Priority Report

Cancer Research

Direct Activation of Emmprin and Associated Pathogenesis by an Oncogenic Herpesvirus

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Abstract

Emmprin (extracellular matrix metalloproteinase inducer) is a multifunctional glycoprotein expressed by cancer cells and stromal cells in the tumor microenvironment. Through both direct effects within tumor cells and promotion of tumor-stroma interactions, emmprin induces tumor cell invasiveness and regional angiogenesis. The Kaposi's sarcoma–associated herpesvirus (KSHV) is a common etiology for cancers arising in the setting of immune suppression, including Kaposi's sarcoma and primary effusion lymphoma. However, whether emmprin expression and function are regulated by KSHV or other oncogenic viruses in the tumor microenvironment to promote viral cancer pathogenesis remains unknown. Fibroblasts and endothelial cells support latent KSHV infection and represent cellular components of Kaposi's sarcoma lesions. Therefore, we used primary human fibroblasts and endothelial cells to determine whether KSHV itself regulates emmprin expression, and whether KSHV-emmprin interactions mediate cell invasiveness. We found that KSHV promotes fibroblast and endothelial cell invasiveness following *de novo* infection through the upregulation of emmprin, and that this effect is mediated by the KSHV-encoded latency-associated nuclear antigen. We also found that emmprin promotes invasiveness, as well as colony formation, by primary effusion lymphoma cells derived from human tumors. Collectively, these data implicate KSHV activation of emmprin as an important mechanism for cancer progression and support the potential utility of targeting emmprin as a novel therapeutic approach for KSHV-associated tumors. *Cancer Res; 70(10); 3884–9. ©2010 AACR.*

Introduction

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 (1). However, it remains unknown whether emmprin plays a role in viral cancer pathogenesis, or whether viruses themselves directly regulate emmprin expression. The Kaposi's sarcoma–associated herpesvirus (KSHV) is an important etiologic agent for cancers preferentially arising in the setting of immune suppression, including primary effusion lymphoma (PEL; ref. 2) and Kaposi's sarcoma (3). Following *de novo* infection of target cells, KSHV-encoded proteins induce the expression and secretion of multiple factors, including MMPs, with established importance for tumor cell invasiveness and angiogenesis (4, 5). The KSHV-encoded latency-associated nuclear antigen (LANA) tethers viral episomes to host cell chromatin and acts as a regulator of transcription for various cellular and viral genes (6–8), but a role for LANA in perpetuating cell invasiveness has not been defined. Because fibroblasts and endothelial cells support latent KSHV infection and represent cellular components of Kaposi's sarcoma lesions (9, 10), we used primary human fibroblasts and endothelial cells, as well as PEL cells derived from human tumors, to determine whether KSHV, and possibly LANA, regulates emmprin expression and associated cell invasiveness.

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Materials and Methods

Cell culture and infection assays. Two KSHV-infected, patient-derived PEL cell lines, BCP-1 and BCBL-1 cells, were kindly provided by Dr. Dirk Dittmer (University of North Carolina, Chapel Hill, NC), and both reverse transcription-PCR and immunofluorescence assays were used to verify the uniform presence of KSHV episomes within these cells through the identification LANA expression as described elsewhere (11). PEL cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal

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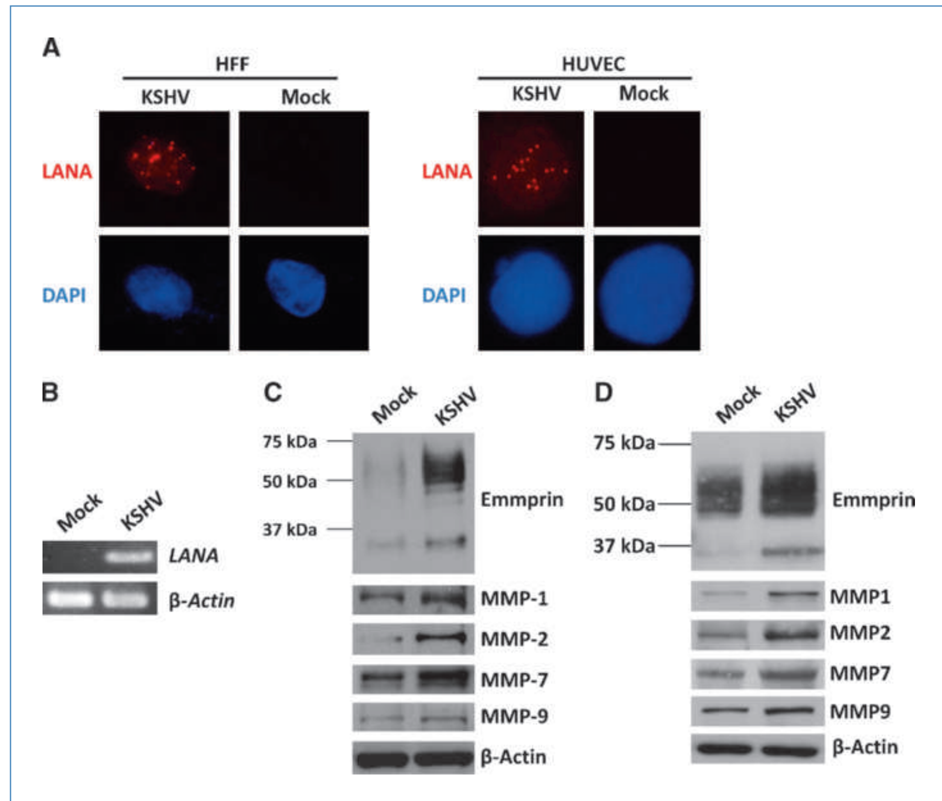

Figure 1. KSHV induces the expression of emmprin and MMPs following *de novo* infection. A, HFF and HUVEC were incubated either with medium (mock) or purified KSHV for 2 hours, and following a subsequent 24-hour incubation, immunofluorescence assays were performed (see Materials and Methods) for identification of LANA expression indicated by typical intranuclear, punctate staining (red dots). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to identify nuclei (blue). B, LANA transcripts were identified by reverse transcription-PCR (data shown for HFF). C and D, immunoblots were performed using HFF (C) and HUVEC (D) 24 hours after viral incubation to identify expression of high molecular weight (~65 kDa) and low molecular weight (~35 kDa) glycoforms of emmprin, representative MMPs associated with emmprin activation, and β-actin as an internal control.

bovine serum, 10 mmol/L HEPES (pH 7.5), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 0.05 mmol/L β-mercaptoethanol, and 0.02% (wt/vol) sodium bicarbonate. Human foreskin fibroblasts (HFF) were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Human umbilical vein endothelial cells (HUVEC) were grown in DMEM/F-12 50/50 medium (Cellgro) supplemented with 5% fetal bovine serum and 0.001 mg/mL puromycin (Sigma). To obtain KSHV for infection experiments, BCBL-1 cells were incubated with 0.6 mmol/L valproic acid for 6 days, and the concentration of infectious viral particles within concentrated culture supernatants was determined prior to infection experiments as described previously (12). Using a multiplicity of infection of 10, immunofluorescence assays revealed that ~80% to 90% of HFF and HUVEC exhibited positive LANA staining 12 to 24 hours after viral incubation (Supplementary Fig. S1).

Immunofluorescence assays. Briefly, 1 × 10^4 cells per well of either HFF or HUVEC were seeded in eight-well chamber slides (Nunc) and incubated with purified virions at a multiplicity of infection of 10, in the presence of 8 μg/mL polybrene (Sigma-Aldrich) for 2 hours at 37°C. Following overnight culture, 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 hour at 25°C with 1:1,000 dilution of a rat anti-LANA monoclonal antibody (ABI) followed by 1:100 dilution of a goat anti-rat secondary antibody conjugated to Texas red (Invitrogen). For nuclear localization, cells were subsequently counterstained with 0.5μg/mL of 4′,6-diamidino-2-phenylindole (DAPI, Sigma) in 180 mmol/L Tris-HCl (pH 7.5). Slides were washed once in 180 mmol/L ris-HCl for 15 minutes and prepared for visualization using a Leica TCPS SP2 AOBS confocal microscope.

PCR. Total RNA was isolated using the RNeasy Mini kit according to the instructions of the manufacturer (Qiagen). cDNA was synthesized from equal total RNA using SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) according to the procedures of the manufacturer. The primers designed for targeting the genes included the following: LANA sense, 5′ TCCCTCTACACTAAACCCAATA 3′; LANA antisense, 5′ TTGCTAATCTCGTTGTCCC 3′; β-actin sense, 5′ GGAAATCGTGCGTGACATT 3′; β-actin antisense, 5′ GACTCGTCATACTCCTGCTTG 3′. The PCR was performed in a DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) under conditions of 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 60 seconds.

Transfection assays. HFF and HUVEC were transfected with control pcDNA3.1 or pcDNA3.1-LANA vectors in 12-well plates for 48 hours using Lipofectamine 2000 (Invitrogen)

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according to the instructions of the manufacturer. 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 instructions of the manufacturer (Promega). For RNA silencing, HFF, HUVEC, and PEL cells were transfected for 48 hours with either emmprin- or control non-target short interfering RNAs (siRNA; ON-TARGET plus SMART pool; Dharmacon) using a commercially available transfection reagent (Dharmacon) according to the instructions of the manufacturer. Three independent transfections were performed for each experiment, and all samples were analyzed in triplicate for each transfection.

Immunoblotting. Total cell lysates (20 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies for emmprin (BD PharMingen), MMP1, MMP2, MMP7, and MMP9 (Santa Cruz), and β-actin (Sigma) as a loading control. Immunoreactive bands were developed using an enhanced chemiluminescence reaction (Perkin-Elmer), and visualized by autoradiography.

Transwell invasion/migration assays. Matrigel Invasion Chambers (Becton Dickinson) were hydrated for 4 hours at 37°C with culture medium. Following hydration, media in the bottom of the well was replaced with fresh media, then 5×10^4 HFF or HUVEC were plated in the top of the chamber. After 24 hours, cells were fixed with 4% formaldehyde for 15 minutes at room temperature and chambers rinsed in PBS prior to staining with 0.2% crystal violet for 10 minutes. After washing the chambers, cells at the top of the membrane were removed and cells at the bottom of the membrane were counted using a phase contrast microscope. For PEL cell migration assays, procedures were performed as described above except that 5×10^6 cells were plated in the top of the chamber, and 10 ng/mL human vascular endothelial growth factor (Lonza) was added to the lower well as a chemoattractant as previously described (5). Relative invasion/migration was determined for cells in experimental groups as follows: relative invasion = number of invading cells in experimental group / number of invading cells in control group.

Colony formation assays. Colony formation assays were performed as previously described (13). Briefly, 96-well plates were seeded first with 2×10^4 HFF per well, then subsequently with either BCP-1 or BCBL-1 cells ranging from 1 to 100 cells per well (eight replicates per dilution). After 14 days, colony formation was tabulated as the percentage of wells containing individual colonies comprised of >1,000 cells.

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

Results and Discussion

We first verified the establishment of KSHV infection within HFF and HUVEC using LANA expression as a surrogate marker for infection (Fig. 1A and B; Supplementary Fig. S1). Subsequent immunoblot analyses revealed

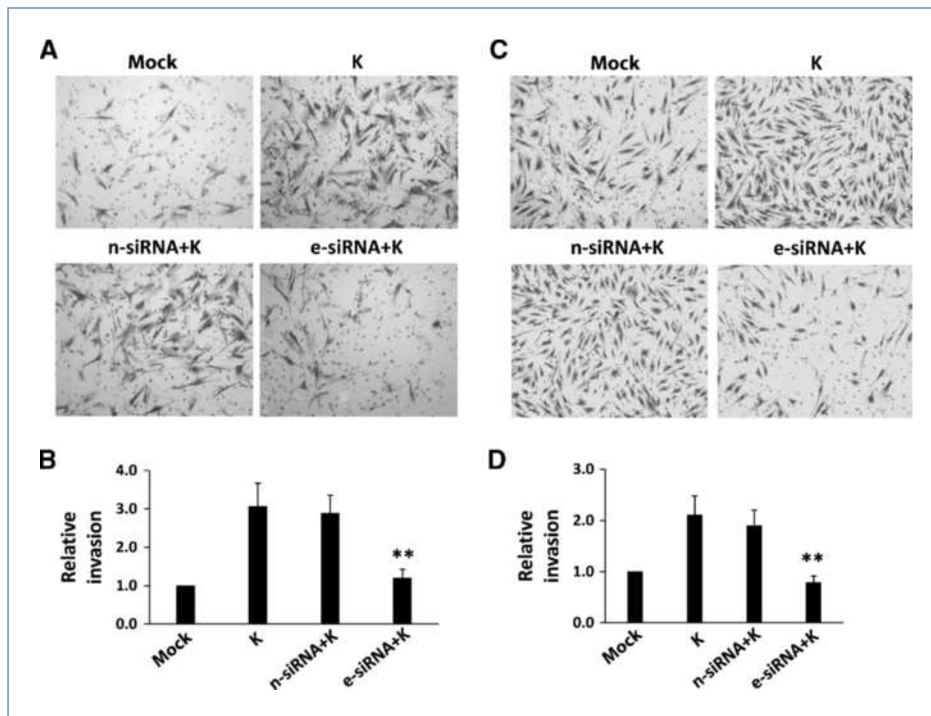

Figure 2. Targeting emmprin suppresses KSHV promotion of cell invasiveness. Cells were transfected with either control non-target (n-siRNA) or emmprin-specific siRNA (e-siRNA), and after 48 hours, HFF (A) or HUVEC (C) were incubated with media (mock) or KSHV (K) for 2 hours. Subsequent transwell invasion assays were performed 24 hours later, and invasiveness for HFF (B) and HUVEC (D) in each group was determined relative to control cells (see Materials and Methods). Bars, SEM for three independent experiments; **, $P < 0.01$ for comparison of invasion for e-siRNA–transfected/KSHV-infected cells versus n-siRNA–transfected/KSHV-infected cells.

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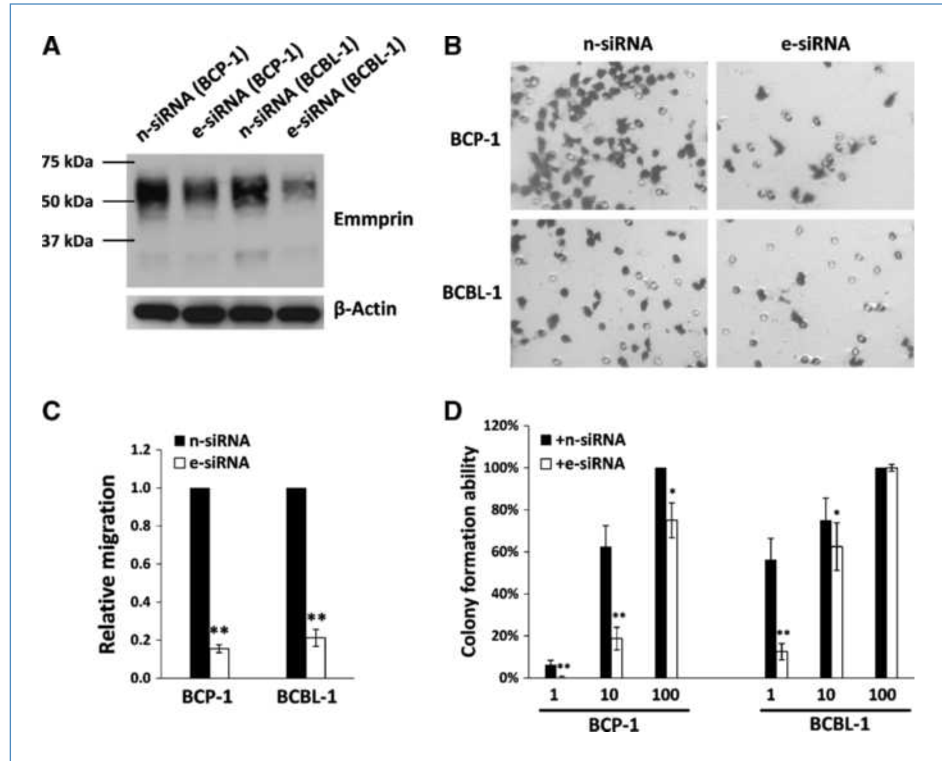

Figure 3. Targeting emmprin reduces migration and colony formation for PEL cells. A, BCP-1 and BCBL-1 cells were transfected with either control (n-siRNA) or emmprin-specific siRNA (e-siRNA) for 48 hours, and emmprin expression subsequently identified by Western blot. B and C, the invasiveness of emmprin-siRNA–transfected PEL cells was assessed relative to control transfectants using a modified transwell invasion assay and calculations detailed in Materials and Methods. D, PEL cells were transfected as above, and wells seeded with either BCP-1 or BCBL-1 cells ranging from 1 to 100 cells per well. Colony formation assays were performed as in Materials and Methods. Bars, SEM for three independent experiments; *, $P < 0.05$; **, $P < 0.01$ for comparison of colony formation for e-siRNA–transfected cells versus n-siRNA–transfected cells.

increased expression of emmprin, as well as MMPs associated with emmprin activation, in both of these cell types (Fig. 1C and D). Of note, both high molecular weight (~65 kDa) and low molecular weight (~35 kDa) emmprin glycoforms were elevated in KSHV-infected cells, particularly the mature high molecular weight glycoform associated with the biological activity of emmprin and induction of MMP secretion (14). After establishing an efficient RNA interference protocol for reducing the expression of emmprin in HFF and HUVEC, we confirmed that targeting of emmprin resulted in a corresponding reduction in MMP expression (Supplementary Fig. S2). Subsequent transwell invasion assays confirmed that KSHV infection significantly increased invasiveness for HFF and HUVEC, and that emmprin-siRNA significantly reduced this effect (Fig. 2A–D). To validate these findings using human KSHV-associated tumor cells, we established an RNA interference protocol for reducing emmprin expression in two independent KSHV-infected PEL cell lines (Fig. 3A). Using modified transwell migration and colony formation assays validated previously for virus-infected lymphoma cells (5, 13), we found that emmprin-siRNA significantly reduced the capacity for both migration (Fig. 3B and C) and colony formation (Fig. 3D) in both PEL cell lines.

Because others have shown that *de novo* infection of primary human cells by KSHV results in the predominant expression of latent viral genes including LANA (7, 8), we hypothesized that LANA induces emmprin expression. After establishing transient transfection assays conferring intranuclear expression of LANA within HFF (Fig. 4A), we found that ectopic LANA expression significantly increased emmprin expression in these cells, especially the high molecular weight glycoform (Fig. 4B). Subsequent assays revealed that LANA expression significantly increased HFF invasiveness, and that emmprin-siRNA suppressed this effect (Fig. 4C and D).

These data provide the first evidence that upregulation of emmprin by KSHV, and more specifically, KSHV-encoded LANA, plays an important role in promoting KSHV-associated cancer pathogenesis. Existing data support the role of emmprin as a cofactor for infection or pathogenesis related to HIV (15), coronaviruses (16), and hepatitis C (17), but to our knowledge, data supporting the direct regulation of emmprin by oncogenic viruses has not yet been reported. In addition, LANA has not been previously implicated in the induction of cell motility and invasiveness by KSHV. Cooperative interactions between emmprin and the hyaluronan receptor CD44 on the cell surface (18) initiate the activation of downstream signal transduction events relevant to cell mobility and proliferation and which coincidentally are initiated following *de novo* KSHV infection (1, 9). Future studies should, therefore, confirm whether targeting emmprin directly or through competitive inhibition of hyaluronan-CD44 interactions, as previously described (19), would selectively disrupt cancer pathogenesis associated with KSHV.

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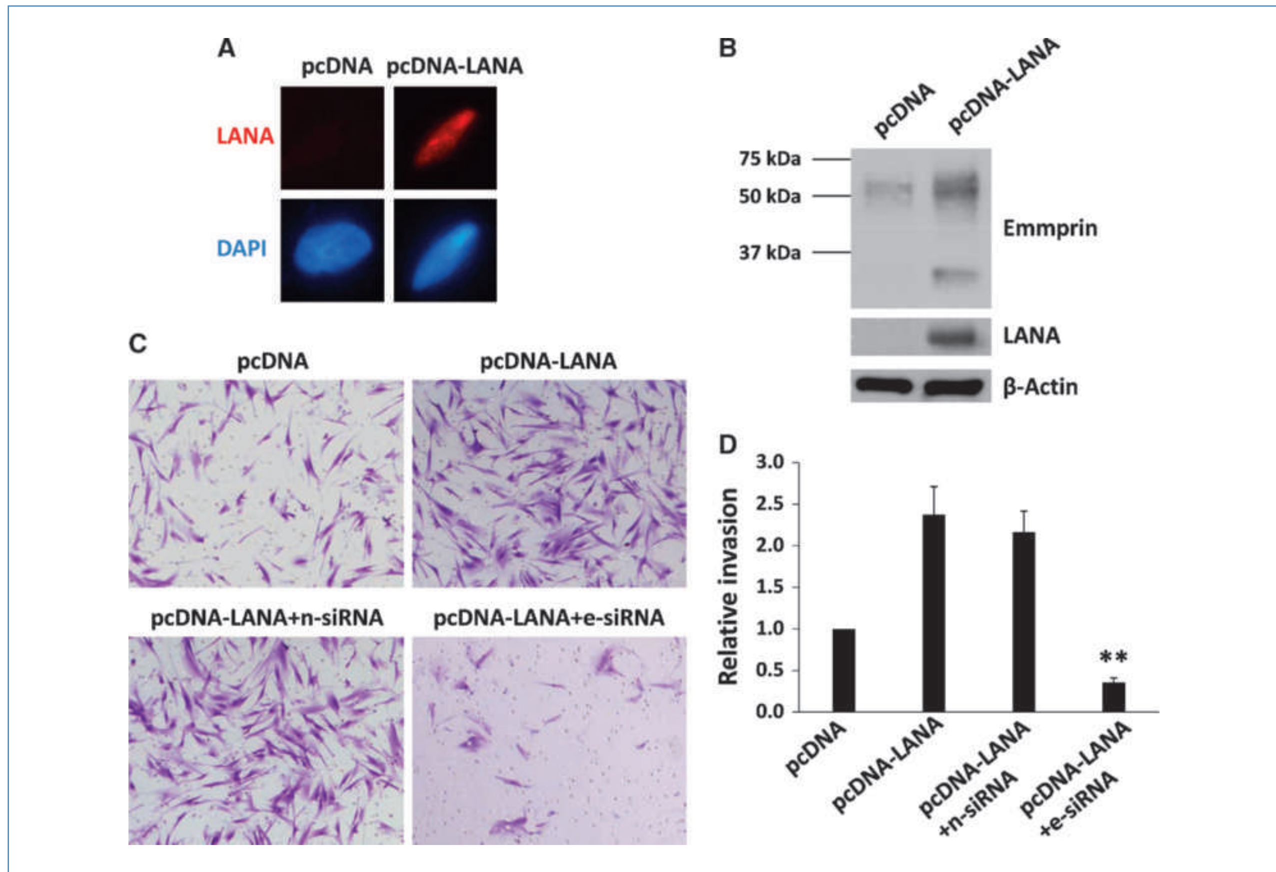

Figure 4. LANA induces emmprin expression and cell invasiveness. A and B, HFF were transfected with either control (pcDNA) or LANA-encoding (pcDNA-LANA) vectors for 48 hours, and LANA or emmprin expression subsequently confirmed by immunofluorescence assay (A) and/or Western blot (B). C and D, HFF were cotransfected with pcDNA-LANA and either control non-target (n-siRNA) or emmprin-specific siRNA (e-siRNA), and invasion assessed as previously described. Bars, SEM for three independent experiments; **, $P < 0.01$ for comparison of invasiveness for e-siRNA/pcDNA-LANA cotransfected cells versus n-siRNA/pcDNA-LANA cotransfected cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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