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Induction of Hyaluronan Production by oncogenic KSHV and the Contribution to Viral Pathogenesis in AIDS Patients

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Abstract

Kaposi sarcoma-associated herpesvirus (KSHV) is the etiologic agent for Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), malignancies arising primarily in immunocompromised patients particularly AIDS-patients, while which are still lack of effective therapy. Hyaluronan (HA) is a large glucuronic acid and has been found closely related to multiple functions in cancer cells, although its role in viral oncogenesis remains largely unknown. Here we provide first evidence that KSHV *de novo* infection induces HA production from primary endothelial cells through upregulation of HA synthase gene 1 (*Has1*) and a multifunctional glycoprotein, CD147. Further data demonstrate that KSHV-induced HA production requires viral latent protein, LANA (in particular functional domain A) and MAPK/ERK signaling activities. In functions, HA production is necessary for KSHV/LANA-induced primary endothelial cell invasion, a hallmark feature for KS development. For clinical relevance, our data indicate that KSHV+ group has higher levels of HA and Has1 activities in their plasma than the KSHV- group of cohort HIV-infected patients. Together, our findings provide innovative insights into the mechanisms of oncogenic virus activation of HA production and its role in virus-associated malignancies

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pathogenesis, which may help to develop novel therapeutic strategies by targeting HA and related signaling.

Keywords

KSHV; hyaluronan; CD147; LANA

1. Introduction

Hyaluronan (HA) is a very large, linear glycosaminoglycan composed of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine [1]. In addition to its structural role through interaction with other extracellular matrix (ECM) components, HA binds to several cell surface receptors such as CD44, LYVE-1 and RHAMM that induce the transduction of a range of intracellular signals and contribute to multiple cellular functions such as embryonic development, healing processes and inflammation [2]. HA is overproduced by many types of tumors, and in some cases, HA levels are prognostic for malignant progression [3]. Moreover, HA and related signaling transductions have been involved in many malignant behaviors of cancer cells, including migration/invasion, angiogenesis, epithelial–mesenchymal transition (EMT), multidrug resistance, and metastasis [4-6]. Currently, ~20% of human cancers have been attributed to virus infection [7], however, there are limited data describing the role of HA production in viral oncogenesis or how oncogenic viral protein regulate HA level and related signaling transductions.

Kaposi sarcoma-associated herpesvirus (KSHV) is the etiologic agent for Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), malignancies arising primarily in patients infected with the human immunodeficiency virus (HIV) or in those receiving organ transplants [8,9]. Furthermore, despite the reduced incidence of KS in the era of highly active antiretroviral therapy (HAART) for HIV infection, KS still remains the most common Acquired immunodeficiency syndrome (AIDS)-associated tumor and a leading cause of morbidity and mortality in this setting [10]. Another KSHV-caused malignancy, PEL, comprises transformed B cells harboring KSHV and arises preferentially within the pleural or peritoneal cavities of immune-suppressed patients [9]. PEL is a rapidly progressing malignancy with a median survival time of approximately 6 months, even under the combinational chemotherapy [11]. Our recent study demonstrates that the glycoprotein, CD147, interacts with the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and the drug transporter, breast cancer resistance protein (BCRP)/ABCG2, to promote multidrug chemoresistance in KSHV+ PEL cells [12]. Moreover, we found higher levels of HA and HA synthase gene (*Has1–3*) transcripts in chemoresistant PEL cell-lines than in chemosensitive ones. In addition, small HA oligosaccharides (oHA) that interact monovalently with HA receptors, and competitively blocking polyvalent interactions between receptors and endogenous HA, sensitize drug resistant PEL cells to chemotherapeutic agents [12]. In the current study, we investigate the role of HA production in KSHV-infected primary endothelial cells, which represent the major cellular component

of KS tumors, and identify the underlying mechanisms whereby oncogenic viral proteins regulate HA production.

2. Materials and methods

2.1. Cell culture, reagents and infection protocol

KSHV-infected PEL cells (BCBL-1) was kindly provided by Dr. Dean Kedes (University of Virginia) and maintained in RPMI 1640 medium (Gibco) with supplements as described previously [12]. Human umbilical vein endothelial cells (HUVEC) were grown in DMEM/F-12 50/50 medium (Cellgro) supplemented with 5% FBS. Selective inhibitors targeting the mitogen-activated protein kinase (MEK; U0126) and NF-κB (Bay11-7082) were purchased from Sigma. Hyaluronan oligosaccharides (oHA) were prepared as described previously [13]. To obtain KSHV for infection experiments, BCBL-1 cells were incubated with 0.6 mM valproic acid for 6 days, purified virus concentrated from culture supernatants and infectious titers were determined as described previously [14].

2.2. Cell transfection

HUVEC were transfected with control vector pcDNA3.1, pcDNA3.1-LANA (pcLANA) or LANA deletion fragments (pcLANA-A, pcLANA-AB, pcLANA-AC, pcLANA-BC and pcLANA-C) or pcDNA3.1-ERK (pcERK) in 12-well plates for 48 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Transfection efficiency was determined through co-transfection of a lacZ reporter construct and quantified as described previously [14]. For RNA interference assays, *Has1* or *CD147* ON-TARGET plus SMART pool siRNA (Dharmacon), or negative control siRNA, were delivered using the DharmaFECT transfection reagent according to the manufacturer's instruction.

2.3. Immunofluorescence assays

Cells were incubated in 1:1 methanol-acetone at 20°C for fixation and permeabilization, followed by a blocking reagent (10% normal goat serum, 3% bovine serum albumin, and 1% glycine) for an additional 30 min. Cells were then incubated for 1 h at 25°C with 1:1000 dilution of a rat anti-LANA monoclonal antibody (ABI, for LANA wt) or a mouse anti-V5-Tag monoclonal antibody (Cell Signaling, for LANA deletion fragments) followed by 1:100 dilution of a goat anti-rat or goat anti-mouse secondary antibody conjugated to Texas Red (Invitrogen). For intracellular HA detection, cells were permeabilized for 20 min at room temperature with 0.1% Triton-X-100 in 1% BSA, and incubated overnight at 4 °C with bHABC (biotinylated hyaluronan binding complex, Sigma) (1.25μg/mL) in 1% BSA. To remove the pericellular HA, fixed cells were treated with Streptomyces hyaluronidase (1 turbidity reducing unit/mL, Seikagaku Kogyo) before permeabilization. After washing, the cells were incubated for 1 h with Alexa 488-labeled streptavidin (1:1000) (Invitrogen) for bHABC staining. Cells were counterstained with 0.5 μg/mL 4’,6-diamidino-2-phenylindole (DAPI, Sigma) in 180 mM Tris-HCl (pH 7.5) for nuclear localization. Slides were washed once in 180 mM Tris-HCl for 10 min and prepared for visualization using a Leica TCPS SP5 AOBS confocal microscope.

2.4. Immunoblotting

Total cell lysates (20μg) were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies for CD147 (BD), LANA (ABI), phospho-p44/42 ERK (Thr202/Tyr204), t-p44/42 ERK (Cell Signaling) and β-Actin (Sigma) for loading controls. Immunoreactive bands were identified using an enhanced chemiluminescence reaction (Perkin-Elmer), and visualized by autoradiography.

2.5. 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 HUVEC 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. Relative invasion was determined for cells in experimental groups as follows: relative invasion = # invading cells in experimental group / # invading cells in control groups.

2.6. ELSA for HA

Concentrations of HA in culture supernatants or plasma from patients were determined using HA ELSA kit (Echelon) according to the manufacturers' instructions.

2.7. qRT-PCR

Total RNA was isolated from infected or uninfected cells using the RNeasy Mini kit according to the manufacturer's instructions (QIAGEN). cDNA was synthesized from equal total RNA using SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) according to the manufacturer's procedures. The primers designed for target genes are displayed in Supplemental Table 1. Amplification experiments were carried out using an iCycler IQ Real-Time PCR Detection System, and cycle threshold (Ct) values were tabulated in duplicate (cDNA) for each gene of interest for each experiment. "No template" (water) controls were also used to ensure minimal background contamination. Using mean Ct values tabulated for different experiments and using Ct values for β-actin as loading controls, fold changes for experimental groups relative to assigned controls were calculated using automated iQ5 2.0 software (Bio-rad).

2.8. Patients and ethics statement

The study was approved by the Institutional Review Boards for Human Research (IRB, No. 8079) at Louisiana State University Health Science Center – New Orleans (LSUHSC-NO). All subjects were provided written informed consent. In the current study, a total of 28 HIV + patients with antiretroviral treatment (ART) in our HIV Outpatient (HOP) Clinic are involved. There are 15 females and 13 males, the average age is 48.6 y (range 21-65 y). The average CD4 T cell counts is 539/mL (range 35-1773/mL), and the average HIV viral loads is 5928 copies/mL (range 25-66681 copies/mL).

2.9. Plasma and PBMC preparation

Whole blood was collected in heparin-coated tubes, and peripheral blood mononuclear cells (PBMCs) were isolated over a Ficoll-Hypaque cushion. Plasma was isolated by centrifugation. The KSHV infection status was determined by using quantitative ELISAs for identifying circulating IgG antibodies to KSHV proteins (LANA and K8.1) as previously described [15,16].

2.10. Statistical Analysis

Significance for differences between experimental and control groups was determined using the two-tailed Student's t-test (Excel 8.0). The linear analyses were determined using SPSS Statistics 20.0.

3. Results

3.1. KSHV de novo infection induces HA production from primary endothelial cells through CD147

By using an enzyme-linked sorbent assay (ELSA), we found that KSHV *de novo* infection induced a significant increase in extracellular HA produced by human umbilical vein endothelial cells (HUVEC); accumulation of extracellular HA continued for at least 96 h post-infection (**Fig. 1A**). Furthermore, immunofluorescence assay (IFA) data indicated that KSHV *de novo* infection also induced intracellular HA accumulation within the HUVEC, when compared to uninfected mock cells (**Fig. 1B**). There are three human HA synthase genes (*Has1-3*) responsible for HA production [17], and our data indicated that KSHV infection prominently increased *Has1* transcripts, while having little or no effect on *Has2* or *Has3* transcripts (**Fig. 1C**). To confirm the role of *Has1* in KSHV-induced HA production, we directly targeted *Has1* by RNAi and showed that it significantly reduced HA production from KSHV-infected HUVEC (**Fig. 1D**).

Previous studies have shown that HA production is regulated by a multifunctional glycoprotein, CD147 (emmprin; basigin), in several types of tumor cells [12,18,19]. Our data confirmed that directly targeting CD147 by RNAi significantly reduced HA production from KSHV-infected HUVEC (**Fig. 2A**). Interestingly, targeting CD147 by RNAi also decreased *Has1* transcription but not *Has2* and *Has3* (**Fig. 2B**), while knock-down of *Has1* by RNAi decreased CD147 transcription but not *Has2* and *Has3* (**Fig. S1**), implying a positive feedback for regulation of CD147 and *Has1*. In comparison, oHA treatment caused reduction of *Has1* and*Has2* transcription but did not affect CD147 or *Has3* (**Fig. 2C**).

3.2. HA production is induced by the viral latent protein LANA and requires LANA functional domain A and MAPK/ERK signaling activities

In more than 90% of KSHV-infected host cells, the virus exists at latency stage [20], implying that some viral latent proteins are probably responsible for inducing HA production. Latency-associated nuclear antigen (LANA) is one of the only viral proteins consistently expressed in all KS-associated malignancies [21], and its major function is to maintain viral episome in latently-infected cells [22], as well as to modulate expression of a variety of viral and cellular genes [23,24]. To determine the role of LANA in inducing HA

production, we ectopically expressed LANA by transfection of HUVEC with a recombinant construct [25] (**Fig. 3A**), and showed that LANA was sufficient for induction of HA production through upregulation of *Has1* transcription (**Fig. 3B-C**).

The LANA protein sequence can be divided into three functional domains: a conserved proline- and serine-rich N-terminal region (domain A), a central region composed of several acidic repeats (domain B), and a conserved C-terminal domain containing a proline-rich region and a region rich in charged and hydrophobic amino acids (domain C) [26]. Both N- and C-terminal domains contain a nuclear localization sequence (NLS, **Fig. 4A**). Further IFA data indicated that the LANA-A fragment containing the N-terminal NLS localized to the nucleus in similar fashion to the full-length control, while the C-terminal NLS within the LANA-BC fragment was non-functional (**Fig. 4B**), which is in accordance with previous findings [27,28]. By using a variety of LANA deletion fragment and full-length control constructs, we found that the LANA domain A (LANA-A) was sufficient to induce CD147 expression with equivalent efficiency to the full-length control (**Fig. 4C**).

Our published data have indicated that LANA upregulates CD147 expression, although the underlying mechanisms remain unclear [25]. Here we showed that LANA-A was sufficient to induce *has1* transcription and HA production, both of which were blocked by RNAi targeting CD147 (**Fig. 4D-E**). We have previously shown that the mitogen-activated protein kinase (MAPK) pathway is involved in KSHV upregulation of CD147 [29]. Here we found that ectopic expression of LANA increased the phosphorylation of MAPK/ERK (**Fig. 5A)** but did not affect NF-κB p65 phosphorylation (data not shown) in HUVEC. Blocking the activities of MAPK by its specific inhibitor U0126 significantly reduced *Has1* transcription and HA production by LANA-transfected cells and KSHV-infected cells, while NF-κB inhibitor Bay11-7082 had no such effects (**Fig. 5B-E**). Immunoblot analysis indicated that U0126 treatment greatly reduced CD147 expression (**Fig. S2A**). Moreover, overexpression of ERK by a recombinant vector [14] significantly upregulated *Has1* transcripts but slightly increasing *CD147* transcripts, while additional silencing of CD147 by RNAi partially reduced *Has1* transcripts, implying other mechanisms independent of CD147 are probably involved in MAPK-ERK upregulation of *Has1* (**Fig. S2B-C**).

3.3. Targeting HA production represses KSHV/LANA-induced endothelial cell invasiveness

Acquisition of a migratory or invasive phenotype represents one hallmark of KSHV-infected endothelial cells, with implications for both viral dissemination and angiogenesis within KS lesions [30]. Here we found that either targeting *CD147* or *Has1* by RNAi or inhibiting HA-receptor interactions by oHA treatment significantly blocked KSHV-induced endothelial cell invasiveness, as measured by the transwell assays (**Fig. 6A**). Further analysis indicated that RNAi against *CD147* or *Has1* or oHA treatment also reduced VEGF, a major pro-migratory and proangiogenic factor production for endothelial cells (**Fig. 6B**). These results suggest that the effects of HA and CD147 on invasiveness are potentially mediated by VEGF. We further found that LANA-A induced endothelial cell invasiveness with a similar efficiency as the full-length LANA, and that the effect of LANA on invasiveness was significantly blocked by targeting *CD147* or *Has1* with RNAi or by inhibiting HA-receptor interactions via oHA treatment (**Fig. 6C**). Interestingly, we found that silencing of *Has1* also partially

reduced the transcripts of VEGF receptor 1 and 2 (VEGFR1 and VEGFR2), while silencing of *CD147* mainly reduced the transcripts of VEGFR2 (**Fig. S3**). Taken together, our data indicate that HA production is required for KSHV- or viral protein-induced invasiveness of primary endothelial cells.

3.4. Upregulation of HA production and Has1 in KSHV+ HIV-infected patients

To explore the clinical relevance of HA production within KSHV+ HIV-infected patients, we tested plasma HA levels by ELSA in a small collection of our cohort HIV-infected patients; KSHV infection status in these patients was determined as described in the Methods. We found that the KSHV+ group (n=16) had higher HA concentrations in their plasma than those from the KSHV- group (n=12) of HIV-infected patients (**Fig. 7A**). Further analysis of a subset of these same patients indicated that the KSHV+ group had higher *Has1-3* transcripts within their peripheral blood mononuclear cells (PBMCs) than those from the KSHV- group (**Fig. 7B**). Only the difference in *Has1* level was highly statistically significant, whereas *Has2* level was moderately significant and *Has3* level had no significance. Moreover, in the KSHV+ group, only the *Has1* level was highly linearly correlated with HA concentration whereas *Has2* and *Has3* levels had moderate or no linear correlation, respectively (**Fig. 7C**). In contrast, there was no linear correlation between HA concentrations and HIV viral loads or CD4 counts from these patients (**Fig. S4**), implying that HA production was potentially induced by KSHV co-infection.

4. Discussion

As mentioned above, HA binds with a number of cellular receptors, leading directly or indirectly to activation of downstream signaling pathways [2]. Although the current study does not address the levels of HA receptors on KSHV-infected primary endothelial cells, we have observed upregulation of HA receptors including CD44 and LYVE-1 by either KSHV *de novo* infection or ectopic expression of LANA (**Fig. S5**). Interestingly, LYVE-1 has been found highly expressed by KSHV-infected cells and within KSHV-associated tumors [31,32], although its role in HA-mediated signaling and functions remains largely unknown. Another major HA receptor, CD44, is also a well-known cancer stem cell (CSC) marker for many tumors [33], while its functions in KSHV pathogenesis and tumorigenesis still remains unclear. Therefore, future work is required to understand the contribution to KSHV pathogenesis by regulation of these HA receptors.

Here we have shown that HA and related signaling pathways are involved in KSHV-infected cell invasiveness. Previous studies suggest that they may also regulate other functions within these cells, such as multidrug resistance and endothelial-to-mesenchymal transformation (EndMT). We and others have found that, in response to interaction with HA, HA receptors interact with many signaling and transporter proteins such as ErbB2, EGFR, BCRP, P-glycoprotein, monocarboxylate transporters (MCTs) to mediate cancer cells multidrug resistance [12,13,34-36]. EndMT is an important pathophysiologic correlate for cancer progression, and two recent studies demonstrate that EndMT is induced by KSHV infection and contributes to viral tumorigenesis [37,38]. Interestingly, HA has been found as a critical regulator of EndMT during cardiac valve formation [39,40].

We found a higher level of HA production and *Has* genes transcripts in KSHV+ than KSHV- groups of HIV-infected patients. Moreover, no linear correlation was observed between HA concentrations and HIV viral loads or CD4 counts from the same patients, indicating the importance of KSHV co-infection for regulation of HA production. However, due to the small number of samples analyzed here, the final conclusion still requires analyses of additional samples from HIV-infected patients. Therefore, we are now working with clinicians within LSUHSC HIV Outpatient (HOP) Clinic to collect more plasma and PBMC samples from HIV-infected patients to continue this analysis.

Published data have reported a higher level of plasma HA within HIV/HCV co-infected patients, and patients with liver fibrosis and/or cirrhosis [41,42]. Therefore, we cannot exclude the possibility of other pathogen co-infection or pathological conditions contributing to upregulation of HA levels in our cohort HIV-infected patients. These factors should be resolved by further investigation in future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

1) KSHV *de novo* infection induces HA production from primary endothelial cells through upregulation of HA synthase gene 1 (Has1) and a multifunctional glycoprotein, CD147.

2) KSHV-induced HA production requires viral latent protein, LANA (in particular functional domain A) and MAPK/ERK signaling activities.

3) HA production is necessary for KSHV/LANA-induced primary endothelial cell invasion, a hallmark feature for KS development.

4) KSHV+ group has higher levels of HA and Has1 activities in their plasma than the KSHV- group of cohort HIV-infected patients.

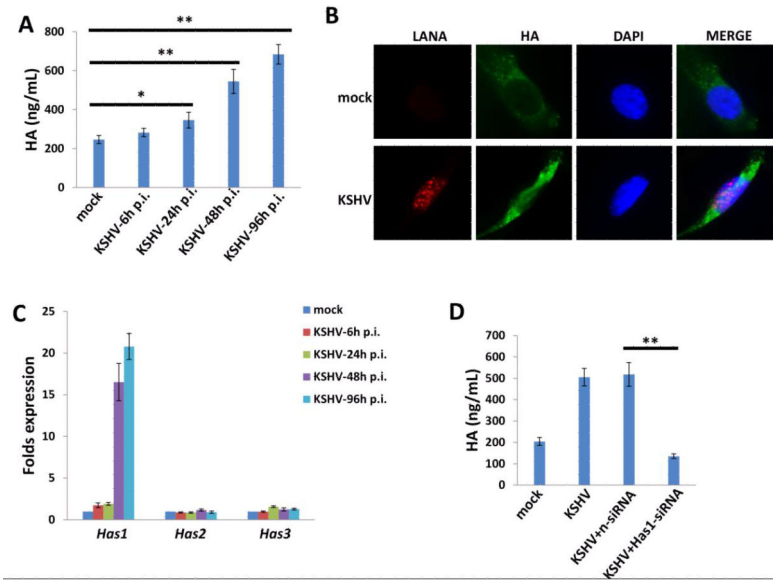

Figure 1. KSHV *de novo* infection induces HA production through upregulation of *Has1*

(**A**) Human umbilical vein endothelial cells (HUVEC) were infected with purified KSHV (MOI~10) for 2 h, then the extracellular HA production at indicated time-points post-infection (p.i.) was measured and compared with mock cells control by ELSA. (**B**) Cells were infected as above, and immunofluorescence (IFA) was used to detect endogenous HA production at 48 h p.i. (**C**) Transcripts representing the 3 HA synthase genes (*Has1-3*) were quantified by qRT-PCR. (**D**) Cells were infected with KSHV for 2 h, then transfected with either negative control siRNA (nsiRNA) or *Has1*-siRNA for 48 h, and the extracellular HA production was measured by ELSA. Error bars represent the S.E.M. for three independent experiments. **=p<0.01.

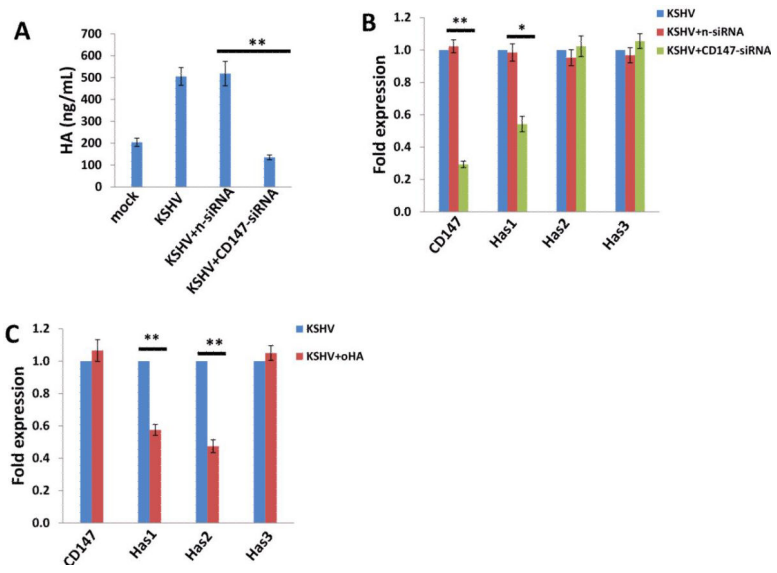

Figure 2. CD147 is involved in KSHV-induced HA production

(**A-B**) HUVEC were infected with KSHV for 2 h, then transfected with either negative control siRNA (n-siRNA) or *CD147*-siRNA for 48 h, and the extracellular HA production was measured by ELSA. Gene expression was quantified by qRT-PCR. (**C**) Cells were infected with KSHV for 2 h, then treated with or without oHA (150 μg/mL) for 48 h, and gene transcripts were quantified by qRT-PCR. Error bars represent the S.E.M. for three independent experiments. **=p<0.01.

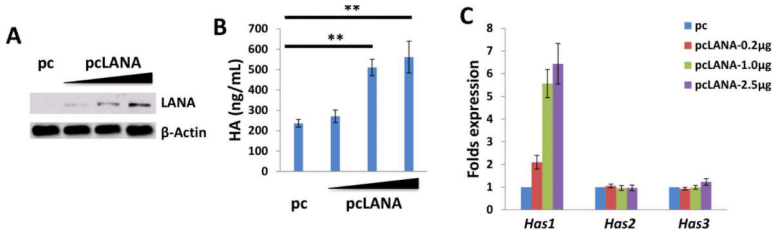

Figure 3. KSHV-encoded LANA protein is sufficient to induce HA production from primary endothelial cells

(**A-C**) HUVEC were transfected with control vector (pc), or vectors encoding LANA (pcLANA) at 0.2, 1.0 or 2.5 μg, respectively, for 48 h, then protein expression, HA production and *Has* genes transcription were measured by immunoblots, ELSA, qRT-PCR, respectively. Error bars represent the S.E.M. for three independent experiments. **=p<0.01.

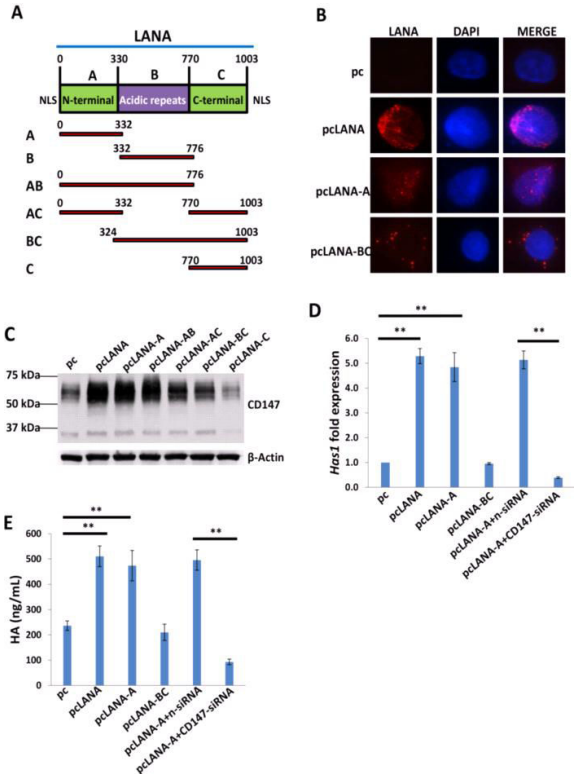

Figure 4. LANA induces HA production requiring functional domain A and CD147

(**A**) Putative domain structure of LANA based on primary sequence features. The N-terminal region (domain A) is rich in prolines and serines and contains a putative nuclear localization sequences (NLS). The central region of LANA (domain B) is comprised of several repeats and is very acidic. The C-terminal region (domain C) also contains a putative NLS. All fragment variants and their coordinates are depicted below the domain model of LANA. (**B**) HUVEC were transfected with control vector pc, full-length LANA construct pcLANA, and fragment variants pcLANA-A, pcLANA-BC, respectively, for 48 h. IFA was used to detect their cellular localization, and the nuclear shown by DAPI. (**C-E**) Cells were transfected with control vector pc, full-length LANA construct and fragment variants, respectively, for 48 h. Immunoblots, qRT-PCR and ELSA were used to detect CD147 expression, *Has1* transcription and extracellular HA production, respectively. Error bars represent the S.E.M. for three independent experiments. **=p<0.01.

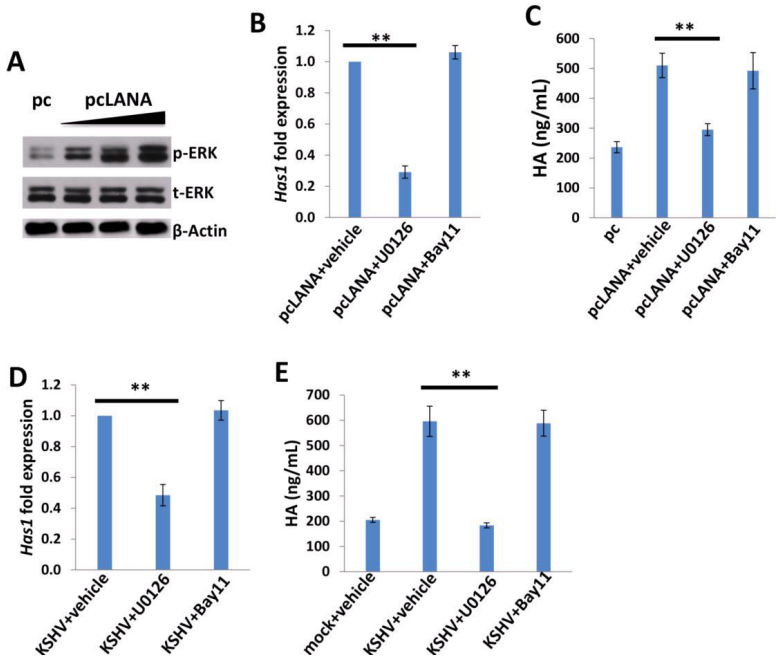

Figure 5. The MAPK/ERK pathway is involved in LANA-induced HA production from primary endothelial cells

(**A-C**) HUVEC were transfected with control vector (pc), or vectors encoding LANA (pcLANA) at 0.2, 1.0 or 2.5 μg, respectively, for 48 h, then some cells were treated with vehicle, MAPK inhibitor U0126 (10 μM), or NF-κB inhibitor Bay11-7085 (10 μM) for 2 h followed by additional 24 h-culture. (**D-E**) Cells were infected with KSHV for 2 h, then treated with vehicle or signaling inhibitors as above. Protein expression, *Has1* gene transcription and HA production were measured as above. Error bars represent the S.E.M. for three independent experiments. **=p<0.01.

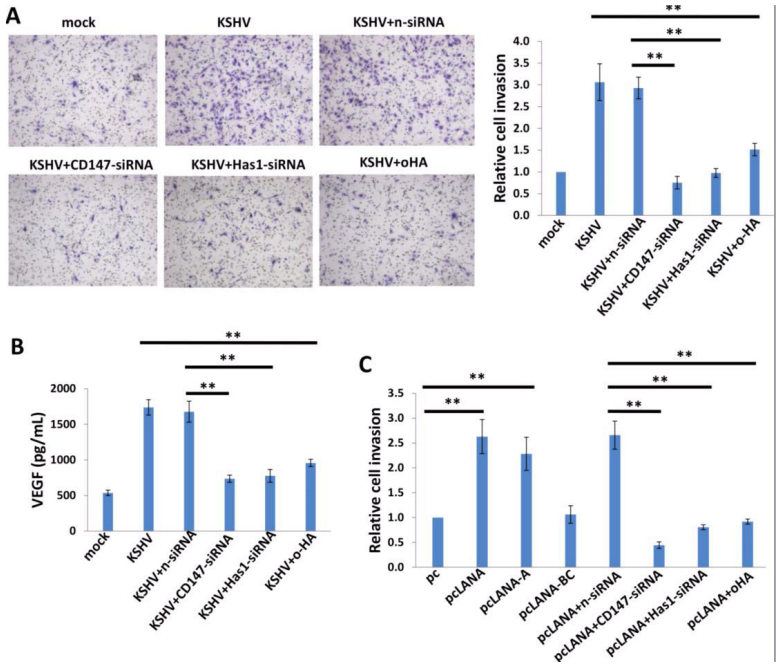

Figure 6. Targeting HA production represses KSHV/LANA-induced endothelial cell invasiveness

(**A**) HUVEC were infected with KSHV for 2 h, then transfected with either negative control siRNA (n-siRNA) or *Has1*-siRNA, *CD147*-siRNA, or treated with oHA (150 μg/mL) for 48 h, and cell invasion were assessed using the transwell assays and calculations detailed in the Methods. (**B**) VEGF concentrations within culture supernatant were quantified by ELISA. (**C**) Cells were transfected with control vector pc, full-length LANA construct pcLANA, and fragment variants, respectively, for 48 h. Some cells were then transfected with either negative control siRNA (n-siRNA) or *Has1*-siRNA, *CD147*-siRNA, or treated with oHA (150 μg/mL) for additional 48 h. Cell invasion were assessed as above. Error bars represent the S.E.M. for three independent experiments. **=p<0.01.

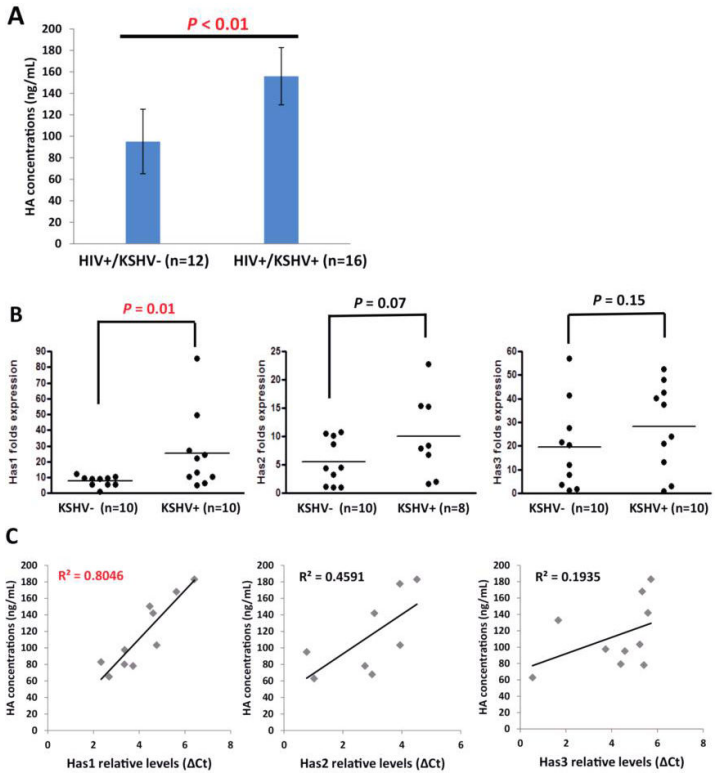

Figure 7. Upregulation of HA production and *Has1* in KSHV+ HIV-infected patients

(**A**) The HA concentrations within plasma from HIV-infected patients were quantified using ELSA. KSHV infection status was identified as described in the Methods. (**B**) The transcriptional levels of *Has1-3* within PBMCs from the same patients were quantified by qRT-PCR. (**C**) The linear analysis of correlation between HA concentrations and *Has1-3* transcriptional levels were performed by SPSS.