GENERATION OF A ΔMIR-K12-7 KSHV MICRORNA MUTANT AND A VIRUS PRODUCER CELL LINE

By

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To my parents, family, and friends

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AIDS	acquired immune deficiency syndrom
ATP	adenosine tri-phosphate
BAC	bacterial artificial chromosome
bp	base pair
cDNA	cloned DNA
DMEM	dulbecco’s modified eagle medium
DNA	deoxyribonucleic acid
HAART	highly active antiretroviral theray
HHV-8	human herpesvirus 8
IL	interleukin
iSLK	inducible SLK
Kb	kilo base
KLAR	KSHV latency-associated region
KS	kaposi’s sarcoma
KSHV	kaposi’s sarcoma-associated herpesvirus
LANA	latency-associated nuclear antigen
MCD	multicentric castleman’s disease
MiRNA	microRNA
ORFs	open reading frames
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
qPCR	quantitative PCR
RISC	RNA induced silencing complex
rKSHV	recombinant KSHV

RNA	ribonucleic acid
RTA	replication and transcriptional activator
TPA	tetradecanoyl phorbol acetate
TRs	terminal repeats
UTR	untranslated region
VA	valproic acid
WT	wild type

Abstract of Thesis Presented to the Graduate School
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The current approach to identify gene targets of Kaposi's sarcoma-associated herpesvirus (KSHV) miRNAs utilizes bioinformatics algorithms to predict sequence complimentary to 3' UTRs followed by experimental verification of targets. Thus far, this approach has led to the identification of a very few miRNA targets. Additionally, only a few of the miRNA targets have been experimentally proven to date. This strategy imposes limitations as miRNA dependent phenotypes may not readily be seen in the context of infection. Hence, generating miRNA deletion-mutants of KSHV are imperative to study their function in the context of infection. The latency-associated region is a genomic region which is abundantly expressed during latency. As all KSHV miRNAs reside in the latency-associated region, simply removing a miRNA might lead to loss of hairpin structure in the adjacent miRNAs. Loss of hairpin structure would affect miRNA expression. Here, a KSHV miR K12-7 deletion mutant was generated in a bacterial artificial chromosome (BAC) backbone containing the JSC-1 KSHV virus using two-step red recombination. A recombinant virus was also reconstructed in mammalian 293T cells. Further, this recombinant virus will be used to infect iSLK cells to generate a

producer cell line that can be stored, re-grown and induced to produce a high titer of recombinant virus.

CHAPTER 1
INTRODUCTION

MicroRNA

In 1993, Victor Ambros' group discovered that a gene, lin-4, produces small RNAs with antisense complementarity to another gene, lin-14 (15). Further study demonstrated that lin-4 small RNA targets the 3' untranslated region (UTR) of lin-14 mRNA to post-transcriptionally silence it. This ground-breaking study unearthed an entirely novel mechanism for gene expression regulations by a class of small RNAs, mainly micro-RNAs (miRNAs). Small interfering RNAs (siRNAs) and miRNAs, essentially serve similar functions but differ in their stringency of complimentarity to the target mRNA, the former having 100% complimentary while the later, stringency of complimentarity is somewhat relaxed. Since the discovery of the lin-4, more than 900 miRNAs have been identified in humans to date (MIRNA SANGER).

MicroRNA Biogenesis

miRNA genes are mostly encoded from intronic regions of protein coding genes but a few miRNAs have also been found to transcribe from exons as well. Genes for human miRNAs are generally transcribed by RNA polymerase II into primary-miRNA (Pri-miRNA) stem-loop structure. The pri-miRNA structure is subsequently recognized by Drosha/DGCR8 which is an RNase III type endonuclease, which cleaves off the 5' and 3' ends leaving a two-nucleotide 3' overhang. This cleaved structure, also called the pre-miRNA hairpin, is quickly exported from nucleus to the cytoplasm by the exportin 5/Ran GTPase pathway. The pre-miRNA is recognized and processed by cytoplasmic Dicer, an RNase III type endonuclease. Dicer cleaves off the bulgy head of the pre-miRNA hairpin to generate a short-dsRNA with 3' overhang on both ends. Finally, one

strand of ds-RNA is destined to be incorporated into the RNA-induced silencing complex (RISC). Once the mature miRNA is incorporated into RISC, the 3' UTR of the transcript containing the complementary sequence is targeted (3).

MicroRNA Mechanism

miRNAs can direct RISC to target a transcript by two mechanisms; transcript degradation or translational repression. The fate of the transcript being targeted relies on the level of complementarity with the miRNA. Low complementarily exerts translational silencing while high complementarily leads to degradation of the message. Thus, the major function of miRNAs seems to be the regulation of gene expression through either of the described mechanisms. In both cases, positions 2-8 from the 5' end of the miRNA are the most important basess that exert post-translational inhibition of the message. This sequence of the miRNA is referred as the seed miRNA sequence (4).

MicroRNA Regulates Fundamental Processes in All metazoan

When the first miRNA was discovered in C. elegans, it was thought to be specific to worm biology. However, in 2000, when Pasquinelli et al. demonstrated that C. elegans miRNA, let-7, was 100% conserved in mice and humans, it triggered research to identify novel miRNAs across species. More than 15,000 different miRNAs have been identified in all metazoan and plant species investigated so far (miRBase V.17) and these have been implicated in central biological processes including, but not limited to, development, organogenesis, cell cycle control, and apoptosis(1, 3).

Kaposi's Sarcoma-Assocoated Herpesvirus and Its Associated Diseases

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is the causative agent of Kaposi's sarcoma (KS), an AIDS-

defining malignancy. The genomic DNA fragments of KSHV were identified in KS-lesions, but not in unaffected skin, by Chang et al. in 1994 using a PCR-based representational difference analysis (10). This pioneering work subsequently lead to the cloning and sequencing of the KSHV (20). KSHV is found in AIDS-patients as a result of secondary infection or affecting immune-suppressed individuals. In addition to KS, which is of endothelial origin, KSHV is also associated with two lymphoproliferative diseases: primary effusion lymphoma (PEL) and multicentric Castlemen's disease (MCD). Although the use of highly active antiretroviral therapy (HAART) has reduced the number of AIDS-KS and PEL cases significantly, KSHV is still rampant in the developing world and causes severe mortality(32).

KSHV Life Cycle

KSHV contains a double stranded DNA genome encoding 90 Open reading frames (ORFs) (19). KSHV ORFs are classified as lytic genes or latent genes. Lytic genes are required for productive viral infection and include those encoding viral proteins required for DNA replication, viral gene expression, and viral structural proteins. KSHV has the ability to establish and maintain latent infection, characteristic of most gamma-herpesviruses. It has been demonstrated that the majority of cells in KS tumors and PELs are latently infected with KSHV. Upon infection, the KSHV genome circularizes and persists as an episome in the nucleus of infected cells. Only a few genes are expressed during latency from the same genomic region, called latency-associated region (LAR), and this region expresses latency-associated nuclear antigen (LANA), vcyclin, vFLIP and 12 miRNAs. Restricted gene expression during latency along with nonimmunogenic miRNAs targeting the components of cell-mediated immunity is very elegant mechanism to evade immune detection. This state of infection

exhibits a remarkable balance between host immune surveillance and KSHV's ability to evade the host immune system (6, 19).

KSHV MicroRNAs

Early research had shown that miRNAs can serve as major modulators of gene expression. Virologists soon demonstrated that DNA-viruses also encode miRNAs (9, 22, 25). Researchers have found that DNA-viruses are able to exploit miRNA-dependent gene regulations to further their life-cycle. This finding makes sense from an evolutionary perspective as a virus exerts itself to co-evolve with a host that it depends on.

cDNA cloning from PEL cell lines latently-infected with KSHV revealed a 12 hairpin cluster in the region of the genome that corresponds to latency. This KSHV latency-associated region (KLAR) encodes 12 pre-miRNAs, giving rise to a total of 17 mature miRNAs (25). Ten of the 12 hairpins are expressed from a single transcript, while the rest were found in the Kaposin/K12 open reading frame (6). Although miRNAs are shown to target the 3' UTR of mRNAs, there are a few exceptions of targeted sites residing within coding regions or the 5' UTR (12, 18). miRNA target specificity, as described earlier, depends on the mRNA seed sequence. Therefore a single miRNA can target many mRNAs and it is believed that about half of all mammalian transcripts are regulated by miRNAs (4, 13).

KSHV MicroRNAs and Latency

Analysis of KSHV miRNAs expression in PEL cell lines has revealed that all of the miRNAs are expressed abundantly during latency. As latency is the default life cycle of KSHV upon infection, it is logical to hypothesize that KSHV modulates host gene expression to establish and maintain latency using viral miRNAs. Cell-mediated

immunity is an important component of the host response against intracellular pathogens. KSHV miRNAs inhibit effector cell recognition by T-cell and NK cell, or by miRNA induced expression of IL-6 and IL-10 cytokines (7). KSHV miRNAs also target pro-apoptotic factor BCLAF1, thus extend the life of infected cells. The use of viral miRNAs instead of viral proteins to establish and/or maintain latency provides the virus with an additional advantage as only few, if any, antigens would be presented to the host immune system. Understanding functions of the KSHV miRNAs will require mutant KSHV lacking one or more miRNAs. These viral mutants have to be examined by transcriptome and proteomic profiling of infected cells to reveal corresponding targets. Currently virologists can only study phenotypic effects in cell culture given the limitations imposed by the absence of model systems to study KSHV pathogenesis. Current estimate from genome-wide microarrays and proteomics profiling suggests that any given miRNA can regulate more than 100 targets (2, 16, 27).

KSHV MicroRNAs Regulate Cellular and Viral Targets

In 2005, several laboratories identified 12 pre-miRNAs within KSHV, which are expressed during latency in tumors of endothelial and lymphoid origin (9, 21, 25). This finding presented a reason to hypothesize that KSHV miRNAs would regulate both cellular and viral target genes. Samols et al. further expressed the KSHV miRNA cluster in 293 cells to profile a list of potential target genes that were down-regulated in cells expressing the KSHV miRNA cluster (26). Recent reports have confirmed that viral RTA, a switch required to initiate lytic replication, is targeted by miR-K12-9* (5)) and miR-K12-7-5p (17). During latency, KSHV must also protect the infected cells from destruction and arbitrary proliferation for maintaining latency and spreading infection further. Samols et. al described that KSHV miRNAs can repress thrombospondin, an

inhibitor of angiogenesis (26). Taken together, KSHV miRNAs can potentially regulate key cellular processes such as apoptosis, angiogenesis, innate and adaptive immunity, and cell cycle control (Table: 2-2; for review see (6, 29)). This suggests that they play important roles in viral biology and potentially pathogenesis. Thus, the central objective to studying KSHV miRNAs is to identify their targets, both, cellular and viral. Target identification will help to better explain viral biology and pathogenesis.

Current Approaches for MicroRNA Target Identification

Identification of miRNA targets can be complicated by the fact that a single miRNA can bind to multiple targets with a different degree of complementarity. The major factor that contributes to the target specificity of a miRNA is the seed sequence, usually located between positions 2 to 8 from the 5′ end of an miRNA. The location within the 3' UTR, number of target sites within the mRNA, and additional base-pairing and AU-content of the RNA flanking a seed sequence all contribute to the target efficiency (6). Current approaches to determine miRNA targets utilize bioinformatics based tools using the above mentioned criteria to predict targets. The predicted targets are then experimentally verified using either miRNA over-expression in non-physiological cells or antagomir-based miRNA inhibition to knock down the expression of the gene.

Bioinformatics tools such as PicTar, TargetScan, DIANA-microT, and miRanda are currently used to predict miRNA targets. Because these tools use different criteria for target selection, targets predicted for any given miRNA are never consistent. Bioinformatics tools may predict targets that are biologically irrelevant as numerous factors including expression levels of miRNA and that of targets dictate miRNA targeting. Defining a miRNA target is also contextual because only those genes that can be expressed in a given cell type can be considered as legitimate targets.

Predicted targets also have to be validated experimentally. Genome wide transcriptome analyses are performed after ectopic expression of a given miRNA or sequence-specific inhibition of the same by antagomirs. Targets can also be confirmed by cloning their respective 3' UTR downstream of a reporter gene and assessing the ability of the miRNA to bring down the reporter expression level. Additionally, if antibodies against the predicted targets are available, it can provide strong support for target validation by western blot analysis.

Notwithstanding the approaches taken so far to validate miRNA targets, function of the given viral miRNAs can only be understood in the context of infection. Therefore, an experimental system that will allow the systemic removal of individual miRNAs will prove to be a great tool to study the functions of viral miRNA. We adopted the two step Red-mediated recombination to specifically delete miR-K12-7 and create a markerless recombinant KSHV BAC (Figure 1-1).

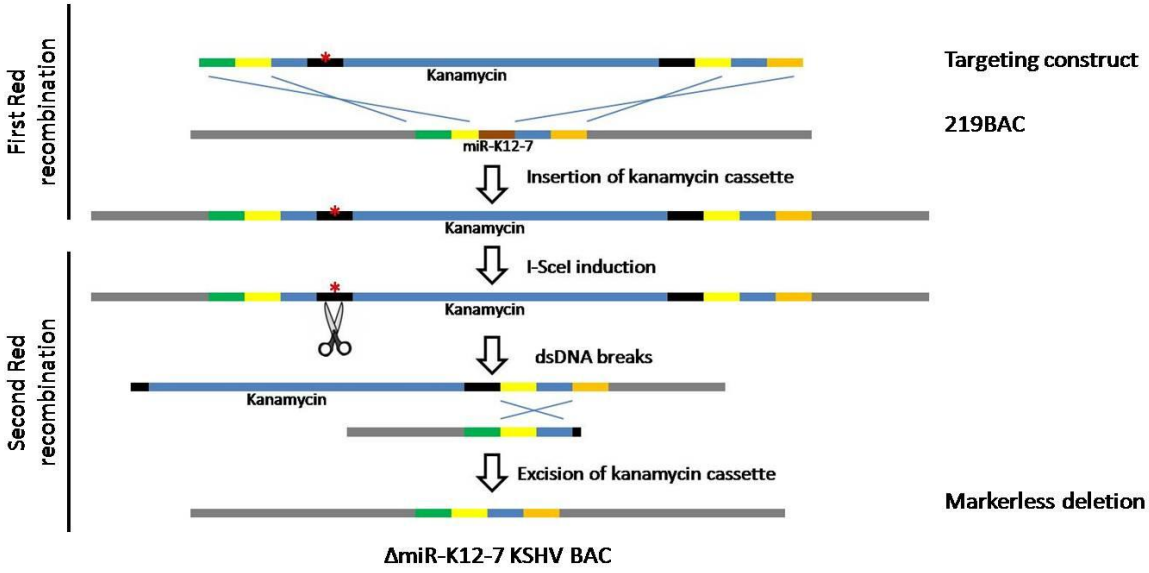

Figure 1-1. Two step ds-Red mediated recombination. During the first recombination, the targeting fragment replaces miR-K12-7 with the kanamycin marker. Second recombination induces I-SceI mediated ds-DNA breaks followed by excision of kanamycin marker. This experimental approach produces markerless recombinant KSHV BAC with the desired deletion. This rKSHV BAC will be transfected in 293T cells to produce infectious virus.

CHAPTER 2
GENERATION OF ΔMIR-K12-7 KSHV BAC MIRNA

Background

MicroRNAs (miRNAs) are approximately 22 nucleotide-long non-coding RNA molecules found in all metazoan eukaryotes. miRNAs bind to the 3' untranslated regions of the target mRNAs leading to translational silencing of the targets. To date, only a few genes have been experimentally proven to be miRNA targets. More than 200 miRNAs have been discovered in double stranded DNA viruses, mainly herpesviruses and polyomaviruses. Kaposi's sarcoma (KS)-associated herpes virus (KSHV), also known as human herpes virus 8 (HHV8), is a gammaherpesvirus that encodes 12 pre-miRNAs, all located in the latency associated region. Upon infection, KSHV predominantly maintains latency wherein a few vial genes are transcribed while the rest are transcriptionally silent. Contrarily, a high level of expression of viral miRNAs is found during latency (8). In the past few years, several viral and cellular targets of viral miRNAs were discovered implicating their role in many fundamental cellular processes like promoting angiogenesis, inhibition of apoptosis, and regulation of transcription factors (6, 35). Table 2-2 lists some of the experimentally verified KSHV miRNA targets. Notably among those are cellular targets including THBS1 (angiogenesis inhibitor), BACH1 (transcriptional suppressor), BCLAF1 (pro-apoptotic factor), Rbl2 (repressor of DNA methyl transferase), C/EBPβ (transcription factor), and viral target RTA (major switch for lytic replication). Recently, roles for many miRNAs in maintaining latency and regulating the switch to lytic replication have also been established. Thus, identification of more targets of viral miRNAs will provide more information on viral pathogenesis and possible therapies.

Mutation Background

Until very recently, mutants of KSHV have been made in the BAC36 background. A recent publication, however, revealed that BAC36 has a large duplication of the KSHV genome in its long terminal repeats (LTR) (34). This finding prompted researchers to generate another BAC mid system without duplications or deletions in the KSHV genome because KSHV mutants generated in the BAC36 background would not be ideal.

Recently, Dr. Jae Jung's laboratory created and provided the 219BAC which was derived from JSC-1, a PEL cell line with high virus titer. The 219BAC was created by inserting the pBelo45 backbone in between vIRF-1 and ORF57. This backbone has a GFP marker and provides hygromycinB resistance to mammalian cells. It also contains chloramphenicol resistance and an origin of replication for bacmid selection in bacteria. Two-step DS-double recombination was used to delete miR-K12-7 in the 219BAC background.

Mutation Strategy

Mature miRNAs are derived from Pre-microRNA hairpins as described earlier. A pre-microRNA hairpin is the substrate for Dicer, an RNaseIII, which recognizes the overall shape of the RNA duplex to produce mature miRNAs. To create a miR-K12-7 miRNA mutant, the 20 base pair region encoding one arm of the miRNA hairpin was deleted from the viral genome (Figure. 2-1). This deletion destroys the formation of the Pre-microRNA hairpin; resulting in the loss of substrate specificity for Dicer, and leading to the deletion of the microRNA from the virus. Ideally, this strategy should also not affect the expression of the other microRNAs found within the cluster.

Materials and Methods

Two-step Red-mediated Recombination

The protocol of two-step Red-mediated recombination was developed by Tischer et al (31). We modified the protocol to create a miR-K12-7 deletion mutation in the 219BAC background (Figure 1-1). E.coli GS1783 was used to facilitate two rounds of homologous recombination as it contains the temperature-inducible Red recombination system and arabinose inducible I-SceI.

In the first round of recombination, the targeting fragment containing the kanamycin selectable marker was generated and inserted in to the KSHV genome in the place of miR-K12-7. Deletion primers to amplify the kanamycin selectable marker were designed to carry flanking sequences of miR-K12-7. The resultant targeting fragment would replace miR-K12-7 via inter-molecular homologous recombination when Red expression is induced at 42 °C. Clones were screened for the kanamycin cassette insertion and the quality of BAC terminal repeats (TRs) were determined.

For the second round of recombination, clones containing both the kanamycin cassette and intact TRs, were selected. Clones were grown in LB-chloramphenicol and arabinose was added to induce the expression of I-SceI restriction enzyme which would linearize the viral bacmid at the insertion site of the kanamycin cassette. This linearized bacmid could then use itself as the substrate for an intra-molecular homologous recombination resulting in the excision of the marker. Clones were further selected for chloramphenicol-resistance but kanamycin-sensitivy as the marker cassette should be removed. Clones were further verified for deletion of the 20 bases of the miRNA arm and quality of BAC TRs was checked again.

Deletion Primers for miR-K12-7 Deletion

Deletion primers are listed in table 2-1. Deletion primers were designed with sequences flanking the miR-K12-7 region which was targeted for deletion. Figure 2-2 describes the strategy to design deletion primers. The forward primer miR-K12-7 delF, contained sequences 40 bp of upstream and 20 bp downstream of the miR-K12-7 deletion target. 20 bp corresponding to the kanamycin cassette was added to make 80 bp primer.

The reverse primer was similar except it contained 20 bp of genomic sequence upstream of the miR-K12-7 deletion followed by 40 bp of sequence corresponding to the region downstream of the miR-K12-7 deletion.

Targeting fragment generated using deletion primers would contain duplication of 20 bp upstream and downstream sequences of miR-K12-7 at both ends of the fragment. Upon linearization of KSHV BAC with arabinose-inducible I-SceI, substrates for second Red-mediated intra-molecular homologous recombination would be generated for the excision of kanamycin marker.

Primers for Colony PCR and Verification PCR

K11test and KanR primers were used for colony PCRs (Table 2-1). K11test primer corresponds to a region upstream of miR-K12-7 in the KSHV genome while KanR targets the end of the kanamycin marker. Successful insertion of the kanamycin cassette would result in 1.3 Kb amplification products.

The first step to verify the miR-K12-7 deletion from the chloramphenicol-resistant and kanamycin-sensitive second recombinants was done using colony PCR primers. The lack of amplification reflects the excision of the kanamycin cassette. The second step to verify miR-K12-7 was done using verification primers, K7 verifyF and K7 verifyR.

These primers target flanking sequences of miR-K12-7. Successful deletion of miR-K12-7 results in the amplification of a 60 bp product while amplification of the wildtype virus results in the production of an 80 bp product.

Generation of Targeting Fragment for first Red Recombination

PCR was carried out using the Phusion High-Fidelity PCR kit (Finnzymes) according to manufacturer's instructions except for the following changes in conditions: No DMSO was used, only 1 ng of pEPKan-S template was used per 50 µL of PCR reaction, and PCR was run for only 20 cycles. The primers used in this reaction were obtained from IDT and designed according to the procedure explained in the previous section. After the run, three 50 µL PCR reactions were pooled together and 3 µL DpnI was added to digest the template for 2 hours at 37 ºC. The PCR reaction was run on a 1% agarose TBE gel and gel purified using a Qiagen Gel purification kit.

Generation of Electrocompetent GS1783 *E.coli*

A 2 mL overnight culture of *E. coli* strain GS1783 containing the KSHV 219BAC was inoculated in to 40 mL of LB-chloramphenicol (15 µg/mL) in a sterile 250 mL flask. Bacteria were grown at 220 rpm at 30 ºC until 0.5 O.D. Induction of Red expression was carried out by shaking the flask vigorously in a 42 ºC water-bath for 10 minutes. Immediately after, the culture flask was placed on ice slurry for 10 minutes. The culture was transferred with ice cold pipettes into 15 mL ice-cold culture tubes and centrifuged at 3000x g at 0 ºC for 8 minutes. Cells were spun and washed three times with sterile ice cold ddH_2O. At the end of the last wash, cells were resuspended in the water remaining in the tubes. These electro-competent cells were then used for electroporation of the targeting fragment to achieve the first red recombination.

First Red Recombination

100 ng of gel purified targeting fragment was mixed and finger tapped with 40 µL of GS1783 electro-competent *E.Coli* in a pre-chilled 1mm cuvette. Electroporation was performed using a Bio-Rad gene pulser XCell set at 1.5kV, 25 µF and 200Ω. The time constant of the pulse was observed in the range of 3.5 to 4.5 to achieve successful electroporation. 250 µL of SOC media was added to the cuvette after the pulse and cells were recovered from pulse shock in an eppendorf tube at 30 ºC at 220 rpm for 1 hour. After recovery, 200 µL of the recovered cells were plated on LB-kanamycin plates and incubated at 30 ºC for 24 hrs.

Screening of First Red recombinants

Positive integrates after the first Red recombination were identified using colony PCR and lengths of the terminal repeats were confirmed using pulse field gel electrophoresis (PFGE). Colonies from the first Red recombination were grown overnight in 5 mL LB-kanamycin to verify the insertion of kanamycin marker. Overnight mini-cultures were also streaked on kanamycin plates to prepare the master plate. BAC DNAs were isolated using Qiagen mini-prep kit as per the manufacturer's instructions. Colony PCRs to determine insertion of kanamycin cassette were performed by Taq polymerase according to the manufacturer's instructions using K11test and KanR primers. PCR products were run on a 1% agarose TBE gel to visualize the length of amplification product to confirm the insertion of targeting fragment.

Second Red Recombination

A clone from the first recombination plate that contained the kanamycin insertion cassette and intact TRs was grown overnight in kanamycin containing LB media. 2 mL of the culture was inoculated in 40 mL of LB-chloramphenicol and grown at 30 ºC at 220

rpm. When the culture reached O.D. 0.5, arabinose was added to a final concentration of 2% and cells were further grown for 45 minutes to induce I-SceI expression. I-SceI was used to linearize the virus at the site of the kanamycin cassette insertion. Cells were then shaken vigorously on a 42 ºC water-bath for 10 minutes to induce Red recombinase expression. Cells were further grown for 2 hours to facilitate intra-molecular homologous recombination leading to the excision of the kanamycin cassette. A 10-fold serial dilution series of the culture was plated on LB plates with 15 µg/mL chloramphenicol and 1% arabinose. Plates were incubated at 30 ºC for 24-48 hours.

Qualities of terminal repeats

Recombinant BAC DNAs were NheI digested for 2 hours at 37 ºC and subsequently run on PFGE. 1% Megabase agarose gel ladder was prepared in 0.5X TBE buffer and run in 0.5X TBE at 47 ºF (8 ºC) for 16 hours. Quality of the TRs was determined by comparing digestion patterns of BAC clones with that of WT 219BAC. Clones that possessed kanamycin cassette insertion, intact terminal repeats, and a similar banding pattern to that of the wildtype BAC were chosen for second Red recombination.

Verification of mir-K12-7 deletion

Clones from the second Red recombination were replica plated on LB agar plates supplemented with 1% arabinose along with chloramphenicol or kanamycin. BAC DNAs of chloramphenicol resistant but kanamycin sensitive clones were isolated and PCRs were performed using verification primers. PCR reactions were run on a 6% TBE acrylamide gel and the 20 bp deletion of miR-K12-7 was verified. BAC DNAs were also subjected to NheI digestion and run on PFGE to confirm that the terminal repeats were intact.

Stocking E. coli GS1783 Harboring ∆miR-K12-7 KSHV

Verified clones with intact terminal repeats were grown overnight in LB-chloramphenicol. An equal volume of 80% glycerol was added to make glycerol stocks. These glycerol stocks were stored at -80 ºC.

Results

The kanamycin targeting fragment was successfully generated and size verified on a TBE agarose gel (Figure 2-3). The gel purified targeting fragment was delivered into freshly made electrocompetent WT KSHV BAC harboring E.coli GS1783 cells at 4.3 mS. More than 100 clones were observed on the LB-kanamycin plate 48 hours post electroporation. Kanamycin resistance is conferred due to the inter-molecular homologous recombination between the targeting fragment and WT KSHV BAC.

While all of the clones under screening have the kanamycin cassette insertion in their respective BACs as a result of inter-molecular recombination (Figure 2-4), only clones 1-5 have intact terminal repeats when compared to WT219BAC (Figure 2-5). Terminal repeats of clones 6-10 are either degraded or lost. Clone #4 was selected for a second round of Red recombination, however this time; SceI was induced to linearize the virus and promote the removal of the Kanamycin cassette by homologous recombination. Half of the clones screened after the second Red recombination by replica plating show kanamycin sensitivity (Figure 2-6). Clones sensitive to kanamycin confirm the excision of the targeting fragment. When verified for the 20 bp deletion of miR-K12-7 from the KSHV BAC, all clones except clone #1 show the deletion (Figure 2-7). Further, all of the clones except #4 have intact terminal repeats (Figure 2-8). Because the two-step recombination strategy excises the marker after the deletion

mutation, a markerless and quality-verified miR-K12-7 KSHV BAC is created. Two clones were chosen and glycerol stocks were frozen at -80 ºC.

All first Red-recombinants had kanamycin cassette insertion because clones were selected for kanamycin resistance. Half of the second Red-recombinant clones screened by replica plating were kanamycin sensitive and chloramphenicol resistant. Thus, it is evident that the two-step Red-mediated recombination strategy is highly efficient for molecular manipulation of the KSHV virus cloned in to 219BAC. Not only has it provided a highly efficient tool for deletion of miRNAs without leaving any “foreign” DNA sequences behind but it also enables us to manipulate the viral genome in a quality-controlled manner at each step.

Table 2-1. List of primers

Primers	Sequences
miR-K12-7 delF	5’ TGCGTGCTTTCGACGTCCAG GCGGCTGGCACACGGGCCGT TGGTAGTAAGATACAGCATA AGGATGACGACGATAAGTAGGG 3’
miR-K12-7 delR	5’ AGCGCCACCGGACGGGGATT TATGCTGTATCTTACTACCA ACGGCCCGTGTGCCAGCCGC AACCAATTAACCAATTCTGATTAG 3’
K11test	5’ CATGCTTTGTCACCCCAGCGC 3’
KanR	5’ AA CCAATTAACCAATTCTGATTAG 3’
miR-K12-7verifyF	5’ TGCGTGCTTTCGACGTCCAG 3’
miR-K12-7verifyR	5’ TATGCTGTATCTTACTACCA 3’

This table lists the primers used in this study. miR-K12-7 delF and miR-K12-7 delR were used to amplify targeting fragment. Insertion of targeting fragment in WT KSHV BAC219 was confirmed by K11test and KanR primers. miR-K12-7 verifyF and miR-K12-7 verifyR confirmed the deletion of 20 bp region from BAC219.

Table 2-2. List of published targets of KSHV miRNAs

KSHV miRNAs	Target	Function
miRNA Cluster	THBS1	Angiogenesis Inhibitor
K12-11	BACH1	Transcriptional Suppressor
K12-5	BCLAF1	Pro-apoptotic Factor
K12-3 & K12-7	C/EBPβ	Transcription Factor
K12-4-5p	Rbl2	Repressor of DNA Methyl Transferase
Cluster (K12-6 & K12-11)	MAF	Transcription Factor
K12-1	IκBα	NFκB Inhibitor
K12-9*	RTA (Viral Gene)	Major Transctivator of Lytic Replication
K12-5	RTA (Viral Gene)	Major Transctivator of Lytic Replication
miRNA Cluster	THBS1	Angiogenesis Inhibitor
K12-11	BACH1	Transcriptional Suppressor
K12-5	BCLAF1	Pro-apoptotic Factor
K12-3 & K12-7	C/EBPβ	Transcription Factor
K12-4-5p	Rbl2	Repressor of DNA Methyl Transferase
Cluster (K12-6 & K12-11)	MAF	Transcription Factor

This table lists the published target of KSHV miRNAs. Notably, KSHV viral miRNAs also target viral genes apart from cellular genes (Adapted from Boss, Plaisance and Renne, 2009, Trends in Microbiology).

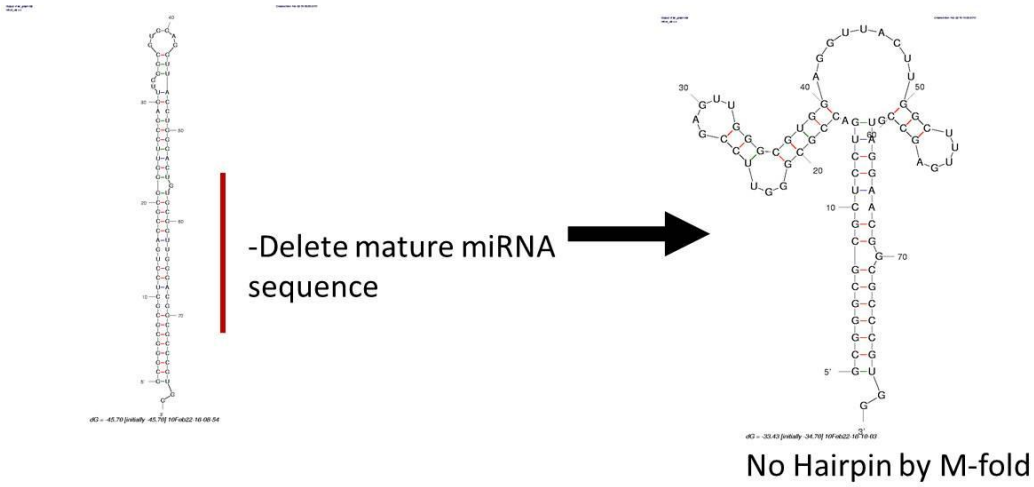

Figure 2-1. Mutagenesis strategy. Hairpin structure is highly important for recognition by Drosha and its ability to further process the hairpin into pre-miRNA. Deletion of 20 bases from one arm of the mature miRNA hairpin structure would disrupt hairpin structure formation.

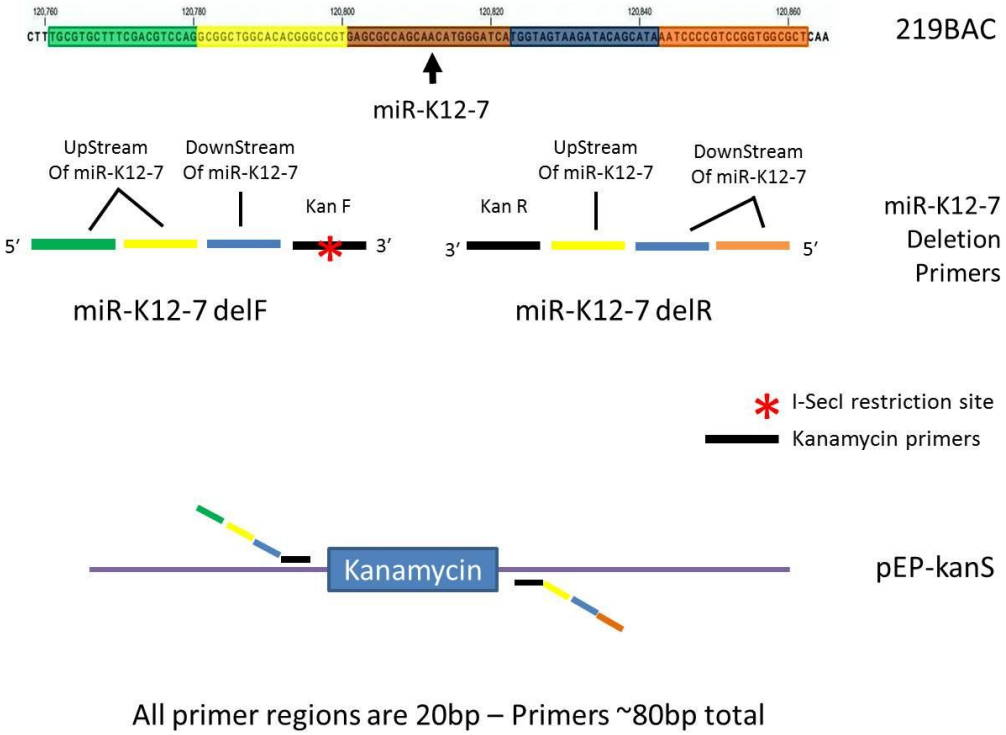

Figure 2-2. Generation of targeting fragment. A markerless mutation strategy was employed by using E. coli GS1783 harboring the KSHV 219BAC bacmid and the Red recombination system. Color coding of sequences is as follows: yellow – 20 bp upstream of deletion, green – 40 bp upstream of deletion, blue – 20 bp downstream of deletion, orange – 40 bp downstream of deletion, black with red star – kanamycin selection marker sequence with I-SceI restriction site, black – kanamycin selection marker reverse sequence.

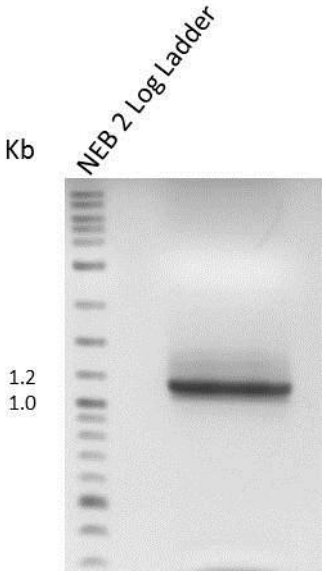

Figure 2-3. Generation of targeting fragment. The kanamycin targeting fragment is visualized at 1.1 Kb as expected.

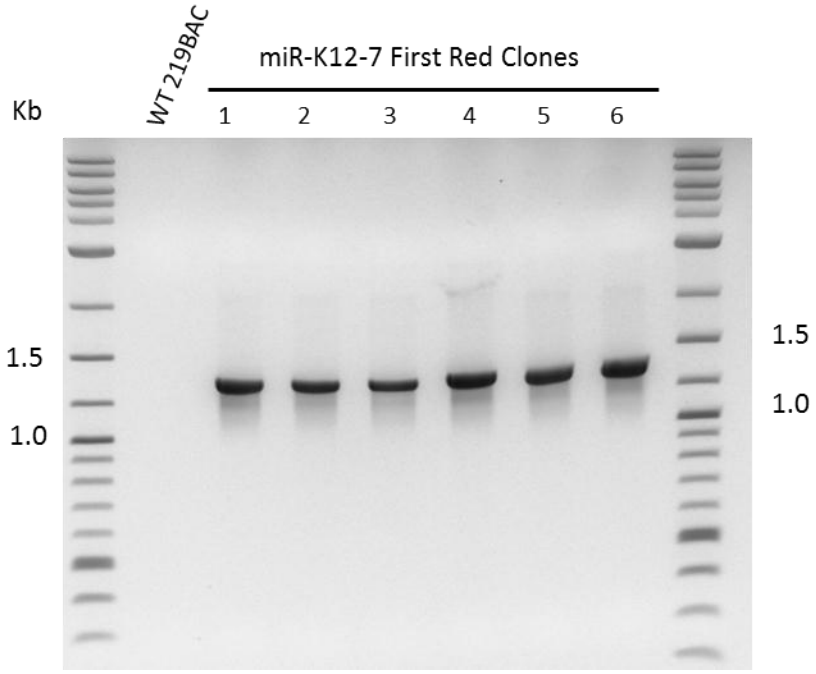

Figure 2-4. Insertion of kanamycin cassette: After first Red recombination, clones were screened for the kanamycin cassette insertion by colony PCR. WT 219BAC is the negative control. All of the clones screened were successful first recombinants.

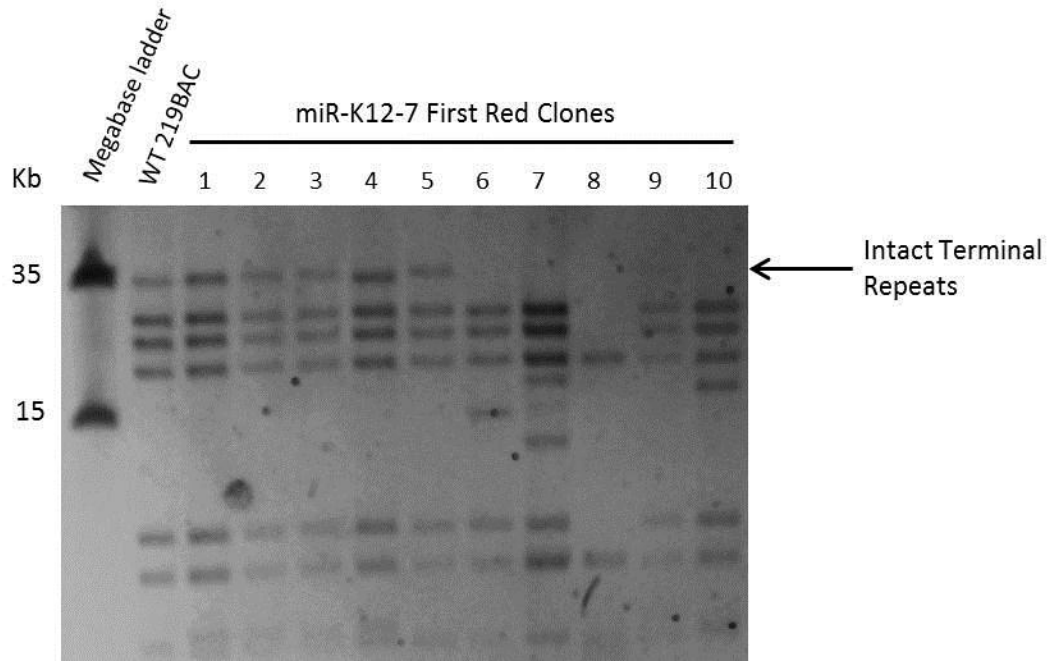

Figure 2-5. Pulse field gel electrophoresis of First Recombinants. BAC clones from the first-recombination plates were NheI digested along with WT 219BAC as control and run on PFGE. Intact terminal repeats of the clones are determined by comparing their digestion pattern to that of WT 219BAC control. Clones 1-5 show intact terminal repeats as WT 219BAC control.

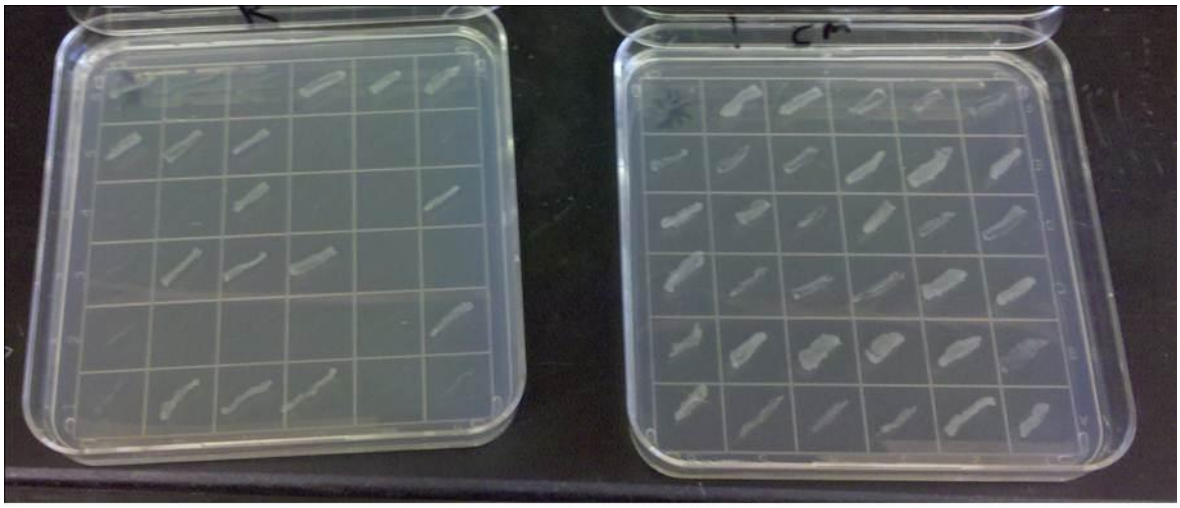

Figure 2-6. miR-K12-7 replica plates. Clones from the second red-recombination were streaked on Kanamycin or chloramphenicol replica plates.

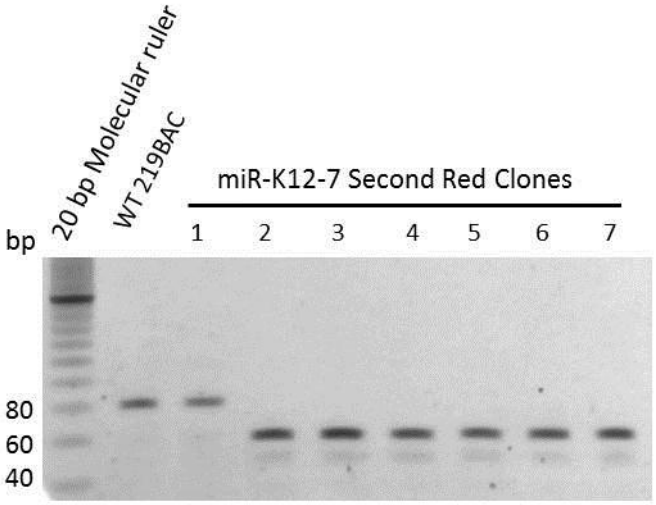

Figure 2-7. Deletion of miR-K12-7. Second Red clones were subjected to verification PCR and run on a 0.6 % TBE acrylamide gel. Deletion of miR-K12-7 in BAC clones was confirmed by the visualization of the 20 bp shorter amplification product as compared to that of WT 219BAC control.

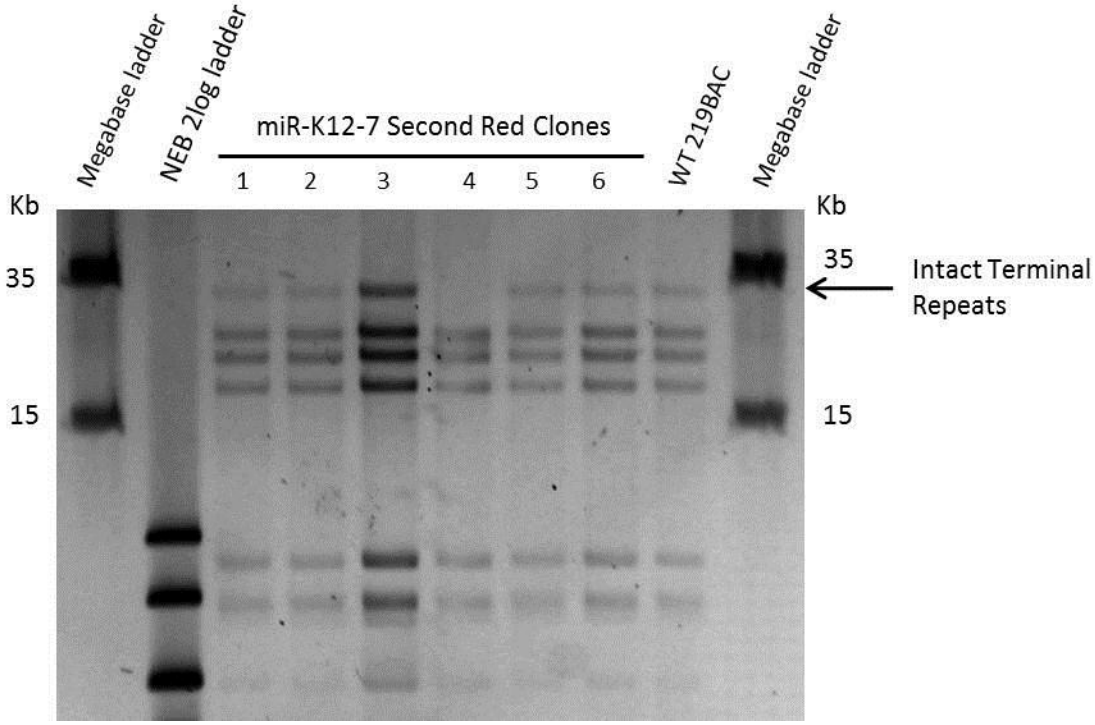

Figure 2-8. Pulse field gel electrophoresis of Second Recombinants. Cam-resistant but Kan-sensitive BAC clones were NheI digested along with WT 219BAC as control and run on PFGE. Intact terminal repeats of the clones were determined by comparing their digestion pattern to that of WT 219BAC control.

CHAPTER 3
RECONSTRUCTION OF ΔMIR-K12-7 KSHV FROM ΔMIR-K12-7 KSHV BAC

Background

Following the creation of the miR-K12-7 mutant bacmid, live and infective virus was generated to further study how this mutation affects viral pathogenesis. The mammalian cell line 293T is an ideal system for transfecting large viral BACmids Previous work in the Renne lab has demonstrated that viral particles can be produced using these cells. 12-O-tetradecanoylphorbol-13-acetate (TPA)- a phorbol ester which activates protein kinase C (PKC), and Valproic acid- a HDAC inhibitor which makes chromatin transcriptionally accessible, were used to reactivate the virus from latency. TPA activates an extensive signal transduction pathway and VA makes an entire chromatin transcriptionally accessible. Even though these chemical inducers of viral lytic replication have broad effects, the aim here was to produce virus particles to help generate a stable virus producer cell line rather than to study any miRNA-deletion based phenotypic changes at this time. 293T cells will provide a short-term latency state for transfected BAC mutants and provide us with enough virus particles to create a stable cell line. Recombinant virus produced after induction of lytic replication by TPA and VA will be used to infect iSLK cells to create a virus producing cell line.

Materials and Methods

Cell Culture

293T cells were freshly thawed from a liquid nitrogen frozen stock. Cells were suspended in serum-free Dulbecco's Modified Eagle's Medium (DMEM) and centrifuged for 5 minutes at 1100 rpm at 4 °C. Cells were resuspended and maintained in complete-DMEM (DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin).

Cells were grown at 37 °C in a CO_2 incubator. Cells were split and maintained every two days by first trypsinizing them with 1 mL trypsin and 1:10 diluting them with fresh complete-DMEM. ΔmiR-K12-7 KSHV BAC transfected 293T cells were grown under 100 µg/mL hygromycin B selections.

Isolation of ΔmiR-K12-7 KSHV BAC for Transfection

Glycerol stock of clone harboring ΔmiR-K12-7 KSHV BAC was thawed and inoculated in 500 mL LB-chloramphenicol overnight. BAC DNA was prepared using the Qiagen Large-construct Maxi-Prep kit according to manufacturer's instructions.

Transient Transfection of 293T Cells

293T cells were plated in 6-well plates at the cell density of 300,000 in a total of 2 mL DMEM-complete media. Plates were incubated in the CO_2 incubator at 37 ºC for 24 hours prior to transfection. Transfection was performed using 293 Mirus Trans-IT according to manufacturer's direction. Briefly, 3 µl of Mirus Trans-IT was mixed well with 200 µl of serum-free DMEM and incubated at room temperature for 20 minutes. 3 µg of ΔmiR-K12-7 KSHV BAC DNA was added to the mixture and mixed well by pipetting up and down. After 30 minutes of incubation at room temperature, 200 µl of the transfection reaction mixture was added drop wise to each well. The plate was rocked back and forth and from side to side to distribute the transfecting complex evenly on cells. The plate was incubated for 24-48 hours in a CO_2 incubator at 37 ºC. At 24-48 hours post-transfection, cells were checked for GFP expression to determine transfection efficiency. Once BAC DNA was transfected in to 293T cells, as confirmed by GFP expression, 100 µg/mL hygromycin B was added to select BAC transfected cells.

Propagation of Transfected 293T Cells

Transfected 293T cells were harvested from 6-well plates by disrupting cells with trypsin. Cells were then transferred to 10cm dish containing fresh DMEM-complete in the presence of 100 µg/mL hygromycin B to provide selection of BAC infected cells over non-infected cells. Infected 293T cells were allowed to recover for a few days under antibiotic selection. Once the 10cm plate was 40-60% confluent with green cells, cells were transferred and propagated in 15cm plates. Virus was ready to induce from latency when plates were 40-60% confluent.

Reactivation of Recombinant Virus

Recombinant virus was induced to reactivate from latency by addition of 20 ng/mL TPA and 1mM valproic acid. Virus was harvested 4 days post induction from cell culture media and cleaned using a 0.45 micron filter.

Isolation & Quantification of Recombinant Virus

Filtrate media containing recombinant virus was pipetted drop-wise on top of a 25% sucrose cushion and subjected to ultracentrifugation at 25,000rpm for 1 hour. Pellets containing virus were resuspended in 1% of the original filtrate volume using serum-free DMEM to make the virus stock and it was stored at -80ºC. 25µL of the recombinant virus was used to isolate DNA for further quantification using DNAzole according to the Invitrogen's recommendations. Virus DNA was resuspended in 25µL of ddH_20 and 1µL was used per qPCR reaction. Real-Time qPCR was performed using five 10-fold serial dilutions of pcDNA3.1-Orf73 plasmid as standards along with primers specific for the N-terminus of LANA (Orf73). Real-Time qPCR was performed using Fast SyBr Green according to the manufacturer's recommendations (Applied

Biosystems). Viral genome copy number was determined by comparing the threshold cycle (Ct) of sample DNA to the plasmid standard curve.

Results

The 293T cell line is a highly transfectable mammalian cell line. 293T cells were successfully transfected with ΔmiR-K12-7 rKSHV BAC as a considerable number of cells expressed GFP 24 hours post-transfection. Transfected 293Tcells were maintained and transferred to 10cm plates and scaled-up to harvest virus (Figure 3-1). When induced with TPA and VA, transfected 293T cells yielded an average 2.8×10^5 copies of WT KSHV and 1.73×10^6 copies of ΔmiR-K12-7 rKSHV when quantified with Real-time qPCR (Figure 3-2). Table 3-1 lists viral copies per mL for 3 preparations of each, WT and recombinant KSHV. Although WT219 BAC transfected cells displayed faster growth compared to ΔmiR-K12-7 rKSHV BAC transfected cells, virus yields were six fold higher in ΔmiR-K12-7 rKSHV BAC transfected cells. Even though titers for recombinant and WT viruses were not normalized to measure viral production per cell, it was observed that ΔmiR-K12-7 rKSHV was induced to lytic replication at a higher level.

ΔmiR-K12-7 rKSHV will be used to infect iSLK cells to create a doxycycline-inducible producer cell line in the next chapter.

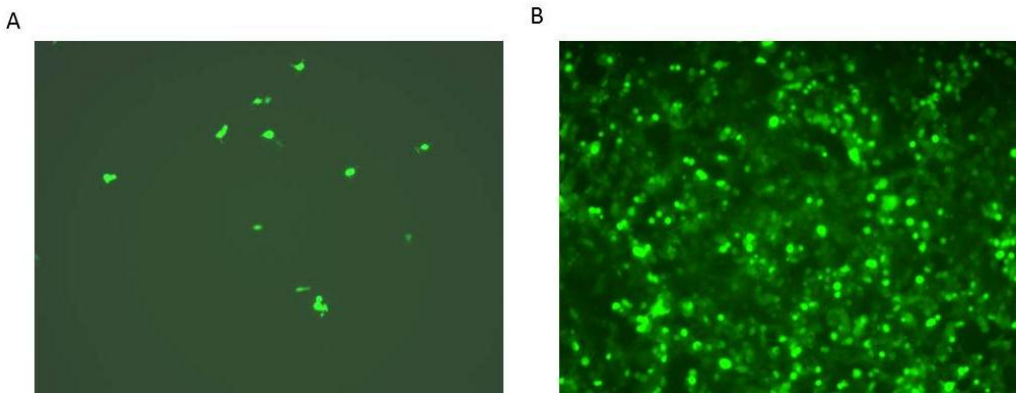

Figure 3-1. Transient Transfection of 293T cells. 293T cells were transfected with 3 µg of ΔmiR-K12-7 rKSHV BAC and GFP expression was observed 24 hours post-transfection. ΔmiR-K12-7 KSHV BAC transfected 293T cells A) 3 days post-transfection and B) expanded and grown to confluence in a 10 cm plate

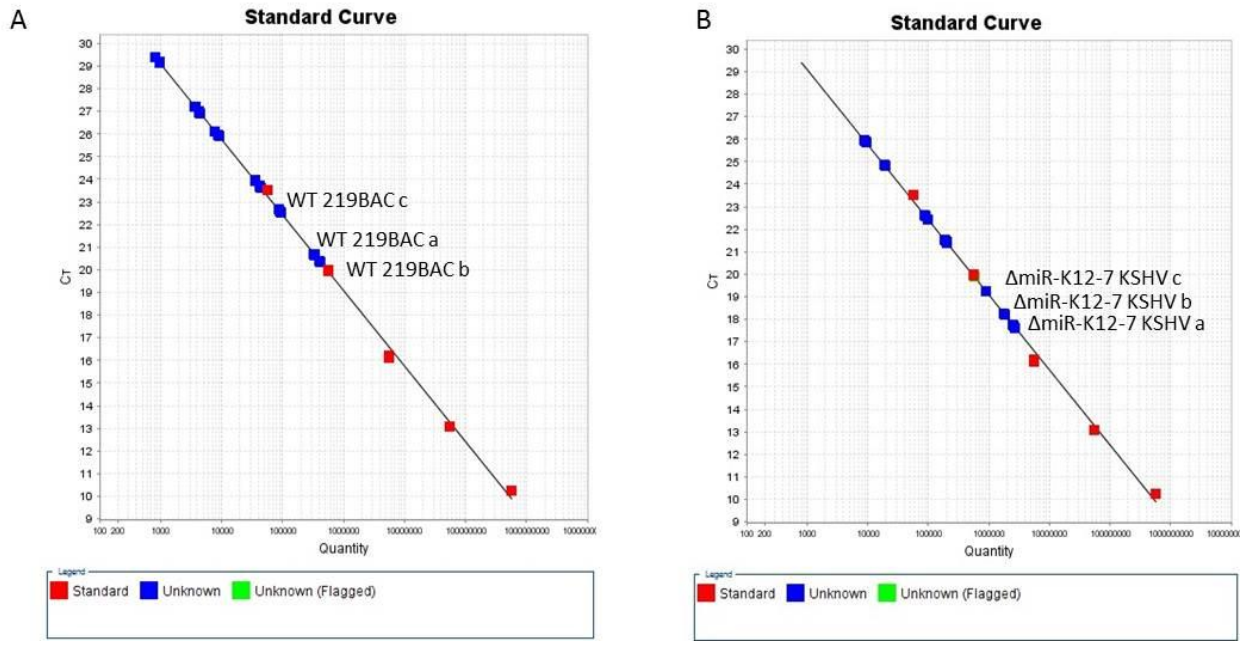

Figure 3-2. Quantification of virus. Viruses from infected 293T cells were purified and resuspended in 1% original volume. Viral copy numbers were quantified by qPCR using LANA as standard. Virus quantification from 3 different sets of A) WT 219BAC transfected cells and B) ΔmiR-K12-7 KSHV BAC transfected cells

Table 3-1. List of viral copy number

Sample	Viral copy per mL
219 BAC a	3.36×10^5
219 BAC b	4.14×10^5
219 BAC c	8.96×10^4
ΔmiR-K12-7 KSHV a	2.6×10^6
ΔmiR-K12-7 KSHV b	1.8×10^6
ΔmiR-K12-7 KSHV c	7.97×10^5

Viruses were induced to reactivate from latency with TPA and VA. Viral copy numbers were determined by qPCR.

CHAPTER 4
ESTABLISHING A PRODUCER CELL LINE

Background

Currently available KSHV-infected cell lines have many experimental limitations. Spontaneous lytic reactivation of virus occurs at a very significant level in most of the existing PEL cell lines (24). Earlier, the Renne Laboratory observed that recombinant virus cannot be produced from transfected 293T cells after thawing from frozen stocks. Also, the amount of virus production decreases over longer passages of the transfected 293T cells. Thus, the absence of recombinant virus producer cell lines has led to mandatory repetition of the entire procedure i.e., from creating recombinant KSHV BAC to transiently transfecting 293T cells followed by virus induction, if more recombinant virus is needed. Constant repetition of the entire procedure leads to additional problems like mutations within the virus and inconsistent amounts of virus production.

iSLK cells, uninfected endothelial cells of gingival KS lesion of an HIV-negative renal transplant recipient. SLK cells, which contain a doxycycline inducible RTA expression cassette, was created and provided by Don Ganem. RTA (Replication and Transcriptional Activator), the major immediate early lytic gene needed for reactivation, is the latent-lytic switch encoded by ORF50. Ectopic expression of RTA leads to induction of latently KSHV-infected cells (33). The inducible RTA cells were produced by transducing SLK endothelial cells (28) with a RTA expression construct which is tightly regulated by a promoter bearing a tet operator sequence. Cells were also transduced with Tet-On transactivator which can be activated by doxycycline. iSLK cells have been also demonstrated as a good model for maintaining KSHV latency. When

virus is required in sizeable quantity, infected iSLK cells or frozen-thawed stock can be induced by addition of doxycycline and sodium butyrate.

Materials and methods

Cell Line

iSLK cells were maintained in complete DMEM media (supplemented with 10% FBS and 1% penicillin/streptomycin) with 100 µg/mL G418 and puromycin. Infected iSLK cells were under additional selection of 100 µg/mL hygromycin B.

De novo Infection of iSLK cells

iSLK cells were seeded 14-16 hours prior to infection in 24-well plates at 100,000 cells in 500 µL DMEM-complete media. 500µL of 293T virus stock was added to cells along with 4µg/mL of polybrene. Polybrene is a small, positively charged polymer that increases efficiency of viral infection by neutralizing surface charges on the cell. Cells were incubated for 12 hours, then virus was removed and fresh media was added. Cells were observed for GFP expression 24 hours post infection. Infected iSLK cells were then incubated and monitored for several days post infection. Cells were washed with PBS and trypsinized to dislodge and transferred to 6-well plates in the presence of 1200µg/mL of hygromycin B for selection of infected cells. Hygromycin B inhibits protein synthesis of only uninfected iSLK cells while rKSHV confers hygromycin B resistance in infected cells Cells were allowed to recover from selection, and then expanded and frozen cell stocks were made.

Induction of virus

Once infected iSLK cells reached 40-60% confluency in 15 cm plates, cells were induced using 1µg/mL doxycycline, to induce RTA expression, and 1mM of sodium

butyrate, another HDAC inhibitor. Virus was harvested 4 days post induction from cell culture media using a 0.45μM filter and quantified by Real-Time qPCR.

Results

iSLK cells were infected with recombinant virus and I observed few GFP expressing cells 4 days post-infection. Infected cells were put under hygromycin B selection upon visualizing GFP expressing cells. Earlier attempts to expand infected iSLK cells had failed so we repeated the infection. Currently infected cells are under hygromycin B selection and expansion. Once we expand infected iSLK cells successfully, we will freeze them. This frozen stock will be tested for its ability to serve as a producer cell line which can be frozen, thawed and induced to produce recombinant virus. An important step in the quality control of generating this viral mutant will be whole genome sequencing. The mutant virus will be sequence verified by Illumina solexa sequencing to further confirm the miR-K12-7 deletion and to determine if there are any other mutations in the viral genome. Further, real time PCR will be performed to show that only miR-K12-7 has been deleted and that the miR-K12-7 deletion does not affect the expression of the other KSHV miRNAs.

In summary, I have successfully created and validated a markerless recombinant KSHV BAC containing miR-K12-7 deletion. Upon transfection into 293T cells, infectious virus was reconstituted

Figure 4-1. Generating a ΔmiR-K12-7 KSHV producer cell line. iSLK cells were infected with ΔmiR-K12-7 KSHV. Image was taken 3 days post-infection. Only few GFP-expressing iSLK cells were observed post infection. Left, phase contrast image; Center, fluorescent image; Right, merged image.

CHAPTER 5
DISCUSSION AND FUTURE DIRECTIONS

The mutation strategy employed in this study has proven to be an ideal system to systematically delete an individual KSHV miRNA. Each step during the mutagenesis procedure ensures that the quality of BAC TRs is not compromised. Skalsky et.al identified that TRs contain two LANA binding sites and a GC-rich replication element (30). Not only LANA binds to LANA-binding sites in order to tether the episome to the chromosome, it also recruits cellular replication components at the origin of replication for latent DNA replication. Thus, functions of LANA and TRs are crucial for episome maintenance.The mutagenesis strategy used here will ensure that TRs of recombinant BACs are neither degraded nor lost. Most importantly, we can produce KSHV miRNA mutants that do not contain any marker or extra “foreign” sequence. Thus, utilizing this approach will greatly benefit virology researchers to create and produce good molecular biology reagents.

In the context of the viral mutant generated here, Lin et. al experimentally confirmed that miR-K12-7-5p, a miRNA derived from miR-K12-7, targets replication and transcription activator (RTA) (17). As RTA is the main protein switch that induces lytic replication, miR-K12-7 targeting of RTA may promote the maintenance of latency. Additionally, bioinformatic analysis by Qin et. al lead to the identification of binding sites for miR-K12-7 within the 3'UTR of the basic region/leucine zipper motif transcription factor C/EBPbeta which is a known regulator of IL-6 and IL-10 transcriptional activation (23). IL-6 and IL-10 have been implicated in KSHV-associated cancer pathogenesis.

To identify other potential targets of miR-K12-7, comparative array-based transcription profiling of recombinant KSHV and WT KSHV can be very useful. Those

genes that upregulate upon infection of mutant virus in comparisons to WT KSHV infected cells, and also contain seed sequence homology, can be further tested by reporter assays to validate them as genuine targets of miR-K12-7. Target identification using recombinant virus generated through the two-step Red mediated recombination offers advantages, as described below, over ectopic over-expression of a miRNA and antagomir-based inhibition approach. Because physiological expression levels of a miRNA and target genes are important for targeting, ectopically over-expressing a miRNA might result into targeting of a transcript which otherwise would not be targeted at physiological concentration. Transfecting miRNA mimics may also increase off-target effects. As there can be more than 100 targets of a given miRNA, it will be difficult to determine targets that are relevant in the context of infection. Hence, recombinant virus generated here will be crucial for miR-K12-7 target identification. Once potential miR-K12-7 targets have been identified by microarray profiling, genes showing changed expression levels can be validated as genuine targets by reporter assays. 3' UTRs of potential target genes will be cloned downstream of a luciferase reporter gene to check miRNA-dependent inhibition of the reporter gene. Subsequently the seed sequence can be mutated to abrogate miRNA-dependent inhibition of the reporter gene to validate a given gene as a genuine target.

Recently developed techniques like HITS-CLIP (high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation) (11), and PAR-CliP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) (14) have proved to be great tools to determine miRNA targets. These techniques are developed based on the enrichment of Ago/miRNA/mRNA complexes from cells. HITS-

CLIP uses UV-irradiation to crosslink RNA-protein complexes having the direct contact in cells, followed by stringent immunoprecipitation of these complexes and partial RNA digestion. Resulting small RNAs in the RNA-protein complexes are sequenced through high throughput PAR-CLIP relies on incorporation of photoreactive ribonucleoside analogs in RNAs by living cells. These methods exploit RNA-protein interactions to identify miRNA targets in physiological conditions. These methods can also be employed to compare miRNA targets in wt and recombinant KSHV as additional means to identify and validate miR-K12-7 targets.

It is evident from the literature that KSHV miRNAs target host and viral transcripts to contribute to the establishment and maintainance of latency (6, 17). It is imperative to identify and experimentally prove the targets for miR-K12-7 and those of the other KSHV miRNAs. Finding and experimentally proving KSHV miRNA targets will require quality-controlled KSHV miRNA deletion mutants. By using this two-step BAC recombination protocol, one can generate miRNA deletion KSHV mutants. Identification of crucial targets will lead to the emergence of a unifying theme pertaining to KSHV pathogenesis and potential therapies.

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BIOGRAPHICAL SKETCH

Rajni Sangani was born in Jamnagar, India. The youngest of three siblings in a farming family, he grew up in a rural village Majoth, India. He moved to boarding school at the age of 12, to finish higher secondary education. He earned his B.S. in biotechnology, in 2002 from Virani Science College, Saurashtra University–India. He further earned his post–graduate diploma in applied biochemistry from Maharaja Sayajirao University of Baroda in 2004. Further, he earned MS in biochemistry from Maharaja Sayajirao University–Baroda in 2005.

He entered the interdisciplinary program in biomedical sciences at UF in 2007. He changed his mentor and concentration in 2010 and joined Renne Core laboratory, as a technician and continued to work with Dr. Thomas O'Brien, to overexpress and purify the human mitochondrial ribosme small-subunit protein 22. In Spring of 2011, Dr. Rolf Renne agreed to carry over his project of miRNA deletion mutant of KSHV in bacmid and extend it into a master's thesis project.