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Efeitos de miR-BARTs do vírus de Epstein-Barr na modulação de vias de sinalização intracelular em células derivadas de linfomas humanos

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientador: Prof. Dr. Deilson Elgui de Oliveira

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Effects of Epstein-Barr virus miR-BARTs in modulating intracellular pathways in human lymphoma cells

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Thesis Supervisor: Deilson Elgui de Oliveira, Ph.D.

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Palavras-chave: CRISPR/Cas9, ; Edição gênica; Linfoma de Burkitt; Vírus de Epstein-Barr; microRNAs virais.

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"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make because they lead little by little to the truth."

Jules Verne, A Journey to the Centre of the Earth.

"We're forever teetering on the brink of the unknowable and trying to understand what can't be understood. It's what makes us men."

Isaac Asimov, The Caves of Steel

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List of Abbreviations

4-HT	4-hydroxytamoxifen
AmBic	Ammonium bicarbonate buffer
BCR	B cell receptor
BL	Burkitt lymphoma
CBP	CREB-binding protein
CDT1	Chromatin licensing and DNA replication factor 1
cHL	Classic Hodgkin lymphoma
Cp	C-latency promoter
CR2 or CD21	Complement receptor 2
DLBCL	Diffuse large B cell lymphoma
DOX	doxycycline
DS	Dyad symmetry
DTT	Dithiothreitol
EBNAs	EBV-encoded nuclear antigens
EBV	Epstein-Barr virus
ESCRT	Cellular endosomal sorting complexes required for transport
FBS	Fetal bovine serum
FDR	False discovery rate
FR	Family of repeats
GC	Gastric carcinoma
gp350	Glycoprotein 350
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
IA-LPD	Immunodeficiency-associated lymphoproliferative disorders
IAA	2-iodoacetamide
IgA	Immunoglobulin A
IM	Infectious mononucleosis

LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LCV	Lymphocryptovirus
LG	Lymphomatous granulomatosis
LMPs	Latent membrane proteins
MAPKs	MAP kinases
MCD	Multicentric Castleman's disease
MHC class II	Major histocompatibility complex class II
miRNAs	Micro-RNAs
NaB	Sodium butyrate
NPC	Nasopharyngeal carcinoma
ORC2	Origin recognition complex subunit 2
ORF	Open Reading Frame
OriP	Replication origin region
PBL	Plasmablastic lymphoma
PEL	Primary effusion lymphomas
PI3K	Phosphatidylinositol 3 kinase
PL-HIV	People living with HIV
plgR	Polymeric immunoglobulin receptor
PTLD	Post-transplant lymphoproliferative disorders
RBP	RNA binding protein
Rp	Rta promoter
sncRNAs	Small non-coding RNAs
STR	Short tandem repeat
T7EI	T7 endonuclease I
TCF-3	Transcription factor 3
TFA	Trifluoroacetic acid
TFIIA	Transcription factor IIA
TIDE	Track of indels by decomposition
TPA	Tetradecanoylphorbol-13-acetate
VCRs	Viral replication compartments
VRC	Viral replication compartments

VZV	Varicella-Zoster virus
Wp	W promoter
WT	Wilde type
Zp	Zta promoter
ZRE	Z-responsive elements

Resumo

O linfoma de Burkitt (LB) é um linfoma não-Hodgkin de células B altamente agressivo, associado à infecção pelo vírus de Epstein-Barr (EBV) em virtualmente todos os casos de forma endêmica da doença. O EBV foi o primeiro vírus humano descoberto codificar miRNAs no genoma viral, agrupados em duas regiões distintas: *BamHI fragment H rightward open reading frame 1* (miR-BHRFs) e *BamHI-A rightward transcripts* (miR-BARTs). Embora extensivamente estudado no contexto de neoplasias epiteliais, o papel dos EBV miR-BARTs na patogênese de linfomas associados à infecção pelo EBV é essencialmente desconhecido. Este estudo tem como objetivo investigar os efeitos da supressão dos EBV miR-BART7 e miR-BART9 em células Akata EBV-positivas através da edição dirigida do genoma viral por CRISPR/Cas9. Ambos os mutantes gerados apresentaram baixos níveis de expressão dos miRNAs virais, demonstrando a eficácia da edição mediada por CRISPR/Cas9. A supressão de ambos miRNAs nos mutantes acarretou em uma diminuição drástica de viabilidade e proliferação celular, levando ao aumento da expressão de genes líticos virais. A análise do perfil protêomico dos mutantes demonstrou que a supressão do EBV miR-BART7 aumentou a expressão de diversas proteínas de ligação de RNA, enquanto a supressão de miR-BART9 levou ao aumento da expressão de DNA topoisomerases e proteínas associadas a via ubiquitina-proteossoma. Nossos resultados demonstram importantes papéis e mecanismos associados a expressão de EBV miR-BART7 e miR-BART9 no aumento da proliferação celular e manutenção da latência viral, sequestrando vias celulares importantes associadas ao ciclo lítico viral.

Palavras-chave: Vírus de Epstein-Barr, linfoma de Burkitt, microRNAs virais, ebv-miR-BART7, ebv-miR-BART9, CRISPR/Cas9, edição gênica.

Abstract

Burkitt Lymphoma (BL) is a highly aggressive B-cell non-Hodgkin lymphoma associated with the Epstein-Barr virus (EBV) infection in virtually all cases of its endemic African form. EBV was the first human virus found to encode viral miRNAs, clustered in two distinct regions of the viral genome: the BamHI fragment H rightward open reading frame 1 (miR-BHRFs) and the BamHI-A rightward transcripts (miR-BARTs). Although extensively studied in epithelial cancers, the role of EBV miR-BARTs on the pathogenesis of EBV-associated lymphomas is essentially unknown. Therefore, this study sought to investigate the effects of EBV miRs BART7 and BART9 suppression in Akata EBV-positive cells using CRISPR/Cas9-targeted mutagenesis. Overall, both Akata mutants harboring the edited EBV genomes exhibited low levels of viral miRNAs expression, demonstrating the efficacy of the CRISPR/Cas9-mediated knockdown of EBV miR-BART7 and 9. A reduction in cell viability, proliferation, and increased expression of viral lytic genes was found in both mutants. The knockdown of EBV miR-BART7 significantly increased the expression of several RNA binding proteins (RBPs), while the knockdown of EBV miR-BART9 increased the expression of DNA topoisomerases and ubiquitin/proteasome proteins. Our results unravel potential roles for EBV miR-BART7 and miR-BART9 increasing cell proliferation and maintaining viral latency by hijacking critical cellular pathways associated with the viral lytic cycle.

Keywords: Epstein-Barr Virus, Burkitt lymphoma, viral microRNAs, ebv-miR-BART7, ebv-miR-BART9, CRISPR/Cas9, knocking down.

Chapter I – Introduction

1 Cancer and infection

Cancer is a leading cause of morbidity and mortality worldwide, accounting for more than 9.5 million deaths in 2018 (1). The development of cancer is a multistage process known as carcinogenesis, usually taking years or even decades to develop in the cases of sporadic cancers affecting humans (2). Environmental factors such as diet, lifestyle, and occupational exposures play a crucial role in the etiopathogenesis of most cancers. Genetic factors also influence the risk of developing cancers, especially malignant neoplasms affecting people at young ages (3).

Environmental factors influencing the development of human cancers include a growing number of physical, chemical, and infectious agents, most of them potentially preventable (4). Among these are, for instance, the frequent intake of ultra-processed foods, tobacco and alcohol abuse, sedentary habits, and some infections. According to GLOBOCAN, about 2.2 million (15.4%) of all new cancer cases worldwide in 2018 were associated with infectious agents, notably *Helicobacter pylori*, followed by human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HBC), and Epstein-Barr virus (EBV) (5). Overall, viruses are the most important infectious agents associated with cancer development in humans, particularly in developing countries where the incidence of cancers attributed to infections is significantly higher (6,7).

Oncogenic viruses contribute to carcinogenesis by various mechanisms, including direct DNA damage/mutagenesis (*e.g.*, via viral integration into the host genome) and dysregulation of cellular pathways by viral products, culminating in increased cell survival, cell proliferation, and immune evasion (8,9). Infection by oncogenic viruses is necessary but insufficient to cause cancers because their etiopathogenesis includes additional constitutive and environmental risk factors. Malignancies induced by oncogenic viruses typically develop in chronic infection scenarios, arising many years or even decades after primary viral infection (8). Landmark studies on the role of oncogenic viruses in the development of human cancers include the seminal works on EBV, the first human oncovirus described infecting neoplastic cells in Burkitt lymphoma (10), discussed in more detail in the next section.

2 Burkitt lymphoma

In 1958, the British surgeon Denis Parsons Burkitt reported the first cases of an endemic sarcoma affecting children in equatorial Africa, usually shown as debilitating tumors growing around the jaw or facial bones (11). Several years later, this neoplastic disease was recognized as an aggressive form of non-Hodgkin B-cell lymphoma, named Burkitt lymphoma (BL). After attending a lecture by Dr. Burkitt on "The Commonest Children's Cancer in Tropical Africa," the British pathologist Michael Anthony Epstein and his mentees Yvone Barr and Bert Geoffrey Achong started to investigate BL-derived lymphoblasts using electron microscopy. Upon identifying viral particles in BL cells showing structural features equivalent to herpesviruses, Epstein and colleagues reported the first EBV description in 1965 (12,13).

BL is a highly aggressive non-Hodgkin B cell lymphoma composed of monomorphic, medium-sized B cells, found in extranodal sites or eventually mimicking acute leukemia (14). Three forms of this cancer are commonly recognized, named endemic, sporadic, and immunodeficiency-associated BL. These forms share morphologic and immunophenotypic features, but they differ regarding geographic distribution, clinical presentation, association with EBV infection, and chromosomal rearrangements involving the *MYC* proto-oncogene (15,16) (**Table 1.1**).

Endemic BL is essentially confined to equatorial Africa and accounts for up to 50% of all pediatric cancers diagnosed annually in the region (17). The average incidence of endemic BL in Sub-Saharan Africa was estimated at 1.21 cases per 100,000 children, corresponding to 3,900 new cases in 2018. African countries outside the malaria belt show BL incidence rates similar to those of developed countries; this data agrees with the occurrence of sporadic BL in the great majority of these cases (18). Endemic BL is predominant in male children aged 4 to 6 years, and jaw tumors are the most common clinical presentation of the disease. Other sites of involvement include the kidneys, liver, gonads, and breast, with relatively common cases of acute leukemia (19). The environmental factors associated with endemic BL include co-infection with malaria, arboviruses, and exposure to ingenol esters, present in some plants of the genus Euphorbia (22,23). Co-infection with malaria (*Plasmodium falciparum*) strongly increases the risk of developing endemic BL via synergistic mechanisms that culminate in the increase of EBV load and impairment of anti-viral immune surveillance (20).

Table 1.1 – Characteristic features of BL subtypes

	Endemic Burkitt Lymphoma (eBL)	Sporadic Burkitt Lymphoma (sBL)	Immunodeficiency-associated BL
Geographic distribution	Equatorial Africa	Worldwide	Worldwide
Incidence	Children	Children and adults	Adults
Sites	Jaws, facial bone, kidney, liver, gonads, and breast	Ileocecal region, lymphoid tissue in Waldeyer's ring, gonads, breast	Nodal, central nervous system
EBV infection	100%	20-30%	25-40%
Other environmental factors implicated	Malaria, arboviruses, *Euphorbia*	-	HIV infection
c-MYC breakpoint in t(8;14)(q24;32)	Far 5' (centromeric) of c-MYC (class III)	Exon and intron I (class I) and 5' (centromeric) of c-MYC (class UU)	Exon and intron 1 (class)
Predominant IGH's genes breakpoint in t(8;14)(q24;32)	VDJ region	Switch region	Switch region

Adapted from Leoncini & Stein, 2013 **(16)**.

Sporadic BL is the most common subtype in North America and Western Europe. In the United States, sporadic BL cases represent up to 30% of pediatric lymphomas and less than 1% of non-Hodgkin lymphomas in adults, with an estimated incidence of 0.4 cases per 100,000 people (21). Although relatively rare, sporadic BL is considered one of the most common types of pediatric lymphoma in the United States, predominant in male children and young adults with an average of 30 years of age (22). The sites of involvement frequently include bowel or mesenteric lymph nodes but can also involve the kidneys, pancreas, liver, spleen, and gonads. Bone marrow involvement is more commonly seen in progressive disease, with acute leukemia manifestations also admissible (19).

Immunodeficiency-associated BL is often linked to human immunodeficiency virus (HIV) infection, being much less frequent in other contexts, such as post-transplant lymphoproliferative disorders (PTLD). About 20% of all cases of BL occur in people living with HIV (PLHIV), accounting for up to 40% of the lymphomas associated with HIV infection. The

pathogenesis of HIV-associated BL is still unclear, but it may involve viral cooperation mechanisms involving HIV and EBV that culminate in cell transformation, proliferation, immunosuppression, and tumor development involving c-MYC-associated pathways (23). Immunodeficiency-associated BL typically presents as abdominal masses or localized lymphadenopathies; less frequently, the central nervous system and bone marrow might be the primary tumor site. PLHIV are more likely to present B symptoms (fever, night sweats, weight loss, itching, tiredness, and loss of appetite) and be diagnosed with advanced disease; hence, HIV-associated BL is a leading cause of death among this population (24).

There is insufficient data on the incidence and prevalence of BL in Brazil, but different areas have incidence rates similar to those observed in regions with endemic and sporadic forms of BL. During 1997-2007, a significant increase in the incidence of BL was observed in the northeast southeast regions of Brazil, with about 63% of the new cases associated with EBV infection (25). The number of non-Hodgkin lymphomas expected in the country during 2020-2022 was estimated for 6,850 new cases in men and 5,450 in women, with an incidence rate of 6.31 and 5.07 new cases per 100,000 men/women, respectively (26). Overall, lymphomas rank third among the most frequent causes of cancers in children in Brazil, representing 13.7% of the 12,600 new cases of pediatric cancer estimated for the year 2017 (29).

Chromosomal translocations involving the proto-oncogene c-MYC (MYC, gene ID: 4609) are critical events in the pathogenesis of BL in all forms of this disease, culminating in the overexpression of the c-MYC protein, an essential transcriptional regulator. These translocations cooperate with other oncogenic events to initiate BL tumorigenesis, and they can also occur sporadically during cancer progression in other lymphomas (27). The most common translocation found in BL is the reciprocal t(8;14)(q.24.1;q32.3), causing juxtaposition of the *MYC* gene sequence within enhancer elements of the *IGH* gene, encoding immunoglobulin heavy chain (28). Although the t(8;14) is present in almost 80% of BL cases, other recurrent chromosomal abnormalities are also found in BL (29).

EBV infection is associated with the immortalization of B cells and plays a vital role in the activity of several signaling mechanisms involved in B-cell biology, including *MYC*-associated pathways, the phosphatidylinositol 3-kinase (PI3K, gene ID: 5291), and transcription factor 3 (TCF-3, gene ID: 6929) signaling (30). The frequent association of BL

cases with EBV infection, notably in the endemic form, indicates a critical role of viral infection in the pathogenesis of this disease. The following section will discuss relevant mechanisms of EBV biology involved in the pathogenesis of EBV-associated lymphomas.

3 Epstein-Barr virus

Officially designated *human gammaherpesvirus type 4* (HHV-4), EBV is a ubiquitous virus that causes lifelong latent infection in over 90% of the human adult population worldwide. The EBV infection is regarded as carcinogenic to humans, and it is associated with a variety of human cancers, notably endemic BL and undifferentiated nasopharyngeal carcinomas (31). The phylogeny and evolution of EBV are closely related to viruses that infect non-human primates of the old-world (Cercopithecidae family monkeys), including herpesviruses from chimpanzees (*Pan troglodytes*) and rhesus monkeys (*Macaca mulatta*). For instance, the EBV and Rhesus lymphocryptovirus (LCV) share similarities regarding their genome sequences and genetic organization, as well as their life cycles (32,33).

The EBV genome consists of a linear, double-stranded DNA molecule of approximately 172kb and encodes around 100 viral proteins. The viral DNA is enclosed in an icosahedral nucleocapsid, immersed in tegument proteins, and surrounded by a lipidic envelope derived from the nuclear membrane of the infected cell. Upon infection, the viral genome circularizes into an episomal form, replicates by the host DNA polymerase during cell division, and exhibits a complex regulation of the viral gene expression (34).

The primary route for EBV transmission is via the saliva of an infected host and commonly occurs during childhood, where the infection is typically asymptomatic. Upon primary infection, the virus infects epithelial cells in the oropharynx and adjacent structures, culminating in the lytic cycle's activation, viral progeny production, and cytopathic effects in lytic-infected cells. Although the EBV entry into epithelial cells remains to be better understood, some *in vitro* studies have shown that the virus can infect epithelial cells using the polymeric immunoglobulin receptor (pIgR), exploiting immunoglobulin A (IgA) transport mechanisms via the formation of IgA-EBV complexes (35). Other mechanisms may involve glycoproteins present in the viral envelope that binds to a wide variety of cell membrane receptors, promoting the fusion of viral and cell membranes, followed by EBV entry via the endocytic route (36,37).

The epithelial cells are essential targets for productive lytic replication, fostering the viral shedding in saliva and allowing the transmission by the oral-oral route (38). The adhesion and entry of EBV particles in epithelial cells occur by binding the viral envelope transmembrane glycoprotein gp350 with the complement receptor 2 (CR2 or CD21, gene ID: 1380) (39). Alternatively, EBV can use the viral envelope transmembrane protein BMRF-2 or the gH/gL viral capsid complex to bind to the αvβ1, αvβ6, or αvβ8 integrins expressed in CR2-negative epithelial cells (40). The BMRF-2 and BDLF2 viral proteins also participate in rearrangements of cellular actin, increasing intracellular contacts and promoting the transmission of viral particles via cell-to-cell communication mechanisms (41).

While the classical model shows that EBV primary infection first occurs in oropharyngeal epithelial cells to subsequently infect regional naïve B cells, there is also sufficient evidence showing that the *naïve* B cells compartment in the oropharynx may also be a primary target of EBV infection (42,43). The virus infects tonsillar B cells via the interaction of the gp350/220 envelope glycoprotein complex with the CR2 and the Major Histocompatibility Complex class II (MHC-II) molecules, present on the B cell surface (44,45). EBV persists in a subset of resting memory B cells in a quiescent infection state, with its genome in a nuclear episomal form showing a restrictive gene expression program that characterizes the latent phase of the viral lifecycle (38). The virus establishes a lifelong persistent infection within its hosts, with a small proportion of EBV-infected memory B cells eventually reactivating to the lytic cycle, promoting the production and release of new virions into the saliva (46).

Figure 1.1 shows the main features of the EBV life cycle: lytic and latent. In the lytic cycle, the viral genome is duplicated hundreds to thousands of times using both viral and cellular replication machinery components. Viral DNA replication during the lytic cycle occurs at discrete sites in the nucleus of the EBV-infected cells known as viral replication compartments (VRC). It follows the expression of viral proteins, inhibition of the cell cycle, and substantial changes in the cellular microenvironment (47). The replication of the EBV genome during the latent cycle relies on chromosomal initiating factors known as origin recognition complex subunit 2 (ORC2, gene ID: 4999) and the chromatin licensing and DNA replication factor 1 (CDT1, gene ID: 81620) (48). During the latent cycle, the EBV genome is in

its episomal form, and the expression of viral products is limited to a set of genes that configures distinct viral latency programs (see Section 3.2).

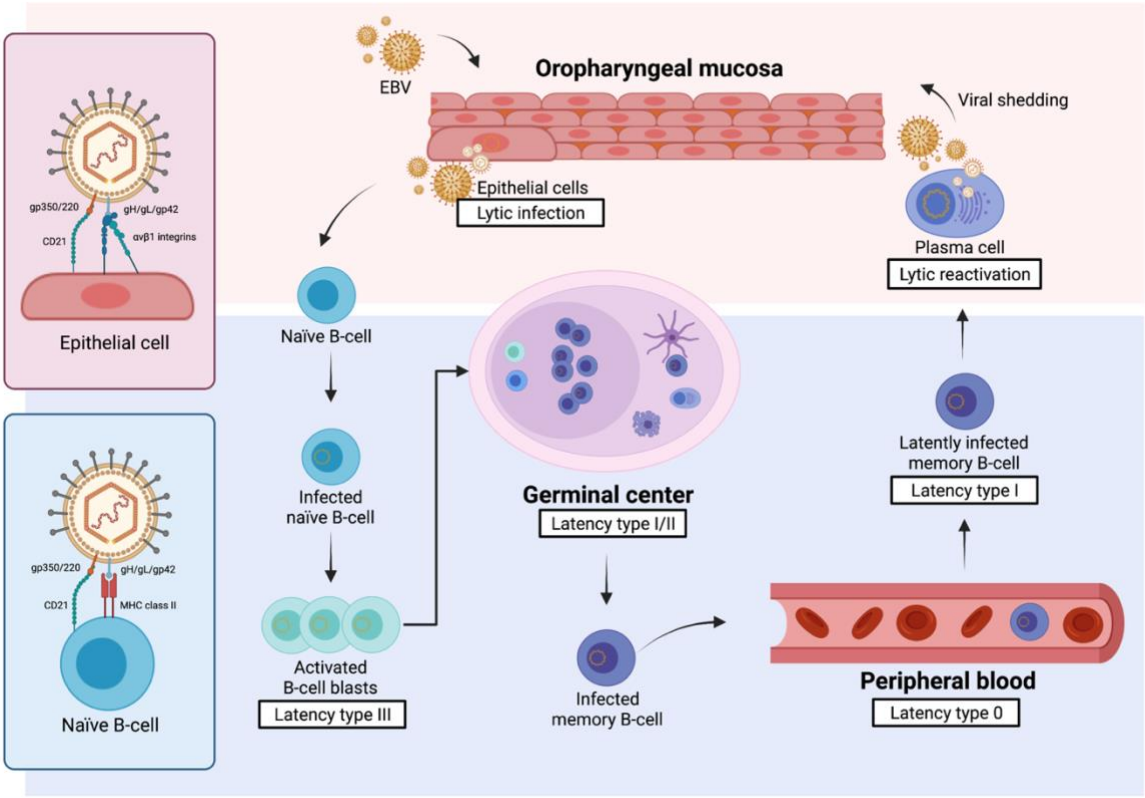

Figure 1.1 – Overview of the Epstein-Barr virus (EBV) life cycle. EBV's transmission by saliva infects the oropharyngeal mucosa epithelial cells through the interaction of viral glycoproteins (gp350/gp220 and gH/gL/gp42) with cellular surface molecules (CD21 and integrins). The EBV lytic cycle is typically found in epithelial cells and causes the production of virions that infects local *naïve* B-cells mediated by the viral glycoproteins gp350/gp220 and gH/gL/gp42, and cellular CD21 and MHC-class II molecules. The virus establishes viral latency (type III) in activated B-cell blasts, and more restricted profiles of viral expression latency genes (latency types I/II) are observed during B-cell maturation in the germinal center, favoring immune evasion. Also, quiescent memory B-cells latently infected by EBV may lack expression of any viral genes (latency type "zero"). Upon differentiation into plasma cells, memory B-cells present EBV lytic reactivation, culminating in new infectious virions and viral shedding via saliva. Created with BioRender.com.

3.1 EBV lytic cycle

The lytic cycle is required for EBV horizontal transmission, contributes to primary infection, and exerts an essential role in establishing latent infection (52). Overall, most EBV

genes are expressed during the lytic cycle, allowing replication of the viral genome, synthesis of structural viral proteins, capsid assembly, and production of new viral particles. Essentially, the EBV lytic products may have immediate-early (IE), early (E), or late (L) expression profiles, being expressed in a coordinated fashion, as shown in **Figure 1.2**.

Two EBV immediate-early genes, BZLF1 (a.k.a., Zta, Z, ZEBRA or EB1) and BRLF1 (a.k.a., Rta or R), induce the expression of early genes, promoting the synthesis of the viral DNA polymerase BALF5 and the protein complexes required for replication of the viral genome. Late lytic genes are then expressed, encoding viral structural proteins and glycoproteins necessary to assemble nucleocapsids and viral particles (49). Nucleocapsids are assembled in the nucleus of infected cells and exported to the cytoplasm. This process is mediated by the viral nuclear egress complex BFRF1/BFLF2 proteins, the cellular endosomal sorting complexes required for transport (ESCRT), and NEDD4-like ubiquitin ligases (50). Subsequently, the viral tegument proteins incorporate into nucleocapsids, which are then enveloped at the juxtanuclear membrane regions, followed by the release of newly-formed virions into the extracellular space (51).

The expression of the immediate-early genes BZLF1 and BRLF1 are required and sufficient to induce the EBV lytic cycle. Although complete lytic reactivation requires the presence of both Zta and Rta, only one of these immediately-early proteins is enough to promote and regulate the expression of the other one (52). The Zta protein can activate the Rta promoter via direct biding to Z-responsive elements (ZRE) (53). On the other hand, Rta binds into GC-rich sequences in the enhancer elements of several promoters that regulate the expression of EBV early lytic genes, but not to Zta (54).

The synergistic activation of EBV proteins Zta and Rta promote a robust induction of the lytic cycle, a result of the promoters' promiscuous activation, causing amplification of lytic cycle-inducing signals. Zta activates its promoter (Zp) directly due to binding to the ZIIIA and ZIIIB elements and the Rta promoter (Rp) via three ZREs (ZRE1, ZRE2, and ZRE3) present in the Rp region (55). Conversely, Rta activates its promoter by an indirect mechanism that involves interaction with the specific protein 1 (Sp1) transcription factor. Rta can also activate Zp indirectly due to the activation of transcription factors participating in the MAP kinases (MAPKs) and PI3K signaling pathways, binding to their ZII-responsive element (56).

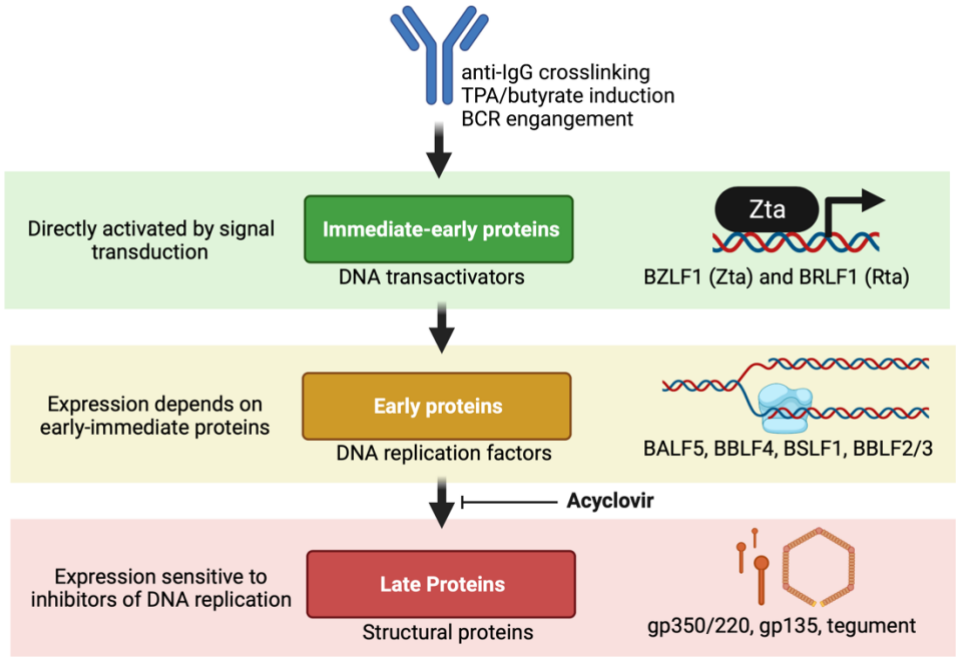

Figure 1.2 – Overview of the viral products expressed during the EBV lytic cycle. The viral Zta and Rta immediate-early proteins are transcription factors directly activated by several exogenous and endogenous factors, including the activation of B-cell receptors (BCR), calcium ionophores, phorbol esters (e.g., tetradecanoyl phorbol acetate, TPA), and the transforming growth factor β (TGFβ). The expression of Zta and Rta causes activation of early-immediate proteins (such as viral DNA replication enzymes, e.g., DNA polymerase, helicase) and cellular regulatory factors. Upon viral genome replication, EBV expresses late viral genes encoding structural and transport proteins necessary for assembly and transport of the viral capsid and glycoproteins. Acyclovir [9-(2-hydroxy ethoxy methyl)guanine inhibits EBV lytic cycle, blocking the replication of the viral DNA by hindering the activity of the viral DNA polymerase BALF5. Created with BioRender.com.

The replication of the viral genome during the lytic cycle requires the expression of essential viral proteins encoded by EBV, including Zta, the single-stranded DNA-binding protein BALF2, and five DNA replication enzymes: the viral BBLF4 helicase, BSLF1 primase, primase-associated factor BBLF2/3, BALF5 DNA polymerase, and BRMF1 DNA polymerase processivity factor (57). The replication of the EBV genome occurs in membraneless intranuclear assembly sites termed viral replication compartments (VRCs) and requires binding of Zta to responsive elements present in the oriLyt site, with subsequent recruitment of other viral proteins (58). During the viral DNA replication, EBV expresses several early lytic proteins involved in the regulation of nucleotide metabolism and synthesis (*e.g.*, BKRF3,

BXLF1 BORF2), along with some late-gene regulatory proteins (*e.g*., BcRF1, BDLF3.5, BFRF2), viral homologs of the cellular BCL-2 (*e.g*., BHRF1 and BALF1), and other viral proteins that exert diverse regulatory roles (*e.g*., BGLF4, BFLF2, and BFRF1) (49).

EBV can express up to 36 late genes that encode structural proteins (e.g., capsid, tegument, and viral glycoproteins), in addition to the homologous viral interleukin 10 (vIL-10) and other regulatory proteins with unknown functions (49). Late lytic genes play a fundamental role in the productive infection, participating in capsid assembly and maturation, viral DNA packaging into the capsid, viral adhesion, fusion, and internalization during *de novo* infection, as well as in immunoevasion mechanisms (56). Nonetheless, a substantial number of EBV late lytic genes remain uncharacterized.

3.2 EBV latent cycle

During the latent infection, EBV runs restrictive gene expression programs based on a subset of viral products to keep the viral episome and manipulate the expression of cellular genes. The viral episomes behave like a host chromosome, packed with cellular histones, replicated during the S phase, and segregated evenly to the daughter cells during the mitosis (47). This silent infection is advantageous for viral persistence since the latent phase of the EBV life cycle avoids the expression of potentially immunogenic viral products (59). The maintenance and stability of viral episomes in the nucleus of infected cells require only the expression of EBV EBNA1 protein and the replication origin (OriP) region, a 1.7-kb region of the viral genome consisting of a dyad symmetry (DS) and a family of repeats (FR) elements, both containing multiple EBNA1 bindings sites (60). The DS seems to play a role in replicating the OriP region, while the FR acts in conjunction with the EBNA1 protein to prevent the loss of EBV episomes during host cell proliferation (61,62).

EBV infects *naïve* B-cells releasing the linear viral DNA into the cytoplasm, followed by nuclear transport and circularization in the nucleus of infected cells. A pre-latent phase occurs shortly after viral infection and before the first cell division. During this pre-latent phase, EBV shows a promiscuous gene expression program that includes the expression of transcripts of both latent and lytic cycles, but it does not culminate in a productive infection (63,64). This transient program is commonly referred to as “abortive lytic infection” and occurs concomitantly with the activation of the W promoter (Wp), triggering the expression of

latency viral products (65). The pre-latent phase plays a crucial role in lymphomagenesis, especially in transforming B-cells (66).

After the pre-latent phase, EBNA2 and EBNA-LP proteins accumulated in the nucleus of infected cells, activating the latency-associated C promoter (Cp). EBNA-2 and EBNA-LP are the first nuclear antigens expressed in B-cells during the latent infection. EBNA2 is a transcriptional regulator of both viral and cellular genes and is essential for the immortalization of B cells, stimulating their cellular activation and proliferation and regulating the expression of viral latent membrane proteins (67). EBNA-LP also plays an essential role in the B-cell immortalization, cooperating with EBNA2 and facilitating the recruitment of cellular transcription factors (68).

The Cp activation by EBNA2 and EBNA-LP causes expression of the viral proteins EBNA1, EBNA3A, EBNA3B and EBNA3C (69). EBNA1 facilitates the replication of the EBV genome, while EBNA3 proteins antagonize cellular tumor suppressor proteins, increasing cell proliferation and survival. Concomitantly with the EBNAs expression, the viral microRNAs (miRNAs) clustered within the BamHI-A rightward transcripts (BART) and BamHI fragment H rightward open reading frame 1 (BHRF1) regions of the EBV genome, as well as the small non-coding RNAs (sncRNAs) EBER1 and EBER2, are abundantly expressed during viral latency (70).

Several *in vitro* models indicate that EBV latent cycle plays a fundamental role in the immortalization of naïve B cells, contributing to the pathogenesis of B cell lymphomas associated with EBV infection (66,71). B-cell immortalization involves the expression of six EBV-encoded nuclear antigens (EBNA1, 2, 3A, 3B, 3BC, and LP) and two latent membrane proteins (LMP1 and LMP2), in a coordinated gene expression program named latency type III (**Table 1.2**). Viral products in latency type III express before the first cell division and EBV lytic replication. However, this model excludes other viral latency programs identified in several EBV-associated lymphoid disorders (72).

The EBV-mediated immortalization and malignant transformation of B cells *in vivo* are complex processes that encompass all viral latency programs described as potentially relevant to developing different diseases associated with the viral infection. The ability to coordinate distinct latency viral gene expression programs is unique to EBV (73). **Table 1.2** shows the viral latency programs described so far (types I, II, III, and zero) and their associated EBV-induced lymphoproliferative diseases.

Table 1.2 – Most relevant viral products expressed by EBV during viral latency

	EBV Latency			
	Type I	**Type II**	**Type III**	**Type "zero"**
Main viral proteins	EBNA1	EBNAs 1 & LP 1; LMPs 1, 2A, 2B	EBNAs 1, 2, 3A, 3B, 3C, LP; LMPs 1, 2A, 2B	none
Viral miRNAs	miR-BARTs	miR-BARTs	miR-BARTs and mirBHRF1	miR-BARTs
Other ncRNAs	EBERs	EBERs	EBERs	EBERs
Associated diseases[1]	BL, PBL, PEL	cHL, DLBCL, GC, NPC, PBL, LG, PTDL	DLBCL, IM, LG, PTDL	BL

[1]Abbreviations: BL - Burkitt Lymphoma; cHL - classic Hodgkin Lymphoma; DLBCL - Diffuse Large B-cell Lymphoma; GC - Gastric Carcinoma; IM - Infectious Mononucleosis; LG - Lymphomatous Granulomatosis; MCD - Multicentric Castleman's disease; NPC - Nasopharyngeal Carcinoma; PBL - Plasmablastic Lymphoma; PEL - Primary Effusion Lymphoma; PTDL - Post-Transplant Lymphoproliferative Disease.

EBV-positive BL and primary effusion lymphomas (PEL) exhibit latency type I (expression of EBNA1, EBER1/2, and BART miRNAs), while classic Hodgkin's lymphomas (cHL) and plasmablastic lymphomas (PL) have type II latency (EBNA1, EBNA-LP, LMP1, LMP2A/B, EBERs and BART miRNAs). Immunodeficiency-associated lymphoproliferative disorders (IA-LPD), including diffuse large B cell lymphomas (DLBCL), and post-transplant lymphoproliferative disease (PTDL), show latency type III, in which all the EBV latent genes are expressed (74). The BART miRNAs are expressed in all forms of EBV latency, playing essential roles in viral life cycle control and pathogenesis of lymphomas.

4 EBV-encoded miRNAs

Herpesviruses are well-known for their ability to establish a lifelong infection in their hosts. Furthermore, another distinguishing feature includes the ability of these viruses to encode and express viral non-coding RNAs (ncRNAs). Viral ncRNAs are advantageous for regulating the viral life cycle, exerting a crucial influence in the biology and evolutive success of herpesviruses (75,76). The first viral miRNAs were discovered in 2004 for EBV, leading to the investigation and discovery of viral miRNAs in other viruses (77). Except for the Varicella-

Zoster virus (VZV), a substantial repertoire of viral miRNAs has been identified so far, mostly in human, simian, murine, and avian herpesviruses, as shown in **Table 1.3**.

Table 1.3 – Viral miRNAs encoded by herpesviruses

Viral Family	Viral Species	Host	# of Pre-miRs[1]
α-herpesvirus	Herpes simplex virus 1 (HSV-1)	Human	16
	Herpes simplex virus 2 (HSV-2)	Human	18
	Marek's disease virus (MDV)	Avian	14
β-herpesvirus	Human cytomegalovirus (HCMV)	Human	11
	Murine cytomegalovirus (MCMV)	Murine	18
γ-herpesvirus	Epstein-Barr virus (EBV)	Human	25
	Kaposi's sarcoma-associated herpesvirus (KSHV)	Human	12
	Rhesus lymphocryptovirus (rhLCV)	Simian	35
	Rhesus macaque rhadinovirus (RRV)	Simian	15
	Murine gammaherpesvirus-68	Murine	15

[1]Precursor miRNAs.

Initially, Pfeffer and colleagues reported five EBV miRNAs in human B-cells infected by the viral genotype B95.8, clustered in two distinct regions of the EBV genome: the BamHI fragment H rightward open reading frame 1 (miR-BHRFs 1, 2 and 3), and the BamHI-A rightward transcripts (miR-BARTs 1 and 2) (77). To date, 25 precursor miRNAs (pre-miRNA) encoded in the EBV BHRF1 and BART regions were described, resulting in 48 functional mature miRNAs (78). Interestingly, ebv-miR-BARTs locate within intronic regions in a viral genome segment that is extensively spliced and expressed in all forms of EBV latency programs (79).

To efficiently run viral latency, EBV mastered its control over gene protein by expressing viral-encoded miRNAs. The expression of viral regulatory proteins such as HPV early proteins E1 to E7, or HBV HBx are examples of common strategies used for the control of gene expression and life cycle, although a relatively inefficient strategy for the regulation of viral persistence since regulatory proteins are commonly cytopathic or immunogenic to the cells (80,81). Conversely, viral RNAs are non-immunogenic regulators and, therefore,

undetectable by the host immune system (82). Overall, viral-encoded miRNAs regulate the translation of both viral and cellular transcripts, influencing pathways related to the viral life cycle and cancer. The biogenesis of EBV-encoded miRNAs and the mechanisms involved in the functions of viral life cycle regulation and carcinogenesis will be discussed below.

4.1 Biogenesis, maturation, and mechanisms of action of viral miRNAs

A miRNA is a single-stranded RNA molecule, 19-24 nucleotides long. These molecules are encoded into introns of either protein-coding or non-coding genes and in exons of non-coding genes. The biogenesis of viral miRNAs follows the conventional cellular pathway described for endogenous miRNAs in eukaryotic cells. Essentially, viral miRNAs are transcribed by the RNA polymerase II, forming the primitive miRNA (pri-miRNA) cleaved by the Drosha-DGCR8 complex to produce a precursor miRNA duplex (pre-miRNA), which binds to the Exportin-5 protein into the cytoplasm of the infected cell. Subsequently, the pre-miRNA duplex is cleaved by the Dicer-TRBP complex, originating two mature miRNAs derived from the 3’ (3p) and 5’ (5p) strands that can be conjugated with Argonaut proteins to exert their functions (83).

Mature miRNAs bind to messenger RNAs (mRNAs) due to sequence complementarity of their seed region, a conserved heptameric sequence present in between the second and eighth nucleotides of the miRNA. The miRNA seed region can bind to the 3’-untranslated region (3’-UTR) of targeted mRNA transcripts. In the case of high complementarity, the miRNA binding causes the degradation of the target mRNA via the RNA-induced silencing complex (RISC), or its deadenylation mediated by the complex named C-C Motif Chemokine Receptor 4 and negative on TATA-less (CCR4-NOT). Low complementarity miRNA binding can cause translation repression due to reduced elongation or ribosomal drop-off (84). **Figure 1.3** summarizes the biogenesis, maturation, and effects of viral miRNAs.

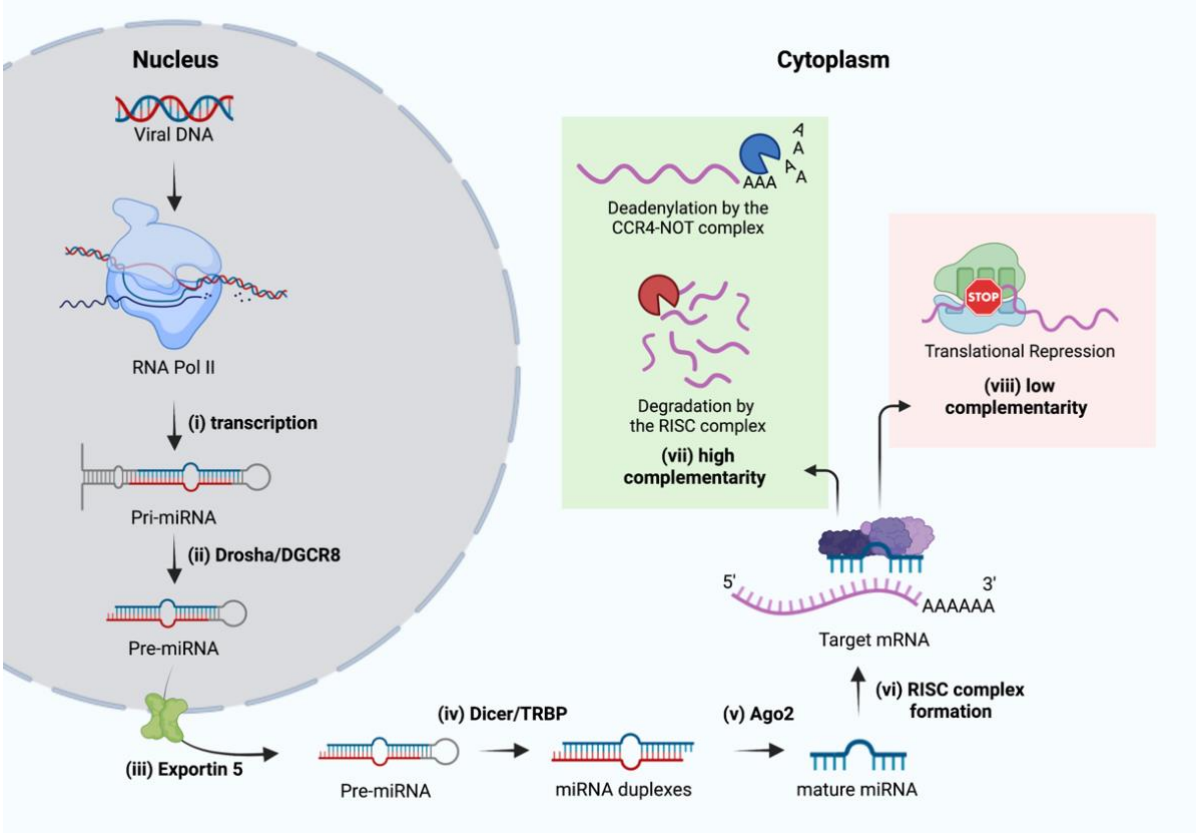

Figure 1.3 – Biogenesis, maturation, and effects of viral miRNAs. Viral miRNAs are transcribed by the RNA Polymerase II (i), forming a pri-miRNA loop that is cleaved by the Drosha-DGCR8 complex into pre-miRNA (ii), and exported to the cytoplasm via Exportin-5 (iii). The Dicer-TRBP complex processes the pre-miRNA into mature miRNAs (iv) that are recruited by the argonaute-2 protein (v), forming the RNA-induced silencing complex (RISC). Mature miRNAs bind to mRNAs to exert their functions. Seed sequences that exhibit high complementarity induce mRNA degradation via the RISC complex or deadenylation via the CCR4-NOT complex (vii). On the other hand, low complementary seed sequences still impact protein expression due to induction of ribosomal drop-off and translational repression (viii). Created with BioRender.com.

4.2 EBV-encoded miRNAs regulate the viral life cycle and cancer

EBV miRNAs are not required to produce viral particles during the lytic cycle, but there is evidence that they play a fundamental role in the viral pathogenesis, contributing to the establishment of a reservoir of latently infected cells (85). Some key roles of viral miRNAs include the regulation of the viral cycle, viral cytopathic effects, and the expression of immunogenic proteins (86). For instance, the ebv-miR-BART6-3p and ebv-miR-BART6-5p regulate the expression of Zta and Rta, inhibiting the lytic cycle (87), whereas ebv-miR-BART9

regulates the expression of the viral oncoprotein LMP1, modulating the survival and proliferation of infected neoplastic cells in EBV-associated nasal NK/T cell lymphomas (NKTCL) (88).

EBV miRNAs are also essential for the survival and transformation of B-cells in the early stages of viral infection, as demonstrated by the effects of ebv-miR-BHRF1 inactivation (89,90). Strikingly, ebv-miR-BHRF1 transcripts can promote the transformation of primary B lymphocytes and sustain BL cells in the absence of EBV viral oncogenes (91). Although the lack of the BART region in the EBV genome does not adversely affect the virus ability to immortalize B-cells, EBV miR-BARTs exert a significant influence on lymphomagenesis due to activities already described regarding the regulation of cell growth and survival, apoptosis, local immune response, microenvironment and viral latency (92–94).

The ebv-miR-BARTs are highly expressed in epithelial cancers, including nasopharyngeal and gastric carcinomas, playing putative roles in malignant transformation and tumor progression (95). On the other hand, miR-BARTs express at relatively reduced levels in lymphoid malignancies, and their impact on the development of lymphomas is mainly unknown (96). In 2011, Ramakrishnan and colleagues reported that the expression of miR-BART9 was associated with increasing levels of LMP1 (both mRNA and protein), and this was associated with increased cell proliferation in nasal NK/T-cell lymphomas (88). Furthermore, ebv-miRs-BART 7 and 9 reduce the expression of the *BCL6* gene in DLBCL, an event associated with modulation of the cell cycle, differentiation, apoptosis, and inflammation (97). These and other published results point towards relevant functions for EBV miR-BARTs on lymphomagenesis; nevertheless, the evidence available so far is still scarce.

5 Research Rationale and Objectives

This thesis seeks to address two main topics on the biology of EBV and their role in the pathogenesis of lymphomas: **(1)** the relevance and role of selected ebv-miR-BARTs in BL cell lines latently infected with EBV and **(2)** the role of early lytic proteins on the degradation of the B-cell receptor (BCR) during lytic reactivation induced by EBV lytic genes in EBV-positive and EBV-negative BL cell lines.

(1) EBV-encoded miRNAs are abundantly expressed during latency and participate in the modulation of viral life cycle phenomena and intracellular signaling pathways relevant to

carcinogenesis (98). Because EBV-encoded miRNAs are expressed in relatively low levels in lymphomas compared to EBV-associated carcinomas, their putative effects in lymphomagenesis may be neglected, and data on this topic are scarce (93,99). Hence, we aimed to investigate whether the expression of EBV miR-BART7 and miR-BART9 affects selected cellular phenotype parameters in vitro and crucial molecules of signaling pathways relevant to lymphoma development in humans.

We generated mutants for miR-BART7 and miR-BART9 by editing the EBV genome in Akata cell lines using the CRISPR/Cas9 technology. High genome editing efficiency and a significant reduction in the expression of EBV miR-BART7-3p and miR-BART9-3p were found in Akata-EBV/Cas9 mutants. Subsequently, we investigated the effects of knocking-down of EBV miR-BART7 and miR-BART9 on the viability, proliferation, gene, and protein expression of EBV-infected Akata cells. Overall, suppressing these viral miRNAs reduced cell viability and proliferation, increasing the expression of the lytic genes BZLF1 (Zta) and BLLF1 (gp350). EBV mutants also exhibited an increased expression of the immune checkpoint genes TIM3 and PDL1. The proteomics profiles of Akata-EBV/Cas9 mutants show the expression of several cellular proteins, including RNA binding Proteins (RBPs), DNA topoisomerases, and ubiquitin/proteasomes. These proteins and pathways can be closely associated with the regulation of the EBV lytic cycle.

(2) Following infection, EBV employs a transient lytic program before establishing latent infection. Although the viral latency products are commonly oncogenic, the viral lytic cycle also plays an essential role in developing and maintaining cancers associated with EBV infection, even though the mechanisms remain unclear. Some EBV early lytic factors might target cell-surface and intracellular BCR for ubiquitination and proteasomal degradation (100). We aimed to investigate whether the expression of early-immediate genes by EBV impact the expression and activity of the BCR. This preliminary study was a collaboration with Prof. Benjamin Gewurz from Harvard Medical School (Boston, Massachusetts, USA) during the Research Internship Abroad (BEPE) training in the USA sponsored by the São Paulo Research Foundation (FAPESP).

We developed a knockout panel for this study containing 30 immediate-early EBV genes edited by CRISPR/Cas9 in constitutively infected Akata cells. Akata cells harboring EBV mutants were then induced to lytic reactivation with IgG-crosslinking, and the Igα expression was assessed by flow cytometry. No differences in the Igα profile expression across the

mutants were observed. The induction of lytic reactivation of EBV mutants in Akata cells yielded a low percentage of gp350-positive cells (15-25%). A different approach was then defined, aiming to evaluate the effects of EBV early lytic genes on BCR expression.

Lentiviral vectors with a doxycycline-inducible expression cassette containing the ORFs of EBV early lytic genes were produced to transduce EBV-positive (P3HR1-ZHT/RHT) and EBV-negative (BJAB) cells. After induction of EBV early lytic genes with doxycycline, we found that the overexpression of BRRF1 (a viral transcript factor associated with the Rta-mediated activation of Zp) significantly reduced the levels of Igα in both EBV-positive and EBV-negative cells. Downregulation of Igα was confirmed by western blot; nonetheless, no differences in the expression of IgM, Stat2, and AIM2 were observed. Finally, we found that the overexpression of BRLF2 also induces Igα degradation in P3HR1-ZHT/RHT EBV-positive cells.

In conclusion, both studies presented herein led to the discovery of novel targets and pathways closely related to the viral biology and the pathogenesis of lymphomas associated with EBV infection. We found that EBV miR-BART7 and miR-BART9 increase cell proliferation and maintenance of the viral latency, hijacking critical cellular pathways associated with the lytic cycle induction. Finally, we found two EBV lytic genes involved in the BCR degradation during the lytic reactivation, unraveling exciting new roles for a subset of viral proteins that are still poorly understood.

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Chapter II – Epstein-Barr virus-encoded miR-BART7 and miR-BART9 regulate cell proliferation and viral latency in Akata cells

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Abstract

Burkitt Lymphoma (BL) is a highly aggressive B-cell non-Hodgkin lymphoma associated with the Epstein-Barr virus (EBV) infection in virtually all cases of its endemic African form. EBV was the first human virus found to encode viral miRNAs, clustered in two distinct regions of the viral genome: the BamHI fragment H rightward open reading frame 1 (miR-BHRFs) and the BamHI-A rightward transcripts (miR-BARTs). Although described in epithelial cancers, the role of EBV miR-BARTs on the pathogenesis of EBV-associated lymphomas is essentially unknown. Therefore, this study sought to investigate the effects of EBV miRs BART7 and BART9 suppression in Akata EBV-positive cells using CRISPR/Cas9-targeted mutagenesis. Overall, both Akata mutants harboring the edited EBV genomes exhibited low levels of viral miRNAs expression, demonstrating the efficacy of the CRISPR/Cas9-mediated knockdown of EBV miR-BART7 and 9. A reduction in cell viability, proliferation, and increased expression of viral lytic genes was found in both mutants. The knockdown of EBV miR-BART7 significantly increased the expression of several RNA binding proteins (RBPs), while the knockdown of EBV miR-BART9 increased the expression of DNA topoisomerases and ubiquitin/proteasome proteins. Our results unravel potential roles for EBV miR-BART7 and miR-BART9 increasing cell proliferation and maintaining viral latency by hijacking critical cellular pathways associated with the viral lytic cycle.

Keywords: Epstein-Barr Virus, Burkitt lymphoma, viral microRNAs, ebv-miR-BART7, ebv-miR-BART9, CRISPR/Cas9, knocking down.

1 Introduction

The Epstein-Barr Virus (EBV) is a ubiquitous gammaherpesvirus implicated in the pathogenesis of lymphoproliferative disorders and several human cancers, notably epithelial and lymphoid tumors. These include the undifferentiated nasopharyngeal carcinoma (NPC) and Burkitt lymphoma (BL), but also a variable number of cases of other diseases, such as the classical form of Hodgkin lymphoma (cHL), various non-Hodgkin lymphomas (NHL), notably in immunocompromised subjects, and a small fraction of gastric carcinomas (1). Epithelial cells and B-cells are the primary targets of EBV infection, and the virus latently infects memory B cells to establish a life-long, persistent infection of its human hosts. Several viral products expressed during the EBV latent cycle have known oncogenic activities, contributing to EBV-associated malignancies. These include the latent membrane proteins (LMPs) 1 and 2, the Epstein–Barr virus nuclear antigen 1 (EBNA1), the Epstein-Barr virus-encoded small RNAs (EBERs), and EBV-encoded microRNAs (miRNAs), described more recently (2).

EBV was the first human virus identified to encode miRNAs, clustered in two regions of the viral genome: the BamHI fragment H rightward open reading frame 1 (miR-BHRFs) and the BamHI-A rightward transcripts (miR-BARTs) (3). To date, 25 precursor miRNAs (pre-miRNA) were described, resulting in 48 mature viral miRNAs (4). The EBV miR-BARTs locates within intronic segments of the genomic locus encoding BART transcripts, a region that is extensively spliced and expressed in all forms of viral latency programs. EBV-encoded miRNAs are not necessary for producing viral particles; nonetheless, they play a crucial role in the viral lytic cycle and the pathogenesis of some EBV-associated cancers (5,6).

The miR-BARTs are highly expressed in epithelial cancers, such as the nasopharyngeal and gastric carcinomas, playing putative roles in malignant transformation, tumor growth, and metastasis (7). However, EBV miR-BARTs express at reduced levels in lymphoid malignancies, and their impact on lymphomagenesis remains to be appropriately elucidated (8). In 2011, Ramakrishnan and colleagues reported that the expression of EBV miR-BART-9 was associated with increasing levels of LMP1 (both mRNA and protein), and this was associated with increased cell proliferation in nasal NK/T-cell lymphomas (NKTL) (9). Furthermore, EBV miRs BART-7 and BART-9 reduce the expression of the *BCL6* gene in EBV-associated Diffuse Large B cell Lymphoma (DLBCL), which was associated with modulation of the cell cycle and differentiation, apoptosis, and local inflammation (10). Therefore, in this

study, we sought to assess the expression of EBV miRs BART-7 and BART-9 in a panel of lymphoid cells constitutively infected by EBV and investigate the *in vitro* effects of knocking down these viral miRNAs in EBV-positive Akata cells.

2 Material and Methods

2.1 Cell lines and other resources

Akata, MutuI, and P3HR1 cells were a gift from Benjamin Gewurz from Harvard Medical School. BC1, BC2, BC3, Daudy, IBL-1, Jiyoye, and Rael were a gift from Ethel Cesarman from Cornell University. All cell lines were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS; Sigma-Aldrich, St Louis, MO, USA) and 0.4% gentamicin (Thermo Fisher) at 37°C in 5% CO_2. EBV-positive Akata cell line with stable *Streptococcus pyogenes* expression was generated by lentiviral transduction and blasticidin selection. For the stable selection of EBV-positive Cas9-expressing clones, Akata cells were continuously cultured in a medium supplemented with 500 µg/mL geneticin (Thermo Fisher) and 10 µg/mL blasticidin (Thermo Fisher). All cells were confirmed to be free of mycoplasma (PCR-based method for detection) and authenticated by Short Tandem Repeat (STR) analysis using the GenePrint® 10 System (Promega, Madison, WI, USA; Supplementary Material, **Table S2.1**). A list of other research resources used in this study can be found in Supplementary Material (**Table S2.2**).

2.2 CRISPR/Cas9 edition of EBV miR-BARTs 7 and 9

2.2.1 Design and synthesis of CRISPR/Cas9 single-guide RNAs (sgRNAs)

The sgRNAs sequences used to target EBV miR-BARTs 7 and 9 were selected using the CRISPick tool (Broad Institute, **https://portals.broadinstitute.org/gppx/crispick/public**), from hairpin sequences retrieved from miRbase (**http://www.mirbase.org**). Customized DNA oligonucleotides with additional nucleotides for restriction enzyme cloning were synthesized with Thermo Fisher (sequences in Supplementary Material, **Table S2.3**). Oligonucleotides for sgRNAs resuspended in Tris-EDTA buffer (TE, 10 mM Tris, 0.1 mM EDTA, pH 8.0) were annealed using the T4 Ligase Reaction Buffer (New England Biolabs, Ipswich, MA, USA).

2.2.2 Production of lentiviral vectors for sgRNA expression

For the CRISPR/Cas9-mediated edition of EBV miRs BARTs 7 and 9, the recombinant vectors LentiGuide-Puro-BART7 and LentiGuide-Puro-BART9 were constructed by cloning annealed oligonucleotide sequences into the LentiGuide-Puro backbone (RRID: Addgene_52963, **http://n2t.net/addgene:52963**), a gift from Feng Zhang (11). Briefly, the backbone vector was digested with the restriction enzyme BsmBI (New England Biolabs), followed by gel purification using the QIAquick PCR & Gel Cleanup Kit (Qiagen, Valencia, CA, USA). The linearized vector and the sgRNAs heteroduplexes were ligated overnight at 16°C using T4 DNA ligase (New England Biolabs), and the ligation product used for the transformation of competent One Shot™ Stbl3™ cells (Thermo Fisher), plated on LB plates with 100 µg/mL ampicillin and left to grow overnight at 37°C. Transformed bacteria colonies subjected to plasmidial isolation and purification had their constructs sequences confirmed by Sanger sequencing using the hU6.F primer (Supplementary Material, **Figure S2.1**).

Lentiviral particles harboring the sgRNAs for CRISPR/Cas9 edition of EBV BARTs 7 and 9 were obtained after transfection of HEK293FT cells with the LentiviralPuro-based constructs and the packaging vectors psPAX2 (RRID: Addgene12260; http://n2t.net/addgene:12260) and VSVG (RRID: Addgene_14888; **http://n2t.net/addgene:14888**). The transfection was carried out with Lipofectamine™ 3000 (Thermo Fisher) in 6-well plates; DNA-liposome complexes were suspended in OptiMEM™ (Gibco), homogenized, and then added dropwise into each well containing attached HEK293FT cells cultivated in DMEM medium (Gibco), afterward incubated for 24h at 37°C in 5% CO_2. The transfection media was then replaced with fresh RPMI; the lentiviral particles were then collected at 48 and 72 h post-transfection, filtered through a 0.45 µM PES syringe filters, and directly used for lentiviral transduction.

2.2.3 Lentiviral transduction in EBV-infected Akata cells expressing Cas9

EBV-positive Akata cells expressing Cas9 (Akata-EBV/Cas9) were transduced with fresh stocks of lentiviral particles harboring the sgRNA vectors for CRISPR-Cas9 edition of EBV miRs BARTs 7 and 9. After 48 h post-transduction, fresh RPMI medium was supplemented with 10 µg/mL puromycin (Invitrogen) for cell selection. Stably transduced cell lines obtained after 2 weeks (Supplementary Material, **Figure S2.2**) were then kept in RPMI medium supplemented with 10% FBS, 1 µg/mL puromycin, 500 µg/mL geneticin, and 10 µg/mL blasticidin.

2.2.4 T7 endonuclease I assay and TIDE analysis to evaluate CRISPR/Cas9 edition

The T7 endonuclease I (T7EI) assay was used to assess the efficiency of the CRISPR/Cas9 edition of the EBV genome (12). Briefly, stably transduced Akata-EBV/Cas9 cells subjected to the edition of the viral EBV miR-BARTs 7 and 9 were cultivated, and the genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega). Specific primers for amplification of a region flanking the predicted target sites for CRISPR/Cas9 edition were used designed and used for PCR amplification (Supplementary Material, **Table S2.3**) with the Platinum™ High Fidelity Taq DNA Polymerase (Thermo Fisher) enzyme. The PCR product was separated into 1.5% agarose gels and purified using the QIAquick PCR & Gel Cleanup Kit (Qiagen). Purified PCR products (300 ng) were then denatured at 95°C for 5 min and allowed to cool at room temperature to form heteroduplexes in a reaction mix containing nuclease-free water and NEBuffer2.1 1x (New England Biolabs). The T7 endonuclease I (New England Biolabs) was then added to the heteroduplexes and incubated for 30 min at 37°C. DNA fragments were separated on 1% agarose gel, and the cleavage products were evaluated using a Reverse Mass DNA Ladder in Gel Loading Dye Orange (New England Biolabs).

We used the Tracking of Indels by DEcomposition (TIDE) analysis to determine the spectrum and frequency of targeted mutations generated in Akata-EBV/Cas9 cells by CRISPR/Cas9, following previously published instructions (13). Genomic DNA was amplified, and the PCR product was purified as previously described, then submitted to Sanger sequencing on a 3500 Series Genetic Analyzer (Thermo Fisher) device. The samples sequenced in parallel using both forward and reverse primers (indicated in Table S2) had their sequence trace files uploaded to the online TIDE tool (**https://tide.nki.nl**) for analysis of the indel/HDR spectrum.

2.3 Assessment of expression of human and viral genes by qPCR

Total RNA was isolated using the TRIzol™ (Invitrogen) reagent, followed by purification with the Qiagen RNeasy column (Qiagen), treatment with DNAse (Sigma-Aldrich), and quantification by spectrophotometry using the NanoDrop® device (Thermo Fisher). Reverse transcription to first-strand complementary DNA (cDNA) was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher), according to the manufacturer's instructions. To detect EBV miRNAs, custom reverse transcription primers (Canopy Biosciences, St. Louis, MO, USA) were added to total RNA samples before cDNA synthesis.

The quantitative real-time Polymerase Chain Reaction (qPCR) to assess gene expression was performed on the 7500 Fast Real Timer PCR system (Applied Biosystems®) using the GoTaq® qPCR Mix (Promega) with the following cycling conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. SNORD47 and SNORD48 or HSP90 and RPS13 were used as reference endogenous controls for miRNA and mRNA expression, respectively. Relative expression levels were calculated using the comparative Ct method. The expression of the viral lytic proteins BZLF1 (*i.e*., Zta, gene ID: 3783744) and BLLF1 (*i.e*., gp350, gene ID: 3783713) were assessed in parental Akata-EBV/Cas9 (WT) and its mutants. To access putative effects of knocking down EBV miR-BARTs 7 or 9 in the expression of molecules involved in immune checkpoint regulation, a panel consisting of the following targets was used: B7-H3 (gene ID: 80381), B7-H4 (gene ID: 79679), BTLA (gene ID: 151888), CTLA-4 (gene ID: 1493), LAG3 (gene ID: 3902), PD-L1 (gene ID:29126), PD-L2 (gene ID: 80390), TIM-3 (gene ID: 84880) and VISTA (gene ID: 64115) (14). The primers sequences and qPCR conditions for these assays are indicated in **Table S2.3**

2.4 Luciferase reporter assays

The activity of EBV miR-BARTs 7-3p and 9-3p was evaluated in Akata-EBV/Cas9 cells by luciferase reporter gene assay with constructs designed based on the psiCheck-2 backbone vector (Promega), which encodes genes for two luciferase enzymes: the renilla luciferase (RLuc, experimental reporter), and firefly luciferase (FLuc, control reporter). 3'-UTR sequences (Supplementary Material, **Table S2.3**) with miRNA-binding sites and the respective negative controls (scramble, SCR) were cloned into the psiCheck-2 backbone vector according to manufacturer's instructions. Furthermore, a Luciferase reporter assay to assess the NFκB activity was performed using a pcDNA3.1-NFκB-luc (Firefly) construct, a gift of Ethel Cesarman (Weill Medical College, Cornell University, NY, USA), and the pGL4.73 (hRluc/SV40, renilla) vector (Promega). The pcDNA3.1-NFκB-luc contains a sequence reporter with the luciferase gene under the control of a kappa enhancer element. Akata-EBV/Cas9 cells expressing psiCheck-2-BART7-3p, psiCheck-2-BART9-3p, pcDNA3.1-NFκB-luc, and the pGL4.73 were generated by transfection with Lipofectamine™ 3000 (Thermo Fisher). The firefly and renilla luciferase activity assessment were conducted 48h post-transfection using the Dual-Glo® Luciferase Assay System (Promega), according to the manufacturer's protocol and the GloMax® Discover Microplate Reader (Promega). Normalized ratios were obtained by dividing

the luciferase activities of the reporter by the control, and the results were expressed in terms of arbitrary Relative Luciferase Units (RLU).

2.5 Cell viability and proliferation in vitro

The MTS-based assay was performed to assess cell viability and proliferation using the CellTiter 96® Aqueous One Solution (Promega, Madison). Briefly, $2x10^3$ cells were seeded onto 96-well plates in complete medium and 20 µL of the CellTiter reagent. The cells were incubated for 4h to assess cell viability, and for 4, 24, 48, and 72 h to assess cell proliferation *in vitro*. After incubation, the formazan dye produced by viable cells was quantified by measuring the absorbance at 490 nm on the Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.6 Proteomic analysis

Whole-cell lysates from Akata-EBV/Cas9 WT and mutants were extracted using a detergent-free urea buffer containing 8 M urea, 75 mM NaCl, 50 mM Tris-HCl pH 8.2, 50 U/mL benzonase, 2 mM $MgCl_2$, and protease inhibitors (cOmplete™, EDTA-free Protease Inhibitor Cocktail; Roche Life Science, Indianapolis, IN, USA), as previously reported (15). Briefly, cell pellets were washed twice with DPBS, resuspended in lysis buffer (100 µL in $1x10^6$ cells), and sonicated (30 sec, 5 sec on and 5 sec off) at 20% amplitude with the Vibra-Cell™ VCX 750 (Sonics & Materials Inc., Newtown, CT, USA). Samples were then incubated at 4°C for 30 min and centrifuged at 16,000 x *g* for 30 min at 4°C. The supernatant was collected and quantified using the Bio-Rad Protein Assay Kit and the Quick Start Bovine Serum Albumin Standard Set (Bio-Rad) and stored at -80°C until use.

Protein samples (50 µg) were diluted in 50 mM ammonium bicarbonate buffer (AmBic, NH_4HCO_3), 0.2% RapiGest SF (Waters™, Milford, MA, USA), and incubated for 60 min at 37°C. After surfactant incubation, 100 mM dithiothreitol (DTT, 2.5 µL; Sigma-Aldrich) and 300 mM 2-iodoacetamide (IAA, 2.5 µL; Sigma-Aldrich) were added into the samples, followed by incubation for 30 min at room temperature. Samples were then digested with Sequencing Grade Modified Trypsin (0.1 µg/µL, Promega) for 16 h at 37°C. Upon digestion, reactions were acidified with 5% trifluoroacetic acid (TFA, v/v) and incubated for 90 min at 37°C to stop proteolysis. Samples were centrifuged at 16,000 x *g* at 4°C for 30 min to remove insoluble debris. The supernatant containing soluble peptides was collected and dried overnight using

the Vacufuge® Concentrator Plus (Eppendorf, Hamburg, HH, Germany). Peptides were desalted, purified, and concentrated using the Pierce™ C18 Spin Columns (Thermo Fisher) according to the manufacturer's instructions.

2.6 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Mass spectrometry was performed to identify and quantify differentially expressed proteins in Akata-EBV/Cas9 mutants. The obtained peptides were subjected to MS followed by sequencing analysis using a liquid chromatography-tandem mass spectrometry system consisting of the UltiMate 3000 LC liquid nanochromatography system (LC Packings DIONEX, Sunnyvale, CA, USA) coupled to the Q-Exactive™ Plus (Thermo Fisher). LC-MS/MS was conducted according to parameters previously described (16). Expressed proteins were identified with the PatternLab software version 4.0.0.84 (17) using the UniProt proteome of *Homo sapiens* (UniProt ID: UP000005640) as reference. Fragment mass tolerance was set to 40 ppm with a false discovery rate (FDR) of ≤1%. Identified proteins were submitted to parsimony analysis, and metabolomic data was processed using the MetaboAnalyst (**https://www.metaboanalyst.ca)** web-based tool (18).

3 Results

3.1 Expression of EBV miRs BARTs 7 and 9 in human lymphoma cell lines

The expression levels of EBV miR-BARTs 7 and 9 in a panel of human-derived lymphoma cell lines were assessed by qPCR to get insights into their relevance in EBV-induced lymphomagenesis. There was substantial heterogeneity in the expression of the viral miRNAs across the cell lines evaluated, with Rael (BL), Jiyoye (BL), and BC-1 (PEL) exhibiting the highest levels of viral miRNAs expression compared to the Akata EBV/Cas9 cell line, used as reference (**Figure 2.1**). In a preliminary experiment, we observed that the Akata EBV/Cas9 cell line expresses low levels of EBV miR-BARTs 7-5p and 9-5p relative to the expression of their 3p forms (data not shown).

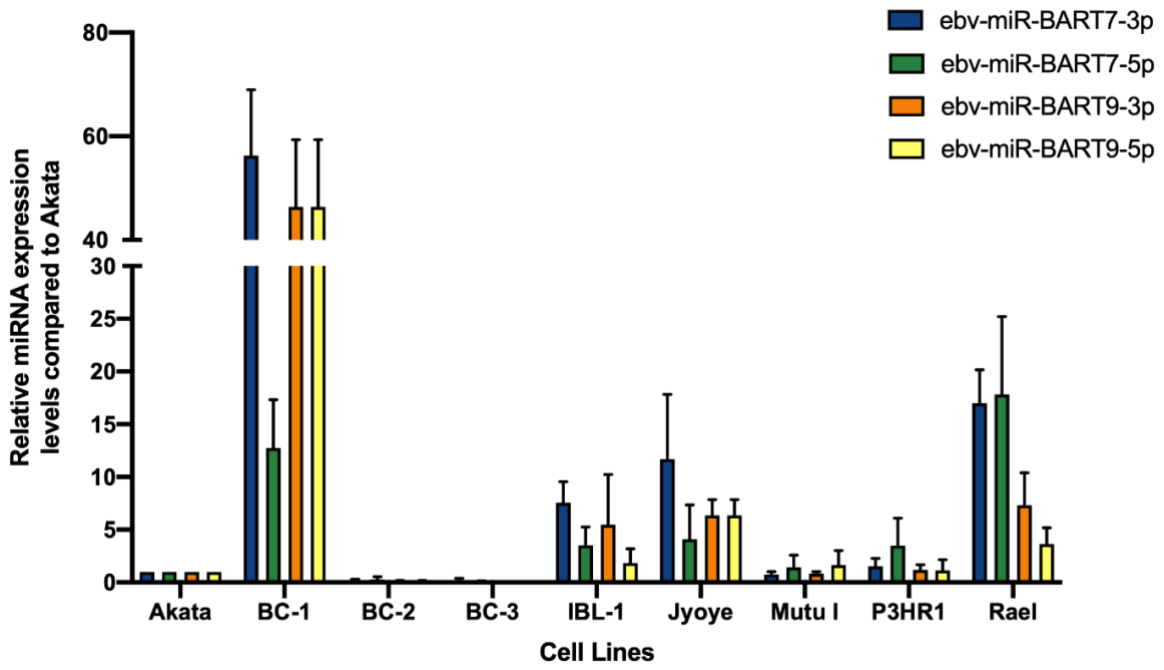

Figure 2.1 – Expression of EBV miRs BARTs 7 and 9 in human-derived lymphomas associated with EBV infection. BC3 (EBV-negative PEL) and Akata EBV/Cas9 are negative control and reference (expression = 1), respectively. The human miRNAs Snord47 and Snord48 were used as endogenous controls. Data expressed as mean ± SD.

3.2 Validation of Akata EBV/Cas9 knocked down for miRs BARTs 7 and 9

We sought to inhibit the expression of EBV miR-BARTs 7 and 9 using CRISPR/Cas9 technology to edit the EBV episomes in Akata EBV/Cas9 cells. These viral miRNAs locate within intronic regions of the BART cluster 2 (**Figure 2.2A**). Two sgRNAs for each miR-BART were designed with different sequences targeting the 5'-Dicer processing sites to impair the miRNA processing to generate their respective mature miR-BART. DNA sequencing of the flanking region within the EBV miR-BARTs 7 and 9 was conducted using the primers indicated in **Table S2.2**. Cas9/sgRNA-induced deletions were observed for both sgRNAs designed for their respective EBV miR-BART (**Figure 2.2B**). The T7 endonuclease I (T7EI) mismatch cleavage assay was used to validate the CRISPR/Cas9-mediated mutagenesis, and products ranging from 200 to 300 bp were found in EBV miRs BARTs 7 and 9 mutants following the T7EI treatment (**Figure 2.2C**).

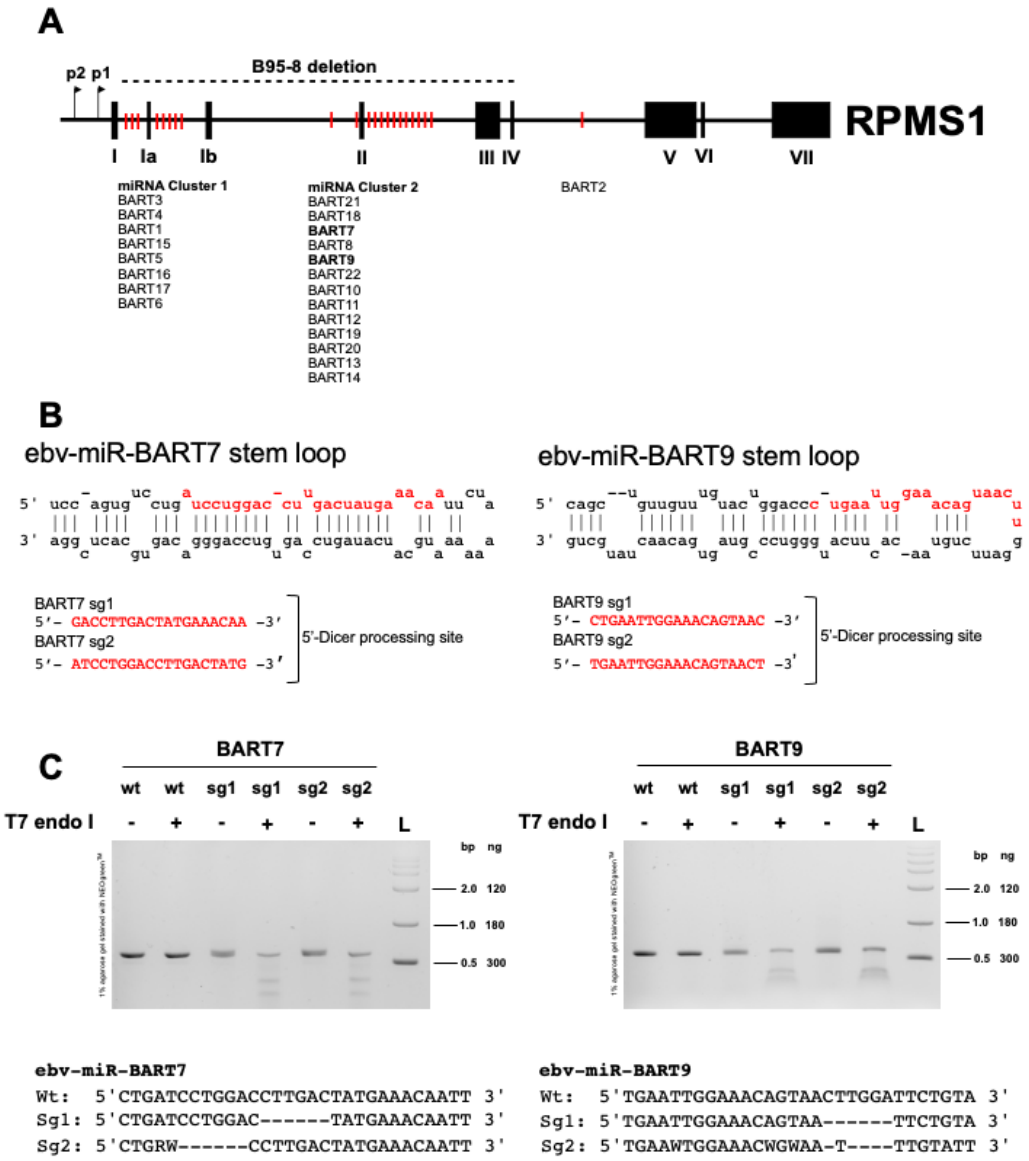

Figure 2.2 – (A) Schematic representation of the EBV miR-BARTs clusters within the intronic region of the BART transcripts. (B) Sequences of the pre-miRNAs and sgRNAs designed for Cas9/sgRNA-directed mutagenesis of EBV miR-BART7 and miR-BART9. Both sgRNAs target the 5'-Dicer processing site, impairing the maturation of pre-miRNAs. (C) T7EI assay and sequence analysis of Akata WT and mutants after transduction with sgRNAs and stable selection.

The Tracking of Indels by Decomposition (TIDE) platform, used to estimate the efficiency of CRISPR/Cas9 edition and the profile of indels in the Akata EBV/Cas9 mutants, demonstrated a high genome editing efficiency for both sgRNAs designed, ranging from 72 up to 83.4% (**Figure 2.3**). The decomposition analysis showed that deletions spanning 1-6 nucleotide(s) were the most frequent mutations found in cells transduced with the lentiviral vectors harboring the designed sgRNAs, with a low percentage of insertions or large deletions

(**Figure 2.3**). These data demonstrate that the designed sgRNAs efficiently caused deletions in the targeted sequences of EBV miRs BARTs 7 and 9, impacting their maturation.

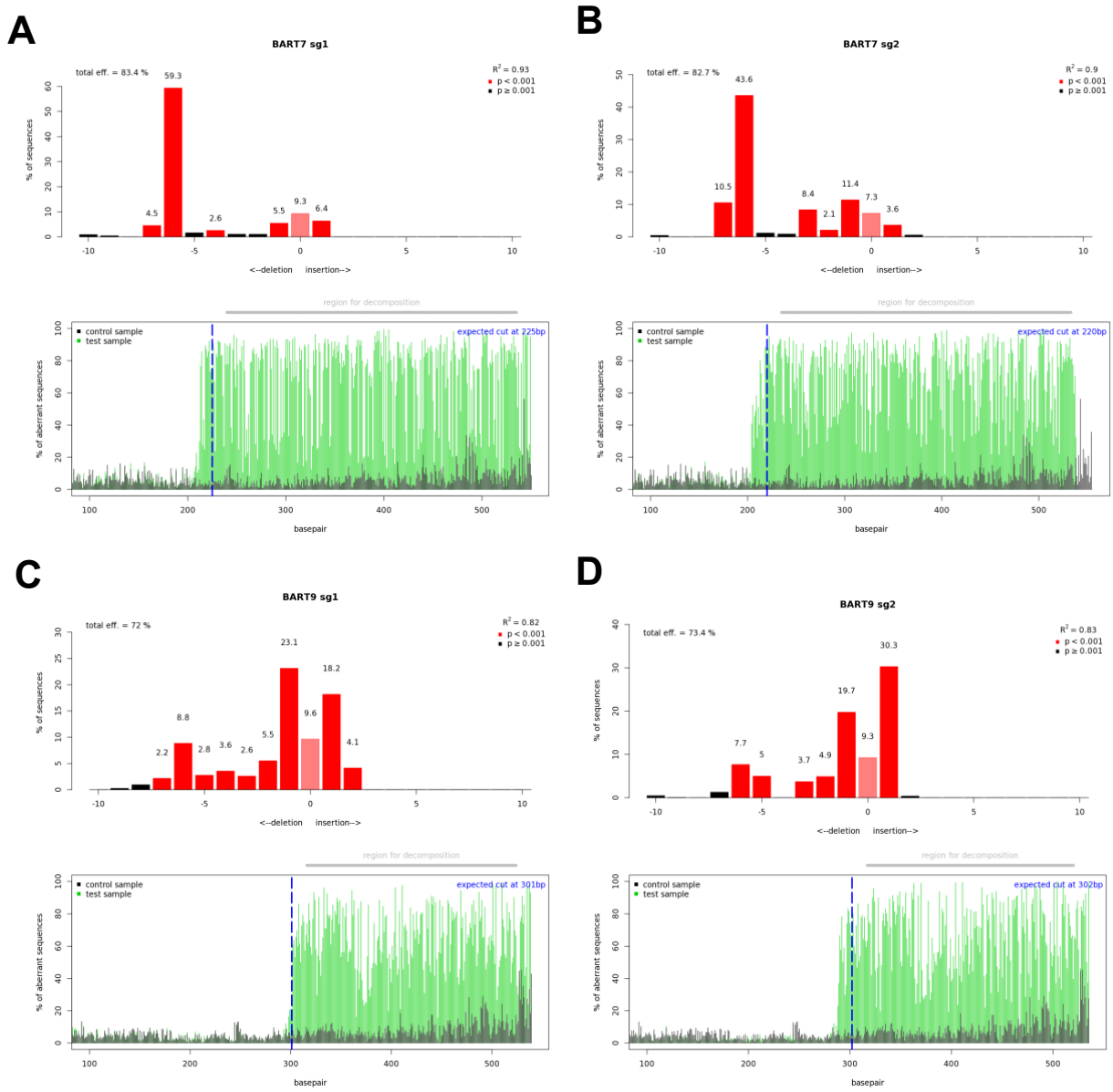

Figure 2.3 – Genome editing efficiency and INDEL profiles of Akata mutants using the Tracking of Indels by Decomposition (TIDE) analysis. Sequences of (**A**) ΔBART7_sg1, (**B**) ΔBART7_sg2, (**C**) ΔBART9_sg1 and (**D**) ΔBART9_sg2, compared to Akata WT.

3.3 Expression of miRs BARTs 7 and 9 in Akata EBV/Cas9 cells

The expression of EBV miR-BARTs 7-3p and 9-3p was evaluated in Akata EBV/Cas9 mutants by RT-qPCR. As expected, the mutants transduced with either of their specific sgRNAs showed a significant reduction in the levels of the viral miR BART when compared to

the Akata EBV/Cas9 wild-type cells, and **Figure 2.4A** shows that higher levels of inhibition observed in the ΔBART7_sg2 and ΔBART9_sg2 mutants.

The CRISPR/Cas9-mediated edition of EBV miR-BARTs 7 and 9 was also validated by luciferase reporter assay using the constructs psiCheck-2-BART7-3p and psiCheck-2-BART9-3p, which harbor 3'-UTR sequences targeted by the respective EBV miRNAs. Mutants for either miR-BARTs display an increased luciferase activity when compared to the control (**Figure 2.4B**), indicating a reduction in the expression of the EBV miRNAs targeted. In agreement with the qPCR results, higher levels of miRNA inhibition were found in mutants for miR-BARTs 7 and 9 (both with sg2).

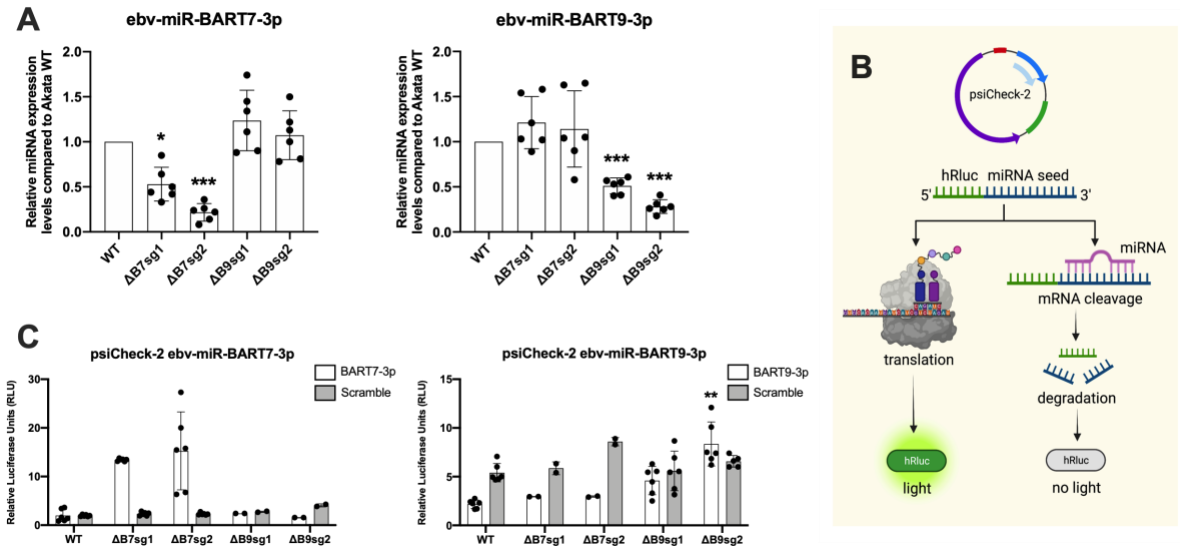

Figure 2.4 – Effects of CRISPR/Cas9-targeted mutagenesis on the expression of EBV miR-BART7 and miR-BART9 in Akata mutants. (A) Relative expression of EBV miR-BART7-3p and miR-BART9-3p in Akata mutants assessed by RT-qPCR. The human miRNAs Snord47 and Snord48 were used as endogenous controls. *p=0.0003 and ***p<0.0001 when compared to Akata WT; unpaired two-sample t-test. (B) Mechanism of psiCheck-2 luciferase reporter vector. (C**)** Luciferase activity in Akata WT and mutants transfected with report vectors for EBV miR-BART7-3p and miR-BART9-3p. **p=0.00017 when compared to Akata WT; one-way ANOVA followed by Dunnet's test.

3.4 Cell viability and proliferation

The MTS assay was used to assess the effects of knocking down either the EBV miR BART-7 or BART-9 on the viability and proliferation of the Akata EBV/Cas9 cells cultivated *in vitro*. The CRISPR/Cas9-mediated edition of both EBV miR-BARTs caused a significant decrease in cell viability (**Figure 2.5A**). Although not statistically significant, the proliferation

rates of the Akata EBV/Cas9 mutants were reduced compared to the wild-type control (**Figure 2.5B**). These results suggest that both EBV miRs BARTs 7 and 9 contribute to the viability and the proliferation rates of the BL cells in the Akata EBV/Cas9 cell line model used.

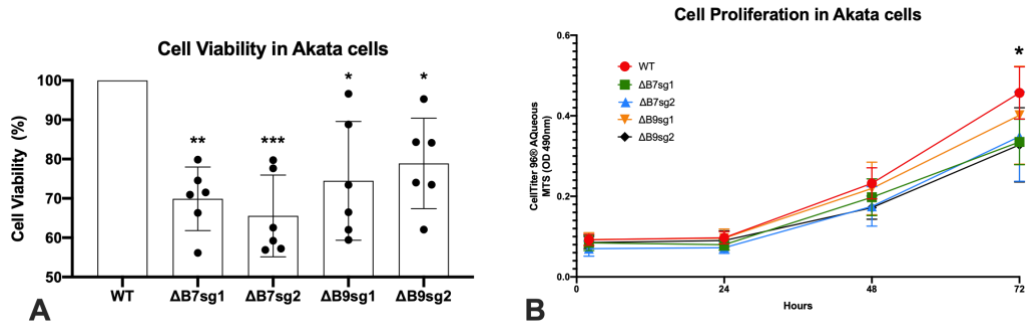

Figure 2.5 – Cell viability and proliferation in Akata WT and mutants assessed by the CellTiter 96® AQueous One Solution Cell Proliferation Assay. (A) Cell viability in Akata WT and mutants; *p=0.0001, **p=0.0009 ***p<0.0001 when compared to Akata WT cells; one-way ANOVA followed by Dunnet's test. (B) Cell proliferation in Akata WT and mutants following 4, 24, 48, and 72h after plating. *p=0.0320, ΔBART9_sg2 when compared to Akata WT; one-way ANOVA followed by Dunn's test.

3.5 NF-κB activity

Because EBV infection is known to upregulate the NF-κB signaling pathway with critical consequences for EBV-mediated carcinogenesis, notably lymphoma development (19,20), the NF-κB activity was assessed with a luciferase reporter assay in Akata EBV/Cas9 cells (wildtype and mutants). We used a recombinant vector based on the pLVX-IRES-ZsGreen1 backbone (Takara Bio Inc., San Jose, CA, USA) to constitutively express EBV LMP1, previously constructed by our research group, here referred as ZsGreen-LMP1 (21). As expected, the cells transfected with this vector exhibited a significant increase in the NF-κB activity induced by LMP1, compared to basal activity observed for the Akata EBV/Cas9 wild-type cells. There was no difference in the levels of NF-κB activity in the EBV miRs BARTs 7 and 9 mutants compared to the negative control (**Figure 2.6**). Thus, the knockdown of both EBV miR-BARTs 7 and 9 did not affect the expression of NF-κB in Akata-EBV/Cas9 cells.

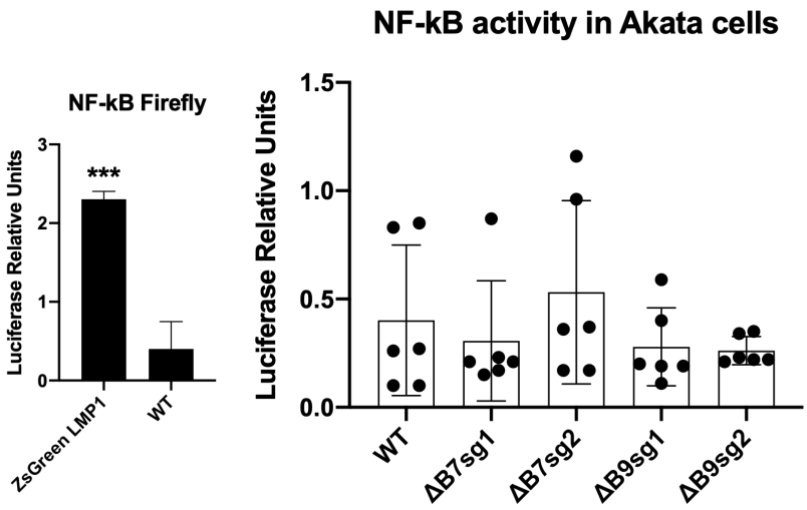

Figure 2.6 – Expression of NF-κB in Akata WT and mutants. (A) NF-κB luciferase reporter assay in Akata WT and mutants. Akata WT transfected with the vector ZsGreen LMP1 used for positive control.

3.6 Expression of EBV and human immune checkpoint genes

To gain insight into the effects of downregulating the EBV miR-BARTs 7 and 9 in our model, we evaluated the transcriptional levels of the EBV lytic genes BLZF1 and gp350 and selected human genes encoding proteins involved in immune checkpoint regulation. There was a remarkable increase in the expression of the EBV lytic genes BZLF1 and gp350 in the Akata-EBV/Cas9 mutants for viral miRs BARTs 7 and 9 compared to the non-edited (WD) control (**Figure 2.7A** and **Figure 2.7B**). Considering a previously established qPCR panel of immune checkpoint genes including B7-H3, B7-H4, BTLA, CTLA-4, LAG3, PD-L1, PD-L2, TIM-3, and VISTA (14), only TIM-3 and PD-L1 were found to be expressed at detectable levels in the Akata-EBV/Cas9 cells (data for undetected gene targets not shown). We found a significant increase in the expression of TIM-3 and PD-L1 in Akata-EBV/Cas9 cells knocked down for EBV miRs BARTs 7 (sg1) and 9 (sg2) (**Figure 2.7C** and **Figure 2.7D**). For PD-L1, the expression levels found in the cell line Rael were used as reference control, as the non-mutated Akata-EBV/Cas9 cells did not express this checkpoint regulator at detectable levels in our assays.

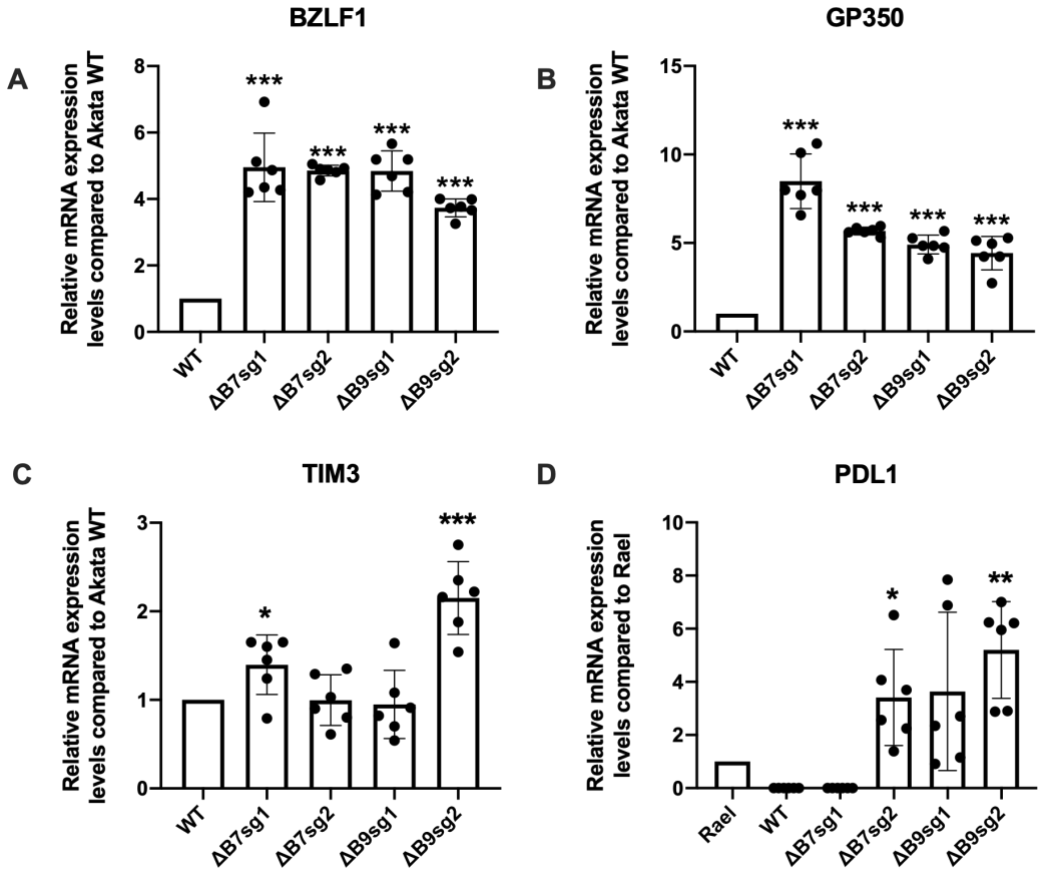

Figure 2.7 – Expression of the viral lytic genes (A) BZLF1 and (B) GP350 by RT-qPCR. Expression of human immune checkpoint genes (C) TIM3 and (D) PDLF1 by RT-qPCR. *p=0.0161, **p=0.0002 and ***p<0.0001 when compared to Akata WT; unpaired two-sample t-test.

3.7 Proteomic profiles of Akata mutants

The global expression of proteins in the Akata-EBV/Cas9 cells was assessed and quantified using LC-MS/MS, followed by *in silico* pathway enrichment analysis based on the uniquely-expressed proteins in the mutants harboring the deletion of EBV miR-BARTs 7 or 9, compared to the parental (non-mutant) cell line. **Table 2.1** and **Table 2.2**. show the fold change values of proteins significantly up- and downregulated in the EBV miRs BARTs 7 and 9 mutants (sg2). A list of significantly up-regulated proteins present in both mutants is also shown in **Table 2.3**.

Table 2.1 – List of proteins significantly up- and down-regulated in ΔBART7_sg2 mutant compared to the Akata-EBV/Cas9 wild-type.

UniProt ID	Protein	Description	FC[1]	p-value
P46459	VFA	Vesicle-fusing ATPase	7.75	1.49E-02
Q06203	PPAT	Amidophosphoribosyltransferase	7.38	5.38E-05
E9PF82	CAMK2D	Calcium/calmodulin-dependent protein kinase	6.56	3.82E-04
Q16762	TST	Thiosulfate sulfurtransferase	6.56	3.82E-04
P00403	MT-CO2	Cytochrome c oxidase subunit 2	5.58	2.23E-06
Q96AG4	LRRC59	Leucine-rich repeat-containing protein 59	4.62	2.98E-02
P00505	GOT2	Aspartate aminotransferase, mitochondrial	4.60	6.23E-02
Q9BZZ5	API5	Apoptosis inhibitor 5	4.12	3.58E-02
P35527	KRT9	Keratin, type I cytoskeletal 9	3.74	5.47E-04
P31323	PRKAR2B	cAMP-dependent protein kinase type II-beta subunit	3.43	8.85E-02
P50995	ANXA11	Annexin A11	3.11	5.76E-02
Q13422	IKZF1	DNA-binding protein Ikaros	3.11	5.76E-02
O75475	PSIP1	PC4 and SFRS1-interacting protein	2.78	1.98E-03
Q9Y6C9	MTCH2	Mitochondrial carrier homolog 2	2.72	8.94E-02
B4DLN1	2 SV	cDNA FLJ60124	2.63	5.04E-03
P62495	ETF1	Eukaryotic peptide chain release factor subunit 1	2.63	5.04E-03
Q04760	GLO1	Lactoylglutathione lyase	2.63	5.04E-03
Q96FZ2	HMCES	Abasic site processing protein HMCES	2.63	5.04E-03
Q9Y2V2	CARHSP1	Calcium-regulated heat-stable protein 1	2.63	5.04E-03
P55327	TPD52	Tumor protein D52	2.59	5.92E-05
Q02880	TOP2B	DNA topoisomerase 2-beta	2.47	2.05E-02
P23921	RRM1	Ribonucleoside-diphosphate reductase large subunit	2.38	6.18E-02
P30049	ATP5F1D	ATP synthase subunit delta, mitochondrial	2.31	1.45E-03
Q4G176	ACSF3	Malonate--CoA ligase ACSF3, mitochondrial	2.27	2.38E-02
A0A2R8Y7X9	3 SV	GLOBIN domain-containing protein	2.23	4.62E-05
O60888	CUTA	Protein CutA	2.23	4.62E-05
O75533	SF3B1	Splicing factor 3B subunit 1	2.23	4.62E-05
P08133	ANXA6	Annexin A6	2.23	4.62E-05
P08575	PTPRC	Receptor-type tyrosine-protein phosphatase C	2.23	4.62E-05
P15927	RPA2	Replication protein A 32 kDa subunit	2.23	4.62E-05
P54819	AK2	Adenylate kinase 2, mitochondrial	2.23	4.62E-05

UniProt ID	Protein	Description	FC[1]	p-value
Q92922	SMARCC1	SWI/SNF complex subunit SMARCC1	2.23	4.62E-05
P78527	PRKDC	DNA-dependent protein kinase catalytic subunit	2.22	2.66E-04
P27694	RPA1	Replication protein A 70 kDa DNA-binding subunit	2.18	4.03E-02
P48735	IDH2	Isocitrate dehydrogenase [NADP], mitochondrial	2.14	4.53E-04
Q9NSD9	FARSB	Phenylalanine--tRNA ligase beta subunit	2.09	7.61E-03
P51649	ALDH5A1	Succinate-semialdehyde dehydrogenase,	2.08	3.57E-02
P62753	RPS6	40S ribosomal protein S6	2.08	5.69E-02
O95433	AHSA1	Activator of heat shock protein ATPase homolog 1	2.07	2.77E-02
O60343	TBC1D4	TBC1 domain family member 4	2.03	6.18E-04
P61221	ABCE1	ATP-binding cassette sub-family E member 1	2.03	2.14E-02
P61158	ACTR3	Actin-related protein 3	0.48	1.65E-04
Q01081	U2AF1	Splicing factor U2AF 35 kDa subunit	0.47	1.28E-02
A0A087WYT3	PTGES3	Prostaglandin E synthase 3	0.47	6.15E-02
P08243	ASNS	Asparagine synthetase [glutamine-hydrolyzing]	0.47	5.71E-03
E9PAV3	NACA	Nascent polypeptide-associated complex subunit A	0.46	6.87E-02
Q96G03	PGM2	Phosphoglucomutase-2	0.45	4.21E-02
P24539	ATP5PB	ATP synthase F(0) complex subunit B1, mitochondrial	0.44	2.38E-02
P23381	WARS1	Tryptophan-tRNA ligase, cytoplasmic	0.43	8.49E-04
P49257	LMAN1	Protein ERGIC-53	0.43	2.40E-02
E9PR30	FAU	40S ribosomal protein S30	0.42	1.45E-03
O76003	GLRX3	Glutaredoxin-3	0.42	1.45E-03
P33176	KIF5B	Kinesin-1 heavy chain	0.42	1.45E-03
Q9Y3F4	STRAP	Serine-threonine kinase receptor-associated protein	0.42	1.45E-03
Q9UKY7	CDV3	Protein CDV3 homolog	0.41	2.43E-02
Q92598	HSPH1	Heat shock protein 105 kDa	0.41	7.83E-02
A0A087WZE9	HMGN3	High mobility group nucleosome-binding protein 3	0.40	9.18E-02
O75534	CSDE1	Cold shock domain-containing protein E1	0.37	2.01E-05
Q9UH65	SWAP70	Switch-associated protein 70	0.37	2.01E-05
Q9Y490	TLN1	Talin-1	0.36	1.40E-04
C9JVQ0	SNRPG	Small nuclear ribonucleoprotein G	0.34	6.76E-03
Q9Y617	PSAT1	Phosphoserine aminotransferase	0.33	2.39E-02
O00231	PSMD11	26S proteasome non-ATPase regulatory subunit 11	0.33	1.11E-03
P20042	EIF2S2	Eukaryotic translation initiation factor 2 subunit 2	0.33	1.11E-03

UniProt ID	Protein	Description	FC[1]	p-value
A0A7I2V3L8	NDUFA5	Complex I subunit B13	0.27	4.32E-02
P62633	CNBP	CCHC-type zinc finger nucleic acid binding protein	0.27	4.32E-02
P12270	TPR	Nucleoprotein TPR	0.24	3.59E-02
B0QY89	EIF3L	Eukaryotic translation initiation factor 3 subunit L	0.22	3.83E-06
Q9GZL7	WDR12	Ribosome biogenesis protein WDR12	0.22	3.83E-06
S6AU73	HLA-B	HLA class I histocompatibility antigen	0.22	3.83E-06
Q08257	CRYZ	Quinone oxidoreductase	0.10	1.60E-07

[1]Fold change.

Table 2.2 – List of proteins significantly up- and down-regulated in ΔBART9_sg2 mutant compared to Akata Akata-EBV/Cas9 wild-type.

UniProt ID	Protein	Description	FC[1]	p-value
P54727	RAD23B	UV excision repair protein RAD23 homolog B	11.13	2.06E-03
P62993	GRB2	Growth factor receptor-bound protein 2	8.01	4.27E-04
Q16762	TST	Thiosulfate sulfurtransferase	5.77	6.24E-04
P46459	NSF	Vesicle-fusing ATPase	5.48	2.58E-04
Q02880	TOP2B	DNA topoisomerase 2-beta	4.89	3.31E-06
Q13177	PAK2	Serine/threonine-protein kinase PAK 2	4.89	3.31E-06
Q13422	IKZF1	DNA-binding protein Ikaros	4.89	3.31E-06
Q92922	SMARCC1	SWI/SNF complex subunit SMARCC1	4.89	3.31E-06
O43684	BUB3	Mitotic checkpoint protein BUB3	4.21	5.16E-02
A0A2R8Y7X9	3 SV	GLOBIN domain-containing protein	4.12	3.43E-02
O75533	SF3B1	Splicing factor 3B subunit 1	4.12	3.43E-02
Q06203	PPAT	Amidophosphoribosyltransferase	3.55	4.98E-02
P46013	MKI67	Proliferation marker protein Ki-67	3.29	1.05E-03
E9PF82	CAMK2D	Calcium/calmodulin-dependent protein kinase	3.18	7.18E-02
P55809	OXCT1	Succinyl-CoA:3-ketoacid coenzyme A transferase	3.18	7.18E-02
P11388	TOP2A	DNA topoisomerase 2-alpha	3.11	2.72E-04
Q96PK6	RBM14	RNA-binding protein 14	2.85	4.37E-02
O75475	PSIP1	PC4 and SFRS1-interacting protein	2.72	2.57E-04
A0A7P0TB04	BSG	Basigin	2.69	8.29E-02
P11387	TOP1	DNA topoisomerase 1	2.67	7.61E-03
O60343	TBC1D4	TBC1 domain family member 4	2.65	1.46E-02

UniProt ID	Protein	Description	FC[1]	p-value
P62191	PSMC1	26S proteasome regulatory subunit 4	2.58	5.92E-04
Q9BZZ5	API5	Apoptosis inhibitor 5	2.58	5.92E-04
P62140	PPP1CB	Serine/threonine-protein phosphatase PP1-B	2.36	1.52E-02
B4DLN1	2 SV	cDNA FLJ60124	2.31	1.04E-02
P54687	BCAT1	Branched-chain-amino-acid aminotransferase,	2.31	1.04E-02
P61081	UBE2M	NEDD8-conjugating enzyme Ubc12	2.31	1.04E-02
P62495	ETF1	Eukaryotic peptide chain release factor subunit 1	2.31	1.04E-02
Q9BX68	HINT2	Adenosine 5'-monophosphoramidase HINT2	2.31	1.04E-02
Q9P0M6	MACROH2A	Core histone macro-H2A.2	2.31	1.04E-02
Q9Y2V2	CARHSP1	Calcium-regulated heat-stable protein 1	2.31	1.04E-02
A0A1C7CYX9	DPYSL2	Dihydropyrimidinase-related protein 2	2.21	6.02E-02
O00567	NOP56	Nucleolar protein 56	2.20	6.12E-02
P29144	TPP2	Tripeptidyl-peptidase 2	2.07	3.78E-03
A0A0A0MQX8	MBNL1	Muscleblind-like protein 1	0.49	7.72E-05
C9JVQ0	SNRPG	Small nuclear ribonucleoprotein G	0.49	7.72E-05
F5H1U9	MPDZ	Multiple PDZ domain protein	0.49	7.72E-05
O00231	PSMD11	26S proteasome regulatory subunit 11	0.49	7.72E-05
O76003	GLRX3	Glutaredoxin-3	0.49	7.72E-05
P35520	CBS	Cystathionine beta-synthase	0.49	7.72E-05
P62851	RPS25	40S ribosomal protein S25	0.49	7.72E-05
Q05048	CSTF1	Cleavage stimulation factor subunit 1	0.49	7.72E-05
Q53EL6	PDCD4	Programmed cell death protein 4	0.49	7.72E-05
Q8WW12	PCNP	PEST proteolytic signal-containing nuclear protein	0.49	7.72E-05
Q9BXP5	SRRT	Serrate RNA effector molecule homolog	0.46	1.03E-02
P61158	ACTR3	Actin-related protein 3	0.42	2.91E-04
Q9Y490	TLN1	Talin-1	0.42	3.57E-05
P13284	IFI30	Gamma-interferon-inducible lysosomal thiol	0.36	1.88E-02
P12270	TPR	Nucleoprotein TPR	0.23	2.85E-02
Q08257	CRYZ	Quinone oxidoreductase	0.23	8.27E-04
Q9Y696	CLIC4	Chloride intracellular channel protein 4	0.20	2.92E-06

[1]Fold change.

Knocking down the EBV miR-BART7 by CRISPR/Cas9-targeted mutagenesis affected 71 proteins ($p<0.05$) while the edition of miR-BART9 affected 51 proteins ($p<0.05$), when

compared to the control (non-mutated Akata-EBV/Cas9 cells). For the EBV miR-BART7 mutant, 41 upregulated and 30 downregulated proteins were identified, while 34 upregulated and 17 downregulated proteins were found in the miR-BART9 mutant. The volcano plots with the 50 most expressive hits and the clustering results for both Akata-EBV/Cas9 mutants are shown in **Figure 2.8** and **Figure 2.9**

Table 2.3 – List of proteins significantly upregulated[1] in both ΔBART7_sg2 and ΔBART9_sg2 mutants compared to Akata-EBV/Cas9 wild-type.

UniProt ID	Protein	Description
B4DLN1	2 SV	cDNA FLJ60124
A0A2R8Y7X9	3 SV	GLOBIN domain-containing protein
Q9BZZ5	API5	Apoptosis inhibitor 5
E9PF82	CAMK2D	Calcium/calmodulin-dependent protein kinase
Q9Y2V2	CARHSP1	Calcium-regulated heat-stable protein 1
E9PF82	CAMK2D	Calcium/calmodulin-dependent protein kinase
P62495	ETF1	Eukaryotic peptide chain release factor subunit 1
Q13422	IKZF1	DNA-binding protein Ikaros
Q06203	PPAT	Amidophosphoribosyltransferase
O75475	PSIP1	PC4 and SFRS1-interacting protein
O75533	SF3B1	Splicing factor 3B subunit 1
Q92922	SMARCC1	SWI/SNF complex subunit SMARCC1
O60343	TBC1D4	TBC1 domain family member 4
Q02880	TOP2B	DNA topoisomerase 2-beta
Q16762	TST	Thiosulfate sulfurtransferase

[1]No down-regulated proteins in both ΔBART7_sg2 and ΔBART9_sg2 were found.

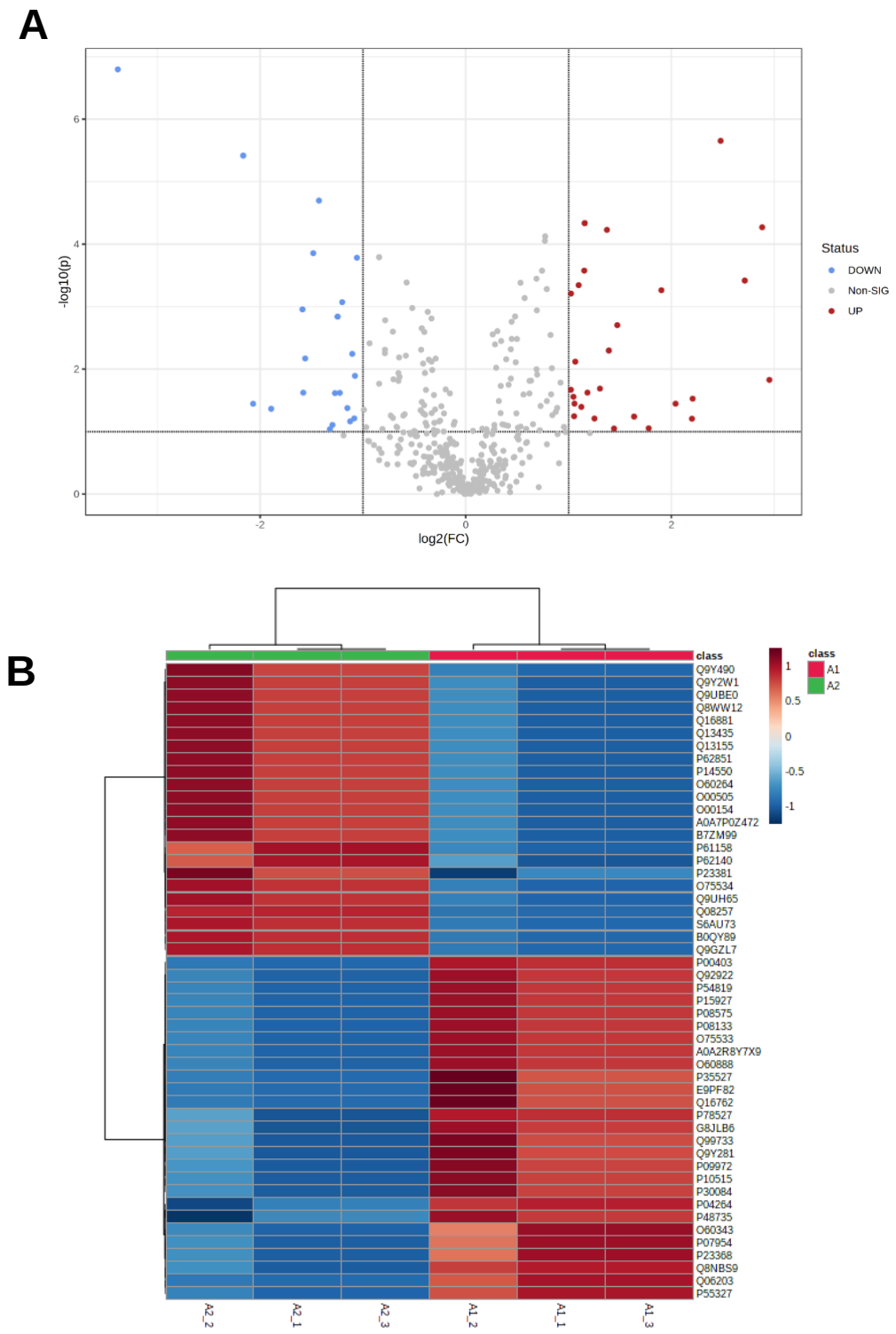

Figure 2.8 – LC/MS results of differently expressed proteins in Akata-EBV/Cas-9 cells with knock-down of EBV miR-BART7 expression by CRISPR/Cas9 genomic edition, compared to the parental, non-edited cell line. **(A)** Volcano plot and **(B)** clustering results of proteins significantly up- and down-regulated, shown as heatmap[1]. A1 = Akata WT; A2 = EBV miR-BART7 mutant (sg2). Distance and clustering measured using ward.D algorithm. Proteins were considered regulated if fold-change values were ≥1.5 or ≤0.5 (log2 FC) with p-value < 0.05 (-log10[p]).

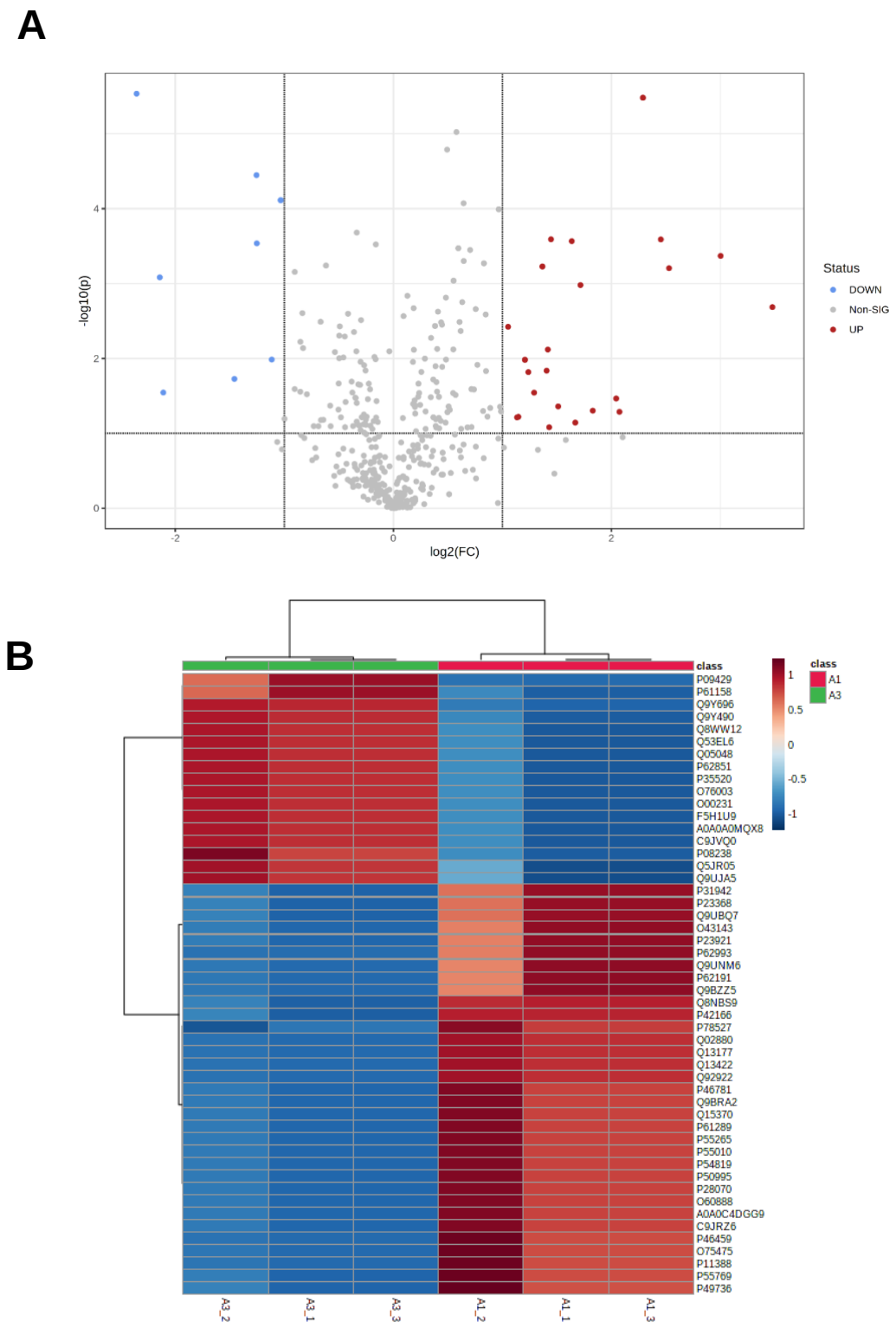

Figure 2.9 – LC/MS results of differently expressed proteins in Akata-EBV/Cas-9 cells with knock-down of EBV miR-BART9 expression by CRISPR/Cas9 genomic edition, compared to the parental, non-edited cell line. **(A)** Volcano plot and **(B)** clustering results of proteins significantly up- and down-regulated, shown as heatmap[1]. A1 = Akata WT; A3 = EBV miR-BART9 mutant (sg2). Distance and clustering measured using ward.D algorithm. Proteins were considered regulated if fold-change values were ≥1.5 or ≤0.5 (log2 FC) with p-value < 0.05 (-log10[p]).

For the EBV miR-BART7 mutant (sg2), several hits on pathways related to protein and RNA metabolism, mRNA binding, and other regulatory molecules were identified (**Table 2.4**). The pathway enrichment analysis identified several proteins that play essential roles in cell cycle regulation and proliferation, splicing of mRNAs, cellular trafficking, and metabolism. We also identified hits on DNA topoisomerases and protein binding pathways in the EBV miR-BART9 mutant (sg2) (**Table 2.5**). These molecules are associated with metabolic and cellular trafficking pathways, DNA topology changes, cell cycle, and proliferation regulation.

Table 2.4 – Gene Ontology pathway enrichment analysis of proteins uniquely expressed in ΔBART7_sg2 mutant compared to Akata wild-type.

Pathway ID[1]	Description	Hits on pathways	FDR[2]
GO:0097159	Organic cyclic compound binding	47 of 5916	6.61E-08
GO:1901363	Heterocyclic compound binding	47 of 5831	6.61E-08
GO:0003723	RNA binding	23 of 1649	5.01E-06
GO:0003676	Nucleic acid binding	32 of 3947	5.40E-04
GO:0005488	Binding	60 of 12516	2.10E-03
GO:0036094	Small molecule binding	22 of 2516	1.77E-02
GO:0043168	Anion binding	23 of 2805	2.58E-02
GO:0032559	Adenyl ribonucleotide binding	16 of 1522	2.95E-02
GO:0043167	Ion binding	37 of 6188	2.95E-02
GO:0000166	Nucleotide binding	19 of 2119	3.10E-02
GO:0003729	mRNA binding	7 of 289	3.10E-02
GO:0005524	ATP binding	15 of 1464	4.09E-02

[1] Gene Ontology Pathway ID (**http://geneontology.org**); [2] False discovery rate.

Table 2.5 – Gene Ontology pathway enrichment analysis of proteins uniquely expressed in ΔBART9_sg2 mutant when compared to Akata wild-type.

Pathway ID[1]	Description	Hits on pathways	FDR[2]
GO:0003723	RNA binding	21 of 1649	2.27E-07
GO:0097159	Organic cyclic compound binding	33 of 5916	3.55E-05
GO:1901363	Heterocyclic compound binding	33 of 5831	3.55E-05
GO:0003676	Nucleic acid binding	26 of 3847	1.60E-04
GO:0003916	DNA topoisomerase activity	3 of 7	1.60E-03
GO:0005515	Protein binding	31 of 7026	2.43E-02

Pathway ID[1]	Description	Hits on pathways	FDR[2]
GO:0003918	DNA topoisomerase type II (double-strand cut)	2 of 3	4.18E-02
GO:0003729	mRNA binding	6 of 289	4.60E-02
GO:0005488	Binding	42 of 12516	4.85E-02

[1]Gene Ontology Pathway ID (**http://geneontology.org**); [2] False discovery rate.

4 Discussion

This study sought to investigate the effects of CRISPR/Cas9-knocking down of miR-BARTs 7 and 9 in EBV infecting Akata-EBV/Cas9 cells. For a permanent knock-down of EBV miRs BARTs 7 and 9, we have edited the EBV genome in Akata-EBV/Cas9 using a CRISPR/Cas9-targeted mutagenesis protocol. The generated Akata-EBV/Cas9 mutants showed a remarkable reduction in the expression of the viral miRNAs, as assessed by qPCR and luciferase reporter assays. Furthermore, the gene-editing was also confirmed by Sanger sequencing and T7EI mutation assays. The decomposition analysis of DNA sequences from Akata mutants also shows a high editing efficiency, predominately deletions of five or more base pairs. CRISPR/Cas9-targeted mutagenesis is an efficient, simple, and versatile tool for editing the viral genome with high specificity and research potential (22). This study is one of the few published in the literature that has reported a successful edition of the EBV genome using the CRISPR/Cas9 technology, enabling new advances to investigate unknown and overlooked viral genes (23–25).

The analysis of viral miRNAs expression by qPCR in lymphoid and lymphoblastoid cell lines revealed heterogeneity in EBV miR-BART7 and miR-BART9, with higher viral miRNAs levels found in BC-1, Jyoye, and Rael cells. These differences may account for the distinctive biological features of these cell lines. The expression levels of EBV miR-BARTs vary across tumors, showing particular profiles in response to growth stimuli and tumor microenvironment (6,26). To date, only a few studies have estimated the expression levels of EBV miR-BARTs in both BL cell lines and fresh tumors; thus, the influence of these viral miRNAs in the pathogenesis of EBV-induced lymphomas is unknown. A study comparing the molecular profile of endemic, sporadic, and immunodeficiency-associated BL reported that EBV-positive tumors express high viral miRNAs levels associated with the activation of proliferation pathways (27).

Compared to the non-mutated Akata-EBV/Cas9 cells, we found that knocking down the EBV miRs BARTs 7 or 9 significantly reduced the viability and proliferation of mutants. In NPC-derived cell lines, previous studies reported that the expression of miR-BART7 was associated with increased cell proliferation, resistance to apoptosis, and tumorigenesis *in vitro* and *in vivo* (28,29). The expression of EBV miR-BART9 was also associated with increased cell proliferation and resistance to apoptosis in NPC and BL-derived cells, leading to changes in the regulation of the proteins PTEN, BIM, PUMA, and increasing expression of the EBV LMP1 oncoprotein (9,30). The results presented here strengthen the hypothesis that the viral miR-BARTs 7 and 9 may promote cell proliferation and reduce the susceptibility to apoptosis of BL-derived cells *in vitro*.

EBV miRNAs contribute to regulating the viral life cycle, for instance, supporting the viral latency by suppressing the expression of lytic genes. In this study, mutants for miRs BARTs 7 and 9 exhibited an increased expression of the genes encoding the early-immediate lytic proteins Zta (BZLF1) and gp350 (BLLF1). The viral miR-BART9 can regulate EBV lytic cycle by attenuating cell signaling events triggered by the activation of the B-cell receptor (BCR) (31). Interestingly, a recent study also showed that the expression of miR-BART7 and miR-BART9 increases in the BL-derived MutuI cell line after lytic induction by anti-IgG crosslinking. The study reports that EBV miR-BART9 has sequence homology to the human miRNA miR-141 (hsa-miR-141) and regulates the EBV lytic cycle via transcriptional inhibition of several components of the FOXO3 signaling pathway (32).

The proteomic analysis showed that the suppression of EBV miR-BART7 significantly increased the expression of the RNA binding proteins (RBPs) TST, ETF1, RPS6, FARSB, and PRKDC, while reducing the expression of STRAP, EIF2S2, and FAU. The pathway enrichment analysis resulted in several hits for RNA binding, mRNA binding, and nucleic acid binding, corroborating our preliminary findings; in fact, 60 out of 71 proteins identified are related to binding pathways. RBPs are master regulators of mRNA splicing, polyadenylation, stability, localization, and translation. They exert crucial activities in normal hematopoiesis, and changes in their expression can be associated with cancer development and progression (33). RBPs participate in pathways related to differentiation and self-renewal of B cells while also playing a critical role in the pathogenesis of hematological malignancies (34–36).

RBPs are an emerging class of potential targets for cancer treatment (37), but the significance of these proteins in the context of lymphomas is currently unexplored. Human RBPs are not only essential for the EBV lytic infection, but the virus also encodes its own RBP, the SM protein, which increases and enhances EBV lytic gene expression by binding viral mRNAs and recruiting nuclear export proteins. The EBV SM protein also increases mRNA stability, modulates the viral RNA splicing, and induces the transcription of several viral genes during lytic reactivation (38,39). EBV also recruits the host's RBPs isoforms XBP1 and TCF4 to restrict BZLF1 activation and control the lytic cycle induction in primary B cells and Burkitt lymphoma cells (40), contributing to the maintenance of the latent cycle. Nonetheless, the suppression of EBV miR-BART7 in Akata-EBV/Cas9 cells led to a significant increase in the expression of the Zta and gp350 lytic genes; thus, it is plausible that the viral miR-BART7 might selectively target host RBPs to enhance lytic activation in our model.

The suppression of EBV miR-BART9 in Akata-EBV/Cas9 cells significantly increased the expression of the DNA topoisomerases TOP1, TOP2A, and TOP2B. Several other proteins associated with DNA topology and remodeling complexes, such as BUB3, SMARCC1, TPR, and MKI67, were also found to be upregulated. DNA topoisomerases are a family of enzymes that modulate the DNA topology by the transient introduction of DNA strand breaks (41). TOPO1 plays an essential role in introducing positive supercoils, aiding the resolution of topological entanglements, and promoting cell proliferation (42). TOPO2A is critical for DNA replication and cellular viability, while TOPO2B has a crucial role in DNA repair (43). DNA topoisomerases exert essential roles in the pathogenesis of various cancers, commonly targeted by drugs used for cancer treatment such as doxorubicin, irinotecan, and etoposide (44). Topoisomerases inhibitors block the ligation step during DNA replication, resulting in single- and double-strand DNA breaks that lead to cell death by either apoptosis or necrosis (45)

TOPO1 and 2 are essential for lytic induction, and their inhibition affects the viral replication, suppressing the EBV lytic cycle (46). Inhibition of TOPO1 with camptothecin reduces the binding of EBV Zta and the human DNA helicase RecQL1 in the OriLyt region of the viral genome (47). Furthermore, the EBV tegument protein BPLF1 may also regulate the activity of type II topoisomerases during productive lytic infection (48). These reports support our findings since the suppression of EBV miR-BART9 led to an increase in the transcription of the viral lytic genes Zta and gp350. The increased expression of topoisomerases found in

Akata-EBV/Cas9 cells edited for miR-BART9 (sg2) might be associated with EBV DNA replication and not directly related to cell pathways associated with EBV-induced lymphomagenesis. Therefore, we hypothesize that the expression of EBV miR-BART9 might regulate the viral lytic cycle by targeting and regulating the expression of human topoisomerase.

Knocking down EBV miR-BART9 also modified the expression of several human proteins and regulators related to the 26S proteasome pathway, such as the upregulation of the UBE2M, PSMC1, and TPP2 proteins, and downregulation of the PSMD11 protein. The proteasome is vital for cellular viability and immune responses, targeted by various unrelated viruses, including HBV, HPV, HIV, and CMV. The ubiquitin/proteasome pathway is a complex proteolytic system regulating several critical cellular processes, including signal transduction, cell cycle progression, apoptosis, and MHC class I loading complex (49). EBV can exploit the ubiquitin/proteasome pathway by either inhibiting proteasomal degradation to avoid the loss of critical viral proteins or inducing degradation of unwanted cellular proteins (50–52). The ubiquitin/proteasome pathway is pivotal for generating antigenic peptides of viral proteins for presentation to T cytotoxic cells mediated by MHC-I molecules. In this regard, EBV EBNA-1 can inhibit the proteasome pathway, contributing to the immune escape of EBV-infected cells (53).

Several studies indicate that EBV miRNAs have a preeminent role in assisting immune escape, as reviewed by Li et al. (2020) (55). Ungerleider and colleagues (2021) reported that the expression of EBV miRNAs, including the miR-BART9, interfere with several pathways related to anti-viral immunity and tumor microenvironment, suppressing the immune system (54). Worth noting, EBV miR-BART9 targets the LMP1 3'-UTR, regulating the levels of this viral oncoprotein (9). Thus, our study unravels potential new targets for EBV miR-BART9 in the ubiquitin/proteasome pathway, providing new insights to understand the immune-suppressive properties of this viral miRNA.

Several minor pathways and differentially expressed proteins found after knocking down EBV miRs BARTs 7 and 9 were not discussed. The identification of viral proteins expressed in Akata-EBV/Cas9 mutants will also shed some light on the interactions of human and viral proteins/pathways discussed in the present study. These proteomic analyses are currently in development. In summary, this study shows that EBV miR-BARTs 7 and 9 exert an

essential role in the lytic cycle regulation in Akata-EBV/Cas9 cells, increasing cell proliferation and viability and repressing the lytic cycle activation by hijacking several critical cellular pathways, including RBPs, DNA topoisomerases, and ubiquitin/proteasome (**Figure 2.10**).

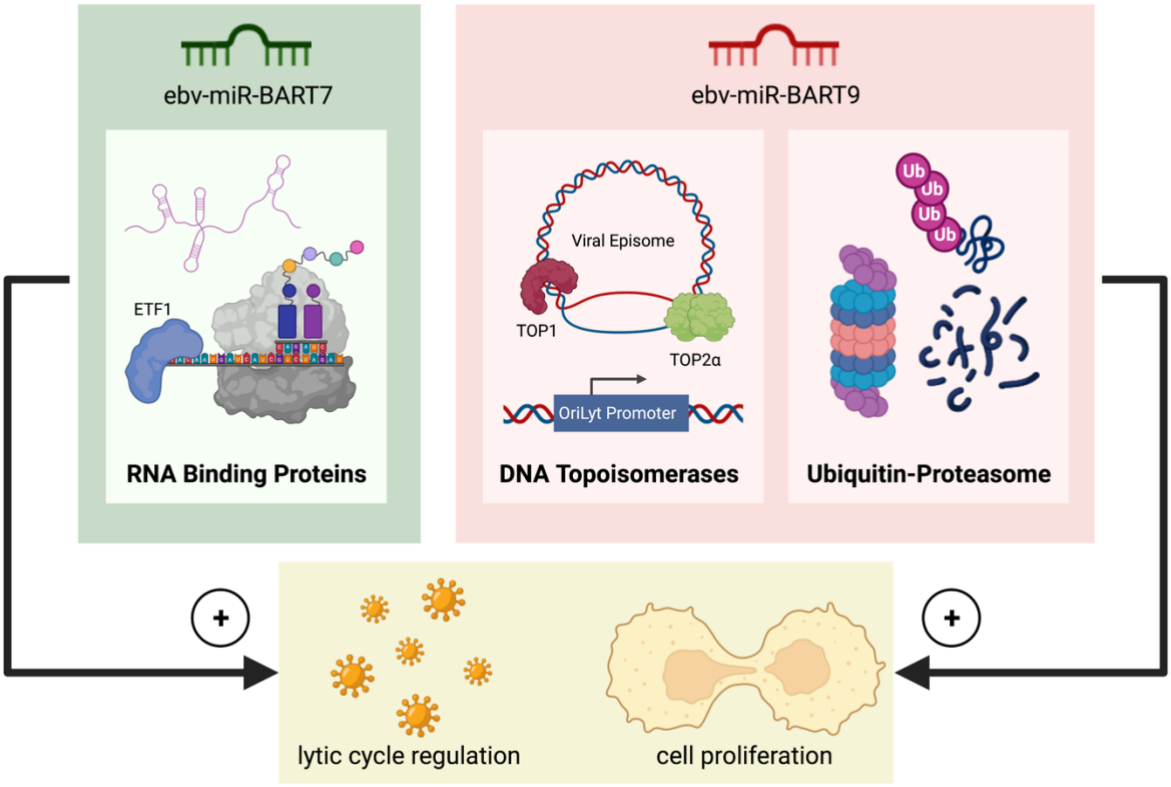

Figure 2.10 – Proposed mechanisms of potential targets for ebv-miR-BART7 and ebv-miR-BART9. EBV miR-BART7 may target host RNA binding proteins (RBPs) involved in lytic reactivation, maintaining viral latency in Akata-EBV/Cas9 cells. EBV-miR-BART9, on the other hand, may target DNA topoisomerases and proteins from the ubiquitin-proteasome pathway, also involved in regulating the lytic cycle and cell proliferation.

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6 Supplementary Material

Table S2.1 – STR profiles of the cell lines used in the study

Cell Line (RRID)	Description	STR profile
Akata-Cas9[a] (CVCL_1856)	Burkitt lymphoma cells, stably infected by EBV (Latency type I) and expressing of Cas9.	AMEL: X,X; CSF1PO: 11; D5S818: 11,12; D7S820: 10,12; D13S317: 8,12; D16S539: D21S11: 30,32.2, 10,11; TH01: 9; TPOX: 8,11; vWA: 14,16
BC-1[b] CVCL_1079	Primary effusion lymphoma from ascitic fluid; stably infected by EBV (Lat I); coinfected by KSHV and HIV.	AMEL: X, Y; CSF1PO: 10,11; D13S317: 8; D16S539: 12, 13; D21S11: 28, 30; D5S818: 11, 12; D7S820: 11; TH01: 9.3; TPOX: 8, 9; vWA: 16,20
BC-2[b] CVCL_1856	Primary effusion lymphoma from pleural fluid; stably infected by EBV (Lat I); coinfected by KSHV and HIV.	AMEL: X,Y; CSF1PO: 10,12; D13S317: 11,12; D16S539: 11,12; D21S11: 29,30; D5S818: 11,13; D7S820: 8,9; TH01: 7; TPOX: 10,11; vWA: 14,19
BC-3[b] CVCL_1080	Primary effusion lymphoma from pleural fluid; EBV negative, infected by KSHV and HIV.	AMEL: X CSF1PO: 11, 12; D13S317: 11; D16S539: 12; D21S11: 29, 30; D5S818: 11,12; D7S820: 10, 12; TH01: 6, 9; TPOX: 8, 11; vWA: 14,18
IBL-1[b] CVCL_9638	Diffuse large B cell lymphoma; stably infected by EBV (Lat II/III); coinfected by HIV.	AMEL: X,Y; CSF1PO: 12,12; D5S818: 11,12; D7S820: 12,13; D13S317: 11,11; D16S539: 9,12; D21S11: 28,29; TH01: 9,9.3; TPOX: 8,11; vWA: 17,19
Jiyoye[b] CVCL_1317	Burkitt lymphoma; stably infected by EBV (Lat I).	AMEL: X,Y; CSF1PO: 10,11; D5S818: 12,12; D7S820: 8,10; D13S317: 12,12; D16S539: 10,11; D21S11: 28,36; TH01: 7,9; TPOX: 6,8; vWA: 15,19
MutuI-Cas9[a] CVCL_7202	Burkitt lymphoma, stable expression of Cas9 and infection by EBV (Lat I).	AMEL: x,y; CSF1PO: 10; D5S818: 12,14; D7S820: 8,10; D13S317: 12,13; D16S539: 13; D21S11: 30,32.2; TH01: 7; TPOX: 8,11; vWA: 15,17
P3HR1-Cas9[a] CVCL_2676	Burkitt lymphoma, stable expression of Cas9 and infection by EBV (Lat I)	AMEL: X,Y; CSF1PO: 10,11; D5S818: 12,12; D7S820: 8,10; D13S317: 12,12; D16S539: 10,11; D21S11: 28,36; TH01: 7,9; TPOX: 6,8; vWA: 15,19

[1]EBV latency type (I, II or III); [a]A gift from Benjamin Gewurz at the Harvard Medical School; [b]A gift from Ethel Cesarman at the Weill Medical College, Cornell University.

Table S2.2 – Reagents and resources

Reagent	Source	Cat. No.
2-Iodoacetamide	Sigma-Aldrich	161125
3500 Series Genetic Analyzer	Thermo Fisher Scientific	4406017
Ammonium bicarbonate	Sigma-Aldrich	A6141
Ampicillin sodium salt	Sigma-Aldrich	A0166
Bio-Rad Protein Assay Kit	Bio-Rad	5000001
Blasticidin S HCl (10 mg/mL)	Thermo Fisher Scientific	A1113903
BsmBI-v2 Restriction Enzyme	New England Biolabs	R0739S
CellTiter 96® AQueous One Solution	Promega	G3582
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	11873580001
Countess™ 3 FL Automated Cell Counter	Thermo Fisher Scientific	AMQAF2000
Dithiothreitol	Bio-Rad	161-0611
DMEM, powder, high glucose	Thermo Fisher Scientific	12100061
DNase I, Amplification Grade	Sigma-Aldrich	AMPD1-1KT
Dual-Glo® Luciferase Assay System	Promega	E2920
Fetal Bovine Serum	Sigma-Aldrich	F7524
Filtropur S, PES, pore size: 0.45 µm	Sarstedt Inc	83.1826.001
Gel Loading Dye, Orange (6X)	New England Biolabs	B7022S
GenePrint® 10 System	Promega	B9510
Geneticin™ Selective Antibiotic (G418 Sulfate)	Thermo Fisher Scientific	10131035
Gentamicin (10 mg/mL)	Thermo Fisher Scientific	15710064
GloMax® Discover Microplate Reader	Promega	GM3000
GoTaq® qPCR Mix	Promega	A6001
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4374966
LB Agar, powder (Lennox L agar)	Thermo Fisher Scientific	22700041
Lipofectamine 3000 transfection reagent	Thermo Fisher Scientific	L3000015
Luria Broth Base (Miller's LB Broth Base)™, powder	Thermo Fisher Scientific	12795084
One-Shot Stbl3 Chemically Competent E. coli	Thermo Fisher Scientific	C737303
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	31985070
pGL4.73[hRluc/SV40] Vector	Promega	E6911
Pierce™ C18 Spin Columns	Thermo Fisher Scientific	89870
Platinum™ Taq DNA Polymerase High Fidelity	Thermo Fisher Scientific	11304029
Puromycin Dihydrochloride	Thermo Fisher Scientific	A1113803
QIAquick PCR & Gel Cleanup Kit	Qiagen	28506
Quick Start Bovine Serum Albumin Standard Set	Bio-Rad	5000207
RapiGest SF	Waters	186001861

Reagent	Source	Cat. No.
Reverse Mass DNA Ladder	New England Biolabs	N3240S
RNeasy Mini Kit	Qiagen	74104
RPMI 1640 Medium, powder	Thermo Fisher Scientific	31800105
Sequencing Grade Modified Trypsin	Promega	V5111
T4 DNA Ligase	New England Biolabs	M0202S
T4 DNA Ligase Reaction Buffer	New England Biolabs	B0202S
T7 Endonuclease I	New England Biolabs	M0302S
TaKaRa PCR Mycoplasma Detection Set	Takara Bio Inc	6601
Trifluoroacetic acid	Sigma-Aldrich	302031
TRIzol™ Reagent	Thermo Fisher Scientific	15596026
Vacufuge® Concentrator Plus	Eppendorf	EP5305000169
Vibra-Cell™ VCX 750	Sonics & Materials Inc.	VCX 750
Wizard® Genomic DNA Purification Kit	Promega	A1120

Table S2.3 – Sequences of oligonucleotides used in this study

Name	Sequences (5'-3')
sgRNA sequence cloned into LentiGuide-Puro	
BART7_sg1	F: CACCGTTGTTTCATAGTCAAGGTCC / R: AAACGGACCTTGACTATGAAACAAC
BART7_sg2	F: CACCGCATAGTCAAGGTCCAGGATC/ R: AAACGATCCTGGACCTTGACTATGC
BART9_sg1	F: CACCGCTGAATTGGAAACAGTAACT / R: AAACAGTTACTGTTTCCAATTCAGC
BART9_sg2	F: CACCGAAGTTACTGTTTCCAATTCA / R: AAACTGAATTGGAAACAGTAACTTC
Sequencing of LentiGuide-Puro constructs	
hU6.F	GAGGGCCTATTTCCCATGATT
Luciferase Reporter Assays	
psiCheck-2-BART7.3p	AAACCATCATAGTCCAGTGTCCAGGGTCTAGAC TCGAGTCTAGACCCTGGACACTGGACTATGATGGTTT
psiCheck2 BART7.3p SCR	AAACGTAAGACTAGGCCATCGTGCTCTCTAGAC TCGAGTCTAGAGAATACTCCGTATCGTGAGAAGCGTTT
psiCheck-2-BART9.3p	AAACTAACACTTCATGGGTCCCGTAGTTCTAGAC TCGAGTCTAGAACTACGGGACCCATGAAGTGTTAGTTT
psiCheck-2-BART7.3p SCR	AAACGCTTCTCACGATACGGAGTATTCTCTAGAC TCGAGTCTAGAGAATACTCCGTATCGTGAGAAGCGTTT
Sequencing of EBV miRs BARTs 7 and 9 within the EBV genome	
BART7_seq	F: ATTCTGTTCTATGACCCCGT / R: CTAGGAAACCGTAATCAGTG
BART9_seq	F: GTATTTTCCCATCAGCACCT / R: ACCATGACTTTGTAACCGAG
EBV lytic cycle evaluation by RT-qPCR	
Zta (BZLF1)	F: TACAAGAATCGGGTGGCTTC / R: GCACATCTGCTTCAACAGGA
gp350 (BLLF1)	F: TGTTACAGTGACTGCCTTTTGGG / R: GGTGTCCCCGAGGTGAGAGT
Analysis of Immune-checkpoint molecules by RT-qPCR	
Tim3	F: CTTTCCAAGGATGCTTACCAC / R: CAGATCCCTGCTCCGATGTA
PDL1	F: GCCCCATACAACAAAATCAACC / R: GCTTGTCCAGATGACTTCGG

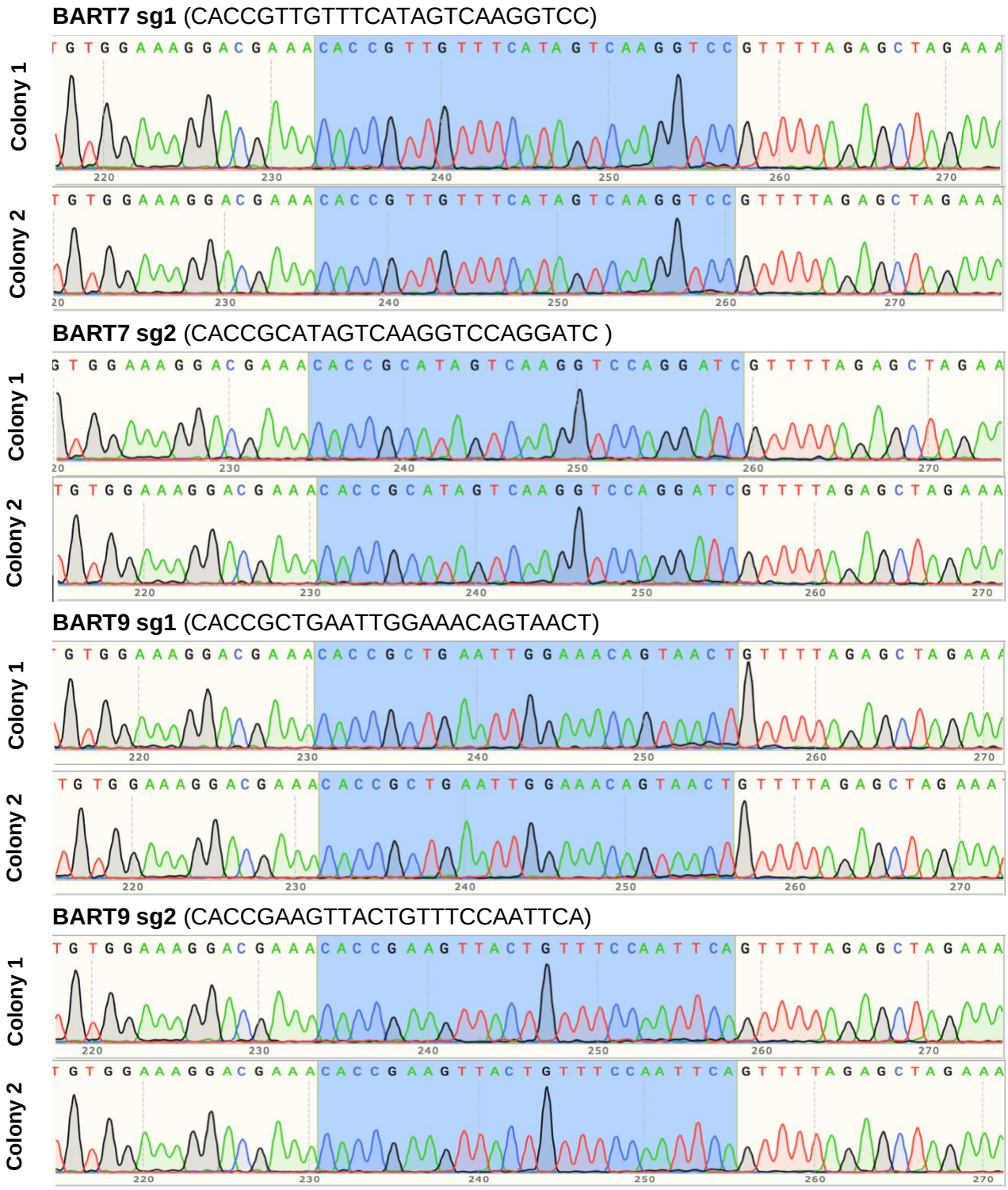

Figure S2.1 – Electropherograms produced by Sanger sequencing of LentiGuide-Puro vectors containing the desired sequences of sgRNAs for ebv-miR-BART7 and ebv-miR-BART9.

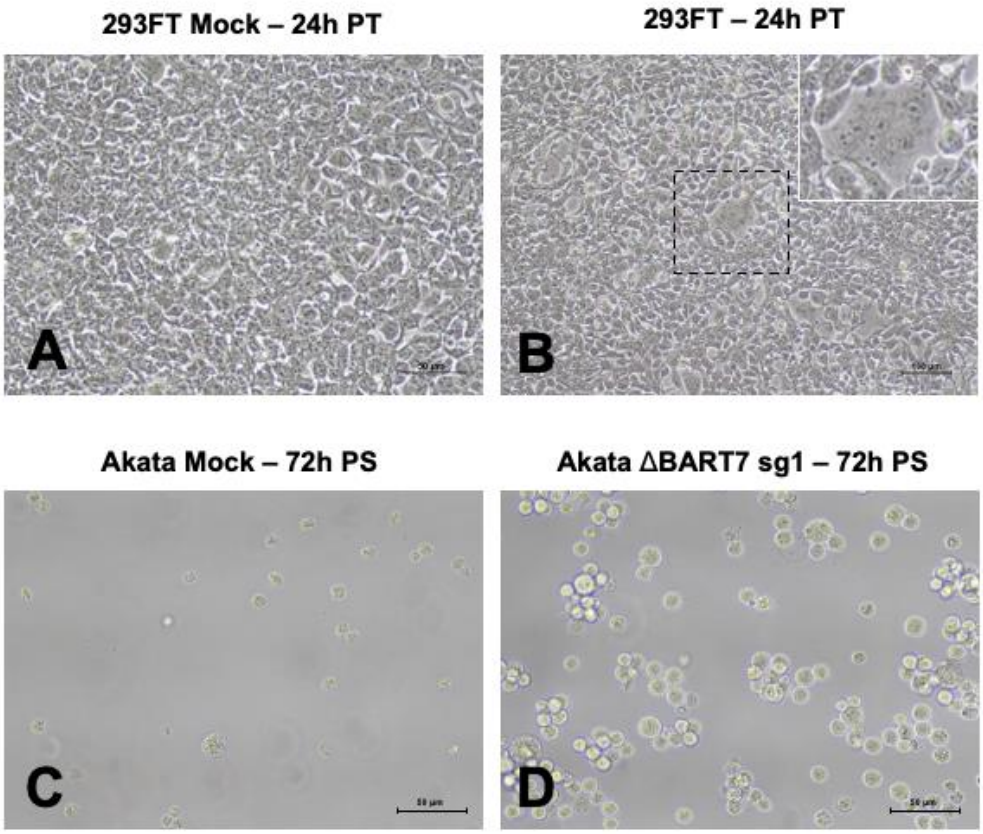

Figure S2.2 – Production of lentiviral particles in HEK293FT cells transfected with LentiGuide-Puro-BART7 and LentiGuide-Puro-BART9 and transduction in Akata-Cas9 cells. (A) HEK293FT cells without transfection (mock). (B) HEK293FT cells transfected with LentiGuide and packaging vectors, showing increased syncytia formation, consistent with viral particle production. (C) Akata-Cas9 cells mock-treated with puromycin 72 PS. (D) Akata-Cas9 cells transduced with LentiGuide-PuroBART7_sg1 treated with puromycin and showing antibiotic-resistant cells 72 PS. PT: post-transfection; PS: post-selection.

Chapter III – Identification of Epstein-Barr Virus (EBV) lytic products involved in B Cell Receptor (BCR) degradation

Abstract

The Epstein-Barr virus (EBV), formally designated human gammaherpesvirus 4 (HHV-4), is a ubiquitous virus that causes lifelong latent infection in over 90% of human adults worldwide. The EBV infection is regarded as carcinogenic to humans and is associated with various human cancers, especially the endemic form of Burkitt Lymphomas (BL) and nasopharyngeal carcinomas (NPC). EBV employs a transient lytic program following primary infection before establishing latency. Although the EBV oncogenic effects are mostly regarded to viral products expressed during the latency, the lytic phase of the viral life cycle also plays an essential role in EBV-induced carcinogenesis, but the mechanisms remain unknown. Previously, EBV early lytic products were found to target cell-surface and intracellular B-cell receptor (BCR) for ubiquitination and proteasomal degradation. Thus, this study aimed to investigate whether the expression of EBV early lytic genes plays a role in BCR degradation. A panel of EBV inducible ORFs was generated, and the expression of IgM and CD79a under the controlled expression of EBV ORFs was assessed in vitro in P3HR1-ZHT/RHT and BJAB cells. We found that the EBV early lytic gene BRRF1 targets CD79a and may degrade the BCR complex in both EBV-positive and EBV-negative cell lines. Furthermore, the EBV late lytic gene BLRF2 also downregulates CD79a and can be closely associated with BRRF1. The preliminary results of this study shed some light on possible EBV lytic genes associated with immunoevasion mechanisms during the viral lytic reactivation.

+ Keywords: Epstein-Barr Virus, Viral lytic cycle, B-cell receptor, BRRF1, BLRF2, lymphomagenesis

1 Introduction

Formally designated *human gammaherpesvirus 4* (HHV-4), EBV is a ubiquitous virus that causes lifelong latent infection in over 90% of human adults worldwide. The infection by EBV is regarded as carcinogenic to humans, and it is associated with a variety of cancers, notably the endemic form of Burkitt lymphoma (BL) and nasopharyngeal carcinomas (1). The primary infection by EBV typically occurs very early in life, and it is asymptomatic. The virus is transmitted by the saliva, infecting the epithelial cells of the oropharynx, where it replicates. EBV virions spread to adjacent structures, infecting local B cells. Upon infection, the viral genome assumes a circular form, and it is kept indefinitely as an episome in the nucleus of latently-infected B cells (2).

EBV continuously shuttles between B cells and epithelial cells during infection, maintained by checks and balances with the immune system. Following primary infection, EBV employs a transient lytic program before entering the latent phase of the viral life cycle. Although latent infection prevails in most neoplastic cells of EBV-associated cancers, the lytic phase of the viral life cycle also plays a vital role in EBV-induced carcinogenesis (3), stimulating the secretion of viral/cellular cytokines and growth factors and contributing to cell proliferation, resistance to apoptosis, inflammation, and angiogenesis (4). EBV requires the expression of immediate-early (IE), early (E), and (L) proteins in a coordinated fashion to complete the lytic phase, and the IE genes BZLF1 and BRLF1 play an essential role in the latent-to-lytic switch (5). Early lytic genes are transcribed to produce proteins required for EBV replication, such as the viral DNA polymerase and other associated factors. The expression of late lytic genes allows the production of structural proteins necessary to assemble new viral particles within the infected cells (6).

In a study published in 2017, Ersing and colleagues reported that an unknown EBV early lytic factor might target the cell-surface and intracellular B-cell receptor (BCR) for ubiquitination and proteasomal degradation (7). The BCR pathway is essential for cell-fate decisions in the B-cell lineage, and it was previously reported that the BCR signaling promotes survival of malignant B cells in BL and diffuse large B cell (DLBCL) lymphomas (8). Given the scarcity of data on the role of viral lytic products for the elucidation of EBV-induced lymphomagenesis, this study aimed to investigate whether the expression of EBV early lytic genes impacts the BCR levels, aiming to shed light on possible mechanisms of EBV-mediated

immunoevasion during lytic reactivation.

2 Material and Methods

2.1 Designing and cloning sgRNAs for EBV early lytic genes knockouts

A knocked-outs (KOs) panel comprising 35 early lytic genes was produced using EBV-positive Akata cells (**Table S3.1**) subjected to EBV genome edition by CRISPR/Cas9. The list of early lytic genes was according to the classification provided by Murata (9), with a fraction of the Akata-KOs already produced by former members in the lab. Specific single guides RNAs (sgRNAs) were designed and subsequently cloned into the pLentiGuide-hygro, a gift from Kristen Brennand (Addgene plasmid #99375). The sgRNAs were designed using the GPP sgRNA Designer CRISPRko (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). Briefly, coding sequences (CDS) from selected EBV genes were retrieved from GenBank (#KC207813.1, *Human herpesvirus* 4 strain Akata, complete genome) (10) and subjected to analysis in the GPP sgRNA designer software. Two sgRNAs sequences for each EBV gene were selected based on the on-target scoring performed using Azimuth 2 (11). Adaptor sequences for cloning into pLentiGuide-hygro were then added and submitted to oligonucleotide synthesis (Integrated DNA Technologies, Newark, NJ). The list of the sgRNAs oligos is shown in **Table S3.2**.

The sgRNAs oligos were resuspended in nuclease-free water, diluted, and annealed according to the reaction setup indicated in **Table S3.3**. For conventional cloning of sgRNAs duplexes, the pLentiGuide-hygro vector was linearized using the restriction enzyme Esp3 (BsmBI). Upon digestion, fragments were subjected to separation by electrophoresis and purification using the QIAquick® Gel Extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. The ligation reaction was performed with the linearized vector and diluted duplexes using the T4 DNA ligase (**Table S3.3**). Stbl3™ cells (Thermo Fisher Scientific, Waltham, MA) were transformed with the ligation product, plated in LB agar plates with Ampicillin (100 µg/mL), and incubated at 37°C for 24h.

Bacteria colonies were then isolated, grown in LB liquid with Ampicillin (100 µg/mL) for 24h, and subsequently submitted to plasmid purification using the QIAprep® Spin Miniprep Kit (Qiagen, Germany). The purified plasmids were validated by Sanger sequencing using the universal primer LKO.1.5' forward (**Table S3.4**; Eton Biosciences, Boston, MA).

Sequences were analyzed for the presence of the insert using an oligo annealing tool with the SnapGene Software (GSL Biotech LLC, Chicago, IL). Validated plasmids were then used for transfection in 293T cells to produce lentiviral particles.

2.2 Production of sgRNA lentiviral particles

To produce lentiviral particles, 293T cells were seeded onto 6-wells plates ($5x10^5$ cells/mL) and grown at 37°C with 5% CO_2 for 24 h in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Waltham, MA) supplemented with fetal bovine serum (10% v/v), and penicillin/streptomycin (Gibco). Cells were transfected using the TransIT®-LT1 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions. The packaging vectors psPAX2 (Addgene plasmid #12260; **http://n2t.net/addgene:12260**), VSV-G (Addgene plasmid #14888; **http://n2t.net/addgene:14888**), and the destination vector were mixed in Opti-MEM to form the Lipid-DNA complexes, added drop-wise to different areas of the wells. After a 24-h incubation, the medium was removed and replaced by RPMI-1640 (Thermo Fischer Scientific, Waltham, MA). Lentiviral supernatants were collected 24- and 48-h post-transfection and filtered in 0.45µm syringe filters (Merck Millipore, Burlington, MA).

2.3 CRISPR/Cas-9 editing and induction of EBV lytic cycle

Akata cells constitutively expressing Cas9 were seeded at $5x10^5$ cells/mL onto T25 flasks and grown in a complete RPMI 1640 medium. After 24 h, fresh-filtered lentiviral supernatants from 293T cells were added directly into Akata cells for two days. Cells were then selected with 500 µg/ml hygromycin (Calbiochem, Merck Millipore, Burlington, MA) for 3 weeks before further experiments. The induction of the EBV lytic cycle in EBV-positive Akata cells (wild-type and mutant EBV genomes) was performed by IgG crosslink with anti-human IgG antibody (**Table S3.5**; Dako Agilent, Santa Clara, CA). Cells were seeded at $5x10^5$ cells/mL onto 6-wells plates and treated with 10 µg/mL anti-human IgG, as previously described **(12)**. The cells were then incubated at 37°C with 5% CO_2 for 24 h before subsequent assays.

2.4 Analysis of CD79a (Igα) and gp350 expression in Akata cells

The intracellular expression of CD79a (Igα) and viral gp350 was analyzed by Flow Cytometry in IgG-induced EBV-positive Akata cells. Induced and non-induced (control) cells

were fixed with BD Cytofix™ buffer at 4˚C for 20 min and permeabilized in BD Perm/Wash™ buffer (Becton Dickinson, Franklin Lakes, NJ). Cells were then incubated with conjugated APC anti-human CD79a antibody at 4˚C for 30 min. For EBV gp350, cells were washed once in PBS and then incubated with APC conjugated anti-gp350 72a1 antibody at 4˚C for 30 min. Cells were washed with BD Perm/Wash™ or PBS buffer twice before analysis on a FACSCalibur™ instrument (Becton Dickinson, Franklin Lakes, NJ). Data were processed and analyzed using FlowJo software (FlowJo LLC, Becton Dickinson, Franklin Lakes, NJ). The antibodies used are indicated in **Table S3.5**.

2.5 Cloning EBV ORFs library into the DOX-inducible vector PLIX-402

We aimed to create a panel with doxycycline (DOX)-inducible vectors containing EBV ORFs from a library available in our lab using the pLIX-402 vector, a gift from David Root (Addgene plasmid #41394). The plasmid was transformed in One Shot™ ccdB Survival™ 2 T1R Competent Cells (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. After transformation, the competent bacteria were plated in LB-agar plates with ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) and incubated at 37°C for 24 h. Growing colonies were isolated, grown in LB liquid with ampicillin (100 μg/mL) and chloramphenicol (25 μg/mL) for 24 h, and plasmid purification was conducted using the QIAprep® Spin Miniprep Kit (Qiagen, Germany). The vector was validated according to its size and digestion using the BsrGI enzyme.

EBV ORFs were cloned into the pLIX-402 vector using Gateway cloning technology (Thermo Fisher Scientific, Waltham, MA), using the conditions indicated in **Supplementary Table 3.2**. Briefly, after LR Reaction with entry and destination vectors, Stbl3™ cells (Thermo Fisher Scientific, Waltham, MA) were transformed with the recombination reaction, plated in LB agar plates with ampicillin (100 μg/mL), and incubated at 37°C for 24 h. Colonies were isolated, grown in LB liquid with ampicillin (100 μg/mL) for 24 h, and subsequently submitted to plasmid purification using the QIAprep® Spin Miniprep Kit (Qiagen, Germany). The recombinant vectors were validated by BsrGI digestion. Furthermore, purified plasmids were validated by Sanger sequencing using the LNCX_seq forward primer (**Table S3.4**). The sequences obtained were evaluated using the NCBI BLAST tool (**https://blast.ncbi.nlm.nih.gov/Blast.cgi**) for matching sequences.

2.6 Effect of EBV ORFs in P3HR1-ZHT/RHT and BJAB cells

Upon validation, lentiviral particles with EBV ORFs on the pLIX-402 backbone were produced in 293T cells as described in **Section 3.2**. P3HR1-ZHT/RHT and BJAB cells were transduced with fresh-filtered lentiviral supernatants from 293T cells for two days. The cells were then selected in 500 µg/ml puromycin (Thermo Fisher Scientific, Waltham, MA) for 2 weeks before further experiments.

Single-clone P3HR1-ZHT/RHT and BJAB cells transduced with EBV ORFs were analyzed for CD79a expression by FACS after 24h induction with doxycycline. Briefly, cells were fixed with BD Cytofix™ buffer at 4˚C for 20 min and permeabilized in BD Perm/Wash™ buffer (Becton Dickinson, Franklin Lakes, NJ). Cells were then incubated using the conjugated CD79a anti-mouse primary antibody for 30 min (**Table S3.5**). The expression of selected proteins was also analyzed by western blot. For that purpose, transduced cells were treated with doxycycline for 24 h and then total protein lysates were used in western blots with antibodies against IgM, Igα, Stat2, Aim2, and HA-tag (**Table S3.5**).

3 Results

3.1 IgG-mediated induction of EBV lytic cycle decreases CD79a (Igα)

Consistent with previous findings, IgG-crosslinking significantly decreased the expression of CD79a and increased the expression of GFP (**Figure 3.1**). Two distinct cell subpopulations were identified: $CD79a^{low}/GFP^{high}$ and $CD79a^{low}/GFP^{high}$. The EBV-positive Akata cells used have a GFP expression cassette inserted downstream of the right oriLyt of the AK-BAC (12). Consequently, an increased expression of GFP fluorescence is observed during lytic reactivation upon replication of the EBV genome by the BALF5 viral DNA polymerase. In our model, IgG-crosslinking induced lytic reactivation in up to 30% of the cells, with high variability among experimental replicates (**Figure 3.1**).

3.2 Effects of EBV early lytic genes on EBV-positive Akata cells

EBV-positive Akata cells (wild type and CRISPR/Cas9 mutants) were subjected to FACS analysis of CD79a expression by flow cytometry after IgG treatment for lytic reactivation. We hypothesized that viral genes possibly involved in CD79a degradation would show a distinctive feature during the screening of EBV earl lytic genes KOs, with total or partial

restoration of CD79a expression. No distinctive phenotype regarding CD79a expression in the Akata mutants was observed since all the genes assessed showed 15-40% of CD79a^{low} cells (**Figure 3.2**). To overcome this issue, we have created a new panel with inducible expression of EBV early lytic ORFs transduced in EBV-positive P3HR1-ZHT/RHT and EBV-negative BJAB cells to investigate possible viral lytic genes involved in CD79a degradation.

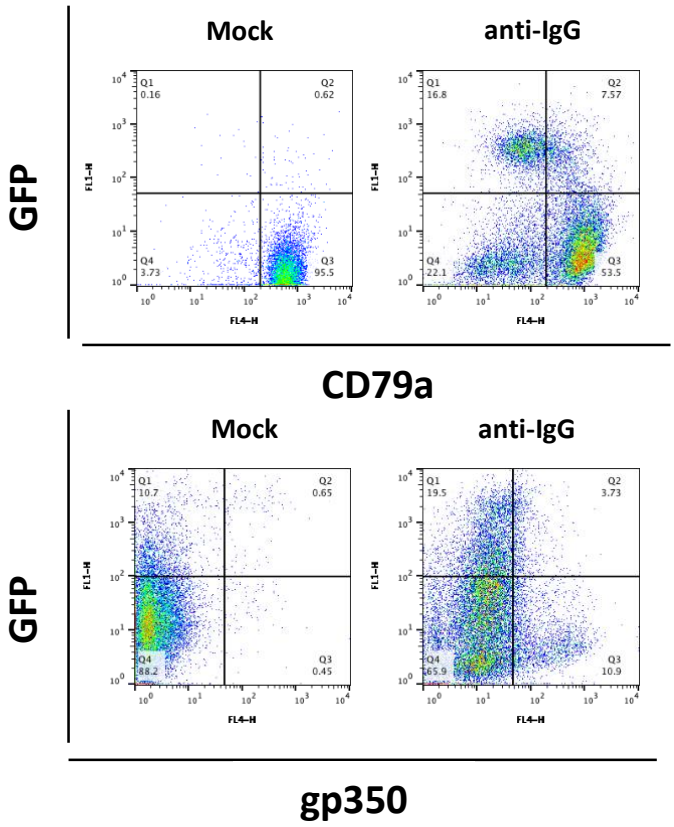

Figure 3.1 – Effects of lytic cycle induction with anti-human IgG crosslinking on the expression of CD79a (Igα) and gp350 in Akata EBV-positive cells.

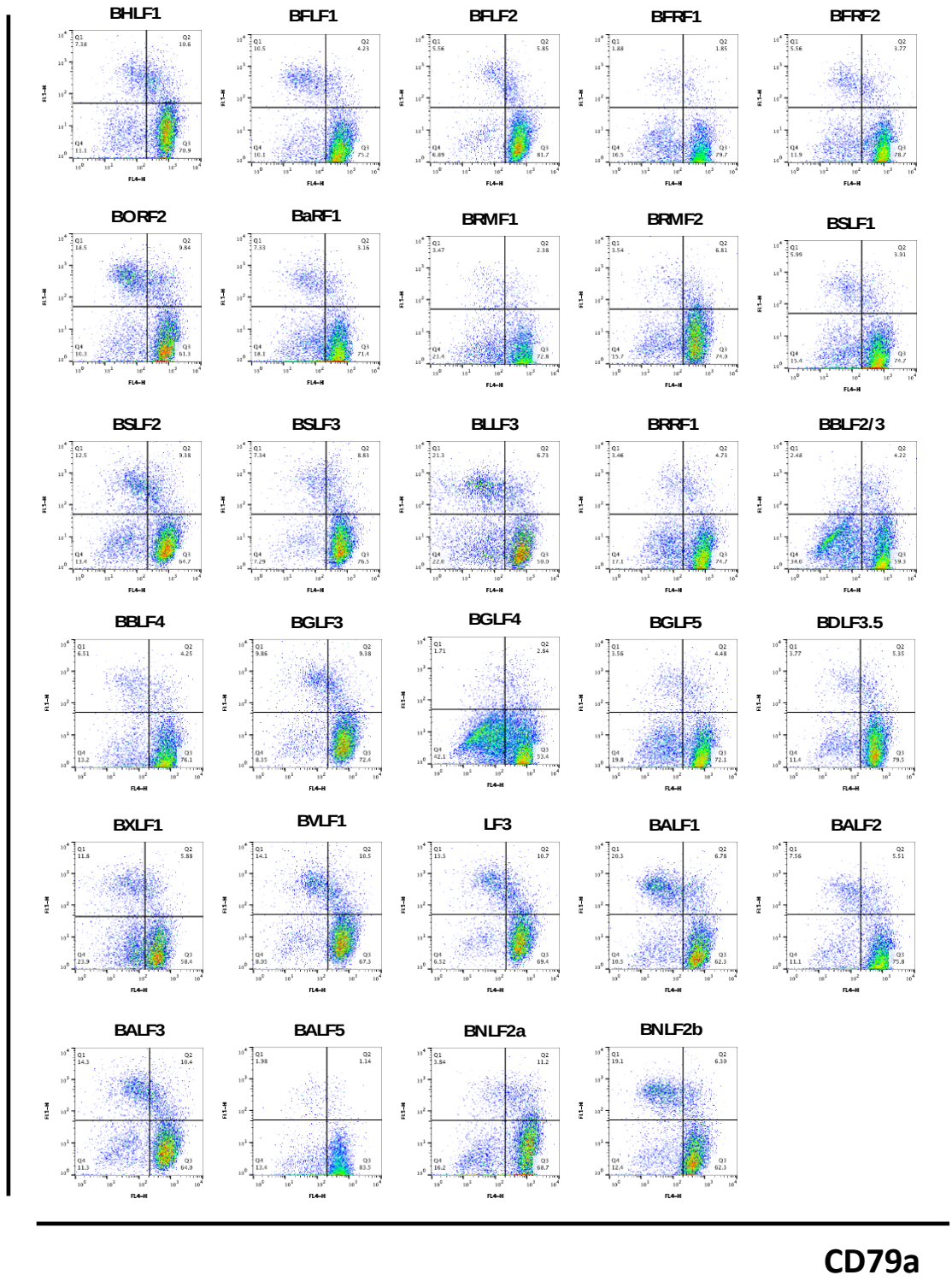

Figure 3.2 – Effects of EBV early lytic genes knocking-outs (KOs) on CD79a (Igα) expression in Akata EBV-positive cells induced with anti-human IgG cross-linking.

3.3 Effects of EBV early lytic genes on the expression of CD79a (Igα)

The P3HR1-ZHT/RHT cells transduced with EBV early lytic ORFs were induced with 5 µg/mL doxycycline for 24 h and then collected, fixed, and stained for CD79a detection according to the steps described previously. During the screening of EBV early lytic ORFs in

P3HR1-ZHT/RHT cells, we found one possible candidate involved in the degradation of CD79a, the gene BRRF1 encoding the Na protein (**Figure 3.3**). In early pilots, we also observed degradation of CD79a by BZLF1, an early-immediate gene that codifies the Zta protein, a transactivator involved in the initiation of the lytic cycle in EBV-positive cells. Hence, we sought to investigate whether the expression of BZLF1 in EBV negative cells (BJAB) can also cause CD79a degradation.

The effects of the remaining EBV early lytic genes ORFs on the expression of CD79a are also shown in **Figure 3.3**. We did not observe any changes in CD79a profiles, except for a very slight degradation in the BRLF2 gene. Following next, we proceeded to the validation of the potential targets involved in CD79a in EBV-positive P3HR1-ZHT/RHT and EBV-negative BJAB cells using cell cytometry and analysis of the expression of proteins by western blot.

3.4 BRRF1 induces CD79a (Igα) degradation in EBV-positive and EBV-negative cells

As shown in **Figure 3.4**, the expression of BZLF1 reduces CD79a in EBV-positive P3HR1-ZHT/RHT cells but not in EBV-negative BJAB cells. Interestingly, in both EBV-negative and positive cells lines, the expression of BRRF1 decreases CD79a expression. The BJAB and P3HR1-ZHT/RHT cells induced with 5 μg/mL doxycycline were also harvested for protein extraction and protein expression analysis by western blot. We assessed the expression of IgM, CD79a (Igα), Stat2, Aim2, and HA-tag in both EBV-negative and EBV-positive cells lines, as shown in **Figure 3.5**. Although IgM expression remained unchanged in both cell lines, we observed a significant reduction in CD79a expression in BJAB EBV-negative cells lines transduced with the EBV BRRF1 ORF. Furthermore, a reduction in CD79a expression was also found in P3HR1-ZHT/RHT EBV-positive cells expressing both BZLF1 and BRRF1. No differences in the expression of Stat2 and Aim2 can be observed in both BJAB and P3HR1-ZHT/RHT cells. The HA-tag staining shows that BZLF1 (37 kDa), BRRF1 (34kDa), and GFP (27 kDa) are being expressed in both cell lines. Therefore, our preliminary results support the activities of BRRF1 in the degradation of the BCR-complex during lytic reactivation.

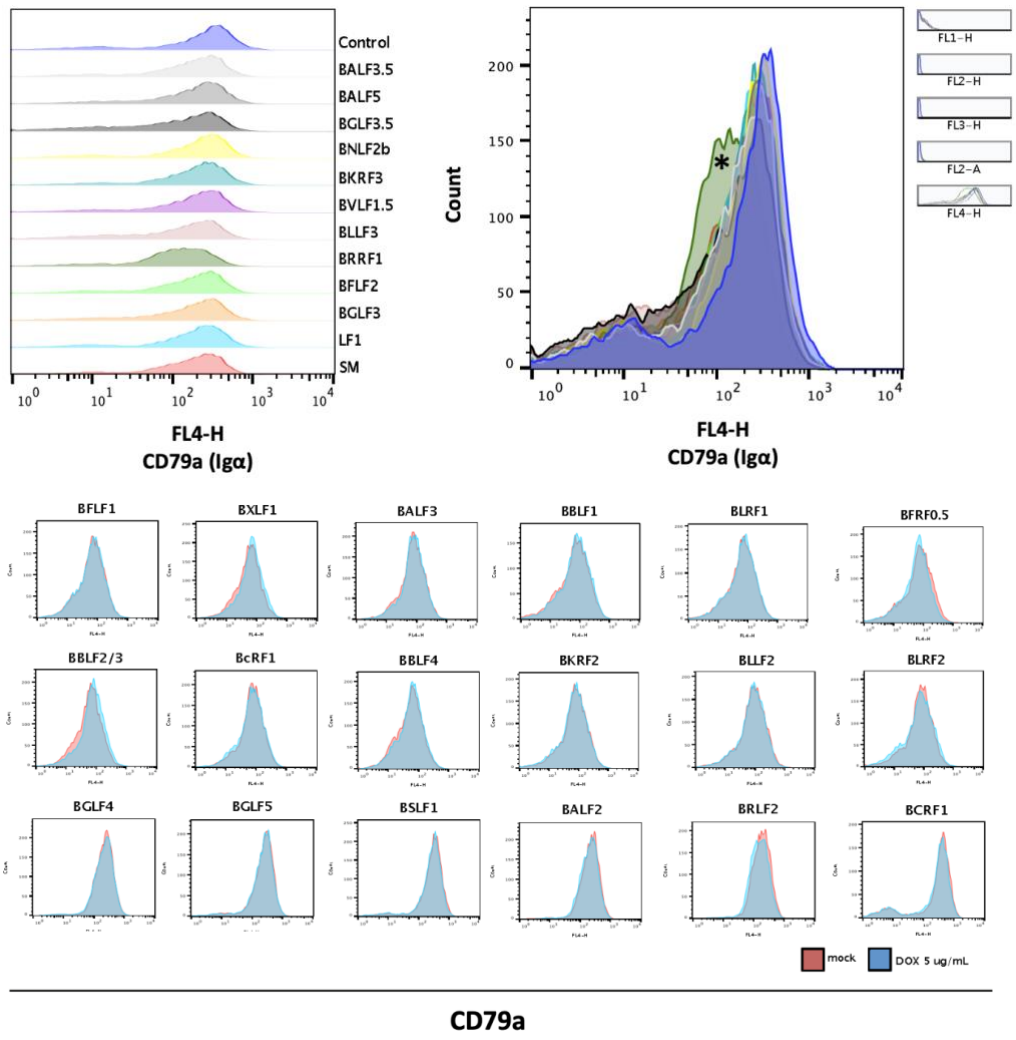

Figure 3.3 – Effects of EBV early lytic genes on the expression of CD79a in P3HR1-ZHT/RHT cells induced with 5 µg/mL Dox for 24 hours. BRRF1 shows decreased expression of CD79a (asterisk) compared to controls and other EBV ORFs induced in P3HR1-ZHT/RHT cells. Dox: doxycycline; 4-HT: 4-hydroxytamoxifen; NaB: sodium butyrate.

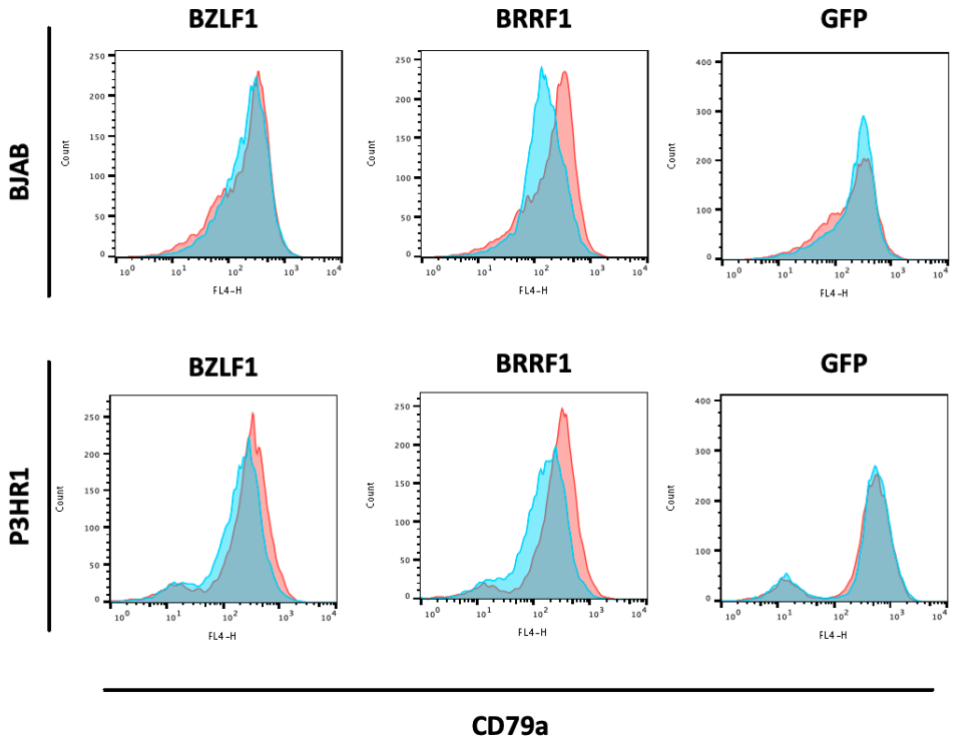

Figure 3.4 – Expression of CD79a in BJAB and P3HR1-ZHT/RHT cells transduced with pLIX-BZLF1, pLIX-BRRF1, and pLIX-GFP vectors. The expression of BZLF1 in EBV-negative BJAB cells does not decrease the levels of CD79a, while BRRF1 expression alone significantly decreases CD79a expression. Dox: doxycycline; 4-HT: 4-hydroxytamoxifen; NaB: sodium butyrate.

3.5 BLRF2 induces CD79a (Igα) degradation in P3HR1-ZHT/RHT cells

The effects of BLRF2 expression on CD79a profiles in EBV-positive, single-clone P3HR1-ZHT/RHT cells transduced with the pLIX-BZLF1, pLIX-BLRF2, and pLIX-GFP were also analyzed. Single clone cells kindly provided by Stephanie Yiu were induced with doxycycline, 4-hydroxytamoxifen (4-HT), and sodium butyrate (NaB) for 24 hours. GFP cells were used as a positive control for the induction. The expression of BRFL2 decreases CD79a expression in comparable levels to that of BZLF1 (**Figure 3.6**).

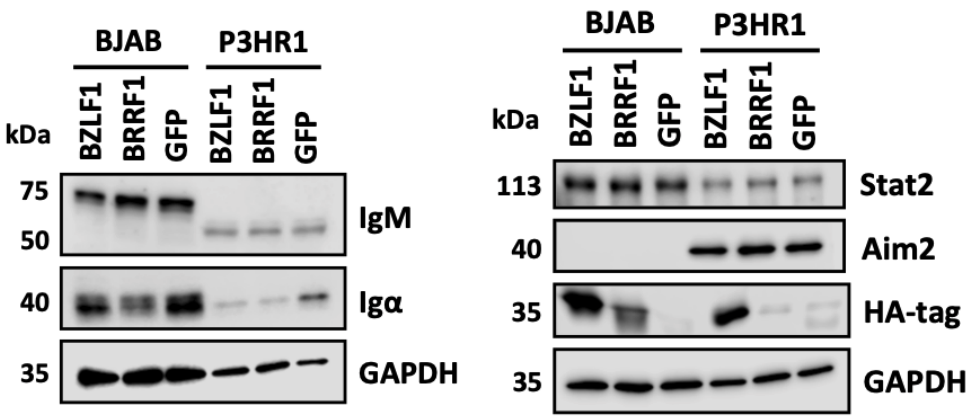

Figure 3.5 – Effects of BZLF1, BRRF1, and GFP expression on IgM, Igα, Stat2, Aim2, and HA-tag protein expression assessed In BJAB EBV-negative and P3HR1 EBV-positive cells by western blot.

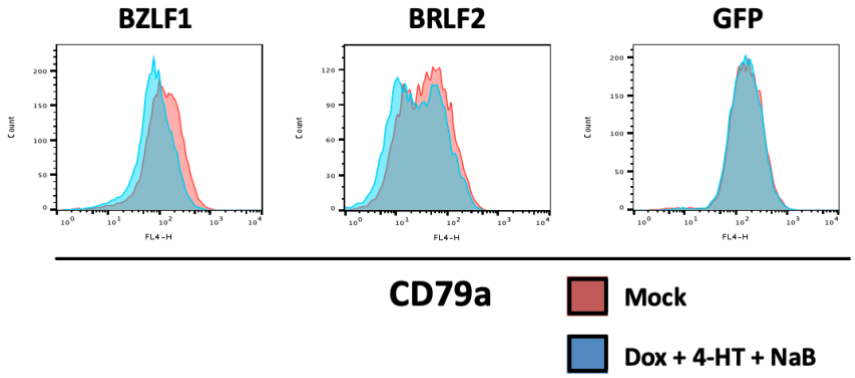

Figure 3.6 – Expression of CD79a in P3HR1-ZHT/RHT single-clone cells transduced with pLIX-BLZF1, pLIX-BLRF2, and pLIX-GFP vectors. The expression of both BZLF1 and BRLF2 in EBV-positive cells significantly decreases CD79a expression in cells induced with Dox. Dox: doxycycline; 4-HT: 4-hydroxytamoxifen; NaB: sodium butyrate.

4 Discussion

The objective of this study was to identify early lytic genes encoded by EBV related to the BCR degradation during lytic induction. We constructed a panel with EBV inducible ORFs in EBV-positive P3HR1-ZHT/RHT cells and EBV-negative BJAB cells and analyzed the expression of IgM and CD79a under the controlled expression of EBV ORFs. Our results show that the EBV early lytic gene BRRF1 targets CD79a and may degrade the BCR complex. We also found that a late protein associated with viral tegument proteins, BLRF2, also downregulates CD79a and can be closely associated with BRRF1.

The activation of the lytic cycle involves the sequential expression of immediate-early

(IE), early (E), and (L) proteins. The immediate-early genes BZLF1 and BRLF1 play an essential role in switching EBV from the latent to lytic cycles (5). BZLF1 encodes Zta, a lytic immediate-early transcription factor that enhances the expression of early EBV genes. Zta recruits CREB-binding protein (CBP), promoting histone acetylation and stabilizing the association of transcription factor IIA (TFIIA) to the TATA element, also functioning as an origin-binding protein to the EBV ori-Lyt. BRLF1 encodes Rta, another essential lytic immediate-early transcription factor protein responsible for the transcriptional activation of viral lytic genes by directly binding to the BRFL1-responsive elements or indirectly binding through other transcript factors. Rta can also enhance specific signal transduction cascades such as MAPK signaling (13).

Furthermore, the immediate-early locus of EBV contains an ORF within the first intron of the Rta gene that is oriented in the opposite direction and encodes the protein BRRF1 (also designated Na). BRRF1 is a transcription factor able to increase the ability of Rta to activate Zta expression via interaction with the Zta promoter (Zp) in 293 cells infected with the Rta-KO EBV genome (14). The promoter region of the BRRF1 gene was also shown to be activated by the EB1/Zta protein, producing two different mRNAs species translated into a 34kDa protein found in the nucleus of HeLa cells transfected with the expression vector (15). The expression of BRRF1 in the infected cells results in increased hyperphosphorylated c-Jun (14). Interestingly, TPA induction also activates the ERK/c-Jun signaling pathway, facilitating Tet1 to bind to Zp, increasing DNA demethylation, EBV gene expression, and viral reactivation (16). Thus, BRRF1 plays a crucial role in an auto-regulatory circuit for Rta-mediated EBV lytic induction, although the expression of BRRF1 alone is dispensable for viral replication in 293 cells (14,17).

The BLRF2 gene encodes a 23kDa capsid-associated tegument protein, also known as capsid antigen (VCA)-p2. BLRF2 is a late protein that shares homology with the Kaposi's sarcoma-associated herpesvirus (KSHV) and the Murine gammaherpesvirus-68 (MHV-68) ORF52. As an associated-tegument protein, BLRF2 is a Rta-responsive late gene required for the maturation and egress of viral particles mediated by vesicle trafficking, and its transcription is modulated via repression by phosphorylated Zta (18). Although BLRF2 is not well studied in the context of EBV, ORF52-null viral particles associate with decreased incorporation of ORF45 (EBV's BKRF4 homolog) in KSHV, including other teguments proteins. ORF52 is crucial for the packaging of viral proteins and is required for the optimal production

of viral progenies (19). Interactome studies of BLRF2 ORF show a strong interaction with BRRF1 homolog, the KSVH ORF49 (20). Hence, it is plausible that BCR degradation is caused by a complex formed by BRRF1 and BLRF2 during lytic activation, with both proteins involved in the Rta-mediated gene expression.

The BCR complex control is pivotal for B-cell fate decisions, including cell survival, differentiation, and proliferation (21). Activation of the BCR pathway occurs through antigen triggers that induce the phosphorylation of the cytoplasmatic subunits CD79a (Igα) and CD79b (Igβ) by Src-family tyrosine kinases, recruiting signalosome components including Syk, Btk, Vav, Grb2 to transduce downstream signals involving the PLC-γ2, PI3K and MAPK pathways. The induction of BCR signaling ultimately leads to the activation of multiple transcription factors such as the nuclear factor kappa-B (NF-κB) and Jun, inducing genes that promote B cell proliferation and survival (22). A study published in 2017 assessed the temporal proteomics expression of EBV-positive B-cells induced to the lytic cycle. It showed that EBV early lytic factors might target cell-surface and intracellular B-cell receptor (BCR) for ubiquitination and subsequently proteasomal degradation (7). One of the hypotheses mentioned for the BCR degradation during lytic activation considers that lytic B cells may produce Ig-reactive viral proteins that could trap EBV lytic proteins by binding to these antibodies via either the secretory pathway or at the cell surface (7). It was also previously suggested that BCR downregulation by autoreactive B cells is closely associated with pathways involved with immunological anergy (23).

In conclusion, our study shows that CD79a is targeted for degradation by BRRF1, an early transcript factor responsible for Rta regulation, and BLRF2, a late protein associated with viral tegument proteins (**Figure 3.7**). Although preliminary, our findings shed some light on the underlying mechanisms of BCR degradation during lytic activation in BL cells, pointing towards a complex and intricated regulation exerted by EBV immediate-early and late lytic genes.

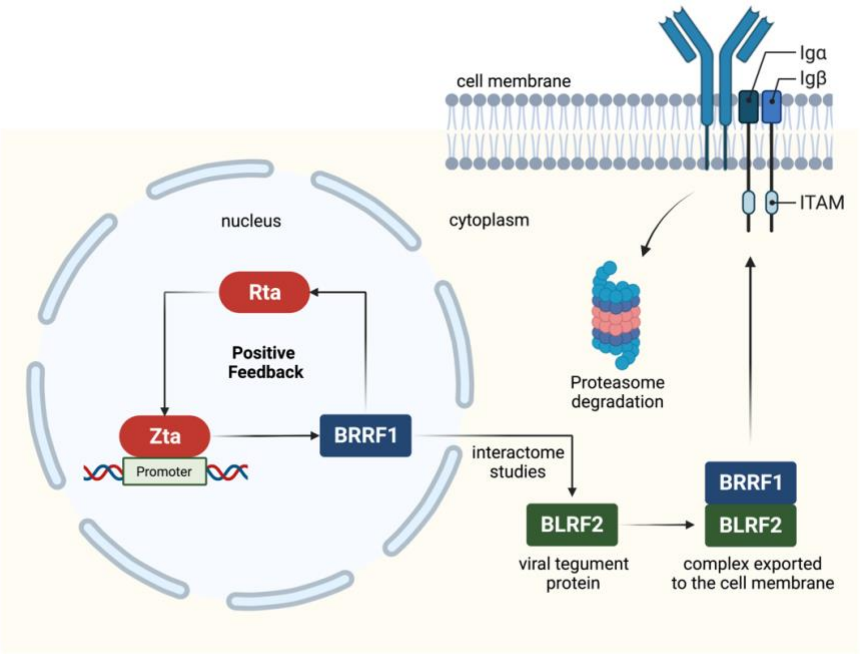

Figure 3.7 – A proposed mechanism for BCR degradation induced by BRRF1 and BLRF2. BRRF1 is a transcription factor that interacts with Rta, binding in the Zp and increasing the transcription of Zta, favoring the lytic cycle induction. BLRF2, on the other hand, is a late protein associated with the viral tegument. Interactome studies have shown that BRRF1 has a strong affinity for BLRF2 and might form a complex exported to the cell membrane. Thus, we hypothesize that BRRF1 and BLRF2 complexes might interact and degrade the BCR receptor during lytic cycle activation.

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6 Supplementary material

Table S3.1 – List of early lytic genes encoded by EBV

#	Gene Name	Properties
1	BHLF1	Involved in viral DNA synthesis
2	BHRF1	Protect cells from cell death under certain conditions
3	BFLF2	Interacts with BFRF1 and regulates nuclear egress of nucleocapsid
4	BFLF1	Packaging of viral DNA
5	BFRF1	Interacts with BFLF2 and regulates nuclear egress of nucleocapsid
6	BFRF2	Transactivation for late genes
7	BORF2	Large subunit of ribonucleotide reductase
8	BaRF1	Small subunit of ribonucleotide reductase
9	BMRF1	Processability subunit of DNA polymerase BALF5
10	BRMF2	Membrane protein attaches to integrins, interacts with BDLF2
11	BSLF2	Post-transcriptional regulator produced by splicing
12	BSLF1	Primase for viral DNA synthesis
13	BLLF3	Hydrolyzes of dUTP to prevent misincorporation
14	BRRF1	Dispensable for lytic replication
15	BKRF3	Removes misincoporated uracil from DNA
16	BBLF4	Helicase for viral DNA synthesis
17	BBLF2/3	Binds to BSLF1 primase
18	BGLF5	Alkaline exonuclease, host shutoff
19	BGFL4	Phosphorylates viral and cellular proteins
20	BGLF3.5	Possible tegument protein
21	BGLF3	Transactivation for late genes
22	BDLF4	Transactivation for late genes
23	BDLF3.5	Transactivation for late genes
24	BcRF1	Transactivation for late genes, TATA-binding protein
25	BXLF1	Thymidine kinase
26	BVRF1	Cork protein that confines viral genome DNA in the capsid
27	BVLF1	Transactivation for late genes
28	LF3	Abundant transcript involved in viral DNA synthesis
29	BALF5	Viral DNA polymerase catalytic subunit
30	BALF3	Terminal ligase subunit
31	BALF2	single-stranded DNA-binding protein

#	Gene Name	Properties
32	BALF1	Protect cells from cell death under certain conditions
33	BARF1	Soluble decoy for CSF-1, a role in cell immortalization
34	BNLF2b	Not reported
35	BNLF2a	Downregulates HLA through inhibition of TAP

[1]Adapted from Murata 2018.

Table S3.2 – List of sgRNAs oligos cloned into the pLentiGuide-hygro vector

Gene Name	sense (5'-3') / anti-sense (3'-5')
BALF3_sg1	ACACGAAACGGAAGAAATCAATTCGG AAAACCGAATTGATTTCTTCCGTTTC
BALF3_sg2	AAAACCGACGGAGTTAACATCACCGC AAAACCGACGGAGTTAACATCACCGC
BARF1_sg1	ACACGCATGAAACTGGGCGAGACCGG AAAACCGGTCTCGCCCAGTTTCATGC
BARF1_sg2	ACACCGCTGCCAACATCTCCCATGAG AAAACTCATGGGAGATGTTGGCAGCG
BFLF2_sg1	ACACGCGACGAAGATGCCATCTACGG AAAACCGACGAAGATGCCATCTACGC
BFLF2_sg2	ACACCGCTGGCCGTGGACCTAGTAGG AAAACCTACTAGGTCCACGGCCAGCG
BGLF3_sg1	ACACGTATGGAGCCGAGATCATACGG AAAACCGTATGATCTCGGCTCCATAC
BGLF3_sg2	ACACGACATGGTGTATAAACCGCAGG AAAACCTGCGGTTTATACACCATGTC
BGLF3.5_sg1	ACACGATGTTCCTCAAATGGCTCGAG AAAACTCGAGCCATTTGAGGAACATC
BGLF3.5_sg2	ACACGAGCTAATTTCTACCTATCAGG AAAACCTGATAGGTAGAAATTAGCTC
BHLF1_sg1	ACACCGCAACAGTGCCACCAACCCGG AAAACCGGGTTGGTGGCACTGTTGCG
BHLF1_sg2	ACACCGCCACCCCCCACCCGGAGCGG AAAACCGCTCCGGGTGGGGGGTGGCG
BHRF1_sg1	ACACGTTTGTAGAGATATTTCACCGG AAAACCGGTGAAATATCTCTACAAAC
BHRF1_sg2	ACACGACCTCTCCGCCTTTCGCCAGG AAAACCTGGCGAAAGGCGGAGAGGTC
BKRF3_sg1	ACACGTCACGGCTGTCTAGACGCGTG AAAACACGCGTCTAGACAGCCGTGAC
BKRF3_sg2	ACACGCTGGCATTCAGCGTCGCATAG AAAACTATGCGACGCTGAATGCCAGC
BMLF1_sg1	ACACCGCCCCTCGAGATTCTGACCGG AAAACCGGTCAGAATCTCGAGGGGCG
BMLF1_sg2	ACACCGGGAATCACAAACGATGCTGG AAAACCAGCATCGTTTGTGATTCCCG
BRMF2_sg1	ACACCGTACAGGCTTGGATTCCCGAG AAAACTCGGGAATCCAAGCCTGTACG

Gene Name	sense (5'-3') / anti-sense (3'-5')
BRMF2_sg2	ACACCGAGTCTACTCGACCAGCACGG AAAACGAGTCTACTCGACCAGCACGG
BSLF2_sg1	ACACGTACATCAAGAATTACAACCCG AAAACGGGTTGTAATTCTTGATGTAC
BSLF2_sg2	ACACGCTGCGCCATGAGTCTTAGCAG AAAACTGCTAAGACTCATGGCGCAGC
BVLF1_sg1	ACACGCAATTACCCACTCCAGTGCGG AAAACCGCACTGGAGTGGGTAATTGC
BVLF1_sg2	ACACGTAAATAAAGTCATCGCACGGG AAAACCCGTGCGATGACTTTATTTAC
BVRF1_sg1	ACACGAGCCCCTCTCAACCGACAGGG AAAACCCTGTCGGTTGAGAGGGGCTC
BVRF1_sg2	ACACCGGGACCGAGACCTGGTGTACG AAAACGTACACCAGGTCTCGGTCCCG
LF3_sg1	ACACGCGCTGCCCCGCTCCGGCGGGG AAAACCCCGCCGGAGCGGGGCAGCGC
LF3_sg2	ACACGCGGCCGCCCCCCGAGCTCCAG AAAACTGGAGCTCGGGGGGCGGCCGC

Table S3.3 – Reaction components and conditions

Experiment Performed	Reaction Components	Reaction Conditions
pLentiGuide-hygro vector digestion with Esp3 (BsmBI) restriction enzyme	Esp3 Buffer (33 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate), 0.1 mg/mL BSA and 1.0 mM DTT	Incubation at 37°C for 5 hours, followed by thermal inactivation at 65°C for 20 min.
Annealing sgRNAs oligos into duplexes	Ligation Buffer (50 mM Tris-HCl, 10 mM MgCl2, 10 mM Dithiothreitol 1 mM ATP, pH 7.5) and 50nM sense and anti-sense oligos.	Incubation at 37°C for 30 min, 95°C for 5 min and then ramping down to 25°C at 5°C/min.
Ligation of sgRNA oligos into a digested pLentiGuide-Hygro vector with T4 DNA ligase	T4 DNA reaction Buffer (50 mM Tris-HCl, 10 mM MgCl2, 10 mM Dithiothreitol 1 mM ATP, pH 7.5), 50 ng previous digested vector, diluted oligos (100 mM), 40U T4 DNA ligase.	Incubation at room temperature for 30 min, followed by thermal inactivation at 65°C for 10 min.
Validation of pLIX-TRC402 vector by digestion with BsrGI restriction enzyme	NEB Buffer 2.1 (50 mM NaCl. 10 mM Tris-HCl. 10 mM MgCl2 100 µg/ml BSA. pH 7.9), 500 ng DNA, 10 U BsrGI.	Incubation at 37°C for 30 min, followed by thermal inactivation at 80°C for 20 min.
LR Clonase II Recombination for pLIX-TRC402 vectors	Tris-EDTA Buffer (10 mM Tris-Cl, EDTA 0.51 mL 10 mM e 1 mM EDTA, pH 8.0), LR Clonase II Mix, 150 ng destination vector and 100 ng entry vector.	Incubation at 22°C for 16h followed by thermal inactivation with proteinase K at 37°C for 10min

Table S3.4 – DNA sequencing primers used to validate pLentiGuide-Hygro and pLIX-TRC402 constructs by Sanger Sequencing

Primer	Description	Sequence (5'-3')
LKO.1 5'	hU6 promoter, forward primer	GACTATCATATGCTTACCGT
LNCX_seq_F	Human CMV promoter, forward primer	AGCTCGTTTAGTGAACCGTCAGATC

Eton Biosciences, Boston, MA.

Table S3.5 – List of antibodies used for FACS, Western Blot (WB), and lytic induction experiments

Antibody	Manufacturer	Cat #
Anti-CD79A rabbit mAb (D1X5C)	Cell Signaling Technology, Danvers, MA	13333
Anti-EBV gp350 (72A1)	BioXCell, Lebanon, NH	n/a
Anti-GAPDH mouse mAb	Proteintech, Rosemont, IL	60004-1-1g
Anti-goat bovine IgG	Jackson ImmunoResearch, West Grove, PA	805-035-180
Anti-IgM goat F(ab')2	Southern Biotech, Birmingham, AL	2020-02
anti-Mouse IgG (H+L), APC	Thermo Fischer Scientific, Waltham, MA	A-865
Anti-Mouse IgG, IgM (H+L), AF 488	Thermo Fischer Scientific, Waltham, MA	A-10684
Anti-Stat2 rabbit mAb (D9J7L)	Cell Signaling Technology, Danvers, MA	72604S
APC anti-mouse CD79a (Igα)	BioLegend Way San Diego, CA	133106
HRP-conjugated anti-mouse	Cell Signaling Technology, Danvers, MA	7076
HRP-conjugated anti-rabbit	Cell Signaling Technology, Danvers, MA	7074
Polyclonal rabbit anti-human IgG	Agilent Dako, Santa Clara, CA	A0423
Purified anti-AIM2 Antibody	BioLegend Way San Diego, CA	652802

Appendix 1 – Review Paper: Epstein-Barr Virus (EBV)-encoded microRNAs in the pathogenesis of human cancers

Abstract

The Epstein-Barr Virus (EBV) is a gamma-herpesvirus involved with a variety of human cancers, notably the endemic Burkitt lymphoma and nasopharyngeal carcinoma. In 2004, EBV was described as the first known human oncovirus to encode viral microRNAs (miRNAs), and these molecules were found to interact with viral and host targets. EBV miRNAs modulate biological processes that are critical for carcinogenesis, contributing to cell transformation and tumor progression of EBV-associated cancers. Herein we review and discuss EBV miRNAs as modulators of viral biology and carcinogenesis, as well as their usefulness as putative markers to monitor the onset, progression, and recurrence of cancers associated with the EBV infection.

+ Keywords: Epstein-Barr Virus,

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1 Epstein-Barr virus: an overview

Cancers accounted for more than 18.1 million new cases and 9.6 million deaths in 2018 and are a leading cause of morbidity and mortality worldwide (1). The incidence and prevalence rates for specific cancers vary worldwide among different geographical regions and socioeconomic contexts. The risk of developing cancers is influenced by constitutive factors (genetic background, for instance), lifestyle, and environmental factors, such as exposition to some physical, chemical, or infectious agents, most of them preventable.

The Epstein-Barr-Virus (EBV) deserves special attention compared to other infectious agents associated with human cancers for several relevant reasons. EBV is a ubiquitous virus that causes lifelong latent infection in over 90% of the human adult population worldwide. In 2018, 2.2 million new cancer cases (about 13% of all cancers diagnosed) were estimated to be associated with infectious agents, mostly in developing countries (2). Also, EBV infection is associated with various human cancers, especially the endemic form of Burkitt lymphoma (BL) and nasopharyngeal carcinoma (NPC). Nonetheless, it also has an etiopathogenic role in a variable number of other malignancies, such as classical Hodgkin lymphomas (cHL), non-Hodgkin lymphomas (NHL), and gastric carcinomas (GC) (3).

EBV is a gammaherpesvirus, formally designated Human Herpesvirus 4 (HHV-4). As for other herpesviruses, the EBV life cycle is divided into lytic and latent infection phases. Either in vivo or in vitro, most of the EBV-infected cells are in viral latency, a phase in which only a subset of viral genes is expressed. Conversely, most of the viral genes are expressed to produce new particles during the lytic cycle, allowing persistent infection and viral dissemination to other hosts. The lytic reactivation occurs sporadically in vivo, usually triggered by changes in the status of the host's immune responses. It also occurs spontaneously in a small fraction of latently infected cells in vitro, but higher levels of lytic reactivation can be obtained by treating the infected cells with phorbol esters tetradecanoylphorbol-13-acetate (TPA) or sodium butyrate (4). The EBV proteins Zta and Rta (encoded by BZLF1 and BRLF1 genes, respectively) are critical activators of viral lytic infection.

Three profiles of viral gene expression are typically observed in cells latently infected by EBV, described as latency types I, II, and III (see Table 1). Some EBV products are expressed in all latency programs, such as the EBV-encoded small RNAs (EBERs), BamHI-A rightward transcripts (BARTs), and the EBV nuclear antigen 1 (EBNA1). This minimum set of viral

products expressed during the latent phase of the viral life cycle characterizes the EBV latency type I, commonly found in BL cells in vivo. Latency type II also encompasses the expression of EBV latent membrane proteins (LMPs 1, 2A, and 2B), and it is typically observed in NPC neoplastic cells, for instance. In latency type III, all the latency viral products are expressed: six EBNAs (1, 2, 3A, 3B, 3C, and LP) and three EBV LMPs (1, 2A, and 2B) proteins, along with EBERs, miRNAs encoded in the BART and the BamHI fragment H rightward open reading frame 1 (BHRF1) segments of the viral genome. Latency type III is usually observed in EBV-associated lymphoproliferative diseases and lymphoblastoid cell lines constitutively infected by EBV (5).

In the first years of the XXI century, the discovery that EBV encodes non-coding RNAs (ncRNAs) in its genome provided valuable insights to understand the viral carcinogenesis mechanisms (6). In this regard, recent evidence shows that the contribution of EBV in the pathogenesis of human cancers is a result of the activity of viral oncoproteins and viral ncRNAs. Thus, we will review the current state of the knowledge on the role of viral ncRNAs in the development and progression of human cancers associated with EBV infection.

2 EBV encodes viral ncRNAs

Both EBV and the Kaposi Sarcoma Herpesvirus (KSHV) – the two known herpesviruses recognized as oncogenic to humans so far – encode and express their ncRNAs (6), which are critical for the viral life cycle and the infection pathogenesis. Overall, viruses rely on a subset of their viral products to regulate and take advantage of cellular gene expression. Nonetheless, viral proteins may trigger immune defenses, which impose costly limitations to use them as a leading strategy for modulation of cellular gene expression. This is especially important in the context of viral carcinogenesis because viral proteins may elicit immune responses against cells transformed by oncogenic viruses. The activity of viral ncRNAs may enhance viral control over cellular gene expression without significantly eliciting host defenses against the infected cells (7).

The EBV-encoded RNAs (EBERs) 1 and 2 are two non-polyadenylated non-coding RNAs encoded in the viral genome that have been known to contribute to EBV-induced malignant transformation for a few decades (8). Both EBERs are generated by the RNA Polymerase III (Pol III), processed to either short 5’-derived RNAs or 3’ fragments, and expressed in all EBV latency types. Some authors refer to EBERs as EBV “small non-coding RNAs” (sncRNAs).

However, based on their distinctive biogenesis compared to typical sncRNAs, others designated EBERs as “long non-coding RNAs” – even though they do not meet the usual definition criterion for lncRNAs (longer than 200 nucleotides), as they have 167 and 172 nucleotides in length, respectively. Even though there is no agreement in the literature regarding the proper classification of EBERs, in this review, we will discuss EBERs in addition to EBV microRNAs (miRNAs) due to their importance in EBV pathogenesis. EBV also encodes a stable intronic sequence RNA (ebv-sisRNA), mainly derived from the W repeat region of the viral genome (9). It is suspected that ebv-sisRNA plays a role in EBV-related diseases, although their virological and pathogenetic activities require further elucidation (10).

2.1 Biogenesis of EBERs and EBV-encoded miRNAs

Similar to that found in EBERs, EBV miRNAs are abundantly expressed in cells latently infected by the virus (see Table 1). There are two groups described of EBV miRNAs: ebv-miR-BHRFs and ebv-miR-BARTs, encoded within the BHRF1 and BART regions of the viral genome, respectively. To date, 25 EBV precursor miRNAs (pre-miRNAs) were identified, giving rise to 48 mature miRNAs (11). EBV miRNAs are generated from either protein-coding or non-coding regions of the viral genome by the RNA polymerase II (Pol II) and subsequently processed by Drosha and Dicer for miRNA maturation (12).

The miRNAs encoded by EBV hijack intracellular pathways implicated in the synthesis and maturation of miRNAs of either viral or host origin. For instance, ebv-miR-BART6-5p targets four sites within the 3’-UTR of the human Dicer mRNA, affecting the miRNAs maturation in general and causing global suppression of these molecules. This is a key regulatory phenomenon in the viral life cycle, contributing to the maintenance of the latent infection (13). Dicer downregulation and the global suppression of miRNAs were previously described in malignant cells, often associated with cancer progression (14). EBV products other than viral miRNAs also contribute to Dicer downregulation, such as the EBNA1 oncoprotein (15).

The biogenesis and effects of EBV miRNAs are also affected by adenosine to inosine (A-to-I) RNA editing. Briefly, adenosine in RNA molecules (double-stranded, both coding and non-coding) can be converted to inosine due to the activity of adenosine deaminases acting on RNA (ADARs) enzymes, which results in negative regulation of specific mature RNAs, including ncRNAs (16). These A-to-I conversions affect the stability of miRNAs or may even

create alternative 3'UTR targets, impacting the properties and activities of well-known mature miRNAs (17,18). In cells latently infected by EBV, A-to-I editing of ebv-miR-BART6 precursors elicits the activation of viral genes encoding EBNA2, LMP1, ZTA, and RTA, leading to viral cycle transition either to type III latency or lytic reactivation (13). In NPC cell lines, the ebv-miR-BART3 is also subjected to A-to-I editing, disrupting its maturation (19). Altogether, this data shows that the EBV life cycle takes advantage of both EBV miRNAs and viral proteins to regulate the biogenesis, maturation, and activity of the ncRNAs repertoire in infected cells, with significant consequences for the pathogenesis of EBV-associated cancers, as discussed later in this review.

2.2 EBV miRNAs and EBERs are critical for the regulation of the viral life cycle

The life cycle of herpesviruses evolved alongside their hosts, allowing these viruses to develop ways for efficient regulation of the expression of both viral and cellular genes in the infected cells. The EBV lytic reactivation allows the expression of highly immunogenic viral proteins repressed during latency. Nonetheless, while most viral proteins are suppressed during viral latency, the EBV ncRNAs are abundantly expressed, as they play essential roles in inhibiting lytic reactivation and maintaining persistent infection in the host (20). A recent target prediction analysis in-silico using the seeding sequences of EBV miR-BARTs showed that the EBV genes encoding EBNA1, BFRF2, BILF2, BALF5, and BRRF2 are putative candidates for viral autoregulatory inhibition. Noteworthy, the respective EBV proteins are crucial for the lytic cycle, involved in duplicating the viral genome and the production of new virions (21).

Experimentally, it was demonstrated that the ebv-miR-BART2 inhibits the replication of the EBV genome by targeting the viral DNA polymerase BALF5, while ebv-miR-BART6 suppresses EBNA2 and inhibits ZTA and RTA (13,22), two essential EBV lytic transactivators that are also directly targeted by ebv-miR-BART20-5p (23). In EBV-positive gastric carcinoma cells, the ebv-miR-BART20-5p was described to exert an essential role in latency and viral persistence via inhibition of the Bcl-2-Associated Death gene (BAD), which regulates BRLF1 and BZLF1, two EBV immediate-early genes that are essential to trigger the lytic reactivation (24).

Recently, it was described that the latent-to-lytic switch is regulated in EBV-infected cells by ebv-miR-BHRF1-2-5p and ebv-miR-BART2-5p, which target key molecules involved in the B-cell Receptor (BCR)-mediated signaling, including Growth Factor Receptor Bound

Protein 2 (GRB2), MALT1 Paracaspase (MALT1), Rac family Small GTPase 1 (RAC1), and Rac Guanine Nucleotide Exchange Factor 1 (SOS1). The BCR signaling pathway also plays a crucial role in B-cell activation, differentiation, cellular proliferation, and EBV lytic reactivation. The inhibition of ebv-miR-BHRF1-2-5p and ebv-miR-BART2-5p cause viral lytic reactivation triggered by BCR engagement, highlighting the importance of these EBV miRNAs in antagonizing extracellular stimuli that elicit EBV reactivation (25). The regulation of the EBV lytic cycle by viral miRNAs remains to be appropriately elucidated; the available evidence discloses essential yet overlooked mechanisms by which EBV explores a repertoire of viral miRNAs for self-regulation of its life cycle.

2.3 EBV miR-BARTs target cell proliferation and apoptosis

The EBV miRNAs are also instrumental in maintaining persistent infection by reducing cytopathic effects associated with the microenvironment, modulating cell survival, and apoptosis. In this regard, EBV-encoded miRNAs were found to regulate multiple molecules involved in the Wnt signaling, one of the vital intracellular pathways that regulate cell fate, development, and stemness. For instance, in AGS-EBV and SNU-719 EBV-positive gastric carcinoma cells, the EBV-miR-BART10-3p was found to downregulate the expression of the Dickkopf WNT Signaling Pathway Inhibitor 1 gene (*DKK1*), which encodes a soluble secreted protein involved in the suppression and control of the Wnt signaling (26). Another study reported that ebv-miR-BART1-3p suppresses apoptosis and promotes cell migration via the downregulation of the Disabled Homolog 2 (DAB2), a putative tumor suppressor that contributes to attenuation of the Wnt/β-Catenin signaling (27). More recently, the expression of ebv-miR-BART19-3p was reported to induce cell growth and suppress apoptosis in EBV-negative Jurkat and CNE2 cell lines. Similar effects were also found in cells transfected with ebv-miR-BART19-3p mimics and were attributed to the downregulation of the Adenomatous Polyposis Coli gene (*APC*), another key regulator of the Wnt signaling (28).

EBV-encoded miRNAs' contribution to cell survival and proliferation may also be related to post-transcriptional regulation of the Tumor suppressor gene TP53. This was shown for ebv-miR-BART5-3p, which directly target the TP53 transcript, increasing cell proliferation in vitro and in vivo, in xenograft models using gastric cancer cell lines SGC7901 (EBV-positive) and AGS (EBV-negative). Ebv-miR-BART5-3p was found highly expressed in human specimens of EBV-positive gastric cancer (29). Furthermore, an inverse correlation between the

expression of ebv-miR-BHRF1-1 and TP53 was reported in patients with chronic B-cell leukemia, B-cell leukemia MEC1 and JVM3 cells in vitro. Inhibition of ebv-miR-BHRF1-1 upregulated TP53 while expressing the viral miRNA decreased cell cycle arrest and apoptosis and caused an increase in cell proliferation. These effects were reported to be due to the binding of ebv-miR-BHRF1-1 to the 3'-UTR region of the TP53 transcript (30).

The modulation of cell survival pathways by EBV miRNAs is an essential matter for cancer treatment, as it may be explored to understand and manage radio- and chemoresistance phenotypes in EBV-associated malignancies. In this regard, the expression of ebv-miR-BART8-3p in 5-8F and HONE1 EBV-positive NPC cells was associated with apoptosis resistance after radiotherapy, an effect associated with modulation of the Ataxia-Telangiectasia Mutated (ATM) / Ataxia Telangiectasia Mutated and Rad3-related (ATR) signaling pathway. A tumor xenograft model using 5-8F cells confirmed that EBV-miR-BART8-3p significantly promotes radioresistance and tumor growth in nude mice. EBV was also shown to increase phosphorylated forms of ATM and ATR proteins in irradiated cells, favoring DNA repair and apoptosis resistance (31). Also, in NPC cells, ebv-miR-BART22 has been shown to induce cisplatin chemoresistance via direct downregulation of the Mitogen-Activated Protein Kinase 4 (MAP2K4) transcript, inducing Myosin Heavy Chain 9 (MYH9) expression and GSK3β ubiquitination, promoting tumor stemness, metastasis, and chemotherapy resistance (32). Therefore, the foregoing evidence highlights pivotal roles for EBV miRNAs in cell survival and proliferation pathways, contributing to viral persistence and, ultimately, with expected consequences for the development and progression of EBV-associated cancers.

2.4 EBV ncRNAs contribute to the subversion of host immune responses

The successful persistence of viral infection depends on checks and balances with the host immune system. In the case of EBV, this can be addressed with stringent regulation of highly immunogenic viral proteins such as LMP2A, which interferes with the B Cell Receptor (BCR) signaling and is crucial for viral persistence in B-cells. The robust and specific cytotoxic responses mediated by T CD8+ cells expression elicited by EBV LMP2A can be mitigated by ebv-miR-BART22 (33). Similar results were reported recently in a study using NOD-SCID γc null (NSG) and HLA-A2 transgenic NSG mice with infection by EBV variants lacking viral miRNAs. B-cells from mice infected with miRNA-deficient virus exhibited a drastic reduction in tumorigenic potential, reduced cell proliferation, and lower viral load when compared to wild-

type EBV. Furthermore, depletion of T CD8+ cells resulted in lymphoma formation and increased viral load in most mice infected with a miRNA-deficient virus. These results strengthen the idea that EBV-encoded miRNAs support tumorigenesis via immune evasion in vivo (34).

Other mechanisms for immune regulation by EBV-encoded miRNAs are also described. For instance, ebv-miR-BART6-3p mediates downregulation of the receptor for Interleukin-6 (IL-6R) and the RIG-I-Like Receptor (RIG-I), a Pattern Recognition Receptor (PRR) important for virus recognition by the innate immune system (35,36). Recently it was reported that ebv-miR-BART7 targets the MHC-I Polypeptide-Related Sequence A (MICA) transcript, causing impairment activity of Natural Killer (NK) cells (37). EBV miRNAs may also regulate immune checkpoints: for instance, in EBV-negative gastric carcinoma cells, the expression of Ebv-miR-BART5-5p inhibited the Protein Inhibitor of Activated Stat3 (PIAS3) transcript, increasing PD-L1 upregulation and ultimately causing cell resistance to apoptosis, increased cell proliferation, cellular invasion, migration, and immune escape (38). In a similar study, ebv-miR-BHRF1-2-5p was shown to exert a context-dependent counterregulatory role by targeting LMP1-driven PD-L1 and PD-L2 expression, supporting immunoevasion viral persistence (39).

There are scarce data regarding the immunomodulatory effects of EBERs, essentially investigated in a few pioneer works. In EBV-positive BL cell lines, the expression of EBERs induces IL-10 production mediated by the Retinoic Acid-Inducible Gene I (RIG-I)/IRF-3 pathway, decreasing local inflammation and other immune responses (40). EBER1 also inactivates the interferon-induced RNA-dependent Protein Kinase (PKR), a key component in the host cell mechanism against viral infection (41,42).

3 EBV ncRNAs in infection-associated cancers

Compelling evidence indicates that viral ncRNAs may influence many biological phenomena in various cancers associated with EBV infection. Because a significant number of the studies relevant to this topic rely on cell lines cultivated in vitro, their results should be carefully evaluated based on these models' known biological features and limitations (see Table 2).

EBV-associated cancers may express a variable set of viral ncRNAs, including EBERs, Small Nucleolar RNAs (snoRNAs), Stable Intronic-Sequence RNA (ebv-sisRNA), and viral miRNAs encoded in the BHRF1 and BART regions of the EBV genome (43). Besides EBERs, EBV miR-BARTs are the most studied ncRNAs so far, but relevant data have also been published for EBV miRNAs encoded in the BHRF1 region of the viral genome lately.

As discussed in the previous section, EBV-encoded miRNAs exert a preeminent role in regulating the viral life cycle, targeting both viral and human transcripts to support viral persistence in the infected cells. While these mechanisms primarily regulate the EBV life cycle, they can eventually contribute to cancer development and progression. The following sections will delineate the current state of knowledge regarding the role of EBV ncRNAs, notably microRNAs, in the pathogenesis of human cancers associated with viral infection. For better understanding, the selected studies will be discussed considering significant contributions of their results in three complementary, non-exclusive scenarios in cancer biology: 1) cell survival, proliferation, and tumorigenesis; 2) cancer microenvironment and immunobiology; 3) cancer progression and aggressiveness.

3.1 Cell survival, proliferation, and tumorigenesis

Several EBV ncRNAs are being reported as putative regulators of cell proliferation and survival, ultimately contributing to the development of EBV-associated cancers. For instance, the expression of ebv-miR-BARTs was found to provide a growth advantage for xenografted tumors derived from the EBV-positive gastric carcinoma cells SNU-719 (44,45). Furthermore, in the EBV-negative SGC7901 and AGS gastric carcinoma cells, the ectopic expression of ebv-miR-BART3-3p supported cell growth and inhibited cell senescence both in vitro and in in vivo (46). In another scenario, it was demonstrated that the expression of both ebv-miR-BHRF1-2 and ebv-miR-BHRF1-3 was positively correlated with an increase in the rate of cell growth for EBV-positive lymphoblastoid cell lines (LCLs) spontaneously established from peripheral blood cells of transplanted patients under therapeutic immunosuppression (47). Although the specific mechanisms remain to be fully clarified, this data supports the concept that the expression of EBV ncRNAs impacts the regulation of cell proliferation and may support either tumorigenesis or lymphomagenesis associated with EBV infection.

Tumor suppressor genes encoding regulators of cell proliferation and cell death pathways are essential targets of EBV ncRNAs. In this regard, in the epithelial cells HNE1* and

the gastric carcinoma cells SGC7901, the transcriptional and protein levels of the TP53 tumor suppressor gene were adversely affected by the ebv-miR-BART5-3p ectopic expression. In these models, cells expressing the viral miRNA showed accelerated TP53 degradation and reduced levels of TP53-responsive genes, such as the cell-cycle regulator Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A), as well as the apoptosis-related genes Fas Cell Surface Death Receptor (FAS), BCL2 Associated X, apoptosis regulator (BAX), and BCL2 Binding Component 3 (BBC3), also known as PUMA. Ultimately, cells expressing the ebv-miR-BART5-3p were more resistant to ionizing radiation and etoposide-induced apoptosis, showing increased growth both in vitro and in vivo (48). In another study, the expression of ebv-miR-BARTs 1, 2, 7, 8, and 22 in HEK293 cells caused downregulation of the caspase-3 pro-apoptotic protein (49), which was also affected by ebv-miR-BARTs 1-3p and 16 in the 293 cell line and Oku-BL (EBV-positive BL cells) (50). In the AGS-EBV cell line, the expression of ebv-miR-BART20-5p was also implicated in apoptosis inhibition by targeting the 3'-UTR of the Bcl2-associated Agonist of Cell Death (BAD) (24). The tumor suppressor gene TP53 is also targeted directly by ebv-miR-BART3-3p, as demonstrated by Wang and colleagues (2019), reporting that the expression of ebv-miR-BART3-3p was negatively correlated with TP53 expression in cases of EBV-positive gastric carcinomas. Furthermore, the expression of ebv-miR-BART3-3p in SGC7901 and AGS gastric carcinoma cell lines caused an increase in cell growth in vitro and tumorigenesis in vivo (51).

Together, these results demonstrate that EBV miRNAs can downregulate tumor suppressor genes, including those encoding regulators of the cell cycle and apoptosis pathways, although this data has emerged more recently, and its clinical significance requires further investigation.

3.2 Cancer microenvironment and immunobiology

The expression of EBV ncRNAs is also relevant in terms of tumor metabolism and tumoral microenvironment. For instance, ebv-miR-BART1-5p stably expressed in CNE1* and HONE1* cells increased the expression of genes involved in the Warburg effect (aerobic glycolysis), causing neoangiogenesis in vitro (chorioallantoic membrane model) and in in vivo (xenograft mouse tumor). These effects were associated with the downregulation of the α1 catalytic subunit of AMP-Activated Protein Kinase (AMPK), which causes an increase in the mammalian-Target of Rapamycin (mTOR) signaling pathway and the expression of the

Hypoxia-Inducible Factor 1-alpha (HIF-1α) (52). Recently, it was reported that primary endothelial cells (HUVEC) either transfected with EBV EBERs or exposed to exosomes derived from EBV-positive C666-1 NPC cells showed an increase in angiogenic response linked to EBERs-mediated activation of the Toll-Like Receptor 3 (TLR3) and RIG-I, causing vascular remodeling mediated by the Vascular Cell Adhesion Protein 1 (VCAM1) (53).

EBV ncRNAs can also be instrumental in setting up an inflammatory microenvironment favorable to EBV-positive malignant cells. The expression of the LMP1 oncoprotein and EBERs in EBV-negative HNE2* cells cooperatively induces the expression of the pro-inflammatory cytokines TNFα, IL-6, and IL-1α, with high levels of EBERs correlated to higher levels of TNF-α and poorer survival rates in NPC patients (54). Additionally, ebv-miR-BARTs induce features of tumor-associated macrophages (TAMs) when transferred into regular macrophages via exosomes derived from umbilical cord blood cell lines transformed with the EBV strain B95.8 or Akata. This effect was attributed to inhibition of the Myocyte Enhancer Factor 2C gene (MEF2C) translation, causing an increase in pro-tumoral molecules, such as IL-10, TNF-α, and Arginase 1 (AGR1) (55). Furthermore, EBV EBER2 was reported to increase Toll-Like Receptor 7 (TLR7) levels, known to be involved in inflammation and carcinogenesis (56).

As mentioned earlier in this review, EBV-infected cells may express immunogenic proteins tightly regulated by viral ncRNAs. Beyond favoring the EBV persistence, this regulation can provide adaptative advantages for the infected malignant cells. For instance, it was previously shown that LMP1 expression upregulates the immune checkpoint ligand PD-L1 via STAT3, AP-1, and NF-κB, supporting the immunoevasion of infected cells (57). The PD-L1 and PD-L2 transcripts can be directly targeted by ebv-miR-BHRF1-2-5p, disclosing a context-dependent counterregulatory mechanism to fine-tune the LMP1-driven upregulation of these immune checkpoints molecules (39). This typifies the complex regulatory roles that EBV ncRNAs may exert in the immunobiology of EBV-associated cancers, providing relevant information to consider when using therapies based on immune checkpoints to manage these diseases.

As shown herein, by increasing the expression of cytokines, immune mediators, and other pro-tumoral molecules, EBV miRNAs and EBERs contribute to setting up an inflammatory microenvironment that promotes vascular remodeling and immunoevasion. Although relatively scarce, the available data provides valuable insights into the mechanisms

underlying the modulation of the tumor microenvironment and immunobiology by ncRNAs encoded by EBV.

3.3 Cancer progression and aggressiveness

The traditional paradigm that implicates some viruses in the pathogenesis of cancers only recently evolved to consider the participation of oncoviruses not only in malignant cell transformation but also as modulators of the aggressiveness and progression of their associated cancers (58). On top of that, compelling evidence published in the past decade unraveled important roles of EBV ncRNAs interfering in critical pathways associated with cancer invasion, epithelial-to-mesenchymal (EMT) transition, and metastasis.

In that regard, increased cell migration rates and invasion in vitro were attributed to the expression of ebv-miR-BART8-3p in CNE-1*, SUNE1, and C666-1 cells, associated with a higher metastatic potential to the lungs of nude mice. The transcript for the gene encoding for RING Fingerrotein 38 gene (RNF38) was implicated as a direct target of ebv-miR-BART8-3p in these NPC cells; moreover, downregulation of RNF38 was associated with activation of the NF-κB and Erk1/2 signaling pathways, increasing the phosphorylation of TAK1, p65, IKKα, IKKβ, and IKKα/β, while reducing the expression of both non-phosphorylated and phosphorylated forms of IκB proteins α and β (59). The activation of the NF-κB was also reported to be a result of ebv-miR-BART13-mediated downregulation of NF-κB inhibitor interacting Ras-like 2 gene (NKIRAS2) (60). Additionally, miR-BART8-3p was recently implicated in radiotherapy resistance in NPC, as this viral miRNA promotes activation of the ATM/ATR signaling, leading to DNA repair after irradiation in vivo in EBV negative cell lines (HONE1 and 5-8F) and increased xenograft tumor size in nude mice (61).

The activity of EBV miRNAs eventually reflects changes in the aggressiveness of EBV-infected malignant cells in vivo due to its impact on various cancer progression phenomena. In patients with preclinical NPC, high levels of ebv-miR-BART2-5p were correlated with disease progression, adversely impacting the prognosis. The ectopic expression of ebv-miR-BART2-5p in EBV-negative NPC cell lines CNE2 and HONE-1 significantly increased the cell migration rate in vitro; otherwise, downregulation of this viral miRNA in EBV-positive NPC cells decreased the migration and invasion of these cells. Mechanistically, the ebv-miR-BART2-5p negatively regulates the transcript for Rho Family GTPase 3 gene (RND3), leading to an increase in cell motility due to the activation of the Rho signaling pathway(62). In

another recent study, the metastatic potential and stemness for NPC cell line 5-8F, HONE1, and SUNE1, as well as CNE1* cells, were correlated to the activity of the endogenous oncomir miR-7421 induced by ebv-miR-BART22 via the PI3K/AKT/c-JUN/Sp1 signaling, ultimately inhibiting the expression of GSK3β and causing the activation of the Wnt/β-catenin pathway (63).

EBV ncRNAs may also modulate the cancer progression phenomena due to their activity over the Epithelial-to-Mesenchymal (EMT) program in epithelial cells. For instance, ebv-miR-BART10-3p downregulates the transcript for the gene encoding the Beta-Transducing Repeat Containing E3 Ubiquitin Protein Ligase (BTCR), involved in the Wnt signaling pathway, cell cycle checkpoints, as well as EMT regulation (64). The Wnt/β-catenin axis is also affected by ebv-miR-BART9, which targets the E-cadherin transcripts (65). Alternatively, EMT activation can be a result of inhibition of the Phosphatase and Tensin Homolog (PTEN) by ebv-miR-BART1, upregulated in NPC at advanced stages (e.g., TNM N2-3 and clinical stages III-IV), as well as linked to an increase in the metastatic potential in nude mice tumor model (66).

In primary EBV-positive human gastric carcinoma samples, the ebv-miR-BART10-3p and ebv-miR-BART22 were highly expressed and associated with lymph node metastasis and worse overall survival. The expression of these viral miRNAs was also implicated in activating the Wnt pathway by targeting the transcripts for the genes APC and DKK1, causing an increase in cell migration and invasion of EBV-positive gastric carcinoma cells (67). In EBV-positive gastric carcinoma cell lines SNU-719 and AGS-EBV cells, the ebv-miR-BART10-3p increased cell proliferation and migration by directly targeting the DKK1 transcript, a negative regulator of the Wnt/β-catenin signaling (68). Similarly, the expression of ebv-miR-BART1-3p in gastric carcinoma cells promoted cell migration due to the downregulation of the Disabled Homolog 2 (DAB2), an adaptor phosphoprotein implicated in the regulation of clathrin-mediated endocytosis and a variety of signaling pathways, including MAPK, WNT, and TGF-β (69). The overexpression of ebv-miR-BART11 was shown to also increase the EMT phenotype in human gastric cell lines by targeting the transcript for the Forkhead Box P1 gene (FOXP1), a transcriptional repressor involved in the regulation and expression of embryonic genes and cell differentiation (70). These results strengthen the idea that signaling pathways that participate in the EMT program, such as Wnt, are targeted by multiple EBV miRNAs, with

expected consequences in modulating the aggressiveness of carcinomas associated with EBV infection, such as EBV-positive gastric cancers and NPC.

In a study published in 2020, Min and colleagues reported that ebv-miR-BART1-5p downregulates the O-glycan Synthase Glucosamine (N-acetyl) Transferase 3 (GCNT3) enzyme, which catalyzes the formation of O-linked glycosylation in proteins, a putative marker of poor prognosis in non-small cell lung carcinoma (NSCLC). Intriguingly, EBV-associated gastric cancer showed lower levels of the GCNT3 protein compared to EBV-negative cases. This was linked to a suppressive activity of ebv-miR-BART1-5p, ultimately causing inhibition of cell proliferation and migration in vitro(71). Even though the mechanisms of GCNT3 regulation by ebv-miR-BART1-5p remain to be better described, this data may suggest that differences in the activity of viral ncRNAs could be context-dependent, stressing the importance to discriminate pathogenetic properties of EBV ncRNAs in NPC and EBV-associated gastric carcinomas, for instance.

Besides Wnt, the TGF-beta signaling is also a critical pathway for EMT and the stemness phenotype. The TGF-β signaling pathway may be activated by ebv-miR-BART7-3p, which is abundantly expressed in NPC clinical samples and inhibits the Mothers Against Decapentaplegic Homolog 7 gene (SMAD7) by binding the 3'-UTR portion of its transcript. The inhibition of SMAD7 by ebv-miR-BART7-3p was inversely correlated with the potential of CNE2* and 5-8F cells to form tumorspheres in vitro, an experimental proxy to estimate the cell stemness phenotype. Moreover, the ectopic and constitutive expression of ebv-miR-BART7-3p in CNE2* cells increased their tumorigenic potential in xenografted nude mice (72). Furthermore, patients with advanced-stage NPC, compared to patients with early-stage tumors, express higher levels of ebv-BART2-5p in serum, which is associated with worse progression-free survival. In this study, the ectopic expression of ebv-BART2-5p caused an increase in the metastatic potential of SUNE1 and HK1 cells in nude mice, an effect that was mechanistically linked to direct suppression of the transcript for the Rho Family GTPase 3 gene (RND3) – a negative regulator of the Rho-associated protein kinases (ROCK) signaling pathway (73). Therefore, the expression of EBV miR-BARTs plays a role in the progression and metastatic phenotype of EBV-associated carcinomas due to modulation of the EMT program, the stemness of EBV-infected carcinoma cells, or even disrupting critical signaling networks involved in cell motility, such as the Rho Kinases (ROCK) pathway.

Another important mechanism for the activity of EBV ncRNAs in cancer development and progression could be either by mimicking cellular ncRNAs or interfering with their activity. The sequencing of NPC samples has shown that EBV miRNAs can share seed sequences with some human miRNAs while interacting with others (74). For instance, by mimicking the human miR-513b, ebv-miR-BART8-3p expressed in NPC cells supports disease progression due to its activity on the IFN pathway (59). Also, ebv-miR-BART6-3p binds and downregulates the LOC553103 lncRNA. The overexpression of this viral miRNA or knocking down of the LOC553103 via siRNA caused a decrease in the migration and invasion rates in vitro for HNE2* and other cell lines derived from NPC (5-8F and C666-1) or gastric carcinoma (AGS), and these strategies also inhibited metastasis of 5-8F-derived tumors in nude mice in vivo (75). In EBV-positive NPC cells, C666-1, and the EBV-negative AGS gastric carcinoma cell line, the expression of ebv-miR-BART6-3p was associated with inhibition of cell proliferation C666-1 cells transfected with ebv-miR-BART6-3p inhibitors showed an increase in cell proliferation and cell cycle progression. These effects were attributed to the downregulation of the transcripts for the Stathmin 1 gene (STMN1) and the lncRNA LOC553103 mediated by ebv-miR-BART6-3p, ultimately impacting the expression of cell-cycle associated proteins (76).

In summary, the available data indicates that EBV ncRNAs may have either a positive or negative effect on cancer development, interfering in key oncogenic pathways related to cell cycle regulation, cellular migration and invasion, activation of the EMT program stemness, and regulation of immune responses. Considering EBV miRNAs, the available data in the literature on the activity of miR-BARTs are more frequent and robust, while the effects and importance of viral miRNAs derived from the BHRF1 still are poorly understood.

4 Concluding remarks and future perspectives

The data presented in this review discloses the activities of EBV ncRNAs in the virus-induced carcinogenesis by a variety of mechanisms, with effects spanning from the malignant transformation itself to all over the cancer progression phase – at the cellular, microenvironmental, or even systemic levels. A summary of known activities of viral ncRNAs in phenomena occurring during EBV-induced carcinogenesis is presented in Figure 1.

Several EBV ncRNAs are emerging in the spotlight due to their potential application for diagnosis and prognosis for some virus-induced cancers. In this regard, we may recall that

the detection of EBV EBERs by in situ hybridization has been the gold standard method for diagnosing EBV-associated malignancies for a few decades now (77). Moreover, new data in the literature also provide evidence for using EBV-encoded miRNAs as biomarkers for cancer detection/progression using either blood or tumor samples. As shown in Table 3, several EBV miR-BARTs were suggested as putative markers to monitor the onset, progression, and recurrence of EBV-associated cancers.

The ncRNAs encoded by EBV are crucial regulators of gene expression in cells infected by this virus. Their activities are not limited to regulating the viral life cycle, latency, and persistence of infection, as these ncRNAs also impact cellular immortalization, malignant transformation, and cancer progression. Overall, EBV-encoded ncRNAs are emerging as putative biomarkers for early detection, disease monitoring, and assessment of treatment response or disease recurrence for cancers associated with the viral infection, such as NPCs. Remarkably, the EBV ncRNAs may act within the cell, in a paracrine fashion, or even systemically, transported via exosomes. For instance, both EBERs 1 and 2 were detected in exosomal fractions of EBV-infected cells (78,79); nonetheless, the effects of EBERs in the phenotype infected cells and the tumor microenvironment remain to be elucidated. Also, interactions of EBERs and EBV miRNAs in the pathogenesis of EBV-associated cancers are essentially unknown.

Therapeutic approaches targeting either human or EBV ncRNAs are still a long shot because they impose important challenges, such as methodological limitations and admissibly unpredictable effects in the clinical setting. Nonetheless, the technological advances and the increasing knowledge about cancer pathways affected by EBV ncRNAs are expected to shed more light on important and perhaps overlooked molecules that could be targeted in new therapies.

Finally, a note of caution is warranted when interpreting the new or previously published data because the effects of the expression of specific EBV ncRNAs may be substantially different considering models using the sole expression of these molecules compared to the simultaneous activity of multiple viral ncRNAs in the natural scenarios of EBV-driven carcinogenesis in vivo. Furthermore, it is essential to consider the features and limitations of the experimental models used for these analyses, notably when experimental data in vitro is used to conclude the role of the viral ncRNAs in the pathogenesis and evolution of specific cancers associated with EBV infection.

5 Figures

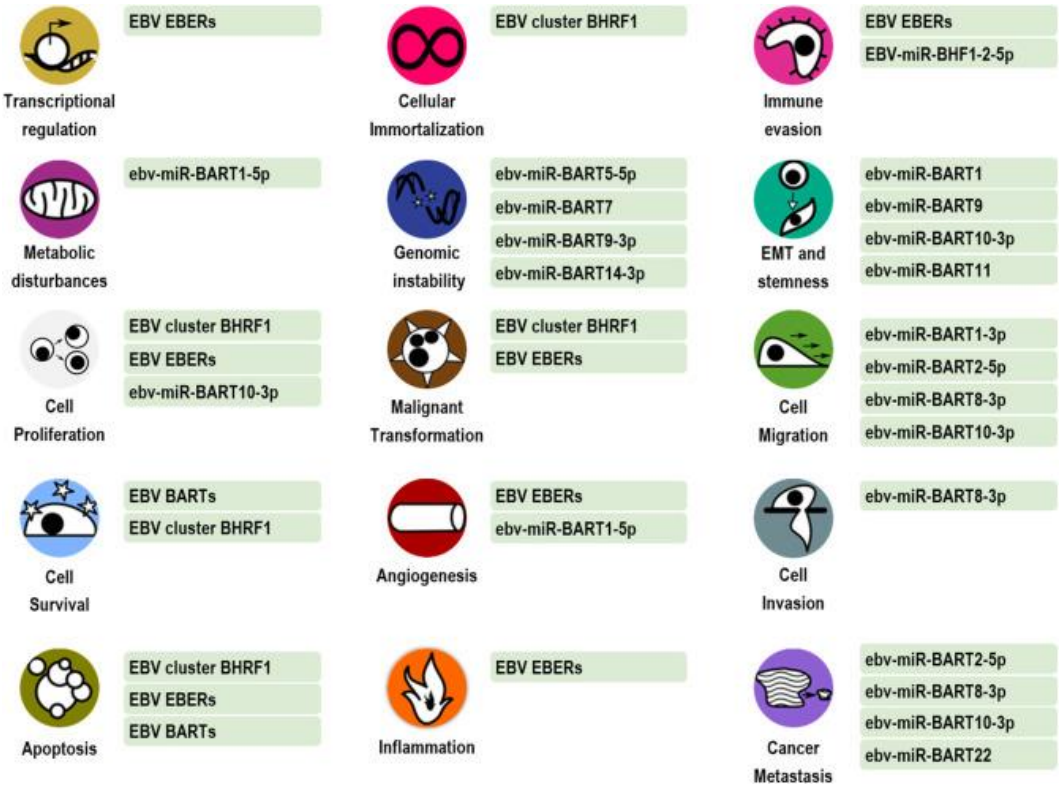

Figure 4.1 – Cancer-associated biological phenomena associated with the expression of EBV ncRNAs. The ebv-miR-BHRF1 cluster has a crucial role in immortalizing B-cells and its expression alongside EBERs contributes to malignant transformation. The expression of some ebv-miR-BARTs was also shown to increase genomic instability, favoring tumor progression. EBV miR-BHRF1 and EBERs increase cell proliferation, contributing to tumorigenesis, while the expression of EBERs, ebv-miR-BHRF1, and ebv-miR-BARTs is associated with increased resistance to apoptosis and cell survival. Furthermore, EBERs and ebv-miR-BARTs were shown to promote immune evasion, alter cell metabolism, and increase angiogenesis, facilitating tumor growth and metastasis. Several ebv-miR-BARTs have been associated with increased cell migration and invasion, fostering EMT and cancer metastasis. The current evidence discloses the essential roles of EBV ncRNAs on the initiation and progression of infection-associated cancers.

6 Tables

Table 4.1 - Cell line models used in cited studies investigating the role of EBV ncRNAs in human cancers

Designation	Origin and biological remarks	ID code(s)1
5-8F	Human, nasopharyngeal carcinoma, EBV-negative	CVCL_C5
AGS	Human, gastric adenocarcinoma, EBV-negative	CVCL_0139
C666-1	Human, undifferentiated nasopharyngeal carcinoma, EBV-positive. Presence of murine leukemia virus (MuLV) **(80)**	CVCL_7949
CNE-1*	Originally described as well-differentiated human nasopharyngeal carcinoma, EBV-positive, but evidence of HeLa genome **(81,80)**	CVCL_6888; ICLAC-00473.
CNE-2*	Originally described as derived from a poorly differentiated human nasopharyngeal carcinoma, EBV-positive, but evidence of HeLa genome **(81,80)**	CVCL_6889 ICLAC-00474
HEK293 (T)	Human, embryonic kidney cells (neuronal), with incorporated adenovirus 5 genome. HEK293T cells are transformed with the SV40 large T antigen	CVCL_0045; CVCL_0063
HeLa	Human, uterus adenocarcinoma, HPV-18-associated.	CVCL_0030
HNE1*	Originally described as derived from a human nasopharyngeal carcinoma, EBV-negative, but evidence of HeLa genome **(81)**	CVCL_0308
HNE2*	Originally described as derived from an EBV-negative human nasopharyngeal carcinoma, but evidence of the HeLa genome **(81)**.	CVCL_FA07
HONE1*	Originally described as derived from a poorly differentiated human nasopharyngeal carcinoma, but evidence of HeLa genome **(81,80)**	CVCL_8706
MUTU1	Human, Burkitt lymphoma, EBV-positive.	CVCL_7202
NIH-3T3	Mouse, embryonic fibroblasts.	CVCL_0594
NPC/HK1	Human, differentiated nasopharyngeal squamous cell carcinoma	CVCL_7084

Designation	Origin and biological remarks	ID code(s)1
NP69	Human, nasopharyngeal epithelial cells, immortalized by large-T antigen expression for SV40 virus, EBV-negative.	CVCL_F755
Oku-BL	Human, Burkitt lymphoma, EBV-positive.	CVCL_7206
SGC7901	Human, gastric adenocarcinoma, EBV-negative	CVCL_0520
SNU-719	Human, gastric adenocarcinoma, EBV-positive	CVCL_5086
SUNE-1	Human, nasopharyngeal carcinoma, EBV-negative.	CVCL_6946

[1]Cell lines indicated with (*) are flagged as problematic in the Register of Misidentified Cell Lines (82), curated by the International Cell Line Authentication Committee (ICLAC), or elsewhere in the literature. 2 CVCL - Accession ID in Cellosaurus database (83), maintained by the Swiss Institute of Bioinformatics (SIB) (refer to https://web.expasy.org/cellosaurus/). When available, ICLAC Registration ID is also indicated (refer to https://iclac.org/databases/cross-contaminations/).

Table 4.2 – Summary of selected studies showing the usefulness of EBV ncRNAs to monitor the onset, progression, and recurrence of EBV-associated cancers in the last 5 years

Study	Key study features	Summary of conclusions
(30)	RT-qPCR[1] from peripheral blood mononuclear cells from patients with chronic lymphocytic leukemia (CLL)	The expression of ebv-miR-BHRF1-1 was correlated with aberrant p53 and prognostic marker for overall survival in patients with CLL; inhibition of ebv-miR-BHRF1-1- upregulated p53 protein expression, induced cell cycle arrest, and apoptosis.
(84)	RT-qPCR[1] from plasma samples of NPC patients and non-cancer donors	Ebv-miR-BART19-5p levels used in blood samples were useful for the detection of primary NPC; ebv-miR-BARTs 8-3p and 10-3p were correlated with disease recurrence; ebv-BART13-5p in serum was associated with NPC progression and regional lymph node metastasis.
(85)	RT-qPCR[1] from serum samples of NPC patients and non-cancer donors	The levels of ebv-miR-BART2-5p to rise before the NPC diagnosis; particularly valuable for the screening of high-risk patients
(86)	Small RNA sequencing from serum, nasopharyngeal brushing, and frozen tumor biopsy samples	Expression of ebv-miR-BART13-3p had high specificity (97%) to distinguish sera from NPC patients and healthy controls. Superior diagnostic performance compared to both anti-EBNA1 IgA serology and EBV-DNA load.
(87)	Small RNA sequencing obtained from patient-derived xenografts (PDX) and the NPC cell line C666-1	Ebv-miR-BARTs 5-5p, 7-3p, 9-3p, and 14-3p synergistically target the ATM gene. ATM downregulation increased the radiosensitivity of transfected NP69 and Hela cells and contribute to EBV latency.
(48)	RT-qPCR from qPCR from formalin-fixed paraffin-embedded (FFPE) tumor samples of EBV-associated gastric cancer.	Expression ebv-miR-BART5-3p was found in 10 out of 20 EBV-associated gastric carcinoma tumors, but none of the 4 EBV-negative tumors evaluated

Study	Key study features	Summary of conclusions
(88)	RT-qPCR from formalin-fixed paraffin-embedded (FFPE) tumor samples of EBV-associated gastric cancer.	High intratumoral levels of ebv-miR-BART20-5p were correlated with poorer recurrence-free survival rates in EBV-associated gastric cancers.
(89)	RT-qPCR from serum samples NPC patients and non-cancer donors	Circulating ebv-miR-BART17-5p after NPC treatment was associated with poor prognosis and treatment outcomes
(90)	RT-qPCR from blood samples of newly diagnosed NPC patients	Ebv-miR-BARTs 7 and 12 diminished after radiotherapy treatment in NPC patients, highlighting their usefulness for the prediction of treatment efficacy.
(91)	RT-qPCR from tissue samples of NPC, chronic nasopharyngitis, lymphoma, and non-NPC head, and neck tumors	Detection of ebv-miR-BART1-5p in nasopharyngeal brush samples was effective for diagnosis of early-stage NPC and disease progression, even in false-negative cases based on antibody titers, EBV DNA load, and histopathological analyses.

[1] Reverse-Transcription Quantitative Polymerase Reaction (RT-qPCR).

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Appendix 2 – Ethics Committee Certificate of Approval

Certificado de Apresentação para Apreciação Ética (CAAE) #82316118.0.0000.5411, sob o parecer CEP FMB #2499053 transcrito a seguir.

UNESP -FACULDADE DE MEDICINA DE BOTUCATU

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Título Principal: Efeitos in vitro de RNAs não-codificantes (ncRNAs) humanos e do vírus de Epstein-Barr (EBV) em células imortalizadas derivadas de epitélio nasofaríngeo.Subprojeto I: "Efeitos de microRNAs endógenos humanos no repertório de células tronco cancerígenas e progressão de cânceres in vivo em modelo Zebrafish". (Pesquisadora:Barbara Grasiele Muller Coan)Subprojeto II: "Efeitos de miR-BARTs do vírus de Epstein-Barr nas propriedades in vitro de células derivadas de linfomas humanos e na modulação de vias de sinalização intracelular envolvidas na linfomagênese." (Pesquisador: Bruno Felipe Ramos Caetano)

Pesquisador: Deilson Elgui de Oliveira
Área Temática:
Versão: 1
CAAE: 82316118.0.0000.5411
Instituição Proponente: Instituto de Biotecnologia
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 2.499.053

Apresentação do Projeto:

A infecção pelo vírus de Epstein-Barr (Epstein-Barr vírus - EBV) está associada a patogênese de distúrbios proliferativos e diversos cânceres humanos, notadamente a forma endêmica do Linfoma de Burkitt e a variante indiferenciada do carcinoma de nasofaringe. O EBV foi o primeiro vírus no qual pequenos RNAs não codificantes (miRs) foram encontrados codificados no genoma viral. Por outro lado, o importante papel que miRs endógenos humanos desempenham na carcinogênese tem sido sistematicamente verificado nas últimas duas décadas. Assim, além de sofrer a influência de miRs humanos, o desenvolvimento de cânceres humanos associados ao EBV pode ainda ser influenciado pelos miRs virais. O presente estudo visa verificar a hipótese de que mudanças nos níveis de expressão de determinados miRs endógenos humanos ou miR-BARTs do EBV em células imortalizadas de epitélio nasofaríngeo (CIEN) humano podem modificar suas propriedades biológicas e comportamento in vitro, de modo a favorecer a transformação
celular ou aquisição de características relacionadas à agressividade biológica em cânceres. Para tanto serão gerados modelos baseados em linhagens de CIENs EBV-negativas com alteração na

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expressão de miRs endógenos humanos (miR-100, miR-192 e miR-574), e CIENs infectadas com EBV cujo genoma foi editado por CRISPR/Cas9 para deleção de miR-BARTs (miR-BART 7 e 9). As CIENs EBV-positivas e EBV-negativas indicadas serão então avaliadas quanto as taxas de proliferação, migração, invasão, e transformação celular in vitro. As alterações moleculares subjacentes aos efeitos possivelmente verificados nas CIENs com alteração na expressão de miRs endógenos (EBV-negativas) ou dos miR-BARTs virais (EBV-positivas) também serão avaliadas no que se refere à modulação de vias de sinalização intracelular relevantes na carcinogênese, tais como NF-B, PI3K/AKT e JAK/STAT.

O presente estudo visa verificar a hipótese de que mudanças nos níveis de expressão de determinados ncRNAs humanos ou miR-BARTs do EBV em células humanas imortalizadas derivadas de epitélio de nasofaringe podem modificar suas propriedades biológicas e comportamento in vitro, de modo a favorecer a transformação celular ou aquisição de características relacionadas à agressividade biológica em cânceres.

Para o presente estudo serão empregadas células imortalizadas de epitélio de nasofaringe humano (CiEN) (Li et al., 2006) e células embrionárias de rim humano HEK293, todas autenticadas pela análise do perfil genético STRs. As CiENs e células HEK293 serão cultivadas em seus meios apropriados e mantidas a 37oC em estufa com atmosfera úmida e 5% CO2. Será efetuado monitoramento de rotina para averiguação de viabilidade e concentração celular.

Objetivo da Pesquisa:

Objetivos:

1. Gerar modelos baseados em linhagem de células humanas imortalizadas de epitélio de nasofaringe com alteração na expressão basal dos miRs humanos miR-100, miR-192 e miR-574;
2. Efetuar a edição genética in vitro do genoma do EBV de genótipo M81 para produção de partículas virais com deleção dos miR-BARTs 7 ou 9, para análise dos efeitos da supressão desses miRs virais em células humanas imortalizadas de epitélio de nasofaringe infectadas
experimentalmente pelo EBV;
3. Avaliar as taxas de proliferação, migração, invasão, e transformação celular in vitro nos modelos experimentais estabelecidos:
a. células imortalizadas de nasofaringe EBV-negativas com alterações na expressão dos miRs endógenos humanos -100, -192 e -574;
b. células imortalizadas de nasofaringe EBV-positivas com comprometimento da expressão dos miR-BARTs virais 7 e 9 virais;

4. Dissecar as alterações moleculares subjacente aos efeitos possivelmente verificados nas células imortalizadas de nasofaringe com alteração na expressão de miRs endógenos (EBV-negativas) ou dos miR-BARTs virais (EBV-positivas), particularmente no que se refere à modulação de vias de sinalização intracelular relevantes na carcinogênese, tais como NF-B, PI3K/AKT e JAK/STAT.

Avaliação dos Riscos e Benefícios:

Riscos:

O presente trabalho não oferece riscos a sujeitos humanos recrutados ou à população em geral. Seus riscos são inerentes à experimentação laboratorial e estão devidamente equacionados face as diretrizes vigentes de biossegurança para a manipulação dos microorganismos empregados no estudo (bactérias E. Coli, lentivírus em sistema de transdução gênica de 3a. geração e partículas virais do EBV). As manipulações experimentais são efetuadas em instalações laboratoriais de biossegurança nível 2, localizadas no Instituto de Biotecnologia (IBTEC), UNESP Botucatu, SP.

Benefícios:

A execução desta pesquisa propiciará a geração de dois modelos baseados em cultura in vitro de células imortalizadas derivadas de epitélio nasofaríngeo (CiEN) humanas: A) CiENs sem infecção pelo EBV (CiENs EBV-negativas), com expressão aumentada de miR humanos selecionados (miR-100, miR-192 ou miR-574), induzível por tratamento com tetraciclina; B) CiENs infectadas com EBV (CiENs EBV-positivas), (1) com genoma viral M81 selvagem; (2) com deleção em EBV miR-BART-7 (CiEN-EBV-miR-BART-7) ; e (3) com deleção miR-BART-9 (CiEN-EBV-miR-BART-9). Os experimentos empregando esses modelos possibilitarão levantar as seguintes informações para as CiEN e suas derivadas EBV-negativas (CiENmiR-100, CiEN-miR-192 e CiEN-miR-574) e EBV-positivas (CiEN-EBV-miR-BART-7 e CiEN-EBV-miR-BART-9):

1. Taxas de viabilidade, citotoxicidade e apoptose in vitro;
2. Taxa de transformação celular em ensaio de clonogênese;
3. Taxas de migração e invasão celular in vitro;
4. Níveis de expressão e status de ativação de proteínas-chave das vias de sinalização intracelular com papel relevante na carcinogênese (PI3K/AKT, NF-B e JAK/STAT, à priori).

Com base nessas informações pretende-se verificar o impacto de alterações dos ncRNAs endógenos humanos miR-100, miR-192 e miR-574, bem como da supressão dos miR-BARTs 7 e 9 do EBV, nas propriedades biológicas in vitro de células imortalizadas de nasofaringe. Esses

dados contribuirão para um melhor entendimento do papel desses ncRNAs na patogênese de carcinomas humanos, em particular o carcinoma de nasofaringe associado ao EBV.

Comentários e Considerações sobre a Pesquisa:

A pesquisa apresenta título adequado, objetivos claros, metodologia bem descrita, avaliação de riscos e benefícios, sendo de importância pois seus dados contribuirão para o melhor entendimento do papel desses RNAs não codificantes na patogênese de carcinoma de nasofaringe associado ao EBV.

Considerações sobre os Termos de apresentação obrigatória:

- Folha de rosto: preenchida corretamente;
- Informações básicas do projeto e projeto completo: adequados e completos;
- TCLE: foi proposta a dispensa do TCLE, pois o estudo utilizará exclusivamente linhagens celulares comercialmente disponíveis ou cedidas por pesquisadores colaboradores na comunidade científica internacional;
- Cronograma de execução: adequado, programado para iniciar 02/04/2018.

Recomendações:

Após a realização do projeto de pesquisa, enviar o Relatório Final de Atividades via Plataforma Brasil.

Conclusões ou Pendências e Lista de Inadequações:

Aprovado sem pendências.

Considerações Finais a critério do CEP:

Conforme deliberação do Colegiado em reunião ordinária do Comitê de Ética em Pesquisa da FMB/UNESP, realizada em 05 de fevereiro de 2018, o projeto encontra-se APROVADO, sem necessidade de envio à CONEP.

O Comitê de Ética em Pesquisa, no entanto, informa que ao final da execução da pesquisa, seja enviado o "Relatório Final de Atividades", na forma de "Notificação", via Plataforma Brasil.

Atenciosamente,

Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu - UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

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Continuação do Parecer: 2.499.053

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1023523.pdf	23/01/2018 08:12:34		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Deilson.pdf	22/01/2018 15:45:10	Deilson Elgui de Oliveira	Aceito
Outros	Subprojeto_Brunno.pdf	22/01/2018 15:43:48	Deilson Elgui de Oliveira	Aceito
Outros	Subprojeto_Barbara.pdf	22/01/2018 15:34:49	Deilson Elgui de Oliveira	Aceito
Folha de Rosto	Folha_rosto_Deilson.pdf	22/01/2018 15:32:55	Deilson Elgui de Oliveira	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BOTUCATU, 19 de Fevereiro de 2018

Assinado por:
SILVANA ANDREA MOLINA LIMA
(Coordenador)

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