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Kaposi sarcoma-associated herpesvirus (KSHV) induces a functional tumor-associated phenotype for oral fibroblasts

Lu Dai^{1,2,3,5}, Zhiqiang Qin^{1,2,5,*}, Michael Defee², Bryan Toole³, Keith L. Kirkwood^{2,4}, and Chris Parsons^{1,2,4,*}

¹Department of Medicine, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas St., Charleston, SC 29425, United States

²Department of Craniofacial Biology, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas St., Charleston, SC 29425, United States

³Department of Regenerative Medicine & Cell Biology, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas St., Charleston, SC 29425, United States

⁴Center for Oral Health Research, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas St., Charleston, SC 29425, United States

⁵Key Laboratory of Arrhythmias, Ministry of Education, China (East Hospital, Tongji University School of Medicine), Shanghai, Shanghai 200120, China

Abstract

The Kaposi sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi sarcoma (KS), the most common HIV/AIDS-associated tumor worldwide. Involvement of the oral cavity portends a poor prognosis for patients with KS, but mechanisms for KSHV regulation of the oral tumor microenvironment are largely unknown. Infiltrating fibroblasts are found with KS lesions, and KSHV establishes latent infection within human primary fibroblasts *in vitro*, but contributions for KSHV-infected fibroblasts to the KS microenvironment have not been previously characterized. Secretion of pro-migratory factors and intratumoral invasion are characteristics of tumor-associated fibroblasts (TAF) found in the microenvironment of non-viral malignancies. In the present study, we show that latent KSHV infection of primary human fibroblasts isolated from the oral cavity enhances their secretion of KS-promoting cytokines and intrinsic invasiveness through VEGF-dependent mechanisms. Moreover, we find that KSHV induces these effects through Sp1- and Egr2-dependent transcriptional activation of the extracellular matrix metalloproteinase inducer (emmprin). These data implicate KSHV activation of emmprin in the induction of a “TAF-like” phenotype for oral fibroblasts in the KS microenvironment and support the potential utility of targeting TAFs and/or emmprin in the treatment of oral KS.

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*Correspondence: Mailing address: Medical University of South Carolina, Hollings Cancer Center, Room 512G, 86 Jonathan Lucas St., Charleston, SC 29425. Phone: (843)-792-3644. Fax: (843)-792-6680. parsonch@musc.edu or qinzh@musc.edu.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Keywords

KSHV; pathogenesis; Kaposi sarcoma

1. Introduction

KSHV is one of the most common etiologic agents for cancers arising in the setting of immune suppression, including Kaposi sarcoma (KS)—the most common HIV/AIDS-associated tumor worldwide and a leading cause of morbidity and mortality in this population [1]. Oral involvement occurs in a substantial proportion of patients with KS [2]. KSHV also persists in the oral cavity despite highly active antiretroviral therapy (HAART) for HIV infection [3]. In addition, available data suggest that oral KS is more resistant to chemotherapy [4] and portends a less-favorable prognosis [5]. These data imply that unique KSHV-host cell interactions in the oral cavity provide a foundation for viral persistence and KS progression in this location. KS involvement of the palate, gingiva, and tongue has been described [6], but our current understanding of KSHV-host protein interactions within cells from the oral cavity remains limited.

Tumor-associated fibroblasts (TAF) enhance tumor progression in part through their secretion of pro-migratory factors, including vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), which promote angiogenesis and invasiveness for tumor cells [7,8]. In addition, invasion of the extracellular matrix (ECM) by TAF supports tumor cell invasion through direct modification of the ECM [9]. KSHV-infected “spindle cells,” likely of endothelial origin, represent the characteristic component of KS lesions, but fibroblasts are also found in the KS microenvironment and support *de novo* KSHV infection [10]. We have found previously that KSHV-infected, skin-derived fibroblasts promote endothelial cell invasion of ECM through paracrine mechanisms [11], highlighting the potential role for KSHV-infected fibroblasts in promoting KS pathogenesis. To begin to define putative roles for KSHV-infected oral fibroblasts (OF) in KS pathogenesis and mechanisms for KSHV regulation of OF function, we sought to: 1) determine whether primary human OF support *de novo* KSHV infection; 2) determine whether KSHV infection induces a “TAF-like” phenotype for OF; and 3) identify mechanisms for KSHV regulation of OF function.

2. Materials and methods

2.1 Cell culture and infection assays

KSHV-infected PEL cells (BCBL-1) were maintained in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.5), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, and 0.02% (wt/vol) sodium bicarbonate. Primary human gingival fibroblasts (HGF) and periodontal ligament fibroblasts (PDLF) were originally purchased from ScienCell (www.sciencellonline.com) by Dr. Amy Bradshaw (Medical University of South Carolina) and kindly provided to our laboratory. These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 10% FBS, 10 mM HEPES (pH 7.5), 100 U/mL of penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 µg/ml streptomycin.

2.2 Virus purification and infection assays

To obtain KSHV for infection experiments, BCBL-1 cells were incubated with 0.6 mM valproic acid for 6 days. Following 2 low-velocity centrifugation steps to remove BCBL-1 cells, KSHV was purified from culture supernatants through ultracentrifugation at 20,000 g for 3 h, 4°C. Light microscopy was used subsequently to ensure that no intact BCBL-1 cells were retained during viral purification. The viral pellet was resuspended in 1/100 original volume in the appropriate culture media, and aliquots frozen at -80°C. Six-well plates containing HeLa cells were then incubated with serially diluted virus for 2 h, washed, and incubated in media for an additional 18–24 h. Immunofluorescence assays (IFA) to quantify expression of the KSHV latency-associated nuclear antigen (LANA; see IFA methods below) were then used to determine infectious viral titers by examining slides at 63X magnification using a Nikon TE2000-E fluorescence microscope. LANA exhibits punctate expression (“dots”) within the nucleus of infected cells using this IFA protocol, and the number of LANA dots correlates with viral episome copy number, as LANA tethers the viral episome to host cell chromosomes [12,13]. Therefore, assuming that one LANA dot corresponds to a single viral episome in these assays, titers of our KSHV stocks approximated $4-5 \times 10^6$ infectious particles/ml. HGF or PDLF were incubated with dilutions (MOI~10) of freshly prepared viral stocks in the presence of 8 µg/mL Polybrene (Sigma) for 2 h at 37°C, and LANA IFA (see below) were used to quantify viral episomes within cells within 24 h of viral incubation with visualization at least 200 cells. For negative controls using ultraviolet light-inactivated KSHV (UV-KSHV), viral aliquots were exposed to 1200 J/cm² UV light for 10 minutes using a CL-1000 Ultraviolet Crosslinker.

2.3 Immunoblotting

Cells were lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM EDTA, 5 mM NaF and 5 mM Na₃VO₄. Total cell lysates (30 µg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using 100–200 µg/mL monoclonal antibodies, including emmprin (BD), LANA (ABI), MMP-2 and MMP-9 (cell signaling), BACH-1, Nrf-2, MMP-1, Sp1 and Egr2 (Santa Cruz). For loading controls, blots were reacted with antibodies detecting β-Actin (Sigma). Immunoreactive bands were developed using an enhanced chemiluminescence reaction (Perkin-Elmer) and visualized by autoradiography.

2.4 Immunofluorescence Assays (IFA)

Briefly, 1×10^4 HGF or PDLF per well were seeded in eight-well chamber slides (Nunc) and incubated with serial dilutions of freshly prepared viral stocks in the presence of 8 µg/mL Polybrene (Sigma) for 2 h at 37°C. After remaining in culture overnight, cells were incubated in 1:1 methanol-acetone at 20°C for fixation and permeabilization, then with a blocking reagent (10% normal goat serum, 3% bovine serum albumin, and 1% glycine) for an additional 30 minutes. Cells were then incubated for 1 h at 25°C with 1:1000 dilution of a rat monoclonal antibody (ABI) recognizing the latency-associated nuclear antigen (LANA) of KSHV, followed by 1:100 dilution of a goat anti-rat secondary antibody conjugated to Texas Red (Invitrogen). For identification of nuclei, cells were subsequently counterstained with 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma) in 180 mM Tris-HCl (pH 7.5), washed and prepared for visualization using a Nikon TE2000-E fluorescence microscope.

2.5 Transfection assays

HGF were transfected with control pcDNA3.1 or pcDNA3.1-LANA vectors in 12-well plates for 48 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was determined through cotransfection of a lacZ

reporter construct and quantification of β -galactosidase activity using a commercially available enzyme assay according to the manufacturer's instructions (Promega). For RNA silencing, HGF were transfected for 48 h with either emmprin-, Sp1-, Egr2-, Bach1-, Nrf2- or control non-target-siRNAs (ON-TARGET plus SMART pool, Dharmacon) using a commercially available transfection reagent (Dharmacon) according to the manufacturer's instructions. 3 independent transfections were performed for each experiment, and all samples were analyzed in triplicate for each transfection.

2.6 Transwell invasion assays

Matrigel Invasion Chambers (Becton Dickinson) were hydrated for 4 h at 37°C with culture media. 5×10^4 HGF or PDLF were plated in the top of the chamber. For experiments to determine the role of VEGF in cell invasiveness, 5 ng/mL recombinant human VEGF (LONZA) were added to the top of chambers. After 24 h, cells were fixed with 4% formaldehyde for 15 min and chambers rinsed in PBS prior to staining with 0.2% crystal violet for 10 min. After washing, 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.

2.7 ELISA

Concentrations of IL-1 β , IL-6, IL-10, TNF- α , IL-8 and VEGF in culture supernatants were quantified using human IL-1 β /IL-6/IL-10 (eBioscience), TNF- α /IL-8 (Becton Dickinson) and VEGF (Pierce Biotechnology) ELISA kits according to the manufacturers' instructions.

2.8 PCR

For qRT-PCR experiments, total RNA was isolated 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. Primers designed for amplification of target genes are provided 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 for each gene of interest for each experiment. "No template" (water) controls were 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).

2.9 Transduction assays

HGF were transduced using a recombinant adenoviral vector (MOI ~ 20) encoding emmprin, or a control vector, as previously described [14] for 48 h prior to experimental analyses.

2.10 Statistical analyses

Significance for differences between experimental and control groups was determined using the two-tailed Student's t-test (Excel 8.0).

3. Results

3.1 KSHV establishes latent infection within OF

Latent KSHV infection is dependent upon intranuclear expression of KSHV-encoded LANA which tethers the viral episome to host cell chromatin [15], and immunofluorescence assays

(IFA) for identifying LANA are used commonly to quantify KSHV genome equivalents [12]. Therefore, to first establish whether OF are susceptible to KSHV infection, we incubated HGF and PDLF with purified KSHV and used IFA to quantify LANA expression within individual cells. Evaluation of both high (Fig. 1) and low (Fig. S1) magnification images using confocal microscopy revealed LANA expression within >98% of HGF and PDLF following 24 h of viral incubation using an MOI~10. To further validate these results, and to quantify expression of a larger number of representative latent and lytic viral genes following viral incubation, qRT-PCR was performed using KSHV-infected HGF and PDLF. In agreement with observations using other primary cell types [16], we observed predominant expression of latent genes and relatively minimal expression of lytic genes over 72 h in our cell culture experiments (Fig. 1B, C). Of note, PDLFs exhibited approximately 2–3-fold greater expression of virus-encoded IL-6 (vIL-6) relative to HGF (Fig. 1C).

3.2 KSHV infection induces a TAF-like phenotype for OF

TAF are defined by their capacity for secretion of pro-migratory cytokines and their intrinsic invasiveness [7–9]. Cytokines and chemokines, including VEGF, IL-8, IL-6, IL-10, IL-1 β and TNF- α , support KS pathogenesis through multiple complimentary mechanisms, including induction of migration for KSHV-infected cells and angiogenesis [17]. TAF secretion of VEGF and IL-8 likely facilitates pathogenesis for many tumors [7, 8], and KSHV induces secretion of these factors following *de novo* infection of several host cell types, including endothelial cells, macrophages, and skin-derived fibroblasts [11, 18]. To first explore whether KSHV infection induces OF secretion of soluble factors associated with KS pathogenesis, we used ELISAs to quantify OF secretion of several cytokines and chemokines following *de novo* infection of OF. We found that KSHV infection significantly increased OF secretion of VEGF, IL-8, IL-6 and IL-10 (Fig. 2), but not IL-1 β or TNF- α (data not shown). Subsequent transwell invasion assays further revealed that KSHV infection enhanced intrinsic invasiveness for OF (Fig. 3).

3.3 KSHV induces OF secretion of pro-migratory factors and invasiveness through upregulation of emmprin transcription

The membrane-associated protein emmprin (extracellular matrix metalloproteinase inducer; CD147; basigin) induces matrix metalloproteinase (MMP) synthesis by both fibroblasts and tumor cells, thereby promoting tumor cell invasiveness and angiogenesis in the local environment [19]. We have reported previously that emmprin and associated MMPs are upregulated in KSHV-infected endothelial cells and induce intrinsic endothelial cell invasiveness [20]. Therefore, we hypothesized that KSHV-induced secretion of soluble pro-migratory factors and invasiveness for OF was mediated through KSHV activation of emmprin. Immunoblots revealed that KSHV infection of HGF and PDLF induced expression of emmprin, MMP-1 and MMP-9, although not MMP-2 (Fig. 4A, B). Furthermore, RNA interference assays targeting emmprin confirmed that KSHV induction of MMP-1 and MMP-9 expression by HGF was mediated through viral activation of emmprin (Fig. 4C).

The emmprin promoter contains putative binding sites for several transcription factors, including Sp1 and Egr2 [21], and site-directed mutagenesis has confirmed a significant role for Sp1 in transcriptional activation of emmprin within certain cell types [21, 22]. However, the role of specific transcription factors in the transactivation of emmprin within KSHV-infected cells has not been described. Therefore, to determine whether Sp1 and/or Egr2 regulate transcriptional activation of emmprin in KSHV-infected cells, we performed RNA interference assays targeting Sp1 or Egr2 in KSHV-infected HGF and quantified emmprin expression in these cells. Knockdown of either Sp1 or Egr2 expression suppressed KSHV upregulation of emmprin protein (Fig. 4C) and transcript (Fig. 4D) expression, as well as

KSHV-induced expression of MMP-1 and MMP-9 (Fig. 4C). Interestingly, KSHV infection also increased expression of Egr2, but not Sp1, by HGF (Fig. 4C). In contrast, suppression of other transcription factors lacking binding sites within the emmprin promoter [23, 24] had no impact on KSHV induction of emmprin expression for OF (Fig. S2).

Since we found that emmprin mediates OF invasiveness, we next hypothesized that KSHV induced secretion of soluble pro-migratory factors and intrinsic invasiveness for OF was regulated through Sp1-, Egr2-, and/or emmprin-dependent mechanisms. Using RNA interference, we found that knockdown of emmprin, Sp1 or Egr2 expression significantly suppressed KSHV-induced secretion of VEGF-A (Fig. 5A) but had no effect on KSHV-induced secretion of IL-8 (Fig. 5B). Next, we used a recombinant adenovirus to overexpress emmprin (AdV-em) in the context of Sp1 and Egr2 knockdown in HGF and found that emmprin overexpression was sufficient to restore secretion of VEGF-A, but not IL-8, in these experiments (Fig. 5A–B). Transwell invasion assays further revealed that knockdown of emmprin, Sp1 or Egr2 significantly suppressed KSHV-induced invasiveness for HGF, and that provision of exogenous human recombinant VEGF restored invasiveness under these conditions (Fig. 6). Finally, we transduced KSHV-infected OF with AdV-em in the context of Sp1 or Egr2 knockdown and found that emmprin overexpression was sufficient to restore intrinsic invasiveness for these cells (Fig. 6). Collectively, these data support Sp1- and Egr2-mediated transcriptional activation of emmprin as a mechanism for KSHV upregulation of VEGF-A and invasiveness for OF.

3.4 KSHV-encoded LANA induces emmprin expression and cell invasiveness for OF

Given our observation of predominant latent viral gene expression in KSHV-infected OF, as well as our previous observation of LANA upregulation of emmprin expression by endothelial cells [20], we hypothesized that LANA induced emmprin expression and invasiveness for OF. To test this hypothesis, we transiently transfected HGFs using a recombinant construct for ectopic LANA overexpression and found that LANA increased emmprin expression and HGF invasiveness in these assays (Fig. 7). Moreover, these effects were suppressed using RNA interference targeting emmprin (Fig. 7).

4. Discussion

TAF support cancer cell invasion through the secretion of soluble mediators of inflammation and cell migration [7, 8]. Moreover, TAF invasion and modification of the extracellular matrix facilitates cancer cell invasion [9]. Direct targeting of TAF also reduces cancer progression *in vivo* and sensitizes tumors to chemotherapy [25], further justifying additional mechanistic studies to understand the role of TAF in cancer pathogenesis. Unique clinical features of oral KS, including its propensity for chemotherapeutic resistance and its prediction of less desirable outcomes for patients [3–5], warrant further mechanistic studies to elucidate novel KSHV-oral host cell interactions, including those involving OF. KSHV induces production of pro-inflammatory and pro-migratory factors by skin-derived fibroblasts following *de novo* infection [11, 26], and KSHV-infected, skin-derived fibroblasts promote intrinsic invasiveness for uninfected endothelial cells through paracrine mechanisms [11]. However, determination of OF susceptibility to KSHV infection and the functional consequences of KS-OF interactions have not been previously described.

We found both HGF and PDLF permissive for KSHV infection, with predominant expression of latent genes in both cell types. Interestingly, although expression of KSHV lytic genes was relatively limited, PDLF exhibited an increase in expression of vIL-6 relative to HGF. Although the meaning and functional consequences of this observation were not addressed in our studies, it suggests a need for broader characterization of KSHV gene expression in OF, and that different KSHV-infected OF may serve different roles in

oral KS pathogenesis. KS involvement of the periodontal ligament is exceedingly rare [27]. However, vIL-6 induces VEGF secretion [28, 29], and it is interesting to speculate whether KSHV infection of PDLF may provide a supportive role for pathogenesis in the oral KS microenvironment.

We found that KSHV infection induced OF secretion of several soluble factors associated with KS pathogenesis, including VEGF, IL-8, IL-6 and IL-10. We and others have previously demonstrated mechanisms for KSHV induction of these cytokines in other cell types [11, 18, 26, 30]. Although not addressed in our study, secretion of IL-6 and IL-10 by KSHV-infected OF has implications for suppression of KSHV-specific immune responses and proliferation of KSHV-infected tumor cells in the oral cavity [31]. We did not observe an effect of KSHV infection on secretion of IL-1 β or TNF- α by OF. In addition, secretion of VEGF by OF was emmprin-dependent while secretion of IL-8 was not. Furthermore, we noted KSHV/emmprin-dependent increases in OF expression of MMP-1 and MMP-9, but not MMP-2 which contrasts our observed increases in KSHV/emmprin-dependent activation of all three MMPs in endothelial cells [20]. Additional work will determine whether KSHV induction of emmprin-dependent pathogenesis differs for OF due to unique KSHV-host cell interactions occurring in these cells.

We found that direct targeting of emmprin, Sp1, or Egr2 suppressed KSHV-induced VEGF secretion and invasiveness for OF, and that targeting either Sp1 or Egr2 suppressed KSHV-induced expression of emmprin and related MMPs. Moreover, OF secretion of VEGF-A and invasiveness were restored with emmprin overexpression in the context of Sp1 or Egr2 targeting. Taken together, these data implicate KSHV induction of Sp1- and Egr2-mediated transcriptional activation of emmprin as an important mechanism for OF pathogenesis following KSHV infection of these cells. Additional experiments are needed to confirm direct interactions between these transcription factors and emmprin in KSHV-infected OF, as we cannot categorically exclude the possibility that Sp1 and/or Egr2, rather than binding the emmprin promoter directly, induce transcriptional activation of other cellular or viral genes that subsequently regulate the activation of emmprin. Interestingly, we also observed that KSHV infection of HGF increased expression of Egr2, but not Sp1. In contrast, we have not observed an increase in expression of either Egr2 or Sp1 following infection of primary endothelial cells (data not shown). Our data revealing upregulation of emmprin and OF invasiveness with ectopic expression of LANA is consistent with results from a recent study indicating that LANA is capable of direct interaction with Sp1 and facilitating Sp1-mediated transcriptional activation of telomerase [32]. However, additional work is needed to determine whether LANA facilitates direct binding of Sp1 and Egr2 to the emmprin promoter in OF. Collectively, these observations support the concept that KSHV regulates TAF pathogenesis through unique transcription control mechanisms, and that elucidation of these pathways may provide clues for observed clinical differences between oral KS and KS localized elsewhere.

In summary, this report provides the first evidence for successful establishment of latent KSHV infection within human primary OF and the induction of a TAF-like phenotype for these cells following *de novo* infection. Moreover, we provide evidence for KSHV regulation of emmprin transcription as one mechanism for this process. These results support a putative role for TAF in KS pathogenesis, provide rationale for *ex vivo* studies to determine whether primary OF from oral KS lesions harbor infectious KSHV, and rationale for development of novel studies to determine whether targeting TAF and emmprin-related pathways reduces progression of “KS-like” lesions *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Establishment of latent KSHV infection within primary oral fibroblasts
(A) HGF and PDLF were incubated with purified KSHV (MOI~10), or UV-inactivated KSHV as a negative control, for 2 h. After cells were incubated for an additional 24 h in fresh media, IFA were performed as in Methods to quantify expression of KSHV-encoded LANA as indicated by the typical intranuclear, punctate staining pattern (red dots). Nuclei were identified using DAPI (blue). **(B–C)** HGF (B) and PDLF (C) were incubated with KSHV as in (A). After 72 h, qRT-PCR was used to quantify expression of transcripts encoding latent (LANA, vFLIP, and vCyclin) and lytic (RTA, vGPCR, K8.1, and vIL-6) viral proteins as detailed in Methods. Error bars represent the S.E.M. for three independent experiments.

Figure 2. KSHV increases cytokine/chemokine secretion by oral fibroblasts following *de novo* infection

HGF and PDLF were incubated with either virus-free media (mock) or purified KSHV for 2 h, followed by fresh media for an additional 24 h. Secreted factors were quantified within culture supernatants using commercial ELISA kits as described in Methods. Error bars represent the S.E.M. for three independent experiments. **= $p < 0.01$.

Figure 3. KSHV induces intrinsic invasiveness for oral fibroblasts following *de novo* infection HGF and PDLF were incubated with purified KSHV as above, then relative invasiveness quantified 24 h later using matrigel-based transwell invasion assays as described in Methods. Error bars represent the S.E.M. for three independent experiments. **= $p < 0.01$.

Figure 4. KSHV induces Sp1- and Egr2-dependent transcriptional activation of emmprin (A–B) HGF (A) and PDLF (B) were incubated with purified KSHV as described previously, then immunoblots were performed 24 h later to identify expression of high MW (~65 kDa) and low MW (~35 kDa) glycoforms of emmprin, as well as emmprin-associated MMPs. β -Actin was also identified for loading controls. (C–D) HGF were transfected with either control non-target (n-siRNA), emmprin-siRNA (em-siRNA), Sp1-siRNA or Egr2-siRNA for 48 h prior to their incubation with KSHV. 24 h later, protein (C) and transcript (D) expression were determined by immunoblots and qRT-PCR, respectively.

Figure 5. KSHV induces VEGF secretion by oral fibroblasts through Sp1- and Egr2-mediated activation of emmprin

HGF were transfected with either control non-target (n-siRNA), emmprin-siRNA (em-siRNA), Sp1-siRNA, or Egr2-siRNA for 48 h prior to their incubation with KSHV, then VEGF (A) and IL-8 (B) secretion were quantified within culture supernatants using ELISAs.

In some groups, transfected cells were transduced with a recombinant adenoviral vector encoding emmprin (AdV-em) or a control vector (AdV) for 48 h prior to virus incubation.

** = $p < 0.01$ relative to n-siRNA+K group. ## = $p < 0.01$ relative to Sp1-siRNA+AdV+K or Egr2-siRNA+AdV+K groups. Error bars represent the S.E.M. for three independent experiments.

Figure 6. KSHV induces VEGF-mediated invasiveness for oral fibroblasts through activation of emmprin

HGF were transfected with either control non-target (n-siRNA), emmprin-siRNA (em-siRNA), Sp1-siRNA, or Egr2-siRNA for 48 h prior to their incubation with either virus-free media (mock) or purified KSHV, then cell invasiveness quantified 24 h later using transwell invasion assays as described previously. In some groups, transfected cells were transduced with a recombinant adenoviral vector encoding emmprin (AdV-em) or a control vector (AdV) for 48 h prior to virus incubation. For some groups, 5 ng/mL recombinant human VEGF was added to the top of chambers. Error bars represent the S.E.M. for three independent experiments. ** = $p < 0.01$.

Figure 7. LANA induces emmprin expression and cell invasiveness for oral fibroblasts
(A) HGF were transfected with either control (pcDNA) or LANA-encoding (pcDNA-LANA) vectors for 48 h. LANA and emmprin expression were subsequently confirmed using immunoblots. **(B)** HGF were transfected as in (A) in the presence of either control non-target (n-siRNA) or emmprin-specific siRNA (e-siRNA), and invasion assessed as previously described. Error bars represent the S.E.M. for three independent experiments. ** = $p < 0.01$ for comparison of invasiveness for e-siRNA/pcDNA-LANA and n-siRNA/pcDNA-LANA cotransfectants.