FUNCTIONAL ANALYSIS OF MIR-K12-11, A KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS-ENCODED MIRNA, AND ITS ROLE IN VIRAL PATHOGENESIS

By

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To my family, especially my parents, Harold and Sonja Boss, and the loves of my life, Natasha Moningka and Mochi

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Kaposi's sarcoma-associated herpesvirus (KSHV), a B cell-tropic virus associated with Kaposi's sarcoma (KS) and the B cell lymphomas, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), encodes 12 miRNA genes that are highly expressed in these tumor cells. MicroRNAs are small non-coding RNA molecules that function as post-transcriptional regulators of gene expression. One viral miRNA, miR-K12-11, shares 100% seed sequence homology with hsa-miR-155, an oncogenic human miRNA that functions as a key regulator of hematopoiesis and B cell differentiation. *In vitro* studies have shown that both miRNAs can regulate a common set of cellular target genes suggesting that miR-K12-11 may mimic miR-155 function. To comparatively study miR-K12-11 and miR-155 function *in vivo*, we used a foamy virus vector to express the miRNAs in human hematopoietic progenitors and performed immune reconstitutions in NOD/LtSz-scid IL2Rγ^{null} mice. We found that ectopic expression of miR-K12-11 or miR-155 leads to a significant expansion of the CD19+ B cell population in the spleen. Subsequent qPCR analyses of these splenic B cells revealed that C/EBPβ, a transcriptional regulator of IL-6 that is linked to B cell lymphoproliferative disorders, is downregulated when either miR-K12-11 or miR-155 is

ectopically expressed. In addition, inhibition of miR-K12-11 function, using antagomirs in KSHV infected human primary effusion lymphoma (PEL) B cells, resulted in derepression of C/EBPβ transcript levels.

Both PEL and MCD resemble B cells that are frozen at a plasmablast stage of differentiation. While the aeitology of these B cell malignancies is unclear, we propose that miR-K12-11 mimics miR-155 function to promote plasmablast differentiation and potentially block plasma cell differentiation. To study the role of miR-K12-11 in B cell differentiation, we utilized an *in vitro* model of human plasma cell differentiation and searched for B cell regulatory genes that can be regulated by both miRNAs. In our model system, transfection of synthetic miR-K12-11 or miR-155 mimics into purified human B cells did not induce plasmablast differentiation or inhibit plasma cell differentiation. However, we identified the B cell genes MYB, IgJ, and SHIP1 as valid targets of both miRNAs, whose regulation may influence B cell maturation and function during *de novo* KSHV infection. Together, these studies indicate that miR-K12-11 phenocopies miR-155 function in human hematopoiesis by mimicking miR-155 regulation of B cell targets, and provides important insights into the role of this KSHV miRNA in B cell pathogenesis.

CHAPTER 1
INTRODUCTION

Discovery and Classification of Kaposi's Sarcoma-Associated Herpesvirus

Kaposi's sarcoma-associated herpesvirus (KSHV) was first discovered as the etiological agent of the rare human vascular tumor Kaposi's sarcoma (KS) (Chang et al., 1994). KS was originally described in 1872 by the preeminent Hungarian dermatologist, Moritz Kaposi, and called "an idiopathic multiple pigmented sarcoma of the skin" (Kaposi 1872). Kaposi characterized KS as brown-red or blue-red nodules that develop first on the skin of hands and feet, and later spread to other external and internal areas of the body, leading to skin deformation. Because Kaposi failed to notice any spread in the lymph vessels he believed that the cause of KS was a pre-existing systemic disease (Sanders, 1997), an observation later proven inaccurate (Chang et al., 1994).

While the classical form of KS, described by Kaposi, is a rare disease, KS was later found to be endemic in areas of sub-Saharan Africa and is now separated into 4 clinical subtypes: classical KS; endemic; iatrogenic, associated with organ transplantation and immunosuppressive therapy; and epidemic or AIDS-related (Antman and Chang, 2000). Each subtype of KS shares a similar histopathology, with lesions containing a mixture of spindle cells, representing the main proliferating cell type, and inflammatory cells (lymphocytes and monocytes) (Ganem, 2006).

While the histopathology of all subtypes are similar the target population and severity of disease differs. Classical KS is normally displayed as an indolent skin tumor, mainly affecting elderly men of Eastern European, Mediterranean, Italian, or Jewish descent. Endemic KS, in sub-Saharan Africa, not only affects the elderly as an indolent disease, but is frequently seen in young children as an aggressive lymphadenopathic

tumor with high mortality rates (Ziegler and Katongole-Mbidde, 1996). Iatrogenic KS affects mostly men from the same ethnic groups which are found in classical KS, but is much more aggressive than the classical form, spreading into the lymph nodes, mucosal surfaces, and internal organs (Antman and Chang, 2000). In the United States, AIDS-related KS mainly affects homosexual men infected with HIV and its progression can vary from indolent to aggressive (Sanders et al., 2004).

After the description of KS by Kaposi, the causative agent of KS was widely debated as being of infectious origin (Sanders, 1997). Inoculation studies with KS tissues in 1910 induced tumors in mice, and in 1938, similar studies in a human patient resulted in a bright red plaque similar to early KS; however these results were controversial (Sanders, 1997). When endemic KS was discovered in Africa (1940-1960), it was further suggested, based on its geographical restriction, that the disease was caused by an infectious agent (Mesri et al., 2010). In 1981, a dramatic increase of KS tumors, observed in HIV positive men from New York and Los Angeles, brought awareness to the AIDS epidemic, leading many scientist to believe that KS was caused by HIV (CDC, 1981; Gottlieb et al., 1981). Later evidence showed that the incidence of KS was highest in homosexual and bisexual men who contracted HIV through sexual contact versus other means (intravenous drug use or blood transfusion), indicating that a virus unrelated to HIV could be the agent (Beral et al., 1990). Then in 1994, biopsies of KS taken from AIDS patients, were studied by representational difference analysis (RDA), a technique that compares DNA sequences from diseased and normal tissues, leading to the discovery of unique genetic material of viral origin (Chang et al., 1994). Closer analysis showed that the unique sequences were similar to members of

Gammaherpesvirinae: the human Epstein-Barr virus (EBV) and the primate virus herpesvirus saimiri (HVS) (Chang et al., 1994). Phylogenetic analyses of KSHV open reading frames (ORFs) placed it into the genus rhadinovirus, along with HVS, making it the first human pathogen of this genus (Moore et al., 1996). Since the implementation of highly reactive antiretroviral therapy (HAART) for the treatment of AIDS, the rate of KSHV induced disease has dropped in most of the Western World. However, in underdeveloped countries, the incidence of KS and KSHV associated lymphomas still remains a serious health risk (Mbulaiteye and Engels, 2006; Mosam et al., 2009).

KSHV is a Lymphotropic Virus

A hallmark of gammaherpesviruses is their ability to infect lymphocytes (B cells or T cells). The human gammaherpesviruses, EBV and KSHV, are lymphotropic for B cells. Both viruses most likely utilize B cell compartments as reservoirs for persistent infection and in a small percentage of individuals infection sometimes leads to lymphoproliferative disorders (LPDs). In addition to KS, KSHV has now been associated with two LPDs, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Cesarman and Knowles, 1999; Soulier et al., 1995). These KSHV associated neoplasms predominantly occur in immunocompromised patients that are also co-infected with HIV (Boshoff and Weiss, 2002; Cesarman, 2011).

The association of KSHV and PEL was made soon after the initial finding of KSHV in lymphoma samples from AIDS patients (Cesarman et al., 1995; Chang et al., 1994). These B cell lymphomas are normally found as effusions within pleural, peritoneal, and pericardial body cavities, usually with no solid tumor mass. They have been classified as a distinct subgroup of AIDS-related non-Hodgkin lymphomas (NHL), and are extremely rare, only accounting for 4% of AIDS-related NHLs and 0.3% in HIV-negative

patients (Carbone and Gloghini, 2008). KSHV is present in all PEL cells, with the majority also harboring EBV (Cesarman et al., 1995; Nador et al., 1996). Based on their large cell size and other common morphologic features, PEL cells bridge immunoblastic and anaplastic large-cell lymphomas (Brimo et al., 2007; Cesarman et al., 1995; Nador et al., 1996).

Phenotypically, PELs are hard to characterize because they generally lack expression of surface B-cell associated antigens and immunoglobulin (Ig). However, they do express the hematopoietic marker CD45, as well as markers linked to plasma cell differentiation (CD138/syndecan-1 and MUM/IRF4) and activation (CD30, CD38, CD71), suggesting a late B cell origin (Carbone et al., 2000; Cesarman et al., 1995; Nador et al., 1996). In addition, sequence analysis of PEL cell Ig genes show high levels of somatic mutation, in comparison to the germline, and also show evidence of antigen selection, indicating that these cells have already transited through the germinal center (GC) (Fais et al., 1999; Matolcsy et al., 1998). Lastly, gene expression by PEL is more similar to a post-GC or plasma cell, than a naïve or GC B cell (Jenner et al., 2003; Klein et al., 2003). Based on these immunophenotypic and immunogenotypic properties, it is believed that PELs represent a mature B cell that has exited the GC and is arrested at a stage of post-GC development (Carbone et al., 2010).

Before the discovery of KSHV, it was observed that ~13% of patients with MCD developed KS for unexplained reasons (Peterson and Frizzera, 1993). When the association between KS and KSHV was uncovered, the link between MCD and KSHV infection was quickly made (Soulier et al., 1995). In 100% of HIV-positive patients with MCD, KSHV infection is found; this is reduced to 50% when MCD patients are HIV-

negative (Gessain, 1997; Luppi et al., 1996). MCD is a rare atypical lymphoproliferative disorder consisting of two separate subtypes: the hyaline vascular type and the more common plasma cell type. Both types have been shown to involve KSHV, but the majority of KSHV-positive cases involve the plasma cell variant (Larroche et al., 2002). In KSHV-positive cases of MCD, large plasmablasts containing KSHV genomes are located in the mantle zones of GCs, a feature absent from KSHV-negative cases (Dupin et al., 2000). Based upon these morphological differences it has been proposed that KSHV-positive MCD is a separate plasmablastic variant of the disease (Dupin et al., 2000).

Unlike PEL cells, which resemble a mature B cell origin, plasmablasts from MCD represent a naïve B cell origin. This is based on the lack of somatic mutations in their rearranged Ig genes, suggesting that they have not undergone Ig selection in the GC (Du et al., 2001). Interestingly, MCD plasmablasts have a mature phenotype based on expression of the memory B cell marker CD27 and high expression of cytoplasmic IgM, two features absent in PEL cells (Du et al., 2001; Dupin et al., 2000). This mature phenotype may suggest that KSHV infection might be driving naïve B cells to mature without a GC reaction. MCD plasmablasts do not express the plasma cell associated marker CD138 and the activation marker CD30, two markers commonly expressed by PEL (Du et al., 2001). Additionally, unlike the majority of PEL cells, KSHV infection in MCD is not associated with EBV co-infection (Du et al., 2001; Dupin et al., 2000). These differences highlight the fact that PEL and MCD represent two distinct types of B cell lymphomas associated with KSHV.

KSHV Lifecycle

Like all herpesviruses, KSHV is an enveloped DNA virus that exists in two distinct phases of infection termed latent and lytic. During latency the viral genome is found in the nucleus of the host cell as multiple circularized episomes, which do not undergo productive replication and whose expression is limited to a small subset of viral genes that modulate host cell growth and inhibit immune recognition. In contrast, the lytic phase of KSHV infection is characterized by the regulated expression of the entire viral genome, leading to productive replication of the viral episomes into linear genomes, which are packaged into progeny virions and released by cell lysis, resulting in cell death. While latency is the default pathway in most KSHV infected cells, a small percentage undergo lytic replication (Lieberman et al., 2007).

Transmission of KSHV is believed to occur mainly via saliva from infected individuals (Koelle et al., 1997; Mayama et al., 1998). Once inside the new host, KSHV establishes lifelong persistent infection by remaining hidden from the host immune response, mainly through latent gene expression. The interplay between the host immune response and KSHV associated disease is highlighted by the fact that, while 2%-7% of the North American population is seropositive for KSHV, only a small fraction will ever develop KSHV associated disease (Ganem, 2006). Furthermore, KSHV tumorigenesis is strongly correlated with compromised immune systems, as tumors regress with immune restoration by HAART (Pellet et al., 2001; Wilkinson et al., 2002).

The KSHV Genome, Latency, and MiRNA Production

KSHV shares a common genome structure with all known rhadinoviruses including a genome size of ~170 kbp that contains a unique internal sequence (~140 kbp) encoding 87 open reading frames (ORFs), which is flanked by GC-rich terminal repeats

(TR) (Russo et al., 1996). The KSHV internal sequence encodes 66 ORFs that have homologues in the closely related New World primate rhadinovirus, HVS (Russo et al., 1996). KSHV also encodes at least twenty genes with homology to cellular genes, a common characteristic of rhadinoviruses. The high number of cellular homologues is believed to give KSHV an advantage in hijacking host cellular pathways without eliciting an immune response (Neipel et al., 1997). Additionally, KSHV encodes a number of unique genes designated K1-K15, not found in other rhadinoviruses (Russo et al., 1996) (Figure 1-1).

Early studies showed that KSHV has limited gene expression in PEL and KS tumors (Renne et al., 1996; Zhong et al., 1996). Furthermore, when virus-infected cells were isolated from these tumors and treated with the phorbol ester 12-O-tetradecanoyl phorbol13-acetate (TPA), a known inducer of lytic replication in EBV, there was a dramatic increase in gene expression, indicating that the majority of virus is latent in these tumors (Renne et al., 1996; Zhong et al., 1996). TPA induction in PEL cells was also used to analyze latent/lytic gene expression, and revealed a cluster of three latently expressed genes: LANA (ORF73), v-cyclin (ORF72), and vFLIP (ORF71/K13) (Sarid et al., 1998). These three ORFs were found to be expressed from two major polycistronic mRNAs, latent transcript 1 and 2 (LT1 and LT2) (Dittmer et al., 1998; Sarid et al., 1999; Talbot et al., 1999). The region in which these transcripts are encoded is designated the KSHV latency associated region (KLAR).

KLAR is under the control of two promoters LTc and LTd, which are constitutively active. The two mRNAs expressed from KLAR are spliced into a 5.4 kbp transcript (LT1), which encodes LANA, and a 1.7 kb transcript (LT2) that encodes two

homologues of cellular proteins v-cyclin and vFLIP (Dittmer et al., 1998; Sarid et al., 1999; Talbot et al., 1999). Functional studies have demonstrated that these latent proteins promote cell growth, either through inhibiting apoptosis (v-Flip and LANA) or inducing cell cycle progression (v-cyclin and LANA). LANA also plays an essential role in the establishment of latency by replicating KSHV DNA, acting as a transcriptional activator of the LTc promoter, inhibiting expression of the reactivation transcriptional activator (RTA), and maintaining viral episomes by tethering them to host chromsomes during mitosis (Ballestas et al., 1999; Hu et al., 2002; Lan et al., 2004; Renne et al., 2001).

The other latent proteins expressed from KLAR consist of the unique kaposin family of proteins (kaposin A, B, and, C). The kaposin proteins have varying functions, with kaposin A having transforming potential and kaposin B promoting increased secretion of pro-proliferative cytokines (McCormick and Ganem, 2005; Muralidhar et al., 1998). Together, the function of these latently expressed proteins, to stimulate proliferation and inhibit apoptosis, indicate that they play a major role in KSHV-induced pathogenesis.

In addition to latent proteins encoded in KLAR, non-coding miRNAs have also been discovered (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005) (Figure 1-2). In total, 12 miRNA genes were identified, all of them within the major latency-associated region of the genome, giving rise to at least 17 mature miRNAs. Ten of the 12 genes were found in a single cluster and mapped to a 3.6 kbp intragenic region between K12 and ORF 71 , whereas the remaining two were located within the kaposin/K12 locus (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). Expression of the primary-

miRNA transcripts (pri-miRNAs) is controlled by three promoters, one latent and two lytic . In PEL cells all miRNAs are highly expressed during latency, and induction of lytic replication has only moderate effects on miRNA expression, with the exception of miR-K10 (Cai and Cullen, 2006; Pearce et al., 2005). These miRNAs, like the other latent gene products, function to modulate host cell growth, immunity, and maintain latent infection.

MiRNA Discovery and Function

MicroRNAs (miRNAs) are short RNAs of about 22 nucleotides in length that post-transcriptionally regulate gene expression by binding to 3' untranslated regions of mRNAs, thereby inducing translational silencing. The first discovered microRNA (miRNA), *lin-4* of *Caenorhabditis elegans*, was found because of its role in a developmental timing defect (Lee et al., 1993; Wightman et al., 1993). Functional analysis of the *lin-4* gene revealed that it does not encode a protein, but instead produces two short transcripts (60 and 24 nucleotides in length) (Lee et al., 1993). It was demonstrated that the *lin-4* RNA induced post-translational silencing of *lin-14*, a developmental control gene whose protein product is involved in the temporal regulation of cell lineage patterning in *C. elegans*. Further work showed that the mechanism of this post-transcriptional regulation was mediated through complementary binding of the *lin-4* miRNA to sequences within the 3' UTR (untranslated region) of *lin-14* (Lee et al., 1993; Ruvkun et al., 1991; Wightman et al., 1993). This novel RNA-based inhibition was thought to be specific to *C. elegans*, until the discovery that the *let-7* miRNA was conserved in many metazoans, including humans and flies (Pasquinelli et al., 2000; Reinhart et al., 2000; Slack et al., 2000). MicroRNAs have now been isolated from

every metazoan and plant species examined thus far, and to date, more than 900 human miRNAs have been identified (Ambros, 2004; Griffiths-Jones, 2004).

In mammalian species, it has been estimated that greater than half of all protein coding genes contain a miRNA target site, indicating their heavy impact on gene regulation (Friedman et al., 2009). Functionally, miRNAs are known to play key regulatory roles in many biological processes including, but not limited to, hematopoiesis, immune response, and apoptosis. They have also been associated with tumor formation, where their aberrant expression can be used as a signature for the clinical diagnosis of different types of leukemia and lymphomas (Garzon et al., 2008). Sequences of miRNAs are highly conserved through evolution, leading to shared miRNA homologs between vastly different animal lineages. For example, a third of the miRNAs expressed by *C. elegans* have homologs found in humans (Lim et al., 2003). Recent discoveries have also shown that human miRNAs can be mimicked by viral miRNA orthologs, leading to the regulation of identical target genes (Gottwein et al., 2007; Skalsky et al., 2007).

MiRNA Biogenesis and Mechanisms of Gene Regulation

The first step of miRNA biogenesis occurs in the nucleus, where RNA polymerase II transcribes miRNA coding genes into primary miRNAs (pri-miRNAs) (Figure 1-3). Structurally, the pri-miRNA consists of a double-stranded RNA hairpin loop with a 5'-end cap structure and a polyA-tail sequence. Following transcription, the pri-miRNA is processed in the nucleus by Drosha, an RNase III endonuclease, along with its cofactor DiGeorge syndrome critical region gene 8 (DGCR8). Processing occurs when the Drosha-DGCR8 complex binds to and cleaves the pri-miRNA, leaving a ~70nt pre-miRNA hairpin loop that contains a 5' phosphate and ~2nt 3' overhang (Lee et al.,

2003). The pre-miRNA is exported out of the nucleus and into the cytoplasm by the export receptor Exportin-5 (Yi et al., 2003). Once in the cytoplasm, processing of the pre-miRNA is carried out by the enzyme Dicer, a cytoplasmic RNase III endonuclease. Dicer cleaves the double stranded portion of the pre-miRNA close to the base of the stem loop, thereby removing the terminal loop, and leaving a ~22nt miRNA imperfect duplex with a 5' phosphate and ~2nt 3' overhang at each end. This miRNA duplex consists of the mature miRNA, or guiding strand, while the opposite strand is considered the passenger strand and is normally not used in targeting. Generally, the strand in the duplex that is less stable at its 5' end will be the one selected for incorporation into the RNA induced silencing complex (RISC), which is composed of Argonaute proteins (Schwarz et al., 2003; Tomari et al., 2004).

Targeting of mRNA by RISC is mediated through Watson-Crick base pairing between the miRNAs seed sequence, nucleotides 2 to 7 at the 5' end, and complementary nucleotides found in the 3'UTR of the mRNA (Lewis et al., 2005; Stark et al., 2003). Other parameters that influence targeting and silencing include: the number of miRNA seed targets within the 3'UTR, location of the seed region within the 3'UTR, position of the seed region target site in an AU rich environment, and flanking of the target site by adenosines (Grimson et al., 2007). Unlike siRNAs, which mediate target cleavage and degradation, miRNA silencing is believed to occur through translational inhibition. The difference between siRNA and miRNA mediated silencing is based on the level of complementarity between the small non-coding RNAs sequence and its target, with full complementarity leading to cleavage (siRNA) and partial leading to translation inhibition (miRNA) (Hutvagner and Zamore, 2002). Metazoan miRNA

mediated translation inhibition is believed to occur by one of four separate mechanisms: inhibition of translation initiation; inhibition of translation elongation; co-translational protein degradation; or premature termination of translation (Huntzinger and Izaurralde, 2011).

While much debate still exists on the exact mechanism of translation inhibition, increasing evidence indicates that, like siRNAs, miRNAs predominately induce target degradation (Baek et al., 2008; Guo et al., 2010; Selbach et al., 2008). Mass spectrometry studies showed that regulation of miRNA target proteins and degradation of target mRNAs are strongly correlated, with only a small number of targets showing a change in protein level without a reduction in mRNA (Baek et al., 2008; Selbach et al., 2008). Furthermore, ribosomal profiling of target mRNAs was used to measure target protein abundance, during ectopic and endogenous miRNA targeting, results indicated that reduced target mRNAs are associated with decreased protein production, indicating that mRNA destabilization and not translation inhibition is the predominant mechanism (Guo et al., 2010).

Viral MiRNAs

In 2004, Tuschl and colleagues reported the molecular cloning of five EBV miRNAs from Burkitt's lymphoma (BL) cells, a finding that started a new field in virology (Pfeffer et al., 2004). Since then, more than 140 herpesvirus miRNAs have been identified. Initially, three EBV miRNAs were found located within the BHRF gene (BamHI fragment H rightward open reading frame 1), and two within the BART gene (BamHI-A region rightward transcript). Bioinformatic approaches, in combination with the use of tiled arrays and molecular cloning, revealed 17 additional miRNA genes in the BART region of EBV; these genes are located within a 12 kbp region that was

absent in the EBV strain studied in the original report (Cai and Cullen, 2006; Grundhoff et al., 2006). Recently, two additional BART miRNA genes were identified in EBV-positive nasopharyngeal carcinoma (NPC) tissue samples, bringing the total number of miRNA genes to 25 (Zhu et al., 2009). BART and BHRF miRNAs are differentially expressed in lymphoid and epithelial cells and furthermore under different programs of viral latency. In epithelial cells BART miRNAs are expressed more abundantly than in B cells, while BHRF miRNAs have only been detected during type III latency, when all known latency-associated genes are expressed. Induction of lytic replication in latently-infected BL cell lines leads to induction of a subset of EBV miRNAs. (Cai and Cullen, 2006; Cosmopoulos et al., 2009; Edwards et al., 2008; Xing and Kieff, 2007). EBV miRNAs are also expressed early after *de novo* infection of primary B cells, which might suggest roles in the establishment of latency (Pratt et al., 2009). In the EBV-related lymphocryptovirus (LCV) of the rhesus macaque, 16 miRNAs were identified, eight of which show sequence homology to EBV miRNAs, suggesting conservation of miRNAs in this subfamily (Cai et al., 2006).

After the identification of EBV miRNAs, other members of the gammaherpesvirus subfamily were found to encode miRNAs. In KSHV, four independent groups cloned 12 miRNAs from PEL-derived cell lines (as discussed in the above section). The genome of the closely related rhesus rhadinovirus (RRV), was found to encode seven miRNAs (Schafer et al., 2007). Like KSHV miRNAs, the RRV miRNAs are encoded within the latency-associated region of the genome; however, their sequences are not homologous to those of KSHV. Another related gammaherpesvirus, Murine gammaherpesvirus type 68 (MHV68), encodes fifteen miRNA genes, most of which are

embedded within tRNA-like genes at the 5' end of the genome; these genes have been suggested to be transcribed by RNA polymerase III (polIII) (Pfeffer et al., 2005).

The presence of miRNA genes in betaherpesviruses has, so far, been restricted to cytomegaloviruses (CMV). Nine miRNA genes were initially found in human cytomegalovirus (HCMV) scattered throughout the viral genome and all being expressed from multiple promoters (Pfeffer et al., 2005). This number was later expanded to 11, when two additional miRNAs were identified, both by cloning and bioinformatic prediction of conserved hairpins between HCMV and chimpanzee CMV (Dunn et al., 2005; Grey et al., 2005). HCMV miRNAs are readily detectable by Northern blot after *de novo* infection of epithelial, endothelial, and neuronal cells, even in the presence of cycloheximide, indicating that HCMV miRNAs are expressed as immediate-early gene transcripts (Dunn et al., 2005). The genome of murine cytomegalovirus (MCMV) encodes 18 miRNA genes. Quantitative analysis of viral miRNA expression after MCMV infection revealed that at early time points post infection the majority of expressed miRNAs were of viral origin (Pfeffer, 2007). CMV latency *in vivo* affects multiple tissues, including bone marrow. Unfortunately, due to the lack of latent tissue culture models, miRNA expression during CMV latency has not been investigated.

Among alphaherpesviruses, miRNAs have been identified in herpes simplex viruses 1 and 2 (HSV-1 and -2), and Marek's disease viruses 1 and 2 (MDV-1 and -2) (Burnside et al., 2006; Cui et al., 2006; Morgan et al., 2008; Tang et al., 2008; Tang et al., 2009; Umbach et al., 2008; Yao et al., 2007; Yao et al., 2008). Interestingly, like in KSHV, alphaherpesvirus miRNA genes are located within a region expressed during

latency. HSV latency is characterized by the expression of the latency-associated transcript (LAT), a non-coding transcript that is antisense to two lytic genes: ICP0, a transcriptional regulator, and ICP34.5, a neurovirulence factor (see (Bloom et al., 2010) for a review on LAT). Recently, four miRNAs (miR-H2 to miR-H5) were cloned from a variety of sources: (i) human endothelial kidney (HEK) 293 cells that ectopically express LAT, (ii) productively infected Vero cells (a cell line derived from African green monkey kidney), and (iii) latently infected trigeminal ganglia in mice (Umbach et al., 2008). One additional miRNA gene (miR-H6) was located upstream of LAT in HSV-1, and 11 miRNAs were predicted to be encoded elsewhere in the viral genome but to date have not been cloned (Cui et al., 2006; Umbach et al., 2008). Most recently, Umbach and colleagues confirmed the expression of miR-H2 to miR-H6 in human trigeminal ganglia, and also identified two novel miRNAs (miR-H7 and miR-H8) also located within LAT (Umbach et al., 2009). The genome of HSV-2 encodes three miRNAs within LAT that are positionally conserved, as compared to its close relative, HSV-1 (Tang et al., 2008; Tang et al., 2009).

Burnside and colleagues used 454 deep sequencing to identify 13 miRNAs expressed from the genome of MDV-1 (Burnside et al., 2006). These miRNAs were mapped to the inverted repeat short and long regions (IR_S and IR_L) of the MDV-1 genome. Eight of these miRNA genes are located within the meq oncogene region, whereas the others map to the LAT region (Burnside et al., 2006; Morgan et al., 2008). In the closely related MDV-2 virus, conventional cloning techniques identified 17 miRNAs which, like MDV-1, were mapped to the IR_S and IR_L genomic regions (Yao et al., 2007; Yao et al., 2008).

With the exception of varicella zoster virus (VZV) (Umbach et al., 2009), all herpesviruses examined to date express miRNAs. However, the use of mass parallel sequencing to analyze small RNA libraries from virus-infected cells might uncover new, less abundantly expressed, miRNAs.

Despite high-throughput sequencing attempts, RNA viruses (e.g. Influenza, HIV, and HCV) and cytoplasmic replicating DNA viruses (Poxviruruses) have not been found to encode miRNAs. The absence of viral miRNAs from these viruses may reflect their inability to access nuclear Drosha and the requirement for RNA viruses to protect their genome from Drosha/Dicer processing.

Herpesvirus MiRNAs Closely Resemble Their Host Cellular Counterparts

With respect to gene organization, viral miRNA genes recapitulate their cellular counterparts. They are organized either as single genes (e.g. in CMV) or in clusters (e.g. in alphaherpesviruses and gammaherpesviruses), the latter allowing for co-regulated expression. To date there is no evidence that herpesviral proteins are involved in viral miRNA maturation, which is strictly dependent on Drosha/DGCR8 and Dicer processing (Figure 1-3). Viral miRNA and host miRNA sequences can be located within introns or exons of protein encoding genes. The relative genomic location of the pre-miRNA and surrounding splice-donor/acceptor sites might lead to competition between miRNA maturation and mRNA splicing, like it occurs for cellular genes. For example, the EBV BART miRNAs, which are located within introns of a multiple spliced transcript, are processed prior to splicing, thereby suppressing the usage of surrounding exons. It is not clear whether a single BART transcript can give rise to an intron-encoded miRNA and a fully processed mRNA (Edwards et al., 2008).

One hallmark of cellular miRNAs is that ~30% are highly conserved across species. For instance, eight of 29 EBV miRNAs showed sequence similarity to those of its close relative LCV (Cai et al., 2006). In contrast, no homology was noted between KSHV and RRV miRNAs (Schafer et al., 2007). In this case, one possible explanation is that, in rhesus macaques and chimpanzees two different rhadinoviruses (RRV1 and RRV2) exist, while to date only one human rhadinovirus strain (KSHV) has been identified.

If miRNA function is important for viral biology then the corresponding sequences would likely co-evolve with their respective host target sequences (Sood et al., 2006). Hence, answering the question whether viral miRNAs are conserved will be greatly aided by understanding their targets and function. Thus far, sequence analysis of both EBV and KSHV miRNA gene loci from a large number of cell lines and primary isolates revealed very few polymorphisms, which suggest *in vivo* selection for intact miRNA genes; albeit indirect, this constitutes a genetic argument for biological function (Marshall et al., 2007).

KSHV MiRNA Targets and Function

The majority of identified viral miRNAs are encoded by herpesviruses, suggesting that they play an essential role in the herpesvirus lifecycle. Because KSHV miRNAs are nonimmunogenic and have the capacity to regulate a large number of targets, they represent ideal tools for hijacking the host cellular responses to viral infection. Understanding the functions of KSHV miRNAs requires the determination of target genes, which can be viral and/or cellular. To identify a valid target, bioinformatic approaches are usually used in combination with experimental functional assays. However, targets for KSHV miRNAs have largely been determined by unbiased gene

expression profiling studies rather than bioinformatic prediction (Gottwein et al., 2007; Samols, 2007; Skalsky et al., 2007; Ziegelbauer et al., 2009). While the initial reports identifying KSHV miRNAs predicted many gene targets (Cai et al., 2005; Gottwein et al., 2007; Pfeffer et al., 2005; Samols, 2007; Skalsky et al., 2007; Ziegelbauer et al., 2009) the number of experimentally validated targets is still modest (Table 1-1). Based on the current list of validated targets for KSHV miRNAs, it is apparent that they function to modulate several fundamental cellular processes: angiogenesis, cell cycle, immunity, apoptosis, and key steps in the herpesvirus life cycle; latency and the switch from latent to lytic replication.

The first published host cell target for KSHV miRNAs was the gene coding for thrombospondin 1 (THBS1), a tumor suppressor and antiangiogenic factor that is reported to be down-regulated in KS lesions (Samols, 2007). Samols and colleagues generated HK 293 cells expressing 10 KSHV miRNAs and found 65 genes that showed decreased mRNA levels compared to vector controls. The 3' UTRs of down-regulated genes had a high frequency of seed-sequence matches, including the 3' UTR of THBS1 that contained 34 potential binding sites for multiple KSHV miRNAs. By using 3' UTR luciferase reporter assays and Western blot analysis, direct targeting and repression of THBS1 expression by several KSHV miRNAs (miR-K12-1, miR-K12-3-3p, miR-K12-6-3p and miR-K12-11) was demonstrated. This was the first example of multiple viral miRNAs regulating a single host gene. Because THBS1 activates latent TGFβ, Samols and colleagues used TGFβ responsive reporter assays to demonstrate that KSHV miRNA repression of THBS1 translates into decreased TGFβ activity. Given that angiogenesis is a hallmark of KS, the finding that KSHV miRNAs target a strong

inhibitor of angiogenesis suggests that KSHV miRNAs contribute to pathogenesis (Samols, 2007).

To promote cell viability and proliferation during infection, herpesviruses not only inhibit apoptosis but also modulate cell cycle regulation. The first evidence for KSHV miRNA cell cycle regulation was the finding that KSHV miR-K1 targets p21, a p53-inducible gene that functions as a cell cycle inhibitor and tumor suppressor (Gottwein and Cullen, 2010). Knockdown of endogenous miR-K1, with miRNA sponges in KSHV infected cells, resulted in a modest increase of p53 mediated cell cycle arrest, implicating miR-K1 in cell cycle regulation and pathogenesis.

The ability of KSHV to repress host-immune responses is essential for persistent infection. The importance of immune regulation is underscored by the fact that almost a quarter of the KSHV genome (22 ORFs) has an immune modulatory function (Areste and Blackbourn, 2009). Experimental evidence now suggests that KSHV miRNAs also play an important role in immune modulation by directly inhibiting cytokine expression, the antiviral interferon response, and immune cell recognition.

KSHV miRNAs miR-K12-3 and miR-K12-7, when ectopically expressed in human myelomonocytic and murine macrophage cell lines can increase secretion of host cytokines IL-6 and IL-10, which are highly expressed in KS lesions (Qin et al., 2010). Bioinformatic analysis in combination with antagomir-based derepression assays demonstrated that miR-K12-3 and miR-K12-7 downregulates LIP, an isoform of C/EBPβ that functions as a negative transcriptional regulator of IL-6. Although these cytokines have broad functions in suppressing the activity of multiple immune cell types including

T-cells, NK cells, and dendritic cells, their impact during natural KSHV infection needs to be further tested (Cirone et al., 2008; Moore et al., 2001; Mosmann, 1994).

An additional study demonstrated that ectopic expression of KSHV miR-K10a, in primary endothelial cells, markedly reduced production of the pro-inflammatory cytokine IL-8 and monocyte chemoattractant protein 1 (MCP-1) by targeting tumor necrosis factor (TNF)-like weak inducer of apoptosis receptor (TWEAKR) (Abend et al., 2010). Curiously, 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 integrate these paradoxical observations, 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).

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). To test the impact of IKKε targeting by miR-K12-11 without any confounding effects of other KSHV immune regulatory proteins, miR-K12-11 transduced lung cancer cells were infected with two RNA viruses, Sendai virus (SeV) and vesicular stomatitis virus (VSV), which strongly induce the interferon response. Results showed that upon infection, miR-K12-11 expressing cells had markedly attenuated interferon signaling and enhanced VSV titers.

Elimination of virally infected cells by NK cells or CD8+ T-cells involves cell receptor recognition of ligands expressed by KSHV infected target cells. KSHV miR-K12-7 was shown to target the major histocompatibility complex class I-related chain B

(MICB), a stress-induced ligand recognized by the NKG2D receptor expressed by NK cells and CD8+ T-cells (Nachmani et al., 2009). The functional impact of this regulation was tested using a miRNA sponge to inhibit miR-K12-7 targeting in virally infected cells, which resulted in increased NK cell killing. Interestingly, it has also been shown that miRNAs encoded by HCMV and EBV regulate MICB expression to inhibit NK cell killing (Nachmani et al., 2009; Stern-Ginossar et al., 2007). These data strongly suggest that miRNA-dependent regulation of MICB is important for herpesviral persistence, which is further underscored by the fact that both HCMV and KSHV encode proteins that inhibit MICB surface expression (Dunn et al., 2003; Thomas et al., 2008). In addition, the observation that HCMV-, EBV-, and KSHV- encoded miRNAs target the MICB gene by completely different sequences raises a very interesting question about the co-evolution of viral miRNAs and their corresponding cellular targets.

Recently, Ganem and colleagues reported a highly comprehensive tandem-array approach to identify miRNA targets, utilizing gene expression profiling in endothelial cells after *de novo* infection, and in B cells that ectopically expressed individual, or various sets of, miRNAs (Ziegelbauer et al., 2009). For a gene to be recognized as a potential target, its expression had to be reduced in the cells expressing ectopic miRNA, but increased in latently-infected PEL cells transfected with the corresponding antagomir (Ziegelbauer et al., 2009). For each KSHV miRNA, about 10 to 30 host genes fulfilled these criteria. Analysis of miR-K5 revealed 11 gene targets, including Bcl-2-associated factor (BCLAF1), and this was studied in more detail. BCLAF1 functions as a transcriptional repressor and can mediate apoptosis when over-expressed (Kasof et al., 1999). In addition to miR-K5, both miR-K12-9 and miR-K12-10b were also found to

target and regulate the expression of BCLAF1 (Ziegelbauer et al., 2009). Ziegelbauer and colleagues demonstrated that miR-K5, miR-K12-9 or miR-K12-10b transfected into human umbilical vein endothelial cells (HUVEC) were able to inhibit etoposide-induced caspase activation, thereby suggesting that miRNA repression of BCLAF1 inhibits apoptosis. Interestingly, increased etoposide-induced apoptosis was observed when HUVEC cells were plated at a lower density and then transfected with the same KSHV miRNAs, indicating that BCLAF1 can have anti-apoptotic activity under particular growth conditions. Although the suggestion that BCLAF1 might have an anti-apoptotic function was at first counterintuitive, the researchers went on to find that BCLAF1 expression in latently infected PEL cells could inhibit lytic virus replication. In addition, inhibiting KSHV miRNA targeting of BCLAF1 with antagomirs resulted in decreased lytic reactivation in KSHV-infected endothelial cells (SLK). Together, these data suggest that targeting BCLAF1 sensitizes latently infected cells to signals that induce reactivation from latency. Hence, miR-K5 provides the first example by which targeting of a host gene contributes to latency control.

In contrast to a lytic role, KSHV miRNAs have also been reported to promote latency. The viral replication and transcription activator (RTA), a master regulator of lytic reactivation, has been shown to be regulated either directly or indirectly by multiple viral miRNAs. Two independent studies, using similar KSHV bacmid 36-derived recombinant viruses that lack 10 of 12 miRNA genes, reported elevated expression of lytic genes, including RTA, during *de novo* infection in separate cell lines (Lei et al., 2010; Lu et al., 2010b). To determine the mechanism leading to increased lytic gene expression, Lu et al. screened the individual KSHV miRNAs, using miRNA expression

plasmids, for their ability to target a RTA luciferase construct and found that miR-K5 can repress RTA expression, albeit the 3′UTR of RTA lacks a canonical miR-K5 seed sequence. Additionally, Lu et al. carried out genome wide epigenetic analysis of the miRNA knockout virus and found drastically reduced repressive marks on histones along with a global reduction of DNA methylation, suggesting that epigenetic modifications induced by viral miRNAs may contribute to the maintenance of latency. Searching for a mechanism to explain these modifications, Lu et al. found that miR-K12-5p targets retinoblastoma (Rb)-like protein 2 (Rbl2), a negative regulator of DNA methyltransferases, thereby leading to an increase in DNA methylation. This is the first reported evidence that viral miRNAs can directly impact the epigenetic status of herpesvirus genomes during latency.

In the second study, Lei et al. also found an increase in RTA mRNA expression in cells infected with a very similar KSHV miRNA knockout virus, but they did not identify direct targeting of the RTA 3′UTR by miR-K12-5p or any other KSHV miRNA (Lei et al., 2010; Lu et al., 2010b). Instead, Lei et al. showed that miR-K1 targets the host gene IκBα, an inhibitor of NFκB, leading to activation of NFκB, which is known to inhibit lytic reactivation and, in the case of PELs, contributes to cell survival (de Oliveira et al., 2010).

In addition to targeting IκBα, two independent studies reported that lytic reactivation can be regulated by KSHV miR-K12-11 targeting of IKKε and nuclear factor I/B (NFIB) (Lei et al., 2010; Lu et al., 2010a). As mentioned before miR-K12-11 targeting of IKKε leads to attenuation of the interferon response (Lei et al., 2010). This same study found that inhibiting miR-K12-11, with an anti-miR-K12-11 sponge, leads to an

increase in lytic gene expression (RTA and ORF65) in bacmid-infected A549 cells. The authors also showed that IKKε overexpression enhanced lytic replication when TPA, a chemical agent that triggers lytic reactivation, was used.

Using lentiviruses to express individual KSHV miRNAs in BC3 cells, Lu et al. found that miR-K1, K3, K7, and K11 were all capable of moderately decreasing RTA mRNA levels (Lu et al., 2010a). MiR-K3 showed the greatest effect on RTA, and further investigation found that it directly targets NFIB, a cellular transcription factor that had previously been shown to reactivate KSHV when overexpressed (Yu et al., 2007). Further analysis identified that the promoter of RTA contains a putative NFIB binding site and that ectopic NFIB expression could activate an RTA promoter construct. Additionally, shRNA knockdown of NFIB resulted in decreased RTA expression. This study provides indirect evidence that miR-K3 maintains latency by targeting NFIB, but further experiments using anti-miR-K3 antagomirs or a miR-K3 knockout virus are needed to prove this mechanism.

In addition to indirectly regulating RTA expression two separate studies have demonstrated that miR-K12-9* and miR-K12-7-5p can directly target and regulate RTA expression through seed match binding (Bellare and Ganem, 2009; Lin et al., 2011). Using luciferase constructs, containing the 3′UTR of RTA, and KSHV miRNA mimics, Bellare et al. identified that miR-K9* directly targets RTA through a canonical 6mer seed match site. Furthermore, when miR-K9* function in latently infected cells was inhibited with specific antagomirs a moderate increase in lytic reactivation, was observed. In a separate study by Lin et al., which used KSHV miRNA expression plasmids instead of miRNA mimics, miR-K9* and miR-K12-7-5p were also found to target RTA (Lin et al.,

2011). Lin et al. further show that miR-K12-7-5p targeting of RTA is mediated through a 7mer seed match site and that ectopic expression of miR-K12-7-5p in latently infected cell lines reduces the amount of progeny virus produced. In summary, these studies lend further credence that KSHV miRNAs directly regulate RTA expression during latency. However, while some studies hypothesize that KSHV miRNAs function as major regulators of latency, Bellare et al. suggest that these miRNAs may provide a mechanism for fine tuning and/or sensitizing latently infected cells to stimuli that trigger lytic replication.

KSHV miRNA regulation is an emerging component of the complex relationship that governs viral–host interactions. From the targets identified to date (Table 1-1) it is apparent that viral miRNAs play an important role in the biology of the virus and contribute to overall pathogenesis associated with KSHV infection. However, determining the targets of these miRNAs is only one step in understanding their function. Because KSHV miRNA regulation is likely dependent on the context of infection (i.e. cell-type and viral genome expression), future studies using recombinant viruses, appropriate cell lines, and where available animal models are needed to further understand their impact on viral pathogenesis *in vivo*.

KSHV MiR-K12-11 is an Ortholog of Human MiR-155

The ability of herpesviruses to pirate host cellular genes into their genome for biological benefit is a hallmark of herpesvirus evolution. Although only one example of a herpesvirus pirating a host pre-miRNA has been reported (Waidner et al., 2009), statistical analysis of seed sharing between human herpesvirus miRNAs and human miRNAs revealed a high probability of conservation (Grundhoff and Sullivan, 2011). Because the seed sequence is the most important parameter for miRNA target binding,

it is not surprising that herpesvirus miRNAs may co-evolve their seeds with host miRNAs in order to hijack their function. Currently, the only functional evidence of this seed sharing exists for the human miR-155 and KSHV miR-K12-11.

Data from our lab and the Cullen group revealed that KSHV miR-K12-11 shares 100% seed sequence homology with human miR-155 (Gottwein et al., 2007; Skalsky et al., 2007). Because the seed sequence is the most important parameter in mRNA target recognition (Grimson et al., 2007), it was predicted that both miRNAs might target an overlapping set of host genes. Gene expression profiling in two separate cell lines, HEK293 cells and an EBV negative Burkitt's lymphoma cell line, BJAB, that stably express either miR-155 or miR-K12-11 identified a common set of downregulated gene targets. Further computational analysis found that one gene, BACH1, contained four target sites within its 3'UTR (Gottwein et al., 2007; Skalsky et al., 2007). Targeting and inhibition of BACH1 by both miRNAs was validated by 3'UTR reporter assays, mutagenesis and Western blot analysis. Importantly, PEL-derived cell lines that express high levels of miR-K12-11, but not miR-155, expressed very low BACH1 levels. BACH1 is a transcriptional repressor that has been shown to repress expression of heme-oxygenase 1 (HMOX1), a protein that enhances cell survival and proliferation (Igarashi and Sun, 2006). Because KSHV has been reported to directly increase HMOX1 levels during endothelial cell infection, these studies suggest that miR-K12-11 could contribute to HMOX1 upregulation by inhibiting the expression of its transcriptional repressor BACH1. The other potential targets found in the computational analysis include genes involved in cell signaling, cell division, T cell activation, and apoptosis. While the impact

of miR-K12-11 regulation on these targets needs further investigation, it is apparent that miR-K12-11 can hijack cellular pathways regulated by miR-155.

While many metazoan miRNAs share complete sequence homology across closely related species, viral miRNAs do not appear to share this conservation (Grundhoff and Sullivan, 2011). However, in addition to KSHV miR-K12-11, the lymphotropic alphaherpesvirus, MDV, also encodes a miRNA (mdv1-miR-M4) that functions as a miR-155 ortholog. *In vivo* functional analysis of mutant MDV viruses which contain a non-functional or deleted miR-M4 revealed that this miRNA plays an essential role in the induction of T-cell lymphomas in birds (Zhao et al., 2011). Based on these separate findings, in two unrelated herpesviruses, it appears that the development of herpesvirus miR-155 orthologs is an important adaption. How miR-K12-11 phenocopies miR-155 function during KSHV B cell infection and how it promotes pathogenesis will be further discussed in Chapters 2 and 3.

MiR-155 in Hematopoietic Development and Disease

Systematic analysis of miRNA expression, using microarrays and high-throughput sequencing, has revealed insights into the expression patterns of specific miRNAs during hematopoiesis (Chen et al., 2004; Georgantas et al., 2007). One miRNA in particular, miR-155, has been shown to be differentially expressed during lineage specific cell differentiation. Mir-155 is processed from the non-protein-coding gene *bic* (B cell integration cluster), which is a common retroviral integration site originally identified in chicken B cell lymphomas (Tam et al., 1997). Moderate expression of miR-155 was detected in early human CD34+ hematopoietic stem-progenitor cells (HSPCs) analyzed by microarray (Georgantas et al., 2007). In mature peripheral B cells, T-cells, monocytes, and granulocytes the expression of miR-155 is detected at much lower

levels compared to their progenitors (Merkerova et al., 2008; Ramkissoon et al., 2006). *In vitro* colony forming assays revealed that overexpression of miR-155 in human CD34+ progenitor cells, using lentiviral transduction, can cause a decrease in myeloid and erythroid colony formation (Georgantas et al., 2007). Based on miR-155's differential expression during hematopoiesis and its ability to influence cell lineage specification, it appears that miR-155 plays an important role at different stages of hematopoiesis.

Recent studies have shown that miR-155 is an important component of immune activation and function in mature B cells. *Bic* expression was originally detected in human germinal center (GC) B cells and activated T-cells by Northern blot and RNA *in situ* hybridization (RNAish) (Tam, 2001; van den Berg et al., 2003). Studies investigating the mechanisms for *bic*/miR-155 induction revealed that murine B cells, activated by *in vitro* BCR, CD40, or Toll-like receptor (TLR) stimulation, show increased transient production of this miR-155 (Thai et al., 2007). An increase in miR-155 was also detected in primary murine macrophages, after *in vitro* stimulation of their antigen receptors (O'Connell et al., 2007). This enhanced upregulation of bic/miR-155, in response to events mimicking innate or adaptive immune activation, points to a cellular role for miR-155 in which its expression might be needed to regulate immune pathways. However, to date there are no studies that have examined this pattern of miR-155 upregulation in human B cells or macrophages.

To further define the *in vivo* function of miR-155 during these immune responses, loss of function experiments using miR-155 germline deficient mice and B cell specific miR-155 knockout mice were developed (Rodriguez et al., 2007; Thai et al., 2007;

Vigorito et al., 2007). Both mouse models appeared to have normal B cell development during steady-state (non-inflammatory) conditions, but after immunization with either T-cell dependent (TD) or T-cell independent (TI) antigen, B cells were impaired in their ability to form germinal centers (GC) and to produce class switched antibodies (Rodriguez et al., 2007; Thai et al., 2007). In order to define the cellular mechanisms that were contributing to these defects, gene expression profiling was performed on activated B cells isolated from these transgenic mice (Vigorito et al., 2007). Approximately 60 upregulated genes were identified that contained a miR-155 binding site (Vigorito et al., 2007). The authors of this study predicted that direct targets of miR-155 are those which have higher mRNA expression in the absence of miR-155. However, this prediction does not eliminate the possibility that some of these upregulated genes could actually be indirect targets, whose expression is not regulated by the miRNA itself, but by other proteins that are direct targets of miR-155. Regardless of this fact, further analysis of two miR-155 targets identified by this study, PU.1 and activation induced deaminase (AID), has provided insight into the B cell gene regulatory pathways regulated by miR-155.

Based on its overexpression pattern in a number of B cell lymphomas and its ability to induce both myeloproliferative and B cell lymphoproliferative malignancies in separate mouse models; miR-155 has been characterized as an oncomir, a miRNA with tumorigenic activity (Costinean et al., 2006; Eis et al., 2005; Kluiver et al., 2005; O'Connell et al., 2008; van den Berg et al., 2003). Insights into the mechanisms of miR-155 induced tumorigenesis have been provided by studies that identified CCAAT enhancer-binding protein β (C/EBPβ) and Src homology 2 domain-containing inositol-5-

phosphotase (SHIP1) as miR-155 targets in tumor cells (Costinean et al., 2009; O'Connell et al., 2009). In both studies, miR-155 was shown to reduce expression of 3'UTR reporter constructs *in vitro*, which correlated with reduced C/EBPβ and SHIP1 protein levels in leukemic B-cells.

C/EBPβ is a regulator of IL-6, a cytokine that promotes B cell proliferation and is involved in plasma cell differentiation (Jego et al., 2001). IL-6 has also been shown to promote the growth of malignant B cells in PEL, MCD, and multiple myeloma (Asou et al., 1998; Foussat et al., 1999; Klein et al., 1995; Oksenhendler et al., 2000). Translation of C/EBPβ mRNA leads to the production of three separate protein isoforms: two separate liver-enriched transcriptional activator proteins (LAP-1 and -2) and the negative repressor liver inhibitory protein (LIP). Regulation of IL-6 expression by C/EBPβ occurs by two separate mechanisms, transcriptional activation of the IL-6 promoter by LAP and transcriptional repression by LIP (Zahnow et al., 1997). Deregulated IL-6 in C/EBPβ knockout mice promotes the development of B cell lymphomas identical to MCD, indicating that C/EBPβ is directly linked to IL-6 dependent B-cell lymphomagenesis (Screpanti et al., 1996; Screpanti et al., 1995). SHIP1 also negatively regulates IL-6 expression in hematopoietic cells, but only one study has examined this function in B cells (Khaled et al., 1998). The ability of miR-155 to target two regulators of IL-6 expression and signaling, suggests that one component of miR-155's oncomir activity may be through deregulated IL-6 activity. Because IL-6 also plays an essential role in KSHV associated PEL and MCD, IL-6 deregulation initiated by KSVH miR-K12-11 hijacking of miR-155 in these malignancies is an intriguing premise.

Subsequent studies have also identified and validated the following miR-155 targets: SMAD2, SOCS1, Ets-1, and Meis1 (Ceppi et al., 2009; Jiang et al., 2010; Louafi et al., 2010; Lu et al., 2008; Romania et al., 2008). From these targets only the tumor suppressor SOCS1 has been implicated in tumorigenesis, with the other targets playing roles in cell signaling and differentiation pathways in macrophages and dendritic cells. While these target genes are also expressed in B cells, the impact of their regulation by miR-155 has not been reported. From the increasing amount of validated miR-155 targets, it is apparent that miR-155 exerts its function by regulating a large set of gene targets, leading to the modulation of a variety of B cell development, pro-growth, and anti-apoptotic pathways. It still remains to be seen if these targets involved in normal cell function are also involved in promoting tumorigenesis. Nonetheless, miR-155 has a heavy impact on the regulation of the cellular transcriptome during hematopoiesis, suggesting that miR-K12-11 hijacking of miR-155 function may be an invaluable tool for KSHV during B cell infection.

Does MiR-K12-11 Share a Homologues Function with MiR-155?

Based upon miR-155's physiological function in B cell activation and differentiation, as well as its oncogenic potential, we hypothesize that miR-K12-11 can play a similar role during KSHV B cell infection, thereby contributing to PEL and MCD pathogenesis and potential transformation. To investigate the oncogenic potential of miR-K12-11 as a functional mimic of miR-155, I have developed an *in vivo* approach discussed in Chapter 2. Because miRNA function is dictated by their targets, I have used a combination of *in vitro* approaches discussed in Chapter 2 and 3, to identified overlapping miR-155 and miR-K12-11 targets whose dysregulation during B cell differentiation might directly contribute to KSHV pathogenesis. The ability of miR-K12-

11 to directly inhibit B cell differentiation is investigated using *in vitro* B cell models of plasma cell differentiation discussed in Chapter 3. Lastly, in Chapter 4, I will discuss ongoing studies to examine how miR-K12-11 manipulates IL-6 production to promote KSHV pathogenesis.

Table 1-1. KSHV miRNA targets

miRNA	Target	Function
miR-K12-11	Host BACH1	Transcriptional regulator
miR-K12-7	Host MICB	NK cell ligand
miR-K12-3 miR-K12-7 miR-K12-11	Host C/EBPβ (LIP)	Inhibits IL6 and IL10 expression
miR-K12-5 miR-K12-9 miR-K12-10b	Host BCLAF1	Promotes Lytic reactivation
miR-K12-1 miR-K12-3-3p miR-K12-6-3p miR-K12-11	Host THBS1	Tumor Suppressor
miR-K1	Host p21	Cell cycle inhibitor
miR-K1	Host IκBα	Inhibits NF-κβ
miR-K10a	Host TWEAKR	Tumor necrosis factor receptor
miR-K12-11	Host IKKε	Interferon signaling molecule
miR-K12-4-5p	Host Rbl2	Transcriptional repressor
miR-K3	Host NFIB	Transcriptional activator
miR-K5 miR-K9* miR-K12-7-5p	Viral RTA	Master lytic switch

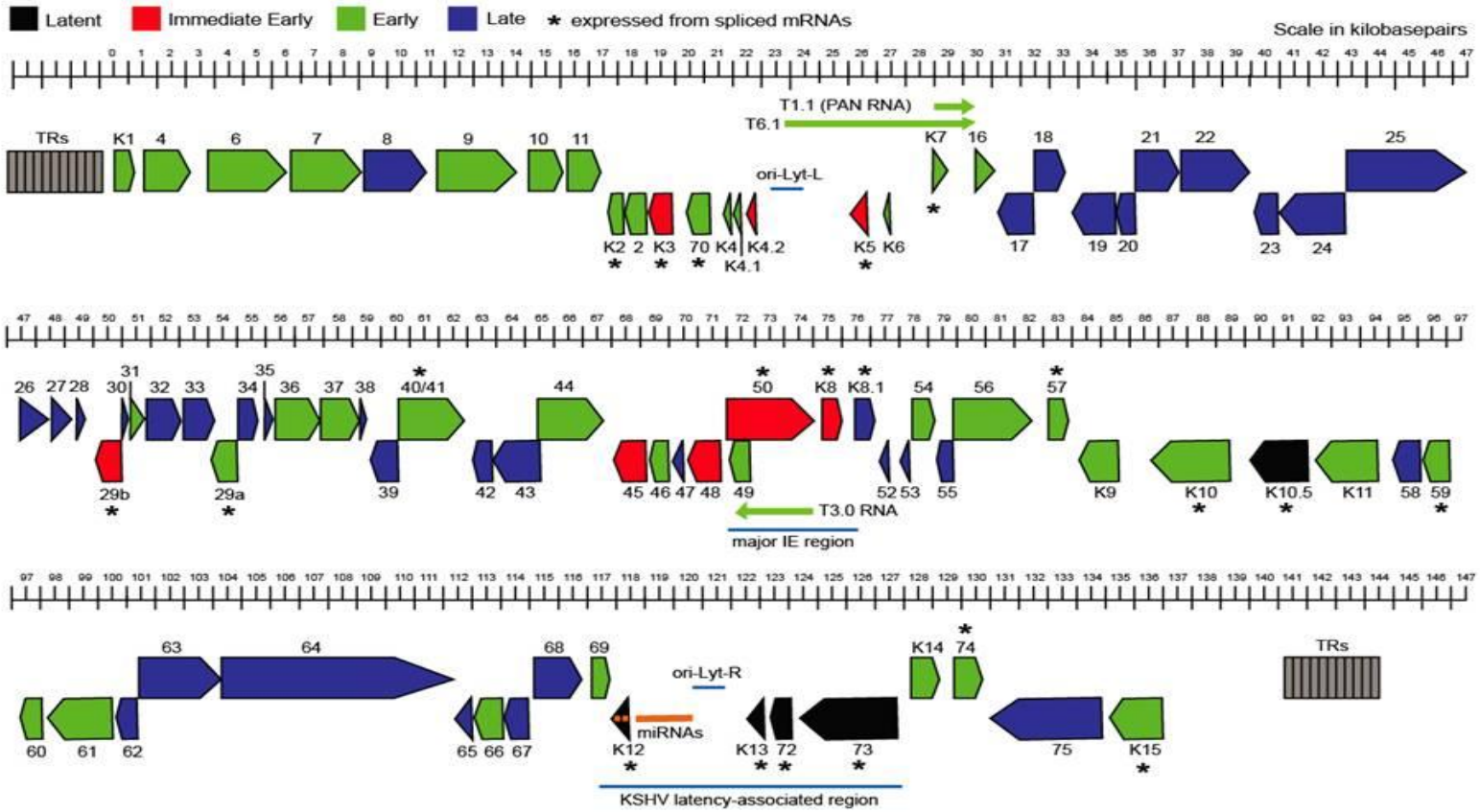

Figure 1-1. The KSHV Genome. Open reading frames (ORFS) are labeled in color based on their expression pattern during latent, immediate early, early, or late infection. The KSHV latency-associated region (KLAR) is underlined in blue and the miRNA genes are labeled in orange.

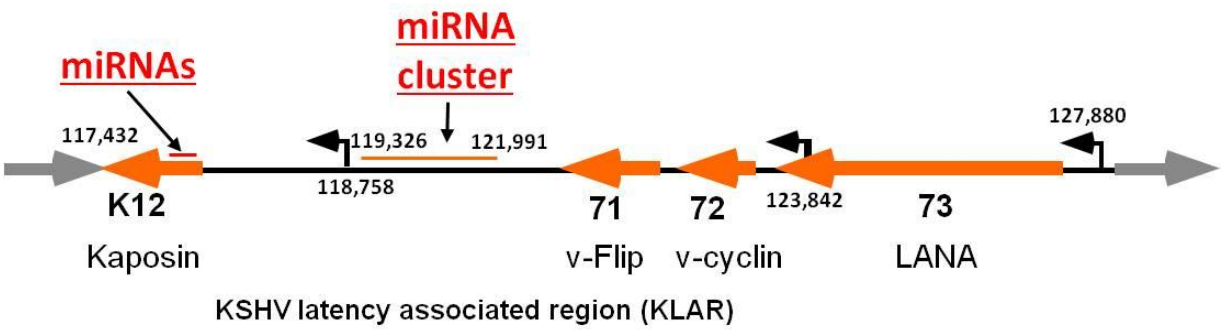

Figure 1-2. KSHV miRNAs are encoded in the KSHV latency associated region (KLAR). The latent genes in KLAR are in orange with the direction of latent transcription denoted by orange arrows. Latent promoters are indicated by the black directional arrows. The miRNA cluster contains 10 miRNA genes and downstream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

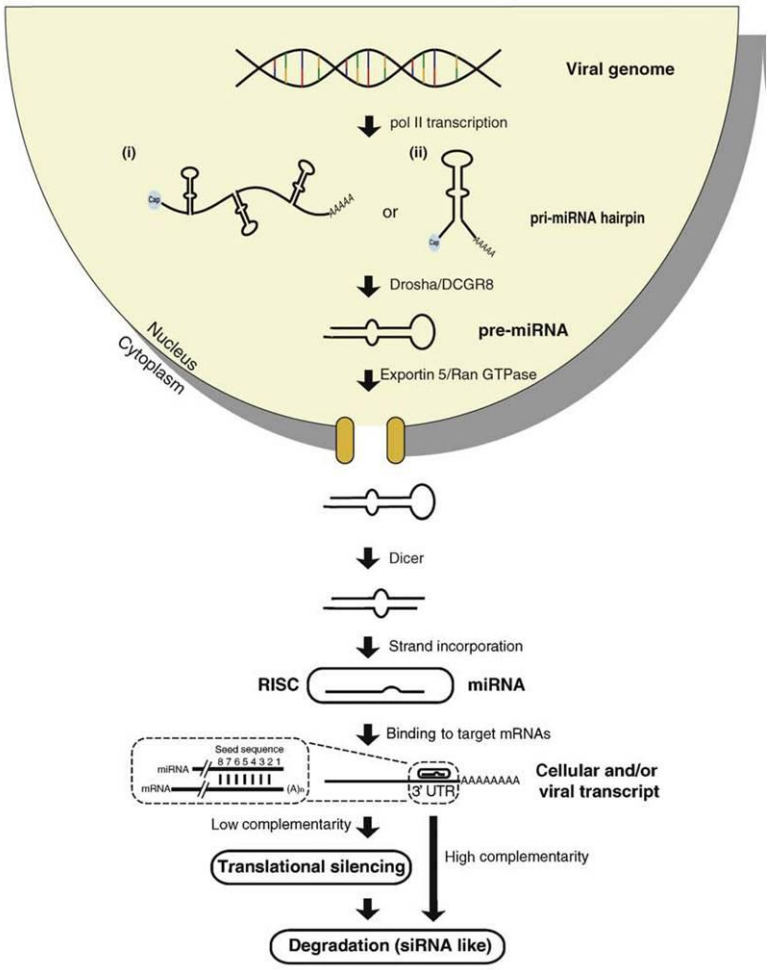

Figure 1-3. Biogenesis of miRNAs. Genes encoding miRNAs are generally transcribed from polII promoters. The majority of miRNAs are encoded in introns, but a small percentage are encoded in exons of protein coding genes. MiRNA genes can occur either as (i) clusters of multiple hairpins or as a (ii) single hairpin structure. The hairpins in primary transcripts (pri-miRNAs) are recognized by Drosha/DGCR8, a RNase III type endonuclease, which cleaves off the 5' and 3' ends, leaving a two nucleotide 3' overhang. The 60-80 nt hairpin, termed pre-miRNA, is rapidly exported from the nucleus to the cytoplasm via the Exportin5/RAN-GTPase pathway. The pre-miRNA is now recognized by a cytoplasmic RNase III type endonuclease, Dicer, which is also known to cleave dsRNA to create siRNA. Dicer cleaves off the bulged end of the hairpin now forming a short dsRNA with each end having a two nucleotide 3' overhang. The final step in miRNA biogenesis is the incorporation of one strand of the short RNA duplex into the RNA Induced Silencing Complex (RISC) to form a mature miRNA. Both strands can be incorporated into RISC and as a consequence many miRNA genes encode two mature miRNAs. Once the mature miRNA is incorporated into RISC, it targets the 3' UTR of mRNAs that contain complementary sequences. It has been observed that positions 2-8 of the miRNA are most important for targeting of mRNAs; this site is referred to as the miRNA seed sequence.

CHAPTER 2
A KSHV ENCODED ORTHOLOG OF MIR-155 INDUCES HUMAN SPLENIC B-CELL EXPANSION IN NOD/LTSZ-SCID IL2Rγ^{NULL} MICE

MicroRNAs (miRNAs) are small non-coding RNAs, 22-24 nucleotides in length, that mediate post-transcriptional gene repression by binding to the 3' untranslated region (UTR) of target mRNAs (Bartel, 2009). MiRNAs are expressed by a diverse range of organisms which include all metazoa and many plant species (Grimson et al., 2007). Functionally, miRNAs are key regulators of many biological processes including but not limited to embryonic development, hematopoiesis, immunity, and apoptosis. Their importance in regulating these processes is further underscored by their association with oncogenesis; for example, aberrant expression of miR-155 and members of the miR-17-92 family contribute to tumor formation in multiple types of leukemia and lymphomas (Garzon et al., 2008).

Recently, DNA viruses were found to encode miRNAs, including all three families of herpesviruses (α, β, and γ) (for review see (Boss et al., 2009)). Our group and others identified that the gammaherpesvirus KSHV, encodes a total of 12 miRNA genes all located within the KSHV latency associated region (KLAR) (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005). KSHV is lymphotropic, establishes latency in B-cells (Whitby et al., 1995), and is associated with the vascular tumor KS and two B-cell lymphoproliferative malignacies: primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Cesarman and Knowles, 1999; Chang et al., 1994; Du et al., 2002; Soulier et al., 1995). The majority of cells in these malignancies are latently infected, and during this stage the viral genome expresses only a limited number of genes, including the viral miRNAs (Dittmer et al., 1998;

Staskus et al., 1997). KSHV latent proteins regulate cellular pathways to inhibit apoptosis, induce cellular proliferation, and modulate cytokine responses, but the roles of KSHV miRNAs in pathogenesis are still being characterized (for review see (Dourmishev et al., 2003)). Insights into the pathogenic nature of these viral miRNAs have been provided by findings that they target host genes involved in tumorigenesis, cellular differentiation, immunity and apoptosis (Hansen et al., 2010; Nachmani et al., 2009; Qin et al., 2010; Samols, 2007; Ziegelbauer et al., 2009).

The most essential parameter for miRNA regulation of mRNA expression is complementary base pairing between the miRNA 'seed' sequence (5' nucleotides 2-7) and the target transcript (Bartel, 2009). Recently, we and others reported that KSHV miR-K12-11 shares 100% seed sequence homology with the human oncomir miR-155 and can regulate an overlapping set of genes in cell lines engineered to express miR-155 or miR-K12-11 (Gottwein et al., 2007; Skalsky et al., 2007). This was an important finding because miR-155-dependent regulation is important during hematopoiesis of different lineages, including B-cells (for review see (Baltimore et al.)), and deregulated miR-155 expression has been implicated in the formation of B-cell tumors (Costinean et al., 2006). In addition to KSHV, the oncogenic avian alphaherpesvirus, Marek's disease virus (MDV), also encodes a miRNA (mdv1-miR-M4) that shares seed sequence homology with miR-155 and, like miR-K12-11, is capable of regulating an overlapping set of miR-155 mRNA targets (Morgan et al., 2008; Zhao et al., 2009). Moreover, *in-vivo* functional analysis of mutant MDV viruses which contain a non-functional or deleted miR-M4 revealed that this miRNA plays an essential role in the induction of T-cell lymphomas in birds (Zhao et al., 2011). Interestingly, two separate viruses that

cause B-cell lymphomas; Epstein-Barr virus (EBV), a transforming human gammaherpesvirus closely related to KSHV, and the oncogenic retrovirus, reticuloendotheliosis virus strain T (REV-T), do not encode miR-155 orthologs but induce miR-155 expression during infection (Bolisetty et al., 2009; Cameron et al., 2008; Gatto et al., 2008; Jiang et al., 2006; Mrazek et al., 2007). Furthermore, a recent study found that inhibiting miR-155 function in two EBV positive B-cell lines resulted in decreased proliferation and increased apoptosis, providing evidence that miR-155 plays an important role during B-cell immortalization (Linnstaedt et al., 2010). While these studies have confirmed the oncogenic potential of miR-155 and miR-M4 during viral infection, the miRNA targets responsible for these phenotypes have not been reported.

Based on the roles of miR-155 and its ortholog miR-M4 in virally induced immortalization and lymphomagenesis we hypothesize that KSHV miR-K12-11 also plays a similar role in promoting KSHV pathogenesis. To directly address this, we examined the effects of ectopic miR-K12-11 and miR-155 expression in human hematopoietic stem cells (HSCs) during immune reconstitution using the NOD/LtSz-scid IL2Rγ^{null} mouse model. This is the first *in vivo* study using a humanized mouse model to examine the function of miR-K12-11 during hematopoiesis. In brief, human cord blood (CB) derived CD34+ progenitors were retrovirally transduced with miRNA/GFP expression vectors and transplanted into sublethally irradiated mice. FACS and histology results show that ectopic expression of either miR-K12-11 or miR-155, leads to a significant expansion of the hCD19+ B-cell population in the spleen. To gain further insight into the mechanisms contributing to this expansion we analyzed RNA from harvested splenocytes for expression of validated miR-155 targets involved in

lymphomagenesis and B-cell development and found that CCAAT enhancer-binding protein β (C/EBPβ), a negative regulator of IL-6, is repressed (Costinean et al., 2009). Moreover, inhibiting miR-K12-11 function with specific antagomirs in two separate PEL cell lines (BCBL1 and BC3) resulted in derepression of C/EBPβ. These data suggest that miR-K12-11 contributes to human B-cell expansion in part by regulating the miR-155 target C/EBPβ and provides further evidence that this miRNA plays an important role in promoting KSHV B-cell pathogenesis.

Results

Transduction of human CB CD34+ cells with miR-K12-11 and miR-155 expressing foamy virus vectors and their engraftment into NOD/LtSz-scid IL2Rγ^{null} mice. To ectopically express the miRNAs in human CB CD34+ progenitors, we constructed foamy virus vectors that contain miR-K12-11 or miR-155 pri-miRNA sequences downstream of EGFP (Figure 2-1A). MiRNA expression from these vectors was analyzed in 293 cells by performing luciferase reporter assays, as previously described (Skalsky et al., 2007). Transfection of the miR-K12-11 or miR-155 expression vectors resulted in a dose-dependent inhibition of luciferase activity, while transfection of a control vector did not, confirming that miR-K12-11 and miR-155 pre-miRNAs are efficiently processed into mature miRNAs (Figure 2-1C).

Human CB CD34+ progenitors were retrovirally transduced and monitored for GFP expression in colony forming assays. GFP expressing colonies were detected 14 days later indicating successful transduction *in vitro*. For immune reconstitution, 2 x 10^5 transduced CB progenitors were transplanted by tail vein injection into groups of sublethally irradiated NOD/LtSz-scid IL2Rγ^{null} mice (8 mice for each miRNA and 4 for

EGFP vector control). At 14 weeks post reconstitution bone marrow (BM) and spleen were harvested from mice for fluorescence-activated cell sorting (FACS) and histological analysis. GFP expression was detected in hCD45+ leukocytes harvested from both the bone marrow and spleen, indicating successful human hematopoietic engraftment of transduced cells in all mice (Figure 2-2).

Ectopic expression of miR-K12-11 and miR-155 in cells harvested from BM and spleen. To validate miR-K12-11 and miR-155 expression in the BM and spleen of engrafted mice total RNA was analyzed by stem-loop qRT-PCR assays. As expected, miR-K12-11 was only detected in the BM and spleen of miR-K12-11 engrafted mice (Figure 2-3A), while ectopic miR-155 expression was highest in the BM and spleens of miR-155 engrafted mice (Figure 2-3B). Interestingly, the relative increase in ectopic miR-155 expression in the spleen was higher (1 - 1.5 fold) than the increase detected in the BM (0.4 - 1.2 fold), possibly indicating that the majority of cells ectopically expressing miR-155 had already migrated to the spleen at this time point. We next compared the absolute levels of miR-K12-11 expression in splenocytes (hCD19+ GFP-positive) versus PEL cells. MiR-K12-11 miRNA expression was at similar or lower levels than those observed in BCBL1 (Figure 2-3C). These data confirm ectopic expression of miR-K12-11 and miR-155 in the engrafted mice and furthermore, demonstrate that we are not overexpressing these miRNAs in our model system.

Expression of either miR-155 or miR-K12-11 does not affect cell lineage populations in the bone marrow at 14 weeks post-transplantation. To ask whether ectopic expression of miR-155 or miR-K12-11 affects hematopoiesis in the bone marrow we performed cell lineage analysis by FACS. Results indicated that the

majority of cells in all mice were human CD45+ leukocytes indicating successful engraftment. Although we observed a modest increase of hCD45+ leukocytes in miR-K12-11 (80.2 ± 10.6%) and miR-155 (83.7 ± 4.5%) expressing mice when compared to vector controls (70.8 ± 18.3%), these differences were not statistically significant across all animals (Figure 2-4A).

We further characterized the various subpopulations of human leukocytes based on cell surface expression of hCD19 (B-cells), hCD33 (myeloid cells), and hCD3 (T-cells) (Figure 2-4 B, C, and D). The hCD19+ B-cell population represented the predominant lineage with higher levels found in mice expressing miR-K12-11 (61 ± 12.6%) and miR-155 (62.1 ± 4.9%) as compared to the vector control (52.2 ± 17.4%), but again this trend was not statistically significant across all animals (Figure 2-4B). In contrast to the large number of hCD19+ B-cells in the BM, the fraction of hCD33+ myeloid cells in the miR-K12-11 (14 ± 5.7%), miR-155 (13.4 ± 3%), and empty vector control mice (12.7 ± 3.1%) were much lower, regardless of miRNA expression (Figure 2-4C). Across all animals we detected less than 1% of hCD3+ T-cells (Figure 2-4D). Except for the modest, but non-significant, increase of hCD45+ leukocytes and hCD19+ B-cell populations in the miR-K12-11 and miR-155 expressing mice, these values represent a normal distribution of hematopoietic cell lineages as previously reported after engraftment of human CB CD34+ progenitors into NOD/LtSz-scid IL2Rγ^{null} mice (Giassi et al., 2008; Shultz et al., 2005).

Because miR-155 has been implicated in B-cell development (Costinean et al., 2009; Costinean et al., 2006), we also analyzed B-cells for expression of CD10 (B-cell precursors) and surface IgM (mature B-cells) (Figure 2-5). In all animals the majority of

hCD19+ cells expressed CD10+ [miR-K12-11 (98.5 ± 0.9%), miR-155 (97.5 ± 1.0%), and vector control (97.7 ± 1.2%)] compared to lower levels of IgM expression [miR-K12-11 (66.2 ± 11.2%), miR-155 (55.7 ± 11.6%), and vector control (63.5 ± 11.6%)]. These data indicate that the majority of hCD19+ B-cells in the BM represent an immature phenotype whose differentiation was not affected by ectopic miRNA expression.

Ectopic expression of miR-K12-11 and miR-155 induces B-cell proliferation in the spleen. To further evaluate human hematopoietic development in the engrafted mice we removed the spleens for histology and harvested splenocytes for cell lineage analysis by FACS. Results indicated a significant increase of hCD45+ leukocytes in the miR-K12-11 (49.6 ± 8.7%) and miR-155 (46.3 ± 9.5%) expressing mice compared with the empty vector control (33.6 ± 5.7%) (Figure 2-6A). Furthermore, splenocytes were significantly enriched for hCD19 (B-cells) in the miR-K12-11 (45.7 ± 12.6%) and miR-155 (42.6 ± 10.1%) expressing mice compared to the vector control (29.3 ± 6.1%) (Figure 2-6A). In contrast, the hCD33+ monocyte and hCD3+ T-cell populations were not significantly altered in the presence of miRNA expression (Figure 2-6A). The increased percentages observed in the hCD45+ and hCD19+ populations were due to an increase in the absolute cell numbers for these populations and not a reduction in the absolute cell numbers of the hCD33+ and hCD3+ populations (data not shown). Based on these observations the increased hCD45+ leukocyte counts in the spleen are caused by an expansion of the hCD19+ B-cell population. This was further supported by the observation that the percentage of GFP-positive miRNA expressing cells in the hCD45+ and hCD19+ populations represented a significantly higher fraction of the total cell population in mice expressing miR-K12-11 (14.4 ± 5.2% CD45+ and 14.3 ± 4.0%

CD19+) and miR-155 (17.0 ± 5.4% CD45+ and 17.1 ± 5.4% CD19+) as compared to the empty vector control (6.5 ± 0.9% CD45+ and 7.9 ± 1.2% CD19+) (Figure 2-7). Interestingly, there was also an increase in the GFP-negative hCD45+ and hCD19+ populations in mice ectopically expressing miR-K12-11 or miR-155 but this increase was not statistically significant (data not shown). Together these data show that ectopic miR-K12-11 and miR-155 expression during hematopoiesis in NOD/LtSz-scid IL2Rγ^{null} mice lead to a marked increase in B-cell proliferation within the spleen.

Next, we asked whether the observed expansion of hCD19+ B-cells in the spleen was due to increased frequencies of B-cell subsets expressing CD10 or surface IgM. In all animals, regardless of ectopic miRNA expression, the hCD19+ B-cell population was significantly enriched for IgM expression [miR-K12-11 (84.8 ± 4.4%), miR-155 (87.9 ± 4.1%), and vector control (88.1 ± 3.4%)] indicating that the majority of cells had differentiated into a more mature phenotype after migrating from the BM to the spleen (Figure 2-8A). Furthermore, when hCD19+ cells were gated for GFP (miRNA expressing) and analyzed for IgM expression there was no significant difference between the groups [miR-K12-11 (86.9 ± 3.6%), miR-155 (86.7 ± 3.5%), and empty vector control (89.8 ± 3.1%)] (Figure 2-8B). Compared to IgM, expression of CD10 was lower in the hCD19+ B-cells, but again there was no difference between groups [miR-K12-11 (67.9 ± 6.6%), miR-155 (69.7 ± 8.1), and vector control mice (68.2 ± 8.8%)] (Figure 2-8C). Gating for GFP also revealed no significant difference in CD10 expression between the miR-K12-11 (67.3 ± 9.6%), miR-155 (73.6 ± 8.2%), and empty vector control mice (76.0 ± 7.1%) (Figure 2-8D). Together these data suggest that while ectopic expression of both miR-K12-11 and miR-155 had a significant effect on B-cell

proliferation, B-cell differentiation as assessed by the distribution of CD10 and IgM expressing cells was not affected in this model.

MiR-155 and miR-K12-11 expression leads to hCD19+ B-cell infiltrates in splenic red pulp. Histopathological examination of bone marrow from femurs and tibias after hematoxylin eosin (H&E) staining revealed no major differences in cellularity, with the majority of animals displaying large numbers of nucleated cells. We also found no significant differences in the hCD19+ B-cell population in the BM of mice when examined by immunohistochemistry (IHC) using an hCD19 antibody, which supports the FACS data. Initial gross analysis of the spleen did not indicate any abnormalities in weight or size in any of the mice examined. However, H&E and IHC staining of the spleen for hCD19+ B-cells confirmed the significant expansion of human B-cells in the miRNA expressing mice (Figure 2-9), as observed by FACS analysis (Figure 2-5A). Furthermore, we observed peculiar differences in the splenic localization of hCD19+ B-cells in the miRNA expressing mice. While the majority of B-cells from the empty vector control mice were localized interior to the periarteriolar lymphoid sheaths (PALS), reflecting normal spleen architecture, we observed large numbers of hCD19+ cells from the miR-K12-11 and miR-155 expressing mice infiltrating and expanding into the splenic red pulp regions outside the PALS (Figure 2-9). These B-cell infiltrates appear to disrupt the normal architecture of the PALS and may indicate either a homing defect or are a direct result of aberrant B-cell proliferation. Interestingly, a similar immunophenotype of splenic red pulp B-cell infiltrates was previously reported for studies where miR-155 was overexpressed in the Eμ-miR-155 transgenic mouse model (Costinean et al., 2009; Costinean et al., 2006).

C/EBPβ is targeted by miR-K12-11 in splenocytes and PEL cells. A number of miR-155 targets have previously been identified including C/EBPβ, a transcription factor involved in B-cell lymphomagenesis (Costinean et al., 2009). C/EBPβ is a negative regulator of IL-6, a cytokine associated with proliferation of KSHV infected B-cell malignancies (Asou et al., 1998; Foussat et al., 1999; Hassman et al., 2011; Oksenhendler et al., 2000; Sin et al., 2007). Hence, we investigated whether miR-K12-11 also targets C/EBPβ, thereby providing a possible mechanism for the observed splenic B-cell expansion.

The 3'UTR of C/EBPβ contains one putative binding site for both miR-K12-11 and miR-155 (Figure 2-10A). Previous studies have shown that miR-155 can directly target and repress reporter constructs containing portions of the C/EBPβ 3'UTR with the miR-155 binding site (Costinean et al., 2009; O'Connell et al., 2008; Yin et al., 2008). To test the ability of miR-K12-11 to target and repress C/EBPβ, we inserted the full length C/EBPβ 3'UTR into a reporter vector downstream of the firefly luciferase gene. Co-transfection of the C/EBPβ reporter construct with either miR-K12-11 or miR-155 expression vectors in 293 cells resulted in a 50% repression of luciferase activity compared to the no miRNA control, indicating that both miRNAs can target C/EBPβ (Figure 2-10B).

Next, we wanted to determine if ectopic miR-155 and miR-K12-11 expression correlated with reduced endogenous C/EBPβ mRNA levels in harvested splenocytes. Using qRT-PCR we found that C/EBPβ transcripts were reduced [miR-K12-11 (0.4 fold) and miR-155 (0.5 fold)] compared to empty vector control mice indicating that these miRNAs regulate C/EBPβ expression in our mice (Figure 2-10C). To investigate the

ability of endogenous miR-K12-11 to regulate C/EBPβ in PEL cells, we inhibited miR-K12-11 function with specific antagomirs. Inhibition of miR-K12-11, in two PEL cell lines (BCBL1 and BC3), resulted in moderate derepression of C/EBPβ mRNA levels [BCBL1 (0.25 fold) and BC3 (0.26 fold)] measured by qRT-PCR (Figure 2-10D). These analyses validate C/EBPβ as a miR-K12-11 target and suggest one possible mechanism to explain the observed splenic B-cell expansion.

Discussion

MiR-155 was one of the first described "oncomirs" (a miRNA with tumorigenic activity) based on its aberrant expression in B-cell lymphomas (Eis et al., 2005). Within this context, the finding that miR-K12-11 and miR-155 have identical seed sequences immediately lead to the hypothesis that miR-K12-11 could mimic miR-155, thereby contributing to KSHV tumorigenesis (Gottwein et al., 2007; Skalsky et al., 2007)

To determine whether miR-K12-11 can phenocopy miR-155 activity *in vivo*, we utilized the humanized NOD/LtSz-scid IL2Rγ^{null} mouse model. In summary, we demonstrate that ectopic expression, of miR-K12-11 or miR-155 lead to an increased expansion of human B-cells in the spleen. Furthermore, this increase was accompanied by B-cell infiltrates within the splenic red pulp, a phenotype which was previously described in miR-155 overexpressing mice using the Eμ-miR-155 transgenic mouse model (Costinean et al., 2009; Costinean et al., 2006).

This study describes the first phenotype for a KSHV-encoded miRNA in the context of human hematopoiesis and more specifically B-cell development. The ability of miR-155 to induce lymphoproliferative diseases when overexpressed in hematopoietic cells during differentiation has been previously documented in studies

using non-humanized mouse models (Costinean et al., 2006; O'Connell et al., 2008). Interestingly, the observed phenotypes in these studies differed depending on the type of progenitor cell, and mouse model used. MiR-155 overexpression in a B-cell restricted manner induced B-cell proliferation, while ubiquitous expression in adult murine HSCs induced deregulated myeloproliferation (Costinean et al., 2006; O'Connell et al., 2008) suggesting that miR-155 plays a role in regulating several differentiation pathways during hematopoiesis (for review see (Baltimore et al.)).

In our NOD/LtSz-scid IL2Rγ^{null} mouse model, ectopic miR-155 or miR-K12-11 expression, but not overexpression, in human CB CD34+ progenitors induced a splenic expansion of mature B-cells without a marked inhibition of myeloid lineages. Our observations resemble the splenic B-cell proliferation reported in the Eμ-miR-155 transgenic mouse but do not correlate with the reduction of mature IgM+ B-cells seen in that model (Costinean et al., 2009; Costinean et al., 2006). We also observed no increase in myelopoeisis, which was previously reported during inflammatory responses and during ectopic expression of miR-155 in murine bone marrow-derived HSCs (O'Connell et al., 2009; O'Connell et al., 2008). In our model the absence of an increased B-cell population in the BM may suggest that the cells ectopically expressing either miR-K12-11 or miR-155 had already migrated from the bone marrow at this point of differentiation and/or that the miRNAs in our system might only be affecting later time points of differentiation in the spleen.

Since KSHV is a human pathogen, we chose to transduce human CB CD34+ progenitors and not murine BM derived adult HSCs. Furthermore, the context of our experiment was carried out under steady state conditions without the use of either

inflammatory inducers or IL-6, which has been shown to increase myelopoiesis and suppress lymphopoiesis at early stages of differentiation in the bone marrow (Nakamura et al., 2004). Importantly, in our system, miR-K12-11 and miR-155 were not overexpressed but expressed at levels similar to those in the PEL cell line BCBL1, eliminating potential off-target consequences due to miRNA oversaturation.

Although the consequences of miR-155 expression on the hematopoietic system vary depending on the model system used, our study clearly demonstrates that miR-K12-11 can phenocopy the lymphoproliferative activity of miR-155 during hematopoiesis *in vivo*. The ability to induce B-cell proliferation strongly indicates a role for miR-K12-11 in promoting KSHV lymphomagenesis and provides supporting evidence to previous studies in MDV, EBV and REV-T that targeting of the miR-155 regulatory pathway is conserved among transforming herpesviruses (Bolisetty et al., 2009; Linnstaedt et al., 2010; Lu et al., 2008; Morgan et al., 2008; Yin et al., 2008; Zhao et al., 2011; Zhao et al., 2009) .

To delineate the underlying molecular mechanisms contributing to the observed B-cell expansion/proliferation we searched for miR-155 targets that could also be regulated by miR-K12-11 in B-cell malignancies. Our search identified C/EBPβ as a potential candidate based on its regulation by miR-155 in B-cell lymphoproliferation (Costinean et al., 2009). We confirmed direct targeting of the C/EBPβ 3'UTR by miR-K12-11 using luciferase reporter constructs and correlated repression of C/EBPβ mRNA in splenocytes ectopically expressing miR-K12-11 or miR-155. Lastly, regulation of C/EBPβ in PEL cell lines was validated by inhibiting miR-K12-11 with antagomirs, leading to derepression of C/EBPβ mRNA.

C/EBPβ is a negative regulator of IL-6 and its deficiency in mice has been shown to induce a B-cell lymphoproliferative disorder that closely resembles human MCD, a malignancy closely associated with KSHV infection (Screpanti et al., 1995; Soulier et al., 1995). The development of MCD in C/EBPβ deficient mice has been linked to dysregulated IL-6 production (Screpanti et al., 1995); while the clinical presentation of KSHV associated MCD is correlated with high plasma levels of IL-6 and IL-10 (Oksenhendler et al., 2000; Yoshizaki et al., 1989). Both IL-6 and IL-10 are cytokines that function in an autocrine and paracrine fashion to promote proliferation and survival of B-cells, including PEL (Asou et al., 1998; Foussat et al., 1999; Hassman et al., 2011; Jego et al., 2001; Oksenhendler et al., 2000; Rousset et al., 1992; Sin et al., 2007). To our knowledge, there has been no reported correlation between KSHV B-cell lymphomagenesis and C/EBPβ repression but a recent study bioinformatically predicted that C/EBPβ could be targeted by multiple KSHV miRNAs, including miR-K12-11 (Qin et al., 2010). Qin et al also showed that these KSHV miRNAs induce IL-6 and IL-10 production in monocytes and macrophages but did not confirm that this was due to direct miRNA regulation of C/EBPβ. Because lack of C/EBPβ has been shown to lead to deregulated IL-6 in MCD, we believe that miR-K12-11 induces IL-6 expression in KSHV infected B-cells by repressing C/EBPβ, thereby promoting B-cell proliferation. The ability of IL-6 to stimulate B-cell proliferation may also explain the increase of GFP-negative CD19+ cells that we observed in our mice. Further studies are ongoing to determine the potential role of miR-K12-11 induction of IL-6 in B-cell proliferation. In this study we have shown that C/EBPβ is indeed a direct target of miR-K12-11 and

further establish a direct correlation between KSHV miRNA regulation of C/EBPβ and KSHV B-cell lymphomagenesis *in vivo*.

In addition to C/EBPβ, a number of other miR-155 targets that play roles in hematopoietic malignancies and B-cell function have been identified (Bolisetty et al., 2009; Gottwein et al., 2007; Lu et al., 2008; O'Connell et al., 2009; Rai et al., 2010; Skalsky et al., 2007; Teng et al., 2008; Yin et al., 2010). While we have identified one gene regulated by miR-K12-11 in both our mouse model and in PEL cells it is highly probable that this is not the only miR-155 gene deregulated by miR-K12-11 that contributes to KSHV B-cell lymphomas. Additional work is still needed to identify those targets which have functional relevance in KSVH associated malignancies.

During latency KSHV expresses a small set of viral genes including V-cyclin, a cyclin D homolog, V-Flip, a potent inducer of NFkB, LANA, a modulator of host gene expression, and Kaposin, which stabilizes cytokine mRNAs (for reviews see (Dourmishev et al., 2003)). While ectopic expression has unmasked limited transforming potential for each of these genes, *in vitro* KSHV infection of either lymphoid or endothelial cells rarely leads to outgrowth of transformed cells (Flore et al., 1998; Watanabe et al., 2003). Since all KSHV miRNAs and the above proteins are co-expressed during latency it is plausible that they work synergistically to deregulate host transcriptional networks promoting cell proliferation and transformation (Boss et al., 2009; Hassman et al., 2011). Here, we show that miR-K12-11 expression alone induces human B-cell proliferation in the context of hematopoiesis. Other KSHV miRNAs have been found to repress pro-apoptotic, anti-angiogenic, and immune stimulatory factors, thereby potentially contributing to lymphomagenesis, a notion that is

testable using our NOD/LtSz-scid IL2Rγ^{null} mouse model (Gottwein et al., 2007; Hansen et al., ; Nachmani et al., 2009; Qin et al., 2010; Samols, 2007; Skalsky et al., 2007; Ziegelbauer et al., 2009).

In summary, this *in vivo* study further validates miR-K12-11 as a functional mimic of miR-155. The discovery that miR-K12-11 can promote B-cell proliferation suggests a novel mechanism by which a KSHV miRNA contributes to lymphomagenesis. This work was published in October 2011.

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Materials and Methods

Cell culture. The 293T cell line (human embryonic kidney fibroblasts) was obtained from American Type Culture Collection (Rockville, Md.). Cryogenically preserved primary human cord blood CD34+ cells were purchased from StemCell Technologies, Vancouver, BC. The 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (100 µg/ml), penicillin (100 µg/ml), and 10% fetal calf serum. Human cord blood CD34+ cells were cultured for transduction in Iscove's modified Dulbescco's medium (IMDM) supplemented with 10% FBS containing 1ug/ml each human stem cell factor, human thrombopoietin (Tpo), human Flt-3 ligand, and IL-11 (Peprotech, Rocky Hill, NJ).

Foamy virus vector construction. To produce miR-155 or miR-K12-11 vector constructs we first amplified the miR-K12-11 or miR-155 miRNA sequences containing

a region of approximately 200nts surrounding each pre-miRNA hairpin from pCDNA3.1/V5/HisA expression vectors that were previously described (Samols, 2007). We inserted individual miRNA cassettes downstream of EGFP into an SFV-1 simian foamy virus vector backbone (pCCEGFPL) previously described (Gartner et al., 2009; Zucali et al., 2002).

Luciferase assays and reporter construction. MiRNA sensor vectors were created using the pGL3 promoter vector from Promega (http://www.promega.com). Synthetic oligonucleotides containing two complete complementary copies of a miRNA sequence separated by a 9-bp-long spacer were inserted into the 3'UTR of the luciferase gene upstream of the poly-adenylation signal as previously described (Skalsky et al., 2007). To construct a luciferase reporter plasmid containing the full length 3'UTR sequence of C/EBPβ, the following primers were first designed with Vector NTI (Invitrogen), the forward primer contained an Nde1 site and the reverse an Fse1 site: fwd 5'-CATATGGAACTTGTTCAAGCAGCTGC-3' and rev 5' GGCCGGCCGGCTTTG TAACCATTCTC-3'. PCR products were cloned into pCRII-TOPO (Invitrogen), excised, and inserted into the 3'UTR of pGL3 promoter at Nde1 and Fse1 sites. All constructs were confirmed by sequencing. 293 cells were co-transfected with luciferase reporter constructs, foamy virus vectors, and/or miRNA expression vectors in 24 well plates for 72 h using Mirus TransIT-293 reagent (Madison , WI) according to manufacturer's instructions. Luciferase activity was quantified using the Luciferase assay system (Promega) according to the manufacturer's protocols. Briefly, transfected 293 cells were lysed in cell culture lysis reagent (Promega), and 20% of

each cell lysate was assayed for firefly luciferase activity. Light units were normalized to *Renilla* luciferase, using a dual luciferase reporter kit (Promega).

Foamy virus production, human cord blood CD34+ cell transduction. To generate infectious viral particles we co-transfected 293T cells with the individual miRNA expression vectors and the packaging plasmid pCIenv previously described (Gartner et al., 2009). Transfections were carried out in T75 cell culture flasks (5 X 10^6 293T cells per flask) by the calcium phosphate method. Viral supernatants were harvested 4 days post-transfection and clarified by centrifugation at 5000 rpm for 20 min then by passaging through a 0.45 Am filter. The vector particles were further concentrated 100-fold by using the Apollo Centrifugal Spin Concentrators, 70 kDa (Orbital Biosciences, Topsfield, MA). The amounts of SFV-1 vector produced were titered on fresh 293T cells plated at a density of 2.5 X 10^4 per well in 24 well plates. Seventy-two hours after infection, cells were monitored and scored for GFP fluorescence under a microscope with UV light source. Transduction of CD34+ cells was carried out by spin-inoculation as previously described (Zucali et al., 2002). Briefly, 3 X 10^6 CD34+ cells (a heterogeneous mixture from two separate donors) were seeded into 15 wells of a 24 well Human Fibronectin plate (BD Biosciences, San Jose, CA) at a density of 1.5 X 10^5 cells per well. 24 hours after initial seeding, viral supernatant was added to cells at a multiplicity of infection of 50. The plates were then spun at 1200 rpm for 1.5 hours and the infection procedure was repeated 24 hours later. Following the last transduction, 2 X 10^5 transduced CD34+ cells were transplanted by lateral tail vein injection into each of 4–8 sublethally irradiated (250 rads from Cesium 137 source at 65.7 rads/minute) NOD/LtSz-scid IL2Rγ^{null} mice. For colony forming assays transduced

cells were plated in serum-free methylcellulose culture (Methocult 04236, StemCell Technologies, Vancouver, BC) in the presence of 1ug/ml each human Flt-3 ligand, human stem cell factor, human GM-CSF, human IL-3, and human erythropoietin (Epo) for 14 days.

Mice. NOD/LtSz-scid IL2Rγ^{null} mice were obtained from The Jackson Laboratory. All experiments involved male mice and were performed according to IACUC-approved protocols.

MiRNA detection and absolute quantification of miR-K12-11. RNA was extracted from samples using the RNA-Bee kit per manufacturer's instructions (AMS Biotechnology, Milton, UK). cDNA was synthesized from 10ng total RNA using the TaqMan MicroRNA Reverse Transcription Kit (AppliedBiosystems, Foster City, CA). To detect miR-155 and miR-K12-11 the TaqMan miRNA detection assay was run in triplicates using human miR-155 and KSHV miR-K12-11 TaqMan probes according to manufacturer's instructions (AppliedBiosystems, Foster City, CA). MiRNA relative expression was determined using the Applied Biosystems Relative Quantification (RQ) Manager Software v2.1 with human miR-16 set as the endogenous control. The absolute copy number of miR-K12-11 in both splenocytes and BCBL1 was calculated by using a standard curve of known quantities of a miR-K12-11 synthetic miRNA mimic (ThermoScientific, Lafayette, CO). To determine miR-K12-11 copy number in GFP-positive CD19+ splenocytes we assumed that 10 ng of total RNA equals 10,000 cells. Furthermore, we expressed the absolute copy number per GFP-positive CD19+ splenocyte by taking into account the percentage of GFP-positive CD19+ cells as determined by FACS.

Flow Cytometry Cell lineage analysis. GFP expression and phenotypic markers were analyzed by flow cytometry using a LSR-II cytometer and FacsDiva software (BD Biosciences, San Jose, CA). Fluorophor-conjugated monoclonal antibodies specific for human CD45, CD19, CD33, and CD3 (BD Biosciences, San Jose, CA BD) were used to stain RBC-depleted splenocytes and bone marrow cells. Background staining was determined using a murine monoclonal IgG1 isotype control (BD Biosciences, San Jose, CA).

Necropsy, histology and immunohistochemistry. Mice were necropsied and all tissues were evaluated for gross lesions. Portions of the spleen, liver, and femur were fixed in 10% buffered formalin for 18 to 24 hours, dehydrated, and embedded in paraffin. Sections were cut at 5 microns for routine hematoxylin and eosin (H&E) staining and 3 microns onto positively charged slides (Probe On Plus, Fisher Scientific, Springfield, NJ) for immunohistochemistry (IHC) against CD19, a marker for human B-lymphocytes. Deparaffinized tissue sections were subjected to heat-induced antigen retrieval by microwaving in citrate buffer solution (Antigen Unmasking Solution, Vector Laboratories, Burlingame, CA). The primary antibody for IHC was mouse monoclonal anti-human CD19 (BIOCARE Medical, LLC; Concord, CA) used at a dilution of 1:150. Sites of primary antibody binding were identified by high affinity immunocytochemistry STAT-Q (Innovex Biosciences; Richmond, CA) using a secondary antibody and strept-avidin-horseradish peroxidase. The chromagen was diaminobenzidine (DAB) with Mayer's hematoxylin counterstain.

Antagomir de-repression assays and real-time qRT-PCR analysis. For inhibition of miR-K12-11, 2'OMe RNA antagomirs, previously described (Skalsky et al.,

2007), were used. PEL cells ($1x10^6$) were transfected with 25nM of antagomir in 24 well plates using Mirus TransIT-TKO (9ul / 250ml total media). After 6 h of incubation cells were pelleted, transfection media was removed, and cells were plated in fresh RPMI 1640 supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin (Gibco) for 48 h before RNA was harvested. RNA from splenocytes, BC-3, and BCBL1 cells was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) in the presence of random hexamers according to the manufacturer's protocols. qPCR was carried out using an ABI StepOne Plus system along with ABI Fast SYBR (Applied Biosystems, Carlsbad California). Primers for C/EBPβ were designed across exon boundaries and were previously described (Lu et al., 2010c). Primer pair efficiencies for GAPDH, β-actin, and C/EBPβ were validated before analyzing for C/EBPβ expression. PCR signals were normalized to both GAPDH and β-actin to check for accuracy and reported as relative quantitation (RQ) values using StepOne software.

Statistics. All statistical analyses used a Student's two-tailed *t* test performed on Microsoft Excel software.

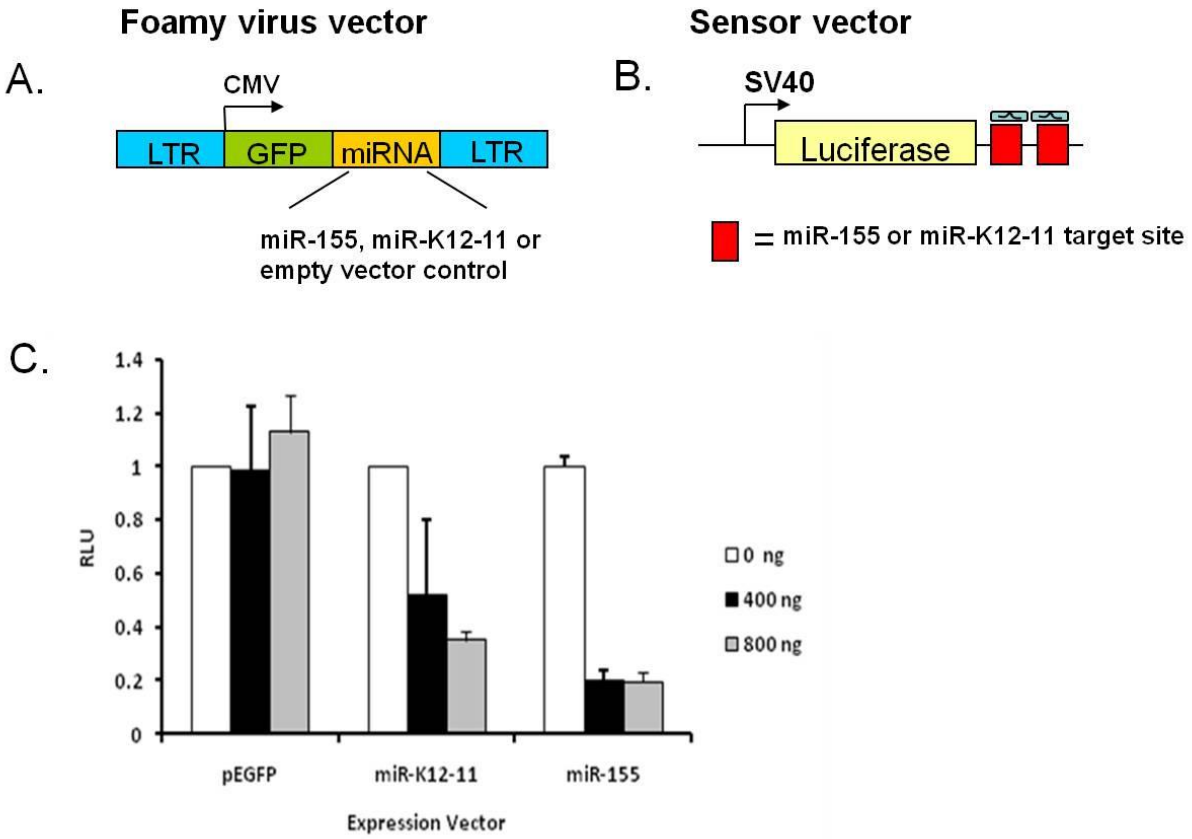

Figure 2-1. Foamy virus vectors express miR-K12-11, miR-155, or empty vector control. A. Foamy virus vectors were constructed by inserting the pri-miRNA sequence downstream of a GFP cassette and CMV promoter. B. Schematic of miRNA sensor vectors containing two perfectly complementary binding sites. C. MiRNA expression and sensor vectors were co-transfected into 293 cells and luciferase activity was measured 72 hours post-transfection. Results show that both miR-K12-11 and miR-155 expression vectors repressed luciferase activity >2 fold compared to no repression by the control.

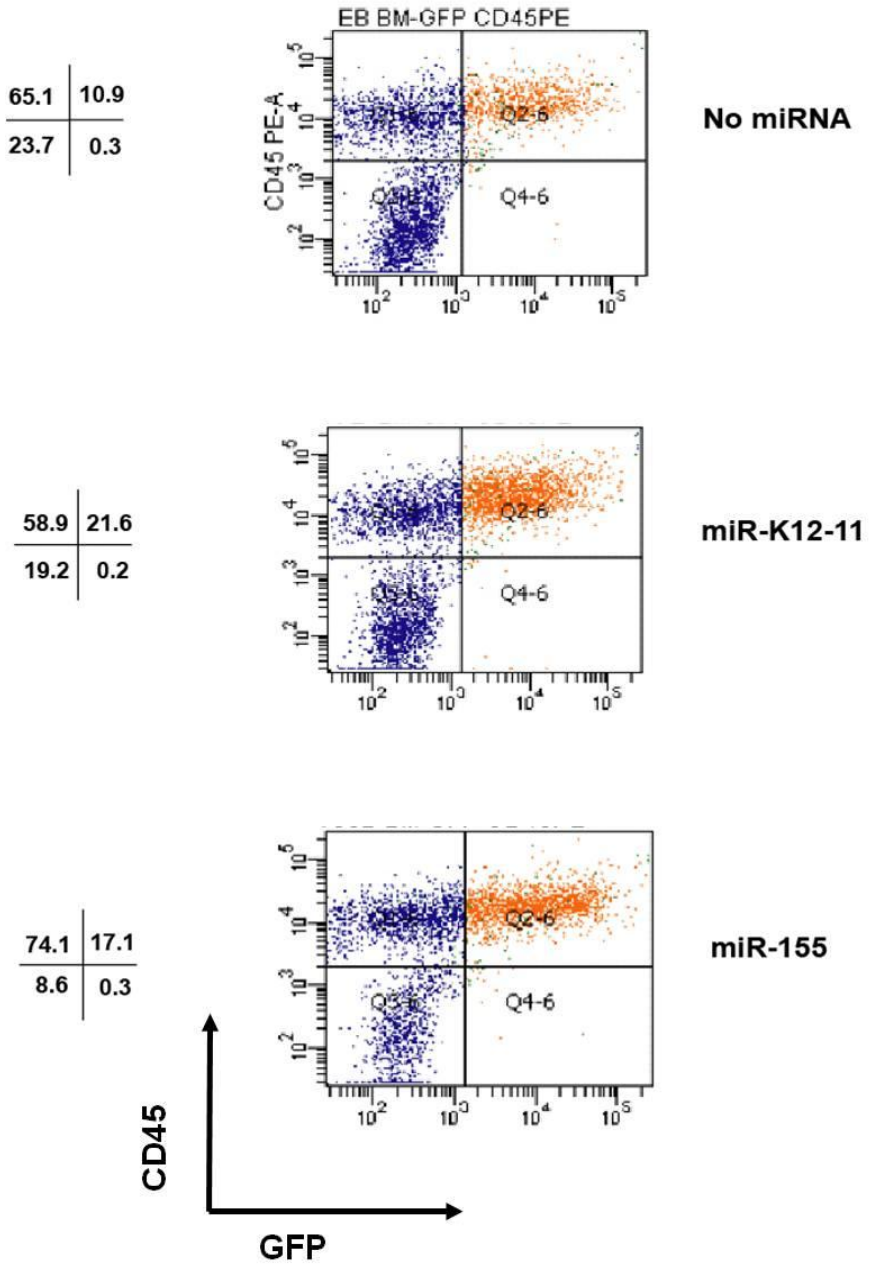

Figure 2-2. Engraftment of transduced CB CD34+ cells. Cells harvested from the bone marrow were analyzed by FACS using a human CD45-specific antibody. Human CD45+ cells were detected in all mice reconstituted with human CB CD34+ progenitors expressing either miR-K12-11, miR-155, or empty vector control. A large percentage of the CD45+ cells also expressed GFP, shown in the upper right quadrant of each histogram. Shown are representative dot plots for one animal from each group.

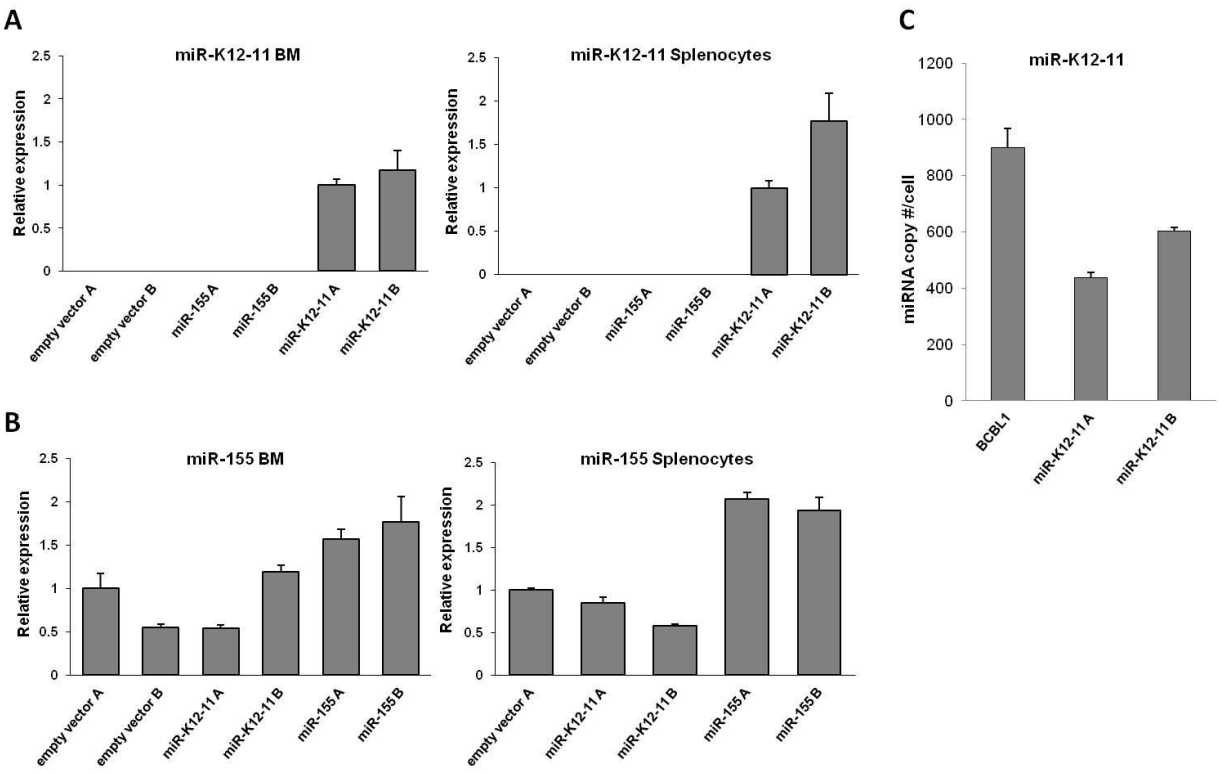

Figure 2-3. Ectopic miR-K12-11 and miR-155 expression in engrafted mice. A. Ectopic miR-K12-11 was only detected in the miR-K12-11 engrafted animals in both the BM and spleen. B. Ectopic miR-155 was detected above endogenous levels in the miR-155 engrafted animals in both the BM and spleen. C. Absolute miR-K12-11 copy number in the GFP-positive CD19+ splenocyte populations from engrafted mice is comparable to or lower than endogenous miR-K12-11 expression in the PEL cell line BCBL1 and therefore is not overexpressed.

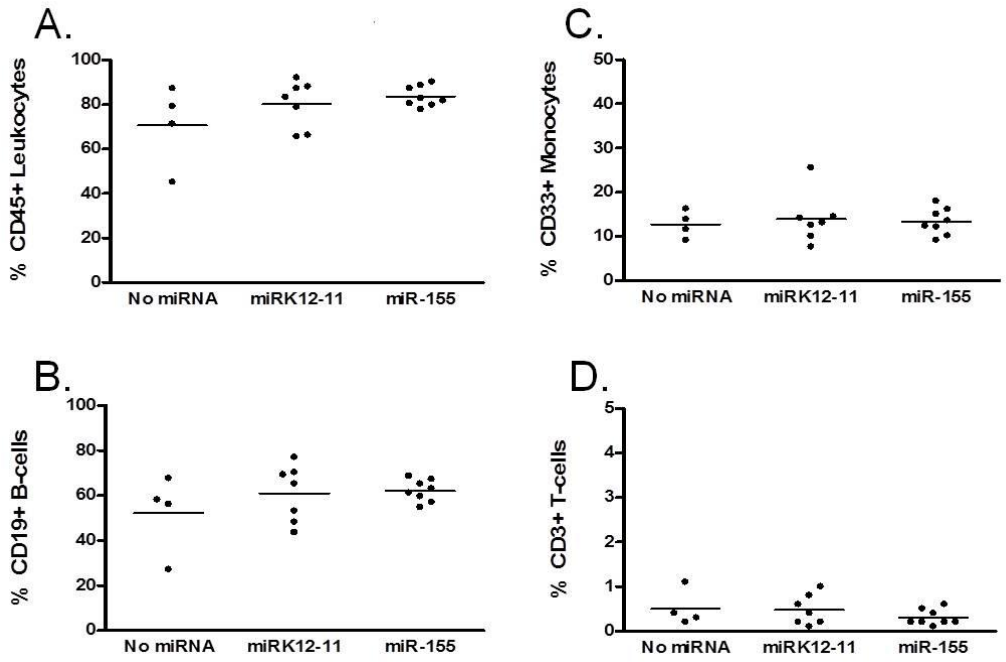

Figure 2-4. Cell lineage differentiation of human progenitors was not significantly altered by miRNA expression in the bone marrow. Cells harvested from the bone marrow of mice expressing empty vector (n=4), miR-K12-11 (n=7), and miR-155 (n=8) were stained with antibodies specific for human (A) CD45+ leukocytes, (B) CD19+ B-cells, (C) CD33+ monocytes, and (D) CD3+ T-cells and analyzed by FACS. Each dot represents FACS analysis of one animal from each group and the mean score for each group is shown as the solid horizontal line.

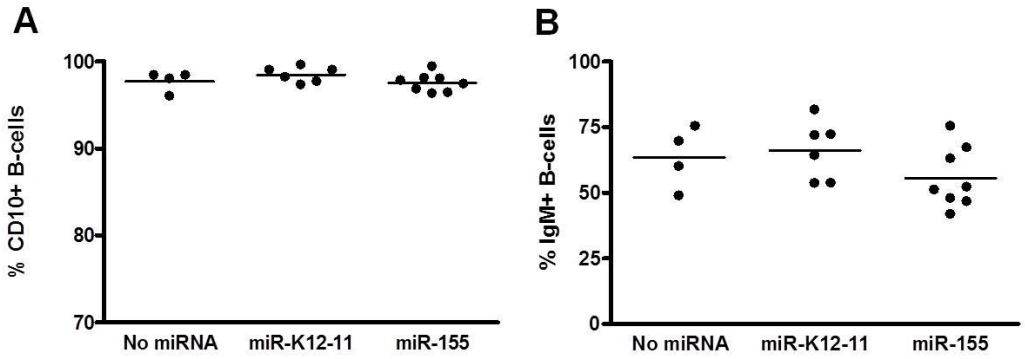

Figure 2-5. B cell subsets in the bone marrow are mostly CD10+ precursors. Cells harvested from the bone marrow of mice expressing empty vector (n=4), miR-K12-11 (n=6), and miR-155 (n=8) were stained with antibodies specific for human CD19+ and then analyzed for surface expression of (A) CD10 (precursor B cells) and (B) IgM (mature B cells). Each dot represents FACS analysis of one animal from each group and the mean score for each group is shown as the solid horizontal line.

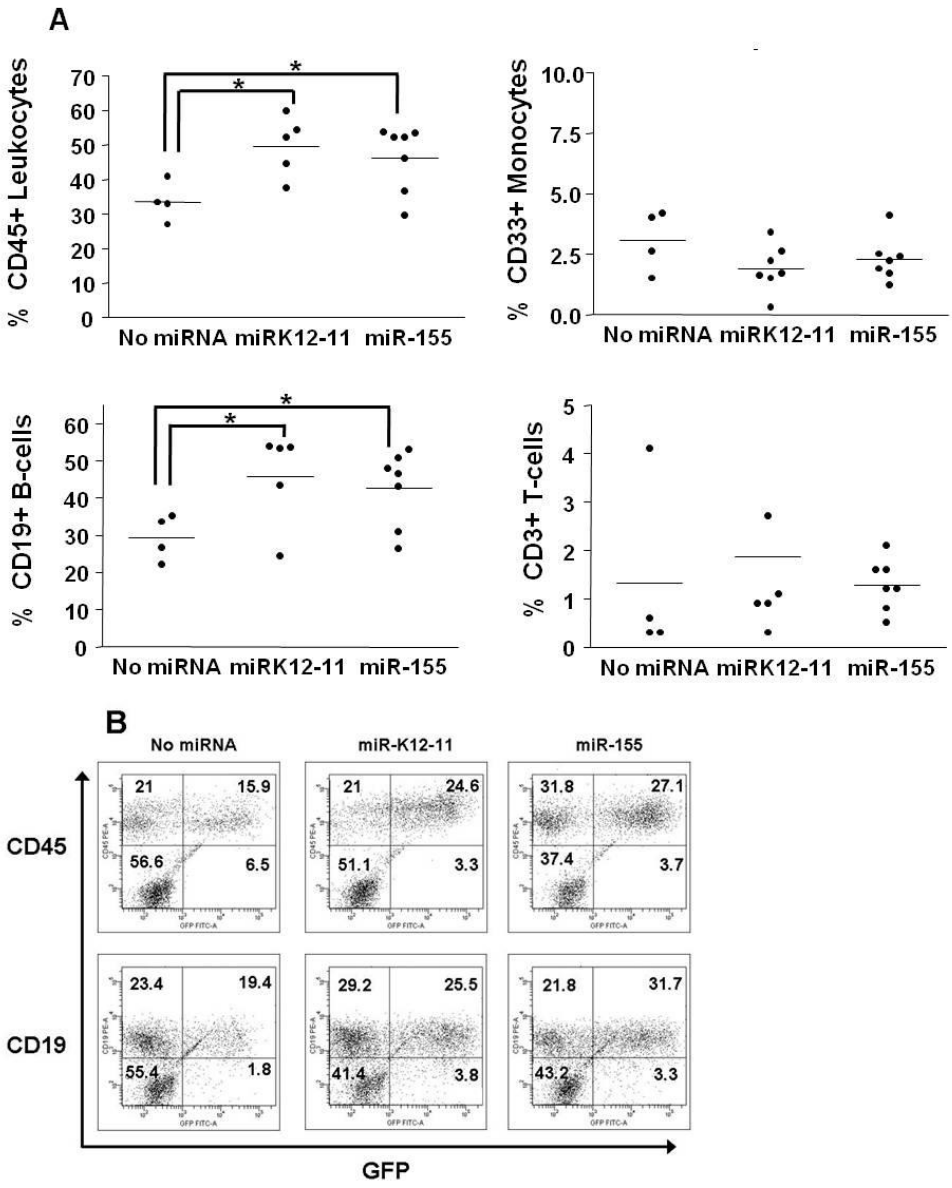

Figure 2-6. Ectopic expression of miR-K12-11 or miR-155 in human leukocytes during hematopoiesis leads to increased CD19+ B-cell expansion in the spleen. Splenocytes harvested from mice expressing empty vector (n=4), miR-K12-11 (n=5), and miR-155 (n=7) were stained with antibodies specific for human CD45+ leukocytes, CD19+ B-cells, CD3+ T-cells, and CD33+ monocytes and analyzed by FACS. A. The fraction of human CD45+ leukocytes and CD19+ B-cells was significantly higher (*$p<0.05$) in mice expressing either miR-K12-11 or miR-155 compared to empty vector control. No change was detected in the CD33+ monocyte or CD3+ T-cell populations when either miRNA was expressed. Each dot represents FACS analysis of one animal from each group and the mean score for each group is shown as the solid horizontal line. A p-value (*) of 0.05 or less after a Student's two-tailed t-test was considered statistically significant. B. Representative dot plots for flow cytometry analysis of splenocytes using hCD45+ and hCD19+ antibodies.

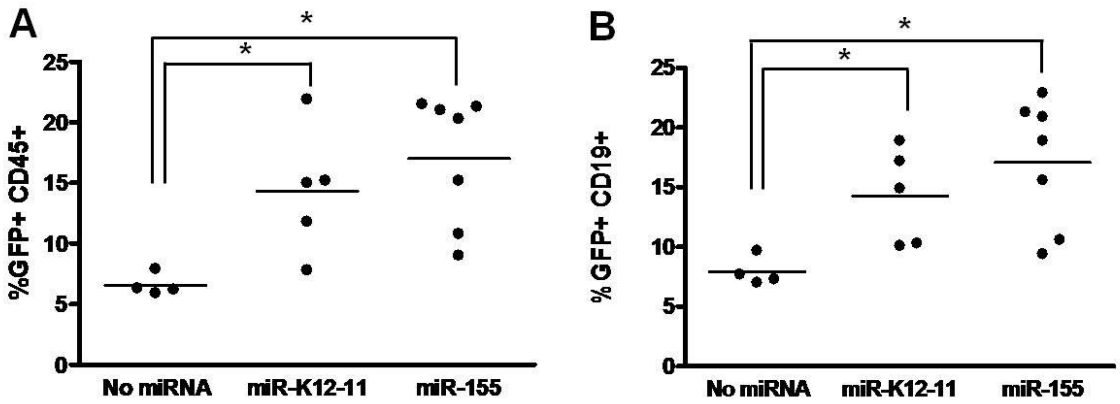

Figure 2-7. GFP-positive (miRNA expressing) accounted for the overall increase in human CD45+ leukocytes and CD19+ B-cells. Splenocytes harvested from mice expressing empty vector (no miRNA control, n=4), miR-K12-11 (n=5), and miR-155 (n=7) were stained with antibodies specific for human CD45+ leukocytes or CD19+ B-cells and analyzed for GFP-positive expression by FACS. A. The fraction of GFP-positive CD45+ human leukocytes was significantly higher (*$p<0.05$) in mice expressing either miR-K12-11 or miR-155 compared to empty vector control. B. The fraction of GFP-positive CD19+ human B-cells was significantly higher (*$p<0.05$) in mice expressing either miR-K12-11 or miR-155 compared to empty vector control. Each dot represents FACS analysis of one animal from each group and the mean score for each group is shown as the solid horizontal line. A p-value (*) of 0.05 or less after a Student's two-tailed t-test was considered statistically significant.

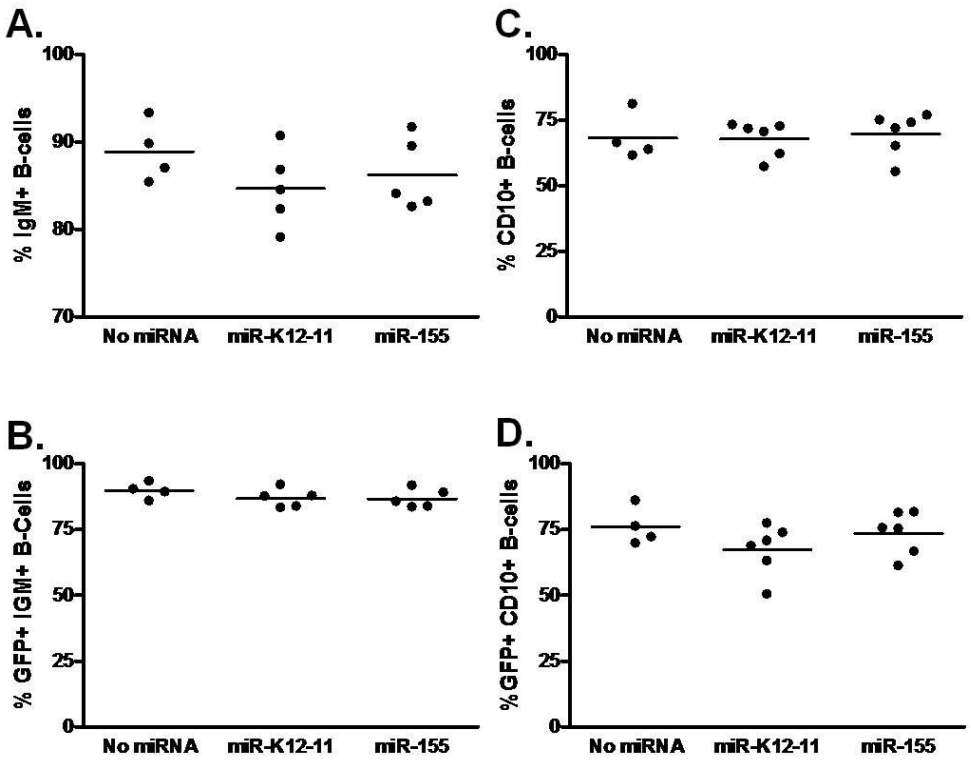

Figure 2-8. Ectopic expression of miR-K12-11 or miR-155 did not affect B cell differentiation in the spleen. Cells harvested from the spleens of mice expressing empty vector (n=4), miR-K12-11 (n=6), and miR-155 (n=8) were stained with antibodies specific for human CD19+, (A) IgM (mature B cells), and (C) CD10 (germinal center B cells). MiRNA expressing cells were gated by GFP expression and analyzed for surface expression of (B) IgM and (D) CD10. Each dot represents FACS analysis of one animal from each group and the mean score for each group is shown as the solid horizontal line.

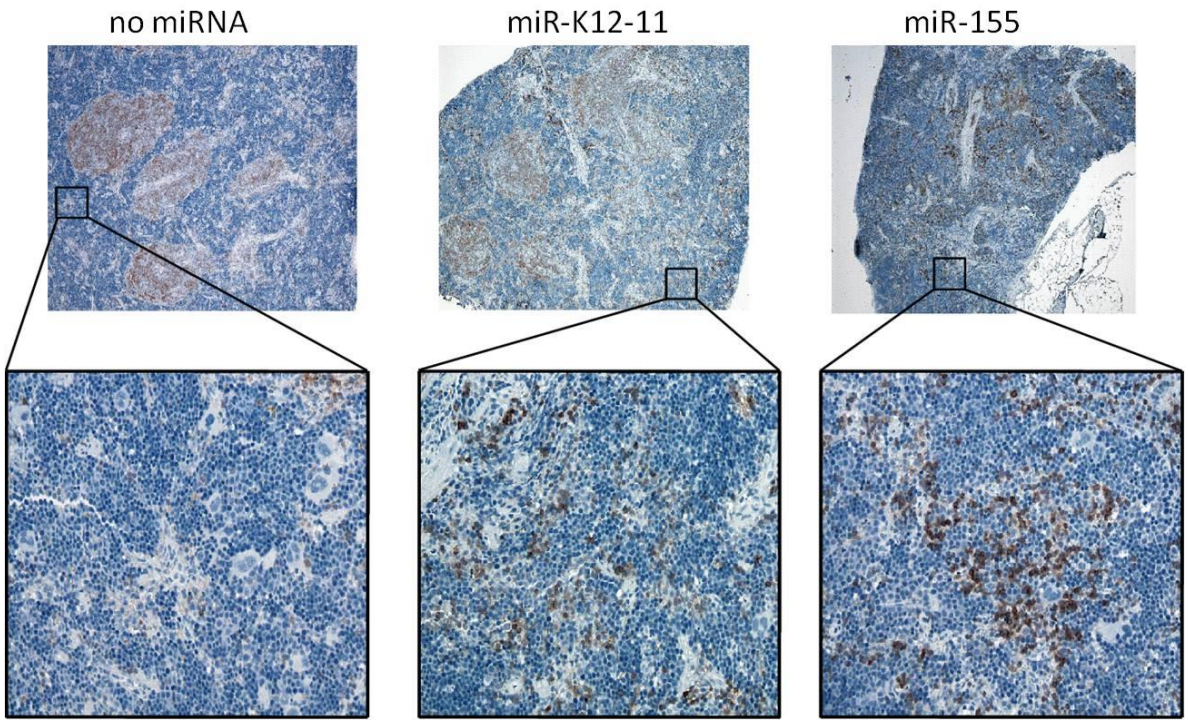

Figure 2-9. Immunohistochemical analysis of spleens revealed an increase in human CD19+ B-cell infiltrates in the splenic red pulp of mice expressing miR-K12-11 or miR-155. For immunohistochemistry, spleens were fixed, sectioned, and stained with a monoclonal antibody against human CD19. Photomicrographs of splenic sections at 40X magnification are shown in the panel of pictures at the top. The splenic red pulp regions are further magnified (200X) in the bottom panels to show the increased hCD19+ B-cell infiltrates (red staining) in the miRNA expressing animals versus the no miRNA control. Shown are representative sections from one animal from each group.

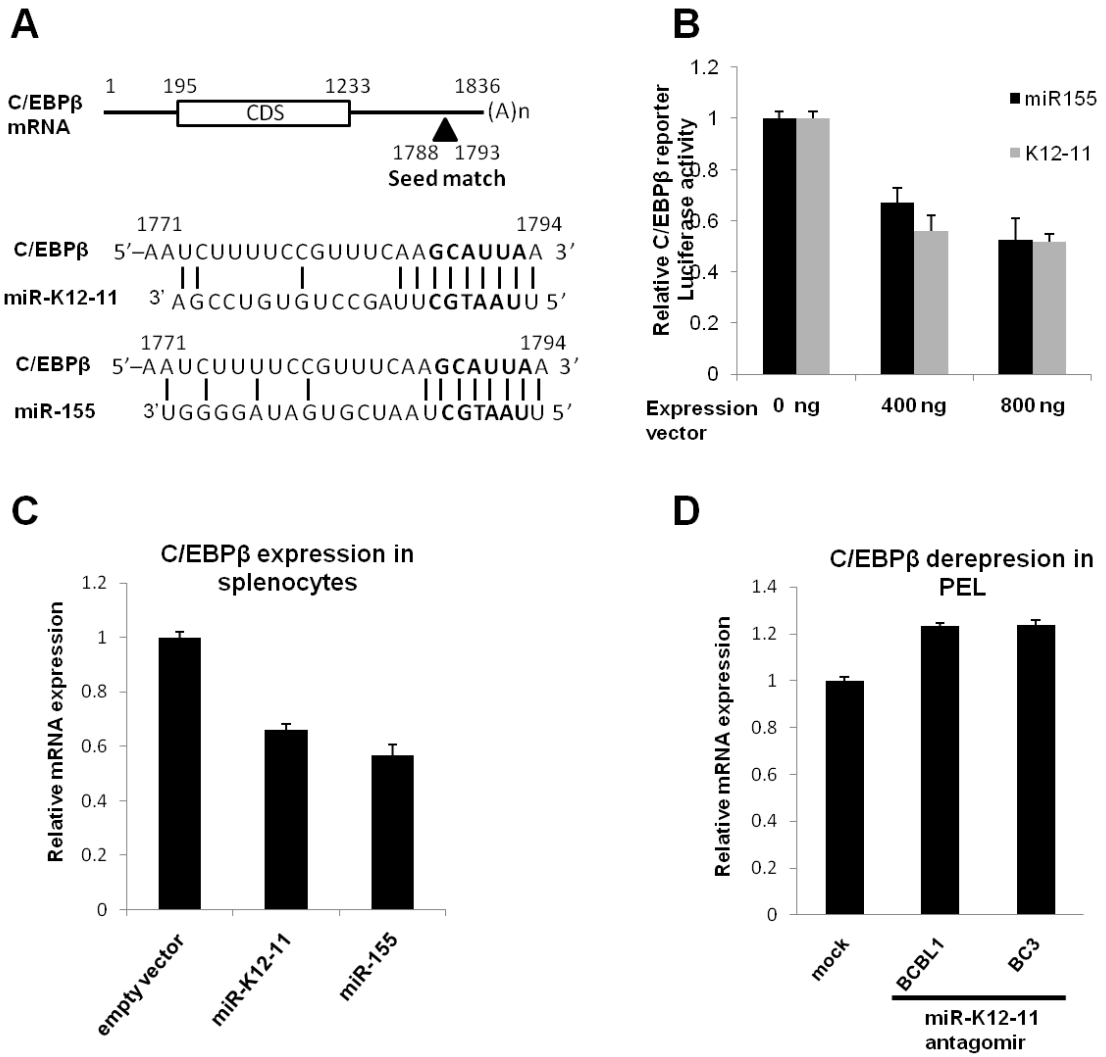

Figure 2-10. C/EBPβ is targeted by both miR-K12-11 and miR-155 in splenocytes and is regulated by miR-K12-11 in PEL. A. The C/EBPβ 3'UTR contains 1 seed match site for miR-K12-11 and miR-155. B. The full length C/EBPβ 3'UTR was cloned (nt 1233-1836) downstream of luciferase (pGL3-C/EBPβ) and co-transfected into 293 cells with increasing amounts (400ng and 800ng) of miR-155 or miR-K12-11 expression plasmids and a renilla luciferase control vector. Transfection was normalized to renilla values and firefly values were graphed as relative light units. C. RNA harvested from splenocytes from two separate animals from each group (empty vector control, miR-K12-11, and miR-155) was analyzed by qRT-PCR for expression of C/EBPβ mRNA and normalized to GAPDH. D. miR-K12-11 function in the PEL cell lines BCBL1 and BC1 was inhibited using 25nM 2'OMe antagomir specific for miR-K12-11. RNA was harvested from these cells and derepression of C/EBPβ mRNA was analyzed by qPCR and normalized to GAPDH. Mock transfected cells were used as a control. All experiments represent the average of three independent replicates and were repeated at least two times.

CHAPTER 3
DEFINING THE ROLE OF KSHV MIR-K12-11 ON TERMINAL B CELL DIFFERENTIATION

KSHV is a lymphotropic virus that infects B cells *in vivo* and can promote the B cell lymphoporliferative diseases PEL and MCD. While PEL and MCD differ morphologically and phenotypically, they both resemble B cells that are arrested at a pre-plasma stage of differentiation. Because suitable models to study KSHV B cell infection have been extremely limited, the mechanisms governing KSHV inhibition of plasma cell differentiation are not well understood. We have previously found that the KSHV miRNA, miR-K12-11, functions as a homolog of miR-155, a human miRNA that regulates B cell differentiation. To determine if miR-K12-11 can hijack miR-155 function to inhibit plasma cell differentiation, we transfected human naïve B cells with synthetic miRNAs and stimulated them *in vitro* with IL-21, anti-CD40, and anti-IgM to induce plasma cell differentiation. While the results of this experiment did not show any miRNA mediated inhibition of differentiation, we identified a number of B cell terminal differentiation targets regulated by both miRNAs. These results indicate that miR-K12-11 can regulate miR-155 B cell targets, but this regulation alone does not inhibit IL-21-induced plasma cell differentiation *in vitro*.

Introduction to KSHV and terminal B cell differentiation

KSHV infection of B cells in some immunocompromised individuals can induce two types of B cell tumors, PEL and MCD (Cesarman et al., 1995; Soulier et al., 1995). PEL cells are believed to be derived from a late stage of B cell differentiation based on somatic mutations in their immunoglobulin genes and the expression of the plasma cell marker CD138. In contrast, MCD carries no immunoglobulin somatic mutations and does not express CD138, indicating that these tumors are derived from a naïve B cell

origin. Although the tumors differ phenotypically, both express PR domain containing 1 with zinc finger domain /B lymphocyte-induced maturation protein 1 (PRDM1/BLIMP1), considered the master regulator of plasma cell differentiation (Chadburn et al., 2008; Shaffer et al., 2002; Turner et al., 1994). So it appears that KSHV infects different subtypes of B cells, either at early or late stages of differentiation, and somehow manipulates the B cell differentiation program to undergo terminal differentiation but stalling at a pre-terminal stage. The precise mechanisms governing KSHV control of B cell differentiation programs are largely unknown.

In addition to B cell tumors, KSHV genomes are detected in circulating CD19-positive B cells from infected individuals, suggesting that B cells represent the reservoir for persistent KSHV infection (Ambroziak et al., 1995; Mesri et al., 1996). Although KSHV infects B cells *in vivo*, B cells are resistant to infection *in vitro* (Bechtel et al., 2003; Blackbourn et al., 2000; Renne et al., 1998). This limitation has restricted study on the mechanisms governing KSHV B cell infection. Very recently, two separate groups have shown that tonsillar B cells can be infected *in vitro,* but without any immortalizing or transformation events (Hassman et al., 2011; Myoung and Ganem, 2011). In one study it was found that KSHV specifically targets IgMλ tonsillar B cells, and that infection drives these cells to proliferate and express CD27, IgM, and IL-6R, an immunophenotype closely related to MCD (Hassman et al., 2011). These studies suggest that KSHV may target a tonsillar B cell subtype for infection, inducing proliferation and differentiation. More work is needed to examine how changes in B cell differentiation programs might contribute to this process during *in vitro* infection.

Potential insight into KSHV regulation of B cell differentiation comes from studies on closely related EBV, which is believed to establish persistent infection by driving naïve B cells to activate and undergo germinal center reactions where the virus expresses its “growth transcription programme” to induce differentiation into resting memory B cells (Thorley-Lawson, 2001). This expression programme consists of three viral proteins that mimic B cell host proteins including; LMP1, which shares functional homology with CD40, allowing for the activation of anti-apoptotic and proliferation signals; and LMP2 which enhances B cell receptor (BCR) signaling (Caldwell et al., 1998; Panagopoulos et al., 2004). EBV also induces miR-155 expression during in vitro B cell infection, which contributes to immortalization by inhibiting apoptosis and promoting episomal maintenance of the EBV genome (Linnstaedt et al., 2010; Lu et al., 2008). In contrast to EBV, no KSHV latent protein has been identified that can activate resting B cells and promote their differentiation. However, KSHV expresses two lytic proteins, K1 and K15, which share structural homology to LMP1 and LMP2 respectively, and can activate similar cell signaling pathways (Brinkmann and Schulz, 2006; Lagunoff et al., 1999). Additionally, KSHV expresses miR-K12-11 during latent B cell infection, which we have shown is a functional ortholog of miR-155. Based on miR-155’s function during EBV infection, we speculate that miR-K12-11 may play a similar role during KSHV infection.

In normal B cells, activation of BCR and toll-like receptor (TLR) signaling induces transient miR-155 expression (Thai et al., 2007). Expression profiling of miR-155 in human tonsillar B cells indicates that upon activation resting naïve B cells increase miR-155 expression, hitting a peak in the GC, followed by a decrease in the memory B cells

and plasma cell populations (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009). It is possible that after leaving the GC, B cells must shut down expression of miR-155 in order to switch on a transcription program for memory B cell or plasma cell differentiation. However, no targets of miR-155 have been found which repress the terminal differentiation program in B cells. The PEL and MCD phenotype of an arrested post-GC plasmablast suggests that the B cell terminal differentiation program is being blocked. Because we have shown that miR-K12-11 is a functional homolog of miR-155 (discussed in Chapter 1 and 2) it is possible that KSHV constitutively expresses miR-K12-11 during B cell infection to promote activation, differentiation, and immortalization. At the same time, miR-K12-11 expression after germinal center transit could prevent plasma cell differentiation**.** This would be advantageous for the virus because it could disrupt an anti-viral humoral response by inhibiting plasma cell differentiation. Additionally, KSHV episome replication has been shown to occur only in dividing cells, in which the host DNA replication machinery is accessible for viral replication (Grundhoff and Ganem, 2003). Because plasma cells no longer divide, KSHV may drive infected cells into proliferating plasmablasts and freeze them at this stage to establish a pool of blasting cells for persistent infection. To further explore the ability of miR-K12-11 to regulate B cell differentiation and inhibit plasma cell differentiation I designed the following *in vitro* differentiation system and also identified targets that may play important roles in these differentiation pathways.

In vitro model of plasma cell differentiation

To study miR-K12-11's ability to inhibit plasma cell differentiation we have designed an *in vitro* system that uses human naïve and memory B cells purified from peripheral blood (Appendix 1). This system was previously developed to study the

ability of IL-21, a T-cell cytokine, to drive B cell proliferation and plasma cell differentiation (Ettinger et al., 2005). B cell differentiation is controlled by a complex series of transcription programs which are turned on and off by regulatory networks as the B cell transits into and out of the germinal center (Lin et al., 2003; Schebesta et al., 2002). In the germinal center, cytokines play an important role in orchestrating B cell differentiation by activating JAK-STAT signaling pathways which control the regulatory networks (Shuai and Liu, 2003). IL-21 drives plasma cell differentiation by activating STAT-3, which in turn activates BLIMP1 expression, the master regulator of terminal differentiation (Diehl et al., 2008). In addition to IL-21, contact-dependent BCR and CD40 activation are also essential for resting B cell proliferation and differentiation (Ettinger et al., 2008). *In vitro* differentiation of resting naïve B cells to plasma cells is induced by activation signals from IL-21, anti-IgM (to mimic BCR antigen cross-linking), and anti-CD40 (to mimic T-cell CD40L interaction), while differentiation of memory B cells only requires IL-21 and anti-CD40 (Bryant et al., 2007). To measure the capacity of differentiation using these signals, cells are analyzed for increased expression of CD38 and loss of IgD, the immunophenotype of a terminally differentiated plasma cell. Additionally, because plasma cells are characterized by their ability to produce class switched antibodies (IgM to IgG), supernatant is analyzed for secreted IgG.

To verify our ability to recapitulate plasma cell differentiation using this system, we first purified naïve and memory B cells from human peripheral blood by negative selection. Flow cytometry analysis indicated that our enriched CD19+ B cell population was at least 95% pure (Figure 3-1). These cells were further analyzed for surface expression of IgD and CD38, indicating that the majority (95%) represented a mixture of

naïve (IgD+CD38$^{low/int}$) and memory B cells (IgD-CD38$^{-/low}$) (Figure 3-2). To confirm the ability of this system to produce fully differentiated plasma cells, we cultured the purified CD19+ B cells with or without IL-21, anti-CD40, and anti-IgM. After seven days the cells were analyzed by flow cytometry for expression of CD38 and IgD. B cells cultured without the stimulatory factors resulted in very few IgD-CD38^{high} plasma cells (3.4%) (Figure 3-3). In contrast, B cells cultured with the factors exhibited a significantly higher fraction of IgD-CD38^{high} plasma cells (14.6%) (Figure 3-3). This indicated that phenotypically these cells resembled fully differentiated plasma cells. Further analysis of the culture supernatant for secreted IgG indicated that the stimulated B cells produced dramatically more IgG than the unstimulated B cells (Figure 3-4). These data indicate that *in vitro* plasma cell differentiation was successful.

Ectopic miR-K12-11 expression during plasma cell differentiaton

To deliver miR-K12-11 into the purified B cells, I needed to determine the most efficient and non-toxic method. Transduction of B cells using the foamy virus miRNA vectors, previously described in Chapter 2, resulted in high cell death and low transduction rates (<8.6%) (Figure 3-5). In comparison, transient transfection of synthetic miRNA mimics was less toxic and resulted in a high percentage of successfully transfected cells (44.8%) (Figure 3-5), therefore I used transfection for miR-K12-11 delivery.

To test the ability of miR-K12-11 and miR-155 mimics to inhibit plasma cell differentiation, B cells were stimulated for 3 days and then transfected with miR-K12-11, miR-155, or a non-specific miRNA control, and analyzed 4 days post-transfection for changes in phenotype and IgG secretion. Phenotype analysis by flow cytometry did not indicate any significant inhibition of plasma cell differentiation by miR-K12-11 or miR-

155 (Figure 3-6). Analysis of the culture supernatant for IgG indicated no significant differences between the control miRNA, miR-K12-11, or miR-155 transfected B cells (Figure 3-7). Although there was a slight increase of IgG secretion in the non-transfected B cells, this difference is likely due to a decrease in viable antibody producing B cells caused by the transfection. Lastly, to determine if miR-K12-11 or miR-155 promoted plasmablast proliferation we measured the proliferation capacity of the stimulated B cells at day 6 by EdU incorporation, using the Click-IT Edu flow cytometry assay kit. Results showed low proliferation rates in all conditions, indicating that the miRNAs alone were not promoting the differentiation of proliferating plasmablasts (Figure 3-8).

To investigate miR-K12-11's ability to promote activation or proliferation independent of other stimulation factors, unstimulated B cells were transfected with miR-K12-11, miR-155, or a non-specific miRNA control 24 hours after plating. Activation of resting B cells was analyzed by measuring CD38 expression, a marker for activation, with flow cytometry 24, 48, and 72 hours post-transfection. No significant increase of CD38 expression was observed in the miR-K12-11 or miR-155 transfected B cells at either time point, indicating that these miRNAs were not inducing activation (Figure 3-9). Furthermore there was no significant proliferation in the transfected B cells, measured by EdU incorporation 48 hours after miRNA transfection (Figure 3-9). Without BCR and anti-CD40 co-stimulation purified B cells rapidly die in culture. While miR-155 was reported to inhibit apoptosis in B cells newly infected with EBV (Linnstaedt et al., 2010), we did not observe any anti-apoptotic affect in miR-K12-11 or miR-155 transfected B cells 5 days post-transfection (Figure 3-10). These results indicate that,

at least in vitro, miR-K12-11 and miR-155 alone do not affect resting B cell activation or proliferation and also do not promote resistance to apoptosis. It still remains possible that miR-K12-11 contributes to B cell activation and proliferation while inhibiting terminal differentiation during KSHV infection *in vivo*. However, based on the results in this model system, the mechanisms contributing to miR-K12-11's role in these processes appear to be complex and may require additional viral factors and/or other model systems for adequate investigation. Two possible viral proteins that may also contribute to KSHV B cell regulation are K1 and K15, based on their similiarity to the EBV B cell regulatory proteins LMP1 and LMP2. Future experiments designed to co-express these proteins along with miR-K12-11 in primary B cells may reveal important phenotypes.

Identification and validation of miR-K12-11 targets involved in B cell regulatory pathways

To identify potential miR-K12-11 cellular gene targets involved in B cell activation, proliferation, and differentiation, I utilized published reports of validated miR-155 B cell targets. The first gene profiling arrays from miR-155 deficient B cells identified 60 upregulated genes with miR-155 seed matches in their transcript's 3'UTR (Vigorito et al., 2007). Subsequent studies found that two candidate genes from this list, Pu.1 and AID, are indeed regulated by miR-155 and that this regulation is important for germinal center formation (Dorsett et al., 2008; Teng et al., 2008; Vigorito et al., 2007). This set of genes also included; Jarid2, a cell cycle regulator; Bach1, a transcriptional regulator and target of miR-K12-11; MYB, an important regulator of hematopoiesis; and SMAD5, a transcription factor; all of which are now experimentally validated miR-155 targets (Bolisetty et al., 2009; Rai et al., 2010; Skalsky et al., 2007; Yin et al., 2008). Seperate

studies have identified and validated CEBP/β and SHIP1 as miR-155 targets that play a crucial role in B cell tumorigenesis (Costinean et al., 2009; O'Connell et al., 2009). Based on their functions in B cells, I choose to further analyze PU.1, MYB, CEBP/β, and SHIP1 for functional targeting by miR-K12-11. In addition, I have also identified three potential novel miR-K12-11 targets; nuclear factor κβ inducing kinase (NIK), a transducing kinase essential for B cell activation; immunoglobulin J chain (IgJ), which is essential for secretory IgA and IgM production; and IFN regulatory factor (IRF)8, a transcription factor involved in B cell development.

To confirm that the 3'UTRs of these potential gene targets contained miR-K12-11 target sites I used miR target finder, a bioinformatic program designed by Dr. Alberto Riva. This program utilizes a set of previously defined miRNA binding parameters to assess a miRNA's ability to repress transcription of a target gene (Grimson et al., 2007). Analysis by miR target finder revealed that the 3'UTRs of PU.1, CEBP/β, SHIP1, NIK, IgJ, and IRF8 all contained one miR-K12-11 binding site, while MYB's 3'UTR contained two. To confirm that both miR-155 and miR-K12-11 can target and repress these genes we carried out *in vitro* luciferase reporter assays, using vector constructs that contained the genes 3'UTR inserted downstream of the luciferase cassette. The reporter construct was co-transfected into HEK293 cells with increasing amounts of miR-155 or miR-K12-11 expression vectors. Results of the assay demonstrated that both miRNAs mediated a dose-dependent knockdown of luciferase expression for all reporter constructs, except NIK and IRF8, indicating 3'UTR miRNA targeting for PU.1, CEBP/β, MYB, SHIP1, and IgJ (Figure 3-11).

To confirm that miR-K12-11 can regulate expression of PU.1, CEBP/β, MYB, SHIP1, and IgJ in the latently infected PEL cell lines, BCBL-1 and BC3, I carried out antagomir de-repression assays. This assay utilizes antagomirs to specifically inhibit miR-K12-11 function, resulting in de-repression of targets that can be measured by RT-qPCR. Results indicated modest de-repression for MYB [BCBL1 (0.3 fold) and BC3 (0.35 fold)], CEBP/β [BCBL1 (0.25 fold) and BC3 (0.2 fold)], SHIP1 [BCBL (0.26 fold) and BC3 (0.3 fold)], and IgJ [BCBL1 (0.3 fold) and BC3 (0.4 fold)] (Figure 3-12). In contrast, PU.1 transcript levels remained unchanged in response to miR-K12-11 inhibition. Interestingly, a previous study revealed that PEL cells do not express the PU.1 B cell specific transactivator Oct-2, possibly explaining the low levels of PU.1 transcript and lack of derepression observed in PEL cells (Arguello et al., 2003). However, Oct-2 is expressed in MCD, so it remains plausible that PU.1 is a target for miR-K12-11 in this context of KSHV infection.

These findings confirm that miR-K12-11 can target an overlapping set of miR-155 targets involved in diverse B cell regulatory pathways. As discussed in Chapter 2, the functional relevance of miR-K12-11 targeting of C/EBPβ in the regulation of IL-6 expression suggests a possible mechanism for promoting B cell proliferation in KSHV pathogenesis. SHIP1, which was implicated in miR-155 induced B cell lymphomas, is also targeted by miR-K12-11 and may contribute to KSHV lymphomagenesis, a link that will need to be further examined. MiR-K12-11 targeting of IgJ may inhibit the ability of KSHV infected B cells to produce secreted antibody and will need to be further examined. While the function of MYB targeting in B cell development is unclear, a recent study showed that MYB can activate the KSHV lytic switch transactivator RTA

(Lacoste et al., 2007). This suggests that miR-K12-11 regulation of MYB may be important for preventing lytic reactivation, a role currently being investigated by another graduate student in the lab, Karlie Plaisance. Based on the modest levels of target derepression in PEL cells shown in these experiments, miR-K12-11 may function as a fine tuning mechanism, instead of a strong repressor to regulate protein expression, an observation that has been made for many miRNAs (Baek et al., 2008; Selbach et al., 2008). However, it is apparent from our *in vivo* study discussed in Chapter 2, as well as the growing list of validated miR-K12-11 targets, that this miRNA can impact the biology of virally infected cells by regulating multiple cellular pathways.

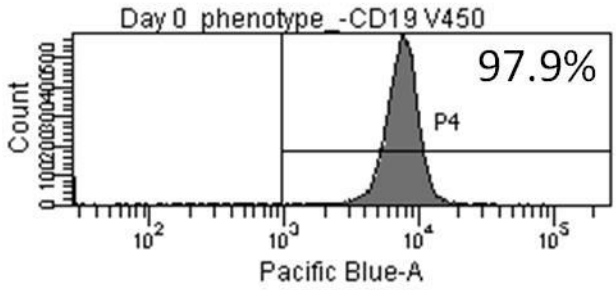

Figure 3-1. Phenotype analysis of freshly purified human B cells. Human purified B cells were negatively enriched from peripheral blood and analyzed for CD19+ surface expression by flow cytometry. Enrichment of B cells resulted in at least a 95% pure population of CD19+ B cells.

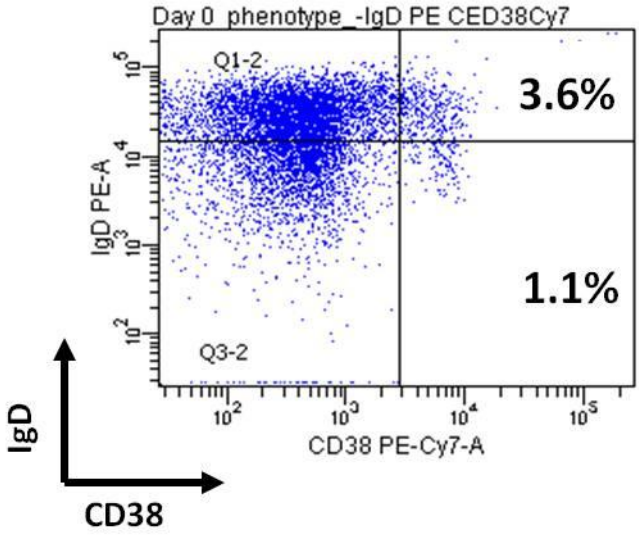

Figure 3-2. Plasma cell phenotype analysis before stimulation. Freshly purified B cells were analyzed for surface expression of CD38 and IgD by flow cytometry. 95.3% of the B cells were naïve (IgD+CD38$^{low/int}$) or memory B cells (IgD-CD38$^{-/low}$).

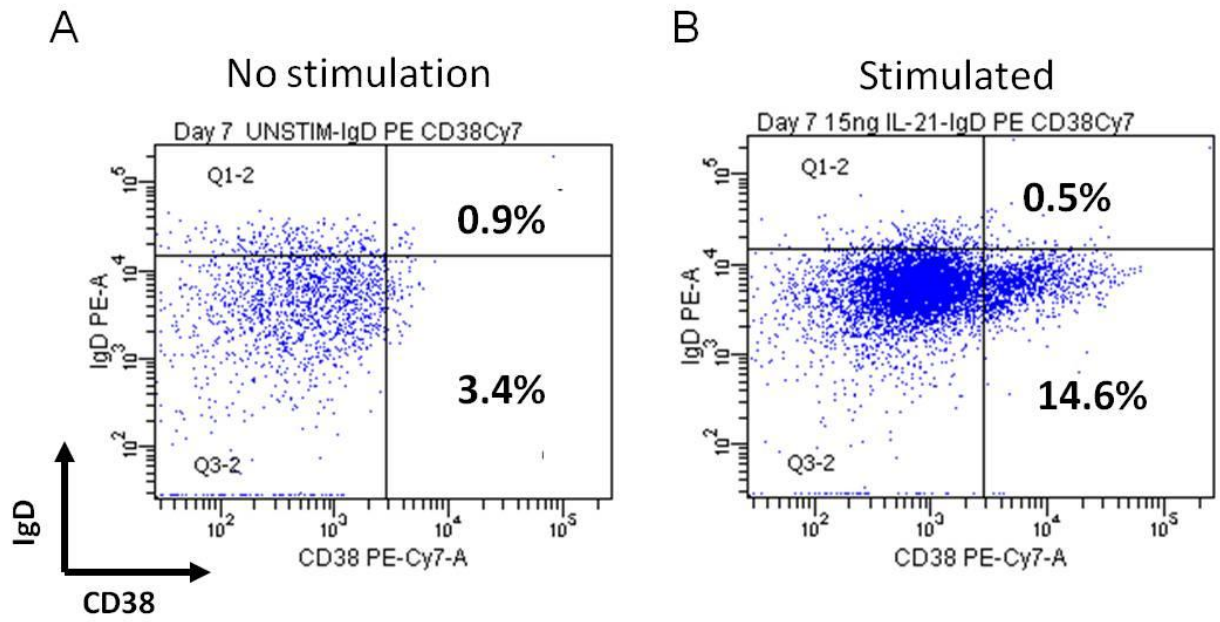

Figure 3-3. Stimulated B cells undergo plasma cell differentiation. Purified B cells were stimulated with (A) no activators or (B) with the combination of IL-21, anti-IgM, and anti-CD40 for 7 days and analyzed for expression of CD38 and IgD by flow cytometry. A. Unstimulated B cells showed very little plasma cell differentiation (IgD-CD38^{high}) in the lower right quadrant. B. 14.6% of stimulated B cells fully differentiated into plasma cells.

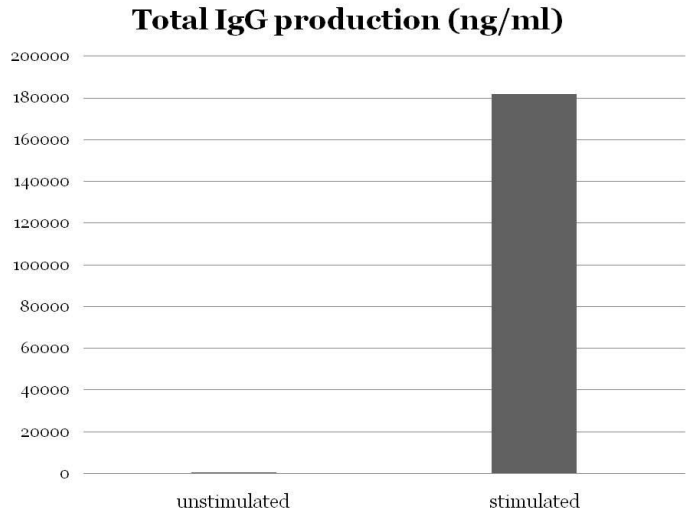

Figure 3-4. Stimulated B cells secrete class switched IgG antibody. Cell supernatant was removed from unstimulated and stimulated B cells after 7 days in culture and analyzed for the presence of IgG with the BD Cytometric Bead Array (CBA) for Human Immunoglobulin assay. The stimulated B cells produced large quantities of IgG compared to unstimulated B cells. We note this experiment was carried out one time.

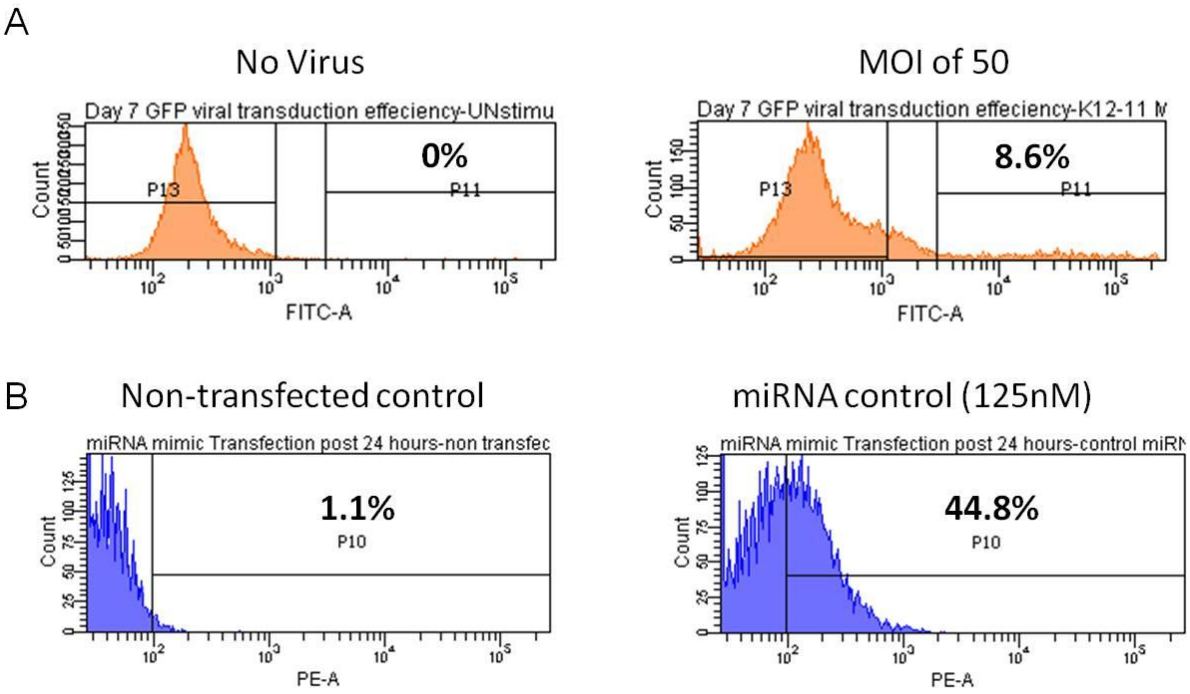

Figure 3-5. MiRNA transfection of human B cells is more efficient and less toxic than foamy virus transduction. A. Purified B cells were stimulated with IL-21, anti-IgM, and anti-CD40 for 48 hours and then infected with foamy virus at an MOI of 50. 4 days post-infection transduction was measured by GFP expression using flow cytometry. 8.6% of infected cells were GFP+. B. Purified B cells were stimulated with IL-21, anti-IgM, and anti-CD40 for 24 hours and then transfected with a Cy3 labeled siRNA control. 44.8% of B cells were successfully transfected after 24 hours.

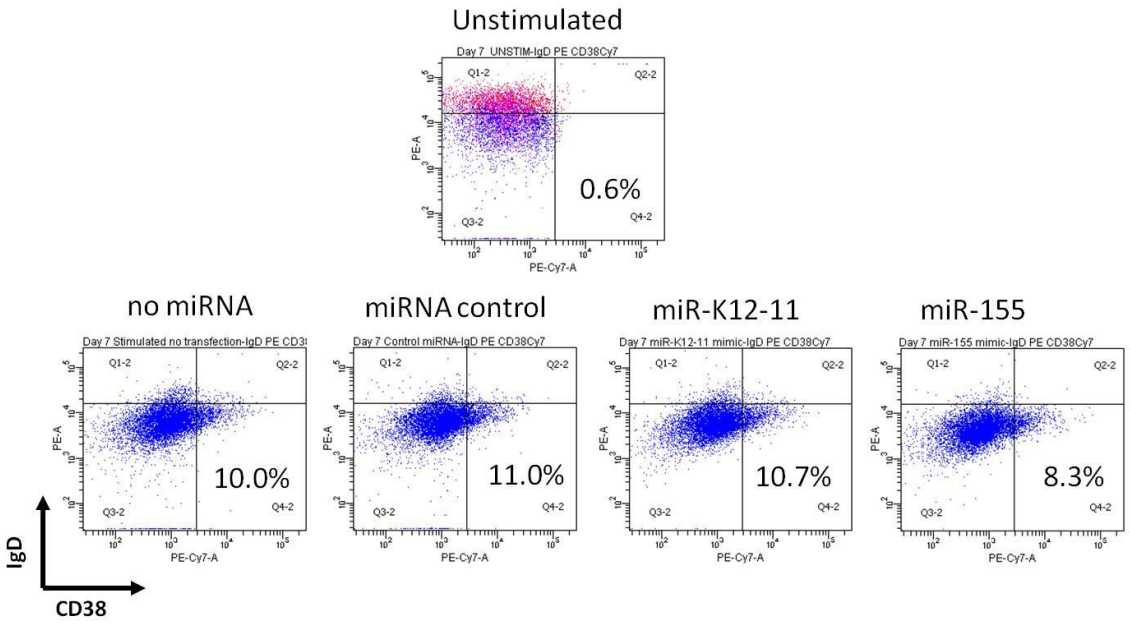

Figure 3-6. MiR-K12-11 and miR-155 does not inhibit in vitro plasma cell differentiation. Purified B cells were stimulated with IL-21, anti-IgM, and anti-CD40 for 3 days and then transfected with miRNA control, miR-K12-11, or miR-155. 4 days post-transfection the B cells were analyzed for expression of CD38 and IgD by flow cytometry. There was no significant difference in plasma cell differentiation when comparing the miR-K12-11 and miR-155 transfected cells to the miRNA control or untransfected cells.

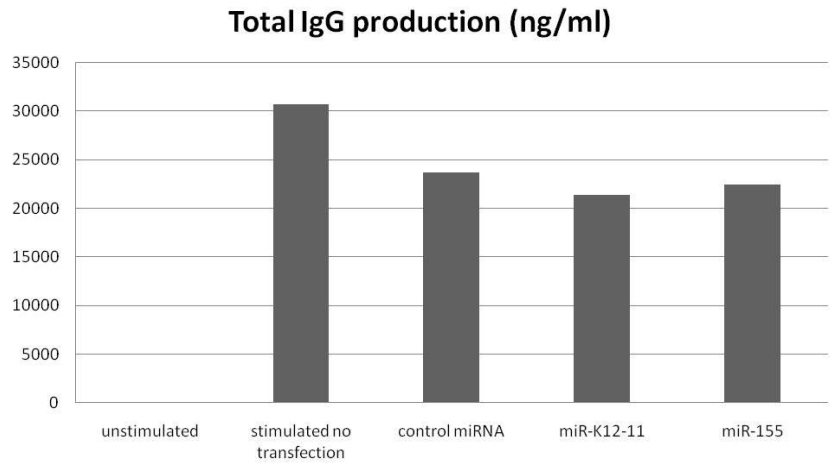

Figure 3-7. MiR-K12-11 and miR-155 does not inhibit IgG class switching. Cell supernatant was removed from three wells of unstimulated, stimulated, control miRNA, miR-K12-11, and miR-155 transfected B cells after 7 days in culture and analyzed for the presence of IgG with the BD Cytometric Bead Array (CBA) for Human Immunoglobulin assay. The stimulated B cells produced larger quantities of IgG compared to the unstimulated B cells and miRNA transfected cells but there was no difference in IgG secretion between the cells transfected with miR-K12-11, miR-155, or the control miRNA . We note this experiment was carried out one time.

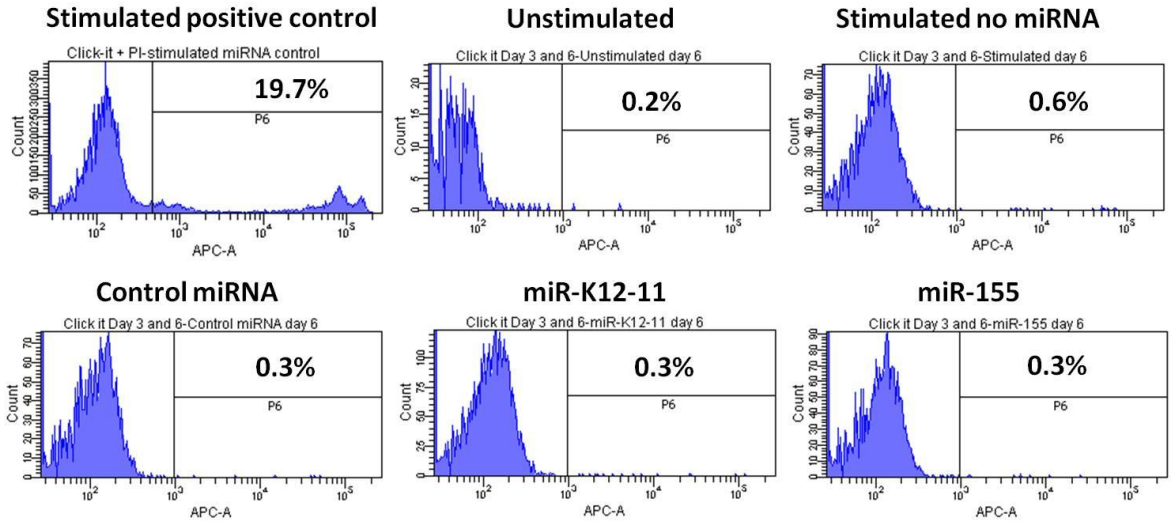

Figure 3-8. MiR-K12-11 and miR-155 does not promote plasmablast proliferation. B cells were stimulated to differentiate with IL-21, anti-IgM, and anti-CD40 for 3 days and then transfected with miRNA control, miR-K12-11, or miR-155. Edu was added to the culture 2 days post-transfection and Edu incorporation was measured by the BD Click-IT assay by flow cytometry. There were no significant differences in proliferation in the B cells transfected with either miR-K12-11 or miR-155 when compared to the control miRNA and non-transfected conditions.

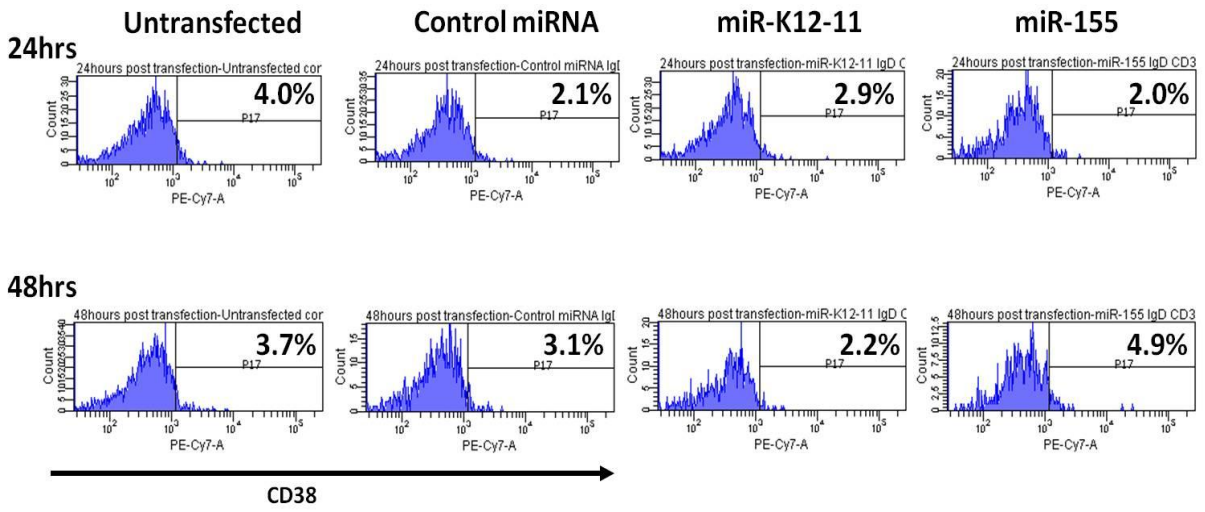

Figure 3-9. MiR-K12-11 and miR-155 do not induce activation in resting naïve or memory B cells. Unstimulated B cells were transfected 48 hours after plating with miRNA control, miR-K12-11, and miR-155. CD38 expression was analyzed by flow cytometry 24 and 48 hours after transfection. There was no significant induction in CD38 expression in the B cells transfected with either miR-K12-11 or miR-155 when compared to the control miRNA and non-transfected conditions.

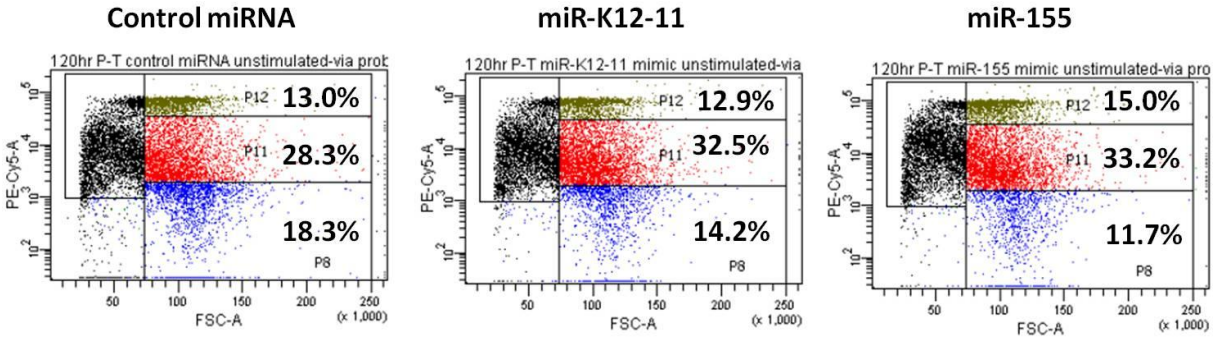

Figure 3-10. MiR-K12-11 and miR-155 do not inhibit B cell apoptosis. Unstimulated B cells were transfected 48 hours after plating with miRNA control, miR-K12-11, and miR-155. B cell apoptosis was measured 5 days post-transfection with BD via-probe and flow cytometry. There was no observed increase in viable cells (gate P8) in either the miR-K12-11 or miR-155 conditions compared to the control miRNA or a decrease in early apoptotic cells (gate P11) in either the miR-K12-11 or miR-155 conditions compared to the control miRNA.

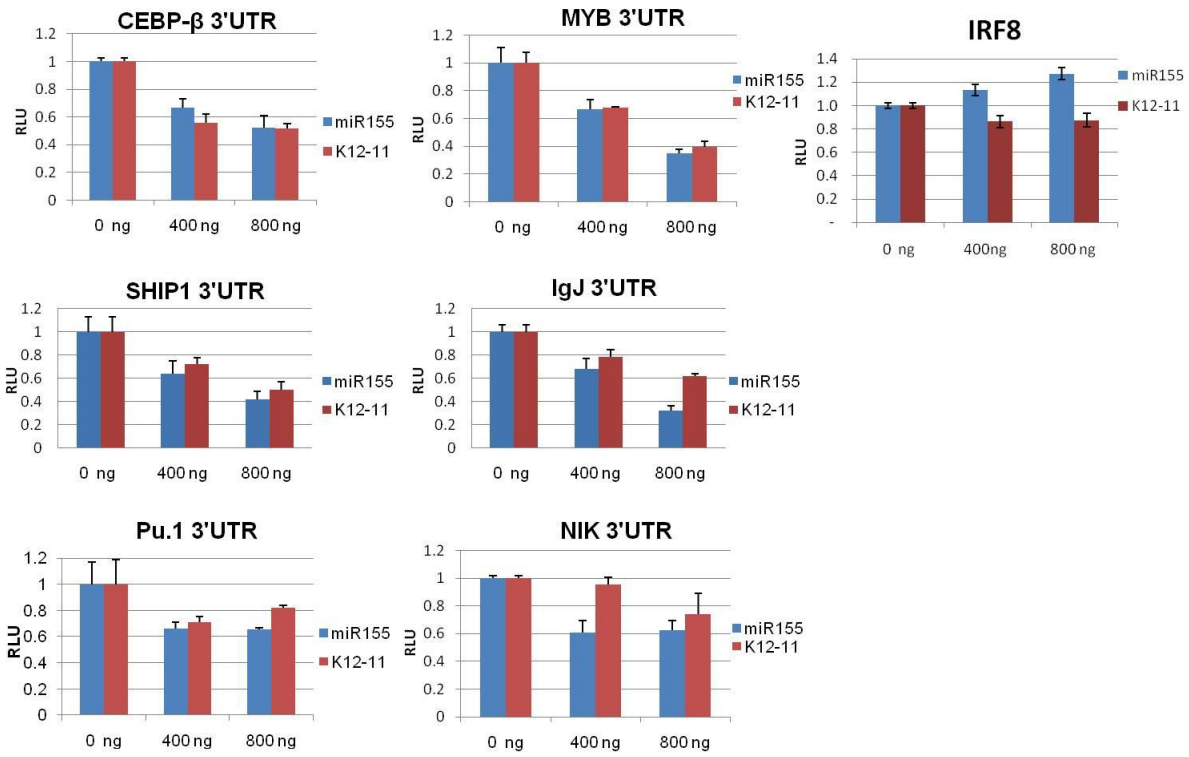

Figure 3-11. MiR-K12-11 and miR-155 can target the 3'UTR's of genes involved in B cell regulatory pathways. Full length 3'UTRs for C/EBPβ, SHIP1, PU.1, MYB, IgJ, NIK, and IRF8 were cloned downstream of firefly luciferase (pGL3) and co-transfected into 293 cells with increasing amounts (400ng and 800ng) of miR-155 or miR-K12-11 expression plasmids and a renilla luciferase control vector. Transfection was normalized to renilla values and firefly values were graphed as relative light units. A dose dependent decrease in luciferase was observed for C/EBPβ, SHIP1, PU.1, MYB, and IgJ.

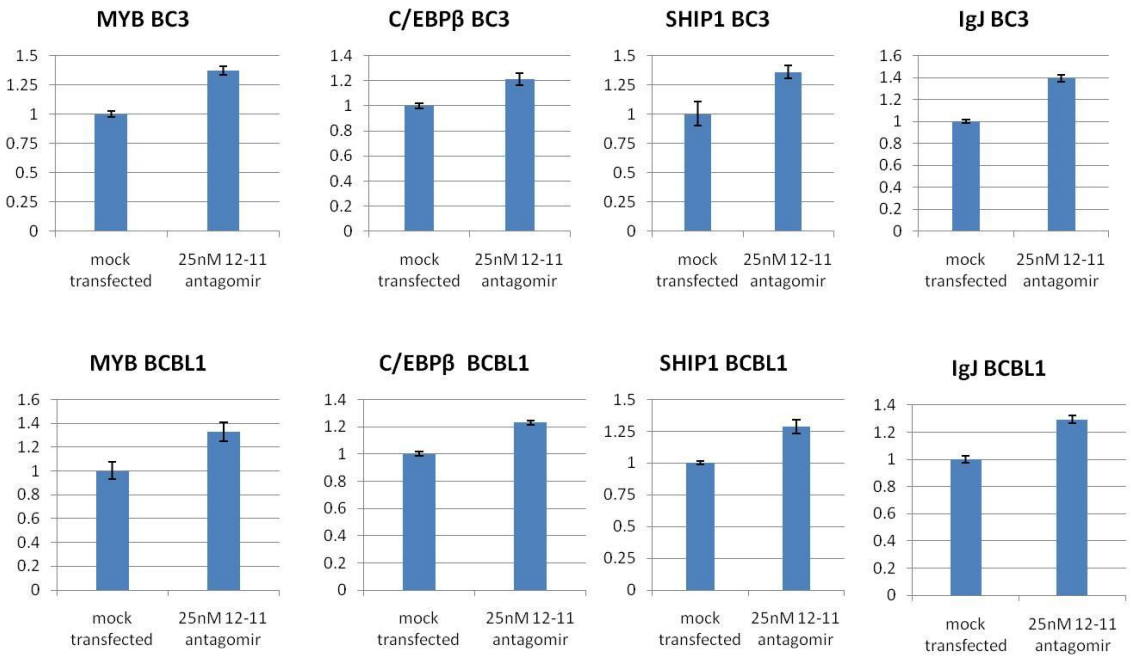

Figure 3-12. MiR-K12-11 targets MYB, C/EBPβ, SHIP1, and IgJ in PEL cells. Antagomir de-repression assays were carried out in the PEL cell lines BC3 and BCBL1 using 25nM of 2'OMet antagomir specific to miR-K12-11. 48 hours post-transfection RNA was harvested from the PEL cell lines and derepression of each target was analyzed by qPCR and normalized to GAPDH. Mock transfected cells were used as a control. All experiments represent the average of three independent replicates and were repeated at least two times.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

KSHV miR-K12-11 is a latently expressed miRNA that shares seed sequence homology with the human miRNA, miR-155. KSHV miRNAs have been shown to regulate a number of cellular processes including immune evasion, apoptosis, angiogenesis, and cell cycle regulation indicating that these miRNAs play an important function in modulating the cellular environment during infection. The focus of my studies in Chapter two was to evaluate miR-K12-11's ability to function as a homolog of miR-155 during hematopoiesis *in vivo*. Findings from this study revealed that miR-K12-11 promotes splenic B cell proliferation, phenocopying miR-155 function, and identifying an important role for this miRNA in KSHV B cell pathogenesis. To determine functional mechanisms underlying the B cell proliferation, I identified C/EBPβ, an IL-6 and IL-10 regulator, as a target in both splenic B cells and PEL cells. In Chapter three I examined the ability of miR-K12-11 to phenocopy miR-155 function in the processes of B cell activation and differentiation *in vitro*. While I did not observe any functional consequences of miR-K12-11 or miR-155 on human B cell activation or differentiation, I did identify a number of targets that could contribute to de-regulation of B cell regulatory pathways. Together these results prove that miR-K12-11 is a functional mimic of miR-155 and provide further insights into the role of miR-K12-11 on KSHV B cell pathogenesis.

KSHV miR-K12-11 functions as a miR-155 ortholog *in vivo*

To determine whether miR-K12-11 can phenocopy miR-155 activity *in vivo*, we utilized the humanized NOD/LtSz-scid IL2Rγ^{null} mouse model. This was the first functional study of a KSHV miRNA in the context of *in vivo* human hematopoietic cell

development. Results showed that ectopic expression of either miR-K12-11 or miR-155 during hematopoiesis induced an increase in the human CD19+ B cell population in the spleens of mice, without altering the CD33+ monocyte or CD3+ T cell populations. The finding that a single viral miRNA may promote B cell pathogenesis is an important discovery because KSHV is a B cell tropic virus associated with B cell lymphomas.

For this study, it was important that miR-K12-11 and miR-155 was expressed at similar levels to endogenous expression in latently infected PEL cells, in order to eliminate any potential off-target consequences due to miRNA oversaturation. Interestingly, we did not observe B cell tumor formation in our mouse model, a phenotype previously reported when miR-155 is overexpressed in the Eμ-miR-155 transgenic mouse (Costinean et al., 2006). It is possible that miR-K12-11 overexpression can lead to oncomir addiction; a process where the miRNA alone is essential for the initiation, maintenance, and survival of tumors *in vivo*; which has been shown for human miRNAs miR-155 and miR-21 (Costinean et al., 2009; Medina et al., 2010). While studies measuring the expression levels of miR-K12-11 in PEL were done with a population of infected cells (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005), individual KSHV infected B cells within the population may express significantly more miR-K12-11, thus becoming sensitive to oncomir addiction. Based on differences in DNA methylation patterns found on latent and lytic promoters within a population of latently infected B cells, it appears that differential patterns of gene expression exist (unpublished work by Irina Haeker). Determining if there is a threshold of expression where miR-K12-11 becomes an oncomir in some infected cells, but not others, is important and can be further investigated using stronger promoters in our mouse model.

While we observed a splenic B cell expansion induced by ectopic miRNA expression, we did not analyze B cells for increased proliferation capacity or reduced apoptosis. Both of these functions are promoted by miR-155 expression in EBV infected B cells, suggesting that miR-K12-11 may play a similar role (Linnstaedt et al., 2010). To further examine this potential role we could purify transduced B cells from mice spleens, culture them *ex vivo*, and analyze them for changes in cell cycle and apoptosis.

In addition to the splenic B cell expansion, we observed abnormal B cell infiltrates in the splenic red pulp regions of mice expressing either miR-K12-11 or miR-155. This phenotype was also observed in the Eμ-miR-155 transgenic mouse model and suggests that aberrant miR-155 or miR-K12-11 expression may produce homing defects in these B cells. Additionally, the miRNAs may be inducing pre-plasma cell differentiation while inhibiting the final stage of differentiation, thereby stimulating them to leave the PALS but blocking their exit from the spleen into circulation. Interestingly, miRNAs have been shown to influence tumor invasion and metastasis (Asangani et al., 2008; Zhu et al., 2008). One potential target of miR-K12-11, MYB, is a transcriptional activator of CXCR4, a chemokine receptor specific for stromal cell derived factor 1 that is involved in normal cell migration, which is also downregulated by the latent protein v-FLIP (Liu et al., 2006; Punj et al., 2010). Repression of CXCR4 expression, mediated by miR-K12-11 targeting of MYB, could be one component of dysfunctional homing and will need to be further investigated. Future studies examining additional miR-K12-11 targets involved in B cell migration may help to explain the abnormal splenic infiltration.

In this study, we focused on the impact of a single KSHV miRNA on human hematopoiesis. Because KSHV expresses additional miRNAs, future studies are needed to examine the impact of all viral miRNAs, independently and co-dependently, on human immune development. Additionally, investigating the synergistic activity of these miRNAs with other KSHV latent gene products, such as the pro-proliferative v-Cyclin and anti-apoptotic v-FLIP, may reveal stronger impacts on human B cell biology, leading to a better understanding of how KSHV latency promotes pathogenesis. Because systems to study the direct impact of KSHV pathogenesis on human cells are lacking, this model represents an important tool for further examining the impact of KSHV miRNAs *in vivo*.

MiR-K12-11 targets C/EBPβ in B cells

To identify the mechanisms contributing to the splenic B cell expansion, I examined possible miR-K12-11 targets and found that C/EBPβ is regulated in splenic B cells and PEL cells. Because C/EBPβ is a regulator of the inflammatory cytokine IL-6, a potent inducer of human plasmablast proliferation and survival, I proposed that its repression by miR-K12-11 or miR-155 may lead to increased production and secretion of IL-6 (Jego et al., 2001). C/EBPβ has also been shown to regulate expression of IL-10, another inflammatory cytokine that induces B cell proliferation and differentiation (Jego et al., 2001; Liu et al., 2003; Rousset et al., 1992). Based on the pro-proliferative properties of these cytokines it is not surprising that they have also been found to promote KSHV pathogenesis. Studies of cytokine expression in PEL cells, *in vitro* and *in vivo*, indicate that they produce high levels of IL-6 and IL-10 (Asou et al., 1998; Drexler et al., 1998; Jones et al., 1999; Sin et al., 2007). Moreover, both cytokines were found to promote PEL cell growth activity in an autocrine fashion (Foussat et al., 1999;

Sin et al., 2007). While the autocrine effect of IL-6 on PEL cell growth appears to be dependent on the cell line (BC1 and BC3 are affected but not BCBL1) or culture condition used, IL-6 and IL-10 overproduction in patients with MCD is directly correlated with disease manifestation (inflammatory symptoms) and progression (Beck et al., 1994; Oksenhendler et al., 2000; Yoshizaki et al., 1989). More recently, it was found that HIV+ KSHV infected patients who exhibited inflammatory symptoms of MCD, but do not display clinical MCD, produce high levels of circulating IL-6 and IL-10 (Uldrick et al., 2010). These studies suggest that KSHV directly influences overproduction of these cytokines to promote pathogenesis.

The mechanisms that KSHV utilizes to induce IL-6 and IL-10 in PEL and MCD are still unclear. In KS tumors IL-6 expression is induced in part by the latent protein v-FLIP and the lytic protein v-GPCR (Montaner et al., 2004; Sakakibara and Tosato, 2009). The lytic protein vIL-6, a poorly secreted homologue of human IL-6, has also been shown to induce IL-6 expression in non-KSHV-infected cell lines (Mori et al., 2000). Evidence that KSHV miRNAs induce IL-6 expression was first provided by studies using human myelomonocytic and murine macrophage cell lines (Qin et al., 2010). While a role for miR-155 in the regulation of cytokines was initially indicated by observations that miR-155 deficient CD4+ T-cells express increased levels of IL-4, IL-5, and IL-10 (Vigorito et al., 2007). To further investigate if miR-K12-11 targeting of C/EBPβ in B cells leads to aberrant IL-6 and/or IL-10 expression, we could analyze purified transduced B cells *ex vivo* for cytokine expression and secretion. Serum levels from transduced mice could also be analyzed for increased levels of circulating IL-6 and IL-10. To examine if miR-K12-11 affects IL-6 and IL-10 expression in PEL, we could

inhibit miR-K12-11 function with antagomirs and then analyze for changes in cytokine expression and secretion. Interestingly, two other KSHV miRNAs, miR-K12-3 and miR-K12-7, were shown to increase IL-6 and IL-10 secretion when ectopically expressed in human myelomonocytic and murine macrophage cell lines (Qin et al., 2010). Therefore, testing the synergistic impact of multiple KSHV miRNAs, on cytokine production in B cells, will also be important.

In the centrocyte region of the germinal center, B cells receive stimulation from IL-21, IL-10, or IL-6 in order to activate STAT3, leading to increased BLIMP1 expression which induces plasmablast differentiation (Diehl et al., 2008; Ettinger et al., 2005; Jourdan et al., 2009). Because I have shown that miR-K12-11 may induce IL-6 and IL-10 expression, it is possible that this action may contribute to plasmablast differentiation in KSHV infected B cells. Furthermore, it has been shown that miR-155 can promote STAT3 activity by targeting SOCS1 in breast cancer cells (Jiang et al., 2010). Further investigation is needed to determine if miR-K12-11 can also promote plasmablast differentiation either by activating STAT3 through induction of IL-6 production, or by directly targeting SOCS1.

In order for IL-6 to promote plasmablast differentiation B cells must express the IL-6R (receptor). Interestingly, KSHV positive plasmablasts in PEL and MCD express high levels of hIL-6R (Asou et al., 1998; Du et al., 2001). Recently, it was found that KSHV infection of tonsillar IgMλ B cells induced IL-6R expression (Hassman et al., 2011). Furthermore, adding exogenous IL-6 to the culture of these infected B cells promoted a blasting phenotype (Hassman et al., 2011). To investigate if miR-K12-11 induction of IL-6 contributes to plasmablast differentiation we could infect purified IgM memory B

cells with miR-K12-11 KO recombinant viruses and analyze these cells for reductions in IL-6 production and plasmablast differentiation.

KSHV miR-K12-11 does not inhibit *in vitro* plasma cell differentiation

KSHV infected B cells in PEL and MCD appear to be stalled in a plasmablast stage of differentiation. Currently, no viral mechanism has been uncovered to explain how KSHV inhibits B cell terminal differentiation in these malignancies. Because miR-155 plays essential roles in B cell differentiation and activation, I examined miR-K12-11's ability to hijack miR-155 function, potentially blocking plasma cell differentiation while promoting proliferation and survival of human plasmablasts. To differentiate purified resting B cells *in vitro*, I utilized a system that is dependent on the cytokine IL-21 to drive differentiation. Results indicated no inhibition of plasma cell differentiation based on phenotype (expression of CD38 and loss of IgD) and IgG secretion. Although I observed no inhibition of differentiation in this model system, it is possible that it does not recapitulate the type of differentiation that occurs during KSHV infection *in vivo* and is therefore not influenced by miR-K12-11. For example, miR-K12-11 may only be capable of regulating differentiation at specific stages or in certain B cell subtypes, when its targets are expressed.

During latency it is believed that KSHV miRNAs are constitutively expressed and processed, therefore the miRNAs are most likely present during most stages of KSHV B cell latent infection. In our model system, miR-K12-11 is not constitutively expressed throughout the differentiation process; therefore we may not be reproducing what occurs during natural KSHV infection. Moreover, we transfected B cells with miR-K12-11 3 days after differentiation was initiated, thus missing any potential regulatory functions early during differentiation. To address these issues, lentiviral transductions

of B cells with miRNA vectors, before stimulation, could allow for sustained miRNA expression throughout differentiation. This system has been successfully used to express B cell specific proteins during plasma cell differentiation (Diehl et al., 2008).

B cell differentiation into plasma cells is defined by several different stages (activation, germinal center reaction, post-germinal center differentiation, and terminal differentiation) and sometimes does not involve a germinal center reaction. These stages are determined by the expression patterns of various sets of transcription factors and transcriptional regulators, including miRNAs. The impact of a miRNA on differentiation, either as a fine tune regulator or molecular switch, is dependent on the miRNAs abundance, as well as the abundance of its target (Mukherji et al., 2011). Because an increase in target transcript abundance has been shown to saturate miRNA repression, miRNA regulation at some stages of B cell differentiation is likely redundant (Mukherji et al., 2011). Therefore, the impact of miR-155 and miR-K12-11 on B cell pathway regulation may only be significant during specific points of developmental transition, when the pool of available targets is low and miRNA expression is high. In turn, other latently expressed proteins and miRNAs could also inhibit expression of miR-K12-11 targets, therefore increasing the pool of available miR-K12-11 that is free to regulate other targets. Overall, host cell gene regulation during KSHV infection is a complex and dynamic process, especially in the context of B cell differentiation pathways. Because we have only tested the affects of miR-K12-11 on B cell differentiation in a static manner (one time point and one stage of differentiation), pinpointing the exact stage when miR-K12-11 targeting affects differentiation requires more investigation.

In my differentiation experiments, a heterogeneous population of purified B cells was used that consisted mostly of naïve B cells and a smaller percentage of memory B cells. While both memory and naïve B cells respond to IL-21 stimulation and undergo plasma cell differentiation *in vitro*, differentiation differs for these two subsets *in vivo* (Ettinger et al., 2005). To undergo plasma cell differentiation *in vivo*, naïve B cells are first activated and either undergo a GC reaction for selection and further differentiation into a memory or plasma B cell, or differentiate into short lived plasma cells without a GC reaction. In contrast to naïve B cells, memory B cells are already preactivated and can rapidly differentiate without going into a GC response (Carsetti et al., 2004). Recently, it was shown that KSHV specifically targets a subset of tonsillar IgMλ B cells for latent infection *ex vivo* (Hassman et al., 2011; Myoung and Ganem, 2011). These cells are hypothesized to be memory B cells based on cytoplasmic expression of IgM and variable levels of surface CD27, a phenotype very similar to KSHV infected B cells in MCD (Hassman et al., 2011). Because of the increasing evidence that KSHV may specifically target memory B cells, rather than naïve B cells, this subset may be more sensitive to miR-K12-11 regulation. Interestingly, a recent study examining human memory B cell differentiation *in vitro* reported that IL-21 did not affect differentiation of these cells into plasmablasts, instead IL-6 and IL-10 in combination with IL-2 and IL-15 promoted their differentiation (Jourdan et al., 2009). Therefore it is plausible that KSHV infection of memory B cells may promote plasmablast differentiation through induction of IL-6 and IL-10. Future experiments using purified memory B cells should be used to examine the affects of miR-K12-11 on the differentiation of this subset, without IL-21,

and to further explore any functional differences miR-K12-11 may have on naïve B cell differentiation.

Interestingly, PEL cells do not express miR-155 but do express miR-K12-11. Currently the mechanisms of miR-155 inhibition and its importance are unclear, but it is possible that KSHV inhibition of miR-155 is required for the hijacking of miR-155 regulatory pathways. In our model system endogenous miR-155 is still present and is most likely induced during BCR and CD40 co-stimulation, this may affect miR-K12-11's ability to mimic miR-155. In order to recapitulate miR-K12-11 hijaking of miR-155 we would need to use B cells with miR-155 gene deletions, or possibly use antagomirs specific for miR-155 to block its function. Future studies using recombinant KSHV viruses, discussed below, are needed to further examine the importance of KSHV inhibition of miR-155 and its impact on miR-K12-11 function during B cell infection.

KSHV miR-K12-11 did not affect human B cell activation, proliferation, or apoptosis *in vitro*

To further investigate miR-K12-11's and miR-155's function in human B cells, I analyzed their ability to induce activation and proliferation. Transfection of synthetic miRNAs into resting or stimulated B cells did not indicate any changes in activation or proliferation, when measured by CD38 expression and Edu incorporation respectively. While miR-155 overexpression in murine models can induce both B cell and myeloproliferative disorders, *ex vivo* studies of miR-155 deficient murine naïve B cells; stimulated with anti-BCR, anti-CD40, IL-4, and IL-5 have indicated no effects on proliferation (Thai et al., 2007; Vigorito et al., 2007). This suggests that, at least in normal resting naïve B cells, miR-155 is not directly involved in promoting activation or proliferation, which correlates with our results.

The ability of miR-155, when overexpressed, to promote B cell lymphomagenesis is not well understood. Recently, it was shown that inhibiting miR-155 function in EBV infected B cells, LCLs and DBLCLs, resulted in a significant reduction of proliferation (Linnstaedt et al., 2010). However, inhibiting miR-155 function in other established EBV+ B cell lines had no effect on proliferation. In a separate study, miR-155 was also shown to impart a proliferative advantage in DLBCLs by regulating a non-canonical pathway that is absent in normal B cells (Rai et al., 2010). This indicates that miR-155's effect on proliferation is likely dependent on the overall cellular environment, including target expression and appropriate signaling pathways. It is possible that miR-K12-11 only enhances proliferation in the context of viral infection, when other viral products, like the pro-proliferative vFLIP and the cell signaling regulators K1 and K15, are co-expressed. Current studies by Karlie Plaisance, a graduate student in our lab, have shown that BJAB cells, an EBV negative BL cell line, infected with a miR-K12-11 knockout recombinant KSHV, displays a reduction in proliferation compared to wt KSHV infected cells. Although these results require more detailed analysis with the click-it EDU proliferation assay, it appears that miR-K12-11 may also enhance proliferation in the context of KSHV infection.

MiR-155 has also been shown to inhibit apoptosis in EBV infected LCLs early during infection, in our model miR-K12-11 and miR-155 did not inhibit apoptosis of resting B cells in culture (Linnstaedt et al., 2010). During infection EBV expresses a number of proteins which modulate the cellular environment by turning on or repressing cellular gene expression. This suggests that the ability of either miRNA to inhibit apoptosis and promote cell survival may require other viral factors or is dependent on a

specific stage of B cell activation/differentiation. Because our model system examines miRNA function independent of other viral factors, we would miss any potential synergistic activity of miR-K12-11. Future studies using recombinant viruses will help to overcome this limitiation.

Recombinant KSHV and miRNA knockouts

To fully examine the impact of miR-K12-11 on KSHV B cell infection and pathogenesis appropriate model systems are needed. Recombinant miRNA knockout viruses are currently available in our lab and will be an important tool that can be used to decipher viral miRNA function during *de novo* infection. Recently, Karlie Plaisance designed a KSHV miR-K12-11 knockout virus, and has begun to produce infectious virus for future experiments. The ability to create and use this virus opens the possibility of many new functional studies for miR-K12-11 in the context of viral *de novo* infection.

Direct evidence that KSHV promotes B cell transformation is lacking, mostly due to the lack of appropriate *in vitro* models to study this process. With the recent development of *in vitro* B cell infection models, it is now possible to examine KSHV transformation potential (Hassman et al., 2011; Myoung and Ganem, 2011). Using recombinant miR-K12-11 knockout viruses combined with these B cell infection systems may reveal phenotypes relating to transformation, including decreased proliferation and increased apoptosis. Other unanswered questions that can be studied with this system include what B cell subtypes does KSHV target, does KSHV directly regulate cytokine expression, and how does KSHV affect B cell differentiation? While these model systems have yet to reproduce KSHV-induced B cell transformation, they offer an

invaluable tool to examine how individual viral products, including miR-K12-11, may contribute to overall pathogenesis.

Currently, the KSHV miRNA expression profile during *de novo* B cell infection is unknown because, until very recently, there have been no systems to recapitulate B cell infection *in vitro*. With the development of B cell infection systems, it should now be possible to determine patterns of KSHV miRNA expression. Determining miR-K12-11's expression pattern in B cells can provide insights into its functional relevance at different stages of B cell differentiation. Overcoming the challenges of non-existent model systems to study KSHV B cell infection and potential transformation has been a major hurdle for answering basic questions regarding KSHV miRNA regulation of B cell biology. With these new model systems and the creation of recombinant KSHV viruses, the aetiology of events that promote KSHV pathogenesis can be further understood.

KSHV miR-K12-11 targets and the future for miRNA target mining

I have shown that miR-K12-11 phenocopies miR-155 to produce a splenic human B cell expansion, indicating that miR-K12-11 targets genes involved in the growth and development of these cells. Using a combination of bioinformatic and *in vitro* approaches I identified several genes involved in B cell function that can be regulated by both miRNAs including PU.1, MYB, C/EBPβ, SHIP1, and IgJ. Analysis of transcript abundance for PU.1, MYB, C/EBPβ, and SHIP1 in splenocytes, by qPCR, indicated that only C/EBPβ is significantly reduced. However, inhibition of miR-K12-11 by antagomirs in PEL cell lines revealed a modest derepression for all targets, except PU.1, suggesting that MYB, SHIP1, and IgJ are valid miR-K12-11 targets. The apparent inability of miR-K12-11 to reduce target transcripts in harvested splenocytes could be

due to increased expression of these genes, leading to an over abundance of target transcripts, thus limiting the level of miR-K12-11 repression. Because B cells express genes at varying levels throughout differentiation, determining the valid miR-K12-11 targets at specific points of differentiation and latent infection remains a challenge. In addition to the six miR-K12-11 targets I have identified, other miR-155 validated targets have now been found including SOCS1, SMAD5, and ETS-1, which play roles in B cell regulatory pathways. While no functional implications for miR-K12-11 regulation of these targets were revealed by the *in vitro* model of plasma cell differentiation, other models, such as miR-K12-11 knockout recombinant viruses, may be better suited to elucidate the mechanisms of miR-K12-11 targeting.

To definitively define the KSHV miRNA targetome in PEL cells, Irina Haecker, a post-doc in the laboratory, is using new techniques that combine *in vivo* UV crosslinking with RISC-specific immunoprecipitation to probe for miRNA/mRNA interactions. HITS-CLIP (High throughput sequencing UV cross linking Immunoprecipitation) uses 254 nm UV to directly cross-link RNA protein complexes prior to immunoprecipitation (Chi et al., 2009). In a second method, PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation), cells are first labeled with photoreactive nucleoside analogs that are incorporated into nascent mRNAs in living cells (Hafner et al., 2010). An advantage of PAR-CLIP over conventional HITS-CLIP is that upon cDNA cloning of the recovered RNA, the cross-linking induces base transition, which creates a RISC footprint within the recovered mRNA tag (Hafner et al., 2010). Analyzing RISC complexes from virus infected cells will help to catalogue the miRNA/target gene

interactions within specific cell types and from Irina's initial results it appears that miR-K12-11 targets a large set of gene transcripts in PEL.

One major limitation to these strategies is that the targets identified in PEL only represent a static picture of miRNA regulation. For example PEL cells are cultured cell lines which have undergone many cellular changes and have already undergone transformation. The process of PEL transformation is most likely a multistep process that includes miRNA regulation of targets early during infection, which would be missed by PAR-CLIP/HITS-CLIP analysis of PEL. To better analyze the dynamic process of KSHV miRNA targeting, these techniques, in combination with *de novo* KSHV B cell infections with recombinant viruses, may help to reveal miRNA targets that promote early transformation events.

In summary, my studies have shown that miR-K12-11 can hijack miR-155 to regulate an overlapping set of genes involved in B cell regulatory pathways. Determining the functional relevance of miR-K12-11 mimicing miR-155 target regulation requires the identification of phenotypes, which I have shown in the NOD/SCID mouse model. Future work using new models of infection and miR-K12-11 knockout viruses should also reveal functional phenotypes for miR-K12-11. Together with new methods of miRNA data mining, the functional roles for miR-K12-11 and all other viral miRNAs in the processes underlying KSHV pathogenesis can be further elucidated.

Future prospective on KSHV miRNAs

KSHV miRNAs have been shown to target a diverse list of genes that play roles in latency, proliferation, immunity, cell signaling, and transcription (Table 1-1). In addition, the data from my work shows that miR-K12-11 alone can regulate a number of genes involved in B cell biology (discussed in Chapter 3). From this ever expanding list of

targets it appears that KSHV miRNAs play important functions in promoting KSHV infection and possibly pathogenesis. However, it is still unclear if these miRNAs are utilized merely as an auxiliary tool or if they are essential for the lifecycle and pathogenesis of the virus. Furthermore, while most studies have focused on individual miRNAs, the synergistic impact that all KSHV miRNAs have on the biology of the virus is unknown. A recent study using recombinant EBV, in which the BHRF1 miRNA cluster was deleted, revealed that these miRNAs enhance B cell transformation potential but are not absolutely required for this process (Feederle et al., 2011). Because EBV and KSHV are closely related, it is possible that the KSHV miRNAs behave in a similar manner to enhance transformation potential. If this is indeed the case, KSHV miRNAs may represent a novel therapeutic target for the treatment of KSHV tumors. Future recombinant KSHV viruses with miRNA cluster deletions are currently being developed in our lab and will be useful in understanding the combined impact of viral miRNAs.

In addition to viral miRNAs, it has been shown that many cellular miRNAs are upregulated in PEL (O'Hara et al., 2008). Some of the cellular miRNAs identified include members of the oncogenic miR-17-92 family. Therefore, it is possible that these host miRNAs also contribute to KSHV pathogenesis. Future studies are needed to determine the functional relevance of these host miRNAs and how they may work together with viral miRNAs to regulate the host transcriptome.

Because miRNAs have been linked to the formation of many human tumors, the use of miRNA antagomirs as a possible cancer treatment are being extensively developed. The potential of this therapy has shown some promise in pre-clinical

studies, where tumor growth was suppressed by specific antagomirs delivered into mice (Fontana et al., 2008; Ma et al., 2010). Inhibiting viral miRNA function using this strategy may also offer a potential therapy to treat KSHV-associated tumors. However, for this strategy to work it will be important to define the KSHV miRNAs which directly promote tumorgenesis. Based on my work, miR-K12-11 appears to be a good candidate for anti-miR therapy because it functions as an orthologue of the oncomir miR-155. While studies in non-human primates have shown that anti-miR strategies can work to inhibit miRNA function (Elmen et al., 2008) limitations still exist, mainly effective delivery into specific target cells or tissue. So, while the future of anti-miR therapy to treat KSHV-associated malignancies offers tantalizing potential, many questions still remain. Future experiments using newly developed recombinant viruses in combination with appropriate models to test the efficacy of these treatments, will provide a strong platform to better understand and treat KSHV pathogenesis.

APPENDIX
PROTOCOLS AND PRIMERS

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

- Aliquot 15 ml room temperature Ficoll-Paque Plus (StemCell Technologies) into 4 50 ml conical tubes.
- Aliquot 10ml of buffy coat using a 30 ml sterile syringe into 4 new 50 ml conical tubes.
- With room temperature 1x PBS (w/o Ca and Mg) bring the volume up to 30 ml and mix by inverting tube several times.
- Carefully overlay the diluted buffy coat onto the Ficoll using the slowest speed on the autopipette.
- Centrifuge samples @ 423 RCF with the brake off for 30 minutes.
- With the autopipette, remove the top layer (containing most of the platelets) very carefully within about 1 cm of the interphase (white fluffy band that contains B cells). Make sure not to disturb the interphase.
- With the autopipette and a sterile 1ml stripette slowly remove the interphase (white fluffy band) while making sure that you are not sucking up any material below the interphase. Once all lymphocytes have been collected split the cells evenly into two 50 ml conical tubes.
- Wash 1: Bring the volume of both tubes up to 40 ml with 1xPBS (w/o Ca and Mg)/1 mM EDTA/2% FBS.
- Centrifuge @ 311 RCF for 10 minutes.
- Aspirate off as much supernatant as possible without disturbing the cell pellet. The supernatant will be cloudy at this point.
- Wash 2: Separate the pellet by hitting the tube on a table top. Bring the volume of each tube up to 40 ml with 1xPBS (w/o Ca and Mg)/1 mM EDTA/2% FBS and carefully resuspend the pellet.
- Centrifuge @ 311 RCF for 10 minutes.
- Wash 3: Wash 2: Separate the pellet by hitting the tube on a table top. Bring the volume of each tube up to 40 ml with 1xPBS (w/o Ca and Mg)/1 mM EDTA/2% FBS and carefully resuspend the pellet.
- Centrifuge @ 216 RCF for 10 minutes.

- Again carefully remove as much supernatant as possible without disturbing the pellet.

- Separate the pellet by hitting the tube on a table top. Resuspend both pellets in 2.5 ml 1xPBS (w/o Ca and Mg)/1mM EDTA/2% FBS and combine into one 50 ml concial tube (so cells will be in 5 ml total PBS).

- PBMCs are now ready for counting and B cell enrichment.

Human B cell Enrichment

- Count PBMCs with a hemocytometer (I usually dilute cells 100X before counting).

- Prepare PBMCs at a concentration of 5×10^7 cells/ml in room temperature 1xPBS (w/o Ca and Mg)/1 mM EDTA/2% FBS.

- Place 2 ml of PBMCs in 5 ml polystyrene round-bottom tubes to properly fit into the purple EasySep magnet (STEMCELL Tech. Catalog #18000).

- Using the Human B cell enrichment kit (STEMCELL Tech. Catalog #19054) add 100 ul (50 ul/ml of cells) Human B cell enrichment cocktail. Mix well and incubate at room temperature for 10 minutes.

- Vortex EasySep D Magnetic Particles to ensure that they are in a uniform suspension. Add 150ul (75 ul/ml of cells) D Particles. Mix well and incubate at room temperature for 5 minutes.

- Add 250 ul 1xPBS (w/o Ca and Mg)/1 mM EDTA/2% FBS (Brings total volume to 2.5 ml). Mix cells by gently pipetting up and down 2-3 times. Place the tube (without the cap) into the magnet. Set aside for 5 minutes.

- Pick up the EasySep Magnet, and in one continuous motion invert the magnet and tube, pouring off the desired fraction (B cells) into a new 5ml polystyrene tube. The magnetically unwanted cells will remain bound inside the original tube, held by the magnetic field of the magnet. Leave the magnet and the tube inverted for 2-3 seconds (do not shake or blot off any drops that remain hanging from the mouth of the tube!!!) and then return to upright position.

- The negatively selected B cells are now ready to analyze.

- Count B cells with a hemocytometer (I usually dilute cells 10X before counting).

- Analyze purity of B cells (50,000 cells/facs tube) using 5ul V450 Mouse Anti-Human CD19 antibody (BD Biosciences Catalog #560353). B cells should be a 95-98% pure population.

- You can also analyze cells for IgD expression (20 ul PE Mouse Anti-Human IgD BD Biosciences Catalog #555779) and CD38 expression (5 ul APC clone HB7 Catalog #340439).

- Keep the cells for analysis in 1xPBS (w/o Ca and Mg)/1 mM EDTA/2% FBS. Pellet the remaining cells @311 RCF for 10 minutes.

- Resuspend pellet in enough B cell medium (described in protocol below) to keep the concentration around 6 x 10^6 cells/ml for B cell differentiation.

B cell medium

- 500 ml 1640 RPMI

- 5 ml 100 mM Sodium Pyruvate (1 mM final concentration)

- 50 ml FBS (10% final concentration)

- 5 ml Penicillin/Streptomycin (1% final concentration)

In vitro plasma cell differentiation

- Count purified B cells with a hemocytometer.

- Prepare B cell media with the following: 15 ng/well IL-21 (Peprotech Recombinant Human IL-21 Catalog #200-21), 500 ng/well anti-IgM (Jackson ImmunoResearch AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgM Catalog #109-006-129), 10 ng/well anti-CD40 (R&D Systems anti-human CD40/TNFRSF5 Antibody Catalog#AF632)

- Plate cells in 96 well round bottom plates (BD Falcon clear tissue culture treated with lid Catalog #353227) at a density of 5 x 10^4 cells/well in 100 ul room temperature B cell medium + stimulatory factors (IL-21, anti-CD40, and anti-IgM).

- Anti-CD40 removal: 3 days post-stimulation harvest cells into 1.5 ml eppendorf tubes, pellet @ 500 RCF for 5 min, remove media, and replate in 100 ul room temperature B cell media with 15 ng/well IL-21 and 500 ng/well anti-IgM but without anti-CD40.

- 7 days post-stimulation harvest cells, pellet @ 500 RCF for 5 min, resuspend in 100 ul 1xPBS (w/o Ca and Mg) and add to facs tubes.

- Add 20 ul anti-IgD and 5ul anti-CD38 to facs tubes with cells, mix, and protect tubes from light until you analyze them by flow.

- For flow analysis add ~1 ml 1xPBS (w/o Ca and Mg) to the tube, vortex, and analyze.

MiRNA mimic transfection

- Transfection is carried out in 96 well round bottom plates (BD Falcon clear tissue culture treated with lid Catalog #353227) containing 100 ul total media/well.
- 1st make transfection mixture containing:
- 9 ul/well room temperature Opti-MEM Reduced Serum Medium (Invitrogen Catalog #31985-062).
- 0.35 ul/well room temperature Mirus TransIT-TKO transfection reagent (Mirus Bio LLC Catalog #MIR 2152).
- Vortex transfection mixture and incubate 10 minutes @ room temperature.
- Add 125 nM/well miRNA mimic (Thermo Scientific miRIDIAN microRNA mimic), for a transfection control use Dy547 conjugate mimic (Catalog #CP-004500-01-05), to the transfection mixture and mix by gentle pipetting, incubate 10 minutes @ room temperature.
- Add transfection mixture dropwise to cells.
- If worried about toxicity of transfection mixture (especially for primary B cells) change media 6 hours post-transfection

B cell proliferation assay

- Proliferation is measured using the Click-iT Edu Flow Cytometry Assay Kit with Alexa Fluor 647 azide (Invitrogen Catalog #C10424).
- To measure rapidly proliferating cell types (PEL and BJAB) cells should be incubated with 10 uM Edu for 2 hours. For slowly proliferating cells, longer incubations with lower concentrations of Edu may be required.
- I followed the protocol for the assay with the following minor adjustments:
- The amounts of all components can be reduced by half, for example instead of 500 ul of the Click-IT reaction cocktail you can use 250 ul, therefore you will get twice the number of reactions from each kit.
- Wash the cells with 1ml of 1% BSA in PBS (w/o Ca and Mg) instead of 3 ml.
- After fixing the cells and washing them in 1 ml of 1% BSA in PBS (w/o Ca and Mg) you can store the fixed cells up to one week @ 4°C in 100 ul 1% BSA in PBS (w/o Ca and Mg).

Primers for qPCR

- BACH1 Forward primer: CACCGAAGGAGACAGTGAATC
- BACH1 Reverse primer: TGTTCTGGAGTAAGCTTGTGC
- SHIP1 Forward primer: AGTACAACTTGCCTTCCTGG
- SHIP1 Reverse primer: TGACTCCTGCCTCAAATGTG
- MYB Forward primer: TCAGGAAACTTCTTCTGCTCACA
- MYB Reverse primer: AGGTTCCCAGGTACTGCT

LIST OF REFERENCES

Abend, J.R., Uldrick, T., and Ziegelbauer, J.M. (2010). Regulation of tumor necrosis factor-like weak inducer of apoptosis receptor protein (TWEAKR) expression by Kaposi's sarcoma-associated herpesvirus microRNA prevents TWEAK-induced apoptosis and inflammatory cytokine expression. Journal of virology *84*, 12139-12151.

Ambros, V. (2004). The functions of animal microRNAs. Nature *431*, 350-355.

Ambroziak, J.A., Blackbourn, D.J., Herndier, B.G., Glogau, R.G., Gullett, J.H., McDonald, A.R., Lennette, E.T., and Levy, J.A. (1995). Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science *268*, 582-583.

Antman, K., and Chang, Y. (2000). Kaposi's sarcoma. N Engl J Med *342*, 1027-1038.

Areste, C., and Blackbourn, D.J. (2009). Modulation of the immune system by Kaposi's sarcoma-associated herpesvirus. Trends Microbiol *17*, 119-129.

Arguello, M., Sgarbanti, M., Hernandez, E., Mamane, Y., Sharma, S., Servant, M., Lin, R., and Hiscott, J. (2003). Disruption of the B-cell specific transcriptional program in HHV-8 associated primary effusion lymphoma cell lines. Oncogene *22*, 964-973.

Asangani, I.A., Rasheed, S.A., Nikolova, D.A., Leupold, J.H., Colburn, N.H., Post, S., and Allgayer, H. (2008). MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene *27*, 2128-2136.

Asou, H., Said, J.W., Yang, R., Munker, R., Park, D.J., Kamada, N., and Koeffler, H.P. (1998). Mechanisms of growth control of Kaposi's sarcoma-associated herpes virus-associated primary effusion lymphoma cells. Blood *91*, 2475-2481.

Baek, D., Villen, J., Shin, C., Camargo, F.D., Gygi, S.P., and Bartel, D.P. (2008). The impact of microRNAs on protein output. Nature *455*, 64-71.

Ballestas, M.E., Chatis, P.A., and Kaye, K.M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency- associated nuclear antigen. Science *284*, 641-644.

Baltimore, D., Boldin, M.P., O'Connell, R.M., Rao, D.S., and Taganov, K.D. (2008). MicroRNAs: new regulators of immune cell development and function. Nature immunology *9*, 839-845.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Basso, K., Sumazin, P., Morozov, P., Schneider, C., Maute, R.L., Kitagawa, Y., Mandelbaum, J., Haddad, J., Jr., Chen, C.Z., Califano, A., and Dalla-Favera, R. (2009). Identification of the human mature B cell miRNome. Immunity *30*, 744-752.

Bechtel, J.T., Liang, Y., Hvidding, J., and Ganem, D. (2003). Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. Journal of virology *77*, 6474-6481.

Beck, J.T., Hsu, S.M., Wijdenes, J., Bataille, R., Klein, B., Vesole, D., Hayden, K., Jagannath, S., and Barlogie, B. (1994). Brief report: alleviation of systemic manifestations of Castleman's disease by monoclonal anti-interleukin-6 antibody. N Engl J Med *330*, 602-605.

Bellare, P., and Ganem, D. (2009). Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation. Cell host & microbe *6*, 570-575.

Beral, V., Peterman, T.A., Berkelman, R.L., and Jaffe, H.W. (1990). Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet *335*, 123-128.

Blackbourn, D.J., Lennette, E., Klencke, B., Moses, A., Chandran, B., Weinstein, M., Glogau, R.G., Witte, M.H., Way, D.L., Kutzkey, T.*, et al.* (2000). The restricted cellular host range of human herpesvirus 8. Aids *14*, 1123-1133.

Bloom, D.C., Giordani, N.V., and Kwiatkowski, D.L. (2010). Epigenetic regulation of latent HSV-1 gene expression. Biochim Biophys Acta *1799*, 246-256.

Bolisetty, M.T., Dy, G., Tam, W., and Beemon, K.L. (2009). Reticuloendotheliosis virus strain T induces miR-155, which targets JARID2 and promotes cell survival. Journal of virology *83*, 12009-12017.

Boshoff, C., and Weiss, R. (2002). AIDS-related malignancies. Nat Rev Cancer *2*, 373-382.

Boss, I.W., Plaisance, K.B., and Renne, R. (2009). Role of virus-encoded microRNAs in herpesvirus biology. Trends Microbiol *17*, 544-553.

Brimo, F., Michel, R.P., Khetani, K., and Auger, M. (2007). Primary effusion lymphoma: a series of 4 cases and review of the literature with emphasis on cytomorphologic and immunocytochemical differential diagnosis. Cancer *111*, 224-233.

Brinkmann, M.M., and Schulz, T.F. (2006). Regulation of intracellular signalling by the terminal membrane proteins of members of the Gammaherpesvirinae. J Gen Virol *87*, 1047-1074.

Bryant, V.L., Ma, C.S., Avery, D.T., Li, Y., Good, K.L., Corcoran, L.M., de Waal Malefyt, R., and Tangye, S.G. (2007). Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol *179*, 8180-8190.

Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B.C., Green, P.J., Jain, N., Isaacs, G., and Morgan, R.W. (2006). Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript. Journal of virology *80*, 8778-8786.

Cai, X., and Cullen, B.R. (2006). Transcriptional origin of Kaposi's sarcoma-associated herpesvirus microRNAs. Journal of virology *80*, 2234-2242.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C.M., Damania, B., and Cullen, B.R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. Proceedings of the National Academy of Sciences of the United States of America *102*, 5570-5575.

Cai, X., Schafer, A., Lu, S., Bilello, J.P., Desrosiers, R.C., Edwards, R., Raab-Traub, N., and Cullen, B.R. (2006). Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog *2*, e23.

Caldwell, R.G., Wilson, J.B., Anderson, S.J., and Longnecker, R. (1998). Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity *9*, 405-411.

Cameron, J.E., Fewell, C., Yin, Q., McBride, J., Wang, X., Lin, Z., and Flemington, E.K. (2008). Epstein-Barr virus growth/latency III program alters cellular microRNA expression. Virology *382*, 257-266.

Carbone, A., Cesarman, E., Gloghini, A., and Drexler, H.G. (2010). Understanding pathogenetic aspects and clinical presentation of primary effusion lymphoma through its derived cell lines. Aids *24*, 479-490.

Carbone, A., and Gloghini, A. (2008). KSHV/HHV8-associated lymphomas. Br J Haematol *140*, 13-24.

Carbone, A., Gloghini, A., Cozzi, M.R., Capello, D., Steffan, A., Monini, P., De Marco, L., and Gaidano, G. (2000). Expression of MUM1/IRF4 selectively clusters with primary effusion lymphoma among lymphomatous effusions: implications for disease histogenesis and pathogenesis. Br J Haematol *111*, 247-257.

Carsetti, R., Rosado, M.M., and Wardmann, H. (2004). Peripheral development of B cells in mouse and man. Immunological reviews *197*, 179-191.

CDC (1981). Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. In Mmwr, pp. 305-308.

Ceppi, M., Pereira, P.M., Dunand-Sauthier, I., Barras, E., Reith, W., Santos, M.A., and Pierre, P. (2009). MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proceedings of the National Academy of Sciences of the United States of America *106*, 2735-2740.

Cesarman, E. (2011). Gammaherpesvirus and lymphoproliferative disorders in immunocompromised patients. Cancer Lett *305*, 163-174.

Cesarman, E., Chang, Y., Moore, P.S., Said, J.W., and Knowles, D.M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med *332*, 1186-1191.

Cesarman, E., and Knowles, D.M. (1999). The role of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in lymphoproliferative diseases. Semin Cancer Biol *9*, 165-174.

Chadburn, A., Hyjek, E.M., Tam, W., Liu, Y., Rengifo, T., Cesarman, E., and Knowles, D.M. (2008). Immunophenotypic analysis of the Kaposi sarcoma herpesvirus (KSHV; HHV-8)-infected B cells in HIV+ multicentric Castleman disease (MCD). Histopathology *53*, 513-524.

Chang, Y., Cesarman, E., Pessin, M.S., Lee, F., Culpepper, J., Knowles, D.M., and Moore, P.S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma [see comments]. Science *266*, 1865-1869.

Chen, C.Z., Li, L., Lodish, H.F., and Bartel, D.P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science *303*, 83-86.

Chi, S.W., Zang, J.B., Mele, A., and Darnell, R.B. (2009). Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. Nature *460*, 479-486.

Cirone, M., Lucania, G., Aleandri, S., Borgia, G., Trivedi, P., Cuomo, L., Frati, L., and Faggioni, A. (2008). Suppression of dendritic cell differentiation through cytokines released by Primary Effusion Lymphoma cells. Immunology letters *120*, 37-41.

Cosmopoulos, K., Pegtel, M., Hawkins, J., Moffett, H., Novina, C., Middeldorp, J., and Thorley-Lawson, D.A. (2009). Comprehensive profiling of Epstein-Barr virus microRNAs in nasopharyngeal carcinoma. Journal of virology *83*, 2357-2367.

Costinean, S., Sandhu, S.K., Pedersen, I.M., Tili, E., Trotta, R., Perrotti, D., Ciarlariello, D., Neviani, P., Harb, J., Kauffman, L.R*., et al.* (2009). Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. Blood *114*, 1374-1382.

Costinean, S., Zanesi, N., Pekarsky, Y., Tili, E., Volinia, S., Heerema, N., and Croce, C.M. (2006). Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proceedings of the National Academy of Sciences of the United States of America *103*, 7024-7029.

Cui, C., Griffiths, A., Li, G., Silva, L.M., Kramer, M.F., Gaasterland, T., Wang, X.J., and Coen, D.M. (2006). Prediction and identification of herpes simplex virus 1-encoded microRNAs. Journal of virology *80*, 5499-5508.

de Oliveira, D.E., Ballon, G., and Cesarman, E. (2010). NF-kappaB signaling modulation by EBV and KSHV. Trends Microbiol *18*, 248-257.

Diehl, S.A., Schmidlin, H., Nagasawa, M., van Haren, S.D., Kwakkenbos, M.J., Yasuda, E., Beaumont, T., Scheeren, F.A., and Spits, H. (2008). STAT3-mediated up-regulation of BLIMP1 Is coordinated with BCL6 down-regulation to control human plasma cell differentiation. J Immunol *180*, 4805-4815.

Dittmer, D., Lagunoff, M., Renne, R., Staskus, K., Haase, A., and Ganem, D. (1998). A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. Journal of virology *72*, 8309-8315.

Dorsett, Y., McBride, K.M., Jankovic, M., Gazumyan, A., Thai, T.H., Robbiani, D.F., Di Virgilio, M., Reina San-Martin, B., Heidkamp, G., Schwickert, T.A.*, et al.* (2008). MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. Immunity *28*, 630-638.

Dourmishev, L.A., Dourmishev, A.L., Palmeri, D., Schwartz, R.A., and Lukac, D.M. (2003). Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. Microbiol Mol Biol Rev *67*, 175-212, table of contents.

Drexler, H.G., Uphoff, C.C., Gaidano, G., and Carbone, A. (1998). Lymphoma cell lines: in vitro models for the study of HHV-8+ primary effusion lymphomas (body cavity-based lymphomas). Leukemia *12*, 1507-1517.

Du, M.Q., Diss, T.C., Liu, H., Ye, H., Hamoudi, R.A., Cabecadas, J., Dong, H.Y., Harris, N.L., Chan, J.K., Rees, J.W.*, et al.* (2002). KSHV- and EBV-associated germinotropic lymphoproliferative disorder. Blood *100*, 3415-3418.

Du, M.Q., Liu, H., Diss, T.C., Ye, H., Hamoudi, R.A., Dupin, N., Meignin, V., Oksenhendler, E., Boshoff, C., and Isaacson, P.G. (2001). Kaposi sarcoma-associated herpesvirus infects monotypic (IgM lambda) but polyclonal naive B cells in Castleman disease and associated lymphoproliferative disorders. Blood *97*, 2130-2136.

Dunn, C., Chalupny, N.J., Sutherland, C.L., Dosch, S., Sivakumar, P.V., Johnson, D.C., and Cosman, D. (2003). Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med *197*, 1427-1439.

Dunn, W., Trang, P., Zhong, Q., Yang, E., van Belle, C., and Liu, F. (2005). Human cytomegalovirus expresses novel microRNAs during productive viral infection. Cell Microbiol *7*, 1684-1695.

Dupin, N., Diss, T.L., Kellam, P., Tulliez, M., Du, M.Q., Sicard, D., Weiss, R.A., Isaacson, P.G., and Boshoff, C. (2000). HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. Blood *95*, 1406-1412.

Edwards, R.H., Marquitz, A.R., and Raab-Traub, N. (2008). Epstein-Barr virus BART microRNAs are produced from a large intron prior to splicing. Journal of virology *82*, 9094-9106.

Eis, P.S., Tam, W., Sun, L., Chadburn, A., Li, Z., Gomez, M.F., Lund, E., and Dahlberg, J.E. (2005). Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proceedings of the National Academy of Sciences of the United States of America *102*, 3627-3632.

Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjarn, M., Hansen, H.F., Berger, U.*, et al.* (2008). LNA-mediated microRNA silencing in non-human primates. Nature *452*, 896-899.

Ettinger, R., Kuchen, S., and Lipsky, P.E. (2008). The role of IL-21 in regulating B-cell function in health and disease. Immunological reviews *223*, 60-86.

Ettinger, R., Sims, G.P., Fairhurst, A.M., Robbins, R., da Silva, Y.S., Spolski, R., Leonard, W.J., and Lipsky, P.E. (2005). IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. J Immunol *175*, 7867-7879.

Fais, F., Gaidano, G., Capello, D., Gloghini, A., Ghiotto, F., Roncella, S., Carbone, A., Chiorazzi, N., and Ferrarini, M. (1999). Immunoglobulin V region gene use and structure suggest antigen selection in AIDS-related primary effusion lymphomas. Leukemia *13*, 1093-1099.

Feederle, R., Linnstaedt, S.D., Bannert, H., Lips, H., Bencun, M., Cullen, B.R., and Delecluse, H.J. (2011). A viral microRNA cluster strongly potentiates the transforming properties of a human herpesvirus. PLoS Pathog *7*, e1001294.

Flore, O., Rafii, S., Ely, S., O'Leary, J.J., Hyjek, E.M., and Cesarman, E. (1998). Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. Nature *394*, 588-592.

Fontana, L., Fiori, M.E., Albini, S., Cifaldi, L., Giovinazzi, S., Forloni, M., Boldrini, R., Donfrancesco, A., Federici, V., Giacomini, P.*, et al.* (2008). Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. PloS one *3*, e2236.

Foussat, A., Wijdenes, J., Bouchet, L., Gaidano, G., Neipel, F., Balabanian, K., Galanaud, P., Couderc, J., and Emilie, D. (1999). Human interleukin-6 is in vivo an autocrine growth factor for human herpesvirus-8-infected malignant B lymphocytes. European cytokine network *10*, 501-508.

Friedman, R.C., Farh, K.K., Burge, C.B., and Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. Genome research *19*, 92-105.

Ganem, D. (2006). KSHV infection and the pathogenesis of Kaposi's sarcoma. Annual review of pathology *1*, 273-296.

Gartner, K., Wiktorowicz, T., Park, J., Mergia, A., Rethwilm, A., and Scheller, C. (2009). Accuracy estimation of foamy virus genome copying. Retrovirology *6*, 32.

Garzon, R., Volinia, S., Liu, C.G., Fernandez-Cymering, C., Palumbo, T., Pichiorri, F., Fabbri, M., Coombes, K., Alder, H., Nakamura, T.*, et al.* (2008). MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. Blood *111*, 3183-3189.

Gatto, G., Rossi, A., Rossi, D., Kroening, S., Bonatti, S., and Mallardo, M. (2008). Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-kappaB pathway. Nucleic Acids Res *36*, 6608-6619.

Georgantas, R.W., 3rd, Hildreth, R., Morisot, S., Alder, J., Liu, C.G., Heimfeld, S., Calin, G.A., Croce, C.M., and Civin, C.I. (2007). CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. Proceedings of the National Academy of Sciences of the United States of America *104*, 2750-2755.

Gessain, A. (1997). [Human herpesvirus 8 and associated diseases: Kaposi's sarcoma, body cavity based lymphoma and multicentric Castleman disease: clinical and molecular epidemiology]. Bulletin de l'Academie nationale de medecine *181*, 1023-1034.

Giassi, L.J., Pearson, T., Shultz, L.D., Laning, J., Biber, K., Kraus, M., Woda, B.A., Schmidt, M.R., Woodland, R.T., Rossini, A.A., and Greiner, D.L. (2008). Expanded CD34+ human umbilical cord blood cells generate multiple lymphohematopoietic lineages in NOD-scid IL2rgamma(null) mice. Experimental biology and medicine (Maywood, N.J *233*, 997-1012.

Gottlieb, G.J., Ragaz, A., Vogel, J.V., Friedman-Kien, A., Rywlin, A.M., Weiner, E.A., and Ackerman, A.B. (1981). A preliminary communication on extensively disseminated Kaposi's sarcoma in young homosexual men. The American Journal of dermatopathology *3*, 111-114.

Gottwein, E., and Cullen, B.R. (2010). A human herpesvirus microRNA inhibits p21 expression and attenuates p21-mediated cell cycle arrest. Journal of virology *84*, 5229-5237.

Gottwein, E., Mukherjee, N., Sachse, C., Frenzel, C., Majoros, W.H., Chi, J.T., Braich, R., Manoharan, M., Soutschek, J., Ohler, U., and Cullen, B.R. (2007). A viral microRNA functions as an orthologue of cellular miR-155. Nature *450*, 1096-1099.

Grey, F., Antoniewicz, A., Allen, E., Saugstad, J., McShea, A., Carrington, J.C., and Nelson, J. (2005). Identification and characterization of human cytomegalovirus-encoded microRNAs. Journal of virology *79*, 12095-12099.

Griffiths-Jones, S. (2004). The microRNA Registry. Nucleic Acids Res *32*, D109-111.

Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell *27*, 91-105.

Grundhoff, A., and Ganem, D. (2003). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus permits replication of terminal repeat-containing plasmids. Journal of virology *77*, 2779-2783.

Grundhoff, A., and Sullivan, C.S. (2011). Virus-encoded microRNAs. Virology *411*, 325-343.

Grundhoff, A., Sullivan, C.S., and Ganem, D. (2006). A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. Rna *12*, 733-750.

Guo, H., Ingolia, N.T., Weissman, J.S., and Bartel, D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466*, 835-840.

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano, M., Jr., Jungkamp, A.C., Munschauer, M.*, et al.* (2010). Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell *141*, 129-141.

Hansen, A., Henderson, S., Lagos, D., Nikitenko, L., Coulter, E., Roberts, S., Gratrix, F., Plaisance, K., Renne, R., Bower, M.*, et al.* (2010). KSHV-encoded miRNAs target MAF to induce endothelial cell reprogramming. Genes Dev *24*, 195-205.

Hassman, L.M., Ellison, T.J., and Kedes, D.H. (2011). KSHV infects a subset of human tonsillar B cells, driving proliferation and plasmablast differentiation. J Clin Invest *121*, 752-768.

Hu, J., Garber, A.C., and Renne, R. (2002). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA replication in dividing cells. Journal of virology *76*, 11677-11687.

Huntzinger, E., and Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet *12*, 99-110.

Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056-2060.

Igarashi, K., and Sun, J. (2006). The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. Antioxidants & redox signaling *8*, 107-118.

Jego, G., Bataille, R., and Pellat-Deceunynck, C. (2001). Interleukin-6 is a growth factor for nonmalignant human plasmablasts. Blood *97*, 1817-1822.

Jenner, R.G., Maillard, K., Cattini, N., Weiss, R.A., Boshoff, C., Wooster, R., and Kellam, P. (2003). Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. Proceedings of the National Academy of Sciences of the United States of America *100*, 10399-10404.

Jiang, J., Lee, E.J., and Schmittgen, T.D. (2006). Increased expression of microRNA-155 in Epstein-Barr virus transformed lymphoblastoid cell lines. Genes, chromosomes & cancer *45*, 103-106.

Jiang, S., Zhang, H.W., Lu, M.H., He, X.H., Li, Y., Gu, H., Liu, M.F., and Wang, E.D. (2010). MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. Cancer Res *70*, 3119-3127.

Jones, K.D., Aoki, Y., Chang, Y., Moore, P.S., Yarchoan, R., and Tosato, G. (1999). Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. Blood *94*, 2871-2879.

Jourdan, M., Caraux, A., De Vos, J., Fiol, G., Larroque, M., Cognot, C., Bret, C., Duperray, C., Hose, D., and Klein, B. (2009). An in vitro model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. Blood *114*, 5173-5181.

Kasof, G.M., Goyal, L., and White, E. (1999). Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. Mol Cell Biol *19*, 4390-4404.

Khaled, A.R., Butfiloski, E.J., Sobel, E.S., and Schiffenbauer, J. (1998). Functional consequences of the SHP-1 defect in motheaten viable mice: role of NF-kappa B. Cellular immunology *185*, 49-58.

Klein, B., Zhang, X.G., Lu, Z.Y., and Bataille, R. (1995). Interleukin-6 in human multiple myeloma. Blood *85*, 863-872.

Klein, U., Gloghini, A., Gaidano, G., Chadburn, A., Cesarman, E., Dalla-Favera, R., and Carbone, A. (2003). Gene expression profile analysis of AIDS-related primary effusion lymphoma (PEL) suggests a plasmablastic derivation and identifies PEL-specific transcripts. Blood *101*, 4115-4121.

Kluiver, J., Poppema, S., de Jong, D., Blokzijl, T., Harms, G., Jacobs, S., Kroesen, B.J., and van den Berg, A. (2005). BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. J Pathol *207*, 243-249.

Koelle, D.M., Huang, M.L., Chandran, B., Vieira, J., Piepkorn, M., and Corey, L. (1997). Frequent detection of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) DNA in saliva of human immunodeficiency virus-infected men: clinical and immunologic correlates. J Infect Dis *176*, 94-102.

Lacoste, V., Nicot, C., Gessain, A., Valensi, F., Gabarre, J., Matta, H., Chaudhary, P.M., and Mahieux, R. (2007). In primary effusion lymphoma cells, MYB transcriptional repression is associated with v-FLIP expression during latent KSHV infection while both v-FLIP and v-GPCR become involved during the lytic cycle. Br J Haematol *138*, 487-501.

Lagunoff, M., Majeti, R., Weiss, A., and Ganem, D. (1999). Deregulated signal transduction by the K1 gene product of Kaposi's sarcoma-associated herpesvirus. Proceedings of the National Academy of Sciences of the United States of America *96*, 5704-5709.

Lan, K., Kuppers, D.A., Verma, S.C., and Robertson, E.S. (2004). Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen inhibits lytic replication by targeting Rta: a potential mechanism for virus-mediated control of latency. Journal of virology *78*, 6585-6594.

Larroche, C., Cacoub, P., Soulier, J., Oksenhendler, E., Clauvel, J.P., Piette, J.C., and Raphael, M. (2002). Castleman's disease and lymphoma: report of eight cases in HIV-negative patients and literature review. American journal of hematology *69*, 119-126.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415-419.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C.G., Huang, Y., and Gao, S.J. (2010). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. Nat Cell Biol *12*, 193-199.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15-20.

Liang, D., Gao, Y., Lin, X., He, Z., Zhao, Q., Deng, Q., and Lan, K. (2011). A human herpesvirus miRNA attenuates interferon signaling and contributes to maintenance of viral latency by targeting IKKvarepsilon. Cell research *21*, 793-806.

Lieberman, P.M., Hu, J., and Renne, R. (2007). Maintenance and replication during latency. In Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis, A. Arvin, G. Campadelli-Fiume, and E. Mocarski, eds. (Cambridge University Press).

Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B., and Bartel, D.P. (2003). The microRNAs of Caenorhabditis elegans. Genes Dev *17*, 991-1008.

Lin, K.I., Tunyaplin, C., and Calame, K. (2003). Transcriptional regulatory cascades controlling plasma cell differentiation. Immunological reviews *194*, 19-28.

Lin, X., Liang, D., He, Z., Deng, Q., Robertson, E.S., and Lan, K. (2011). miR-K12-7-5p encoded by Kaposi's sarcoma-associated herpesvirus stabilizes the latent state by targeting viral ORF50/RTA. PloS one *6*, e16224.

Linnstaedt, S.D., Gottwein, E., Skalsky, R.L., Luftig, M.A., and Cullen, B.R. (2010). Virally induced cellular microRNA miR-155 plays a key role in B-cell immortalization by Epstein-Barr virus. Journal of virology *84*, 11670-11678.

Liu, F., Lei, W., O'Rourke, J.P., and Ness, S.A. (2006). Oncogenic mutations cause dramatic, qualitative changes in the transcriptional activity of c-Myb. Oncogene *25*, 795-805.

Liu, Y.W., Tseng, H.P., Chen, L.C., Chen, B.K., and Chang, W.C. (2003). Functional cooperation of simian virus 40 promoter factor 1 and CCAAT/enhancer-binding protein beta and delta in lipopolysaccharide-induced gene activation of IL-10 in mouse macrophages. J Immunol *171*, 821-828.

Louafi, F., Martinez-Nunez, R.T., and Sanchez-Elsner, T. (2010). MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-{beta}. J Biol Chem *285*, 41328-41336.

Lu, C.C., Li, Z., Chu, C.Y., Feng, J., Feng, J., Sun, R., and Rana, T.M. (2010a). MicroRNAs encoded by Kaposi's sarcoma-associated herpesvirus regulate viral life cycle. EMBO Rep *11*, 784-790.

Lu, F., Stedman, W., Yousef, M., Renne, R., and Lieberman, P.M. (2010b). Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway. Journal of virology *84*, 2697-2706.

Lu, F., Weidmer, A., Liu, C.G., Volinia, S., Croce, C.M., and Lieberman, P.M. (2008). Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. Journal of virology *82*, 10436-10443.

Lu, G.D., Leung, C.H., Yan, B., Tan, C.M., Low, S.Y., Aung, M.O., Salto-Tellez, M., Lim, S.G., and Hooi, S.C. (2010c). C/EBPalpha is up-regulated in a subset of hepatocellular carcinomas and plays a role in cell growth and proliferation. Gastroenterology *139*, 632-643, 643 e631-634.

Luppi, M., Barozzi, P., Maiorana, A., Artusi, T., Trovato, R., Marasca, R., Savarino, M., Ceccherini-Nelli, L., and Torelli, G. (1996). Human herpesvirus-8 DNA sequences in human immunodeficiency virus-negative angioimmunoblastic lymphadenopathy and benign lymphadenopathy with giant germinal center hyperplasia and increased vascularity. Blood *87*, 3903-3909.

Ma, L., Reinhardt, F., Pan, E., Soutschek, J., Bhat, B., Marcusson, E.G., Teruya-Feldstein, J., Bell, G.W., and Weinberg, R.A. (2010). Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. Nature biotechnology *28*, 341-347.

Malumbres, R., Sarosiek, K.A., Cubedo, E., Ruiz, J.W., Jiang, X., Gascoyne, R.D., Tibshirani, R., and Lossos, I.S. (2009). Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. Blood *113*, 3754-3764.

Marshall, V., Parks, T., Bagni, R., Wang, C.D., Samols, M.A., Hu, J., Wyvil, K.M., Aleman, K., Little, R.F., Yarchoan, R.*, et al.* (2007). Conservation of virally encoded microrna in Kaposi sarcoma--associated herpesvirus in primary effusion lymphoma cell lines and in patients with Kaposi sarcoma or multicentric castleman disease. J Infect Dis *195*, 645-659.

Matolcsy, A., Nador, R.G., Cesarman, E., and Knowles, D.M. (1998). Immunoglobulin VH gene mutational analysis suggests that primary effusion lymphomas derive from different stages of B cell maturation. Am J Pathol *153*, 1609-1614.

Mayama, S., Cuevas, L.E., Sheldon, J., Omar, O.H., Smith, D.H., Okong, P., Silvel, B., Hart, C.A., and Schulz, T.F. (1998). Prevalence and transmission of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in Ugandan children and adolescents. International journal of cancer *77*, 817-820.

Mbulaiteye, S.M., and Engels, E.A. (2006). Kaposi's sarcoma risk among transplant recipients in the United States (1993-2003). International journal of cancer *119*, 2685-2691.

McCormick, C., and Ganem, D. (2005). The kaposin B protein of KSHV activates the p38/MK2 pathway and stabilizes cytokine mRNAs. Science *307*, 739-741.

Medina, P.P., Nolde, M., and Slack, F.J. (2010). OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature *467*, 86-90.

Merkerova, M., Belickova, M., and Bruchova, H. (2008). Differential expression of microRNAs in hematopoietic cell lineages. European journal of haematology *81*, 304-310.

Mesri, E.A., Cesarman, E., Arvanitakis, L., Rafii, S., Moore, M.A., Posnett, D.N., Knowles, D.M., and Asch, A.S. (1996). Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. J Exp Med *183*, 2385-2390.

Mesri, E.A., Cesarman, E., and Boshoff, C. (2010). Kaposi's sarcoma and its associated herpesvirus. Nat Rev Cancer *10*, 707-719.

Montaner, S., Sodhi, A., Servitja, J.M., Ramsdell, A.K., Barac, A., Sawai, E.T., and Gutkind, J.S. (2004). The small GTPase Rac1 links the Kaposi sarcoma-associated herpesvirus vGPCR to cytokine secretion and paracrine neoplasia. Blood *104*, 2903-2911.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annual review of immunology *19*, 683-765.

Moore, P.S., Gao, S.J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D.M., Garber, R., Pellett, P.E., McGeoch, D.J., and Chang, Y. (1996). Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae. Journal of virology *70*, 549-558.

Morgan, R., Anderson, A., Bernberg, E., Kamboj, S., Huang, E., Lagasse, G., Isaacs, G., Parcells, M., Meyers, B.C., Green, P.J., and Burnside, J. (2008). Sequence conservation and differential expression of Marek's disease virus microRNAs. Journal of virology *82*, 12213-12220.

Mori, Y., Nishimoto, N., Ohno, M., Inagi, R., Dhepakson, P., Amou, K., Yoshizaki, K., and Yamanishi, K. (2000). Human herpesvirus 8-encoded interleukin-6 homologue (viral IL-6) induces endogenous human IL-6 secretion. Journal of medical virology *61*, 332-335.

Mosam, A., Carrara, H., Shaik, F., Uldrick, T., Berkman, A., Aboobaker, J., and Coovadia, H.M. (2009). Increasing incidence of Kaposi's sarcoma in black South Africans in KwaZulu-Natal, South Africa (1983-2006). International journal of STD & AIDS *20*, 553-556.

Mosmann, T.R. (1994). Properties and functions of interleukin-10. Advances in immunology *56*, 1-26.

Mrazek, J., Kreutmayer, S.B., Grasser, F.A., Polacek, N., and Huttenhofer, A. (2007). Subtractive hybridization identifies novel differentially expressed ncRNA species in EBV-infected human B cells. Nucleic Acids Res *35*, e73.

Mukherji, S., Ebert, M.S., Zheng, G.X., Tsang, J.S., Sharp, P.A., and van Oudenaarden, A. (2011). MicroRNAs can generate thresholds in target gene expression. Nat Genet *43*, 854-859.

Muralidhar, S., Pumfery, A.M., Hassani, M., Sadaie, M.R., Kishishita, M., Brady, J.N., Doniger, J., Medveczky, P., and Rosenthal, L.J. (1998). Identification of kaposin (open reading frame K12) as a human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) transforming gene. Journal of virology *72*, 4980-4988.

Myoung, J., and Ganem, D. (2011). Infection of primary human tonsillar lymphoid cells by KSHV reveals frequent but abortive infection of T cells. Virology *413*, 1-11.

Nachmani, D., Stern-Ginossar, N., Sarid, R., and Mandelboim, O. (2009). Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell host & microbe *5*, 376-385.

Nador, R.G., Cesarman, E., Chadburn, A., Dawson, D.B., Ansari, M.Q., Sald, J., and Knowles, D.M. (1996). Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood *88*, 645-656.

Nakamura, K., Kouro, T., Kincade, P.W., Malykhin, A., Maeda, K., and Coggeshall, K.M. (2004). Src homology 2-containing 5-inositol phosphatase (SHIP) suppresses an early stage of lymphoid cell development through elevated interleukin-6 production by myeloid cells in bone marrow. J Exp Med *199*, 243-254.

Neipel, F., Albrecht, J.C., and Fleckenstein, B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? Journal of virology *71*, 4187-4192.

O'Connell, R.M., Chaudhuri, A.A., Rao, D.S., and Baltimore, D. (2009). Inositol phosphatase SHIP1 is a primary target of miR-155. Proceedings of the National Academy of Sciences of the United States of America *106*, 7113-7118.

O'Connell, R.M., Rao, D.S., Chaudhuri, A.A., Boldin, M.P., Taganov, K.D., Nicoll, J., Paquette, R.L., and Baltimore, D. (2008). Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. J Exp Med *205*, 585-594.

O'Connell, R.M., Taganov, K.D., Boldin, M.P., Cheng, G., and Baltimore, D. (2007). MicroRNA-155 is induced during the macrophage inflammatory response. Proceedings of the National Academy of Sciences of the United States of America *104*, 1604-1609.

O'Hara, A.J., Vahrson, W., and Dittmer, D.P. (2008). Gene alteration and precursor and mature microRNA transcription changes contribute to the miRNA signature of primary effusion lymphoma. Blood *111*, 2347-2353.

Oksenhendler, E., Carcelain, G., Aoki, Y., Boulanger, E., Maillard, A., Clauvel, J.P., and Agbalika, F. (2000). High levels of human herpesvirus 8 viral load, human interleukin-6, interleukin-10, and C reactive protein correlate with exacerbation of multicentric castleman disease in HIV-infected patients. Blood *96*, 2069-2073.

Panagopoulos, D., Victoratos, P., Alexiou, M., Kollias, G., and Mosialos, G. (2004). Comparative analysis of signal transduction by CD40 and the Epstein-Barr virus oncoprotein LMP1 in vivo. Journal of virology *78*, 13253-13261.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Muller, P.*, et al.* (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86-89.

Pearce, M., Matsumura, S., and Wilson, A.C. (2005). Transcripts encoding K12, v-FLIP, v-cyclin, and the microRNA cluster of Kaposi's sarcoma-associated herpesvirus originate from a common promoter. Journal of virology *79*, 14457-14464.

Pellet, C., Chevret, S., Blum, L., Gauville, C., Hurault, M., Blanchard, G., Agbalika, F., Lascoux, C., Ponscarme, D., Morel, P.*, et al.* (2001). Virologic and immunologic parameters that predict clinical response of AIDS-associated Kaposi's sarcoma to highly active antiretroviral therapy. J Invest Dermatol *117*, 858-863.

Peterson, B.A., and Frizzera, G. (1993). Multicentric Castleman's disease. Seminars in oncology *20*, 636-647.

Pfeffer, S. (2007). Identification of virally encoded microRNAs. Methods Enzymol *427*, 51-63.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F.A., van Dyk, L.F., Ho, C.K., Shuman, S., Chien, M.*, et al.* (2005). Identification of microRNAs of the herpesvirus family. Nat Methods *2*, 269-276.

Pfeffer, S., Zavolan, M., Grasser, F.A., Chien, M., Russo, J.J., Ju, J., John, B., Enright, A.J., Marks, D., Sander, C., and Tuschl, T. (2004). Identification of virus-encoded microRNAs. Science *304*, 734-736.

Pratt, Z.L., Kuzembayeva, M., Sengupta, S., and Sugden, B. (2009). The microRNAs of Epstein-Barr Virus are expressed at dramatically differing levels among cell lines. Virology *386*, 387-397.

Punj, V., Matta, H., Schamus, S., Tamewitz, A., Anyang, B., and Chaudhary, P.M. (2010). Kaposi's sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 suppresses CXCR4 expression by upregulating miR-146a. Oncogene *29*, 1835-1844.

Qin, Z., Kearney, P., Plaisance, K., and Parsons, C.H. (2010). Pivotal advance: Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded microRNA specifically induce IL-6 and IL-10 secretion by macrophages and monocytes. Journal of leukocyte biology *87*, 25-34.

Rai, D., Kim, S.W., McKeller, M.R., Dahia, P.L., and Aguiar, R.C. (2010). Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. Proceedings of the National Academy of Sciences of the United States of America *107*, 3111-3116.

Ramkissoon, S.H., Mainwaring, L.A., Ogasawara, Y., Keyvanfar, K., McCoy, J.P., Jr., Sloand, E.M., Kajigaya, S., and Young, N.S. (2006). Hematopoietic-specific microRNA expression in human cells. Leuk Res *30*, 643-647.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901-906.

Renne, R., Barry, C., Dittmer, D., Compitello, N., Brown, P.O., and Ganem, D. (2001). Modulation of cellular and viral gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. Journal of virology *75*, 458-468.

Renne, R., Blackbourn, D., Whitby, D., Levy, J., and Ganem, D. (1998). Limited transmission of Kaposi's sarcoma-associated herpesvirus in cultured cells. Journal of virology *72*, 5182-5188.

Renne, R., Zhong, W., Herndier, B., McGrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat Med *2*, 342-346.

Rodriguez, A., Vigorito, E., Clare, S., Warren, M.V., Couttet, P., Soond, D.R., van Dongen, S., Grocock, R.J., Das, P.P., Miska, E.A.*, et al.* (2007). Requirement of bic/microRNA-155 for normal immune function. Science *316*, 608-611.

Romania, P., Lulli, V., Pelosi, E., Biffoni, M., Peschle, C., and Marziali, G. (2008). MicroRNA 155 modulates megakaryopoiesis at progenitor and precursor level by targeting Ets-1 and Meis1 transcription factors. Br J Haematol *143*, 570-580.

Rousset, F., Garcia, E., Defrance, T., Peronne, C., Vezzio, N., Hsu, D.H., Kastelein, R., Moore, K.W., and Banchereau, J. (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proceedings of the National Academy of Sciences of the United States of America *89*, 1890-1893.

Russo, J.J., Bohenzky, R.A., Chien, M.C., Chen, J., Yan, M., Maddalena, D., Parry, J.P., Peruzzi, D., Edelman, I.S., Chang, Y., and Moore, P.S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proceedings of the National Academy of Sciences of the United States of America *93*, 14862-14867.

Ruvkun, G., Wightman, B., Burglin, T., and Arasu, P. (1991). Dominant gain-of-function mutations that lead to misregulation of the C. elegans heterochronic gene lin-14, and the evolutionary implications of dominant mutations in pattern-formation genes. Dev Suppl *1*, 47-54.

Sakakibara, S., and Tosato, G. (2009). Viral Interleukin-6: Role in Kaposi's Sarcoma-Associated Herpesvirus-Associated Malignancies. J Interferon Cytokine Res.

Samols, M.A., Hu, J., Skalsky, R.L., and Renne, R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. Journal of virology *79*, 9301-9305.

Samols, M.A., Hu, J., Skalsky, R.L., Maldonado, A.M., Riva, A., Lopez, M.C., Baker, H.V., and R. Renne (2007). Identification of cellular genes targeted by KSHV-encoded microRNAs. PLoS Pathog.

Sanders, C.J. (1997). Kaposi's sarcoma in retrospect. Genitourinary medicine *73*, 571-574.

Sanders, C.J., Canninga-van Dijk, M.R., and Borleffs, J.C. (2004). Kaposi's sarcoma. Lancet *364*, 1549-1552.

Sarid, R., Flore, O., Bohenzky, R.A., Chang, Y., and Moore, P.S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). Journal of virology *72*, 1005-1012.

Sarid, R., Wiezorek, J.S., Moore, P.S., and Chang, Y. (1999). Characterization and cell cycle regulation of the major Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) latent genes and their promoter. Journal of virology *73*, 1438-1446.

Schafer, A., Cai, X., Bilello, J.P., Desrosiers, R.C., and Cullen, B.R. (2007). Cloning and analysis of microRNAs encoded by the primate gamma-herpesvirus rhesus monkey rhadinovirus. Virology *364*, 21-27.

Schebesta, M., Heavey, B., and Busslinger, M. (2002). Transcriptional control of B-cell development. Curr Opin Immunol *14*, 216-223.

Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199-208.

Schwarz, M., and Murphy, P.M. (2001). Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-kappa B and induces proinflammatory cytokine and chemokine production via a C-terminal signaling determinant. J Immunol *167*, 505-513.

Screpanti, I., Musiani, P., Bellavia, D., Cappelletti, M., Aiello, F.B., Maroder, M., Frati, L., Modesti, A., Gulino, A., and Poli, V. (1996). Inactivation of the IL-6 gene prevents development of multicentric Castleman's disease in C/EBP beta-deficient mice. J Exp Med *184*, 1561-1566.

Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., Lattanzio, G., and et al. (1995). Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. Embo J *14*, 1932-1941.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. Nature *455*, 58-63.

Shaffer, A.L., Lin, K.I., Kuo, T.C., Yu, X., Hurt, E.M., Rosenwald, A., Giltnane, J.M., Yang, L., Zhao, H., Calame, K., and Staudt, L.M. (2002). Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity *17*, 51-62.

Shuai, K., and Liu, B. (2003). Regulation of JAK-STAT signalling in the immune system. Nat Rev Immunol *3*, 900-911.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J.*, et al.* (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol *174*, 6477-6489.

Sin, S.H., Roy, D., Wang, L., Staudt, M.R., Fakhari, F.D., Patel, D.D., Henry, D., Harrington, W.J., Jr., Damania, B.A., and Dittmer, D.P. (2007). Rapamycin is efficacious against primary effusion lymphoma (PEL) cell lines in vivo by inhibiting autocrine signaling. Blood *109*, 2165-2173.

Skalsky, R.L., Samols, M.A., Plaisance, K.B., Boss, I.W., Riva, A., Lopez, M.C., Baker, H.V., and Renne, R. (2007). Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. Journal of virology *81*, 12836-12845.

Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R., and Ruvkun, G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell *5*, 659-669.

Sood, P., Krek, A., Zavolan, M., Macino, G., and Rajewsky, N. (2006). Cell-type-specific signatures of microRNAs on target mRNA expression. Proceedings of the National Academy of Sciences of the United States of America *103*, 2746-2751.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M.F., Clauvel, J.P., Raphael, M., Degos, L., and et al. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood *86*, 1276-1280.

Stark, A., Brennecke, J., Russell, R.B., and Cohen, S.M. (2003). Identification of Drosophila MicroRNA targets. PLoS Biol *1*, E60.

Staskus, K.A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D.J., Ganem, D., and Haase, A.T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. Journal of virology *71*, 715-719.

Stern-Ginossar, N., Elefant, N., Zimmermann, A., Wolf, D.G., Saleh, N., Biton, M., Horwitz, E., Prokocimer, Z., Prichard, M., Hahn, G.*, et al.* (2007). Host immune system gene targeting by a viral miRNA. Science *317*, 376-381.

Sun, Q., Matta, H., Lu, G., and Chaudhary, P.M. (2006). Induction of IL-8 expression by human herpesvirus 8 encoded vFLIP K13 via NF-kappaB activation. Oncogene *25*, 2717-2726.

Talbot, S.J., Weiss, R.A., Kellam, P., and Boshoff, C. (1999). Transcriptional analysis of human herpesvirus-8 open reading frames 71, 72, 73, K14, and 74 in a primary effusion lymphoma cell line. Virology *257*, 84-94.

Tam, W. (2001). Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA. Gene *274*, 157-167.

Tam, W., Ben-Yehuda, D., and Hayward, W.S. (1997). bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. Mol Cell Biol *17*, 1490-1502.

Tan, L.P., Wang, M., Robertus, J.L., Schakel, R.N., Gibcus, J.H., Diepstra, A., Harms, G., Peh, S.C., Reijmers, R.M., Pals, S.T.*, et al.* (2009). miRNA profiling of B-cell subsets: specific miRNA profile for germinal center B cells with variation between centroblasts and centrocytes. Lab Invest *89*, 708-716.

Tang, S., Bertke, A.S., Patel, A., Wang, K., Cohen, J.I., and Krause, P.R. (2008). An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. Proceedings of the National Academy of Sciences of the United States of America *105*, 10931-10936.

Tang, S., Patel, A., and Krause, P.R. (2009). Novel less-abundant viral microRNAs encoded by herpes simplex virus 2 latency-associated transcript and their roles in regulating ICP34.5 and ICP0 mRNAs. Journal of virology *83*, 1433-1442.

Teng, G., Hakimpour, P., Landgraf, P., Rice, A., Tuschl, T., Casellas, R., and Papavasiliou, F.N. (2008). MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. Immunity *28*, 621-629.

Thai, T.H., Calado, D.P., Casola, S., Ansel, K.M., Xiao, C., Xue, Y., Murphy, A., Frendewey, D., Valenzuela, D., Kutok, J.L.*, et al.* (2007). Regulation of the germinal center response by microRNA-155. Science *316*, 604-608.

Thomas, M., Boname, J.M., Field, S., Nejentsev, S., Salio, M., Cerundolo, V., Wills, M., and Lehner, P.J. (2008). Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. Proceedings of the National Academy of Sciences of the United States of America *105*, 1656-1661.

Thorley-Lawson, D.A. (2001). Epstein-Barr virus: exploiting the immune system. Nat Rev Immunol *1*, 75-82.

Tomari, Y., Matranga, C., Haley, B., Martinez, N., and Zamore, P.D. (2004). A protein sensor for siRNA asymmetry. Science *306*, 1377-1380.

Turner, C.A., Jr., Mack, D.H., and Davis, M.M. (1994). Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. Cell *77*, 297-306.

Uldrick, T.S., Wang, V., O'Mahony, D., Aleman, K., Wyvill, K.M., Marshall, V., Steinberg, S.M., Pittaluga, S., Maric, I., Whitby, D.*, et al.* (2010). An interleukin-6-related systemic inflammatory syndrome in patients co-infected with Kaposi sarcoma-associated herpesvirus and HIV but without Multicentric Castleman disease. Clin Infect Dis *51*, 350-358.

Umbach, J.L., Kramer, M.F., Jurak, I., Karnowski, H.W., Coen, D.M., and Cullen, B.R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature *454*, 780-783.

Umbach, J.L., Nagel, M.A., Cohrs, R.J., Gilden, D.H., and Cullen, B.R. (2009). Analysis of human alphaherpesvirus microRNA expression in latently infected human trigeminal ganglia. Journal of virology *83*, 10677-10683.

van den Berg, A., Kroesen, B.J., Kooistra, K., de Jong, D., Briggs, J., Blokzijl, T., Jacobs, S., Kluiver, J., Diepstra, A., Maggio, E., and Poppema, S. (2003). High expression of B-cell receptor inducible gene BIC in all subtypes of Hodgkin lymphoma. Genes, chromosomes & cancer *37*, 20-28.

Vigorito, E., Perks, K.L., Abreu-Goodger, C., Bunting, S., Xiang, Z., Kohlhaas, S., Das, P.P., Miska, E.A., Rodriguez, A., Bradley, A.*, et al.* (2007). microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. Immunity *27*, 847-859.

Waidner, L.A., Morgan, R.W., Anderson, A.S., Bernberg, E.L., Kamboj, S., Garcia, M., Riblet, S.M., Ouyang, M., Isaacs, G.K., Markis, M.*, et al.* (2009). MicroRNAs of Gallid and Meleagrid herpesviruses show generally conserved genomic locations and are virus-specific. Virology *388*, 128-136.

Watanabe, T., Sugaya, M., Atkins, A.M., Aquilino, E.A., Yang, A., Borris, D.L., Brady, J., and Blauvelt, A. (2003). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen prolongs the life span of primary human umbilical vein endothelial cells. Journal of virology *77*, 6188-6196.

Whitby, D., Howard, M.R., Tenant-Flowers, M., Brink, N.S., Copas, A., Boshoff, C., Hatzioannou, T., Suggett, F.E., Aldam, D.M., and Denton, A.S. (1995). Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet *346*, 799-802.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855-862.

Wilkinson, J., Cope, A., Gill, J., Bourboulia, D., Hayes, P., Imami, N., Kubo, T., Marcelin, A., Calvez, V., Weiss, R.*, et al.* (2002). Identification of Kaposi's sarcoma-associated

herpesvirus (KSHV)-specific cytotoxic T-lymphocyte epitopes and evaluation of reconstitution of KSHV-specific responses in human immunodeficiency virus type 1-Infected patients receiving highly active antiretroviral therapy. Journal of virology *76*, 2634-2640.

Xing, L., and Kieff, E. (2007). Epstein-Barr virus BHRF1 micro- and stable RNAs during latency III and after induction of replication. Journal of virology *81*, 9967-9975.

Yao, Y., Zhao, Y., Xu, H., Smith, L.P., Lawrie, C.H., Sewer, A., Zavolan, M., and Nair, V. (2007). Marek's disease virus type 2 (MDV-2)-encoded microRNAs show no sequence conservation with those encoded by MDV-1. Journal of virology *81*, 7164-7170.

Yao, Y., Zhao, Y., Xu, H., Smith, L.P., Lawrie, C.H., Watson, M., and Nair, V. (2008). MicroRNA profile of Marek's disease virus-transformed T-cell line MSB-1: predominance of virus-encoded microRNAs. Journal of virology *82*, 4007-4015.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev *17*, 3011-3016.

Yin, Q., McBride, J., Fewell, C., Lacey, M., Wang, X., Lin, Z., Cameron, J., and Flemington, E.K. (2008). MicroRNA-155 is an Epstein-Barr virus-induced gene that modulates Epstein-Barr virus-regulated gene expression pathways. Journal of virology *82*, 5295-5306.

Yin, Q., Wang, X., Fewell, C., Cameron, J., Zhu, H., Baddoo, M., Lin, Z., and Flemington, E.K. (2010). MicroRNA miR-155 inhibits bone morphogenetic protein (BMP) signaling and BMP-mediated Epstein-Barr virus reactivation. Journal of virology *84*, 6318-6327.

Yoshizaki, K., Matsuda, T., Nishimoto, N., Kuritani, T., Taeho, L., Aozasa, K., Nakahata, T., Kawai, H., Tagoh, H., Komori, T., and et al. (1989). Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease. Blood *74*, 1360-1367.

Yu, F., Harada, J.N., Brown, H.J., Deng, H., Song, M.J., Wu, T.T., Kato-Stankiewicz, J., Nelson, C.G., Vieira, J., Tamanoi, F.*, et al.* (2007). Systematic identification of cellular signals reactivating Kaposi sarcoma-associated herpesvirus. PLoS Pathog *3*, e44.

Zahnow, C.A., Younes, P., Laucirica, R., and Rosen, J.M. (1997). Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. J Natl Cancer Inst *89*, 1887-1891.

Zhao, Y., Xu, H., Yao, Y., Smith, L.P., Kgosana, L., Green, J., Petherbridge, L., Baigent, S.J., and Nair, V. (2011). Critical role of the virus-encoded microRNA-155 ortholog in the induction of Marek's disease lymphomas. PLoS Pathog *7*, e1001305.

Zhao, Y., Yao, Y., Xu, H., Lambeth, L., Smith, L.P., Kgosana, L., Wang, X., and Nair, V. (2009). A functional MicroRNA-155 ortholog encoded by the oncogenic Marek's disease virus. Journal of virology *83*, 489-492.

Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996). Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proceedings of the National Academy of Sciences of the United States of America *93*, 6641-6646.

Zhu, J.Y., Pfuhl, T., Motsch, N., Barth, S., Nicholls, J., Grasser, F., and Meister, G. (2009). Identification of novel Epstein-Barr virus microRNA genes from nasopharyngeal carcinomas. Journal of virology *83*, 3333-3341.

Zhu, S., Wu, H., Wu, F., Nie, D., Sheng, S., and Mo, Y.Y. (2008). MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell research *18*, 350-359.

Ziegelbauer, J.M., Sullivan, C.S., and Ganem, D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. Nat Genet *41*, 130-134.

Ziegler, J.L., and Katongole-Mbidde, E. (1996). Kaposi's sarcoma in childhood: an analysis of 100 cases from Uganda and relationship to HIV infection. International journal of cancer *65*, 200-203.

Zucali, J.R., Ciccarone, T., Kelley, V., Park, J., Johnson, C.M., and Mergia, A. (2002). Transduction of umbilical cord blood CD34+ NOD/SCID-repopulating cells by simian foamy virus type 1 (SFV-1) vector. Virology *302*, 229-235.

BIOGRAPHICAL SKETCH

Isaac Wayne Boss was born in Tacoma, Washington. In Florida, he received his high school diploma from Oviedo High School in 1992. He attended Florida Community College Jacksonville where he received his Associate of Arts degree in 2003. He next graduated Summa Cum Laude from the University of Florida in 2006 where he received his B.S. in microbiology and cell science. He continued at the Universtiy of Florida as a Ph.D student in the Interdisciplinary Program in biomedical sciences where he joined the lab of Dr. Rolf Renne to study the biology of Kaposi's sarcoma-associated herpesvirus. He will earn his Ph.D. in medical sciences with a concentration in genetics.