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Original Article

KSHV co-infection regulates HPV16+ cervical cancer cells pathogenesis *in vitro* and *in vivo*

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Abstract: High-risk human papillomavirus (HPV) infection is the etiological agent of cervical, oral and oropharyngeal cancers. Another oncogenic virus, Kaposi sarcoma-associated herpesvirus (KSHV) can cause several human cancers arising in those immunocompromised patients. KSHV DNA has been detected in the oral cavity and the female genital tract, although its detection rate in cervical samples is relatively low. Therefore, it remains unclear about the role of KSHV co-infection in the development of HPV-related neoplasia. We recently report that KSHV infection of HPV16+ cervical cancer cell line SiHa induces several pro-inflammatory factors production while reducing HPV16 E6 and E7 expression through the manipulation of cellular microRNA function. In the current study, we focus on determining the influence of KSHV co-infection on cervical cancer cells pathogenesis *in vitro* and *in vivo*. We found that KSHV co-infection is able to maintain SiHa and/or CaSki cells pathogenesis and tumorigenesis, although hijacking HPV oncogenic proteins expression. In mechanisms, KSHV co-infection is capable of increasing Macrophage migration inhibitory factor (MIF) and its receptor CXCR2 expression from cervical cancer cells, which may contribute to cervical cancer development. Our data indicate that KSHV co-infection may act as a potential co-factor to promote HPV-related neoplasia development.

Keywords: HPV, KSHV, SiHa, MIF

Introduction

Cervical cancer represents one of the most common malignancies in females worldwide. The pathogenesis of cervical cancer occurs following persistent infection with high-risk human papillomavirus (HPV) in particular subtype 16 and 18 [1]. E6 and E7 proteins represent the major high-risk HPV-encoded oncoproteins, which are closely associated with cervical carcinogenesis [2]. Mechanistically, E6 and E7 proteins can bind to the p53 and retinoblastoma (Rb) family proteins, respectively, resulting in the regulation of cell cycle and final transformation [3]. In addition, high-risk HPV infection is prevalent in oral cavity and related to oral and oropharyngeal cancer development [4-6].

Kaposi sarcoma-associated herpesvirus (KSHV) represents a principal causative agent of several human cancers arising in those immunocompromised patients, including Kaposi's Sarcoma (KS) and Primary Effusion Lymphoma (PEL) [7, 8]. Published literatures have reported that KSHV DNA sequences are detected in the prostate, semen, oral cavity and the female genital tract [9-13]. In contrast to the high prevalence of KSHV shedding in oral cavity, the detection rate of KSHV DNA or virus infection in cervical samples are relatively low (< 2%), even in those high-risk population such as sex workers and HIV+ patients [13, 14]. Furthermore, currently there are few studies reporting the co-infection of KSHV and HPV in cervical samples or cervical cancer cells. Therefore, it remains unclear about the role of KSHV co-infection in the development of HPV-related neoplasia. Recently, we have reported that HPV16+ cervical cancer cell line SiHa is susceptible to KSHV initial infection and supports virus replication [15]. Interestingly, we have found that KSHV *de novo* infection or ectopic expression of viral

latent proteins can significantly reduce HPV16 E6 and E7 expression (~50%-70% of reduction) through the up-regulation of one cellular microRNA, miR129-5p [15, 16]. In the current study, we focus on determining the influence of KSHV co-infection on HPV16+ cervical cancer cells pathogenesis *in vitro* and *in vivo*.

Materials and methods

Cell culture and KSHV purification/infection

Body cavity-based lymphoma cells (BCBL-1, $KSHV^{+}/EBV^{neg}$) were kindly provided by Dr. Dean Kedes (University of Virginia) and maintained in RPMI 1640 medium (Gibco) with supplements as described previously [17]. SiHa and CaSki cells were purchased from ATCC and maintained in Eagle's Minimum Essential Medium or RMPI 1640 medium (ATCC) supplemented with 10% FBS, respectively. All cells were incubated at 37°C in 5% CO_2. All experiments were carried out using cells harvested at low passages (< 20). To obtain KSHV for infection experiments, BCBL-1 cells were incubated with 0.6 mM valproic acid for 6 days, and purified virus was concentrated from culture supernatants and infectious titers were determined as described previously [18].

Cell proliferation and apoptosis assays

Cell proliferation was measured by using the WST-1 assays (Roche) according to the manufacturers' instructions. Flow cytometry was used for quantitative assessment of apoptosis using the FITC-Annexin V/propidium iodide (PI) Apoptosis Detection Kit I (BD Pharmingen).

Transwell invasion assays

Matrigel Invasion Chambers (BD) were hydrated for 4 h at 37°C with culture media. Following hydration, media in the bottom of the well was replaced with fresh media, then 2×10^4 tumor cells were plated in the top of the chamber. After 24 h, cells were fixed with 4% formaldehyde for 15 min at room temperature and chambers rinsed in PBS prior to staining with 0.2% crystal violet for 10 min. After washing the chambers, cells at the top of the membrane were removed and cells at the bottom of the membrane counted using a phase contrast microscope.

Soft agar assays

A base layer containing 0.5% agarose medium and 5% FCS was poured into six-well plates. Then, 1×10^5 cells were mixed with 0.4% agarose in Earl's minimal essential medium (EMEM) containing 5% FCS to form a single-cell suspension. After being seeded, the plates were incubated for 2 weeks. Colonies were stained with 0.005% crystal violet and photographed under a phase-contrast microscope (Leica DFC320).

ELISA

Concentrations of MIF in culture supernatants were determined using the human MIF ELISA kit (R&D Systems), according to the manufacturer's instructions.

qRT-PCR

Total RNA was isolated using the RNeasy Mini kit (QIAGEN), and cDNA was synthesized from equivalent total RNA using a SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) according to the manufacturer's instructions. Primers used for amplification of HPV16 E6 and E7 are described previously [15]. Amplification was carried out using an iCycler IQ Real-Time PCR Detection System, and cycle threshold (Ct) values were tabulated in duplicate for each gene of interest in each experiment. "No template" (water) controls were used to ensure minimal background contamination. Using mean Ct values tabulated for each gene, and paired Ct values for β-actin as a loading control, fold changes for experimental groups relative to assigned controls were calculated using automated iQ5 2.0 software (Bio-rad).

Nude mouse xenograft model

Cells were counted and washed once in ice-cold PBS, and 2×10^6 cells in 50 μL PBS plus 50 μL growth factor-depleted Matrigel (BD Biosciences) were injected subcutaneously into the flanks of nude mice (Jackson Laboratory). The mice were observed and measured every 2~3 d for the presence of palpable tumors. At the end of experiment, the tumors were excised for subsequent analysis such as hematoxylin & eosin (H&E) and immunohistochemistry staining as described previously [19]. Images were collected using an Olympus BX61 microscope

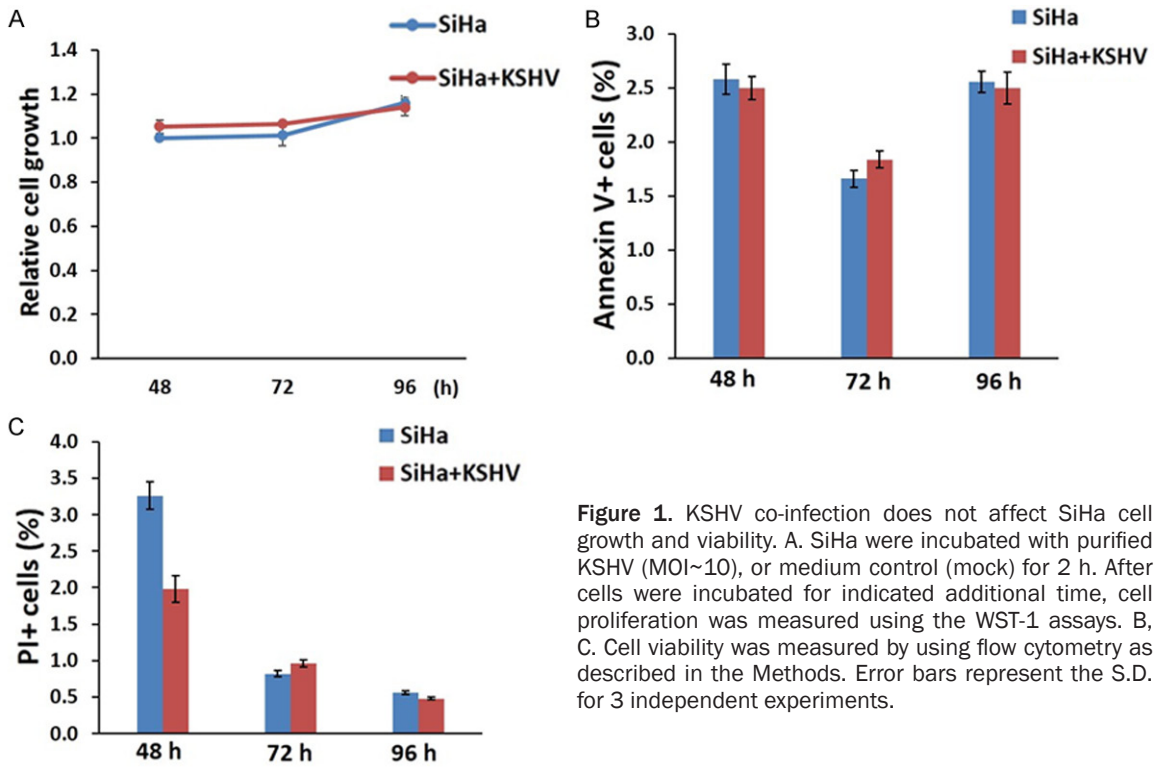

Figure 1. KSHV co-infection does not affect SiHa cell growth and viability. A. SiHa were incubated with purified KSHV (MOI~10), or medium control (mock) for 2 h. After cells were incubated for indicated additional time, cell proliferation was measured using the WST-1 assays. B, C. Cell viability was measured by using flow cytometry as described in the Methods. Error bars represent the S.D. for 3 independent experiments.

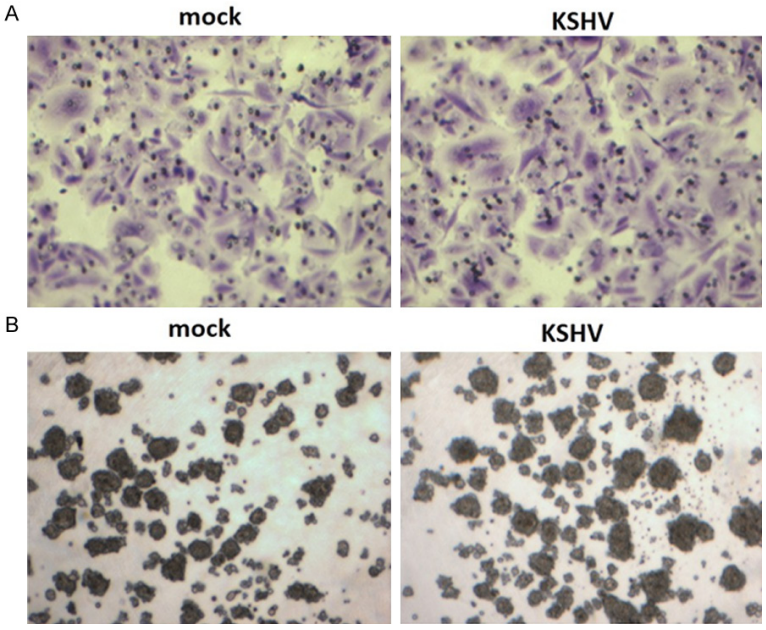

Figure 2. Comparison of cell invasiveness and anchorage-independent growth abilities between mock and KSHV co-infected SiHa cells. A. SiHa were incubated with or without purified KSHV (MOI~10) for 2 h, after additional 48 h incubation, the transwell assays were performed to determine cell invasiveness ability as described in the Methods. B. The anchorage-independent growth ability was determined using the soft agar assays as described in the Methods.

equipped with a high resolution DP72 camera and CellSense image capture software. All protocols were approved by the LSUHSC Animal Care and Use Committee in accordance with national guidelines.

Statistical analyses

Significance for differences between experimental and control groups was determined using the two-tailed Student's t-test (Excel 8.0), and p values < 0.05 or < 0.01 were considered significant or highly significant, respectively.

Results and discussion

During a time-course culture, we first have confirmed that KSHV co-infection does not affect SiHa cell growth and viability when compared to non-infected mock cells (**Figure 1**), therefore which are not responsible for any differences between these two groups of cells in cellular functional assays if have. Next, by using the transwell and soft agar assays, respectively, we

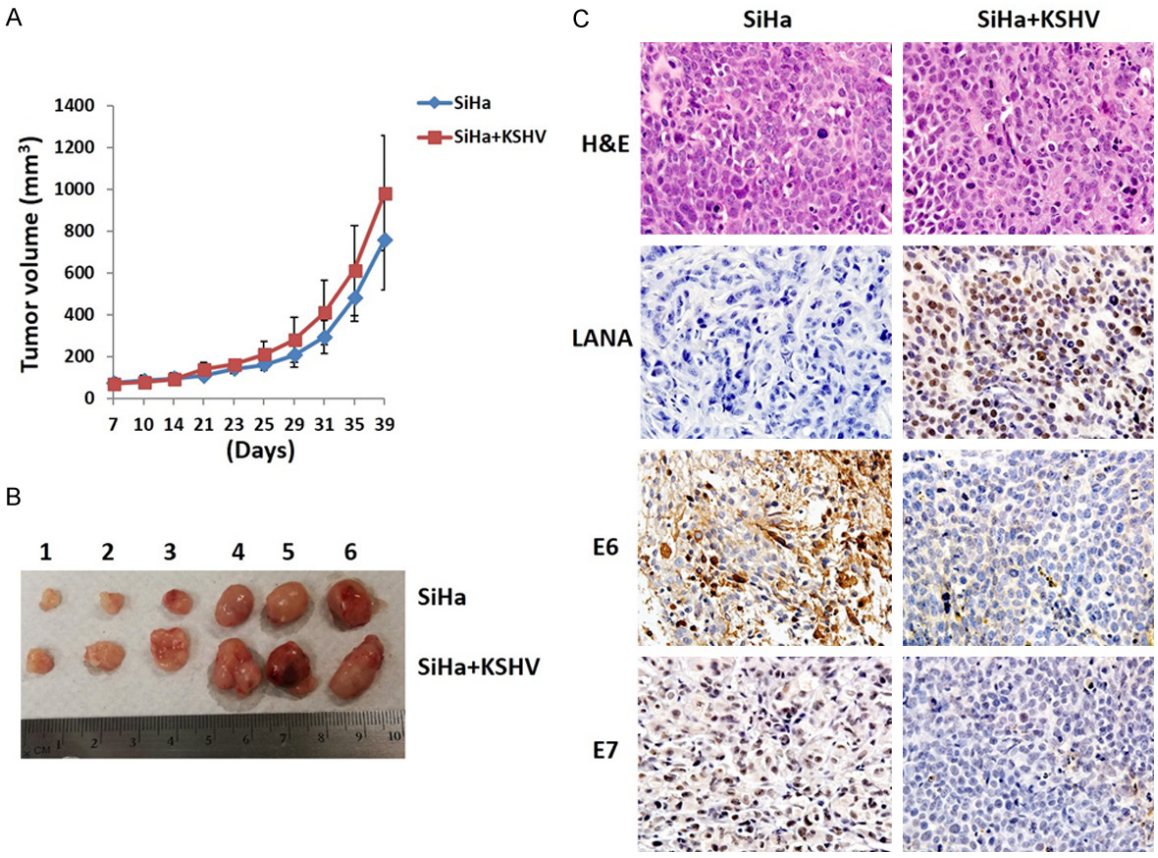

Figure 3. Comparison of tumorigenesis ability between mock and KSHV co-infected SiHa cells in a nude mice xenograft model. A, B. The mock and KSHV co-infected SiHa cells (approximately 5 × 10^5 cells were mixed at a ratio of 1:1 with growth factor-depleted Matrigel) were injected subcutaneously into the right flanks of nude mice, respectively. The mice were observed and measured every 2~3 d for the presence of palpable tumors for ~40 d. Error bars represent the S.D. from 2 independent experiments. C. Protein expression within tumor tissues from representative injected mice was measured by using immunohistochemistry staining as described in the Methods.

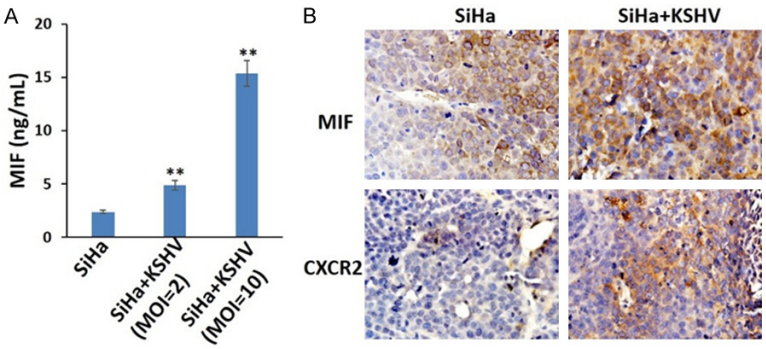

Figure 4. The up-regulation of MIF and its receptor by KSHV co-infection *in vitro* and *in vivo*. A. SiHa were incubated with purified KSHV (MOI~2 or 10), or medium control (mock) for 2 h. After cells were incubated for additional 72 h, MIF concentrations in supernatant were determined by using ELISA. Error bars represent the S.D. for 3 independent experiments, **=P < 0.01. B. The mock and KSHV co-infected SiHa cells were injected subcutaneously into the nude mice as described previously. Protein expression within tumor tissues from representative injected mice was measured by using immunohistochemistry staining.

have observed that there is no difference in cell invasiveness and anchorage-independent growth abilities between mock and KSHV co-infected SiHa cells (**Figure 2**), although KSHV co-infected cells seem to form a little larger size of colonies. Therefore, we think that KSHV or viral latent proteins can hijack HPV-encoded oncogenic proteins expression in cervical cancer cells, however, the new co-infected virus may use its unique mechanisms to maintain cervical cancer cells pathogenesis.

We next have compared the tumorigenesis ability between

mock and KSHV co-infected SiHa cells in a nude mice xenograft model. Our data indicate that the tumors formed by KSHV co-infected SiHa cells are a little bigger than those from mock cells during ~40 d growth in mice, but with no statistical significance (**Figure 3A**, **3B**). There are no architecture difference in the H&E staining tumor tissues between these two groups of mice (**Figure 3C**). By using immunohistochemistry staining, we confirm that more than 90% of tumor cells from KSHV co-infected SiHa injected mice are LANA+, which means these cells are still latently infected by KSHV [20]. In contrast, none of tumor cells from SiHa mock cells injected mice are LANA+ (**Figure 3C**). We also confirm that E6 and E7 proteins expression are dramatically repressed in tumor cells from KSHV co-infected SiHa injected mice (**Figure 3C**), which is consistent with what we have previously observed *in vitro* cultures (a 50%-70% of reduction of HPV16 E6 and E7 expression in KSHV co-infected SiHa cells) [15].

By using a cytokine/chemokine array, we recently have identified a global signature altered within KSHV co-infected SiHa when compared to the control mock cells [15]. We have found that KSHV co-infection increases several pro-inflammatory factors production, including an induction of ~8-fold increasing of Macrophage migration inhibitory factor (MIF) [15]. By using ELISA in the current study, we confirm that KSHV co-infection significantly increases MIF secretion from SiHa cells in a dose-dependent manner (**Figure 4A**). MIF is well recognized as a cancer biomarker protein [21-23], since its expression in normal cells is several orders of magnitude lower than levels observed in cancer cells [24]. For example, MIF protein levels can be 100-fold higher in lung cancer tissue over normal lung tissue, and MIF mRNA levels rise 7- to 24-fold in tumors [24]. Soluble MIF produced by cancer cells is imported into the cytoplasm and nucleus of its target cancer cells via an autocrine loop [25, 26]. MIF enters its target cells by binding to its cellular receptor such as CXCR2 and CD74 [26, 27]. Our data indicate that the obvious up-regulation of MIF and its receptor CXCR2 in tumor tissues from KSHV co-infected SiHa injected mice (**Figure 4B**). Interestingly, published literature has reported the overexpression of MIF in invasive cervical cancer samples compared to cervical dysplasias samples [28].

To test the cell line relevance, we also used purified KSHV to infect another HPV16+ cell line, CaSki. Similarly, we have also found that KSHV co-infection maintains CaSki cell growth, pathogenesis (invasion), increase MIF secretion while decreasing HPV16 E6 and E7 expression (Figure S1).

In conclusion, our data indicate that KSHV may manipulate some unique mechanisms (e.g., MIF/CXCR2) to maintain cervical cancer cells pathogenesis, although its co-infection hijacking HPV-encoded oncogenic proteins expression. Therefore, we still cannot exclude the possibility that KSHV as one of co-factors for cervical cancer development, even the detection rate of its co-infection in cervical samples are relatively low.

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Disclosure of conflict of interest

None.

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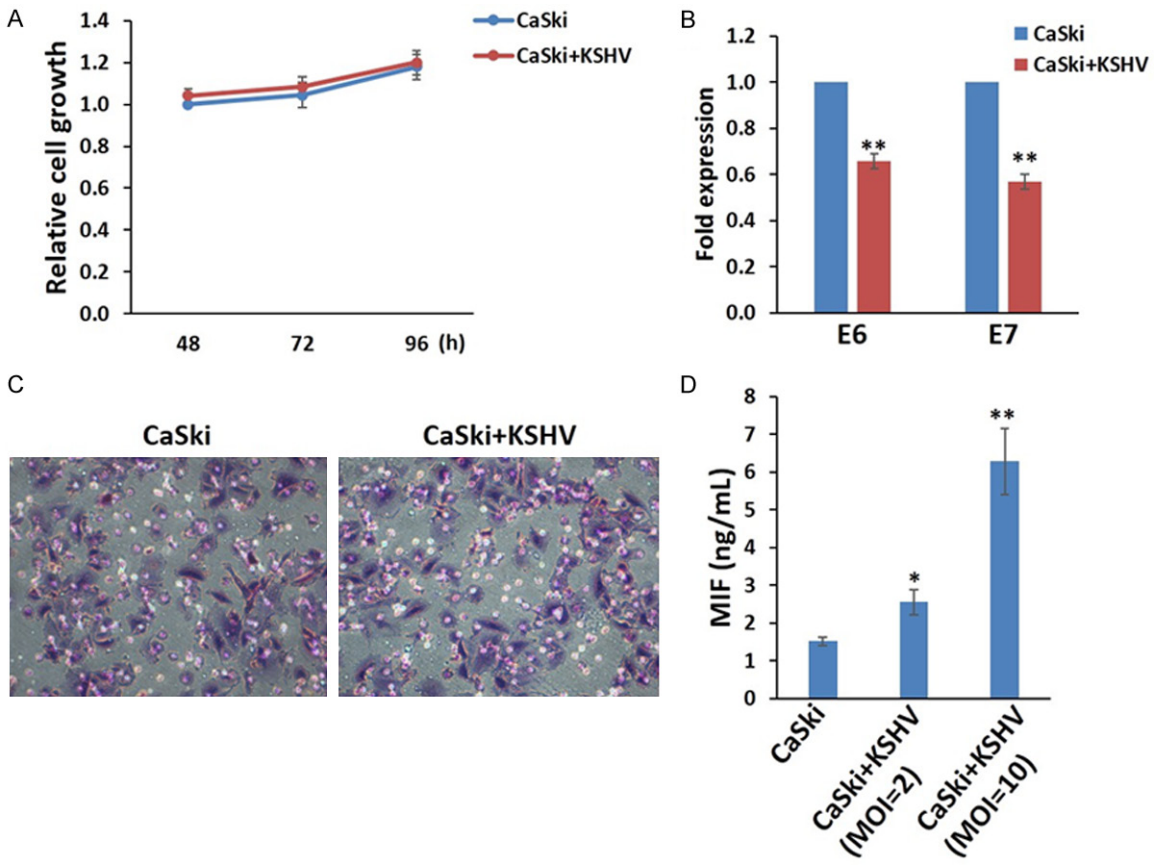

Figure S1. KSHV co-infection maintains CaSki cell pathogenesis although hijacking HPV-encoded oncogenic proteins expression. A. CaSki cells were incubated with purified KSHV (MOI~10), or medium control for 2 h. After cells were incubated for indicated additional time, cell proliferation was measured using the WST-1 assays. B, C. The gene transcripts were quantified by using qRT-PCR and the transwell assays were performed to determine cell invasiveness ability. D. CaSki were incubated with purified KSHV (MOI~2 or 10), or medium control for 2 h. After cells were incubated for additional 72 h, MIF concentrations in supernatant were determined by using ELISA. Error bars represent the S.D. for 3 independent experiments, *=P < 0.05; **=P < 0.01.