TARGETS AND FUNCTIONS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS MICRORNAS IN ENDOTHELIAL CELLS

By

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To my parents, who instilled in me a lifelong love of learning, and to my husband, whose patience and encouragement have been indispensable
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LIST OF ABBREVIATIONS

Ago  Argonaute
BR   biological replicate
CLASH crosslinking and sequencing of hybrids
CLIP crosslinking immunoprecipitation
ECAR extracellular acidification rate
HITS-CLIP high-throughput sequencing crosslinking immunoprecipitation
KS   Kaposi’s sarcoma
KSHV Kaposi’s sarcoma-associated herpesvirus
miRNA microRNA
mRNA messenger RNA
OCR oxygen consumption rate
PAR-CLIP photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation
PCR polymerase chain reaction
qCLASH quick crosslinking and sequencing of hybrids
qPCR quantitative polymerase chain reaction
RT   reverse transcription
RTA replication and transcription activator
TARGETS AND FUNCTIONS OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS MICRORNAS IN ENDOTHELIAL CELLS

By
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August 2017

Kaposi’s Sarcoma-Associated Herpesvirus (KSHV) is the causative agent of Kaposi’s Sarcoma (KS), one of the most common cancers in those infected with HIV. KSHV has a lytic phase, in which the virus actively replicates, and a latent phase, during which the virus remains quiescent and expresses only a limited set of genes. Among these genes are the viral microRNAs (miRNAs). miRNAs are short, 19-22 nucleotide long RNAs which posttranscriptionally suppress gene expression. Since KS tumors consist mainly of cells of endothelial origin which are latently infected with KSHV, and the viral miRNAs are expressed during latency, the role of the miRNAs in oncogenesis needs to be clarified. To this end, we have taken a two-part approach.

In order to understand the function of a given miRNA it is necessary to elucidate the genes which are targeted by it. Ribonomics approaches have made it possible to do this in a high-throughput manner. We have developed a ribonomics protocol, quick Cross-Linking and Sequencing of Hybrids (qCLASH), and successfully used it to identify numerous targets of KSHV miRNAs in wild-type KSHV-infected endothelial cells. A significant number of these target genes play a role in cellular processes related to
cancer, such as cell cycle control, angiogenesis, and glucose metabolism. This list of targets may be used to inform research in the KSHV field in the future.

We have also taken a classical genetics approach to understanding the functions of KSHV miRNAs. We utilized a panel of KSHV mutants, each lacking one of the twelve miRNA genes. Wild-type KSHV is known to cause several cancer-related phenotypic changes in infected endothelial cells, such as enhanced cell migration, angiogenesis, and proliferation, as well as alterations to cellular metabolism. Indeed, the results from the qCLASH experiment not only confirm the importance of these cellular processes to KSHV biology, but also reinforce the role which the KSHV miRNAs have in them. We therefore characterized KSHV-induced changes to cellular phenotype in the absence of specific miRNAs. The elimination of certain miRNAs did have an impact on infected cell phenotype.
Kaposi’s Sarcoma

The angioproliferative cancer Kaposi’s sarcoma (KS) was initially described by Moritz Kaposi, a Hungarian doctor living and working in Austria. He encountered several elderly male patients with highly vascularized tumors on the lower extremities (1). This type of KS, which presents mainly in older men and progresses quite slowly, has come to be known as classic KS. Other forms of KS have since been recognized. In the early 1980s, doctors unexpectedly began to see cases of KS in young, homosexual men in New York City and San Francisco (2). The sudden emergence of the disease in an atypical patient population was the first indication that the Acquired Immune Deficiency Syndrome (AIDS) epidemic had begun (3). Shortly thereafter, the Human Immunodeficiency Virus (HIV), the causative agent of AIDS, was discovered (4, 5). AIDS-KS is far more aggressive than the classic form, and it was a leading cause of mortality in the HIV-infected population prior to the introduction of Highly Active Anti-Retroviral Therapy (HAART) (6). Even in the HAART era, HIV-positive individuals in the United States are approximately 200 times more likely to be diagnosed with KS than HIV-negative individuals (7). In parts of the world where HAART is largely unavailable and HIV infection rates are high, such as sub-Saharan Africa, AIDS-KS remains a significant problem. KS is the most common type of cancer in males in many countries located within this region (8). Sub-Saharan Africa is also burdened with endemic KS, which affects mainly HIV-negative children presenting with lymphadenopathy (9). Lastly, iatrogenic KS is principally a problem in countries bordering the Mediterranean. It arises in individuals who are medically immunosuppressed following an organ transplant (10).
Kaposi’s Sarcoma-Associated Herpesvirus

One-hundred and twenty-two years after its initial description, the etiological agent of KS was discovered. This was accomplished when researchers performed representational difference analysis on a KS biopsy and healthy tissue from the same patient (11). In doing so, they found sequences of a previously unknown herpesvirus. Named Human Herpesvirus 8 (HHV-8), or, alternatively, Kaposi’s Sarcoma-Associated Herpesvirus (KSHV), the new virus was also found to be the causative agent of Primary Effusion Lymphoma (PEL) and some forms of Multicentric Castleman’s Disease (MCD) (12, 13).

KSHV falls within the Gammaherpesvirinae subfamily of the Herpesviridae family of viruses (11). A defining feature of this family is a bipartite infectious cycle consisting of latent and lytic phases. The lytic phase is characterized by active replication of the viral genome and the release of progeny virions from cells. While the lytic phase is essential for the spread of the virus between hosts, latency is actually the default program of KSHV. During the latent phase, the viral genome exists as a circular episome associated with host chromatin (14). Episomes replicate only once per cell cycle, faithfully segregating with the host chromosomes in order to ensure that all daughter cells inherit the KSHV genome.

The study of latency is of particular significance because cells from KSHV-induced malignancies are latently infected with the virus. Early on, researchers observed that the circular (episomal) form, but not the linear form, of the KSHV genome was present in KS biopsies. It was already known at the time that the episomal form of the closely-related Epstein-Barr Virus (EBV) was associated with latency. This indicated that KS tumors were latently infected with KSHV (15). KS lesions are made up of cells
of endothelial origin (16, 17), however KSHV is also responsible for several B-cell malignancies (12, 13). In B-cells, too, KSHV was found to be predominantly latent (18, 19).

Another important difference between latency and lytic replication is the pattern of gene expression. During the lytic phase most of the genome is actively transcribed. In contrast, much of the genome is silenced when the virus is in the latent state (Fig. 1-1). Only a select few genes, mainly residing within the KSHV Latency-Associated Region (KLAR), are expressed (20). Since all KSHV-associated malignancies are latently infected with the virus, and only a small subset of genes is expressed during latency, it is reasonable to argue that these few genes are the drivers of malignancy.

Indeed, there is copious evidence that this is the case (reviewed in reference 21). KSHV has been shown to manipulate the cell cycle, prevent apoptosis, induce angiogenesis, and alter cellular glucose metabolism, all processes related to oncogenesis. For example, viral cyclin (vCyclin) is a homolog of cellular cyclin D2 and is therefore capable of binding to and activating cyclin-dependent kinase 6 (cdk6), leading to cell cycle dysregulation (22, 23). Viral FLICE inhibitory protein (vFLIP), prevents the activation of the pro-apoptotic caspase 8 (24). Another homolog of a cellular gene, vIL-6 has been shown to promote angiogenesis (25). Finally, it has been demonstrated that KSHV induces the Warburg effect, a fundamental alteration in the way cells derive energy from glucose, in latently infected endothelial cells (26). Apart from protein-coding genes, the KLAR also contains twelve microRNA (miRNA) genes (27, 28, 29). These also have the potential to be quite significant to the KSHV-induced oncogenic process.
miRNAs

The first miRNA was identified in *C. elegans*, while researchers were attempting to identify a gene which, when deleted, resulted in postembryonic developmental disruption. What they discovered was that the gene, rather than encoding a protein, gave rise to a small, untranslated RNA (30). Although it was initially thought that miRNAs were a phenomenon unique to *C. elegans*, more miRNAs were eventually discovered in other organisms, including humans (31, 32). miRNAs are small RNAs, typically only 19-22 nucleotides in length, which serve as posttranscriptional controls on gene expression. They bind to imperfectly complementary regions of mRNA transcripts, thereby preventing translation (reviewed in reference 33).

**miRNA Biogenesis**

The biogenesis of miRNAs begins when miRNA genes are transcribed by RNA polymerase II (34). The initial transcript is known as the primary-miRNA (pri-miRNA). Along the length of the pri-miRNA one or more secondary structures form which consist of a short, base-paired stem connected by a loop (35). The stem-loops, which are also called hairpins, are recognized by the microprocessor complex. This complex consists of the proteins Drosha and DGCR8 (DiGeorge syndrome chromosomal region 8) (36). Drosha releases each hairpin from its parent transcript by cleaving it at the base of its stem, leaving a two nucleotide overhang at the 3’ end (37). The free hairpins, which are called precursor-miRNAs (pre-miRNAs), are transported from the nucleus to the cytoplasm by Exportin 5 in conjunction with Ran small GTPase (38). Once in the cytoplasm, pre-miRNAs encounter the Dicer complex. Dicer is an RNase III type endonuclease which removes the loop portion of the pre-miRNA, leaving behind the double-stranded stem region (39).
The RNA duplexes left behind by Dicer become incorporated into the RNA induced silencing complex (RISC). This occurs when the Hsc70/Hsp90 complex induces a conformational change in Argonaute (Ago), the major protein component of the RISC, opening up its nucleic acid binding channel (40). The guide strand of the double-stranded RNA binds the MID domain of Ago with its 5’ end (41). The other strand, known as the passenger strand, is ejected from the complex (42). The miRNA bound within the RISC, which is now considered a mature miRNA, recruits a target RNA. The mechanisms by which the RISC interferes with target RNA expression include both the prevention of translation and the precipitation of target degradation (reviewed in reference 43).

**miRNA Targeting Rules**

The way that miRNAs recruit target mRNAs has to do with the complementarity between the two. According to current understanding, the most significant determinant of targeting is perfect complementarity between the mRNA and the seed sequence of the miRNA, defined as nucleotides 2-7 or, alternatively, nucleotides 2-8 from the 5’ end. This is based on observations that the 5’ ends of miRNAs tend to be the most highly conserved regions when related miRNAs from different species are compared (44). Computational approaches to predicting miRNA targets have a lower false positive rate if perfect seed pairing is required (45). In addition, there are many examples in which miRNA regulation is abrogated when seed pairing is disrupted (46, 47). Since the seed sequence is only seven nucleotides long, there could potentially be numerous complementary sites across the transcriptome. Since miRNAs do not regulate every mRNA with a seed sequence match, this implies that other mechanisms may be responsible for imparting greater specificity to the miRNA (48). One possible
mechanism is 3’ compensatory base-pairing, although it is thought to have only a modest role (reviewed in 49).

There are many ways to identify the targets of miRNAs. Purely in silico tools, such as Targetscan (50), rely on conventional miRNA binding rules to locate likely targets. While this type of method provides us with a best guess of what the miRNA targetome looks like, it ultimately only produces a picture of what occurs under idealized, or at least very narrowly defined, conditions. There are numerous indications that the set of mRNAs a given miRNA targets can vary greatly between cell types and cell states. Additionally, since our understanding of miRNA behavior is continually evolving, it is not possible to produce an algorithm which exactly recapitulates what is occurring in reality.

**Ribonomics Approaches for miRNA Target Analysis**

Chi et al. developed the first high-throughput method for determining miRNA targets, called High-Throughput Sequencing, Crosslinking, and Immunoprecipitation (HITS-CLIP) (51). In this protocol, living cells are UV-irradiated, causing protein and nucleic acid in close proximity to become covalently bound. The cells are then lysed and Ago is immunoprecipitated with a specific antibody. Since Ago forms a complex with a miRNA and a target mRNA, both species are pulled down with the protein. Once Ago has been digested away, the RNAs can be used to prepare libraries for high-throughput sequencing. This creates a picture of which miRNAs and which mRNAs were present in RISC complexes at the time of crosslinking. It does not give any indication of which miRNA was interacting with a given mRNA. This must be determined bioinformatically. To do so, sequencing reads are aligned to the appropriate genome and clusters of reads are identified. The region of the transcript covered by a cluster is then searched
for sequences complementary to the seed sequences of the miRNAs which are known to have been in RISC complexes.

HITS-CLIP is an incredibly powerful tool for miRNA target identification. Even so, assumptions must be made in order to assign miRNAs to targets. It is assumed that the Ago binding site could be anywhere within a sequence covered by a cluster of reads. In truth, clusters can be longer than the footprint of the Ago protein. This can result in a false positive if a miRNA seed match is found within the cluster but outside of the area actually bound by Ago. In order to remove this source of ambiguity, Hafner et al. developed Photoactivatable-Ribonucleoside-Enhanced (PAR)-CLIP (52). In this protocol, cells are given 4-thiouridine, which will be incorporated into new mRNA transcripts, prior to crosslinking. Any 4-thiouridine which has been crosslinked to Ago will be reverse-transcribed into a cytosine. By looking for T to C mutations in the resulting sequencing reads, it is possible to pinpoint where Ago was bound. While this removes one potential source of inaccuracy, both HITS-CLIP and PAR-CLIP rely on the major assumption that miRNAs will have canonical or near-canonical seed-pairing. Noncanonical interactions, which have been found to represent a significant portion of miRNA-mRNA interactions, are missed by HITS-CLIP and PAR-CLIP. Ultimately, these methods cannot provide direct experimental evidence for specific interactions between miRNAs and mRNAs and thus will always be subject to a certain level of ambiguity.

Building on the foundation of HITS-CLIP, Helwak and Tollervey introduced an important innovation to the method which removes this ambiguity (53,54). Their new method, which they named Crosslinking and Sequencing of Hybrids (CLASH), offers the elegant solution of adding RNA ligase to the purified RNA-Ago complexes. This causes
the two species of RNA which are bound to the same Ago molecule to join together, creating one chimeric, or hybrid, RNA. CLASH is the first ribonomics method to provide direct experimental evidence of interactions between specific miRNAs and specific mRNA targets.

**The KSHV miRNAs**

It was discovered in 2005 that KSHV encodes miRNAs (27, 28, 29). The twelve miRNA genes are located within the KLAR. Ten out of twelve are arranged as a cluster, while miR-K12-10 and miR-K12-12 are located within the open reading frame of Kaposin (29). The functions of the KSHV miRNAs can roughly be divided into five classes. These are: cell cycle control, prevention of apoptosis, angiogenesis, immune evasion, and the maintenance of viral latency (55).

Skalsky et al. noted that one of the KSHV miRNAs, miR-K12-11, is an ortholog of cellular miR-155, a known oncogene (56, 57). Further studies into the function of miR-K12-11 have revealed that this miRNA plays a role in promoting cell proliferation. For instance, the overexpression of either miR-K12-11 or miR-155 in NOD/LtSz-scid IL2RYnull mice resulted in significant expansion of CD19-positive splenocytes (58, 59).

Another KSHV miRNA, miR-K12-1, was found to suppress levels of the cyclin-dependent kinase (CDK) inhibitor p21, thereby promoting cell division (60). Both miR-K12-1 and miR-K12-11, along with miR-K12-4, were found to rescue the proliferative phenotype of a KSHV mutant which was lacking the entire miRNA cluster (61). Overall, there are strong indications that miR-K12-1 and miR-K12-11, in particular, have an important role in driving the increased proliferation of KSHV-infected cells.

The KSHV miRNAs are also involved in the prevention of apoptosis. miR-K12-1, -3, and -4-3p directly target caspase 3, preventing it from actuating cell death (62).
Tumor necrosis factor receptor superfamily member 12A (TNFRSF12A) is targeted by miR-K12-10a, resulting in the inhibition of caspase activity as well as the suppression of proinflammatory cytokine production (63). Another mechanism by which KSHV miRNAs prevent apoptosis is through interference with the transforming growth factor beta (TGF-beta) signaling pathway. Suppressing signaling through this pathway promotes cell survival. miR-K12-10 has been shown to downregulate the TGF-beta type II receptor. In addition, miR-K12-11 targets SMAD5, an intermediate in the TGF-beta pathway (64). KSHV miRNAs are also known to cause the upregulation of the cellular miR-17-92 cluster which in turn leads to the downregulation of TGF-beta signaling (65).

Angiogenesis is an essential process in cancer, as tumors require sufficient oxygen in order to sustain themselves. The KSHV miRNAs have been shown to repress breakpoint cluster region protein, thus upregulating Rac1 and promoting angiogenesis (66). They also promote the expression of pro-angiogenic cytokines (67). Li et al. found that two KSHV miRNAs in particular have very important roles in the process of angiogenesis. On the one hand, miR-K12-6-3p directly targets SH3 domain binding glutamate-rich protein (SH3BGR), promoting cell migration and angiogenesis (68). At the same time, miR-K12-6-5p targets CD82, a metastasis suppressor. Ectopic expression of this miRNA downregulated CD82 and induced endothelial cell invasion and angiogenesis (69).

Undeniably, the ability to evade the host immune system is an asset for most viruses. KSHV avoids detection in several ways. Interleukin 1 receptor-associated kinase 1 (IRAK1) and myeloid differentiation primary response 88 (MYD88), two components of the Toll-like receptor signaling pathway, are targeted by miR-K12-9 and
miR-K12-5, respectively, to reduce the production of inflammatory cytokines (70). miR-K12-7 binds to the 3' UTR of MHC class I polypeptide-related sequence B (MICB), leading to a reduction in natural killer cell activity (71). It has also been found that miR-K12-11 suppresses type I interferon signaling by targeting Inhibitor of nuclear factor kappa B kinase subunit epsilon (IKBKE).

KSHV has both latent and lytic phases and there is an appropriate time for each. Bellare et al. discovered that miR-K12-9* directly targets the KSHV gene responsible for switching on lytic reactivation, replication and transcription activator (RTA) (72). The miRNAs also promote latency by targeting specific cellular genes. For example, miR-K12-3 and miR-K12-11 downregulate MYB proto-oncogene (MYB), CCAAT/enhancer binding protein alpha (C/EBPα), and ETS1 proto-oncogene (Ets-1), which are all known activators of RTA (73). The DNA methyltransferase repressor RB transcriptional corepressor like 2 (RBL2) is targeted by miR-K12-4, leading to the epigenetic maintenance of latency (74). NFKB inhibitor alpha (NFKBIA) is downregulated by miR-K12-1 and this causes enhanced signaling through the Nuclear Factor Kappa B (NFKB) pathway and the promotion of latency (75). Finally, mir-K12-3 targets nuclear factor 1 B (NFIB), an activator of RTA (76).

While much is known about the KSHV miRNAs and their relevance to various cancer-related processes, that knowledge is somewhat piecemeal. It has been obtained in a variety of cell types which have varying degrees of relevance to KS pathogenesis. In particular, many experiments have been carried out in B-cells. Since KS arises from endothelial cells, it is this cell type which is the most appropriate for study of KSHV-induced tumorigenesis in KS. To date, large-scale studies aimed at elucidating the
targets of KSHV miRNAs in endothelial cells are lacking. The following chapters detail work aimed at rectifying this situation using two distinct approaches.

Figure 1-1. Diagram of the KSHV genome.
CHAPTER 2
MODIFIED CROSS-LINKING, LIGATION, AND SEQUENCING OF HYBRIDS (qCLASH) IDENTIFIES KSHV MICRORNA TARGETS IN ENDOTHELIAL CELLS

Background

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi’s sarcoma (KS), primary effusion lymphoma, and a subset of Multicentric Castleman’s disease (11, 12, 13). KS is a disease of global significance (77), yet much work remains to be done to better understand the mechanisms by which KSHV infection leads to cancer. KS tumors are composed of spindle cells that are latently infected with the virus (78, 79). During latency, KSHV expresses only a small subset of genes, which are mainly confined to the KSHV Latency Associated Region (KLAR) of the genome (20, 80). Encoded in this region are twelve microRNA (miRNA) genes that give rise to 25 mature miRNAs (27, 28, 29). miRNAs are 19-22 nucleotide RNAs which perform important regulatory functions by exerting posttranscriptional control over gene expression. They bind to imperfectly complementary sequences of target mRNAs, often found within the 3’ UTR, thereby preventing translation and mediating eventual degradation of certain transcripts (reviewed in reference 33). There is already evidence that the KSHV miRNAs contribute to oncogenesis. One of the earliest targets of the KSHV miRNAs identified was THBS1, a tumor suppressor and antiangiogenic factor (81). miR-K12-5 is known to target BCLAF1, which has been shown to promote apoptosis (82). It was also discovered that one of the KSHV miRNAs, miR-K12-11, is an ortholog of the cellular oncomiR miR-155 (56, 57). Exogenous expression of miR-K12-11 or miR-155 in hematopoietic progenitors leads to expansion of splenic B-cells in NOD/LtSz-scid IL2Rγ(null) mice (58). Most of the work aimed at identifying KSHV miRNA targets has been performed in B cells, yet KS tumors are composed of cells
principally of endothelial origin (16). KSHV miRNA expression has been shown to vary widely between different cell types (83), thus there is a need to determine which mRNAs are targeted by the virus miRNAs in this cell type. Doing so will help us to better understand the process by which KSHV infection leads to KS.

Studies to determine which mRNAs are targeted by miRNAs on a genome-wide scale were pioneered by Chi et al. with the development of High-Throughput Sequencing, Cross-Linking, and Immunoprecipitation (HITS-CLIP) (51). Put simply, protein and nucleic acid are crosslinked in living cells, the RNA-binding protein Argonaute (Ago) is immunoprecipitated, and the bound RNA is isolated, reverse-transcribed, and sequenced. This yields two datasets: Ago-bound miRNAs and Ago-bound mRNAs. mRNAs and miRNAs must then be assigned to each other bioinformatically based on whether the mRNA contains a perfect or near-perfect complement to the seed sequence (nucleotides 2-8) of the miRNA (51). Although miRNAs and mRNAs may interact in this way much of the time, there is also evidence that noncanonical base-pairing is significant as well (reviewed in reference 49). These nonstandard interactions are not detected by HITS-CLIP or the related Photo-Activatable Ribonucleoside Cross-Linking and Immunoprecipitation (PAR-CLIP) method (52). Recently, Helwak et al. developed Cross-Linking and Sequencing of Hybrids (CLASH), a method for the identification of miRNA targets which builds on the HITS-CLIP approach. CLASH adds an RNA ligation step prior to the removal of the Ago protein. Separate RNAs (miRNA and mRNA target) which are both bound to the same Ago are joined to become a single hybrid RNA. This removes the ambiguity from miRNA target discovery (53, 54).
While CLASH is an improvement upon HITS-CLIP, both methods contain several cleanup steps after immunoprecipitation which result in large losses of RNA and as a result require large numbers of cells as input. However, in CLASH, the hybrids are formed prior to the cleanup steps (54). We reasoned that, given current increased sequencing capabilities, it was not strictly necessary to separate extraneous RNAs from the hybrids; they could all be sequenced and the nonhybrids could simply be ignored. In keeping with this line of thinking, we have developed a shortened CLASH protocol which omits the steps resulting in the largest losses of RNA. We have named the new variant protocol quick CLASH, or qCLASH, as it takes less time than the original method. It can also be completed using fewer cells as input, making it preferable for cell types which are difficult to grow in large quantities and for clinical samples. We successfully used qCLASH to identify targets of KSHV miRNAs in endothelial cells. Almost 1,500 genes were identified as high-confidence targets of the viral miRNAs, of which some have been previously shown to be targeted by KSHV miRNAs in B-cells. Additionally, we identified 54 high-confidence targets of the viral miRNA miR-K12-11. A number of the target genes were found to be components of several cancer-related pathways, including cell cycle control, angiogenesis, and glycolysis. These results provide a unique resource, broaden our understanding of the roles played by the KSHV miRNAs in endothelial cells, and point to a number of targets that can be studied more extensively in the future.

**Materials and Methods**

**Cell Lines, Tissue Culture, and Generation of TIVE-EX-LTC Cells**

TIVE-LTC cells (48) which had been passaged many times (exact passage number not known) were maintained in DMEM containing 10% FBS and 1% P/S. Cells
were trypsinized, diluted to a concentration of 10 cells per mL, and plated in a 96-well plate at 100 μL per well. Cells from wells which did not express GFP were expanded. The absence of virus was confirmed by qPCR for LANA (forward primer 5'-GCGCCCTTAACGAGAGGAAGTT-3', reverse 5'-TTCCTTCGCGTTGTAGATG-3') using the plasmid LANA-pcDNA3.1 as a standard. One KSHV-negative culture was selected for all future experiments and the cell line is henceforth referred to as TIVE-EX-LTC. Cells were maintained in DMEM containing 10% FBS and 1% P/S.

qCLASH

TIVE-EX-LTC cells were expanded in 15 cm plates until they reached approximately 80-90% confluence. Working with 6 plates at a time, cells were trypsinized, resuspended in medium, and rinsed twice with PBS. After the second rinse the cells were resuspended in 10 mL PBS and transferred to a clean 10 cm cell culture plate on ice. Cells were UV-irradiated in a crosslinker at a wavelength of 250 nm, receiving a total energy of 600 joules/cm². After crosslinking, the cells were counted using a hemocytometer and distributed into aliquots of 50 million cells. The cells were pelleted at 4° C, supernatants were removed, and the cells were frozen at -80° C until further use.

The preparation of cell lysates and antibody-coated beads, and immunoprecipitation were performed as in Haecker et al. with only slight modifications (83). All quantities given are for one experiment with 5 x 10⁷ cells. Six mg Dynabeads® Protein G (Invitrogen 10004D) were rinsed 3x with PBS-T, pH 7.2 (1x PBS, pH 7.4 w/o Ca²⁺/Mg²⁺, 0.02% Tween-20). Beads were briefly pelleted between rinses at ≤ 3000 rpm. The beads were resuspended in PBS-T and 72 μL AffinPure rabbit anti-mouse IgG antibody (Jackson ImmunoResearch 315-005-008) was added for a final concentration
of 200 μg/mL. The mixture was incubated while spinning for 50 min at RT. The beads were rinsed 3x with PBS-T, resuspended in PBS-T, and 10 μL of 2A8 anti-Ago antibody (a generous gift of Dr. Zissimos Mourelatos) was added. The mixture was incubated while spinning at 4°C overnight. The next day the beads were rinsed 4x with 1x PXL (1x PBS, pH 7.4 w/o Ca^{2+}/Mg^{2+}, 0.1% SDS, 0.5% Na-deoxycholate, 0.5% NP-40), resuspended in 1x PXL, and stored on ice until the cell lysates were ready.

To prepare cell lysates, 5 x 10^7 UV-crosslinked TIVE-EX-LTC cells were thawed on ice and resuspended in 500 μL Lysis Buffer (50 mM Hepes-KOH, pH 7.5, 150 mM KCl, 2mM EDTA, 1mM NaF, 0.5% NP-40, *0.5 mM DTT, *1x Complete Protease Inhibitor (Roche 11836170001), *: added immediately before use). Cells were allowed to lyse for 15 min on ice. Ten μL RQ1 DNase (Promega M610A) was added and the lysate was incubated 5 min at 37° C while shaking at 1000 rpm. Afterwards the lysate was centrifuged for 15 min at 21000 x g and 4° C. The supernatant was transferred to a new tube while the pellet was discarded. 0.5 μL of RNase T1 (ThermoFisher Scientific EN0541) was added to the lysate and it was incubated for 15 min at 22° C. A 5 μL aliquot was transferred to a new tube and stored at -20° C to be used later in a Western blot.

For immunoprecipitation, the 1x PXL buffer was removed from the antibody-coated beads and the lysate was added. The mixture was incubated while spinning at 4° C overnight. After incubation, the supernatant was removed and stored at -20° C for Western blot analysis. The beads were rinsed 3x with Lysis Buffer and resuspended in 1 mL Lysis Buffer. After the addition of 0.5 μL RNase T1, the beads were incubated at 22° C for 12 min while shaking continuously at 700 rpm. The beads were washed 4x.
with each of the following buffers: 1x PXL, 5x PXL (5x PBS, pH 7.4 w/o Ca\(^{2+}/\)Mg\(^{2+}\), 0.1% SDS, 0.5% Na-deoxycholate, 0.5% NP-40), High Stringency Buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 120 mM NaCl, 25 mM KCl), High Salt Buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1 M NaCl), and PNK (50 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 0.5% NP-40). The beads were left in the final wash buffer while the T4 PNK mixture was prepared.

Phosphorylation and intermolecular ligation were performed as follows. To prepare the T4 PNK mixture, the following components were combined: 8 μL 10x PNK Buffer, 2 μL RNasin Plus (40U/μL) (Promega N2615), 0.8 μL 100 mM ATP (ThermoFisher Scientific R0041), 65.2 μL autoclaved ddH\(_2\)O, and 4 μL T4 PNK (10U/μL) (NEB M0201). Eighty μL of this mixture was added to the beads and they were incubated 40 min at 10° C. After incubation the beads were washed 3x with PNK. To prepare the ligation mixture, the following components were combined: 50 μL 10x T4 RNA Ligase Buffer, 60 μL 50% PEG-8000, 1.25 μL 4 M KCl, 12.5 μL RNasin Plus, 5 μL 100 mM ATP, 321.25 μL autoclaved ddH\(_2\)O, and 50μL T4 RNA Ligase 1 (NEB M0204). Five-hundred μL of the ligation mixture was added to the beads. The beads were incubated while rotating at 4° C overnight.

Dephosphorylation and 3’ linker addition were performed as follows. The beads were washed 3x with PNK. The dephosphorylation mixture was prepared with the following: 8 μL 10x Dephosphorylation Buffer, 2 μL RNasin Plus, 67 μL autoclaved ddH\(_2\)O, and 3 μL Alkaline Phosphatase (1U/μL) (Roche cat. # 10713023001). Eighty μL of the dephosphorylation mixture was added to the beads and they were incubated at
10° C for 40 min, shaking every 2 min for 15 s at 1000 rpm. The beads were washed 2x with PNK-EGTA (50 mM Tris-HCl, pH 7.5, 20 mM EGTA, 0.5% NP-40) and 3x with PNK. The ligation mix was prepared by combining the following: 42 μL autoclaved ddH₂O, 8 μL 10x T4 RNA Ligase Buffer, 16 μL 50% PEG-8000, 2 μL RNasin Plus, 8 μL 10 μM miRCat-33 3'-linker (5′-TGGAATTCTCGGGTGCCAAGG-3'), 4 μL T4 RNA Ligase 2, truncated, K227Q. Eighty μL of the mixture was added to the beads and they were incubated overnight at 16°C, shaking every 2 min for 15 s at 1000 rpm.

To elute complexes, the beads were washed 3x with PNK. The elution buffer was prepared by combining the following: 40 μL 500 mM NaHCO₃, 20 μL 10% SDS, and 140 μL autoclaved ddH₂O. One-hundred μL was added to the beads and they were incubated 15 min at RT while shaking continuously at 1400 rpm. This step was repeated a second time. On both occasions the supernatant was transferred to a new tube. Two μL of the supernatant was transferred to a new tube and stored at -20° C for later Western blot analysis.

For proteinase K treatment and RNA extraction, the following components were combined: 10 μL 5x PK Buffer (500 mM Tris-HCl, pH 7.5, 250 mM NaCl, 50 mM EDTA), 10 μL Proteinase K (final concentration 4 mg/mL) (Roche cat. # 03115887001), and 30 μL autoclaved ddH₂O. The Proteinase K solution was incubated at 37° C for 20 min. Fifty μL of the solution was added to the 200 μL of supernatant from the previous step and the mixture was incubated 20 min at 37° C. Two-hundred fifty μL Phenol/Chloroform/Isoamyl Alcohol (25:24:1) were added and the sample was incubated 8 min at RT, shaking continuously at 1400 rpm. The sample was then centrifuged 10 min at 18,000 x g and 4° C. The aqueous supernatant was transferred to
a new tube and combined with 20 μL 3 M NaOAc, pH 5.2, 2 μL GlycoBlue (Invitrogen cat. # AM9516), and 500 μL of a 1:1 mixture of ethanol and isopropanol. The sample was incubated overnight at -20° C.

The sample was centrifuged for 30 min at 21,000 x g and 4° C. The supernatant was removed and the RNA pellet was washed once with 950 μL and once with 200 μL of 80% ice-cold ethanol. The sample was centrifuged at 18,000 x g for 10 min at 4° C after each wash. The pellet was allowed to air-dry for approximately 10 min before being resuspended in 10.5 μL autoclaved ddH₂O. The T4 PNK mix was prepared with the following: 1.5 μL 10x T4 PNK Buffer, 0.5 μL RNasin Plus, 1.5 μL 10 mM ATP, and 1 μL T4 PNK. 4.5 μL of the mixture was added to the RNA and it was incubated 40 min at 10° C. A ligation solution was prepared by combining the following: 0.5 μL 10x T4 RNA Ligase Buffer, 2 μL BSA, 0.5 μL 10 mM ATP, 1 μL 5’ RNA linker (100 pM/μL), and 1 μL T4 RNA Ligase. Five μL of the ligation mixture was added to the RNA and the sample was incubated overnight at 16° C. After incubation the RNA was extracted with Phenol C/I as before.

The sample was centrifuged for 30 min at 21,000 x g and 4° C. The supernatant was removed and the RNA pellet was washed twice with 200 μL of 80% ice-cold ethanol. After each wash, the sample was centrifuged at 18,000 x g for 10 min at 4° C. The pellet was air-dried for approximately 10 min before it was resuspended in 11 μL autoclaved ddH₂O. One μL of 10 μM reverse transcription primer (Illumina TruSeq® Small RNA Sample Prep Kits RTP) and 1 μL of 10 mM dNTPs were added to the RNA. The mixture was incubated at 65° C for 5 min and then chilled on ice and centrifuged briefly. The RT mix was prepared with the following: 4 μL 5x SuperScript RT Buffer, 1
μL 0.1 M DTT, 1 μL RNasin Plus, and 1 μL SuperScript III (200U/μL) (Invitrogen cat. # 18080093). Seven μL of the RT mix was added to the RNA. The mixture was incubated at 50°C for 45 min, 55°C for 15 min, 95°C for for 5min, and then chilled on ice while the PCR mix was prepared. The following were combined to make the PCR mix: 10 μL 2x Phusion High-Fidelity Master Mix, 1 μL 10 μM Primer 1 (Illumina TruSeq® Small RNA Sample Prep Kits RP1), 1 μL Index Primers 1, 2, or 3 (Illumina TruSeq® Small RNA Sample Prep Kits RPI1, RPI2, or RPI3), and 6 μL autoclaved ddH2O. Eighteen μL of the PCR mix were combined with 2 μL of the RT product. The PCR block was preheated to 98° C before running the following cycle: 98° C for 30 s, (98° C for 10 s, 52° C for 30 s, 72° C for 30 s) for 19-24 cycles, and 72° C for 5 min.

A 30% PEG-8000 and 29.25 mM MgCl2 mixture was prepared by combining 19.2 μL 50% PEG-8000, 0.94 μL 1M MgCl2, and 11.86 μL autoclaved ddH2O. Sixty μL of TE buffer was added to the PCR product followed by 40 μL of the PEG-8000 mixture. The sample was vortexed, incubated 10 min at RT, and then centrifuged 15 min at 10,000 x g and RT. The supernatant was transferred to a new tube, to which was added 12 μL 3 M NaOAc, 300 μL 100% ethanol, and 1 μL GlycoBlue. The sample was incubated overnight at -20° C and precipitated the following day as described above. The pellet was resuspended in 15 μL autoclaved ddH2O. The sample was submitted for sequencing on an Illumina HiSeq 2500 with a read length of 100 bp.

The efficacy of the Ago immunoprecipitation was confirmed by Western blot. The equivalent of material from 5 × 10^5 cells from before immunoprecipitation ("input"), after immunoprecipitation ("flow-through"), and after elution ("eluate") were run side-by-side on a polyacrylamide gel and transferred to a PVDF membrane. The membrane was
probed with the anti-Ago2 antibody 11A9 (Millipore cat. # MABE253) diluted 1:1000 followed by HRP-tagged anti-rat secondary antibody (Jackson ImmunoResearch cat. # 312-036-045) diluted 1:5000.

**Mimic Transfections and qPCR**

TIVE-EX-LTC cells infected with KSHV-ΔmiR-K12-11 were grown to confluence in 10 cm plates, trypsinized, and resuspended in medium without serum at a density of $2 \times 10^5$ cells/mL. $2 \times 10^6$ cells were used per transfection treatment. Cells were transfected with a miR-K12-11-3p mimic (Qiagen cat. # MSY0002181) using Lipofectamine RNAiMax Transfection Reagent (Invitrogen cat. # 13778-150) following the manufacturer’s instructions for reverse transfection, and plated in a clean 10 cm plate. Each transfection was performed in duplicate. Forty-eight hours posttransfection, the cells were harvested and RNA was extracted using RNA-Bee (Amsbio cat. # CS-501B). cDNA was prepared using SuperScript III and treated with DNase prior to qPCR. qPCR for selected transcripts was performed using Fast SYBR® Green Master Mix (Applied Biosystems cat. # 4385610). Primers were designed using IDT PrimerQuest.

**Sequencing and Bioinformatics Analysis**

The libraries were sequenced on a HiSeq 2500 with a read length of 100 bp. The raw sequences were preprocessed with Trimmomatic (85) to remove adapter sequences and then analyzed with Hyb, a bioinformatics pipeline developed by Travis et al. specifically for the analysis of CLASH data (86). Determination of base-pairing along the length of the miRNA, categorization of miRNA seed-pairing and 3’ end pairing, and determination of mRNA transcript region origin were all carried out with custom scripts.
Results

qCLASH

The interactions between KSHV miRNAs and cellular mRNAs were investigated by performing qCLASH on cells latently infected with KSHV, with $5 \times 10^7$ endothelial cells per sample. The endothelial cells used were derived from Telomerase-Immortalized human umbilical Vein Endothelial cells, Long-Term Culture (TIVE-LTC). TIVE-LTC cells are KSHV positive and relatively fast-growing. This makes them easier to work with than primary endothelial cells or their parent TIVE cells, which are slow-growing and dependent on exogenous growth factors for survival. At very high passages, TIVE-LTC cells begin to lose the KSHV genome. We performed single-cell dilutions on high passage TIVE-LTC cells in order to isolate KSHV-negative clones. A single clone was selected for all future experiments. We named the new cells, which grow quickly and can be re-infected with KSHV, TIVE-EX-LTC cells.

The TIVE-EX-LTC cells used for qCLASH analysis were either uninfected, infected with WT KSHV, or infected with KSHV miR-K12-11 knockout virus, with three biological replicates for each treatment. The basic procedure for qCLASH (Fig. 2-1) was derived from Haecker et al., with the important addition of intermolecular ligation as in Helwak and Tollervy (53, 54, 83). Cell lysates were thawed, treated with DNase, treated with a low concentration of RNase T1, and then incubated with beads coated in an Ago-specific antibody. This made it possible to pull down the endogenous protein rather than overexpress tagged Ago. After immunoprecipitation, the samples were again treated with RNase T1. Two RNase digestions were performed in order to remove as much non-Ago bound RNA from the samples as possible. The samples were 5’ phosphorylated with T4 PNK and then incubated with T4 RNA ligase. The
intermolecular ligation was performed in a large volume as in Moore et al. (87). This was to ensure that Ago-RNA complexes were well separated from each other and from any remaining extraneous RNAs in solution, making it more likely that hybrids would only be formed by RNAs bound to the same Ago. After intermolecular ligation, the samples were treated with alkaline phosphatase before ligation to the 3’ adapter. At this point the qCLASH protocol diverges sharply from the original CLASH protocol. Normal CLASH calls for radiolabeling, SDS-PAGE, and transfer of Ago-RNA complexes to nitrocellulose, after which labeled RNA running larger than 110 kDa are cut and eluted from the membrane. Rather than perform these steps, which result in the loss of large amounts of RNA, we proceeded directly to proteinase K digestion of Ago, phosphorylation, and 5’ adapter ligation. From there RT and PCR were performed, followed by Illumina sequencing (Fig. 2-1).

**Hybrid Library Characteristics**

The cDNA libraries were sequenced on a HiSeq 2500 with a read length of 100 nt. There were three libraries per flow cell lane and each library yielded between ten and twenty million reads. Sequencing reads were preprocessed using Trimmomatic (85) and then analyzed with Hyb (86). Approximately 0.3-1% of unique, filtered reads were classified as hybrids (Table 2-1). On average, approximately 23% of hybrids consisted of miRNA and mRNA. Roughly 95% of the time the miRNA was located at the 5’ end of the hybrid. This is consistent with what has been observed by others (53, 87).

Approximately 6% of miRNA-mRNA hybrids consisted of a KSHV miRNA and a cellular mRNA in WT-infected cells (Table 2-1). Even though only a minute percentage of total reads were KSHV miRNA-cellular mRNA hybrids, this still resulted in a range of approximately 6,000-12,000 such hybrids per WT biological replicate, and 2,200-4,000
per ΔmiR-K12-11 biological replicate. There were on average ten KSHV hybrids identified in each biological replicate performed on uninfected cells. This indicates that the level of misidentification by Hyb is very low. Furthermore, none of the hybrids found in uninfected cells occurred in more than one biological replicate. Only one hybrid containing miR-K12-11 was identified across all biological replicates of ΔmiR-K12-11-infected endothelial cells.

The frequencies of miRNA-mRNA hybrids involving specific KSHV miRNAs are shown in Figure 2-1 for cells infected with WT KSHV and with KSHV ΔmiR-K12-11, and are expressed as the number of hybrids obtained from each biological replicate. The five KSHV miRNAs most commonly found in WT-infected cell hybrids were, in descending order, miR-K12-3, K12-4-3p, K12-12*, K12-10a, and K12-6-3p (Fig. 2-2A). The same miRNAs made up the top five in ΔmiR-K12-11-infected cells (Fig. 2-2B). Three of these miRNAs, miR-K12-3, K12-10a, and K12-4-3p, were previously found to be highly expressed in two B-cell lymphoma lines, BCBL-1 and BC-3, using HITS-CLIP (83). The KSHV miRNAs were present in hybrids at a similar frequency relative to each other in ΔmiR-K12-11-infected cells as in WT-infected cells, but the number of viral hybrids was lower overall in ΔmiR-K12-11-infected cells. This is consistent with our previous observation that the expression of all KSHV miRNAs is lower in cells infected with this knockout virus (73).

**Binding Region**

Next, we investigated which region of the mRNAs was targeted by the viral and cellular miRNAs. First, we considered all the miRNAs present in WT KSHV-infected cells, in ΔmiR-K12-11-infected cells, and in uninfected cells (Fig. 2-3A). When the mRNA portion of each hybrid was mapped to an ENSEMBL transcript database, more
than 50% of hybrid mRNAs mapped to the CDS of their respective transcripts in each case. This is similar to what has been noted by others based on CLASH data (53, 87). The targeting was assessed for all KSHV miRNAs pooled from the WT and ΔmiR-K12-11-infected cells, and for all cellular miRNAs pooled from all three samples (Fig. 2-3B). mRNAs from KSHV miRNA-cellular mRNA hybrids had a slightly greater likelihood of mapping to the CDS than did those from cellular miRNA-cellular mRNA hybrids. For KSHV hybrids, 27.7% contained an mRNA which mapped to a 3' UTR and 65% contained an mRNA which mapped to a CDS. These figures were 33.6% and 61.2%, respectively, for cellular hybrids (Fig. 2-3). It is unclear why KSHV miRNAs would have a greater propensity than cellular miRNAs to target the CDS of mRNAs as opposed to the 3' UTR.

**Seed Pairing Characteristics**

We plotted the frequency with which each nucleotide of each miRNA was base-paired with the mRNA portion of the hybrid (Fig. 2-4). This yielded a broad picture of intramolecular base-pairing within the datasets. Plots were generated for cellular and viral miRNAs, with 5' and 3' hybrids (miRNAs at the 5' and 3' end of the hybrids, respectively) combined and for 5' and 3' hybrids alone. In general, nucleotides 2-8, the canonical seed region, were bound at relatively high frequencies when the results of all cellular and viral miRNAs were considered together (Fig. 2-4A–Fig. 2-4D). Canonical seed pairing was somewhat more common in cellular hybrids than it was for KSHV hybrids. There was also a high frequency of base-pairing toward the 3' ends of the miRNAs, particularly between nucleotides 11-18. An appreciable number of 3' interactions have been observed in other, similar experiments (53, 87, 88). Interestingly, a greater proportion of hybrids with the miRNA 3' to the mRNA had noncanonical
binding (compare Figure 2-4C–Figure 2-4F with Figure 2-4B–Figure 2-4E). This agrees with what is thought to occur at the molecular level. If the extreme 5’ end of the miRNA is bound to the mRNA, steric hindrance should prevent it from joining with the 3’ tail of the mRNA. If the 5’ end is unbound, however, this could provide enough flexibility of movement for the ligation to proceed.

When the KSHV miRNAs were considered separately, distinct patterns of seed or nonseed base-pairing became evident. Plots are shown for miR-K12-1, miR-K12-3, and miR-K12-6-5p (Fig. 2-4G, Fig. 2-4H, Fig 2-4J). For miR-K12-1, a clear M-shaped plot was generated. However, miR-K12-3 showed a distinct propensity for 3’ interactions to the exclusion of most 5’ binding. This is similar to what was reported by Grosswendt, et al. when they reanalyzed existing CLIP datasets in KSHV-infected cells in order to look for naturally occurring hybrids (88). miR-K12-6-5p, in contrast, had mainly canonical seed interactions (Fig. 2-4).

In order to look at miRNA-mRNA interactions in a different way, hybrids were placed into groups based on how their miRNA seed region is predicted to bind to the mRNA portion of the hybrid. The categories were: pairing at nt 2-8 with no mismatches; pairing at nt 2-7 with no mismatches; pairing at nt 2-8 with one mismatch; pairing at nt 2-8 with two mismatches; and other, for interactions that did not fall into any of the previous categories (Fig. 2-5). The seed-pairing characteristics of the hybrid miRNAs were considered in several different ways. First, hybrids from WT-infected cells, hybrids from ΔmiR-K12-11-infected cells, and hybrids from uninfected cells were compared side-by-side (Fig. 2-5A). In order to understand how KSHV and cellular hybrids differ, all KSHV hybrids from WT- and ΔmiR-K12-11-infected cells were compared with all cellular
hybrids from WT- and ΔmiR-K12-11-infected cells as well as uninfected cells (Fig. 2-5B). Finally, all hybrids with the miRNA located at the 5’ end were compared with all hybrids with the miRNA located at the 3’ end (Fig. 2-5C). Interestingly, a large proportion of miRNAs fell into the other category, i.e., showed noncanonical binding (Fig. 2-5). This was true for both uninfected and infected cells (Fig. 2-5A), although when hybrids containing viral and cellular miRNAs were considered separately (Fig. 2-5B), viral miRNAs were somewhat more likely than cellular miRNAs to lack a canonical seed match. The proportion of cellular miRNAs in the other category was 46%, compared with 56% for viral miRNAs. Additionally, hybrids with the miRNA at the 5’ end fell into the other category 44% of the time while 3’ miRNA hybrids did so 68% of the time. The prevalence of noncanonical seed-pairing in our hybrids agrees with the recent findings of others indicating that canonical seed matches are not necessarily the strongest determinant of which genes a given miRNA will target (53, 87).

In light of this information, we chose to examine more closely how the 3’ ends of the miRNAs, which we classified as anything downstream of nucleotide 8, interacted with the mRNA ends of the hybrids. We wanted to ask if compensatory base pairing toward the 3’ end of the miRNA was more prevalent in cases where a traditional seed sequence was absent. Interactions involving the 3’ end of the miRNA were considered “absent” if they had no paired bases from nt 8 to the 3’ end of the miRNA; “weak” if they had between one and four bound nucleotides; “moderate” if they had between five and eight bound nucleotides; and “strong” if they had more than eight bound nucleotides. The proportions of the four classes of pairing at the 3’ end of the miRNA are shown in Figure 2-6 for each of the seed-pairing classes. miRNAs with perfect 2-8 or perfect 2-7
seed pairing had proportionally more weak and absent 3’ interactions than the other categories of seed pairing. Conversely, miRNAs falling into the “other” category had a greater propensity for strong 3’ interactions and fewer interactions classified as weak or absent (Fig. 2-6). It would appear that the lack of canonical binding to the seed sequence is compensated for with a greater amount of base-pairing at the 3’ end of the miRNA.

**KSHV miRNA Targets**

In total, there were 27022 KSHV miRNA-cellular mRNA hybrids across all three biological replicates in WT-infected endothelial cells, representing 6497 unique genes. Three-thousand three-hundred twenty-four of these genes were found in at least two of three biological replicates while 1433 genes were found in three of three biological replicates. Since it was impractical to follow up on all of these targets, we chose to focus specifically on targets of miR-K12-11. We selected genes which were present in hybrids in at least two of three biological replicates of WT-infected cells and absent in at least two of three biological replicates of ΔmiR-K12-11-infected cells. This yielded fifty-four genes which we considered to be high-confidence miR-K12-11 targets. The list necessarily excluded any genes targeted by miR-K12-11 and one or more additional KSHV miRNAs. One of the genes on our list, STIP1, has already been validated as a target of miR-K12-11 elsewhere (83). Two more genes, CTNND1 and PKN2, were also previously confirmed to be targeted by a KSHV miRNA, although it was miR-K12-10 (89). In order to validate our targets, ΔmiR-K12-11-infected TIVE-EX-LTC cells were transfected with a miR-K12-11 mimic. RNA was prepared from the cells and the transcript levels of the candidate targets were determined by RT-qPCR.
Hybrids in B-cells

We ran Hyb on earlier HITS-CLIP data (83) in two KSHV-infected B-cell lymphoma lines in order to search for hybrids formed by endogenous ligases, a phenomenon first observed by Grosswendt et al. (88). On average 0.01% of reads were identified as hybrids, indicating that the natural formation of hybrids is a vanishingly rare event. Even so, KSHV miRNA hybrids made up a much greater percentage of hybrids overall in B-cells than in endothelial cells. There were a total of 833 KSHV miRNA-cellular mRNA hybrids in BCBL-1 cells and a total of 3065 such hybrids in BC-3 cells. These hybrids were analyzed in the same way as hybrids from endothelial cells. In contrast to hybrids in endothelial cells, it was found that more than 50% of mRNAs from B-cell hybrids originated from the 3’ UTR. This also differs from the percentage of mRNAs from 3’ UTRs in the original HITS-CLIP analysis, which was closer to 30% (83). Another surprising finding was that approximately 90% of B-cell hybrids lacked canonical seed-pairing. It is unclear whether this actually represents the reality of miRNA-mRNA interactions in B-cells, is a characteristic of hybrids formed by endogenous ligases, or is simply an artifact of having a small sample size. When the hybrids were analyzed for binding towards the 3’ ends of the miRNAs, a pattern similar to that in endothelial cells was observed, with noncanonical seed-pairing correlating with strong 3’ interactions.

Comparison with Targets in B-cells

KSHV miRNA targets previously identified through HITS-CLIP on two types of KSHV-infected B-cells, BCBL-1 and BC3 (83), were compared with targets found with CLASH on KSHV-infected endothelial cells. It was expected that there would be at least some overlap between the results of the different experiments. Indeed, when lists of
genes identified as targets of KSHV miRNAs in three out of three biological replicates of CLIP on BCBL-1 or BC3 cells were compared with genes found in three of three biological replicates of CLASH in WT-infected cells, 223 and 169 targets, respectively, were shared. There were 105 shared targets when all three cell lines were compared simultaneously. It would seem that it is important for KSHV to reduce the levels of these particular mRNAs regardless of the cell type infected. Additionally, since these targets were identified in three different cell types in all biological replicates using two different protocols, they are particularly promising for further study.

In addition to the targets discovered by Haecker et al., KSHV miRNA targets identified through CLASH in endothelial cells were also compared with previously published, validated targets of the KSHV miRNAs from a variety of sources (83). A number of the published targets were also found in our CLASH dataset (Table 2-2). It is interesting to note that some of these genes, while targeted in both datasets, were associated with a larger number of KSHV miRNAs in the CLASH data. The additional miRNAs were most likely missed by other methods because they either do not undergo canonical seed-pairing or they target a region of the mRNA other than the 3’ UTR.

Nevertheless, we deem it highly likely that the genes listed in Table 2, which were initially identified by a variety of methods in different cell types, are highly significant to KSHV regardless of the cell type infected.

Results of Pathway and Gene Enrichment Analysis

The 1433 genes which appeared in three of three biological replicates of WT-infected cells were further examined in order to determine if there are any major cellular processes which are targeted by KSHV miRNAs. Using Enrichr, the KEGG pathways which contain a significant number of our genes were identified. Among these pathways
were several with particular relevance to cancer and/or the biology of the virus. These were cell cycle control, glycolysis, angiogenesis, and the AKT pathway.

It is well known that controls on the cell cycle become suppressed or inactivated in cancer, allowing for the unimpeded proliferation of cells. As an oncogenic virus, it is therefore unsurprising that KSHV has the ability to dysregulate the cell cycle. It has been demonstrated that this can occur in multiple ways. For example, the latency-associated nuclear antigen (LANA) has the ability to inhibit both tumor protein 53 (p53) and RB transcriptional corepressor 1 (Rb), two important checks on cell cycle progression (90). vCyclin, the KSHV homolog of cellular cyclin D2, can also inhibit Rb (91). Viral FLICE-inhibitory protein (vFLIP) prevents the formation of active caspase 8, a pro-apoptotic protein (92, 24). KSHV miRNAs have also been implicated in cell cycle dysregulation. miR-K12-1 was previously found to target cyclin dependent kinase inhibitor 1A (CDKN1A, also called p21), thereby preventing cell cycle arrest (60).

We, too, found an interaction between miR-K12-1 and p21. Indeed, we observed that suppressors of cyclins and cyclin-dependent kinases were frequent targets of KSHV miRNAs. These included glycogen synthase kinase 3 beta (GSK3B), a suppressor of Cyclin D1 (93); cyclin dependent kinase inhibitor 1B (CDKN1B, also called p27), which blocks the activation of cyclin E-CDK2 and cyclin D-CDK4 complexes (94); and WEE1 G2 checkpoint kinase (WEE1), an inhibitor of mitosis (95). We also found that miR-K12-3 and miR-K12-8, like LANA and vCyclin, target Rb. Overall, the KSHV miRNA targets identified through CLASH appear to interact broadly with many components of the cell cycle control machinery.
The Warburg effect is the preference of cancer cells to produce ATP through glycolysis rather than oxidative phosphorylation. It has been shown that KSHV induces the Warburg effect in infected cells (26). Indeed, exogenous expression of the KSHV miRNA cluster by itself in lymphatic endothelial cells was sufficient to induce this effect (96). Consistent with this, our findings show extensive involvement of the KSHV miRNAs in the glycolysis pathway.

**Discussion**

We have successfully completed a shortened version of CLASH, named qCLASH, and established that the resulting libraries have a very similar composition to those generated by the standard CLASH protocol. There are two major advantages to our approach. First, it can be completed more quickly than the original, in as few as four days. Even if time is not a concern, our protocol has eliminated the steps that involve the use of radionuclides and the issues involved with their handling and disposal. Second, qCLASH requires fewer cells as input. To date it has been successfully performed with as few as 1 x 10^7 cells. This opens up the important possibility of using the protocol on patient biopsies, which would be an unprecedented opportunity to examine KSHV miRNA targets *in vivo*.

The establishment of the TIVE-EX-LTC cell line also creates more opportunities in the field of KSHV research. Since the discovery that SLK cells are not actually endothelial cells, the relevance of KSHV studies in this cell type has become more limited (97). TIVE-EX-LTC cells grow quickly, require no exogenous growth factors, and can readily be infected with KSHV. As such they have the potential to fill the void left by SLK cells.
Using qCLASH on TIVE-EX-LTC cells infected with WT KSHV, we identified a number of high-confidence cellular targets of KSHV miRNAs. There were 1,433 genes which appeared in three of three biological replicates of WT-infected cells. While following up on all of these targets is well beyond the scope of the present study, this information has the potential to inform the work of other researchers. We also performed qCLASH on ΔmiR-K12-11-infected TIVE-EX-LTCs. As the viral ortholog of the known human oncomir miR-155, miR-K12-11 is of particular interest to studies of KSHV and cancer (57). Our experiment allowed us to look for miR-K12-11 targets found in WT-infected cells which were absent in ΔmiR-K12-11-infected cells. There were 54 such high-confidence targets of miR-K12-11.

Figure 2-1. Schematic of the qCLASH method.
Figure 2-2. Frequencies of the KSHV miRNAs determined by qCLASH analysis. A) The number of hybrids containing each of the 25 mature KSHV miRNAs was determined for each biological replicate of WT. B) The number of hybrids containing each of the 25 mature KSHV miRNAs was determined for each biological replicate ΔmiR-K12-11-infected TIVE-EX-LTC cells.
A majority of miRNAs target sites within the CDS of mRNA transcripts. The transcript region of origin was ascertained for each mRNA portion of each hybrid. The regions were categorized as 5' UTR, 5' UTR-CDS, CDS, CDS-3' UTR, or 3' UTR. Hybrid mRNAs for which the transcript was not annotated in the database are omitted.

A) Comparison of WT-infected, ΔmiR-K12-11-infected, and uninfected TIVE-EX-LTC cells.

B) Comparison of hybrids containing KSHV miRNAs from either WT- or ΔmiR-K12-11-infected cells with hybrids containing cellular miRNAs from WT- and ΔmiR-K12-11 infected cells and uninfected cells.

Figure 2-3.
miRNAs exhibit binding in both the seed region and at the 3’ end. Based on the Vienna diagrams generated through Hyb, the status of each nucleotide (bound or unbound) along the length of the miRNA portion of each hybrid was determined. Only KSHV hybrids from WT-infected TIVE-EX-LTC cells were included in this analysis. BR = biological replicate. A) All cellular miRNAs. B) All cellular miRNAs located at the 5’ end of the hybrid. C) All cellular miRNAs located at the 3’ end of the hybrid. D) All KSHV miRNAs. E) All KSHV miRNAs located at the 5’ end of the hybrid. F) All KSHV miRNAs located at the 3’ end of the hybrid. G) KSHV miR-K12-1 hybrids. H) KSHV miR-K12-3 hybrids. I) KSHV miR-K12-6-5p hybrids.
Figure 2-5. Nearly half of miRNAs do not have any recognizable seed-pairing. Hybrids were sorted into categories based on the type of seed pairing determined for the miRNA portion of each hybrid. These categories were no mismatches nucleotides 2-8, no mismatches nucleotides 2-7, one mismatch nucleotides 2-8, 2 mismatches nucleotides 2-8, and other. A) Comparison of hybrids (viral and cellular) from WT-infected, ΔmiR-K12-11-infected, and uninfected TIVE-EX-LTC cells. B) Comparison of all hybrids containing KSHV miRNAs from WT- and ΔmiR-K12-11-infected TIVE-EX-LTC cells and all hybrids containing cellular miRNAs from WT- and ΔmiR-K12-11-infected as well as uninfected TIVE-EX-LTC cells. C) Comparison of all hybrids (viral and cellular) with the miRNA at the 5’ end with all hybrids with the miRNA at the 3’ end.
Figure 2-6. miRNAs with no recognizable seed-pairing have stronger interactions at the 3’ end. Hybrids in each of the seed pairing categories were further sorted based on the number of nucleotides bound in the miRNA portion of the hybrid downstream from the seed sequence. These categories were strong (> 8 bound nucleotides), moderate (5-8 bound nucleotides), weak (1-4 bound nucleotides), and absent (0 bound nucleotides). A) KSHV hybrids from WT- and ΔmiR-K12-11-infected cells. B) Cellular hybrids from all cell types.
Table 2-1. Hybrid statistics.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% of Reads which are Hybrids</th>
<th>% of Hybrids which are miRNA-mRNA</th>
<th>% of miRNA-mRNA Hybrids which have 5' miRNA</th>
<th>% of miRNA-mRNA Hybrids which have 3' miRNA</th>
<th>% of miRNA-mRNA Hybrids which have Cellular miRNA</th>
<th>% of miRNA-mRNA Hybrids which have KSHV miRNA</th>
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<tbody>
<tr>
<td>WT BR1</td>
<td>1.02</td>
<td>31.13</td>
<td>95.65</td>
<td>4.35</td>
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<td>WT BR2</td>
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<td>WT BR3</td>
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<td>94.26</td>
<td>5.74</td>
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<td>ΔmiR-K12-11 BR1</td>
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<td>95.15</td>
<td>4.85</td>
<td>97.68</td>
<td>2.32</td>
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<td>ΔmiR-K12-11 BR2</td>
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<td>19.58</td>
<td>94.78</td>
<td>5.22</td>
<td>97.88</td>
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<td>5.18</td>
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<td>UI BR3</td>
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<td>95.11</td>
<td>4.89</td>
<td>99.99</td>
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Table 2-2. CLIP-CLASH target comparison.

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<th>Targeting miRNA (qCLASH)</th>
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<tr>
<td>BACH1</td>
<td>miR-K12-11</td>
<td>miR-K12-4-3p, miR-K12-10a, miR-K12-12*</td>
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<td>miR-K12-4-3p, miR-K12-10a, miR-K12-10b</td>
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<tr>
<td>Validated Target (CLIP)</td>
<td>Targeting miRNA (CLIP)</td>
<td>Targeting miRNA (qCLASH)</td>
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<td>-------------------------</td>
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CHAPTER 3
PHENOTYPIC CHARACTERIZATION OF ENDOTHELIAL CELLS INFECTED WITH KSHV MICRONIA KNOCKOUT VIRUSES

Background

Kaposi’s sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi’s sarcoma (KS) (11), an endothelial cell tumor, as well as primary effusion lymphoma (12) and multicentric Castleman’s disease (13), two B cell proliferation disorders. KS affects millions worldwide, being the most common cancer in several sub-Saharan African countries, and it frequently occurs in those with AIDS (98). KSHV undergoes both lytic and latent phases of replication, but it is when the virus establishes latency in endothelial cells that KS occurs (78). Among the few genes expressed during latency are the viral microRNAs (miRNAs) (99). miRNAs are short RNAs, only 19-22 nucleotides in length, yet they function as important post-transcriptional controls on gene expression. They prevent the translation of target mRNAs by binding to imperfectly complementary sequences which are often found within the 3’ UTRs of the transcripts (reviewed in reference 33 and reference 49). Dysregulation of human miRNAs is often associated with cancer (reviewed in reference 100).

There is evidence that the KSHV miRNAs are drivers of tumorigenesis. For example, KSHV miR-K12-11 is the ortholog of the cellular oncogene miR-155 and it has been shown to induce splenic B-cell expansion when expressed in mice (57, 58). KSHV also drives phenotypic changes in endothelial cells which are hallmarks of transformation. Infection with the virus has been shown to induce angiogenesis through upregulation of hypoxia-induced factor 1α (HIF1α) (101). It also has been shown to induce the Warburg effect, a shift in cellular glucose metabolism, in latently infected endothelial cells (26). In fact, later experiments demonstrated that transducing lymphatic
endothelial cells (LECs) with a lentivirus expressing the KSHV miRNA cluster was sufficient to recapitulate this phenotype (96). It was also found that KSHV can induce greater invasiveness in cells, although this work was carried out in oral fibroblasts (102). We sought to characterize the contributions of the individual KSHV miRNAs to the previously noted phenotypic changes which cells undergo upon infection with KSHV. To this end, we utilized a panel of miRNA knockout mutants in the BAC 16 background which we had generated previously (103). Telomerase Immortalized Vein Endothelial (TIVE) cells were infected separately with each of the mutant viruses and allowed to attain latency. Cell proliferation, migration, angiogenesis, and induction of the Warburg effect were evaluated for each of the mutant-infected cell lines and compared to the characteristics of WT-infected cells. We found broadly varying effects of the different mutant viruses on endothelial cell phenotype. For example, the ΔmiR-K12-11 virus had a significant defect in proliferation. This same mutant, along with ΔmiR-K12-7, also had a lessened ability to migrate when compared to WT-infected cells. The ΔmiR-K12-5, -6, and -7 viruses had a profound defect in their ability to form tubules in Matrigel, a measure of angiogenesis. When evaluating induction of the Warburg effect, it was found that ΔmiR-K12-10-infected cells showed an aberrant phenotype. Taken together, these results indicated that the KSHV miRNAs do play a significant role in the KSHV-induced transformation of endothelial cells.

**Methods**

**Viruses**

The KSHV mutant viruses used in this study were developed previously (103). Briefly, mutations were made to KSHV in the BAC16 background. Each single miRNA deletion mutant contains only a small deletion which prevents the selected miRNA from
forming a hairpin. This prevents the expression of both the leading and passenger strands from the locus. For the ΔmiR-K12-10 and ΔmiR-K12-12 viruses, both of which are located within the open reading frame of kaposin, synonymous mutations were introduced in order to prevent hairpin formation but keep the coding sequence of kaposin intact. Both the Δcluster and Δall viruses have the entire cluster region of the genome deleted. The Δall virus additionally has mutations in the miR-K12-10 and miR-K12-12 genes.

Establishment of Mutant-infected TIVE Cell Lines

TIVE cells were seeded at high density in 6-well plates and infected separately with each virus at approximately 1000 genome copies per cell. Seventy-two hours after infection, the medium was replaced with medium containing 50 ug/ml of hygromycin in order to selectively kill any noninfected cells. Subsequently, cells were maintained in medium containing 100 ug/ml of hygromycin.

Cell Proliferation Assay

Ninety-six-well plates were seeded at a density of 5,000 cells per well and grown in the absence of hygromycin selection for 72h. At that time cell proliferation was measured using a CellTiter 96 ® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

Wound Healing Assay

Cells were seeded at a density of $2 \times 10^5$ cells per ml in 48-well plates for a total of $5 \times 10^4$ cells per well. Approximately twenty-four hours later, each monolayer was scratched with a 1000 ul pipet tip. Photographs of each scratch were taken at 0 h and 12 h after scratching. The distance between the edges of each scratch was measured
for both time points and the 0 h distance was divided by the 12h distance to yield the proportion of the scratch that was filled during the time period.

**Tubule Formation Assay**

BD Matrigel was diluted to 4 mg/ml of protein and 100 ul of this solution was distributed to each well of a 48-well plate. The Matrigel was allowed to solidify at 37 °C for 30 min before adding $5 \times 10^4$ cells per well. Photographs were taken of two fields per well at 6 and 12 h after plating. The number of junctions between three or more tubules was counted for each field.

**Cell Metabolism Assay**

Seahorse assay plates were seeded at a density of $2.5 \times 10^4$ cells per well. Approximately 24 h later, the oxygen consumption rate and extracellular acidification rate of the cells were determined on a Seahorse XF96 machine. In a follow-up experiment, uninfected TIVE cells were seeded in an assay plate at the same density as before. One group of wells was infected with WT KSHV BAC at 1000 genome copies per cell every 6 h over a 48 h period. The extracellular flux assay was performed 48 h after the first infection, which was approximately 72 h after the cells were plated.

**Results**

TIVE cells infected with each of the fourteen miRNA knockout viruses, in conjunction with WT-infected and uninfected cells, were characterized in four assays. The lone exception is the cell line infected with the ΔmiR-K12-9 virus, which was not evaluated in some of the experiments. Working with this cell line posed several unique challenges which will be discussed in greater depth below. The different assays were chosen in order to characterize the relative contribution of each KSHV miRNA to the
major phenotypic changes induced by KSHV. In this way we measured cell proliferation, cell migration, angiogenesis, and induction of the Warburg effect.

**Cell Proliferation**

MTS assays were performed in order to measure cell growth rate. There were three biological replicates per cell line with each biological replicate consisting of eight technical replicates. MTS is a colorimetric assay which looks for the accumulation of a metabolic compound in cell culture medium over time. The absorbance was measured for each cell line and compared with that of WT-infected cells. It was found that cells infected with the ΔmiR-K12-11 virus had the most profound defect in proliferative ability. The ΔmiR-K12-3, -4, -6, -7, -8, and -10 cell lines had a modest but significant deficit in this metric as well (Fig. 3-1). Since cells multiply more slowly when these miRNAs are absent, this suggests that these miRNAs play a role in the KSHV-induced enhancement of cell proliferation. Indeed, the findings for ΔmiR-K12-11 cells were expected based on the previous work mentioned above. miR-K12-4 was previously identified as capable of rescuing a proliferative phenotype in Δcluster-infected rat mesenchymal precursor cells (61). The MTS assay does not actually distinguish between cell proliferation and prevention of apoptosis. Therefore it is instructive that miR-K12-3 and miR-K12-4-3p have been found to directly target the pro-apoptotic caspase 3 (62). miR-K12-10 and miR-K12-11 both downregulate the transforming growth factor β (TGFβ) pathway, thereby encouraging cell survival (104, 64). To our knowledge, miR-K12-6 and miR-K12-8 have not previously been identified as potential players in cell proliferation or cell survival enhancement.
**Migration**

Wound healing assays are designed to measure the rate at which cells are able to migrate. The assay itself is quite simple. Cells are grown to confluency and the monolayer is scratched with a pipette tip in order to create a “wound.” Endothelial cells, being the component cells of blood vessels, will naturally migrate to patch any injury. In cancer, cell migration rate is enhanced, contributing to the invasiveness of tumors.

Wound healing assays were performed on each of the mutant-infected cell lines as well as WT-infected and uninfected cells. Photographs were taken of the cells at 0 and 12 h after scratching. The ratio between the scratch area at 0 h and at 12 h was determined using TScratch (105). It was found that ΔmiR-K12-7 and ΔmiR-K12-11-infected TIVE cells, as well as Δall-infected cells, moved into the cell-free area far more slowly than WT-infected cells. ΔmiR-K12-1, -10, and -12-infected cells also showed a deficiency in this respect, although to a lesser extent (Fig. 3-2). miR-K12-11 has previously shown to indirectly downregulate dual-specificity phosphatase 1 (DUSP1), an inhibitor of mitogen-activated protein kinase (MAPK) signaling. The restoration of MAPK signaling has a number of downstream effects, including the release of promigratory factors (106). This may explain why the absence of miR-K12-11 leads to a reduction in the migration speed of infected cells.

**Angiogenesis**

In order to evaluate the angiogenic abilities of each mutant virus, the virus-infected cell lines were used in tubule formation assays. TIVE cells are blood endothelial cells, so they are able to form vessels in culture as long as they are provided with an appropriate extracellular matrix. The cells were plated on Matrigel, which serves as the matrix, and photographed at 6 and 12 h after plating. The number of junctions
between three or more tubules was counted. Cells infected with ΔmiR-K12-5, -6, and -7 showed a significant defect in the ability to form tubules (Fig. 3-3, parts C, D, and E). They formed fewer junctions than even uninfected cells. ΔmiR-K12-9-infected cells also showed a similar defect, but due to the unique characteristics of this cell line it is hard to make a direct comparison to the other cell lines (Fig. 3-3, part F). Cells infected with ΔmiR-K12-1, -10, and -all also formed fewer junctions that WT-infected cells. By contrast, the cells infected with ΔmiR-K12-2 and -8 actually showed a significant increase in the number of junctions which formed (Fig. 3-4). This suggests that these two miRNAs may actually suppress angiogenesis, perhaps serving as necessary counterbalance to the proangiogenic miRNAs. Overall, there is a strong indication that miR-K12-5, -6, and -7 are indispensable to KSHV-induced angiogenesis. miR-K12-6, in particular, has already been strongly implicated in angiogenesis. It was found that miR-K12-6-3p targets SH3 domain binding glutamate rich protein (SH3BGR), resulting in enhanced cell migration and angiogenesis (68). The same researchers also noted that miR-K12-6-5p targets the metastasis suppressor CD82. Downregulation of CD82 resulted in greater invasiveness and angiogenic ability in KSHV-infected endothelial cells (69). Others showed that miR-K12-6-5p targets breakpoint cluster region protein (BCR), leading to derepression of ras-related C3 botulinum toxin substrate 1 (Rac1) and the subsequent enhancement of angiogenesis (66).

**The Warburg effect**

The Warburg effect is a phenomenon in cancer cells which is characterized by an increased rate of glycolysis in conjunction with increased use of lactic acid fermentation as a source of ATP (107). Cells experiencing the Warburg effect do not rely on oxidative phosphorylation as their main source of ATP, so they require less oxygen than normal.
cells and would be expected to have a lower oxygen consumption rate (OCR). Another characteristic of these cells is the accumulation of lactic acid, which leads to an increase in the extracellular acidification rate (ECAR). KSHV infection is known to induce the Warburg effect in endothelial cells (26). In order to assess whether or not the various KSHV mutant-infected cell lines were capable of inducing the Warburg effect, a Seahorse Metabolic Analyzer was used to simultaneously measure the OCR and ECAR of living cells. In addition to uninfected and WT-infected cells, we used an additional cell line in this assay. Telomerase immortalized vein endothelial long-term culture (TIVE-LTC) cells are both KSHV positive and fully transformed (84). As a transformed cell line, we expected that TIVE-LTC cells would show the strongest induction of the Warburg effect and would therefore make an appropriate positive control.

Much to our surprise, the TIVE-LTC cells had a much higher OCR than uninfected TIVE cells. This is the opposite of what was anticipated based on results obtained by others (26). WT-infected cells had an OCR which was intermediate between these two extremes. Cells infected with the virus from which all miRNAs were deleted had a similar OCR to uninfected cells. The cells infected with the ΔmiR-K12-10, -11, and -cluster viruses had OCR readings on par with that of TIVE-LTC cells (Fig. 3-5, part B).

The ECAR results were more in line with expectations. TIVE-LTC cells had the highest ECAR while uninfected and Δall-infected cells had the lowest. WT-infected cells once again had an intermediate phenotype, as did ΔmiR-K12-11-infected cells. Both ΔmiR-K12-10- and Δcluster-infected cells had an ECAR more similar to that of TIVE-LTC cells (Fig. 3-5, part A).
Apart from the unusual OCR results, there was another conundrum created by these experiments: the ΔmiR-K12-10- and Δcluster-infected cells behaved very similarly. While the ΔmiR-K12-10 virus lacks only miR-K12-10, the Δcluster virus only expresses two miRNAs, miR-K12-10 and miR-K12-12. It is unclear why there would be such a strong phenotypic effect when miR-K12-10 is deleted and the same strong effect when miR-K12-10 is expressed alongside miR-K12-12. When miR-K12-10 and miR-K12-12 are expressed together along with other miRNAs the effect is not observed.

**TIVE cells infected with ΔmiR-K12-9**

There were pronounced differences between ΔmiR-K12-9-infected TIVE cells and those infected with WT or, indeed, any of the thirteen other mutant viruses. These differences were apparent from the very beginning, when the initial infections were being performed. It took multiple separate attempts before cells could finally be infected with this virus. After infection, it took an abnormally long amount of time to grow sufficient cells for use in the phenotypic assays. We were only able to complete two of the four assays using the ΔmiR-K12-9-infected TIVE cells. Another unusual feature of these cells was their size. When observed through a microscope, it was readily apparent that the ΔmiR-K12-9-infected cells were much larger than any of the other infected cell lines. Because of their exaggerated size, special considerations had to be made when performing phenotypic assays. For example, a density of $1 \times 10^5$ cells/mL was sufficient to generate a confluent monolayer of cells 24h post-seeding for all of the TIVE cells lines with the sole exception of ΔmiR-K12-9-infected cells. At this concentration of cells, the ΔmiR-K12-9-infected TIVEs formed a hyper-dense monolayer which would detach from the plate. It was found that only when the density of the ΔmiR-K12-9-infected cells was reduced by half would the resulting monolayer remain...
adherent and resemble those of the other cell lines. Even with this adjustment, the outcome of scratching a monolayer of Δ9-infected TIVEs was atypical. While the scratches through most of the cell lines tended to have fairly straight edges, the scratches through ΔmiR-K12-9-infected cells had very jagged edges, as if additional cells which were not in the path of the pipet tip had been torn away by the removal of their neighbors. It was also not uncommon for the cells at the edge of the scratch to begin to detach somewhat. So ultimately, even though the results show no significant difference in the distance ΔmiR-K12-9-infected and WT-infected cells migrated post-scratching, it is unclear how reliably these measurements can be compared.

There was a significant difference between WT- and ΔmiR-K12-9-infected TIVE cells with respect to tubule formation. We were able to use the same number of cells from each cell line in this assay. The ΔmiR-K12-9-infected cells had significantly fewer tubule junctions per field than did WT-infected cells, suggesting the former has a deficiency in angiogenesis. However, since the ΔmiR-K12-9-infected cells are larger, the overall tubule network created by these cells is larger than normal. It seems they may have fewer junctions than WT-infected cells simply because fewer junctions are able to fit in one microscope field.

Overall, the deletion of miR-K12-9 produced the most profound phenotypic changes in infected cells. This mutant virus expresses neither miR-K12-9 nor miR-K12-9*. miR-K12-9* is known to downregulate RTA, the KSHV gene responsible for initiating lytic replication of the virus, thereby contributing to the maintenance of latency (72). It is possible that in the absence of miR-K12-9/9* KSHV-infected cells may undergo more frequent lytic reactivation. Lysis of cells due to lytic replication may have contributed to
the perceived slow growth of the ΔmiR-K12-9-infected cell line; however we did not observe an increased number of dead cells compared to other cell lines. The unusual phenotype of the ΔmiR-K12-9-infected endothelial cells warrants further investigation of this miRNA.

Discussion

The phenotypic changes which occur in endothelial cells latently infected with KSHV are characteristic of the process of oncogenesis. Since the KSHV miRNAs are expressed during latency, they may play a role in the observed changes. In order to evaluate what contributions the individual KSHV miRNAs make to changes in cellular phenotype, we infected endothelial cells with a panel of miRNA knockout viruses. We looked for differences in cell proliferation, migration, angiogenesis, and metabolism between cells infected with WT and the mutant viruses.

Cells infected with the mutant lacking miR-K12-11 showed a pronounced deficit in proliferative ability, indicating that this miRNA is needed in order to sustain the levels of cell proliferation seen in WT KSHV infection. This agrees well with the work of others showing that ectopic expression of miR-K12-11 can enhance B-cell proliferation (58, 59). Two miRNAs whose absence had a modest impact on cell proliferation, miR-K12-6 and miR-K12-8, are not currently well understood in this context and may be a subject for future study. Through recent ribonomics analysis of the KSHV miRNA targetome, we identified RB1, a negative regulator of the cell cycle, to be a target of miR-K12-8. We also found that miR-K12-6 may disrupt TGF-β signaling and thereby promote cell survival by targeting SMAD4. These potential targets will need to be validated in the future.
Wound healing assays demonstrated that miR-K12-7 and miR-K12-11 are important drivers of the increased migration seen in KSHV-infected cells. While miR-K12-11 was previously shown to be indirectly involved in the enhancement of cell migration (106), the relationship of miR-K12-7 to this process will need to be elucidated in the future. One putative target of miR-K12-7 identified through quick cross-linking sequencing and analysis of hybrids (qCLASH) is cytoplasmic FMR1 interacting protein 1 (CYFIP1), which is thought to have a role in suppressing tumor invasion (108). This interaction will require verification.

Three of the KSHV miRNAs, miR-K12-5, -6, and -7, were found to be significant factors in virally-induced angiogenesis. Others have identified miR-K12-6 as a component of this process (66, 68, 69), but miR-K12-5 and -7 need to be studied further.

It is difficult to draw any solid conclusions from the OCR/ECAR data other than that miR-K12-10 should be thoroughly characterized for its role in changes to cellular metabolism.

The unusual phenotype of TIVE cells infected with the ΔmiR-K12-9 virus is particularly intriguing in light of work which has been done aimed at understanding the significance of single-nucleotide polymorphisms (SNPs) in the KSHV miRNAs. Marshall et al. discovered that miR-K12-9 pre-miRNA sequences from a variety of cell lines and patients showed the greatest variation out of all the miRNAs (109). It has been shown that SNPs within the pre-miRNA sequence can impact miRNA processing by Drosha and Dicer, leading to changes in mature miRNA expression. In fact, as a result of SNPs, the PEL-derived BC-3 cell line does not express mature miR-K12-9 (110). Although this
made clear that variation in this miRNA is well tolerated by the virus, it did not immediately indicate what the functional consequences of this variation might be. This was further clarified when researchers discovered that certain SNPs in the miR-K12-9 coding region correlate with specific clinical diagnoses. In particular, patients with MCD have divergent miR-K12-9 sequences (111).

Taken together, this study has laid the groundwork in characterizing which KSHV miRNAs are responsible for the oncogenesis-related phenotypic changes observed in endothelial cells latently infected with KSHV.

Figure 3-1. Results of Promega CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay. Error bars show standard deviation. p values vs. WT, 2-tailed Student’s T-test *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001.
Figure 3-2. Wound healing assays. A) Representative images of uninfected (UI), wild type (WT)-infected, ΔmiR-K12-9-infected, and ΔmiR-K12-11-infected TIVE cells at 0 and 12h after scratching. B) Combined data from all wound healing assays. Data are the average of 3 independent experiments consisting of 3-6 replicates per treatment and have been normalized to WT. Error bars show standard deviation. p values vs. WT, 2-tailed Student’s T-test*: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001.
Figure 3-3. Representative images of uninfected (UI), wild type (WT)-infected, ΔmiR-K12-5-infected, ΔmiR-K12-6-infected, ΔmiR-K12-7-infected and ΔmiR-K12-9-infected TIVE cells 6h after plating on Matrigel. Magnification is 50x.
Figure 3-4. Tubule formation assays. Data are the average of 3 independent
experiments consisting of 12 replicates per treatment and have been
normalized to WT. Error bars show standard deviation. p values vs. WT, 2-
tailed Student’s T-test *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001.
Figure 3-5. Extracellular acidification rate and oxygen consumption rate of uninfected, WT-infected, or mutant infected cell lines. Error bars show standard deviation. A) Extracellular acidification rate. B) Oxygen consumption rate.
CHAPTER 4
CONCLUSION

The work described herein contributes to the larger understanding of KSHV. More specifically, it has provided greater insight into the KSHV miRNAs, their targets, and their functions. This work is unique from other large-scale studies of KSHV miRNAs in that all of the experiments were completed in endothelial cells, the cell type from which KS lesions originate. In addition, several important tools for the further study of KSHV were developed, namely the qCLASH method and TIVE-EX-LTC cells.

Chapter 2 described the establishment of the qCLASH method, which has several advantages over other, similar protocols. As in the original CLASH method, the ambiguity of assigning miRNAs to mRNAs, a problem encountered in CLIP, has been removed. Beyond this, qCLASH can be performed with many fewer cells than its predecessor. This is because the steps in the CLASH protocol which result in the greatest losses of RNA have been eliminated. These steps were initially designed to remove any irrelevant RNAs from the sample so that they would not overwhelm the signal of the relatively rare hybrids. In the short space of a few years, however, high-throughput sequencing technology has improved to the point where this is no longer a real concern. qCLASH takes advantage of this. Even without the cleanup steps after immunoprecipitation, we identified hundreds of thousands of hybrids per biological replicate of qCLASH in endothelial cells. The shortened protocol has the added benefits of being quicker than the original and not requiring the use of radionuclides.

There is another important distinction between qCLASH and CLASH. The original CLASH relies on the overexpression of tandem tagged Ago. While this allows for stringent purification of Ago-RNA complexes, it is a highly artificial system which
does not faithfully represent the conditions in ordinary cells. qCLASH uses a high-affinity antibody to pull down native Ago, thereby capturing Ago-RNA interactions in a more natural state. In this respect it is more similar to CLEAR-CLIP, another CLASH variant (87). The crosslinking of Ago to RNA coupled with the potent affinity of the antibody for Ago allow for reasonably stringent purification conditions.

Overall, the two most important features of qCLASH, namely the lower cell input and the pulldown of native Ago, set the stage for comprehensive analysis of KSHV miRNA targets in KS patient biopsies. This type of experiment, which previously would not have been feasible, could yield our most complete understanding to date of the interaction of KSHV miRNAs with their targets in a natural tumor context. Beyond the study of KS, qCLASH could be a valuable asset for elucidating miRNA targets in any situation where the number of cells available is limited.

Also discussed in Chapter 2 was the establishment of the TIVE-EX-LTC cell line. The intent in isolating and cultivating these cells was to create a convenient, fast-growing, KSHV-negative endothelial cell line. In the past, this niche had been filled by SLK cells, an endothelial cell line which was initially isolated from a KS tumor but which was not infected with KSHV. Unfortunately, it was discovered relatively recently that SLK cells had been contaminated early on by a renal carcinoma cell line (97). This meant that the cells, which had been used by laboratories around the world for countless experiments, were no longer as applicable to the study of KS and KSHV as once thought. TIVE-EX-LTC cells, which we have confirmed to be of endothelial origin and uncontaminated by other cell types, could potentially fill the void in the KSHV field left by SLK cells.
By performing qCLASH in TIVE-EX-LTC cells, we were able to discover a number of KSHV miRNA targets. We also noted several interesting phenomena which may have broader implications for the study of miRNAs in general. Perhaps most significantly, we observed that a majority of the hybrid miRNAs had no discernible seed matches in the target mRNAs. While this goes against the established understanding of the way that miRNAs interact with their targets, it agrees with the few other CLASH studies which have been published to date. Since CLASH and qCLASH offer direct evidence for binding, studies of this type merit particular weight when it comes to the nature of miRNA-mRNA interactions. This therefore seems to indicate that the importance of non-canonical seed matches may have been underestimated in the past. Nevertheless, our observations must be informed by the recent work of Agarwal et al, who undertook a large-scale study to ascertain the functionality of canonical and non-canonical seed matches. They found that canonical seed matches were significantly more likely to be functional than non-canonical ones (112). Functionality in this sense refers to the ability of a miRNA to repress translation of the target mRNA, leading to lower expression of the encoded protein. While target repression is certainly important, it may represent only part of the overall miRNA regulatory landscape. Indeed, numerous hybrids from our qCLASH dataset consisted of miRNAs bound to noncoding RNAs. Since reduced protein expression cannot result from this type of interaction, this hints at a larger role for miRNAs in the complex milieu of living cells. One salient example of a miRNA which carries out an unusual role is miR-122. miR-122 is a liver-specific miRNA which is required for hepatitis C virus (HCV) replication (113, 114). It interacts with the 5' UTR of the viral genome, stimulating translation and protecting the uncapped 5' end
of the genome from degradation (115, 116). At the same time, this has the effect of sequestering miR-122, preventing it from carrying out its normal cellular functions (117). Looking at the broader picture, our data add to the accumulation of evidence suggesting that miRNAs are far more complex than initially thought.

Chapter 3 followed up on the findings that the KSHV miRNAs were particularly involved in the processes of cell cycle control, cell migration and angiogenesis, and glycolysis. Studying the relationship of the KHSV miRNAs to these processes, which all have particular relevance to cancer, could elucidate the role the miRNAs have in the development of KS. To this end, TIVE cells, immortalized endothelial cells which faithfully maintain many of the phenotypic characteristics of primary endothelial cells, were infected with a panel of KSHV mutants. There were fourteen mutants in all, twelve which each lacked one of the miRNAs, one which lacked the ten miRNAs found within the KSHV miRNA cluster, and one from which all of the miRNAs had been deleted (103). WT-infected and uninfected TIVE cells were used alongside the mutant-infected cells in all experiments. The experiments were aimed primarily at evaluating the phenotypic differences between WT-infected cells and mutant-infected cells. Observable differences between cells infected with WT versus cells infected with any given mutant virus could logically be attributed to the miRNA or miRNAs absent from the mutant.

In the cell proliferation assay, intended to characterize the role of KSHV miRNAs in cell cycle control and related processes, several mutants were observed to have a deficiency when compared to WT. Most notable among these was the mutant lacking miR-K12-11/11*. The finding that the absence of miR-K12-11 leads to inhibited cell
proliferation agrees well with previous work showing that the overexpression of miR-K12-11 in B-cells in the mouse leads to enhanced proliferation (58). The miR-K12-11 mutant, along with the miR-K12-7 mutant, also showed decreased cell migration speed. This indicates that these two miRNAs may play some role in the enhancement of cell migration speed seen in the context of WT-infection. The cells infected with miR-K12-7, in turn, along with those infected with miR-K12-5 and miR-K12-6, displayed a pronounced defect in tubule formation. From this, one could reasonably expect that these three miRNAs have particular relevance to angiogenesis. Finally, miR-K12-10-infected cells displayed greatly increased extracellular acidification and oxygen consumption, two measurements related to how cells produce energy after glycolysis. This strongly implicates miR-K12-10a/10a*/10b in the metabolic changes observed in cells infected with WT KSHV.

In summation, the KSHV miRNAs, with a particular emphasis on their cellular targets and functions in cancer-related processes, have been studied extensively in endothelial cells, a model of particular relevance to KS. This was undertaken with a bipartite approach. First, modern ribonomics techniques coupled with next-generation sequencing were used to identify numerous cellular targets of the KSHV miRNAs. Second, a classical genetics approach was employed in which mutant viruses were used to evaluate the roles that KSHV miRNAs have in driving infected cells through phenotypic changes related to oncogenesis. Bringing the two arms of our research together, we are now poised to connect the pathways which are perturbed by KSHV miRNAs in endothelial cells with the endothelial cell-specific KSHV miRNA targets identified by qCLASH. The tools developed as part of this work will make it possible to
examine the interactions between KSHV miRNAs and host mRNAs in KS biopsies. In summary, we believe that our work has contributed and will continue to contribute to the understanding and identification of the KSHV miRNAs which are directly involved in pathogenesis and tumorigenesis.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Lauren Appleby Gay received her Bachelor of Science in biology from the University of Georgia in 2007. After working as a laboratory technician for one year, she returned to school in 2008 and obtained her Master of Science in veterinary and biomedical sciences from the University of Georgia in 2010. For her master's work, she developed a reverse-genetics system for an H6N1 avian influenza virus and used the system to characterize a mutation in one of the viral polymerase genes. Lauren began her doctoral work in 2010, opting to enroll in the Interdisciplinary Program in Biomedical Sciences offered through the College of Medicine at the University of Florida. She was awarded both the Alumni Fellowship and the Grinter Fellowship from the University. Lauren soon joined the laboratory of Dr. Rolf Renne, where she began research on the targets and functions of KSHV miRNAs in endothelial cells. In 2013, she received the Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship from the National Institutes of Health. Lauren obtained her Doctor of Philosophy in biomedical sciences from the University of Florida in 2017.