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The Cellular Mirna, Mir-190, is Upregulated in Type I Ebv Latency by Ebers and Modulates Cellular Mrnas involved in Cell Survival and Viral Reactivation

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Abstract
Epstein-Barr Virus (EBV) is a highly prevalent human pathogen infecting over 90% of the population. Much of the success of the virus is attributed to its ability to maintain latency through different programs in host cells. MicroRNAs (miRNA) are small, non-coding RNAs capable of post-transcriptionally regulating mRNA expression. A microarray comparison of EBV type I latency and type III latency infected cells yielded evidence of differential cellular microRNA expression. I hypothesized that one of these differentially upregulated type I latency miRNAs, miR-190, is important in maintenance of latency I, and miR-190 upregulation is due to viral gene expression. Lentiviral overexpression systems were used to overexpress miR-190 and a microarray of gene expression revealed candidate miR-190 targets, including: TP53INP1 and NR4A3. The modulation of these targets by miR-190 was confirmed through evaluating mRNA and protein level changes in the presence or absence of miR-190. In the case of TP53INP1, a 3'UTR target site was identified through mutagenesis. The effect of miR-190 expression was evaluated for markers of cell cycle and cell death by flow cytometry, western blot and RT-PCR. Measures of viral reactivation were lowered in the presence of miR-190 after induction by anti-IgG stimulation. I also observed upregulation of miR-190/Talin2 promoter activity or miR-190 expression in the presence of EBERs, Epstein-Barr encoded RNAs. Interestingly, a panel of type I latency cell lines had higher EBER1 expression compared to their type III latency counterparts. Work by others has indicated that EBERs activate the double-stranded RNA (dsRNA) sensor, retinoic acid-inducible gene 1 (RIG-I). Transiently expressed, constitutively activated RIG-I induced miR-190 expression and promoter activity. Knockdown of RIG-I in the type I latency cells yielded lowered miR-190 expression levels. To investigate how miR-190 is upregulated I generated miR-190/Talin2 promoter reporters that lacked YinYang1 (YY1) and Nuclear factor-κB (NF-kB) binding motifs. In the presence of EBERs, promoters with these deleted binding motifs had lowered activation compared to the full miR-190/TLN2 promoter. This work describes a mechanism by which EBERs upregulate a cellular miRNA, miR-190, which aids in type I latency preservation by preventing apoptosis, promoting cell cycle and maintaining virus in its latent state.

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THE CELLULAR MIRNA, MIR-190, IS UPREGULATED IN TYPE I EBV LATENCY BY EBERS AND MODULATES CELLULAR MRNAS INVOLVED IN CELL SURVIVAL AND VIRAL REACTIVATION

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DEDICATION

This thesis is dedicated to my family. They have supported me through my many, many years of graduate school. If I had been told in my first year that I would be married and have a one year old when I defended my thesis I would not have believed it. My husband, Steve, has been my rock. Commuting to New York City while living in Philadelphia is a crazy thing to do, but Steve did just that so we could be together. And for a blessed period of time we both lived and worked in Philadelphia. Now it is my turn to make a move for Steve.

Our daughter, Laura, is the best thing that ever happened to us. I want her to know that all things are possible with the love of family and lots and lots of support (both moral and financial). I cannot wait for our next adventures as a family!

I owe a great deal to my parents as well. Geoffrey and Lorett Cramer are strong supporters of my graduate school education. When the going gets tough they encourage me to work harder and persevere, which is important in grad school and life. My father is always ready to spend time in Philadelphia and encourages me to relax, live my life and not just stress about what's going on in lab. My mother has never hesitated to help me in any way possible—with childcare, planning a wedding or deciding what step to take next in life. She also knows when I need some babying myself, but most of the time she encourages me to toughen up. She is the strongest woman I know, a great mom/Bashie and the best grad school cheerleader ever! I love you all and I couldn't have done it without you!

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ABSTRACT

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Epstein-Barr Virus (EBV) is a highly prevalent human pathogen infecting over 90% of the population. Much of the success of the virus is attributed to its ability to maintain latency through different programs in host cells. MicroRNAs (miRNA) are small, non-coding RNAs capable of post-transcriptionally regulating mRNA expression. A microarray comparison of EBV type I latency and type III latency infected cells yielded evidence of differential cellular microRNA expression. I hypothesized that one of these differentially upregulated type I latency miRNAs, miR-190, is important in maintenance of latency I, and miR-190 upregulation is due to viral gene expression. Lentiviral overexpression systems were used to overexpress miR-190 and a microarray of gene expression revealed candidate miR-190 targets, including: TP53INP1 and NR4A3. The modulation of these targets by miR-190 was confirmed through evaluating mRNA and protein level changes in the presence or absence of miR-190. In the case of TP53INP1, a 3'UTR target site was identified through mutagenesis. The effect of miR-190 expression was evaluated for markers of cell cycle and cell death by flow cytometry, western blot and RT-PCR. Measures of viral reactivation were lowered in the presence of miR-190 after induction by anti-IgG stimulation. I also observed upregulation of miR-190/Talin2

promoter activity or miR-190 expression in the presence of EBERs, Epstein-Barr encoded RNAs. Interestingly, a panel of type I latency cell lines had higher EBER1 expression compared to their type III latency counterparts. Work by others has indicated that EBERs activate the double-stranded RNA (dsRNA) sensor, retinoic acid-inducible gene 1 (RIG-I). Transiently expressed, constitutively activated RIG-I induced miR-190 expression and promoter activity. Knockdown of RIG-I in the type I latency cells yielded lowered miR-190 expression levels. To investigate how miR-190 is upregulated I generated miR-190/Talin2 promoter reporters that lacked YinYang1 (YY1) and Nuclear factor-κB (NF-kB) binding motifs. In the presence of EBERs, promoters with these deleted binding motifs had lowered activation compared to the full miR-190/TLN2 promoter. This work describes a mechanism by which EBERs upregulate a cellular miRNA, miR-190, which aids in type I latency preservation by preventing apoptosis, promoting cell cycle and maintaining virus in its latent state.

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CHAPTER 1 INTRODUCTION

1.1 Discovery of the first human tumor virus, EBV

Epstein-Barr Virus (EBV) or human herpesvirus 4, is a primate herpesvirus that coevolved with humans for millennia, likely migrating out of Africa with the first human populations over 100,000 years ago (Kieff et al., 2007). EBV is best known as the causative agent of Burkitt's Lymphoma (BL). Dennis Burkitt, a field surgeon working in Uganda, identified a tumor that occurred with alarming frequency in young children. Burkitt described lymphomas causing facial swelling of the jaw and accompanying lymphomas in other organs. This tumor occurred with alarming frequency in young children in parts of Africa. Burkitt coined this area of Africa "the lymphoma belt," although incidences of sporadic BL occurred elsewhere (Kieff et al., 2007). It was later demonstrated that the lymphoma belt and another area with high incidence of BL co-localized with the habitat of the mosquito vector of the malaria parasite, *Plasmodium falciparum*. Furthermore, the high parasite levels which occurred in the first few years of life following parasite exposure were coincident with the appearance of endemic BL in affected individuals (Kufuko and Burkitt, 1970). To date the connection between endemic malaria and BL is not fully understood.

Many researchers sought to identify the causative viral agent of BL. In the 1960s, a new technique using electron microscopy allowed for visualization of sub-cellular structures, including viruses. Andrew Epstein and his colleagues, Yvonne Barr and Bert Achong, were able to first establish a BL derived cell line and then identify a virus in the

BL cell line that we now call the Epstein- Barr Virus (Epstein MA et al., 1964). After its discovery, EBV was recognized to be a prevalent human pathogen, infecting 90% of the population worldwide, (as measured by seropositivity for IgG antibodies to viral capsid antigen (VCA) complex) (Henle et al., 1969). EBV is not only studied for its own pathogenic qualities, but also for its ability to immortalize B cells. In addition to being a valuable tool for establishing cell lines, EBV-driven transformation of cells also indicates the ability EBV may have in vivo to influence cancer cell development.

1.2 Diseases caused by and associated with EBV

EBV is spread orally and most individuals are infected within the first few years of life due to close contact with family members. This early infection appears to be universal and may be clinically silent. Primary infection can be delayed in more developed countries with up to 50% of people remaining unexposed in their first 10 years (Kieff et al., 2007). In the Western World and parts of Asia, infectious mononucleosis, which is directly caused by EBV, is common (Kieff et al., 2007). This disease is commonly seen in adolescents and young adults and presents as glandular fever. The pathology of the disease is mainly immunopathological and corresponds to CD8+ T cell lymphocytosis and pro-inflammatory cytokine release, rather than viral shedding (Kieff et al., 2007).

As a pathogen, the majority of EBV infections do not go on to cause disease in human hosts, yet EBV is still considered medically relevant, as it is capable of directly causing some diseases in a fraction of hosts and is strongly associated with many others where causation is still being investigated. Some diseases directly caused by EBV are due

to genetic abornmalities or immunosuppression in the hosts. X-linked lymphoproliferative syndrome and fatal infection mononucleosis (XLP) occur in young boys and result in massive proliferation of lymphocytes and if survived, B cell lymphoma (Purtilo et al., 1982). Oral hairy leukoplakia results from permissive viral replication in immunocompromised carriers and results in thickening of oral epithelium and lesions (greenspan et al., 1985). B cell lymphoproliferative disease can also occur in immunocompromised patients, perhaps post organ transplant due to immunosuppressive drugs or in AIDS patients, likely due to CD4+ T cell loss and immune system destruction (Crawford et al., 1980; Green and Michaels, 2013).

There are numerous diseases that have no causal association with EBV but have links to EBV infection. These include the endemic BL mentioned earlier and its AIDs-associated BL, but not sporadic BL, which has varying associations with EBV from ~15% in Europe and the US and ~85% in Brazil and Northern Africa. All three types of BL do exhibit hallmark c-myc translocations (Kelly and Rickinson, 2007). While c-myc translocations confer BLs with the ability to grow and divide rapidly, the presence of EBV in BLs is instrumental in rendering them apoptosis resistant (Komano et al.,1998). Nasopharyngeal carcinoma is associated with EBV infection and is common in south-east Asia and Inuit populations. Also, Hodgkin's disease is associated with EBV in latency II programs (Kieff et al., 2007). In addition to infecting B cells and epithelial cells, EBV has also ben found as a latent infection in upwards of 10 percent of gastric carcinomas (Shibata and Weiss, 1992). Many of these EBV associated cancers exhibit EBV infected cells that express certain viral genes that fit within the different latency programs of

EBV. The EBV latency programs and the viral genes expressed within each program will be described in more detail in the following sections.

With its classification as an omnipresent pathogen, EBV can potentially play a role in many diseases. Recently, the role EBV plays in autoimmune disease has come under scrutiny. In a prospective study, blood taken from individuals before onset of multiple sclerosis (MS) displayed higher levels of anti-EBNA antibodies than blood from individuals who did not develop MS (Munger et al., 2011). The link between EBV and MS, a chronic and progressive demyelinating disease of the central nervous system (CNS), has prompted proposals for EBV therapeutics to treat MS (Pender and Burrows, 2014).

1.3 EBV infection and latency establishment

EBV is primarily a B cell lymphotropic virus, however, it can also infect epithelial, natural killer (NK) and T cells. In B cells, infection occurs via the complement receptor CD21. After initial exposure, EBV infects oropharyngeal cells, where the virus enters the lytic cycle and expresses more than 80 genes. During lytic infection or reactivation, production of infectious virus occurs. (Kalla and Hammerschmidt, 2012). While the infection of B cells spurs the development of latency, these cells likely also enter lytic infection (Macsween and Crawford, 2003). Long-term carriers of EBV maintain the virus in memory B cells (IgD- CD27+), while naïve B cells (IgD- CD27-) remain virus free (Babcock et al., 1998).

Two models of B cell infection and latency establishment in vivo prevail. The first, referred to as the germinal center (GC) model, involves the EBV driven

differentiation of naïve B cells to the GC of lymph nodes. This theory is mainly based on EBV gene expression in vivo and the ability of some of these genes to drive B cell into the memory compartment, resembling antigen driven differentiation (Babcock et al., 2000; Hochberg et al., 2004; Hochberg and Thorley-Lawson, 2005). A full complement of EBV latent gene expression (type III latency) allows for proliferation of infected cells and entrance into the GC compartment. Upon entry into the GC, infected cells begin to express a more restricted form of latent gene expression (type II latency) and finally the infected cells can leave for the blood as memory B cells (type I latency or type 0 latency) (Fig. 1). Evidence for this model includes presence of each of these different latency stages in vitro and ex vivo (Babcock et al., 1999; Babcock et al., 1998; Babcock et al., 2000; Laichalk et al., 2002).

The second model involves the direct infection of memory B cells. Evidence for this theory includes the infection of both naïve and memory B cells in vitro and the persistence of EBV in X-linked lymphoproliferative disease (XLP), where patients do not have germinal centers (Chaganti et al., 2008; Ehlin-henriksson and Klein, 2003). But this model does not account for the different observed latency stages in vitro and ex vivo.

Two genes are pivotal in the transition from latency to lytic infection, BZLF1 and BRLF1. Both genes encode transcription factors, with BZLF1 (also known as Zta or Zebra) acting as a master regulator, which can spur the induction of lytic entry in latently infected cells (Countryman et al., 1987). In the absence of BRLF1, full lytic replication cannot occur (Feederle et al., 2000). In concert with expression of these viral immediate early genes, cellular genes are also upregulated. In the case of the cellular immediate

early genes, including early growth response 1-3 (EGR1-3) and nuclear receptor 4A3 (NR4A3), increases in gene expression even precede the upregulation of the master regulator, BZLF1 (Ye et al., 2010). The process of reactivation together with the spectrum of latency programs allows EBV to spread and maintain infection for the lifetime of the human host.

1.4 Type I latency gene products and their actions

EBV latency is accompanied by the expression of EBV nuclear antigen (EBNAs) or latent membrane proteins (LMPs) along with the expression of non-coding RNAs that make up the viral miRNAs and EBERs. Throughout latency, EBNA1 expression along with the viral OriP, or origin of replication, allows for the replication and segregation of the viral genome, or episome. EBNA1 tethers the episome to the host chromatin and can also bind to two clusters within OriP during replication, playing a role in transcriptional regulation at all 3 EBNA promoters Wp, Cp and Qp (Kieff et al., 2007).

The EBV-encoded small RNAs, EBERs, are highly structured RNAs which are transcribed together by pol III and are expressed as EBER1 and EBER2. The EBERs were shown to confer apoptosis protection in BL cells, as EBV- BLs with restored EBERs expression survived apoptotic stimulation better than BLs which had lost their EBV genomes (Komano et al., 1999). EBERs have also been shown to interact with components of the innate immune response—both inhibiting through PKR and stimulating through RIG-I (McKenna et al., 2007; Nanbo et al., 2002; Samanta et al., 2006). These actions are further discussed in section 1.6. Despite years of study, the

molecular mechanism behind the functions of EBERs and their role in tumorigenesis is not completely understood.

In addition to the EBERs, EBV also expresses its own contingent of miRNAs. There are 25 EBV pre-miRNAs, 3 from the BHRF1 rightward open reading frame transcript and 22 from the BART transcript. EBV miRNAs are differentially expressed during latency, with modest expression of BART miRNAs and no expression of BHRF1 from the Wp or Cp promoter in latency I. There is higher expression of all EBV miRNAs in latency II and III (Forte and Luftig 2011). Both BART and BHRF1 miRNAs are expressed during lytic reactivation. Many herpesvirus miRNAs seed sequences are not well conserved across the herpesvirus family, yet they often have conserved targets. For instance, EBV miR-BART3, human cytomegalovirus UL112-1 and KSHV miR-K7 target a component of antigen processing and presentation, MICB making it more difficult for the immune system to recognize viral infection. EBV viral miRNAs have an array of both viral targets (Balf5, lmp1, lmp2a) and cellular targets (CXCL-11 and PUMA). (Forte and Luftig 2011). Viral miRNA expression can impact processes like apoptosis through miR-BART 1-3p and miR-BART16 targeting of caspase 3 (Vereide et al., 2014). EBV uninfected cells may also be influenced by EBV miRNAs, as there is evidence they can be transported by exosome to uninfected cells non-B cells (Pegtel et al., 2010). A diagram of the EBV latency programs and their corresponding gene expression is provided in Fig. 1.

1.5 DNA damage and EBV

During viral infection, EBV promotes genomic instability through numerous mechanisms, which results in the activation of the DNA damage response (DDR) pathway and ultimately leads to cellular senescence or death. To make the host cell a more hospitable environment, many viruses, including EBV, are capable of exploiting or bypassing DNA damage checkpoints. (Cayrol et al., 1996; O'nions and Allday, 2004). Activation of the ATM pathway may occur during EBV lytic replication, causing viral genomes to be recognized as damaged DNA (Kudoh et al., 2005). Although other work has suggested that viral latent gene expression (particularly EBNA2 and EBNALP), not viral DNA is the source of DDR (Nikitin et al., 2010). Three EBV latency proteins, EBNA1, EBNA3C and LMP1 independently promote genomic instability. EBNA-1 promotes chromosomal abnormalities, genomic instability and the DNA damage response by inducing reactive oxygen species (ROS) (Gruhne et al., 2009a, Gruhne et al., 2009b). EBNA-1 also induces the expression of the V(D)J recombinases RAG-1 and RAG-2, which could result in genomic recombination (Srinivas and Sixbey, 1995; Kuhn-Hallek et al., 1995). EBNA3C and LMP1 showed an association with genomic instability assayed by DNA damage, chromosomal aberrations and phosphorylated histone H2AX. These proteins appear to inactivate DNA repair via LMP1 mediated downregulation of ATM, A DNA damage sensing kinase, and EBNA3C mediated downregulation of BubR1 as well as the overrunning of the mitotic spindle checkpoint (Gruhne et al., 2009b). While each of these components is expressed throughout latency, DDR may be activated during the early proliferative stages of EBV infection, perhaps through expression of EBNA2 targets

like c-myc (Nikitin et al., 2010). The end result is an accumulation of DNA damage that is later subverted by the virus.

EBV has numerous mechanisms to promote cell cycle. EBNA2 and EBNALP upregulate cyclin D2, a positive regulator of G1 progression (Sinclair et al., 1994) LMP2A increases the instability of p27 allowing progression beyond G1 (Fish et al., 2014). EBV thwarts the DDR response and anti-viral responses by limiting apoptosis as well. In type III latency, protection from apoptosis is mediated by the viral proteins LMP-1, EBNA-LP and EBNA-3C, which modulate levels of anti-apoptotic Bcl2 (LMP1), bind or inactivate p53 (EBNA-LP) and interact with and stabilize IRF4 (EBNA-3C) (Banerjee et al., 2013; Thompson and Kurzrock, 2004).

In contrast to the permissivity of cell cycle in latency, in lytic reactivation, cell cycle is arrested at G0/G1. This is conducive to viral replication perhaps because it lowers competition for replication resources with cellular DNA (Flemington, 2001). BZLF1 expression alone can result in initiation of the lytic cycle. BZLF1 expression in EBV positive 293T cells also induces a G0/G1 growth arrest (Cayrol et al., 1996; Flemington et al., 2001). Additionally, BZLF1 binds and stabilizes p53 and allows for induction of p21 and p27, both cyclin dependent kinase inhibitors necessary for G0/G1 arrest (Cayrol et al., 1996, Rodriguez et al., 1999).

EBV encoded miRNAs also have roles in apoptosis resistance and cell cycle progression. Recombinant viruses lacking BHRF-1 miRNAs left newly infected cells susceptible to cell cycle arrest and apoptosis compared to the parental virus. (Seto et al., 2010). Additionally a HITS-CLIP analysis of targeted mRNAs in latency III cell lines

revealed that targets of viral miRNAs were significantly enriched for pathways associated with the regulation of apoptosis and cell cycle progression (Riley et al., 2012). Cellular miRNAs also enhance cell survival in the context of EBV infection. miR-155 promotes cell cycle progression and inhibits apoptosis in LCLs (Linnstaedt et al., 2010). Additionally, a role for miRNAs in cell cycle progression is enhanced by the fact that many miRNAs are regulated by cell cycle master regulators, such as c-myc, E2Fs and p53 (Bueno and Malumbres, 2011). Harnessing cellular miRNA allows EBV to manipulate cell cycle progression and suppress apoptosis.

1.6 EBV modulates host immunity

The interaction of the host immune system and EBV in healthy hosts seems to be fine tuned as more pathogenic aspects of infection are tamped down upon establishment of latency with limited viral protein expression and replication. The importance of the interplay between the immune system and EBV infection is illustrated in the numerous examples of diseases that arise in immunocompromised hosts or the link between autoimmunity and EBV infection implying a misregulation by the immune system. While innate response to infection likely occurs, there is not much data on how this might happen. EBV is thought to modulate innate responses based on data indicating that the virus controls pathways that subvert aspects of innate immunity.

EBV can be detected by innate immune pattern recognition receptors (PRR), which recognize pathogen associated molecular patterns or (PAMPs). Among these PRRs are RIG-I and multiple Toll-like receptors (TLRs). These PRRs can be activated within the infected B cells themselves or perhaps sensed in the environment by dendritic cells

(DCs), which are crucial antigen presenting cells to prime the T cell response (Iwakiri et al., 2009; McKenna et al., 2007; Nanbo et al., 2002; Samanta et al., 2006).

Non-coding RNAs, EBERs are transcribed by RNA polymerase III. EBERs have a 5' tri-phosphate moiety and their stem-loop structure makes them appear double stranded, which makes them attractive to the double stranded RNA sensor, retinoic acid-inducible gene I (Ablasser et al., 2009). In BL cells, RIG-I is activated in the presence of EBERs and can induce production of IRF3, IFN-ß, interferon stimulated genes (ISGs) and IL-10 (Samanta et al., 2006; Samanta et al., 2008). EBERs can also stimulate activation of TLR3, but this has been shown to act mostly in conventional DCs (cDCs), after EBERs are released from infected cells in association with La protein. Furthermore, EBERs helps induce DC maturation, which can be reduced by knocking down TLR3. (Iwakiri et al., 2009).

Despite the induction of cytokines, EBV infection or EBERs alone can protect cells from IFN- α induced death. EBERs have been shown to bind to protein kinase R (PKR), which is interferon-inducible and activated by double stranded RNA. PKR induces apoptosis and prevents protein translation through phosphorylation of eukaryotic initiation factor 2 α, (eIF2 α). In response to foreign dsRNA, PKR dimerizes and autophosphorylates and this self-association is inhibited by EBER1. The downstream effect of EBER1 on PKR is inhibition of apoptosis and continued protein translation (McKenna et al., 2007; Nanbo et al., EMBO 2002). However, others have refuted the role of the PKR-EBERs interaction in limiting IFN- α effects after finding that the levels of phosphorylated of nuclear PKR and eIF2 α are similar in EBV positive or negative BL

cells (Ruf et al., 2005). EBV and EBERs do still offer protection from IFN- α induced death (Ruf et al., 2005).

Other PRRs interact with EBV during infection. TLR9, which senses unmethylated CpG motifs, detects virions in plasmacytoid DCs (pDCs) (Fiola et al., 2010). Inactivated virus in naive B cells increases TLR7 expression and decreases TLR9 expression resulting in increased IRF-5 expression, but the variant of IRF-5 expressed is spliced and acts as a dominant negative that is a poor activator of IFN-ß allowing TLR7 to promote B cell proliferation (Martin et al., 2007).

1.7 EBV impact on host miRNA and targets

While EBV expresses its own miRNAs from the BART and BHRF-1 transcripts, EBV could potentially utilize any of the over 700 host miRNAs to modulate gene expression. Co-opting host miRNAs instead of relying on viral proteins is ideal for EBV as it provides a nonimmunogenic mechanism for alteration of gene expression. Studying cellular miRNAs is also important as miRNAs have been shown to play roles in oncogenesis and EBV has associations with numerous cancers.

The importance of altering cellular miRNA expression is evident in the viral miRNAs themselves. Many of the highly expressed BART miRNAs share seed sequence homology with cellular miRNAs at higher percentages than would be predicted by chance. (Chen et al., 2010; Forte and Luftig 2011). Numerous studies have explored altered cellular miRNA expression during latency (Cameron et al., 2008; Forte et al., 2012). Among these studies, focus is primarily on latency III, often using LCLs as experimental models and exploring the role of cellular miRNAs in transformation. Some

commonly identified host miRNAs that are upregulated with transformation or latency III are 146a/b, miR-155, and miR-34a. Mrazek, et al. used BL41 EBV negative line vs. LCLs and subtractive hybridization to identify differential expression in miR-155, 146a, 21, 34a, 29b, 23a and 27a (Mrazek et al., 2007). Cameron et al. identified miR-155, 146a/b, 21, 28, 34a upregulation in LCLs (Cameron et al., 2008). Forte et al. used microarray to evaluate differential miRNA expression between isolated B cells compared to activated B cells early in EBV infection and LCLs to identify miRNAs associated with early infection through transformation. In Forte et al. experiments, LCL formation was associated with miR-155 and 146b, but the authors also identified 34a as upregulated early in infection and in LCLs (Forte et al., 2012).

Viral latency genes play a role in cellular miRNA expression. LMP1 is linked to expression of several miRNAs, including miR-155, miR-146a and 34a (Cameron et al., 2008; Forte et al., 2012; Lu et al., 2008a). miR-155 also decreases bone morphogenic protein signaling perhaps to reduce EBV reactivation (Yin et al., 2010). miR-146a appears to target multiple interferon stimulated genes when overexpressed in latency I (Akata EBV positive) cell lines, perhaps to counteract the effect brought on by stimulation through LMP-1 and NF-kB (Cameron et la., 2008).

Use of PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) and HITS-CLIP (High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation) isolation of argonaute with associated miRNAs and mRNAs allows for a more nuanced study of cellular miRNA operations during latency. In the Steitz lab, HITS-CLIP was used to study both EBV miRNA and cellular miRNA in

latency III Jijoye cells that express all of the viral miRNAs. While viral miRNAs have cellular targets it is clear that the majority of miRNAs associated with argonaute are cellular and that the viral and cellular miRNAs share common targets. In addition to targeting cellular genes, cellular miRNAs also target viral mRNAs, including LMP-1 (miR-17), BHRF1 (miR-17 and miR-142) (Riley et al., 2012). Another group utilized PAR-CLIP in LCLs, which lacked most of the BART miRNAs, and identified LMP-1 as a target of miR-17 as well as confirming several other immunomodulatory targets of both viral and cellular miRNA (Skalsky et al., 2012).

Additional studies focus on other stages of latency as well as the lytic cycle. In one study, miR-127 was upregulated in EBV positive BL isolates but not EBV negative BL isolates (Leucci et al., 2010). Later work from this same group implicates EBNA1 in upregulated miR-127 expression and further suggested a role for miR-127 in impairing B cell differentiation and exit of B cells from the germinal center through targeting BLIMP-1, XBP-1 and IRF-4 in memory B cells (Onnis et al., 2012). Members of the miR-200 family, miR-429 and 200b, induce lytic replication in epithelial and B cells by targeting ZEB1 and 2 and blocking their repressive activity on the BZLF-1 promoter Zp (Lin et al., 2010). Multiple lines of evidence now support EBV's ability to modulate the cellular miRNA network to influence the expression of numerous host pathways.

1.8 miR-190 Background

miR-190 is not well studied in connection to EBV compared to other miRNAs, including miR-146 and miR-155. Many of the miRNAs studied in relationship to EBV latency also play duel roles as oncomiRs, or oncogenic miRNAs. miR-190 expression has

also been examined in relationship to cancer with its expression being high in granulocytes from primary myelofibrosis patients compared to granulocytes from normal patients (Guglielmelli et al., 2007). In normal pancreatic tissue, miR-190 expression is very low, yet the expression of miR-190 increased over 20 fold in pancreatic cancer compared to the control normal pancreatic tissues (Zhang et al., 2009). miR-190 is also upregulated in B- cell chronic lymphocytic leukemias (CLL) (Calin et al., 2004). Along with these observations, miR-190 is also increased in colorectal, bladder, breast and lung cancers and some HBV-positive hepatocellular carcinomas (Ichimi et al., 2009; Lowery et al., 2009; Navon et al., 2009; Ng et al., 2009; Ura et al., 2009. miR-190 is downregulated in some colon carcinoma and melanoma cancer samples (Hao et al., 2014, Mueller et al., 2009). While these associations provide some evidence for the growth promoting properties of miR-190, further understanding of functional targets, regulation and expression patterns are ongoing.

Like many miRNAs, the effects of miR-190 expression seem to be dependent on its cellular or organ environment. In HELA cells, a reduction of miR-190 expression with antisense RNAs slowed cell growth (Cheng et al., 2005). miR-190 has also been studied in rat brains in connection to addiction. miR-190 was downregulated after treatment with synthetic opiod fentanyl. Targeting NeuroD, a key player in repressing neurogenic differentiation also reduced miR-190 (Zheng et al., 2010b,c). In clinical glioma samples, miR-190 expression decreased with advanced tumor grade. Overexpression of miR-190 in a fast growing glioma cell injected into mice resulted in an inhibition of tumor growth. Furthermore, miR-190 expression in a fast growing osteosarcoma model reverted tumors back to dormancy (Almog et al., 2012).

miR-190 is also involved in the cellular response to Arsenic. Arsenic treatment of a bronchial epithelial cell line results in increased miR-190 expression. This in turn enhances tumorigenic potential as assessed by proliferation and soft agar assays. This function was attributed to miR-190's ability to lower PHLPP. PHLPP, an Akt phosphatase works as a tumor suppressor by inactivating Akt, leading to decreased cell growth and increased apoptosis (Beezhold et al., 2011). Further work by another group demonstrated that this Arsenic induced miR-190 upregulation is mediated by p50 (Yu et al., 2014). Like most miRNAs, mir-190 appears to have variable roles and expression patterns depending upon the disease state and type of cell.

1.9 miR-190 regulation

miR-190 is conserved in mice, rats and humans and is located in the intronic region of the talin2 (TLN2) gene of each species. In humans, miR-190 lies in the 52nd intron of TLN2. miR-190 is regulated by the TLN2 promoter (Zheng et al., 2010a; Beezhold et al., 2011). The talins are scaffolding proteins located in the focal adhesions of elongated cells (Critchley and Gingras, 2008). The head of the talin molecule binds integrin to enhance its affinity for the extracellular matrix (ECM) (Calderwood, 2004). For many years only one talin gene was studied, talin1 (TLN1), but expression sequence tag databases indicated another protein with a highly homologous C-terminus distinct from TLN1—TLN2) (Monkley et al., 2001). TLN2 shares about 74% homology with TLN1. The significant sequence homology with TLN1 and the ability of TLN2 to functionally rescue some embryonic knockouts of TLN1 suggests that TLN2 may functionally overlap with TLN1. Both talins bind vinculin and integrin and play roles in cell proliferation, cell migration and homeostasis through these cell-cell and cell–ECM

interactions. While initially thought to be the most abundant in the heart, brain and skeletal muscle, newer evidence suggest a more widespread expression pattern. (Calderwood et al., 2013).

Given the similarity between the two talins, it is likely that TLN2 binds some of the same ligands as TLN1. As a cytoskeletal link, TLN2, like TLN1, mediates interactions between ligand bound integrins and actin. It is also necessary for focal adhesion formation and focal adhesion kinase (FAK) signaling. In undifferentiated embryonic stem cells with a TLN1 knockout, TLN2 restores cellular spreading and adherence phenotypes (Zhang et al., 2008).

While no formal role for TLN2 has been defined in B cells, TLN1 has been shown to play a role in the formation of the immunological synapse of T cells (Wernimont et al., 2011). Additionally, TLN1 plays a role in the entry of B cells into the lymph node and bone marrow and plays a role in activation of VLA-4 and LFA-1 by BCR signaling (Manevich-Mendelson et al., 2010). Given the overlapping roles of TLN1 and TLN2 observed by others, it is possible that TLN2 plays a role in B cells as well.

The TLN2 promoter, which is responsible for both miR-190 and TLN2 expression, contains multiple binding motifs for the transcription factor YinYang1. The transcription factor YinYang1 (YY1) is a member of the GLI-Küppel gene family and is known by the alternative names: δ factor, NF-E1 and UCRBP (Montalvo et al., 1995). YY1 has been identified as a regulator of the germinal center specific program in murine B cells and its expression was confirmed in human germinal center B cells (Green et al., 2010).

Mutation of a conserved YY1 binding site in the mouse TLN2 promoter decreased promoter activation indicating the necessity of YY1 for miR-190 expression. Furthermore, the authors concluded that specific changes in mRNA or protein levels of YY1 did not correlate with increase in miR-190/TLN2, but phosphorylation of YY1 increased while miR-190/TLN2 decreased (Zheng JBC 2010a). This indicates that YY1 promoter binding is regulated by phosphorylation events, where YY1 phosphorylation reduces its DNA binding affinity.

The TLN2 promoter sequence also contains a NF-kB binding sites for both the p50 and p65 subunits, the most common NF-kB dimeric partners. YY1 and NF-kB interact by working in concert with each other or by affecting each other's transcription. The interaction between NF-kB and YY1 influences target gene expression, perhaps because interaction between the transcription factors leads to subsequent interactions with enhancer regions, as is the case for IgH chain genes in B cells (Sepulveda et al., 2004, Gordon et al., 2006). Alternatively, the binding of NF-kB and YY1 to motifs near each other could result in situations where the binding of one inhibits the binding of the other. This is the case with the serum amyloid A promoter, where YY1 binding inhibits NF-kB binding of the promoter (Lu et al., 1994, Gordon et al., 2006). In skeletal myogenesis, subunits subunits of NF-kB, p50 and p65 bind the YY1 promoter and increase its expression (Wang et al., 2007). Additionally, the p50 subunit of NF-kB has been implicated in enhancing miR-190 expression. (Yu et al., 2013).

1.10 Aims and Objectives

EBV uses multiple mechanisms to modify the cellular environment to suit its needs. Previous work identified several cellular miRNAs known to regulate processes in EBV infection or latency. Thus far, most of the focus on cellular miRNAs in EBV infection has been on LCLs and miRNAs upregulated during uninfected/resting B cell to LCL transition (Cameron et al., 2008; Luftig et al., 2012). A limited focus on cellular miRNA expression in type I latency has left a hole in the realm of latency research. miRNAs are regulators of many cellular processes, like cell cycle and apoptosis, that the virus handily modifies during latency. Cellular miRNAs are good candidates for type I latency EBV to use during infection to modify cellular processes as many miRNAs have redundant targets and each miRNA may have multiple targets in the same pathway—so precision is not necessary. Furthermore, miRNAs are nonimmunogenic, so detection of viral infection is thwarted. Along with the type I latency EBV genes, a comprehensive picture describing the maintenance of an optimal latency environment is not known. The goals of my research were to identify the mechanisms by which miR-190 is regulated during type I latency and to determine how miR-190 modulates the latency environment.

The second chapter focuses on characterizing miR-190 expression in multiple type I latency cell lines compared to their type III latency counterparts. Targets for miR-190 are identified and the effect of miR-190 on cellular processes, like cell cycle and apoptosis, and viral mechanisms, such as lytic reactivation, are explored. Furthermore, the role EBV type I latency genes play in modulating miR-190 expression is addressed and EBERs are identified as potential modulators.

In the third chapter, the mechanisms that control mir-190 expression during type I latency I are explored. RIG-I, which is activated by EBERs, appears to be upstream of miR-190 and knockdown studies of RIG-I reduces miR-190 expression (Samanta et al., 2006). Binding motifs for the transcription factors YY1 and NF-kB (subunits p50 and p65) are identified and mutated in the miR-190/TLN2 promoter. Both binding motifs are necessary for miR-190/TLN2 promoter activation indicating an activating role for these transcription factors in miR-190 upregulation.

These findings, while focusing exclusively on one miRNA, provide further evidence of EBV in type I latency having far reaching effects on gene expression and cellular and viral processes. It also provides impetus for further study of the cellular miRNA network in the context of type I latency EBV infection.

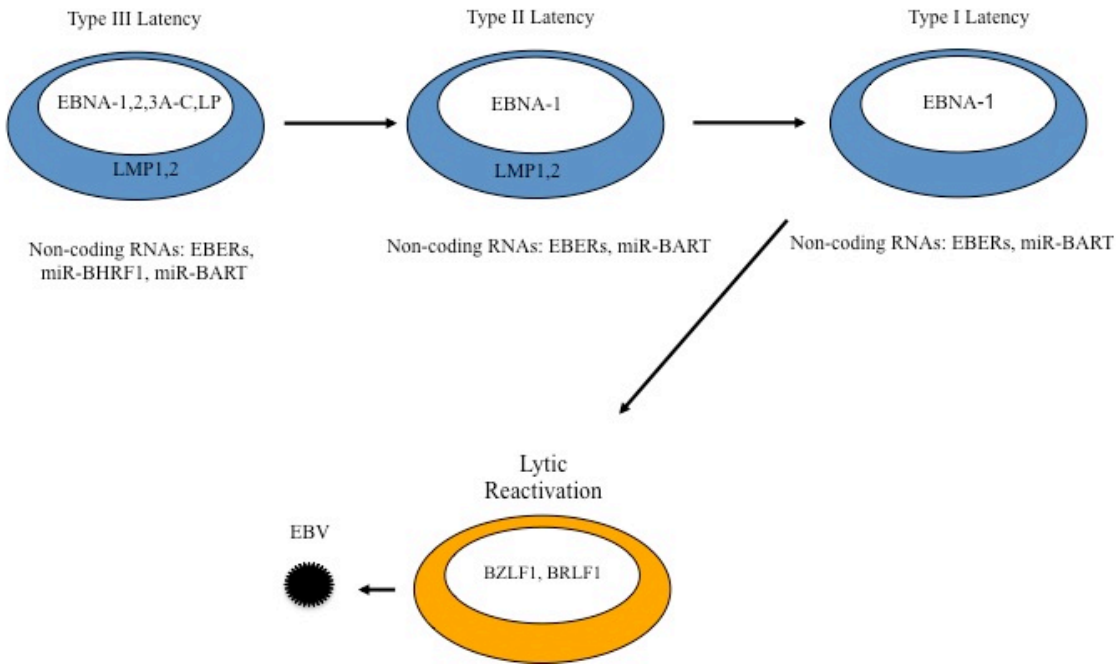

Figure 1: EBV latency programs and the corresponding latency gene expression patterns.

CHAPTER 2- miR-190 Is Upregulated in Epstein-Barr Virus Type I Latency and Modulates Cellular mRNAs Involved in Cell Survival and Viral Reactivation

2.1 Summary

Epstein-Barr Virus (EBV) is a prevalent human pathogen infecting over 90% of the population. Much of the success of the virus is attributed to its ability to maintain latency. The detailed mechanisms underlying the establishment and maintenance of EBV latency remain poorly understood. A microRNA profiling study revealed differential expression of many cellular miRNAs between types I and III latency cells, suggesting cellular miRNAs may play roles in regulating EBV latency. mir-190 is the most differentially up-regulated mRNA in type I latency cells as compared with type III latency cells and the up-regulation appears to be attributed to EBERs, EBV-encoded RNAs, that are expressed at higher levels in type I latency cells than type III cells. With the aide of a lentiviral overexpression system and microarray analysis, several cellular mRNAs are identified as potential targets of mir-190. By targeting TP53INP1, miR-190 enhances cell survival by preventing apoptosis and relieving G0/G1 cell cycle arrest. Additionally, miR-190 down-regulates NR4A3, a cellular immediate-early gene for EBV reactivation, and inhibits the expression of the viral immediate-early gene bzlf1 and viral lytic DNA replication. Taken together, our data revealed a mechanism that EBV utilizes a cellular microRNA to promote host cell survival and prevent virus from entering lytic life cycle for latency maintenance.

2.2 Introduction

Epstein Barr Virus (EBV) is a gamma-herpesvirus, which primarily infects B lymphocytes and establishes latent infection that persists for the life of its human host. Even after decades of research, EBV remains important for study, as it is nearly ubiquitous in the human population and because the virus is associated with numerous human diseases, including Burkitt's lymphoma (BL), Hodgkin's disease, nasopharyngeal carcinoma (NPC), and posttransplant lymphoproliferative disease (PTLD) (Kieff et al., 2007).

In vitro, EBV infects, activates and transforms human B cells into lymphoblastoid cell lines (LCLs) (Kieff et al., 2007). In vivo, the virus physiologically mimics normal B cell activation in naïve cells and provides the survival signals necessary for cellular differentiation into a resting memory phenotype (Thorley-Lawson, 2001). Several different latency programs are associated with this transition. In LCL where EBV establishes type III latency, all nine viral latent proteins (EBNA1, EBNA2, EBNA3A-C, EBNA-LP, LMP1, LMP2A and LMP2B) are expressed, which helps to drive proliferation and survival of the infected B cell. In vivo, type III lymphoblastoid cells have the ability to differentiate to resting memory B cells where the virus has been switched to type I latency with limited viral gene expression. The type I latently infected B cells just express one latent protein EBNA-1, two non-coding RNAs, EBER-1 and EBER-2 (EBV encoded RNAs), as well as BART microRNAs (miRNAs) (Kieff et al., 2007; Qiu et al., 2011). The limited type I latency gene expression pattern is beneficial to the virus as this allows the virus to escape immune recognition and clearance. Thus, EBV takes advantage of the normal biology of B lymphocytes to establish a persistent latent

infection in long-lived memory B cells. Although evidence suggests that the expression pattern of EBV latency genes is dependent upon the diffentiation stage of the infected-primary B cells, the mechanisms utilized by the virus for maintaining type I latency have not been fully described (Babcock, et al. 2000).

MicroRNAs have emerged as important regulators of gene expression affecting diverse cellular processes. EBV expresses its own contingent of viral miRNAs, which can help epithelial or BL cells survive (Marquitz et al., 2011; Seto et al., 2010; Vereide et al., 2013). The virus is also capable of using cellular microRNAs to modulate proliferation, differentiation, reactivation and survival of infected cells (Cameron et al., 2008; Yin et al., 2008, 2010; Forte and Luftig, 2011). miR-155, miR-146a and miR-34a are up-regulated in type III latency cells and are associated with functions that allow for maintenance of latency, including anti-apoptosis, subversion of interferon response and growth promotion (Cameron et al., 2008; Forte et al, 2012; Yin et al., 2008). Additionally miR-155 has the ability of regulating bone morphogeneic (BMP) signaling, thus reducing EBV lytic reactivation in latently infected cells (Yin et al., 2010). Other miRNAs, such as miR-200b and miR-429, regulate the latent/ lytic switch in epithelial cells inducing lytic replication (Ellis-Connell et al., 2010; Lin et al., 2010). Thus, miRNAs contribute to an ideal cellular environment for EBV to establish latency and persistence.

Previous work has focused on the miRNAs that are upregulated in the type III latency program or LCLs (Cameron et al., 2008; Forte et al., 2012; Riley et al., 2012, Skalsky et al., 2012). To identify the miRNAs that may contribute to the EBV-driven B

cell differentiation, switch of EBV latency type and the establishment of type I latency, we attempted to search for the miRNAs that are upregulated in type I latency vs. type III latency. Toward this regard, we compared the microRNA expression profiles between type I and type III latency cells with the aide of miRNA microarray analysis. Among the miRNAs differentially expressed, miR-190 was found to be one of the highest differentially regulated miRNAs in type I latency cells. Confirmation of miR-190 upregulation in multiple type I latency cell lines over type III counterparts provided a possible mechanism for the cellular miRNA in maintaining type I latency. While implicated in various forms of cancer, tumor dormancy of gliomas and osteosarcomas and opiate addiction in neurons (Almog et al., 2013; Calin et al., 2004; Zhang et al., 2009; Zheng et al., 2010), we found that miR-190 also targets apoptosis and viral reactivation pathways, which are critical for maintenance of persistent EBV latent infection.

2.3 Results

2.3.1 miR-190 is highly expressed in type I latency vs. type III latency cells

To identify the microRNAs that are differentially upregulated in type I latency cells, miRNA expression profiles in a pair of genetically identical cell lines, Sav I and Sav III (Jang et al., 2005), representing latency types I and III respectively, were evaluated. RNAs isolated from these cells were subjected to Exiqon^{TM} miRNA array analyses and the comparison of the miRNA profiles in the pair of cells identified several miRNAs that are up-regulated in Sav I cells and another class that are up-regulated in Sav III cells (Fig. 2A). Among the highly differentially expressed miRNAs in Sav I cells was

miR-190 (Fig. 2A and B). The upregulation of miR-190 expression in type I latent cells was confirmed via RT-PCR in multiple corresponding type I and type III latency cell lines as well as Akata EBV positive and negative cell lines. miR-190 is consistently expressed in higher levels in Sav I, Mutu I and Kem I cell lines in comparison to latency III counterparts Sav III, Mutu III and Kem III cells. Additionally, miR-190 is more highly expressed in EBV-positive Akata cells vs. EBV-negative cells, including Akata cells that have lost the virus and two EBV negative Burkitt lines BJAB and BL41, indicating a potential role for type I latent virus in regulating miR-190 expression (Fig. 2C). Sav I, Mutu I, Kem I and EBV-positive Akata cells are derived from BLs and exhibit restricted expression of EBV genes, which allows them to be described as type I latency (Jang et al., 200; Hughes et al., 2011; Ruf et al., 1999).

2.3.2 Identification of miR-190 targets in B lymphocytes

To identify the potential target genes of miR-190, we utilized a mRNA microarray to detect miR-190 expression-associated changes in mRNA expression profile in the cells that ectopically expressed miR-190. A lentiviral vector containing miR-190 (pSIF-mir190) or an empty vector (pSIF) was introduced into two cell lines, namely BJAB, a B cell lymphoma line that does not contain the virus, and Sav III, a type III latency EBV infected lymphoblastoid cell line. Both cell lines have low endogenous expression of miR-190. The miR-190 lentiviral vector transduction raised the expression levels of this miRNA in both BJAB and Sav III cells, respectively (Fig. 3A and B). Total RNA was purified from these cells and subjected to a gene expression profiling analysis with Affymatrix® gene array chips. Using two-way ANOVA, genes were deemed possible targets if there was a decrease in expression with the ectopic expression of miR-

190 in comparison to pSIF and met the criteria of a false discover rate of <0.1 (Table 1). A set of genes with down-regulated expression in response to ectopic expression of miR-190 was identified. Among them, TP53INP1 and NR4A3 were chosen for further studies because of their involvement in maintaining cell survival and preventing viral reactivation, respectively, implicating their potential roles in EBV latency establishment.

2.3.3 miR-190 targets TP53INP1 and influences cell apoptosis and cell cycle arrest

Tumor suppressor p53-inducible nuclear protein 1, TP53INP1, is known to play a pivotal role in induction of cell cycle arrest and apoptosis (Tomasini et al., 2005). To confirm the down-regulation of TP53INP1 by miR-190, TP53INP1 expression levels in miR-190 lentiviral transduced cells and control cells were examined by Western blot analyses. The results confirmed that the ectopic miR-190 expression in two type III latency cell lines, Sav III and Raji (Fig. 3B and C), resulted in decreases in TP53INP1 protein levels (Fig. 4A). Conversely, in Sav I cells, which are type I latency cells with high endogenous levels of miR-190, inhibition of miR-190 expression by a specific miR-190 inhibitor (miR-190 antagomiR) modestly increases TP53INP1 expression. In both protein (Fig. 4B) and mRNA levels (Fig. 4C), TP53INP1 expression was higher in the absence of miR-190.

A potential miR-190 recognition sequence was predicted in the 3'UTR of TP53INP1 (Fig. 4D). In order to confirm that TP53INP1 can be targeted by miR-190 through this site, the 3'UTR of TP53INP1 with the recognition sequence was cloned into a luciferase reporter vector (pMiR). Co-transfection of the reporter with miR-190 expression vector into 293T cells resulted in a significant decrease in luciferase activity

in comparison to the control with the empty vector, pSIF. Furthermore, when the miR-190 target site was deleted from the TP53INP1 3'UTR reporter vector and cotransfected into 293T cells with miR-190 expression vector, the luciferase reporter activity was restored and the reporter was no longer responsive to miR-190 expression (Fig. 4D). These results suggest that miR-190 indeed targets TP53INP1 mRNA through the predicted recognition sequence in the 3'UTR.

TP53INP1 can be induced with DNA damage through either p53 dependent or independent routes (Hershko et al., 2005; Tomasini et al., 2003; Tomasini et al., 2005). In conjunction with HIPK2, TP53INP1 induces p53 phosphorylation at serine 46 and mediates both G0/G1 arrest of cell cycle and cell death (Okamura et al., 2001; Tomasini et al., 2003). In the absence of DNA damage, ectopic expression of miR-190 in the type III latency cell line, Raji, did not result in significant changes to apoptosis. However, when DNA damage was induced with doxorubicin over the course of 72 hours in Raji cells, the expression of miR-190 was found to be able to reduce the apoptosis of the cells in comparison to the control cells with empty vector pSIF as measured by TUNEL (Fig. 5A). Additionally, the effect of miR-190 on apoptosis of Raji cells was also evaluated by PARP cleavage, a protein that binds to single stranded DNA breaks, cleavage of which serves as a marker for apoptosis. The result showed that the cleaved PARP-1 is reduced in Raji cells that overexpress miR-190 (Fig. 5B, lane 4). The p53 phosphorylation at ser46 was also reduced with miR-190 overexpression in Raji cells, which is consistent with the decreased apoptosis in the cells, as TP53INP1 is known to induce p53 phosphorylation at ser46 (Okamura et al., 2001;Tomasini et al., 2003) (Fig. 5B, lane 4).

Cell cycle dysregulation was induced in Raji cells treated with doxorubicin. The treatment brought about a massive increase in G0/G1 arrest observed via propodium iodide cell cycle flow cytometry (Fig. 5C). Yet with miR-190 overexpression, the doxorubicin treatment did not induce a G0/G1 arrest, but rather moves on to the next checkpoint of the cell cycle (Fig. 5C). Consistent with the G0/G1 arrest, p21 mRNA expression was increased in doxorubicin-treated Raji cells. However, with miR-190 overexpression, doxorubicin failed to induce p21 mRNA expression (Fig. 5D), suggesting that mir-190 dampens G0/G1 arrest perhaps through targeting TP53INP1 which in turn regulates p53 transcriptional activity on p21 (Tomasini et al., 2003).

2.3.4 miR-190 down-regulates NR4A3 expression and attenuates bzlf1 expression

Another potential target of miR-190 identified by our microarray data is NR4A3 (nuclear receptor 4, group A, member 3). NR4A3 was recently demonstrated to be one of five cellular genes that are induced during EBV reactivation in Akata EBV-positive cells triggered by anti-IgG. Its expression precedes that of the viral transcription factor, bzlf1, a viral transcription factor that is responsible for EBV reactivation from latent to lytic replication (Ye et al., 2010).

To confirm if miR-190 indeed down-regulates NR4A3 expression, the miR-190 expression vector was introduced into two type III latency cell lines, Sav III and Raji, both with low endogenous miR-190 expression by lentiviral transduction (Fig. 3B and C). The expression levels of NR4A3 in the cells with and without miR-190 over-expression were compared by Western blot analysis. A reduction in NR4A3 protein levels in miR-190 over-expression samples in both cell lines were observed in comparison to pSIF

controls (Fig. 6A). Conversely, in type I latent Akata EBV-positive cells, when mir-190 expression was inhibited with hsa-miR-190-specific antagomiRs, there was a modest increase in NR4A3 expression, both in protein and mRNA levels (Fig. 6B and C). These data suggest that miR-190 alters NR4A3 expression levels. However, no sequence that perfectly match with the miR-190 seed sequence is found in the 3'UTR region of NR4A3 by prediction algorithms. It is possible that miR-190 causes NR4A3 translational repression through either an imperfect match with a seed sequence (perhaps upstream of the 3'UTR) or an indirect targeting.

As NR4A3 expression precedes the expression of bzlf1 in viral reactivation, the down-regulation of NR4A3 expression by miR-190 was hypothesized to be able to dampen bzlf1 expression, a precursor to viral reactivation. To investigate this hypothesis, first, viral reactivation was induced in Raji cells that overexpress miR-190 or were transduced with pSIF (empty vector) (Fig. 3C) by treatment with 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) for various time points up to 12 hours. Induction of both NR4A3 and bzlf1 mRNA were analyzed via RT-PCR. In keeping with the identification of NR4A3 as an immediate early gene, we saw increases in mRNA expression following TPA treatment in Raji cells, yet this expression was decreased in the presence of stable miR-190 expression (Fig. 6D). While the TPA stimulus induced bzlf1 expression in the absence of miR-190, the induction of bzlff1 expression was attenuated in the cells that over-expressed miR-190 expression, significantly so at 8 and 12 hours (Fig. 7A).

The effect of miR-190 on bzlf1 expression in Akata EBV-positive cells, which were originally used to study the role of NR4A3 in bzlf1 induction (Ye et al., 2010), was

also investigated. Although there is endogenous miR-190 expression in Akata EBV-positive cells, we boosted the miR-190 expression by a lentiviral overexpression system. The lentiviral transduction resulted in an increase in miR-190 expression in Akata EBV-positive cells (Fig. 3D). Then Akata EBV-positive cells were treated with anti-IgG for induction of reactivation. Consistent with what was observed previously, the expression of NR4A3 was increased with anti-IgG stimulation in Akata EBV-positive cells, but expression was attenuated in the presence of miR-190 (Fig. 6E). Stimulation for up to eight hours with anti-IgG resulted in increased bzlf1 mRNA expression in control cells (Akata EBV-positive cells transduced with the empty vector. However, the bzlf1 expression was attenuated in the cells that overexpressed miR-190, significantly at hours 3 and 8 (Fig. 7B). In a loss-of-function assay, introduction of miR-190 antagomiRs into Akata EBV-positive and Sav I cells led to increased bzlf1 expression in the absence of stimulation (Fig. 7C and D). These data provide evidence that miR-190 acts to attenuate bzlf1 expression and perhaps ultimately lytic reactivation through down-regulating NR4A3. As a consequence of down-regulation of bzlf1 expression, the presence of miR-190 also provided an inhibition of viral DNA replication in Akata EBV+ cells, as measured by viral genomic DNA content via quantitative PCR. Stimulation for up to 24 hours with anti-IgG resulted in increased EBV intracellular DNA in the presence of empty vector (pSIF), yet miR-190 inhibited this accumulation at 24 hours (Fig. 7E).

2.3.5 miR-190 expression is up-regulated in the presence of EBERs in EBV latently infected Cells

The next question was how miR-190 is regulated in EBV latently infected cells. miR-190 is located in an intron of the Talin2 gene and transcribed with it before

processing to its mature form (Beezhold et al., 2011). Using three pairs of types I and III cell lines, namely Sav I and Sav III; Mutu I and Mutu III; and Kem I and Kem III, we confirmed that Talin2 mRNA expression is higher in type I latency cells than in type III cells, consistent with the expression pattern of miR-190 (Fig. 8). To determine if the up-regulation of miR-190/Talin2 in latency I cell lines was due to an increase in miR-190/Talin2 promoter transcriptional activity, the promoter was cloned into a luciferase reporter. The promoter construct was modeled after that used previously by Beezhold et al., 2011. The promoter reporter was introduced into Sav I (type I latency) and Sav III (type III latency) cells and a high luciferase activity was only detected in the Sav I cells ($p<0.003$) (Fig. 9A). This suggests that miR-190 up-regulation in type I latency cells occurs at the transcriptional level and the promoter-reporter provided a tool to study the differential expression of miR-190 in EBV latently infected B cells.

The differential expression of miR-190 in types I and III latency as well as the different expression between EBV positive and negative Akata cells suggested that an EBV latent component may be responsible for the regulation of miR-190. As in type I latency cells, only one viral latent protein, EBNA1 and several noncoding RNAs, including the EBERs, are known to be expressed. We examined the effect of expression of EBNA-1 and EBERs, respectively, on miR-190 promoter activity in BJAB cells with EBNA-1 and EBER stable expression cell lines. The expression of EBNA1 and EBERs in these stably transfected BJAB cells was confirmed by Western analysis and quantitative RT-PCR, respectively (Fig. 9B-D). There was no significant difference in miR-190 promoter activity between BJAB cells that stably express EBNA1 and the parental BJAB cells (Fig. 9E). However, miR-190 promoter activity was found to be

elevated in the BJAB cells that stably express EBER-1 and EBER-2, respectively, in comparison to the control parental BJAB cells (Fig. 9E).

Additionally the miR-190 expression in these stably transfected BJAB cell lines were also examined and results showed the miR-190 expression level was not significantly altered by the expression of EBNA1, but dramatically elevated in the BJAB cells expressing EBERs in comparison to control BJAB cells (Fig. 9F).

EBERs are expressed in EBV-infected cells of all types of latency. We questioned whether the expression levels of EBER RNAs are very different in type I vs. type III latent cells and tested this hypothesis by examining the EBER RNA levels in three pairs of type I and type III EBV latently infected cells. Interestingly, the expression of EBER-1 is higher in type I latency cell lines (Sav I, Mutu I, Kem I) compared to their type III latency counterparts (Sav III, Mutu III and Kem III) (Fig. 10A). EBER2 expression is robustly higher in Sav I and Mutu I vs. Sav III and Mutu III cells (Fig. 10B). Finally, the effect of EBERs on the miR-190 promoter was examined in a transient transfection study, where the miR-190 / Talin2 promoter-luciferase reporter was introduced into 293T cells along with EBER-1, EBER-2, both EBERs, or control expression (pU6) vectors. A higher luciferase activity of the miR-190 / Talin2 promoter was observed with expression of EBER-1, EBER-2 or EBERs 1 and 2 compared to the control vector (pU6) counterpart (Fig. 10C). Taken together, these results suggest that EBER RNAs have a potential role in the regulation of miR-190 expression in type I latency.

2.4 Discussion

Through identifying miRNAs that are differentially expressed in type I latency cells, we aimed to find miRNAs that play roles in establishing and maintaining EBV latent infection and regulation of latency program switch. In the current study, miR-190 is highly expressed in type I latency cell lines compared to their genetically identical type III latency counterparts. The functions of miR-190 in type I latency cells were explored by identifying and studying its target mRNAs. The results showed that through down-regulating cellular genes TP53INP1 and NR4A3, miR-190 could contribute to evasion of cell apoptosis and prevention of viral reactivation, potentially to maintain the latency I phenotype. These results provide insight into how EBV infection modulates host processes to its advantage.

By targeting TP53INP1, miR-190 promotes cell survival and prevents cell cycle arrest. Both apoptosis and cell cycle arrest are barriers that a latently infected cell may encounter, perhaps due to cellular sensing of EBV. Some of the EBV latency proteins, such as EBNA1, EBNA3C and LMP1, produce genomic instability by inducing DNA damage, inhibiting DNA repair or inactivating checkpoints (Gruhne et al., 2009b). EBNA1, the sole type I latency protein, was shown to promote DNA damage through reactive oxygen species production (Gruhne et al., 2009a). To ensure the success of infection, the virus needs to reduce genomic instability-associated apoptosis at every stage of its life cycle. In early infection, BHRF1 prevents the death of the host cell during virus production through its homology to the human anti-apoptotic protein Bcl-2, which allows BHRF1 binding to the pro-apoptotic protein Bim (Desbien et al., 2009). In type III latency, the viral proteins LMP-1, EBNA-LP and EBNA-3C may provide protection

from apoptosis by modulating levels of anti-apoptotic Bcl2 (LMP-1), by binding and inactivating p53 (EBNA-LP) and by physically interacting with and stabilizing IRF4 (EBNA-3C) (Banerjee et al., 2013; Thompson and Kurzrock, 2004). In type I latency cells where BHRF1, LMP-1, EBNA-LP and EBNA-3C are not expressed, miR-190 could contribute to prevention of apoptosis and cell cycle arrest. This is evident in experiments where over-expression of miR-190 in Raji cells resulted in both lowered apoptosis and decreased cell cycle arrest in G0/G1. This function can be achieved through miR-190 targeting the 3'UTR of TP53INP1, which plays a pivotal role in DNA damage prevention. Under the control of p53, p73 or E2F1 (p73 in p53 deficient and E2F1 in p53 null conditions), TP53INP1 has been shown to induce cell cycle arrest at the G0/G1 checkpoint and apoptosis (Hershko et al., 2005; Tomasini et al., 2003; Tomasini et al., 2005). While the ultimate activity of TP53INP1 is dictated by the tissue type or tumor microenvironment, TP53INP1 is downregulated in multiple cancers, including pancreatic, gastric and colon cancer (Gironella et al., 2007; Jiang et al., 2006; Shibuya et al., 2010). Additionally, TP53INP1 is a robust target of miRNA regulation. It has been known that miR-93, miR-130b (in HTLV-1 transformed T cells) and miR-155 (in pancreatic cancer cells) reduce TP53INP1 allowing for survival of the affected cell (Gironella et al., 2007; Yueng et al., 2008; Zhang et al., 2013). Kaposi's Sarcoma Herpesvirus, a gamma-herpesvirus closely related to EBV, targets TP53INP1 through a viral miRNA, miR-K12-11 (Haecker et al., 2012). In this respect, miR-190 appears to fit the role of other miRNAs, both cellular and viral, which target TP53INP1 to limit apoptosis and enhance survival. Thus our model regarding the role of miR-190 in type I latency establishment is as follows: in type I latently infected cells, EBNA-1 causes

genomic instability that in turn triggers cell cycle arrest or apoptosis. EBV may counteract this adverse effect by inducing miR-190 expression and down-regulating TP53INP1, thus preventing cell cycle arrest and apoptosis and providing a clear benefit to EBV and its latency establishment.

miR-190 overexpression in conjunction with an mRNA transcriptome analysis helped in identification of numerous potential miR-190 targets not suggested by algorithm. These targets included NR4A3, a cellular immediate early gene for EBV reactivation. EBV latency disruption though stimulation of EBV+ cell with anti-IgG resulted in the expression of NR4A3, which preceded viral lytic gene, bzlf1, expression (Ye et al., 2010). miR-190 expression is able to down-regulate NR4A3 and results in dampened bzlf1 expression and reduced EBV intracellular DNA expression. In this manner, miR-190 may contribute to maintenance of type I latency by preventing host cells from entering the lytic cycle.

Additionally, our study provided preliminary evidence indicating that EBV-encoded noncoding RNAs, EBER-1 and EBER-2 may play a role in regulating miR-190 expression. The EBERs transcripts, shown here to be more highly expressed in latency I cells over their latency III counterparts, have been shown to promote soft agar growth and tumorigenicity in nude mice in EBV negative Akata BL cell lines (Komano et al., 1999). The mechanism for EBERs involvement in higher miR-190 expression has yet to be elucidated, but both stable and transient expression of EBER-1 and EBER-2 resulted in increases in miR-190 expression and Talin2 activity. Our preliminary data support a model that high levels of EBER-1 and EBER-2 in type I latency cells stimulate activity in

the promoter of miR-190 /Talin2, perhaps through the YY1 transcription factor, which in turn activates the promoter of miR-190 /Talin2, resulting in expression of both miR-190 and Talin2 mRNA (Zheng 2010a).

In the setting of EBV type I latency with limited viral gene expression, it appears that the virus can influence the expression of cellular miRNA to enhance the survival of the infected cell and maintain long term latency. While a direct effect on the virus may not always be evident, miRNAs appear to contribute to an ideal cellular environment for viral persistence. The current study contributes to revealing the strategy that EBV utilizes to manipulate cellular miRNAs and facilitate EBV latent infection. A full understanding of the detailed mechanism underlying the role of miR-190 and other cellular miRNAs in EBV type I latency development and maintenance requires identification of a whole panel of mRNAs, cellular or viral, that are regulated by miR-190 and other type I associated cellular miRNAs in type I latently infected B cells. A direct and comprehensive identification of all the mRNA target sites bound by miRNAs, such as high-throughput sequencing of RNAs, both miRNA and mRNA, isolated by crosslinking and immunoprecipitation of argonaute (HITS-CLIP) (Riley et al., 2012), could be useful in identifying other miR-190 targeted mRNAs and comprehending the role of miR-190 in EBV latency.

2.5 Materials and Methods

2.5.1 Cell Culture

Multiple type I (Sav I, Mutu I, Kem I, Akata EBV-positive) and type III (Sav III, Mutu III, Kem III) EBV latently infected cell lines were utilized in this study. Sav I, Kem I

Mutu I and Akata EBV-positive cells were derived from BL patients. Sav III and Kem III were derived from same BL patients as their counterparts Sav I and Kem III, respectively (Jang et al., 2005; Hughes et al., 2011). Mutu III cells were derived from peripheral blood mononuclear cells (PBMCs) infected with EBV from Mutu I BL cells. Kem I and III cells were a gift from Dr. Jeff Sample at Penn State Medical Center. Sav I and III cells were provided by Dr. Luwen Zhang at University of Nebraska-Lincoln. BJAB cells stably expressing EBNA1 were a gift from Dr. Bill Sugden at University of Wisconsin at Madison. BJAB cells stably expressing EBER-1 and EBER-2 were developed by transfecting BJAB cells with EBER-1 and EBER-2 expression vectors followed by neomycin selection and cloning from single cells that stably express EBER-1 and EBER-2 RNA, respectively. These cells along with BJAB, BL41, Raji and Akata cell lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-Invitrogen Life Technologies, Carlsbad, Ca) and 1% penicillin-streptomycin-amphotericin (Gibco-Invitrogen). To induce DNA damage, Raji cells were treated with 0.2 µg/µl of doxorubicin for up to 72 hours. To induce bzlf1 expression, Raji cells were treated with 20 ng/ml TPA (Sigma) and Akata EBV-positive cells were treated with 7.5 µg /ml anti-IgG (Jackson Immuno-Research Labs). 293T cells were maintained in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin-amphotericin and were allowed to grow until 80% confluency before passaging. All cells were incubated at 37°C with 5% (v/v) CO2.

2.5.2 Plasmids and Reagents

A pSIF- neo-IRES-GFP plasmid with the miR-190 minigene inserted in the Bam HI and ecoRI site and an empty control were provided by Dr. Yong Li of the University of Louisville. The 3'UTR of TP53INP1 was generated by PCR with the primers (5'-GGACTAGTTTCTGGAACAACCCAAGAGC-3' and 5'-AGCTTTGTTTAAACCTGCACTAATGGGTTAGTTACAGAC-3') and cloned into a pMIR-report miRNA Expression Reporter Vector (Life Technologies) expression plasmid. The suitable mutation was made with oligonucleotides (5'-CACTTTTTCAGATTATTTCTGGAATTACAGTGTTTGGGGGTGTC-3' and 5'GACACCCCCAAACACTGTAATTCCAGAATTATTCTGAAAAAGTG-3') using the Quikchange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, California). The miR-190/talin2 promoter sequence is defined by the region of proximal genomic DNA within 1Kb (1095 bp) of the human Talin2 promoter region and was amplified by PCR with the primers (5' ATCGGCTAGCCACCATGCCAGGCTAATTTT-3' and 5' CAGTCTCGAGACTCGACACGCATCGTACAC-3') and cloned into a pGL3 luciferase repoter vector (Promega) (Beezhold et al, 2011). The EBER-1 expression vector was generated by cloning a 530 bp Sac I-Sau3A restriction fragment of EBV genomic DNAs that contains the EBER-1 genes with the promoter into pGem-3 vector (Promega). The EBER-2 expression vector was generated by cloning a 525 bp Sau3A-EcoRI restriction fragment of EBV genomic DNAs that contains the EBER-2 genes with the promoter into pGem-3 vector (Promega).

2.5.3 RNA and DNA preparation for RT-PCR

RNA was extracted from lymphocyte lines using a modified TRIZOL method (Invitrogen). Briefly, 107 cells were suspended in 1 ml Trizol reagent, homogenized and incubated for 5 min on ice. 0.2 ml of chloroform was added to each sample followed by vigorous vortex for 1 min. The samples were centrifuged for 15 min at 14,000 rpm at 4oC, and the upper aqueous phase was transferred to fresh tubes. The samples were re-extracted with an equal volume of phenol/chloroform/isoamyl alcohol (Fisher Scientific, Pittsburg, PA) once and an equal volume of isopropyl alcohol once again. RNAs were recovered by ethanol precipitation, dissolved in RNase free H20 and stored at 80oC until further processing. For intracellular EBV DNA analysis, DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's protocol.

2.5.4 Microarray and data analysis

Total RNA of Sav I and Sav III cells were submitted to the Microarray Core Facility of University of Pennsylvania for miRNA expression profiling using Exiqon miRCURY LNA™ miRNA array. Total RNA from cell lines expressing pSIF-miR-190 or pSIF were submitted for mRNA expression profiling using the Affymetrix GeneChip Human Exon 1.0 ST Array. mRNA array data analysis was performed with the Partek® Geneomics Suite 6.4 software. The microarray probe result files from individual hybridizations were normalized using the Robust Multi-Chip Average (RMA) algorithm and converted to log values. Data was analyzed by two-way ANOVA with a False Discovery Rate corrected p-value (Benjamini Hochberg). Differentially expressed mRNAs with a false discover rate of less than 0.1 and a fold change of less than -1.3 were

selected for study. TargetScan 5.1 was also used to identify those mRNAs with predicted miR-190 interaction sites.

2.5.6 Real-time RT-PCR for miRNA, mRNA and EBER RNA detection and quantitation

10 ng of total RNA was reverse transcribed using microRNA reverse transcription kit and stem-loop microRNA assay kit specific for miR-190 (Applied Biosystems, Foster City, CA). RT-PCR was done on Roche LightCycler® System using the TaqMan® microRNA assay kit (Applied Biosystems) and LightCycler® TaqMan® master mix (Roche, Mannheim, Germany) following the manufacturer's instructions. Human small nuclear RNA U6 (RNU6B) was used to normalize mature miRNA data. For mRNA quantification, 1 μg of total RNA was reverse transcribed using SuperscriptTM II reverse transcriptase according to manufacture's instructions (Invitrogen). The resulting cDNA was subjected to qPCR using the QuantiTectPrimer Assay SYBR for Talin2 (Hs_TLN2_2_SG), TP53INP1(Hs_TP53INP1_1_SG), NR4A3 (Hs_NR4A3_2_SG), and GAPDH (Hs_GAPDH_2_SG) (Qiagen, Valencia, CA). Custom primers were developed for bzlf1 (5'-TACAAGAATCGGGTGGCTTC-3' and 5'-GCACATCTGCTTCAACAGGA-3') with results being normalized to GAPDH (5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3'). EBER1 and EBER2 were quantitated by qRT-PCR with reverse transcription primer (EBER-1: 5'-ACCACCAGCTGGTACTTGACCGA-3', EBER2: 5'-CAAGCCGAATACCCTTCTCCCAGA-3') and custom-designed TaqMan® assay kits (Applied Biosystems). Probes from Qiagen and Life Technologies offer close to 100% PCR efficiency for reliable relative quantification of expression. Specifically, the amount of relative gene expression of miRNA or mRNA is presented as 2 (−ΔCt), ΔCt=

Ct gene of interest - Ct housekeeping gene.

2.5.7 Analysis of intracellular EBV genomic DNA content

After induction with anti-IgG, total DNAs were purified from the EBV-positive Akata cells (induced and uninduced) using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa). EBV genomic DNA was quantified by real-time PCR with primers for EBNA1 (5'-CATTGAGTCGTCTCCCCTTTGGAAT-3' and 5'-TCATAACAAGGTCCTTAATCGCATC-3') and normalized to GAPDH (5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCAATACGACCAAATCC-3'). Quantitative PCR was done on Roche LightCycler® System using the LightCycler® FastStart DNA master SYBR green I kit® (Roche, Mannheim, Germany) following the manufacturer's instructions.

2.5.8 Western blot analysis

Whole cell extract were prepared in lysis buffer [20 mM Na2HPO4 (PH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.3% (v/v) Triton X-100, 100 μM PMSF with complete protease inhibitor cocktail tablet (Roche). Protein concentrations of the supernatant were determined using Bradford protein assay (Bio-Rad, Hercules, CA). Samples were diluted with NuPAGE® LDS Sample Buffer (Invitrogen), denatured by boiling for 10 min, and loaded on 4-12% Tris-Bis pre-cast gels (Invitrogen). Proteins in the gels were transferred to nitrocellulose membrane (manufacturer). The membranes were blocked with 5% (w/v) non-fat milk in phosphate buffered saline (PBS) containing 0.1% (v/v) Tween-20 (PBS-T), followed by overnight incubation of specific primary antibodies, including rabbit anti-TP53INP1 (Sigma); rabbit anti -p53Ser46, rabbit anti-

Poly (ADP-4 ribose) polymerase 1 (PARP- 1), mouse anti-b-actin (Cell Signaling); rabbit anti-Nor1/NR4A3 (Novus Biologicals) at varying dilutions in blocking buffer at 4°C. The blots were treated with HRP conjugated anti-rabbit or anti-mouse IgG (Pierce, Rockford, IL) and detected with Supersignal West Dura Extended Duration Substrate (Pierce).

2.5.9 Retroviral Transduction

293T cells were transfected with 4.5 μg of pSIF/pSIF-190 microRNA expression vector, 4.5 μg of pFIV-34N lentiviral gag-pol packaging vector and 0.57 μg of pVSV-G envelope vector using the calcium phosphate method. After 72 hours, lentiviral particles were harvested and filtered through a 0.45 micron filter and used to infect 1x 106 suspension cells (BJAB, SAVIII, Raji, Akata EBV-positive). Cells were spinoculated at 2500 rpm for 1 hour in the presence of 8 μg /ml polybrene. Forty-eight hours post infection, cells were selected for vector expression by G418 treatment. After 10-14 days of selection RNA, protein and functional assays were completed.

2.5.10 DNA and miRNA inhibitor Transfection

293T cells were transfected for luciferase assays with Lipofectamine 2000 according to manufacturer's recommendations. Plasmids or miRNA inhibitors, miRIDIAN inhibitors (ThermoScientific), were introduced into cells with the Amaxa Nucleofector II and Kit V (Lonza, Basel, Switzerland). Briefly, 3-5x 106 cells was suspended in 100 μl of nucleofector solution and transferred into cuvettes. In the Nucleofector II system (Lonza), Program T020 was used for BJAB and Sav III cells, program C009 was used for Sav I and program G016 was used for Akata EBV-positive cells.

2.5.11 Luciferase reporter assay

This assay was used in two applications: (i) to assess 3'UTR targeting activities and (ii) to measure promoter activities. Cells were cotransfected 1:1 with pSIF-miR190 (or pSIF) and 3'UTRs of TP53INP1 reporter (or its seed sequence-deletion mutant) along with 1/20 total DNA of the Renilla reporter plasmid OR 2 (treatment):1(reporter) pEBERs (or pU6) and pmiR-190/TLN2(or pGL3 reporter) along with 1/20 total DNA of the Renilla reporter plasmid. After 48 hours cells were lysed and luciferase/renilla activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was normalized to renilla activity for each transfection.

2.5.12 Flow Cytometry

For cell cycle and apoptosis data, cells were washed once in PBS and fixed in 70% (v/v) ethanol overnight at -20°C. Staining for DNA content was performed with 50 μg/ml propidium iodide and 100 μg/ml RNase A for 60 min in the dark at 37°C. Cell were subsequently washed with PBS and re -suspended in 500 ul of PBS for analysis. TUNEL was performed with the In Situ death detection kit (Roche) according to manufacturers suggestions for suspension cells. A minimum of 10,000 events were acquired by LSRII using FACSDiva (BD biosciences, San Jose, CA). Data was analyzed by WinList – TUNEL and ModFit— cell cycle (Verity Software).

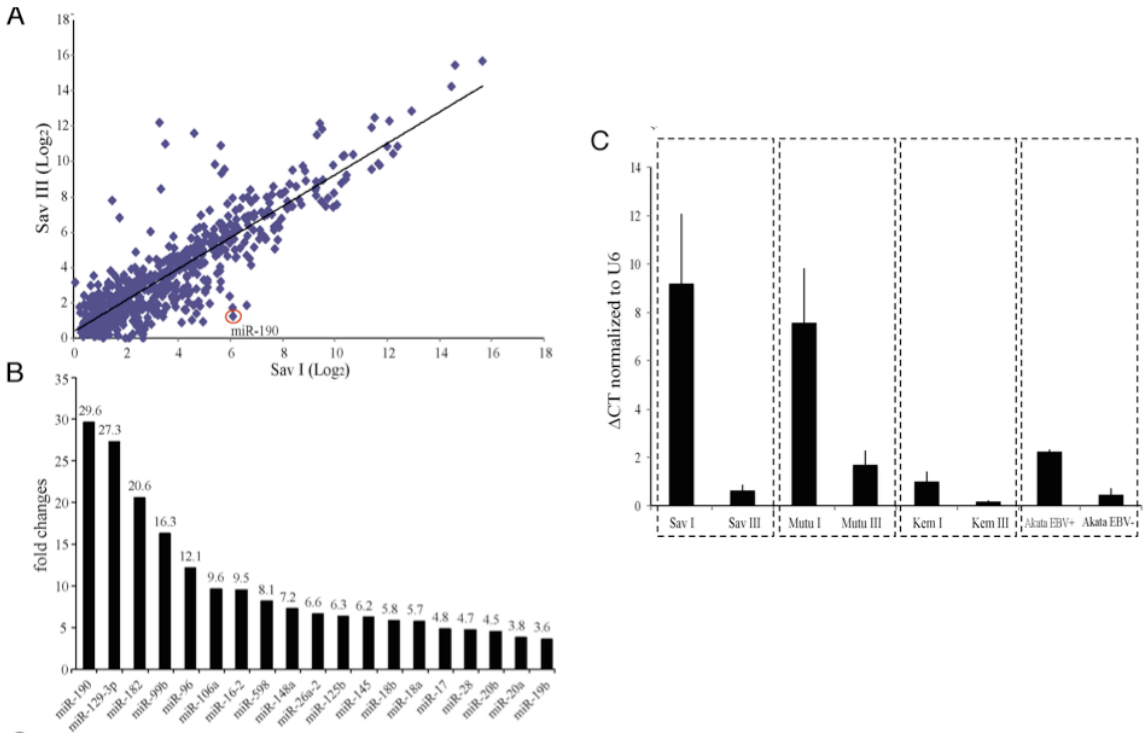

Figure 2: miR-190 is highly expressed in type I latency vs. type III latency cells. Sav I (type I latency) and Sav III (type III latency RNAs were subjected to an ExiqonTM microRNA array. (A) The expression levels of each miRNA in Sav I and Sav III cells are presented on a scatter plot and (B) a bar graph of fold change in Sav I vs. Sav III cells. (C) miR-190 expression levels in three matched type I (Sav I, Mutu I, Kem I) and type III (Sav III, Mutu III, Kem III) latency cell lines as well as EBV-positive (Akata EBV+) BL and EBV- (Akata EBV-, BJAB and BL41) B cell lines were measured by qRT-PCR with a specific TaqMan® kit and normalized to U6 snoRNA. Notable comparisons are in dashed boxes. Error bars indicate standard deviations for miR-190 expression in three separate culture vessels

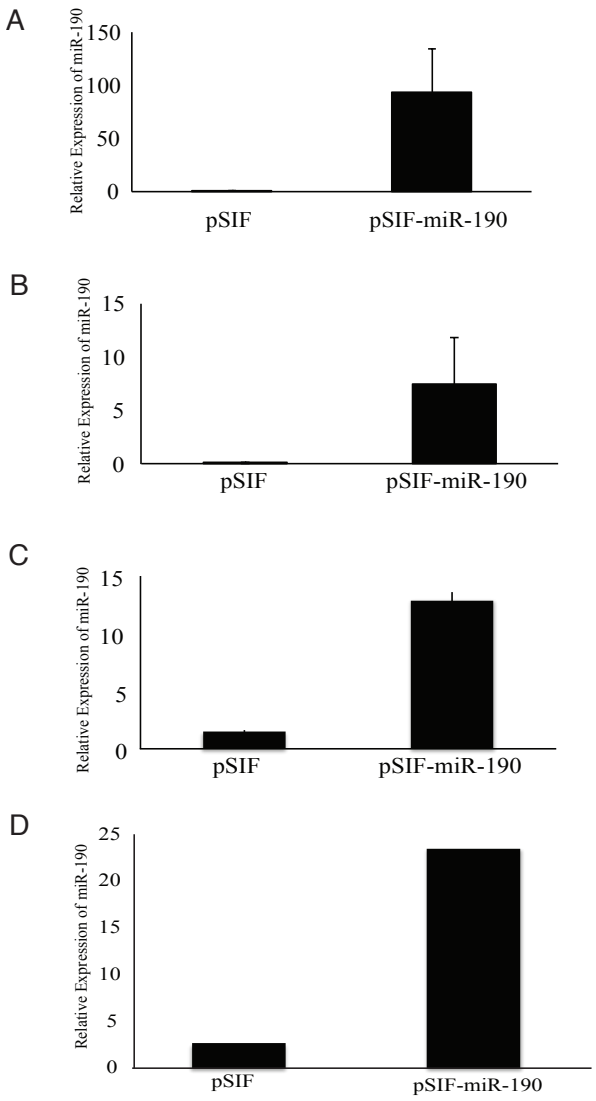

Figure 3: Overexpression of mir-190 in numerous cell lines with a lentiviral expression system.

Following lentiviral transduction of pSIF or miR-190 and antibiotic selection, miR-190 expression in BJAB (A), Sav III (B), Raji (C) and Akata EBV+ (D) cells was evaluated by qRT- PCR with a specific TaqMan® kit and normalized to U6 snRNA.

Table 1: Summary of targets identified by microarray from Sav III and BJAB miR-190 lentiviral overexpression.

	Gene Name	Gene Symbol
Predicted targets of miR-190 and genes down-regulated with miR-190 overexpression in SAVIII	Tumor suppressor p53 inducible nuclear protein 1	TP53INP1
	Fibrillin 1	FBN1
Genes down-regulated with miR-190 overexpression in both BJAB and SAVIII	n-myc downstream regulated 1	NDRG1
	Nuclear receptor subfamily 4, group A, member 3	NR4A3
	GTP binding protein overexpressed in skeletal muscle	GEM

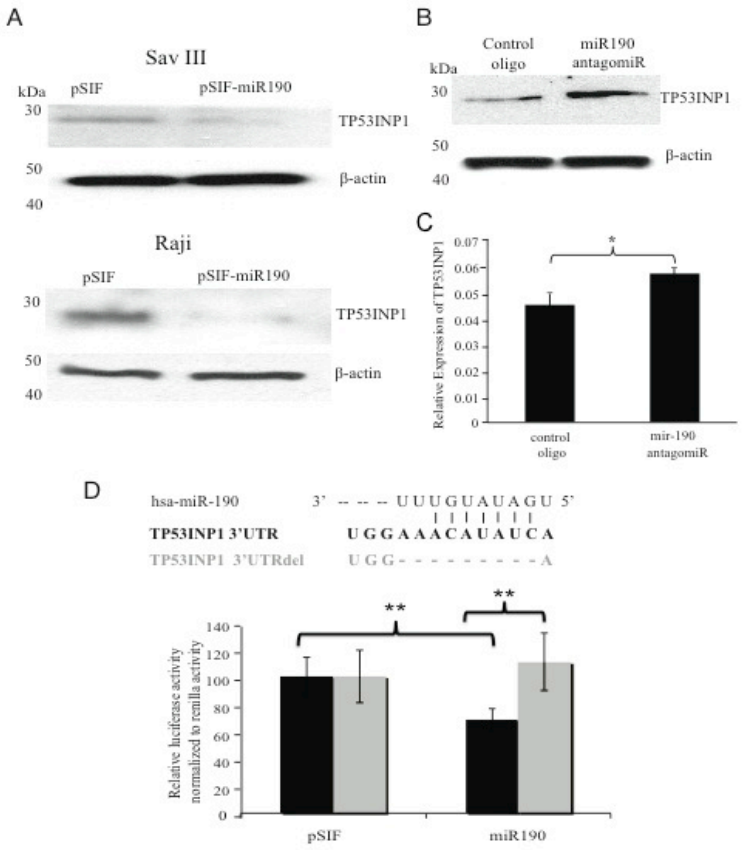

Figure 4: miR-190 reduces TP53INP1 expression.
(A) Lentiviral expression vectors for miR-190 (pSIF-miR-190) and the control empty vector (pSIF) were introduced into Sav III and Raji cells via lentiviral transduction. Cell extracts were subjected to Western blotting for the detection of TP53INP1 and β-actin as a protein loading control. (B) miRNA antagomiR for miR-190 and the control RNA targeting C. elegans RNA were introduced into Sav I cells via nucleofection. 24 h postnucleofection cell lysates were analyzed by Western blot for TP53INP1 (upper panel). RNA was also analyzed for TP53INP1 and GAPDH mRNA expression via RT-PCR. Data are means and standard deviations for three experimental replicates and comparisons are by one-tailed Student′s t-test. $*P \leq 0.05$; $**P \leq 0.01$ (lower panel). (C) To test if miR-190 directly targets the 3'UTR of TP53INP1, the 3'UTR sequence of TP53INP1 (black) or its mutant with the predicted seed sequence deleted (gray) was cloned into a pMiR luciferase reporter vector. The wild type 3'UTR or the seed sequence deletion reporters were transfected into 293T cells along with miR-190 expression vector (or empty vector) and renilla normalization constructs. Data are means and standard deviations for three experimental replicates. Comparisons of data are one-way ANOVA followed by Tukey′s test. $**P \leq 0.01$.

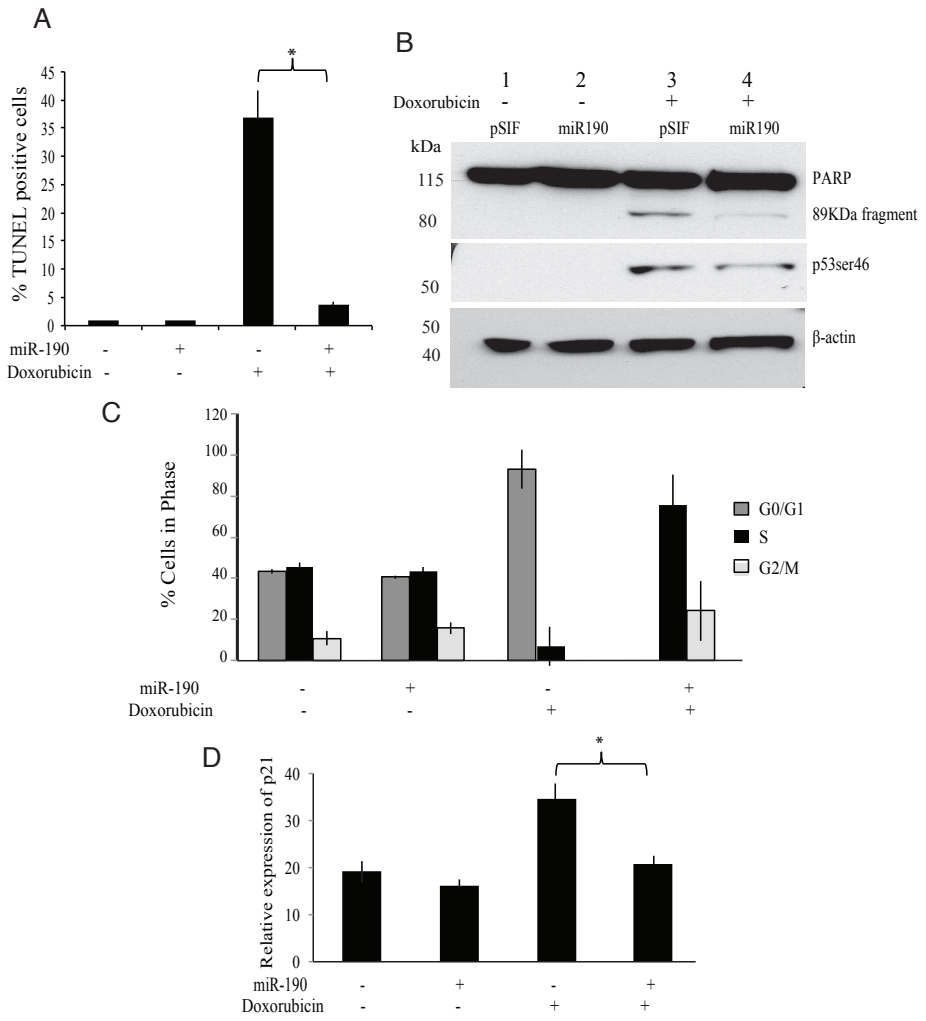

Figure 5: miR-190 protects type III latency cells from cell cycle G1 arrest and apoptosis.

DNA damage was induced in Raji cells overexpressing pSIF-miR-190 or PSIF by treating cells with doxorubicin (Dox) for 72 h. (A) Apoptosis was assayed with TUNEL assay. Data are means and standard deviations for three experimental replicates and comparisons are by Student′s t-test. $*P \leq 0.05$; $**P \leq 0.01$. (B) Cell lysates were collected to evaluate expression levels of PARP cleavage, p53serine46 phosphorylation and β-actin by Western blot. (C) Cells were also collected to assay cell cycle distribution by propidium iodide staining. (D) RNA was analyzed for p21 mRNA by RT-PCR and results were normalized to GAPDH. Data are means and standard deviations for three experimental replicates and comparisons are by Student′s t-test. $*P \leq 0.05$; $**P \leq 0.01$.

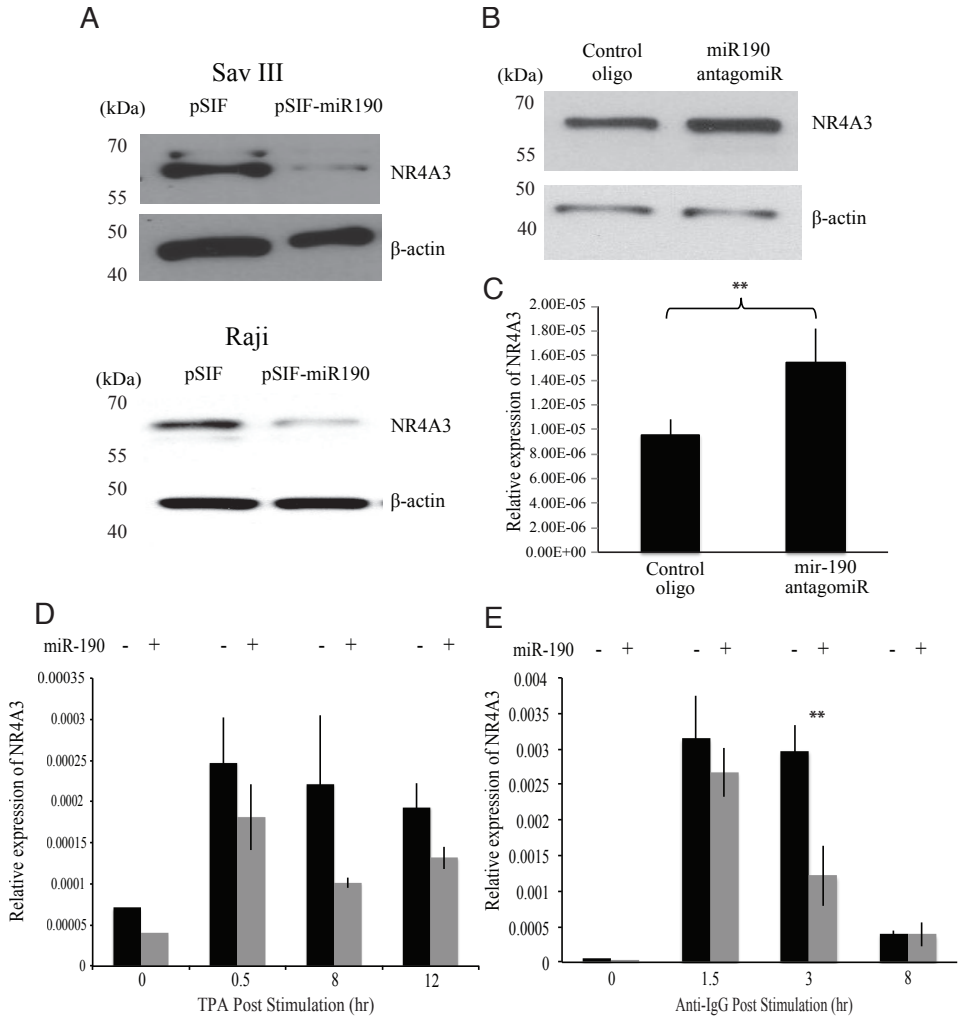

Figure 6: miR-190 reduces NR4A3 expression.
(A) Lentiviral expression vectors for miR-190 and the control empty vector (pSIF) were introduced into Sav III and Raji cells via lentiviral transduction, cell extracts were subjected to Western blotting for the detection of NR4A3. β-actin was included as a protein loading control. (B and C) miRNA antagomiRs for miR-190 and a control RNA targeting C. elegans RNA were introduced into Akata EBV positive cells via nucleofection. Forty-eight hours postnucleofection cell lysates were collected and analyzed via Western blot for NR4A3and β-actin (B). RNA was analyzed for NR4A3 mRNA expression via qRT-PCR and normalized to GAPDH (C). (D and E) NR4A3 mRNA expression was analyzed by RT-PCR in Raji (D) and Akata EBV+ (E) cells expressing empty vector (pSIF) or miR-190 following treatment with reactivation stimuli, TPA (D) or anti-IgG (E), respectively, over the course of several hours.

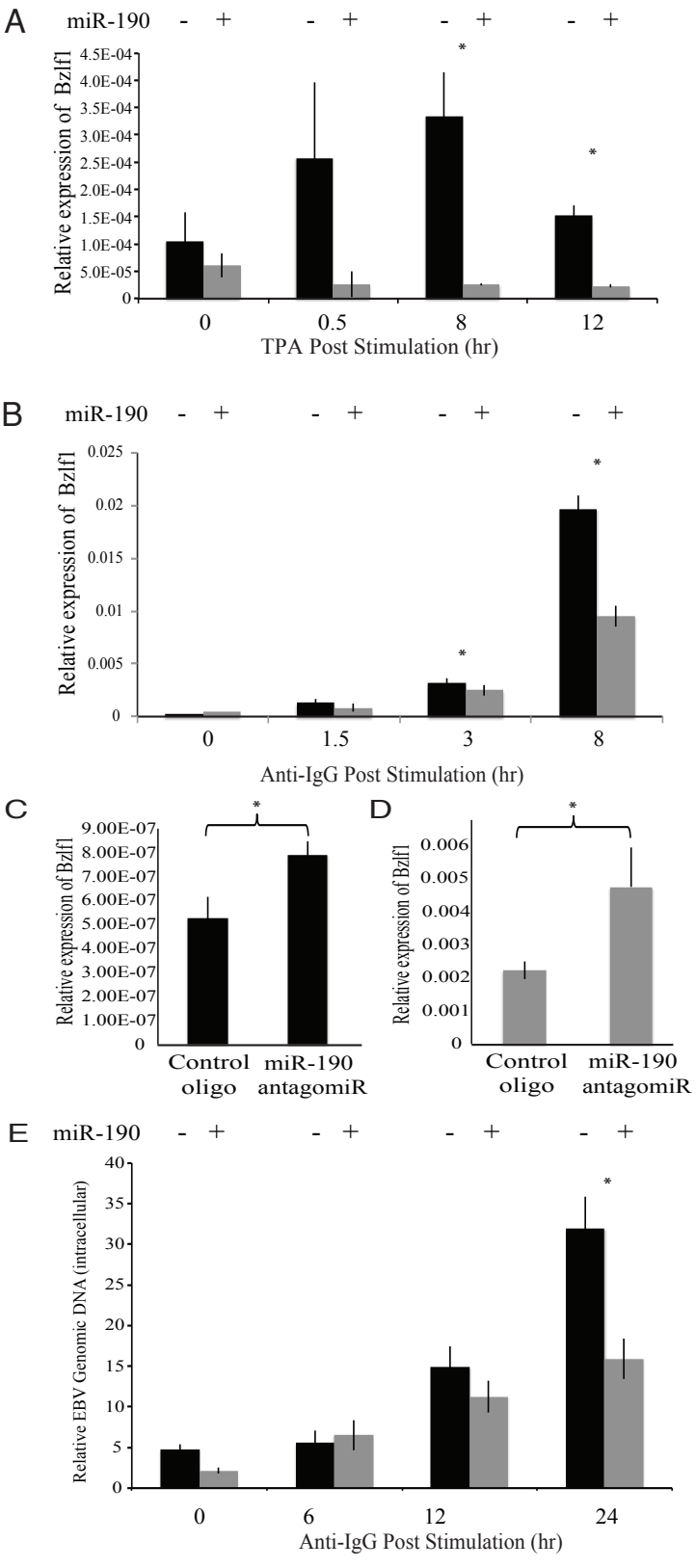
A
miR-190 - + - + - + - +
Relative expression of BzlfI
4.5E-04
4.0E-04
3.5E-04
3.0E-04
2.5E-04
2.0E-04
1.5E-04
1.0E-04
5.0E-05
0
*
*
0
0.5
8
12
TPA Post Stimulation (hr)
B
miR-190 - + - + - + - +
Relative expression of BzlfI
0.025
0.02
0.015
0.01
0.005
0
*
*
0
1.5
3
8
Anti-IgG Post Stimulation (hr)
C
Relative expression of BzlfI
9.00E-07
8.00E-07
7.00E-07
6.00E-07
5.00E-07
4.00E-07
3.00E-07
2.00E-07
1.00E-07
0
*
Control oligo
miR-190 antagomiR
D
Relative expression of BzlfI
0.006
0.005
0.004
0.003
0.002
0.001
0
*
Control oligo
miR-190 antagomiR
E
miR-190 - + - + - + - +
Relative EBV Genomic DNA (intracellular)
40
35
30
25
20
15
10
5
0
*
0
6
12
24
Anti-IgG Post Stimulation (hr)

Figure 7: miR-190 reduces bzlf1 expression and EBV DNA replication.
(A) Viral reactivation was induced in Raji cells overexpressing miR-190 or pSIF (empty vector) by treatment with TPA for various time points. RNA was analyzed for bzlf1 and GAPDH mRNA expression via qRT-PCR. (B) Viral reactivation was induced in Akata EBV-positive cells overexpressing miR-190 or pSIF (empty vector) by treatment with anti-IgG for various time points. (C and D) RNA was analyzed for bzlf1 and GAPDH mRNA expression via qRT-PCR. RNA was collected from type I latency cell lines, Akata EBV-positive (C) and Sav I miR-190 (D) that had been nucleofected with miR-190 antagomiR or control oligo in the absence of reactivation stimulus and analyzed for bzlf1 and GAPDH mRNA expression via qRT-PCR. (E) DNA was isolated Akata EBV positive cells expressing pSIF or miR-190 after anti-IgG treatment up to 24 h and intracellular EBV genomic DNA (normalized to GAPDH) was measured by qPCR. Data are means and standard deviations for three experimental replicates and comparisons are by Student′s t-test. $*P \leq 0.05$; $**P \leq 0.01$.

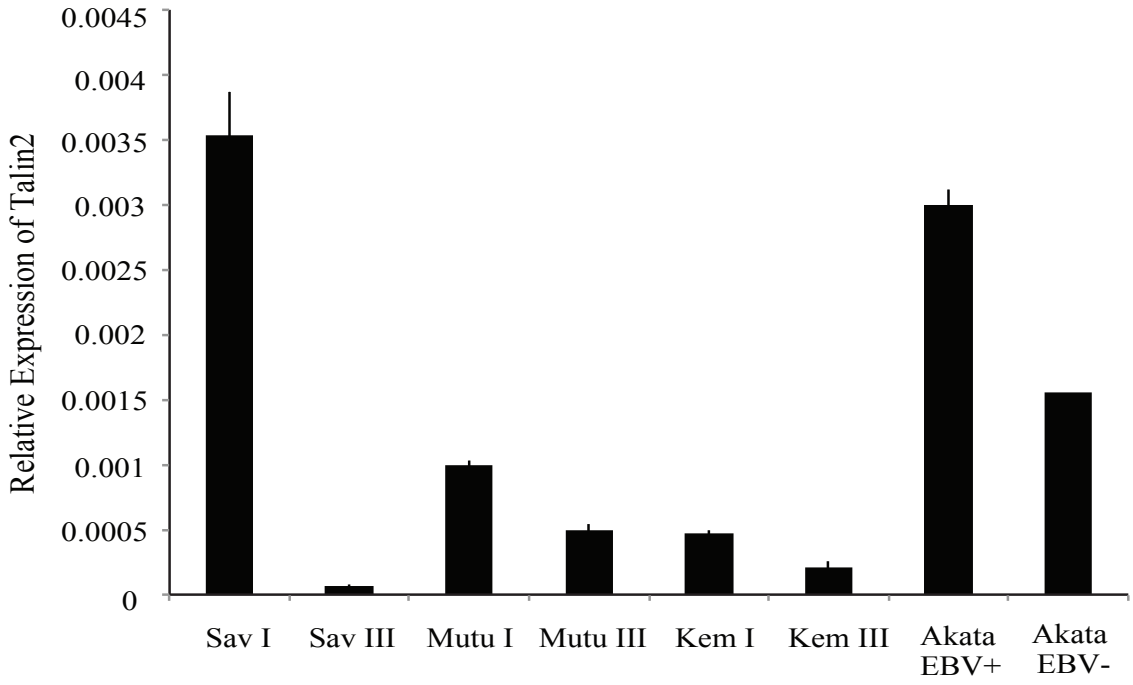

Figure 8: Talin2 expression in type I and type III latency cell lines.
RNA isolated from cell lines representing type I (Sav I, Mutu I, Kem I) and type III (Sav III, Mutu III and Kem III) was analyzed for Talin2 mRNA expression via qRT-PCR and normalized to GAPDH.

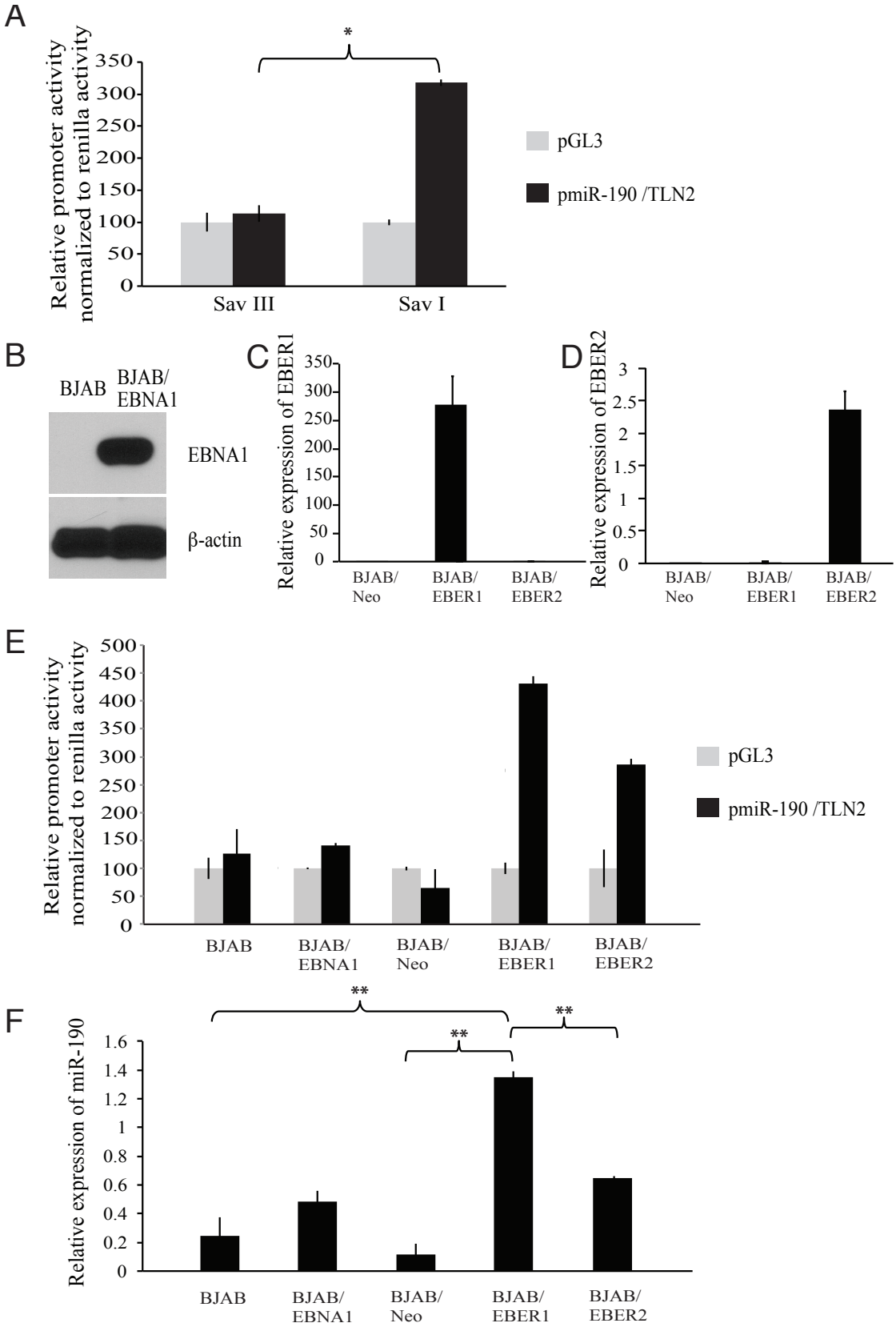
A
Relative promoter activity normalized to renilla activity
350
300
250
200
150
100
50
0
*
Sav III
Sav I
pGL3
pmiR-190 /TLN2
B
BJAB
BJAB/ EBNA1
EBNA1
β-actin
C
Relative expression of EBER1
350
300
250
200
150
100
50
0
BJAB/ Neo
BJAB/ EBER1
BJAB/ EBER2
D
Relative expression of EBER2
3
2.5
2
1.5
1
0.5
0
BJAB/ Neo
BJAB/ EBER1
BJAB/ EBER2
E
Relative promoter activity normalized to renilla activity
500
450
400
350
300
250
200
150
100
50
0
BJAB
BJAB/ EBNA1
BJAB/ Neo
BJAB/ EBER1
BJAB/ EBER2
pGL3
pmiR-190 /TLN2
F
Relative expression of miR-190
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
BJAB
BJAB/ EBNA1
BJAB/ Neo
BJAB/ EBER1
BJAB/ EBER2

Figure 9: miR-190 promoter activity is upregulated in type I latency and responsive to the presence of EBERs.
(A) The promoter for miR-190/Talin2 was cloned into a luciferase reporter vector. The miR-190/Talin2 promoter-reporter or an empty pGL3 vector was nucleofected into either Sav I or SavIII cell lines with a renilla nucleofection control. Luciferase activity was normalized to transfection and pGL3 controls. Data are means and standard deviations for three experimental replicates. $*P \leq 0.05$; $**P \leq 0.01$. (B–E) The miR-190/Talin2 promoter-reporter was introduced into BJAB cells that stably transfected with EBNA1, EBER-1 and EBER-2. The expression of EBNA1 was confirmed by a Western blotting (B) and the expression of EBER-1 and EBER-2 was verified by qRT-PCR (C and D respectively). The luciferase activities were normalized to transfection and pGL3 controls (E). (F) Total RNA was purified from the BJAB cells stably transfected with EBNA1 and EBERs. miR-190 levels in these cells were evaluated by TaqMan® RT-PCR and normalized to U6 snRNA. Data are means and standard deviations for three experimental replicates. Comparisons of data are one-way ANOVA followed by Tukey′s test. $**P \leq 0.01$.

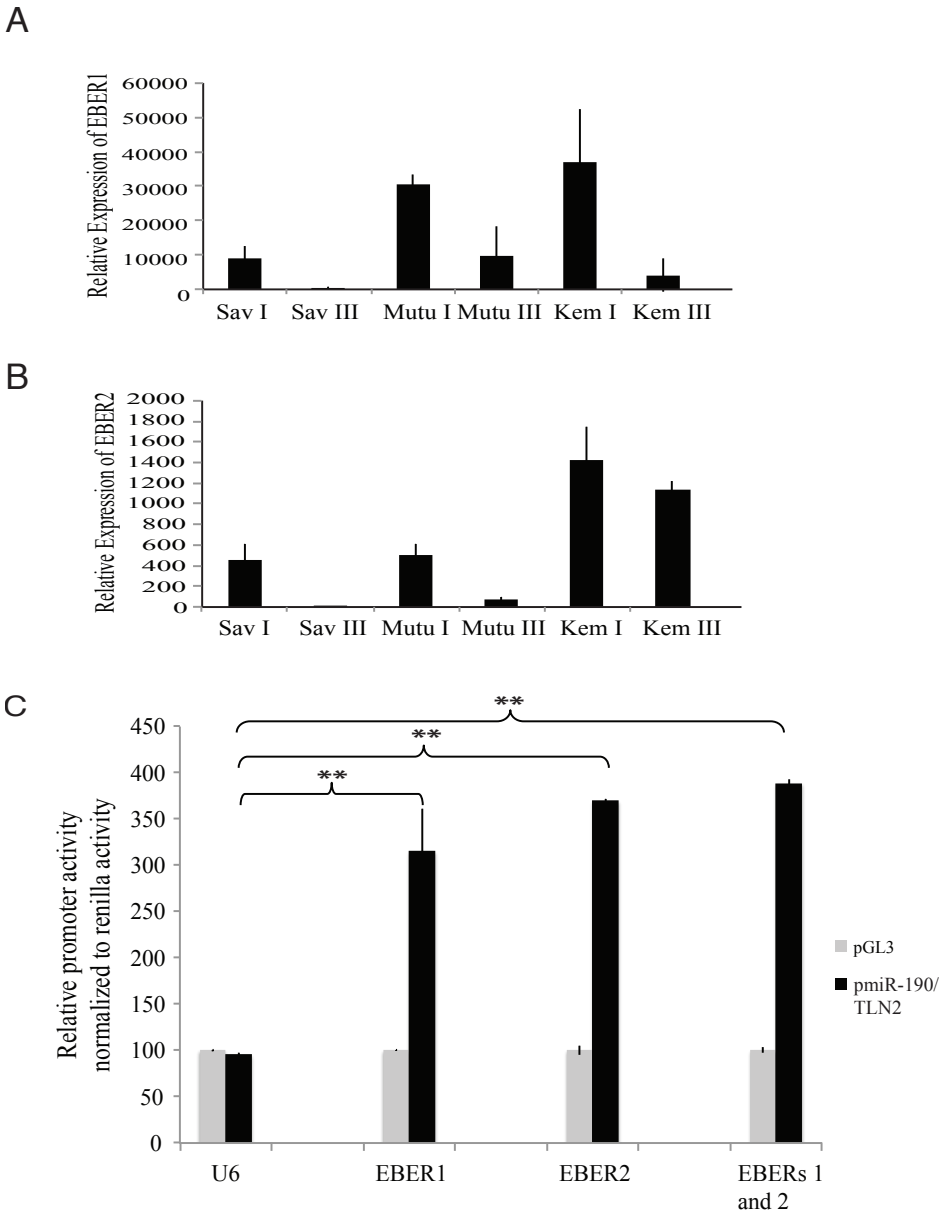

Figure 10: EBERs are expressed in higher levels in type I latency cells compared to type III latency cells and enhance miR-190 promoter activity.
The levels of EBER-1 (A) and EBER-2 RNA (B) in three pairs of types I (Sav I, Mutu I and Kem I) and III (Sav III, Mutu III and Kem III) latency cells were examined using qRT-PCR. (C) miR-190/Talin2 promoter-luciferase reporter or pGL3 (empty vector) was cotransfected into 293T cells with EBER-1, EBER-2, both EBERs or U6 expression vectors. Luciferase activities were normalized to transfection and pGL3 controls. Data are means and standard deviations for three experimental replicates. Comparisons of data are one-way ANOVA followed by Tukey′s test. $**P \leq 0.01$.

Chapter 3- EBERs regulate cellular miRNA expression through triggering RNA sensor signaling

3.1 Summary

Previously, we observed that the cellular miRNA, mIR-190, is upregulated in type I latency. We also observed an increase in miR-190/TLN2 promoter activity in the presence of the EBV-encoded small RNAs (EBERs). EBERs are highly expressed during latency and are more abundant in type I latency vs type III latency (Cramer et al., 2014). This differential expression may have a role in latency maintenance. Here we further establish a connection between EBERs and miR-190 expression and promoter activity. 293-BAC cells expressing WT EBERS exhibit higher miR-190/TLN2 promoter activity compared to EBERs null 293-BAC cells. EBERs are known to interact with the dsRNA sensor, RIG-I (Samanta et al., 2006). We find that in the presence of activated RIG-I, miR-190 expression and promoter activity is increased in a variety of cell backgrounds. Analysis of the miR-190/TLN2 promoter indicated potential binding sites for NF-kB, a downstream effector of RIG-I, and YY1, a known mediator of miR-190 expression. The essential roles of YY1 and NF-kB in miR-190/TLN2 promoter activation are examined through deletion of specific binding motifs in the miR-190/TLN2 promoter.

3.2 Introduction

Epstein Barr-Virus (EBV) is a ubiquitous herpesvirus that is the causative agent of infectious mononucleosis and is associated with numerous diseases; including cancers, like Burkitt's lymphoma and AIDS-associated lymphomas (Kieff et al. 2007). The latency program of EBV infected cells characterizes many EBV associated diseases,

including; endemic Burkitt's lymphoma (latency I), Hodgkin's disease (latency II) and post-transplant lympoproliferative disease (latency III) (Kieff et al 2007; Thorley-Lawson and Gross, 2004). While these programs can be associated with overt disease, EBV utilizes these latency programs to propagate and establish a reservoir in B lymphocytes (Thorley-Lawson, 2001). There are multiple latency programs utilized by EBV. Each of the latency programs displays unique characteristics, which are due to either viral or cellular components. Briefly, these programs are latency III, II, I and 0, listed in order from most viral protein expression to least. Latency III is accompanied by expression of nine latent proteins, (EBNA1, EBNA2, EBNA3A-C, and EBNALP and LMP1, LMP2A, and LMP2B) and is characterized by viral driven proliferation. Latency I, which typically persists in resting mature B cells, has protein expression limited to only EBNA-1 to allow for viral episome maintenance (Babcock et al., 2000; Kieff et al 2007; Thorley-Lawson, 2001).

In addition to protein expression during latency, non-coding viral RNAs are also expressed, including the EBERs (EBV-encoded small RNAs, and upwards of 40 viral miRNAs (Qui et al., 2011). Interestingly, EBERs are the most abundant transcript in latency, with a reduction in expression during the lytic cycle (Rosa et al., 1981). The viral miRNAs also display differential expression during latency, with miRNAs from the BHRF1 and BART fragments expressed in latency III while only BART miRNAs, particularly miR-BART18-5p, are expressed in latency I/II (Qui et al., 2011, Qui et al., 2014) Additionally, the viral non-coding RNAs, EBERs 1 and 2, are more highly expressed in latency I relative to latency III (Cramer et al., 2014). This differential

expression of EBERs and viral miRNAs could indicate a role in latency maintenance or switch.

In conjunction with higher EBERs expression, latency I cells display a different profile of cellular miRNAs than latency III cells. The cellular miRNA, miR-190 is highly expressed in latency I. miR-190 is an intragenic miRNA that is transcribed with the gene Talin2. Our previous work has indicated that miR-190 expression and miR-190 promoter activity increased in the presence of EBERs (Cramer et al., 2014). Modulation of cellular miRNAs by viral non-coding RNAs is a strategy employed by other viruses to subtly alter their cellular environment. However, the means of this modulation varies for each virus and miRNA,

Other viral non-coding RNAs have been shown to affect cellular miRNAs. The primate Herpesvirus Saimiri U RNAs (HSURs) have been shown to bind to the seed sequence of mir-27 resulting in upregulation of miR-27 targets like FOXO1 (Cazalla et al., 2010). In murine cytomegalovirus, the highly abundant m169 transcript is responsible for the degradation of miR-27 and this interaction affected efficiency of viral replication in multiple organ sites (Marcinowski et al., 2012). The adenovirus VA RNAs exhibit miRNA suppressor activity through a variety of mechanisms, like acting as decoy substrates for exportin 5, DICER and RISC, all components necessary for mature miRNA formation. (Andersson et al., 2005).

EBERs have not yet been shown to directly affect cellular miRNA expression. However, multiple consequences of EBERs expression and interactions with cellular proteins have been identified. The EBERs genes render Akata EBV- cells less susceptible

to apoptosis (Komano et al., 1999). More recently, EBER2 has also been shown to localize with Pax5 to the terminal repeats of the EBV genome, and downregulates LMP1, -2A and -2B and enhances viral lytic replication (Lee et al., 2015). EBERs form intra-molecular pairings, which result in stem-loop structures resembling double stranded RNA (Glickman et al., 1988; Rosa et al., 1981). EBERs have been shown to interact with numerous innate immunity dsRNA sensors upstream of type I IFN expression. While EBERs forms complexes with a number of host proteins, including PKR, L22, and La another notable interaction is with innate immune sensors like RIG-I and TLR3. Retinoic acid inducible gene, RIG-I, is a cytosolic sensor of foreign 5' triphosphate double stranded RNAs, like EBERs (Iwakiri et al., 2009; Samanta et al., 2006; Samanta et al., 2008).

EBERs interaction with each of these innate immune factors has activating effects on downstream targets, including the signaling molecules IRF3 and NF-kB (Samanta et al., 2006; Samanta et al., 2008). Ultimate products of this activation include type I IFN and IL-10 production. Upon activation, NF-kB becomes phosphorylated and crosses from the cytoplasm to the nucleus where it is a transcription factor for immune response genes (Yoneyama et al., 2004). The activation of NF-kB in latency I cell lines like Akata EBV+ and Mutu I is attributed to RIG-I, as knockdown ablates this activation (Samanta et al., 2008). Additionally, NF-kB positively regulates the expression of YY1 (Wang et al., 2007). YY1 is also a transcription factor for miR-190/Talin2 promoter (Zheng et al, 2010a). This indicates a potential connection from EBERs to miR-190 through the RIG-I-NF-kB axis. Here we provide evidence that EBERs modulation of the RIG-I pathway upregulates expression of the cellular miRNA, miR-190. We also demonstrate the

importance of the transcription factors YY1 and NF-kB in EBERs-induced upregulation of miR-190.

3.3 Results

3.3.1 Effect of EBER-WT vs. EBER-null BAC-EBV on miR-190 promoter activity in 293T cells

The miR-190/Talin2 promoter was activated in the presence of EBERs in BJAB cells stably expressing EBERS and in 293T cells transiently transfected with EBERs (Cramer et al., 2014). Additionally, 293T-BAC-EBV cell lines with both EBERs, EBER1 only, EBER2 only or no EBERs (EBER null) were transfected with control or miR-190/TLN2 promoters (Fig. 11A). Deletion of EBER1 (2-2), EBER2 (1-2) , or both EBERs (S-15) was confirmed following BamH1 digestion and gel electrophoresis (Fig. 11B). EBERs expression levels were also evaluated by RT-PCR to confirm the 293-BAC-EBV lines expressed both EBER1 and EBER2 (WT), EBER1 only (1-2), EBER2 only (2-2), or no EBERs (S15) (Fig. 11C). The 293-BAC-EBV EBERs WT or null cells were then transfected with a miR-190/talin2 promoter luciferase reporter construct and luciferase activity was normalized to renilla activity (Fig. 11D). We saw that consistent with what we had seen earlier in 293T cells, the 293-BAC EBERs WT cells had higher miR-190/talin2 promoter activity compared to the 293-BAC EBERs null cells. These results indicated the need to further examine the upregulation of miR-190 by EBERs.

3.3.2 RIG-I modulates miR-190 expression

EBERs activate RIG-I due to their dsRNA nature and 5’ triphosphate ends (Ablasser et al., 2009; Samanta et al., 2006). TRANSFAC analysis indicated that the

miR-190/Talin2 promoter has a p65/p50 binding site, so it could be responsive to NF-kB, one of the downstream effectors of RIG-I activation. We tested the effect of RIG-I activation in two EBV negative cell lines, BJAB and Akata EBV negative. Nucleofection of BJAB cells with a constitutively active RIG-I (RIG MIII), that contains mutation in motif III, results in increased miR-190 expression as well as increases in known targets like, IFNß, ISG 56 or IL-10 mRNA expression (Fig. 12A, B, and C). In Akata EBV- cells, nucleofection of the miR-190/Talin2 promoter along with RIG MIII results in higher luciferase activity compared to the control (Fig. 12D). Nucleofection of the synthetic RNA analog, poly I:C, an agonist for RIG-I, results in an increase in miR-190, IFNß and ISG56 expression in Akata EBV- cells (Fig. 12E and F). This indicates that RIG-I activation can increase miR-190 expression. In an EBV+ latency I environment, represented by Akata EBV+ cells, knockdown of endogenous RIG-I as illustrated by decreased protein expression in western blot correlates with a decrease in the known effector IL-10 and decreases miR-190 expression (Fig 13A-C). Thus, RIG-I contributes to the increase in miR-190 expression that is evident in latency I cells.

3.3.3 EBERs expression induces miR-190 promoter activity through YY1 and NF-kB binding sites

miR-190 is an intragenic miRNA which is processed with the TLN2 gene under the TLN2 promoter. Within the TLN2 promoter region, 2 kilobases in front of the transcriptional start site, there is a binding site for two members of the NF-kB family, NfkB1 or p50 and Rel A or p65, and multiple binding sites for YY1(TRANSFAC). We wanted to determine whether these transcription factors played a role in miR-190 expression in the presence of EBERs. To do this we employed a miR-190/Talin2

luciferase reporter construct. We selected one YY1 binding site with the strong consensus sequence ATCCATGTG to mutate. This sequence is also conserved between rats, mice and humans (Zheng et al., 2010a). Additionally, the p50/p65 heterodimer binding site could also alter miR-190 expression in a NF-kB dependent manner. We also deleted this binding site within the miR-190/Talin2 reporter. Another construct containing deleted YY1 and NF-kB binding sites was generated as well (Fig. 14A). These luciferase constructs were introduced into 293T cells by transfection along with EBER1 or empty vector plasmid and readings were normalized to Renilla activity and pGL3 controls. EBER1 cotransfection with the miR-190/TLN2 reporter induced higher promoter activity than empty vector (pU6) and miR-190/TLN2 reporter. Strikingly, -YY miR-190 promoter, - NF-kB miR-190 promoter and –YY1/- NF-kB miR-190 promoter did not induce similar high levels of reporter activity when cotransfected with EBER1 (Fig. 14B). Nucleofection of Akata EBV- cells with WT or mutant promoter reporters and EBERs or empty vector plasmid (pU6) yielded similar results (Fig. 14C). These results indicate not only a role for EBER1 induction of miR-190, but also a role for the transcription factors YY1 and NF-kB in EBER1 mediated miR-190 expression.

3.3.4 miR-190 expression reduced by NF-kB inhibitor Bay-11 7082 in Latency I cells and EBV negative cells expressing EBERs

Previous work has identified EBERs as modulators of higher miR-190 expression (Cramer et al., 2014). Deletion of the p65/p50 binding site in the miR-190/Talin2 promoter reduced promoter activity in the presence of EBER1 compared to wildtype miR-190/Talin2 promoter (Fig. 14B and C) Here we treated Sav I and Sav III cell lines (Latency I and III, respectively) with the NF-kB inhibitor Bay11-7082. The inhibitor

functions by keeping NF-kB bound to its inhibitor IκB-α by blocking IκB-α phosphorylation. We found that under two different inhibitor concentrations, 2μM and 6μM, miR-190 expression was steeply suppressed compared to a DMSO control. miR-190 expression in Sav III cells, which display a very low level of miR-190 expression, was unchanged by treatment (Fig 15A).

In BJAB cells, which are not EBV infected, stable EBERs expression increased miR-190 expression (Fig 15B). This expression is again sensitive to Bay11 treatment with reductions in miR-190 expression evident at higher (6uM) concentrations in Bay11-treated BJAB-EBER1 cells and robust reduction in miR-190 expression in Bay11-treated BJAB-EBER2 cells compared to DMSO controls.

LMP1 is a protein essential for B cell transformation. It is functionally homologous to CD40 and substitutes signaling for it in the growth and differentiation of B cells. It can activate NF-kB signaling (Hammarskjold et al., 1992). We tested LMP1 regulation of miR-190 by nucleofecting empty plasmid (pSG5) or LMP-1 (pSG5-LMP1) into BJAB or Sav I cells and measuring miR-190 expression by RT-PCR. We found that LMP-1 in both of these cellular contexts did not increase miR-190 expression (Fig. 15C).

3.3.5 YinYang-1, a cellular transcription factor, is more highly expressed in cells with EBERs

Previous work has identified YinYang-1, YY1, as a transcription factor for miR-190/TLN2 promoter activity (Zheng et al., 2010a). There are multiple potential binding motifs for YY1 in the miR-190/TLN2 promoter. Here we confirm the activating nature of YY1 on the miR-190/Talin 2 promoter by cotransfecting YY1 and the promoter into

293T cells. The combination of YY1 and miR-190/TLN2 promoter results in higher luciferase activity than pCMV control and miR-190/TLN2 promoter together. (Fig 16A). There is also higher protein expression in both type I cell lines (Sav I and Mutu I) vs. type III cell lines (Sav III and Mutu III) (Fig 16B) and higher expression in EBV negative background (BJAB) when EBERs is present (Fig 16C). Combined with the data from miR-190/TLN2 promoter deletion of the conserved YY1 binding motif, we can conclude that YY1 plays a role in inducing miR-190 expression in the presence of EBERs.

3.4 Discussion

Previous work from our lab explored the biological consequences of miR-190 expression in EBV infected cells. We showed that miR-190 expression was tied to lowered apoptosis, limiting G0/G1 arrest and attenuating lytic reactivation of the virus (Cramer et al., 2014). Along with these findings we showed that miR-190 expression was responsive to EBERs expression in EBV- BJAB cells and that the miR-190/TLN2 promoter was activated by EBERs expression in 293T cells. With these findings regarding miR-190, we wanted to address the mechanism uniting EBERs to miR-190 expression. In the work above we reached the following conclusions: (i) miR-190 expression is increased in the presence of EBERs; (ii) RIG-I activation increases miR-190 expression; and (iii) NF-kB and YY1 binding increase miR-190 promoter activity.

EBERs role in EBV pathogenesis are not yet fully defined. EBERs interact with numerous cellular factors, including; PKR, La, L22, RIG-I and TLR3 (Fok et al., 2006; Lerner et al., 1981; Nanbo et al., 2002; Samanta et al. 2006). We chose to focus on interactions that might induce expression of NF-kB and perhaps the transcription factor

YY1, because of predicted binding motifs for NF-kB and YY1 within the miR-190/TLN2 promoter (our data, Zheng et al., 2010). EBER1 and 2, while only sharing 54% homology, exhibit very similar stem-loop RNA structures and thus may serve similar purposes in the cell. In our hands, miR-190 expression was responsive to both EBER1 and EBER 2, so we examined previously described interactions that did not discriminate between the two (Cramer et al., 2014). For these reasons, RIG-I was a strong candidate for upstream modulator.

We confirmed that RIG-I increases miR-190 expression. Constitutive expression of RIG-I in EBV negative cell lines showed that RIG-I alone induces miR-190 expression and promoter activation. Additionally a RIG-I agonist, poly(I:C), induced miR-190 expression. In Akata EBV+ cells, RIG-I knockdown decreased miR-190 expression. Along with our previously published data, these data suggest that miR-190 expression in EBERs+ cells is downstream of RIG-I activation.

Deletion of binding motifs for NF-kB and YY1 in the miR-190/TLN2 promoter revealed roles for both of these transcription factors in upregulating miR-190 expression. Further work could include localization assays to determine if these transcription factors are in the nucleus when EBERs is present. Since both transcription factors seem to be necessary for miR-190 expression when EBERs is present it would be interesting to see if they are working together perhaps through YY1 recruitment to enhancers (Gordon et al., 2006). Chromatin immunoprecipitation (ChIP) in which protein-protein interactions are preserved would be helpful in determining the presence of interaction between the two transcription factors or recruitment of other proteins on the miR-190/Talin2 promoter.

We also demonstrate that miR-190 is not upregulated in the presence of LMP-1 in BJAB and Sav III cells, and although we did not assess NF-kB activation, these results may indicate LMP-1 driven NF-kB activation alone is not enough for miR-190 upregulation.

The benefit for the virus in activating the innate immune response is tied to the expression of autocrine growth factors that are expressed after RIG-I activation. In type I latency this is IL-10, which promotes infected cell growth and survival (Kitagawa et al., 2000). Our results demonstrate that EBERs increase miR-190 expression. In conjunction with EBERs induced expression of IL-10, miR-190 may permit cells to survive apoptosis and divide more readily (Cramer et al., 2014; Samanta et al., 2008). It is also possible that miR-190 acts in a mechanism to dampen some of the effects brought on by EBERs activation of the innate immune response. An unpublished analysis of the expression of genes following miR-190 overexpression in BJAB (EBV negative) and Sav III (latency III) backgrounds revealed that many of the genes downregulated were from categories of immune response and activation (Table 2). Perhaps in addition to maintaining latency, miR-190, along with many other RIG-I upregulated miRNAs works as a negative feedback on EBERs induced response through RIG-I.

While our work is limited to exploring effects on miR-190 expression, a more expansive look at how EBERs affect cellular miRNA expression in latency I is needed. With the use of antisense oligonucleotides against EBERs that induce endogenous RNase H-mediated degradation, it may be possible to lower EBERs expression sufficiently without perturbing the latency I phenotype of the examined cells (Lee et al., 2015). Another possible experimental method could be the use of RNA polymerase III inhibitors

to lower the expression of EBERs, although this will likely have other effects on the cells and may have unintended effects on miRNA expression (Ablasser et al., 2009).

This study adds yet another layer to this story of EBERs interaction with RIG-I, illustrating another strategy through which EBV can influence cellular microRNA expression that doesn't involve expression of many latency genes. In type I latency, a limited expression of viral proteins does not preclude the virus from having an effect on cellular genes. It is likely RIG-I activation stimulates the expression of many cellular miRNAs through its myriad of downstream effectors.

3.5 Materials and Methods

3.5.1 Cell Culture

Type I (Sav I), type III (Sav III) EBV latently infected cell lines and the EBV negative cell lines, BJAB and EBV positive and negative Akata, were utilized in this study. Akata EBV negative cells were derived from Akata EBV positive cells (Shimizu J. Virol 1994). Sav I and III cells were provided by Dr. Luwen Zhang at University of Nebraska-Lincoln. BJAB cells stably expressing EBER-1 and EBER-2 were developed by transfecting BJAB cells with EBER-1 and EBER-2 expression vectors followed by neomycin selection and cloning from single cells that stably express EBER-1 and EBER-2 RNA, respectively. These cells lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-Invitrogen Life Technologies, Carlsbad, Ca) and 1% penicillin-streptomycin-amphotericin (Gibco-Invitrogen). 293T and 293T-BAC-EBV cells generated by Dr. Ronghua Meng were maintained in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% (v/v)

heat-inactivated fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin-amphotericin and were allowed to grow until 80% confluency before passaging. All cells were incubated at 37°C with 5% (v/v) CO2.

3.5.2 Plasmids and Reagents

The miR-190/talin2 promoter sequence is defined by the region of proximal genomic DNA within 1Kb (1095 bp) of the human Talin2 promoter region and was amplified by PCR with the primers (5' ATCGGCTAGCCACCATGCCAGGCTAATTTT-3' and 5' CAGTCTCGAGACTCGACACGCATCGTACAC-3') and cloned into a pGL3 luciferase repoter vector (Promega) (Beezhold et al, 2011). The suitable deletions were made with oligonucleotides for YY1 binding motif at -141 to -133 (5'-GTACTCTTTATTTGCTGTTTTATTCTCTTAAAGTTGATTTTAAAGTCCAGCACTTTTT-3' and 5'-AAAAAGTGCTGGACTTTAAAATCAACTTTAAGAGAATAAAACAGCAAATAAAGAGTAC-3') and the NF-kB binding motif at -499 to -488 (5'-AAGAGGAGCACCTGAGGATTCTTCTGTCTCCC-3' and 5'GGGACAGAGAAGAATCCTCAGGTGCTCCTCTT3') using the Quikchange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, California). The EBER -1 expression vector was generated by cloning a 530 bp Sac I-Sau3A restriction fragment of EBV genomic DNAs that contains the EBER-1 genes with the promoter into pGem-3 vector (Promega). The pSG5-LMP1 plasmid was from Dr. Erle Robertson (University of Pennsylvania). The pGL3-IFNbeta plasmid was provided by Dr. Fangxiu Zhu, Florida State University. RIG-I motif III mutant, pEF-BOS FLaG RIG, was provided by Dr.

Andy Minn, Abramson Family Cancer Research Center-University of Pennsylvania. YY1 plasmid, pCMV-YY1 was a kind gift from Dr. Yang Shi, Harvard Medical School.

3.5.3 RNA preparation for RT-PCR

RNA was extracted from lymphocyte lines using modified TRIZOL method (Invitrogen). Briefly, 107 cells were suspended in 1 ml Trizol reagent, homogenized and incubated for 5 min on ice. 0.2 ml of chloroform was added to each sample followed by vigorous vortex for 1 min. The samples were centrifuged for 15 min at 14,000 rpm at 4oC, and the upper aqueous phase was transferred to fresh tubes. The samples were re-extracted with an equal volume of phenol/chloroform/isoamyl alcohol (Fisher Scientific, Pittsburg, PA) once and an equal volume of isopropyl alcohol once again. RNAs were recovered by ethanol precipitation, dissolved in RNase free H20 and stored at 80oC until further processing.

3.5.4 Real-time RT-PCR for miRNA, mRNA and EBER RNA detection and quantitation

10 ng of total RNA was reverse transcribed using microRNA reverse transcription kit and stem-loop microRNA assay kit specific for miR-190 (Applied Biosystems, Foster City, CA). RT-PCR was done on Roche LightCycler® System using the TaqMan® microRNA assay kit (Applied Biosystems) and LightCycler® TaqMan® master mix (Roche, Mannheim, Germany) following the manufacturer's instructions. Human small nuclear RNA U6 (RNU6B) (Applied Biosystems) was used to normalize mature miRNA and EBERs data. For mRNA quantification, 1 μg of total RNA was reverse transcribed using SuperscriptTM II reverse transcriptase according to manufacture's instructions (Invitrogen). The resulting cDNA was subjected to qPCR with the SYBR green I kit®

(Roche, Mannheim, Germany) following the manufacturer's instructions. Custom primers were developed for: IFN-ß (5′-GAT TCA TCG AGC ACT GGC TGG-3’; 5′-CTT CAG GTA ATG CAG AAT CC-3’, ISG56 (5′-TAG CCA ACA TGT CCT CAC AGA C-3’; 5′-TCT TCT ACC ACT GGT TTC ATG C-3’with results being normalized to GAPDH (5’-AGCCACATCGCTCAGACAC-3’ and 5’-GCCCAATACGACCAAATCC-3’). Or the resulting cDNA was subjected to qPCR using the QuantiTectPrimer Assay SYBR kit for IL-10 (Hs_IL10_1_SG) and GAPDH (Hs_GAPDH_2_SG) (Qiagen, Valencia, CA). EBER1 and EBER2 were quantitated by qRT-PCR with reverse transcription primer (EBER-1: 5’-ACCACCAGCTGGTACTTGACCGA-3’, EBER2: 5’-CAAGCCGAATACCCTTCTCCCAGA-3’) and custom-designed TaqMan® assay kits (Applied Biosystems). Probes from Qiagen and Life Technologies offer close to 100% PCR efficiency for reliable relative quantification of expression. For other probes efficiency of 100% is assumed. Specifically, the amount of relative gene expression of miRNA or mRNA is presented as 2 (−ΔCt), ΔCt= Ct gene of interest - Ct housekeeping gene.

3.5.5 Western blot analysis

Whole cell extract were prepared in lysis buffer [20 mM Na2HPO4 (PH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.3% (v/v) Triton X-100, 100 µM PMSF with complete protease inhibitor cocktail tablet (Roche). Protein concentrations of the supernatant were determined using Bradford protein assay (Bio-Rad, Hercules, CA). Samples were diluted with NuPAGE® LDS Sample Buffer (Invitrogen), denatured by boiling for 10 min, and loaded on 4-12% Tris-Bis pre-cast gels (Invitrogen). Proteins in the gels were transferred to nitrocellulose membrane (manufacturer). The membranes

were blocked with 5% (w/v) non-fat milk in phosphate buffered saline (PBS) containing 0.1% (v/v) Tween-20 (PBS-T), followed by overnight incubation of specific primary antibodies, including rabbit anti-RIG-I (Cell Signaling Technologies), rabbit anti-YY1 (Cell Signaling Technologies) and mouse anti-ß-actin (Cell Signaling) at varying dilutions in blocking buffer at 4°C. The blots were treated with HRP conjugated anti-rabbit or anti-mouse IgG (Pierce, Rockford, IL) and detected with Novex® ECL Chemiluminescent Substrate Reagent Kit or Supersignal West Dura Extended Duration Substrate (Pierce).

3.5.6 shRNA-mediated knockdown of RIG-I gene expression

Mission shRNAs against human RIG-I were purchased from Sigma-Aldrich. There were two individual shRNA lentiviral vectors in pLKO.1-puro plasmids against different target sites in RIG-I (Clone ID: NM_014314.2-505s1c1 (1) NM_014314.2-2024s1c1 (2). Each of the shRNA vectors and a control vector were used to prepare lentiviral stocks by cotransfecting 293T cells with the shRNA vector and two packaging vectors (pHR'8.2DR and pCMV-VSV-G) at a ratio of 4:3:1 respectively using the calcium phosphate method. Three days post-transfection the supernatant medium was harvested. Viral stocks were centrifuged (500xg for 10 mins at 4°C) and filtered through a 0.45 um filter (ensuring removal of nonadherent cells) and stored at 4°C or immediately used in infection. Logarithmic phase BJAB, Akata EBV+ cells were transduced with the shRNA stocks (RIG-I/control) in the presence of polybrene (8ug/ml). Transduced cells were selected with puromycin (2ug/ml) and tested for effective knockdown of RIG-I by western blot performed on whole cell lysates. RNA was also collected for analysis.

3.5.7 DNA Transfection

293T cells were transfected with Lipofectamine 2000 according to manufacturer's recommendations. Plasmids or poly (I:C) were introduced into suspension cell lines with the Amaxa Nucleofector II and Kit V (Lonza, Basel, Switzerland). Briefly, 3-5x 106 cells was suspended in 100 µl of nucleofector solution and transferred into cuvettes. In the Nucleofector II system (Lonza), Program T020 was used for BJAB and Sav III cells and Program G016 was used for Akata EBV negative cells.

3.5.8 Luciferase reporter assay

Cells were transfected with the full amount of DNA suggested for method (Lipofectamine 2000 or Amaxa Kit V), cell number and vessel volume. Luciferase reporter plasmid transfection alone was accompanied by 1/20 of the total DNA of renilla reporter plasmid. pEBER1/pU6 or pRIG-IMIII/pCR3.1 were cotransfected with luciferase reporter in a ratio of 2(treatment):1(reporter) with 1/20 of the total DNA of renilla reporter plasmid. After 48 hours cells were lysed and luciferase/renilla activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was normalized to renilla activity and pGL3 controls for each transfection.

3.5.9 Bay11-7082 treatment

BJAB background, Sav I or Sav III cells were cocultured with the NF-kB inhibitor, Bay 11-7082 in DMSO carrier at 2 µM or 6 µM or DMSO control alone for 30 minutes before media was refreshed. 24 hours after culture RNA was harvest and analyzed for miR-190 expression.

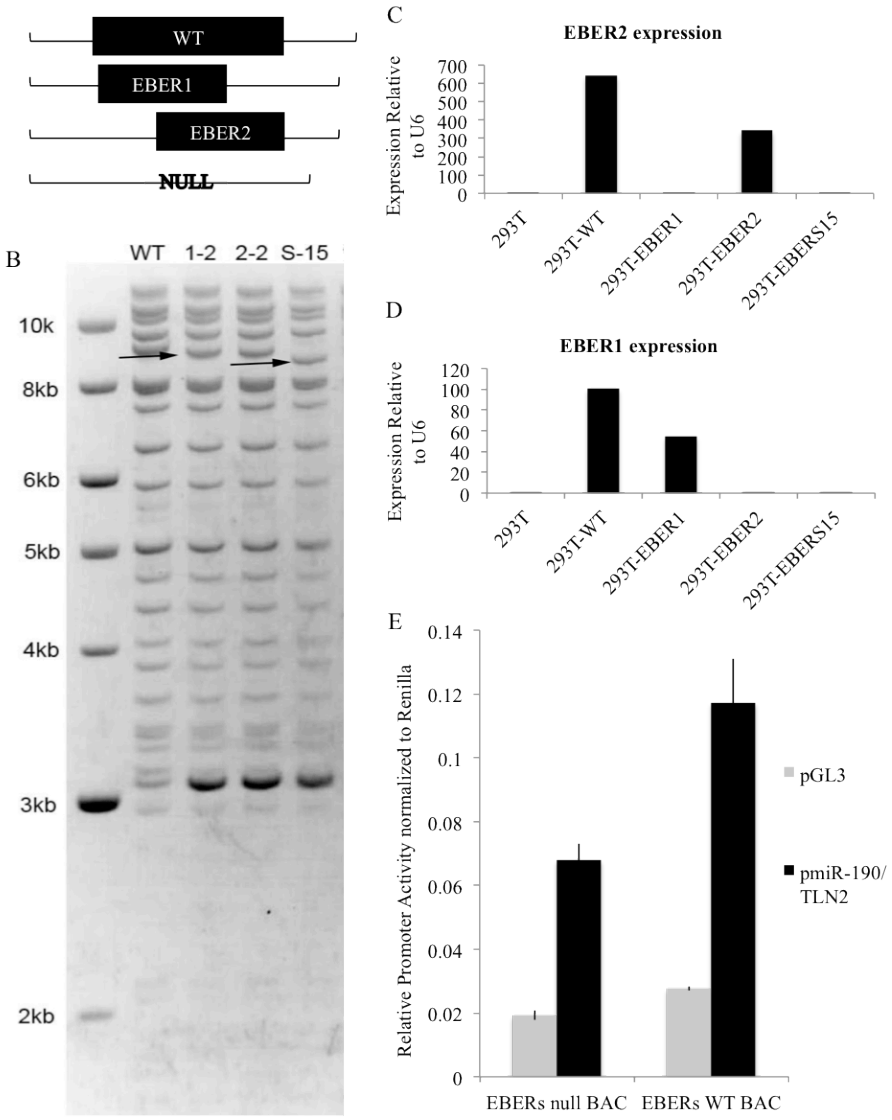

Figure 11: Effect of EBER-null BACMID on miR-190 promoter activity.
(A) Schematic structures of EBERs WT or mutant BACMIDs. (B) Electrophoretic analysis of viral genomes digested with BamH1, resolved on a 0.8% agarose gel and stained with ethidium bromide. (C) EBER2 expression analyzed via RT-PCR in 293-BAC cell lines and normalized to U6. (D) EBER1 expression analyzed via RT-PCR in 293-BAC cell lines and normalized to U6. (E) 293-BAC EBERs WT and null cells were transfected with pGL3 or miR-190/Talin2 promoter and luciferase activity was normalized to transfection (renilla) controls.

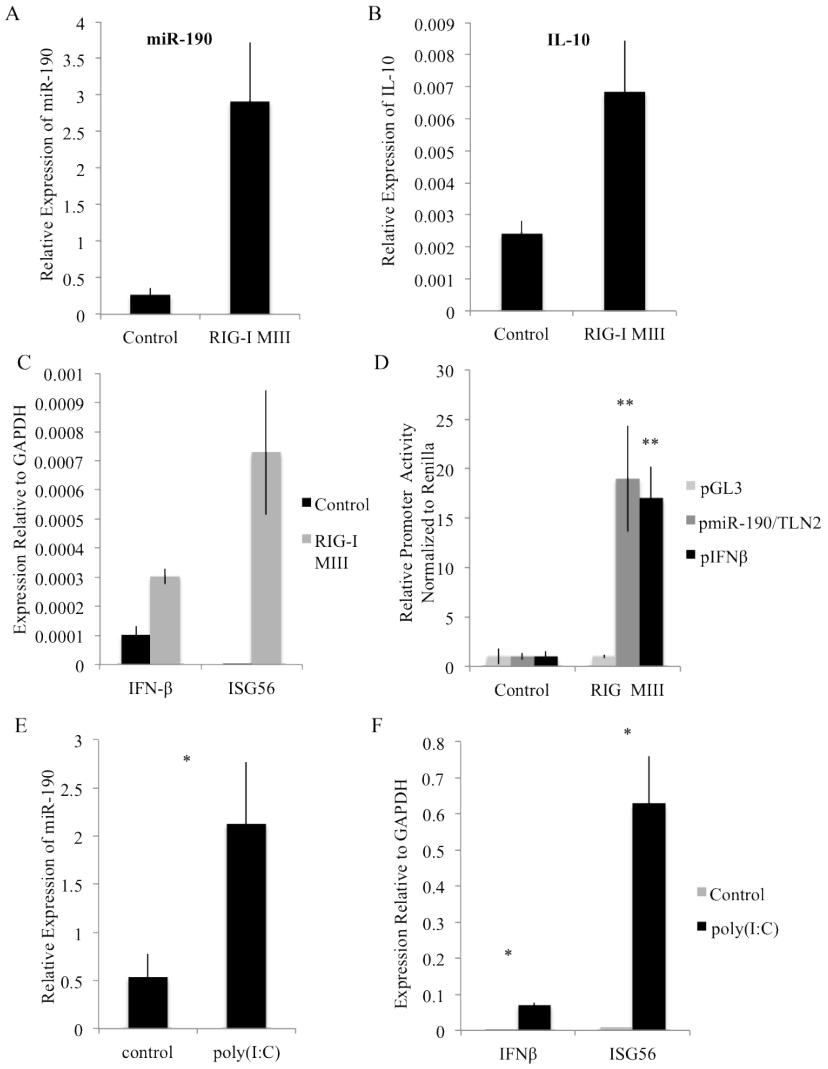

Figure 12: RIG-I activation induces miR-190 expression.
(A, B, and C) In BJAB cell lines, nucleofection of constitutively active RIG-I induces miR-190 expression and the expression of IFN response elements like IFNBeta, ISG56 and IL-10 as measured by RT-PCR. (D) In Akata EBV- cells, constitutively active RIG-I increases pmiR-190/TLN2 and IFNbeta expression as measured by luciferase activity normalized to renilla and pGL3 controls. (E and F) In Akata EBV- cells nucleofection of poly(I:C) increases miR-190, IFNbeta and ISG56 expression over control plasmid (pU6) as measured by RT-PCR. Data are means and standard deviations for three experimental replicates and comparisons are by Student's t-test*P< 0.05, **P<0.01

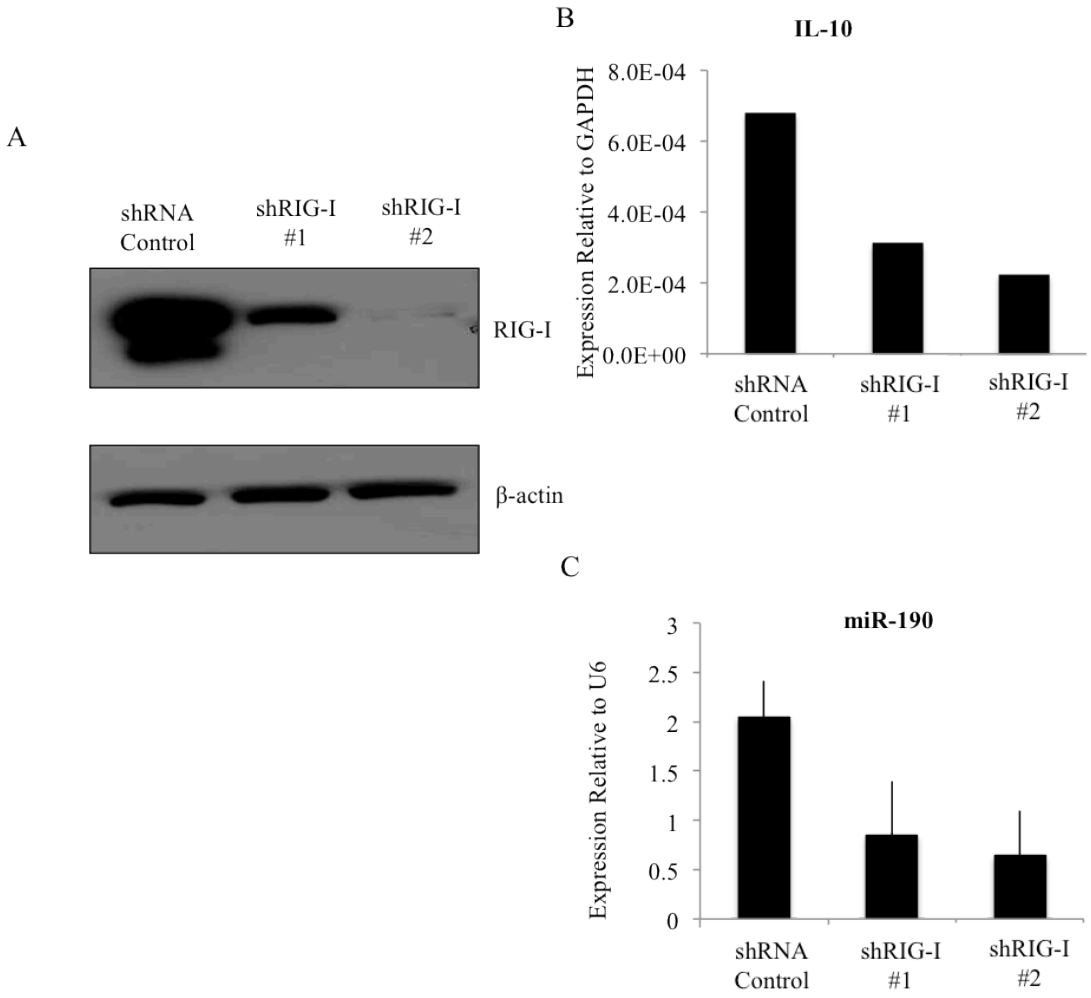

Figure 13: RIG-I knockdown decreases miR-190 expression in Akata EBV positive cells.
(A) In Akata EBV positive cells, the knockdown of RIG-I with shRNA results in reduction of RIG-I protein levels. (B) sh-RNA knockdown of RIG-I reduces expression of downstream effectors, like IL-10 as measured by RT-PCR normalized to GAPDH. (C) sh-RNA knockdown of RIG-I reduces miR-190 expression normalized to U6 as measured by RT-PCR

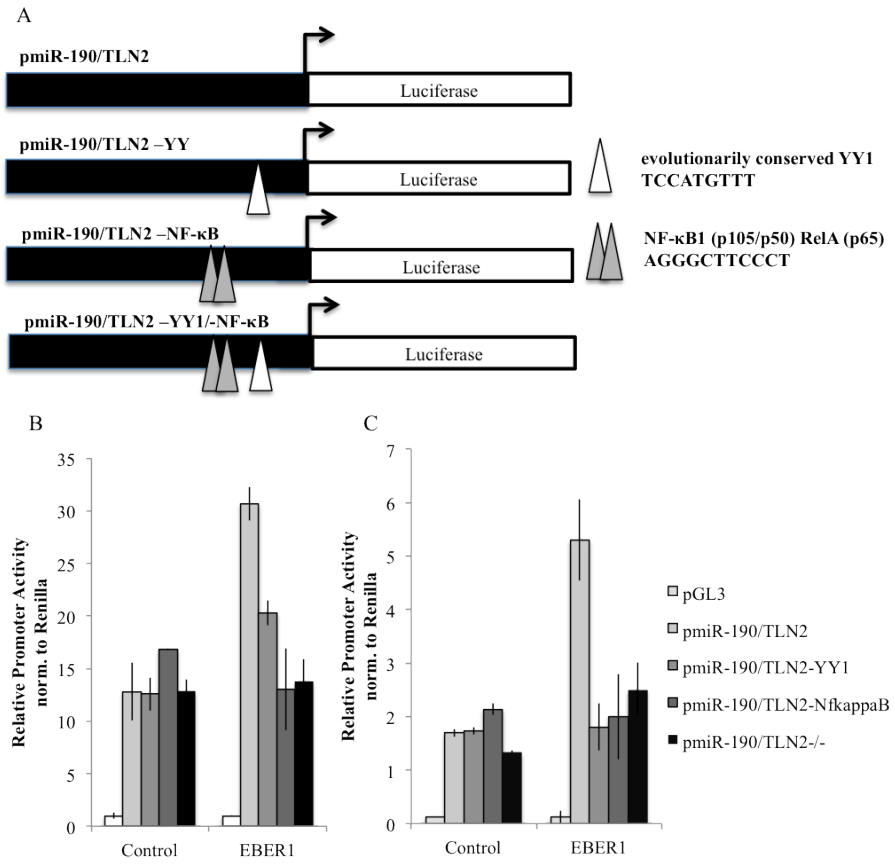

Figure 14: EBERs induces miR-190/TLN2 promoter activity through YY1 and NF-kB binding sites.
(A) mutant miR-190/Talin2 promoter luciferase constructs that lack the conserved YY1 binding site (-YY1), NF-kB heterodimer site (-NF-kB) or both sites (-YY1/-NfkappaB) (B) Relative luciferase activity normalized to renilla of reporter constructs (pGL3 control, WT, -YY1, - NF-kB or –YY1/- NF-kB miR-190 Talin2 promoter) transfected with empty vector or EBER1 plasmid DNA in 293T cells. (C) Relative luciferase activity normalized to renilla of reporter constructs (pGL3 control, WT, -YY1, - NF-kB or –YY1/- NF-kB miR-190 Talin2 promoter) transfected with empty vector or EBER1 plasmid DNA in Akata EBV- cells.

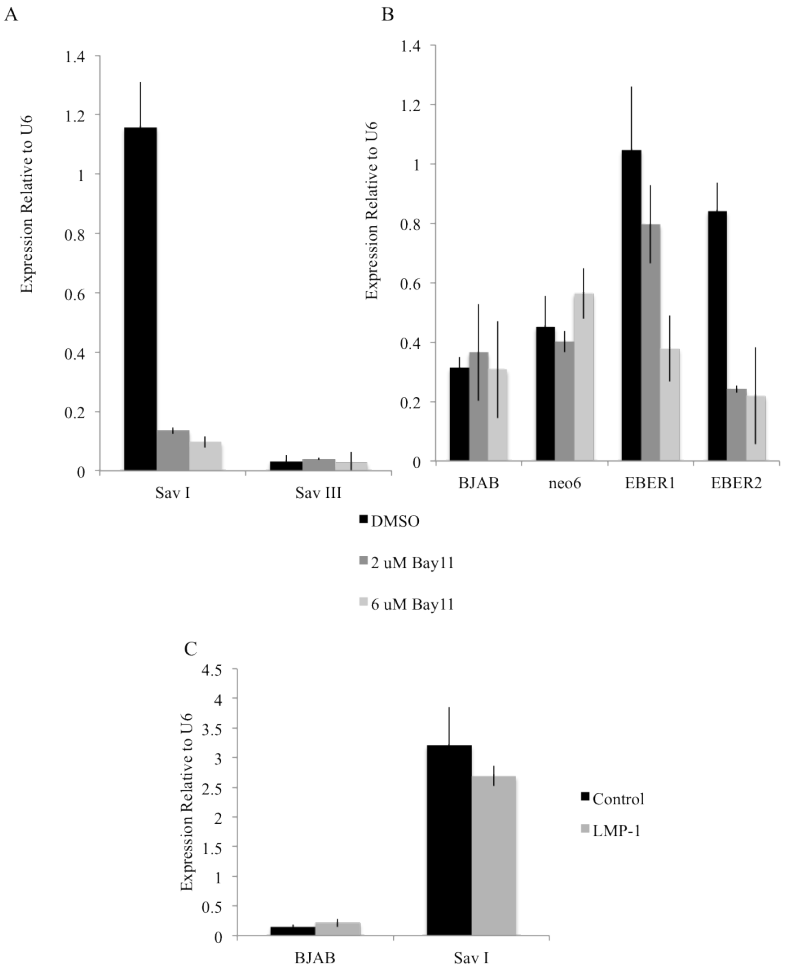

Figure 15: Induction of miR-190 expression is inhibited by Bay11 in Type I latency and EBERs expressing BJAB cells.
A) Bay-11 inhibitor of miR-190 expression measured by RT-PCR expression of miR-190 normalized to small nuclear RNA U6 in Sav I vs Sav III cells (B) and in BJAB cells with (EBER1 or EBER2) or without EBERs (BJAB or neo6). (C) miR-190 expression measured by RT-PCR and normalized to U6 in BJAB and Sav I cells nucleofected with control plasmid (pSG5) or pSG5-LMP1

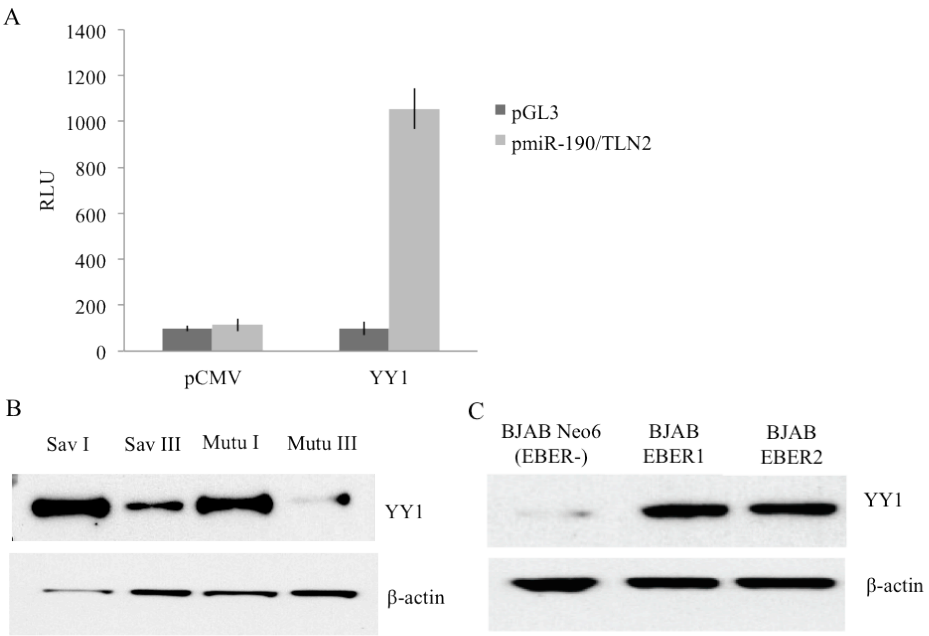

Figure 16: YY1 expression high in type I latency and EBERs expressing cell lines. (A) In a transient transfection of 293T cells, co-transfection of YY1 expression vector with the miR-190/Talin2 promoter report enhanced promoter activity. (B) Type I latency (Sav I or Mutu I) and type III latency (Sav III and Mutu III) cell lines were examined for YY1 expression by western blot. (C) BJAB cells that stably transfected with EBER-1, EBER-2 and empty plasmid (Neo) were analyzed for YY1 expression level by Western blot.

CHAPTER 4 General Discussion

miRNAs are small non-coding RNAs which can modulate gene expression, by either degrading mRNAs or preventing mRNA translation into proteins. Ideally, viruses in latency will reside in a cell that successfully proliferates, divides, differentiates and/or evades cell death— these processes are heavily regulated by microRNAs. Viruses have evolved strategies to harness miRNA-mediated regulation by producing their own miRNAs or modulating host miRNAs. EBV employs both strategies—with a host of its own miRNAs and by influencing cellular miRNA expression. The microRNAs of viruses and EBV, in particular, are well studied as they provide a mechanism whereby the virus can modulate host gene expression. For instance, EBV can target the cellular proteins PUMA and CXCL-11 through its miRNAs, miR-BART5 and miR-BHRF1-3, respectively (Choy et al., 2008; Xia et al., 2008). There are also cellular miRNAs that are upregulated during type III latency (Cameron et al., 2008; Forte et al., 2012; Mrazek et al. 2007). The viral latency protein, LMP1, is a regulator of many cellular miRNAs including miR-34a, miR-155 and miR-146a (Forte et al., 2012; Gatto et al., 2008; Motsch et al., 2007).

The physiological status of cells in the various programs of latency are different—with major differences between type III latency, a more proliferative state driven by the virus, and type I latency, a quiescent homeostatic division mostly attributed to cellular factors (Hochberg et al., 2004; Hochberg and Thorley-Lawson, 2005). There is differential miRNA expression between type III latency and type I latency (Cramer et al. 2014). This differential expression could be attributed to the difference in B cell compartment in which these programs reside or from the different viral gene expression

that accompanies each latency program. Given this differential expression, cellular miRNAs may play critical roles in regulating latency type determination and switch. Little focus has been placed on studying cellular miRNAs in the context of latency I. Perhaps this is due to the difficulty of establishing a good base of comparison, while type III latency LCLs are easily compared to uninfected B cells before primary infection and the greater interest in fully understanding the transformation process (Cameron et al., 2008; Forte et al., 2012). It is not surprising given the vastly different viral and cellular gene expression and phenotypes of the various programs of latency, that there is divergent miRNA expression between one or more programs (Cramer et al., 2014). Utilizing a miRNA expression comparison between type I latency and type III latency, I described the mode of induction and action of one miRNA, miR-190, in latency I (Cramer et al., 2014). While my focus has been trained on understanding the upregulation and function of miR-190, it is important to study other cellular miRNAs within the latency I program as my broader hypothesis is that latency establishment and switch are influenced by many different cellular miRNAs.

The primary aims of this thesis were: to identify targets of the latency I upregulated miRNA, miR-190, and to examine the mechanisms by which EBV in latency I may control miR-190 expression. Several conclusions were reached. First, miR-190 is upregulated in latency I compared to latency III. There is upregulation of miR-190 in Sav I (type I latency) vs. Sav III (type III latency) cells and these results were confirmed by looking in multiple paired type I and type III cells lines (Cramer et al., 2014). Next, miR-190 expression results in reduced expression of TP53INP1 and NR4A3. I overexpressed miR-190 in low miR-190 background cells and utilized a miR-190 antagomiR in high

miR-190 background cells for loss of function and observed changes in mRNA and protein levels. A target site in the 3'UTR of TP53INP1 for miR-190 was also described and verified. Third, through overexpression experiments, I was able to observe miR-190 influenced reductions in G0/G1 cell cycle arrest, apoptosis and viral reactivation. Fourth, using stable or transient EBERs expression I saw an increase in miR-190 expression in the presence of EBERs. Additionally, through constitutively active RIG-I expression, RIG-I agonist nucleofection and RIG-I knockdown I determined that RIG-I activation increases miR-190 expression and promoter activity. Finally, mutation of predicted NF-kB and YY1 binding motifs in the miR-190/Talin2 promoter indicated the importance of these transcription factors in miR-190 promoter activity. In the following, I address questions raised by the experiments in this thesis, discuss the future directions this project may take and speculate on the broader implications of this work.

In the second chapter through use of microarray profiling I identified several potential miR-190 targets. This identification was based off of a gene expression analysis in two different cell types, BJAB and Sav III following overexpression of miR-190. I focused primarily on TP53INP1 and NR4A3 for their potential relevance to latency I maintenance and did not investigate the remaining three potential targets; GEM (GTP binding protein overexpressed in skeletal muscle), NDRG1 (N-myc downstream regulated 1) or FBN1 (fibrillin1) in depth. Of these three, NDRG1 and GEM were downregulated in both SAV III and BJAB cells overexpressing miR-190, while FBN1 was only downregulated in Sav III, but was identified in TargetScan 5.1 as a predicted target. (Table 1)

All three genes could potentially be important to study as they may contribute to the effect exerted by miR-190 in latency I cells. GEM is upregulated following mitogenic stimuli in T cells (Maguire et al., 1994) and was a predicted target of miR-190 in the miRNA.org database. NDRG1 plays a role in p53-mediated apoptosis and suppresses proliferation (Ellen et al., 2008; Stein, et al., 2004). NDRG1 is also targeted by EBV BART miRNAs in epithelial cells (Kanda, et al., 2015). FBN1, an extracellular matrix glycoprotein, is involved in forming microfibrils and connective tissue. In mice, FBN1 mutation is associated with increased inflammation (Gerber et al., 2013). I can only speculate at this point on what impact miR-190 targeting of these genes would have on latency I. NDRG1 seems to serve a similar purpose as TP53INP1 and its targeting by miR-190 could contribute to the decrease in cell cycle arrest and death seen with miR-190 overexpression. It is also possible that these genes are just a few of the targets that are grouped within immune response and activation. Potentially, miR-190 could dampen immune activation or response—either making EBV infected B cells less attractive to cytotoxic T cells, inhibiting proliferation and/or minimizing trafficking to other tissues. Furthermore when I analyzed the miR-190 overexpression gene expression data in DAVID, which extracts biological meaning from large gene lists and maps those lists to associated biological annotation and onotology, miR-190 was predicted to target regulators of immune response (including GEM) and activation (including NDRG1) in BJAB cells and immune response (GEM) in Sav III cells (Table 2).

Through identification of two targets for study, TP53INP1 and NR4A3, I questioned what effect miR-190 had on cell cycle and death and viral reactivation. I focused on describing the manner in which miR-190 overexpression affected these

biological processes. I chose to focus on the impact that miR-190 had instead of specifically knocking down each of these targets in the latency I backgrounds. Though this approach makes it impossible to be certain if TP53INP1 or NR4A3 alone are the reasons for G0/G1 arrest, and cell death (in the case of TP53INP1) or viral reactivation (in the case of NR4A3) the effect could be more readily subscribed to miR-190 through my experiments. In the case of viral immediate early genes, it is possible that reductions in expression of multiple genes are needed to reduce viral reactivation. In BAC-EBV containing 293T cells, introduction of NR4A3 alone had no effect on viral reactivation—although transfection of another immediate early cellular gene, EGR1, increased both BZLF1 and BRLF1 expression (Ye et al., 2010).

George Miller's group identified several other immediate early genes, which exhibit peaks in expression before the induction of BZLF1 and BRLF1 (Ye et al., 2010). The miR-190 overexpression microarray identified decreases in four of these previously described immediate early genes, including NR4A3. The other three immediate early genes were EGR1, NR4A1 and EGR3 and all exhibited decreases in expression with miR-190 overexpression in both Sav III and BJAB cells. I was unable to confirm a direct binding site for miR-190 in NR4A3's 3'UTR, but the decrease in multiple cellular immediate early genes in the presence of miR-190 suggests that there may be a master regulator of these genes targeted by miR-190. Potentially these genes share a common transcription factor that contains a miR-190 target site in its 3'UTR. Several transcription factors, including SMAD2, SMAD4 and IGF-1 were downregulated by miR-190 through 3'UTR targeting in 293T cells (Hao et al., 2014), In mouse adipocytes, IGF-1 itself

induces the expression of several identified cellular immediate early genes, including: NR4A1, EGR1 and EGR2 (Boucher et al., 2010).

As shown in chapter two, the initial observation of higher expression of miR-190 in latency I vs. latency III cells and in Akata EBV+ vs Akata EBV- cells indicated that a viral factor might influence miRNA expression. To my knowledge, this work is the first to identify a relative difference in EBERs expression between latency I vs latency III cell lines, with latency I lines expressing far more EBERs. It is unclear why this differential expression of EBERs exists between type I and type III latency cell lines. It may be worthwhile to look at whether type I latency cells exhibit higher levels of the polymerase III-specific transcription factors, like TFIIIC and Bdp1 (a subunit of TFIIIB), which have been shown to stimulate EBERs transcription, than their type III latency counterparts (Felton-Edkins et al., 2006). It is also possible that EBERs have a longer half-life in type I latency cells vs. their type III counterparts. Due to the limited viral gene expression in latency I cell lines, I was able to identify EBERs as potential regulators of miR-190 expression. Further investigation of EBERs and miR-190 expression or miR-190/Talin2 promoter activity yielded a positive association between the two. This led me to further describe the mechanism for EBERs based upregulation of miR-190 in chapter three.

In the third chapter, our lab was able to utilize 293T cell line transfected with BAC-EBV with or without EBERs. This system while providing some data for the current study will also be a useful future tool to establishing the role EBERs play in upregulating other miRNAs. Combined with data from chapter two, we were able to show that both transient expression of EBERs and BACMID-based expression of EBERs

increased miR-190 promoter activity (Cramer et al., 2014). While our work with the 293-BAC-EBV lines focused on 293-BAC EBERs null vs. 293-BAC EBERs WT cells, we also have 293-BAC-EBV cells, which express only EBER1 or only EBER2. My current work makes no distinction between EBER1 and EBER2 and often utilizes EBER1 only for experiments, but if a differential effect of EBER1 or 2 on miR-190 expression could be explored with these cell lines.

EBERs have numerous associations with cellular proteins, providing multiple possible candidates for activators upstream of miR-190. EBERs inhibits IFN- α mediated apoptosis, perhaps through its association with and inhibition of PKR, which is a key mediator of the IFN- α response to viral dsRNA (Nanbo et al., 2002). Although other reports suggest that PKR remains active and phosphorylated in BL cells with EBERs expression (Ruf et al., 2005). RIG-I, a RNA sensor molecule, is activated by both EBERs in BL cells, La protein also associates with both EBERs, while L22 proteins primarily associate with EBER1 and Pax5 is limited to EBER2 interaction (Fok et al., 2006; Lee et al., 2015; Lerner et al., 1981; Samanta et al., 2006). Other RNA sensor molecules, like TLR3 have been shown to respond to EBERs, but this association is mostly described in the context of EBERs released with La extracellularly (Iwakiri et al., 2009). I established that miR-190 is induced with RIG-I activation through experiments in BJAB and Akata EBV-positive cells utilizing constitutively active RIG-I, the RIG-I agonist poly (I:C) and RIG-I knockdown. Since RIG-I shares downstream signaling with other RNA sensors, including MDA5 and TLR3 it would be interesting to see if constitutive activation or knockdown of these sensors has any effect on miR-190 expression. Additionally it would be interesting to investigate whether other viral RNAs, which activate RIG-I, like

Adenovirus virus associated RNA (VA), have an effect on miR-190 expression (Minamitani et al., 2011)

While my work focuses on the consequences of RIG-I activation in the context of miR-190 expression, I acknowledge that RIG-I and its resulting downstream activation of NF-kB, IFN-ß and ISGs will likely have a robust effect on many other miRNAs. To address the role that NF-kB plays in modulating miR-190 expression I deleted a p65/p50 binding site in the miR-190/Talin2 promoter. This motif deletion did robustly reduce miR-190 promoter activity. In Sav I latency I cells and in EBERs expressing BJAB cells, treatment with Bay-11 7082 a NF-kB inhibitor also reduced miR-190 expression—although this method likely has a drastic effect on multiple genes, including many miRNAs. In the context of EBV infection alone, NF-kB is identified as a regulator of expression in numerous miRNAs. Global analysis of miRNAs modulated during resting B lymphocytes to LCL transition revealed a role for NF-kB in both the upregulation and downregulation of many miRNAs, with histone modification marks found around the transcriptional start sites of these miRNAs (Vento-Tormo et al., 2014). Several miRNAs, including the well studied latency III miRNAs, miR-155 and miR-146a are downstream of NF-kB (Gatto et al., 2008; Motsch et al., 2007).

The latency protein, LMP-1 is a robust inducer of NF-kB activity. As such, I have also examined if LMP-1 increases miR-190 expression in both BJAB and Sav I cells. In my hands, I saw no significant changes in miR-190 expression in either cell lines. This may be an indicator of the importance of having YY1 present in the cell to increase miR-190 expression. YY1 could perhaps enhance miR-190 expression through interaction

with NF-kB. There is evidence of interactions between YY1 and NF-kB in B cells (DLBCL), with YY1 binding to the p65 (RelA) subunit and binding to enhancer regions of IgH (Sepulveda et al., 2004). There is differential expression of YY1 in the presence or absence of EBERs or type I vs type III cells, with YY1 expression higher both in latency I cells and in the presence of EBERs. The true importance of YY1 in miR-190 expression in BL cells could be described by further experiments. YY1 has multiple binding sites within the promoter and this complicates determining the role of YY1 on miR-190 expression, as different binding sites may have different effects on expression. Knockdown of YY1 could potentially indicate the importance of this transcription factor in miR-190 expression in the presence of EBERs and in type I latency cells. Although in my hands, attempts to knockdown YY1 in type I latency cell lines have yet to be successful. Another course of action is through the use of dominant negative mutant YY1. Perhaps amino-terminal activation domain deletion mutants, could be utilized to address the role of YY1 in miR-190 expression.

EBERs induced NF-kB activation within an environment where YY1 is prevalent could potentially result in higher miR-190 expression. Within germinal center B cells, YY1 is known to play an important role in the expression of many genes and is essential for pro-B to pre-B cell development (Liu et al., 2007; Green et al., 2011). Additionally higher YY1 protein levels were found in both BL and DLBCL cells (Castellano et al., 2010). Perhaps the availability of YY1 is the determining factor in the differential expression of miR-190 between type I and type III latency cells. Increasing YY1 expression exogenously in latency III cells and then assaying miR-190 promoter activity could address this question. However, it may also be true that phosphorylation state of

YY1 is the true indicator of its ability to interact with the miR-190/TLN2 promoter. In the case of the miR-190/TLN2 promoter, YY1 phosphorylation reduces its DNA binding affinity (Zheng et al, 2010a). YY1 phosphorylation levels in type I and type III latency cells and cells expressing EBERs should be investigated as well. Beyond this observation, the responsible phosphorylation sites in YY1 would have to be mapped and ultimately mutated to determine if YY1 phosphorylation is a key event in miR-190 regulation.

EBV has evolved with its human hosts to occupy a cellular niche, which is ideal for the virus and in the case of type I latency does not perturb the host immune system. Through this evolution, EBV has acquired the means to establish a lifelong infection, keep infected cells alive and avoid immunological detection by employing multiple programs of latency. EBV latency maintenance is aided by the differential upregulation of cellular miRNAs during latency. EBERs upregulated miR-190 may play a small role in EBV's success in type I latency through dampening apoptosis and reactivation and is a paradigm for type I latency upregulated miRNAs (Fig. 17). More type I latency miRNAs could be identified by utilizing a broader screen of multiple type I latency vs type III latency cell lines. Multiple cellular miRNAs may respond to latency I gene expression, particularly through the mechanism that I identify in chapter three, and may share effects or targets with miR-190. In combination with other upregulated miRNAs, miR-190 may have effects that outweigh anti-viral response and promote survival of the virus within the type I latency program.

Table 2: DAVID gene ontology categories of downregulated genes in BJAB and Sav III cells after miR-190 overexpression.

Gene expression data for genes with a fold changes less than -1.3 and a significance with False Discovery rate of less than 0.1 in BJAB and SAVIII lines overexpressing miR-190. Genes were normalized to Background for HuGene1_st v1 array to eliminate any existing bias. Bolded genes are downregulated with miR-190 overexpression in both BJAB and Sav III cells

	Gene Ontology Description	% genes from list downregulated by miR-190 overexpression	Genes	Fold Enrichment over pSIF control	Benjamini
BJAB	leukocyte activation	16.95	EGR1, ICAM1, GPR183, KLF6, CXCR4, BCL2, **NDRG1**, IRF4, TLR7, LCP2	11.42	1.39E-04
	immune response	23.73	GPR183, ICAM1, IL2RA, **GEM**, TLR7, CXCL10, CCR7, RGS1, CXCR4, FTHL3, BCL2, FAIM3, EBI3, LCP2	5.96	1.20E-04
	cell activation	16.95	EGR1, ICAM1, GPR183, KLF6, CXCR4, BCL2, **NDRG1**, IRF4, TLR7, LCP2	9.66	1.91E-04
	lymphocyte activation	11.86	EGR1, ICAM1, GPR183, KLF6, CXCR4, BCL2, IRF4	9.72	0.016352174
SAVIII	immune response	13.45	HLA-DQB1, GALNT2, IL1R2, IL1R1, C3, OAS1, LY9, IL7R, CCL28, CFP, IGF1R, LILRA1, LILRA2, CCL20, CD24, FYB, LY75, GBP6, IL18RAP, LY96, IL1RN, CTSS, **GEM**, PDCD1LG2, CD55, CXCL16, TREM1, XCL1, GBP3, XCL2, GBP2, DMBT1	3.92	1.31E-07

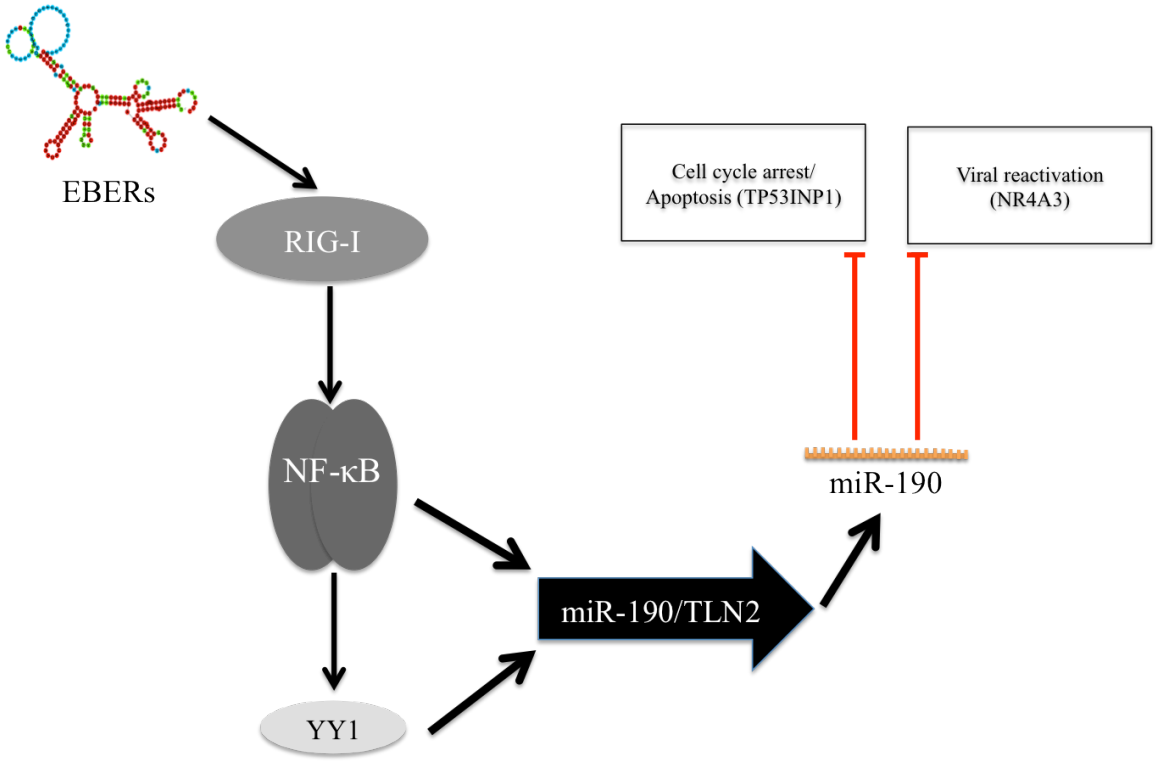

Figure 17 Proposed mechanism for EBERs upregulation of miR-190 and its effects in type I latency

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