A human herpesvirus miRNA attenuates interferon signaling and contributes to maintenance of viral latency by targeting IKK ϵ

Deguang Liang¹, Yuan Gao¹, Xianzhi Lin¹, Zhiheng He¹, Qinglan Zhao¹, Qiang Deng¹, Ke Lan¹

¹Key Laboratory of Molecular Virology and Immunology, Institute Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China

Type I interferon (IFN) signaling is the principal response mediating antiviral innate immunity. IFN transcription is dependent upon the activation of transcription factors IRF3/IRF7 and NF- κ B. Many viral proteins have been shown as being capable of interfering with IFN signaling to facilitate evasion from the host innate immune response. Here, we report that a viral miRNA, miR-K12-11, encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) is critical for the modulation of IFN signaling and acts through targeting I-kappa-B kinase epsilon (IKK ϵ). Ectopic expression of miR-K12-11 resulted in decreased IKK ϵ expression, while inhibition of miR-K12-11 was found to restore IKK ϵ expression in KSHV-infected cells. Importantly, expression of miR-K12-11 attenuated IFN signaling by decreasing IKK ϵ -mediated IRF3/IRF7 phosphorylation and by inhibiting the activation of IKK ϵ -dependent IFN stimulating genes (ISGs), allowing miR-K12-11 suppression of antiviral immunity. Our data suggest that IKK ϵ targeting by miR-K12-11 is an important strategy utilized by KSHV to modulate IFN signaling during the KSHV lifecycle, especially in latency. We also demonstrated that IKK ϵ was able to enhance KSHV reactivation synergistically with the treatment of 12-*O*-tetradecanoylphorbol 13-acetate. Moreover, inhibition of miR-K12-11 enhanced KSHV reactivation induced by vesicular stomatitis virus infection. Taken together, our findings also suggest that miR-K12-11 can contribute to maintenance of KSHV latency by targeting IKK ϵ .

Keywords: KSHV; IFN; IKK ϵ ; miR-K12-11

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Introduction

Type I interferons, including IFN α and IFN β , are key players in the innate antiviral response. Upon viral infection, IFN expression can be rapidly induced via heterogeneous signaling pathways which are dependent on types of invading pathogens and host immune cells. Generally, viral products, such as the viral genome and surface glycoproteins, are recognized by pattern recognition receptors, like the Toll-like receptors (TLRs), to trigger various downstream cascades leading to the activation

of the transcription factors NF- κ B and IRF3/IRF7, ultimately resulting in IFN transcription [1]. In order to establish successful infection, many viruses have evolved mechanisms to interfere with IFN signaling and evade the host innate immune response [2].

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), a human gamma herpesvirus, is the etiological agent of KS, the most common malignance in AIDS patients, primary effusion lymphoma (PEL) and multicentric Castleman's disease [3–5]. As with other herpesviruses, KSHV is characterized by a life cycle consisting of a latent phase and a lytic phase. During the primary lytic infection, IFN signaling modulation is required to suppress the innate immune response and establish successful latent infection. Multiple viral proteins, such as vIRFs, ORF45, RTA and K-bZIP, are involved in this process [6–9]. Unfortunately, IFN signaling modulation in viral la-

Correspondence: Ke Lan

Tel: +86 21 63851321; Fax: +86 21 63843571

E-mail: lanke@sibs.ac.cn

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tency remains poorly studied and underestimated. During latency, KSHV-infected cells can experience secondary infection with other viruses, including HCMV, HSV1 and HHV6, which will induce the innate immune response and KSHV reactivation [10–12]. Moreover, TLR7/8 signaling has been implicated in secondary pathogen-induced KSHV reactivation [13]. These reports suggest that controlling the innate immune response during the latent stage of infection is not only important in fighting host antiviral activity, but also contributes to KSHV latency maintenance.

KSHV encodes 17 mature miRNAs, which are derived from 12 pre-miRNAs that are located in a latent locus in the KSHV genome [14–17]. To date, some studies have shown that KSHV-encoded miRNAs are involved in regulation of viral gene expression, thereby playing a role in maintenance of viral latency [18–20]. However, the functions of these miRNAs remain largely unknown. By using the DIANA microT v3.0 web server (<http://diana.cslab.ece.ntua.gr/microT/>), IKKε (I-kappa-B kinase epsilon) was predicted as a potential target of the miR-K12-11 encoded by KSHV. IKKε is a non-canonical IKK (I-kappa B kinase)-related kinase, which is responsible for IRF3 and IRF7 activation in multiple IFN signaling pathways [21, 22]. Moreover, IKKε is required for the activation of a subset of IKKε-dependent IFN-stimulating genes (ISGs) [23]. Therefore, we hypothesized that miR-K12-11 could attenuate IFN signaling and aid in pathogen evasion of the innate immune response by targeting IKKε.

Through the study described herein, we were able to demonstrate that IKKε is a direct target of miR-K12-11. Ectopic expression of miR-K12-11 decreased IKKε expression, while inhibition of miR-K12-11 partially restored IKKε expression in KSHV-infected cells. More importantly, expression of miR-K12-11 was found to attenuate IFN signaling and lead to suppression of antiviral immunity. Thus, our findings provide the first evidence that viral miRNA is capable of modulating IFN signaling and facilitating viral infection. Since the innate immune response is related to viral reactivation, and miR-K12-11 is expressed both in latent and lytic phase, we also sought to define whether miR-K12-11 was involved in KSHV latency control by targeting IKKε. We found that IKKε could synergistically enhance KSHV reactivation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [24]. Moreover, inhibition of miR-K12-11 enhanced KSHV reactivation induced by vesicular stomatitis virus (VSV) infection [13]. Thus, our findings suggest that miR-K12-11 contributes to KSHV latency maintenance by targeting IKKε.

Results

IKKε is a direct target of miR-K12-11

In order to study whether any of the KSHV miRNAs are involved in modulation of the host innate immune response, we employed DIANA microT v3.0 web server (<http://diana.cslab.ece.ntua.gr/microT/>, The National Technical University of Athens, Greece) to predict potential viral miRNA targets [25]. We employed the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway filter to screen TLR signaling pathway-related genes among the predicted results, and we identified IKKε (gene symbol IKBKE) as a potential target of miR-K12-11. IKKε is a known multifunctional IKK-related kinase responsible for IRF3 and IRF7 activation in IFN signaling pathways. Interestingly, IKKε had been previously implicated as a target of miR-K12-11 and its human homologue miR-155 [26, 27], so we sought to confirm whether IKKε was a direct target of miR-K12-11. Two putative miR-K12-11 binding sites were predicted within the IKKε 3' UTR (untranslated region) by DIANA microT. We designated the sites as MRE1 (miRNA response element 1) and MRE2 (Figure 1A). An IKKε 3' UTR reporter (pGL3-IKKε-UTR) and a positive control reporter (pGL3-BACH1-UTR) were constructed by sub-cloning the appropriate 3' UTR sequence (sequence information is provided in supplementary information, Table S1) into the pGL3 control vector downstream of the luciferase gene [26, 27]. We also constructed lentiviral vectors to co-express pre-miR-K12-11 or pre-miR-155 along with copGFP (indicator of infection), designated as pCDH-miR-K12-11 and pCDH-miR-155, respectively. We found that both pCDH-miR-K12-11 and pCDH-miR-155 were able to repress the pGL3-IKKε-UTR-driven luciferase activity by about 40%, as compared to that of the control vector pCDH-copGFP. pGL3-BACH1-UTR luciferase activity was also repressed by both pCDH-miR-K12-11 and pCDH-miR-155 (Figure 1B). Furthermore, we found that pGL3-IKKε-UTR luciferase activity was repressed by pCDH-miR-K12-11 in a dose-dependent manner (Figure 1C).

In order to demonstrate that IKKε is a direct target of miR-K12-11, we constructed several IKKε 3' UTR reporter mutants: N, wild type; M1 with mutation in MRE1; M2 with mutation in MRE2; and M12 with mutations in both MRE1 and MRE2. Compared to wild-type reporter N, reporter M1 was able to partially resist the miR-K12-11 repression effect; reporter M2 and reporter M12 totally abolished the miR-K12-11 repression effect (Figure 1D). These results indicate that MRE1 and MRE2 are both *bona fide* binding sites of miR-K12-11, and the match between miR-K12-11 seed sequence and

Figure 1 miR-K12-11 targets the 3' UTR of IKKε. **(A)** Diagram of two miRNA responding elements (MREs) within the 3' UTR of IKKε. The seed match sites of miR-K12-11 in IKKε 3' UTR were predicted by DIANA MicroT. **(B)** miR-K12-11 down-regulates IKKε 3' UTR reporter activity in HEK293T cells. HEK293T cells were co-transfected with pRL-SV40 (5 ng), pGL3-IKKε-UTR (100 ng) and either pCDH-miR-K12-11, pCDH-mir-155 or pCDH-copGFP (500 ng). pGL3-BACH1-UTR (100 ng) was co-transfected as positive control. **(C)** miR-K12-11 down-regulates IKKε 3' UTR reporter in a dose-dependent manner. pRL-SV40 (5 ng) and pGL3-IKKε-UTR (100 ng) were co-transfected with either 0, 100, 200, or 500 ng of pCDH-miR-K12-11 (compensated to 500 ng with pCDH-copGFP) into HEK 293T cells. **(D)** Mutations of MREs in IKKε 3' UTR reporter abolish miR-K12-11 down-regulation effect. N: wild type, M1: mutated MRE1, M2: mutated MRE2, M12: mutated MRE1 and MRE2. The reporter luciferase activity was normalized to Renilla luciferase activity. **(E)** Inhibition of miR-K12-11 reverses its repression effect on IKKε 3' UTR reporter. HEK293T cells were co-transfected with pRL-SV40 (5 ng), pGL3-IKKε-UTR (100 ng) pCDH-miR-K12-11 (500 ng), together with pCDH-sponge/GFP(500 ng) or pCDH-copGFP (500 ng). Data are presented as mean ± SEM, $n = 3$.

MREs in IKKε 3' UTR is critical for miR-K12-11 function.

Then, we designed and constructed an miR-K12-11 sponge in lentiviral vector, designated as sponge/K12-11 (Figure 2E, top). The miRNA sponge is a type of long-effect competitive miRNA inhibitor, and is a transcript expressed from strong promoters that contains multiple, tandem binding sites to an miRNA of interest [28]. We found that sponge/K12-11 partially reversed the repression effect of miR-K12-11 on IKKε 3' UTR reporter

activity (Figure 1E). Thus, we conclude that IKKε is a direct target of miR-K12-11.

Ectopic expression of miR-K12-11 decreases the IKKε protein level

Since miR-K12-11 was able to repress IKKε 3' UTR reporter activity, we next sought to determine whether ectopic expression of miR-K12-11 would decrease exogenous and endogenous IKKε expression. To this end, we constructed an IKKε expression vector containing both

the coding sequence and the 3' UTR of IKKε, designated as Flag-IKKε-UTR. Flag-IKKε-UTR was co-transfected with either pCDH-miR-K12-11 or pCDH-copGFP in HEK293T cells and the exogenous IKKε expression was evaluated 48h later by western blot probing with anti-Flag antibody. miR-K12-11 obviously decreased the expression of exogenous IKKε by about 50%, as compared to the vector only control (Supplementary information, Figure S1).

In order to determine whether miR-K12-11 was able to regulate endogenous IKKε expression, we prepared lentivirus for pCDH-miR-K12-11 and pCDH-copGFP and transduced miR-K12-11 or vector control into A549 cells, a lung cancer cell line commonly used to study innate immune responses. At 72 hours post-infection, more than 90% of A549 cells were found to be copGFP-positive. We designated these cells as A549/Ctrl and A549/K12-11. Then, we carried out bulge-loop qRT-PCR to confirm miRNA expression. We found that mature miR-K12-11 was only detected in A549/K12-11 cells – the expression of which was comparable to KSHV-positive BCBL1 cells but was completely absent in A549/Ctrl cells (Figure 2A). Importantly, miR-155 was barely detected in both A549/K12-11 and A549/Ctrl cells (Figure 2B). Thus, the potential confounding effect of miR-155 could be excluded from our experiments. Then, we used immunofluorescence assay (IFA) to visually evaluate IKKε expression in single cells. We found that the copGFP-positive (Figure 2C, arrow) A549/K12-11 cells (about 80%) always exhibited decreased IKKε expression, as compared to the adjacent copGFP-negative cells (Figure 2C, bottom), while no such difference was observed in the A549/Ctrl cells (Figure 2C, top). These data indicated that miR-K12-11 was able to decrease endogenous IKKε expression in A549 cells.

In order to determine whether miR-K12-11 regulates IKKε expression at the transcriptional or translational level, we used western blot and RT-PCR or qRT-PCR, respectively. We found that IKKε protein level was decreased by about 40% in A549/K12-11 cells as compared to the A549/Ctrl cells ($P < 0.01$), while IKKε mRNA was slightly increased in A549/K12-11 cells (Figure 2D). These data suggested that miR-K12-11 could target endogenous IKKε expression by translational inhibition, but not by direct mRNA degradation.

Then, we transduced sponge/K12-11 into A549/K12-11 cells to determine whether competitive binding of sponge/K12-11 to miR-K12-11 was able to rescue IKKε expression. Indeed, the sponge-miR-K12-11 rescued IKKε expression by about 20%, as compared to vector control ($P < 0.05$; Figure 2E). We also demonstrated that the rescue effect was not due to differential miR-K12-11

expression in these cell lines (Figure 2F). Based on these experiments, we conclude that IKKε is an authentic target of miR-K12-11.

IKKε is down-regulated in KSHV-infected cell lines by miR-K12-11

As expression of miR-K12-11 can regulate either exogenous or endogenous IKKε expression in HEK293T cells and A549 cells, we sought to determine whether IKKε was similarly regulated by miR-K12-11 in KSHV naturally infected cells. First, we evaluated the endogenous expression level of IKKε in KSHV-infected PEL cells, and performed comparisons to levels found in KSHV-negative lymphoma cells. IKKε protein expression was remarkably lower in KSHV-infected PEL cells than in KSHV-negative lymphoma cells (Figure 3A, top), while IKKε mRNA levels varied (Figure 3A, bottom and Figure 3B). Based on these data, we inferred that IKKε was down-regulated in PEL cells by a post-transcriptional mechanism.

Since IKKε is targeted by both miR-K12-11 and miR-155, we sought to detect the expression of these two miRNAs. Using bulge-loop qRT-PCR, we found that the expression of miR-155 varied among those B cell lines. Ramous and Loukes have high miR-155 expression; BCBL1, BJAB, DG75 have low miR-155 expression; and JSC-1 has hardly any miR-155 expression (Figure 3C), while miR-K12-11 is only detected in KSHV-positive JSC-1 and BCBL1 cells (Figure 3D). Therefore, we speculated that miR-K12-11 played an important role in regulating IKKε expression in PEL cells, through its translation inhibition function on IKKε. In order to test this hypothesis, we transduced sponge/K12-11-copGFP or pCDH-copGFP into BCBL1 cells by lentivirus infection. At 72 h post-infection, we used IFA to screen single-cell IKKε expression. We found that about 80% of copGFP-positive cells (Figure 3E, arrow) in the sponge group had higher IKKε expression in comparison to adjacent copGFP-negative cells (Figure 3E, bottom); no such difference was observed in the control group (Figure 3E, top). These data showed that inhibition of miR-K12-11 was able to rescue IKKε expression in BCBL1 cells. Therefore, we conclude that miR-K12-11 is likely to be responsible for the regulation of IKKε expression in KSHV-infected PEL cells.

We next sought to determine whether miR-K12-11 could also regulate IKKε expression in a KSHV *de novo* infection system. KSHV/Bac36 virus was used to infect A549 cells. We designated this cell line as K/A549. We found that IKKε protein was dramatically down-regulated in K/A549 cells, in comparison to the A549 cells ($P < 0.01$; Figure 4A). In contrast, IKKε mRNA expression

Figure 2 Ectopic expression of miR-K12-11 decreases the IKK ϵ protein level. (**A**, **B**, **F**) miR-K12-11 or miR-155 expression was detected in indicated cells. Bulge-loop qRT-PCR was used to detect mature miRNA expression of indicated cells. (**C**) IKK ϵ expression was decreased in miR-K12-11-overexpressing cells. IFA was used to screen single-cell IKK ϵ expression in A549/K12-11 cells or in A549/Ctrl cells. copGFP was used as the marker of successful transduction of the indicated vector. 3 to 4 random fields are subjected to statistics of IKK ϵ repressed cell population among GFP-positive cells. (**D**) IKK ϵ expression was decreased at the protein level in A549/K12-11 cells. Endogenous protein expression of IKK ϵ was detected by western blot using the IKK ϵ antibody. mRNA level was detected by RT-PCR and qRT-PCR (**E**). Sponge/K12-11 rescues IKK ϵ expression in A549/K12-11 cells. A diagram shows the design of sponge/K12-11 (top). The band intensities were quantified using NIH ImageJ. Data are presented as mean $\pm$ SEM, $n = 3$. * $P < 0.05$; ** $P < 0.01$.

Figure 3 IKKε is down-regulated by miR-K12-11 in KSHV-positive PEL cells. **(A)** Screen of endogenous IKKε expression in various KSHV-positive and -negative B cells. Western blot and RT-PCR were used to detect IKKε expression at the protein and mRNA levels, respectively. **(B, C, D)** IKKε, miR-K155 or miR-K12-11 expression was detected in indicated cells by qRT-PCR. **(E)** Sponge/K12-11 can rescue IKKε expression in BCBL1 cells. BCBL1 cells were infected by sponge/K12-11 or control virus. copGFP acted as the indicator of successful infection. IFA was used to detect IKKε expression at the single-cell level. 3 to 4 random fields are subjected to statistics of IKKε rescued cell population among GFP-positive cells.

was only slightly decreased in the K/A549 cells (Figure 4A). We found that mature miR-K12-11 was only detect-

ed in K/A549 cells – the expression of which was comparable to BCBL1, but was completely absent in A549

Figure 4 IKKε is down-regulated by miR-K12-11 in KSHV *de novo* infected A549 cells. **(A)** IKKε expression was down-regulated in response to KSHV *de novo* infection. Western blot, RT-PCR and qRT-PCR were used to detect IKKε expression at the protein and mRNA levels, respectively. **(B, C, E)** miR-K155 or miR-K12-11 expression was detected in indicated cells by qRT-PCR. **(D)** Sponge/K12-11 can rescue IKKε expression in K/A549 cells. The band intensities were quantified using NIH ImageJ. Data are presented as mean ± SEM, $n = 3$. * $P < 0.05$; ** $P < 0.01$.

cells (Figure 4B), while miR-155 was hardly detected in both cell lines (Figure 4C). To determine whether inhibition of miR-K12-11 could rescue IKKε expression, K/A549 cells were infected with lentivirus of sponge/K12-11-puro or control pCDH-puro. We found that IKKε expression was rescued in sponge/K12-11-expressing cells, as compared to control cells ($P < 0.05$; Figure 4D). We also demonstrated that the rescue effect was not due to differential miR-K12-11 expression in these cell lines (Figure 4E). These data demonstrated that miR-K12-11 can also regulate IKKε expression in a KSHV *de novo* infection system.

Expression of miR-K12-11 attenuates the IFN signaling pathway

The above experiments showed that IKKε was an authentic target of miR-K12-11; however, the functional significance of the down-regulation of IKKε by miR-K12-11 remained uncharacterized. IKKε is known to be a critical modulator of the IFN signaling pathway, which acts by phosphorylating IRF3 and IRF7. We hypothesized that down-regulation of IKKε by miR-K12-11 could attenuate the IFN signaling pathway by decreasing IRF3 phosphorylation, allowing the virus to interfere with antiviral immunity. To test this hypothesis, we employed the Sendai virus (SeV) infection as a model pathogen to compare subsequent IFN signaling between A549/Ctrl and A549/K12-11 cells. The cells were infected with SeV for 1 h. We used qRT-PCR to check the mRNA level of various genes in the IFN signaling pathway. We found that *IFNB* was activated by 6 h, while *IFNA1*, *IFNA2* and *IFNA14* were activated by 12 h. The relative mRNA expression for these four IFNs was decreased to about 50% at 12 h in the A549/K12-11 cells as compared with the A549/Ctrl cells (Figure 5A). In addition, ISGs (like *IP-10*, *ISG15* and *ISG56*) were relatively less activated to different extents at 6 and 12 h in A549/K12-11 cells, compared with A549/Ctrl cells; *IL8*, an IFN non-related gene, was not activated in either cell line (Figure 5B). In a previous study, IKKε was found to be required for the activation of a subset of IKKε-dependent ISGs in mice [23]. We sought to determine whether IKKε-dependent ISGs also existed in humans, and were affected by IKKε down-regulation. We chose three antiviral ISGs for evaluation: *ADAR1*, *OAS1* and *IFIT3*. We observed that *ADAR1* and *OAS1* were slightly affected by miR-K12-11, while *IFIT3* was dramatically repressed in A549/K12-11 cells, in comparison to A549/Ctrl cells (Figure 5C). Taken together, these results demonstrated that the expression of miR-K12-11 could repress activation of multiple IFN pathway-related genes.

We next examined whether miR-K12-11 could influ-

ence antiviral immunity in A549 cells. To this end, A549/K12-11 and A549/Ctrl cells were challenged with VSV. The VSV titer at 24 hpi (hours post infection) was measured using plaque assay. We found that A549/K12-11 had as high as 2-fold more of virus titer than the control cells ($P < 0.05$; Figure 5D), suggesting that A549/K12-11 had weaker antiviral immune response. These results provided further demonstration that miR-K12-11 was able to attenuate the IFN signaling pathway and interfere with antiviral immunity.

We then tested if miR-K12-11 could inhibit the phosphorylation of IRF3 through targeting IKKε, since IKKε-mediated IRF3 phosphorylation is responsible for the activation of IRF3. We found that A549/K12-11 cells had lower expression of IKKε (Figure 5E, compare lanes 1, 2 and lanes 3, 4) and lower levels of phosphorylated IRF3 upon SeV infection (Figure 5E, compare lanes 2 and 4; the expression of p-IRF3 has been normalized to total IRF3). Thus, it is likely that miR-K12-11 attenuates IFN signaling by down-regulating IKKε-mediated IRF3 activation.

miR-K12-11 inhibits IKKε's synergistic effect on KSHV reactivation

Recently, it has been shown that TLR8 activation or VSV infection can reactivate KSHV lytic replication [13], which suggests that activation of innate immunity may be related to viral reactivation. In addition, it has also been shown that miR155 can stabilize Epstein-Barr virus (EBV) latency by attenuating IKKε-mediated NF-κB activity [29]. Therefore, we sought to test whether miR-K12-11 was involved in maintenance of KSHV latency through its targeting of IKKε. We found that forced expression of IKKε alone was unable to activate RTA (a switch molecule of KSHV reactivation) expression in 293/Bac cells, a HEK293T cell line harboring the KSHV/Bac36 episome (Figure 6A) [30]. However, when we used TPA to induce KSHV reactivation, interestingly, TPA alone was able to induce about 15-fold of RTA activation and in combination with IKKε could induce about 30-fold activation of RTA (Figure 6B). This suggests a synergistic role for IKKε in KSHV reactivation. Furthermore, we transduced sponge/K12-11 or a control vector into K/A549 cells, and then we used VSV to infect these cells and compared the KSHV reactivation rates. We observed that RTA could be induced by VSV infection, by about 4-fold in the control group and by about 6-fold in the sponge group (Figure 6C). The same phenomena were observed in the case of ORF65 (a late gene involved in the KSHV lytic program, and functioning in virion assembly). VSV alone was able to induce ORF65 by 4.5-fold, and when coupled with Sponge/K12-11 it

Figure 5 Overexpression of miR-K12-11 attenuates the IFN signaling pathway. Activation of IFN pathway-related genes was detected using qRT-PCR at 6 and 12 h after SeV infection. **(A)** Type I interferon molecules. **(B)** Common ISGs and IL-8 (control). **(C)** IKKε-dependent ISGs. Delta-delta-CT method was used and all values were normalized to *ACTB* (endogenous control). **(D)** miR-K12-11 enhanced VSV replication in A549 cells. VSV was used to infect A549/K12-11 cells and A549/Control cells at MOI = 0.1. Supernatants were collected at 24 hpi and viral titer was measured using plaque assay. **(E)** miR-K12-11 decreases IRF3 phosphorylation during SeV infection. The band intensities were quantified using NIH ImageJ. Relative p-IRF3 expression was normalized to total IRF3 and GAPDH. Data are presented as mean ± SEM, $n = 3$. * $P < 0.05$.

Figure 6 miR-K12-11 inhibits IKKε synergistic effect on KSHV reactivation. **(A)** IKKε alone is not sufficient to induce RTA expression. Flag-IKKε or control vector was transfected into 293/Bac cells, and RT-PCR was carried out to detect RTA expression 48 h later. **(B)** IKKε can enhance the induction effect of TPA. Flag-IKKε or control vector was transfected into 293/Bac cells, and 24 h later TPA was added to induce lytic replication. An additional 24 h later, RT-PCR was carried out to detect RTA expression. The values were normalized to mock control. **(C, D)** Sponge/K12-11 enhances VSV induction of KSHV lytic replication. Sponge/K12-11 or control vector was induced into K/A549 cells, and VSV infection occurred at MOI=0.1. After 24 hours, RTA **(C)** or ORF65 **(D)** expression was detected by RT-PCR. The values were normalized to mock control. **(E)** A working model of the mechanism of miR-K12-11 action on IFN signaling. miR-K12-11 targets IKKε and decreases its proteins level. miR-K12-11 attenuates IFN signaling triggered by various PRRs by (1) decreasing IKKε-dependent IRF3/IRF7 phosphorylation and attenuating IFN synthesis and (2) by decreasing activation of IKKε-dependent ISG transcription. Meanwhile, miR-K12-11 inhibits IKKε synergistic effect(s) on RTA transcription and KSHV reactivation. Data are presented as mean ± SEM, $n = 3$. * $P < 0.05$; ** $P < 0.01$.

induced ORF65 by about 7.5-fold (Figure 6D). These experiments suggested that inhibition of miR-K12-11 was able to enhance VSV-induced KSHV reactivation. Based on these data, we conclude that miR-K12-11 likely contributes to KSHV latency control by targeting the IKKε synergistic effect on KSHV reactivation.

Discussion

Type I IFN signaling is considered the most important immune response in antiviral innate immunity. KSHV

utilizes multiple strategies to block IFN signaling in order to evade being eliminated by the immune system and to establish long-term latency in the host. Several viral proteins have already been characterized for their abilities to interfere with the IFN signaling pathway, including the vIRFs, ORF45, RTA and K-bZIP [6-9]. Notably, most of these proteins are expressed specifically in the lytic phase, except vIRF3 [31]. It remains largely unclear, however, as to how KSHV is able to control IFN signaling during viral latency. Recently, a model was reported in which a major latent protein, LANA, was able

to inhibit *IFNB* expression by competing with IRF3 for binding to the *IFNB* promoter [32]. Since activation of IFNAs and IFNB are very heterogeneous [33], this proposed model is unlikely to fully explain every molecular scenario.

In this report, we demonstrated that viral miRNA-mediated gene regulation is used by KSHV to control IFN signaling. We found that IKK ϵ , an important modulator in IFN signaling, is an authentic target of miR-K12-11. Ectopic expression of miR-K12-11 decreased exogenous and endogenous IKK ϵ protein levels. Inhibition of miR-K12-11 partially restored IKK ϵ expression in KSHV-infected cells. More importantly, expression of miR-K12-11 attenuated IFN signaling by decreasing IKK ϵ -mediated IRF3/7 phosphorylation and inhibiting the activation of IKK ϵ -dependent ISGs, so that miR-K12-11 could suppress antiviral immunity (Figure 6E). During the preparation of this manuscript, Boshoff *et al.* showed that miR-132 was inducible by KSHV infection and targeted CBP/P300-mediated IFN signaling [34]. This result complements our novel finding of a viral miRNA modulating host IFN signaling. In our experiment, we used KSHV-free A549 cells to study the function of miR-K12-11 in modulating IFN signaling, so that we can exclude the confounding effects of other viral factors. Since miR-K12-11 is largely expressed during viral latency, we believe that miR-K12-11 is important for modulation of IFN signaling during KSHV latency; however, further studies are still needed to clarify the roles of different viral factors in this process.

Generally, IRF3 and IRF7 are phosphorylated by non-canonical IKK kinases TBK1 and IKK ϵ [21, 22], except in some endosomal pathways in pDC [35]. The relationship between IKK ϵ and TBK1 is debatable. TBK1 is universally expressed, while IKK ϵ has some preference for expression in immune cells and can be induced by lipopolysaccharide [36]. Further evidence suggests that the functions of IKK ϵ and TBK1 are not totally redundant in IFN signaling. A subset of antiviral ISGs were found to be transactivated in the presence of IKK ϵ [23]. Moreover, MAVS, an important adaptor in the IFN signaling pathway, was shown to recruit IKK ϵ but not TBK1 to the mitochondria following viral infection [37]. As a result, IKK ϵ appears to play a unique role in the IFN signaling pathway, as opposed to merely acting as a backup of TBK1. In the study described herein, we showed that miR-K12-11 decreased the expression of IFNs, common ISGs and IKK ϵ -dependent ISGs upon SeV infection. Moreover, miR-K12-11 decreased IKK ϵ -mediated IRF3 phosphorylation and therefore attenuated IFN signaling. Thus, our data suggested that deregulation of IKK ϵ by miR-K12-11 exert profound effects on IFN signaling.

Here, we also showed that overexpression of IKK ϵ alone was insufficient to activate RTA transcription, but that overexpression of IKK ϵ could synergistically enhance the reactivation effect of TPA. Meanwhile, inhibition of miR-K12-11 could enhance the induction effect of VSV infection in K/A549 cells. Thus, we have provided evidence that down-regulation of IKK ϵ by miR-K12-11 facilitates KSHV latency maintenance (Figure 6E). Indeed, it has been reported that miR-155 can stabilize the EBV latent program by attenuating NF- κ B activity, possibly mediated by IKK ϵ targeting [29]. We thus infer that IKK ϵ targeting may be a common strategy to facilitate latency maintenance for the gamma herpesviruses.

The event of secondary pathogen infection of KSHV latently infected cells is known to lead to the activation of the innate immune response, which may then promote cell death and hamper KSHV survival in the host. In order to persist in the host, it is reasonable that KSHV is induced to lytic phase and spreads its new virions to new host cells [38]. However, it has been observed that KSHV latently infected PEL cells are always co-infected with EBV [39]; viral co-infections have also been identified in KS cells [40, 41]. This finding suggests that some negative regulatory mechanisms may exist to suppress the secondary infection-induced innate immune response and inhibit viral reactivation, so that KSHV can co-exist with another virus in the same cell and maintain cell homeostasis. In this paper, we have demonstrated that miR-K12-11 is capable of attenuating IFN signaling during the latent stage by targeting IKK ϵ ; moreover, we demonstrated that miR-K12-11 can inhibit KSHV reactivation induced by VSV infection. Thus, IKK ϵ targeting by miR-K12-11 may represent one of the negative regulatory mechanisms in pathogen secondary infection.

Materials and Methods

Cell lines

BCBL1 (KSHV-positive PEL cells), JSC-1 (KSHV- and EBV-positive PEL cells), and BJAB, Ramos, Loukes and DG75 (four KSHV-negative B cells) cell lines were generously provided by Dr Erle S Robertson (University of Pennsylvania, USA).

Vero/Bac36 (a Vero cell line harboring the KSHV genome inserted into a bacterial artificial chromosome) was a gift from Dr SJ Gao (The University of Texas, USA). K/A549 was established through infection of A549 cells with KSHV, as described [30], and selected with 500 μ M hygromycin (Calbiochem, San Diego, CA, USA). A549/K12-11 and A549/Ctrl were established by infection of indicated lentivirus, according to the manufacturer's instructions (System Bioscience). 293/Bac, A549, and Vero cells came from our in-lab stock.

Constructs

Wild-type or mutated 3' UTR reporters were constructed by

cloning the appropriate 3' UTR sequence into the pGL3 control vector (Promega, Madison, WI, USA) downstream of the firefly luciferase gene (primers used are listed in Supplementary information, Table S1). pCDH-miR-K12-11 and pCDH-miR-155 were constructed by cloning pre-miR-K12-11 (amplified from BCBL1 DNA) and pre-miR-155 (amplified from pSIF-BIC; kindly provided by Dr Mofang Liu, Shanghai Institute of Biochemistry and Cell Biology, China) into pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA, USA). pCDH-sponge/K12-11 is constructed by annealing sponge primers (supplementary information, Table S1) and sub-cloning into vectors pCDH-CMV-MCS-EF1-copGFP or pCDH-CMV-MCS-EF1-puro (System Bioscience). Flag-IKKε was a gift from Dr Hongbing Shu (Wuhan University, Wuhan, China). Flag-IKKε-UTR was constructed by sequentially sub-cloning Flag-IKKε CDS (with stop codon) and IKKs 3' UTR into pCDH-CMV-MCS-EF1-puro.

Viruses

SeV and VSV were kindly provided by Dr Bing Sun (Institut Pasteur of Shanghai, China). Lentivirus was prepared according to the manufacturer's instructions (System Bioscience). KSHV was prepared from Vero/Bac36 cells. Briefly, Vero/Bac36 cells were synchronized at G1 phase by serum starvation for approximately 24 hours. Then, 10% bovine serum was added and cultured for 12-16 hours. After that, TPA (P1585; Sigma-Aldrich, St Louis, MO, USA) and VPA (P4543; Sigma) were added to a final concentration of 25 ng/ml and 3 mM, respectively. At 120 hours post-induction, supernatants were collected and filtered through a 0.45 μm filter; filtrates were aliquoted and stored at -80°C until further use.

Reagents and antibodies

Puromycine (P9620) was purchased from Sigma-Aldrich. Anti-actin (A2066) and anti-IKKε (14907) were obtained from Sigma-Aldrich. Anti-IRF3 (4962) and anti-p-IRF3 (Ser396, 4947) were from Cell Signaling (Danvers, MA, USA). Anti-GAPDH (M20006) was from Abmart (China).

In vitro expression reporter assay

Firefly luciferase 3' UTR reporter construct (100 ng per well), miRNA construct or control plasmid (each, 500 ng per well) were co-transfected along with pRL-SV40 Renilla luciferase construct (5 ng per well; Promega) into HEK293T cells in 12-well plates. Cell extracts were prepared 48 hours after transfection, and luciferase activity was measured with the Dual-Luciferase Reporter Assay system (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity. To carry out miRNA dose-dependent experiments, pCDH-miR-K12-11 was transfected at 0, 100, 200, and 500 ng (compensated to 500 ng per well with control vector).

Plaque assay

A549 cells were infected with VSV at MOI=0.1 for 1 h. Cells were washed with PBS and fresh media was added. The supernatants were harvested 24 h later and series diluted for use to infect confluent Vero cells cultured in 12-well plates. At 1 h post-infection, supernatants were discarded and 3% methylcellulose was overlaid. Three days later, the overlay was removed and cells were stained for 20 min with 0.1% crystal violet (prepared with 27%

formaldehyde solution). Stained plaques were counted, averaged and multiplied by the appropriate dilution factor to determine viral titer as PFU/ml.

qRT-PCR

miRNA quantification: Bulge-loop™ miRNA qRT-PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for miR-K12-11 and miR-155 are designed by RiboBio (Guangzhou, China). Briefly, the total RNA was extracted with TRIzol Reagent (Invitrogen). Then, miRNA bulge-loop was reverse transcribed with the First-Strand cDNA Synthesis Kit (Fermentas UAB, Fermentas International Inc., Burlington, Canada) and quantified by qPCR using SYBR Green Real-Time PCR Master Mix Kit (Toyobo, Osaka, Japan) according to the indicated manufacturer's instructions.

mRNA quantification: Similar as miRNA quantification, except that cDNA is reverse transcribed using oligo(dT)₁₈ primer. The primers used are listed in supplementary information, Table S1.

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References

- 1 Kawai T, Akira S. Innate immune recognition of viral infection. *Nat Immunol* 2006; 7:131-137.
- 2 Bowie AG, Unterholzner L. Viral evasion and subversion of pattern-recognition receptor signalling. *Nat Rev Immunol* 2008; 8:911-922.
- 3 Chang Y, Cesarman E, Pessin MS, *et al.* Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994; 266:1865-1869.
- 4 Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N Engl J Med* 1995; 332:1186-1191.
- 5 Soulier J, Grollet L, Oksenhendler E, *et al.* Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castelman's disease. *Blood* 1995; 86:1276-1280.
- 6 Zhu FX, King SM, Smith EJ, Levy DE, Yuan Y. A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc Natl Acad Sci USA* 2002; 99:5573-5578.
- 7 Yu Y, Wang SE, Hayward GS. The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteasome-mediated degradation. *Immunity* 2005; 22:59-70.
- 8 Rezaee SA, Cunningham C, Davison AJ, Blackburn DJ. Ka-

- posi's sarcoma-associated herpesvirus immune modulation: an overview. *J Gen Virol* 2006; **87**(Pt 7):1781-1804.
- 9 Lefort S, Soucy-Faulkner A, Grandvaux N, Flamand L. Binding of Kaposi's sarcoma-associated herpesvirus K-bZIP to interferon-responsive factor 3 elements modulates antiviral gene expression. *J Virol* 2007; **81**:10950-10960.
- 10 Vieira J, O'Hearn P, Kimball L, Chandran B, Corey L. Activation of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) lytic replication by human cytomegalovirus. *J Virol* 2001; **75**:1378-1386.
- 11 Lu C, Zeng Y, Huang Z, *et al.* Human herpesvirus 6 activates lytic cycle replication of Kaposi's sarcoma-associated herpesvirus. *Am J Pathol* 2005; **166**:173-183.
- 12 Qin D, Zeng Y, Qian C, *et al.* Induction of lytic cycle replication of Kaposi's sarcoma-associated herpesvirus by herpes simplex virus type 1: involvement of IL-10 and IL-4. *Cell Microbiol* 2008; **10**:713-728.
- 13 Gregory SM, West JA, Dillon PJ, Hilscher C, Dittmer DP, Damania B. Toll-like receptor signaling controls reactivation of KSHV from latency. *Proc Natl Acad Sci USA* 2009; **106**:11725-11730.
- 14 Umbach JL, Cullen BR. In-depth analysis of Kaposi's sarcoma-associated herpesvirus microRNA expression provides insights into the mammalian microRNA-processing machinery. *J Virol* 2010; **84**:695-703.
- 15 Cai X, Lu S, Zhang Z, Gonzalez CM, Damania B, Cullen BR. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc Natl Acad Sci USA* 2005; **102**:5570-5575.
- 16 Pfeffer S, Sewer A, Lagos-Quintana M, *et al.* Identification of microRNAs of the herpesvirus family. *Nat Methods* 2005; **2**:269-276.
- 17 Samols MA, Hu J, Skalsky RL, Renne R. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J Virol* 2005; **79**:9301-9305.
- 18 Lei X, Bai Z, Ye F, *et al.* Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat Cell Biol* 2010; **12**:193-199.
- 19 Lu F, Stedman W, Yousef M, Renne R, Lieberman PM. Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway. *J Virol* 2010; **84**:2697-2706.
- 20 Bellare P, Ganem D. Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation. *Cell Host Microbe* 2009; **6**:570-575.
- 21 Fitzgerald KA, McWhirter SM, Faia KL, *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 2003; **4**:491-496.
- 22 Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 2003; **300**:1148-1151.
- 23 Tenover BR, Ng SL, Chua MA, McWhirter SM, García-Sastre A, Maniatis T. Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. *Science* 2007; **315**:1274-1278.
- 24 Renne R, Zhong W, Herndier B, *et al.* Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat Med* 1996; **2**:342-346.
- 25 Maragkakis M, Reczko M, Simossis VA, *et al.* DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic Acids Res* 2009; **37**:W273-W276.
- 26 Gottwein E, Mukherjee N, Sachse C, *et al.* A viral microRNA functions as an orthologue of cellular miR-155. *Nature* 2007; **450**:1096-1099.
- 27 Skalsky RL, Samols MA, Plaisance KB, *et al.* Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. *J Virol* 2007; **81**:12836-12845.
- 28 Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods* 2007; **4**:721-726.
- 29 Lu F, Weidmer A, Liu CG, Volinia S, Croce CM, Lieberman PM, *et al.* Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol* 2008; **82**:10436-10443.
- 30 Zhou FC, Zhang YJ, Deng JH, *et al.* Efficient infection by a recombinant Kaposi's sarcoma-associated herpesvirus cloned in a bacterial artificial chromosome: application for genetic analysis. *J Virol* 2002; **76**:6185-6196.
- 31 Joo CH, Shin YC, Gack M, Wu L, Levy D, Jung JU. Inhibition of interferon regulatory factor 7 (IRF7)-mediated interferon signal transduction by the Kaposi's sarcoma-associated herpesvirus viral IRF homolog vIRF3. *J Virol* 2007; **81**:8282-8292.
- 32 Cloutier N, Flamand L. Kaposi sarcoma-associated herpesvirus latency-associated nuclear antigen inhibits interferon (IFN) beta expression by competing with IFN regulatory factor-3 for binding to IFNB promoter. *J Biol Chem* 2010; **285**:7208-7221.
- 33 Genin P, Lin R, Hiscott J, Civas A. Differential regulation of human interferon A gene expression by interferon regulatory factors 3 and 7. *Mol Cell Biol* 2009; **29**:3435-3450.
- 34 Lagos D, Pollara G, Henderson S, *et al.* miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. *Nat Cell Biol* 2010; **12**:513-519.
- 35 Kawai T, Sato S, Ishii KJ, *et al.* Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol* 2004; **5**:1061-1068.
- 36 Shimada T, Kawai T, Takeda K, *et al.* IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases. *Int Immunol* 1999; **11**:1357-1362.
- 37 Paz S, Vilasco M, Arguello M, *et al.* Ubiquitin-regulated recruitment of IkappaB kinase epsilon to the MAVS interferon signaling adapter. *Mol Cell Biol* 2009; **29**:3401-3412.
- 38 West JA, Damania B. Kaposi's sarcoma-associated herpesvirus and innate immunity. *Future Virol* 2010; **5**:185-196.
- 39 Horenstein MG, Nador RG, Chadburn A, *et al.* Epstein-Barr virus latent gene expression in primary effusion lymphomas containing Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8. *Blood* 1997; **90**:1186-1191.
- 40 Giraldo G, Beth E, Huang ES. Kaposi's sarcoma and its relationship to cytomegalovirus (CMV). III. CMV DNA and CMV early antigens in Kaposi's sarcoma. *Int J Cancer* 1980; **26**:23-29.
- 41 Kempf W, Adams V, Wey N, *et al.* CD68+ cells of monocyte/macrophage lineage in the environment of AIDS-associated

and classic-sporadic Kaposi sarcoma are singly or doubly infected with human herpesviruses 7 and 6B. *Proc Natl Acad*

Sci USA 1997; **94**:7600-7605.

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