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miR-K12-7-5p Encoded by Kaposi’s Sarcoma-Associated Herpesvirus Stabilizes the Latent State by Targeting Viral ORF50/RTA

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Abstract

Seventeen miRNAs encoded by Kaposi’s sarcoma-associated herpesvirus (KSHV) have been identified and their functions have begun to be characterized. Among these miRNAs, we report here that miR-K12-7 directly targets the replication and transcription activator (RTA) encoded by open reading frame 50. We found that miR-K12-7 targeted the RTA 3’ untranslated region (RTA3’UTR) in a seed sequence-dependent manner. miR-K12-7-5p derived from miR-K12-7 mediates the inhibition of RTA expression, and the mutation of the seed match site totally abrogated the inhibitory effect of miR-K12-7 on RTA3’UTR. The inhibition of RTA expression by miR-K12-7 was further confirmed in the latently KSHV-infected 293/Bac36 cell line through transient transfection of miR-K12-7 expression plasmid or specific inhibitor of miR-K12-7-5p, respectively. The transient transfection of miR-K12-7 into 293/Bac36 cells reduced RTA expression and the expression of the downstream early genes regulated by RTA, and also the production of progeny virus was significantly reduced after treatment with chemical inducers. Our study revealed that another miRNA, miR-K12-7-5p, targets the viral immediate early gene RTA and that this miRNA contributes to the maintenance of viral latency.

Introduction

miRNAs are a class of small (~21–25 nucleotides) single-stranded noncoding RNAs that posttranscriptionally regulate the expression of specific messenger RNAs by pairing to complementary target sequences within related mRNAs [1]. To date, more than 15,000 mature miRNAs have been discovered in 142 species (http://www.mirbase.org/, Release 16, Sep. 2010), which are involved in development, apoptosis, metabolism, embryonic patterning, and miRNA biogenesis [2]. Many viruses, like their hosts, encode miRNAs, which can be utilized by viruses to inhibit host cell apoptosis, evade the host immune system, regulate the viral life cycle, and control viral gene expression [3].

Kaposi’s sarcoma-associated herpesvirus (KSHV) has been reported to express 17 mature miRNAs from 12 pre-miRNAs during viral latency [4,5,6,7], but the functions of these are as yet largely unknown. Because these miRNAs were identified as components of the viral latency program, we speculate that they may contribute to the maintenance of viral latency. Therefore, we screened these miRNAs using a luciferase reporter construct containing the 3’ untranslated region (3’UTR) of the RTA gene (RTA3’UTR). Among these miRNAs, we found that miR-K12-7, together with miR-K12-9, regulates the switch between viral latency and lytic replication by directly inhibiting the expression of RTA, the major regulator of the viral life cycle. The regulation of RTA by miR-K12-7 and other miRNAs constitutes another mechanism by which KSHV maintains a latent state in host cells.

Results

miR-K12-7 targets the RTA3’UTR in reporter system

To test the hypothesis that the miRNAs expressed during the latent phase participate in the maintenance of viral latency, we decided to investigate the effects of these miRNAs on expression of RTA, the major regulator of the viral life cycle. We first amplified the RTA3’UTR [8] from the BCBL-1 cell line (primers are shown in Materials and Methods). RTA3’UTR was then inserted into the pGL3 vector downstream from the luciferase gene, and we designated this construct pGL3-RTA3’UTR. Each miRNA encoded by KSHV or a cluster of miRNAs (miR-Cluster, containing miR-K12-1 to miR-K12-9 and miR-K12-11) was cloned into the expression vector pCDH3.1+. This system was transfected into HEK293 cells. The Renilla luciferase expression plasmid pRL–SV40 (Promega, Madison, WI) was transfected as an
internal control. We found that the miR-Cluster expression plasmid reduced the expression of firefly luciferase from pGL3–RTA3'UTR by about 30% compared with the reduction caused by the empty vector pCDH-copGFP (Figure 1A). This suggests that these miRNAs, which were expressed in the cluster during viral latency, have an overall negative role in the regulation of RTA expression.

To identify which miRNAs are responsible for downregulation of RTA3'UTR expression, each miRNA expression construct or the corresponding empty vector, or an irrelevant human miRNA has-miR-210 was cotransfected with pGL3–RTA3'UTR into HEK293 cells. Several miRNAs, including miR-K12-1, miR-K12-3, miR-K12-6, miR-K12-7 and miR-K12-9, repressed to varying degrees the expression of the reporter gene that contained RTA3'UTR (Figure 1B). miR-K12-7 reduced expression of the reporter most effectively in our experimental system (Figure 1B). miR-K12-7 reduced expression of the reporter gene bearing RTA3'UTR directly [10], therefore, we focused on miR-K12-7.

Table 1. Primer pairs for cloning KSHV pre-miRNA expression constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer sequence</th>
<th>Genome position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-K12-1</td>
<td>5' primer AATCTGGTGACGGCATCTTC</td>
<td>121783–121802</td>
</tr>
<tr>
<td></td>
<td>3' primer GTACCGGTTGGTTTTACCCAG</td>
<td>121947–121966</td>
</tr>
<tr>
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<td>121620–121640</td>
</tr>
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<td></td>
<td>3' primer GCCTGCACTCCGCTT GCC</td>
<td>121809–121828</td>
</tr>
<tr>
<td>miR-K12-3</td>
<td>5' primer GTTGCCTGACCG CTGGAG</td>
<td>121522–121542</td>
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<td>3' primer GGCAACATCCCTAA CCCAA</td>
<td>121639–121658</td>
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<tr>
<td>miR-K12-4</td>
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<tr>
<td></td>
<td>3' primer CTCCAGTGTGACGGACGAAC</td>
<td>121522–121542</td>
</tr>
<tr>
<td>miR-K12-5</td>
<td>5' primer CCGGTAGGATTTTGGGG</td>
<td>121234–121234</td>
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<tr>
<td></td>
<td>3' primer GAGTGTCTACGC TCTCAAC CTC</td>
<td>121364–121387</td>
</tr>
<tr>
<td>miR-K12-6</td>
<td>5' primer ACACAGAACAAATACGGGACTA</td>
<td>120727–120750</td>
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<td>3' primer TAAAGGGGGGTTGGTGAAG</td>
<td>120861–120880</td>
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<td>120293–120310</td>
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<td></td>
<td>3' primer CTGCCACCGATGAGATAC</td>
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<td>119884–119901</td>
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<tr>
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<td>119224–119243</td>
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<td></td>
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<td>119384–119405</td>
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<td>miR-Cluster</td>
<td>5' primer TCCACAGATGAGTGA CCGAC</td>
<td>119099–119117</td>
</tr>
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<td></td>
<td>3' primer GTACCGGTTGGTTTTACCCAG</td>
<td>121947–121966</td>
</tr>
</tbody>
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*All positions indicated here were referenced in the KSHV genome (accession number: U75698).

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The seed-match site is essential for the regulation of RTA by miR-K12-7-5p

Because the seed sequence (nucleotides [nt] 2–7/8) is critical for miRNA function [11], the seed complementarity for miR-K12-7 at the 3' UTR of the RTA mRNA was determined. Initially, miR-K12-7 is considered to encode only one mature miRNA, designated “miR-K12-7-3p” [4,5,6]. More recently, using deep sequencing technology, two groups separately found that miR-K12-7 can actually gives rise to two mature miRNAs, miR-K12-7-5p and miR-K12-7-3p, in different KSHV-positive cell lines [12,13]. We confirmed this result with bulge–loop quantitative PCR (qPCR) by checking the total RNA samples from KSHV-positive cell lines BCBL-1, BC-3 and JSC-1, or the total RNA from HEK293 cells transfected with an miR-K12-7 expression construct, respectively (Figure S2; the bulge–loop qPCR primers were synthesized by RiboBio, Inc., Guangzhou, China). When we searched the 3' UTR of RTA mRNA for seed complementarity to miR-K12-7, we identified a 7-mer complementary to nt 2–8 of miR-K12-7-5p (Figure 2A).

Since other group had already shown that miR-K12-7-3p didn’t target RTA3'UTR by using either miR-K12-7-3p mimics or inhibitor [10], we supposed that the inhibitory effect of miR-K12-7 expression plasmid on the expression of luciferase gene bearing RTA3'UTR was possibly mediated by miR-K12-7-5p. Mutational analysis confirmed our hypothesis that the observed inhibition of luciferase expression by miR-K12-7 is dependent on the presence of the seed-match site in RTA3'UTR. Mutations were introduced at nt 2–5 of the 3'UTR of RTA mRNA to reduce its Watson–Crick base pairing with miR-K12-7-5p (Figure 2B). HEK293 cells were cotransfected with pGL3–RTA3'UTR or pGL3–RTA3'UTR-mut and miR-K12-7 or miR-K12-9 expression plasmid, or the corresponding empty vector. The Renilla expression plasmid pRL–SV40 was also transfected as an internal control. Firefly luciferase activity was measured and normalized to the amount of Renilla luciferase activity. When HEK293 cells were cotransfected with the wild-type RTA3'UTR, miR-K12-7 and miR-K12-9 significantly reduced luciferase expression (Figure 2C), similar as results shown in Figure 1B and 1C. However, when pGL3–RTA3'UTR-mut and the miR-K12-7 or miR-K12-9 expression plasmid were cotransfected into HEK293 cells, the mutation of the seed-match site totally abrogated the suppressive effect of miR-K12-7 on the expression of the luciferase gene, whereas the effect of miR-K12-9 was not affected (Figure 2C). This is probably attributable to the destruction of the potential pairing between miR-K12-7-5p and the predicted pairing site in RTA3'UTR (Figure 2B). The result also suggests that miR-K12-9 probably targets different sites from those targeted by miR-K12-7 in RTA3'UTR. These data indicate that the seed-match site is critical for the targeting of miR-K12-7-5p to RTA3'UTR.

miR-K12-7 reduces RTA expression in KSHV-positive 293/Bac36 cells

Because the experiments described above showed that miR-K12-7 is a negative regulator of RTA3'UTR, we next examined...
whether miR-K12-7 is an authentic miRNA with inhibitory effect on RTA expression. The 293/Bac36 cell, latently infected with KSHV, was transfected with individual miRNA expression construct or the corresponding empty vector or an irrelevant miRNA hsa-miR-210, and revealed that several miRNAs encoded by KSHV, miR-K12-1, miR-K12-6, miR-K12-7 and miR-K12-9...
were all able to inhibit the expression of RTA to varying degrees (Figure 3A). Similar inhibition of RTA expression by miR-Cluster was also observed (Figure 3A). Quantification of these effects from three independent experiments (Figure S3) indicated that among these miRNAs, miR-K12-7 repressed RTA expression most significantly, by 45% (Figure 3B; analyzed with NIH ImageJ). miR-Cluster reduced the expression of RTA by 60% (Figure 3B), which was stronger than with any of the individual miRNAs, and was probably due to a combined effect of these miRNAs.

To confirm that miR-K12-7-5p is a negative regulator of RTA, we then transfected 293/Bac36 cells with inhibitor specific to miR-K12-7-5p or random inhibitor (purchased from RiboBio, Inc., Guangzhou, China), respectively. miR-K12-7-5p inhibitor increased RTA expression significantly compared with random inhibitor (Figure 3C) and showed about 30% increase of RTA expression (Figure 3D; analyzed with NIH ImageJ). These results demonstrate that miR-K12-7-5p can inhibit expression of RTA in a KSHV latently infected cell line.

miR-K12-7 inhibits RTA expression at the protein level but not at the mRNA level

Because miRNA can repress its target at both the transcriptional and translational levels, we next examined whether KSHV-encoded miRNAs target RTA at the RNA level. Total RNAs were harvested at the same timepoint at which Protein samples were collected from 293/Bac36 cells transfected with the individual miRNA expression constructs or the corresponding empty vector. pRL–SV40 (4 ng) was transfected as the internal control. Luciferase activity was measured 24 h after transfection. The data shown are from six independent transfections. ***P<0.001; n.s., not significant, t-test. doi:10.1371/journal.pone.0016224.g002
the relative quantity of RTA mRNA in 293/Bac36 cells transfected with pCDH-GFP was used as the control value. If the complementarity between the miRNA and the target 3’UTR is low, the miRNA will usually inhibit the expression of the target gene translationally [11]. We found that miR-K12-7 did not affect the levels of RTA mRNA (Figure 3E), which suggests that miR-K12-7 regulates the expression of RTA at the translational level.

miR-K12-7 downregulates RTA downstream genes expression and impairs the production of progeny virus

The experiments described above show that miR-K12-7 negatively modulates RTA’s 3’UTR, and imply that miR-K12-7 has some functional role in the regulation of the KSHV life cycle. As we already know, the immediate early gene product RTA is a master molecule that responds quickly to stimuli [8]. RTA activates downstream genes, including PAN RNA, kaposin (K12), open reading frame 57 (ORF57), ORF59, thymidine kinase, vIL-6, etc., in a cascade fashion [14,15,16,17,18,19]. We then examined the effect of miR-K12-7 on the expression of genes downstream from RTA using quantitative real-time PCR. We found at 48 h that the expression levels of those early downstream genes regulated by RTA were slightly reduced by miR-K12-7. At 26 h after induction with TPA and VPA, the 293/Bac36 cells transfected with individual miRNA expression constructs or the corresponding empty vector were harvested and RNAs were extracted from those cells with TRizol Reagent. RTA mRNA from those cells was assayed by RT-PCR and quantified relative to β-actin mRNA levels. The data shown are from triplicate independent experiments. *P<0.05; ** P<0.01; n.s., not significant. t test.

doi:10.1371/journal.pone.0016224.g003

Figure 3. miR-K12-7 inhibits RTA expression at the protein level but not at the mRNA level. (A) The transient expression of miR-K12-7 repressed RTA protein expression in the latently KSHV-infected 293/Bac36 cell line. Latently KSHV-infected 293/Bac36 cells were synchronized by culture in serum-free medium and pretransfected with 4 μg of individual miRNA-expressing constructs or the corresponding empty vector 12 h after the addition of serum. After a further 4 h, the S-phase 293/Bac36 cells were induced to lytic replication with both TPA (25 ng/mL) and VPA (3 mM). Cell lysates were prepared 26 h after induction and were probed for endogenous RTA or β-actin. (B) A graph of RTA levels normalized to those of β-actin of three independent experiments performed in Figure 3A (analyzed with NIH ImageJ). (C) miR-K12-7-5p inhibitor increases RTA expression. Synchronized 293/Bac36 cells were transfected with 200 nM miR-K12-7-5p inhibitor or random inhibitor and were introduced into lytic replication with both TPA (25 ng/mL) and VPA (3 mM). Cell lysates were prepared 48 h after induction and were probed for endogenous RTA or β-actin. (D) A graph of RTA levels normalized to those of β-actin of three independent experiments performed in Figure 3C (analyzed with NIH ImageJ). (E) RTA mRNA levels were not affected by miR-K12-7. At 26 h after induction with TPA and VPA, the 293/Bac36 cells transfected with individual miRNA expression constructs or the corresponding empty vector were harvested and RNAs were extracted from those cells with TRizol Reagent. RTA mRNA from those cells was assayed by RT–PCR and quantified relative to β-actin mRNA levels. The data shown are from triplicate independent experiments. *P<0.05; ** P<0.01; n.s., not significant. t test.
We next investigated the effects of miR-K12-7 on the production of progeny virus after chemical induction. On day 5 after induction with TPA and VPA, the supernatants of 293/Bac36 cells, pretransfected with the miR-K12-7 expression construct or the corresponding empty vector, were collected. The viral genomes were then extracted from the supernatants and assayed by real-time PCR. The viral copy numbers were calculated according to the standard curve derived from the ORFK9 construct (Figure 4B). We found that miR-K12-7 reduced the production of progeny virus from 293/Bac36 cells by more than four fold compared with that of empty vector (Figure 4C). Interestingly, miR-Cluster also showed more than two fold reduction of progeny virus (Figure 4C). Taken together, these data suggest that miR-K12-7 prevents the production of progeny virus by targeting RTA, which may help to maintain a latent state of viral infection.

**Discussion**

Since the discovery of KSHV, the regulation of its latency–lytic replication status has intrigued researchers in the field. During
latent infection, the viral episome is tethered to the host chromosomes and replicates with the host cell. At this stage, very few viral genes are expressed. Those that are expressed include the genes encoding viral FLIP (vFLIP), viral cyclin (vCYC), and latency-associated nuclear antigen (LANA; see review [20]). LANA has previously been reported to inhibit lytic replication by targeting the immediate early gene RTA4, in a mechanism that controls virus-mediated latency [21].

Recently, studies in several laboratories have shown that besides those typical latency genes, KSHV also encodes 12 pre-miRNAs, which can give rise to 17 mature miRNAs, during latency [4,5,6,7]. Early observations have suggested a role for miRNAs in the regulation of the latency-to-lytic-replication switch in herpes simplex virus 1 (HSV1) [22]; and human cytomegalovirus (HCMV) [23]. One of those studies demonstrated that at least two miRNAs encoded by HSV1, miR-H2-3p and miR-H6, which are expressed in latently infected neurons, facilitate the establishment and maintenance of viral latency by posttranscriptionally regulating the expression of two viral immediate early genes, ICp0 and ICp4-5, respectively [22]. Another study demonstrated that miR-UL112-1, encoded by HCMV, contributes to the control of viral latency by regulating the viral immediate early gene IE1 [23]. Based on the strong similarities among herpesviruses, we speculated that miRNAs encoded by KSHV may also play a similar role. Because RTA is the only gene essential for the switch from latency to lytic replication (see review [24]), we hypothesized that miRNAs encoded by KSHV target this immediate early gene.

In this study, we found that the miRNA encoded by KSHV, miR-K12-7-5p, newly identified by deep sequencing [12,13] and confirmed by bulge-loop quantitative PCR in our laboratory (Figure S1 and S2), targets expression of RTA at translational level. This inhibitory function of miR-K12-7-5p on the expression of RTA is dependent upon the miRNA seed sequence (Figure 2C). We also demonstrated that miR-K12-7-5p slightly reduces the expression of the downstream early genes regulated by RTA (PAN, ORF57, and ORF59) at 48 h post chemical induction (Figure 4A) and significantly reduces the production of progeny virus in the 293/Bac36 cell line (Figure 4C), which is latently infected with KSHV, upon chemical induction. We also confirmed that miR-K12-9 repressed the expression of RTA; both in the reporter system and in KSHV latently infected 293/Bac36 cells. The target site of miR-K12-9 in RTA3’UTR differs from that of miR-K12-7-5p. We also observed that miR-K12-1 and miR-K12-6 repressed the expression of RTA in KSHV latently infected 293/Bac36 cells (Figure 3A) but not in one of our reporter systems (Figure 1C), which suggests that these two miRNAs affect RTA indirectly, as reported previously [25]. Taken together, these data demonstrate that miR-K12-7-5p, encoded by KSHV, can facilitate the maintenance of the latent viral state by translationally regulating the expression of the viral immediate early gene RTA.

While this manuscript was in preparation, several other groups reported some other miRNAs encoded by KSHV also target RTA. Briefly, Bellare et al. reported that RTA expression is inhibited by miR-K12-9*, which directly targets the 3’UTR of RTA mRNA [10]. Two other groups reported that the expression of RTA can be indirectly regulated by both miR-K12-5 [26] and miR-K12-1 [25], although the underlying mechanisms are totally different. In our study, we confirmed that miR-K12-9 was able to repress expression of the reporter gene that contained RTA3’UTR in two different cell lines (Figure 1B and 1C), and miR-K12-9 was also able to repress expression of RTA in KSHV latently infected 293/Bac36 cells (Figure 3A). We did not subsequently study the effect of miR-K12-1 and miR-K12-6 because we thought that both of these miRNAs would target RTA indirectly because they did not show similar inhibition of expression of the reporter gene in DG75 cells (Figure 1C). However, we noticed that miR-K12-1 and miR-K12-6 both repressed RTA expression in both HEK293 cells (Figure 1B) and the KSHV latently infected cell line 293/Bac36 (Figure 3A).

It is interesting to notice that four groups discovered four different miRNAs which all were able to target RTA, directly or indirectly. By comparing the different experimental systems and conditions among us four groups, we think the different results are understandable. The previous analysis from Bellare et al. employed miRNA mimics rather than pre-miRNA expression plasmids used by other three groups [10]. Specifically, for miR-K12-7, Bellare et al. only used mimics or specific inhibitor for miR-K12-7-3p since miR-K12-7-5p had not yet been discovered at the time of their study [10]. Although Lu et al. used the same pre-miRNA expression plasmids as we did, they didn’t carry out a comprehensive screen for the effect of individual miRNA encoded by KSHV on expression of reporter gene bearing RTA3’UTR. Moreover, Lu et al. focused more on identifying those miRNAs that were found to be able to inhibit RTA expression at the transcriptional level [26]. Lei et al. identified NF-κB inhibitor IκBz as a direct target of miR-K12-1 and expression of miR-K12-1 enhanced NF-κB activity [25]. Based on known evidences that NF-κB was a strong negative regulator of RTA transcription through forming complex with RBP-Jκ (a major coactivator of RTA) [27], they proposed a working model that miR-K12-1 suppresses viral lytic replication via activation of NF-κB [25]. This model really makes sense but it doesn’t exclude the possibility that RTA can be directly targeted by other miRNAs encoded by KSHV. And this model could not fully explain that why RTA was highly upregulated in ΔmiRs (Figure 1a and 1b in Ref. [25]) since NF-κB has no direct known effect on RTA expression. Although Lei et al. showed no effects of KSHV miRNAs on all three types of RTA3’UTR in their supporting information [25], we could not repeat their results due to lacking of dosage parameters for plasmids involved in. Taken together, based on different experimental systems, we and Bellare et al. have identified two different miRNAs that are encoded by KSHV, that are both able to inhibit RTA expression translationally, through direct targeting of RTA3’UTR. By using mi-R-Cluster deletion mutant, Lu et al. and Lei et al. have discovered two distinct miRNAs that are encoded by KSHV that can indirectly repress RTA expression through different mechanisms.

Many miRNAs that are encoded by KSHV contribute to regulation of the viral switch between latency-lytic replication by targeting the viral immediate early gene RTA, using different mechanisms simultaneously. A further question is why KSHV encodes so many miRNAs that are all able to downregulate expression of RTA, which is a major regulator of the viral life cycle. One reasonable explanation is that the virus requires backup systems to maintain viral latency safely. This makes sense because, as well as the role played by miRNAs in the regulation of the switch between latency and lytic replication, LANA is known to function in the maintenance of latency by repressing the promoter of RTA, and by binding directly to the RTA protein to prevent its self-reactivation [21]. Another potential explanation is that a single miRNA might not be strong enough to repress RTA expression to maintain viral latency, and combined effects are required. This is supported by the fact that the effects of all miRNAs that are reported to target RTA are marginal (Figure 3A) [10,25,26], and all miRNAs that target RTA expression, directly or indirectly, are expressed at relatively low levels compared with the most abundantly expressed miRNAs, miR-K12-4-3p in BC3 cells or miR-K12-6-3p in TRE-BCBL-1 cells [12,13] (Figure S1). Further
studies are required to elucidate the contributions of individual miRNAs that are encoded by KSHV to regulation of the viral life cycle. Single and/or combined miRNA mutation or deletion in the context of the whole viral genome might be the best way to understand the effect of each miRNA on RTA expression and viral latency maintenance.

We also noted that there are two different facets for the effects of miR-K12-5 on the latency-to-lytic replication switch: one promotes maintenance of latency in cells [26], and the other facilitates lytic reactivation in cells [28]. Further studies are needed to investigate which effect is dominant under different viral life cycle conditions. Studies from different groups that have reported several miRNAs encoded by KSHV that can repress expression of RTA by distinct mechanisms demonstrate the importance of RTA regulation, and add a new dimension to our understanding about the complex regulation networks for the KSHV viral life cycle. This complexity could provide a paradigm for the study of cellular and other viral miRNAs.

Materials and Methods

Cell culture and transfection

The human embryonic kidney (HEK) 293 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 25 U/mL penicillin, and 25 μg/mL streptomycin. A HEK293 cell line that harbors the KSHV genome inserted into a bacterial artificial chromosome, designated 293/Bac36, was grown under the same conditions, except with the addition of 100 μg/mL hygromycin B. The cells were seeded the day before transfection to achieve ~80%–90% confluence on the following day. DG75 (a KSHV-negative cell line) and BCBL-1 (a KSHV-positive cell line) were cultured in RPMI 1640 medium plus 10% fetal bovine serum. All the cells were cultured at 37°C in the presence of 5% CO2. HEK293 cells and 293/Bac36 cells were both transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. DG75 cells were transfected by electroporation with a Bio-Rad Gene Pulser II electroporator (Bio-Rad Laboratories, Hercules, CA). A total of 5×106 cells, harvested in exponential phase, were collected and washed with phosphate-buffered saline (PBS) and then resuspended in 400 μL of serum-free RPMI 1640 with the DNA mixture to be transfected. The resuspended cells were transferred to a 0.4 cm cuvette and electroporated at 975 μF and 220 V. The electroporated cells were transferred to 8 mL of complete medium, and then incubated at 37°C under 5% CO2.

 Constructs

Type I RTA3’UTR [8] was amplified by PCR from the total RNA extracted from the BCBL-1 cell line that had been induced with TPA for 36 h. Primers for RTA3’UTR were as follows: 5’ CCAggtaACGTGTCGAAGGGGTG; 3’ CCAgctagaGATGGAACATAAACACGTTATT. The PCR products were excised with KpnI and XhoI and cloned into pGL3C-M. This plasmid was constructed by removing the multiple cloning site (MCS) from the pGL3 control vector with KpnI and BglII, and infilling it with Klenow fragment, after which the in vitro MCS was inserted into the XhoI site. The new construct was designated pGL3-RTA3’UTR and confirmed by restriction enzyme digestion and DNA sequencing. HDAC4-3’UTR-pGL3 was kind gift from Dr. Changsheng Du (Tongji University, China).

Stem-loop pre-miRNAs were PCR amplified from BCBL-1 genomic DNA, as described previously [9] (all primers for cloning KSHV pre-miRNAs as well as miR-Cluster are listed in Table 1). The PCR products were digested with XhoI and EcoRI, and inserted into pcDNA3.1+ (Invitrogen) or pCDH-copGFP (System Biosciences) at the corresponding sites. All these constructs were confirmed by both restriction enzyme digestion and DNA sequencing. Individual miRNA expression was confirmed by Bulge-Loop qPCR (Supplementary Figure S1).

 Dual-luciferase reporter assay

A Dual-Luciferase Reporter Assay System (Promega) was used to examine the effects of miRNAs on their target genes. Each miRNA-expressing plasmid or its corresponding vector (4 μg) was cotransfected with RTA3’UTR-pGL3C-M (100 ng) and pRL-SV40 (2 μg; Promega) into HEK293 cells grown on a six-well plate using Lipofectamine 2000 transfection reagent (10 μL; Invitrogen). The transfected HEK293 cells were cultured for 24 h before harvesting for the luciferase assay. The cells were washed with PBS and homogenized with 200 μL of Passive Lysis Buffer (Promega, Madison, WI). Luciferase Assay Buffer (25 μL) was transferred into a 96-well plate. The lysate (10 μL) was added to two wells (duplicate) of the 96-well plate and mixed. Firefly luciferase activity was measured on a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). The plate was removed from the luminometer and Stop & Glo Reagent (25 μL) was added and mixed. Renilla luciferase activity was measured on the luminometer. The data recorded on the luminometer were analyzed and graphed using Excel (Microsoft).

 Western blot analysis

Twenty-four hours after transfection, whole-cell lysates of 293/Bac36 cells were prepared with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL pepstatin) for 1 h on ice, with brief vortexing every 15 min. The whole-cell lysates were then heated in sodium dodecyl sulfate (SDS)-mercaptoethanol lysis buffer and analyzed by SDS-polyacrylamide gel electrophoresis. The proteins were transferred from the gel to polyvinylidene difluoride membranes. The membranes were blocked with 5% dry nonfat milk for 1 h and probed with a mouse monoclonal antibody directed against RTA (using a monoclonal antibody raised by Yunhua Liu in our laboratory) at a 1:1000 dilution, and an anti-β-actin antibody (I-19; Santa Cruz Biotechnology) at a 1:1000 dilution. After the secondary antibody reaction, the membranes were washed in TBS–Tween (0.02 M Tris [pH 7.6], 0.1 M NaCl, 0.05% Tween-20) and visualized using a chemiluminescence reaction.

 RNA extraction and quantitative real-time PCR

The cells were harvested 24 h after transfection, and the total RNA was extracted with TRIzol Reagent (Invitrogen), according to the manufacturer’s instructions. The cDNA (20 μL) for real-time PCR was generated from 4 μg of total RNA with the First-Strand cDNA Synthesis Kit (Fermentas UAB, Fermentas International Inc., Burlington, Canada), after priming with 1 μL oligo(dT)18, according to the manufacturer’s instructions. The primer sequences for the real-time PCR system, which were designed and chosen with the Primer Express Software (version 3.0, Applied Biosystems, Inc., Foster City, CA), were as follows: RTA-RT-F, 5’-AGACCCCAGGGGTTTATTAGAAT-3’; RTA-RT-R, 5’-AGGATATCCGATGTGGAGG-3’; ORF57-RT-F, 5’-TGGCAGGTGCAGGTTTACTGC-3’; ORF57-RT-R, 5’-CTTCAGCTTCCACAGGATATTGG-3’; ORF59-RT-F, 5’-CTTCAGCTTCCACAGGATATTGG-3’. The data recorded on the luminometer were analyzed and graphed using Excel (Microsoft).
GACGCA-3'; ORF65-RT-F, 5'-GGCGGCGGTTCGCCG-3'; ORF65-RT-R, 5'-TCATTGTCGGCCGGGCG-3'; β-actin-RT-F, 5'-TGGCCGACAGATGCAGAAGAAG-3'; β-actin-RT-R, 5'-AGTGGACAGGCGCCAGAT-3'.

The real-time PCRs were performed with the SYBR Green Master Mix Kit (Toyobo, Osaka, Japan). The reactions, in a total volume of 15 μL, consisted of 7.5 μL of Mastermix, 1 mM each primer, and 2 μL of the diluted cDNA product. After 2 min at 50°C, the DNA polymerase was activated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The standard curves for RTA and β-actin cDNAs were generated with serial dilutions, and melting curve analysis was used to verify the specificity of the PCR products. The values for the relative quantification were calculated with the ΔΔCt method. The data were normalized to human β-actin levels to compensate for unequal amounts of cDNA in the samples, and are reported as the increase in mRNA accumulation compared to the mock-cell mRNA levels. All reactions were performed three times with a 7900HT Sequence Detection System (Applied Biosystems).

As a negative control, each plate contained a minimum of four mock-cell mRNA levels. All reactions were performed three times with a 7900HT Sequence Detection System (Applied Biosystems). As a negative control, each plate contained a minimum of four mock-cell mRNA levels. All reactions were performed three times with a 7900HT Sequence Detection System (Applied Biosystems).

PCR analysis of progeny virus

To determine whether miRNAs inhibit KSHV replication and reduce the production of progeny viral particles, 293/Bac36 cells were plated in six wells and after their attachment to the dish, the cells were synchronized to G1 phase by culture in serum-free DMEM for 24 h. Serum was then added to a final concentration of 10%. After 12 h, the cells were transfected with miRNAs or their corresponding vectors using Lipofectamine 2000. Four hours after transfection, the medium was refreshed with complete DMEM containing 25 ng/mL phosphor ester and 3 mM valproic acid [29]. The 293/Bac36 cells were incubated for a further five days at 37°C under 5% CO2. The supernatants were then collected and passed through a 0.45 μm filter. The viral particles were spun down at 15,000 g for 3 h. The pellet was resuspended in 50 μL of 0.2x PBS, heated to 95°C for 15 min, and then at 56°C for 1 h with protease K (10 mg/mL). The enzyme was then inactivated by heating at 95°C for 30 min. A 5 μL aliquot of the viral lysate was used for the PCR amplification of the KSHV-specific region of ORFk9. The primer pair for the real-time amplification of ORFk9 was: ORFk9-F, 5'-GTCTCTTGCGCCATTCACAAAC-3'; ORFk9-R, 5'-CCGGACACGCAACATTAAGAA-3'.

Supporting Information

Figure S1 Bulge-loop quantitative PCR confirms mature KSHV miRNA expression by miRNA constructs. (A) PCR products were separated on 1.5% agarose gels. Lane 1, 293/Bac36 cDNA; lane 2, cDNA from HEK293 transfected with miRNAs expression constructs; lane 3, HEK293 cDNA; lane 4, H2O. Molecular standards are DNA marker DL 2000 (Takara). (B) Relative expression level for all mature miRNAs expressed by constructs compared with miRNAs expressed in 293/Bac36 cells. The expression level of the lowest expressed miR-k12-9 was set as 1 for the controls, and the expression level of other miRNAs was calculated in comparison with miR-K12-9. Data shown are from three independent experiments.

Figure S2 Relative expression level of miR-K12-7-5p and miR-K12-7-3p in 293/Bac36 cells and 293/Bac36 cells transiently transfected with miR-K12-7 expression construct. (A) PCR products were separated on 1.5% agarose gels. Lane 1, cDNA from 293/Bac36 transfected with miR-K12-7 expression construct; lane 2, HEK293 cDNA; lane 3, 293/Bac36 cDNA; lane 4, BCBL-1 cDNA; lane 5, H2O. (B) Relative expression levels for miR-K12-7-5p and miR-K12-7-3p expressed in 293/Bac36 cells and 293/Bac36 cells transfected with miR-K12-7 expression construct. The expression level of the lowest expressed miR-k12-7-5p in 293/Bac36 cells was set as 1, and expression level in other cells was calculated in comparison with miR-K12-7-5p expressed in 293/Bac36 cells. Data shown are from three independent experiments.

Figure S3 Raw western blotting data from three independent experiments for Figure 3A and 3B.

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Author Contributions

Conceived and designed the experiments: XL KL. Performed the experiments: XL. Analyzed the data: XL KL. Contributed reagents/materials/analysis tools: DL ZH QD ESR. Wrote the paper: XL KL.

References


