted 48 hours post-transfection and RNA extracted using the mirVana miRNA Isolation Kit (Ambion). miRNA-cDNA was synthesised by polyadenylating RNA using the Poly(A) Polymerase Tailing Kit (Epicentre Biology). cDNA was synthesised via SuperScript III First Strand cDNA Synthesis Kit (Invitrogen) using the RTQ adapter primer (**Table 1C**) to primer first strand synthesis, with final reaction diluted 1/10 with water. For miRNA real time PCR, reactions contained 0.2 µM miRNA-specific primer and RTQ-uniR2 primer, 0.1 µM RTQ-probe, 1x GeneAmp II buffer, 1 U AmpliTaq Gold (Applied Biosystems), 0.2 µM dNTPs (Promega), 2.0 mM MgCl₂, 1.0 M betaine (Sigma) and 5 µL miRNA cDNA. Real time reverse-transcription PCR was performed in a RotorGene RG-3000 thermal cycler (Corbett Research) using the following PCR program: 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute with fluorescence acquired at the end of the 60°C incubation. Relative levels of expression were determined from the amplification curves generated during cycling using the Comparative Quantification analysis contained within the RotorGene 6 software package (Corbett Research). miRNA levels were calculated relative to a WIL EBV-transformed LCL and normalised to U6 expression.

Luciferase assays

Transfected cells were lysed 48 hours after transfection using 100 µL 1X Passive Lysis Buffer (Promega). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System as per manufacturer's instructions (Promega) and measured using the Synergy 4 Plate Reader (Bio Tek). Assays were performed in duplicate and performed with a minimum of three experimental replicates. Firefly luciferase activity was normalised to renilla luciferase activity and expressed relative to cells transfected with empty pcDNA3.1.

Results

EBV-miRNA target prediction

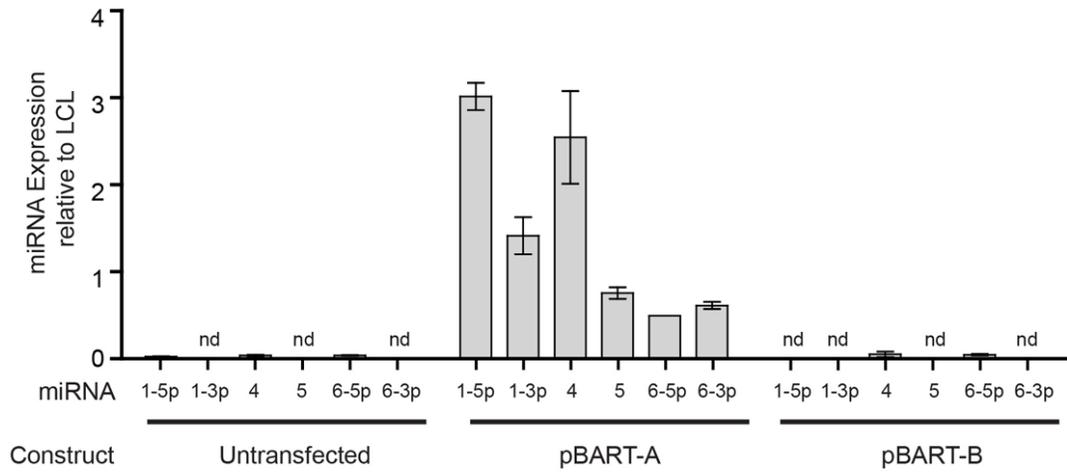
In order to predict potential targets of EBV-miRNAs, an *in silico* analysis was performed,

with a summary of results shown in **Table 3**. Initially the miRanda software package was used to determine predicted targets from the RefSeq 3'UTR library (36985 sequences) using minimum score threshold of 130 and energy threshold (ΔG) of -16 kcal/mol. Here individual miRNAs exhibited a wide range in the number of predicated targets, ranging from 42 hits for BART14* to 1401 for BART12, with an average number of 316 targets per miRNA. This variation in the number of predicted targets per miRNA suggested biases within the miRanda algorithm towards certain sequences. To gain an understanding of this potential bias an analysis was performed using 1000 randomly generated DNA sequences with a G+C content distribution matched to that found in the Refseq 3'UTR population. Analysis of this random set by miRanda using the same parameters as used for the RefSeq 3'UTR library generated a substantial number of hits (**Table 1**). Comparison of the number of hits for each EBV-miRNA in both the RefSeq 3'UTR and random datasets revealed a highly significant correlation (Pearson $r=0.8158$, $p<0.0001$), suggesting a substantial component of the hits from the Refseq 3'UTR set were false positives. Interestingly 18 of the miRNAs had predicted proportionally fewer targets from the RefSeq 3'UTR library than from the random sequences again suggesting a high level of false positive hits. To attempt to overcome these potential false positives an analysis was made excluding all RefSeq 3'UTR predicted targets with scores and ΔG values below that of the highest ranking hit from the random set for each miRNA. This resulted in a significant reduction in the predicted hits as shown in **Table 3** with numbers ranging from 2 to 66 hits, with an average of 12 hits per miRNA.

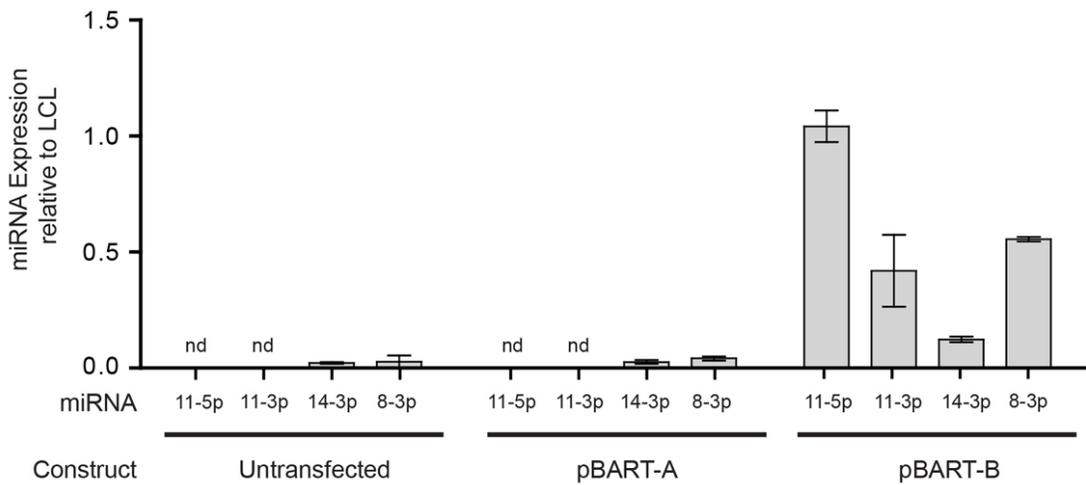
To further filter putative targets and increase the rigour of our analysis, a second program, TargetScan, was used to analyse the RefSeq 3'UTR library. This analysis produced 15 to 446 (average 117) hits per miRNA as shown in **Table 3**. In addition, the free energy binding of the EBV-miRNA against the target 3'UTR was assessed using RNAHybrid (Rehmsmeier et al., 2004). Sequences that had been predicted to have miRNA target sites by both miRanda and TargetScan produced a final list of 154 potential targets with 0 to 30 hits per miRNA and an average of 5 hits per miRNA as shown in **Table 3**. From these genes, ten potential targets for

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A pBART-A localised miRNAs



B pBART-B localised miRNAs



C pBART-B Sub-clones

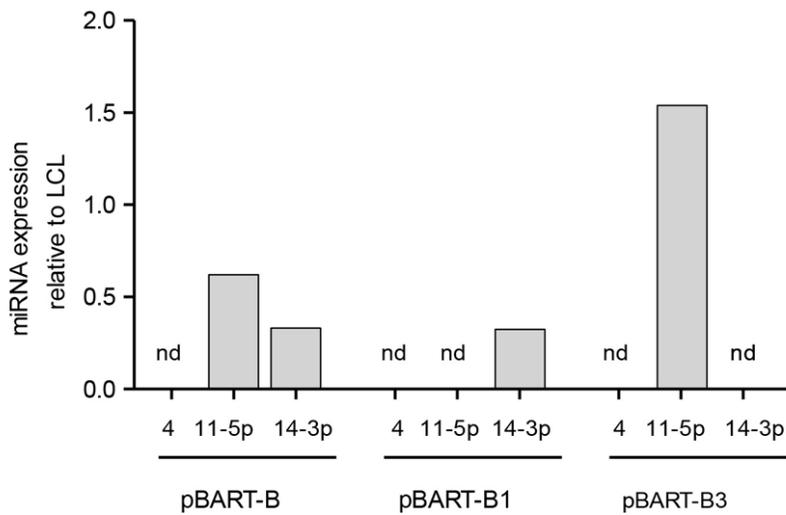


Figure 2. Demonstration of specific miRNA expression from the EBV-miRNA expression constructs. Real time RT-PCR following transfection of miRNA expression constructs. A: Expression levels of BART-A miRNAs. B: Expression of

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BART-B miRNAs. C: Expression levels of EBV-miRNAs from cells transfected with pBART-B, pBART-B1 or pBART-B3. Bars indicate mean and SEM of experimental duplicates. nd=none detected. miRNA expression levels were normalised to U6 expression and reported relative to their expression in an EBV-transformed LCL.

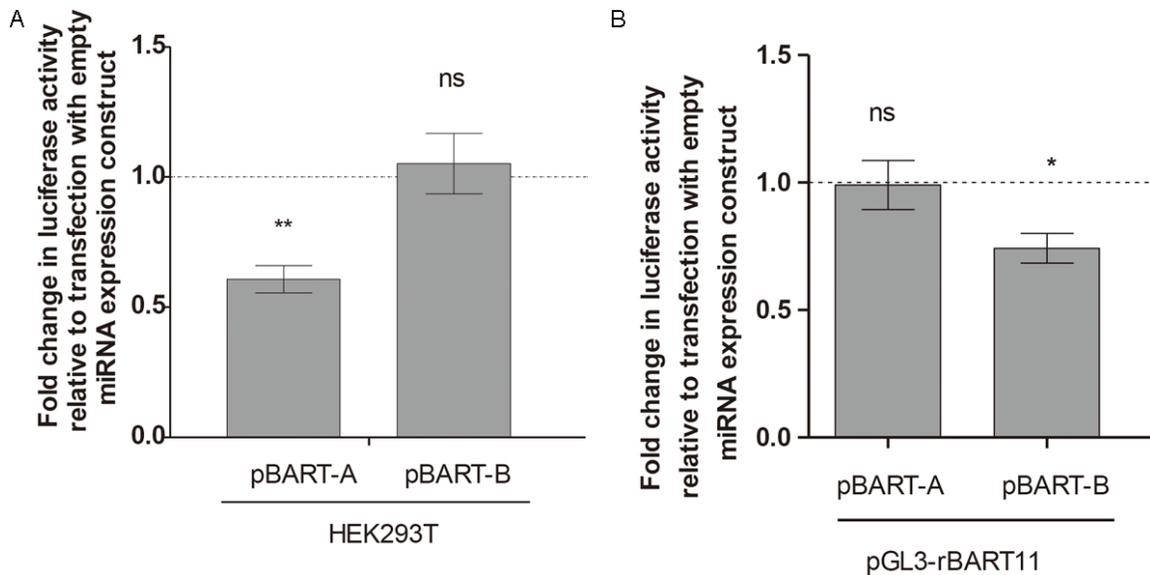


Figure 3. Demonstration of functional miRNA expression from EBV-miRNA expression constructs. A: Firefly luminescence of pGL3-LMP1-UTR reporter construct transfected into HEK293T cells in combination with pBART-A, pBART-B and empty pcDNA miRNA expression constructs. Bars indicate fold level of normalised luminescence relative to that of the pcDNA level for each cell line transfected. Bars indicate the mean of three experimental replicates, each consisting of two technical duplicates. B: Validation of BART11-5p positive control. Bars indicate the firefly luciferase activity of pGL3-rBART11 normalised to the Renilla luciferase activity when transfected with either pBART-A or pBART-B. Bars indicate the mean of four experimental replicates, each consisting of two technical duplicates. Error bars indicate SEM of the experimental replicates. *p*-value of one sample t-test against 1.0 to demonstrate significance of change in luminescence with miRNA expression constructs indicated above bars.

laboratory analysis were selected on the basis of targeting by BART-encoded EBV-miRNAs and an association with EBV, B-cell lymphoma pathogenesis, B-cell differentiation, immunomodulation or apoptosis (**Table 2**).

Validation of EBV-miRNA expression constructs

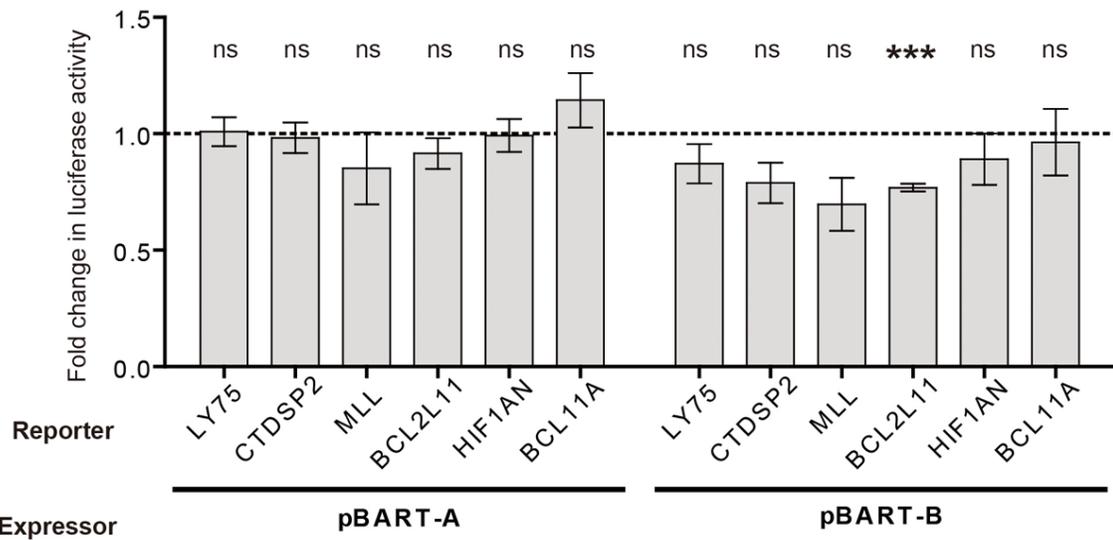
To enable examination of the effects of individual EBV-miRNAs a series of over-expression constructs was produced (**Figure 1**). To confirm specific EBV-miRNA expression quantitative real time RT-PCR analysis was performed on HEK293T cells transfected with the constructs (**Figure 2A** and **2B**). This demonstrated that both pBART-A and pBART-B expressed mature BART miRNAs at levels comparable to those observed from an EBV-transformed LCL, with only very low levels of non-specific amplification detected for some primers. For the constructs designed to produce restricted subsets of the BART-B EBV-miRNAs (**Figure 2C**) neither

pBART-B1 nor pBART-B3 demonstrated expression of BART4, which is encoded within pBART-A. pBART-B1 expressed both BART14-3p and BART13 at levels comparable to an EBV-transformed LCL and did not express BART11-5p. In contrast, pBART-B3 expressed BART11-5p at comparable levels to an EBV-transformed LCL and did not express BART14-3p. In addition, expression levels from pBART-B1 and pBART-B3 were compared to the full pBART-B construct. This confirmed the restricted miRNA expression constructs to express miRNAs at comparable levels to pBART-B.

To confirm functionality of the EBV-miRNA over-expression system the pBART-A construct was over-expression in combination with the EBV LMP1 gene 3'UTR fused to firefly luciferase. LMP1 mRNA is known to be silenced by EBV-miRNAs within BART Cluster 1 [30], contained within pBART-A, and as such co-transfection should demonstrate silencing through reduced

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A BART-A Targets



B BART-B Targets

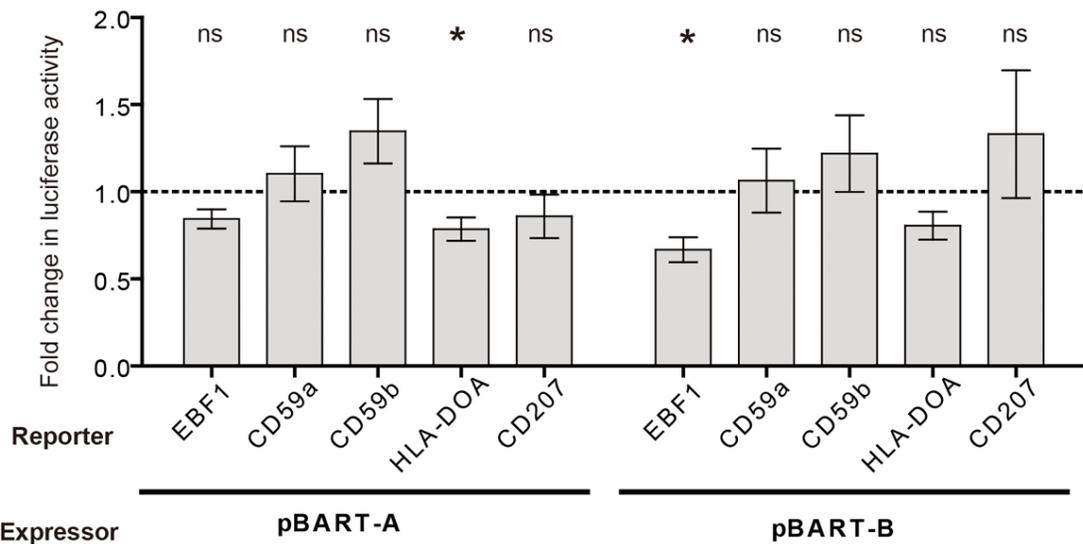


Figure 4. Validation of the bioinformatically predicted (A) BART-A targets and (B) BART-B targets. Activity of firefly luciferase reporter constructs was normalised against the expression of Renilla luciferase and expression was reported relative to luciferase activity in cells not overexpressing BART miRNAs which was arbitrarily defined as 1.0 and is indicated by the dashed lines. Data presented is mean \pm SEM, n=4 except for luciferase assays of BCL11A and CD207 which were performed in triplicate. All experiments were performed with technical duplicates. Significance of fold changes with miRNA expression determined by one-sample T-tests with result summaries indicated above each column. ns=not significant. (*) p<0.05. (***) p<0.001.

luminescence. Transfection was also performed with the empty pcDNA3.1 vector and pBART-B, and results normalized against Renilla luminescence from the constantly expressing pRL-TK construct and presented relative to the empty pcDNA3.1 vector. This revealed the pGL3-LMP1 positive control to exhibit a significant reduction in luciferase

activity ($39.3\% \pm 3.0\%$, $p=0.0029$) when co-transfected with pBART-A with no evidence of silencing when co-transfected with pBART-B (**Figure 3A**).

An additional positive control was developed which is specifically targeted by the BART11 miRNAs, termed pGL3-rBART11. pGL3-rBART-

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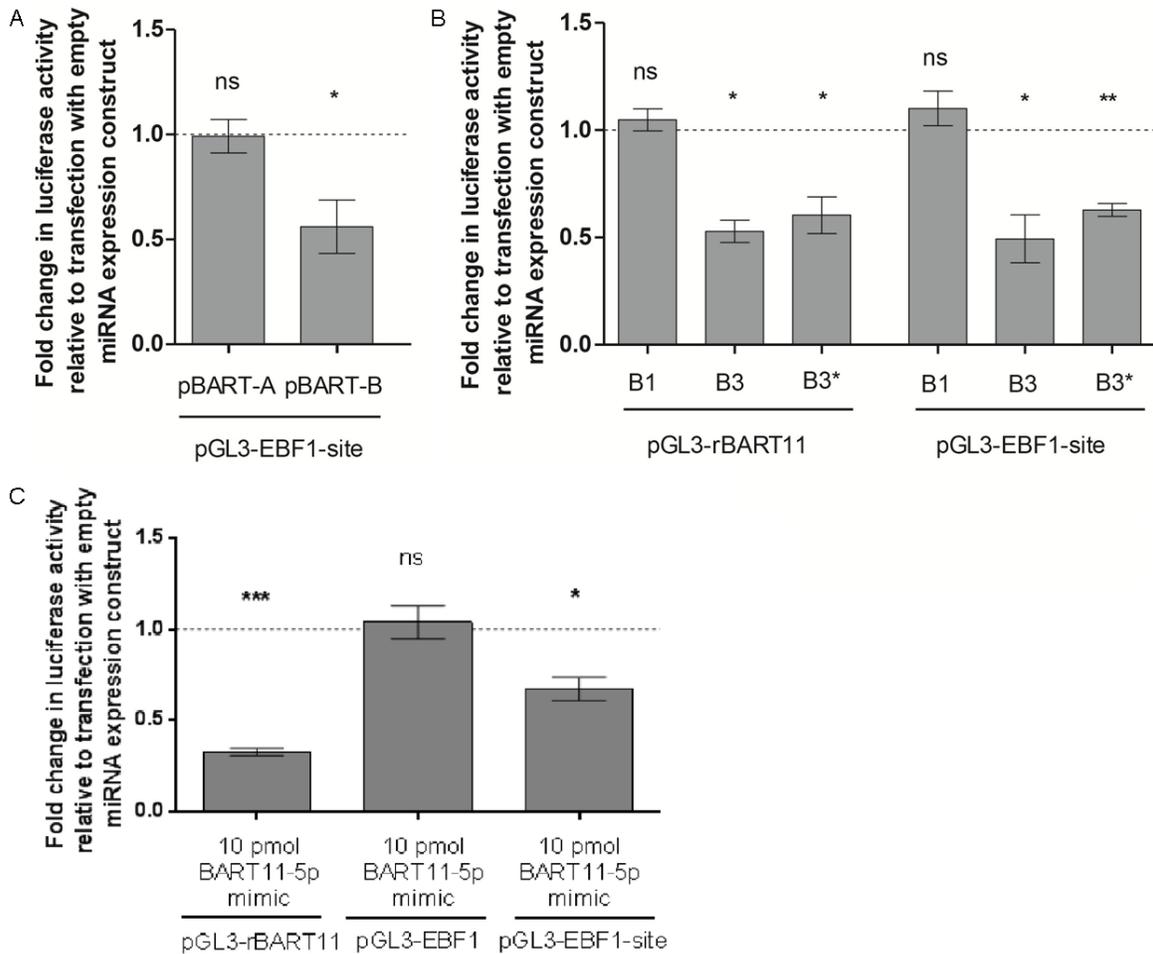


Figure 5. Experimental verification of predicted miR-BART11-5p silencing via predicted EBF1-3'UTR site. A: Bars indicate the firefly luciferase activity of truncated, site-specific EBF1-3'UTR luciferase reporter construct normalised to the Renilla luciferase activity when transfected with either pBART-A or pBART-B miRNA expression constructs relative to cells transfected with an empty pcDNA miRNA expression construct. Bars indicate the mean of five experimental replicates, each consisting of two technical duplicates. B: Bars indicate the firefly luciferase activity of truncated, site-specific EBF1-3'UTR or BART11-specific positive control luciferase reporter constructs normalised to the Renilla luciferase activity when transfected with miRNA expression constructs expressing restricted subsets of pBART-B miRNAs relative to cells transfected with an empty pcDNA miRNA expression construct. Bars indicate the mean of three experimental replicates, each consisting of two technical duplicates. C: Bars indicate the firefly luciferase activity of luciferase reporter constructs normalised to the Renilla luciferase activity when transfected with 10 pmol of synthetic BART11-5p mimic relative to cells transfected with a negative control siRNA. Bars indicate the mean of two experimental replicates for pGL3-EBF1 and pGL3-EBF1-site and three experimental replicates for pGL3-rBART11, each consisting of two technical duplicates. Error bars indicate SEM of the experimental replicates. Results of one sample t-test against 1.0 are indicated above bars, ns=not significant. (*) $p < 0.05$. (***) $p < 0.001$.

11 was co-transfected with pBART-A and pBART-B in order to validate it as a positive control (**Figure 3B**). There was no evidence of silencing upon co-transfection with pBART-A but there was a significant decrease $25.8\% \pm 5.8\%$, $p = 0.0209$ in luciferase activity upon co-transfection with pBART-B. These findings provide validation of the positive control pGL3-rBART11.

Experimental validation of predicted target sites

To determine the functionality of the putative BART miRNA targets identified above luciferase reporter assays were performed (**Figure 4**). For the six selected genes predicted to be targeted by miRNAs contained within the BART-A cluster no significant alteration in expression was

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observed when the pBART-A construct was co-transfected with the reporter constructs. Interestingly when the BART-A target reporter constructs were co-transfected with pBART-B a highly significant, reproducible reduction ($23.2\% \pm 1.7\%$, $p=0.008$) in luciferase activity was observed for the BCL2L11 construct. In addition, pGL3-MLL exhibited a large yet statistically insignificant reduction in luciferase activity ($30.3\% \pm 11.4\%$, $p=0.0763$), when BART-B miRNAs were overexpressed. For the five selected genes predicted to be targeted by BART-B miRNAs, the EBF1 construct was the only target that exhibited a significant reduction in luciferase activity ($33.2\% \pm 7.2\%$, $p=0.0192$) due to expression of BART-B miRNAs. Again unexpectedly a third significant target was identified when the predicted BART-B targets were assayed with the expression of BART-A miRNAs. The HLA-DOA construct exhibited a significant reduction in luciferase activity ($21.49\% \pm 6.7\%$, $p=0.0485$).

Two of the three positive results were not predicted by the bioinformatics analysis. While EBF1 was predicted to be targeted by a miRNA within the BART-B cluster, neither HLA-DOA nor BCL2L11 were down regulated by their predicted BART miRNA clusters. To determine the previously undetermined sites underlying this down regulation the bioinformatics data was reanalysed for these two gene's 3'UTRs. This revealed a second potential target site within the BCL2L11 3'UTR for BART14-3p, a miRNA within the BART-B cluster. This target site had been excluded during filtering of the results using random 3'UTRs. No secondary target site was identified for the HLA-DOA 3'UTR within the miRanda bioinformatics scan and the miRNAs targeting these genes and potential target sites remain unknown.

Experimental verification of predicted miR-BART11-5p silencing of EBF1

As EBF1 was the highest bioinformatically ranked target to exhibit silencing by the predicted BART expression construct, this gene was chosen for further study. The predicted target site within EBF1 was experimentally verified via luciferase silencing assays using the site-specific luciferase reporter pGL3-EBF1-site. This construct contained a 151 bp fragment of the EBF1-3'UTR containing the bioinformatically predicted target site. As expected, there was no

significant reduction in luciferase levels observed when co-transfected with pBART-A. When co-transfected with pBART-B, the luciferase expression of pGL3-EBF1-site was significantly reduced by $43.9\% \pm 12.7\%$ (SEM) ($p=0.0258$) (**Figure 5A**). This strongly support the functionality of the predicted BART11-5p site at position 2137 of the EBF1-3'UTR.

In order to confirm that miR-BART11-5p was responsible for the silencing of pGL3-EBF1-site, additional luciferase assays were performed with co-transfection of pBART-B1 and pBART-B3, miRNA expression constructs expressing reduced subsets of BART miRNAs. Here no silencing of luciferase expression was observed with co-transfection of pBART-B1, however significant levels of silencing were observed upon co-transfection with both pBART-B3 and pBART-B3* (**Figure 5B**). pBART-B3 repressed luciferase expression by $50.6\% \pm 11.2\%$ ($p=0.0458$) compared to co-transfection of pGL3-EBF1-site with an empty miRNA expression construct. pBART-B3* repressed luciferase expression by $37.2\% \pm 3.0\%$ ($p=0.0063$). In addition, an unpaired t test was performed to compare the level of silencing between co-transfections with pBART-B3 and pBART-B3* however there was no significant difference in silencing between these two constructs. These levels of silencing were comparable to that of the positive control pGL3-rBART11 which demonstrated a $47.2\% \pm 5.2\%$ ($p=0.0120$) and a $39.6\% \pm 8.6\%$ ($p=0.0439$) level of silencing when co-transfected with pBART-B3 and pBART-B3* respectively. As both pBART-B3 and pBART-B3* both express BART11-5p and there was no statistical difference in the levels of silencing between the two miRNA expression constructs, these findings provide further support that BART11-5p does repress EBF1 at the predicted site.

In order to test the individual effect of BART11-5p on the predicted target site within the EBF1-3'UTR, luciferase assays were performed using a synthetic miRNA mimic of BART11-5p. The miRNA mimic was first validated against pGL3-rBART11 and significant silencing ($55.1\% \pm 2.1\%$, $p=0.0014$) of luciferase expression was observed (**Figure 5C**). Luciferase assays were then performed with the site specific EBF1 reporter (pGL3-EBF1-site), demonstrating a statistically significant degree of silencing ($32.8\% \pm 6.4\%$, $p=0.0361$) of pGL3-EBF1-site

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by the BART11-5p mimic (**Figure 5C**). In contrast, the whole EBF1-3'UTR shows no evidence of silencing of luciferase activity by BART11-5p (**Figure 5C**).

Discussion

In this study, EBF1 was identified as a novel target of EBV-miRNA BART11-5p. Following bioinformatics analysis (using three separate miRNA prediction tools), we performed luciferase reporter assays with a series of BART-constructs, and confirmed inhibition of the EBF1-3'UTR and identified the target site as 2137 bp after the stop codon. Results were confirmed following transfection of a BART11-5p mimic which was able to inhibit the target site. Our findings highlight a potential role of BART-miRNAs in the regulation of B-cell differentiation.

EBF1 is an important B-cell transcription factor that is crucial for germinal centre formation [31, 32]. Recent studies have demonstrated that EBF1 is expressed during all stages of B-cell differentiation with the exception of plasma cells. In its absence fully functioning, mature B-cells are unable to develop [33]. It targets a number of B-cell specific genes including the B-cell developmental regulator Pax5 (which is necessary for the maintenance of the B-cell phenotype), the B-cell receptor (BCR) and Bim [34]. In addition, the activity of EBF1 is regulated by CD40, a crucial B-cell signalling receptor that is involved in germinal centre differentiation [35].

EBV-driven transformation of B-cells drives an atypical germinal centre reaction [3], indicating that the modulation of EBF1 expression by EBV may play a role in this process. This is supported by the interaction of EBF1 with the latency III viral gene EBV nuclear antigen-2 (EBNA2), which has been found to be enriched at EBF1 transcription sites, suggesting that EBNA2 may associate with EBF1 in order to co-target EBF1-regulated genes [36]. Furthermore Latent Membrane Protein-2A (LMP2A) alters the expression of EBF1 through the Notch signalling pathway [37]. Additionally, binding sites for Pax5, one of the primary and most well-characterised targets of EBF1, have been identified in the Wp sequence, one of the primary promoters governing EBV latency gene expression. Mutation of these binding sites prevents viral

activity in B-cells [38]. This suggests a mechanism ensuring B-cell specificity of EBV latency expression and highlights another possible function of EBF1 targeting by EBV-miRNAs, namely to regulate the expression of EBV latency genes.

Our previous data has confirmed that BART11-5p is expressed in a range of EBV-associated B-cell lymphoma types with a variety of latency profiles including PTLD, DLBCL and BL [21]. BART11-5p is also highly expressed in lymphomas of non-B-cell origin [39], suggesting a contributing to the pathogenesis of these tumors. In contrast, the expression of BART11-5p within the EBV-associated malignancy nasopharyngeal carcinoma (NPC) is barely above background levels [40]. Although BART miRNAs are expressed from the same promoter, the levels of individual miRNAs differ widely from one another [24]. Interestingly, it has been asserted that the expression of miRNA targets protects miRNAs from degradation, resulting in an increase in the levels of the miRNA [41]. Thus one possible explanation for the difference in the expression of BART11-5p between lymphoid and epithelial malignancies is that NPC contains few miRNA targets for BART11-5p. In support of this notion, EBF1 is expressed in both normal and malignant B-cells, whereas the expression of EBF1 in NPC has not been detected [31, 42].

Our study provides evidence of a miRNA target site within the EBF1-3'UTR, 2137 bp after the stop codon, targeted by the EBV-miRNA BART11-5p. This target site (**Table 2B**) shows a high degree of complementarity to BART11-5p but does not exhibit perfect complementarity within the seed region (5' nucleotides 2-7). While the perfect complementarity of the seed region has long been considered one of the defining motifs of potential miRNA target sites, extensive binding of the 3' region of the miRNA, in particular nucleotides 12-16, can compensate for imperfect complementarity of the seed region and a recent study has demonstrated miRNAs which bind to sites with a "centred site" motif [43]. Our study did not explore the specific binding characteristics of BART11-5p to its predicted target site but given the degree of pairing, it is conceivable that the high degree of complementarity within the 3' region compensates for the imperfect complementarity of the seed region.

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It has been demonstrated that several EBV-miRNAs share seed sequences with human miRNAs, possibly in order to regulate existing miRNA regulatory networks [44]. Unfortunately, little is known about miR-1324 and no targets have been identified. miR-1324 was isolated from neuroblastoma samples and, of note EBF1 is not only responsible for regulating B-cell differentiation but also for regulating neuronal differentiation [45].

In summary, we identify EBF1 as a novel target of EBV-miRNA BART11-5p. Our findings highlight a potential role of BART-miRNAs in the regulation of B-cell differentiation. Further investigation into the targeting of EBF1 by EBV-miRNAs during B-cell differentiation and lymphomagenesis is warranted.

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Disclosure of conflict of interest

There are no conflicts to declare.

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