# Epstein-Barr Virus microRNA Expression and Function in Persistent Infection and Oncogenesis

A dissertation submitted by

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# Abstract

Epstein-Barr virus (EBV) infects more than 95% of the adult world population. For most people this is not a problem as EBV seems to be exclusively harbored quiescently in resting B lymphocytes without manifesting any symptoms. EBV has developed elaborate and sophisticated strategies for subverting immune surveillance by the host. In this system, EBV is able to initiate distinct latency programs to trick B cells into accepting the virus. Ultimately the virus is able to persist in a latent state in memory B cells such that it is invisible to the immune system and also harmless. But on the other hand, EBV can be a terribly notorious pathogen. This happens because on its way to quiescent infection it temporarily drives the growth of infected cells. The ability to make cells grow if not tightly regulated can lead to diseases especially cancer. EBV is associated with several cancers of lymphoid and epithelial origin. The exact mechanisms by which EBV influences cancer development are as yet unknown. MicroRNAs (miRNAs) are small noncoding RNAs that can post-transcriptionally regulate gene expression. The EBV genome encodes more than 40 mature miRNAs identified to date. The function of most EBV miRNAs remains to be explored. This thesis work aimed to elucidate the roles of EBV miRNAs in establishing latency and tumor development from three scientific perspectives. First, with a profiling study, I identified a unique pattern of viral miRNA expression by normal persistently infected primary B cells in vivo and a subset of miRNAs that might be associated with cell growth and are deregulated in tumors. Second, using optimized mouse cancer models, I found that the EBV BART miRNAs provide a growth/survival advantage to EBV-associated tumors in vivo therefore promoting tumor growth. Lastly, by focusing on miRNA target identification, I

demonstrated that a miRNA BART18-5p facilitates the maintenance of latency in B cells by inhibiting viral replication by suppressing MAP3K2. In all, these studies on expression profiles and molecular functions of EBV miRNAs provide significant insights into the role of EBV in persistent infection and oncogenesis.

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# Abbreviations

**Ago**: argonaute protein

**APC:** allophycocyanin

**BA:** sodium butyrate

**BARTs**: *Bam*HI A rightward transcripts

**Bp:** Base pair

**BCR:** B cell receptor

**BL:** Burkitt lymphoma

**CD:** Cluster of Differentiation

**cDNA:** complementary DNA

**C. elegans:** Caenorhabditisis elegans

**cHL:** classic Hodgkin lymphoma

**CTL:** cytotoxic T lymphocyte

**DGCR8:** DiGeorge critical region 8

**DLBCL:** diffuse large B cell lymphoma

**DMEM:** Dulbecco’s modified Eagle’s medium

**DNA:** deoxynucleic acid

**DNMT1:** DNA methyltransferase 1

**DPBS:** Dulbecco's phosphate-buffered saline

**EA:** early antigen

**EBV:** Epstein-Barr virus

**EBNA:** EB-virus nuclear antigen

**ECL:** enhanced chemiluminescence

**FACS:** fluorescence-activated cell sorter

**FBS:** Fetal bovine serum

**FITC:** fluorescein isothiocyanate

**GAPDH:** glyceraldehyde-3-phosphate dehydrogenase

**GC:** germinal centre

**GCB:** germinal center B

**GFP:** green fluorescent protein

**Gp:** glycoprotein

**H&E:** hematoxylin and eosin

**HEK 293:** cell Human embryonic kidney 293 cell

**HIV:** human immunodeficiency virus

**HL:** Hodgkin's lymphoma

**Hr:** Hour(s)

**HRS:** Hodgkin-Reed Sternberg cells

**HSV-1:** herpes simplex virus-1

**Ig:** immunoglobulin

**IL:** interleukin

**IM:** infectious mononucleosis

**JAK:** janus kinase

**JNK:** c-Jun N-terminal kinase

**Kbp:** kilobase pair

**KSHV:** Karposi's sarcoma-associated herpesvirus

**Lat I:** Latency I

**Lat II:** Latency II

**Lat III:** Latency III

**LCL:** lymphoblastoid cell line

**LCV:** lymphocryptovirus

**LMP:** latent membrane protein

**LNA:** locked nucleic acid

**MA:** membrane antigen

**MAPK:** mitogen activated protein kinase

**MAP2K:** Mitogen-activated protein kinase kinase

**MAP3K:** Mitogen-activated protein kinase kinase kinase

**MemB:** memory B

**MHC:** Major Histocompatibility complex

**mrna:** Messenger RNA

**min:** Minutes(s)

**ml:** Milliliter

**mm:** Millimeter

**mM:** Millimolar

**MICB:** histocompatibility complex class I related chain B

**miRNA:** microRNA

**Mut:** mutated

**NFκB:** nuclear factor kappa B

**NK cell:** natural killer cell

**NOD:** Non-obese diabetic

**NSG:** NOD SCID IL2r γ KO

**NPC:** nasopharyngeal carcinoma

**ORF:** open reading frame

**p/s/cm2/sr:** photons per second per centimeter squared per steradian

**PBS:** phosphate buffer saline

**PBSA:** phosphate-buffered saline with 1% bovine serum albumin

**PCR:** polymerase chain reaction

**PE:** phycoerythrin

**PI3K:** phosphoinositide 3-kinase

**PKC:** protein kinase C

**PLC γ -1:** phosphor lipase C- γ -1

**PTEN:** phosphatase and tensin homolog

**PTLD:** post-transplant lymphoproliferative disorder

**pmol:** picomolar

**PUMA:** p53 up-regulated modulator of apoptosis

**QPCR:** quantitative polymerase chain reaction

**RISC:** RNA-induced silencing complex

**RNA:** ribonucleic acid

**RNAi:** RNA interference

**RPM:** revolutions per minute

**RT:** reverse transcription

**RT-PCR:** reverse transcribed polymerase chain reaction

**SCID:** severe combined immunodeficient

**SDS:** sodium dodecyl sulphate

**snoRNA:** small nucleolar RNA

**TCR:** T cell receptor

**TLR:** toll like receptor

**TPA:** 12-O-tetradeconoylphorbal-13-acetate

**TR:** terminal repeat

**UTR:** untranslated region

**VCA:** viral capsid antigen

**Wt:** wild-type

**XLP:** X-linked lymphoproliferative disease

**ZEBRA:** RrnnHI-Z-ericoded EBV replication activator

**Zp:** Z ptomoter

# Section I: GENERAL INTRODUCTION

## 1.1 Discovery of EBV

The discovery of EBV can be traced back to 1957 when Dr. Denis Burkitt, an Irish doctor, performing medical services in Uganda, encountered two local children with extraordinarily swelling jaws. Doubting that these two cases could not be coincidental, he carefully examined the medical records of several local children in the same area and postulated that they might have the same diseases. The disease often characterized by giant masses in jaws and abdomens was soon proved to be lymphoma [1, 2]. This type of lymphoma is the most common pediatric cancer in Africa and has a very strong tendency to spread to various organs thus causes a high mortality rate in children without treatment. Burkitt's fascination with lymphomas led him to embark on what he later described as a long safari thoughout equatorial Africa documenting the incidence and distribution of this cancer. He found a definitive pattern in the geographical and climatic distribution of this tumor, later known as Burkitt's Lymphoma, and came with a hypothesis that either environmental factors or infectious agents contributes to its etiology [3].

A milestone in research on EBV occurred in 1964, when Anthony Epstein and his colleagues Bert Achong and Yvonne Barr from Britain observed herpesvirus-like particles in the cultured cells derived from tumor biopsies by electron microscopy, three years after their collaboration with Burkitt who agreed to send tumor samples from Uganda [4]. This so far unannotated virus was named after its finders Epstein-Barr virus (EBV). As such, EBV became the first tumor virus ever described in humans. Soon after, a study showed that blood from BL patients had elevated antibody titers to EBV and EBV is a unique herpes virus distinct from other herpes virus, further suggesting a link

between EBV and this malignancy [5]. The association of EBV with other diseases beyond BL was not established until a laboratory researcher working with the virus developed infectious mononucleosis and subsequent serological tests detected anti-EBV antibodies, hinting that EBV was the etiological agent of infectious mononucleosis (IM) [6]. Further seroepidemiologic investigations revealed that instead of being locally restricted, EBV infection are widespread with more than 90% of the adult population worldwide testing positive for EBV antibodies [5, 7]. It has since become clear that EBV persists in the healthy hosts as a lifelong asymptomatic infection.

## 1.2 Herpesvirus Classification

Herpesviruses are large double-stranded DNA (dsDNA) viruses that infect most species throughout the animal kingdom. With over 130 different herpesviruses identified to date, eight of them are associated with humans [8]. Mammalian herpesviruses are believed to have diverged 180-220 million years ago even before the major mammal radiation [9]. The herpesvirus family is divided into three subfamilies: α-, β-, and γ-herpesvirinae. α-herpesvirinae includes Herpes simplex virus 1 and 2 (HSV-1 and -2), and varicella zoster virus (VZV) also known as the chicken pox virus. Human cytomegalovirus (CMV) and human herpesviruses 6 and 7 (HHV6 and HHV7) are members of β-herpesvirinae. The γ-herpesvirinae subfamily includes two genera: *Rhadinovirus* (RDV) and *Lymphocryptovirus* (LCV). Kaposi's sarcoma-associated herpesvirus (KSHV, also HHV8) is a member of the RDV, whereas EBV also referred to human herpesvirus 4 (HHV4) is the prototype of the LCV. KSHV and EBV are the only γ-herpesvirinae infecting humans. Other LCVs exclusively infect non-human primates including EBV-like viruses of chimpanzees and rhesus monkeys [10]. While the γ-herpesvirinae have a

fairly restricted host range with replication generally occurring in epithelial and fibroblast cells for EBV and KSHV, respectively, the hallmark of infection is their ability to establish a lifelong latent infection in lymphocytes.

## 1.3 Structure and genome of EBV

The EBV particle measures ~180 nm in diameter and consists of a toroid-shaped protein core wrapped with a linear dsDNA genome of 184 kb, a nucleocapsid that is made up of 162 capsomeres, a protein tegument, and an outer envelope containing external glycoprotein spikes [11-13]. EBV has a range of glycoproteins, many of which are homologs of other herpesvirus glycoproteins [14-17]. EBV glycoproteins, often named after their molecular weight, include gp85 (gH), gp84/l13 (gM), gpl5 (gN), gpll0 (gB) and gp25 (gL). The most abundant glycoprotein is gp350/220, encoded by one gene and also unique to EBV, because it is not homologous to any major envelope proteins of other herpesviruses [18, 19]. gp350/gp220 can be alternatively spliced to express proteins at 350kD and 220kD, functioning as a ligand for CD21 a receptor for viral entry in B cells [17, 20-22].

EBV was the first herpesvirus to be completely cloned and sequenced. The genome was sequenced from fragments generated from the prototype laboratory strain B95-8 by digestion with the restriction enzymes BamHI and EcoRI [23-25]. The viral genome contains approximately 100 open reading frames (ORFs). The nomenclature of EBV genes was based on the position and orientation in the BamHI restriction map. The EBV genome is present as a linear DNA in viral particles. The linear genome can be divided into long and short segments by a series of 500 bp tandem terminal direct repeats (TRs)

and 3 kbp, 125 bp, and 102 bp internal direct repeats (IRs) [26-28]. TRs are critical, as upon in vitro infection in B cells EBV DNA circularizes within 16 hour by homologous recombination of its TRs to generate extrachromosomal episomes [29]. After 16 hour, the episome in the infected cell is amplified to become multiple copies [30-32]. The EBV genome is stably maintained in the episomal form in infected cells. It is believed that the number of TRs is unique to the infected cells and progeny cells usually have the same number of TRs in the episome as their parental EBV genome. Therefore, analysis of the TRs by Southern blot can provide information regarding the progenitor of EBV infected cells [33]. IRs are also important since the W fragment promoter (Wp), which controls the expression of all the latent genes, is located in this region.

## 1.4 Viral tropism

EBV is a B lymphotropic virus. The initial step for infection is the binding of glycoprotein gp350/220 to the C3d complement receptor, CD21, which is widely expressed on B cell surface [20]. Two regions of gp350/220 have structural homology with the natural CD21 ligand, C3dg [34-36]. CD21 is expressed on B cells of all developmental stages except for plasma cells, which are terminally differentiated [20, 37-39]. Upon this initial interaction, three additional envelope glycoproteins (gL, gH, gp42) form a heterotrimeric complex to associate with the major histocompatibility complex (MHC) Class II cellular molecules , HLA-DR, DP and DQ, leading to receptor-mediated endocytosis of the virus [40, 41]. The envelope of EBV in the endocytic vesicle fuses with the endosomal membrane followed by the release of the viral nucleocapsid and tegument into the cytoplasm [34, 42, 43]. Most of the time, infection of B cells does not give rise to productive lytic replication instead the virus tends to be latent in the cell.

Although the observation that epithelial-derived malignancies harbor EBV is suggestive of in vivo infection in epithelial cells, and specialised epithelium seems to be the primary site for rapid infection upon exposure of the virus, the infection of epithelial cells in vitro by cell-free virus is still hard to achieve [44]. This is attributed in part to the low expression of CD21 on the surface of epithelial cells [45]. Stable transfection of CD21 in epithelial cells was able to increase the rates of infection [46]. Interestingly, epithelial cells can be efficiently infected in vitro using a system where B cell bound EBV is transferred to the epithelial cells [44, 47], suggesting that direct cell to cell contact may be required for viral entry. The fact that rare lytic replication was seen in mucosal epithelial cells of non-immunocompromised patients [48, 49], and that expression of CD21 in oropharyngeal epithelium was detected by laser capture microdissection [50], indicate that EBV tropism to epithelial cells is possibly through CD21 in vivo. EBV was also shown to infect other CD21-negative cell types such as natural killer (NK), smooth muscle cells and T cells, as evidenced by the direct association of EBV with the corresponding neoplasia [51-54]. The underlying molecular mechanism of EBV infection in these cell types remains unknown. For instance, T lymphocytes with no CD21 are still capable of binding EBV via an unidentified receptor [55]. However, the frequency of EBV infection in these cell types is lower than that of EBV-infected B cells in healthy EBV carriers [56].

## 1.5 EBV infection in vitro

EBV is thought to be a cancer causing agent. This was initially demonstrated in vitro where EBV infects primary B lymphocytes and transforms them into immortalized lymphoblastoid cell lines (LCL) that are capable of long-term growth in culture [57, 58].

After infection, the cells express the full range of latent gene products including 6 nuclear antigens (EBNALP, EBNA1, EBNA2, EBNA3A, EBNA3B and EBNA3C), 3 membrane proteins (LMP1, LMP2A and LMP2B), the small non-polyadenylated RNAs (EBER1 and EBER2), the highly spliced Bam A rightward transcripts (BARTs), and a viral Bcl-2 homolog (BHRF1) [59]. The transcription of viral genes initiates very early upon infection but follows a set sequence. At 12-16 hours of infection, the RNA polymerase II-dependent promoters in the BamHl W (Wp) regions of the viral genome initiates rightward transcription, which gives rise to the expression of the first two proteins EBNA2 and EBNALP [29, 60, 61]. These two proteins contribute to the early stages of the immortalization process and the following viral gene expression. Throughout the process of infection, Wp activity declines and is replaced by BamH1 C region promoter (Cp) which then participates in transcription of the other EBNA genes [62, 63]. Thus in LCL, all EBNA mRNAs are transcribed either from the Cp or Wp promoters. The expression of other genes such as LMP1 and LMP2 is transactivated by EBNA2 via separate promoters. By 72 hour post-infection, all 11 latent proteins are detectable [63]. The oncogenic potential of EBV is probably attributed to latent gene expression. Reverse genetic experiments have shown that nuclear antigens, (EBNA, -2, -3A, 3C), and membrane proteins (LMP1) are required for growth transformation [59]. The molecular basis of EBV-mediated transformation is generally associated with disruption of cellular function by viral genes. For instance, LMP1 inhibits apoptosis by elevating antiapoptotic genes, thus rendering growth advantage to the host cells [64]. In addition to direct infection of EBV in vitro, LCL could also be obtained by spontaneous outgrowth of peripheral leukocytes from EBV-positive patients in medium containing cyclosporine

which inhibits cytotoxic T cells [65]. This further suggests that the conversion of primary B cells into LCLs by either exogenous or endogenous EBV is a strong indicator of the oncogenic potential of the virus.

## 1.6 EBV infection and persistence in vivo

The vast majority of studies on EBV infection in vivo were carried out based on the early observation that EBV is an orally transmitted virus [66]. Although other routes for EBV transmission (i.e sexually transmitted or breast milk-borne) were also documented [67-69], our main focus in this thesis is on the most common route of transmission. Oral contact with infectious saliva is the main way for EBV transmission from individual to individual. The virus from saliva enters through the epithelium of the Waldeyer's ring (tonsils/adenoids) lining the oral cavity. It is believed that epithelial cells of the oropharynx are the primary target for EBV infection and more importantly serve as sites of viral replication and subsequent shedding in healthy EBV carriers. This notion is supported by several studies. It was found that EBV undergoes massive lytic replication in epithelium of the tongue from an AIDS patient with oral hairy leukoplakia [70]. Even in non-HIV-infected EBV-infected individuals, intense lytic infection in the oral epithelial cells and the shedding of virus from the epithelium were observed [71-74]. The progeny viruses from the epithelium then proceed to infect trafficking resting B cells in the nearby lymphoid tissue.

Two models of how the virus continues following infection and finally obtains persistency in B cells have been proposed. One widely accepted model is that EBV exploits the common pathway of B cell differentiation such that a naïve B cell

differentiates to the memory compartment through a germinal center reaction [75] . In this model (Figure 1), EBV initially infects naïve B cells and drives them to undergo transient activation and proliferation. This is simply achieved by the coordinated expression of all latent proteins and transcripts of the growth latency III program in the infected cells, which includes six EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMPs), two small EBER RNAs and the BART [76-78]. Naïve B cells become proliferating lymphoblasts, which are either eliminated by EBV-specific cytotoxic T-lymphocytes (CTLs), or analogous to antigen-activated innate B cells, they continue to differentiate via germinal center (GC) reactions [79]. In the GC, EBV-infected cells proliferate and clonally expand with expression of a more restricted gene profile (EBNA-1, LMP2A, LMP1, EBERs and BARTs, also termed Latency II) [78, 80, 81]. The switch from the type III latency to the type II latency appears to be driven by the actual process of B cell differentiation, because direct infection of tonsillar germinal centre and memory B cells also leads to the classical growth latency III program. However, the objective of down regulation of latent gene expression is not only to permit the differentiation of a GC phenotype, but also to retain expression of some proteins to provide constitutive and ligand-independent signals for B cell proliferation and survival. These proteins are LMP2A and LMP1, which have been shown to mimic the B cell receptor and the CD40 receptor, respectively. The EBV-infected GC cells who receive the signals from these proteins proliferate in an antigen-independent manner, and subsequently differentiate into memory B cells which move into the peripheral blood [82-84]. Once in the resting memory compartment, the virus adopts the most stringently restricted gene expression program (Latency 0) with which all viral protein expression

ceases except ERERs and BART expression remains [80, 85, 86]. Occasionally, when the memory B cells divide, expression of EBNA1 (Latency 1) is required for viral DNA replication in the daughter cells [87]. By limiting viral gene expression in long-lived memory cells, EBV reduces the chance of being recognized by CTLs, thereby escaping immune surveillance and enabling lifelong persistence [85, 86, 88]. Latently infected memory B cells that circulate in the periphery could also return to the lymphoid tissue and undergo antigen driven differentiation to become antibody-secreting plasma cells, leading to the viral lytic replication and the release of infectious virions to infect new targets (Figure1) [75, 89].

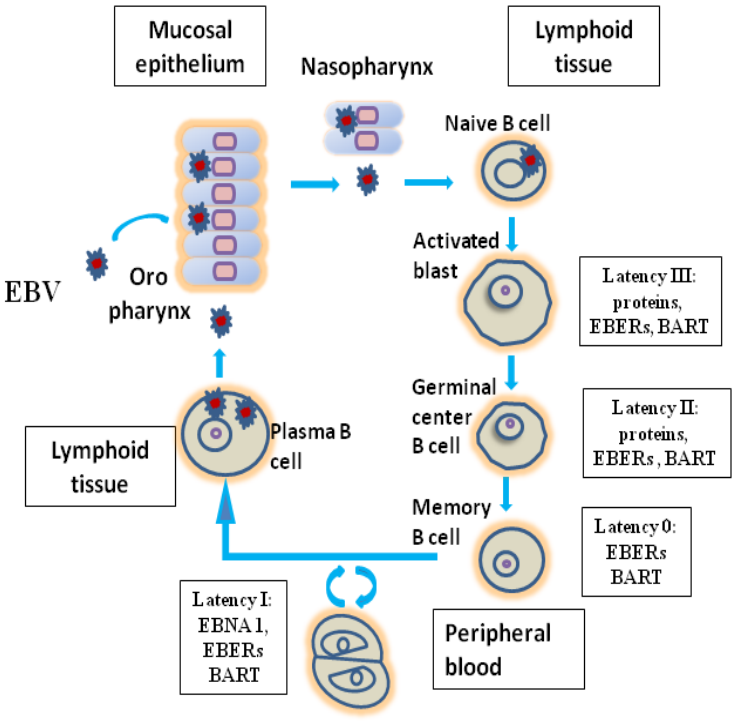



**Figure 1. EBV infection in vivo.** EBV enters the epithelium of the oro-pharynx via saliva. The epithelium is where the virus replicates. EBV then gains access to the secondary lymphoid tissue (e.g, tonsil) and infects naïve B cells leading to expression of the viral latent genes. These B cells become activated lymphoblasts (Latency III) and then go through the germinal center reaction (Latency II). The selected B cells eventually migrate to the peripheral blood as memory cells (Latency 0 or I) – the site of long term latent persistent infection.

An alternative model proposes the direct infection of memory B cells by EBV independent of GC reactions. This model is supported by the fact that B cells from X-linked lymphoproliferative (XLP) patients who don't have fully functional germinal centers are also latently infected with EBV [90]. Both models support the idea that circulating memory B cells are the site of EBV persistent infection.

## 1.7 Lytic replication

Like all viruses, EBV needs to produce progeny and spread within the host as well as to the new host. Distinct from latent infection, this phase of the EBV life cycle is called lytic replication. In vivo, EBV can be detected in the saliva of healthy carriers indicating that lytic replication is a frequent event throughout the lifetime of a host [91-94]. Study of the underlying cellular mechanism for lytic replication in vivo is a daunting challenge. Much of the work has been focused on in vitro cell lines. As yet, there is currently no cell culture system that efficiently supports primary lytic infection, despite the fact that a small fraction of cells in some latently infected B cell lines occasionally undergo spontaneous lytic cycle. Therefore, EBV replication in vitro has been mostly studied by reactivating latently infected cells to enter the lytic cycle with the use of inducing stimuli. A variety of reagents are able to induce the lytic cycle, including sodium butyrate (BA), phorbol esters such as 12-O-Tetradecanoylphorbol-13-acetate (TPA), anti-immunoglobulin (anti-Ig), calcium ionophore, transforming growth factor-B and histone deacetylase inhibitors [95-102]. Different cell lines show distinct lytic permissivity for lytic replication. Marmoset cells (e.g B95.8) and some LCLs seem to be more permissive for lytic replication than BL derived cells. The lytic cycle can be effectively induced by TPA and BA in those cell lines. A BL cell line Akata can be stimulated into the lytic

cycle by crosslinking of the surface immunoglobulin with anti-Ig [103]. Therefore, in vitro models provide a useful and effective means for understanding the molecular and physiological mechanisms of EBV lytic replication.

Regardless of the stimuli, upon lytic induction viral genes are expressed in a temporally ordered fashion, termed the lytic gene cascade, which can be divided into three phases: immediate early (IE), early (E), and late (L) (Figure 2). IE transcripts (BZLF1 and BRLF1) are the first lytic genes to be expressed, which can be induced within an hour after stimulation, even in the presence of protein synthesis inhibitors [104]. BZLF1 and BRLF1 have been found to play essential roles in lytic reactivation. The proteins that these transcripts encode are Zta (or ZEBRA) and Rta. They are DNA-binding transcription factors which activate transcription of the early genes and are therefore crucial for initiating the whole lytic cascade [105-108]. BZLF1 is a viral homologue of the cellular c-jun and c-fos transcription factor that can interact with AP-l-like sequences in both the viral and host genome [109]. Zta and Rta can activate their own promoter and each other's as well, thus even a tiny amount of stimulus is sufficient to amplify the lytic cascade [110-113]. Expression of either BZLF1 or BRLF1 is sufficient to induce lytic replication from latently infected cells in vitro [105, 114, 115]. Deletion of either gene in the EBV genome greatly impaired viral DNA replication or virion production [116]. Early gene transcription is triggered by Zta and Rta. Since it relies on IE gene products, the transcription of E genes does not occur in the presence of protein synthesis inhibitors. E genes encode proteins important for viral genomic DNA replication such as the DNA polymerase (BALF5), the polymerase accessory protein (BMRF1), and helicase (BBLF4) [117]. Following viral genome replication, late gene expression is activated. L

gene transcription can be inhibited by DNA replication inhibitors (e.g. phosphonoformic acid), suggesting that L protein expression is a downstream process regulated by EBV DNA replication [118]. L proteins consist most of structural proteins such as glycoproteins (gp350/220) and nucleocapsid proteins that function in virus assembly and budding [119]. The mechanisms involved in virion packaging and budding are not well characterized. It is believed that viral DNA is replicated as large head-to-tail molecules and subsequently cleaved into small fragments and embraced by the nucleocapsid proteins in the nucleus. The immature virions then are associated with the proteinaceous tegument in the cytoplasm followed by budding at the cellular plasma membrane [120, 121]. In the lytic cells, glycoprotein gp350 is the most abundant protein on the plasma membrane as well as the surface of enveloped virus [119]. The lytic proteins described above are well elucidated. However, a complete lytic cycle can result in expression of more than 80 viral replication associated genes, including functional kinase proteins and viral capsid antigens [119]. The role of most of lytic proteins are as yet undetermined.

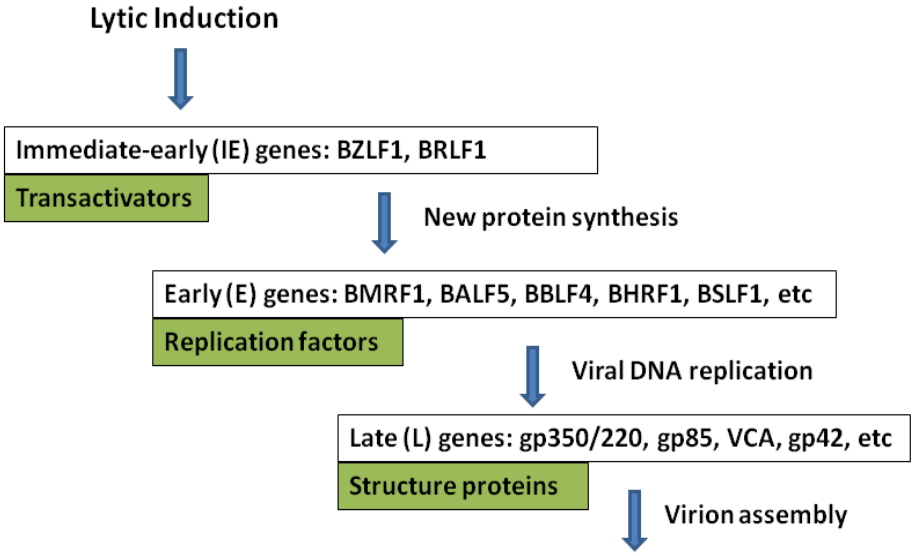



**Figure 2. Cascade of EBV lytic gene expression upon reactivation**. Lytic induction initially results in expression of the IE genes BZLF1 and BRLF1 which encode the viral transactivators. The transactivator proteins act in concert to induce E genes including BALF5, BMRF1, and BHRF1, etc, that are crucial for viral DNA replication. Following replication, L genes are expressed where a number of structure proteins essential for virion assembly are made.

The initial step in viral lytic induction is the activation of promoters Zp and Rp that drives BZLF1 and BRLF1 transcription. In latent EBV-infected B-cells such as memory B cells, both promoters are silent therefore the BZLF1 and BRLF1 transcripts are undetectable [122]. This is largely due to the presence of promoter repressors and the lack of specific activators to initiate the lytic cycle. The activation of these promoters and subsequent lytic gene transcription by stimuli (i.e. TPA/BA or BCR engagement) is most likely regulated by cellular machinery, e.g the critical signaling modules. A variety of signal transduction pathways including PI3 kinase, protein kinase C (PKC), ERK kinase, and MAP kinase have been reported to control the activation of the Zp promoter (Figure 3). Different stimuli-driving lytic cycles are mediated by distinct signaling pathways. For instance, TPA-induced EBV lytic replication is primarily mediated by PKC signaling [123, 124], whereas anti-Ig stimulation in EBV+ Akata cells involves PI3K, PLC, Ras and Rac1 pathways [125]. Downstream transcription factors such as p38, JNK, ERK5 and AP-1 in the MAP kinase cascade are reported to bind to the Zp region for IE gene induction (Figure 3) [126]. MAPK pathways are essential in the induction of IE gene transcription by the inducing stimuli.

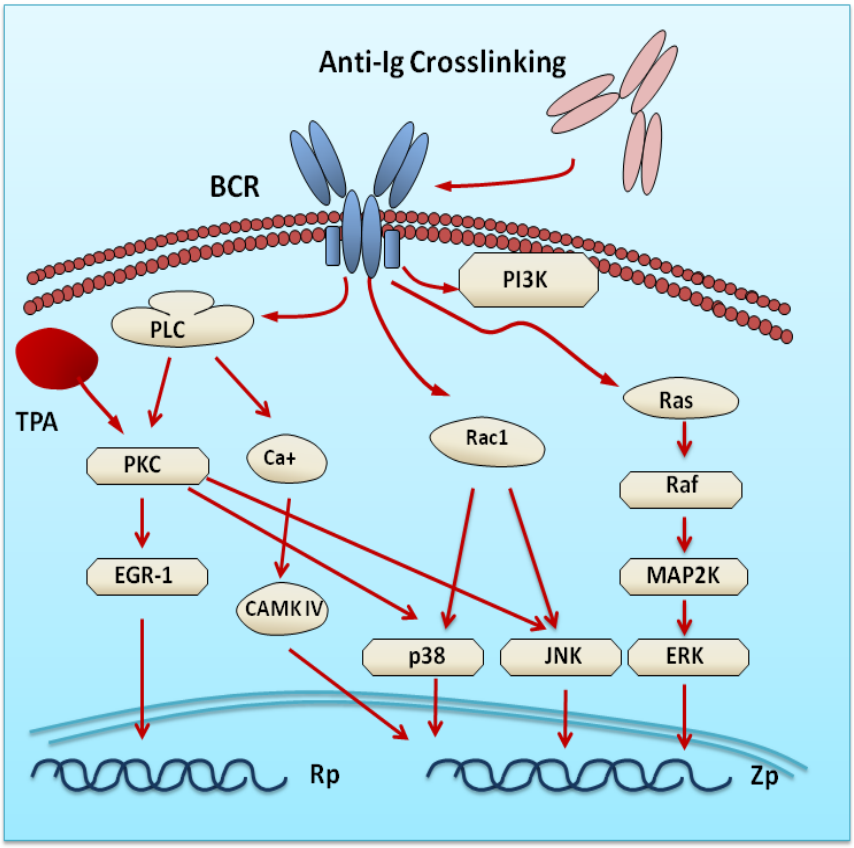



**Figure 3. Signal transduction pathways in EBV lytic induction.** Signaling pathways are induced by either anti-Ig crosslinking on B-cell receptor (BCR) or TPA treatment. BCR stimulation involves the activation of PI3K, PLC, Rac1 and Ras pathways, while TPA mainly activates the PKC pathways. Activated downstream transcription factors such as p38, JNK, ERK, EGR-1, and CAMK IV can bind to the promoter motifs in the two EBV IE promoters (Rp and Zp), followed by BZLF1 and BRLF1 activation. This figure combines several figures or findings in the literature [97, 123, 125-130].

In vivo, primary EBV infection is often accompanied by lytic replication. This will elicit potent T cell responses against viral antigens. Lytic EBV infection is generally restricted to differentiated oropharyngeal epithelial cells and plasma cells in Waldeyer's ring. It is believed that oropharyngeal epithelial cells support primarily lytic infection, whereas latently infected memory B cells will undergo sporadic lytic viral reactivation. Therefore EBV replication occurs most efficiently in epithelial cells in vivo. The reactivation of latent memory B cells leads to lytic replication. The signal that induces reactivation in those cells may originate from the differentiation of plasma cells with involvement of antigen binding [89].

## 1.8 Immune response and immune evasion

EBV infection gives rise to both humoral and cellular immune responses. Due to the lack of symptoms in childhood upon primary infection, research on host immune response has been largely directed towards studies of acute IM patients. The appearance of large numbers of atypical lymphocytes combined with high levels of proinflammatory cytokines is the clinical hallmark of acute IM [131]. The responding T cells that undergo massive expansion express a wide repertoire of T cell receptors (TCRs) specific for EBV epitopes [132, 133]. It is estimated that up to 40% of the entire CD8+ T cell population recognizes lytic epitopes including BZLF1, BRLF1, and BMRF1 [134], while only an estimated 2% of circulating CTLs in IM patients are responsive to the most immunodominant latent antigens [135]. Among those CD8+ T cells against lytic antigens, the majority is directed toward an epitope from BZLF1 [134]. The response to latent antigens mainly focuses on the EBNA 3 family [136]. EBV-specific CD8+ T cells have high levels of perforin and granzyme B thus have strong cytolytic ability. Studies

showed that EBV-specific cytotoxic CD8+ T cells are able to target and kill cells loaded with the relevant peptide epitope ex vivo [134, 136, 137]. Following resolution of primary infection, the EBV-specific CD8+ T cell population, responding to both lytic and latent antigens, decreases but remains detectable over time.

The CD4+ T cell response to EBV is less robust than the CD8+ response in IM patients. Similar to the CD8+ cells' pattern, a small expansion of CD4+ T cells occurs primarily but is followed by a dramatic decrease after recovery [137]. They are responsive to both lytic cycle epitopes (BZLFl, BMLFl, gp350) and latent epitopes (EBNAl, EBNA2, EBNA3C) in patients with IM [137, 138]. During persistent infection, despite low frequency in periphery, memory CD4+ T cell responses for both lytic and latent epitopes can be detected, suggesting that CD4 T cells may perform surveillance for lifelong infection. Upon re-stimulation, memory CD4+ T cells from EBV-positive individuals are able to inhibit B-cell proliferation [139, 140]. CD4+ T cells from healthy carriers have been described to recognize lytic cycle IE, E, and L antigens, indicating a clear memory response [141-143].

Both primary and persistent infection give rise to the production of antibodies. During primary infection, antibodies are produced against viral capsid antigen (VCA), early antigen D (EA-D), and membrane antigen (MA) [59]. VCA consists of nucleocapsid components BcLF1, BFRF3, BLRF2, and the glycoprotein gp110. EA is a composite of immediate-early and early viral proteins (BZLF1, BALF2, BHRF1, BMRF1 and BMLF1 proteins). Much of MA reactivity is against gp350 [144]. High titers of IgM, IgG and IgA antibodies to VCA are found in IM patients, but only the VCA IgG antibodies are detected throughout life in healthy carriers [145]. Antibodies to EA-D are detected in

approximately 80% of IM patients. IgG anti-gp350 and anti-EBNA1 are commonly found in persistently infected individuals. Antibody responses to LMP1 or LMP2 have not been detected during IM but are found in a few healthy carriers with the use of the most sensitive assays [146, 147]. It is believed that the humoral response has a great role in elimination of lytic cells, as virus-neutralizing antibodies (e.g anti-gp350 antibodies) have been shown to hamper spreading of the virus [144].

In order to persists for life in the infected hosts, EBV develops several sophisticated strategies to avoid host defenses. During latent infection, the main goal is to downregulate virtually all potential antigen expression therefore limiting the number of epitopes presented to the immune system. EBNA1 is produced in all forms of latency in vivo, and is the optimal antigen present to T cells. Even in the memory B reservoir, EBNA1 is expressed when cells divide and plays a role in the maintenance of the viral episome [148]. The Glycine/Alanine repeat sequences found in EBNA1 have been shown to affect the interaction of ubiquitinated substrates with the proteasome, therefore consequently stabilizes the EBNA1 protein and inhibits processing of EBNAl by the proteasome [149, 150]. Prior to MHC presentation of antigens, viral proteins are normally cleaved by proteasomes into peptides. Additionally, the Glycine/Alanine repeat sequences also affects the translation of the EBNAl transcript [151]. Therefore, low levels of MHC-I-EBNA1 in the EBV-infected memory B cells are partially attributed to disruption of proteasome interaction by EBNA1 per se. LMP1 is expressed in the Latency II and Latency III program. LMP1 prevents the infected B lymphocytes from apoptosis by upregulating A20 and Bcl-2 [152, 153]. LMP-1 is also the homolog of cellular CD40. It has been shown to bind TNF-receptor-associated factors, induces B cell proliferation,

and increase NFκB and kinase expression [154-157]. LMP1 can be secreted by EBV-positive cells and strongly suppresses activation of T cells and NK cells [158]. The survival advantage conferred by LMP1 to B cells may contribute to the prevention of CTL-induced cell death [159]. LMP2A is shown to inhibit lytic reactivation by blocking the BCR-mediated signal transduction thereby reducing the exposure of EBV antigens to the immune system [160-163]. During lytic infection, a number of lytic proteins have been reported to contribute to the evasion of immune surveillance. EBV E genes BHRF1 and BALF1 are the homologs of the cellular bcl-2 gene, preventing granzyme B-mediated apoptosis [164, 165]. Another E protein BARF1 possibly inhibits the antiviral effects of IFN-a by mimicking a secreted receptor for colony stimulating factor-1 (CSF-1) [166, 167]. The BCRF-1 gene encodes a protein (viral IL-10) with functional homology to human IL-10. vIL-10 is able to abrogate production of IFN-γ by T cells and NK cells thus eliminating their inhibitory capacity, decrease expression of MHC class I and II, and co-stimulatory molecules on B cells, delay the induction of EBV-specific immunity by downregulating pro-inflammatory cytokines, and enhance growth and differentiation of B cells [168-171]. The E protein BNLF2a can block MHC class I antigen presentation by interfering with the TAP1/TAP2 peptide transporter [172, 173]. The E gene BGLF5-encoded EBV DNase is able to promote mRNA degradation and thus blocks the synthesis of new MHC class I molecules [174]. The transmembrane glycoprotein gp42, encoded by the L gene BZLF2, associates intracellularly with HLA class II-peptide complexes and disrupts the interactions with CD4 T lymphocytes through steric hinderence [175]. In addition, gp42 together with gp85 and gp25 participates in the

inhibition of dendritic cell differentiation and promotes apoptosis in CD14+ monocytes [176, 177] .

## 1.9 EBV and cancer

Most people infected with EBV are asymptomatic. However, EBV is also believed to contribute to the development of lymphoid and epithelial malignancies years after primary infection, especially in the immuno-compromised hosts. EBV-associated cancers include Burkitt's lymphoma, Hodgkin's lymphoma, immunoblastic lymphoma, nasopharyngeal carcinoma and gastric carcinomas.

### 1.9.1 Burkitt's lymphoma (BL)

After Burkitt's first description of this tumor in Africa, BL was later found to occur at lower frequencies worldwide. BL can be subdivided into three forms: endemic, sporadic, and AIDS-associated. Endemic BL is the most common form (up to 74%) of childhood malignancies in equatorial Africa and Papua New Guinea. For children up to 15 years of age, the incidence rate is five to ten cases per 100,000 [178]. The distribution of this tumor is associated with geographic and climatic features of holoendemic malaria [179, 180]. BL is clinically characterized by a poorly differentiated mass normally presented at the extranodal sites of jaw, facial bones, kidney, or the abdomen. EBV DNA and EBNA1 expression are consistently detected in more than 90% of endemic BLs [181], with viral genome copy numbers ranging from 10 to 113 per cell [182, 183]. Because of the geographic coincidence with malaria infection, it is believed that co-infection with EBV and the malaria parasite Plasmodium falciparum contributes to endemic BL incidence and development. Sporadic BL does not show geographical restriction but instead is

found worldwide. It occurs at a 100 fold lower frequency than endemic BL, and often develops in older childern. Abdominal tumors are frequently detected in sporadic BL [180]. The association of EBV and this form of BL is weaker than the endemic form, with only 15-25% of cases in Europe and the United States showing EBV positivity [184]. In some areas of North Africa and equatorial South America, EBV association is intermediate between true sporadic and endemic forms [185, 186].

BL is believed to originate from B cells based on gene expression profiling and phenotype [187]. In terms of surface antigens, both forms of BL are phenotypically close to the germinal center B cells, which are CD 10+/CD77+/CD23-/B7- [188]. BL cells also express low level of MHC I, adhesion molecules (ICAM-1, CD58), activation molecules (CD54), and the anti-apoptotic protein Bcl-2 [189-191]. The hallmark of BL is the reciprocal translocation of the c-myc proto-oncogene and an immunoglobulin gene irrespective of geographical origin or EBV association [192]. The translocation occurs most often between chromosomes 8 (c-Myc) and chromosomes 14 (Ig heavy-chain locus), a region that has high transcriptional activity in B cells. This puts c-myc under the control of the Ig promoter and leads to its overexpression in B cells. This may explain the high level of c-myc expression in BL compared to resting B cell counterparts [193]. It is believed that continuous high expression of c-Myc contributes to proliferation of B cells [194]. However, when c-myc reaches a certain level, it will push cells toward apoptosis [195]. Therefore, there must be other mechanisms involved in counteracting the apoptotic signals.

Although the c-myc translocation is the major event leading to tumor formation, the pathogenesis of BL undoubtedly involves multiple factors. EBV infection and malarial

parasitic burden are crucial for BL development. How EBV contributes to the pathogenesis of BL is not completely understood. This is in part because viral genes known to be required for transformation, i.e., EBNA2, EBNA3s, and LMPl are not expressed in BL. BL cells only express EBNA1, EBERs and the BART (Latency I). However, the fact that in endemic BL individual cells arise from a single virally infected cell implies that EBV probably plays a role in oncogenic transformation [33]. In addition, spontaneous loss of the EBV episome in a BL-derived cell line lead to loss of tumorigenicity, but reinfection of this line with EBV restored tumorigenicity in nude mice, suggesting that EBV is directly linked to the tumorigenesis of BL [196, 197]. EBV also seems to favor the c-Myc translocation, as it was shown that EBV infection greatly increases genomic instability [198]. The transforming ability of the virus in the initial infection is also essential for lymphomagenesis, as it would increase proliferation rates of infected B cells. Other latency III proteins EBNA2, EBNA3C and LMP1 may also play roles in the initial transformation event before the cells revert to a latency I gene expression. It is also possible that the ability of malaria to suppress cell-mediated immunity promotes long term B cell expansion thus favoring the accumulation of genetic changes including the chromosomal translocation of c-Myc locus [199-203].

### 1.9.2 Hodgkin's disease (HD)

HD was first described by Sir Thomas Hodgkin in 1832 and defined as a primary lymph node neoplasm followed by enlargement of the spleen and liver [204]. HD emerges as a unifocal lesion in a single lymph node or chain of nodes, followed by spread of the tumor to adjacent nodes. The disease progresses aggressively via the lymphatic system and it can reach to other organs such as spleen, kidneys and liver [205]. The worldwide

incidence of HD varies widely by age, race, gender and geography. HD is the most common lymphoma in Western countries with an incidence of 2-4 new cases per 100,000. Around 8000 new cases are reporting per year representing 1% of all new cancers in the United States [206]. There are two types of HD, classical and lymphocyte predominant. Classical HD includes the nodular sclerosis, mixed cellularity, lymphocyte rich and lymphocyte depleted. The presence of large malignant Hodgkin's Reed-Sternberg (HRS) cells characterizes classical HD. However, HRS cells contained within hyperplastic lymph nodes only account for 2% the total tumor mass. Infiltrating non-neoplastic cells which include B cells, T cells, macrophages, neutrophils and granulocytes, and fibroblasts, make up the rest of the 98%. Due to the low numbers in the tumor, the isolation and characterization of HRS cells has been very challenging.

HRS cells morphologically differ from their non-classical counterparts (lymphocyte predominant HD) in that HRS cells are generally large and multinucleated whereas non-classical HD cells are mononuclear and small. In addition, HRS cells have an abnormal immunophenotype as they express various hematopoietic cell markers such as CD3, CD4, CD15 and CCL17, while non-classical HD cells express the B-cell antigens CD20 or CD19 [207]. Despite controversy, sequence analysis of the immunoglobulin locus in the isolated single HRS cell revealed Ig VH and VL gene rearrangements, suggesting HRS cells originate specifically from germinal center B cells that have undergone somatic hypermutation [208, 209]. Since stop codons are introduced within the mutated V regions during hypermutaiton, the majority of the HRS cells do not have a functional BCR. GCB cells are sensitive to apoptosis in the absence of signals from the BCR.

Therefore, HRS cells are suggested to derive from GCB cells that overcome the death signals rendered by latent proteins e.g LMP2A.

The association of EBV with HD is based upon epidemiological, serological, and molecular evidence. It was initially suggested by seroepidemiological studies that elevated EBV antibody titers were detected in HD patients [210]. Subsequently, individuals with a history of IM were found to have about a three-fold increased risk of developing this disease [211]. The direct link between EBV and HD was suggested by studies that detected EBV genomes in the tumor biopsies which mapped to the malignant HRS cells [212, 213]. In the Western world, 20 to 50% of all classical HD cases are EBV-positive whereas non-classical HDs are consistently EBV-negative [214]. Within classical HDs, 60-90% of mixed cellularity and lymphocyte depleted are EBV positive, while 20 to 40% of nodular sclerosis cases carries EBV DNA. EBV-positive HD is believed to progress from a single virally infected cell [215]. The exact role of EBV in the pathogenesis of HD remains to be fully addressed. EBV is suggested to be responsible for the rescue of pre-apoptotic GCB cells. Immunohistochemistry and PCR analyses have indicated that EBV gene expression in HD is restricted to latency type II where EBNA1, LMP2A, EBERs, BARTs, and high levels of LMP1 are expressed [216-218]. It is believed that EBV contributes to survival of HRS cells via LMP1 and LMP2A signals which lead to activation o f JNK and p38 and transcription factors NF-kB, AP-1 and STATs [219]. NF-kB activity and bcl2, p53, and IL-6 expression levels are constitutively activated in HD [220]. Through these pathways, EBV provides survival signals at a GC checkpoint thus allowing infected precursors to escape apoptosis [221].

### 1.9.3 Nasopharyngeal Carcinoma (NPC)

NPC is an epithelial malignancy that develops in the stratified squamous epithelium of the nasopharynx, clinically characterized by a swelling mass in the neck, hearing loss, and blockage of the nasal pathway. NPC is often diagnosed at late stages and highly metastatic, therefore the prognosis for NPC is poor [222]. NPC is found sporadically in most parts of the world, but it shows geographical preferences. NPC has an exceptionally high incidence in the Cantonese population from the southeast region of China. High incidence is also found in the Pacific Rim countries (e.g Malaysia and Indonesia), Mediterranean Africa, the Inuit populations of Alaska and Greenland, and the Eskimo populations of North America [223-226]. The world health organization (WHO) divides NPC into three categories. WHO type I is keratinizing NPC with squamous cell differentiation. WHO type II is a non-keratinizing NPC with poorly differentiated epithelium. WHO type III is a non-keratinizing undifferentiated carcinoma with massive infiltration with T lymphocytes [227, 228].

Epidemiological and molecular studies suggest that a combination of environmental, genetic and viral factors contributes to the development of NPC. A study based on 732 Singapore Chinese NPC patients indicated that HLA class I haplotypes (A2Bw46, and A19B17) are linked to an increased risk for NPC [229], while others showed that HLA-A11 and HLA-B13 are associated with a decreased risk [230, 231]. Several chromosomal aberration are also detected in NPC samples including deletion of chromosomes 3p, 9p, 11q, 13q, 14q etc. Some of these are associated with tumor suppressor loss of function mutations including genes such as RASSF1A, INK4a, INK4b and ARF [232-235]. Therefore, genetic susceptibility is implicated in the development of NPC. This is further supported by demographic studies of Chinese immigrants in low-risk countries. For

instance, for those Southern Chinese who migrate to North America, the incidence of NPC is higher in the first generation but then declines from generation to generation, indicating that both genetic and environmental factors are associated with the etiology of NPC [236, 237]. A dietary environmental risk factor is believed to contribute to NPC development. For instance, salted fish, a popular food of southern China and also commonly eaten by the Inuit, contains nitrosamines which are an identified chemical carcinogen [238].

The first etiological association between EBV and NPC came from early serological studies in the 70s, which showed that NPC patients have increased antibody titers against EBV [239, 240]. Most of the antibodies are IgG and IgA directed against EBV early antigens and viral capsid antigens, including VCA, EA, BZLF1. Interestingly, antibody titers seem to correlate with tumor progression, as they decline after successful cancer treatment but return if the tumor relapses [239]. Subsequent investigations demonstrated that viral DNA is present in all undifferentiated tumor cells regardless of the geographical origin [241-243]. It is now believed that 100% of undifferentiated NPC, which account for approximately 80% of all NPC cases, is EBV positive. The direct proof for a role of EBV in NPC comes from studies showing that a monoclonal form of EBV DNA was detected in the precancerous lesions of the nasopharynx from patients who finally developed NPC [244, 245]. In addition, EBV has also been detected in high-grade pre-invasive lesions but not in the dysplastic lesions or normal epithelium of nasopharynx [244, 246]. All these findings indicate that EBV infection drives cells to undergo clonal expansion as an early event of NPC pathogenesis. The majority of NPC tumors show a latency II program of gene expression [247, 248]. But unlike HD, a small proportion of

NPC cells also express E proteins, indicating that these cells are permissive for lytic reactivation [249]. The mechanism that EBV infection affects NPC tumor development is not completely understood. EBV infection induces STAT3 and NF-kB activation leading to the activation of pro-survival genes including c-Myc, Bcl-xL, IL-6, VEGF, and COX-2 which can support growth for NPC lines [250, 251]. LMP1 has been shown to induce pleiotropic effects in epithelial cells by activating genes associated with cell proliferation, angiogenesis, invasion and metastasis [252]. EBER is able to inhibit the activation of PKR and p38-MAPK in a nasopharyngeal carcinoma cell line, suggesting that EBER contributes to immune evasion by NPC cells [253]. LMP2 expression in epithelial cells renders a more tumorigenic potential in nude mice [254]. Lytic proteins appear to be crucial for the pathogenesis of NPC. The E protein BARF1 was shown to increase the proliferation of primary epithelial cells infected with an NPC-derived EBV strain [255]. Overexpression of BARF1 in EBV-negative NPC cell lines was further shown to enhance the anti-apoptotic and survival capability of those cells [256].

### 1.9.4 Gastric Carcinoma (GaCa)

GaCa is an epithelial cancer for the stomach, the fourth most common malignancy worldwide following lung, breast and colorectal cancers [257]. It is also the second leading cause of cancer-related death globally. During the last half-decade, the incidence and mortality of this cancer has dropped in many countries, but its prognosis remains poor. Unlike NPC, the geographical distribution of gastric cancer does not suggest any regional and ethnic pattern. The incidence rates vary on the basis of age, gender and location [258]. The average age of patients upon diagnosis is older than 50 years old, and the incidence rate in men is twice as much as in women. The highest incidence of GaCa

is found in East Asia, Eastern Europe, and parts of Central and South America, whereas the incidence rate is low in Southern Asia, Africa, Western and Northern Europe, North America and Australia.

According to the Lauén classification system, GaCa is categorized into two histological subtypes: intestinal and diffuse [259]. Intestinal subtype cancers are often associated with the lower part of stomach (antrum) with a pathological feature of well-defined glandular formation. Diffuse subtype tumors are more commonly localized in the middle or upper part of stomach (corpus) and are clinically characterized by the diffusion of malignant clusters formed by neoplastic cells in the stroma wall without gland formation and cellular adhesion [260, 261]. EBV-associated GaCa is known to occur primarily in the upper middle areas of the stomach [262]. The first association of EBV and GaCa was reported in an undifferentiated lymphoepithelioma-like gastric carcinoma using the polymerase chain reaction (PCR) [263]. Shortly thereafter, more evidence emerged with respect to the presence of EBV in gastric carcinoma [264-266]. It is now estimated that approximately 10% of gastric cancers are monoclonal EBV-infected cells. The worldwide occurrence of EBV-associated GaGc is more than 50,000 cases a year. Histologically, EBV-associated GaCa can be divided in two forms. The more common type of EBV-positive GaCa is the diffuse and intestinal adenocarcinoma, characterized as a poorly differentiated adenocarcinoma with lymphocyte infiltration. The lymphoepithelioma-type is less common, but with a higher frequency of EBV infection (up to 90%) [267]. EBV-positive GaCa is more common in European and Hispanic populations and, men more than women [268, 269]. EBV is detected in all the tumor

cells of EBV-positive GaCa and terminal repeat analysis indicated that GaCa cells originated from a single EBV infected cell [270].

EBV-positive GaCas have very unique molecular characteristics as opposed to the EBV-negative counterparts. For instance, p16 expression is lost and wild-type p53 is expressed [271, 272]. Hypermethylation of CpG islands on tumor suppressor genes is significantly more common when EBV is present, which is a molecular abnormality unique to GaCa [273, 274]. Aberrant methylation patterns are associated with tumorigenesis. The route for EBV infection in the gastric epithelium is unclear. It is postulated that EBV spreads from the nasopharynx to the stomach [275-277]. Similar to NPC, the exact role of EBV in carcinogenesis remains to be elucidated. However, EBV is absent from pre-malignant gastric lesions indicating an oncogenic role for EBV at later stages of pathogenesis of GaCa [278]. EBV-positive GaCa has a modified Type II latency expression pattern such that EBNA1, LMP2A, BARF1, BART, EBERs and a lytic gene BARF1 are expressed, but unlike in NPC, LMP1 is not expressed [279, 280]. Notably, LMP1 and LMP2B are frequently absent from these tumors. A small proportion of tumor cells also express BZLF1, BRLF1, and BHLF1 proteins indicating reactivation occasionally occurs in GaCa [281]. The absence of LMP1 expression in GaCa might suggest a more crucial role for LMP2A in carcinogenesis. In fact, LMP2A is shown to activate DNA methyltransferase 1 (DNMT1) leading to promoter hypermethylation of the phosphatase and tensin homolog (PTEN) gene in gastric carcinoma cell lines [282]. Silencing of PTEN is a key feature found in both infected GC cell lines and in GC tumor samples. In addition, a study showed that LMP2A provides survival advantage for EBV-associated carcinoma cell lines by activating the expression of an apoptosis inhibitor

survivin [283]. Moreover, BARF1 also exerts transforming effects in B cells and fibroblasts by acting as a viral homolog for the antiviral cytokine human colony stimulating factor 1 (CSF-1) [284-287]. This is mainly associated with its ability to prevent apoptosis and promote cell survival via regulation of anti-apoptotic proteins such as Bcl-2 [288].

## 1.10 microRNAs

### 1.10.1 Discovery and biogenesis of miRNAs

MicroRNAs (miRNAs) are small, 19-25nt nucleotides (nt) in length, evolutionarily conserved non-coding RNAs that act posttranscriptionally to regulate gene expression. The first miRNA was described in C. elegans by Lee and colleagues in 1993 [289]. In a search for genes involved in larva development, they found that a small untranslated RNA encoded by a gene termed lin-4 is able to substantially repress lin-14 mRNA, a heterochronic gene that encodes a transcription factor essential for the completion of the L1 embryonic stage. This repression results from the partially complementary RNA-RNA interaction between lin-4 and the 3' untranslated region (UTR) region of lin-14 mRNA [289, 290]. Although these findings presented the first model of endogenous small RNA regulation in gene expression, they did not receive considerable attention in that no homologous miRNA was found in other organisms. It was not until seven years later that the second miRNA in C. elegans, let-7, was identified to negatively regulate the expression of LIN-41, a protein required for the larval stage L4 [291, 292]. Similar to lin-4, let-7 affects protein translation by base-pairing to the 3'UTR of lin-41 mRNA. Given their roles in controlling development in a temporal fashion, they were initially referred to as small temporal RNAs (stRNAs). Unlike lin-4, let-7 homologues were found to exist in a variety of species including Drosophila, humans and others, probably spanning more than 400 million years of evolution [293]. Several labs around the same time using cloning technology identified 20 additional miRNAs in Drosophila, 60 miRNAs in C. elegans and the first human miRNAs [294-296]. In addition, longer RNA species that contain stem-loop like structures were also detected but they turned out to be the

precursors for miRNAs [289, 291, 293, 296, 297]. miRNAs were annotated for the first time in 2001 [296] and these results indicated that miRNAs are a class of evolutionarily conserved molecules widely distributed in a broad range of organisms including plants, mammals, and worms [298, 299]. Today more than 24500 mature miRNAs from a variety of species are annotated at miRBase (version 20, January 2014), a database of miRNA sequences and nomenclature and 27000 publications are listed in PubMed on miRNAs, reflecting the rapid growth of this field of research.

Approximately 50% of all miRNAs are located within the introns of protein-coding genes or in long non-coding RNA transcripts. These miRNAs are often in the same orientation as the mRNA thus under control of the same promoter region and are coexpressed with their host genes [300, 301]. Some miRNAs are located proximal to each other and are arranged in clusters, however the majority are independent and expressed from their own promoters [300, 302, 303]. The current stage of our knowledge concerning miRNA biogenesis is well described in several reviews [304-307]. Briefly, in animals most miRNAs are initially transcribed by RNA polymerase II into long transcripts (pri-miRNAs) from hundreds to thousands of nucleotides in length (Figure 4). These transcripts are normally capped at the 5' end and polyadenylated at the 3' end, followed by folding into a characteristic hairpin secondary structure. Note that a few miRNAs can be transcribed by RNA polymerase III [308]. pri-miRNAs are processed in the nucleus into precursor miRNAs (pre-miRNAs) around 60-70 nucleotides long. This processing is achieved by the heterotetramer microprocessor complex which contains a Ribonuclease III (RNase III) enzyme Drosha and an essential RNA binding protein DiGerorge syndrome critical region 8 (DGCR8). While DGCR8 functions as a molecular

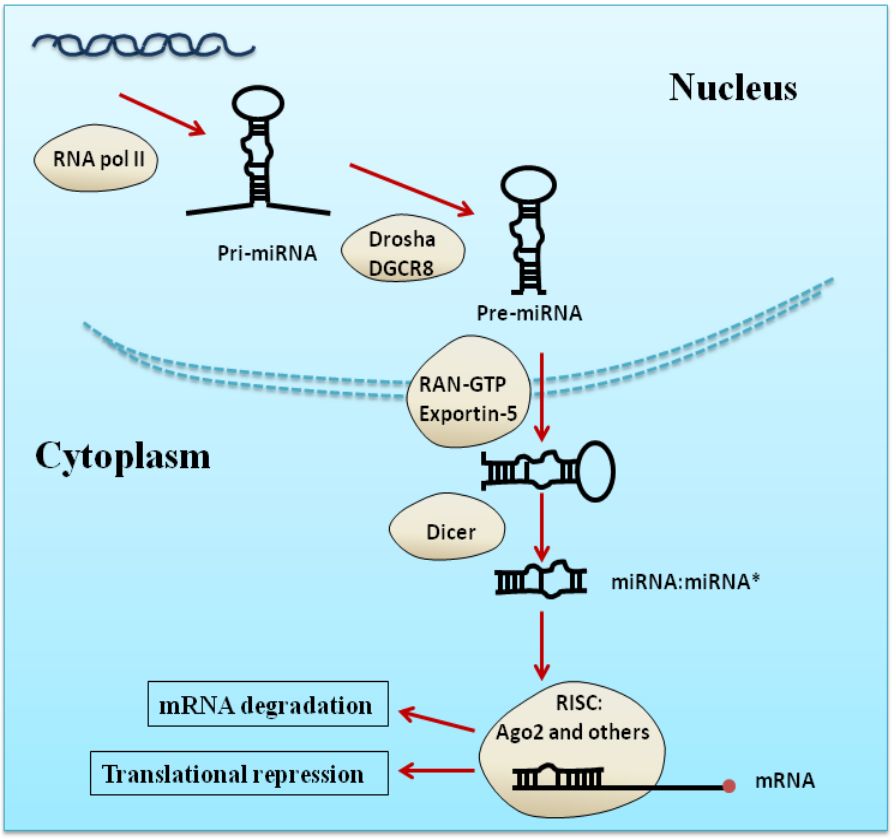



**Figure 4. Biogenesis of miRNAs.** The miRNA gene is first transcribed by RNA polymerase II (RNA pol II) into a stem-loop primary miRNA (pri-miRNA), which is further processed to a pre-miRNA by Drosha and DGCR8. The pre-miRNA is then exported to the cytoplasm by RAN-GTP and Exportin-5, where it is subsequently cleaved by the Dicer enzyme into a shorter RNA dulplex (miRNA: miRNA*). The single stranded miRNA is then incorporated into the RISC complex (Ago2 and TRBP) and interacts with the target mRNA, leading to mRNA degradation and translational repression. This figure is a modified version of figures shown in the literature [309-311].

anchor recognizing the flanking sequences in the stem-loops of the pri-miRNAs, Drosha mediates the cleavage [312, 313]. Pre-miRNAs are subsequently exported from the nucleus to the cytoplasm by binding to the nuclear pore complex, which consists of transport receptor Exportin-5 and Ran-GTP. Exportin-5 recognizes dsRNA hairpins with stem loop structure [314]. Once in the cytoplasm, Ran-GTP is hydrolyzed to Ran-GDP and the pre-miRNAs are released. pre-miRNAs are then bound and cleaved by a second RNase III enzyme Dicer into 18-25 nt double-stranded RNA duplexes. Dicer in combination with TRBP cleaves the pre-miRNAs at the base of the stem loop leading to approximately 22 nt imperfect miRNA:miRNA* duplexes. The duplexes are unstable and one strand will become the mature miRNA. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) consisting of Argonaute 2 (Ago2) and TRBP protein. In humans, there are four Argonaute proteins, but only Ago1 and Ago2 are most commonly associated with miRNAs and functions in the RNAi pathway [315, 316]. Ago2 is unique among these proteins as it has an RNAse H-like domain, which mediates endonucleolytic cleavage. The RISC complexes finally travel and bind to mRNA transcripts via base-pairing interactions. In animals, the binding of miRNAs and target sites is commonly based on partial complementarity but requires perfect base-pairing in the seed region (base 2-8 from the 5' end of mature miRNA) [305]. It is believed that the target sites for miRNAs are most frequently located at the 3' UTR and miRNA binding can lead to either mRNA degradation or translation repression. It is also generally accepted that miRNAs could regulate hundreds of target mRNAs or single mRNA transcript could be targeted by hundreds of miRNAs. However, there are also many reports of exceptions where miRNAs do not obey the common rules in target recognition

and gene regulation [317]. Since their discovery, miRNAs have been shown to play an extensive role in almost all biological processes in animals and plants, including cell cycle, differentiation, development, immunity, metabolism and cancer.

### 1.10.2 EBV miRNAs

Viral miRNAs have been mostly found in DNA viruses which can replicate their genomes in the nucleus. Since Drosha processing takes place in the nucleus, viruses that replicate exclusively in the cytoplasm including RNA viruses and some DNA viruses i.e. poxviruses do not have access to the Drosha complex [318]. EBV was the first virus identified to encode miRNAs. In 2004, Tuschl and colleagues reported the cloning of 5 EBV miRNAs from a BL cell line latently infected with the B95.8 strain of EBV, opening up a new research area in virology [319]. Two years later, 22 additional mature EBV miRNAs were identified in a BL cell line Jijoye using a combined approach of computational prediction of pre-miRNA hairpins and microarray analysis [320]. About the same time, some other novel miRNAs were cloned and sequenced in BC-1 cells, a lymphoma cell line infected with a wild-type strain of EBV [321]. 3 more new mature miRNAs were subsequently detected in the EBV-positive NPC biopsies [322].

To date, more than 40 mature EBV miRNAs have been identified by multiple approaches [319-324]. These miRNAs are located in two major regions within the EBV genome: the BART gene (from mir-BART1 to mir-BART22) and the BamHI fragment H rightward open reading frame 1 (BHRF1) gene (from mir-BHRF1-1 to mir-BHRF1-3). The BART gene expresses multi-spliced transcripts in latently infected cells, NPC tumors, and primary infected B cells [325-331]. The BHRF1 gene is expressed at high levels in lytic infection and it encodes a Bcl-2 homolog [332, 333]. BHRF1 miRNAs are

located in the ORF and 3' UTR of the transcript. miRNAs from the BART region can be further divided into two clusters, cluster 1 and cluster 2, which are located at two separate introns of the locus (Figure 5). The BARTs miRNAs are believed to be expressed prior to splicing of the BART transcript [334]. The abundance of all BART miRNAs has been linked to the transcription of the BART RNA, as miRNA clusters are thought to be transcribed together [334, 335]. In addition, the diversity of splicing pattern may account for the different expression levels of miRNAs as different sized BART RNAs have been detected [324, 334, 336, 337] . It is believed that Cluster 1 lies between BART exon I and IB, and expresses 8 precursor miRNAs (BART1, 3 to 6, and 15 to 17). Cluster 2 is located between exon II and III, and gives rise to 13 precursors (BART 7 to 14, and 18 to 22) . The BART2 miRNA precursor is located between exon IV and V. The prototype B95-8 EBV strain has a 12-kb deletion that spans the BART sequences, leaving only 5 of the BART miRNAs in the genome. EBV lacking the BART region is sufficient to efficiently immortalize B cells, suggesting that the BART miRNAs may not be required for transformation [338, 339].

BART and BHRF miRNAs are differentially expressed in cell lines, LCLs and EBV-positive tumor biopsies, such as NPCs and GaCas, BLs and AIDS-associated BLs, HDs, diffuse large B-cell lymphomas, and primary effusion lymphomas [340, 341]. BHRF1 miRNAs are found in B cells with latency III when all latency-associated genes are expressed, or in Wp-restricted types of latent infection, but are undetectable in B cells or epithelial cells of type I or II latency [321, 342]. The BART miRNAs are expressed in all forms of EBV latency but found to be more abundant in epithelial cancers or cell lines, such as NPC and GaCa as compared to BL. Interestingly, the induction of lytic

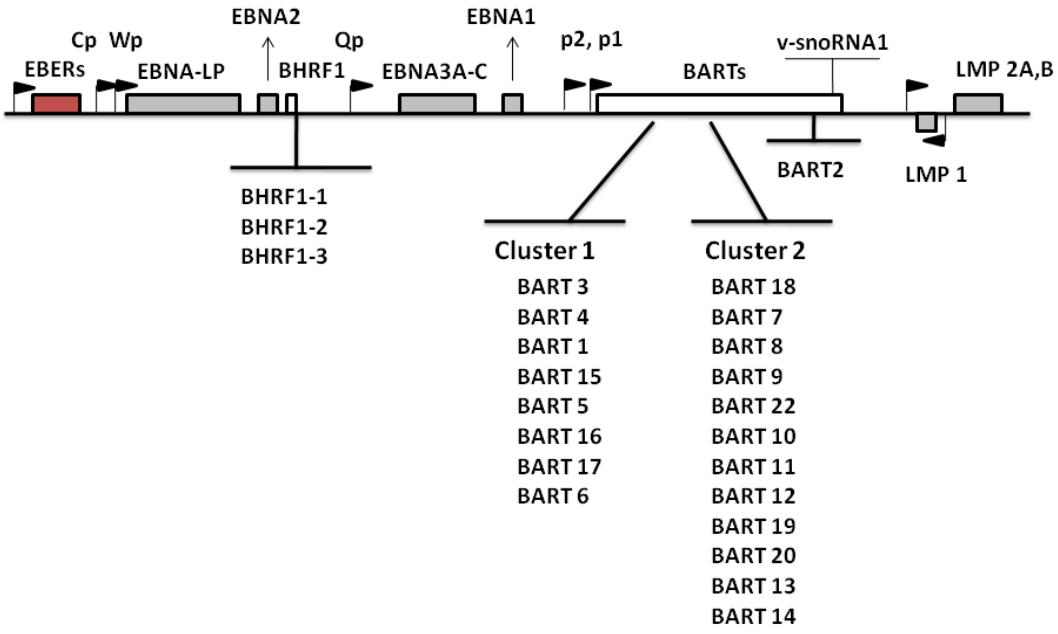



**Figure 5. Schematic representation of the location and ordering of EBV miRNAs within the EBV genome.** EBV miRNAs are derived from two transcripts: BHRF1 and BART (white bars). Precursor miRNAs are listed in the order of genomic location. The location of latent proteins (EBNAs and LMPs –grey boxes), other transcripts found in latency states (EBER – red box) and their promoters (black flags) are also shown.

replication in latently-infected BL cell lines increases the expression of many viral miRNAs [321, 342, 343]. However, this contradicts a report that there is no increased expression of EBV BART miRNAs upon lytic induction [344]. EBV miRNAs are also expressed as early as 2 days after de novo infection of primary B cells with either the B95-8 or the Akata strain of EBV, suggesting a role in the establishment of latency [344].

The function of most EBV miRNAs is largely unknown, but it is suggested that EBV miRNAs may promote viral latency or cancer development by targeting both viral and cellular genes. BART 2 suppresses reactivation from latency by repressing the viral DNA polymerase BALF5 [345]. BART 2-5p also targets the 3'UTR of MHC class I-related chain B (MICB) gene, a stress-induced ligand recognized by NKG2D receptors. The down regulation of MICB allows escape from recognition and consequent elimination by Natural Killer cells [346]. BART 1-5p, -BART 16 and –BART 17-5p target the 3'UTR of the LMP1 gene, supporting immune evasion as LMP1 is immunogenic [347]. In addition, down regulation of LMP1 also facilitates the development of NPC, as it decreases the sensitivity of NPC to the drug cisplatin [347]. BHRF1-3 regulates the expression of the IFN-inducible T-cell attracting chemokine (CXCL-11/I-TAC) by binding to its 3'UTR. CXCL-11/I-TAC has been shown to have a potent antitumor activity, thus down regulation of this protein by EBV miRNAs might help avoid cancer immunosurveillance [348]. Several in vitro studies have suggested that EBV miRNAs also contribute to oncogenesis. BL-derived cell lines with constitutive expression of BART miRNAs are able to inhibit apoptosis induced by the loss of EBV in vitro, implicating a survival role for BART miRNAs in BLs [349]. BART 5 represses the p53 up-regulated modulator of apoptosis (PUMA), thereby protecting EBV-infected cells

from virally-induced apoptosis. High expression of BART 5 also correlates with the low abundance of PUMA in NPC tissues [350] . BART Cluster 1 miRNAs are able to suppress the Bcl-2 interacting mediator of cell death (Bim), thus preventing cells from apoptosis [351]. However, until now no data in support of a role of EBV miRNAs in tumorgenesis in vivo has been presented.

## Section II:

# A NOVEL PERSISTENCE ASSOCIATED EBV MIRNA EXPRESSION PROFILE IS DISRUPTED IN NEOPLASIA

A version of this chapter was published as follows:

Qiu, J., et al., A novel persistence associated EBV miRNA expression profile is disrupted in neoplasia. PLoS pathogens, 2011. 7(8): p. e1002193

## 2.1 Premise and Rationale

Numerous studies have focused on analyzing and interpreting the function of EBV latent proteins in order to better understand their contribution to tumorigenesis. Recently, attention has been directed toward EBV miRNAs that are expressed in latently infected cells. As described above, more than 40 mature EBV miRNAs derived from 25 precursors have been found. BHRF1 miRNAs were reported to be highly expressed in LCL (Latency III), whereas BART miRNAs have been found in all EBV-infected cell lines tested including LCL, BL and NPC and tumor biopsies from NPC, GaCa and DLBCL [319, 344, 352-356]. However a comprehensive comparative accounting is lacking since most studies only examined a limited repertoire of miRNAs (frequently with non- or semi quantitative techniques), a limited range of tissues was studied (frequently employing cell lines instead of fresh infected tissue) and appropriate computational methods for data mining were not employed. Using quantitative multiplex RT-PCR with specific 6'FAM-probes and primers for each miRNA, previous lab member has reported previously an EBV miRNA profile for NPC tissues [340] however a comparable profile does not currently exist for the other EBV associated tumors including BL, HD and GaCa. Therefore it is unknown if there is tumor specific variation in the patterns of EBV miRNA expression. A number of investigations have previously focused on the profiling of human cellular miRNAs in B-cell subpopulations and B cell associated lymphomas (for example, see [357-362]). However, nothing is known about the expression of EBV miRNAs in normal infected B cells in vivo and consequently it is unknown if there are specific changes in their expression associated with tumorigenesis.

Several groups have identified potential functions or targets for EBV miRNAs as described in the general introduction. There are conflicting reports about the possible role of the BARTs. They are suggested to regulate both viral [323, 363, 364] and cellular proteins associated with apoptosis, survival and immune evasion [365-367]. However, they are dispensable for infection and immortalization of B cells in vitro [338] and their absence had no reported effect on susceptibility to apoptosis of infected B cells [368]. This raises the possibility that the BART miRNAs may have an important role to play during normal infection of B cells in vivo that is not required in vitro. This parallels the behavior of LMP2 for example which is believed to play an important signaling and survival role in vivo as a B cell receptor surrogate but is completely dispensable for immortalization in vitro [83, 369].

Therefore there is an immediate need to characterize and understand the expression profiles of EBV miRNAs in normal infected B cell populations and tumors in vivo. In the present study, I aimed to quantitatively assess and computationally analyze the miRNA expression profiles in EBV-associated tumor biopsies (NPC, GaCa BL, HD) and EBV-infected LCL, GCB and MemB cells from normal populations. The goal was to discover if subsets of miRNAs are associated with specific latency programs and if these expression profiles are disrupted during tumor development. I present the first demonstration of deregulation of EBV miRNA expression associated with tumorigenesis. Specifically, I identify a subset of BART miRNAs that are restricted to Latency III in normal infection but are up regulated in tumors that express Latency I and II.

## 2.2 Material and Methods

### Cell lines and culture

The EBV-positive lymphoblastoid cell line (LCL) IB4 (gift of Dr. Elliot Kieff) and the murine CB59 T cell line (gift of Dr. Miguel Stadecker) were used respectively as the positive and negative control for EBV. For miRNA profiling, six spontaneously EBV-infected B LCLs (gift of Dr. Alan Rickinson) with different EBV strains (type 1 or type 2), five EBV-positive Burkitt's lymphoma (BL) cell lines Rael (gift of Dr. Sam Speck), Jijoye, BL36, Raji and Akata 2A8.1 (gift of Dr. Jeff Sample), the gastric carcinoma (GaCa) line AGS/BX1 (gift of Dr Lindsey Hutt-Fletcher), the nasopharyngeal carcinoma (NPC) line C666-1 and the Hodgkin's disease (HD)-derived cell line L591 (gift of Dr. Paul Murray) were included in this study (Table 1). The EBV-negative BL lines Akata, BJAB, DG75, BL2 and BL31, the NPC line HONE-1 (gift of Dr. Ronald Glaser), and the GaCa line AGS were used as the negative controls. The GaCa cell lines were grown in Ham's F-12 medium containing 10% fetal bovine serum (FBS), 2 mM sodium pyruvate, 2 mM glutamine, and 100 IU of penicillin-streptomycin. All other cell lines were maintained in RPMI 1640 medium with the same supplements.

### Ethics Statement - Clinical biopsies and primary cells

The research described herein was approved by the Tufts University Institutional Review Board and our collaborating institutions. Peripheral blood mononuclear cells (PBMCs) of whole blood samples were provided by the University of Massachusetts at Amherst Student Health Service as previously described. Adolescents (ages 17 to 24 years) presenting to the clinic at the University of Massachusetts at Amherst Student

Health Service (Amherst) with clinical symptoms consistent with AIM were recruited for this study. Blood was collected following the obtainment of written informed consent. These studies were approved by the Human Studies Committee at the University of Massachusetts Medical School (Worcester).

Tonsils were collected from patients 18 years of age or younger receiving routine tonsillectomies at the Tufts Medical Center at Boston, MA. Informed consent was not obtained since this was deidentified, discarded material and was deemed exempt by the Tufts University Institutional Review Board.

Tumor biopsy samples for this study were obtained from the archives of the Vrije Universiteit, Amsterdam medical center. Consent was not obtained because I used left over archival material from earlier studies (listed below). This was approved by the Medical Ethical Committee of the VU University medical center, Amsterdam, The Netherlands according to the code for proper secondary use of human tissue of the Dutch Federation of Biomedical Scientific Societies (`http://www.federa.org`). BL samples were collected in Malawi from 1996-1998 under study nr. IC19-CT96-0132. HD samples (all of nodular sclerosing subtype) were collected in Amsterdam from 1994-2002 under studies nr. KWF-VU1994-749 and 2001-2511 GaCa samples were collected in Amsterdam from 1999-2004 under study nr. KWF1999-1990. NPC samples were collected in Indonesia during 2001-2005 in studies KWF-IN2000-02/03. A single case of HD was obtained from the Children's Hospital, Birmingham, UK, with permission from the Childhood Cancer and Leukaemia Group of the United Kingdom. This sample was used in accordance with Trent Research Ethics Committee reference number 05/MRE04/.

Written consent was obtained and the sample taken under ethical approval obtained from South Birmingham Research Ethics Committee.

EBV status of tumor biopsies was assessed based on EBER1/2 in situ hybridization using commercial PNA-based hybridization probes (Dakocytomation, Glostrup, Denmark) and immunohistochemical staining for EBNA1 and LMP1 using previously described monoclonal antibodies[370, 371].

Tonsils were cut into small fragments in phosphate-buffered saline with 1% bovine serum albumin (PBSA) with razor blades. Cells were obtained by filtering the fragment suspensions through a 70 μm mesh size cell strainer. Mononuclear cells were isolated from buffy coats using the standard Ficoll-Paque Plus (Fisher Scientific) centrifugation method and saved for analysis.

**Cell staining and flow cytometry**

Germinal center B (GCB) and memory B (MemB) cell populations were purified by fluorescence-activated cell sorting (FACS). Surface staining for FACS analysis was performed by standard procedures. Monoclonal antibodies against specific cell surface markers including allophycocyanin (APC)-labeled anti-Cluster of Differentiation (CD)19, phycoerythrin (PE)-anti-CD10, and fluorescein isothiocyanate (FITC)-anti-CD27 were used. GCB cells (CD19+CD10+) and MemB cells (CD19+CD27+) were sorted from tonsil and blood PBMCs (Figure 6), respectively as previously reported [372, 373]. For miRNA studies, 5x106 to 107 GCB cells were sorted for RNA isolation. Since the blood samples were small and MemB cells (CD19+CD27+) only account for 0.5-2 % of PBMCs, I sorted all the memory B cells into $10^6$ CB59 cells prior to RNA isolation. EBV

miRNA profiling of CB59 cells indicated that it is appropriate to use them as filler cells since they do not generate detectable signals for any EBV miRNAs (data not shown).

**Limiting dilution and DNA real-time PCR**

Determination of the EBV frequency of infected cells in purified GCB and MemB cells was done by limiting dilution and DNA real-time PCR [374]. Briefly, FACS-gated cells were sorted onto a 96-well plate with 10 replicates each of serially diluted cells. Genomic DNA was isolated by Proteinase K digestion, followed by real time Taqman DNA PCR specific for the W-repeat genome of EBV. The fraction of EBV-negative wells was calculated and the frequency of infected cells was estimated using the Poisson distribution.

**RNA extraction and multiplexed stem-loop RT-PCR**

Total RNA was extracted from frozen sections or sorted cell populations with Trizol (Invitrogen). The quantity and integrity of the RNA was assessed by nanodrop and by the Agilent Bioanalyzer. EBV miRNA expression was assessed by real-time multiplex reverse transcript (RT)-PCR [355]. Multiple stem-loop RT primers specific for the 3' end of each mature miRNA were mixed and applied to RT reaction, followed by Taqman PCR using primers and probes specifically assigned to each miRNA. Synthetic oligonucleotides representing all of the miRNAs were employed to generate the standard curves. The small cellular nuclear RNA U6 was used as the internal control for normalization. EBV-negative materials were used for negative controls. Profiles were performed in duplicate and repeated three times. Since, in our to-be-profiled sample pool the frequency of infected cells in $5x10^6$ to $10^7$ GCB ranged between 6-130 per $10^5$ GCB,

most of the RNA was derived from cellular rather than viral origin. To test if this compromised any of the PCR assays I profiled $5x10^6$ and $10^7$ EBV negative cells. Of the 38 miRNAs tested only four (BARTs 9, 12, 16 and 19-3p) gave detectable and significant signals with EBV negative cells so these were excluded from further analysis involving these cell types.

**Computational analysis**

Please refer to [375].

**Note**

Some data presented in this section have been directly contributed by other people in the lab. In order to retain the entireness of the story, I integrate their data in the following result section. Of note, Dr. Kat Cosmopoulos performed miRNA profiling on tumor biopsies and Dr. Michael Shapiro conducted the bioinformatic data analysis.

## 2.3 Results

### EBV miRNA profiling of normal and neoplastic tissues

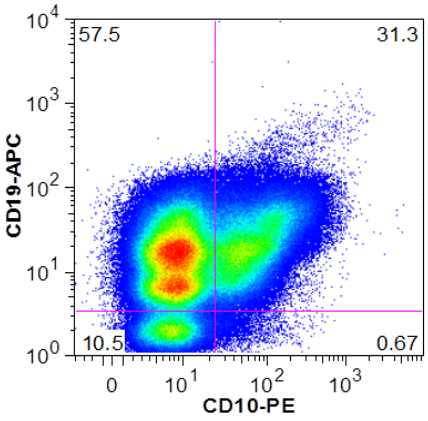
Our laboratory previously described a technique that allows the profiling of EBV miRNAs in small amounts of EBV positive tissue [355]. I and Dr. Cosmopoulos have now used this technique to profile EBV miRNA expression in primary infected normal tissues and tumor biopsies. The tissues tested are listed in Table 1. For the normal tissues, I profiled latently infected germinal center B cells (GCB, Latency II) from the tonsils (n=5) and latently infected memory B cells (MemB, Latency I/0) from the peripheral blood (n=4) all derived from normal, persistently infected individuals. These cell populations were isolated by staining and FACS. While GCBs are CD19 and CD10 double positive, MemBs are CD19 and CD27 double positive (Figure 6). For the tumors, Dr. Kat profiled four types of primary biopsies including Burkitt's lymphoma (BL: n=6, Latency I), gastric carcinoma (GaCa: n=6, Latency II), nasopharyngeal carcinoma (NPC: n=5, Latency II) and Hodgkin's disease (HD: n=3 Latency II) (Figure 7).

| Name | Comment |
|---|---|
| **Peripheral blood MemB cells (CD27+, CD20+)** | |
| MemB1 | # of infected cells tested $=3.8\times10^3$ |
| MemB2 | # of infected cells tested $=2.3\times10^3$ |
| MemB3 | # of infected cells tested $=1.3\times10^3$ |
| MemB4 | # of infected cells tested $=3.0\times10^3$ |
| **Tonsil GCB cells (CD10+, CD20+)** | |
| GCB1 | # of infected cells tested $=6.0\times10^2$ |
| GCB2 | # of infected cells tested $=1.0\times10^3$ |
| GCB3 | # of infected cells tested $=6.5\times10^3$ |
| GCB4 | # of infected cells tested $=4.0\times10^3$ |
| GCB5 | # of infected cells tested $=3.8\times10^3$ |
| **Spontaneous LCL** | |
| LCL1 | A.M from A. Rickinson |
| LCL2 | Angu from A. Rickinson |
| LCL3 | IM43 from A. Rickinson |
| LCL4 | IM86 from A. Rickinson |
| LCL5 | Salina from A. Rickinson |
| LCL6 | IM82 from A. Rickinson |
| **Tumor Biopsies** | |
| HD | Hodgkin's disease |
| NPC | Nasopharngeal carcinoma |
| GaCa | Gastric Carcinoma |
| BL | Burkitt's lymphoma |
| **Tumor Cell Line** | |
| L591 | Origin: Hodgkin's disease |
| C666-1 | Origin: Nasopharngeal carcinoma |
| AGS-BX1 | Origin: Gastric Carcinoma |
| Jijoye | Origin: Burkitt's lymphoma |
| Rael | " |
| BL36 | " |
| Akata 2A8.1 | " |
| Raji | " |

doi:10.1371/journal.ppat.1002193.t001

**Table 1. Tissues and cell lines used in Section 1.**

A




B

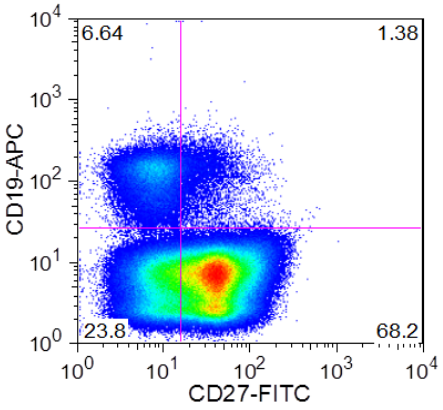



**Figure 6. Separation of GCB and MemB via staining and FACS.**

GCB cells and MemB cells from tonsil and blood PBMCs were stained with antibodies against specific cell surface markers: CD19 & CD10 for GC B cells; CD19 & CD27 for MemB cells. A. GCB cells (CD19+CD10+), and B. MemB cells (CD19+CD27+) were sorted via FACS.

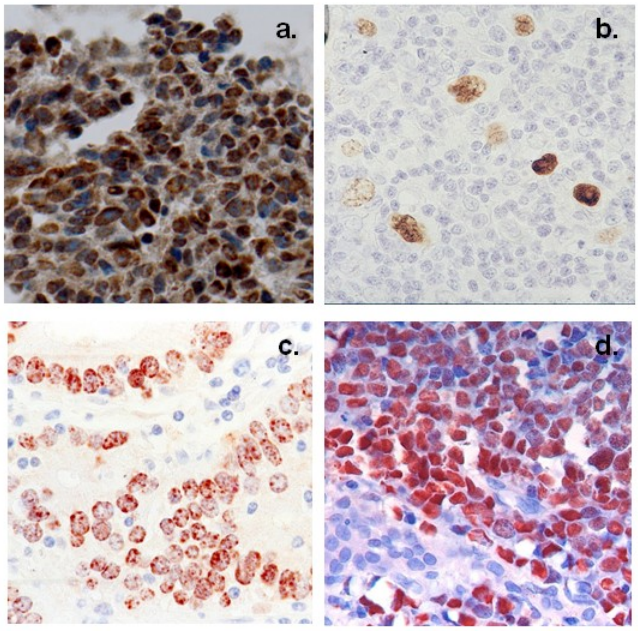



**Figure 7. Histological cross sections of typical tumor biopsies stained for expression of EBV genes.**

Biopsies and slides are from Dr. Jaap Middledrop. Note that with the exception of HD less than half of the cells are non-tumor infiltrating lymphocytes. A. Nasopharyngeal carcinoma stained for the EBV nuclear antigen EBNA1. B. Hodgkin's disease stained for EBNA1 Note the sparse appearance of the tumor cells compared to the other tumor types. C. Gastric carcinoma stained for EBNA1. D. Burkitt's lymphoma stained for the EBV encoded small RNAs EBER.

For the tumor biopsies, I could either normalize the results to the cellular small RNA U6 or by fraction of total viral miRNAs. However, normalization to U6 was not meaningful for the GCB and MemB cells because the fraction of infected cells in these samples was both very low and variable. Therefore to compare tumor biopsies with normal infected tissue, I expressed each miRNA as a fraction of total EBV miRNAs. The results for expression of the BART miRNAs are shown in Figure 8. There were two striking findings. The first was that despite the disparate tissue origins of the biopsies and the viral latency programs they represent, the overall profiles for all four tumor types were similar (Figure 8A). Second the profiles for the two normal infected tissues (GCB and MemB) were markedly different from the tumors but similar to each other (Figure 8B), despite again originating from different tissues and employing different latency program. Therefore the similarity of the profiles was determined by whether or not the tissue of origin was neoplastic not on the latency program or the tissue of origin. The most striking difference was the absence of 11 BART miRNAs from the normal tissues that are highly expressed in the tumors. These included a large subset of the Cluster 2 BART miRNAs. Of the 18 Cluster 2 miRNAs tested, all were present in the tumor biopsies but only 8/18 (44%) were found in the GCB and MemB samples. By comparison, all 10 Cluster 1 BART miRNAs were present in the tumor biopsies but only 1 was absent from the GCB and MemB samples.

I conclude that EBV associated tumors expressing Latency I and II up regulate a subset of BART miRNAs that are silenced in their normal infected counterparts in vivo.

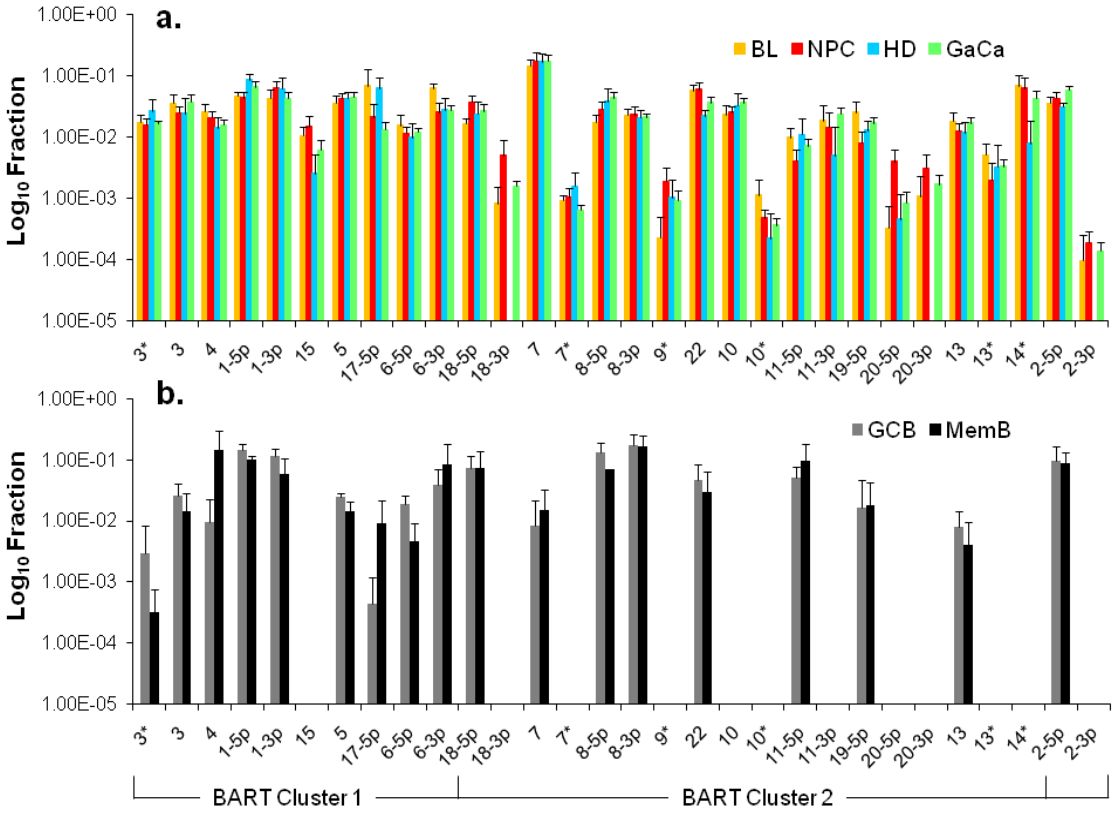



**Figure 8. Profile of EBV BART miRNAs in normal and neoplastic tissue.**

The data is expressed as average and standard deviation of the fraction that each miRNA constitutes of the total of all EBV miRNAs. A. The four tumor types tested. Blue - Hodgkin's disease (n=3): yellow - Burkitt's lymphoma (n=6): green - gastric carcinoma (n=6): red - nasopharyngeal carcinoma (n=6). B. Normal infected tissue. Grey – GCB (n=5), black – MemB (n=4). The y intercept is a conservative approximation to the sensitivity of the assay (this will vary according to the input number of infected cells). It represents the fraction for a miRNA present at 10 copies, the lower limit of sensitivity for detection of all the miRNAs [355]. The miRNAs are presented on the x-axis from left to right in the order that they appear in the viral genome (see Figure 5)

**The Cluster 2 BART miRNAs absent from normal infected tissues are expressed in Latency III**

One possible explanation for the different patterns described above is that the absent 11 BART miRNAs are associated with cellular proliferation and become down regulated when the cells enter a resting state. However, I can exclude this possibility because infected GCB are proliferating [376]. I therefore investigated an alternate hypothesis namely that these miRNAs are specifically expressed only in Latency III (infected lymphoblasts) i.e. with virus driven proliferation, and that their presence in tumors represents aberrant expression. To test this hypothesis I could apply a more quantitative approach since I am able to estimate absolute copy numbers per cell of the miRNAs in all three tissue types namely B cells driven to proliferate by EBV (spontaneous lymphoblastoid cell lines - LCL) derived from infected individuals (Latency III), GCB (Latency II) and MemB (Latency1/0). This was possible for the LCL because they are homogeneous cell lines. For the GCB and MemB samples I could estimate miRNA copy number per cell by first measuring the number of infected cells in the samples to be profiled and then dividing the total copy number of each miRNA by this value. The result for the BART miRNAs is shown as a bar graph in Figure 9 and the actual values are tallied in Table 2. The results for GCB and MemB were again very similar indicating that the profiles were essentially the same both in terms of relative representation (Figure 8B) and absolute copy number (Figure 9B and Table 2) for the BART miRNAs. One notable exception was 17-5p which was almost undetectable in GCB (average 2 copies/cell) but present at a copy number almost 2 logs higher in MemB (average 110 copies/cell). Of the 18 Cluster 2 miRNAs profiled 2 were undetected in all three tissues

(LCL, GCB and MemB). The remaining 16 were all present in LCL. As with the tumors this included the one Cluster 1 and eight Cluster 2 BART miRNAs that were absent from GCB and MemB samples. A second feature to emerge from this comparison is that the BART miRNAs that were detected in GCB and MemB were present at copy numbers that averaged 5-10 fold higher than in LCL.

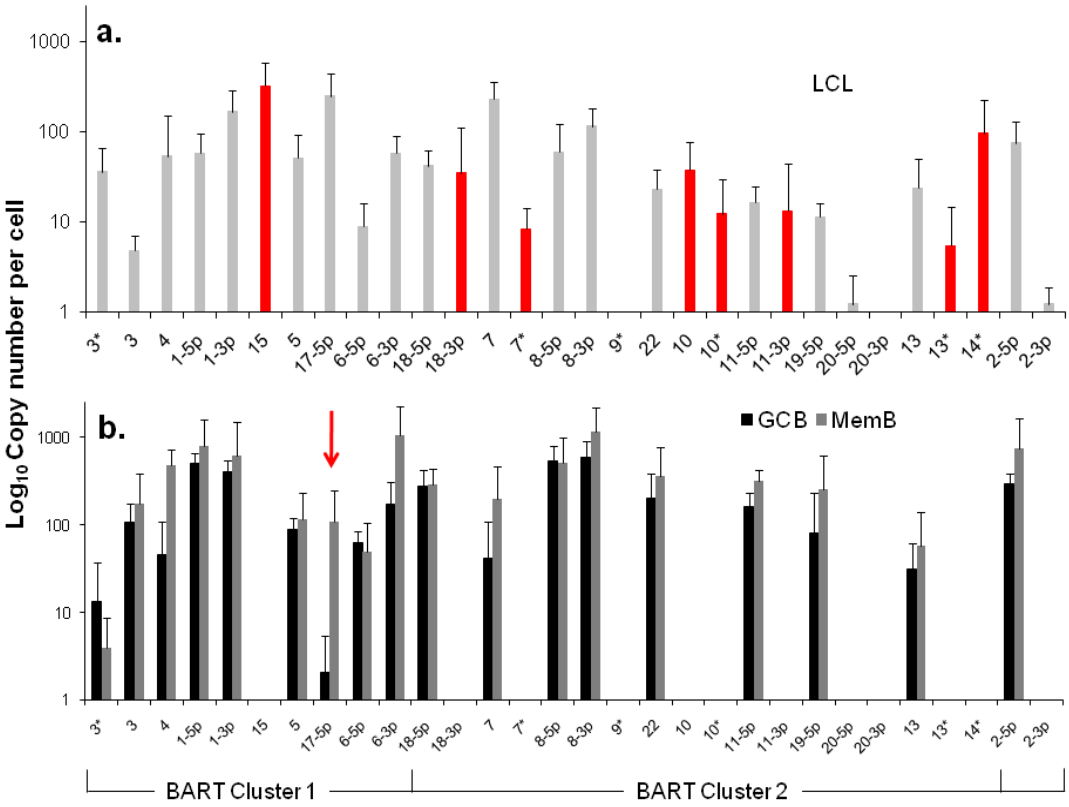



**Figure 9. Profile of EBV BART miRNAs in vivo and in spontaneous lymphoblastoid cell lines (LCL) expressing latency III.**

The data is expressed as copy number of each miRNA per infected cell. A. Spontaneous LCL (n=6). miRNAs that are significantly expressed in LCL but not GCB and MemB are highlighted in red. B. Normal infected tissue. Grey – GCB (n=5), black – MemB

(n=4). This is the same data set as Figure 8A except now expressed as copy number /cell instead of fraction. The y intercept is an approximation to the sensitivity of detection based on the following. The maximum number of infected cells tested for GCB and MemB was 4-6x$10^3$ (Table 1). Approximately 0.1% of the RNA from these samples was used for each PCR i.e. equivalent to ~5 infected cells. Since I can detect as few as 10 copies of each miRNA [355], I conclude that failure to detect a miRNA in any sample indicates it is present at a copy number ≤1/cell. The red arrow indicates miRNA 17-5p which was the only miRNA to show a striking differential expression between GCB and MemB cells.

| miRNA§ | LCL n = 6 | GCB n = 5 | MemB n = 4 | |
|---|---|---|---|---|
| BHRF1-1 | 22 | 64 | 96 | ▼ |
| BHRF1-2 | 34 | 0 | 0 | ▼ |
| BHRF1-2* | 494 | 0 | 0 | ▼ |
| BHRF1-3 | 8 | 0 | 0 | ▼ |
| 2-5p | 76 | 291 | 755 | |
| 2-3p | 1 | 0 | 0 | ▼ |
| 1-5p | 58 | 502 | 795 | |
| 1-3p | 167 | 399 | 617 | |
| 3* | 36 | 13 | 4 | |
| 3 | 5 | 108 | 176 | |
| 4 | 53 | 46 | 476 | |
| 5 | 51 | 88 | 116 | |
| 6-5p | 9 | 63 | 49 | |
| 6-3p | 58 | 170 | 1046 | |
| 15 | 327 | 0 | 0 | ▼ |
| 17-5p | 250 | 2 | 110 | |
| 7 | 230 | 41 | 201 | |
| 7* | 8 | 0 | 0 | ▼ |
| 8-5p | 60 | 532 | 515 | |
| 8-3p | 116 | 598 | 1166 | |
| 9* | 0 | 0 | 0 | |
| 10 | 38 | 0 | 0 | ▼ |
| 10* | 12 | 0 | 0 | ▼ |
| 11-5p | 16 | 164 | 316 | |
| 11-3p | 13 | 0 | 0 | ▼ |
| 13 | 24 | 31 | 57 | |
| 13* | 5 | 0 | 0 | ▼ |
| 14* | 96 | 0 | 0 | ▼ |
| 18-5p | 42 | 279 | 294 | |
| 18-3p | 35 | 0 | 0 | ▼ |
| 19-5p | 11 | 80 | 255 | |
| 20-5p | 1 | 0 | 0 | ▼ |
| 20-3p | 0 | 0 | 0 | |
| 22 | 23 | 203 | 358 | |

**Table 2 Copy number per cell of EBV miRNAs in normal infected tissues and spontaneous lymphoblastoid cell lines.**

§ - The miRNAs are divided into groups based on the annotated location on the genome. Starting from the top BHRF1, BART 2, BART Cluster 1, BART Cluster 2. Arrows (▼) denote miRNAs detected in LCL but not (<0.1 copies/cell) the GCB or MemB cells.

I have investigated whether our failure to detect BART miRNAs from in vivo samples was due to lack of sensitivity of the PCR reaction rather than true absence. Of the 12 BART miRNAs that were not detected in GCB and MemB cells 4 were also either absent or present at marginal levels (~1 copy /cell) in the LCL. Therefore the failure to detect these in the GCB and MemB may not be meaningful. However, the remaining 8 absent miRNAs (highlighted in red in Figure 9) were present in LCL at copy numbers ranging between 5 and 327, identical to the range in LCL for the BART miRNAs that were found in GCB and MemB (range 5-250/LCL cell). Therefore the undetected miRNAs were not consistently those present at low copy numbers in the LCL. Furthermore, when BART miRNAs were detected in GCB and MemB cells they were typically detected at higher levels/cell than in the LCL suggesting that I are not under representing or under detecting miRNAs in these samples. I also performed profiles on samples of $10^6$-$10^7$ EBV negative tonsils into which had been spiked various numbers of LCL cells and on GCB and MemB samples with low numbers of infected cells. I determined that I could quantitatively profile most of the miRNAs in samples that contained $\geq$ 1000 infected cells in a population of $10^6$-$10^7$ uninfected cells (data not shown). As the number of infected cells dropped from 1,000 failure of profiling tended to be associated with drop out of most or all of the miRNAs rather than selective gradual disappearance. In particular I did not see preferential drop out of the miRNAs absent from GCB and MemB. This suggests I may be approaching a general rather than miRNA specific threshold for profiling. The exceptions were mirBARTs 9, 12, 16 and 19-3p. The PCR for these miRNAs consistently gave significant signals due to cross reaction with RNA from the large number of uninfected cells and were therefore excluded from all analysis. With the

exception of one GC sample (600 infected cells) all of the GCB and MemB samples assayed in our study contained >1000 infected cells (Table 1).

I conclude that there is a subset of BART miRNAs that reside predominantly in Cluster 2 and are specifically expressed only during Latency III in normal infected B cells. I refer to these as Latency III associated BARTs. Furthermore there is co-ordinate regulation of the BART miRNAs where approximately one third are extinguished and two thirds are up regulated as the cells traverse out of Latency III into Latency I and II.

**Expression level of BART miRNAs in tumors.**

I have established that there is a subset of Latency III associated BART miRNAs that are also expressed in tumors, irrespective of latency type. I wished to investigate therefore whether the absolute level of expression of BART miRNAs in the tumors also matched those in the LCL i.e. lower compared to normal tissue. To gain insight into this I compared levels of BART miRNA expression in LCL and tumor biopsies after normalization to the ubiquitous small cellular RNA U6. The results are summarized in Figure 10.

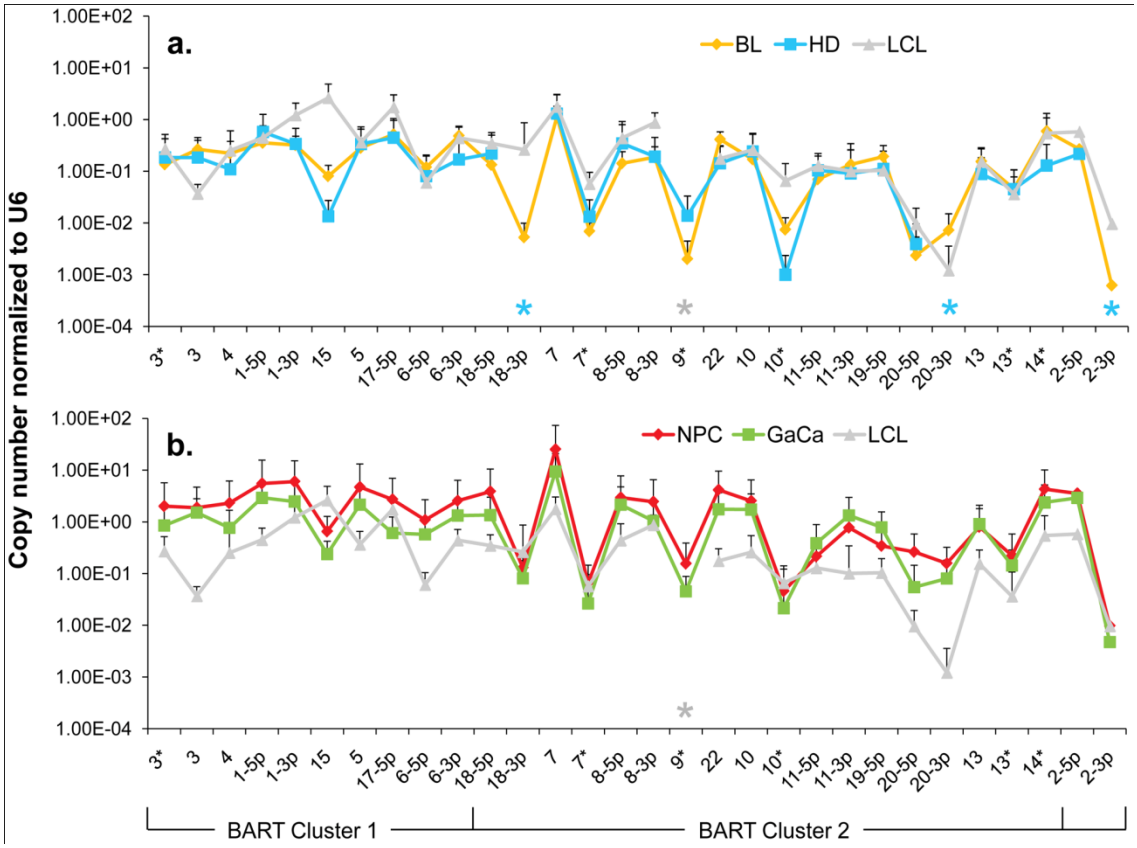



**Figure 10. The profile of EBV BART miRNAs in LCL and neoplastic tissues.**

The average and standard deviation of miRNA expression after normalization to the level of expression of the ubiquitous human small RNA RNU6b is shown. The stars indicate miRNAs that were consistently absent from HD (blue) and LCL (grey) only. A. LCL versus the B cell tumors. Blue - Hodgkin's disease; yellow - Burkitt's lymphoma; grey LCL. B. LCL versus the epithelial tumors. Green - gastric carcinoma; red - nasopharyngeal carcinoma; grey - LCL. This is the same data set for the tumor biopsies as shown in Figure 8.

Overall, the pattern of miRNA expression were similar for all of the tumors (as already shown in Figure 8A when normalized by fraction) and for the LCL. The LCL, HD and BL were closely matched (Figure 10A) both in overall pattern and copy number with the exception that the LCL lacked mirBART 9* and mirBARTs 15 and 18-3p were present at a level more than tenfold higher in the LCL. Also HD lacked mirBARTs 2-3p, 18-3p and 20-3p which were among the group of miRNAs absent from GCB and MemB. However, when LCL were compared with NPC and GaCa (Figure 10B) it was apparent that the epithelial tumors had significantly higher overall expression of the BART miRNAs. When I estimated the average fold increase of the BART miRNAs in the tumors relative to LCL, I found BL 1: NPC 13: HD 0.34: GaCa 8. Note that all of the tumors had some levels of infiltrating non-tumor cells (Figure 7) that would lower the estimates of miRNA expression. However, with the exception of HD, these constituted a small (less than half) fraction of the tumors and would not significantly affect the estimates. Therefore, the much lower levels of BART miRNAs in HD can be explained at least in part from the low abundance of tumor cells in these biopsies. Overall though it appears that the levels of BART miRNAs in the B cell tumors are comparable to those in the LCL but 5-10 fold lower than the epithelial tumors.

I conclude therefore that the BART miRNAs are expressed in all four tumors and that this represents deregulated expression of the Latency III associated BARTs. HD might represent an intermediary state where most but not all of the Latency III associated BART miRNAs are expressed.

**Expression of the BHRF1 miRNAs is not deregulated in the tumors.**

Previous studies have demonstrated that the BHRF1 miRNAs are expressed in Latency III where they are reported to play an anti-apoptotic role [352, 377-379]. This result was confirmed in our profiling (see Table 2 and Figure 11). All four BHRF1 miRNAs were readily detected in the LCL with copy numbers per cell ranging from 10-2000 depending on the miRNA and the cell line tested (Table 2 and not shown). However, they were all absent from GCB (Latency II) and only one (BHRF1-1) was found in MemB (Latency0/1).

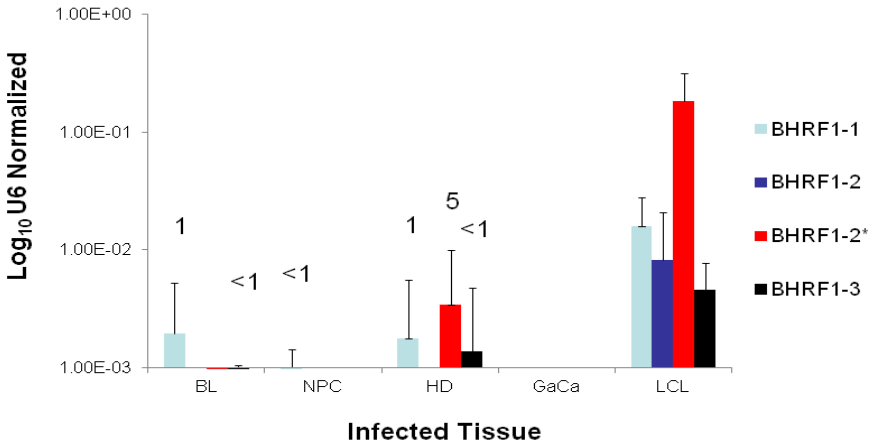



**Figure 11. The profile of EBV BHRF1 miRNAs in LCL and neoplastic tissues.**

The average and standard deviation of miRNA expression after normalization to the level of expression of the ubiquitous human small RNA RNU6b is shown. The miRNAs were undetectable in GaCa. The numbers above the bars indicate estimated copy number per cell based on values from the LCL.

I have demonstrated above that the Latency III associated pattern of BART miRNA expression is deregulated in all four tumor types I have studied. To test if this was also true for the BHRF1 miRNAs, I profiled their expression in all of our tumor samples and the result, compared to LCL, is shown in Figure 11. The BHRF1 miRNAs were not detected at all in GaCa and only sporadically and at low levels in the other tumor biopsies. Using the LCL values as a standard it is possible to estimate that, with the exception of BHRF1-1 in HD which was present in ~ 5 copies, all of the rest were present at ≤ 1 copy per cell. This means that in the tumor samples the levels of all of the BHRF1 miRNAs were at least 10 fold lower in expression than in Latency III (LCL). Thus BHRF1 expression was not consistently deregulated in the tumors.

**EBV miRNA profiles distinguish EBV tumor types.**

I have described a pattern of deregulated BART miRNA expression in EBV associated tumors however at the crude level of our analysis I did not detect tumor type specific miRNA expression. To investigate this more rigorously Dr. Shapiro have performed clustering analysis on our data sets using heat maps and principal component analysis. Figure 12 shows a heat map with clustering dendrograms of EBV miRNA expression for all of the normal and tumor tissue samples I have tested normalized by expressing each miRNA as a fraction of the total EBV miRNAs. The ordering of the samples across the heat map coincided exactly with the three major branches of the miRNA dendrograms which in turn were associated with a functionally distinct group of samples namely normal tissue (GCB and MemB), LCL and the tumor biopsies. This analysis did not distinguish between GCB and MemB cells and the miRNAs responsible for resolving the

GCB+MemB from the LCL (Table 3) coincided with those already identified above. This served as validation for the groupings indicated by the heat map. This was important because the heat map revealed new information namely that the tumors all formed a discrete branch on both the sample and miRNA dendrograms (blue box and Table 3) and that within this group the tumor samples were ordered by tumor type (BL, GaCa, NPC, HD). This was unexpected given the similarity of the profiles as shown in Figures 8A and 10. This result was confirmed when Dr. Cosmopoulos repeated the analysis on a second set of biopsies (not shown). This implies that there are tumor specific patterns of EBV miRNA expression.

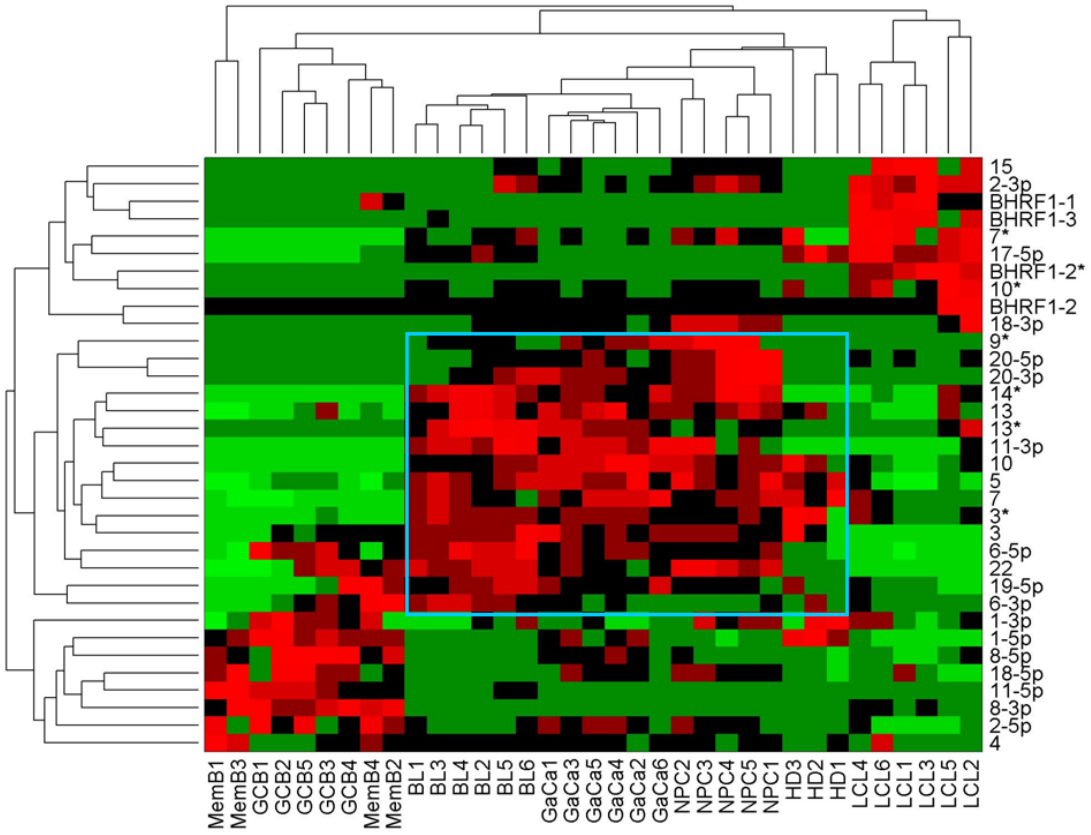



**Figure 12. Heat map of miRNA expression in all tissues tested.**

The data sets used in Figure 8 and 9 were normalized based on the fraction that each miRNA comprised of the total EBV miRNA measured in each sample. A complete description is given in materials and methods. The relative up and down regulation of miRNAs is indicated by red and green respectively. Dendrograms of clustering analysis for samples and miRNAs are displayed on the top and left respectively. Tumors, forming a distinct branch on both the sample and miRNA dendrograms, are boxed in blue.

| Heat map§ | | PCA† | | |
|---|---|---|---|---|
| | Up regulated | PC1 + loading | PC2 + loading | PC2 - loading |
| LCL | BHRF1-1 | | BHRF1-1 | |
| | BHRF1-2 | | BHRF1-2 | |
| | BHRF1-2* | | BHRF1-2* | |
| | BHRF1-3 | | BHRF1-3 | |
| | 2-3p | | 2-3p | |
| | 7* | | 7* | |
| | 10* | | 10* | |
| | 15 | | 15 | |
| | 17-5p | | 17-5p | |
| | 18-3p | | 18-3p | |
| Normal Tissue | 1-3p | | | |
| (MemB +GCB) | 1-5p | | | 1-5p |
| | 2-5p | | | 2-5p |
| | 8-3p | | | 8-3p |
| | 8-5p | | | 8-5p |
| | 11-5p | | | 11-5p |
| | 18-5p | | | 18-5p |
| | | | | 6-5p |
| Tumor biopsies | 3 | 3 | | |
| | 3* | 3* | | |
| | 5 | 5 | | |
| | 6-3p | 6-3p | | |
| | 6-5p | 6-5p | | |
| | 7 | 7 | | |
| | 9* | 9* | | |
| | 10 | 10 | | |
| | 11-3p | 11-3p | | |
| | 13 | 13 | | |
| | 13* | 13* | | |
| | 14* | 14* | | |
| | 19-5p | 19-5p | | |
| | 20-3p | 20-3p | | |
| | 20-5p | 20-5p | | |
| | 22 | 22 | | |
| | | 7* | | |
| | | 18-3p | | |

**Table 3 miRNAs identified by heat map as up regulated in normal infected tissue (GCB and MemB) vs LCL vs tumor biopsies and their counterparts identified by PCA.**

§ - See Figure 11; † - See Figure 12

Curiously though, inspection of the heat maps, failed to identify specific subsets of miRNAs that could account for this resolution. In an attempt to understand the basis for this and possibly identify tumor specific patterns of miRNAs, we analyzed the data by principal component analysis (PCA). Figure 13 shows the result from the same data set used for the heat map in Figure 12. Output for the first three principal components, which account for 55% of all the variation within the normalized data, is shown and confirms that the different tissue types do indeed cluster discreetly. When we examined the contributions of different miRNAs to the first 3 principal components we reproduced the findings from the heat map (Table 3). Of greatest interest was the third principal component which resolved all four tumor types.

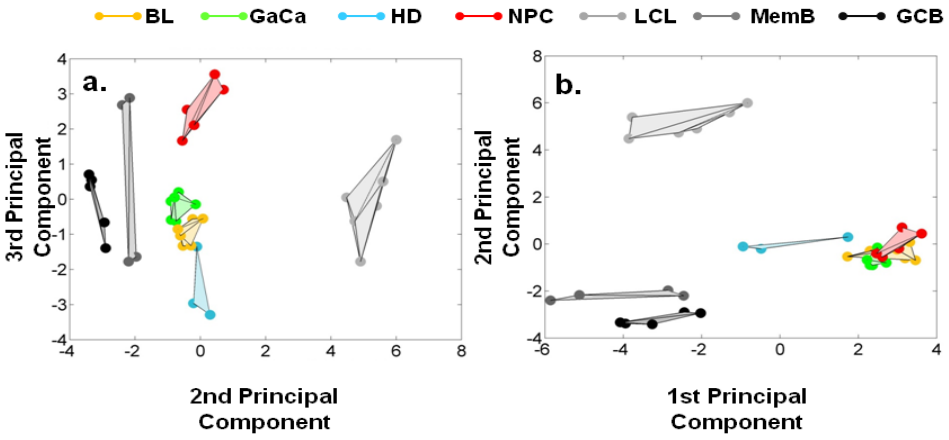



**Figure 13. Principal component analysis (PCA) of miRNA expression in all tissues tested**. PCA was performed on the same data set as shown in Figure 12. A. Second and third principal components. B. First and second principal components.

Resolution of the four tumor types could be shown even more clearly when PCA was performed on the data from the biopsies alone (Figure 14A) and this result was confirmed when we analyzed data from a second completely independent set of biopsies (not shown). We performed two tests of the statistical significance of these results (see Material and methods). Both of these tests showed that successfully separating the four cancer types has a p-value of approximately 0.001.

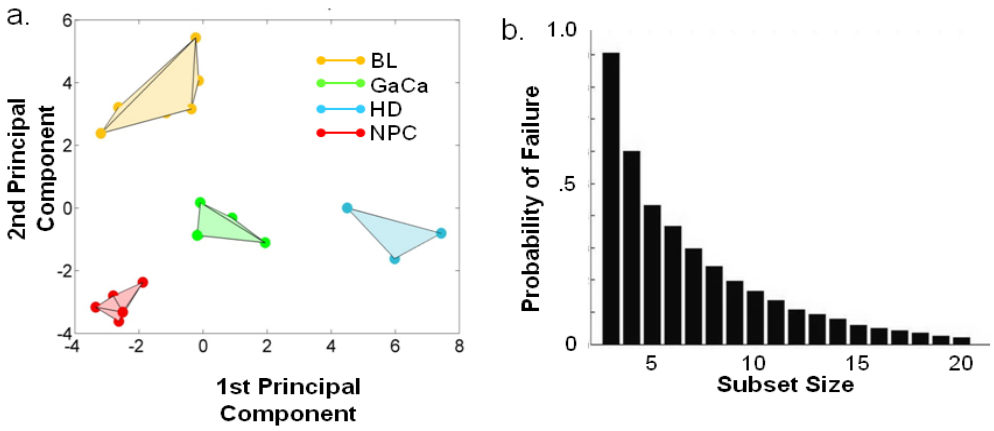



**Figure 14. Principal component analysis of miRNA expression in all tumors tested.**

PCA was performed on the same data set used in Figure 8A. A. The first two principal components resolve the four tumor types. B. Subsets containing as few as 3 miRNAs resolve the tumor types. This figure plots the failure rate of different sized sets of miRNAs. For each set size, 10,000 randomly chosen subsets were tested. N.B. this represents over sampling for the subset n=3

We developed a number of analytical tools to try and extract information about the miRNAs responsible for this effect to no avail. The reason for this became apparent when we attempted to identify which miRNAs were essential and which dispensable for resolving the four tumor types by PCA. To do this we randomly generated subsets of miRNAs and asked if they were capable of resolving all four tumor types by PCA. The surprising result, shown in Figure 14B, was that 10% of such subsets that contained just 3 miRNAs and 60% that contained 5 miRNAs could resolve the tumors. When we then looked at which miRNAs were present in these subsets we found that all of the miRNAs were represented – there was no subset of miRNAs that was uniquely responsible for distinguishing the tumors. Similarly when we asked the question: which miRNAs were dispensable in a given sub-set, i.e. could be removed without affecting resolution of the tumors, we again found that all miRNAs were essential in certain subsets (not shown). This means that the information about the EBV miRNAs which varies between the tumor types and allows their resolution is contained in part by all of the miRNAs such that when combined multiple different subsets of miRNAs contain sufficient information to distinguish the tumors. This phenomenon is reminiscent of a behavior that is well known in computer science referred to a "secret sharing" [380, 381] and represents perhaps a novel and first description of a biological system where information is so distributed across a population. EBV miRNAs cannot be used as the expression signature for the diagnosis of the EBV-positive tumors (for example, to distinguish HD from BL clinically based on EBV miRNA profiles). This is mainly because again in light of PCA above all miRNAs are essential in the subset that distinguishes the tumors therefore no unique EBV miRNA signature is representative of tumors.

**EBV miRNA expression profiles in tumor cell lines are not representative of the tumors.**

We have profiled EBV miRNAs from a number of tumor derived cell lines including 5 BL derived lines and one each of gastric, nasopharyngeal and Hodgkin's origin. When this data was analyzed in a heat map with the data from Figure 12, all of the cell lines clustered with the LCL not with the tumor biopsies they originated from (Figure 15). This was most striking for the BL lines when analyzed by PCA. As shown in Figure 16 the cluster of BL lines completely intersects the cluster of LCL whereas the non BL lines lie just outside. The tendency of the tumor cell lines to drift towards an LCL phenotype was further confirmed when we analyzed expression of the BHRF1 miRNAs ([355] and Table 4). As discussed above these miRNAs are associated with Latency III, the LCL phenotype, and only one is expressed at a significant level in normal infected tissue or tumor biopsies using Latency II or I. As shown in the Table they were all expressed at significant levels in the tumor derived cell lines tested with the exception of the NPC derived C666-1 cells. We conclude therefore that miRNA expression in the cell lines is not fully representative of their tumors of origin.

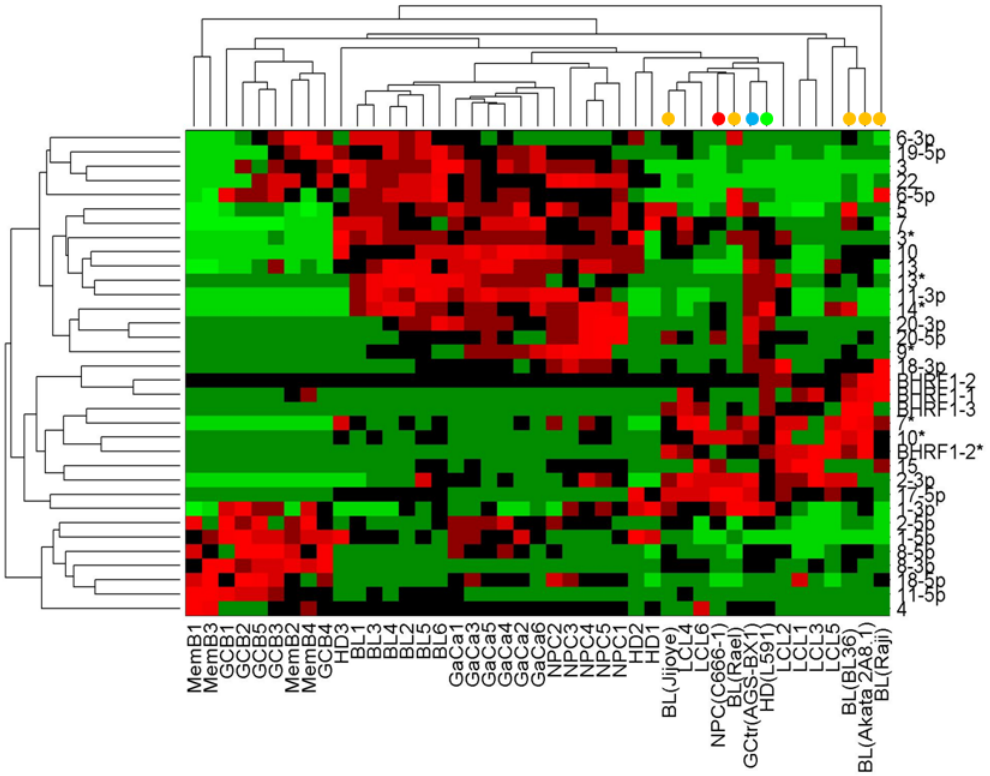



**Figure 15. Heat map of tissue samples from Figure 11 plus cell lines from all four tumor types.**

Cell lines are indicated by colored dots: yellow - BL lines; red - NPC line; blue – HD line; green- GaCa line.

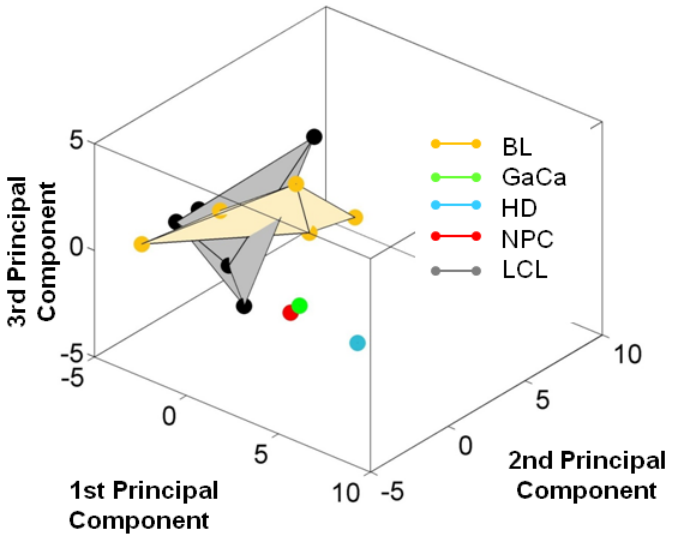



**Figure 16. Unlike BL biopsies, the miRNA expression profile for BL derived cell lines is indistinguishable from LCL by principal component analysis.**

Yellow – BL derived cell lines (n=5); green; GaCa derived cell line; blue – HD derived cell line; red; NPC derived cell line; grey LCL (n=6).

| Cell Line | LCL | Jijoye | BL36 | Rael | Raji | Akata 2A8.1 | L591 | AGS-BX1 | C666-1 |
|---|---|---|---|---|---|---|---|---|---|
| Origin | | BL | BL | BL | BL | BL | HD | GaCa | NPC |
| BHRF1-1 | 22 | 2 | 126 | 0 | 227 | 74 | 114 | 5 | 0 |
| BHRF1-2 | 34 | 88 | 190 | 154 | 644 | 210 | 248 | 27 | 0 |
| BHRF1-2* | 494 | 717 | 401 | 212 | 115 | 407 | 558 | 582 | 12 |
| BHRF1-3 | 8 | 35 | 140 | 45 | 1 | 68 | 56 | 12 | 0 |

**Table 4 Copy number per cell of EBV BHRF1 miRNAs in cell lines.**

## 2.4 Discussion

I have reported the expression profiling of EBV miRNAs in a wide variety of infected normal and neoplastic tissues that express all of the known EBV associated latency transcription programs. I have shown that there are distinct patterns of miRNA expression associated with Latency III and the restricted forms of latency (Latency II/I/0) and that these patterns are deregulated in EBV associated tumors. GCB (Latency II) and MemB (Latency I/0) express the same unique, restricted pattern of miRNAs with the exception that mirBART 17-5p is preferentially expressed in MemB cells. This unique pattern includes the absence of 12 BART miRNAs that include approximately half of Cluster 2. At least 8 of these absent miRNAs are expressed in B cells expressing Latency III (LCL) and in all of the tumor biopsies I have tested although none of the tumors uses Latency III. Thus this is a Latency III restricted pattern of miRNA expression, which I refer to as Latency III associated BARTs, that is deregulated in tumors. Interestingly another group of miRNAs that are associated with Latency III, the BHRF1s [352, 377-379], are not deregulated in the tumors suggesting that viral miRNA deregulation in the tumors is specifically targeted at the BARTs. This is an important conclusion since it represents the first demonstration of Latency III specific gene expression in tumors that are otherwise expressing restricted (Latency I/II) forms of latency [382, 383] and raises the possibility that BART miRNAs may contribute to oncogenesis. The observation of latency program specific miRNA expression could only have been made by studying in vivo derived infected material since the Latency III associated BARTs are expressed in all tumor biopsies and cell lines I have tested.

In the generally accepted model of EBV persistence, newly infected naïve B lymphoblasts (LCL) expressing Latency III, switch to Latency II when transiting the germinal center (GCB) to become resting memory B (MemB) cells, the site of long term persistence where viral latent protein expression is extinguished [383-385]. Our results here suggest that the transit from EBV driven growth into more restricted forms of latency in GCB and MemB cells is associated with turning off expression of the Latency III associated BARTs and up regulation of the remaining BART miRNAs by 5-10 fold. This is not simply related to the cessation of proliferation because latently infected GCB are, like LCL cells, proliferating [376]. Since proliferation in the GC is not driven by Latency III, the viral growth program, I may conclude that the Latency III BART profile (presence of the Latency III associated BARTs and reduced expression of the remaining BARTs) is specifically associated with EBV driven growth. One caveat to this conclusion is that it would have been desirable to confirm the Latency III pattern of miRNA expression on in vivo infected cells rather than spontaneous LCL. Unfortunately newly infected tonsil naïve B cells in vivo are present at a level 5-10 fold lower than infected GCB [386]. This puts them below the threshold of sensitivity and reliability for our profiling and therefore was technically not feasible.

Several studies have reported on potential roles for EBV miRNAs. There is no striking correlation between these reports and the BART miRNAs I have as the Latency III associated BARTs. Marquitz et al [351] have suggested that Cluster 1 and 2 BART miRNAs interact in apoptosis resistance by targeting BIM. However, their observations are not consistent with those of Seto et al, who have reported that BART miRNAs have no impact on LCL growth or survival in vitro [378], or that the entire BART region can

be deleted without impacting the transforming capacity of the virus [387] or that the prototypical laboratory strain B95-8 has most of the BART region, including most of Cluster 2, deleted yet is unimpaired in its transforming ability. The explanation for this discrepancy may lie in the fact that Marquitz et al performed their studies in an epithelial cell line not in B cells. Taken together these results suggest that the Latency III associated miRNAs I have identified, may play a crucial survival role in vivo for newly infected naïve B lymphoblasts activated by the EBV Latency III program but that this role is dispensable for in vitro growth much as has been shown for LMP2 [83, 369]. I assume that the specific up regulation of this group of miRNAs in tumors implies they could play a similar survival role in tumor development.

My results suggest that expression of the Latency III associated BARTs is coordinately regulated. It seems unlikely that this is occurring at the level of transcription/splicing. It is known that the BART miRNAs are derived from the first four introns of the BART transcript prior to the splicing event [388] and the miRNAs absent from GCB and MemB cells are not contiguous but randomly distributed among these introns. For examples, Bart 15 is located in the region between exon 1a and 1b whereas Bart 10 and Bart 20 are in the junction of exon 2 and 3. Therefore, it is unlikely that the differential miRNA expression I have described is related to the selection of splicing patterns. Other possible mechanisms that are known to regulate miRNA expression are differential DNA methylation [389-391] and RNA editing both of which have been shown to function on BART miRNAs [392, 393]. However, these mechanisms defer rather than answer the question as to why or how this particular subset of miRNAs is targeted for coordinate expression. A mechanisms that I favor is based on the observation that the stability of

miRNAs is dependent on the presence of their target mRNA [394]. In this case the absence of miRNAs in GCB and MemB that are present in LCL and the tumors would arise because the mRNAs targeted by those miRNAs were only present in LCL and the cancers.

I was surprised to find that the four tumor types clustered together in the heat map. This was irrespective of the tumor type, tissue of origin or the EBV transcription program that they employed. Perhaps more unexpected was our finding that despite the very similar patterns of miRNA expression the different tumor types were nevertheless clearly distinguished in two separate assays (heat map/clustering and PCA) applied to two completely separate data sets (p in both cases = 0.001). The basis for this resolution is less clear since it is not associated with any particular subset of miRNAs. Rather I discovered that a majority of all subsets of five or more miRNAs and some subsets as small as three were capable of distinguishing all four cancer types. Even more curious was the finding that every miRNA is capable of contributing to one of these subsets. Taken together, these results mean that the signal distinguishing these cancers is highly redundantly encoded across the miRNA expression profiles. Such a distribution of information is uncommon indeed may never have been reported before for a biological system. However, this type of behavior is well known in physics and computer science where there is a close analogy to secret sharing algorithms [380, 381]. For example, it is possible to share a secret message among any number (in our case ~40) people in such a way that if any 5 of them divulge their information to each other, the message can be read. It is interesting to speculate that the signal in our assays, i.e., the causes of or responses to each cancer type is in some similar manner parceled out among the

miRNAs. How this might work at the molecular level is unclear but I assume it must reflect extensive redundancy in the miRNAs both in the number that target the same gene and in targeting genes that lie in the same or parallel signaling pathways related to tumor development. Evidence for such layers of redundancy in miRNA function is well known [395-398] and has recently been reported for both the Cluster 1 and 2 BART miRNAs in reducing apoptosis susceptibility in an epithelial cell line [351].

It has been suggested previously, based on cell lines, that the copy number of BART miRNAs is higher in epithelial cells than B cells. This is consistent with our estimates of the relative abundance of the miRNAs in our tumor biopsy samples versus the LCL. However, the correlation of high expression with epithelial tissue is confounded by our measurement of relative abundance in the MemB and GCB populations where the expressed BART miRNAs are present at comparable levels to the epithelial tumors. The exact meaning of these variable levels of expression is therefore now unclear.

The only EBV latency transcription program that I found to be associated with a specific pattern of miRNA expression was Latency III characterized by up regulation of the Latency III associated BART and BHRF1 miRNAs. The later serves as validation for our approach since it has been reported [379] and confirmed [352] previously. I found BHRF1 expression in some of the tumor biopsies notably BL and HD although they were present at a very low level which I estimate to be generally less than one copy per cell. The absence of BHRF1 miRNAs from EBV associated tumors is consistent with previous findings that BHRF1 miRNAs were not found in biopsies from GaCa [399] and DLBCL tissues [356]. By contrast I found abundant BHRF1 miRNAs in the tumor derived cell lines. This, together with our finding that the BART miRNA expression profile in these

lines tended to resemble LCL more closely than the originating tumor biopsies, casts doubt on the value of using such lines to study EBV miRNAs.

In conclusion, I have presented the first comprehensive profiling of EBV miRNAs from in vivo derived normal and neoplastic tissue. These results demonstrate specific patterns of expression in Latency III versus more restricted forms of latency and deregulation of miRNA expression in tumors.

# Section III:

# EBV BART MIRNAS POTENTIATE TUMOR GROWTH IN VIVO

## 3.1 Premise and Rationale

In the previous section, I have identified different miRNA profiles from four cancer types and have also shown that all of the BART miRNAs are highly expressed in all of the tumor types especially the carcinomas, suggesting a possible role for these miRNAs in carcinomagenesis. I also identified a subset of the BART miRNAs whose expression in vivo is specifically associated only with the Latency III growth program. There are several reports in the literature describing potential gene targets and functions for the BART miRNAs including that they confer some degree of resistance to apoptosis in vitro [349, 400]. However, evidence for the role of BART miRNA in tumorigenesis in vivo is still absent. In addition, the association of EBV miRNAs and metastasis is of particular interest. A wide array of human miRNAs have been studied extensively with identified relevance in metastasis in several cancers [401]. These miRNAs engage in several specific steps in the metastatic pathways by targeting genes with an established role in tumor vascularization, cancer cell detachment, invasion and migration [402]. As yet, no report of the link between viral miRNAs and metastatic tumors in vivo has ever been made. Indeed, I identified differential EBV miRNA profiles from four cancer types but without any concrete knowledge concerning metastatic tumors. Our laboratory has established an NPC mouse model that can visually monitor tumor development and metastasis occurrence with the use of luciferase-transduced C666-1 cells [403]. In order to fully understand the oncogenic aspect of BART miRNAs, I have exploited this in vivo model to study the expression profiles of EBV miRNAs in the xenograft tumors as well as the role of the miRNAs in tumorigenesis in vivo.

## 3.2 Material and Methods

### Cell culture

The following cell lines were used in this study: the EBV-positive BL cell line BL36 (gift of Dr. Jeff Sample); the EBV-positive HD-derived cell line L591 (gift of Dr. Paul Murray); the GaCa cell line AGS and AGS/BX1 (gift of Dr Lindsey Hutt-Fletcher); AGS cells transfected with Orip/EBNA1 vectors; the NPC line C666-1; and mice tumor-derived in vitro lines. For maintenance, the GaCa cell lines and GaCa tumor-derived in vitro lines were cultured in Ham's F-12 medium containing 10% fetal bovine serum (FBS), 2 mM sodium pyruvate, 2 mM glutamine, and 100 IU of penicillin-streptomycin. All other cell lines or tumor-derived in vitro lines were maintained in RPMI 1640 medium with the same supplements.

### Lentiviral infection and cell transfection

BL36, L591, c666-1, AGS, AGS-BX1 cells were separately infected with the pGreenFire1-CMV, TR011VA-1 lentivirus (System Biosciences, Mountain View, CA), which contains green fluorescent protein (GFP) and luciferase expression sequences in the plasmid. GFP positive cells were sorted and collected by Fluorescence Activated Cell Sorting (FACS), and were subsequently cultured with appropriate medium. $2x10^6$ resultant AGS cells were transfected with 5 ug plasmid DNA of either Orip/EBNA1-EMPTY or Orip/EBNA1-BART vector (gift of Dr. Bill Sudgen) using an Amaxa nucleofector (Lonza), the V kit solution, and program B-023. After 24hr, transfected

AGS (AGS-EBNA1-EMPTY and AGS-EBNA1-BART) were then selected and cultured with F-12 medium and 150 ug/ml hygromycin.

**Animal and histology studies**

All animal experiments described in this study were performed according to the Guidelines of the Tufts University Division of Laboratory Animal Medicine, and the animal protocols were approved by the Tufts University Institutional Animal Care and Use Committee (protocol B2011-108). Female NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tm1Wjl}$/SzJ (NSG) mice (the Jackson Lab, ME, USA) ages 6–8 weeks were housed and maintained under sterile conditions with free access to food and water.

For carcinoma models, 2.5-7.5 x $10^5$ cells (c666-1, AGS, AGS-BX1, AGS-EBNA1-EMPTY and AGS-EBNA1-BART) resuspended in 50ul of phosphate-buffered saline (PBS), were injected with a 25-gauge needle into the nasopharyngeal compartment of NSG mice under anesthesia [403]. For lymphoma models, 2.5 x l0$^5$ BL36 and L591 cells in 100 ul ml PBS were injected with an insulin syringe I.V. via the tail vein into NSG mice. For subcutaneous carcinoma growth, 5 x $10^5$ cells (c666-1 and AGS-BX1) in 100 ul of PBS were injected into the subcutis of the right flank. Disease progression was monitored based on overall health and bioluminescent imaging. Mice were intraperitoneally injected with luciferin followed by anesthesia with 3% isoflurane and subsequent measurement of bioluminescence using an IVIS 200 imaging system (Xenogen). Tumor burden (or volume) was presented as radiance (photons per second per centimeter squared per steradian or p/s/cm2/sr) for each tumor by determining photon emission/second of a given tumor within a radius encompassing 5% or greater of

maximal signal intensity. The Kaplan-Meier survival curve analysis was performed with Prism program.

For histology analysis, tissue samples were fixed in 10% formalin buffer and stored in 75% ethanol prior to paraffin wax embedding, sectioning, hematoxylin and eosin (H&E) staining (by the Animal Histology Core at Tufts Medical Center).

**Generation of in vitro lines**

A small proportion of tumor tissue was excised at surgical operation and rinsed with Dulbecco's phosphate-buffered saline (DPBS). Tissue was then transferred to a 100mm Petri dish with appropriate medium. For carcinoma in vitro lines, tissue was minced with sterile scalpels into smaller fragment (< 2mm), and was then pressed under a 3.0 μm PET membrane cell culture inserts (BD Biosciences) followed by incubation at 37°C in 5% CO2-humidified incubator. Carcinoma cells started to grow as a monolayer and attach to the well in a few days. Occasionally, the dish also contained a few clumps of floating epithelial cells. Passage of carcinoma in vitro lines followed the routine protocol for adherent cell culture using trypsinization. For lymphoma in vitro lines, tissue was cut and minced into very small pieces in medium. The finely minced tissue in suspension was then filtered with a 70um cell strainer to remove debris. Suspensions were centrifuged at 1,500 rpm for 5 min and resuspended with RPMI 1640 medium. Lymphoma in vitro lines were maintained routinely as suspension cell cultures.

**Total RNA and genomic DNA isolation**

Tumor tissue was ground to a fine powder by a liquid nitrogen-cooled mortar and pestle. For RNA, tissue power was lysed using Trizol (Invitrogen) extraction according to the manufacturer's instructions. For genomic DNA (gDNA), tissue powder was lysed by DNAzol (Invitrogen). Briefly, 1ml of DNAzol was added to the powder and the cell lysates were obtained by gently passing the mixture through a pipette. several times. The insoluble lysates were removed by centrifugation at 10,000*g* for 10 min at 25°C. The viscous supernatant was transferred into another tube and DNA was precipitated using 0.5 ml of 100% ethanol followed by inverting the tubes several times. The tube was left at room temperature for 20 min and the DNA was pelleted by centrifugation at 14,000g for 15 min at 4 °C. gDNA was finally dissolved in 200ul 8 mM NaOH and the pH adjusted to 7 by adding 20 uL 1 M HEPES buffer. RNA and DNA from cell lines were purified using the method indicated above but without using a mortar and pestle.

**miRNA expression profiling**

EBV miRNA expression was measured using the real-time multiplex reverse transcript (RT)-PCR described in the previous section [340]. For mir-9, 26a and 34a, the same RT kit and the human specific TaqMan MicroRNA assays were used (Life Technologies).

**Quantitative gene and gDNA real-time PCR**

Gene expression was assessed using real time RT-PCR. RNA was reverse transcribed by the iScript cDNA synthesis kit (Biorad), followed by real-time PCR using IQ SYBR Green supermix (BioRad) and specific primers. The housekeeping gene GAPDH was used as an internal control for normalization. The relative expression was expressed as

$2^{\Delta Ct}$ , where ΔCt = mean value Ct (gene of interest) − mean value Ct (GAPDH). For gDNA real time PCR, DNA was diluted 100-fold prior to the PCR amplifications with the IQ SYBR Green supermix and specific primers. All SYBR Green real time PCRs were performed on a Bio-Rad iCycler. The protocol was as follows: step 1, one cycle of 5 min at 95°C; step 2, 40 cycles of 15 s at 95°C and 1 min at 60°C; step 3, one cycle of 1 min at 95°C. Fluorescence was monitored at the end of each extension phase. After amplification, melting curves were generated to verify the specificity of amplification. Primers for amplification are listed in the Appendix.

**Calculation of miRNA and orip/EBNA1plasmid copy number per cell**

Total EBV miRNA copy numbers in tumors were quantified as described above. To determine the cancer cell numbers in the individual tumors, serial dilution of corresponding tumor cell lines (AGS- BX1, c666-1, BL36, L591, AGS-EBNA1-EMPTY and AGS-EBNA1-BART) were made followed by qRT-PCR for expression of the internal control GAPDH. Calibration curves (cell number versus RT-PCR signal (Ct)) for were generated. In parallel, real time qRT-PCR was performed for GAPDH on tumor samples. Ct values were then plotted into calibration curves to calculate total cell numbers in tumors. To measure Orip/EBNA1 plasmid copy numbers per cell, total plasmid copy numbers in AGS-EBNA1-EMPTY and AGS-EBNA1-BART tumors were first determined. Standard curves were generated by using serial 10-fold dilutions of EBV (B95-8 Strain) quantitated viral DNA (Advanced Biotechnologies) followed by gDNA real time PCR on EBNA1 gene. The total cell numbers in tumors were then calculated with calibration curves generated by gDNA real time PCR of GAPDH on serial dilutions of cell lines AGS-EBNA1-EMPTY and AGS-EBNA1-BART.

**Growth curve and colony formation assay**

$3X10^4$ parental AGS-EBNA1-EMPTY, AGS-EBNA1-BART and in vitro line counterparts were seeded in 100mm Petri dish with medium in triplicate. Cell number was counted using the Scepter 2.0 handheld automated cell counter (Millipore) at indicated time points. For soft agar colony formation assays, $1x10^4$ cells were suspended in medium containing 0.4% agarose and overlaid in triplicate onto a solidified layer of medium-containing 0.8% agarose in 6-well plates. After two-three weeks, colonies were counted and photographed in five fields in each well. The results were expressed as the means ± SEM of triplicate counts within the same experiment.

**Apoptosis analysis**

$4\times10^5$ parental AGS-EBNA1-EMPTY, AGS-EBNA1-BART and in vitro counterparts were plated onto 6 well plates. After overnight incubation, cells were treated with 80 uM of etoposide for 72hr prior to harvest. Harvested cells were washed with annexin V binding buffer and subsequently stained with Annexin V-APC (BD Bioscience) for 15min, followed by fixation in 4% buffered paraformaldehyde at room temperature for 5min. Apoptotic cells were analyzed by flow cytometry and defined as positive for annexin V-APC staining. The percentage of cells that had undergone apoptosis in response to etoposide was assessed by subtracting the level of apoptotic cells in the untreated from that of the treated population (%Delta annexin V+).

**Matrigel invasion assays**

In vitro invasion assays were carried out as described previously [404] using complete Matrigel (BD Biosciences). A total of 18 μg Matrigel at a concentration of 0.3 μg/μl was

coated onto a FluoroBlok insert (BD Biosciences) with an 8-μm pore size membrane. The transwell insert was allowed to dry overnight at room temperature and rehydrated with 60ul of serum-free Ham's F-12 medium for 2 hr. AGS-EBNA1-EMPTY and AGS-EBNA1-BART cells were respectively seeded in triplicate at 5x104 per transwell insert. 500 μl of media containing 5% FBS were added to the lower well of the assay chamber to act as a chemoattractant. After 24 hr, the transwell inserts were transferred to wells containing 4 μg/ml calcein AM in Hanks' balanced salt solution and incubated for 30 min at 37 °C in 5% CO2. Cells that had passed through the pores and reached the trans-side of the membrane were counted using the imaging software MetaXpress. The number of invading cells was averaged over triplicate wells and presented as the means ± SD.

## 3.3 Results

### Xenograft models of EBV-positive carcinomas and lymphomas

Inoculation of severe combined immune deficiency (SCID) mice with EBV-positive tumor-derived cell lines has been extensively used to study the role of the virus in tumorigenesis and immune modulation of the diseases [403, 405-413]. Our laboratory has reported the first mouse model that accurately reproduces locally invasive and metastatic EBV-positive nasopharyngeal carcinoma (NPC) using the luciferase-tagged NPC cell line c666-1 [403]. To test if this model can be used for studying other epithelial cancers, I injected luciferase-expressing breast adenocarcinoma cell lines MCF-7 and MDA-MB-231, and the gastric carcinoma cell lines (AGS and AGS-BX1) into the nasopharyngeal

epithelium of the highly immunodeficient mouse strain NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG). While the primary tumors from AGS inoculated mice initially grew at the neck adjacent to the head, some animals developed metastases in distant organs such as lung, lymph nodes, liver, kidney and bones (Figure 17A, panel 1-4; Table 5). Tumor cells generally migrate through the blood vessels followed by invasion of the organs. As in Figure 17B, AGS-BX1 cells accumulated in the lung capillary and proceeded to either invade the alveolar wall or readily localize at the air spaces (panel 1, black arrows). The invasion of tumor cells in organs i.e kidney is often accompanied by a massive infiltration of histiocytes reflecting an inflammatory response (Figure 17B, panel 2. Red arrow: histiocytes). Similar tumorigenic phenotypes were also observed for breast adenocarcinoma cell lines (data not shown), suggesting that this model is broadly useful for studying carcinomas in vivo.

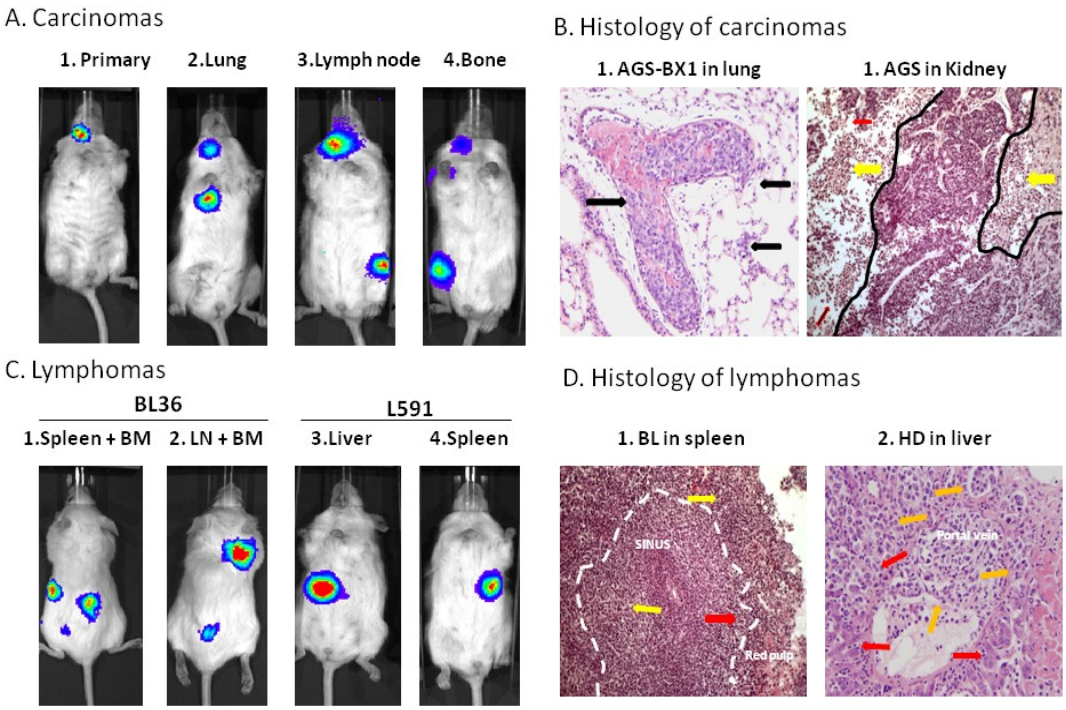



**Figure 17. Xenograft models of EBV-positive carcinomas and lymphomas.**

A. Mice inoculated with either c666-1 or AGS developed metastatic carcinomas in addition to the primary tumor. B. Panel 1: H&E staining revealed that AGS-BX1 cells (black arrow) invaded the lung capillary. Panel 2: the invasion of AGS cells in kidney. The tumor mass is outlined by the black solid line and red arrows indicated the infiltration of histiocytes. C. BL36 and L591 derived-lymphomas were found in many organs. D. Panel 1: BL36 cells packed the sinuses of spleen (dashed line) with interspersed lymphocytes and histiocytes (yellow arrow: lymphocytes; red arrow: histiocytes). Panel 2: L591 infiltrated the hepatic portal veins leading to the destruction of liver structure (Red arrow: hepatocytes; Orange arrow: tumor cells).

No successful engraftment of EBV-positive AGS-BX1 has been reported before [414]. Interestingly, in our hands 4 out of 10 mice injected nasopharyngeally (I.N.) with AGS-BX1 grew tumors, with median time to tumor appearance of 62 days and median survival of 155 days (Table 5). Metastatic dissemination was observed in 3 mice. In parallel, one mouse developed a local tumor with subcutaneous (S.C.) administration in the flank (Table 5). As expected, no metastasis was detected in this animal, suggesting that the I.N. model is superior to the S.C. model for studying metastasis. Surprisingly, 90% of mice receiving the EBV-negative AGS line I.N. developed malignancies. Tumors progressed very robustly because disease onset occurred earlier (30 days) along with mice having a shorter lifespan and more metastases (Table 5). This result is consistent with that of others demonstrating that the in vivo environment in SCID mice may favors tumor growth for AGS over AGS-BX1[414].

| Cell lines | origins | Injection site | Mice with tumors/ total injected | Take rate % | Days to tumor (median) | Survival days (median) | No. with metastases | Organ involved |
|---|---|---|---|---|---|---|---|---|
| C666-1 | NPC | I.N. | 23/24 | 96% | 21 | 40 | 10 | Ref.407 *Ovary (1), spleen (1), liver (2) |
| | | S.C. | 2/2 | 100% | 30 | 47 | 0 | Flank |
| AGS-BX1 | GaCa | I.N. | 4/10 | 40% | 62 | 155 | 3(4) | Lung(2), Lymph node(1), Bone(1), uterus(1) |
| | | S.C. | 1/2 | 50% | 30 | 78 | 0 | Flank |
| AGS | GaCa | I.N. | 9/10 | 90% | 30 | 99 | 6(9) | Lung(5),Thymus(1), lymph node(1), kidney(1),ovary(1), muscle (1) |
| BL36 | BL | I.V. | 10/10 | 100% | 21 | 32 | N/A | Lymph node(4),Lung(2), spleen(1), ovary(2), bone marrow(1) |
| L591 | HD | I.V. | 8/10 | 80% | 21 | 39 | N/A | Liver(6), lymph node(4), spleen(2) |

**Table 5. Xenograft models of EBV-positive carcinomas and lymphomas** .

Cancer origins, injection site, take rate, median days to tumor appearance, median survival days, number with metastases, and involved organs are shown. I.N.: Nasopharygneal injection; S.C.: Subcutaneous injection; I.V.: Intravenous injection.

*: only organs used in this study are shown. N/A: Not available.

It has been shown that both Burkitt and Hodgkin lymphoma-drived cell lines are able to grow in SCID mice [407-411]. Intravenous injection of these lines in mice lead to tumor formation in a disseminated manner analogous to the human diseases [410, 411, 415]. Thus I administrated mice intravenously with EBV-positive Burkitt's lymphoma line BL36 and Hodgkin's disease line L591. As shown in Table 5, injection of BL36 cells gave rise to progressively growing tumors in all 10 animals (100%). The disease onset became evident at approximately 21 days followed by rapid deterioration of the animals, which often showed signs of lymphoma such as tumor cachexia and hind limb paralysis. Tumors were found in many organs but were most common in lymph nodes, spleen, lung and bone marrow (Figure 17C, panel 1-2; Table5). Pathologically, the lymphoma was composed of monomorphic BL cells predominantly of small to medium size with round or irregular nuclei. The neoplastic cells often markedly packed the sinuses of lymphoid organs i.e. spleen interspersed with nonfunctional lymphocytes and histiocytes (Figure 17D, panel 1; yellow: lymphocytes; red: histiocytes). For Hodgkin's lymphoma, mice given L591 developed disseminated tumors in a similar time frame to BL36. Tumors were preferentially localized to the extranodal organs such as livers (75%) and the lymph nodes, particularly the iliac and inguinal nodes (50%) (Table 5). The pattern of dissemination was comparable to the distribution of HD cells in man with involvement of lymph nodes and liver (stages III and IV). Tumors arising from L591 are usually gray-white with ill-defined borders in the local organs. Histological examination of malignancy in liver indicates that neoplastic infiltration of L591 via hepatic portal veins resulted in the destruction of liver structure including distension of sinusoids and dissociation of hepatic plates (Figure 17D, panel 2;red: hepatocytes; yellow: tumor cells).

**Up regulation of EBV BART miRNAs in the in vivo epithelial and lymphoid tumors**

To study the expression level of EBV BART miRNAs in vivo, I profiled tumors from mice inoculated with c666-1, AGS-BX1, BL36 and L591. To compare and discern the in vivo expression, I generated malignancy-derived in vitro cell lines. In vitro cell line preparation, internal control normalization and calculation of miRNA copy number per cell in samples is described in Material and Methods. Tumors, in vitro lines and parental lines included in this study are listed in Table 6. As shown in Figure 18A, there was a significant up regulation of EBV BART miRNA copy numbers in the in vivo NPC tumors compared with parental line c666-1. This increase was up to 1,000-10,000 fold ($p<0.001$). More interestingly, when tumors were cultured in vitro to become cell lines, the miRNA expression declined to a level comparable to that of the parental c666-1. This behavior appeared to be reversible, as when these cultures were re-injected into the mice the miRNA level again increased (not shown). Similar to c666-1, tumors arising in AGS-BX1 mice expressed high level of BART miRNAs (Figure 18B). A substantial increase (100 to 1,000-fold) was observed in the tumors as opposed to the parental line ($p<0.001$). Accordingly, the copy numbers decreased in the AGS-BX1 in vitro lines although the degree of reduction was not as dramatic as c666-1.

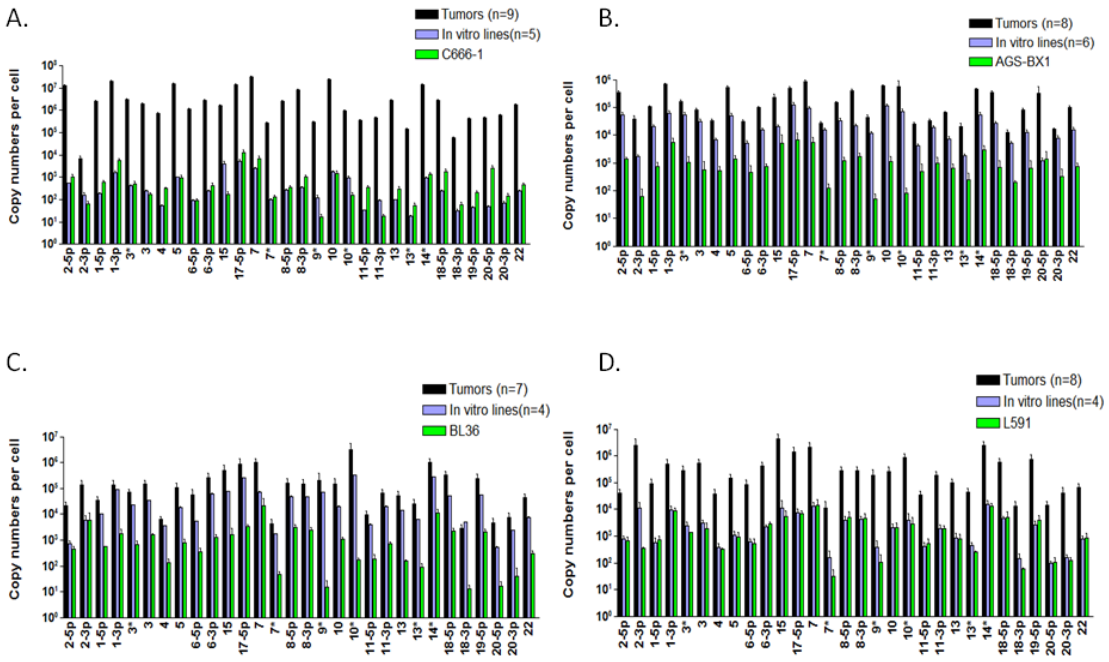



**Figure 18. Up regulation of EBV BART miRNAs in vivo in EBV-positive tumors.**

Comprehensive BART miRNA profiles among xenograft tumors, in vitro lines and parental lines were shown. The data was presented as copy numbers per cell. A significant up regulation of EBV BART miRNA in carcinomas and lymphomas was observed. A. c666-1; B. AGS-BX1; C.BL36; D. L591. Tumors: Black bar; In vitro lines: Blue bar; Parental lines: Green bar.

| Parental lines | In vivo tumors | In vitro lines | In vitro lines culture time |
|---|---|---|---|
| C666-1 | Primary(4),met(4),flank(1) | Primary(1),met(4; including a highly invasive knee met ) | 6 months to a year |
| AGS-BX1 | Primary(2),met(5),flank(1) | Primary(2),met(4) | Up to 1 month |
| BL36 | Lymph node(3),spleen(1),ovary(2),lung(1) | lymph node(1), Spleen(1),ovary(1), lung(1) | Up to 1 month |
| L591 | Liver(5),Lymph node(2),spleen(1) | Liver(4) | Up to 1 month |
| AGS-EBNA1-BART | Primary (3), lymph node(1), bone (1) | Primary (3) | Up to 1 month |

**Table 6. In vivo tumors and in vitro lines used in EBV miRNA profiling.**

In vivo tumors include a number of primary and metastatic tumors. In vitro lines were derived either from primary or metastatic tumors. Note that in vitro lines derived from c666-1 tumors have been cultured longer than others.

To verify the elevation of EBV miRNAs was not an artifact, I examined the expression of cellular miRNAs and genes at the transcription level. Human miRNAs mir-9, mir-34a and mir-26a, were tested. As seen, these miRNAs were expressed at similar levels in AGS-BX1 and c666-1 tumors, in vitro and parental lines (Figure 19 A-B). Two genes that are associated with epithelial mesenchymal transition, CDH1 and Snail1, were also tested. While the level of CDH1 mRNA remained unchanged among the cells tested, Snail mRNA was actually higher in the parental lines for both carcinomas (Figure 19 C-D). The expression of EB viral latent transcripts was also examined. The level of EBER1, another small non-coding RNA, was identical between tumors and cell lines in both cases. Interestingly, EBNA1 and LMP1 transcripts were moderately upregulated in the in vivo tumors, suggesting that these latent proteins might also play a role in tumor growth.

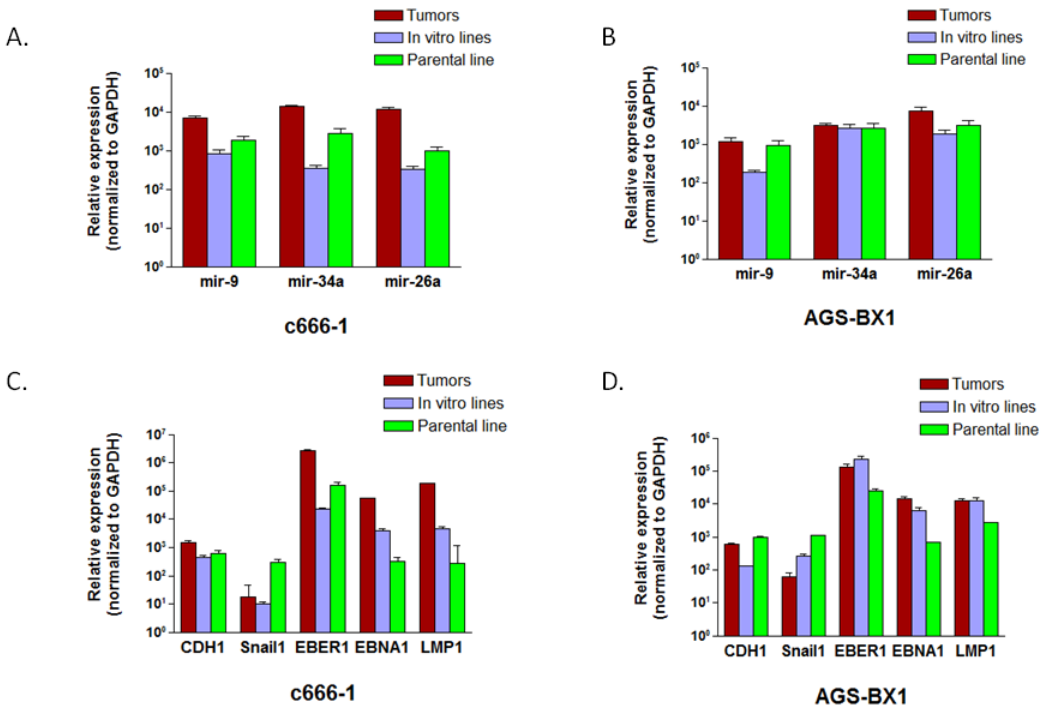



**Figure 19. Relative expression of human miRNAs, cellular and EB viral genes.**

Relative expression of mir-9, mir-34a, and mir-26a in tumors, in vitro lines and parental cell line from c666-1-inoculated mice (A) and AGS-BX1-inoculated mice (B). Relative expression of cellular genes (CDH1 and Snail1) and viral transcripts (EBER1, EBNA1 and LMP1) in tumors, in vitro lines and parental cell line from c666-1-inoculated mice (C) and AGS-BX1-inoculated mice (D).

To validate if the upregulation of BART miRNAs is carcinoma-specific, I evaluated their expression in B cell lymphomas. Similar patterns were observed for BL36 and L591-derived tumors, in which miRNAs were highly upregulated with the increase in copy number per cell reaching up to a marked 100-1000 fold ($p<0.001$) when compared with parental lines (Figure 18C-D). In section II I described a subset of miRNAs (Bart 15, 18-3p, 7*, 10, 10*,11-3p, 13* and 14*) associated with the EBV Latency III program. Surprisingly, upregulation of latency III-associated miRNAs in the in vivo tumors, except for the L591-derived HD, were more profound than that of the rest of miRNAs (Table 7). This finding is in accord with the expression profiles from human cancer biopsies including HPC, GC, BL and HD, suggesting that highly deregulated latency III-associated BART miRNAs may play a more crucial role in tumorigenesis. Taken together, these result show that in vivo growth of tumors selects for higher levels of the BART miRNAs, suggesting that elevated EBV miRNAs may provide a growth/survival advantage in vivo for both lymphomas and carcinomas.

| | Averaged fold change (Copy number per cell in vivo tumors /copy number per cell parental lines) | | | | |
|---|---|---|---|---|---|
| | c666-1 | AGS-BX1 | BL36 | L591 | AGS-EBNA1-BART |
| Latency III associated miRNAs | 9431 | 961 | 2445 | 246 | 300986 |
| The rest of miRNAs | 6197 | 219 | 750 | 526 | 4011 |

**Table 7. Latency III associated EBV miRNAs are greatly increased in vivo.**

The fold increase of Latency III associated miRNAs in vivo and that of the rest of miRNAs were compared in different mouse models. Latency III-associated miRNAs are Bart 15, 18-3p, 7*, 10, 10*,11-3p, 13* and 14*.

**EBV BART miRNAs potentiate tumor growth in vivo**

The remarkable enrichment of BART miRNA in tumor profiles suggests an important role for these miRNAs in tumorigenesis. To identify this role, I transfected AGS cells with either an empty Orip/EBNA1 vector (namely AGS-EBNA1-EMPTY) or one that expresses all of the BART miRNAs (AGS-EBNA1-BART), and subsequently inoculated mice with these cells to monitor tumor growth in vivo. The Orip/EBNA1 vector does not integrate into the host genome but instead persists episomally in cultured cells. Prior to injection, both cell lines were initially cultured with selection medium containing appropriate antibiotics thus stably express the gene of interest. When cells were injected into animals and grown over 60 days, surprisingly all mice with AGS-EBNA1-BART (100% of incidence) were detected with malignancies, whereas only 2 out of 5 AGS-EBNA1-EMPTY had visible tumors (Figure 20A). BART-expressing AGS appeared to be more aggressive in growing tumors in vivo, as most of mice deteriorate rapidly and had to be scarified by around 74 days. In contrast, AGS-EBNA1-EMPTY mice remained healthy and only 3 ultimately showed detectable focal bioluminescent signals. I repeated this experiment with similar results (not shown). Thus BART miRNAs enhance both the rate of tumor formation ($p=0.03$ by Fishers exact test at day 74) and tumor progression/fatality ($p=0.03$ by Fishers exact test at day 88). The tumor-forming capability of BART miRNAs was confirmed by measurement of the tumor burden in two independent experiments. As shown in Figure 20B, differences in tumor burden (represented as luciferase radiance) were not evident between the two groups on day 60, but subsequently difference became apparent as the tumors progressed. By day 74 when most of AGS-EBNA1-BART mice needed to be sacrificed, the average tumor size was

approximately 10-fold higher than that of the AGS-EBNA1-EMPTY (~$1x10^6$ p/s/cm2/sr in BART+ vs $1x10^5$ p/s/cm2/sr in EMPTY), suggesting that BART miRNAs promote tumor growth in vivo. Consistent with this conclusion, Kaplan Meier analysis showed that AGS-EBNA1-BART mice exhibited significantly higher overall mortality rates relative to the AGS-EBNA1-EMPTY mice ($p = 0.02$) (Figure 20C).

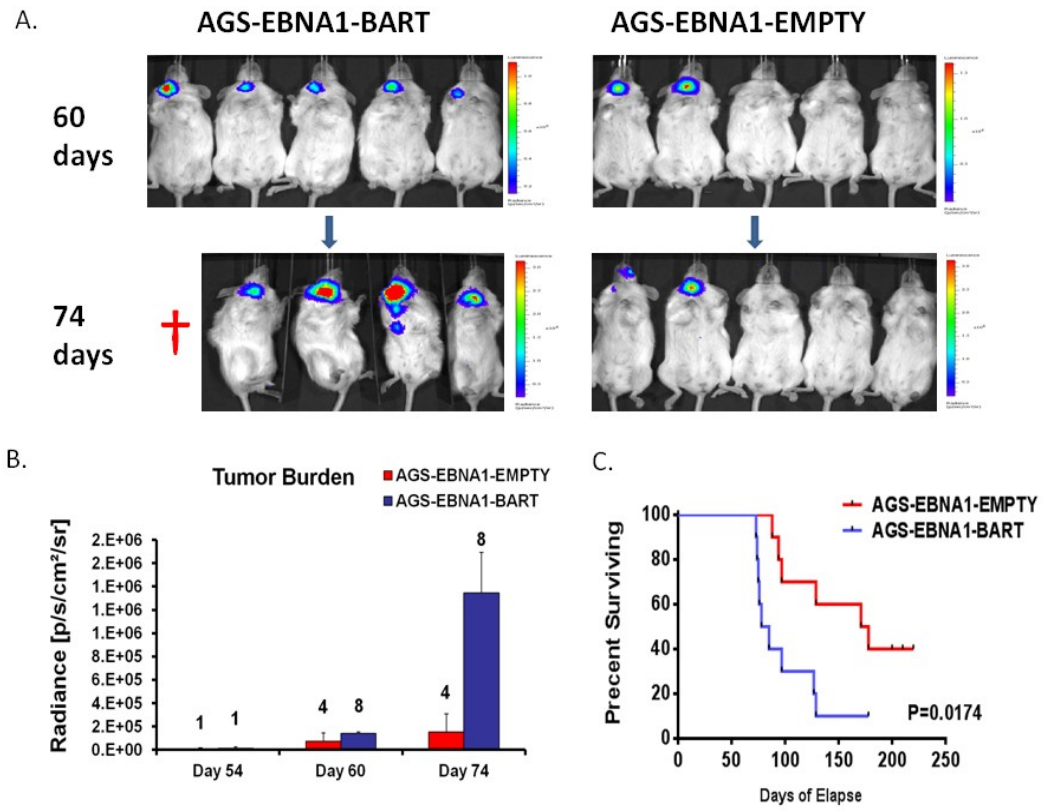



**Figure 20. EBV BART miRNAs potentiate tumor growth in vivo.**

Tumors from AGS-EBNA1-BART and AGS-EBNA1-EMPTY (A) groups on day 60 and day 74. AGS-EBNA1-BART tumors grew faster and larger than AGS-EBNA1-EMPTY in vivo. The red cross represents a mouse that died prior to imaging. C. Tumor burden (numerical representations of tumor size) was presented as Radiance (p/s/cm2/sr). The number of mice with detectable signals was shown above the bar. D. Kaplan-Meier analysis of survival in mice revealed that AGS-EBNA1-BART mice have a higher death rate p = 0.017).

The miRNA expression profiles in the tumors and parental lines were also compared. The expression profile of AGS-EBNA1-BART tumors agreed with engrafted carcinoma and lymphoma data demonstrating that miRNAs were highly elevated (a $10^3$-$10^6$ fold increase) in the in vivo malignancies (Figure 21). This analysis confirmed that the increase is far more dramatic for the Latency III-associated miRNAs than the rest of miRNAs (up to two orders of magnitude), again implying their important roles in tumor formation (Table7).

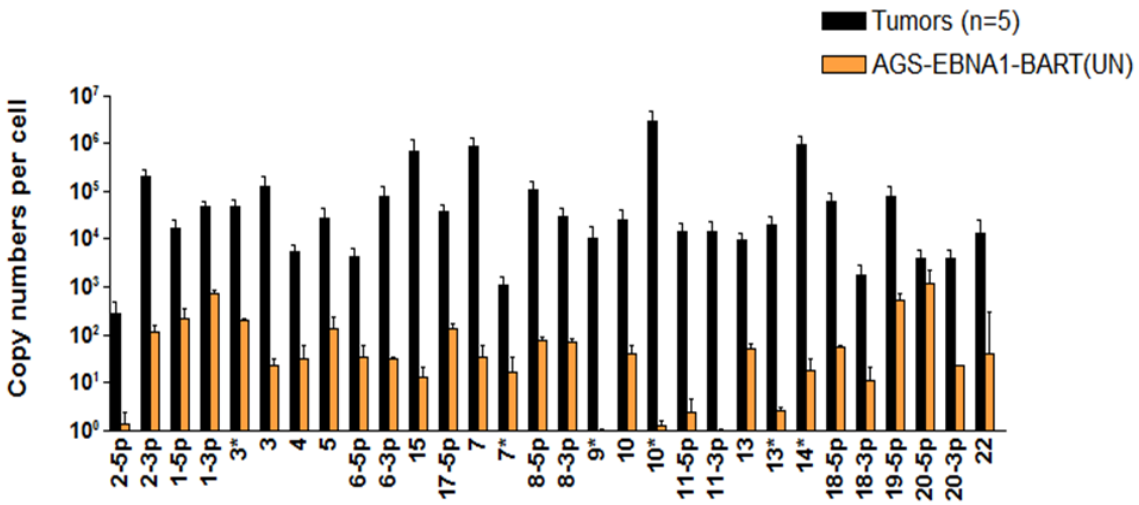



**Figure 21. BART miRNA expression in AGS-EBNA1-BART tumors.**

BART miRNAs were highly increased (a $10^3$-$10^6$ fold increase) in the in vivo tumors compared to the parental line AGS-EBNA1-BART. UN means no drug selection. Cells have been cultured with regular medium without selection regent (hygromycin) for 70 days.

It has been shown that the loss of EBV episomes directly leads to the loss of tumorigenicity [197]. I observed, in agreement with previous studies, that the AGS-EBNA-EMPTY and AGS-EBNA-BART episomes were lost from cells grown without drug selection (not shown). However, analysis of the plasmid copy numbers from the in vivo tumors (Table 8) revealed that the episomal plasmids were retained in the BART miRNA-expressing tumors (n=6 average of 8.9$\pm$3.5 episome copies per cell), but lost from the AGS EBNA1-EMPTY tumors (n=4 average copy number less than 1 per cell; 0.1$\pm$0.1) ($p = 0.0001$). This result indicates that the in vivo tumor environment favorably selects for the retention/expression of EBV miRNAs which, conversely, confer a selective advantage to the tumors.

| Tumor Origin | Plasmid Copy Number/Cell | | | | | | Average | Pvalue |
|---|---|---|---|---|---|---|---|---|
| AGS-EBNA1-BART | 7.2 | 8.9 | 10.7 | 2.4 | 10.5 | 13.9 | 8.9±3.5 | |
| AGS-EBNA1-EMPTY | 0.02 | 0.02 | 0.1 | 0.3 | | | 0.1±0.1 | 0.0001 |

**Table 8. BART expressing plasmids are preferentially retained in tumors.**

Plasmid copy number per cell was calculated as indicated in Material and Methods. 6 AGS-EBNA1-BART -derived tumors and 4 AGS-EBNA1-BART-derived tumors were studied. The episomal plasmids were retained in the BART tumors (8.9±3.5 copies/cell) as opposed to EMPTY tumors (0.1±0.1 copies/cell) ($P<0.0001$).

No direct evidence has ever been found to support the idea that EBV miRNAs confer a growth advantage to miRNA-expressing cells versus vector control cells. Consistent with this I found that the parental AGS-EBNA1-BART cells proliferate at the same rate as the parental AGS-EBNA1-EMPTY in vitro (Figure 22A). However, after "in vivo training" the AGS-EBNA1-BART in vitro lines exhibited a significant growth advantage over the miRNA-negative counterparts (Figure 22B), suggesting that EBV miRNAs might trigger pro-tumorigenic signaling pathways in vivo that are sufficient to enhance the malignant phenotype. This finding was further confirmed by a colony formation assay, which revealed a significantly higher colony-forming capacity in agarose for the AGS-EBNA1-BART in vitro lines (Figure 22C-D). Contrary to recent studies on the effects of BART miRNAs in vitro, I observed no difference in the levels of apoptosis in the parental lines in response to the DNA damaging agent etoposide, regardless of BART miRNA expression (Figure 22E). However, I did observe a moderate level of protection by the BART miRNAs in the vitro lines (13% specific reduction in apoptotic cells) (Figure 22F). This result supports the notion that expression of EBV miRNAs in vitro is not sufficient to provide a growth advantage to cancer cells, but growth in vivo selects for EBV miRNA enhanced tumorigenicity. Taken together, this result suggests that EBV BART miRNAs substantially potentiate tumor growth in vivo.

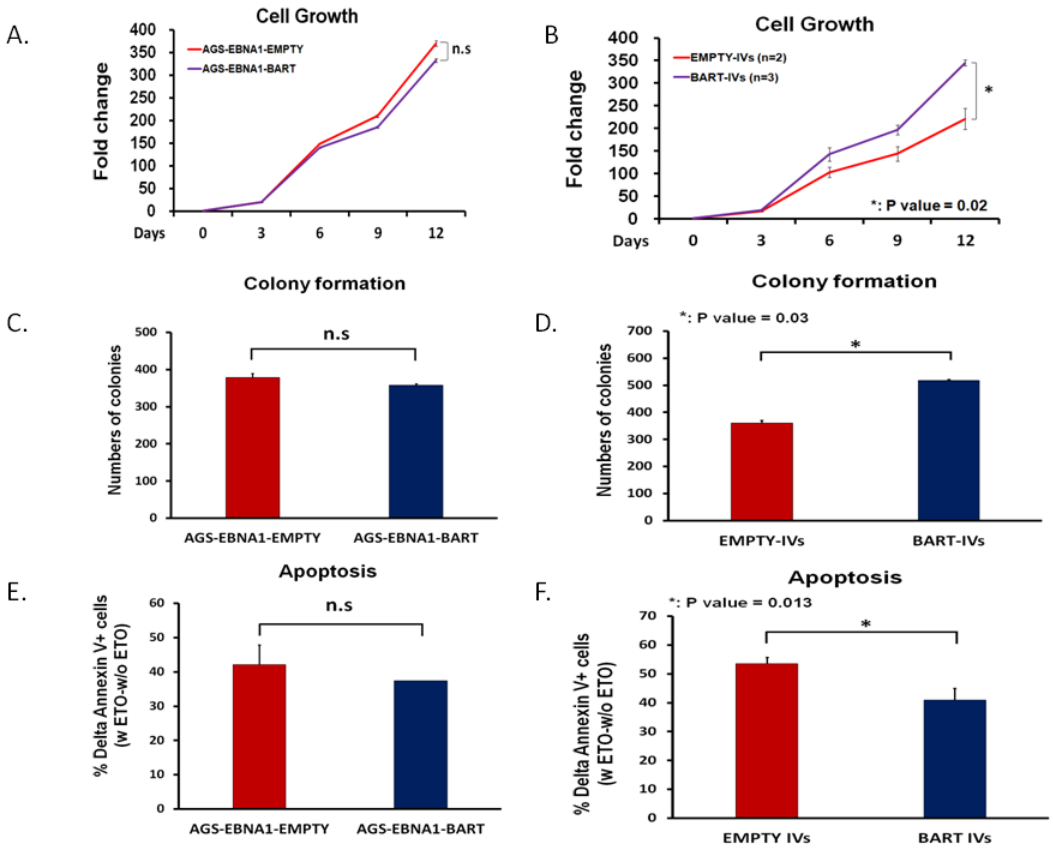



**Figure 22. The BART miRNAs increase tumorigenic potential for cancer cells.**

A. Growth curves for parental lines and in vitro lines. EMPTY-IVs: AGS-EBNA1-EMPTY tumor-derived in vitro lines; BART-IVs: AGS-EBNA1-BART tumor derived in vitro lines. Fold change of cell numbers over initial cell input was presented. B. soft agar colony-forming ability of parental and in vitro lines. Total numbers of colonies were count in triplicate. C. Apoptosis analysis in each group by Annexin V staining and flow cytometry. The percentage of cells that had undergone apoptosis in response to etoposide (%Delta annexin V+) was assessed by subtracting the level of apoptotic cells in the untreated from that of the treated population. All experiments were performed in triplicate and the value was average ± SD. * represents not statistically significant difference ($P > 0.05$). ** represents significant statistical difference ($P < 0.05$).

**No correlation found between EBV BART miRNAs and metastasis**

To investigate the role of EBV BART miRNAs in metastasis, I first studied the EBV miRNA profiles of c666-1 and AGS-BX1 tumors and in the portion of c666-1 and AGS-BX1 mice that developed metastases. For comparison purpose, in addition to the primary (Pri) and metastatic tumors (Met) I also profiled miRNA expression on tumors derived from subcutaneous injection in the dorsal flank of mice. Subcutaneous tumors in the flank are poorly invasive and rarely lead to metastases [403, 414, 416, 417], therefore flank tumors would serve as a control representative of locally restricted malignancies. Metastatic tumors are generally characterized by the signature protein Snail, a key transcriptional factor in epithelial mesenchymal transition that is known to promote metastasis. Elevated expression of Snail is the molecular marker for metastatic tumors. Indeed, Snail expression was greatly increased in the Met versus the Pri or flank tumors (Figure 23 A-B), ensuring that the Mets have attained the characteristics of metastases. When the miRNA profiles were compared, the flank tumors had the highest levels of overall EBV miRNA expression among all the tumors (average 7-fold and 90-fold increase in AGS-BX1 and c666-1 flanks when compared to primary tumors, respectively). The expression however is consistently similar between the local Pri and the Met (Figure 23 C-D). Thus this profiling result does not support a positive correlation between EBV miRNA expression and metastasis at the expression level. In addition, I obtained a highly invasive in vitro line from the c666-1 tumor (a knee met) that displays a 100% metastasis rate in mice (Table 6). Yet, there was no distinction in miRNA expression observed for this line when compared to other lines who have less metastatic potentials (not shown).

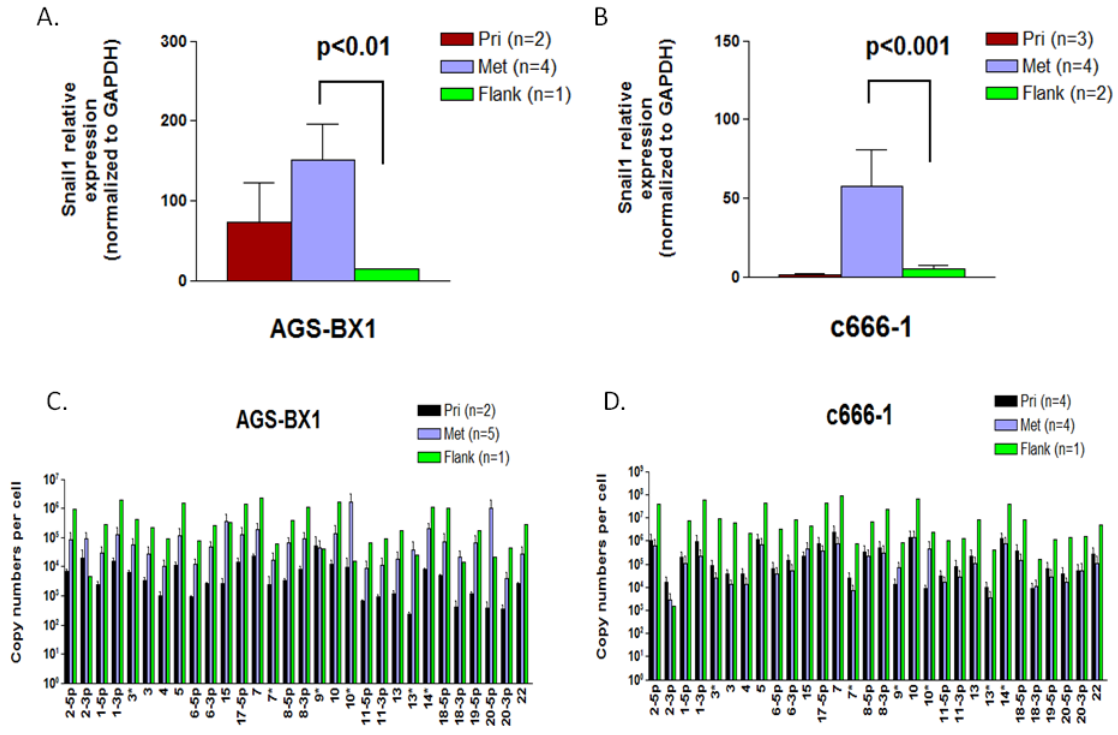



**Figure 23. EBV BART miRNA expression in metastasis.**

Sanil1 gene expression in primary (Pri), metastatic (Met), and flank tumors from AGS-BX1 inoculated mice (A) and c666-1 inoculated mice (B) mice. Comprehensive BART miRNA profiles from Pri, Met and flank tumors in AGS-BX1 inoculated mice (C) and c666-1 inoculated mice (D).

Next, I asked if exogenous expression of EBV miRNAs in AGS cells (AGS-EBNA1-BART) would provide more invasive potential to tumor cells. Since cell invasion is a critical step in metastasis, I performed transwell matrigel invasion assays. In this assay, the ability of cells to invade through an extracellular matrix Matrigel-coated porous membrane in response to chemoattractants was assessed. After a 24 hour incubation, AGS-EBNA1-EMPTY revealed a more invasive phenotype in the assay than did AGS-EBNA1-BART, as the absolute cell numbers of AGS-EBNA1-EMPTY on the trans-side (migrated cells) were around 2-fold more than that of AGS-EBNA1-BART (Figure 24A-B), supporting the proposition that EBV miRNAs may have a negative effect on the invasive growth phenotype. To confirm this phenotype, I also monitored the in vivo metastasis rate for both cell lines. Note that AGS and AGS-BX1 mice had comparable metastasis rate (67% vs 75%) (Table 5, Figure 24C). 4 out of 6 AGS-EBNA1-EMPTY mice with tumors had mets (33%), whereas 3 out of 8 mice with BART+ tumors developed metastasis (20%) (Figure 24D). Although these numbers do not achieve statistical significance, they are consistent with a trend that the BART miRNAs may impede metastasis. The likely interpretation of this phenomenon is that tumors developed much more rapidly in the AGS-EBNA1-BART mice requiring they be sacrificed far earlier than AGS-EBNA1-EMPTY mice. This reduces the time available for metastases to arise in AGS-EBNA1-BART mice compared with tumor bearing EBNA1-EMPTY mice which survive longer with tumors. We conclude from these studies that we find no evidence for BART miRNAs contributing to invasion and/or metastasis.

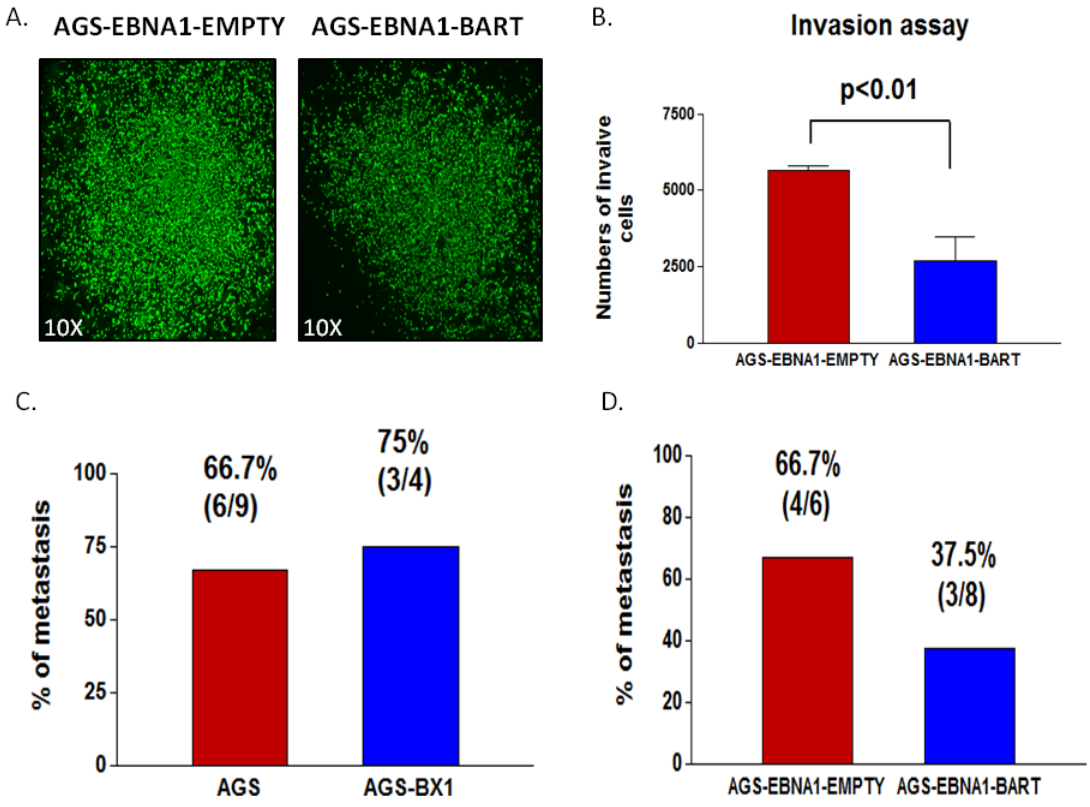



**Figure 24. Association of EBV BART miRNAs and metastasis.**

A. Fluorescent images of cells that had invaded. In vitro invasion was assayed using Matrigel coated inserts as described in Material and Methods. Cells were allowed to invade through the membrane toward FBS, followed by staining with calcein-AM. The images are representatives of triplicates for each group. B. Cells that had migrated to the trans-side were counted, with three wells analyzed per sample. Values are expressed as the means ± SD. AGS-EBNA1-EMPTY (Red bar) had more cells invading on the trans-side than AGS-EBNA1-EMPTY (Blue bar) ($P<0.01$). C. Rate of metastasis in AGS (red bar ) and AGS-BX1 (blue bar) inoculated mice. 6 out of 9 AGS mice showed mets (66.7%) , while 3 out of 4 AGS-BX1 mice had mets (75%). D. Rate of metastasis in

AGS-EBNA1-EMPTY and AGS-EBNA1-BART mice. 4 out of 6 AGS-EBNA1-EMPTY mice showed mets (66.7%), while 3 out of 8 AGS-EBNA1-BART mice had mets (37.5%).

## 3.4 Discussion

In vivo studies of the role of EBV in oncogenesis have been very limited, since EBV only infects New World primates. Xenotransplantation of EBV-positive human cells in SCID mice has provided useful tools to study the biology of the diseases and the function of the virus. Here I described the development and use of an optimized carcinoma xenograft model via nasopharygeal injection to study the in vivo effect of EBV miRNAs. Although nasopharyngeal development of carcinomas from other origins (i.e. gastric cancer) is not necessarily representative of the respective human tumors, the reasons to use this model for studying EBV miRNAs are evident. First, the nasopharynx is considered an epithelial reservoir which provides a favorable location and foundation for the nesting and proliferation of epithelial cancer cells. Second, in contrast to the subcutaneous xenograft model where tumors are usually locally restricted [403, 413, 414, 416-418], our mouse model has a significant level of metastatic tumors. This is probably because the naospharynx is an unique structure containing nasal-associated lymphoid tissue with many underlying HEVs[419]. Tumors are able to grow large enough before they migrate. Indeed, in our study AGS mice showed a steady and constant tumor growth in the time window suited for studying metastasis (Table 5). This model somewhat recapitulated the metastatic properties of GaCa as the common sites of human gastric carcinoma are liver, lung and lymph nodes. Lastly, unlike the experimentally challenging orthotropic GaCa model [403, 420], our ability to generate

cancer-bearing mice via the I.N. route often results in synchronized, easily observable tumors with a high level of reproducibility and predictability. The location for cell injection, except for the study of metastasis, seems not to affect the use of these models for studying the in vivo function of EBV miRNAs (Flank-injected tumors also showed enrichment of EBV BART miRNAs in Figure 23).

Using this model to generate EBV-positive carcinomas (c666-1 and AGS-BX1), I have observed a significant up regulation of EBV BART miRNAs in the in vivo tumors. The increase strikingly was up to 10,000 fold for c666-1-derived tumors, whereas AGS-BX1 tumors had around 1,000-fold induction when normalized to the parental lines (Figure 18A-B). The discrepancy of elevation between those lines might be in part due to the microenvironment which may be more favorable to c666-1 (orthotopic injection). The up regulation of EBV miRNAs in vivo is not carcinoma-specific, as lymphomas arising from BL and HD cell lines (BL36 and L591) exhibited similar level of increase, suggesting that EBV miRNAs play an universal but critical role in tumorigenesis (Figure 18C-D). Surprisingly, relative to the rest of BARTs, up regulation of Latency III-associated BARTs was more prominent in the majority of mouse tumors except for the L591-derived cancers (Table 7). This correlates with the BART expression pattern in human tumors, where the deregulated levels of the Latency III-associated BARTs were identified. Therefore among all the upregulated BARTs, the Latency III-associated BARTs are possibly the most potent viral miRNAs in promoting tumor formation. Indeed, our preliminary data on gene expression of miRNA-transfected cells using gene microarray suggests that a panel of tumor suppressors or apoptosis facilitators are downregulated by Latency III-associated BARTs such as 15 and 10 (not shown).

The function of EBV BART miRNAs has been widely studied in cell lines yet much controversy remains. While some groups showed that BART transcripts are not required for B cell transformation and have no impact on LCL growth in vitro [338, 368], others suggested a direct association between an increased level of BART and the transformed growth properties of EBV-infected cells [349, 421]. In this study, the considerable increase of EBV BART miRNA expression we have observed in tumors raises questions about the etiologic contributions of the miRNAs to cancer formation in vivo. Given EBV generally persists as episomes in tumor cells and EBNA1 and BARTs are co-expressed in every type of tumors, I decided to transfect the Orip/EBNA1 backbone plasmid carrying the whole BART miRNAs into EBV-negative AGS cells and subsequently injected them into mice. The Orip/EBNA1 vector does not integrate into the host genome but instead exists as an episome, mimicking the persistent features of the virus in cells. Analogous to the profiling data, BART miRNA-expressing cells (AGS-EBNA1-BART) have remarkably potentiated tumor formation in mice compared with the control cells (AGS-EBNA1-EMPTY) (Figure 20A-B). BART+ tumors tended to grow larger and faster in vivo leading to shorter survival times and a lower survival rate in animals (Figure 20C-D). It is very clear that acquisition of tumorigenicity in our model is exclusively dependent on the BART miRNAs as the only difference between the two lines is the presence of the BART miRNAs. One interesting observation made with this system is that EBV miRNA positive tumors had a longer retention period for the plasmids (Table 8). Loss of the EBV genome in infected cells and tumors is associated with the eradication of the malignant phenotype. Either spontaneous or hydroxyurea-induced loss of viral episomes in the type I latency BL Akata cells resulted in a significant decrease in

the in vitro cell growth, formation of colonies in soft agar, and generation of tumors in SCID mice [196, 422], but reinfection of these cells with EBV restores tumorigenicity [197, 423]. The molecular mechanisms underlying episome loss in EBV infected cells are largely unknown. It is widely accepted that EBNA1 is essential for the persistence of the EBV genomes through modulating the tethering of the episomes to the host mitotic chromosomes. Whether increased levels of miRNAs contributes to the retention of episomes in vivo thereby facilitating latency of a full virus in tumor or normal cells in general is an open question.

Interestingly, the proliferating and tumorigenic characteristics of miRNA-positive tumors can be passed onto the tumor-derived in vitro lines, as evidenced by in vitro cell growth, a clonogenic assay in soft agar and an apoptosis analysis (Figure 22). High levels of BART miRNAs in the tumor-derived in vitro lines (AGS-EBNA1-BART) retain the tumorigenic potentials for tumor cells (Table 8 and Figure 22). Thus it is believed and is also supported by Vereide et al that BART miRNAs are the major contributors to the survival and proliferation of virally infected tumor cells [349]. Increased EBV miRNAs in vivo might specifically trigger or enhance tumorgenic or survival signaling pathways in response to the tumor microenvironment. It seems unlikely that these pathways are associated with the initiation of transformation because AGS cells are already transformed. Supportive of this concept is the report that the immortalizing function of EBV infection appears not to be associated with tumorigenic capabilities [424]. It is therefore more likely that gene targets for BART miRNAs are linked to specific maintenance of the in vivo malignant phenotype. This could explain why EBV miRNAs do not confer survival advantage to the AGS cells when cultured in vitro (Figure 22). It is

possible that, for instance, signaling pathways that control tumor hypoxia are induced in vivo for the AGS-EBNA1-BART cells therefore resulting in activation of a broad array of mitogenic, pro-invasive and pro-angiogenic genes. Indeed, there is an important cross-talk between virus and angiogenesis in the tumor microenvironment. EBV proteins LMP1 and LMP2A can activate host genes such as VEGF, FGF2, COX-2, IL-8 and MMP- 9 through NF kapa B or Notch signaling pathways to trigger angiogenesis [425-429]. In nasopharyngeal and gastric cancer, expression of LMP-1 in tumor cells correlates with increased expression of COX-2 and PGE2 which have been shown to contribute to neovascularization [270, 429]. How gene profiles are altered in vivo in the BART miRNAs-expressing tumors is as yet unknown. Studies comparing gene expression between miRNA-positive and negative tumors are ongoing.

There are numerous reports in the literature describing the metastasis-promoting capability of viruses. The DNA tumor viruses HPV, HBV, KHSV, polyomavirus and EBV are believed to participate in the regulation of tumor cell invasion and migration to induce a metastatic phenotype [430]. Multiple studies have demonstrated that the EBV oncogenic protein LMP1 is the most critical player in enhancing cell mobility, epithelial-mesenchymal transition and cell invasion [431-435]. Nasopharyngeal carcinomas with high levels of LMP1 expression show more metastatic potentials than those with low levels [436-438]. EBNA1 and LMP2A are also thought to mediate EMT thereby inducing cell mobility, invasion and metastasis [439-443]. The mouse model used here has provided a substantial advantage for studying the role of EBV miRNAs in metastasis. I observed that metastatic tumor cells did not show a distinctly different expression pattern compared to primary tumors in c666-1 and AGS-BX1 mice (Figure 23C-D).

Instead, there was a clear difference in the expression profiles from locally restricted tumors implanted in the flank, evident from the fact that higher levels of overall BART miRNAs expression were found in the flank tumors compared with the primary and metastatic tumors (Figure 23C-D). This might suggest that EBV BART miRNAs may favor local tumor growth over that of distant metastases, although the possibility that they have a role in the initial stages of metastasis cannot be excluded. Perhaps the most direct evidence suggesting the involvement of EBV miRNAs in invasion and metastasis came from the experiment conducted on AGS-EBNA1-BART transfectants. In vitro transwell invasion assays which reflect the early stage of invasion showed that without BART miRNAs AGS cells (AGS-EBNA1-EMPTY) revealed a more invasive phenotype as evidenced by a stronger ability to invade through the Matrigel (Figure 24A-B). Moreover, in vivo development of metastatic tumors in the AGS-EBNA1-EMPTY mice was shown to be more prevalent than in the AGS-EBNA1-BART mice (Figure 24D). Thus, these lines of evidence sugest that EBV BART miRNAs might exert a negative effect on the metastatic potential of cancer cells. This finding accords with the profiling data of the flank tumors implying that in general EBV BART miRNAs are crucial for tumor development in vivo but show little or no impact for metastases formation. This view is supported by the findings from two clinicopathological reports, one showing that EBV positive GaCa is limited to the submucosa with less propensity for lymphatic and blood vascular metastasis [444], and that EBV positive GaCa exhibits a significant lower frequency of lymph node metastasis than EBV-negative GaCa [445]. However other studies failed to reveal any conclusive association between EBV positive GaCa and lymph node metastasis [446-449]. EBV seems to play a different role in metastasis in

NPC as a meta-analysis on the basis of published case-control studies indicates that LMP1 expression is positively associated with an increased risk of metastasis in NPC [450]. Our data are not wholly sufficient to conclude that EBV miRNAs inhibit metastasis for at least two reasons. First, a bigger sample size is needed for a convincing conclusion. Second, BART miRNAs alone may not be associated with metastasis but it cannot be ruled out that they have collaborative functions with other latent proteins (EBNA1 or LMP1) in promoting metastasis.

In conclusion, using a xenograft mouse model to test the tumorigenic potential of EBV miRNAs not reported previously, I have been able to demonstrate a role for BART miRNAs in promoting tumor formation in vivo.

# Section IV:

# EBV MIRNA BART18-5P FACILITATES LATENCY IN B CELLS BY INHIBITING VIRAL REPLICATION VIA DOWNREGULATING MAP3K2

## 4.1 Premise and Rationale

Like many viruses that persist in the infected host, EBV replicates and resides in different cellular compartments with either lytic or latent expression programs. While the lytic program gives rise to the production of infectious viral particles, the latent program enables the virus to express a limited number of viral genes that maintain the viral reservoir in the face of specific immune responses. Unique for EBV is the expression of three different latency programs representing different levels of viral silencing (See General Introduction 1.6). In MemB cells, all viral protein expression is extinguished (Latency 0) except when the cells divide and express EBNA1 (Latency 1), the protein required for replication of the viral genome. This mechanism is thought to allow EBV infected cells to escape immune surveillance, enabling lifelong persistence.

I reported in Section II that a majority of Cluster 2 BART miRNAs absent from normal infected tissues (GCB and MemB) are highly expressed in Latency III cell lines and neoplastic biopsies. In addition, a subset of miRNAs is abundantly expressed in the GCB and MemB but presents with a relative low copy number in LCLs and tumors. Until recently, very little was known about the viral and cellular targets of EBV miRNAs. In order to further understand the role of EBV miRNAs in both oncogenesis and latency, in this section I aimed to identify the cellular targets for EBV miRNAs. Understanding the targets for miRNAs is critical to understanding the biology and clinical consequences of EBV infection.

## 4.2 Material and Methods

### Cell lines

Burkitt's lymphoma cell line BL2, Akata and Akata2A8.1, diffuse large B-cell lymphoma (DLBCL) cell line BJAB, marmoset B-lymphoblastoid cell line (B95.8), and B95.8 transformed human lymphoblastoid cell line (LCL) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM sodium pyruvate, 2 mM glutamine, and 100 IU of penicillin-streptomycin at 37°C and 5% CO2. HEK239T cells were cultured in complete DMEM (Dulbecco's Modified Eagle Medium).

### MiRNA mimcs, inhibitors and cell transfection

Synthetic miRNA mimics were purchased from Thermo scientific. EBV 18-5p miRNA mimic mature sequence is 5'-UCAAGUUCGCACUUCCUAUACA-3'. Mimic negative control #1 (MC) is based on C. elegan's cel-miR-67 and the mature sequence is 5'-UCACAACCUCCUAGAAAGAGUAGA-3'. Locked nucleic acids (LNA) were purchased from Exqion. The ebv-miR-BART18-5p power inhibitor (anti-18-5p LNA) sequence is GTATAGGAAGTGCGAACTTG. The power inhibitor negative control A (NC LNA) sequence is GTGTAACACGTCTATACGCCCA. 100 pmol miRNA mimics or LNA were transfected into 1.5-2.5x $10^6$ cells using an Amaxa nucleofector (Lonza), the V kit solution, and program C-009. Cells were harvested at 48hr for further analysis. Luciferase plasmids, pSUPER and pGIZ plasmids were transiently transfected into HEK392T cells using lipofectamine 2000 reagent according to the manufacturer's manual. 2x$10^6$ B95.8-LCL cells were electroporated with 3ug pCMV6-MAP3K2 (human origin) and pCMV6-XL5 control plasmids (gift of Dr. Ren Sun) using nucleofector.

**Cloning**

The lentivirus plasmid pGIZ vector (Thermo Scientific) and the retrovirus plasmid pSUPER.retro.puro vector (OligoEngine) were used to stably express miRNAs. The region 145921-146050 on the EBV genome (EMBL:AJ507799.2) encompassing the pri-mir-18-5p sequence was used. For a negative control sequence, to match the sequence of miRNA mimic control (MC), C.elegans miR-67 stem-loop sequence was used (mirBase accession ID: MI0000038). Two complementary DNA oligonucleotides based on these sequence with overhangs for restriction enzyme site (XhoI and MluI for pGIZ and BglII and HindIII for pSUPER) were synthesized, followed by annealing at a concentration of 100um in the NEB buffer 2 (Biorad) in a thermal cycler. The annealing condition was as follows. 4min at 95°C, 4min at 90°C, 4min at 85°C, 4min at 82°C, 4min at 80°C, 4min at 78°C, 4min at 75°C, 10min at 72°C, 4min at 70°C, 4min at 68°C, 4min at 65°C, 4min at 60°C, 10min at 55°C, 4min at 50°C, 4min at 45°C, 4min at 40°C, 10min at 37°C, and 10min at 25°C. After annealing, DNA fragments were inserted into linearized pSUPER and pGIZ vectors respectively.

For luciferase plasmid cloning, MAP3K2 cDNA was first reverse transcribed from the RNA of the BL36 cell line using Iscript select cDNA synthesis kit (Biorad). An oligo(dt) primer was used for the 3'UTR and coding region, while a specific reverse primer (GGCCAGCGGCCGCAATTTGAAAATTAGGAAAAT) was used for the 5'UTR in the RT reaction. The wild-type 3'UTR (3018bp) [451], 5'UTR (457bp) and coding region (1860bp) were then amplified by PCR from the cDNA and inserted into the XhoI/NotI sites (for 3'UTR and 5'UTR) or the PmeI/NotI site (for coding region) of the psiCHECK2 dual luciferase vector (Promega). The following primer sequences were

used: 3'UTR: forward primer 5'-GATCGCTCGAGTAGCAGCCAGTAAC-3' and reverse primer 5'- TATTAGCGGCCGCCCATTTTCCAGCGTCAG -3'; 5'UTR: forward primer 5'- GATCGCTCGAGACGAAAACAACGACCAG -3' and reverse primer 5'- GGCCAGCGGCCGCAATTTGAAAATTAGGAAAAT -3'; Coding region: forward primer 5'- AATTCGTTTAAACCTGGATGATCAGCAAGCTTTGGC-3'and reverse primer 5'- GGCCAGCGGCCGCCTAGTGATAATGCACAAACAT-3'. Cloned products were confirmed by DNA sequencing. The mutated 3'UTR DNA was obtained by PCR on the wild-type 3'UTR using the Q5 polymerase (Biorad) and specific primers containing mutation sites (Forward primer: 5'-CGTAAGTTTTTTTTTTAATTTTTATAATGTACTG-3' and reverse primer 5'-TGGTTTGTAAGGGAAAAAAAGATTAAATATAAAAAATTTAG -3'.

**Retrovirus and lentivirus production and cell infection**

Lentivirus particles were produced by transient cotransfection of the pGIZ plasmid and the packaging plasmids (psPAX2 and pMG2.D, gift of Philip Hinds) into HEK239T cells using lipofectamine 2000 transfection reagent. After 12 hr, cells were cultured in fresh DMEM and the supernatant was collected every 24 hr for 2 days. The virus supernatant was filtered using a 0.45μm syringe filter and concentrated by the Lenti-X Concentrator (Clontech), followed by resuspension with 1 ml sterile Dulbecco's phosphate-buffered saline (DPBS). Retrovirus was generated by cotranfestion of pSUPER plasmid and pCL-Ampho packaging vector (gift of Philip Hinds) into HEK239T cells. The supernatant was harvested at 48h post-transfection and filtered through a 0.45μm syringe filter. For infection, $2 \times 10^6$ Akata cells or B95.8 cells were respectively resuspended in 200ul concentrated lentivirus or 5ml retrovirus-containing supernatant in the presence of

polybrene (8 μg/ml) in a 6-well plate. Plates were centrifuged at 1,000 rpm for 30 min at room temperature then incubated at 37°C in a $CO_2$ incubator. After 48hr, 1 μg/ml puromycin was added to select for the miRNA-expressing cells.

**Microarray analysis**

$2x10^6$ EBV-negative B cells (BL2 and BJAB) were transfected with 100 pmol EBV miRNAs followed by microarray analysis using Affymetrix human HGU133 plus 2.0 array (Board Institute). HGU133 plus 2.0 contains 55, 000 probe sets, representing nearly 40,000 genes. Each miRNA was transfected in duplicate in both cell lines. For data analysis, the dChip software was applied to normalize the arrays and extract gene expression values. The mean expression value was obtained from duplicate experiments and the fold change ratio of averaged EBV mir-transfected experiment over averaged MC was calculated. A ratio of 1 refers to no change; below 1 reflects a down regulation while a ratio above 1 indicates up regulation. Potential targets were the consensus genes extracted from both cell lines. The cluster analysis of potential targets was presented as a heat map generated by Java TreeView 1.1.6 [452].

**qRT-PCR and Virion DNA PCR**

Analysis of mature miRNA expression was performed using miRNA-specific qRT-PCR as described in Section II. Gene expression was assessed by qRT-PCR. RNA was reverse transcribed by the iScript cDNA synthesis kit (Biorad), followed by real-time PCR using IQ SYBR Green supermix (BioRad) and specific primers. The housekeeping gene GAPDH was used as an internal control for normalization. For EB virion PCR, real-time DNA PCR with IQ SYBR Green and specific primers for the gp350 gene were used. Absolute virion copy numbers were calculated based on a standard curve, which was

generated by PCR on the gp350 gene of serial 10-fold dilutions of EBV (B95-8 Strain) quantitated viral DNA (Advanced Biotechnologies). Both qRT-PCR and DNA PCR reactions were performed by the Bio-Rad iCycler using the following program: step 1, one cycle of 5 min at 95°C; step 2, 40 cycles of 15 s at 95°C and 1 min at 60°C; step 3, one cycle of 1 min at 95°C.

**Western blot**

Standard western blot analysis was carried out using whole cell extracts lysed by RIPA buffer (Pierce) with Halt protease & phosphatase Inhibitors (Thermo Scientific). A rabbit polyclonal antibody to MAP3K2 (sc-1088, Stanta Cruz) at a dilution of 1:500 was used to detect the MAP3K2 protein level, and a mouse monoclonal antibody to GAPDH (sc-69778, Santa Cruz) at a dilution of 1:5000 was used to detect the GAPDH protein level. Anti-rabbit IgG-HRP (#7074S, cell signaling) or anti-mouse IgG-HRP (#7076S, cell signaling) were used as secondary antibodies at a dilution of 1:5,000. The immunoreactive bands were detected with the ECL western blotting substrate (Perkin Elmer). Band intensities were quantified by densitometry using ImageJ software.

**Establishment of B95.8 infected lymphoblastoid cell line (LCL)**

Peripheral blood was diluted with an equal volume of 1x PBS, layered onto Ficoll-paque plus (GE Healthcare), and centrifuged for 2,000 RPM for 30 min at room temperature. The mononuclear cells layer (buffy coat) was harvested and washed twice in PBSA and re-centrifuged. The resulting cell pellet which was peripheral blood mononuclear cell (PBMC) was resuspended with complete RPMI 1640 medium. For EBV transformation, $10^6$ of PBMCs were cultured with 10 ml EBV-containing culture supernatant of B95.8

and 1 μg/ml cyclosporin A to inhibit the growth of T lymphocytes. The established B95.8-LCL was obtained after 4 weeks or longer.

**Ago2- immunoprecipitation (IP)**

Ago2-IP was performed using the Magna RIP kit (Millipore) according to the manufacturer's instruction. Briefly, 2-3 x $10^7$ cells were lysed in RIP lysis buffer followed by incubation with magnetic beads conjugated with 5μg of human anti-Ago2 antibody (Millipore) or negative control anti-IgG (Millipore) and rotation overnight at 4°C. After incubation, the complex was washed 8 times with ice cold RIP wash buffer and separated by a magnetic separator every time. The complex was subsequently incubated with Proteinase K to digest the protein. The RNA was recovered by precipitation with 100% ethanol, washing with 85% ethanol and finally resuspended with nuclease-free water.

**Luciferase reporter assay**

Reporter assays were carried out in 96-well plates. 100 ng of the psiCHECK2-3′-UTR wt, -3′-UTR mut, -5′-UTR, -coding region and 5 pmol of either miRNA mimics or LNA were co-transfected into 2x$10^4$ 293T cells. The experiments were performed in triplicate. After 24hr post transfection, luciferase activity was measured using the Dual-Glo® Luciferase Assay System (Promega) according to manufacturer's instructions and a luminometer. The result are presented as the fold change of normalized luciferase (Renilla/Firefly) in the group of insert vector ± miRNA ± LNA relative to the levels of control vector-transfected cells.

**Viral reactivation and EB virion isolation**

B95.8 or B95.8-LCL cells were treated in replicate with 30 ng/ml TPA and 5mM BA for 48 hr, and Akata2A8.1 cells were treated with 2.5 ug/ml anti-Human IgG/IgM antibodies (Jackson ImmunoResearch) for 72hr. RNA was isolated from the cells. The supernatant was collected and filtered through a 0.45 um syringe filter. 100 ul of supernatant was then treated with TURBO™ DNase (Invitrogen) to digest free naked DNAs, and extracted by an equal volume of QuickExtract DNA buffer (Epicentre) at 65°C for 10 min, followed by vortexing and termination of the reaction at 98°C for 2 min. 10ul of final solution containing cell-free virions were used for viral DNA PCR.

## 4.3 Results

### BART18-5p targets cellular genes functionally related to viral replication

Our previous study identified differentially expressed EBV miRNAs in normal infected tissue, spontaneous lymphblastoid cell lines and neoplastic biopsies. These include the miRNAs that are absent from EBV-infected primary B cells but highly expressed in Latency III cell lines and tumors (i.e. BART 10 and 14*) , or vice versa (Table 9).

| Copy numbers per cell | | |
|---|---|---|
| **miRNA** | **LCL** | **MemB** |
| **10** | **38** | **<1** |
| **14*** | **96** | **<1** |
| **18-5p** | **42** | **294** |

**Table 9. EBV miRNA expression in normal infected B cells in vivo.**

Of particular interest is BART 18-5p, a miRNA abundantly expressed in the normal infected memory B cells with approximately 300 copies per cell. In the memory compartment, EBV persists latently with a shutoff of all viral protein expression except when the cells divide and express EBNA1. High levels of 18-5p expression in memory B cells may suggest a potential role in maintenance of viral latency. To understand this role, I attempted to identify the cellular genes regulated by 18-5p by performing transfection of miRNA mimics into the EBV-negative B cell lines BL2 and BJAB followed by microarray analysis. MiRNA mimics are synthetic double-strand duplexes with the unwanted strand chemically modified to avoid its off-target activity. For normalization purpose, I transfected cells with a miRNA mimic control (MC), a non-physiological C. elegans-derived miRNA that has no mammalian gene target. Gene arrays have been used extensively to identify putative targets for miRNAs [453-456]. Microarray data extraction, categories for gene selection and fold change calculations are described in Material and Methods. I generated a list of consensus genes downregulated in both BL2 and BJAB cells by 18-5p and aligned it with the same set of genes from other miRNA-transfected groups (10 and 14*). As presented in the heat map (Figure 25A), it is very clear that 18-5p exhibits a distinct pattern of downregulated genes opposed to 10 and 14* as indicated by the blue color. A number of genes moderately downregulated (-1.2 ~ -1.9 fold) by 18-5p are either upregulated or remain unchanged by 10 and 14* (Figure 25A, Table 10).

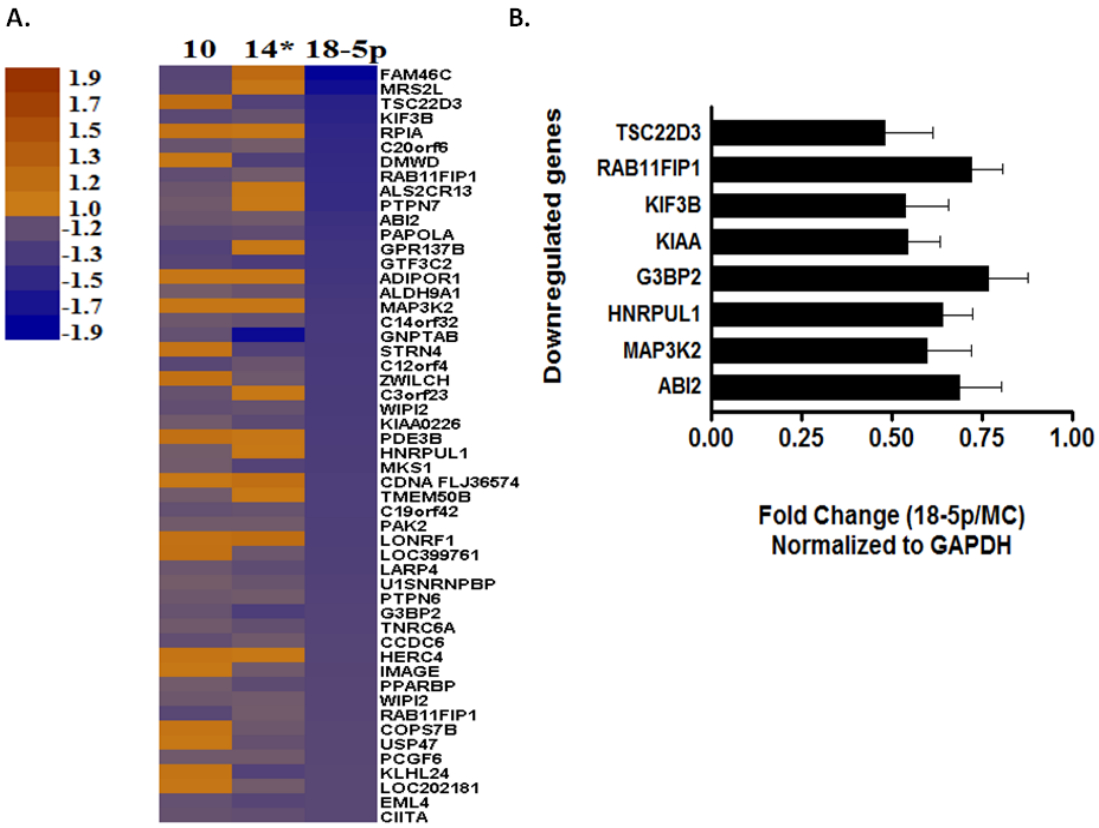



**Figure 25. BART18-5p targets cellular genes related to viral replication.**

A. Heat map of consensus genes both downregulated and upregulated in BL2 and BJAB cells by 18-5p in comparison to the same gene set of 10 and 14*. The fold change was calculated as a ratio of average EBV mir-transfected cells over average MC. A ratio of 1 means no change, below 1 refers to down regulation and above 1 indicates up regulation. The color bar shows the color contrast level of the heat map. Purple and Brown indicate up regulation (up to 1.9 fold) and down regulation (down to -1.9 fold), respectively. 18-5p clearly shows a distinct pattern of down regulated genes compared to other miRNAs. B. Validation of downregulated genes in Akata cells transfected with 18-5p using qRT-PCR.

| Genes | 10 | 14 | 18-5p |
|---|---|---|---|
| FAM46C | -1.21746 | 1.178212 | -1.83805 |
| MRS2L | -1.19671 | 1.03768 | -1.72709 |
| TSC22D3 | 1.148631 | -1.25431 | -1.55084 |
| KIF3B | -1.18647 | -1.1014 | -1.53364 |
| RPIA | 1.070408 | 1.030157 | -1.5049 |
| C20orf6 | -1.08605 | -1.00777 | -1.48934 |
| DMWD | 1.026464 | -1.26817 | -1.4823 |
| RAB11FIP1 | -1.14973 | -1.02284 | -1.47293 |
| ALS2CR13 | -1.07517 | 1.021961 | -1.46412 |
| PTPN7 | -1.03932 | 1.002199 | -1.46244 |
| ABI2 | -1.07755 | -1.03771 | -1.42383 |
| PAPOLA | -1.18595 | -1.15553 | -1.40459 |
| GPR137B | -1.25547 | 1.018358 | -1.38267 |
| GTF3C2 | -1.22147 | -1.30188 | -1.38024 |
| ADIPOR1 | 1.03108 | 1.029391 | -1.37618 |
| ALDH9A1 | -1.00598 | -1.0823 | -1.35338 |
| MAP3K2 | 1.035555 | 1.032586 | -1.33856 |
| C14orf32 | -1.05634 | -1.11492 | -1.338 |
| GNPTAB | -1.12486 | -1.76663 | -1.33563 |
| STRN4 | 1.053008 | -1.26062 | -1.33116 |
| C12orf4 | -1.21184 | -1.08216 | -1.32821 |
| ZWILCH | 1.099257 | -1.05359 | -1.32324 |
| C3orf23 | -1.11264 | 1.012409 | -1.31885 |
| WIPI2 | -1.14295 | -1.11116 | -1.31868 |
| KIAA0226 | -1.03062 | -1.17954 | -1.3122 |
| PDE3B | 1.10632 | 1.032777 | -1.30226 |
| HNRPUL1 | -1.01326 | 1.021578 | -1.29925 |
| MKS1 | -1.02008 | -1.24189 | -1.29861 |
| CDNA FLJ36574 | 1.022552 | 1.120862 | -1.28748 |
| TMEM50B | -1.01891 | 1.019255 | -1.287 |
| C19orf42 | -1.12074 | -1.09238 | -1.28518 |
| PAK2 | -1.02527 | -1.02717 | -1.27763 |
| LONRF1 | 1.091899 | 1.13789 | -1.27661 |
| LOC399761 | 1.104624 | -1.07128 | -1.27154 |
| LARP4 | -1.07095 | -1.16124 | -1.2694 |
| U1SNRNPBP | -1.00976 | -1.08476 | -1.26454 |
| PTPN6 | -1.05648 | -1.0283 | -1.25386 |
| G3BP2 | -1.10417 | -1.29028 | -1.24357 |
| TNRC6A | -1.04262 | -1.13573 | -1.23994 |
| CCDC6 | -1.14097 | -1.0374 | -1.23746 |
| HERC4 | 1.06058 | 1.015186 | -1.23265 |
| IMAGE | 1.023415 | -1.03898 | -1.22901 |
| PPARBP | -1.02507 | -1.16802 | -1.22527 |
| WIPI2 | -1.06253 | -1.03087 | -1.22201 |
| RAB11FIP1 | -1.20401 | -1.01956 | -1.21648 |
| COPS7B | 1.06063 | -1.06146 | -1.21246 |
| USP47 | 1.019035 | -1.11968 | -1.21188 |
| PCGF6 | -1.04453 | -1.03678 | -1.21176 |
| KLHL24 | 1.063089 | -1.25182 | -1.20742 |
| LOC202181 | 1.036173 | -1.01875 | -1.20683 |
| EML4 | -1.12605 | -1.222 | -1.20269 |
| CIITA | -1.11101 | -1.14101 | -1.20208 |

**Table 10. Gene list from microarray data**

Interestingly, most of these genes are functionally related to autophagy, viral replication, viral budding and endocytic protein recycling. For instance, RAB11FIP1, KIF3B and KIAA are the key players in regulating endocytic protein transport and autophagy with potential involvement in virion packaging [457-460]. ABI2 is associated with virus assembly and viral release for Arenavirus, hepatitis B virus, Sendai virus and HIV [461-464]. G3BP2 plays an pivotal role in Sindbis virus replication via interacting with virus-encoded nonstructural proteins [465], while HNRPUL1 is recruited to the viral replication center thus modulates adenovirus replication [466]. Another interesting gene is Mitogen-activated protein kinase kinase kinase 2 (MAP3K2). It was shown that the MAP kinsae signaling transduction pathway is a positive regulator of murine gammaherpesvirus MHV-68 lytic replication [343]. In addition, a study using genome-wide RNAi screens in Jurkat T-cells revealed that MAP3K2 is important for HIV-1 replication [467]. To validate down regulation of these genes, I transfected another EBV-negative B cell line Akata with 18-5p and examined the mRNA level by qRT-PCR. All of these genes were suppressed by 18-5p compared with the MC control, although the reduction only ranged from 20% to 40% (Figure 25B). This observation is consistent with the long-held belief that miRNAs typically exert subtle inhibitory effects on many mRNAs embedded within intricate regulatory networks. This means that individual genes are repressed to a very modest extent but targeting multiple genes within the same pathway or genes that govern the same biological process could augment the effect and lead to a stronger response. Taken together, these results suggests that 18-5p is expressed in EBV-infected primary B cells and targets a panel of host genes associated with viral replication.

**Bart 18-5p specifically downregulates MAP3K2**

We decided to focus on MAP3K2 as MAPK family members are an important signaling molecule in regulating gene expression that is essential for herpes virus replication [110, 123, 128, 343, 468-471]. To test if 18-5p specifically targets MAP3K2, two distinct systems (miRNA mimics and vector-expressing pri-miRNAs) were introduced into Akata cells to express 18-5p. MiRNA mimics that act as a Dicer cleavage product were transiently transfected into Akata B cells for 24 hr by electroporation. The vector-expressing 18-5p (pGIZ-18-5p) is a lentiviral plasmid comprising the 18-5p precursor sequence. The lentiviral vector carries a drug selection marker ensuring stable expression of the miRNAs. The expression of mature 18-5p was validated in pGIZ-18-5p cells using RT-PCR with specific stem-loop primers (not shown). The inhibition of MAP3K2 at both the mRNA and protein levels was measured by real-time qRT-PCR and Western blot, respectively. In contrast to control miRNAs (MC and pGIZ-MC), 18-5p from both systems suppressed MAP3K2 gene expression (Figure 26A). While miRNA mimcs reduced the level of MAP3K2 mRNA by ~40%, plasmid-derived 18-5p only downregulated it 20% when normalized to the control group. A concomitant decrease in MAP3K2 at the protein level (48% and 24% down regulation respectively) was also observed, suggesting that 18-5p represses MAP3K2 by inducing degradation of the specific mRNA, although an impact on translation could not be ruled out (Figure 26B). MAP3K2 appears to be exclusively inhibited by 18-5p as other EBV miRNAs (10 and 14*) did not affect the RNA level of this gene (not shown).

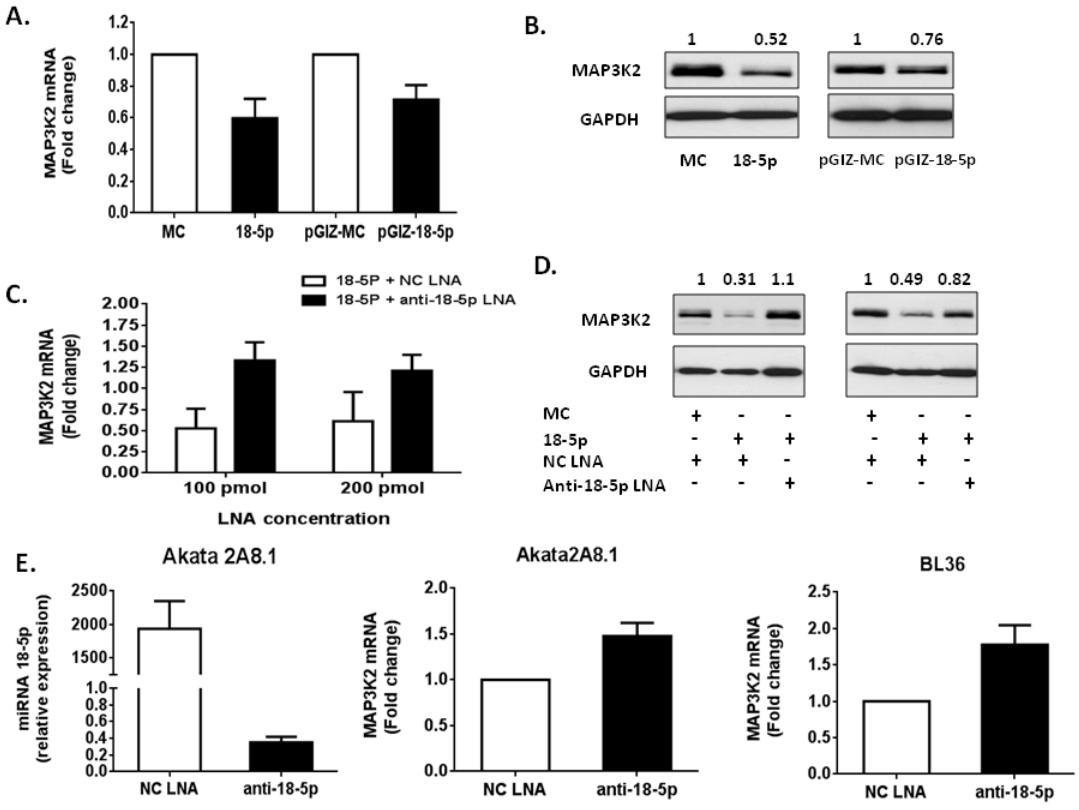



**Figure 26. Bart 18-5p specifically targets MAP3K2 in B cells.**

A. mRNA level of MAP3K2 in Akata cells transfected with miRNA mimics (MC and 18-5p) or infected with lentiviruses that express pri-miRNAs (pGIZ-MC and pGIZ-18-5p). Down regulation was presented as fold change normalized to the control group (MC and pGIZ-MC ). B. Protein level of MAP3K2 from the same experiment as shown by Western Blot. Band intensities were quantified by densitometry using ImageJ software and presented as a ratio to the MC group. C. Knockdown of 18-5p using a 100 or 200 pmol concentration of anti-18-5p LNA reversed the reduction of MAP3K2 by 18-5p at the RNA level. The data are presented as fold change relative to the MC+ NC LNA group. D. Protein level of MAP3K2 in the same experiment as C. E.18-5p miRNA expression in Akata2A8.1 cells treated with the anti-18-5p inhibitor or NC LNA. F and

G. LNA knockdown of 18-5p expression leads to elevated levels of MAP3K2 in the EBV-positive BL cell lines, Akata28.1 and BL36.

To validate that down regulation of MAP3K2 was specific, Locked Nucleic Acids (LNA) were introduced to block 18-5p followed by measurement of MAP3K2 expression. LNA is a conformational RNA analog designed to bind to a complementary miRNA and interfere with its inhibitory function. LNA have been used most frequently to knock down miRNAs [472]. Cotransfection of 18-5p with either 100 or 200 pmol of scrambled LNA (NC LNA) led to a significant decrease in MAP3K2 at the RNA level, but introduction of the specific anti-18-5p LNA into the 18-5p-transfected cells was able to rescue this decrease (Figure 26C). This reversal was even more striking at the protein level, where a 2 or 3 fold increase was observed in MAP3K2 with the two concentration of LNA inhibitors respectively (Figure 26D). To further confirm its ability to rescue MAP3K2, I transfected the anti-18-5p LNA into the EBV-positive BL cell lines Akata2A8.1 and BL36. Akata2A8.1 is an EBV-negative cell line reinfected with recombinant EBV, while BL36 naturally harbors the wild-type virus. Both lines have endogenous expression of 18-5p (shown in Section II). Anti-18-5p LNA dramatically suppressed the endogenous miRNA expression in Akata2A8.1` cells (Figure 26E), leading to a relatively higher level of MAP3K2 (40% increase) in the cells (Figure 26F). The effect of the anti-18-5p LNA seems to be more striking in BL36 cells in which a 70% increase of MAP3K2 mRNA was observed (Figure 26G). Interestingly, overexperssion of 18-5p using miRNA mimics and lentiviral plasmids in Akata 2A8.1 cells continuously downregulated MAP3K2 mRNA at a moderate level (Figure 27A). In

addition, the effect of 18-5p on B95.8 was also tested. B95.8 is a marmoset B cell line transformed by EBV obtained from a patient with infectious mononucleosis. B95.8 lacks a big portion of BART region including 18-5p. Introduction of miRNA mimics or the retroviral vector-expressing 18-5p into B95.8 cells also reduced MAP3K2 expression (Figure 27B). This was further confirmed with a LNA experiment (Figure 27C). Taken together, all the data above suggest that MAP3K2 is a specific cellular target for 18-5p.

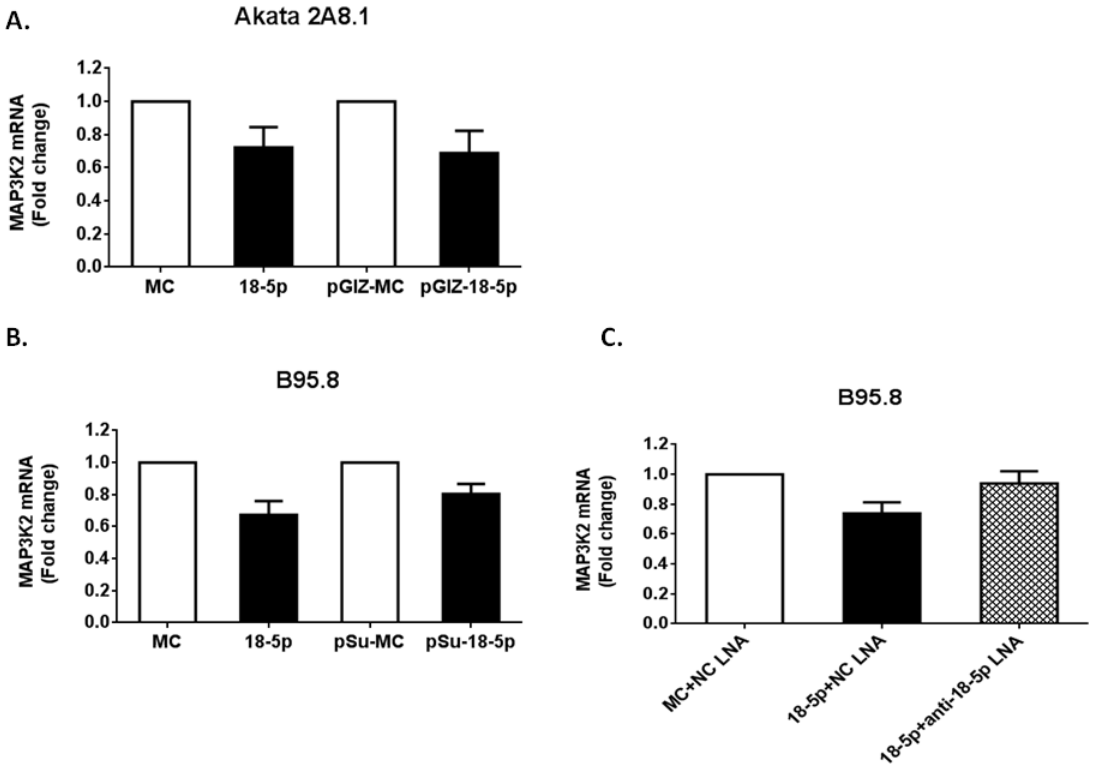



**Figure 27. Overexpression 18-5p in Akata2A8.1 and B95.8 cells.**

A. MAP3K2 mRNA level in Akata2A8.1 transiently transfected with an 18-5p mimic and MC or stably expressing 18-5p or MC using lentiviral plasmids. B. MAP3K2 mRNA level in the marmoset B cell line B95.8 transiently transfected with an 18-5p mimic or MC or stably expressing 18-5p and the MC using retroviral plasmids. C. Knockdown of 18-5p in B95.8 modestly reversed the reduction of MAP3K2 by 18-5p at the RNA level.

**18-5p physically interacts with the MAP3K2 transcript at the 3'UTR**

The silencing of genes requires mature miRNAs to stably aggregate with the Argonaute (Ago) protein, the core protein in the RNA-induced silencing complex (RISC), and then interact with the mRNA. Several studies have reported application of a new biochemical approach to analyze cellular mRNA associated with the RISC [473-477]. Overexpression of exogenous miRNAs followed by immunoprecipitation (IP) of Ago proteins permits the rapid isolation and identification of miRNA-bound mRNAs. To test if MAP3K2 is a real physiological target for 18-5p, I pulled down the endogenous Ago2 from the pGIZ-18-5p and pGIZ-MC Akata cells using magnetic protein-G beads conjugated with appropriate antibodies (anti-Ago2 and anti-IgG negative control). RNA was recovered from the precipitates and subsequently quantified by qRT-PCR. Western blot analysis of Ago2 pull-downs probed with anti-Ago2 antibody showed an intense band with a molecular weight of 95 kDa, indicating that Ago2 complexes were successfully precipitated (Figure 28A). In contrast, there was no visible Ago2 in the IgG control pull-downs. Next, I determined the association of miRNA 18-5p with Ago2. Distinct from the IgG control pull-downs, 18-5p was enriched 400 fold in the Ago2 precipitates from the pGIZ-18-5p cells (Figure 28B).

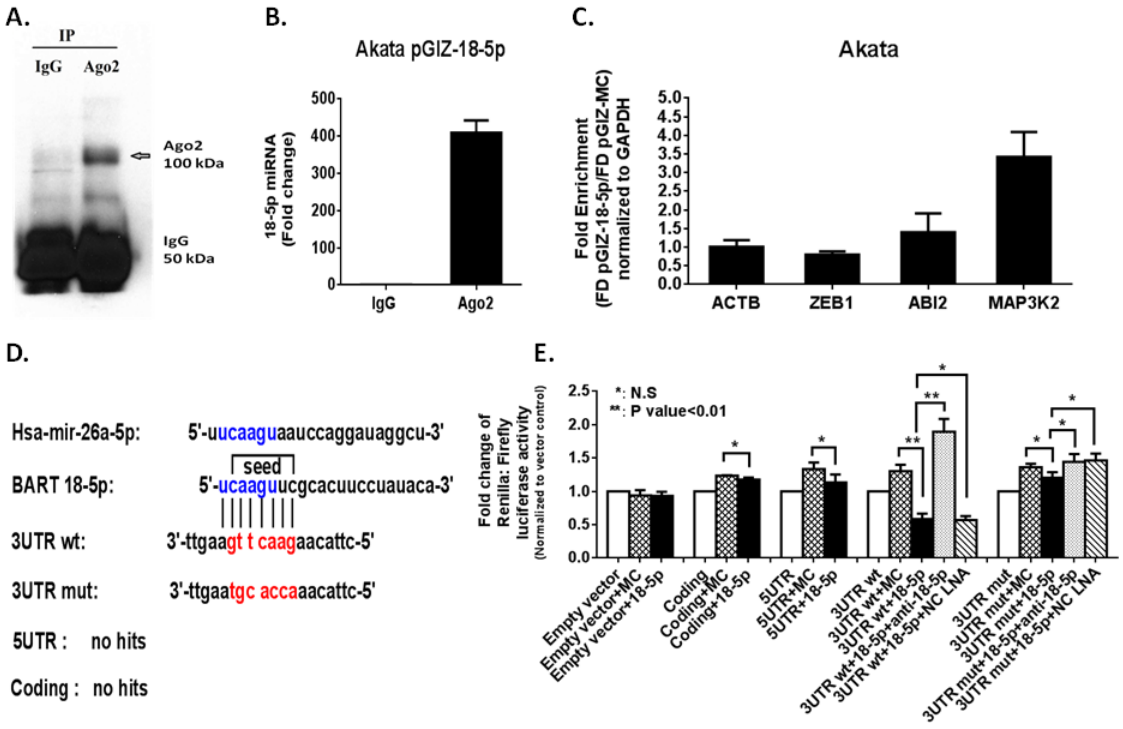



**Figure 28. Bart 18-5p physically interacts with the 3' UTR of MAP3K2.**

A. IP of the Ago2 complex and analysis of the pulldown by western blotting. Ago2-IP was performed using the Magna RIP kit (Millipore) according to the manufacturer's instruction. Complexes were pulled down with 5 ug anti-Ago2 antibody or an anti-IgG negative control antibody. B. qRT-PCR on 18-5p expression indicated a strong association between EBV miRNA and Ago2 (up to 400 fold enrichment). C. Fold enrichment of MAP3K2, ACTB, ZEB1, AB12 in the Akata-pGIZ-18-5p Ago2 pulldowns. The fold enrichment of a potential target in the Ago2 pull-downs was calculated by normalizing to the IgG control pull-downs relative to GAPDH expression

in both cells. Then the fold enrichment was obtained by dividing the resultant fold difference of pGIZ-18-5p cells by that of pGIZ-MC cells.

D. Potential binding sites for 18-5p on the 3' UTR of the MAP3K2mRNA. 18-5p has identical nucleotides (blue) in the seed region to the human miRNA 26a-5p. Target prediction analysis indicated that the seed region (Red) of 18-5p has a complementary binding site in the 3' UTR but not the 5'UTR or coding region. For the mutated 3' UTR, the potential binding nucleotides were replaced to disrupt the complementarity (Red). E. Luciferase assays on HEK293 cells cotransfected by luciferase reporter vectors encoding 3' UTR wt , 3' UTR mut, 5' UTR and coding region with or without LNA inhibitors. The result showed that the 3' UTR wt can be silenced by 18-5p (black bar) but rescued by anti-18-5p LNA (dotted bar). However, 3' UTR mut completely disrupted the interaction between 18-5p and the 3' UTR . Values represent the averages of three replicates with the SD. *: N.S; **: p value<0.01.

The enrichment of miRNAs is specific, since the small non-coding RNA U1 was not associated with Ago2 (Figure 29A). Interestingly, the cellular miRNA mir-200c was also enriched by nearly 130 fold in the Ago2 complexes (Figure 29B). This result indicates that the RISC unbiasedly captured miRNAs regardless of the origin, and since exogenous 18-5p is constitutively expressed it is constantly loaded onto the Ago2 leading to a higher level of enrichment.

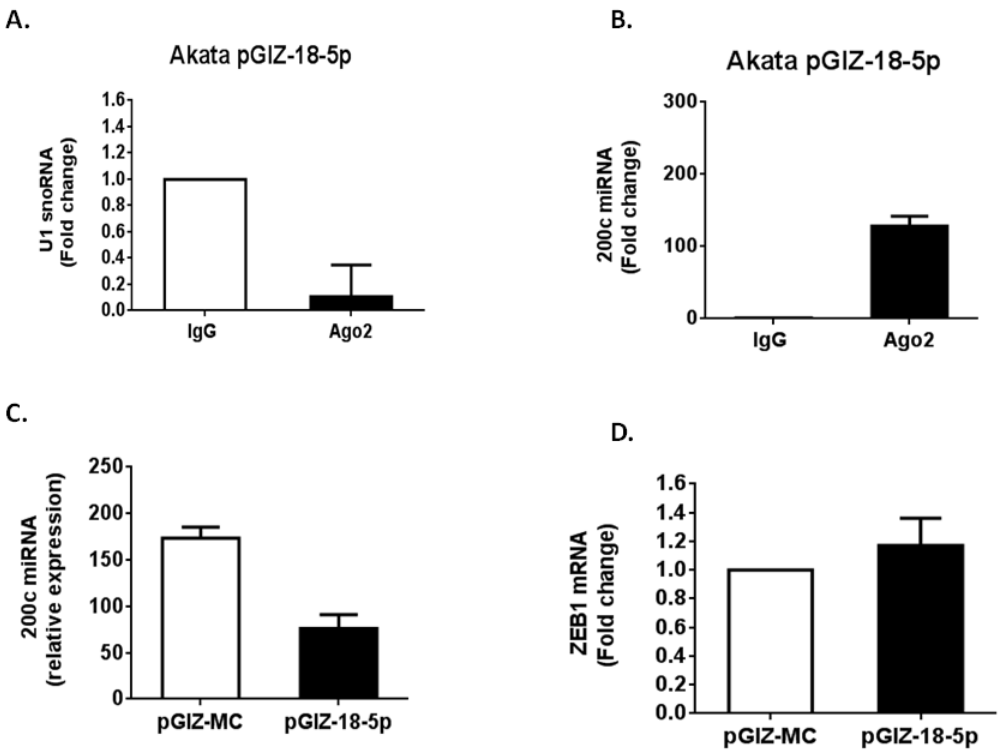



**Figure 29. Ago2-IP on 18-5p expressing Akata and B95.8 cells.**

A. qRT-PCR for the small RNA U1 on the Ago2 and IgG pulldowns from Akata pGIZ-18-5p cells. B. qRT-PCR for 200c miRNA on the Ago2 and IgG pulldowns from Akata pGIZ-18-5p cells. C. Relative expression of 200c miRNA in Akata cells infected with lentiviruses that express MC and 18-5p. D. mRNA level of ZEB1 in Akata cells infected with lentiviruses that express MC and 18-5p.

For target gene enrichment, I assessed the expression level of the gene of interest in both Ago2 and IgG pull-downs from the 18-5p-programmed and MC-programmed cells. Because 18-5p is absent from the Akata pGIZ-MC cells, I was able to discern the abundance of specific mRNAs in the pGIZ-18-5p RISC when compared with the pGIZ-MC RISC. To do that, I first calculated the fold difference of a potential target in the Ago2 pull-downs by normalizing to the IgG pull-downs relative to GAPDH expression in both cells respectively. Then the fold enrichment was obtained by dividing the resultant fold difference of pGIZ-18-5p cells by that of pGIZ-MC cells. As seen, MAP3K2 mRNA was enriched by 3.5 fold in the pGIZ-18-5p precipitates as opposed to the pGIZ-MC, whereas the association of internal control gene beta-actin (ACTB) with Ago2 was not enhanced in the pGIZ-18-5p cells (Figure 28C). ZEB1 is an identified target for human miRNA mir-200c. pGIZ-18-5p Akata cells neither increased the expression of mir-200c nor downregulated the ZEB1 level (Figure 29C-D). ZEB1 was not enriched in the Ago2 complex from the pGIZ-18-5p Akata cells (Figure 28C), confirming that the enrichment of MAP3K2 mRNA in the pGIZ-18-5p cells specifically results from 18-5p expression. Interestingly, a gene AB12 also identified by microarray analysis as a potential 18-5p target was not significantly enriched in the Ago2 complex, meaning that it might be an indirect target for 18-5p (Figure 28C). [478].

To further determine if MA3PK2 is a bona fide target for 18-5p, I performed bioinformatics analysis on 18-5p and the MAP3K2 gene using the miRNA target prediction software MiRanda in an attempt to identify a potential binding site. MiRanda is a commonly used algorithm for miRNA target prediction. It predicts targets based on the whole miRNA sequence complementarity to the 3' UTR, but gives more credit to the

match-up at the 5'end of mature miRNAs rather than the 3' end. It also takes into account the thermodynamics of the miRNA–mRNA duplex and the associated conservation of target genes across species [479]. Computational analysis indicates that the 5' end of the mature 18-5p has eight nucleotides of perfect complementarity within the 3'untranslated regions (3′ UTR) of MAP3K2 (Figure 28D, line alignment). This is the only binding site found in the 3'UTR and it contains a perfectly complementary binding site for the seed region of 18-5p (position 2–8 at 5'end). Perfect complementarity of the seed region to the 3'UTR elements in the mRNA is believed to be the key factor in posttranscriptional repression [480, 481]. Scanning of the 5'UTR and coding sequences of MAP3K2 did not reveal any putative binding sites for 18-5p. Interestingly, 18-5p shares a high degree of sequence homology at the 5' end with the human miRNA mir-26a-5p (Figure 28D, blue nucleotides). mir-26a-5p also downregulates MAP3K2 by targeting its 3'UTR consequently inhibiting JNK-dependent apoptosis in human glioblastoma cells [451]. To validate whether 18-5p could interact with the 3′ UTR of MAP3K2, I performed luciferase reporter assays. I cloned the 3'UTR (3018bp) [451], 5'UTR (457bp) and coding region (1860bp) of MAP3K2 downstream of the Renilla luciferase coding region in the psiCheck2 vector. The psiCheck2 vector expresses firefly and Renilla luciferase as a single transcript and the firefly reporter is used for intraplasmid transfection normalization. HEK293 cells were transfected with either the control (i.e., no miRNA mimic and LNA inhibitor) or the insert vectors with miRNAs and/or LNAs followed by measurement of luciferase activity at 24 hour posttransfection. The result is presented as the fold change of normalized luciferase (Renilla/Firefly) in the group of insert vector ± miRNA ± LNA relative to the levels of control vector-transfected cells. As seen, 18-5p

dramatically reduced the activity of the luciferase vector containing the full-length 3'UTR wt (40% down regulation, $p<0.01$), while MC had no effect on the expression of this reporter (Figure 28E). In contrast, 18-5p mimics did not inhibit luciferase expression in cells cotransfected with the empty reporter vector, 5'UTR or coding region of MAP3K2 as compared to MC (Figure 28E). The reduction of luciferase activity in the 3'UTR group was directly proportional to 18-5p expression, as treatment with a specific anti-18-5p inhibitor but not NC LNA greatly restored luciferase expression (1.89 fold increase, $p<0.01$). In general, miRNAs bind to their target transcripts through a seed region located at position 2–7 or 2–8 in the mature miRNA [482]. To confirm that 18-5p exerts its effect on the 3'UTR-luciferase reporter through the predicted potential binding site, I mutated all 7 bases on the 3'UTR binding site that matched the seed region of 18-5p (Figure 28D, red residues). Mutation of the putative 18-5p binding site on the 3'UTR completely abolished the inhibitory effects of 18-5p, as no distinction was observed on luciferase expression between MC and 18-5p expressing cells (Figure 28E). This suggests that the effect of 18-5p on the luciferase gene is dependent on the presence of the 18-5p binding site on the 3'UTR. Taken together, these results in combination with the Ago2-IP data demonstrate that EBV miRNA 18-5p follows the conventional cellular miRNA biogenesis pathways, within which it is loaded onto the RISC complex, targets and downregulates MAP3K2 via binding to the 3'UTR of the gene.

**18-5p impairs EBV lytic replication upon reactivation**

MAP3K2 has been implicated as a crucial mediator in viral replication for MHV68, a mouse version of EBV [343]. Thus the ability of 18-5p to repress MAP3K2 suggests that 18-5p may be capable of blocking EBV lytic replication. The study of primary EBV lytic

infection is challenging because a permissive culture system for viral replication is lacking [59]. Current experimental models are mainly based on the reactivation of latently infected cell lines with the use of inducing chemicals or B-cell receptor stimulation. To validate the effects of 18-5p in EBV replication I overexpressed 18-5p in B95.8 cells followed by induction with 12-o-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (BA). B95.8 infected with the wild-type EBV is the most potent EBV producer. The transcription of lytic genes including the immediate early (IE) gene BZLF1 and late (L) gene glycoprotein gp350, acting as indicators for viral replication, were examined by qRT-PCR. The gene expression was normalized to the no-induction group. mRNA levels of BZLF1 and gp350 were highly elevated (nearly 900- and 2000-fold, respectively) in B95.8 upon activation by TPA/BA for 48 hr, revealing that robust replication was induced (Figure 30A). However, the induction was significantly disrupted by the presence of 18-5p as evidenced by the dramatic decrease to -500 and -1000 fold for both genes respectively ($p<0.05$) (Figure 30A). The influence appeared to be specific as introduction of anti-18-5p LNA to the cells could reverse the inhibition. In parallel, cell-free virions (DNase resistant) in the culture supernatant, representative of freshly encapsidated virus were measured by gp350 DNA PCR [471]. It is noteworthy that roughly 5% of B95-8 cells are likely to undergo spontaneous viral replication without induction [483], therefore the absolute viral copy numbers in the supernatant after induction were obtained by subtraction of values from non-induced cells. Accordingly, 18-5p expression in B95.8 resulted in decreased titers of virions in supernatant with EBV DNA copies reduced from 106 x $10^6$/ml to 70 x $10^6$/ml ($p<0.005$) (Figure 30B). The reduction in viral titers was correspondingly reversed by the anti-18-

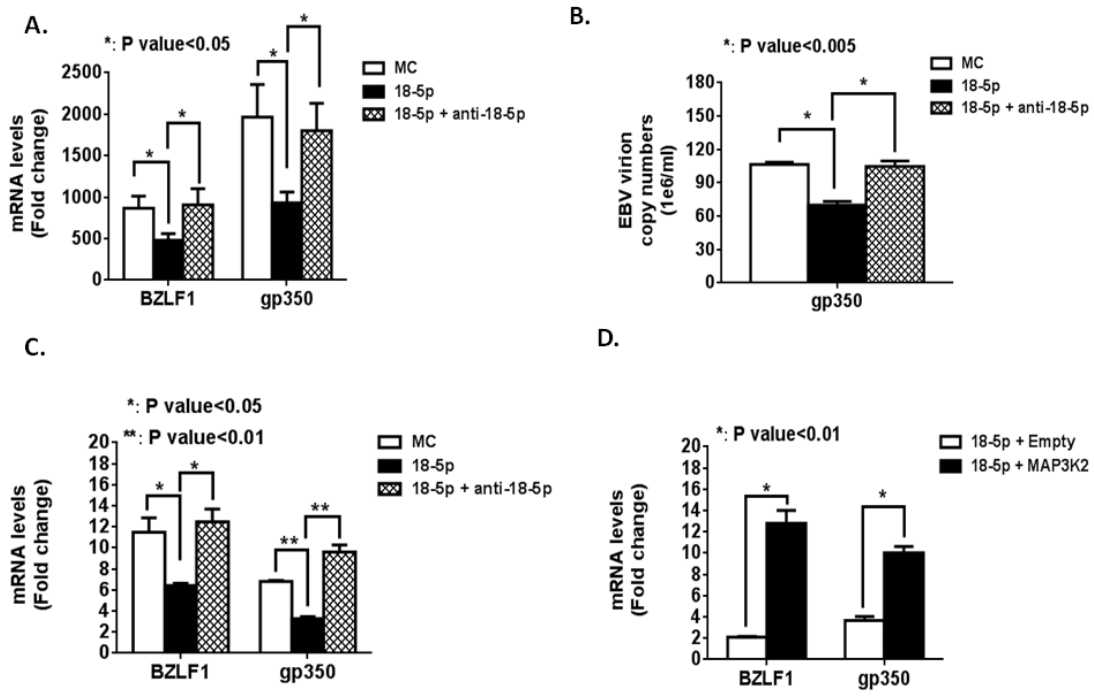



**Figure 30. Bart 18-5p impairs viral lytic replication upon reactivation.**

A. Lytic genes (BZLF1 and gp350) were quantified by qRT-PCR in B95.8 cells transfected with miRNA mimics and LNAs and induced by TPA/BA for 48 hr. The data are presented as fold change normalized to the no-induction group. B. The copy numbers of EBV virions in the supernatant in A. The absolute viral copy numbers in supernatant after induction was obtained by subtraction of values from non-induced cells. The data are presented as $x10^6$ per ml. C. BZLF1 and gp350 expression in a B95.8 infected LCL transfected with miRNA mimics and LNAs upon TPA/BA treatment for 48 hr. D. BZLF1 and gp350 expression in B95.8-LCL transfected with 18-5p + empty vector or 18-5p + MAP3K2 followed by TPA/BA induction.

5p LNA ($10^4$ x $10^6$/ml viral copies). This result suggests that 18-5p is fully responsible for the impaired viral replication.

To further confirm that the inhibitory effect of 18-5p is mediated through MAP3K2, I performed addback experiments in B95.8 with the human MAP3K2. Unfortunately, I was unable to observe the reversible effect in B95.8 (not shown). This result suggests that the human MAP3K2 may not function normally in the marmoset cellular pathways. Similar to many MAP3 kinases, MAP3K2 is a multidomain protein. It is highly possible that either regulatory or catalytic domain of human MAP3K2 do not recognize the upstream or downstream signaling molecules in B95.8. How much sequence homology in MAP3K2 there is between human and marmosets is unknown, as the genome of this particular type of marmoset (cotton-top) is not yet sequenced.

In order to obtain a human EBV positive cell line with no 18-5p expression but lytic-inducible and allowing for MAP3K2 reconstitution, I decided to transform human PBMCs with the B95.8 virus to generate a B95.8-LCLs. It is generally accepted that spontaneous or in vitro transformed LCLs are poorly permissive for EBV lytic replication [483]. However, there is evidence that LCLs show varying degrees of lytic permissivity dependent on passage or donor origin although the entire lytic population is still less than a few percent [471, 483-485]. In fact, lytic replication can be moderately induced in permissive LCLs by TPA/BA treatment [116, 124]. I generated several B95.8-LCLs but only one line was partially permissive for lytic induction. After 48 hr TPA/BA stimulation, the induction of BZLF1 and gp350 genes in this B95.8-LCL was much more modest than that of B95.8, with only an 11.5 and 6.8 fold increase for the respective genes (in the MC-treated group) (Figure 30C). Consistently, cell-free virions from B95.8-

LCL were approximately 1000 times less than B95.8 upon activation (Figure 31A). This however did not change the pattern where it showed that 18-5p potently inhibits viral replication even in the less permissive LCL, as evidenced by reduced levels of lytic gene transcription (declined to 6.4 fold in BZLF1 and 3.3 fold in gp350, $p<0.05$ and $<0.01$, respectively) (Figure 30C), as well as less released viral caspids ($15x10^3$ copies/ml in MC vs $5.1x10^3$ copies/ml in 18-5p, $p<0.05$) (Figure 31A).

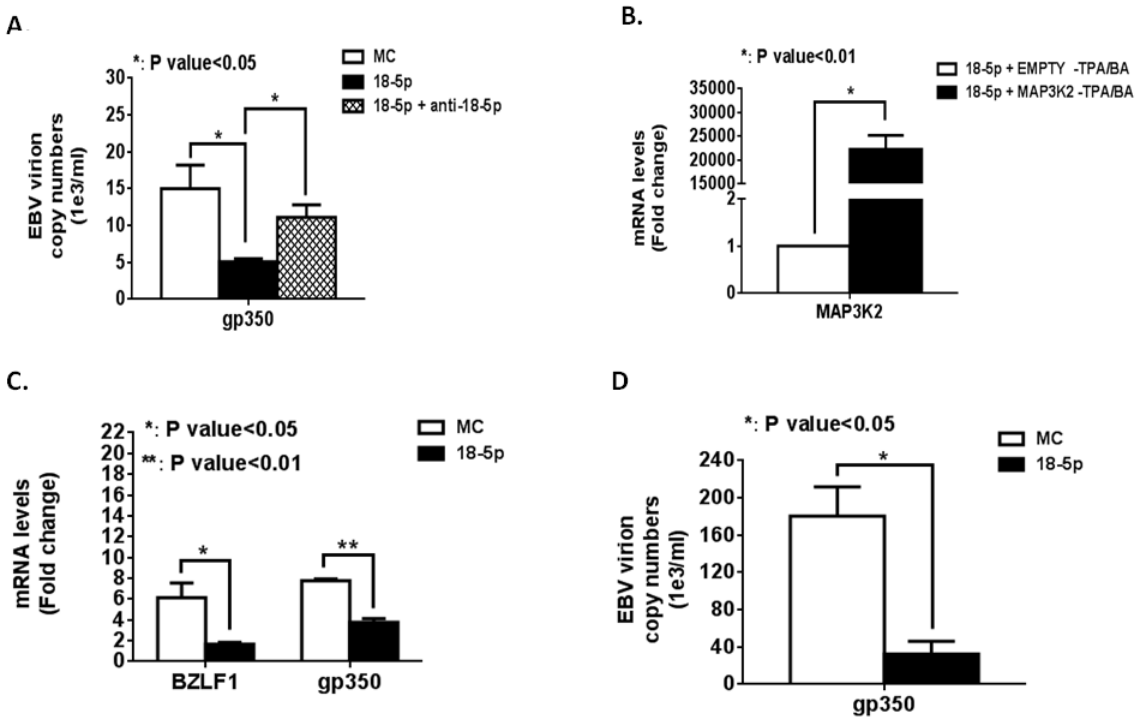



**Figure 31. Overexpression of 18-5p in B95.8-LCL and Akata 2A8.1 inhibits viral replication upon reactivation.**

A. The copy numbers of EBV virions in the supernatant collected in B95.8-LCL from Figure 30C. B. MAP3K2 mRNA levels in B95.8-LCL with overexpression of 18-5p with

or without MAP3K2 prior to reactivation. C. BZLF1 and gp350 expression in Akata 2A8.1 cells transfected with MC or 18-5p mimics upon reactivation by BCR crosslinking for 72 hr. D. The copy numbers of EBV virions in the supernatant collected in Akata2A8.1 from C.

Transient transfection of human MAP3K2 in B95.8-LCL expressing 18-5p resulted in a ~25000 fold increase at MAP3K2 mRNA levels indicting that the gene was successfully overexpressed (Figure 31B). Reconstitution of MAP3K2 significantly restored the expression of BZLF1 (increased from 2.1 fold to 12.8 fold, $p<0.01$) and gp350 (from 3.6 fold to 10 fold, $p<0.01$) (Figure 30D). However, I failed to detect an increase in virions (not shown). It is clear that the effect of 18-5p on abolishment of EBV lytic replication is partially mediated through MAP3K2 since lytic gene transcription was repaired by reconstitution of MAP3K2. MAP3K2 alone also enhanced lytic gene expression and increased virion production in B95.8-LCL upon reactivation (Figure 32A-C). However, It is likely that 18-5p also interferes with other genes associated with viral assembly and budding (Figure 25B) such that add back of MAP3K2 is not sufficient to rescue the phenotypic effects of 18-5p. Our result also support the view that analogous to its role in MHV-68, MAP3K2 is also crucial for EBV lytic replication.

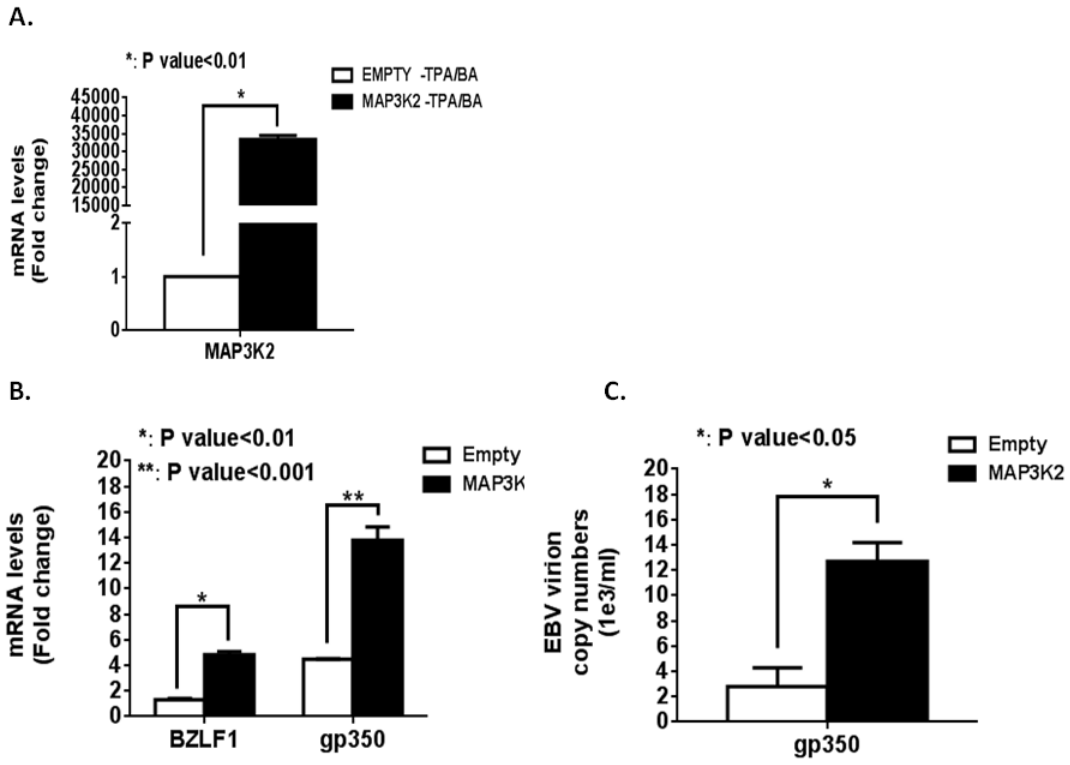



**Figure 32. Overexpression of MAP3K2 enhances viral replication.**

A. MAP3K2 mRNA levels in B95.8-LCL with overexpression of MAP3K2 or empty vector prior to the stimuli induction. B. BZLF1 and gp350 expression in B95.8-LCL was transfected with empty vector or MAP3K2 alone followed by TPA/BA 48 hr induction. C. The copy numbers of EBV virions in the supernatant collected in B95.8-LCL from B.

TPA-induced EBV lytic replication is primarily regulated by the PKC signaling pathway [123, 127]. Yet, PKC activation is not always required in some cell lines nor by some lytic inducers such as histone deacetylase inhibitors [125]. Initiation of the lytic cascade in Akata 2A8.1 after BCR stimulation involves four signaling pathways, PI3K, Rac1, Ras and PLC [97]. A large fraction of Akata cells (~50%) is substantially permissive for viral replication after surface IgG crosslinking [480, 486, 487]. Thus to determine if 18-5p also disrupts the BCR signaling pathway mediated viral replication, I transiently overexpressed 18-5p mimics in Akata2A8.1 followed by anti-immunoglobulin (Ig) treatment for 72hr. Upon reactivation, there was a substantial decrease in lytic gene expression by 18-5p. BZLF1 was reduced from 6.2 fold to 1.7 fold and gp350 declined from 7.8 fold to 3.8 fold ($p<0.05$ and $p<0.01$ respectively)( Figure 31C). The ability of EBV to produce extracellular virus was also significantly reduced in the 18-5p-transfected cells compared to the MC-transfected cells (180x103 copies/ml vs 32.5x103 copies/ml, $p<0.05$) (Figure 31D). In accordance with down regulation of MAP3K2 in AKata2A8.1. This result suggests that the decrease in MAP3K2 caused by 18-5p (Figure 27A) is responsible for the decrease in BCR crosslinking mediated lytic replication. This is further supported by the observation that knockdown of 18-5p by the anti-18-5p LNA enhanced lytic induction in Akata2A8.1 cells stimulated through the BCR. In this case lytic gene transcription was enhanced (5 and 3 fold increase for BZLF1 and gp350, respectively) and virus production was increased (2 fold increase in copy numbers) (Figure 33). Taken together, the data suggest that 18-5p significantly inhibits EBV lytic replication via suppressing MAP3K2 in response to TPA/BA or surface Ig activation.

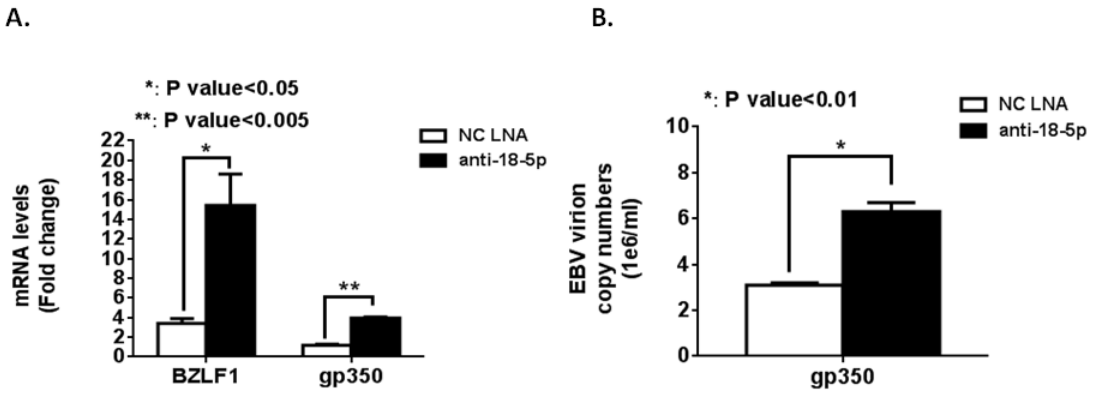



**Figure 33. knockdown of 18-5p in Akata2A8.1 cells enhances viral replication.**

A. BZLF1 and gp350 expression in Akata2A8.1 transfected with NC LNA or anti-18-5p LNA upon reactivation by BCR crosslinking for 72 hr. B. The copy numbers of EBV virions in the supernatant collected in Akata2A8.1 from A.

## 4.3 Discussion

Like other persistent viruses, EBV has two stages in its life cycle: latency and lytic replication. Lytic replication is the phase where the virus produces infectious progeny to infect more cells. This is the scenario seen in vivo when the fully productive lytic cycle occurs in EBV-infected oxyphanrygeal epithelium and plasma B cells, infectious virions are readily released and aggressively proceed to infect new targets [46, 75, 134, 488-492]. The shedding of virus, however, triggers a wide range of immune responses including a massive amount of cytotoxic CD8+ T cells and antibodies which in turn efficiently control virus spread [87, 137, 493-495]. The immune system appears to limit this expansion but provides considerable selective pressure for EBV to develop sophisticated and effective molecular strategies to evade further host attack. These countermeasures, common characteristics of latent infection, include minimized expression of potentially immunogenic viral proteins (e.g. the shutoff of all latent proteins in EBV-infected memory B cells) [149], expression of genes with the ability to downregulate the presentation of antigens (e.g. EBNA1 prevents the processing and presentation of itself on MHC molecules) [496], and avoidance of unnecessary viral replication thereby reducing the chance to be targeted (e.g. BLZF1 is a major target antigen for EBV-specific CTL surveillance) [138, 497].

In my study, the identification of 18-5p with a role in restricting viral replication falls into the last category as a viral strategy. 18-5p is expressed in latently infected B cells in healthy carriers, with especially high copy numbers in the memory compartment, a quiescent environment where only EBERs and BART transcripts (i.e. miRNAs) are expressed [375]. The presence of 18-5p in these cells led to the hypothesis that it might

play an active role in maintaining latency. In this study, with the use of in vitro transfection of 18-5p in EBV negative B cells and the following global gene profiling with microarray analysis, I was able to discern a panel of genes affected by 18-5p that are functionally associated with viral replication documented for other viruses (Figure 25A). It is conceivable that our analysis may underestimate the authentic number of 18-5p-targeting genes that are related to viral replication, as gene microarray only can provide information based on mRNA abundance. It is known that miRNAs posttranscriptionally regulate gene expression by either inducing mRNA degradation or repressing translation. Although the majority of miRNA-mediated repression was attributable to reduced mRNA levels [453], a study of translational repression for target proteins using proteomic approaches could also be considered [497]. Interestingly, 18-5p modulation of most targets is modest in magnitude (downregulated from 20-40%) (Figure 25B). This is however not particularly surprising in view of the generally accepted notion that a typical miRNA may exert only a relatively mild effect on any individual mRNA but the cumulative reduction in expression of multiple targets serving in the same biological process can lead to phenotypic consequences [453, 498].

Of the potential candidates I identified, much of the work was focused on MAP3K2 since it has the most direct evidence of involvement in viral replication. Sylvie et al showed that knockdown of MAP3K2 in HIV-1-infected Jurkat T-cells resulted in decreased virus production [472]. Likewise, overexpression of MAP3K2 in HEK293T cells infected with MHV-68, the closest Herpes relative of EBV in mouse, lead to enhanced viral replication [467]. In addition to MAP3K2, many reports have described the MAPK pathway to be important for herpes virus replication. For instance, increased

RNA levels of MAP2K1, MAP2K3, MAP2K6, and MAP3K12 were positively associated with lytic replication in EBV+ AKata cells induced by IgG cross-linking [59]. Inhibition of MAPK pathway with chemical inhibitors either decreased the TPA-induced expression of BZLF1 and BRLF1 in EBV-positive gastric carcinoma cells [127], diminished expression of BZLF1 at both the mRNA and protein levels in EBV-positive Akata cells stimulated with anti-IgG [469], or reduced the production of KSHV infectous virions during primary infection or in TPA-induced cells [110, 470]. In this study, transient transfection of 18-5p mimics or stable expression of an 18-5p precursor in the EBV negative B cell line Akata reduced expression of MAP3K2 at both the mRNA and protein levels (Figure 26A-B). Conversely introduction of an anti-18-5p LNA inhibitor was able to alleviate this down regulation, revealing that 18-5p specifically targets MAP3K2 (Figure 26C-D). In addition, anti-18-5p LNA treatment of EBV-positive cancer cell lines (Akata2A8.1 and BL36) also increased the expression of MAP3K2 (Figure 26F-G). These results suggest that MAP3K2 is an active and specific target for 18-5p in infected cells. Interestingly, it has been shown that EBV latent infection represses p38-MAPK activity in nasopharyngeal epithelial cells implying that other viral components also surpress this pathway [311].

In general, viral DNA genomes in the nucleus could harness existing host miRNA biogenesis pathways to make their own miRNAs and silence target genes [298, 499-501]. Thus it is reasonable to postulate that the EBV miRNA 18-5p uses the cellular processing and effector machinery. Thus it would be expected that there was an association between 18-5p, MAP3K2 mRNA and Ago2. I recapitulated the retention of 18-5p in the RISC using pGIZ-18-5p Akata cells instead of cells transiently transfected with 18-5p mimics.

This was because pulling down endogenous Ago2 requires at least $2x10^7$ cells. pGIZ-18-5p Akata cells not only express mature 18-5p from the precursor but also could be grown in sufficient amounts to allow for the pull down of Ago2. The enrichment calculation is based on comparison of the ability of a target to bind to Ago2 between pGIZ-18-5p and pGIZ-MC cells. Our method excludes nonspecific targets associated with Ago2 in the pGIZ-18-5p cells and thus provides a reliable identification of bona fide targets. I found that MAP3K2 was significantly enriched in the RISC of 18-5p-programmed cells suggesting that 18-5p utilizes the host effector machinery to bind to MAP3K2 directly (Figure 28C). Complementarity between the 3'UTR of mRNA and the 5' region of a miRNA, particularly the seed region, is the key determinant in their interaction [298, 502, 503]. Yet, it was also shown that a viral miRNA from the human beta-herpesvirus cytomegalovirus predominately binds to the 5′UTRs of many targets [504]. In our study, target prediction analysis showed that only the 3'UTR of MAP3K2 has a predicted binding site for 18-5p and this site is perfectly complementary to the seed region of the miRNA (Figure 28D). Luciferase assays confirmed that the 18-5p seed region binds to the 3' UTR of MAP3K2 as mutation of this region in the 3'UTR abolished luciferase activity (Figure 28E). Our finding accords with the notion that the seed region of 18-5p may be sufficient for inhibition of MAP3K2 expression [298]. Interestingly, 18-5p has sequence homology in the 5' region to a host miRNA mir-26-5p, which also has been shown to target MAP3K2 in the 3'UTR [482]. The coincidence is satisfying, because it not only strengthens our belief that MAP3K2 is a bona fide target for 18-5p, but also supports the prevailing view that EBV miRNAs, like some other virus-encoded miRNAs, exploit their host by possessing homologous seed sequences [114, 345, 505]. This seems

to be a common strategy for human virus as it was estimated that 26% of annotated virus-derived miRNAs have seed regions that are identical to host miRNAs [298].

The full process of EBV lytic replication has three stages: initial induction of the lytic gene cascade, viral assembly and transport, and final egress of infectious progeny. The lytic gene cascade in turn has three stages: inductions of the immediate early transcription factors that in turn activate the early genes responsible for initiating viral DNA replication and finally the late expression of the virion structural proteins. Activation of the MAPK signaling pathway is required for efficient viral replication but especially pertinent to the initiation of the lytic cascade for both EBV and KSHV [59, 110, 127, 469]. Transcription factors such as p38, JNK, ERK5 and AP-1 within the MAPK pathway bind to the promoter regions for IE gene activation [114]. Thus it is assumed that 18-5p mediated down regulation of MAP3K2 at a minimum impacts the immediate early stage of lytic replication. I found that 18-5p retarded both IE and L lytic gene induction in B95.8 and B95.8-LCL cells in response to TPA stimulation (Figure 30A,C). The IE gene BZLF1, encoding the first and foremost transcriptional activator termed Zta, is sufficient to drive the entire lytic replication in both B cells and epithelial cells [126, 506, 507]. Therefore it seems reasonable that the impaired phenotype caused by 18-5p also includes the reduced ability to make new virions (Figure 30B). Our result also showed that the inhibitory effect of 18-5p on viral replication is partially mediated by MAP3K2, as the reconstitution of MAP3K2 in B95.8-LCL only reversed the decreased expression of lytic genes but did not increase the production of virions (Figure 30D and not shown). This clearly implicates that in addition to MAP3K2 18-5p also targets genes functionally related to different stages of viral replication. Indeed, of the potential

candidates I have identified from microarrays, RAB11FIP1, KIF3B, KIAA and ABI2 are involved in autophagy, endocytic protein transport and membrane curvature, fundamental cellular activities known to play critical roles in viral assembly and budding (Figure 25B) [457-462, 464]. Future research is required to determine the molecular function of these proteins in the context of virus production. In concordance with previous studies [467], MAP3K2 expression alone in B95.8-LCL cells was sufficient to increase lytic gene transcription and the resulting virus progeny (Figure 32). Of further interest for the current work is that differential signaling pathways initiate the lytic cycle in response to distinct stimuli. TPA-induced EBV lytic replication is primarily mediated by PKC signaling [123, 127], whereas Ig crosslinking in Akata cells involves PI3K, PLC, Ras and Rac1 pathways [97]. In our study, overexpression of 18-5p inhibited MAP3K2 expression in AKata2A8.1 cells (Figure 27A) and also lead to a substantial decrease in lytic gene transcription and virus production followed by BCR stimulation (Figure 31C-D). Conversely, knocking down the endogenous expression of 18-5p in Akata2A8.1 could enhance the lytic cycle (Figure 33). This result suggests that in addition to the TPA-induced PKC pathway MAP3K2 also engages downstream of BCR signal-regulated lytic replication.

A tentative model for the signaling pathway leading to EBV lytic reactivation in response to stimuli, incorporating these present and previous findings [97, 123, 125-130], is illustrated in Figure 34. It is known that PKC signaling is activated by TPA treatment and BCR stimulation leads to activation of PI3K, PLC, Ras and Rac1 pathways. In view of my observation, the activated PKC and Rac1 would subsequently trigger the activation of the first protein kinase MAP3K2 within the MAPK pathway followed by

phosphorylation and activation of the second kinase, MAP2K. The activated MAP2K further phosphorylates and activates the effector kinase, p38 and JNK. The BCR signal is partially mediated by the Ras pathway, in which activated Ras induces activation of Raf, also a member of the MAP3K family, leading sequentially to activation of MAP2K and ERK. Different MAP2Ks are responsible for activation of p38 (MAP2K3 and MAP2K6), JNK (MAP2K4 and MAP2K7), and ERK (MAP2K1 and MAP2K2) [129, 130, 508]. Finally, the downstream activators bind to the Zp promoter leading to transcriptional activation of BZLF1. The proposed pathway herein requires future experimental validation on several aspects. First, the direct activation of MAP3K2 from TPA-PKC or BCR-Rac1 signals need to be further clarified. Second, the molecular basis of activation of downstream MAP2K and corresponding effecter kinases in lytic replication remains to be established. Third, how 18-5p fine-tunes other signaling modules through a complex network of regulatory interactions (i.e. if 18-5p interferes with Raf or others) to affect viral replication awaits elucidation.

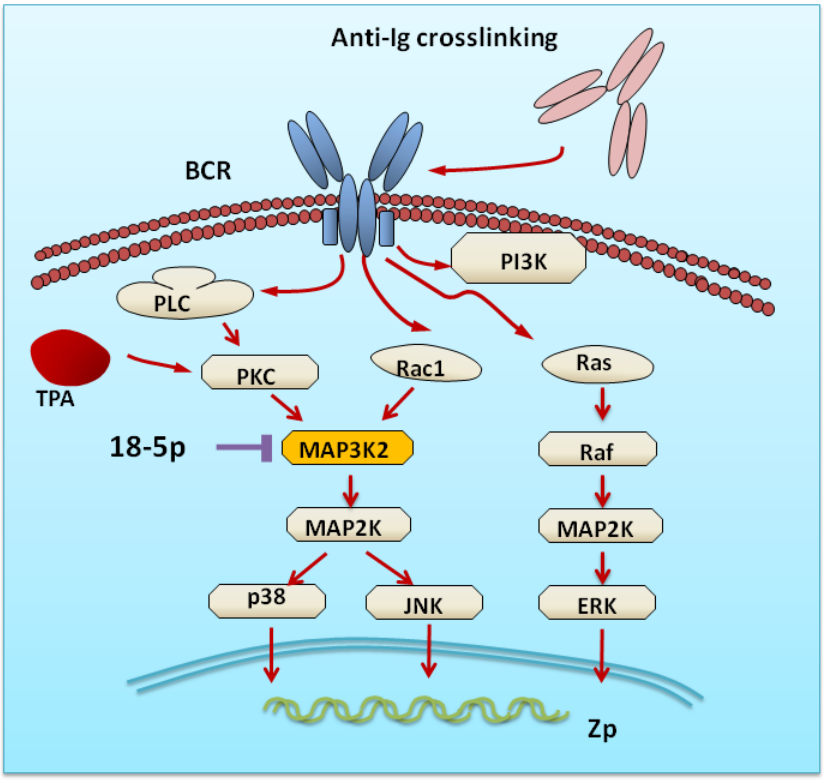



**Figure 34. Proposed model for 18-5p mediated inhibition of viral reactivation through the MAPK signal transduction pathway.**

Tentative model for the mode of action of 18-5p in repression of viral reactivation through the MAPK signal transduction pathway. Briefly, TPA triggers the PKC signaling pathway and BCR stimulation activates the PI3K, PLC, Ras and Rac1 pathways. Both pathways are mediated through MAP3K2 and activate downstream effector transcription factors (e.g. p38 and JNK), which can activate the Zp promoter followed by BZLF1 activation and induction of the lytic cycle gene cascade. Bart 18-5p represses MAP3K2 via binding to its 3'UTR and thereby inhibits viral reactivation. This figure incorporates these present and previous findings [97, 123, 125-130].

It is clear that 18-5p-mediated suppression of the lytic cycle represents a reasonable viral strategy to establish latency. This is one of many mechanisms used by EBV to maintain persistent infection in vitro and in vivo. It has been reported that LMP1 together with activated CD40 inhibits activation of BZLF1 [160, 509]. LMP2A is known to block BCR-mediated signal transduction and inhibit lytic reactivation [161-163, 510]. The EBV miRNA BART2 promotes the mRNA degradation of the viral DNA polymerase BALF5, a core protein with catalytic activity in replication initiation, suggesting a potential role of BART2 in inhibiting virus production [511]. BALF5 is an early viral product turned on by BZLF1. In conjunction with our finding, it demonstrates that EBV miRNAs exert their effects in repression of lytic replication at different phases from inhibiting the essential signaling molecule (MAP3K2) that governs the initiation of reactivation to abrogating the viral DNA polymerase crucial for the ongoing replication. The presence of 18-5p in EBV-infected primary B cells and its role in repression of lytic replication has important implications for viral latency in vivo. First, a fully productive lytic cycle is believed to cause the death of infected cells as new viruses burst out of the host cell. Limiting lytic replication apparently would confer a selective survival advantage to the EBV-infected cells especially in the long-lived memory B populations. This appears to be in accord with the observation that viral reactivation is often restricted in the oropharyngeal lymphoid tissues but is not seen in the peripheral blood of healthy host [46, 75, 488, 490-492]. Indeed, an activated MAP3K2-JNK signaling pathway can induce cell death [512-514]. Disruption of MAP3K2 has been suggested to inhibit JNK-dependent apoptosis in glioblastoma cells and to enhance T cell proliferation in response to TCR stimulation [482, 515]. Second, even in the case of abortive lytic replication, a

condition found in both in vitro and in vivo where no virus is made nor cells are lysed but lytic and latent genes are expressed [516-522], EBV-infected cells benefit from the 18-5p mediated lytic inhibition, because it would greatly diminish the chance of being targeted by CTLs. It is well established that all latent viral proteins except EBNA1 and many of the lytic genes are major targets for CTLs [89, 134, 523-526]. Thus this is undoubtedly considered as EBV's principal evasion strategy in establishing and maintaining latency.

In conclusion, I show in this study that a previously reported but uncharacterized EBV miRNA, BART 18-5p, can inhibit viral replication in EBV latently infected B cells upon in vitro reactivation, through repressing an important cellular signaling molecule MAP3K2. To our knowledge this is the first report providing evidence that a latent virus encodes a miRNA to interact with a host gene embedded within a prevalent signaling transduction pathway that governs a broad spectrum of biological processes. The recognition of crosstalk between viral miRNA and cellular genes is of particular importance as it provides novel insight into the function of viral components in establishing and maintaining latency.

# Section V: CONCLUSION AND FUTURE DIRECTIONS

I began this thesis with a comprehensive profiling study in which I characterized the EBV miRNA expression profile in different types of EBV-associated tumors and EBV-infected normal B cells. This lead to the discovery of a unique pattern of viral miRNA expression by normal persistently infected memory B cells in vivo and the identification of a subset of miRNAs that are associated with cell growth and are deregulated in tumors. I also focused on the specific role of EBV miRNAs in tumorgenesis in which I extended the profiling study of BART miRNAs into a mouse cancer model along with the investigation of tumor formation in vivo by BART miRNAs. I concluded that the EBV BART miRNAs provide a growth/survival advantage to EBV-associated tumors therefore promoting their growth in vivo. Lastly, by pinpointing gene targets and potential functions for EBV miRNAs using in vitro assays, I found that an EBV miRNA, BART18-5p facilitates the maintenance of latency in B cells by inhibiting viral replication via MAP3K2. All of these results--upregulation of Latency III BART miRNAs in tumors, or enhancement of tumor growth in vivo, or inhibition of viral replication by 18-5p--have collectively suggested that BART miRNAs provide a selective growth and/or survival advantage to the infected cells thus contributing to the oncogenesis of EBV-associated cancers or the latent infection of normal cells. Yet, many questions still remain regarding a thorough understanding of EBV miRNAs requiring further in depth investigation in future. First, how are EBV miRNAs transcribed and regulated in different backgrounds (e.g. tumor or normal cell) is unknown. Second, it is not clear why there are so many BART miRNA and what are the cellular targets for EBV miRNAs especially in tumors. Third, how EBV miRNAs interplay with other viral or cellular genes like miRNAs in latency or lytic replication remains elusive. Unraveling

these mysteries will enhance our understanding of not only the basic virology of EBV but also the mechanisms by which it contributes to disease.

# Appendix

## Primers:

ABI2:

F: AGAAACCAAAGCCTACACCAC

R: AGGTTGGCTGGAGCAATAATC

Human b-actin (PrimerBank ID 4501885a1):

F: CATGTACGTTGCTATCCAGGC

R: CTCCTTAATGTCACGCACGAT

Human GAPDH (PrimerBank ID 7669492a3):

F:CATGAGAAGTATGACAACAGCCT

R: AGTCCTTCCACGATACCAAAGT

Human e-cadherin (CDH1) (PrimerBank ID 4757960a3):

F:CCCACCACGTACAAGGGTC

R:ATGCCATCGTTGTTCACTGGA

Human snai1(PrimerBank ID 301336132b1):

F:TCGGAAGCCTAACTACAGCGA

R:AGATGAGCATTGGCAGCGAG

Human ZEB1 (PrimerBank ID 291575187b2):

F:TTACACCTTTGCATACAGAACCC

R:TTTACGATTACACCCAGACTGC

Human tubulin (PrimerBank ID 17921988c3):

F: ACCTCAATCGCCTCATTAGCC

R: CTGCTCGTGGTATGCCTTTT

LMP1:

F:AGCCCTCCTTGTCCTCTATTCCTT

R:ACCAAGTCGCCAGAGAATCTCCAA

EBNA1:

F: GGTCGTGGACGTGGAGAAAA

R:  GGTGGAGACCCGGATGATG

U1:

F: TCCCAGGGCGAGGCTTATCCATT 3'

R: GAACGCAGTCCCCCACTACCACAAAT 3'

MAP3K2:

F: CCCCAGGTTACATTCCAGATGA

R: GCATTCGTGATTTTGGATAGCTC

TSC22D3:

F:AACACCGAAATGTATCAGACCC

R: TGTCCAGCTTAACGGAAACCA

KIAA:

F: TTTGCCAGAAAGCACGAGAG

R: GCTGAGCATAGCTGTTTGGAG

RAB11:

F: GCTCGGCCTCGACAAGTTC

R: ACTTATACCACTGCGTCTTCCT

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