Published in final edited form as:
Curr Opin Virol. 2014 August ; 0: 61–65. doi:10.1016/j.coviro.2014.04.003.

Multiple Functions Are Mediated by the miRNAs of Epstein-Barr Virus

Malika Kuzembayeva[1], **Mitchell Hayes**[2], and **Bill Sugden**[2,*]

[1]HIV Drug Resistance Program, National Cancer Institute, Frederick, Maryland, United States of America

[2]McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin, United States of America

Abstract

Epstein-Barr virus is a gammaherpes virus that is causally associated with several malignancies and expresses multiple miRNAs in both normal and tumor cells. Since the identification of virally-encoded miRNAs, various mRNAs have been identified as targets for regulation by EBV's miRNAs in host cells. We shall summarize these targets, the robustness of their identification, and examine how the regulation of these targets by EBV contributes to the successful infection of its host.

Introduction

Epstein-Barr Virus (EBV) is a successful human pathogen, and is now known to cause at least 200,000 new cancers per year [1]. While initially identified in the cells of a Burkitt's lymphoma, much evidence has implicated EBV as causing several additional lymphomas and carcinomas (reviewed in [2]). A new layer of virus-host interaction has emerged with the discovery of virally-encoded miRNAs. MicroRNAs and their biogenesis have been extensively reviewed previously [3]–[5]. Briefly, they are short (19–24 nt) single-stranded RNA molecules that post-transcriptionally regulate gene expression by recruiting the RNA-induced silencing complex (RISC) to target mRNAs [6]–[8]. Multiple studies using computational and molecular biology techniques as well as deep sequencing have led to the identification of at least 40 viral miRNAs encoded within 25 precursor transcripts [3], [9], [10]. They are encoded within two regions of EBV's genome: BART (Bam HI-A region rightward transcript) and BHRF1 (Bam HI fragment H rightward open reading frame 1) (Figure 1). The BHRF1 transcript also encodes the BHRF1 ORF, while the BART transcript has not been confirmed to express other functional products besides its miRNAs. Since their identification, the expression of these miRNAs has been extensively profiled in various

*Corresponding author: sugden@oncology.wisc.edu.

EBV-infected cells lines including Burkitt's lymphomas, lymphoblastoid cell lines, carcinomas as well as in tumor biopsies [11]–[18]. The abundance of individual miRNAs within cell lines varies widely and is cell type specific. BART miRNAs were found to be expressed in all types of EBV-associated latency, whereas expression of the BHRF1 miRNAs appears to be more restricted (*ibid*). The levels of the miRNAs measured in carcinoma biopsies can exceed those in cell lines by more than 100-fold [18] making it plausible that in vivo EBV's miRNAs have more functions than found in cell culture.

MiRNAs provide a potent mechanism for EBV to modulate the cellular environment: they are thought not to elicit an immunogenic response; they take up little genomic space; and they also have the potential to regulate hundreds of targeted genes. Here we focus on specific cellular processes that appear to be modulated by the currently identified targets of viral miRNAs and explore their possible contributions to EBV's lifecycle. We have also used insights from HITS- and PAR-CLIP experiments to gauge the robustness of these identifications.

Identifying and Validating Targets of EBV's miRNAs

Leads to identifying the targets of cellular miRNAs can be found using bioinformatics programs such as TargetScan [19]. Because these programs use the evolutionary conservation of the seed sequences recognized in the target mRNAs in their identification, they are not readily applicable to studies of EBV's miRNA targets given that EBV has evolved to infect a single host species. Therefore, validating the targets of EBV's miRNAs requires a set of successively more stringent tests. These include assays in which sequences encoding the 3′ UTR of the presumptive mRNA target are ligated to luciferase as a reporter and its regulation recorded in the presence of physiological levels of the miRNAs to be tested. A necessary control experiment is to change the seed sequences in the 3′UTR and demonstrate that the changes abrogate any inhibition of the luciferase activity by the test miRNA. Finally experiments with PAR-CLIP allow identification of sites in mRNAs that are modified by the juxtaposition of Argonaute in the RISC complex. If these sites are found and correspond to the seed sequence recognized by an EBV miRNA, then we can conclude that the mRNA is targeted by that EBV miRNA. Table 1 documents currently identified target mRNAs of EBV miRNAs and the robustness of the experiments validating these identifications. One caveat to consider is that the levels of EBV's miRNAs in cell in culture are far lower than in biopsies isolated from carcinomas so that studies of cells in culture may miss many mRNAs that are targeted by EBV's miRNAs in vivo.

EBV's miRNAs Contribute to its Transformation of B lymphocytes

While the majority of functions ascribed to EBV's miRNAs are to sustain latently infected cells, several studies have expanded on their importance during the initial stages of infection of B-cells. Mature BART and BHRF1 miRNAs were detected in primary B- cells infected with either of two strains of EBV at two days post infection (dpi) and increased in expression through at least the first eight dpi [13]. Cells infected with derivatives of EBV null for the BHRF1 miRNA cluster grew more slowly when exposed to the same multiplicity of infection relative to wild type genomes [20]–[22]. The BHRF1 cluster of

miRNAs appeared to promote both transformation of infected cells and cell-cycle progression [20]–[22]. *In vivo* studies using humanized mice to monitor EBV infection and tumorigenesis revealed significant delays in viremia in mice infected with a derivative of EBV lacking BHRF1 miRNAs [23]. Absence of the BHRF1 miRNAs however had no effect on tumor formation and survival of mice relative to those infected with wild type virus [23].

Regulation of Apoptosis by EBV miRNAs

A common fate for B-lymphocytes *in vivo* is death by apoptosis. EBV infects B-cells and evades apoptosis in its host cell by multiple means including its miRNAs (Table 1). The BHRF1 miRNAs inhibited apoptosis early during infection of primary B cells and promoted their proliferation as shown by infection with derivatives of EBV [22]. The BART miRNAs sustained Burkitt's lymphomas in part by inhibiting Caspase 3 [24]. The BART miRNAs also have been reported to target the pro-apoptotic proteins PUMA (p53-upregulated modulator of apoptosis) and Bim (BCL2L11) [25], [26]. Recent comprehensive HITS- and PAR-CLIP analyses have identified CAPRIN2 and DAZAP2 which are involved in Wnt signaling as targets and which may also function in apoptosis[27], [28]. In contrast, Choi and colleagues reported that miR-BART15-3p promoted apoptosis in part by targeting BRUCE (BIRC6), a member of the inhibitor of apoptosis (IAP) family in gastric carcinoma cells [29]. The functional consequences of BRUCE inhibition are currently unclear but appear inconsistent with the association of EBV with its host cell's survival and proliferation.

Role of EBV's miRNAs in Immune Evasion

While EBV infects the majority of the adult population of the world, most of these infections are asymptomatic and persist for the lifetime of the host. EBV has evolved multiple strategies to avoid immune recognition in order to establish life-long, latent infections in B-cells (reviewed in [30], [31]). New findings indicate that viral miRNAs also attenuate the host's antiviral immune response (Table 1). One of the earliest targets identified for miR-BHRF1-3 was CXCL-11, an IFN-inducible T-cell attracting chemokine [32]. CXCL-11 is one of the more abundantly expressed chemokines that interacts selectively with CXCR3, a chemokine receptor expressed on T cells [32], [33]. These findings show that viral miRNAs may contribute to immune evasion by modulating host cytokine networks.

Nachmani et al. reported that a stress-induced Natural Killer (NK) cell ligand, MICB, was targeted by miR-BART2-5p which could allow EBV-infected cells to escape recognition and subsequent elimination [34]. NK cells play a critical role in detection of virus-infected cells in part by using NKG2D receptors to detect release of molecules such as MICB, MICA and members of ULBP family in response to viral infections ([34] and references therein). A related virus KSHV (Kaposi's Sarcoma-associated herpesvirus) was also found to regulate expression of MICB through its miRNA, miR-K12-7 emphasizing the importance of escaping NKG2D-mediated recognition and NK cell attack. Along with dampening NK cell response, viral miRNAs may also regulate activation of the NLRP3 inflammasome and subsequent production of pro-inflammatory cytokines such as IL-1β and IL-18 [35]. miR-

BART15 was found to decrease expression of NLRP3 in reporter assays; its transient transfection reduced endogenous levels of NLRP3 as well as IL-1β production following inflammasome activation (*ibid*). Notably, miR-BART15 targets NLRP3 through the same site as a cellular miR-223. Several additional targets of EBV's miRNAs that could contribute to viral immune evasion were identified through comprehensive PAR-CLIP analyses and confirmed using reporter assays. Among these are SP100, LY75, PDE7A, PELI1 though functional studies remain necessary to understand their biological significance [28].

Conclusions

EBV is exceptional in encoding so many miRNAs. It is a successful human pathogen that usually persists in people without causing disease. This success likely reflects its evolutionary fitness as a virus to infect human beings. By studying EBV's miRNAs, and identifying their biological targets, we shall gain insights into how EBV succeeds as a pathogen both at the cellular and organismal levels.

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Highlights

- EBV encodes at least 25 pre-miRNAs encoded within two gene clusters.
- Steady-state levels of these miRNAs vary significantly among EBV-positive cell lines and tumor biopsies.
- Targets of EBV's miRNAs mediate various cellular processes to promote cell survival and proliferation.

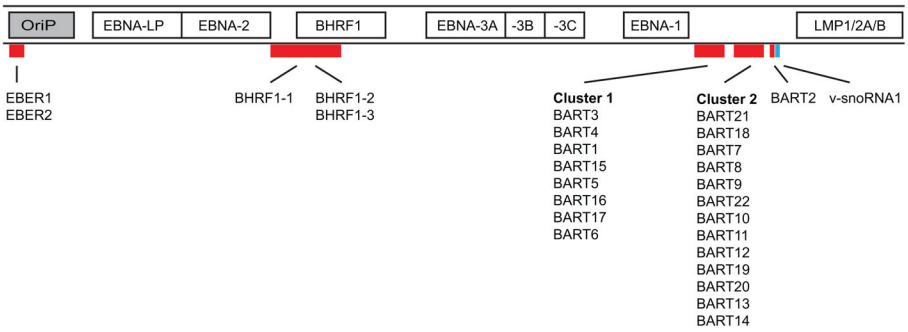

Figure 1. Non-coding EBV RNAs

This schematic illustration shows the locations of non-coding RNAs within EBV's genome. Along with the non-coding nuclear EBER RNAs, and a snoRNA [36], EBV encodes at least 25 pre-miRNAs located within two regions of the genome. The smaller subset of BHRF1 miRNAs is derived from transcripts mapping approximately between 53,000 and 56,000 bps. The majority of EBV miRNAs arise from highly spliced transcripts mapping approximately between 139,000 and 153,000 bps. Also shown are the latent origin of replication (OriP) and genes expressed in latently infected cells. Note: the figure is not drawn to scale and is a linear representation of EBV's circular genome which encompasses approximately 165,000 bps.

Table 1

Possible Validations for Candidate Targets of EBV's miRNAs

MRNA	miRNA	mRNA Functions	PAR-CLIP		Ref.
			Gottwein et al.	Skalsky et al.	
Bim (BCL2L11)	Various (BART9, 11+12)	Pro-apoptotic; inhibits Bcl-2	−	?	[26]
BRUCE (birc6)	BART 15-3p	Inhibitor of apoptosis (IAP)	−	−	[29]
C1orf109	BART 2-5p[#]	Cancer cell proliferation (proposed)	−	+	[38]
CAPRIN2	BART 13-3p	Wnt signaling	−	?	[27]
CASP3	BART 1-3p	Pro-apoptotic; death protease	+	−	[24][†]
CLEC2D(LLT1)	BART 1-3p, 3-3p	Ligand for NK receptor	−	+	[28]
CXCL11	BHRF1-3	IFN-inducible chemokine	?	−	[32]
DAZAP2	BART 3	Wnt signaling	+	+	[28]
DICE1	BART 3*	DEAD box protein; tumor suppressor	−	−	[39]
DICER1	BART 6-5p	RNAi processing (miRNA maturation)	−	?	[40]
IPO7	BART 3	Nuclear import	+	+	[24], [38][†]
LY75	BART 1-5p	Mediates antigen presentation	−	+	[28]
MICB	BART 2-5p	Activates NK cell response	−	−	[34]
NLRP3	BART 11-5p, 15	Pro-apoptotic; release of IL-1B	−	?	[35]
PDCD1LG2	BHRF1-2-5p, BART 1-5p, 15-3p[#]	Ligand for PD-1; Immune regulation	?	+	[38]
PDE7A	BART 3	Cytokine production	−	+	[38]
PELI1	BART 3	E3 ubiquitin ligase; activation of NF-kB	−	+	[28]
PUMA (BBC3)	BART 5	Pro-apoptotic; inhibits Bcl-2	−	?	[25]
SP100	BART 1-5p	Anti-viral response; part of PML-NB	+	+	[28]
T-bet (TBX21)	BART 20-5p	Transcription factor, T cell development	−	?	[41]
TOMM22	BART 16	Mitochondrial import	+	?	[38]
ZNF451	BHRF1-2	Transcriptional regulation?	?	+	[28]

+ Clusters of sequence changes in RNAs in RISC consistent with binding of a given EBV miRNA with high confidence (for details see Gottwein et al. [37] and Skalsky et al. [28])

− No clusters of sequence changes in RNAs in RISC consistent with binding of a given EBV miRNA with high confidence (for details see Gottwein et al. [37] and Skalsky et al. [28])

[†]Reference proposes additional EBV miRNAs target given mRNA that are not confirmed by PAR-CLIP.

? Some EBV miRNAs are not expressed in the cells analyzed by PAR-CLIP making the PAR-CLIP uninformative for this mRNA.

Dolken et al. [38] did not propose a specific EBV miRNA for this target. Skalsky et al. [28] propose the given miRNA(s) based on PAR-CLIP data.