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Transactivation of human endogenous retrovirus K (HERV-K) by KSHV promotes Kaposi's Sarcoma development

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Abstract

Kaposi's Sarcoma-associated Herpesvirus (KSHV) is the causative agent of several human cancers such as Kaposi's Sarcoma (KS), which represents the most common AIDS-associated malignancy that lacks effective treatment options. Despite its clear role in AIDS-malignancies, the fact that only a small set of KSHV-infected patients will eventually develop these tumors implies that additional co-factors are required for the development of KSHV-related cancers. In the current study, we demonstrate for the first time that KSHV *de novo* infection or viral latent proteins are able to transactivate Human Endogenous Retrovirus K (HERV-K) through a variety of cellular signaling pathways and transcriptional factors. Moreover, we found that HERV-K transactivation, particularly activation of its encoded oncogenic NP9 protein, plays an important role in KSHV pathogenesis and tumorigenesis *in vitro* and *in vivo*. Our data provide innovative insights into the

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Competing interests

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mechanisms of HERV-K transactivation contributing to viral oncogenesis, which may represent a promising target for KS treatment.

Keywords

HERV; KSHV; Kaposi's Sarcoma; viral oncogenesis

INTRODUCTION

Approximately 20% of human cancers have been found related to viral infections, including Kaposi's Sarcoma-associated Herpesvirus (KSHV, also named as Human Herpesvirus 8, HHV-8).[1] KSHV is the causative agent of several cancers arising in patients with compromised immune systems, including Kaposi's Sarcoma (KS) and Primary Effusion Lymphoma (PEL).[2,3] Despite the reduced incidence of KS since the invention of Highly Active Antiretroviral Therapy (HAART) for Human Immunodeficiency Virus (HIV), KS remains the most common Acquired Immunodeficiency Syndrome (AIDS)-associated tumor.[4,5] The prevalence of KSHV in the US HIV infected population remains high and incidence of new infections has increased in the HAART era.[6] A longitudinal study of solid organ transplant recipients in the United States reported 15% of KSHV seropositivity in this specific subpopulation.[7] Transplant recipients who develop primary KSHV infection after the transplantation, will have a relatively high probability of developing these KSHV-related malignancies, especially KS.[8, 9] Since its discovery about 25 years ago, KSHV has now become a model pathogen for viral oncogenesis research, but many key questions regarding its mechanisms of pathogenesis and oncogenesis still remain unclear, hindering the identification of rational targets or the development of effective therapeutic strategies against these malignancies. Although KSHV has been closely linked to several human malignancies, only a small portion of KSHV-infected patients will eventually develop these tumors,[1] implying that additional host or environmental co-factors such as co-infecting pathogens are required for the development of KSHV-related malignancies.

Human Endogenous Retrovirus (HERV) sequences occupy ~6% - 8% of the human genome, and have resided in our genome for several million years.[10,11] Due to the accumulation of multiple nonsense mutations, the majority of HERVs are dysfunctional, however, some are still active and may play a role in human disease, in particular the HERV type K (HML-2) family.[12–14] HERV-K transactivation has been observed in a variety of human cancers, such as leukemia,[15] lymphoma,[16] breast cancer[17,18] and melanoma.[19] For instance, the expression of the HERV-K envelope (*env*) protein in malignant breast cancer cell lines have been found higher than non-malignant breast cells, and some anti-HERV-K-specific monoclonal antibodies effectively inhibited breast cancer cells growth and induced their apoptosis of *in vitro* and *in vivo*.[17] Interestingly, several herpesviruses have been reported to induce HERV-K transactivation. For instance, HERV-K18 can be transactivated as a superantigen (SAg) by Epstein–Barr virus (EBV) infection, and subsequently activates TCRVB13 T cells through MHC-II which plays a central role in EBV infection and pathogenesis.[20–22] However, currently there are no data describing the role of HERV-K transactivation in viral oncogenesis, especially KSHV-related malignancies. In the current

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study, we demonstrate for the first time that KSHV *de novo* infection or viral latent proteins are able to transactivate HERV-K through a complex of mechanisms. Moreover, HERV-K transactivation (in particular activation of its oncogenic NP9 protein) are required for KSHV pathogenesis and tumorigenesis *in vitro* and *in vivo*.

RESULTS

KSHV *de novo* infection or encoded latent proteins transactivate HERV-K *in vitro* and *in vivo*

During an infection time course analysis, we found that KSHV *de novo* infection gradually increased HERV-K envelope gene (*env*) transcripts from primary human umbilical vein endothelial cells (HUVEC) when compared to the UV-inactivated KSHV infected cells by using qRT-PCR (Fig. 1A). Currently, the qRT-PCR based detection of HERV-K *env* transcripts is the most common and reliable method to evaluate the level of HERV-K transactivation in host cells.[15–17] Our qRT-PCR primers were designed to measure the total levels of HERV-K *env* transcripts, including type 1 and 2 proviruses. Interestingly, our data indicate that KSHV+ PEL tumor cell lines (BC-1, BC-3, BCP-1 and BCBL-1) also have significantly higher levels of HERV-K *env* transcripts when compared with the virus-negative lymphoma cell line, BL-41 (Fig. S1). To understand the clinical relevance of HERV-K transactivation in KSHV-infected HIV+ patients, we examined the levels of HERV-K *env* transcripts in peripheral blood mononuclear cells (PBMCs) samples collected from a cohort of HIV+ patients prior to undergoing the HAART. KSHV infection status have been determined by measuring the titers of anti-KSHV-encoded LANA and K8.1 circulating IgG as described previously.[26,27] Our results indicated a higher level of HERV-K *env* transcripts in the KSHV+ group (n=11) than those in the KSHV- group (n=10, Fig. 1B). Since there are no significant differences in HIV viral loads and CD4 counts between these two groups (data not shown), we think that KSHV infection may be responsible for the HERV-K transactivation in these patients.

KSHV has two infection-phases: a latent phase with only a limited number of viral genes expressed and a lytic phase in which most viral genes are expressed that ultimately produces infectious virions.[31] In most KSHV-infected host cells (>90%), the virus exists in the latency stage,[32] suggesting that some virus encoded latent proteins are potentially responsible for HERV-K transactivation. We detected the expression of two major KSHV-encoded latent genes, Latency-associated nuclear antigen (*Lana*, *Orf73*)[33] and viral FADD-like interferon converting enzyme (FLICE) inhibitory protein (*vFlip*, *Orf71*)[34] during KSHV *de novo* infection. Notably, we found that the expression of these two latent genes displayed an increase in expression that was relatively concordant with HERV-K *env* expression during the time course of KSHV infection (Fig. 1C). To further determine whether these latent genes are indeed responsible for KSHV-induced HERV-K transactivation, we transfected HUVEC with a recombinant LANA or vFLIP construct,[35,36] respectively. We found that ectopic expression of LANA or vFLIP significantly increased HERV-K *env* transcripts from HUVEC in a dose-dependent manner (Fig. 1D-E). As a comparison, we found that ectopic expression of RTA, a viral lytic protein which initially controlling KSHV “latent to lytic” switch,[59] almost does not induce HERV-K *env* expression (Fig. 1F).

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Identification of cellular mechanisms for KSHV latent proteins induction of HERV-K expression

We next sought to understand the underlying mechanisms for LANA or vFLIP induced HERV-K transactivation in primary endothelial cells. We and others have reported that KSHV latent proteins are capable of activating several intracellular signaling pathways, e.g., LANA can activate the MAPK pathway[37] and vFLIP can activate the NF-κB pathway.[34] Our data here confirmed that ectopic expression of LANA or vFLIP induced the phosphorylation of MAPK-ERK or NF-κB p65, respectively, from transfected HUVEC (Fig. 2A-B). Next, we found that only inhibition of MAPK by U0126 effectively reduced HERV-K *env* transcripts from LANA-transfected cells, while inhibition of NF-κB by Bay11-7082 had no effects (Fig. 2C & E). In contrast, only inhibition of NF-κB but not MAPK effectively reduced HERV-K *env* transcripts from vFLIP-transfected cells (Fig. 2D & E). Furthermore, inhibition of either MAPK or NF-κB can partially reduce HERV-K *env* transcripts from KSHV-infected cells, and dual inhibition of these pathways has synergistic effects on reduction of HERV-K *env* transcripts (Fig. 2F). These data demonstrate that the MAPK and/or NF-κB pathways are indeed required for KSHV or viral latent proteins induced HERV-K transactivation.

In fact, HERV-K transactivation largely depends on the transcriptional regulatory elements within its retroviral long terminal repeats (LTRs), which have potential binding sited for both viral and cellular transcriptional factors (TRs).[38] Currently, there are a few TRs which have been experimentally shown to modulate HERV-K LTR activities, including Sp1 and YY1 proteins.[39,40] A previous study has shown that LANA directly interacts with Sp1 in the nucleus of KSHV+ lymphoma cells.[41] Here we confirmed the interaction of LANA and Sp1 in KSHV-infected HUVEC by using immunofluorescence and co-immunoprecipitation assays (Fig. S2A-B). Moreover, knock-down of Sp1 by RNAi partially reduced HERV-K *env* transcripts from LANA-transfected cells (Fig. S2C). These data provide additional mechanistic insights into viral latent protein mediated induction of HERV-K expression through interaction with cellular TRs.

HERV-K transactivation is closely related to KSHV-induced primary endothelial cell invasiveness

One hallmark of KSHV-infected endothelial cells is displaying a migratory or invasive phenotype, with can facilitate viral dissemination and angiogenesis during KS development.[41] Our data indicated that knock-down of HERV-K *env* by RNAi significantly blocked the invasiveness of KSHV-infected HUVEC by using the transwell assays (Fig. 3A-C). This reduction is independent of cell growth, since we do not observe silencing of HERV-K *env* affecting HUVEC cell growth (data not shown). Our previous study has demonstrated that VEGF is one of the major pro-angiogenic cytokines responsible for KSHV-induced primary endothelial cell invasiveness.[42] Here we found that silencing of HERV-K *env* significantly reduced the VEGF production and the expression of VEGF receptor 1 (VEGFR1) but not the VEGF receptor 2 (VEGFR2) from KSHV-infected HUVEC (Fig. 3D-E). Together, these data indicate that HERV-K transactivation is closely related to KSHV-induced primary endothelial cell malignant behaviors.

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KSHV infection activates HERV-K encoded oncogenic NP9 expression which enhances viral pathogenesis in endothelial cells

Among HERV-K (HML-2) elements, there are two major types of proviruses (type 1 and 2). Unlike type 2 proviruses, type 1 elements share a 292-nt fragment deletion in the *env* region, which gives rise to a difference between two isoforms of regulatory proteins encoded by the double-spliced transcripts. Type 2 proviral transcripts, 1.8 kb long, code the 15-kDa accessory protein Rec,[63] which is a functional homologue of Rex and Rev from other retroviruses.[64] Type 1 specific double-spliced RNA product, NP9, is a 9-kDa protein which shares only the N-terminal 15 amino acid residues with Rec.[18,44,45] Furthermore, the NP9 protein has been found as a oncogenic protein and present in a variety of tumors and transformed cells.[18,44,45] Our data here indicate that KSHV *de novo* infection induced a gradient increase of *Np9* transcripts from HUVEC using qRT-PCR with *Np9*-specific primers,[65] which was subsequently confirmed by immuoblots with a NP9 polyclonal antibody (kindly provided by Dr. Friedrich A. Grasser from Universitatsklinikum des Saarlandes, Germany)[28] (Fig. 4A-B). Notably, NP9 protein is only expressed in KSHV-infected cells, while none in the uninfected cells. In contrast, we found that KSHV *de novo* infection slightly induced the increase of *Rec* transcripts (with no statistical significance) using qRT-PCR with *Rec*-specific primers[65] (Fig. S3). Next, we observed the strong expression of NP9 within AIDS-KS tumor tissues while only low levels of expression in adjacent normal tissues from two HIV+ patients without any HAART treatment (Fig. 4C). Additionally, we found that NP9 was exclusively expressed in the nucleus by transfecting HUVEC with the pDsred-NP9 construct[28] (Fig. 4D), although we observed some cytoplasmic staining of NP9 in AIDS-KS tissues (Fig. 4C). Since this is a self-made polyclonal antibody which has not been tested for immunohistochemistry staining, we cannot exclude the existence of some non-specific staining in the immunohistochemistry assays. The results from co-immunoprecipitation assays in both directions revealed the protein interaction between NP9 and LANA in KSHV-infected HUVEC (Fig. 4E). Moreover, ectopic expression of NP9 from the recombinant construct pSG5-NP9[28] significantly increased HUVEC invasion and anchorage-independent growth (Fig. 4F-H). Interestingly, we found that ectopic expression of NP9 greatly up-regulated the expression of one cellular glycoprotein, CD147 (also named as Emmprin), and its downstream proteins, ADAMTS1 (A disintegrin and metalloproteinase with thrombospondin motifs 1) and ADAMTS9 (A disintegrin and metalloproteinase with thrombospondin motifs 9) (Fig. 4F). Both high and low molecular weight (~65 and ~35 kDa, respectively) CD147 glycoforms were elevated in NP9-transfected cells, in particular the mature high molecular weight glycoform related to biological activities.[46] qRT-PCR analysis indicated that ectopic expression of NP9 also increased the transcripts of these genes (Fig. S4). Our previous studies reported that KSHV infection or ectopic expression of LANA induced CD147 expression, which enhances primary endothelial cell invasiveness.[35] Our recent transcriptomic analysis has determined that ADAMTS1 and ADAMTS9 are two novel CD147-regulated downstream proteins, and they are all highly expressed in AIDS-KS tissues.[30] Moreover, silencing of CD147, ADAMTS1 or ADAMTS9 by RNAi significantly reduced KSHV-induced primary endothelial cell invasiveness.[30,35] Here we also found that silencing of CD147, ADAMTS1 or ADAMTS9 by RNAi significantly blocked the NP9-

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induced HUVEC invasion (Fig. S5), indicating that CD147-ADAMTS1/ADAMTS9 axis is indeed contributed to NP9-mediated cellular functions.

Since KSHV-infected primary endothelial cells (e.g., HUVEC) usually are not able to form tumors in mice,[31] we recently established a KS-like xenograft model using a KSHV long-term-infected *t*elomerase-*i*mmortalized human umbilical *v*ein *e*ndothelial (TIVE-LTC) cell line, which stably supports KSHV latency (kindly provided by Dr. Rolf Renne at University of Florida).[24,30] Our data indicated that TIVE-LTC have much higher levels of NP9, CD147 and downstream protein expression than the parental non-infected TIVE cells (Fig. 5A). Co-immunoprecipitation assays confirmed the interaction of LANA and NP9 in TIVE-LTC (Fig. 5B). We previously showed that TIVE-LTC displayed much stronger abilities of cell invasiveness and anchorage-independent growth than its parental TIVE cells, the latter almost cannot form colonies in soft agar assays.[30] To further study the functional role of NP9 in TIVE-LTC, we first directly silenced it by using lentiviral vector containing shRNA specifically targeting Np9 (Np9-shRNA) to obtain stably "knock-down" cells. A non-silencing (n)-shRNA was used as a negative control, and we do not observe silencing of Np9 affecting TIVE-LTC cell growth (data not shown). Here we demonstrated that silencing of Np9 by RNAi dramatically reduced TIVE-LTC invasion and anchorage-independent growth abilities (Fig. 5C-E).

Targeting HERV-K NP9 significantly suppresses KSHV-induced tumorigenesis *in vivo*

We next seek to determine the role of HERV-K NP9 in KSHV-induced tumorigenesis *in vivo* by using the established KS-like xenograft model.[30] We injected the Np9 stably "knock-down" TIVE-LTC or control cells subcutaneously into the two sides of flanks of nude mice, respectively. These mice were checked and measured every 2~3 days for the presence of palpable tumors for 30 days. Our results indicate that silencing of Np9 significantly repressed KSHV-induced tumorigenesis in nude mice. Mice injected with Np9 stably "knock-down" cells formed much smaller tumors, when compared to mice injected with control "n-shRNA" cells at 30 days (Fig. 6A-C). H & E staining confirmed that there were significantly fewer tumor cells or tumor biomass with more immune cell infiltrated in the tumor tissues from mice injected with Np9 stably "knock-down" cells (Fig. 6D). Of note, we also observed dramatically reduced LANA expression in tumor tissues from mice injected with Np9 stably "knock-down" cells, although the underlying mechanisms remain unclear and we do not observe the similar phenotype *in vitro* cultures (data not shown). Immunoblot results confirmed the reduced levels of NP9, CD147, ADAMTS1 and ADAMTS9 expression in tumor lysates from mice injected with Np9 stably "knock-down" cells (Fig. 6E). Taken together, these data strongly support the important role of HERV-K transactivation (in particular activation of NP9 and related signaling) as the cellular co-factors for KSHV-induced tumorigenesis in this *in vivo* model.

DISCUSSION

In the current study, we demonstrate for the first time that the KSHV-encoded latent proteins LANA and vFLIP can induce HERV-K transactivation through both intracellular signaling pathways (e.g., MAPK and NF-κB) and cellular transactional factors (TRs, e.g., Sp1),

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resulting in enhanced cell invasion, anchorage-independent growth and KSHV-induced tumorigenesis (summarized in Fig. 7). *In silico* analysis of HERV-K 5′ LTR regions has found more than 40 cellular TRs with putative binding sites, including Sp1.[38] Future work will explore which TRs are indeed responsible for KSHV-induced HERV-K transactivation. Besides TRs interacting with HERV-K LTRs, the expression of HERV-K can also be regulated by some epigenetic mechanisms including DNA methylation and histone modification.[47] Interestingly, LANA has been found to interact with or regulate a variety of epigenetic factors such as EZH2, KDM3a and DNMT3a.[48–51] Therefore, it will be interesting to determine whether these epigenetic factors are also involved in KSHV-induced HERV-K transactivation. One recent study reports that the expression of HERV-K correlates with the expression of genes in retinoblastoma (Rb) pathway including p16INK4A-CDK4 in melanoma cells.[52] In fact, LANA can interact with Rb and regulate the Rb/E2F pathway, protecting lymphoid cells from p16 INK4A induced cell cycle arrest and inducing S-phase entry.[53,54] Therefore, it will be important to determine the potential involvement of Rb/E2F pathway in KSHV/LANA-induced HERV-K transactivation.

Based on the types of proviruses, two different isoforms of regulatory proteins are encoded by the double-spliced transcripts of HERV-K *env* region, Rec and NP9, both of which have been reported to link with cancer development.[66, 67] In the current study, we found that KSHV *de novo* infection prominently increases the expression of NP9, which is also highly expressed in AIDS-KS tumor tissues. Interestingly, NP9 has been found to not only activate the Akt, ERK and Notch1 pathways but also to up-regulate β-catenin, which is essential for survival of leukemia stem cells.[45] More importantly, all of these pathways are closely related to KSHV pathogenesis and/or KS development.[55–58] However, it still requires to understand the role of Rec in the KSHV-related tumorigenesis in future studies, since we found that our RNAi silencing of HERV-K *env* caused the reduction of both *Rec* and *Np9* transcripts (data not shown).

We here report that “knock-down” of Np9 by RNAi effectively suppresses KSHV-induced tumorigenesis *in vivo*. Dr. Anil Sood at the MD Anderson Cancer Center has validated the use of 1,2-dioleoyl-snglycero-3-phosphatidylcholine (DOPC) for the efficient systemic delivery of EphA2-specific siRNA (EphA2-siRNA-DOPC) in an established xenograft model for intraperitoneal ovarian cancer.[60] This work has revealed a significant reduction in intra-abdominal tumor expression of EphA2 48 h after intravenous injection of EphA2-siRNA-DOPC, and twice-weekly dosing results in sustained target knock-down and significant anti-tumor efficacy using EphA2-siRNA-DOPC alone or in combination with paclitaxel. Therefore, the use of siRNA-DOPC or siRNA-nano-particles targeting oncogenic NP9 protein may represent a novel and clinically feasible approach for the treatment of KSHV-associated tumors.

Our H & E staining images indicated that there is more immune cells infiltration in the tumor tissues from mice injected with Np9 “knock-down” cells when compared to control mice (Fig. 6D), although the underlying mechanisms need further investigation. Actually, HERVs can promote an immunosuppressive response that may lead to cancer formation and spreading.[61] For instance, HERV Env protein contains an immunosuppressive domain, which was confirmed in animal models as a cause of tumor growth for tumor cells harboring

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the insertion of Moloney MLV and in *env* knockdown in B16 melanoma cells and Neuro-2a neuroblastoma cell lines.[62] Therefore, whether targeting HERV-K transactivation can be part of immunotherapy for KSHV-related malignancies may represent an interesting direction.

MATERIALS AND METHODS

Cell culture and reagents

KSHV+ PEL cell line, BCBL-1 as well as a Burkitt's lymphoma cell line, BL-41 were kindly provided by Dr. Dean Kedes (University of Virginia), which are cultured as described previously.[23] The other PEL cell lines including BC-1, BC-3 and BCP-1 were purchased from American Type Culture Collection (ATCC) and cultured as recommended by the manufacturer. KSHV long-term-infected telomerase-immortalized human umbilical vein endothelial cells (TIVE-LTC) and the parental non-infected TIVE and cells were cultured as previously described.[24] All the cells were cultured at the conditions of 37°C with 5% CO_2.

KSHV purification and infection

BCBL-1 cells were incubated with valproic acid (0.6 mM) for 5 days, and KSHV virions in the culture supernatants was purified using ultracentrifugation as described previously.[25] HUVEC were incubated with purified virus for 2 h at 37°C. The concentration of viral particles (MOI) was calculated as described previously.[25]

Patients and ethics statement

The study was approved by the Institutional Review Boards (IRB) for Human Research at Louisiana State University Health Science Center – New Orleans (No. 8079). All subjects have been provided the written informed consent. A total of 21 HIV+ patients with HAART treatment in our HIV Outpatient (HOP) Clinic are involved. There are 8 females and 13 males, the average age is 50.2 y (range 23-65 y). The average CD4 T cell counts of these patients are 544/mL (range 33-1775/mL), and the average viral loads of HIV is 5904 copies/mL (range 30-63367 copies/mL).

Plasma and PBMC preparation

Whole blood from HIV+ patients was collected and stored heparin-coated tubes, then peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque cushion. Plasma was obtained through the centrifugation. The KSHV infection status is determined by using the quantitative ELISAs as described previously.[26,27]

Immunoblotting and immunoprecipitation

The following antibodies (100-200 μg/mL) were used in immunoblotting: p-ERK/t-ERK, p-p65/t-p65, ADAMTS1 (Cell Signaling, Cat. #4370, #4695, #3033, #8242, #12897), ADAMTS9 (Thermo, Cat. #PA1-1760), CD147 (BD, Cat. #555961), LANA (ABI, Cat. #13-210-100), vFLIP (Ximbio, Cat. #151778) and HERV-K NP9 (kindly provided by Dr. Friedrich A. Grasser from Universitatsklinikum des Saarlandes, Germany).[28] The antibody detecting β-Actin (Cell Signaling, Cat. #4970) was used as the loading control.

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Immunoprecipitation assays were carried out using the Catch and Release Immunoprecipitation Kit (Milipore).

Plasmid transfection and RNA interference

HUVEC were transfected with control vectors, pcDNA3.1-LANA (pcLANA), pcDNA3.1-vFLIP (pcvFLIP), pcDNA3.1-RTA (pcRTA), pDsred-NP9, pSG5-NP9 (both are kindly provided by Dr. Friedrich A. Grasser),[28] in 12-well plates using Lipofectamine 3000 (Invitrogen). Transfection efficiency was determined as described previously.[29] For RNAi assays, ON-TARGET plus SMART pool siRNA for HERV-K *Env, Sp1* (Dharmacon), or the negative control siRNA, were delivered by using the DharmaFECT transfection reagent. To establish stable HERV-K knockdown cells, we used Dharmacon SMARTvector Lentiviral *Np9*-shRNA and a non-silencing (n)-shRNA as a negative control.

qRT-PCR

Total cellular RNA was isolated and purified using the RNeasy Mini kit (QIAGEN). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen). Primers used for amplification of target genes were listed in Table. S1. Amplification was performed on an iCycler IQ Real-Time PCR Detection System, and analyzed as described previously.[23]

Transwell invasion and soft agar assays

Transwell invasion assays were performed using Matrigel Invasion Chambers (BD) and the relative invasion was calculated as describe previously.[25] The anchorage-independent growth abilities were assessed using soft agar assays as described previously.[25]

KS-like Nude mouse model

5×10^5 TIVE-LTC cells in 50 μL PBS plus 50 μL growth factor-depleted Matrigel (BD Biosciences) were together injected subcutaneously into the flanks of nude mice, 6-8 week-old, male (Jackson Laboratory), 4 mice for each group. At the end of experiment, the tumors were excised for immunoblots and immunohistochemistry analyses. All protocols were approved by the LSUHSC Institutional Animal Care and Use Committee (IACUC) in accordance with the national guidelines.

Statistical analyses

Significance for differences among the experimental groups was calculated and determined using the two-tailed Student's t-test (Excel 2016). p values <0.05 or <0.01 were considered significant or highly significant, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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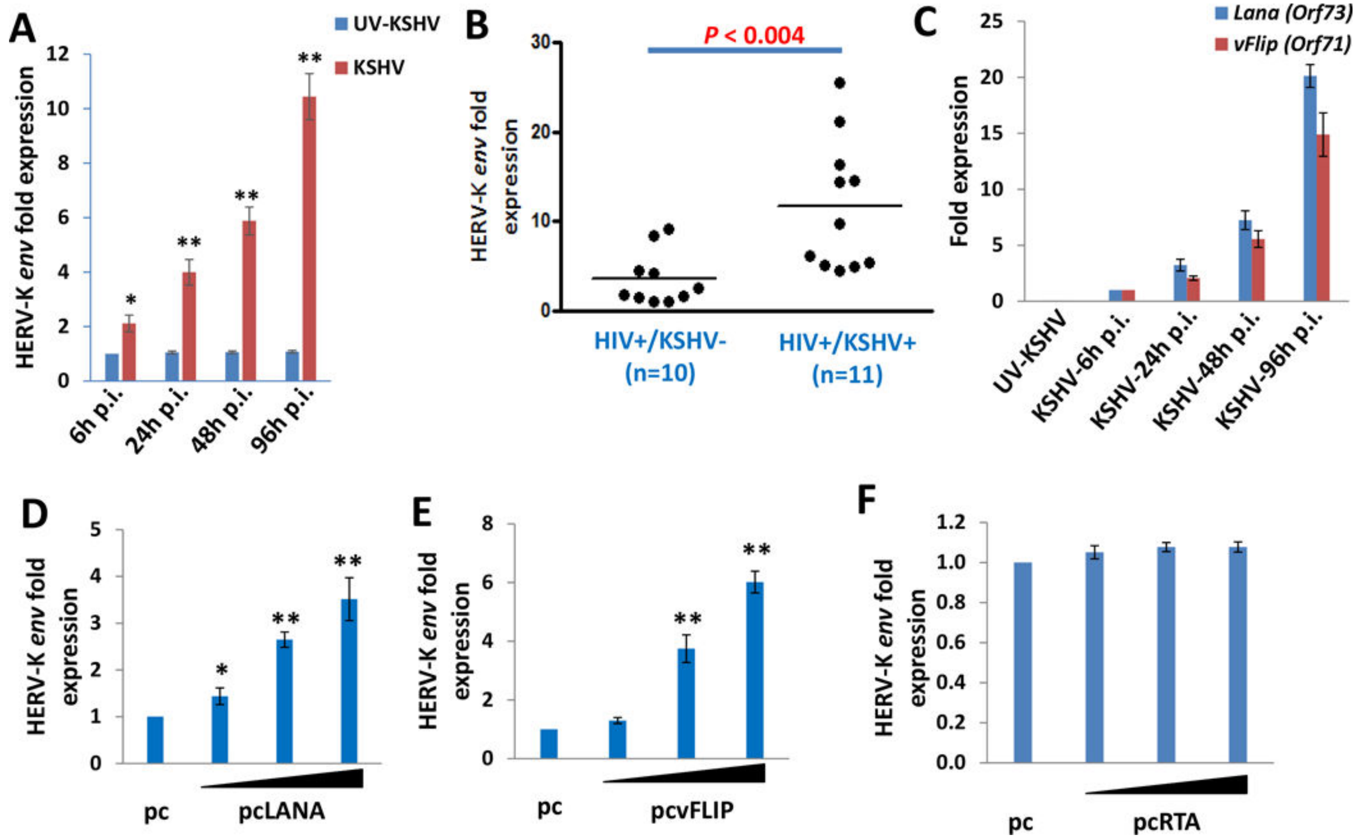

Figure 1. KSHV *de novo* infection or viral latent proteins transactivate HERV-K *in vitro* and *in vivo*

(**A**) Human umbilical vein endothelial cells (HUVEC) were infected with purified KSHV (MOI~10) or UV-inactivated KSHV for 2h, then the induction of HERV-K reactivation at indicated time-points post-infection (p.i.) was measured and compared to UV-inactivated KSHV infected cells control by qRT-PCR with the specific primers for HERV-K *env* gene. (**B**) The levels of HERV-K transactivation within peripheral blood mononuclear cells (PBMCs) from HIV+ patients with or without KSHV co-infection were quantified using qRT-PCR. KSHV infection status was identified using ELISA as described in the Methods. (**C**) HUVEC were infected by purified KSHV as described above, then the transcripts of viral latent genes *Lana* (*Orf73*) and *vFlip* (*Orf71*) at indicated time-points p.i. were measured and compared to control mock cells by using qRT-PCR. (**D-F**) HUVEC were transfected with control vector (pc) or vectors encoding LANA (pcLANA), vFLIP (pcvFLIP) or RTA (pcRTA) at 0.2, 1.0 or 2.5 μg, respectively, for 48 h, then the induction of HERV-K transactivation was quantified by using qRT-PCR. Error bars represent the S.D. from 3 independent experiments. * = p<0.05, ** = p<0.01.

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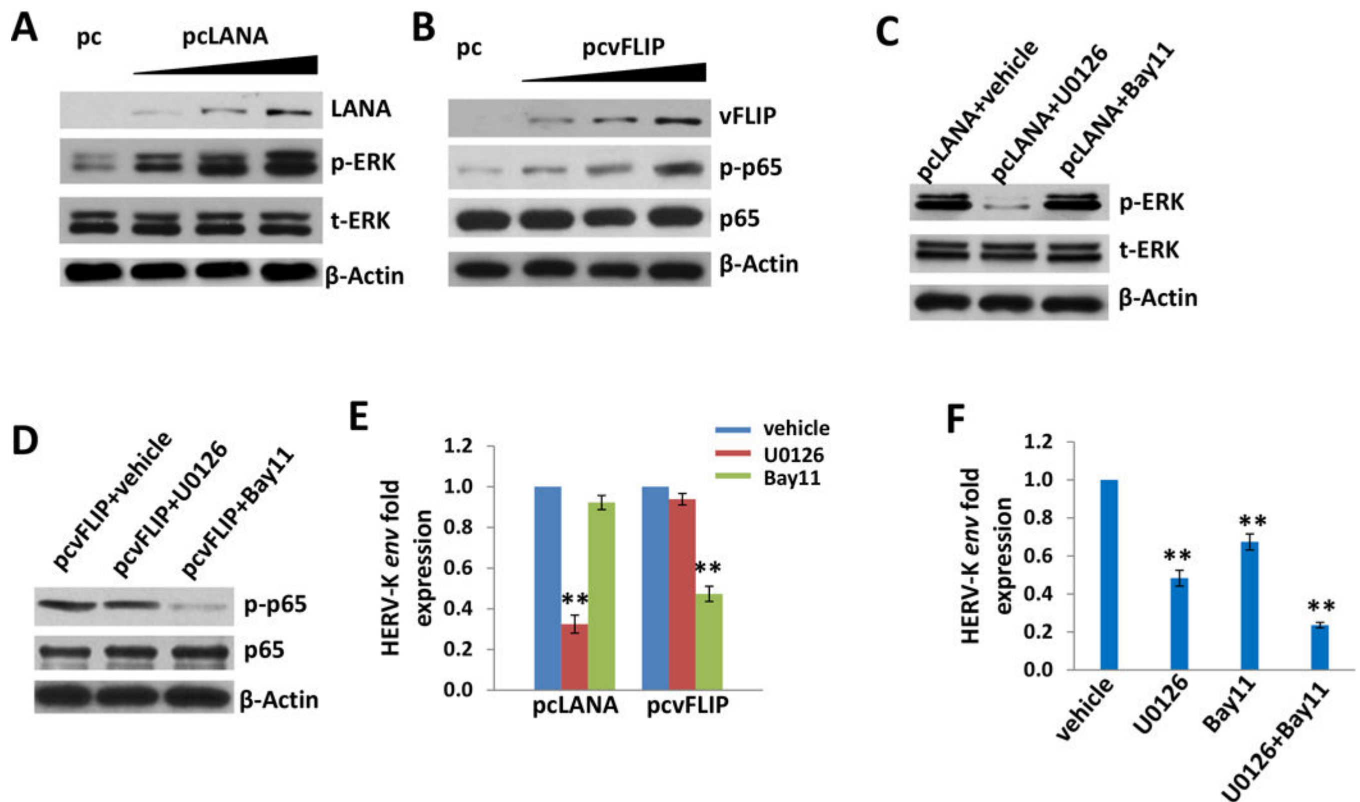

Figure 2. Activation of intracellular signaling pathways is involved in HERV-K transactivation by KSHV

(**A-B**) HUVEC were transfected with control vector (pc) or vectors encoding LANA (pcLANA) or vFLIP (pcvFLIP) at 0.2, 1.0 or 2.5 μg, respectively, for 48 h, then protein expression was analyzed by using immunoblots. (**C-F**) HUVEC were first incubated with either vehicle or MEK inhibitor (10 μM of U0126) or NF-κB inhibitor (10 μM of Bay11-7082) for 1 h, then transfected or infected as described above. The induction of HERV-K transactivation was quantified by using qRT-PCR and protein expression was detected by immunoblots. Error bars represent the S.D. from 3 independent experiments. ** = p<0.01.

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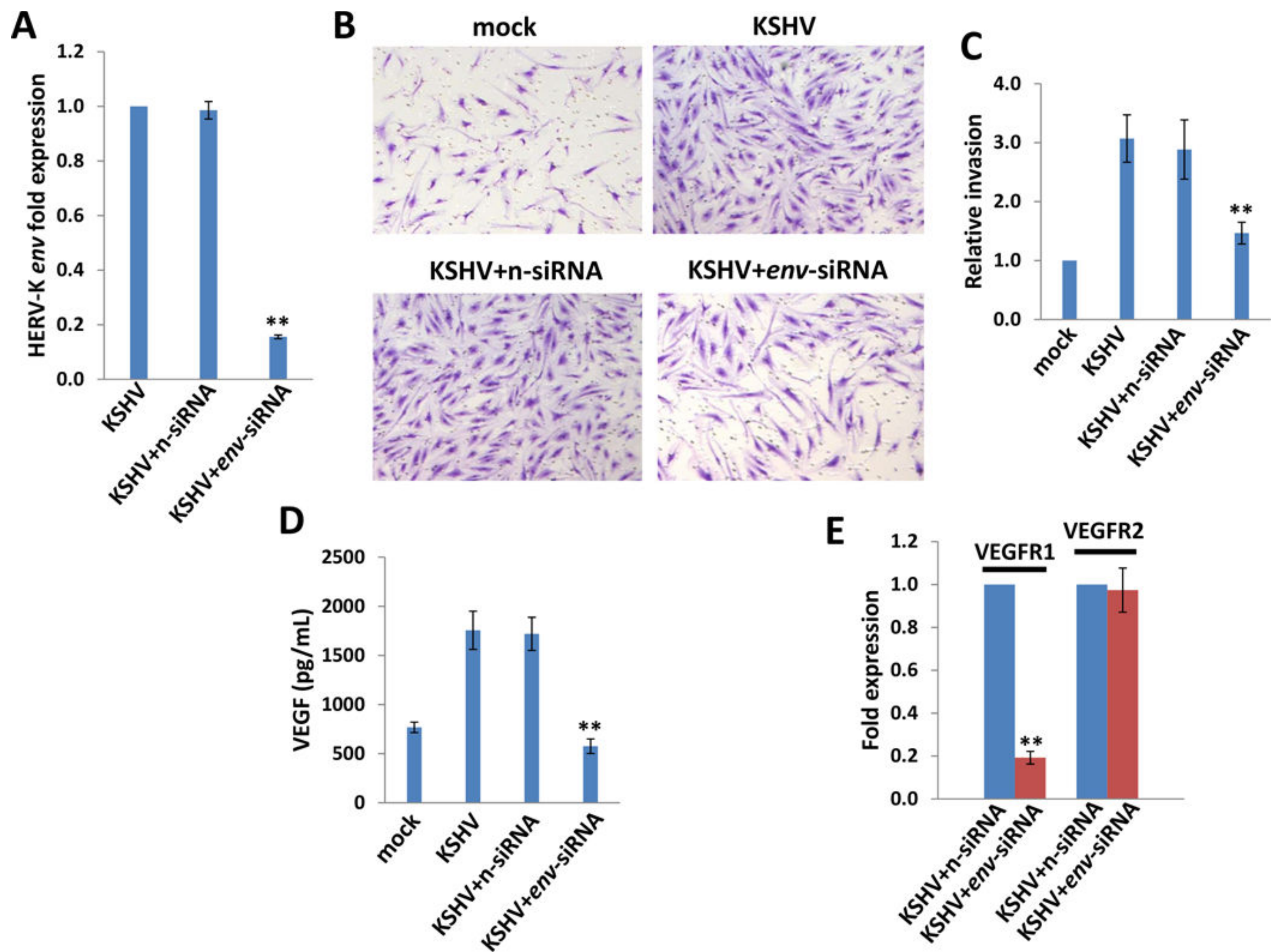

Figure 3. Targeting HERV-K transactivation significantly reduces KSHV-induced primary endothelial cell invasiveness

(**A-C**) HUVEC cells were incubated with purified KSHV (MOI~10) for 2h, then transfected with non-target control siRNA (n-siRNA) or HERV-K *env*-siRNA for additional 48 h. The transwell assays were performed to determine relative invasiveness as described in the Methods. (**D**) The concentrations of VEGF in culture supernatants were determined using ELISA. (**E**) The gene transcripts were quantified by using qRT-PCR. Error bars represent the S.D. from 3 independent experiments. ** = p<0.01.

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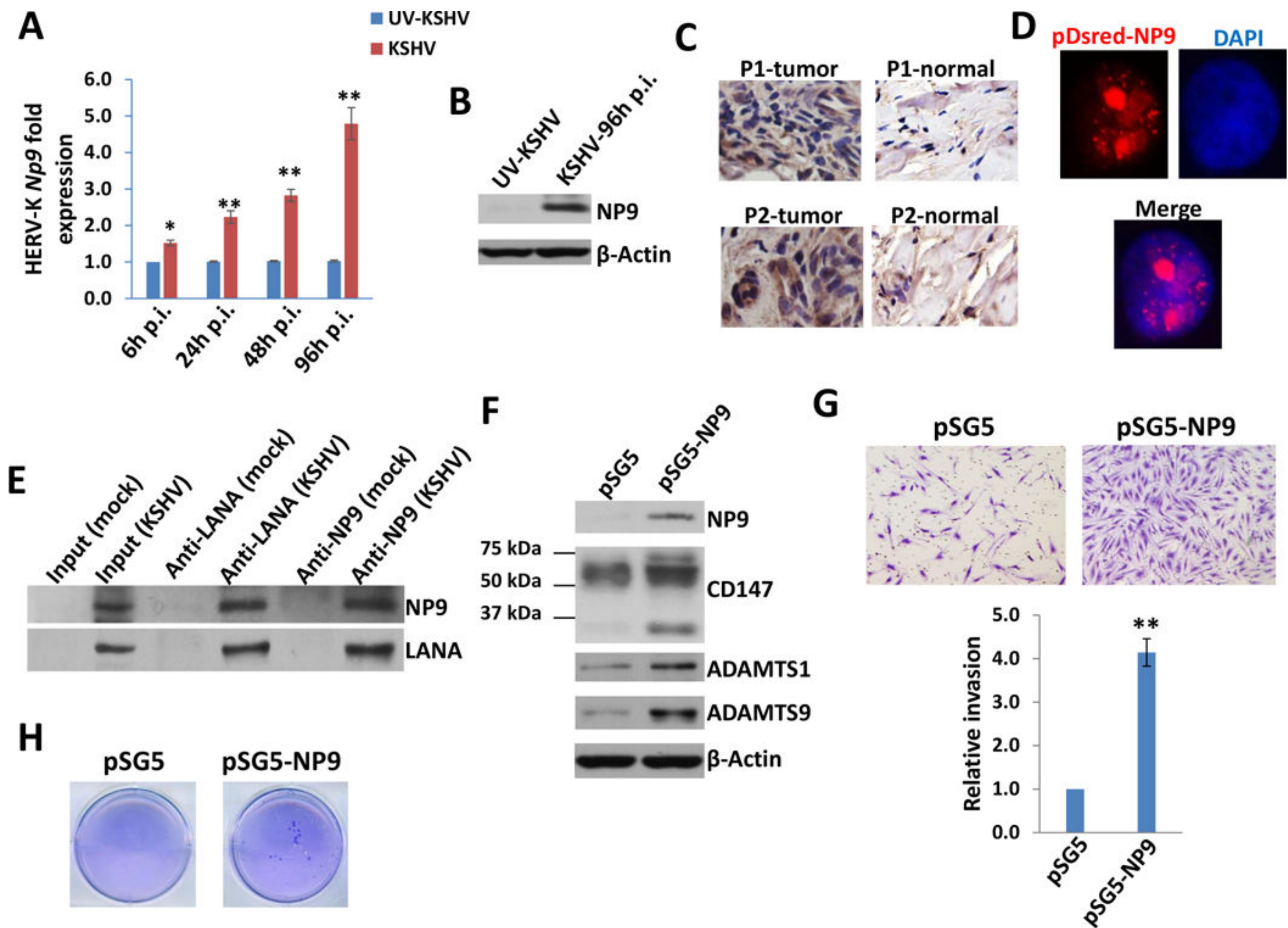

Figure 4. KSHV infection induces HERV-K encoded oncogenic NP9 expression which enhancing primary endothelial cells invasion and colony formation

(**A-B**) HUVEC were infected with purified KSHV (MOI~10) or UV-inactivated KSHV for 2h, then the induction of HERV-K NP9 at indicated time-points post-infection (p.i.) was measured and compared to UV-inactivated KSHV infected cells control using qRT-PCR and immunoblots. (**C**) The strong expression of NP9 protein in KS tissues from our cohort of two AIDS-KS patients without any treatment by immunohistochemistry staining. (**D-E**) HUVEC were transfected with the pDsred-NP9 vector for 48 h, then protein expression was detected by immunofluorescence and nuclear was shown by DAPI. Immunoprecipitation assays in both directions were performed using Catch and Release Immunoprecipitation Kit (Millipore) with anti-LANA or anti-NP9 antibodies, respectively. (**F-H**) HUVEC were transfected with pSG5 control vector or pSG5-NP9 for 48 h, then protein expression was detected by immunoblots. Cell invasiveness was determined using the transwell assays. Anchorage-independent growth ability was determined using the soft agar assays. Error bars represent the S.D. from 3 independent experiments. * = p<0.05, ** = p<0.01.

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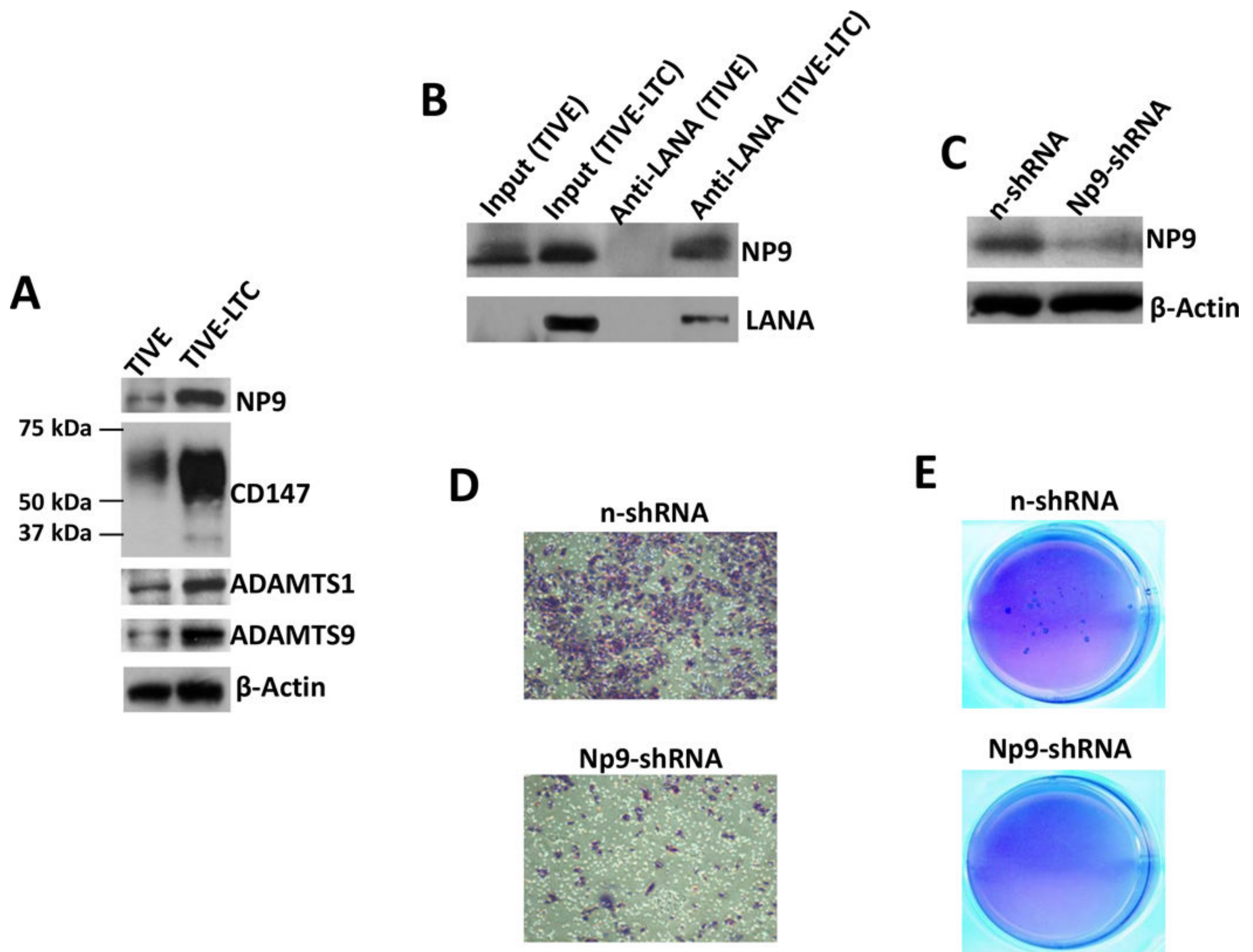

Figure 5. The HERV-K NP9 protein is involved in the pathogenesis of KSHV long-term-infected endothelial cells

(**A**) The protein expression in KSHV long-term-infected telomerase-immortalized human umbilical vein endothelial (TIVE-LTC) and parental uninfected TIVE cells was detected and compared by immunoblots. (**B**) Immunoprecipitation assays were performed with anti-LANA antibody as described previously. (**C-E**) The stably "knock-down" of Np9 in TIVE-LTC were established by using lentiviral vector containing shRNA specifically targeting Np9 (Np9-shRNA) as described in the Methods. A non-silencing (n)-shRNA was used as a negative control. The protein expression, cell invasiveness and anchorage-independent growth abilities were measured as described above.

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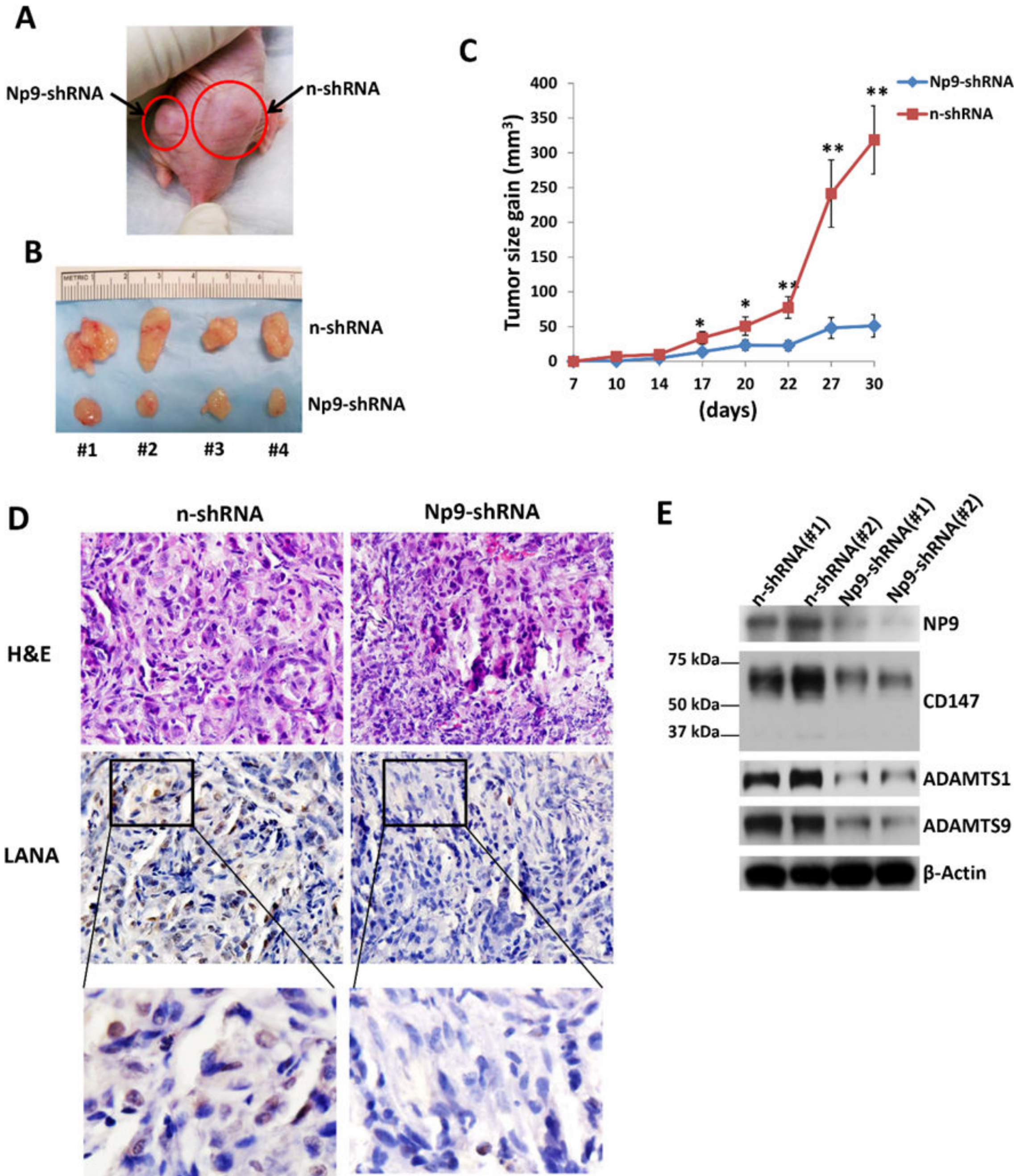

Figure 6. Targeting HERV-K NP9 significantly suppresses KSHV-induced tumorigenesis *in vivo*

(**A-C**) The Np9 stably "knock-down" TIVE-LTC or control 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 and left flanks of nude mice, respectively. The mice were observed and measured every 2~3 d for the presence of palpable tumors for 30 d. Error bars represent the S.D. from 2 independent experiments. * = p<0.05, ** = p<0.01. (**D-E**) Protein expression within tumor tissues from representative injected mice was measured by using immunohistochemistry or immunoblots, respectively.

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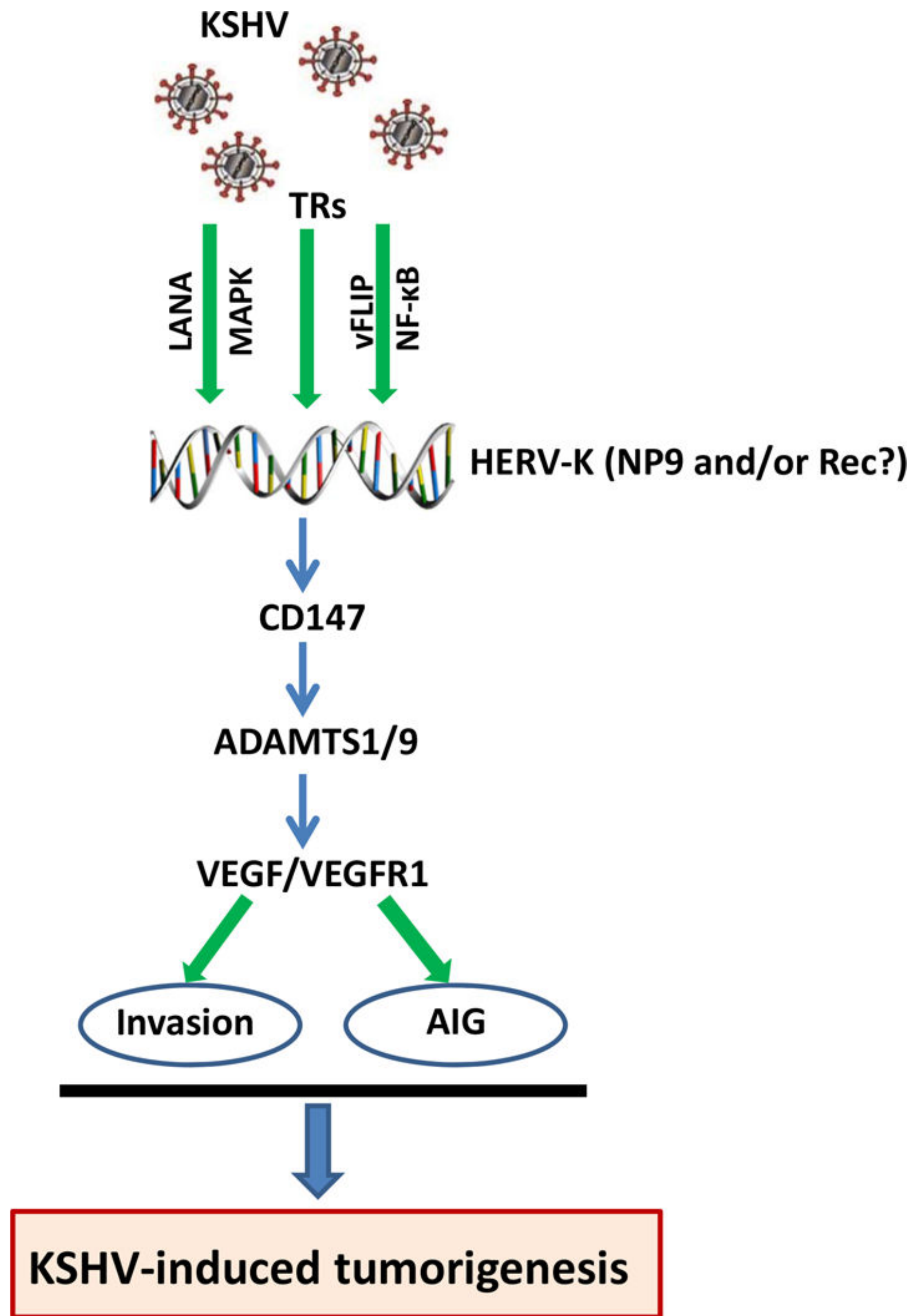

Figure 7. Schematic diagram of potential mechanisms for HERV-K transactivation promoting KSHV-induced tumorigenesis

AIG: anchorage-independent growth. TRs: transcriptional factors.