ANALYSIS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS MIRNAS' ROLE IN THE MAINTENANCE OF VIRAL LATENCY

By

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To my supporting husband and my wonderful family for their constant encouragement throughout my life

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MicroRNAs are short, non-coding RNAs that post-transcriptionally regulate gene expression by targeting 3' UTRs of mRNAs. Kaposi sarcoma-associated herpesvirus (KSHV)-encoded miRNAs are located in the latency-associated region, which plays an important role in the maintenance of viral latency. Several cellular targets of KSHV miRNAs have been discovered implicating roles for KSHV miRNAs in promoting angiogenesis, cell proliferation, and inhibition of apoptosis. Here, we demonstrate that viral miRNAs play a role in the maintenance of latency by targeting cellular transcription factors known to activate the lytic transactivator RTA. BC-3-G, a PEL-derived RTA-reporter cell line, was used to observe the effects of viral miRNA knockdown on reactivation. Our data demonstrate that inhibition of miR-K12-3 and miR-K12-11 leads to increased reactivation. Moreover, both miRNAs are predicted to target cellular transcription factors (TFs) MYB, Ets-1, and C/EBPα, which are known to induce lytic reactivation by activating the RTA promoter. Knockdown of miR-K12-3 and -11 in PEL cells causes de-repression of all three cellular TFs and resulted in increased lytic gene expression and virus egress.

Next, we created miR-K12-3 and -11 deleted recombinant viruses using BAC16. Individual miRNA-deleted recombinant viruses were reconstituted in 293T cells then co-cultured with iSLK cells and induced to release packaged recombinant viruses. This strategy resulted in the first bacmid producer cell lines which generated a significant amount of virus when compared to 293T cells. iSLK cells harboring recombinant virus were confirmed for viral gene expression and miRNA expression. BJAB BAC16 cells were also established and provide the first system with an isogenic control for studying KSHV in lymphoid cells. iSLK cells infected with either of the deletion mutants (ΔmiR-K12-3/ ΔmiR-K12-11) displayed increased spontaneous reactivation and were more sensitive to inducers of reactivation than WT infected cells.

In summary, we have established a new system for studying miRNA function in cells of both endothelial and lymphoid origin through bacmid technology and our data show that the KSHV-encoded miRNAs, miR-K12-3 and miR-K12-11, target Myb, Ets-1, and C/EBPα which in turn regulate key steps in the viral life cycle: the maintenance of latency and the transition from latent to lytic replication.

CHAPTER 1
BACKGROUND

Kaposi's Sarcoma-Associated Herpesvirus Discovery and Associated Diseases

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as Human Herpesvirus 8 (HHV-8), is the etiological agent of Kaposi's sarcoma (KS) in endothelial cells (Chang *et al.*, 1994). In 1872, Moritz Kaposi described a highly vascular abnormal growth in Mediterranean men that is now known as classical KS (Kaposi, 1872). The Hungarian dermatologist described KS as "idiopathic multiple pigmented sarcoma of the skin". Prior to the introduction of the acquired immunodeficiency syndrome (AIDS) epidemic in the 1970s, KS was thought to be a relatively rare tumor form. There are now four defined clinical variants of KS: classical, endemic, iatrogenic, and epidemic or AIDS-KS. All four types of KS have similar histological features being that they are highly vascular lesions and are characterized by proliferating spindle cells among infiltrating inflammatory cells.

Classic KS lesions predominantly are confined to the lower extremities and tend to infect elderly men rather than women. Those patients affected by the disease generally live with KS for 10 years or greater and are usually not killed by it (Dourmishev *et al.*, 2003; Hengge *et al.*, 2002). Endemic KS is primarily located in eastern and central Africa and is a more aggressive subtype of KS. Unlike classical KS, the endemic form is not only found in men but often affects children with disseminated lymphadenopathy and results in a high mortality rate (Dutz and Stout, 1960). Iatrogenic KS, or post-organ transplant KS, is associated with patients after immunosuppressive therapy. Renal transplant patients are the most susceptible to develop of this subtype of KS, which tends to be more aggressive than classical KS (Barozzi *et al.*, 2003). In the late 1970s

upon the AIDS epidemic, KS was initially one of the most common AIDS symptoms, and continues to be the most common malignancy associated with HIV infection and can lead to significantly high mortality rates. AIDS-associated KS is extremely aggressive and displays a more frequent mucosal progression and lymph node spreading than in the other epidemiologic forms. After the introduction of highly active antiretroviral therapy (HAART) in the 1990s, the incidence and fatality of AIDS-KS has dropped significantly in the United States (Tam *et al.*, 2002). However, AIDS-KS continues to have high incidence in developing countries and KS is the most common tumor in African men (Campbell *et al.*, 2003).

Early on it was proposed that an infectious agent caused KS. In 1972 herpesvirus-like particles were found in KS cultures and were believed to be Human cytomegalovirus. However upon the introduction of HIV and AIDS and the dramatic rise in KS incidence in the 1980s, HIV was then believed to be the causative agent. It was not until 1994 that KSHV was first discovered in the lesions of AIDS-KS patients by Chang *et al.* using representational difference analysis (RDA) (Chang *et al.*, 1994). RDA is a technique used to compare two DNA samples by PCR amplification and analyze differences using subtractive DNA hybridization (Schutte *et al.*, 1995). Sequencing analysis of KSHV placed the virus in the gamma-herpesvirus family along with Epstein-Barr virus (Neipel *et al.*, 1997; Russo *et al.*, 1996). KSHV is most related to a primate herpesvirus, herpesvirus saimiri (HVS), thus placing it in the rhadinovirus subfamily (Desrosiers *et al.*, 1997).

Since the discovery of KSHV, two other lymphoproliferative disorders have been linked to the virus. Primary effusion lymphoma (PEL) is a type of non-Hodgkin's

lymphoma also known as body cavity based lymphoma (BCBL) that is more commonly found in immunocompromised AIDS patients (Cesarman et al., 1995a). PEL differs from KS in that it is derived from clonally expanded malignant B cells that are contained in various body cavities such as the pericardium, pleurum, and peritoneum (Arvanitakis *et al.*, 1996). PEL cells have an arrested phenotype between an antigen selected germinal center B cell and a terminally differentiated plasma cell (Nador *et al.*, 1996). PEL cells can either be only KSHV-infected with genome copy numbers ranging from 50-150 genomes per infected cell, or can be co-infected with EBV (Cesarman et al., 1995a; Renne et al., 1996a; Staudt et al., 2004). PEL is much more aggressive than KS, and rapid progression can cause high mortality with an average survival time of 2-6 months (Komanduri *et al.*, 1996).

Along with KS and PEL, KSHV is also variable found in patients with multicentric Castleman disease (MCD) (Soulier *et al.*, 1995). MCD is a rare angiolymphoproliferative disorder that is found mostly in AIDS patients, 90% of which are also infected with KSHV, whereas KS and PEL patients are 100% associated with KSHV infection (Grandadam *et al.*, 1997). MCD also differs from KS and PEL in that infected cells have a more lytic replication program compared to predominantly latent as in KS and PEL, suggesting that the pathogenic role of KSHV in these different diseases might be determined by different gene expression programs of the virus (Staskus *et al.*, 1999).

KSHV Genome and Lifecycle

KSHV is an enveloped, double-stranded DNA virus that has a genome size ranging from 165 to 170 kbp in length. The unique long region (ULR) is ~140 kbp and contains all 87 of the viral open reading frames (ORF). The ULR is flanked by terminal

repeat (TR) sequences at both ends of the linear viral genome. Each TR is 801 bp long, is highly GC rich, and contains the origin of latent DNA replication (Hu et al., 2002; Lagunoff and Ganem, 1997). Although many of the KSHV ORFs are conserved among other herpesviruses, it does contain 15 unique ORFs designated K1 to K15. KSHV also contains several viral genes that have been pirated from the host genome and function as cellular homologues (Swanton *et al.*, 1997).

KSHV, like all herpesviruses, is known for having both lytic and latent modes of infection. Lytic replication involves genome wide activation of viral gene expression, viral polymerase-dependent DNA replication, and production of progeny virions. This phase is associated with death of the infected cell. Latency, on the other hand, involves highly restricted gene expression and does not lead to death of the host cell. Instead, the viral genome remains stably associated as a circular molecule called an episome, which does not integrate in the host chromosome and persists for many generations without virus production (Renne et al., 1998; Renne et al., 1996b; Roizman and Sears, 1993; Zhong et al., 1996).

There are three stages of gene expression during lytic replication: immediate-early, delayed-early, and late. The first protein to be expressed is open reading frame 50 (ORF50) also known as the regulator of transcription activation (RTA). RTA is considered the switch protein essential for lytic reactivation (Lukac *et al.*, 1998; Sun *et al.*, 1998). Once expressed, RTA induces the expression of a number of delayed-early lytic genes which play a role in viral DNA replication, immune evasion, and signaling affecting the host cellular environment. From there, other delayed early and late viral

proteins are expressed leading to the production of progeny virus and subsequently causing lysis of the host cell (Renne *et al.*, 1996b).

Latency can be described as the ability of the virus to lie dormant within a cell. KSHV accomplishes this by forming a circular episome and expressing a very limited number of latency-associated genes that assist in evading immune response and promoting cell proliferation. One particular protein, the latency-associated nuclear antigen (LANA), is crucial for latency. The ORF73/LANA gene is located in the KSHV latency-associated region (KLAR) along with v-FLIP (promotes cell survival), v-cyclin (cyclin D homologue that promotes S phase entry), a cluster of viral-encoded miRNAs (discussed later), and the Kaposin gene family (involved in cytokine mRNA stabilization and cell transformation) (Cai *et al.*, 2005; Dittmer *et al.*, 1998; Grundhoff *et al.*, 2006; Kedes *et al.*, 1997; Pfeffer *et al.*, 2005; Sadler *et al.*, 1999; Samols *et al.*, 2005). LANA is present in all KSHV infected cells of KS, PEL, and MCD and is commonly used as a marker of infection (Verma *et al.*, 2007). Some of LANA's functions include maintaining the viral episome, tethering the viral episome to the host chromosome, supporting latent DNA replication, and promoting cell proliferation and survival (Dourmishev *et al.*, 2003).

Lytic vs. latent replication is tightly regulated by controlling the levels of RTA and LANA expression. In addition to RTA's ability to activate the lytic expression cascade, it also auto-activates itself (Deng *et al.*, 2000; Gradoville *et al.*, 2000). RTA recruits a number of cellular proteins such as RBPJκ, Oct-1, Sp1, and Sp3 to bind and activate its own promoter (Chen *et al.*, 2000; Liang *et al.*, 2002; Sakakibara *et al.*, 2001; Zhang *et al.*, 1998). RTA also interacts with C/EBPα to up-regulate gene expression (Wang *et al.*, 2003). RTA and LANA can also influence each other's expression, resulting in a

negative feedback loop. RTA binds the LANA promoter and activates transcription. On the other hand, LANA can suppress RTA expression through direct interaction with the host cellular binding protein RBPJκ on the RTA promoter (Lan *et al.*, 2004; Lan *et al.*, 2005). During latency the promoter region of RTA displays the histone markings of bivalent chromatin, in which H3K27-me3 repressing marks and H3K9/K14-ac activating marks are present simultaneously so that RTA is in a poised state for reactivation (Gunther and Grundhoff). In tissue culture, RTA can be reactivated into lytic phase by adding TPA or sodium butyrate (NaB) (Miller *et al.*, 1997; Renne *et al.*, 1996b). TPA, a phorbol ester and pleotropic activator of gene expression, can de-methylate RTAs promoter and NaB is an inhibitor of histone deacetylase (HDAC) both of which can allow RTAs promoter to become accessible for activation (Chen *et al.*, 2001).

KSHV Encodes miRNAs

In 2005 and 2006 four independent groups cloned miRNAs from KSHV infected PEL and identified a total of 12 miRNA genes giving rise to 18 mature miRNAs (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005; Umbach and Cullen, 2010). Surprisingly, all KSHV miRNAs are located within the major latency-associated region of the genome with 10 of the 12 miRNAs organized in a cluster in a 2.6 kb intragenic region between v-FLIP and the K12/Kaposin gene. Two additional miRNAs were found to be located within K12 open reading frame. Expression of the 12 pri-miRNA stem-loops (discussed in MiRNA Biogenesis) is controlled by three promoters, 2 latent and 1 lytic. The latent promoters results in expression of all 12 miRNAs whereas the lytic promoter slightly upstream of Kaposin results in increased levels of miR-K12-10 and miR-K12-12 expression during lytic replication (Cai and Cullen, 2006).

MiRNA Discovery

The first miRNA, lin-4 of *C. elegans,* was found through analysis of a strong developmental timing defect. The responsible gene, lin-4, did not contain an ORF, and instead only expressed two short transcripts of 60 and 24 nucleotides in length. It was subsequently shown that the lin-4 RNA was involved in translationally silencing the lin-14 transcript by binding to complementary sequences within the lin-14 3'UTR (Lee *et al.*, 1993; Ruvkun *et al.*, 2004; Wightman *et al.*, 1991; Wightman *et al.*, 1993). This novel RNA-based inhibition was specific to *C. elegans* until the discovery of the let-7 miRNA, which was found to be conserved in metazoans, including humans and flies (Pasquinelli *et al.*, 2000; Reinhart *et al.*, 2000; Slack *et al.*, 2000). MiRNAs have been discovered in every metazoan and plant species tested thus far, and according to estimates, around 30% of all metazoan miRNAs are conserved in all species (Ambros, 2004). There are currently 1,921 human miRNAs known and this number is predicted to expand as more sensitive techniques for discovering miRNAs are developed (http://microrna.org) (Griffiths-Jones, 2004). The major function of miRNAs appears to be regulation of gene expression through translational inhibition and mRNA degradation; however, there are classes of miRNAs discovered which have different mechanisms of action. MiRNAs are an integral part of innate immunity against viruses in plants, and in many organisms, miRNAs are also involved in chromatin silencing (for reviews see (Iorio et al., ; Li et al., 2002; Lippman and Martienssen, 2004). MiRNAs participate in the regulation of apoptosis, cell fate decisions, cell differentiation, stress response, and development (Ambros, 2004; Bartel, 2004). However, those miRNAs where targets are known and the rapid identification of new targets showcase that miRNAs regulate fundamental processes during development and differentiation and

that miRNA expression is tightly regulated in both spatial and temporal manners (Ambros, 2004; Bartel, 2004). This is further supported by the finding that aberrant miRNA expression is associated with pathogenesis including many human malignancies (for review see [Calin and Croce, 2006]).

MiRNA Biogenesis and Function

Viral miRNA genes are expressed from pol II transcripts or in the case of murine g-herpesvirus type 68 (MHV-68), in which miRNA genes are embedded in tRNA-like genes by pol III (Ambros, 2004; Bartel, 2004; Bogerd *et al.*, ; Diebel *et al.*). MiRNAs can occur individually or be organized into clusters and they can exist as stand-alone genes or located within the introns and exons of protein-coding genes (Ambros, 2004; Bartel, 2004). Like metazoan miRNAs, viral miRNA processing begins with the formation of an imperfect stem-loop with a hairpin bulge that forms in a RNA transcript termed the pri-miRNA (Figure 1-3). The dsRNA region of the pri-miRNA is recognized by DGCR8 (Pasha in flies), which recruits the endonuclease Drosha to cleave and release a 60–80-nt long hairpin. This pre-miRNA is then exported to the cytoplasm by the Exportin 5/RAN-GTPase pathway, where it is recognized by Dicer and cleaved to leave a short dsRNA molecule. One strand of this dsRNA product is loaded by Dicer and the dsRNA-binding protein TRBP (also known as R2D2 in flies and RDE-4 in nematodes) into the RNA-induced silencing complex (RISC). The other strand, known as the star (*) strand, is often degraded, but in many cases can also be loaded into RISC with variable efficiency (for review see [Ambros, 2004; Bartel, 2004]). RISC functions by guiding miRNAs to semicomplementary sites within the 3’UTRs of target transcripts and induces translational silencing. For targets that are completely complementary, siRNA-like degradation occurs rather than translational silencing. The 5’ end of the miRNA,

specifically nts 2 through 8 termed the seed sequence, is critical for determining mRNA target binding, but there are rare cases of miRNA-binding sites that have little seed binding but significant 3' compensatory complementarity instead. Many target transcripts have multiple binding sites for a specific miRNA, and it is also common that a single mRNA is targeted by multiple miRNAs. Due to the rather flexible requirements for recognition between the miRNA and its target, a single miRNA can regulate many targets (Grimson *et al.*, 2007). Therefore, miRNAs constitute a large posttranscriptional regulatory network which controls complex processes such as development and cell differentiation.

After binding of RISC to the 3'UTR of the target transcript, silencing is accomplished through a yet not fully deciphered mechanism(s). Current evidence suggests several different mechanisms: inhibition of translational initiation by interfering with the interaction of eIF4E, eIF6E, and the poly(A)-binding protein, premature termination of translation by inducing ribosomal drop off following initiation, and messenger RNA degradation by relocation of the RISC to cytoplasmic Processing (P)-bodies which contain the RNA degradation machinery (Filipowicz *et al.*, 2008).

Viral miRNAs

In 2004, Pfeffer et al. reported the cloning and identification of five miRNAs from Epstein Barr virus (EBV). Initially, three miRNAs were found to be located in the BHRF region and two miRNAs in the BART region of EBV (Pfeffer *et al.*, 2004). This first report of virally encoded miRNAs opened a new field of virology. The possibility that viral miRNAs can regulate hundreds of target genes suggests a novel and extremely complex level of host/virus interaction. A number of herpesvirus proteins, often pirated from host genomes, target specific cellular processes such as immune surveillance,

apoptosis, and proliferation, and in retrospect, it seems obvious that viruses would also utilize miRNAs to regulate these pathways. To date, more than 200 miRNAs have been identified in 20 different DNA viruses (Table 1-1, Figure 1-4).

After EBV was shown to encode miRNAs, four independent groups cloned miRNAs from Kaposi's sarcoma-associated herpesvirus (KSHV)-infected primary effusion lymphoma cells (PEL) and identified a total of 12 miRNA genes giving rise to 18 mature miRNAs (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005; Umbach and Cullen, 2010). Next, a combination of tiled arrays, cloning, and bioinformatic approaches identified 18 additional EBV miRNAs, located within the 12 kb deletion specific to the B95-8 strain analyzed in the original report (Pfeffer *et al.*, 2004) and three more within the BART region outside of the B95-8 deletion (Cai *et al.*, 2006; Grundhoff *et al.*, 2006). Recently, two additional BART miRNA genes were identified in EBV-positive nasopharyngeal carcinomas (NPC) tissue samples (Zhu *et al.*, 2009). This brings the total of EBV miRNA genes to 25. Cai et al. also reported 16 miRNAs within the EBV related Rhesus lymphocryptovirus (rLCV), eight of which are conserved to EBV miRNAs (Cai *et al.*, 2006). Recently, both Grundhoff and Steitz groups have identified additional rLCV-encoded miRNAs bringing the total to 36, 18 of which are conserved to EBV (Riley *et al.*, ; Walz *et al.*). The extent of conservation between EBV and rLCV miRNAs sequences is not found elsewhere in g-herpesviruses. Schaefer et al. reported seven miRNAs within the Rhesus Rhadinovirus (RRV), a g-herpesvirus closely related to KSHV (Schafer *et al.*, 2007). Like KSHV, RRV miRNAs are located within the latency-associated region of RRV; however, their sequences are not evolutionary conserved. Murine g-herpesvirus type 68 (MHV68) encodes nine miRNAs which are located within

transfer RNA-like genes at the 5' end of the genome and have been shown to be transcribed by RNA pol III (Bogerd *et al.*, ; Diebel *et al.*, ; Pfeffer *et al.*, 2005).

Within b-herpesviruses, nine miRNAs were identified from human cytomegalovirus (HCMV), scattered throughout the viral genome (Pfeffer *et al.*, 2005) (Figure 1-4). Dunn et al. cloned a previously unreported miRNA and Grey et al. used a bioinformatics approach to predict conserved hairpins between HCMV and chimpanzee CCMV (Dunn *et al.*, 2005; Grey *et al.*, 2005). Both approaches predicted and confirmed two new HCMV miRNAs for a current total of 11 HCMV miRNAs. These studies also illustrated that bioinformatics approaches alone are not reliable tools for the identification of miRNAs.

In a-herpesviruses, miRNAs have been identified in Herpes simplex virus 1 and 2 (HSV-1 and -2) and Marek's disease virus 1 and 2 (MDV-1 and -2) (Burnside *et al.*, 2006; Burnside *et al.*, 2008; Cui *et al.*, 2006; Jurak *et al.*, ; Tang *et al.*, 2008; Tang *et al.*, 2009; Umbach *et al.*, 2008; Umbach *et al.*, 2009; Waidner *et al.*, 2009; Yao *et al.*, 2007). Latency of HSV is primarily established in the sensory neurons of the trigeminal and sacral ganglia. As seen in KSHV, a-herpesviruses also encode miRNAs during latency. In 2006, Cui et al. predicted several miRNAs within and upstream of the HSV-1 and HSV-2 latency-associated transcript (LAT) (Cui *et al.*, 2006). LAT is a noncoding mRNA and is believed to be the only transcript expressed abundantly during latency. Subsequently, Umbach et al. showed that the HSV-1 LAT functions as a primiRNA giving rise to four miRNAs (Umbach *et al.*, 2008). Additionally, one miRNA is located directly upstream of LAT and was found to be expressed in latently infected mouse trigeminal ganglia (Umbach *et al.*, 2008). Tang et al. first reported LAT-encoded

miRNAs of HSV-2 (Tang *et al.*, 2008; Tang *et al.*, 2009). Umbach and colleagues validated the expression of the HSV-1 and HSV-2 miRNAs in human trigeminal ganglia and sacral ganglia by using sensitive deep sequencing methods. Additionally, two more miRNAs located within LAT of HSV-1 and three novel HSV-2 miRNAs were identified (Umbach and Cullen, 2010; Umbach et al., 2009). Recently, Jurak et al. utilized deep sequencing to confirm previously identified and predicted miRNAs by Cui, Umbach, and Tang, along with identifying 19 new HSV-1 and HSV-2-encoded miRNAs, bringing the total number of HSV-1 miRNAs and HSV-2 miRNAs to 16 and 17, respectively (Table 1-1, Figure 1-4). Interestingly, HSV-1 and HSV-2 share nine miRNAs that are either positional conserved and/or show limited sequence homology (Jurak *et al.*). Burnside et al. used 454 deep sequencing to identify 13 miRNAs from Marek's disease virus type 1 (MDV-1), which map to the inverted repeat short and long regions (IRs and IRL) (Figure 1-4). Eight of these miRNAs are located within the meq oncogene and the remaining map to the LAT region of MDV-1 (Burnside *et al.*, 2006; Burnside *et al.*, 2008). Conventional miRNA cloning, and recently deep sequencing, revealed 18 miRNAs within the closely related MDV-2 virus, 17 of which were clustered within IRL with one additional located in IRS (Waidner *et al.*, 2009; Yao *et al.*, 2007; Yao *et al.*, 2008).

Outside the herpesvirus family, two miRNAs resulting from a single hairpin were identified within the 3'UTR of the SV40 late transcript (Sullivan et al., 2005). Positional homologs of these SV40 miRNAs were also isolated from SA12-infected cells (Cantalupo et al., 2005). Human polyomaviruses BKV and JCV along with Merkel cell polyomavirus were also shown to encode one miRNA each (Seo et al., 2009; Seo et al., 2008). Recently, Sullivan et al. also showed that murine polyomavirus (PyV) encodes

one miRNA gene (Sullivan et al., 2009). All polyomavirus miRNAs are located antisense to the viral mRNAs encoding large T antigen. Hence, while common in herpesviruses and polyomaviruses, miRNA-encoding genes appear to be rare in other virus families. It appears that no RNA virus investigated to date encodes miRNAs. Pfeffer et al. were unable to clone miRNAs from cells infected with either hepatitis C virus, yellow fever virus, or HIV (Pfeffer et al., 2005), and there have been no other reports of miRNAs being predicted and verified in other RNA viruses. Thus, it appears as if miRNAs are a DNA virus-specific phenomenon. Since all RNA viruses (except for retroviruses) progress through a dsRNA intermediate, miRNAs would ultimately act as siRNA and inhibit viral replication. Additionally, transcripts from RNA viruses that often replicate exclusively in the cytoplasm would have to be shuttled into the nucleus to be processed by Drosha. This same issue arises with poxviruses replicating solely in the cytoplasm, which may explain why only very few miRNAs have been predicted within the more than 300 kbp large double-stranded DNA genomes of poxviruses and none have been experimentally confirmed (Pfeffer et al., 2005). At this admittedly early stage in the field, it appears that predominantly herpesviruses encode miRNAs, which may be just another example for host cellular genes that have been successfully captured and subsequently coevolved into herpesvirus genomes.

Targets of KSHV miRNAs

Up to 25% of all herpesvirus genes modulate host cellular functions during both latent and lytic infection and it was hypothesized early on that viral miRNAs will greatly increase the complexity of virus/host interaction (Areste and Blackbourn, 2009). To date, most efforts on identifying cellular genes targeted by viral miRNAs have been focused on KSHV and EBV. In summary, these studies reveal that viral miRNAs target

key cellular pathways, including immunity, proliferation, angiogenesis, and apoptosis (Table 2-2).

The first cellular target genes for viral miRNAs were identified by gene expression profiling of HEK 293 cells stably expressing KSHV miRNA cluster containing 10 miRNAs (Samols, 2007). A total of 65 genes were downregulated in the presence of the miRNAs. SPP1, PRG1, and THBS1 were verified as miRNA targets using luciferase reporter constructs containing the 3'UTRs. Additionally, protein levels of THBS1 were decreased >10-fold in KSHV miRNA expressing cells. This was significant since THBS, a strong tumor suppressor and antiangiogenic factor, had previously been reported to be downregulated in KS lesions (Taraboletti et al., 1999). Interestingly, the 3'UTR of THBS contained seed sequence binding sites for multiple KSHV miRNAs suggesting that viral miRNAs in clusters coordinately regulate host cellular target genes. SPP1 and PRG1 are involved in cell-mediated immunity and apoptosis, respectively. These initial findings, albeit obtained in 293 cells, suggest that KSHV-encoded miRNAs contribute to viral pathogenesis by promoting angiogenesis (a hallmark of KS tumors) and by inhibiting cellular immunity and apoptosis (Samols, 2007).

Viral miRNAs can mimic cellular miRNA function. We and the Cullen group showed that miR-K12-11 and human miR-155 shared complete seed sequence identity (Gottwein et al., 2007; Skalsky et al., 2007). Mir-155 is aberrantly expressed in many human malignancies, and when overexpressed in mice, causes lymphoproliferative disease (Garzon and Croce, 2008). This led to question if miR-K12-11 and miR-155 can target a common set of genes. Bioinformatics identified the BACH1 gene, which has four binding sites for both miR-K12-11 and miR-155 within its 3'UTR. BACH1 is a

transcriptional repressor affecting expression of heme-oxygenase 1 (HMOX1), a protein that promotes cell survival and proliferation. Luciferase reporter assays confirmed regulation of BACH1, and furthermore, BACH1 protein levels were decreased in miR-K12-11 and miR-155 expressing cells. Gene expression profiling also revealed that miR-K12-11 and miR-155 can regulate a common set of genes (Gottwein et al., 2007; Skalsky et al., 2007). In addition, Qin et al. showed that miR-K12-11-dependent regulation of BACH-1 not only affected oxidative stress responses, but also led to an increase of xCT expression, an amino acid transporter, which was previously shown to function as fusion receptor for KSHV (Qin et al., 2010a).

Qin and colleagues also showed that KSHV-encoded miRNAs induce IL-6 and IL-10 secretion in murine macrophages and human myelomonocytic cells (Qin et al., 2010b). C/EBPβ, a known regulator of IL-6 and IL-10 transcription, was shown to be targeted by the KSHV miRNA cluster. Specifically, miR-K12-3 and miR-K12-7 inhibited the LIP isoform of C/EBPb, which functions as transcriptional suppressor. These data suggest that KSHV-encoded miRNAs directly regulate cytokine secretion of latently infected cells (Qin et al., 2010b).

Recently, Isaac Boss from our lab has shown that miR-K12-11 also targets C/EBPβ. As described above, miR-K12-11 is an ortholog of human miR-155. To compare both miRNAs function *in vivo*, miRNAs were expressed in human hematopoietic progenitor cells and reconstituted in NOD/LtSz-scid IL2Rγ^{null} mice. Results showed that B cells within the spleens of miR-K12-11 or miR-155 expressing mice have decreased C/EBPβ expression. MiR-K12-11 targeting of C/EBPβ was further

confirmed by 3'UTR luciferase reporter assay and antagomir knockdown in PEL cells (Boss et al., 2011).

Endothelial cells infected with KSHV have been shown to undergo transcriptional reprogramming, expressing markers for both lymphatic (LECs) and blood endothelial cells (BECs) (Carroll et al., 2004; Wang et al., 2004). Hansen et al. recently showed that KSHV miRNAs directly contribute to this reprogramming by targeting the cellular transcription factor musculoaponeurotic fibrosarcoma oncogene homolog (MAF). MiR-K12-6 and miR-K12-11 together target the 3'UTR of MAF, thereby inducing endothelial cell differentiation and possibly contribute to KSHV oncogenesis (Hansen et al., 2010). Interestingly, miR-K12-11, the ortholog of miR-155, is also involved in B cell differentiation and proliferation in vivo (Boss et al., 2011).

A study by Abend et al. demonstrated KSHV miR-K10a targets tumor necrosis factor (TNF)-like weak inducer of apoptosis receptor (TWEAKR). This was observed by overexpressing miR-K12-10a in primary endothelial cells which resulted in reduced production of the pro-inflammatory cytokine IL-8 and monocyte chemoattractant protein 1 (MCP-1) (Abend et al., 2010). Interestingly, these pro-inflammatory cytokines are induced by KSHV proteins (vFLIP and vGPCR) and may promote tumorigenesis (Schwarz and Murphy, 2001; Sun et al., 2006). To understand this conflicting regulation, the authors hypothesize that miR-K10a-dependent regulation of IL-8 and MCP-1 may provide a mechanism that fine tunes cytokine expression to levels beneficial for the virus, without eliciting a strong immune response (Abend et al., 2010).

KSHV miRNAs have been shown to target genes involved in immune evasion. Recently, it was shown that KSHV miR-K12-11 targets I-kappa-B kinase epsilon (IKKε),

an important signaling molecule in the antiviral interferon response pathway (Liang et al., 2011). Also miR-K12-7 has been shown to target MICB, a stressed-induced ligand that is essential for natural killer cell recognition of virus-infected cells (Nachmani et al., 2009). Interestingly, two other herpesviruses, HCMV and EBV, also encode miRNAs that have been shown to target MICB (Nachmani et al., 2009; Stern-Ginossar et al., 2007). Thus, this coevolution suggests that targeting MICB to prevent virus-infected cells from being recognized by NK cells is a critical step for viral persistence *in vivo.*

The Ganem group devised an elegant tandem-array approach to identify KSHV miRNA targets that were either induced by miRNA knockdown in latently infected PEL cells or inhibited in uninfected B cells ectopically expressing the corresponding KSHV miRNA (Ziegelbauer et al., 2009). This analysis revealed that miR-K12-5, along with K12-9 and miR-K12-10b, targets Bcl-2-associated factor (BCLAF1). BCLAF1 is a transcriptional repressor and overexpression can promote apoptosis. However, BCLAF1 expression in latently infected PEL cells can inhibit viral replication. Antagomir-based inhibition of KSHV miRNAs targeting BCLAF1 resulted in sensitizing latently infected endothelial cells for lytic reactivation. This data suggest that KSHV miRNAs can contribute to latency control by both targeting the viral RTA gene as discussed above and cellular genes like BCLAF1 (Ziegelbauer et al., 2009).

Unlike the above study, there have been several reports that KSHV miRNAs help contribute to latency by targeting cellular factors. Lu et al. found that both human and viral miRNA expression is important for the prevention of lytic reactivation. They further went on to show that by overexpression of miR-K12-3 by lentivirus in BC-3 cells resulted in decreased RTA mRNA levels. Next, miR-K12-3 was shown to directly target

NFIB, a cellular transcription factor. The murine version of NFIB was shown by Yu et al. to reactivate KSHV in BC-3 cells when over expressed by a genome-wide cDNA library screen (Yu et al., 2007). Further analysis by promoter luciferase assays in 293 cells confirmed that human NFIB does activate the RTA promoter, although it is not known if this is a direct interaction (Lu et al., 2010a). This study provides indirect evidence that miR-K12-3 maintains latency by targeting NFIB, but further experiments using antagomirs or a miR-K12-3 knockout virus are needed to prove this mechanism.

The above studies on KSHV were based on either latently infected cell lines or cell lines engineered to express viral miRNAs. Two recent studies addressed the role of KSHV miRNA within the context of the viral genome (Lei et al., 2010; Lu et al., 2010b). Both developed a recombinant KSHVΔmiRNA virus to study viral replication. Lei et al. observed a marked inhibition of NF-kB in 293 cells infected with the virus mutant, which was accompanied by a moderate increase in lytic replication. IkBa, the NF-kB repressor, was subsequently shown to be targeted by miR-K12-1 (Lei et al., 2010). This is the second example of a KSHV miRNA that contributes to latency by targeting cellular genes. Gottwein and colleagues reported another target for miR-K12-1 which like NF-kB is crucial for cell survival and proliferation. Using bioinformatic tools and luciferase assays and mutagenesis, it was shown that miR-K12-1 directly targets p21, a key inducer of cell cycle arrest and tumor suppressor (Gottwein and Cullen).

Using a very similar miRNA knock-out virus, Lu et al. also observed a moderate increase in lytic replication, but identified entirely different mechanisms. In addition to inhibiting RTA through miR-K12-5, Lu et al. observed a drastic inhibition of DNA methylation throughout the KSHV genome after deleting the miRNA cluster.

Subsequently, it was shown that Retinoblastoma (Rb)-like protein 2 (Rbl-2), a potent inhibitor of DNA (cytosine-5-)-methyltransferases (DnmT1, 3a, and 3b), was targeted by several KSHV miRNAs. These data showed for the first time a role of viral miRNAs in epigenetic regulation of latency (Lu et al., 2010b).

The latest technique to identify miRNA targets utilizes immunoprecipitation of RISCs followed by microarray or HTS sequencing analysis of the RISC-bound miRNA targets (Chi et al., 2009; Hafner et al., 2010). Using this technique, Dolken et al. was able to confirm a significant number of the above-discussed targets, and in addition, determined six novel targets of KSHV miRNAs and two targets of EBV miRNAs (Dolken et al., 2010). KSHV miR-K12-3 was shown to target LRRC8D, thought to be involved in proliferation and activation of lymphocytes and macrophages, and NHP2L1, a nuclear protein that binds to U4 snRNA. MiR-K12-4-3p targets GEMIN8, which is required for spliceosomal snRNP assembly in the cytoplasm and pre-mRNA splicing in the nucleus. Also, the KSHV miR-cluster was found to target EXOC6, ZNF684, and CDK5RAP1; however, no functional studies have been presented on these novel target genes (Dolken et al., 2010).

Recently it has been shown that KSHV miRNAs target within the viral genome. To investigate whether KSHV miRNAs target KSHV immediateearly transactivators, Bellare and colleagues utilized luciferase reporter assays in which the 3'UTR of the KSHV reactivation and transcriptional activator gene (RTA) was cotransfected with individual KSHV miRNA mimics. Further analysis of miRNA knockdown using antagomirs (sequence-specific miRNA inhibitors) in latently infected PEL cells showed that miR-K12-9* modulates RTA expression at the protein level (Bellare and Ganem, 2009). Lu et

al. also found that a KSHV miR-K12-5 can inhibit RTA expression. However, this may reflect an indirect effect rather than direct targeting, since the 3'UTR of RTA does not contain a favorable miR-K12-5 seed sequence (Lu et al., 2010b). Later it was demonstrated that miR-7-5p also targets RTA. Lin et al found a 7mer seed match site within the 3'UTR of RTA that mediates targeting and ectopic expression of miR-K12-7-5p in latently infected cell lines reduces the amount of progeny virus produced (Lin et al., 2011).

Although these studies suggest that miRNAs function as major regulators of latency by directly targeting RTA, it is likely that KSHV miRNAs fine tunes latency rather than operate as a major controller of the switch between latency and reactivation. Chapter 2 focuses on two miRNAs, miR-K12-3 and -11, and their role in the regulation of latency by targeting cellular transcription factors known to activate the RTA promoter. Chapter 3 describes the generation of individual miRNA deleted recombinant viruses and how they can serve as tools to study miRNA function in the context of both endothelial and lymphoid cells. Lastly, in Chapter 4, I will discuss ongoing work and future studies on KSHV miRNA functions in latency and possible role as novel therapeutic targets for KSHV malignancies.

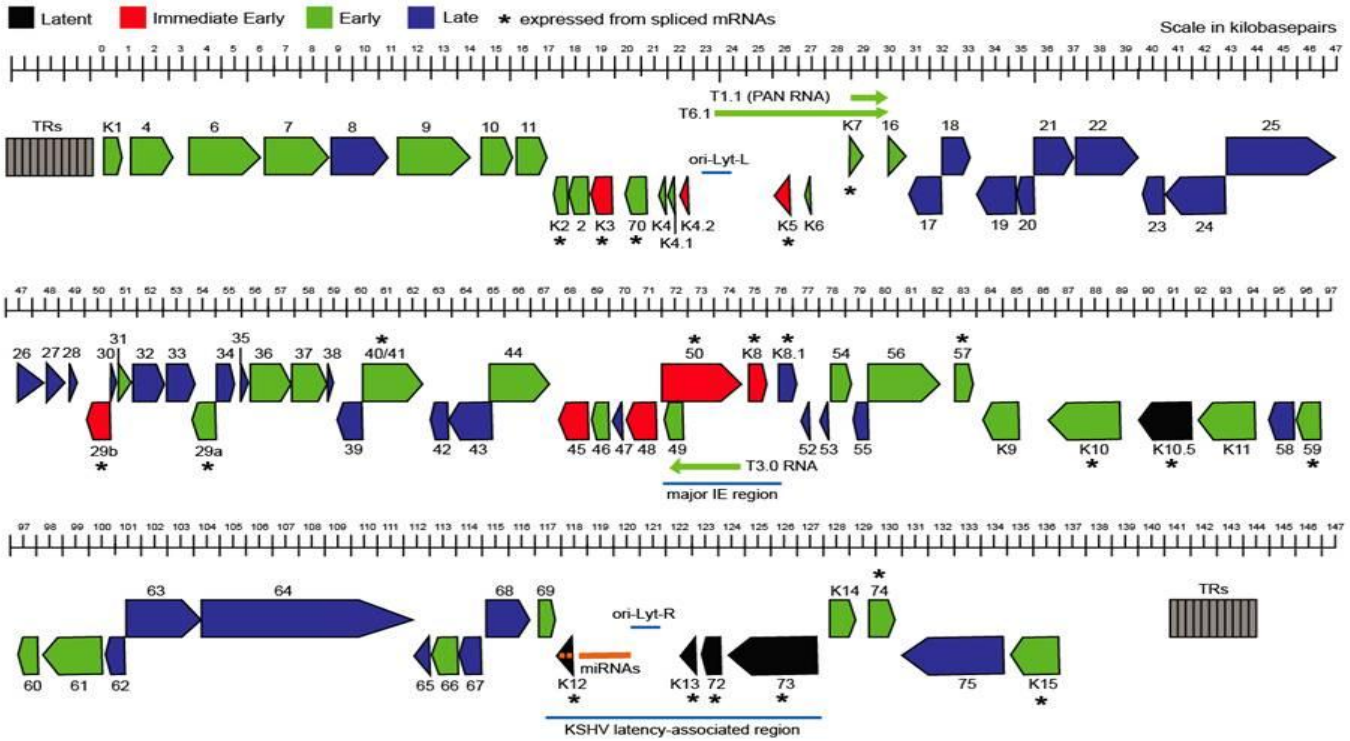


Figure 1-1. The KSHV Genome. Open reading frames (ORFS) are labeled in color based on their expression pattern during latent, immediate early, early, or late infection. The KSHV latency-associated region (KLAR) is underlined in blue and the miRNA genes are labeled in orange.

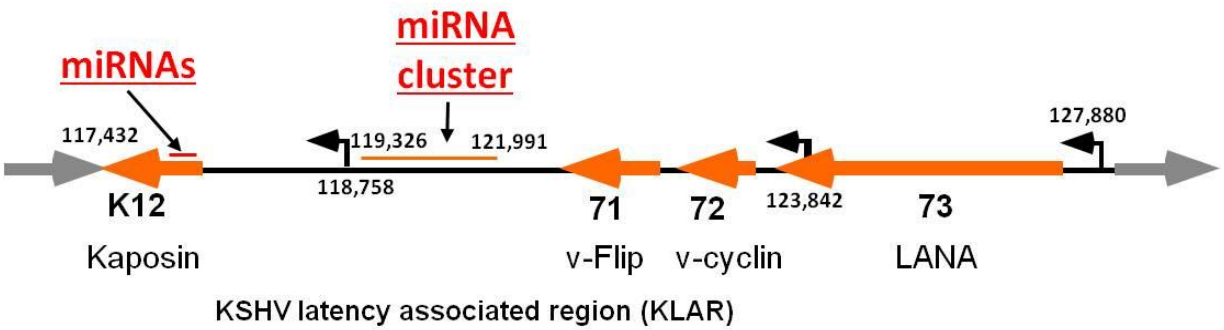


Figure 1-2. MiRNAs are encoded in the KSHV latency-associated region (KLAR). The latent genes in KLAR are in orange with the direction of latent transcription denoted by orange arrows. Latent promoters are indicated by the black directional arrows. The miRNA cluster contains 10 miRNA genes and downstream of the cluster are 2 additional miRNA genes are encoded.

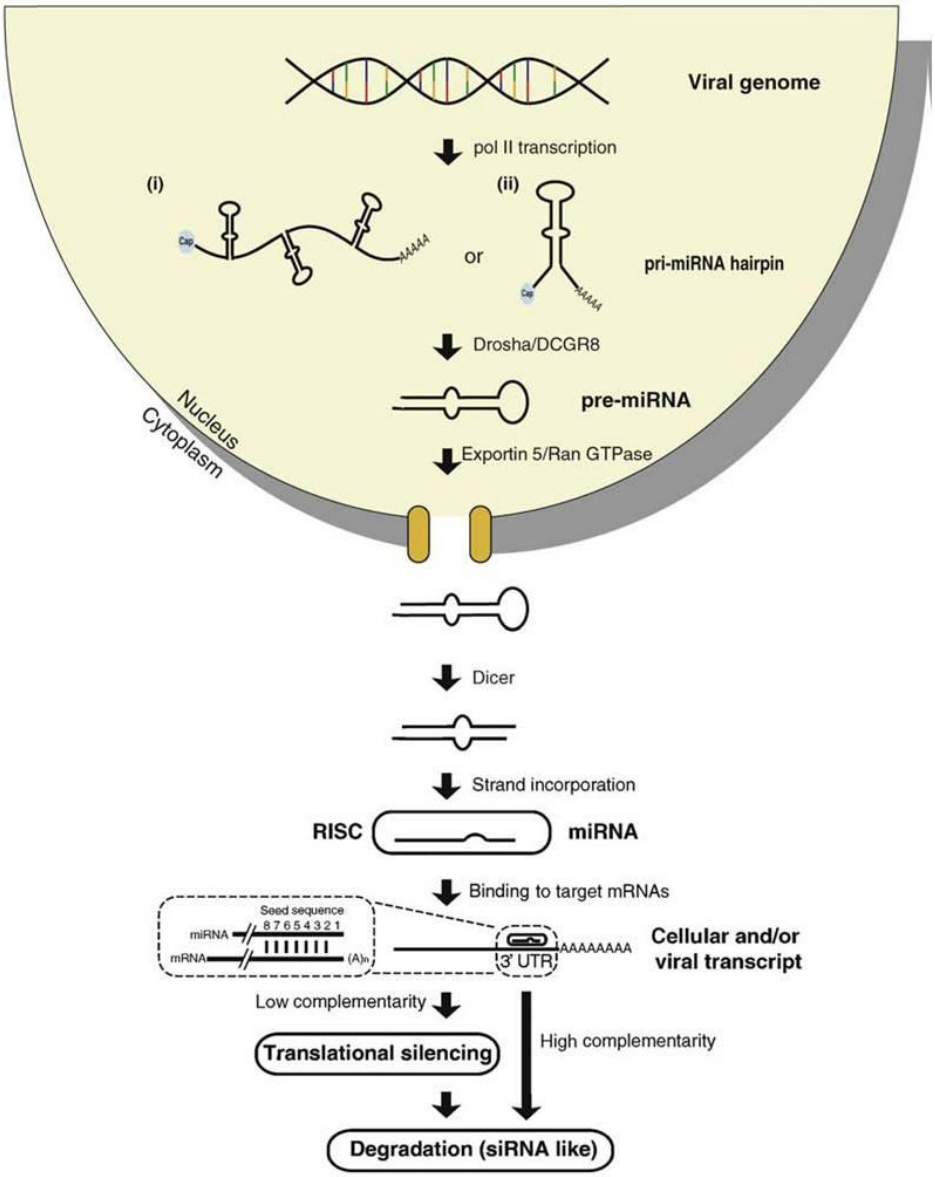


Figure 1-3. Biogenesis pathway for metazoan miRNAs. MiRNA precursors begin as hairpin loops in pol II or pol III transcripts in introns or exons. Drosha cleaves the pri-miRNA transcript leaving a ~80 bp stem loop which is exported into the cytoplasm. Dicer cleaves off the loop structure leaving a 21-24 nt dsRNA molecule. The miRNA is incorporated into the RISC where it binds to the 3'UTR of target transcripts and induces either translational silencing or transcriptional degradation depending on the level of complementarity. The seed sequence of the miRNA, nts 2 through 8, is known to be a critical component of target recognition and binding.

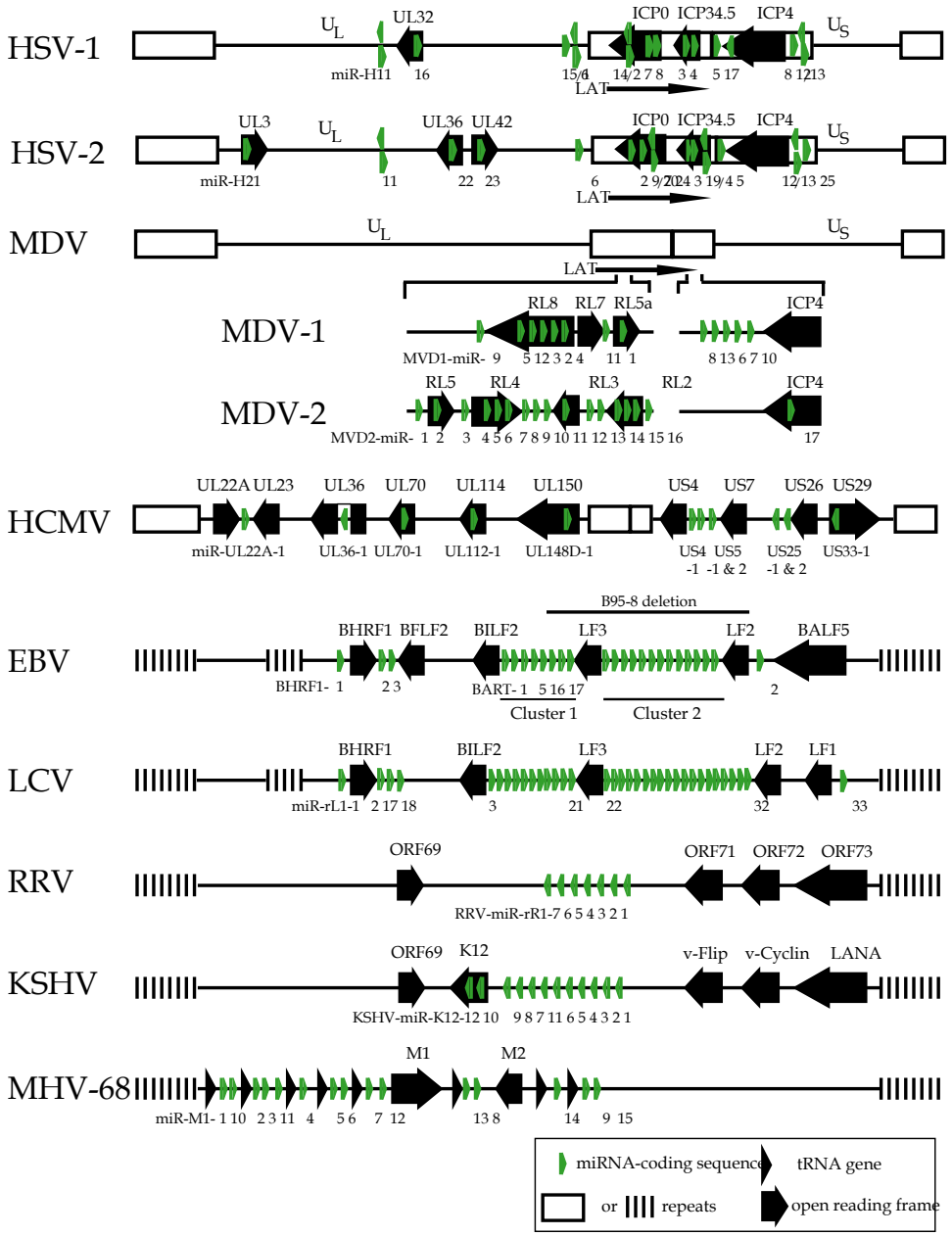


Figure 1-4. Schematic representation of miRNAs found for several herpesviruses. Genomes are represented for HSV-1, -2, MDV-1, -2, HCMV, EBV, LCV, RRV, KSHV and MHV-68 with black arrows for ORFs, black triangles for tRNA genes, and black bars or rectangles for repeat sequences. MiRNA locations are indicated with green arrows. Genomes are not drawn to scale. MDV-1 & -2 are drawn as one complete genome with the respective miRNA coding regions shown in more detail. Abbreviations: U_S, unique short; U_L, unique long; LAT, latency associated transcript.

Table 1-1. Verified known viral miRNAs.

Virus	Number of miRNA genes
HSV-1	16
HSV-2	17
MDV-1	13
MDV-2	17
HCMV	11
MCMV	18
EBV	25
LCV	36
RRV	7
KSHV	12
MHV-68	9
SV40	1
SA12	1
BKV	1
JCV	1
MCV	1
PyV	1

Table 1-2. Experimentally verified viral miRNA targets.

KSHV			
Viral Targets	miR-K12-9* miR-K12-5 miR-K12-7	RTA	Replication and Transcriptional Activator
Cellular Targets	miR Cluster	THBS1	Angiogenesis inhibitor
		EXOC6	SEC15 gene family
		ZNF684	Zinc finger protein
		CDK5RAP1	Regulation of neuronal differentiation
	miR-K12-1	IκBα	NF-κB Inhibitor
		p21	Inducer of cell cycle arrest
	miR-K12-3	LRRC8D	Immune cell activator
		NHP2L1	U4 snRNA nuclear binding protein
		NFIB	Transcriptional Activator
	miR-K12-3 miR-K12-7 miR-K12-11	C/EBPβ	Transcriptional Activator
	miR-K12-4-3p	GEMIN8	Required for splicing
	miR-K12-5	BCLAF1	Pro-apoptotic factor
		Rbl-2	Rb-like protein
	miR-K12-6 miR-K12-11	MAF	Transcription factor
	miR-K12-7	MICB	NK cell ligand
	miR-K12-11	BACH1	Transcriptional suppressor
		IKKε	Inducer of interferon
	miR-K12-10a	TWEAKR	Pro-apoptotic factor

CHAPTER 2
KSHV-ENCODED MIRNAS REGULATE RTA PROMOTER ACTIVATION BY TARGETING CELLULAR TRANSCRIPTION FACTORS MYB, ETS-1, AND C/EBPA

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as Human Herpesvirus 8 (HHV-8) is a DNA tumor virus that is the etiological agent of Kaposi's sarcoma (KS) in endothelial cells (Chang *et al.*, 1994). KSHV has also been linked to two B cell lymphoproliferative disorders, primary effusion lymphoma (PEL) and a subset of multicentric Castleman's disease (MCD) (Cesarman et al., 1995a; Soulier et al., 1995). As with all herpesviruses, KSHV has both lytic and latent modes of replication. During lytic replication/reactivation, genome-wide expression occurs in a temporally regulated cascade of immediate early, early, and late genes, which results in release of progeny virus and lyses of the host cell (Sarid *et al.*, 1998). During latency only a limited number of genes are expressed, the majority residing in the latency-associated region, which encodes the latency-associated nuclear antigen (LANA), v-FLIP, v-cyclin, kaposin, vIRF3, vIL-6, K1, and 12 miRNA genes (Cai et al., 2005; Chandriani and Ganem, 2010; Dittmer et al., 1998; Grundhoff et al., 2006; Pfeffer et al., 2005; Rivas et al., 2001; Samols et al., 2005; Sarid et al., 1998; Talbot et al., 1999).

MicroRNAs (miRNAs) are short, non-coding RNAs 19-23 nucleotides in length that post-transcriptionally regulate gene expression by targeting 3' untranslated regions (UTRs) of messenger RNAs (for review see [Bartel, 2004]). Since the discovery of KSHV-encoded miRNAs are highly expressed in all KSHV-associated tumors (O'Hara *et al.*, 2009; O'Hara *et al.*, 2008), several cellular targets of KSHV-encoded miRNAs have been identified, implicating roles for KSHV miRNAs in promoting angiogenesis, cell cycle regulation, and inhibition of apoptosis (for reviews see [Plaisance-Bonstaff and

Renne, 2011; Skalsky and Cullen]). In addition, viral lytic genes have also been suggested to be targeted by viral miRNAs. Using *in vitro* assays, miR-K12-9*, miR-K12-5, and miR-K12-7 have been suggested to target the 3'UTR of the immediate early lytic gene ORF50, encoding the replication and transcriptional activator (RTA) (Bellare and Ganem, 2009; Lin et al., 2011; Lu et al., 2010b). RTA is the first gene to be expressed during reactivation and initiates the cascade of lytic gene expression by activating several early lytic gene promoters (Lukac *et al.*, 1998; Sun *et al.*, 1998). Hence, direct targeting of RTA by miRNAs would prevent reactivation from latency.

Here we report on an alternative scenario by which KSHV miRNAs contribute to the maintenance of latency by targeting cellular transcription factors that can trigger lytic reactivation. Our data demonstrate that miR-K12-3 and miR-K12-11 are important for preventing lytic reactivation by targeting three cellular transcription factors Ets-1, MYB, and C/EBPα, which have previously been reported as activators of the RTA promoter (Lacoste *et al.*, 2007; Wang *et al.*, 2003; Yu *et al.*, 2007). To confirm the importance of these miRNAs in contributing to latency, recombinant viruses harboring individual miRNA deletions were created. In the absence of miR-K12-3 or miR-K12-11, both spontaneous and induced RTA expression was increased. These data suggest that KSHV miR-K12-3 and miR-K12-11 contribute to the maintenance of viral latency by targeting cellular genes Ets-1, MYB, and C/EBPα thereby acting as a gatekeeper to fine tune latency.

Results

KSHV miRNA Knockdown in BC-3-G Cells

In order to determine which KSHV-encoded miRNAs may be important for preventing lytic reactivation, miRNA knockdown studies were performed in BC-3-G, a

PEL-derived indicator cell line. BC-3-G contains a PAN promoter driven GFP expression cassette, which is highly transactivated by RTA. Upon lytic reactivation, RTA is the first protein expressed, activates the PAN promoter and as a result turns cells green (Yu *et al.*, 2007).

BC-3-G cells were transfected with sequence specific miRNA inhibitors, 2'OMe antagomirs, for each of the 12 KSHV-encoded miRNAs. Cells were observed by fluorescence microscopy for GFP expression at 72 hours post transfection (hpt). Mock transfected control cells displayed a small number of GFP expressing cells representing spontaneous reactivation. After performing a screen by inhibiting all 12 KSHV-encoded miRNAs individually or in combinations, the greatest increase in GFP expression was observed when miR-K12-3 and miR-K12-11 were knocked down (Figure 2-1a). MiR-K12-3 is the most highly expressed miRNA in BC-3 cells (Gottwein *et al.*, 2011) (Haecker et al. under revision). Interestingly, miR-K12-11 is a mimic of hsa-mIR-155 and has been shown to play an important role in B cell proliferation (Boss *et al.*, 2011; Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Reactivation of BC-3-G cells after antagomir knockdown was then quantified by using flow cytometry. Figure 2-1b shows a graph depicting the percent of GFP expressing cells over a time course of 96 hpt post antagomir transfection. MiR-K12-3 knockdown lead to a 26% increase in GFP expressing cells when compared to the control. GFP expression was also induced when both miR-K12-3 and miR-K12-11 were knocked down together, although at a lesser extent (Figure 2-1b).

In silico Target Prediction for miR-K12-3 and miR-K12-11 Identified Ets-1, Myb, and C/EBPα as Potential Targets

Scanning the 3'UTR of RTA revealed no seed sequence matches for miR-K12-3 or -11. We therefore focused on transcription factors known to activate the viral RTA promoter and performed miRNA target prediction (Grimson *et al.*, 2007). Interestingly, we found putative binding sites for miR-K12-3 and miR-K12-11 within the 3'UTRs of Ets-1, MYB, and C/EBPα (Lacoste *et al.*, 2007; Wang *et al.*, 2003; Yu *et al.*, 2007). Ets-1 is a member of the Ets family of transcription factors, which are downstream effectors of the Ras-MAPK signaling cascades (Wasylyk *et al.*, 1998). The Ets family binds to a specific DNA consensus sequence and has been identified to bind and activate genes involved in the regulation of cell proliferation, differentiation, and survival (Dejana *et al.*, 2007). The proto-oncogene MYB acts mostly as a transcriptional activator which plays a central role in hematopoiesis and has been shown to cooperate with a number of transcription factors including the CEBP and Ets families. Targets of the MYB transcription factors include genes involved in development, cell survival, proliferation, and homeostasis (Ramsay and Gonda, 2008). C/EBPα is a member of the CCAAT/ Enhancer-Binding Protein family, which are bZIP nuclear transcription factors. C/EBPα is an important transcription factor involved in controlling tissue-specific gene expression in myeloid tissues and growth arrest (Koschmieder *et al.*, 2009).

Ets-1 has three predicted binding sites for miR-K12-3 and for miR-K12-11. Myb has two potential sites for miR-K12-11, and C/EBPα has two potential sites for miR-K12-3. All sites consist of at least a 6mer seed match with some extending to 8mer seed matches (Figure 2-2a). 3'UTR luciferase reporter assays were performed to confirm miR-K12-3 and/or miR-K12-11 targeting of these transcription factors. The

3'UTRs of Ets-1, MYB, and C/EBPα were cloned downstream of firefly luciferase and co-transfected into 293 cells with either a miR-K12-3 or miR-K12-11 expression vector. As shown in Figure 2-2b, MYB was targeted by miR-K12-11 since increased miRNA expression resulted in reduced luciferase activity. The 3.6 kb long 3'UTR of Ets-1 has a number of additional putative binding sites for KSHV miRNAs. Hence, we tested miR-K12-3 and -11 alone and in combination, as well as the pcDNA3.1/cluster, which expresses 10 KSHV miRNAs (Samols, 2007). While expression of miR-K12-3 had no significant effect, miR-K12-3 and -11 in combination as well as the cluster lead to significant repression suggesting that Ets-1 is targeted by multiple KSHV miRNAs. Equally C/EBPα contained additional seed sequence matches and miR-K12-3 expression alone did not lead to significant repression levels; however transfection of the miRNA cluster significantly reduced luciferase expression. Furthermore, independent Ago HITS-CLIP data demonstrate that miR-K12-3 directly targets C/EBPα in BCBL-1 cells (Figure 2-2c) (Haecker, 2012). In summary, these *in vitro* data show that all three transcription factors can be targeted by KSHV miRNAs.

Ets-1, MYB and C/EBPα Expression is De-repressed upon miR-K12-3/miR-K12-11 Knockdown in PEL cells

To investigate whether ETS-1, MYB, and C/EBPα are regulated by KSHV miRNAs in latently infected PEL cells, BCBL-1 and BC-3 cells were transfected with increasing amount of miR-K12-3 or miR-K12-11 antagomirs. To validate efficiency and specificity, miRNA knock-down was measured by stem-loop TaqMan qPCR which showed that antagomir transfection specifically inhibited miRNA expression up to 80% (Figure 2-3). RT-qPCR was performed 48 hpt which revealed increased gene expression in a dose dependent manner upon miRNA knockdown (Figure 2-4). Ets-1 and C/EBPα showed 2-

fold increases and MYB a 1.5-fold increase in expression compared to control. Ets-1 was also significantly de-repressed in the presence of antagomirs for miR-K12-3 and miR-K12-11, confirming that both miRNAs are important for targeting.

Lytic Gene Expression and Virus Production Increases upon miR-K12-3 and miR-K12-11 Knockdown in PEL cells

We next asked whether miR-K12-3 and miR-K12-11 knockdown affects virus lytic gene expression beyond RTA and subsequently virus production. Following the same method as in Figure 2-4, BCBL-1 and BC-3 cells were transfected with increasing amounts of miR-K12-3 antagomir, or a combination of miR-K12-3 and miR-K12-11. RT-qPCR was performed 48 hpt to monitor RTA (immediate early gene), ORF59 (early gene involved in DNA replication), and ORF19 (late glycoprotein) expression. Significant de-repression of lytic gene expression was observed at all stages of reactivation upon knockdown of miR-K12-3 (Figure 2-5a). Moreover, lytic gene expression was induced more efficiently when both miR-K12-3 and miR-K12-11 were knocked down simultaneously.

Next, we wanted to confirm that the increase in lytic gene expression translates into production of progeny virus. Cell-free virus was isolated from BCBL-1 supernatants 6 days post antagomir transfection, viral DNA was extracted, and viral genome copy number was determined by qPCR. Figure 2-5b shows that virus production increased in a dose dependent manner when both miR-K12-3 and miR-K12-11 were knocked down. Collectively, these data demonstrate that KSHV miR-K12-3 and miR-K12-11 contribute to the maintenance of viral latency presumably by targeting Ets-1, MYB, and C/EBPα.

Generation of miRNA Deleted Recombinant KSHV BACmids and Latently Infected iSLK cells

In order to validate the role of miR-K12-3 and miR-K12-11 in the context of the viral genome, we generated two miRNA deletion mutants using the recently described KSHV BAC16, which was derived from the PEL cell line JSC-1 (Campbell *et al.*). Briefly, short 20 to 25 bp regions were deleted from one arm of each pre-miRNA, destroying pre-miRNA hairpin formation without affecting neighboring miRNA expression. A modified version of the protocol detailed by Tischer *et al* was used to create markerless microRNA deletions within BAC16 (Tischer *et al.*, 2006). Recombinant bacmids were validated by PCR and pulse field gel electrophoreses to monitor intact terminal repeats. Further details on the generation of recombinant bacmids in iSLK cells will be discussed in Chapter 3. Briefly, miRNA deletion mutants and wild-type (WT) bacmids were transfected into 293T cells. After selection, BAC16-containing 293T cells were induced with TPA and valproic acid, and co-cultured with iSLK cells, which harbor a doxycyclin-inducible RTA and produce high levels of progeny virions (Myoung and Ganem, 2011). Co-culturing of 293T and iSLK resulted in higher infection efficiency than either cell-free virus or direct transfection of bacmid DNA into iSLK cells. WT BAC16, KSHV∆miRK-12-3, KSHV∆miRK-12-11-containing iSLK cells produce high titer progeny virus after doxycyclin induction (up to $1.14x10^7$ genome copies/ ml). As a final quality control, episomal DNA isolated from latently infected iSLK cells containing miRNA deletion mutants or WT BAC16 were analyzed by Illumina-based genome-wide sequencing, which confirmed the presence of the appropriate deletion and only detected a very small number of mutations in each genome (Table 2-2).

To test whether deletion of miR-K12-3 or miR-K12-11 had an effect on the maintenance of latency in iSLK cells, we analyzed spontaneous lytic gene expression during latent infection and reactivation upon induction with sodium butyrate by RT-qPCR. During latency, both wild-type BAC16 and miRNA deleted BAC16 infected iSLK cells (KSHVΔmiR-K12-3 and KSHVΔmiR-K12-11) expressed similar amounts of LANA (Figure 2-6b). In contrast, KSHVΔmiR-K12-11 infected iSLK cells displayed ≥2-fold higher levels of lytic gene expression for RTA, ORF59, and ORF19 when compared to WT (Figure 2-6b). KSHVΔmiR-K12-3 iSLK cells did not display increased levels of spontaneous lytic gene expression.

To test whether KSHVΔmiR-K12-3 and KSHVΔmiR-K12-11 are more sensitive to induction of lytic replication we treated bacmid infected iSLK cells with two different sub-optimal concentrations of sodium butyrate (NaB), and analyzed RTA expression by RT-qPCR at 12 and 72 hours post induction (hpi). As shown in Figure 2-6c, at 12 hpi KSHVΔmiR-K12-11 iSLK cells demonstrated increased RTA expression compared to WT iSLK cells, and at 72 hpi both KSHVΔmiR-K12-3 and KSHVΔmiR-K12-11 iSLK cells displayed increased RTA expression. These results confirm that KSHVΔmiR-K12-3 and KSHVΔmiR-K12-11 expression contribute to the maintenance of latency in iSLK cells and are in agreement with the antagomir inhibition experiments in PELs (Figure 2-4). In summary, our results point to a mechanism in which the KSHV miRNAs miR-K12-3 and miR-K12-11 function as gatekeepers of the RTA promoter by modulating cellular transcription factors in cells of both endothelial and lymphoid origin.

Discussion

Early after the discovery of herpesvirus-encoded miRNAs it was hypothesized that these novel viral post-transcriptional regulators may play a role in the regulation of

latency by targeting lytic genes (Murphy *et al.*, 2008). Indeed, one EBV miRNA gene miR-BART2 is encoded antisense to BALF5, the EBV DNA polymerase, and targeting and cleavage of the BALF5 mRNA has been experimentally confirmed (Barth *et al.*, 2008; Pfeffer *et al.*, 2004). For KSHV, elegant work from the Ganem lab utilizing miRNA mimic- and antagomir-based screens provided evidence that KSHV miRNAs can modulate latent/lytic transition through direct targeting of RTA by miR-K12-9*, and by miR-K12-5 and -11 targeting a host factor BCLAF1, which was shown to sensitize cells for reactivation (Bellare and Ganem, 2009; Ziegelbauer et al., 2009). Based on *in silico* prediction and *in vitro* luciferase assays direct targeting of RTA expression was also demonstrated for miR-K12-5 and -7 (Lin et al., 2011; Lu et al., 2010b). Here we used BC-3-G cells, that report RTA activity using the highly RTA responsive PAN promoter driving GFP, to ask whether antagomir-based inhibition of KSHV miRNAs activates RTA. Inhibition of single or multiple miRNAs in combination revealed that in this experimental system only miR-K12-3 and mir-K12-11 lead to significantly increased RTA expression levels (Figure 2-1). It is not clear why inhibition of miR-K12-5 and -7 did not activate RTA expression in BC-3-G, however, we note that miR-K12-3 and -11 expression levels are higher in BC-3 cells compared to BCBL-1 cells used in previous screens (Gottwein *et al.*, 2011). Additionally, miR-K12-9* is not expressed at all in BC-3 cells due to the presence of a highly polymorphic miR-K12-9 pre-miRNA (Marshall *et al.*, 2007). Hence, BCLAF1 targeting may not play a role in BC-3 cells. We note that recent ribonomics reports on KSHV miRNA targetomes using PAR-CLIP and HITS-CLIP did not reveal KSHV miRNA targeting of RTA in two different PEL cell lines including BC-3 during latency. In contrast, BCLAF1, which was reported to sensitize

cells for reactivation, was validated as a viral miRNA target in BCBL-1 (Gottwein *et al.*, 2011; Haecker, 2012; Ziegelbauer *et al.*, 2009). Since neither the 3'UTR of RTA nor its coding region contains any mir-K12-3 and -11 seed sequence matches, miRNAs in BC-3-G cells must indirectly regulate RTA by targeting cellular genes that positively regulate RTA. A number of cellular transcription factors have been identified to activate the RTA promoter including MYB, Ets-1, and C/EBPα (Lacoste *et al.*, 2007; Wang *et al.*, 2003; Yu *et al.*, 2007), which all contain seed sequence matches for miR-K12-3 and -11 and where de-repressed in antagomir transfected PEL cells (Figure 2-4). Lacoste and colleagues demonstrated that MYB transactivates the RTA promoter in the absence of any KSHV protein expression and furthermore that v-FLIP and v-GPCR induction of NF-κB leads to downregulation of MYB expression in PEL cells (Lacoste *et al.*, 2007). Thus, miR-K12-11 fine tunes MYB expression post-transcriptionally in conjunction with virally encoded proteins that regulate MYB through NF-κB signaling. Interestingly, the NFκB pathway or more specifically the IκBα super repressor, was shown to be regulated by miR-K12-1, thereby also inhibiting lytic growth (Lei *et al.*, 2010). Ets-1 was originally identified as activator of RTA by utilizing BC-3-G to perform a genome-wide screen to identify cellular proteins and pathways that reactivate KSHV (Yu *et al.*, 2007). The Raf/MEK/ERK pathway was demonstrated to mediate KSHV reactivation and Ets-1, which is a downstream effector of this pathway, directly activates the RTA promoter. Ets-1 contains multiple putative KSHV miRNA binding sites in addition to 6 seed sequence matches for miR-K12-3 and -11, which was validated by 3'UTR luciferase assays (Figure 2-2b). Hence, miR-K12-3 and -11 targeting of Ets-1 contributes to maintenance of latency by inhibiting Raf/MEK/ERK induced activation of RTA. Wang

and colleagues observed that C/EBPα activates RTA directly and mapped three C/EBPα binding sites within the RTA promoter (Wang *et al.*, 2003). In addition, C/EBPα functions during lytic reactivation by interacting with RTA to bind and activate the K8 promoter, an early replication-associated protein. K8 then interacts with and stabilizes C/EBPα leading to upregulation of C/EBPα, in turn leading to further activation of RTA and K8 gene expression. It was also shown that C/EBPα activates additional early lytic genes PAN and ORF57 (Wang *et al.*, 2003). Therefore, miR-K12-3 targeting of C/EBPα represents a central regulatory node to negatively modulate several immediate early and early genes.

A common feature of these transcription factors is their involvement in signaling pathways as part of cellular stress responses. The RTA promoter contains multiple transcription factor binding sites thereby sensing multiple stress response pathways which link cell stress to lytic reactivation. We believe that KSHV miRNAs fine-tune the regulation of MYB, Ets-1, and C/EBPα, which creates a threshold of mRNA copies that has to be overcome before the lytic cascade is initiated. Rather than acting like an on/ off switch, viral miRNAs serve as gatekeepers of latency by controlling multiple host cellular pathways that when activated lead to RTA activation. In contrast to directly regulating RTA, targeting multiple cellular genes allows for greater flexibility in regulation of latency in different tissues and cell types where both the viral miRNAs and their cognate targets are expressed differentially (O'Hara *et al.*, 2009; O'Hara *et al.*, 2008). For example Ets-1 levels are higher in endothelial cells while MYB is a master regulator of hematopoietic cells. In this context, we demonstrated that iSLK cells infected with ΔK12-3 BAC16 and ΔK12-11 BAC16 were able to reactivate at a

significantly higher rate under sub-optimal induction when compared to cells infected with WT BAC16 (Figure 2-6c). Hence, miR-K12-3 and miR-K12-11 contribute to maintenance of latency in cells of endothelial and lymphoid origin, and their knock-down or absence is associated with a more lytic phenotype. In summary, our data on miRK-12-3 and -11 as well as other reports (Ziegelbauer *et al.*, 2009) suggest a model whereby viral miRNAs contribute to the regulation of a key step in the herpesviral life cycle, the transition from latency to lytic replication by post-transcriptionally modulating multiple signaling pathways. It is noteworthy that again some of these targets are co-regulated by other latency-associated genes as in the case of MYB and NFκB signaling. Therefore, we propose that the miRNA regulation contributes to the ability to quickly respond to environmental stimuli based on small changes in RNA expression levels, which can overcome the intricate balance between miRNA copy number and their cognate targets (Baccarini *et al.*, 2011; Mukherji *et al.*, 2011). A similar miRNA-based "spring-loaded" regulatory model was first proposed for OCT and SOX transcription factors that regulate pluripotency during differentiation (Marson *et al.*, 2008). In conclusion, our data strongly suggest that miR-K12-3 and miR-K12-11 contribute to viral latency by targeting the host cellular transcription factors MYB, Ets-1, and C/EBPα in cells of lymphoid and endothelial origin.

Materials and Methods

Cell Lines

BC-3-G cells were kindly provided by Ren Sun (UCLA) (Yu *et al.*, 2007). BC-3-G, BC-3 and BCBL-1 cells were cultured in RMPI supplemented with 10% FBS, 1% P/S, and 1% sodium pyruvate. HEK293 and HEK293T cells were cultured in DMEM with

10% FBS and 1% P/S. iSLK cells were kindly provided by Don Gamen (UCSF) (Myoung and Ganem, 2011) and were cultured under the same conditions as 293 cells.

Flow Cytometry

BC-3-G cells ($1x10^6$) were pelleted and resuspended in 1XPBS with 2% FBS. Cells were analyzed for GFP expression using the Accuri C6 flow cytometer in the ICBR, University of Florida. 10,000 cells were counted in triplicates and cells were normalized to background fluorescence from BC-3 cells.

Luciferase Assays and Reporter Construction

MYB, Ets-1, and C/EBPα full length 3'UTR sequences were cloned downstream of the luciferase gene in pGL3 promoter (Promega) using pCRII-TOPO (Invitrogen) for MYB and GeneArt Seemless Cloning (Invitrogen) for Ets-1 and C/EBPα. Primers for 3'UTR cloning can be found in Table 2-1. HEK293 cells were transfected using TransIT-293 reagent (Mirus) in 24-well cell culture dishes according to the manufacturer's protocol. Each transfection reaction contained 2 ng of the pCMV-Renilla (Promega) control vector, 20 ng of the pGL3 promoter 3'UTR reporter construct and 0, 400 or 800 ng of the pcDNA3.1 miRNA expression vector complemented with 800, 400, or 0 ng of empty pcDNA3.1 vector as filler to reach 800 ng total pcDNA3.1 in each transfection (Samols, 2007). Cells were harvested 72 hrs post transfection and luciferase activity was quantified using the Dual Luciferase Reporter kit (Promega) according to the manufacturer's protocol. Each lysate was assayed for firefly luciferase activity using a FLUOstar OPTIMA reader (BMG Labtech). Firefly luciferase activity for each sample was normalized to Renilla expression and samples were compared to the miRNA mock transfection control. Transfection assays were performed in triplicate and repeated at least 3 times. Standard deviation was calculated for triplicates and

displayed as error bars in the figures. Significance of the repression of the reporter construct relative to the 0 ng miRNA expression vector was tested by one-tailed, unpaired t-test.

Antagomir Derepression Assays and Quantitative Reverse Transcription-PCR (RT-qPCR) Analysis

For inhibition of KSHV miRNAs, 2'OMe RNA antagomirs were used as previously described (Skalsky *et al.*, 2007). PEL cells ($1x10^6$) were transfected with 50-400 nM of antagomir using TransIT-TKO transfection reagent (Mirus) as described (Boss *et al.*, 2011). At 48 hours post transfection (hpt), cells were harvested using RNA-Bee (Tel-Test) according to the manufacturer's instructions. 1 µg of DNase treated RNA was reverse transcribed using SuperScript III (Invitrogen) according to the manufacturer's suggestions. Quantitative PCR (qPCR) analysis was carried out using an ABI StepOne Plus system along with ABI Fast SYBR reagent (Applied Biosystems, Carlsbad, CA). Expression of all genes was normalized to β-actin expression and student t-tests were performed to determine statistical significance compared to the mock control. Primers for genes can be found in Table 2-1.

Virus Isolation and Quantitation

Virus particles were harvested from PEL cells 6 days post transfection (dpt) of antagomirs. Cells were pelleted at 1100 RPM for 5 minutes and media supernatant was passed through a 0.45 µM filter. Virus particles were then pelleted by ultra-centrifugation using a Beckman SW-40 rotor at 100,000 x g for 1 hr on a 25% sucrose cushion. Virus pellets were then resuspended in 1% of its original volume using serum-free RPMI. DNA was extracted from 25 µL of virus stocks using DNAzole (Molecular Research Center, Inc.) then resuspended in 25 µL of ddH_2O. Viral genome copy

number was determined by qPCR assay using serial diluted LANA expression plasmid as a standard curve.

Generation of iSLK KSHV BAC Cells

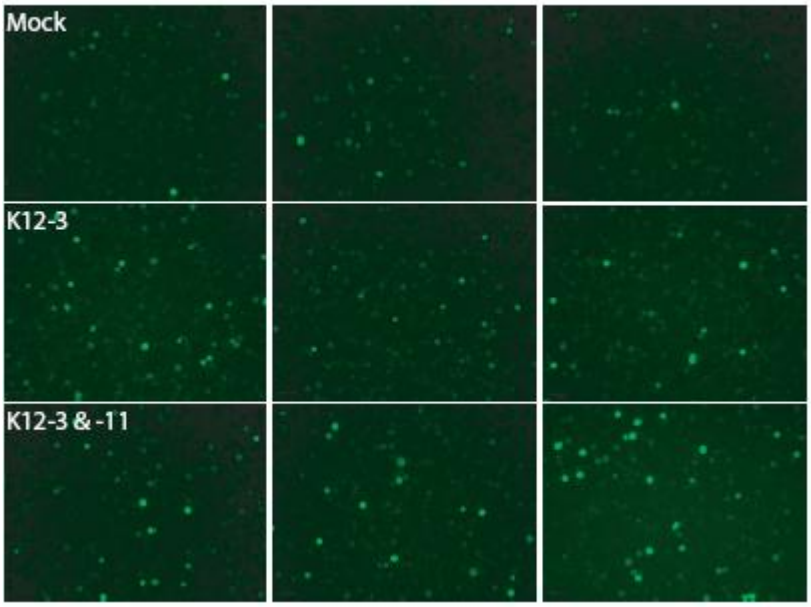
KSHV BAC16 was kindly provided by the Jung lab (USC). A detailed modified version of the protocol by Tischer et al was used as described in the results section (Tischer *et al.*, 2006). BAC DNA was isolated from bacteria using the Large-Construct Kit (Qiagen) according to the manufacturer's recommendations. 293T cells were then transfected with 2 µg of DNA using TransIT-293 reagent (Mirus) according to the manufacturer's instructions. Cells underwent selection using 100 µg/mL of hygromycin B and were expanded for 10-15 days. Transfected cells, which express GFP, were monitored under fluorescence microscope. Once the expanded cell population was 100% GFP positive, cells were co-cultured with iSLK cells (Myoung and Ganem) and induction was performed using 20 pg/mL of TPA and 1 mM valproic acid. Cells were washed 4 days post induction (dpi), and infected iSLK cells were selected using 1 µg/mL puromycin, 250 µg/mL G418, and 1.2 mg/mL hygromycin B. Stable KSHV∆miRNA BAC16 iSLK cells were expanded and induced using 1 µg/mL DOX and 1 mM NaB. Virus was collected and quantified 4 days later using the protocol described above.

Immunofluressence Assay

KSHV∆miRNA BAC16 iSLK cells was plated on coverslips in 24 well plates and fixed using 1% formaldehyde 12-16 hrs later. After a brief washing in PBS cells were permeabilized using 0.2% triton X-100 in PBS for 15 min. on ice followed by two washings with PBS. Primary antibody Rabbit-anti-LANA was applied 1:300 for 1 hour at room temperature as described previously (An *et al.*, 2006). Cells were washed and

incubated for 45 min. in Alexa 594 conjugated goat-anti-rabbit antibody at 1:1000 dilution. After washing, coverslips were mounted using Vector Shield which provided DAPI staining. Cells were then observed under a fluorescence microscope to visualize staining.

a



b

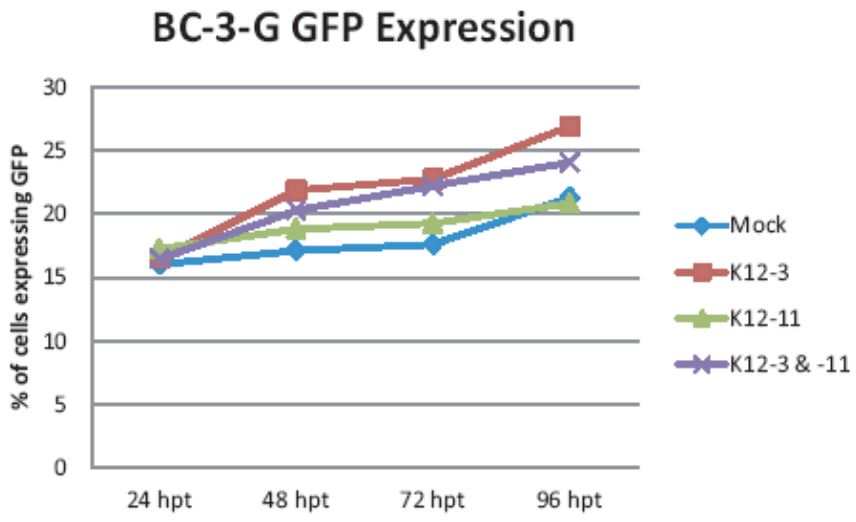


Figure 2-1. Antagomir screen in BC-3-G cells. (a) BC-3-G cells 72hpt of antagomirs. $1x10^6$ BC-3-G cells were transfected with 1μM of miR-K12-3 or miR-K12-3 and miR- K12-11 combined antagomirs. The mock sample underwent transfection in the absence of antagomir. Cells were observed 72hpt under a fluorescence microscope. Three different fields are shown for each transfection. (b) miR-K12-3 inhibition increases reactivation by 26% compared to mock control. Flow cytometry results of 3 independent experiments. Transfections were carried out as explained above. GFP expression was scored in triplicates from each sample every 24 hours until 96 hpt.

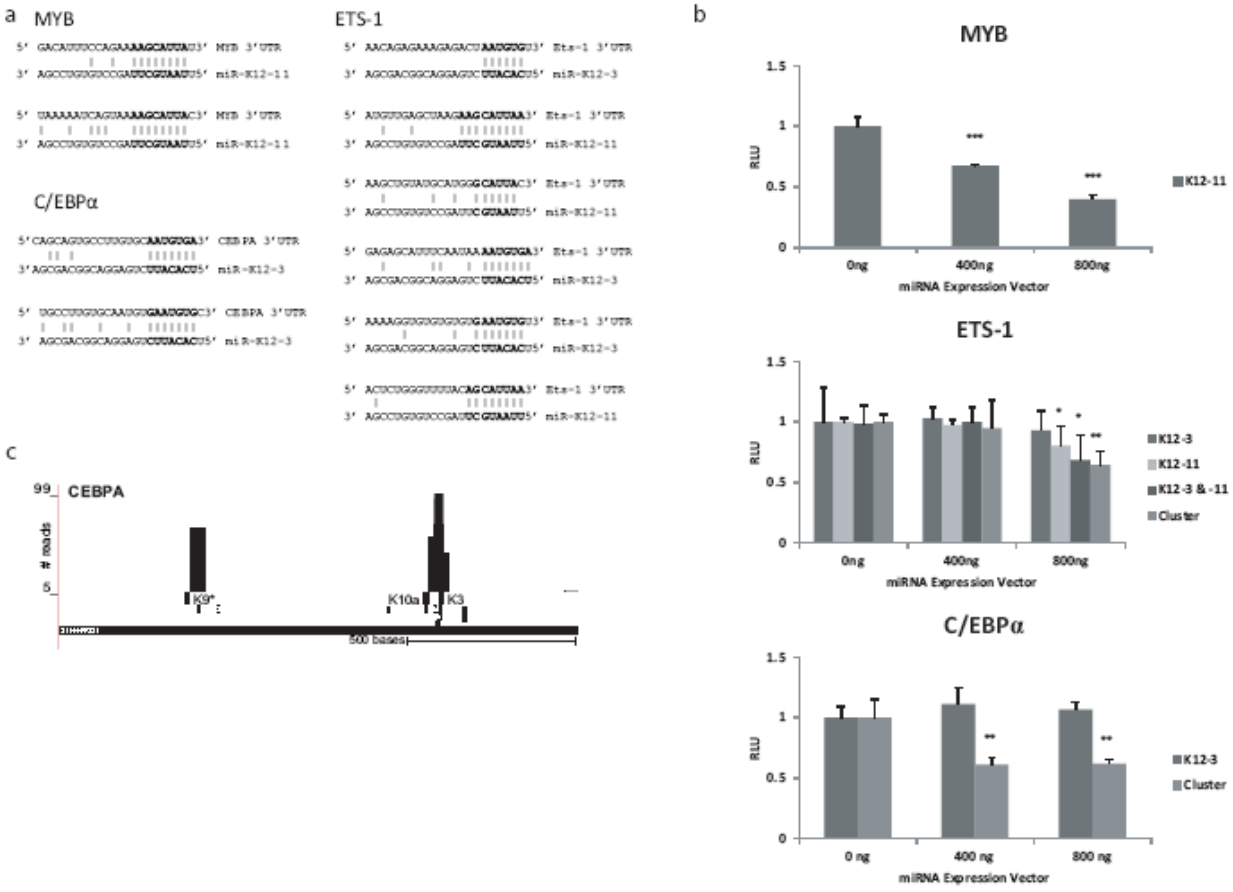


Figure 2-2. MYB, Ets-1, and C/EBPα are targeted by miR-K12-3 & miR-K12-11. (a) 3'UTRs of cellular genes known to activate the promoter of RTA were scanned for potential miRNA binding sites. (b) Dual reporter luciferase assays using MYB, Ets-1, and C/EBPα 3'UTRs in pGL3 promoter co-transfected with increasing amounts of miRNA expression vectors into 293 cells. Cell lysates were collected 72 hpt firefly readings were normalized to the corresponding renilla luciferase values. (c) Ago- miRNA-mRNA clusters in the 3'UTR of C/EPBα. mRNA-derived clusters of reads are visualized in UCSC genome browser as wiggle tracks. Shown are the positions of read clusters overlapping with miRNA seed match sites within the 3'UTR of C/EBPα. KSHV miRNA seed match positions are indicated by colored bars; those of human miRNAs as predicted by TargetScan are shown as black bars (Haecker 2012 under review).

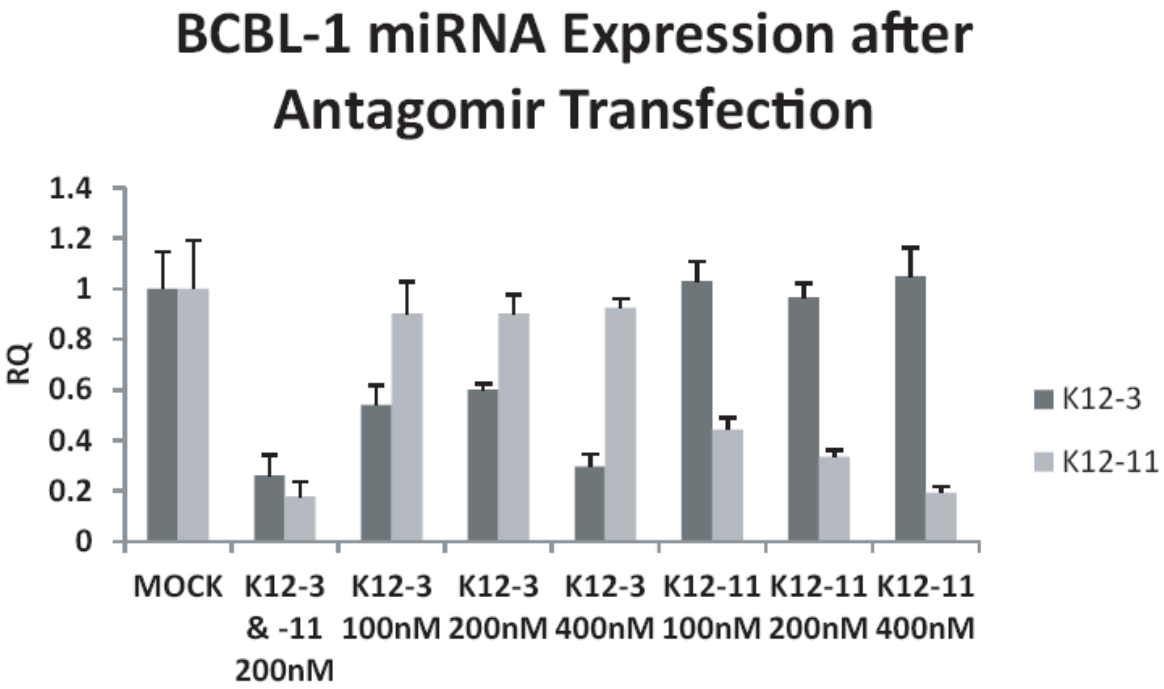


Figure 2-3. Confirmation of miRNA knockdown after antagomir transfection. $1x10^6$ BCBL-1 cells were transfected with increasing amount of miR-K12-3 or miR-K12-11 antagomir or a combination of both. RNA was harvested 48 hpt and TaqMan miRNA-RT and qPCR was performed using primers and probes specific to miR-K12-3 and miR-K12-11. Primers and a probe specific to RNU66 were used as a loading control and all samples were normalized to mock transfected controls.

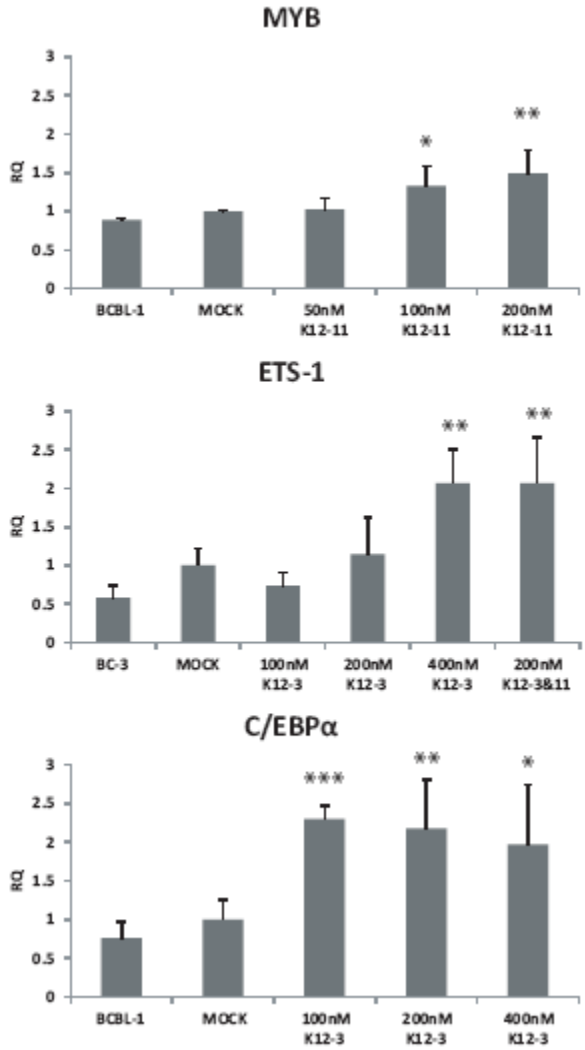


Figure 2-4. MYB, Ets-1, & C/EBPα expression increases upon miR-K12-3 and miR-K12-11 knockdown in PEL cells. $1x10^6$ BC-3 and BCBL-1 cells were transfected with varying amounts of miRNA-specific antagomir. Total RNA was collected 48 hpt and reverse transcribed. qPCR was performed using primers specific for MYB, Ets-1, and C/EBPα. All samples were normalized to β-actin expression and compared to gene expression in the mock transfected control. $p<0.05$(*), $p<0.01$(**), $p<0.001$(***)

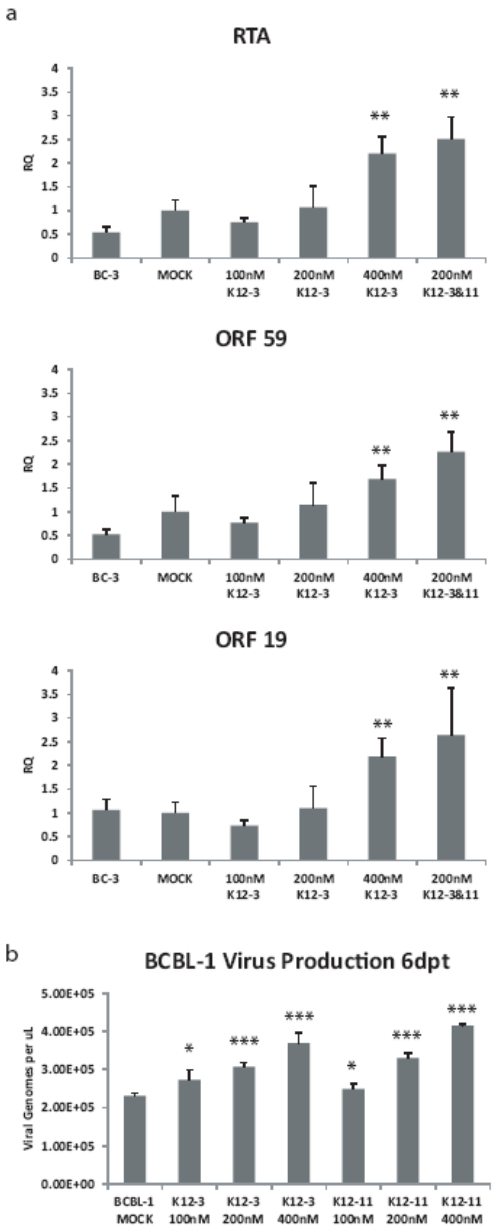


Figure 2-5. Lytic gene expression and virus production increases upon miR-K12-3 and miR-K12-11 knockdown in PEL cells. (a) $1x10^6$ BC-3 cells were transfected with varying amounts of miRNA-specific antagomir. Total RNA was collected 48 hpt and reverse transcribed. qPCR was performed using primers specific for RTA, ORF59, and ORF19. All samples were normalized to β-actin expression and compared to gene expression in the mock transfected control. (b) $1x10^6$ BCBL-1 cells were transfected with varying amounts of miRNA-specific antagomir. Cell media harboring progeny virus was collected and filtered (0.45μM) 6 dpt. DNA was collected from the virus stock and was used for qPCR along with a plasmid standard to determine genome copy number per μL. $p<0.05$(*), $p<0.01$(**), $p<0.001$(***)

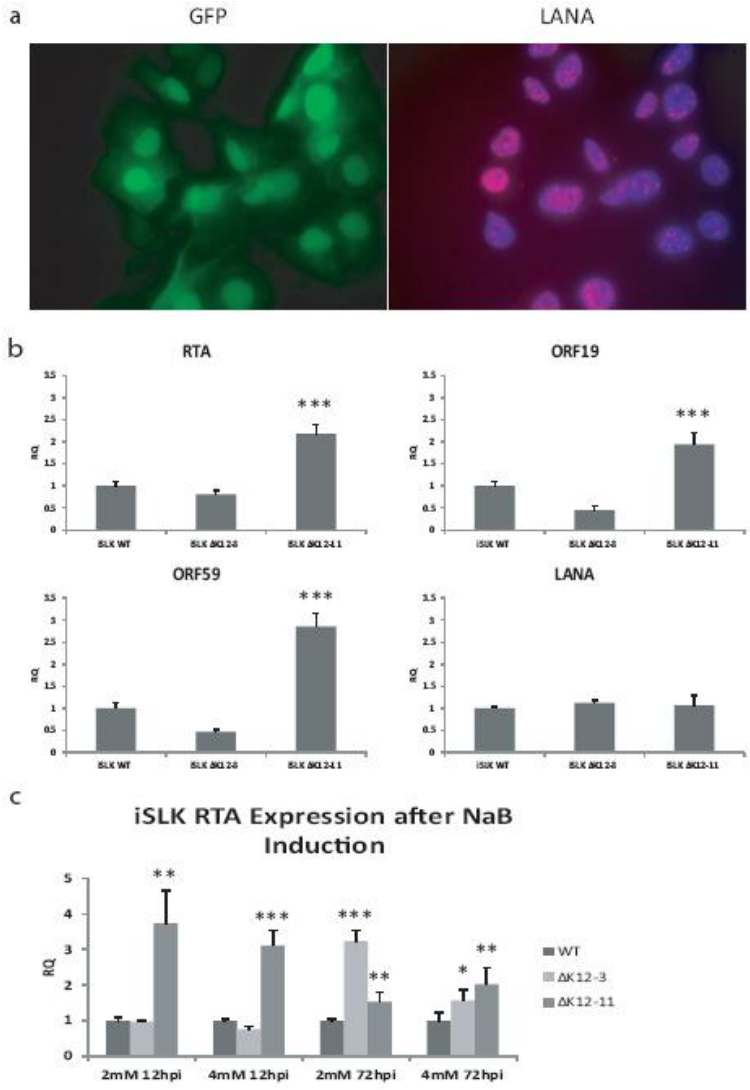


Figure 2-6. Analysis of KSHV miRNA deleted recombinant bacmid viruses. (a) miR-K12-3 and miR-K12-11 deleted virus was generated individually using BAC16. ΔmiR-K12-3 BAC16 was used to infect iSLK cells to make a bacmid producer cell line. Infection was monitored by observing GFP expression (left panel) and infected cells were selected using hygromycin B. IFA for LANA was performed using rabbit- polyclonal antibody and nuclear staining with DAPI (right panel). (b) iSLK cells latently infected with WT, ΔmiR-K12-3, and ΔmiR-K12-11 BAC16 were harvested for RNA and RT-qPCR was performed using primers specific for LANA, RTA, ORF59, and ORF19. All samples were normalized to β-actin expression. (c) iSLK cells infected with and selected for WT, ΔmiR-K12-3, and ΔmiR-K12-11 BAC16 were induced with 2mM and 4mM NaB. Total RNA was harvested 12 and 72 hours post induction. RT-qPCR was performed using primers specific for RTA. All samples were normalized to β-actin expression. Samples were compared to the WT control for each corresponding concentration and time point. $p<0.05$(*), $p<0.01$(**), $p<0.001$(***)

Table 2-1. Primer Sequences

Gene	Primer Sequence 5'-3'	Ref
MYB 3'UTR	FWD- GATGGAGGAGCAGATGACATC REV- AGGTAAAATAAGGGCAC	
Ets-1 3'UTR	FWD- CGTGTTGGTTGGACTCTGAA REV- TCTCCAGCAAAATGATGTGC	
C/EBPα 3'UTR	FWD-CTTGTGCCTTGGAAATGCAAACTCACC REV- AAGAAGAGAACCAAGCCGTCCTTC	
MYB	FWD- TCAGGAAACTTCTTCTGCTCACA REV- AGGTTCCCAGGTACTGCT	
Ets-1	FWD- AAGGGAGATCGAAGGAGGAA REV- TCTGCTATAGGAACTGCAGGAG	
C/EBPα	FWD- TGTATACCCCTGGTGGGAGA REV- TCATAACTCCGGTCCCTCTG	
RTA	FWD- CACAAAAATGGCGCAAGATGA REV- TGGTAGAGTTGGGCCTTCAGTT	(Lei et al., 2010)
ORF59	FWD- TTAGAAGTGGAAGGTGTGCC REV- TCCTGGAGTCCGGTATAGAATC	(Majerciak et al.)
ORF19	FWD- GGCGAAAAAGTCAGCGGTGGT REV- CGGCGCGTCTTCCCTAAAGA	(Persson and Wilson)
LANA N-Terminus	FWD- GCGCCCTTAACGAGAGGAAGTT REV- TTCCTTCGCGGTTGTAGATG	
β-actin	FWD- CATGTACGTTGCTATCCAGGC REV- CTCCTTAATGTCACGCACGAT	Primerbank ID 4501885a1

Table 2-2. iSLK BAC16 Sequencing Results

Position	Ref	Variants	Allele Variations	Freq	Counts	Coverage	Overlapping Annotations	Amino Acid Change
Wildtype								
137761	T	2	T/G	60.7/39.3	17/11	28	Repeat region: direct	
137795	G	1	C	66.7	18	27	Repeat region: direct	
miR-K3 Mutant								
8061	AG	2	T/C	63.6/36.4	56/32	88	CDS: ORF7	Arg485Ser
23787	G	2	A/G	64.3/35.7	9/5	14	Repeat region: LIR1	
23789	A	2	A/T	52.6/47.4	10/9	19	Repeat region: LIR1	
138021	A	1	C	100	16	16	Repeat region: direct	
miR-K1 Mutant								
137795	G	2	G/C	60.0/40.0	27/18	45	Repeat region: direct	
miR-K12-11 mutant (miR-K6)								
66571	C	1	T	68.5	902	1317	CDS: ORF44	Leu622Phe
138021	A	1	C	100	5	5	Repeat region: direct	

CHAPTER 3
GENERATION AND ANALYSIS OF KSHV MIRNA DELETED RECOMBINANT VIRUSES

Since the discovery of KSHV-encoded miRNAs, our lab has focused on functional studies to determine the roles of these miRNAs. Prediction algorithms are used to scan 3'UTRs of mRNAs for presence of miRNA seed match sites (Grimson et al., 2007). Luciferase reporter assays in 293 cells are used to confirm targeting of a gene (as described and shown in Chapter 2), followed by site-directed mutagenesis of seed match sites to relieve targeting or co-transfection with antagomirs to show de-repression (Samols, 2007; Skalsky et al., 2007). 293 cells stably expressing viral miRNAs have been established and gene expression profiling has been performed to compare empty vector vs miRNA expressing cells to determine possible cellular targets (Samols, 2007; Skalsky et al., 2007). Recently Isaac Boss has generated a humanized mouse model expressing miR-K12-11 or human miR-155 to determine the *in vivo* phenotype of these two miRNAs, which share the same seed sequence (Boss et al., 2011). Current work in the lab by Irina Haecker is focused on determining targets of KSHV miRNAs in PEL cells by HITS-CLIP, a new technique in which RNA-protein complexes are UV-crosslinked, followed by immunoprecipitation of Ago-miRNA-mRNA complexes and high throughput sequencing (HTS) of isolated RNA to determine the miRNA target profile of cells (Haecker, 2012). While all of these approaches have improved our understanding of miRNA function and targeting, they lack the ability to investigate miRNA function in the context of the viral genome. This limitation can be overcome by developing a genetic system to mutate viral miRNA and generate recombinant viruses. However, studying KSHV genetics has been difficult due to gamma-herpesviruses having low replication turnover in latently infected cells and lacks an efficient infection system. This

hinders the ability of mutant viruses to be generated by homologous recombination in mammalian cells as is routinely done in alpha herpesviruses such as HSV-1 (Bloom, 1998). Therefore we collaborated with the Jung lab at USC and set up a core facility here at UF to produce individual miRNA deleted recombinant viruses using bacterial artificial chromosomes (BACs also bacmids).

BACs facilitate the propagation and manipulation of large DNA viruses, where previous attempts at genetic manipulation were dependent on rare recombination events in eukaryotic cells. The first herpesvirus BAC system was developed in MCMV which is one of the largest herpesvirus with a genome size of 230 kbp (Messerle et al., 1997). Cloning of such large viral genomes requires the insertion of a minimal fertility factor replicon (mini-F) vector which is flanked by sequences of the desired insertion site and allows up to 300 kb of DNA to be cloned after homologous recombination. The BAC vector also contains a bacteria origin of replication and antibiotic resistance along with selection and visualization cassettes for use in mammalian cells. Currently there are BAC systems for all human herpesviruses with the exception of HHV-7 (for review see (Tischer and Kaufer, 2012)). The first KSHV BAC was generated by Delecluse et al. using BC-3 cells which were recombined with the bacmid backbone at ORF56, resulting in a replication deficient virus. Upon introduction of ORF56 expressing in KSHV BAC 293 cells, virus replication was restored and viral progeny was produced (Delecluse et al., 2001). Shortly after the creation of the ORF56 disrupted KSHV BAC, Zhou and colleges generated another KSHV BAC using BCBL-1 cells, termed BAC36. Unlike the previous study, the bacmid backbone was designed to be inserted between ORF18 and ORF19, therefore not disrupting viral replication and able to complete

abortive lytic replication when induced (Zhou et al., 2002). BAC36 provided the first tool in which KSHV genetic analysis could be performed due to its ability to be shuttled between bacteria and mammalian cells. However, recent work has shown that BAC36 contain duplications and deletions in both the unique coding region and terminal repeats of the KSHV genome (Yakushko et al., 2010). Therefore the Jung Lab established BAC16, which has been confirmed by HTS sequencing to contain no mutations to the KSHV genome (Campbell et al., 2012). Unlike BAC36 which is derived from BCBL-1 cells, BAC16 was cloned from JSC-1 cells which are believed to yield a higher titer virus than BCBL-1 cells. BAC16 was created by inserting the pBelo45 backbone in between vIRF-1 and ORF57, a region of the virus known to be non-coding (Campbell et al.). pBelo45 allows the virus to replicate in bacteria, encodes antibiotic resistance genes to be used in both bacteria and eukaryotic cells, and contains a GFP marker to monitor transfection/infection in cell culture (Figure 3-1). Thanks to the Jung Lab providing this useful tool to us, we now have a new approach to studying KSHV miRNA function. In 2009 a core facility, run by post-doc Brian Krueger, was established and primarily focused on the generation of individual miRNA deleted viruses. Below is a brief description of the work done by Brian Krueger and the core facility on the creation of the miRNA mutation in E. coli harboring BAC16. Next I describe my contribution of reconstituting the bacmid DNA in mammalian cells and establishing a highly robust producer cell line for the efficient harvesting of recombinant virus.

Mutational Strategy

Short 20-25 bp regions of the virus representing the mature miRNA sequence were deleted in order to create the KSHV miRNA mutant viruses. By deleting this short sequence, pre-miRNA stem-loop structure should be effectively destroyed, and both 5p

and 3p strands of the miRNA should not be processed (Figure 3-2). Also this small deletion should have minimal to no effect on the processing of other nearby miRNAs. This strategy was used for 10 miRNAs that are located in the intragenic region between v-FLIP and Kaposin. MiR-K12-10 and -12 are located in the Kaposin open reading frame and therefore point mutations were inserted into the sequence containing the mature miRNA sequence, destroying pre-miRNA hairpin formation but still maintaining the protein coding sequence of the Kaposin gene.

As mentioned in Chapter 2, a modified version of the protocol detailed by Tischer et al was used to create the miRNA deleted viruses (Tischer et al., 2006). The mutations were generated in a two-step process using *E. coli* GS1783 which is a rec minus strain containing the Red recombination system and contains WT KSHV BAC16 (Figure 3-3). In the first step, a mutation cassette containing a kanamycin selectable marker was created and recombined into the KSHV genome at the exact position where the miRNA of interest is deleted. In the second step, recombination is performed again to remove the kanamycin selection marker and the deletion of the target miRNA sequence. Primers were created flanking the region targeted for deletion and included sequence corresponding to the kanamycin marker. These primers were then used to amplify the kanamycin selection marker out of a plasmid resulting in the creation of a mutation cassette (Figure 3-2). *E. coli* was cultured, induced to express red recombinase, then electroporated with the mutation cassette. The electroporated cells were incubated for 1 hour then plated on a kanamycin positive agar plate to select for colonies containing the inserted mutation cassette into BAC16.

Clones from the first step of recombination were screen by colony PCR to confirm insertion of the kanamycin selection marker in the correct position within BAC16. The integrity of the terminal repeats was confirmed to be intact after recombination by digesting bacmid DNA using NheI and performing pulse field gel electrophoresis (PFGE) (Figure 3-4). Only clones passing these screens were used in the next recombination step.

The second step of recombination was performed by growing high quality clones then inducing I-secI expression by the addition of arabinose. This resulted in linearization of the bacmid and allows for recombination to occur, removing the kanamycin selection marker and generates a markerless virus mutant. A second round of screens were performed to confirm sensitivity to kanamycin, loss of kanamycin cassette and miRNA sequence by colony PCR, and integrity of terminal repeats by PFGE. Lastly, bacmid DNA was isolated from bacteria using the Large-Construct Kit (Qiagen). All of the above work was done by Brian Krueger and our core facility.

Reconstitution of KSHV BAC16ΔmiRNA in 293T Cells

In order to produce packaged recombinant KSHV virus, KSHV BAC16 DNA was transfected into 293T cells. For this cells were seeded into 6-well plates and the next day were transfected with 2μg of bacmid DNA using TransIt-293 reagent (Mirus). Transfection efficiency was determined by observing GFP expression 48 hpt. The highest transfection efficiency, between 40-60%, was seen when freshly made bacmid DNA along with newly thawed 293T cells were used. Once cells were observed to express GFP, confirming successful transfection of bacmid DNA, cells were expanded and transferred in a 10cm dish containing fresh media along with 100 μg/mL hygromycin B to select for BAC16 containing cells. 293T cells were continuously

cultured for several days to recover from antibiotic selection and expanded to 15 cm plates. This process usually took 10-15 days post transfection.

RTA Inducible SLK cells Serve as a Recombinant Virus Producer Cell Line

Previously, if more recombinant virus was needed, the above procedure would have to be repeated and virus was induced and collected from 293T cells. Constantly repeating this inefficient procedure may lead to possible mutations within the viral genome and is also inconsistent in the amount of virus produced. The longer the transfected 293T cells are passaged, the smaller amount of virus produced. Moreover, transfected 293T cells cannot be reactivated to make recombinant virus after being thawed from frozen stocks. iSLK cells were generated and provided by the Ganem Lab (Myoung and Ganem, 2011). These cells were produced by transducing SLK uninfected endothelial cells, which were derived from a gingival KS lesion of an HIV-negative renal transplant recipient (Siegal et al., 1990), with a RTA expression construct which is tightly regulated by a promoter bearing a tet operator sequence. The cells were also transduced with the rtTA (Tet-On) transactivator, which requires doxycycline as a cofactor for activation. Efficient and controlled RTA expression allows iSLK cells to serve as a producer cell line for recombinant bacmid KSHV. Attempts were made to directly transfect BAC16 DNA into iSLK cells, bypassing the use of 293T cells, but transfection efficiency was very low. Our next approach was to use virus stock that was collected from induced BAC16 293T cells to infect iSLK cells, and this worked in varying efficiencies with each of the mutated and WT recombinant viruses. Recent work by Myoung and Ganem demonstrated that cell-cell interaction is needed for higher infection efficiency rather than infecting with cell-free virus (Myoung and Ganem). Therefore BAC16 293T cells were co-cultured with iSLK cells at a ratio of 1:1. BAC16

293T cells were then induced by the addition of 20ng/mL TPA and 1mM valproic acid. Cell media was replaced 4 days post induction, and antibiotic selection specific for infected iSLK cells was added (1μg/mL puromycin, 250μg/mL G418, and 1.2 mg/mL hygromycin B). BAC16 iSLK cells were allowed to recover from selection and dead 293T cells were washed away. Infection efficiency was monitored by GFP expression using fluorescence microscopy. Once cells reached 40-60% confluency in 15cm plates, cells were induced using 1μg/mL doxycycline, to induce RTA expression, and 1mM of NaB. Virus was harvested 4 days post induction and filtered using a 0.45μM membrane.

Collection and Analysis of BAC16 iSLK Virus

Filtered media from bacmid infected cells was pipetted upon a 25% sucrose cushion and subjected to ultracentrifugation at 100,000g for 1 hour using a Beckman Coulter SW-28 rotor. Virus pellets were resuspended in 1% of original volume using serum free media and stored at -80ºC. DNA from 25μL of recombinant virus stocks was isolated using DNAzol according to the manufacture's recommendations. Virus DNA was resuspended in 25μL of ddH_20 and 1μL was used per qPCR reaction. Real-Time qPCR was performed using pcDNA3.1-Orf73 plasmid as a standard along with primers specific for the N-terminus of LANA. Viral genome copy number was determined by comparing viral DNA to the plasmid standard curve. Results show that BAC16 iSLK cells are able to reactivate at significantly higher levels than BAC16 293T cells and can produce up to 20 times more virus. Also, infected iSLK cells that had been frozen for 2 months, when thawed, expanded, and induced they produced recombinant virus at significantly higher levels than 293T cells (Figure 3-5). These data confirm the ability of

iSLK cells to serve as a producer cells line for the production of recombinant KSHV virus.

To confirm the deletion of individual miRNA from each recombinant virus, ABI TaqMan miRNA RT-qPCR was performed to detect the expression of the specific miRNA in the mutant virus infected cells. RNA from iSLK cells infected with BAC16ΔmiRNA was isolated using RNA-Bee according to the manufacturer's suggestions. 10ng of total RNA was used for reverse transcription (ABI). RT-looped primers specific for each individual KSHV miRNA was used to make cDNA pools. Parallel experiments were performed with RNU66 which served as an endogenous control. TaqMan miRNA qPCR was performed using specific primers and probes for each KSHV-encoded miRNA along with the RNU66 control. Figure 3-6 shows miRNA expression levels of BAC16ΔK12-1, BAC16ΔK12-3, and BAC16ΔK12-11 virus compared to WT BAC16. Results confirm the deletion of the corresponding miRNA and that expression of the other 11 KSHV miRNAs is intact.

Infection of Endothelial Cells using iSLK BAC16 Virus

Since latency is the default pathway after KSHV infection, the genome copy number of virus stock does not necessarily correlate with the number of infectious particles. In order to show that iSLK BAC16 cells are able to produce infectious virus, virus stocks were used to infect two different endothelial cell lines. Telomerase immortalized vascular endothelial (TIVE) cells and SLK cells were infected with various amount of cell-free virus stock in the presence of polybrene, a cationic polymer used to increase the efficiency of infection (An, 2005). Infection efficiency was monitored by observing GFP expression by both fluorescence microscopy and flow cytometry (Figures 3-7 and 3-8). Results show that iSLK-derived BAC16 virus stocks are

infectious at varying degrees, with WT usually having a slightly higher infectious rate when compared to ΔK12-3 and ΔK12-11 virus stocks. Also, SLK cells show overall greater susceptibility to infection when compared to TIVE cells. Infection was further confirmed by detection of LANA expression by IFA. Using this method we also saw that SLK cells harbor more copies of the viral genome than TIVE cells (Figure 3-9), which is consistent with our previous finding that TIVE cells contain less copies of the viral genome (An et al., 2005),pointing to SLK cells being more susceptible to infection than TIVE cells.

Generation of BJAB BAC16 Cells through Co-culturing

Although there are a limited number of cell types that are infected by KSHV in vivo, in vitro KSHV virions can infect a wide variety of cell lines. Unfortunately, no established B cell line being tested has been able to be infected with KSHV. This has greatly limited the study of KSHV infection in lymphoid compartment. Using again the proven methodology of co-culturing developed by Myoung and Ganem, iSLK BAC16 cells were induced and co-cultured with BJAB cells to establish BJAB BAC16 cells. BJAB is an EBV negative Burkitt's lymphoma cell line. PEL cells are commonly used for the study of KSHV latent infection; however there is no isogeneic control, or non-infected PEL cells, to use as a comparison. With the establishment of BJAB BAC16, we now have a system in which not only can we compare non-infected BJAB to BJAB BAC16, but we can also look at the effects of miRNA deletion on infection in B cells.

iSLK BAC16 cells were plated on a 6 well plate and induced with doxycycline and NaB the next day. BJAB cells were added at a 1:1 ratio on top of the iSLK BAC16 cells 2 days post induction and cells were co-cultured for an additional 4 days. Cell media containing BJAB cells were spun down and resuspended in fresh RPMI with 100μg/mL

hygromycin b. BJAB BAC16 cells were allowed to recover from selection for 2 weeks and dead cells were diluted out by passaging. Once cells were 100% GFP positive, total RNA and cell lysates were collected for analysis.

RT-qPCR was performed to detect viral gene expression. LANA, RTA, ORF59, and ORF19 expression was normalized to β-actin levels and results showed that these viral genes are expressed at similar levels in WT, ΔmiR-K12-3, and ΔmiR-K12-11cells with the exception of ΔmiR-K12-11 expressing higher amounts of RTA than both WT and ΔmiR-K12-3 (Figure 3-10). These results are similar to what we observed in iSLK ΔmiR-K12-11 cells discussed in Chapter 2.

Expression of viral miRNAs in BJAB BAC16 cells was confirmed by TaqMan miRNA qPCR as previously described with iSLK BAC16 cells in BJAB WT, BJABΔK12-3 and BJABΔK12-11 cells (Figure 3-11). Both mutants had complete abolishment of their corresponding miRNA mutants, and similar expression of the other miRNA when compared to BJAB WT. However, miR-K12-3 and -11 expression in all BJAB BAC16 cells was ~75% lower compared to BCBL-1 miRNA expression levels. PEL cells such as BCBL-1 normally have between 50-80 copies of the viral genome in each cell. qPCR results show that BJAB BAC16 cells express significantly lower copies of intracellular LANA when compared to BCBL-1, which translates to ~10-15 genome copies per cell (Figure 3-12). These results explain the reduced miRNA expression in BJAB BAC16 vs BCBL-1.

Lastly, as discussed in Chapter 2, we investigated the expression of the B cell transcription factor MYB, which is regulated by miR-K12-11, in BJABΔK12-11 cells. MYB expression in the absence of miR-K12-11 compared to WT virus infected cells was

tested by RT-qPCR and western blot was performed (Figure 3-13). Results show that MYB expression does not increase when miR-K12-11 is deleted from the KSHV genome. This may be due to low genome copy and miRNA expression in BJAB BAC16 cells; therefore the miRNA expression level may not be high enough to see a significant change in MYB gene and protein expression.

In summary, we were able to utilize BAC16 to generate individual miRNA deleted recombinant viruses. Through the use of iSLK cells, bacmid producer cell lines were established for the first time. These developments have allowed increased productivity using recombinant virus and a more efficient way to produce large amounts of recombinant virus for future work. Also through co-culturing techniques, BJAB BAC16 cells were established, producing the first isogeneic control for studying KSHV infection in B cells.

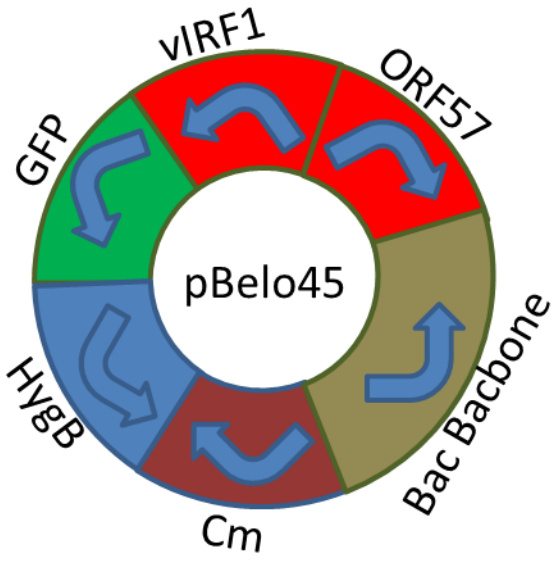


Figure 3-1. pBelo45 plasmid construct. Inserted in between vIRF1 and ORF57 in the KSHV genome. The plasmid contains bacterial origin of replication and chloramphenicol resistance gene for growth in bacteria. It also contains GFP cassette and hygromycin b resistance gene for selection in eukaryotic cells.

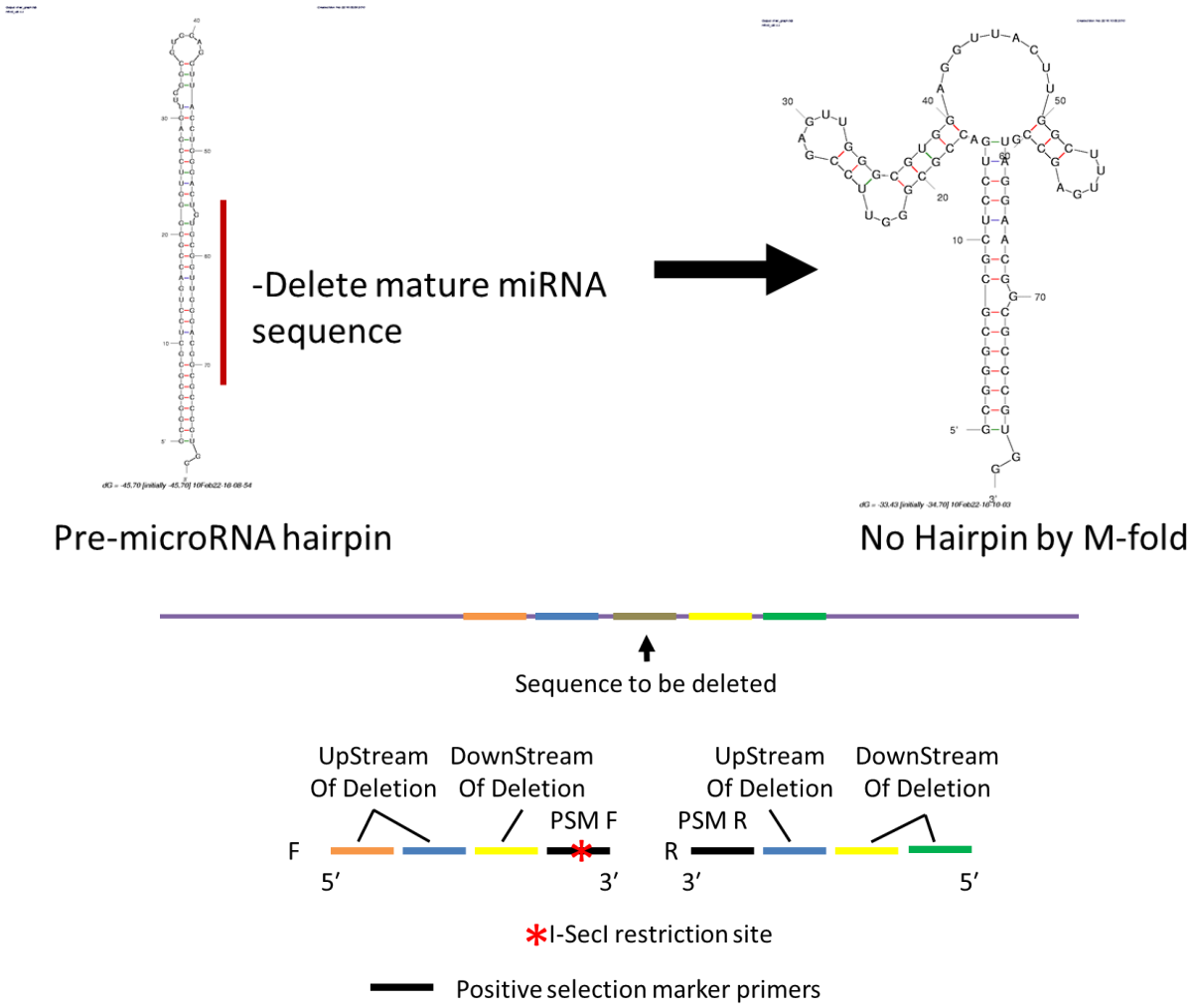


Figure 3-2. Mutational strategy. 20 base pairs were deleted from one arm of each pre-miRNA hairpin. Hairpin disruption was confirmed by M-fold. Primers were designed to amplify kanamycin positive selection marker (PSM). Forward primer contained 40 bp sequence upstream of deletion, 20 bp downstream of deletion, and sequence specific for PSM that include and I-SecI restriction enzyme site. Reverse primer contained sequence specific for PSM, 20 bp sequence upstream of deletion, and 40 bp downstream of deletion. PSM was amplified by PCR to generate the mutation cassette which is used in the next recombination step.

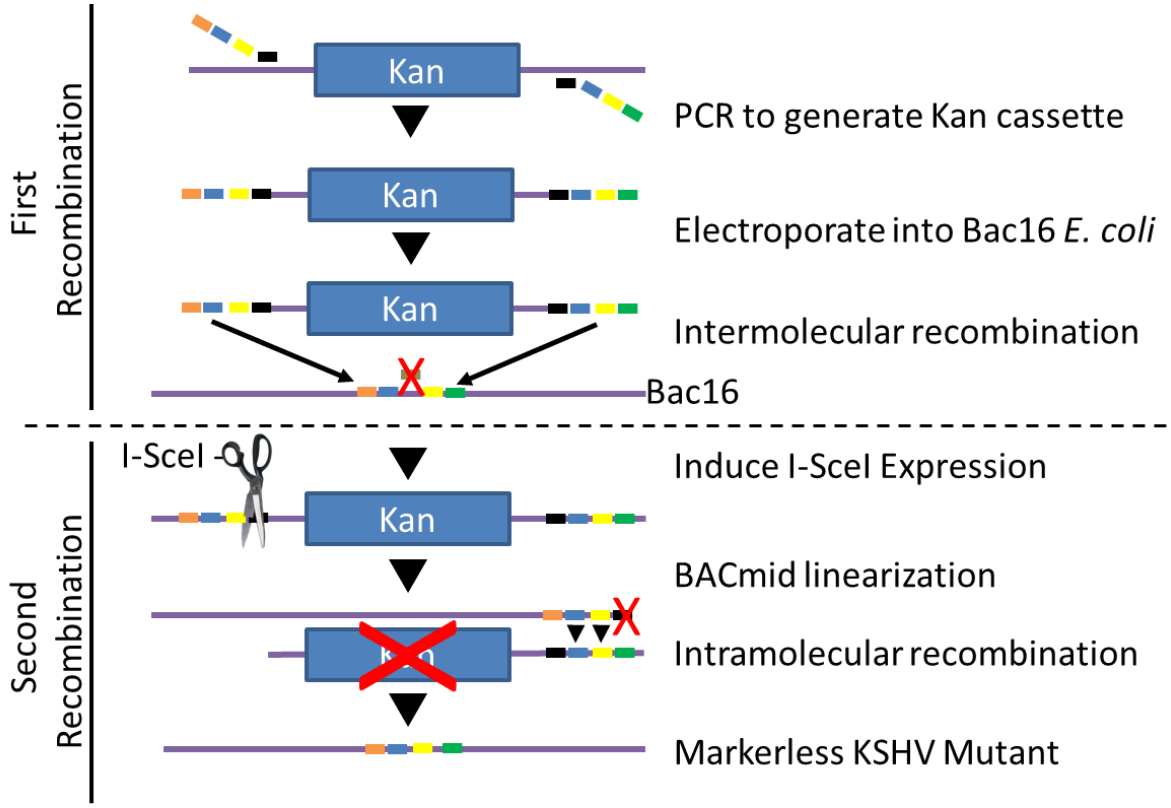


Figure 3-3. Two-step red recombination. Mutation cassette was electroporated into E. coli GS1783 containing BAC16. Red recombinase was induced to facilitate intermolecular recombination between mutation cassette and BAC16. High quality clones were used in the second recombination step. I-SceI expression was induced to linearize bacmid. Red recombinase was induced to facilitate intramolecular recombination which resulted in a markerless KSHV mutant.

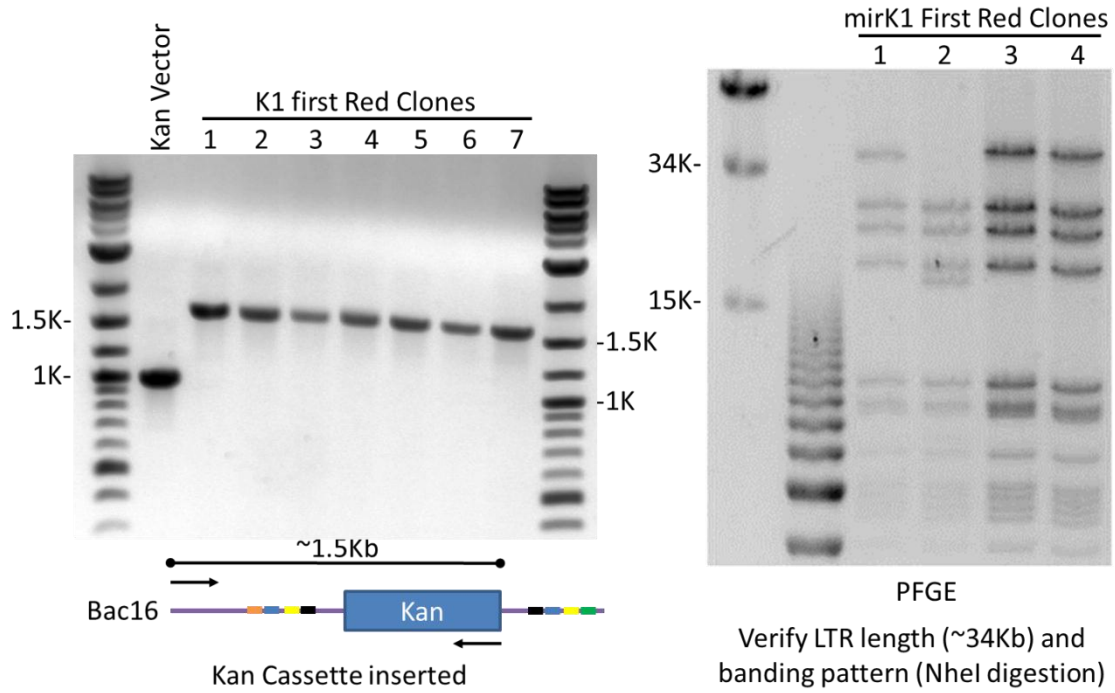


Figure 3-4. Screening of clones. All clones were screened after the first recombination event for the incorporation of the mutation cassette into BAC16 by performing colony PCR using primers containing sequences from both BAC16 and the PSM. DNA was then digested with NheI and terminal repeat integrity was tested by PFGE. Clones 1,3,and 4 are examples of having intact terminal repeats.

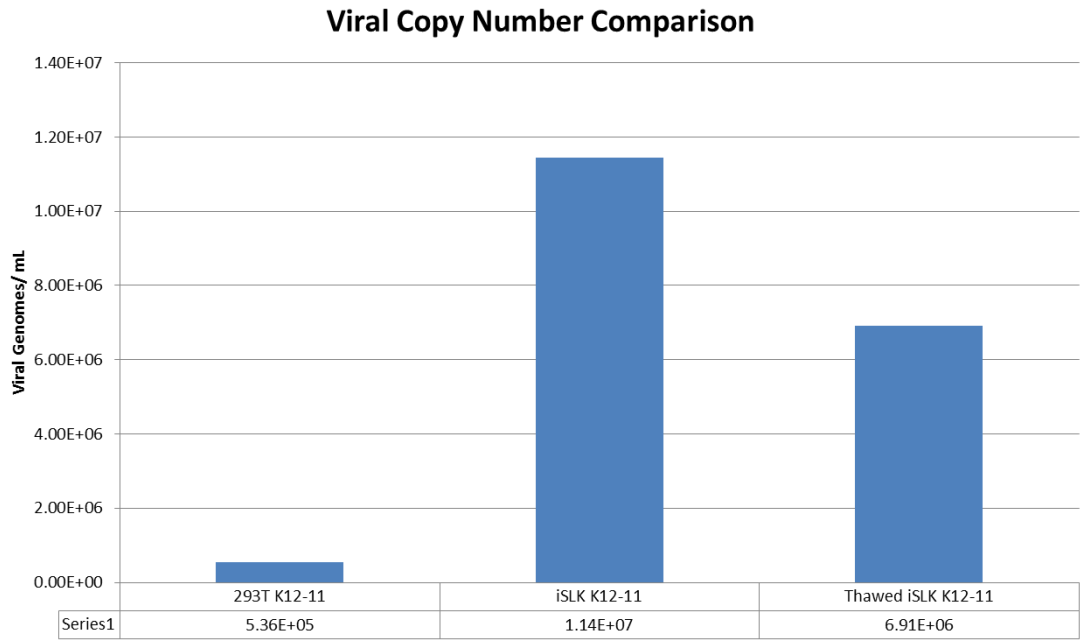


Figure 3-5. Viral copy number comparison. 293T, iSLK and iSLK frozen stock cells were induced to release progeny virus. Cell media harboring progeny virus was collected and filtered (0.45μM) 4 dpi. DNA was collected from the virus stock and was used for qPCR along with a plasmid standard to determine genome copy number per mL.

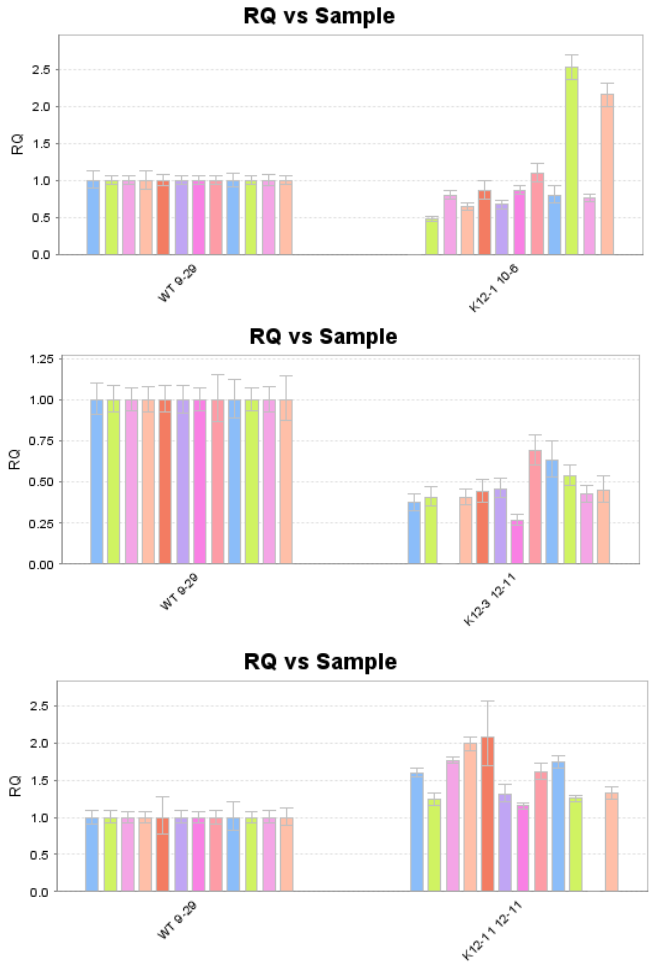


Figure 3-6. miRNA expression in iSLK BAC16ΔmiRNA compared to WT. Total RNA was harvested from iSLK BAC16 cells and TaqMan miRNA-RT and qPCR was performed using primers and probes specific for all 12 KSHV miRNAs along with RNU66 as a loading control. All samples were normalized to WT miRNA expression.

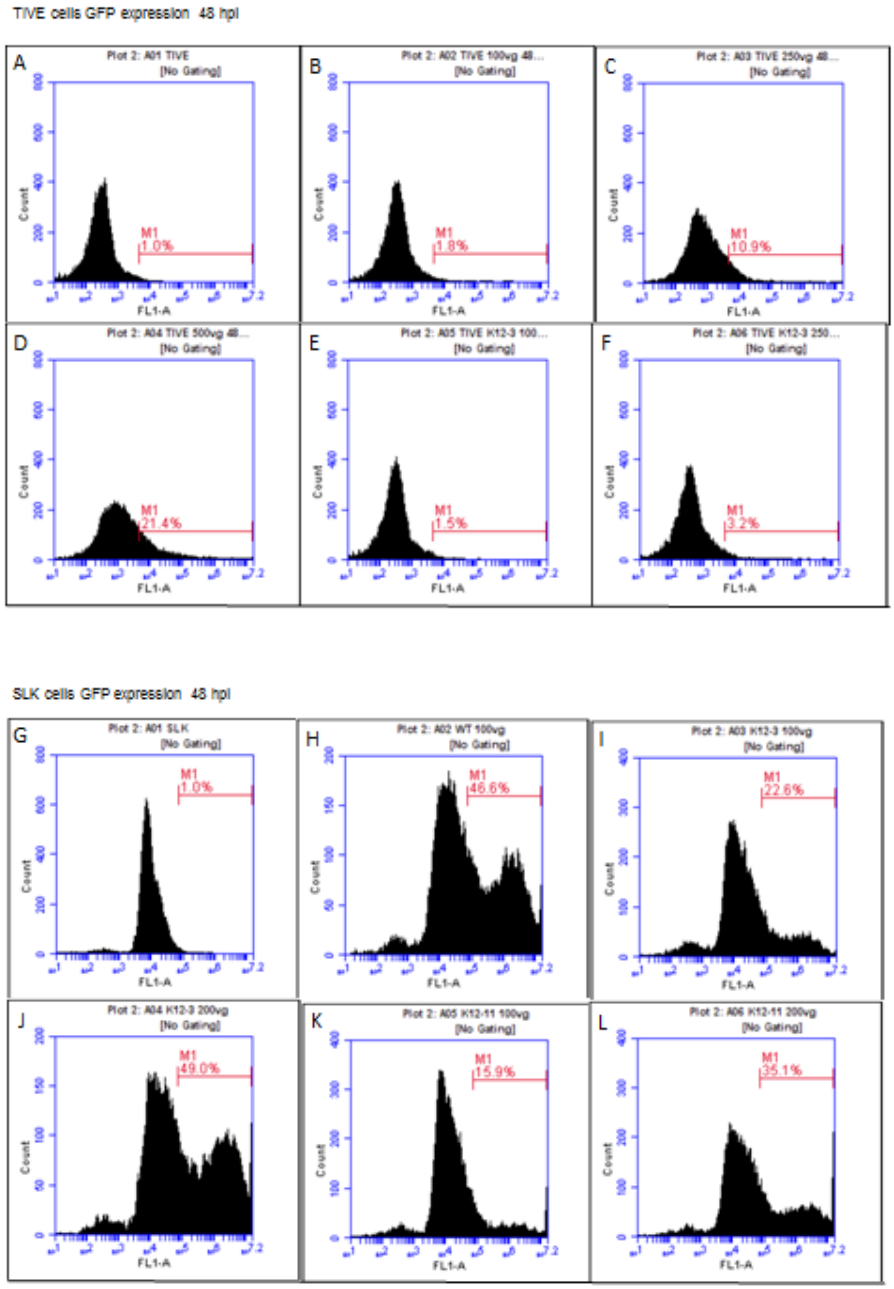


Figure 3-7. TIVE and SLK infection flow cytometry of GFP expression. (A) Non-infected TIVE cells, M1 set at 1%. (B) TIVE WT 100 vg/cell. (C) TIVE WT 250 vg/cell. (D) TIVE WT 500 vg/cell. (E) TIVE ΔK12-3 100 vg/cell. (F) TIVE ΔK12-3 250 vg/cell. (G) Non-infected SLK cells, M1 set at 1%. (H) SLK WT 100 vg/cell. (I) SLK ΔK12-3 100 vg/cell. (J) SLK ΔK12-3 200 vg/cell. (K) SLK ΔK12-11 100 vg/cell. (L) SLK ΔK12-11 200 vg/cell. vg= copies of viral genome. All readings were done 48 hpi.

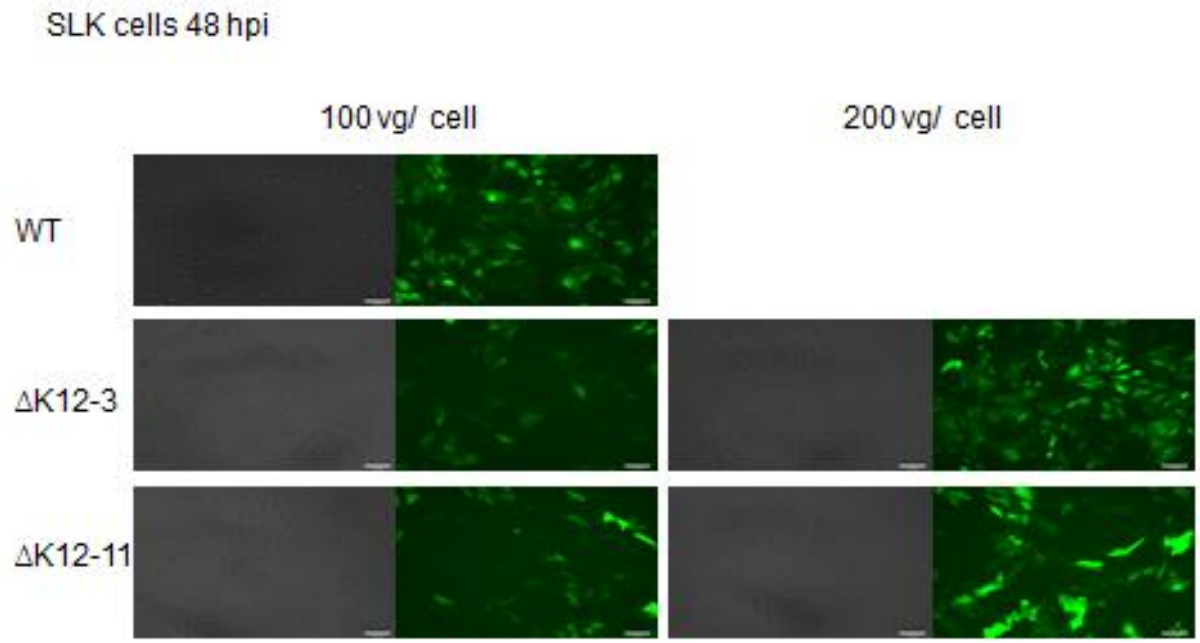


Figure 3-8. GFP expression in SLK cells 48 hpi with BAC16 recombinant viruses. SLK cells were infected with recombinant virus and observed for GFP expression by fluorescence microscopy 48 hours later.

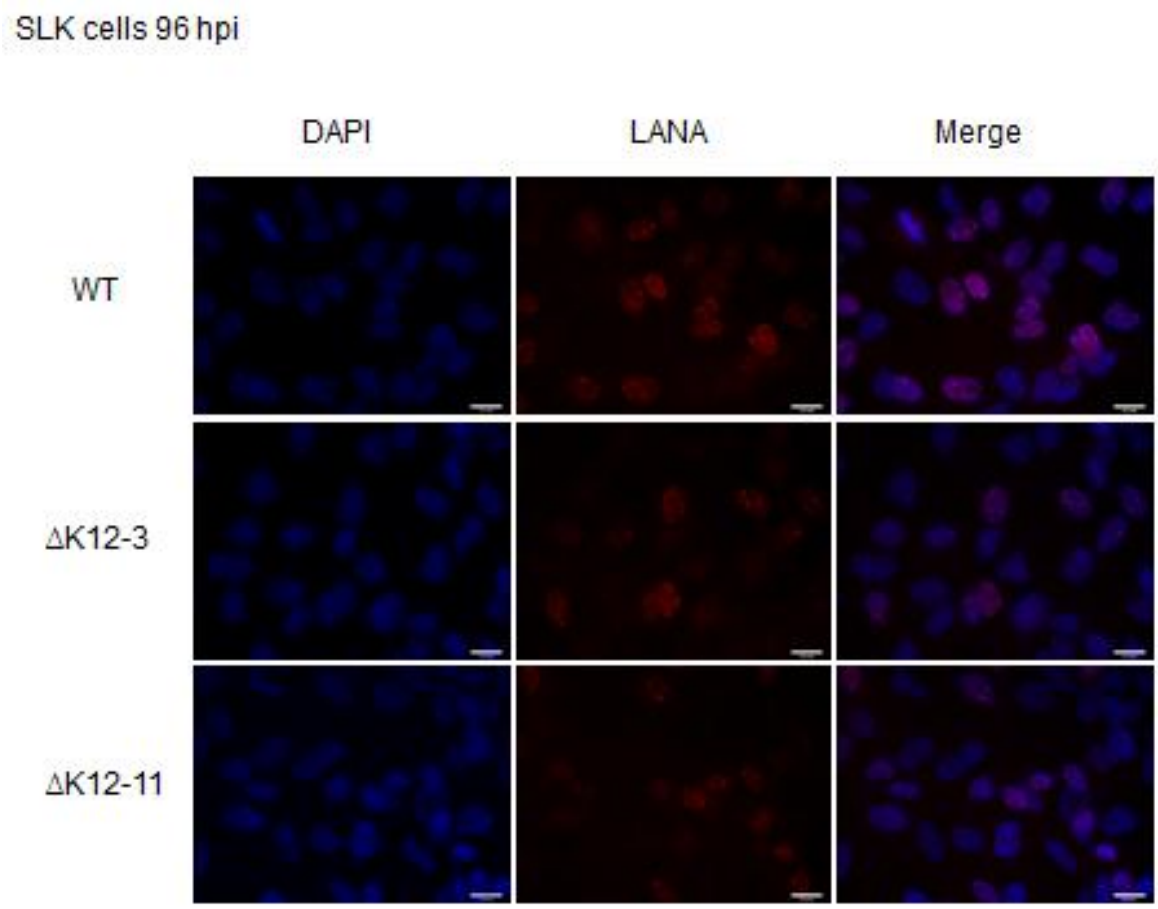


Figure 3-9. IFA for LANA expression in SLK cells 96 hpi. SLK cells were infected with recombinant virus and 96 hpi cells were fixed and stained using a rabbit-anti-LANA polyclonal antibody. Cells were mounted and observed by fluorescence microscopy. DAPI shows nuclear staining.

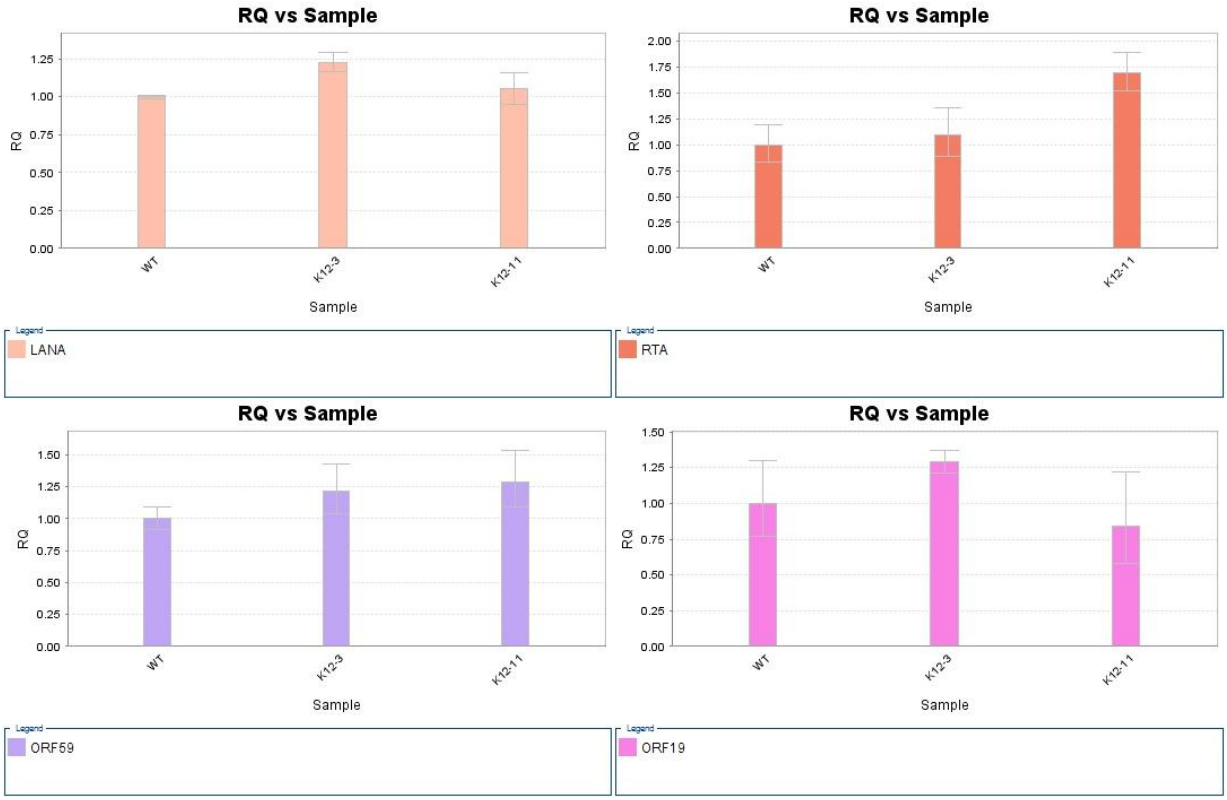


Figure 3-10. Lytic gene expression in BJAB BAC16 infected cells. Total RNA was harvested from BAC16 infected BJAB cells. RT-qPCR was performed using primers specific for LANA, RTA, ORF59, and ORF19. All samples were normalized to cellular β-actin levels. ΔK12-3 and ΔK12-11 samples were compared to WT BAC16 gene expression.

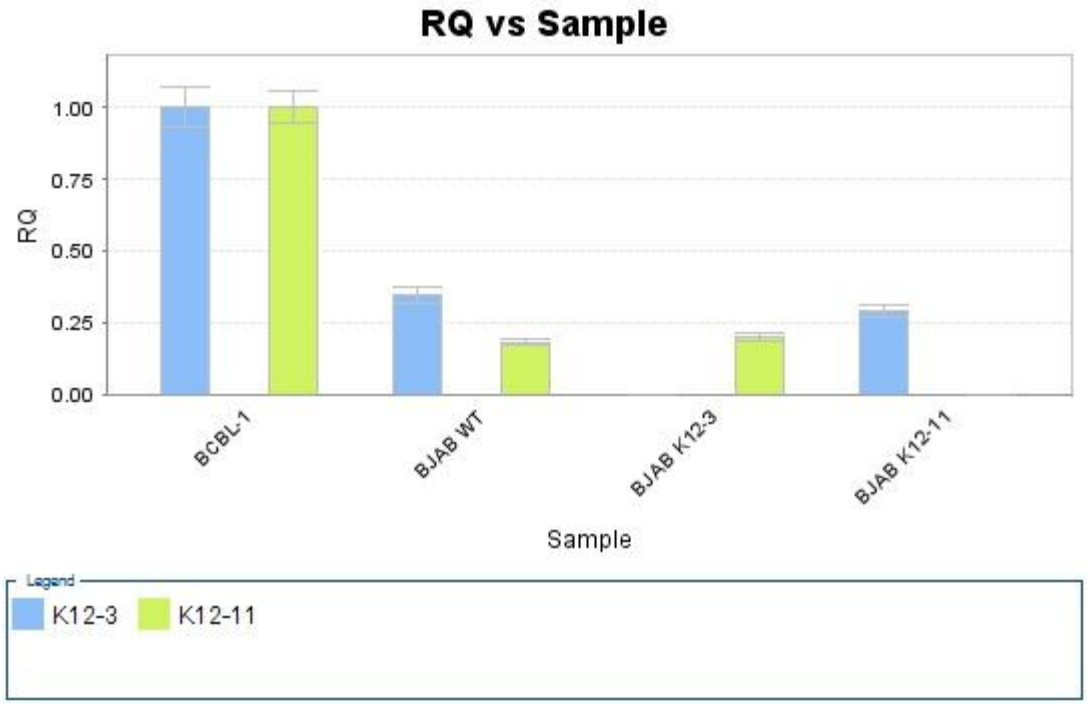


Figure 3-11. BJAB BAC16 vs BCBL-1 miRNA expression. TaqMan miRNA-RT and qPCR was performed using primers and probes specific for miR-K12-3 and miR-K12-11 along with RNU66 as a loading control. All samples were normalized to BCBL-1 miRNA expression.

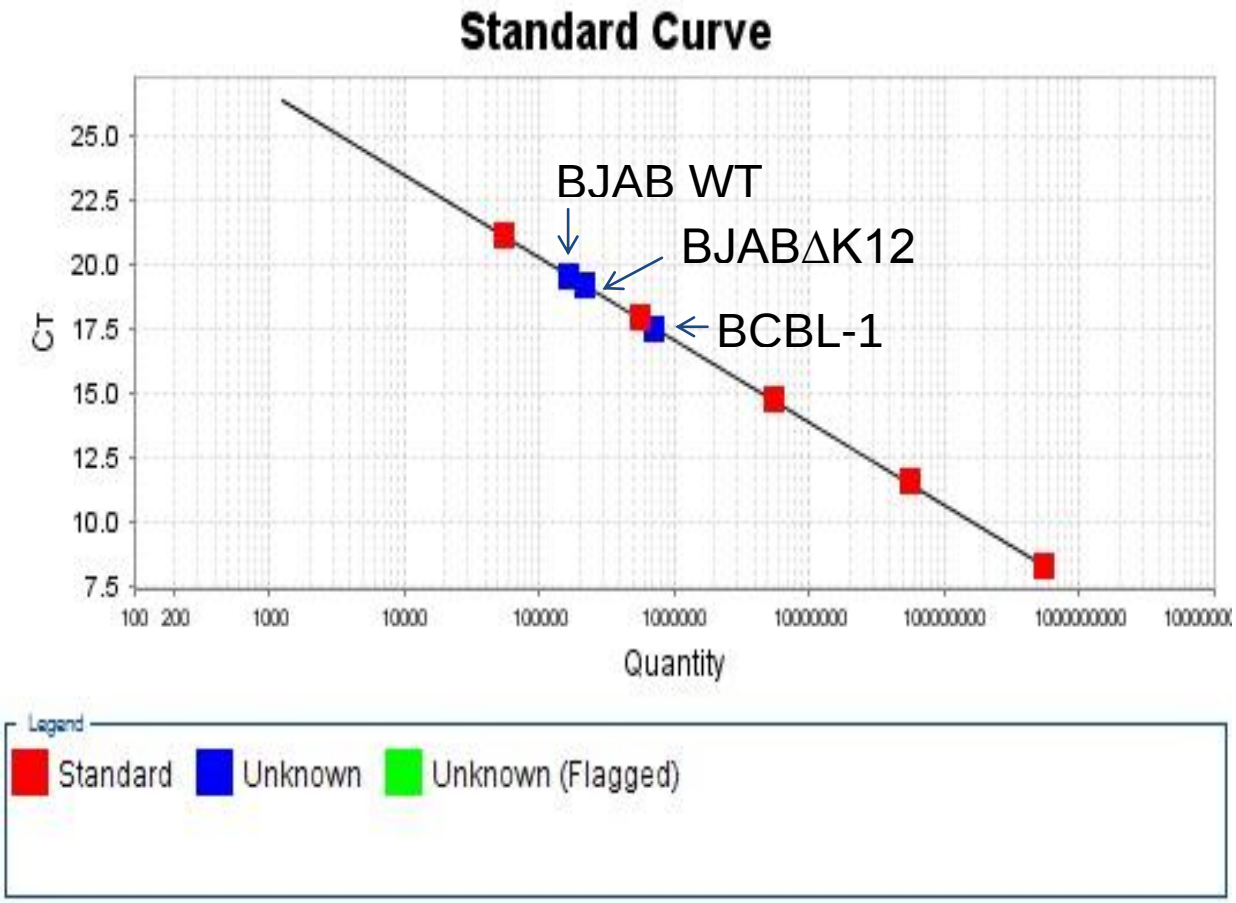


Figure 3-12. BCBL-1 vs BJAB BAC16 intracellular LANA expression. Genomic DNA was collected from BCBL-1, BJAB WT, and BJABΔK12-11 cells. 100 ng was used in qPCR along with a standard plasmid to determine viral genome copy number per cell. BCBL-1= 44 copies/cell, BJAB WT= 10 copies/cell, and BJABΔK12-11= 15 copies/cell.

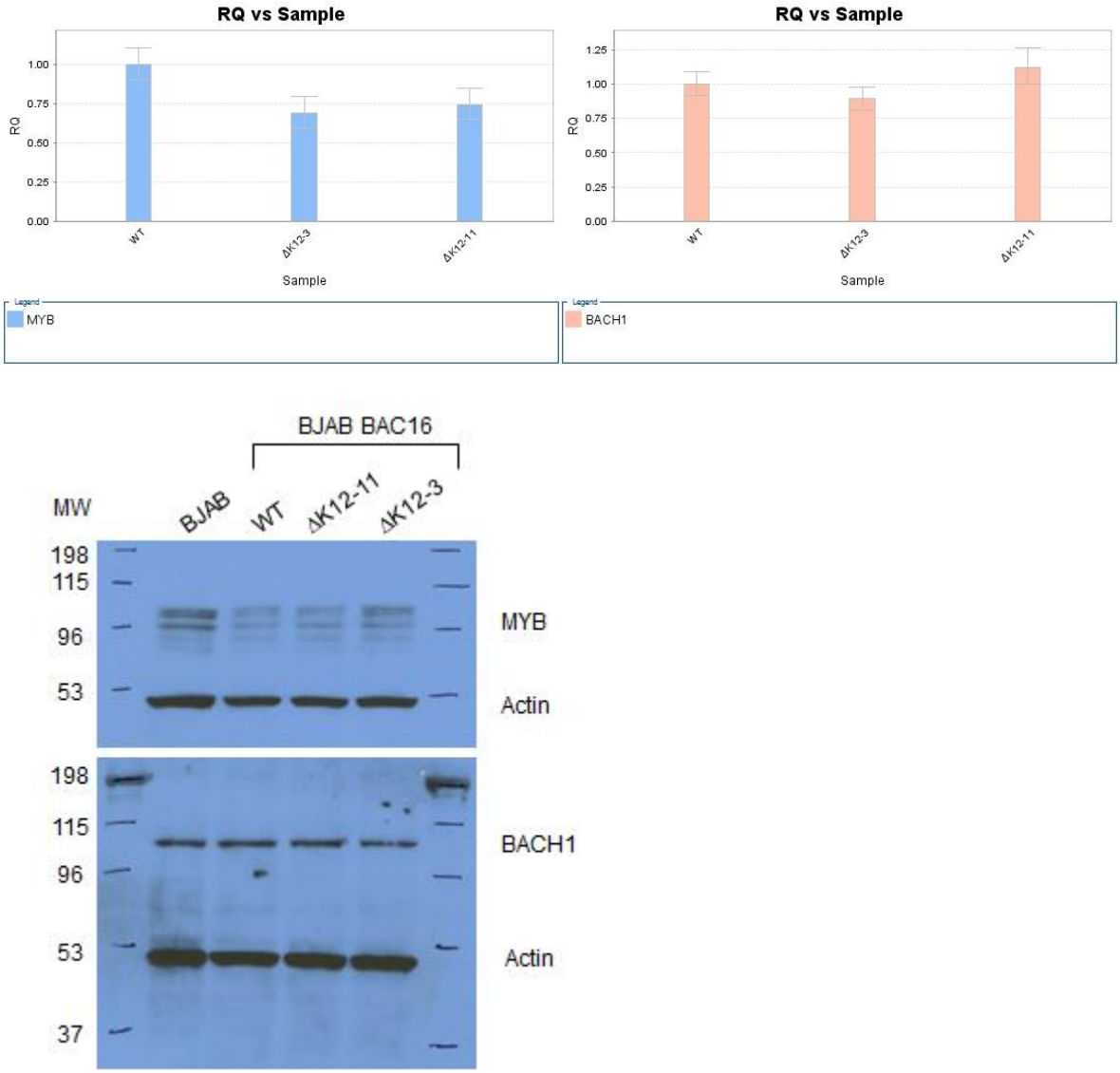


Figure 3-13. BJAB BAC16 MYB and BACH1 expression. Top panel shows RT-qPCR results from BJAB BAC16 cells using primers specific for MYB and BACH1. All samples were normalized to β-actin and compared to WT infected cells. Bottom panel is a western blot probing for MYB, BACH1, and β-actin in non-infected BJAB along with BAC16 infected BJAB cells. Loading is normalized to β-actin levels.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

KSHV-encoded miRNAs are located in the latency-associated region, the area of the genome required for the establishment and maintenance of viral latency. The focus of my research has been to determine which KSHV miRNAs are important for the maintenance of latency and prevention of lytic reactivation. In Chapter 2, I describe miRNA knockdown studies performed in PEL cells and miR-K12-3 and -11 were shown to be important for the prevention of reactivation. Next, MYB, Ets-1, and C/EBPα were determined to be targets of these two miRNAs. These three transcription factors have been previously shown to activate the promoter of RTA, leading to lytic reactivation. Chapter 3 describes the generation and analysis of miRNA deleted recombinant viruses. These viruses were also used in studies in Chapter 2. Individual miRNA knockout viruses are able to infect both endothelial and lymphoid cells and allows for miRNA functional studies in both cell types. MiRNA knockdown studies together with recombinant miRNA knockout viruses show that miR-K12-3 and -11 are important for the maintenance of latency and when deleted from the viral genome, present a more lytic phenotype.

KSHV miR-K12-3 and miR-K12-11 Help to Prevent Reactivation by Targeting Cellular Transcription Factors

To determine which KSHV miRNAs are important for the prevention of lytic reactivation, antagomir knockdown studies were performed in BC-3-G indicator PEL cells. After a panel of individual and combining antagomirs, it was shown by fluorescence microscopy and flow cytometry that knockdown of miR-K12-3 had a 26% increase in GFP, therefore RTA, expression (Figure 2-1). Mir-K12-11 and the combination of -3 and -11 also had an increased effect on lytic reactivation. Since it has

been already shown that miR-K12-9*, -5, and -7 target the 3'UTR of RTA and we did not find any other potential miRNA binding sites, we focused our target analysis on known activators of RTA (Bellare and Ganem, 2009; Lin et al., 2011; Lu et al., 2010b). The 3'UTRs of cellular transcription factors known to activate the promoter of RTA were scanned for potential KSHV miRNA binding sites and several were found to have seed match sites for both miR-K12-3 and -11(Figure 2-2). We decided to focus on MYB, Ets-1 and C/EBPα. Other RTA activating transcription factors such as HIF1α, HMGB1, and EGR-1 also contain KSHV miRNA seed match sites and need to be further investigated (Dalton-Griffin et al., 2009; Dyson et al., 2012; Harrison and Whitehouse, 2008). MYB, Ets-1, and C/EBPα were all shown to be directly targeted by KSHV miRNAs by luciferase reporter assays (Figure 2-2). For Ets-1 and C/EBPα, the effect was not as substantial as seen in MYB. Ets-1 displayed only ~35% decrease in luciferase when both miR-K12-3 and -11 were expressed. However when compared to the no miRNA expressing control, it was statistically significant. Moreover human miR-155, an ortholog of miR-K12-11, was shown to target Ets-1 by 3'UTR luciferase reporter assay and they saw ~45% decrease in luciferase activity when miR-155 mimics were expressed (Zhu et al., 2011). Through 3'UTR mutational analysis, we determined that site 2 was the most important in targeting. Therefore miR-155 targeting of Ets-1 suggest that miR-K12-11 is also capable of regulating Ets-1 expression. C/EBPα did not show any decrease in luciferase expression in the presence of miR-K12-3, however when the entire cluster was expressed I observed ~40% decrease in expression. Besides miR-K12-3, C/EBPα is predicted to be targeted by several other KSHV miRNAs for a total of 14 seed match sites within it's 3'UTR. So although miR-K12-3 may not

strongly target the 3'UTR of C/EBPα, the KSHV miRNA cluster does. Further evidence of C/EBPα being a target of KSHV miRNAs is seen in HITS-CLIP data provided by Irina Haecker in the lab and is shown in figure 2-2c.

In order to determine if miR-K12-3 and -11 target MYB, Ets-1, and C/EBPα in PEL cells, antagomir knockdown studies were performed in BCBL-1 and BC-3 cells. Results show that upon increasing amounts of antagomir, gene expression increased 48 hpt for all three transcription factors by 1.5 to 2 –fold (Figure 2-3). I also attempted to look at protein expression at 48 and 72 hpt, but no visible changes in protein level were detected by western blot (data not shown). There are a few possible reasons for the lack in protein expression changes seen in antagomir transfected PEL cells. First, later time points should be observed for changes in protein expression. 48 and 72 hpt may not be enough time to see significant increases in protein expression. One complication in looking at later time points is that the antagomir affect is diluted out as cells expand, therefore no changes can be seen unless cells are continuously transfected to maintain antagomir concentrations within cells. Recent developments in miRNA inhibition have shown that short complementary sequences of as little as 8 nucleotides are sufficient to inhibit miRNAs (ref). These "tinymers" are complementary to the seed sequence of the miRNA and have locked nucleic acid (LNA) modifications. LNAs contain a biochemical modification where the 2'-oxygen and 4'-carbon atoms of the ribose rings are chemically bridged. This "locked" confirmation confers high thermal stability and resistance to exo- and endonucleases (Chan et al., 2005). An advantage of using tinymers vs antagomirs is that tinymers are able to be taken up by the cell without needing the aid of a transfection reagent. The use of tinymers will allow miRNAs to be inhibited over a long

period of time without the need to re-transfect cells. Stein et al. have shown that by maintaining tinymer concentrations, they see significant protein repression at 7 and 10 days post tinymer addition. By using this strategy, MYB, Ets-1, and C/EBPα expression levels can be observed by western blot 7-10 days post miRNA inhibition in PEL cells. These results may confirm the ~2 fold increase in mRNA expression that we observed by qPCR. Additionally, recent data has indicated that miRNAs primarily act to decrease target mRNA levels. Guo et al. performed ribosome profiling while simultaneously measuring mRNA levels and determined that miRNAs downregulate gene expression mostly through mRNA destabilization with a small effect on translational efficiency. Thus even though western blots were not able to detect changes in protein levels 48 hpt, the results we obtained by qPCR are representative of the miRNAs effect on target mRNA levels (Guo et al., 2010).

Antagomir knockdown of miR-K12-3 and -11 in PEL cells also led to increased viral gene expression and production of progeny virus (Figure 2-4). Results show that upon increasing amount of miR-K12-3 antagomir, viral gene expression increased in a dose dependent response. Also when both miR-K12-3 and -11 antagomirs were transfected at the medium dosage, the greatest increase in viral gene expression was seen. Lastly, progeny virus was measured 6 dpt in antagomir transfected PEL cells. Standard plasmid qPCR revealed that as miR-K12-3 or-11 is knocked down, progeny virus released into the media increases. These data suggest that both miR-K12-3 and -11 are important for the maintenance of viral latency in PEL cells.

KSHV miR-K12-3 and -11 Deleted Recombinant Viruses have a More Lytic Phenotype in Endothelial Cells

In order to study KSHV miRNA function in the context of the viral genome, individual miRNA deleted recombinant viruses were generated using KSHV BAC16 and producer cell lines were established in iSLK endothelial cells (generation discussed in Chapter 3). Upon analysis of iSLK BAC16 cells, RT-qPCR results show that iSLK BAC16ΔmiR-K12-11 cells have increased lytic gene expression. Further investigation through sub-optimal induction with NaB showed that both miR-K12-3 and -11 deleted viruses reactivated better than WT BAC16 infected iSLK cells (Figure 2-5). These data are in agreement with the results in PEL cells treated with miRNA antagomirs, confirming that miR-K12-3 and -11 expression are important for the maintenance of viral latency.

Further analysis of MYB, Ets-1, and C/EBPα expression in iSLK BAC16ΔmiR-K12-3 and -11 cells did not show significant changes in gene or protein expression (data not shown). However MYB is a B-cell transcription factor and is therefore not expressed in endothelial cells, and Ets-1 and C/EBPα are expressed at higher levels in iSLK cells than in PEL cells. Furthermore, PEL cells express KSHV miRNAs at a much higher level than BAC16 infected iSLK cells. The combination of high mRNA transcript levels and low miRNA expression levels in iSLK BAC16 WT infected cells may be the cause for not seeing a difference in expression when compared to ΔmiRNA infected cells. Recent studies by Mukerji and colleges have determined that miRNAs can generate thresholds in target gene expression. Results using single cell analysis of miRNA mediated protein repression suggest that if the target pool of a miRNA is below saturation, then all the targets will be repressed to the same degree regardless of

expression level. However if the target pool size grows, then it is possible to saturate the pool of miRNAs, therefore protein repression is no longer seen (Mukherji et al., 2011). I believe this to be the case when comparing miRNA targeting of MYB, Ets-1, and C/EBPα in PEL and iSLK BAC16 cells. Due to miRNA expression being in lower in iSLK BAC16 cells, they may be saturated with the highly abundant Ets-1 and C/EBPα transcripts, hence no changes in transcript or protein level can be detected. These data suggest there are other targets of miR-K12-3 and -11 in endothelial cells that are important for prevention of lytic reactivation and this needs to be further investigated, which is described below in future directions.

Generation of Endothelial and Lymphoid Cells Harboring miRNA Deleted Recombinant Virus

Bacmids are a very useful tool to study specific gene function in herpesviruses. Previously, KSHV BAC36 was widely used for genome studies, including a miRNA cluster knockout to investigate the role of KSHV miRNAs. Lei et al. deleted the ~2.9 kb region within the latency associated region containing 10 of the 12 KSHV miRNA hairpins. The miRNA cluster deleted virus was then reconstituted in 293 cells and were shown to have higher RTA expression than WT infected 293 cells. Next, NFκB activity was observed to be lower in the miRNA cluster knockout virus infected cells when compared to WT KSHV infected cells. It was determined that miR-K12-1 targets IκBα resulting in activation of NFκB activity and inhibition of viral lytic reactivation (Lei et al., 2010). These data do suggest a role for miR-K12-1 in the maintenance of latency, however these experiments were done in cells that are not naturally infected by the virus and further investigation of miR-K12-1 targeting IκBα in PEL and endothelial cells is needed. Furthermore, BAC36 was recently shown to contain duplications and

deletions within the viral genome (Yakushko et al., 2010). Therefore we set out to use a new bacmid system, BAC16, to investigate individual miRNAs by generating 12 KSHV miRNA deleted recombinant viruses.

As explained in Chapter 3, short sequences were deleted from each mature miRNA sequence, disrupting pre-miRNA formation and drosha recognition. This was performed in a two-step red recombination process that resulted in a markerless mutant. I was able to establish reconstitution of bacmid DNA into mammalian cells and developed the protocol for the generation of iSLK producer cell lines for the first four bacmids made in the core laboratory: WT, ΔK12-1, ΔK12-3, and ΔK12-11. BAC16 transfected 293T cells were co-cultured with iSLK cells then induced to release recombinant virions. BAC16 infected iSLK cells were selected, expanded, and analyzed for GFP expression by fluorescence microscopy, LANA expression by IFA, miRNA expression by TaqMan qPCR, and viral gene expression by RT-qPCR. As discussed in Chapter 2, miR-K12-3 and miR-K12-11 expression are important for the prevention of lytic reactivation, therefore I focused on these two recombinant viruses. BJAB BAC16 cells were established using co-culturing techniques with iSLK producer cells. Viral gene expression, miRNA expression, and viral genome copy number per cell were confirmed by qPCR in BAC16 infected BJAB cells. Results showed that although BJAB cells are of lymphoid origin like PEL cells, they harbor about ¼ of viral genomes when compared to PEL cells (Figure 3-12). BJAB cells harboring less copies of the viral genome also results in lower miRNA expression when compared to PEL cells. These results may explain why no significant increase in MYB or BACH1 expression was seen in BJAB BAC16 WT cells when compared to BJABΔmiR-K12-11

cells. KSHV miRNA expression is not high enough in BJAB BAC16 cells to overcome MYB transcript levels; however this does not mean miR-K12-11 is not still binding and targeting MYB's 3'UTR in BJAB BAC16 cells. In summary, BAC16 has served as a tool for the generation of individual miRNA deletion mutant viruses and through co-culturing, we have established endothelial and lymphoid cells harboring recombinant KSHV virions. This has also for the first time generated the ability to study KSHV infection in lymphoid cells while having an isogeneic control.

Future Directions using KSHV BAC16ΔmiRNA Recombinant Viruses

iSLK BAC16ΔmiR-K12-3 and iSLK BAC16ΔmiR-K12-11 cells displayed a more lytic phenotype than WT infected cells, however I did not see an increase in MYB, Ets-1, and C/EBPα expression in the cells. This may be due to the lower miRNA expression in iSLK cells when compared to PEL cells. Nevertheless, these cells infected with miRNA knockout virus still reactivate better than cells infected with WT KSHV suggesting that there may be other targets of miR-K12-3 and -11 that are important for the maintenance of latency. Also because iSLK cells are of endothelial origin and PEL cells are of lymphoid origin, there may be different cellular factors contributing to activation of the RTA promoter. In order to investigate this further, several different experiments can be performed. The first approach can be the comparison of gene expression profiling among uninfected, WT infected, and miRNA deleted virus infected cells. Our lab has conducted these types of analysis in the past and has found several KSHV targets using this approach which were later confirmed by 3'UTR luciferase reporter assays (Samols, 2007; Skalsky et al., 2007). With this approach not only will we see gene expression changes that occur upon infection with KSHV in endothelial cells, but we will also see how the deletion of miR-K12-3 and -11 affects gene expression and how that

compares back to uninfected cells. A second approach to determining viral miRNA targets in recombinant KSHV infected endothelial cells is to perform HITS-CLIP in uninfected, WT infected, and miRNA deleted KSHV infected iSLK cells. This technique was recently established in the lab by Irina Haecker and is discussed in Chapter 2 (Figure 2-2). Basically, miRNA/mRNA complexes are isolated and sequenced to determine which mRNAs are actually being targeted in a population of cells. Using this method, we are able to determine how often and where a message is targeted by a specific miRNA. This will allow us to determine specific targets of KSHV miRNAs in endothelial cells; also this may give insight as to whether MYB, Ets-1 and C/EBPα are targeted in iSLK cells by miR-K12-3 and -11, yet not at high capacity.

PEL cells have been the model for studying latent KSHV infection in B-cells since their establishment in 1996 (Cesarman et al., 1995b; Komanduri et al., 1996; Renne et al., 1996b). Unlike EBV, KSHV is not able to stably infect primary B-cells, nor is it able to infect cultured lymphoid cells efficiently (Bechtel et al., 2003; Blackbourn et al., 2000; Renne et al., 1998). Because of this, there has never been an isogenic non-infected control for KSHV infection in B-cells. However a recent report by Myoung and Ganem has introduced co-culturing as a method for infected BJAB cells (Myoung and Ganem). Using this technique we now have uninfected and BAC16 infected BJAB cells. Although BJAB BAC16 cells harbor less copies of the KSHV genome than PEL cells, and therefore express miRNAs at a lower level, gene expression profiling and HITS-CLIP can still be performed in order to determine KSHV miRNA targets. The establishment of BJAB BAC16 cells provides for the first time an isogeneic control for

studying KSHV infection in B-cells. It will be interesting to perform HITS-CLIP and compare profiling in BCBL-1, other PEL cells, and BJAB BAC16 infected cells.

As discussed in Chapter 2, antagomir knockdown studies were performed in BC-3-G indicator cells. Results showed that miR-K12-3 knockdown had the greatest effect on GFP, representing RTA expression. It would be interesting to confirm this study in iSLK BAC16ΔmiRNA cells. I have already looked at lytic gene expression and reactivation in ΔmiR-K12-3 and ΔmiR-K12-11 infected cells, however I believe the rest of the individual miRNA deleted virus infected cells should be tested. As mentioned above, miR-K12-1 was shown to target IκBα which leads to reactivation. I did not see a significant increase in GFP expression in BC-3-G cells when miR-K12-1 was knocked down, however miR-K12-1 is highly expressed in PEL cells and therefore a greater effect may be seen when using BAC16ΔmiR-K12-1 infected cells. Also other interesting miRNAs to observe lytic reactivation in are those that are known to target the 3'UTR of RTA; miR-K12-9*, -7, and -5 (Bellare and Ganem, 2009; Lin et al., 2011; Lu et al., 2010b).

Lastly, BAC16 can be used to make new mutations in the miRNA encoding region to further investigate the role of KSHV miRNAs. A double knockout of miR-K12-3 and miR-K12-11 would be noteworthy to observe in both iSLK and BJAB cells for reactivation phenotypes. Knockdown studies in PEL cells show that when antagomirs for both miRNAs were transfected into cells, the highest increase in lytic gene expression was observed. There is current work in progress by the core facility to generate a KSHV complete miRNA knockout virus. Recent studies by Lei et al. has used BAC36 containing a 2.9 kb deletion of the miRNA cluster region, which includes 10 of the 12 KSHV miRNAs (Lei et al., 2010). However, such a large deletion may have

an effect on other viral gene expression. The mutational strategy used to make individual miRNA deletions can be used to make an entire KSHV miRNA knockout virus with minimal deletions to the viral genome. Each miRNA hairpin can be disrupted by deleting very few base pairs, and miRNAs located in the coding region of Kaposin can be mutated to disrupt pre-miRNA formation but keep protein coding intact. This recombinant virus would be the first that would allow studies to be performed on all 12 KSHV encoded miRNAs.

Future Prospective on KSHV miRNAs

KSHV-encoded miRNAs have been shown to play an important role in the biology and pathogenesis of KSHV by targeting genes involved in promoting proliferation, preventing apoptosis, evading the immune system, promoting oncogenesis, and contributing to latent/ lytic control (Table 1-2). The work described in this thesis provides additional insight on the role of two specific miRNAs, miR-K12-3 and -11, in helping to maintain viral latency through targeting cellular transcription factors that are known to activate the RTA promoter. In addition I have also had the opportunity to collaborate on several projects involved in determining KSHV miRNA targets and functions other than maintaining latency. I worked with previous lab member Rebecca Skalsky to determine which of the four predicted miR-K12-11/ miR-155 predicted binding sites were important for targeting of BACH1. Through 3'UTR mutational analysis, we determined that site 2 was the most important in targeting (Skalsky et al., 2007). Next I collaborated with Chris Parsons at MUSC and helped with target analysis of KSHV miRNAs. Using a prediction algorithm that was made in collaboration with Alberto Riva in bioinformatics here at UF, I scanned the 3'UTRs of a set of interesting genes for KSHV miRNAs. This work resulted in two publications with the Parsons lab,

one showing that miR-K12-3 and -7 target C/EBPβ and the other demonstrates that xCT is upregulated in KSHV infected cells as a result of K12-11 targeting BACH1 (Qin et al., 2010a; Qin et al., 2010b). Lastly, I collaborated with Amy Hanson from the Boshoff lab at UCL to help determine that MAF is targeted by KSHV miRNAs. I performed antagomir transfections in our 293 cells stably expression miR-K12-6 or miR-K12-11 and collected total RNA and cell lystates used to confirm MAF derepression upon miRNA knockdown. These data helped to confirm that MAF is indeed a target of KSHV miRNAs (Hansen et al., 2010).

To date, the majority of viral miRNAs identified are encoded in the herpesvirus family. Although there is no sequence homology found between miRNAs in herpesviruses, there are common themes between them. One is the targeting of the same gene in different herpesviruses by miRNAs that have no sequence homology: HCMV, EBV, and KSHV all encode a miRNA that targets MICB, which is important for NK cell recognition and therefore aids in immune evasion (Nachmani et al., 2009; Stern-Ginossar et al., 2007). Another theme is that both KSHV and MDV encode a miR-155 ortholog, KSHV-miR-K12-11 and MDV-miR-M4, which share a common set of targets as the oncomir miR-155 (Gottwein et al., 2007; Skalsky et al., 2007; Zhao et al., 2011). Interestingly, miR-155 is downregulated in KSHV-infected PEL cells and miR-K12-11 seems to take over miR-155 functions (Boss et al., 2011). In contrast however, in EBV-transformed lymphoblastoid cells, miR-155 is upregulated and essential for the survival and growth (Linnstaedt et al., 2010). It is possible that these miRNAs have co-evolved the same seed sequence to target host key pathways. However, it could be that similar to other viral genes like vFLIP and vCyclin, these viral miRNAs have been pirated from

the host genome as yet another means of ensuring viral persistence and evasion of host defenses. Further investigation of the overall function of miRNAs in the context of the viral genome is still needed and the generation of a complete miRNA knockout virus may shed further light on their essential roles in herpesviral biology. The targeting of the same host pathways by seemingly unrelated miRNAs in different herpesviruses strongly indicates that these miRNAs, and possibly viral miRNAs in general, are involved in key regulatory functions for viral life cycle, which makes them interesting and important targets for therapeutics development.

Aberrant miRNA expression has been demonstrated for several diseases and cancers (Iorio and Croce, 2012). Recently, miRNA inhibitors have been used for treatment of such diseases *in vivo*. MiR-132 inhibition was shown to prevent angiogenesis in an orthotopic mouse model of ovarian and breast carcinoma (Anand et al., 2010). In addition, miR-21 inhibition led to regression of malignant pre-B-lymphoid tumors *in vivo* (Medina et al., 2010). Hepatitis C Virus (HCV) is known to hijack miR-122 and is required for virus replication. miR-122 binds two sites upstream of an HCV IRES and drives synthesis of the viral polyprotein, therefore miR-122 is a candidate for drug targeting in HCV infected individuals with hepatocellular carcinoma (Jopling et al., 2005). Santaris Pharma has developed a specific inhibitor of miR-122 called SPC3649 which is a locked nucleic acid antisense molecule 15 bases in length. A phase I clinical trial consisted of SPC3649 administered intravenously to chimpanzees and demonstrated reduced serum concentrations of HCV. The study showed that effective doses (5mg/kg) of antisense molecule can be delivered to liver for 12 weeks without causing major toxic effects (Hildebrandt-Eriksen et al., 2012). However due to miR-122

being a host cellular miRNA, inhibition may disrupt normal physiology and a cause metabolic change, which needs to be further investigated. KSHV miRNAs are a prime candidate for antisense targeted therapy because sequences are specific to the virus and therefore should not disrupt any cellular physiology or have pleotropic effects. Specific miRNAs, such as miR-K12-3 and -11 can be targeted by LNAs to inhibit function, therefore increase lytic reactivation in infected cells. This would sensitize KSHV infected tumor cells to anti-viral drugs such as gancyclovir which would need to be administered together with miRNA inhibitors. Furthermore, miRNA inhibitors can be delivered ectopically for KS lesions of the skin which would be more cost effective and easier to administer than intravenous injections. Another KSHV miRNA that can be targeted through antisense therapy is miR-K12-7, which is known to target MICB, a natural killer cell ligand (Nachmani et al., 2009). This would assist in immune recognition of an infected cell. In summary, KSHV miRNAs may serve as a novel therapeutic target, and together with currently available anti-viral drugs, provide and alternative treatment of KSHV associated malignancies.

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BIOGRAPHICAL SKETCH

Karlie Belle Plaisance-Bonstaff was born in New Orleans, LA and raised in Laffite, LA where she attended Fisher High School and graduated in 2001. She then moved to Baton Rouge, LA and attended Louisiana State University where she received her B.S. in Biological Sciences with a minor in Chemistry in 2006. Upon graduation, she continued her education at the University of Florida as a Ph.D. student in the Interdisciplinary Program in Biomedical Sciences where she joined the laboratory of Dr. Rolf Renne. The focus of her project was studying the role of Kaposi's sarcoma-associated herpesvirus miRNAs. She received her Ph.D. in Medical Sciences with a concentration in Genetics from the University of Florida in the summer of 2012. Upon completion of her Ph.D. program, Karlie will explore post-doctoral opportunities in the gamma-herpesvirus field. She has been married to Christopher Bonstaff, a registered nurse, for nearly 3 years and they welcomed their first child Abigail on July 2nd, 2012.