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VIRUS-CELL INTERACTIONS

Kaposi's Sarcoma-Associated Herpesvirus MicroRNAs Target GADD45B To Protect Infected Cells from Cell Cycle Arrest and Apoptosis

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ABSTRACT Kaposi's sarcoma is one of the most common malignancies in HIV-infected individuals. The responsible agent, Kaposi's sarcoma-associated herpesvirus (KSHV; HHV8), expresses multiple microRNAs (miRNAs), but the targets and functions of these miRNAs are not completely understood. After infection in primary endothelial cells with KSHV, *growth arrest DNA damage-inducible gene 45 beta* (*GADD45B*) is one of the most repressed genes using genomic expression profiling. *GADD45B* was also repressed in mRNA expression profiling experiments when KSHV miRNAs were introduced to uninfected cells. We hypothesized that KSHV miRNAs target human *GADD45B* to protect cells from consequences of DNA damage, which can be triggered by viral infection. Expression of GADD45B protein is induced by the p53 activator, Nutlin-3, and KSHV miRNA-K9 inhibits this induction. In addition, Nutlin-3 increased apoptosis and cell cycle arrest based on flow cytometry assays. KSHV miR-K9 protected primary endothelial cells from apoptosis and cell cycle arrest following Nutlin-3 treatment. Similar protective phenotypes were seen for targeting GADD45B with short interfering RNAs (siRNAs), as with miR-K9. KSHV miR-K9 also decreased the protein levels of cleaved caspase-3, cleaved caspase-7, and cleaved poly(ADP-ribose) polymerase (PARP). In B lymphocytes latently infected with KSHV, specific inhibitors of KSHV miR-K9 led to increased GADD45B expression and apoptosis, indicating that miR-K9 is important for reducing apoptosis in infected cells. Furthermore, ectopic expression of GADD45B in KSHV-infected cells promoted apoptosis. Together, these results identify a new miRNA target and demonstrate that KSHV miRNAs are important for protecting infected cells from DNA damage responses.

IMPORTANCE Kaposi's sarcoma-associated herpesvirus is a leading cause of cancers in individuals with AIDS. Promoting survival of infected cells is essential for maintaining viral infections. A virus needs to combat various cellular defense mechanisms designed to eradicate the viral infection. One such response can include DNA damage response factors, which can promote an arrest in cell growth and trigger cell death. We used a new approach to search for human genes repressed by small nucleic acids (microRNAs) expressed by a gammaherpesvirus (KSHV), which identified a gene called *GADD45B* as a target of microRNAs. Repression of GADD45B, which is expressed in response to DNA damage, benefited survival of infected cells in response to a DNA damage response. This information could be used to design new treatments for herpesvirus infections.

KEYWORDS DNA damage, Kaposi's sarcoma-associated herpesvirus, cell cycle, microRNA

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Kaposi's sarcoma-associated herpesvirus (KSHV) is the responsible agent for Kaposi's sarcoma and a subset of lymphoproliferative malignancies, including primary effusion lymphoma and multicentric Castleman disease, and is often associated with HIV infection. In addition to viral proteins, KSHV also expresses a total of 25 mature microRNAs (miRNAs; 18 of the 25 are relatively more abundant) (1, 2), which are short RNAs that target mRNAs and generally lead to repression of gene expression (3). These miRNAs are newly appreciated molecules that can aid the viral infection in the face of host responses to restrict infection. Some recently identified functions of viral miRNAs include immune evasion and restricting apoptosis (reviewed in references 4–6). A couple of KSHV miRNA targets relevant to this study include p21 (7) and caspase-3 (8), which have similar effects of protecting KSHV-infected cells from apoptosis. We sought host genes that are repressed after infection with KSHV or with introduction of KSHV miRNA mimics and identified the *growth arrest and DNA-damage-inducible 45 beta* (*GADD45B*) gene as one of the most repressed genes under these two conditions. Some of the host factors involved in repairing host DNA damage are also involved in sensing DNA from viral infections, and viral infection can induce a DNA damage response, which can result in activation of an interferon response (9). This led to the hypothesis that KSHV represses specific DNA damage factors in order to protect infected cells from antiviral responses stimulated by these host factors.

The growth arrest and DNA damage-inducible gene family plays roles in stress responses after a cell encounters growth arrest conditions and DNA-damaging agents. *GADD45* family proteins are commonly repressed in multiple types of cancers (10). This family of proteins can cooperate to repress cell growth in response to various stress inducers (11, 12). GADD45B can also regulate inflammatory responses from interleukin-6 (IL-6), IL-18, and IL-12, tumor necrosis factor (TNF), and transforming growth factor β1 (TGF-β1) (13–16). Furthermore, *GADD45B* can be induced by the innate immune activator lipopolysaccharide (LPS) (17). GADD45B has also been shown to be important for production of gamma interferon in response to cytokines (14). Mice deficient for GADD45B have granulocytes and macrophages that are defective in their chemotactic responses to lipopolysaccharide and IL-8 (18). Hypoxia, which is an inducer of GADD45B expression (19), can also stimulate lytic replication in KSHV infections (20). KSHV infection can also upregulate hypoxia inducible factor (HIF), and both hypoxia and HIF-2α have been shown to induce GADD45B expression (21).

Here, we report that *GADD45B* is targeted by multiple KSHV miRNAs for repression. We show that repression of *GADD45B* by KSHV miRNAs can inhibit apoptosis induced by a p53 activator, Nutlin-3. In the context of KSHV infection, both an antisense inhibitor of a specific KSHV miRNA and ectopic expression of GADD45B promote apoptosis. These results suggest that some KSHV miRNA functions include modulating DNA damage response factors to promote survival of infected cells in the face of stress signals.

RESULTS

GADD45B expression is repressed by KSHV infection and specific KSHV miRNAs. We utilized our previously published data sets that investigated changes in human gene expression in response to KSHV infection or in separate assays with cells transfected with KSHV miRNA mimics. We focused on mRNA expression changes after KSHV infection (22) or after transfection of a pool of KSHV miRNAs (23) and found that the *growth arrest and DNA-damage-inducible 45 beta* (*GADD45B*) gene was the 9th most repressed gene out of 13,795 microarray probes when combining these two data sets (Fig. 1A). We note that previous data demonstrated miRNA mimics are not overrepresented in the RNA-induced silencing complex, as determined by argonaute immunoprecipitation experiments (23, 24). The reduced expression of *GADD45B* suggested that this gene was directly targeted for repression by KSHV miRNAs. Transfection of individual KSHV miRNAs in primary endothelial cells revealed that multiple KSHV miRNAs repressed endogenous GADD45B protein (Fig. 1B and C).

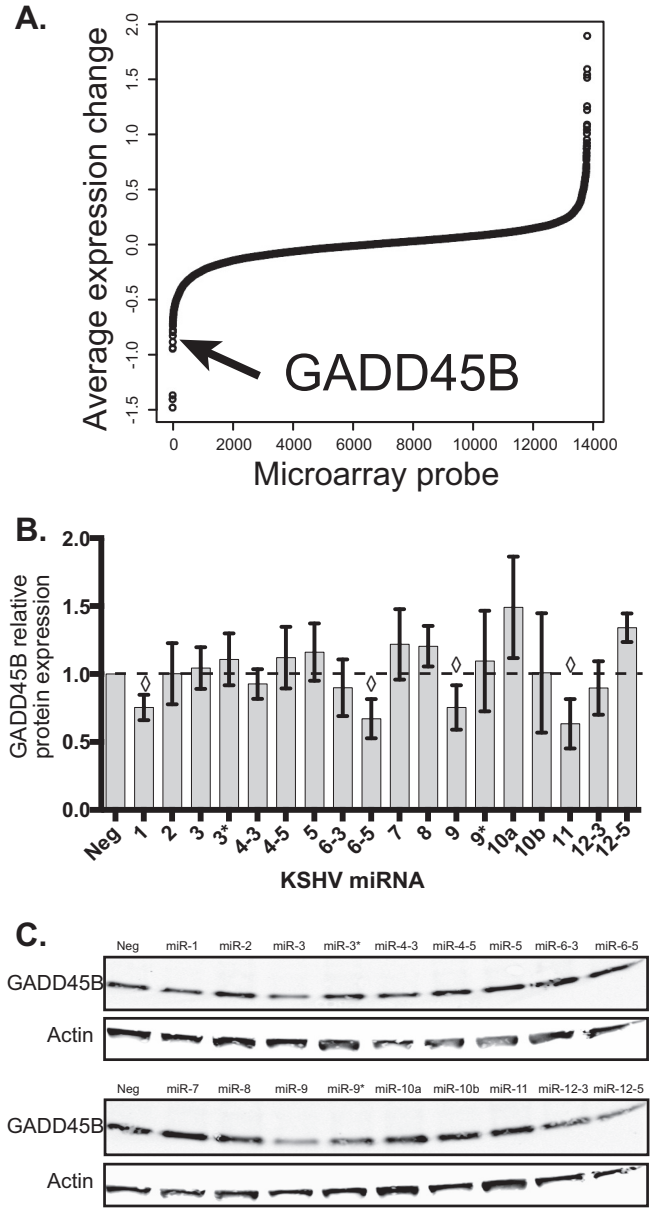

FIG 1 GADD45B expression is repressed by KSHV infection and specific KSHV miRNAs. (A) Microarray data were analyzed for changes after KSHV infection (48 h) or transfection of KSHV miRNAs (30 h). Average expression changes are shown from the two conditions and sorted by expression change. The arrow shows the location of the probe corresponding to GADD45B. (B) Primary endothelial cells were transfected with individual miRNA mimics and harvested 48 h after transfection. Protein expression changes of GADD45B (normalized to the loading control beta-actin) were obtained by immunoblotting using fluorescently labeled secondary antibodies and normalized to a nontargeting negative-control miRNA (miR-Neg). ♢, *P* value of <0.05 compared to miR-Neg using the Student *t* test. (C) A representative Western blot is shown for GADD45B (18 kDa) and beta-actin (45 kDa). The loading control was beta-actin.

KSHV miRNAs target the 3′UTR of *GADD45B*. We sought to determine if specific KSHV miRNAs directly targeted *GADD45B*. Investigation of the 3′untranslated region (UTR) of *GADD45B* for miRNA seed-matching sequences revealed multiple potential target sites for KSHV miRNAs (Fig. 2A). We cloned the full-length 3′UTR into a luciferase reporter and cotransfected cells with the luciferase reporter and a negative-control miRNA or KSHV miRNAs. Additional control conditions included the parental luciferase reporter lacking the *GADD45B* 3′UTR. We found the *GADD45B* 3′UTR luciferase reporter activity was specifically repressed by kshv-miR-K12-9-3p (miR-K9) as well as other KSHV

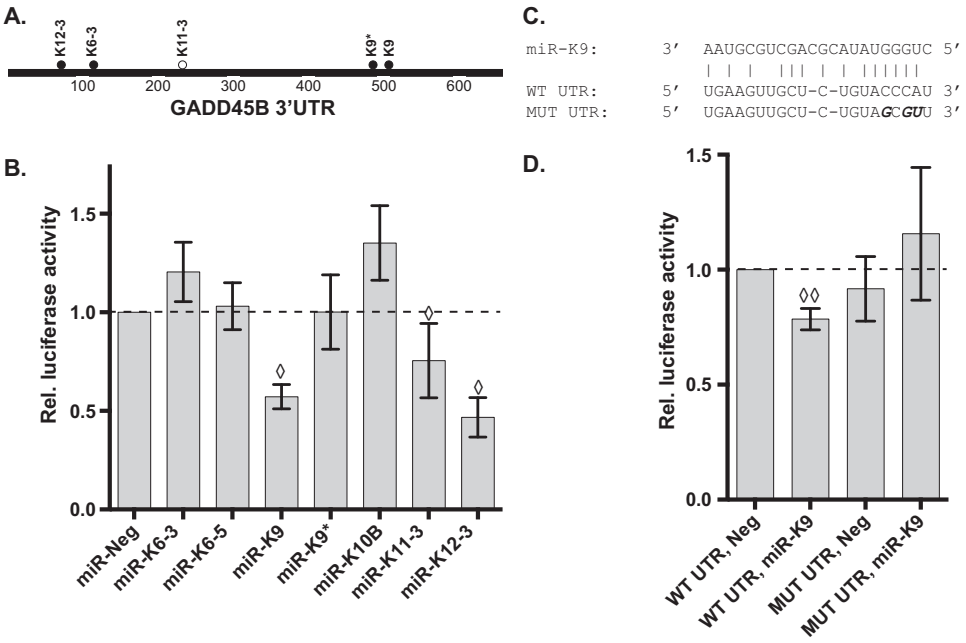

FIG 2 KSHV miRNAs target the 3′UTR of *GADD45B*. (A) Sequences show the predicted target site for miRNAs. Filled circles are for perfect seed matches, and the open circle denotes an imperfect seed-matching site. (B) Cells were transfected with a *GADD45B* 3′UTR reporter and various KSHV miRNAs. Data are presented compared to an internal control luciferase reporter and normalized to the miR-Neg control. (C) Point mutations to disrupt miR-K9 interaction with the 3′UTR are shown. (D) Luciferase assay results are shown with the wild-type *GADD45B* 3′UTR (WT UTR) on the left and the mutant 3′UTR (MUT UTR) on the right with either the control miRNA (Neg) or miR-K9. *P* values of <0.05 (◊) and <0.01 (◊ ◊) were determined compared to miR-Neg using the Student *t* test.

miRNAs (Fig. 2B). We mutated the specific site suspected to be targeted by miR-K9, and this mutant 3′UTR reporter was no longer repressed by miR-K9 (Fig. 2C and D). Based on the combined immunoblot data (Fig. 1B and C) and the *GADD45B* 3′UTR luciferase data, we focused on miR-K9 and associated phenotypes in subsequent experiments.

Repression of GADD45B results in lower levels of apoptosis markers in response to Nutlin-3 treatment. Previously, it has been shown that p53 activators like Nutlin-3 can induce GADD45B protein expression and promote apoptosis (25). We hypothesized that miR-K9 represses *GADD45B* expression to inhibit apoptosis in response to certain stress inducers. We transfected miR-K9 into primary endothelial cells (human umbilical vein endothelial cells [HUVECs]) and measured protein expression changes in GADD45B and standard markers of apoptosis [cleaved poly(ADP-ribose) polymerase (PARP), caspase-3, and caspase-7]. We found that miR-K9 reduced expression of GADD45B and these markers of apoptosis (Fig. 3A). As expected, Nutlin-3 increased expression of GADD45B and these apoptosis markers in cells transfected with the control miRNA. However, in the presence of miR-K9, Nutlin-mediated activation of GADD45B and these specific apoptosis markers was dramatically reduced (Fig. 3A), suggesting that miR-K9 protected cells from Nutlin-induced apoptosis. Similar results were observed using short interfering RNAs (siRNAs) that directly target *GADD45B* (Fig. 3B).

Repression of miR-K9 results in increased apoptosis markers in KSHV-infected cells. In addition to introducing miR-K9 to uninfected cells, we inhibited endogenous miR-K9 in KSHV-infected cells (BCBLs) using antisense inhibitors with locked nucleic acids (LNAs). In cells transfected with the antisense inhibitors to miR-K9, we observed an increase in protein levels of GADD45B, cleaved PARP, cleaved caspase-3, and cleaved caspase-7 (Fig. 4). These data suggest that miR-K9 plays a role in inhibiting apoptosis in a patient-derived KSHV-infected cell line. It should be noted that LNA inhibition of miR-K9 results in increased expression of multiple miR-K9 target genes. The phenotypes observed with LNA inhibitors of miR-K9 are likely the result of changes in expression of multiple miR-K9 target genes.

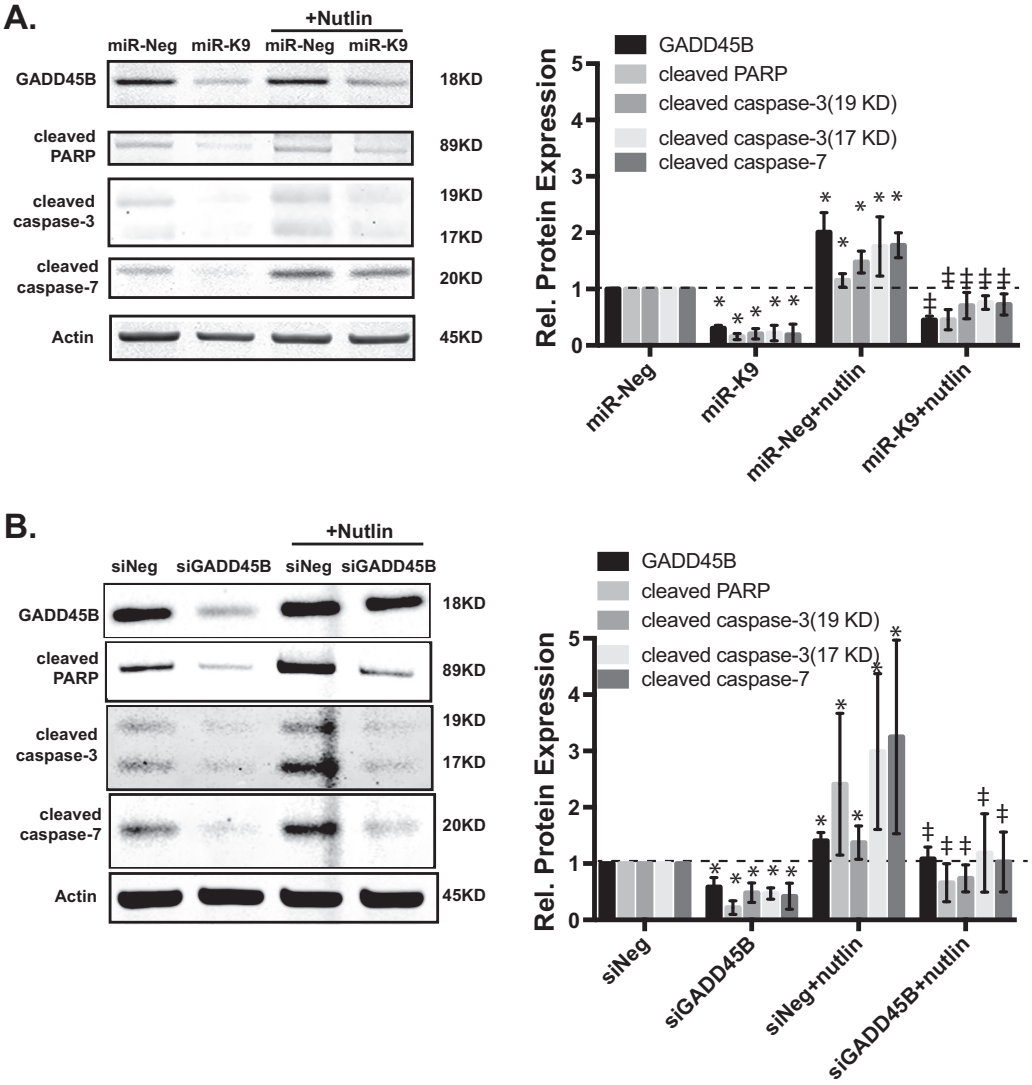

FIG 3 Repression of GADD45B results in lower levels of apoptosis markers in response to Nutlin-3 treatment. (A) Primary endothelial cells were transfected with miRNAs (24 h) and then treated with Nutlin-3 for 24 h. The protein expression of GADD45B and apoptosis markers was analyzed by Western blotting ($n = 3$). (B) Assays similar to those performed for panel A, but cells were transfected with siRNAs targeting GADD45B ($n = 5$). Graphs from biological replicates are shown on the right. *, P value of <0.05 compared to miNeg/siNeg without Nutlin-3; ‡, P value of <0.05 compared to miNeg/siNeg with Nutlin.

Repression of GADD45B inhibits G_2/M cell cycle arrest induced by Nutlin-3. Nutlin-3 treatment is also known to induce cell cycle arrest at G_2/M (26). We wanted to measure how Nutlin-induced cell cycle changes are perturbed by the presence of miR-K9. Primary endothelial cells without Nutlin treatment displayed an average of 16.6% of cells in G_2/M, and this portion nearly doubled to 29% after Nutlin-3 treatment (Fig. 5B). However, in cells transfected with miR-K9 prior to Nutlin-3 treatment, the portion of G_2/M cells increased to only 20.9% (compared to 29% in the control) in Nutlin-treated cells (Fig. 5B). Again, we noticed a correlation between the observations in miR-K9-transfected cells and cells with siRNAs directly targeting GADD45B (Fig. 5C and D). These similar changes were anticipated, since both miR-K9 and the siRNAs repress GADD45B. Together, these data suggested that miR-K9 can also protect cells from Nutlin-induced cell cycle arrest.

Repression of GADD45B inhibits apoptosis induced by Nutlin-3. We also analyzed apoptosis using flow cytometry and staining of primary endothelial cells with 7-aminoactinomycin D (7-AAD) and annexin V-phycoerythrin (PE). As expected, Nutlin-3

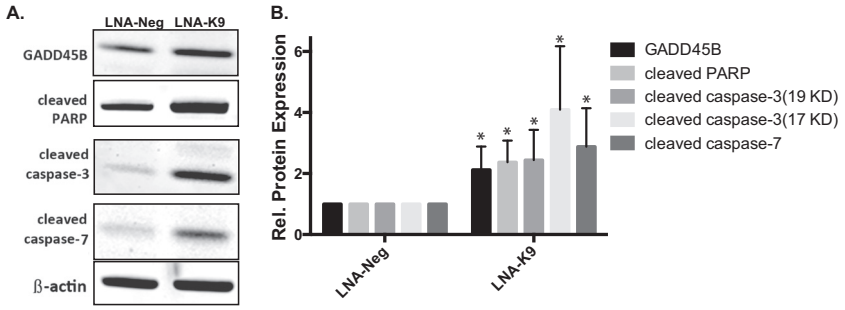

FIG 4 Repression of miR-K9 in KSHV-infected cells. (A) Infected B lymphocytes (BCBL-1) were transfected with control miRNA inhibitors (LNA-Neg) or inhibitors of miR-K9 (LNA-K9). GADD45B protein and apoptosis markers were measured using Western blotting, and the results from at least three biological replicates are graphed on the right. A representative Western blot is shown on the left.

treatment induced apoptosis in primary endothelial cells. In the control cells, we observed a 16% increase in the percentage of apoptotic cells after treatment with Nutlin-3 (Fig. 6A and B). However, in the cells transfected with miR-K9, there was only a 2% increase in the amount of apoptotic cells. Cells transfected with siRNAs to repress GADD45B showed a similar resistance to Nutlin-induced apoptosis (Fig. 6D and E).

Inhibitors of miR-K9 induce apoptosis in KSHV-infected cells with miR-K9 expression. Additionally, we sought to determine changes in the amount of apoptotic cells in infected cells when treated with the LNA inhibitor of miR-K9 (LNA-K9). In the absence of Nutlin-3 treatment, KSHV-infected cells were transfected with inhibitors to miR-K9 to measure the consequences of inhibiting the specific miRNA in KSHV-infected cells. After delivery of the miRNA inhibitor, similar flow cytometry assays were per-

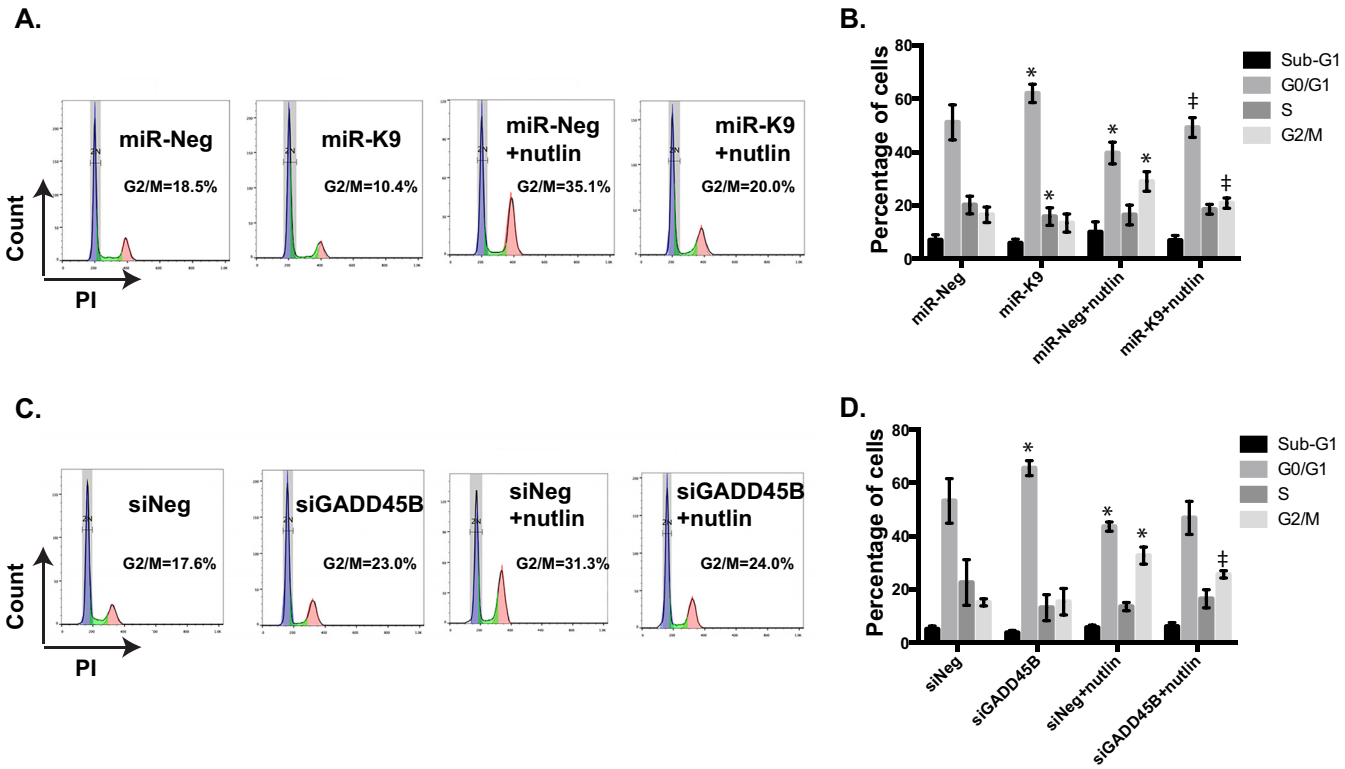

FIG 5 Repression of GADD45B inhibits G_2/M cell cycle arrest induced by Nutlin-3. Primary endothelial cells were transfected with miRNAs or siRNAs and then treated with Nutlin-3 for 24 h. Cell cycle kinetics were measured using propidium iodide staining and flow cytometry analysis software. (A and C) The G_2/M numbers in the graphs on the left are from a representative experiment. (B and D) The average values from three replicates are shown in the graphs on the right. *, *P* value of <0.05 compared to miR-Neg without Nutlin-3; ‡, *P* value of <0.05 compared to miR-Neg with Nutlin-3.

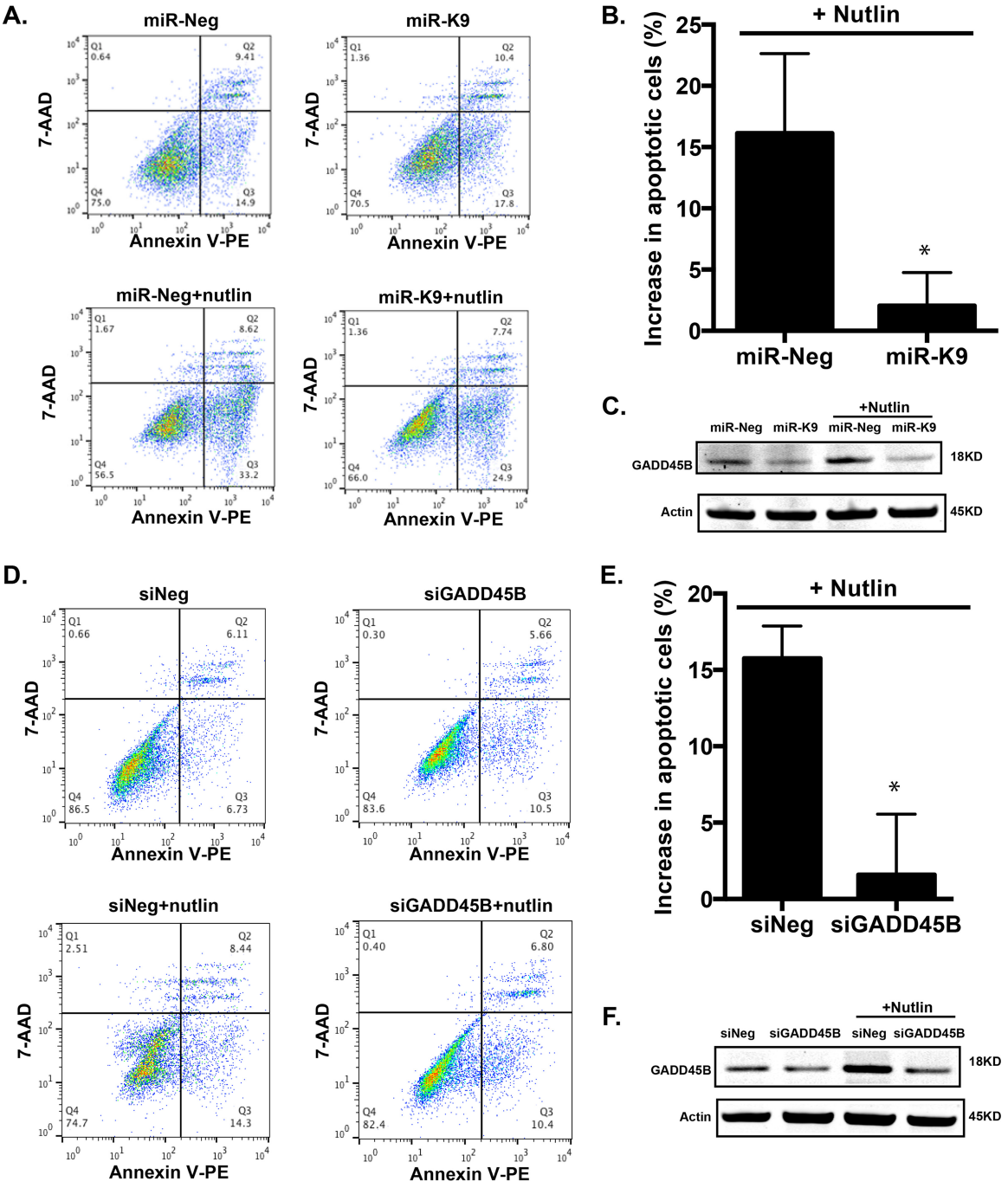

FIG 6 Repression of GADD45B inhibits apoptosis induced by Nutlin-3. Primary endothelial cells were transfected with miRNAs (A, B, and C) or siRNAs (D, E, and F) and then treated with Nutlin-3. Apoptotic and dead cells were measured using annexin-V and 7-AAD staining. (B and E) The increase in the percentage of apoptotic or dead cells due to Nutlin-3 treatment (percentage of apoptotic cells with Nutlin-3 minus percentage of apoptotic cells without Nutlin-3) is shown for three biological replicates. Representative Western blots are shown in panels C and F. *, *P* value of <0.05 compared to miRNA or siRNA negative controls.

formed and the average percentage of apoptotic and dead cells in the control cells (LNA-Neg) was 6.3%, but this increased to 23.4% in the cells treated with the inhibitor to the KSHV miRNA miR-K9 (LNA-K9) (Fig. 7B). As a control, we utilized the KSHV-infected cell line BC-3, which does not express miR-K9 (1). As expected, we did not observe any significant changes in apoptosis when we introduced the inhibitor to miR-K9 compared to the control inhibitor in this cell line that lacks miR-K9 expression (Fig. 7C and D). It was previously shown that BC-3 cells expressed increased *GADD45B*

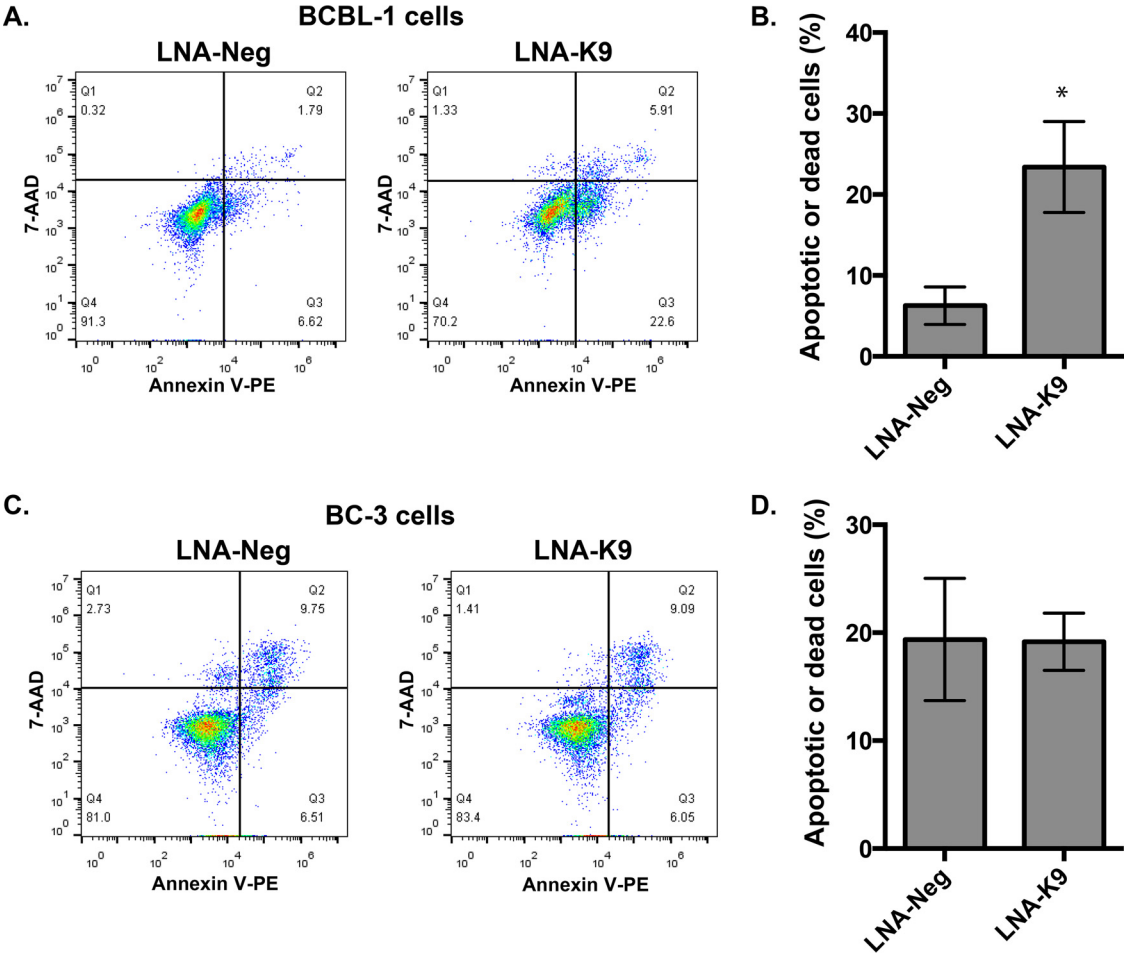

FIG 7 Inhibitors of miR-K9 induce apoptosis in KSHV-infected cells that express miR-K9 (BCBL-1, not BC-3). (A and B) KSHV-infected B lymphocytes (BCBL-1) were transfected with control inhibitors or inhibitors to miR-K9. Apoptotic and dead cells were measured using annexin-V and 7-AAD staining. The percentages of quadrants 2 and 3 were summed, and the average percentages from three biological replicates are plotted in panels B and D. (C and D) Similar assays were conducted with the KSHV-infected cell line BC-3, which does not express miR-K9.

mRNA compared to BCBL-1 cells (27). We also noted that BC-3 cells displayed a higher baseline level of apoptosis than BCBL-1 cells, consistent with higher *GADD45B* expression. These data indicate that miR-K9 performs a role in inhibiting apoptosis in KSHV-infected cells that express miR-K9.

Transduction with lentiviruses expressing GADD45B induces apoptosis in BCBL-1 cells. In addition to inhibition of miR-K9, we also attempted to reverse the viral miRNA-induced changes with ectopic expression of GADD45B with an expression vector that lacks the 3′UTR, which is targeted by miR-K9. This assay sought to determine if increased expression of GADD45B in KSHV-infected cells had an effect on apoptosis. KSHV-infected cells (BCBL-1) were transduced with control lentiviruses or lentiviruses expressing GADD45B. Overall, the infected cells with increased GADD45B expression displayed an increase in the apoptotic markers (Fig. 8A and B). Flow cytometry assays in similar experiments also demonstrated a decrease in the percentage of living cells when GADD45B is ectopically expressed (Fig. 8C and D).

DISCUSSION

In summary, we found that KSHV miRNAs repress expression of GADD45B, and this confers antiapoptotic advantages to infected cells. Using gain and loss of miRNAs and GADD45B expression, these results suggest that *GADD45B* is an important gene for repression by KSHV in order to promote the survival of infected cells. Here, the focus

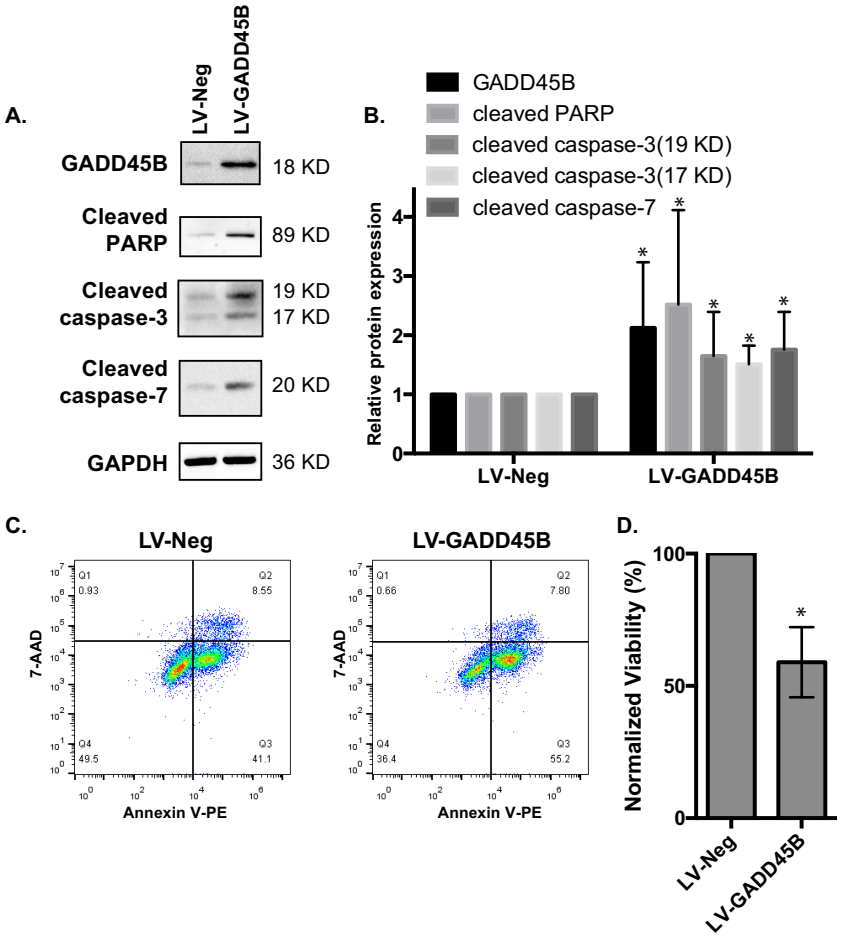

FIG 8 BCBL-1 cells were transduced with lentiviral particles that express GADD45B (LV-GADD45B) or the control (LV-Neg). After transduction, cells were harvested and analyzed using Western blotting (A and B) or flow cytometry assays (C and D). Living cells were defined as the cells in quadrant 4 (negative for both annexin-V and 7-AAD in lower left of plots in panel C). The percentage of living cells in the LV-GADD45B condition was normalized to the percentage in the LV-Neg condition to calculate normalized viability, and these average values from three biological replicates are shown in panel D.

has been on miR-K9 and GADD45B, but it is important to note that multiple KSHV miRNAs repress GADD45B expression (complicating the use of genetic deletions of individual miRNAs) and miR-K9 targets multiple genes. We have tested two commonly utilized PEL lines in this report, but future assays with additional PEL lines would determine if these observations are widespread among additional patient-derived cell lines. The effects of miR-K9 on the response to p53 activation are likely due to the combination of multiple genes being repressed by miR-K9. Previously, it has been shown that KSHV miRNAs can also target caspase-3 and inhibit apoptosis induced by staurosporine (8). A previous report also showed that miR-K9 inhibited PARP cleavage (22). Related viruses have also been shown to regulate apoptosis similarly. Multiple miRNAs encoded by Epstein-Barr virus target caspase-3 (28). Furthermore, others reported that infection with hepatitis C virus caused repression of *GADD45B* expression (but not *GADD45A* or *GADD45G*) not by miRNAs but through hypermethylation of the *GADD45B* promoter by HCV viral proteins via an unknown mechanism (29). In agreement with our data, *GADD45B* was in the group of the most highly repressed genes after KSHV infection in endothelial cells in a previous study; however, the repression mechanism was not determined (30). Together, these findings indicate that a broad range of viruses are using several strategies to repress expression of multiple cellular genes that normally suppress growth in order to promote survival of infected cells.

These recent findings continue to support the observation that KSHV-infected cells possess survival advantages (31), and viral miRNAs are one of the tools employed by KSHV to gain survival advantages.

GADD45 family members GADD45A, GADD45B, and GADD45G are closely related, with an overall amino acid sequence similarity of 55%. Family members can associate with each other and form homo- and heterodimers. *GADD45A*, a gene closely related to *GADD45B*, was shown to induce the lytic cycle in KSHV infection (32). In the same expression data from latently infected HUVECs 48 h after infection as described for Fig. 1, GADD45A expression was down −0.202 ($\log_2$) compared to mock-infected cells (GADD45B was down −0.807). It is unknown if *GADD45B* can also reactivate the lytic cycle. If it can, repression of this GADD45B by viral miRNAs could be a mechanism to maintain latency.

There exists a complicated relationship between DNA viruses and host DNA damage responses (9, 33–36). Some responses can trigger an antiviral response, while other interactions between the cellular DNA damage response factors can promote viral replication. One important cellular factor activated in the context of DNA damage is p53 (37). While p53 status is generally wild type in patient-derived cells infected with KSHV (38–40), the activity of p53 is repressed during latency (38, 39, 41–46). Given these observations, multiple groups have reported interesting anti-KSHV effects upon p53 activation (47, 48). It remains to be determined which DNA damage response factors are activated by which viral products and how the virus prevents host DNA damage responses from combating infection. Inducing expression or activity of multiple DNA damage factors may be a useful strategy to deal with the repression of multiple DNA damage factors by viral proteins and miRNAs.

GADD45B was first identified as a gene elevated after IL-6 treatment in M1D$^+$ myeloid precursor cells (49). This gene was also increased in the absence of protein synthesis following exposure to IL-1, lipopolysaccharide (LPS), and leukemia inhibitory factor (LIF) in these cells. KSHV infection had been found to induce human IL-6 expression (50), and it was strongly upregulated in the expression profiling data (22) from latently infected endothelial cells used in this study. Together, these data suggest that IL-6 is one of the cellular signals that upregulates *GADD45B* expression upon KSHV infection, but this induction is suppressed by the activity of KSHV miRNAs.

As mentioned above, *GADD45B* expression can be induced by multiple factors. Peroxisome proliferator-activated receptor alpha (PPARA) has been shown to activate transcription of *GADD45B* (19). Another downstream effect of PPARA is increased production of reactive oxygen species, including hydrogen peroxide. Lytic reactivation of KSHV can be stimulated with hydrogen peroxide (51), and hydrogen peroxide can lead to various modifications of KSHV latency-associated nuclear antigen (LANA) (52). It remains to be determined if GADD45B can influence KSHV lytic reactivation and LANA modifications in the context of PPARA signaling and oxidative stress.

Given that mature KSHV miRNA sequences are conserved among varied geographical clinical isolates (53) and that KSHV miRNAs are distinct from human miRNAs and expressed during latent and lytic infections, inhibiting specific viral miRNAs may represent a new therapeutic strategy to combat latent infections. Successful delivery of antisense inhibitor of human miR-122 has been achieved in humans (54). Delivery of antisense inhibitors of miR-K9 to KSHV-infected cells induced apoptosis (Fig. 7), which could be applicable to KSHV infections in humans with the proper delivery strategy. In addition, inhibition of antiapoptotic viral miRNAs could be used in combination with p53 activators to achieve a larger amount of apoptosis in virus-infected cells.

MATERIALS AND METHODS

Cell culture and reagents. Human umbilical vein endothelial cells (HUVECs; Lonza) were maintained for up to five passages with a complete EGM-2 bullet kit (Lonza). The latently KSHV-infected body cavity-based lymphoma cell line BCBL-1 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1× penicillin-streptomycin, and 55 μM β-mercaptoethanol. 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1× penicillin-

streptomycin. Nutlin-3, a p53 pathway activator, was purchased from Sigma-Aldrich. HUVECs were treated with 5 μM or 7 μM Nutlin-3 for another 24 h after transfection.

miRNA or siRNA transfections. miRVana miRNA mimics and siRNAs for GADD45B were purchased from Ambion and Qiagen, respectively. For the transfection of miRNAs and siRNAs, HUVECs were seeded at 1.2×10^5/well onto 6-well plates overnight. Transfections were performed with 10 nM miRNAs or 16.5 nM siRNAs using 1.5 μg/well DharmaFECT1 (Dharmacon) according to the manufacturer's instructions. The transfected cells were harvested at 48 h posttransfection (hpt) for further assays.

3′UTR Dual-Luciferase reporter assay. 293 cells were reverse transfected in 96-well plates by using Lipofectamine 2000 (Invitrogen) with 13 nM each KSHV miRNA (or a negative-control miRNA [miR-Neg]) and a luciferase reporter plasmid (pD765-GADD45B), which expresses herpes simplex virus TK (HSV-TK) promoter-driven firefly luciferase as an internal control and simian virus 40 (SV40) promoter-driven *Renilla* luciferase fused to the 3′UTR of GADD45B as the reporter (Protein Expression Laboratory, Leidos, Frederick, MD). A reporter plasmid lacking any 3′UTR adjacent to the *Renilla* luciferase served as a control for nonspecific responses of luciferase expression to the KSHV miRNAs. Site-directed mutagenesis was performed on the pD765-GADD45B reporter plasmid by using the QuikChange II kit (Stratagene) according to the manufacturer's instructions. The following primer and its reverse complement were used to introduce mutations into the *GADD45B* 3′UTR for the GADD45B mutant: 5′-GCAAACTGGGAGT TCAACGCTACAGAGCAACTTCAG CCC-3′ (underlining indicates mutated bases compared to the wild type). Assays were performed by using the Dual-Luciferase reporter system (Promega) at 48 hpt. The ratio of the activities of renilla luciferase to those of firefly luciferase in each well was used as a measure of total reporter activation. The results shown are averages of data from three independent experiments, assayed in triplicate.

Inhibition of endogenous KSHV miRNAs. Power locked nucleic acids (LNAs; Exiqon) against miR-K12-K9 (LNA-9) or negative-control LNA (LNA-Neg), at a total of 100 pmol, were electroporated into 1×10^6 BCBL-1 or BC-3 cells in nucleofection solution V using program T-01 according to the manufacturer's instructions (Amaxa Inc.). Cells were harvested at 48 h postelectroporation or posttransfection for further Western blot assay and apoptosis assay.

Construction of GADD45B cDNA overexpressing lentiviral vector and lentiviral transduction on BCBL-1 cells. The lentiviral vectors overexpressing GADD45B cDNA were constructed by the GeneChem Company (Shanghai, China). Briefly, the GADD45B cDNA product and the negative control were inserted into the linearized eukaryotic GV358 vector. All of these vectors were transformed into DH5α chemically competent *Escherichia coli*, and the desired expression clones were identified by PCR amplification and electrophoresis and then were confirmed by sequencing (TaKaRa). The recombinant GADD45B cDNA vectors and the packaging vectors (pHelper 1.0 and pHelper 2.0) (GeneChem) were cotransfected into 293 cells with Lipofectamine 2000. The culture supernatants were collected at 48 h after transfection, concentrated, and used as virus stocks named LV-GADD45B. All of the lentiviral vectors expressed enhanced green fluorescent protein (GFP), which allowed for determining of titers and measuring their infection efficiency in infected cells.

BCBL-1 cells (5×10^5/vial) were sequentially infected with lentiviral particles at a multiplicity of infection (MOI) of 10 in the presence of 5 μg/ml Polybrene (Sigma), followed by centrifugation at 2,000 rpm, 37°C, for 1 h. Cells were harvested at 48 h postransduction for further Western blot assay and apoptosis assays.

Western blot assay. Total protein was extracted in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing 1× Halt protease and phosphatase inhibitors (Thermo Scientific). For Western blotting, equal amounts of total proteins were electrophoresed by SDS-PAGE, and protein levels were quantified by using a Li-Cor Odyssey infrared imaging system. Primary antibodies against GADD45B were obtained from Santa Cruz, Inc. Primary antibodies against cleaved PARP, caspase-3, and caspase-7 were purchased from Cell Signaling, Inc. Mouse anti-β-actin and -glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies were obtained from Sigma Inc. and Santa Cruz Biotechnology. Secondary antibodies conjugated to the infrared fluorescing dyes IRDye 800CW and IRDye 680 were obtained from Li-Cor. Changes in protein levels were measured relative to the β-actin or GAPDH level, and fold changes were obtained relative to values for the respective negative control.

Apoptosis assay using annexin V-PE/7-AAD. At 48 hpt, cells were harvested and resuspended in 100 μl of 1× annexin-V binding buffer. The supernatant was incubated with 5 μl of phycoerythrin (PE)-conjugated annexin V (BD Pharmingen) and 5 μl of 7-aminoactinomycin D (7-AAD; BD Pharmingen) for 15 min at room temperature in the dark, and then 400 μl of 1× buffer was added to each tube. Flow-cytometric analysis (BD FACSCalibur; BD Biosciences) was performed within 1 h of staining. Each experiment was performed with at least three biological replicates.

Cell cycle assay using PI. At 48 hpt, cells were harvested and fixed with 70% cold ethanol. Cells were resuspended in 500 μl RNase (200 μg/ml) for 30 min and stained with propidium iodide (PI; 50 μg/ml) for 15 min at room temperature in the dark. After washing with phosphate-buffered saline, cells were subjected to flow-cytometric analysis by using a BD FACSCalibur flow cytometer (BD Biosciences). Numbers of cells in different cell cycle phases were determined based on DNA content. Each experiment was performed with at least three biological replicates.

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