



HPV-18 E7 Interacts with Elk-1 Leading to Elevation of the Transcriptional Activity of Elk-1 in Cervical Cancer

Sung-Ho Go^{1,†}, Seung Bae Rho^{2,†}, Dong-Wha Yang¹, Boh-Ram Kim³, Chang Hoon Lee^{3,*} and Seung-Hoon Lee^{1,*}

¹Department of Life Science, Yong In University, Yongin 17092,

²Division of Cancer Biology, Research Institute, National Cancer Center, Goyang 10408,

³College of Pharmacy, Dongguk University, Goyang 10326, Republic of Korea

Abstract

The human papillomavirus (HPV)-18 E7 (E7) oncoprotein is a major transforming protein that is thought to be involved in the development of cervical cancer. It is well-known that E7 stimulates tumour development by inactivating pRb. However, this alone cannot explain the various characteristics acquired by HPV infection. Therefore, we examined other molecules that could help explain the acquired cancer properties during E7-induced cancer development. Using the yeast two-hybrid (Y2H) method, we found that the Elk-1 factor, which is crucial for cell proliferation, invasion, cell survival, anti-apoptotic activity, and cancer development, binds to the E7. By determining which part of E7 binds to which domain of Elk-1 using the Y2H method, it was found that CR2 and CR3 of the E7 and parts 1–206, including the ETS-DNA domain of Elk-1, interact with each other. As a result of their interaction, the transcriptional activity of Elk-1 was increased, thereby increasing the expression of target genes EGR-1, c-fos, and E2F. Additionally, the colony forming assay revealed that overexpression of Elk-1 and E7 promotes C33A cell proliferation. We expect that the discovery of a novel E7 function as an Elk-1 activator could help explain whether the E7 has novel oncogenic activities in addition to p53 inactivation. We also expect that it will offer new methods for developing improved strategies for cervical cancer treatment.

Key Words: HPV-18 E7, Elk-1, Cervical cancer, Yeast two-hybrid, Protein-protein interaction, Transcriptional activity

INTRODUCTION

Cervical cancer is the second leading cause of cancer mortality among women worldwide (Łuczak and Jagodzinski, 2008; Chang *et al.*, 2010; Rho *et al.*, 2022). Over the past two decades, numerous studies have suggested that high-risk HPV plays an important role in the development and pathogenesis of cervical cancer (Guo *et al.*, 2011; Arbyn *et al.*, 2021). Currently, more than 100 HPV genotypes exist, and ~40 of them infect the genital mucosa. These HPVs are classified into low-risk or high-risk types based on their malignant lesion level in the cervix. The high-risk type is associated with more than 90% of cervical cancers (Dell and Gaston, 2001; Gyöngyösi *et al.*, 2012; Davies-Oliveira *et al.*, 2021).

High-risk HPV encodes two transforming genes, E6 and E7, both of which play important roles in malignant transformation

(Zhou *et al.*, 2012; Malla and Kamal, 2021). HPV E6 binding to p53 is mediated by the E6-associated protein ligase (E6-AP), which targets p53 for ubiquitination and subsequent proteasomal degradation (Nuber *et al.*, 1998; Zhang *et al.*, 2022).

E7 is a major transforming protein of HPV and its transforming activity is due to its interaction with retinoblastoma tumor suppressor (Rb) (Thierry, 2009; Moody and Laimins, 2010; Xiong *et al.*, 2022). The conserved LXCXE motif of the E7 protein is crucial for E7/Rb binding, and it accelerates the proteasomal degradation of Rb (Putta *et al.*, 2022). The NH2-terminal segment of the E7 protein is required for Rb degradation, but it is not required for Rb binding (Patrick *et al.*, 1994; Gonzalez *et al.*, 2001; Fiedler *et al.*, 2004; Sima *et al.*, 2008; Zhou *et al.*, 2022) and the carboxyl terminus of the E7 can bind to many cellular proteins and can be an additional determinant for cellular transformation (Wu *et al.*, 1993a; Vats *et al.*, 2022).

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*Corresponding Authors

E-mail: uatheone@dongguk.edu (Lee CH), shlee@yongin.ac.kr (Lee SH)

Tel: +82-31-961-5213 (Lee CH), +82-31-8020-2780 (Lee SH)

Fax: +82-31-961-5206 (Lee CH), +82-31-8020-2886 (Lee SH)

[†]The first two authors contributed equally to this work.

Table 1. List of primer sequences used for the deletion mutants or site-directed mutagenesis

Gene	Primer sequences
E7 (1~20)	Forward: 5'-GATCGAATTCATGCATGGACCTAAGGCA-3' Reverse: 5'-GATCCTCGAGTTCAT-TTTGGGGCTCTAA-3'
E7 (21~40)	Forward: 5'-GATCGAATTCATGATTCCGGTTGA-CCTTCTA-3' Reverse: 5'-GATCCTCGAGTTCATCGTTTTCTTCCTC-3'
E7 (41~106)	Forward: 5'-GATCGAATTCATGATAGATGGAGTTAATCA-3' Reverse: 5'-GATCCTCGAG TTA CTG-CTGGGATGCACA-3'
Elk-1 (1~117)	Forward: 5'-ATGCGGATCCCGATGGACCCATCTGTGACG-3' Reverse: 5'-ATGCCTCGAGTCAAGCAGGGGCCACATT-3'
Elk-1 (118~199)	Forward: 5'-ATGCGGATCCCGATGGCTATACATGCCGCC-3' Reverse: 5'-ATGCCTCGAGTCAGGTGCTCCTGCTCCC-3'
Elk-1 (200~286)	Forward: 5'-ATGCGGATCCCGATGAGTCCAAGCCCTTG-3' Reverse: 5'-ATGCCTCGAGTCACAGCCGGGCTGGCAC-3'
Elk-1 (287~339)	Forward: 5'-ATGCGGATCCCGATGCCCGCGGTTGTTATG-3' Reverse: 5'-ATGCCTCGAGTCACGATCCTGGGGTCCG-3'
Elk-1 (340~428)	Forward: 5'-ATGCGGATCCCGATGGGAAGTGGCTCCGGC-3' Reverse: 5'-TAATCTCGAGTCATGGCTTCTGGGG-3'
E7 (Δ 25~29)	Forward: 5'-ATGATTCCGGTTGACCAATT -AAGCGACTCA-3' Reverse: 5'-TGAGTCGCTT-AATTGGTCAACCGGAATCAT-3'
E7 (L25A)	Forward: 5'-TCCGGTTGACGCTCTA -TGTCACGAGCAATTAAGCG-3' Reverse: 5'-CGCTTAA-TTGCTCGTGACATAG-AGCGTCAACCGGA-3'

High-risk HPV E7 protein binds to pRb with a higher affinity when compared to low-risk HPV. High-risk HPV E7 binding to pRb results in hyperphosphorylation of pRb and release of the E2F transcription factor, which activates cell proliferation genes and reverses G1-S arrest (Hwang *et al.*, 2002; Minoni *et al.*, 2020). Furthermore, E7 modulates cell cycle regulation through the inhibition of cyclin-dependent kinase inhibitors (p21 and p27), stimulation of cyclins, and direct activation of cyclin-dependent kinases (CDKs) (Narisawa-Saito and Kiyono, 2007; Ci *et al.*, 2020). Recent findings suggest that E7, which is independent of pRb, may contribute to the transformation of target cells (Balsitis *et al.*, 2005; Hatterschide *et al.*, 2022; Wendel *et al.*, 2022).

In this study, the Elk-1 transcription factor was identified as an interaction partner for the E7 oncoprotein. We demonstrated *in vitro* and *in vivo* interactions between Elk-1 and the E7 protein using glutathione S-transferase (GST) pull-down assays, co-immunoprecipitations, Western blot analyses, and Y2H assays. We also investigated the specific binding sites between these two proteins and the physiological consequence of their interaction. We discovered that E7 enhanced the transcriptional activity of Elk-1 and induced the expression of the target gene products.

We suggest that Elk-1 transcriptional activity should be increased for the formation of a novel oncogenic action of E7 in cervical cancer through its interaction with E7. Our findings may provide a new logic for inhibiting E7-induced carcinogenesis.

MATERIALS AND METHODS

Plasmid construction

The pCMV-GAL4/Elk-1 plasmid was constructed by cloning *EcoRI* fragments from pCDNA3.1/Elk-1 into same site as pC-

MV-GAL4 (Stratagene, La Jolla, CA, USA). To construct His-E7, *EcoRI/XhoI* polymerase chain reaction (PCR) fragments were cloned into the same sites as pCDNA4-His (Invitrogen, Waltham, MA, USA). The following plasmids were constructed for use in the GST pull-down assays: GST-E7 was constructed by cloning *EcoRI/XhoI* fragments from pCDNA4-His/E7 into same sites as pGEX-4T-1. For the E7 deletion mutants, GST-E7 (1-20), GST-E7 (21-40), and GST-E7 (41-106) were constructed by cloning *EcoRI/XhoI* PCR fragments into same sites as pGEX-4T-1. Various deletion mutant fragments were cloned by PCR using the appropriate sets of primers (Table 1). GST-E7 (Δ 25-29) and GST-E7 (L25A) mutants within the pRb-binding domain of E7 were constructed using the Quick-Change™ Site-Directed Mutagenesis Kit (Stratagene).

Cell culture and transfection

Human cervical cancer cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in recommended medium supplemented with 10% fetal bovine serum (JBI, Seoul, Korea) and 1% penicillin/streptomycin in a humidified 5% CO₂ incubator at 37°C. Transfections were performed using FuGENE6 (Promega, Madison, WI, USA) with indicated amounts of expression plasmids.

Yeast two-hybrid screening and β -galactosidase assay

Yeast two-hybrid screening was performed using the Matchmaker LexA two-hybrid system (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions (Rho *et al.*, 2021b). Briefly, E7 cDNA was cloned into the pGilda bait vector as described above and human ovary cDNA library used as prey vector. Bait and prey vectors were co-transformed in the EGY48 yeast strain using the lithium acetate method, and positive clones were selected on synthetic dropout medium lacking leucine, tryptophan, histidine, and uracil. Posi-

tive clones were further confirmed using the β -galactosidase assay with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal).

GST pull-down assay

Equal amounts of GST and GST fusion proteins bound to glutathione-linked agarose beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) were incubated *in vitro* with translated radiolabeled proteins for 1 h at room temperature in a binding buffer containing 50 mM Tris-HCl (pH 8.0), 1% NP-40, and 2 mM EDTA. The [35 S]-Met labeled proteins were produced using the TNT[®] Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. Bound proteins were washed three times in buffer (binding buffer plus 10% glycerol and 150 mM NaCl). SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and autoradiography were used to analyze the patterns of bound proteins.

Luciferase assay

The luciferase assays were performed using the Dual-Luciferase[®] Reporter Assay System (Promega) (Rho *et al.*, 2021a). Twenty-four hours after transfection, the cells were cultured in 35-mm plates and lysed in 100 μ L of passive lysis buffer; 20 μ L of the cell lysate was mixed with 100 μ L of luciferase assay reagent II (LAR II) in a 96-well white plate. Then, 100 μ L of Dual-Glo[®] Stop & Glo[®] Reagent (Promega) was added to each sample being assayed at 405 nm in a luminometer (PerkinElmer Inc., Waltham, MA, USA). Experiments were carried out in triplicate, and normalized luciferase activity was shown as the fold increase relative to the basal activity.

Co-immunoprecipitation

At 48 h post-transfection, cells were harvested and lysed on ice in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% Na-deoxycholate] (Rho *et al.*, 2022). After centrifugation at 13,000 rpm, the supernatant was incubated with the appropriate antibodies [anti-His (H-3) and anti-E7 (N-19); Santa Cruz Biotechnology, Dallas, TX, USA] overnight at 4°C and then with protein A/G beads (Upstate Biotechnology, Lake Placid, NY, USA) for 3 h. The beads were washed three times with buffer containing 50 mM Tris-HCl (pH 8.0), 1% NP-40, and 2 mM EDTA. Immunoprecipitates were suspended in Laemmli sample buffer and boiled for 5 min. For

the immunoprecipitation of endogenous proteins, cells were treated with 5 mM DTBP (dimethyl 3,3'-dithiobispropionimide-2HCl); Pierce Biotechnology, Waltham, MA, USA) at 4°C for 30 min before harvesting. The cross-linking reaction was stopped by the addition of a wash buffer containing 150 mM NaCl and 100 mM Tris-HCl (pH 8.0). Subsequent procedures were the same as those described above.

Western blotting

Cells were lysed and equal amounts of cell extracts (20–40 μ g) or the samples from the co-immunoprecipitations were electrophoresed on 10% SDS polyacrylamide gel, electrotransferred onto a nitrocellulose membrane (Yoon *et al.*, 2020). The membrane was incubated with the appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (ECL Western blotting detection; Bio-Rad, Hercules, CA, USA).

Colony formation assay

For the colony formation assay, 1,000 cells transfected with E7-, Elk-1-, and En-expressing vectors and a control vector plasmid were seeded on 100-mm plates and allowed to grow for 14 days in culture medium (Choi *et al.*, 2020). The cell colonies were stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 15 min and washed with distilled water before quantitation.

Data analysis and statistics

Values are presented as the mean \pm SD or \pm SE. Statistical comparisons between groups were performed using the Student's t-test. $p < 0.05$ was considered statistically significant.

RESULTS

E7 interaction with Elk-1

E7-binding proteins were screened using the Y2H system to identify novel interaction proteins of the E7. The pGilda-E7 was used as bait to screen a human cDNA library. Based on the results, Elk-1 was identified as an interacting partner. Growth and β -galactosidase assays were conducted to verify the result of the Y2H screening (Fig. 1).

Strains transformed with pGilda/Elk-1 and pB42AD/E7

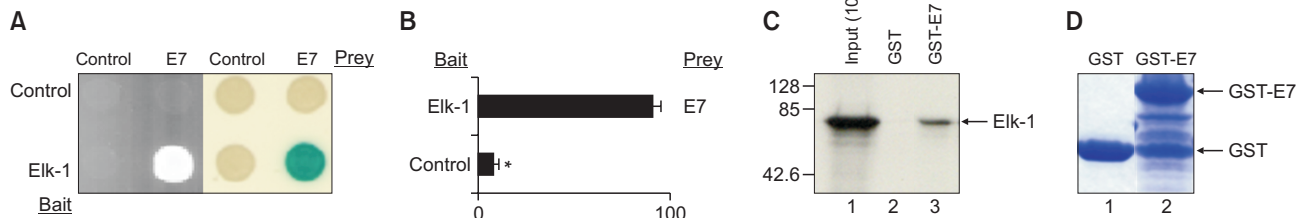


Fig. 1. Interaction of Elk-1 with HPV-18 E7 (E7) *in vivo* and *in vitro*. (A) Interactions between Elk-1 and E7 are represented by the relative activity of β -galactosidase expression. (B) The growth of transformants was measured by their ability to grow on Leu medium and by β -galactosidase expression. (C) GST fusion proteins from induced lysates of *Escherichia coli* expressing recombinant pGEX-4T-1 vectors encoding E7 were incubated with the *in vitro*-translated Elk-1 protein. The bound Elk-1 protein was detected using autoradiography. Input indicates one-fifth of the labeled Elk-1 used in the assay. (D) The amount of GST and GST-E7 proteins. To show that the amounts of GST fusion proteins used in this assay were similar, proteins were separated on 12% SDS-PAGE gels and stained in Coomassie Blue solution. * $p < 0.05$ versus control.

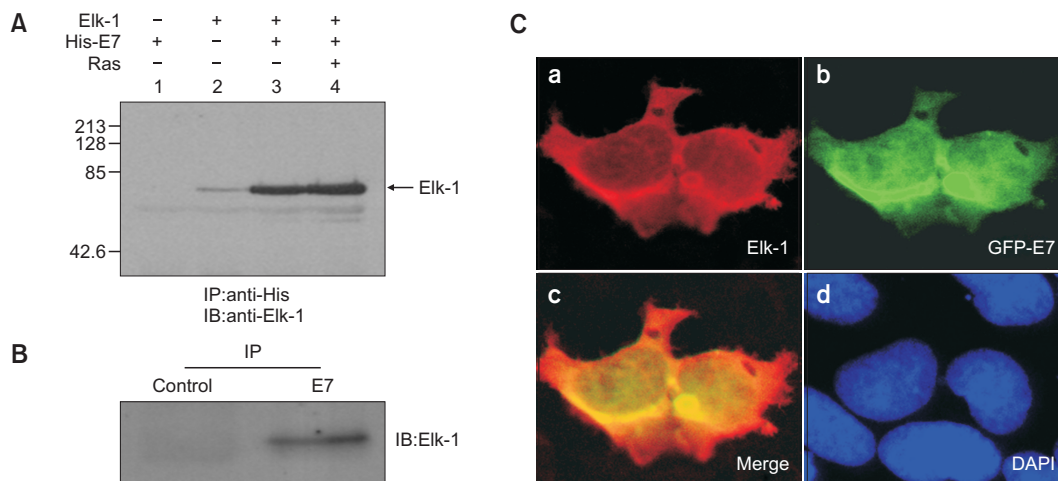


Fig. 2. Interaction and co-localization of E7 and Elk-1. (A) HEK293a cells were transiently transfected with 5 µg of pCDNA3.1/Elk-1 (lanes 2-4) and 5 µg of pCDNA4-His/E7 (lanes 1, 3 and 4) in the absence or presence of RasV12 (lanes 4). Cells were harvested from 48 h post-transfection and whole-cell lysates were immunoprecipitated with an anti-His antibody (Santa Cruz Biotechnology). Immunoprecipitations were separated on 10% SDS-PAGE gels and blotted with an anti-Elk-1 antibody (Santa Cruz Biotechnology). (B) HeLa cells were transiently transfected with 10 µg of pCDNA3.1/Elk-1. Cells were harvested 48 h post-transfection and endogenous E7 was co-immunoprecipitated with the anti-Elk-1 antibody (Santa Cruz Biotechnology) from HeLa cells. Immunoprecipitations were separated on 10% SDS-PAGE gels and blotted with an anti-Elk-1 antibody (Santa Cruz Biotechnology). (C) HEK293a cells were transiently transfected with pCDNA3.1/Elk-1 and pEGFP-C1/E7. Cells were fixed and subjected to indirect immunofluorescence using an anti-Elk-1 antibody (a, Santa Cruz Biotechnology) followed by DAPI (d) and GFP antibody (b) counterstaining. A merged image of Elk-1 and GFP-E7 immunostaining is shown in (c). Images were representative of three independent experiments.

were unable to grow in normal culture media, but those grown in a Leu-deficient culture medium were able to grow (Fig. 1A). The culture medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) turned blue after X-Gal had been decomposed by β-galactosidase (Fig. 1B), demonstrating a strong interaction between the E7 and Elk-1.

To further investigate this interaction, an *in vitro* GST pull-down assay was performed. Bacterially expressed GST and GST-E7 fusion proteins were immobilised on glutathione-linked agarose beads, and a radioactively labelled Elk-1 protein was prepared by *in vitro* transcription after translation using 35S-methionine (Fig. 1C). The GST-E7 fusion protein was incubated with the radiolabelled Elk-1 protein. The complexes were washed, separated on SDS-PAGE gels, and analysed by autoradiography, and the GST fusion proteins were separated on gels for quantitation. As seen in Fig. 1D, the GST-E7 fusion proteins successfully pulled down 35S-Elk-1 in the assay, whereas GST alone failed to interact with 35S-Elk-1. These results showed that Elk-1 interacted directly with the E7 oncoprotein *in vitro*.

Elk-1 interaction and co-localisation with E7 in cells

To determine the feasibility of Elk-1 and E7 association in mammalian cells, Elk-1 and His-E7 were transiently co-transfected into human HEK293a cells for immunoprecipitation analysis.

After 48 h of transfection, cell lysates were prepared and co-immunoprecipitation assays were performed using an anti-His (His-E7) antibody. As seen in Fig. 2, Elk-1 co-precipitated with the His-E7 antibody. However, Ras overexpression slightly increased the interaction between Elk-1 and E7 in the HEK293a cells (Fig. 2A, lane 4).

The co-immunoprecipitation assays were repeated in HeLa

cells to express the endogenous E7 protein. The HeLa cells were transfected with Elk-1 and treated with 5 mM DTBP before cell lysis to preserve the intracellular protein complexes in the lysates. Cell lysates from the HeLa cells underwent co-immunoprecipitation with the E7 antibody, and the immune complexes were subsequently analysed by Western blotting using the Elk-1 antibody. As shown in Fig. 2B, Elk-1 co-precipitated with the E7 antibody, but not with a control IgG antibody (Fig. 2B, lane 1).

To further confirm the interaction between Elk-1 and E7 *in vivo*, we investigated Elk-1 and E7 localisation within the same cellular compartments. To this end, HEK293a cells were co-transfected with Elk-1 and GFP-E7 (green fluorescent E7 protein). The anti-Elk-1 antibody identified Elk-1 in a widespread pattern of expression throughout the cytoplasm and nucleus (Fig. 2C-a). A similar localisation pattern was also observed for the GFP-E7 (Fig. 2C-b). Combined images showed the pattern of Elk-1 and GFP-E7 co-localisation, which was more evident in the endoplasmic reticulum connected to a nuclear membrane where both proteins were overexpressed (Fig. 2C-c). The location of the nucleus was determined by DAPI staining, as shown in Fig. 2C-d.

These results revealed the *in vivo* interaction between Elk-1 and E7 in mammalian cells, and Elk-1 and E7 co-localised in the same cellular compartments, further confirming the inter-cellular interactions.

Identification of the Elk-1-binding domain from E7

To investigate the specific Elk-1 binding domain of E7, three E7 truncation fragments were designed, as shown in Fig. 3A. In the Y2H system, full-length human Elk-1 cDNA and a plasmid containing either the full-length E7 cDNA (1-106) or three deletion fragments (CR1 (1-20), CR2 (21-40), or CR3

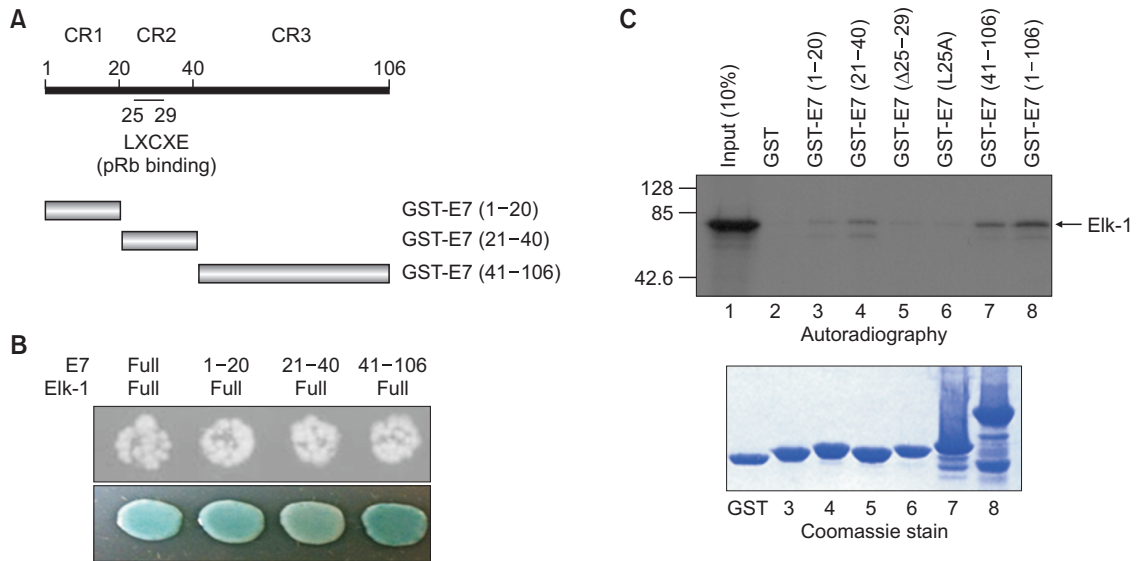


Fig. 3. Mapping of the Elk-1-binding domain on E7. (A) Schematic representation of cDNA constructs indicating the full-length GST-E7 and each deletion construct. (B) β -galactosidase assays were performed in the presence of X-gal to determine the binding activity of the constructs. Positive interactions were revealed based on cell growth on Leu-depleted medium (upper panel), as well as the formation of blue colonies on medium containing X-gal (lower panel). (C) Results of protein interactions using a GST pull-down assay (each protein was detected by autoradiography). Coomassie Blue-stained SDS-PAGE gel showing the *in vitro* interactions between GST-E7 and Elk-1 proteins. Each protein was separated on a 12% SDS-PAGE gel.

(41–106)) were co-transformed into EGY48 yeast cells. Cells containing the full-length and deletion fragments of E7 grew on plates without Ura, His, Trp, and Leu. To confirm these results, the binding activity of these constructs was determined by measuring the relative expression of β -galactosidase.

As shown in Fig. 3B (lower panel), the β -galactosidase assay results confirmed that all the fragments were able to bind to Elk-1. For further confirmation, GST pull-down assays were conducted using various GST-E7 deletion proteins bound to glutathione-linked agarose beads and *in vitro*-translated Elk-1 (Fig. 3C bottom). GST fusion proteins were separated on gels for quantitation. Elk-1 showed strong binding to GST-E7 (41–106), which encompassed the carboxy-terminal zinc finger (CR3) E7 domain (Fig. 3C top). For E7 to bind pRb with high affinity, the CR3 domain is required. A weaker interaction was observed in the CR2 domain. Notably, the Δ 25–29 (containing deletions of the pRb-binding motif) and L25A mutants (deficient in pRb binding) within the CR2 domain showed greatly reduced binding to Elk-1, suggesting that the pRb-binding motif of E7 was essential for Elk-1 and E7 interaction. Furthermore, it was discovered that Elk-1 and GST-E7 (1–20) had weak interactions. The E7 CR1 domain was crucial for pRb destabilisation, although this region was not essential for pRb binding. Together, these results indicated that Elk-1 interacted with a wide range of E7 proteins, but its interactions were stronger with the E7 CR3.

Identification of the E7-binding domain in Elk-1

The E7 domains that interacted with Elk-1 were identified, but the potential Elk-1 motifs involved in E7 interaction were yet to be identified. To this end, a series of GST-Elk-1 derivative proteins were constructed (Fig. 4A). First, to confirm that full-length Elk-1 interacted with E7, (His6)-Elk-1 (immobilised on Ni²⁺-NTA agarose beads) was used in the pull-down as-

says. The bound E7 was examined by autoradiography (Fig. 4B).

GST pull-down assays were then performed using GST-Elk-1 deletion proteins and *in vitro* translated E7. As shown in Fig. 4C, E7 had a higher affinity for binding GST-Elk-1 (1–206) than GST-Elk-1 (1–89) (Fig. 4C top, lanes 3 and 4). Additionally, GST-Elk-1 (148–260) showed weak binding to E7 (Fig. 4C top, lane 5), while no significant binding was observed for Elk-1 spanning residue fragments 230–260 and 307–428 (Fig. 4C top, lanes 6 and 7). The interaction sites between E7 and Elk-1 *in vivo* were also analysed, and the results of the growth and β -galactosidase assays with the yeast system were consistent with the GST pull-down assay result (Fig. 4D).

Together, these results suggest that the N-terminal domain of Elk-1 including the ETS DNA-binding domain and serum response factor (SRF) binding domain was required for E7 association.

Enhancement of Elk-1 transcriptional activity by E7 for the elevation of Egr-1 and c-fos protein expression

To clarify the functional consequences of the interaction between E7 and Elk-1, the role of E7 in controlling the transactivation capacity of Elk-1 was investigated. First, the promoter activity of the Elk-1 target genes, *egr-1* and *c-fos*, were evaluated. The *egr-1* or *c-fos* promoter-reporter gene construct was co-transfected into cells together with plasmid vectors that express E7, Elk-1, and Elk-En (a dominant-negative form of Elk-1) (Fig. 5).

In HeLa cells, transient transfection of Elk-1 stimulated the promoter activity of both *egr-1* and *c-fos*, as expected (Fig. 5A, 5B). However, E7 also stimulated the promoter activity of the two Elk-1 target genes. The intensity of activation of the *egr-1* promoter by Elk-1 and/or E7 was much stronger than that of the *c-fos* promoter (Fig. 5A, 5B). Moreover, when E7 and Elk-1

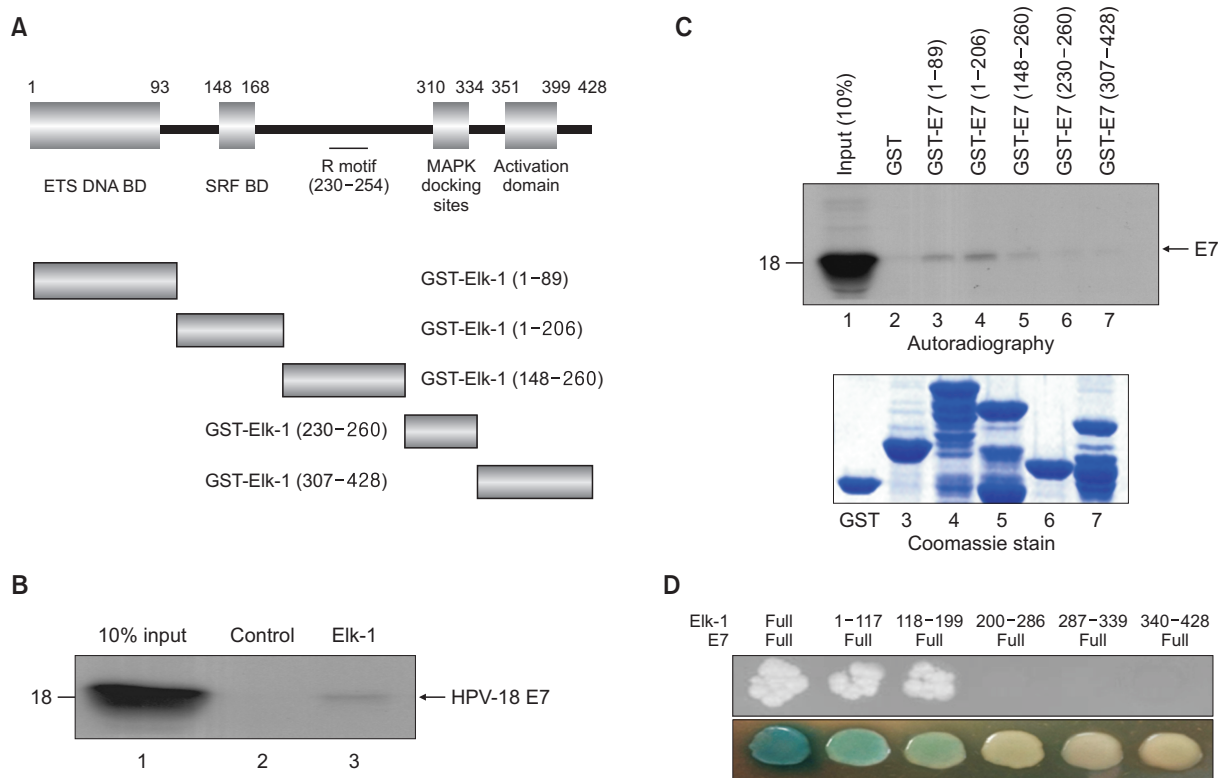


Fig. 4. Mapping of the interaction region of E7 on Elk-1. (A) Schematic representation of cDNA constructs indicating the full-length Elk-1 and deletion constructs. (B) Binding of E7 to the Elk-1 (FL) protein. Pull-down analysis of *in vitro*-translated E7 with full-length Elk-1 immobilized onto Ni-NTA agarose beads (lane 3). The beads on their own were used as a control (lane 2). Input indicates one-tenth of the labeled E7 used in the assay (lane 1). (C) Binding of E7 to Elk-1 derivative proteins. GST fusion proteins from induced lysates of *E. coli* expressing recombinant pGEX-4T-1 vectors encoding five types of Elk-1 deletion fragments were incubated with *in vitro*-translated E7 protein (lanes 3-7). The bound E7 protein was detected using autoradiography. Input indicates one-tenth of the labeled E7 used in the assay (lane 1). To show that the amount of GST and GST-Elk-1 derivative proteins used in this assay were similar, six proteins were separated on a 10% SDS-PAGE gel and stained in Coomassie Blue solution (lower panel). (D) β -galactosidase assays were performed in the presence of X-gal to determine the binding activity of the constructs. Positive interactions were revealed based on cell growth on Leu-depleted medium (upper panel), as well as the formation of blue colonies on medium containing X-gal (lower panel).

were co-transfected, a synergistic effect was observed on the activation of the Elk-1 target gene promoter. Egr-1 promoter activity was increased by ~35-fold, and c-fos promoter activity was increased by ~4-fold. These results suggested that E7 enhanced Elk-1 transactivation activity. To confirm that E7 binding to Elk-1 enhanced transactivation, Elk-En was transiently transfected with E7. The results showed that upregulation of Egr-1 and c-fos promoter activity by E7 was significantly downregulated by Elk-En, suggesting that E7 interacted with Elk-1 to enhance transcriptional activity. In contrast, the expression of the E2F transcription factor activated by E7 (not the target gene of Elk1) was not increased by co-transfection with Elk-1 and E7 or decreased by co-transfection with Elk1-EN and E7 (Fig. 5C).

After transiently transfecting E7, the expression of the egr-1 and c-fos proteins was investigated since E7 increased the transcriptional activity of Elk-1. Cells were co-transfected with 2 μ g of the Elk-1-expressing vector and various amounts of the E7-expressing vector (0, 2, 5, and 8 μ g), and the protein expression levels of egr-1 and c-fos were examined using a Western hybridisation assay. In both HeLa (HPV positive) and C33A (HPV negative) cell lines, cells transiently transfected with E7 exhibited increased protein expression levels of egr-1

and c-fos in a dose-dependent manner (Fig. 5D, 5E). These results suggest that E7 upregulated the expression of target gene products egr-1 and c-fos while activating the transcriptional activity of Elk-1.

Effects of the co-transfection of E7 and Elk-1 on cancer cell proliferation

To determine the synergistic effects of the E7 and Elk-1 on cervical cancer cell proliferation, a colony formation assay was performed in the C33A cells. Transfection of E7 or Elk-1 induced colony formation of C33A cells. More importantly, the co-transfection of E7 and Elk-1 resulted in increased colony formation (Fig. 6A, 6B). However, compared to the untreated control and E7-treated cells, Elk-En significantly reduced colony formation in C33A cells. These results suggest that E7 significantly increased cell proliferation.

DISCUSSION

High-risk HPV encodes oncogenes E6 and E7, which have transforming properties. According to several studies, E6 and E7 are potent oncogenes that promote cervical cancer devel-

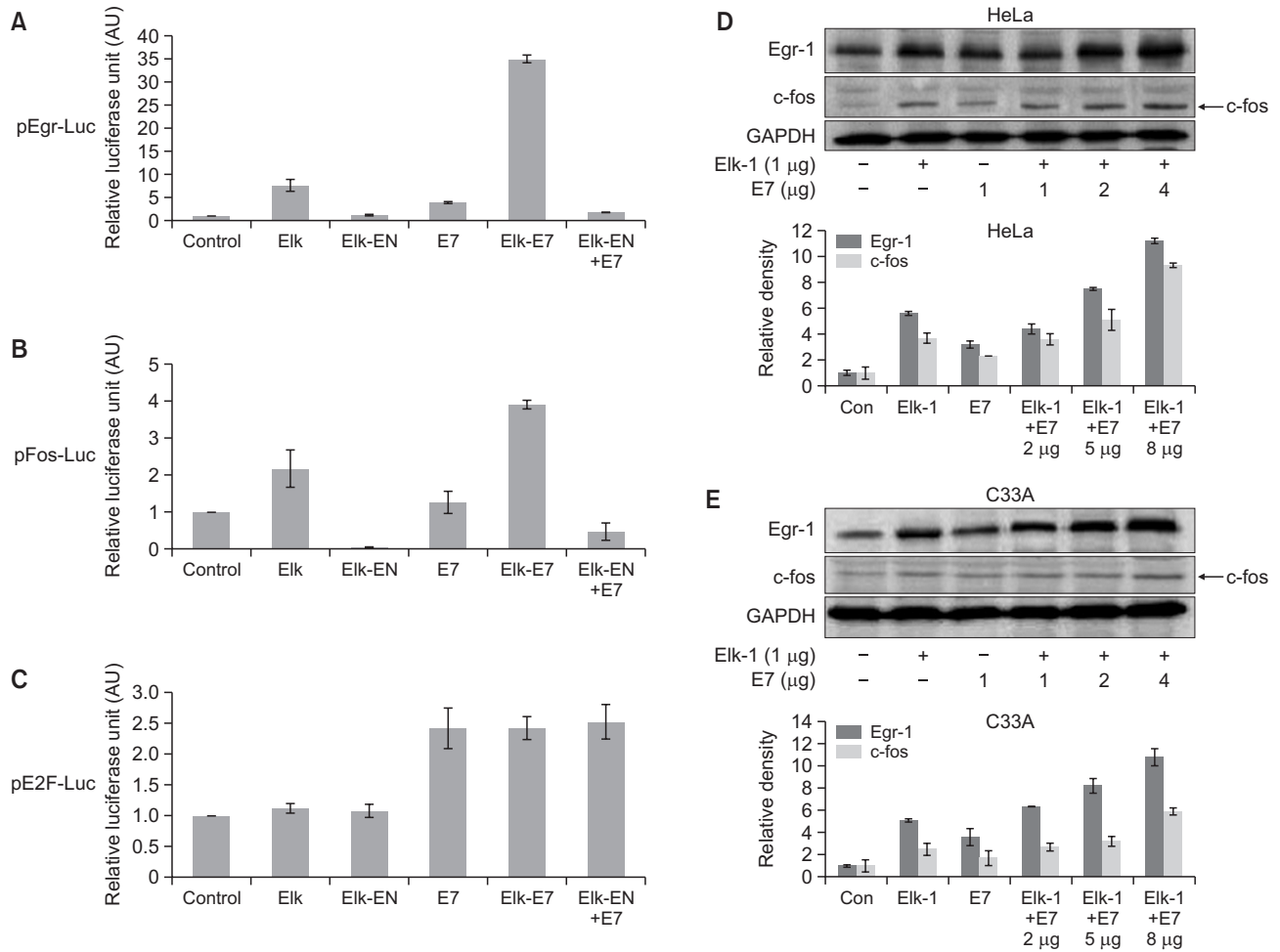


Fig. 5. E7 enhances the transcriptional activity of Elk-1. Luciferase reporter assays for the activity of *egr-1* (A), *c-fos* (B), and the E2F (C) promoter were performed with the expression vectors encoding Elk-1, Elk-En, and E7, as indicated. At 48 h post-transfection, the cells were harvested. Luciferase activity was normalized with respect to the *Renilla* luciferase activity. Values are expressed as the means \pm SD from three experiments. (D, E) Effects of E7 on the expression levels of *egr-1* and *c-fos* in HeLa and C33A cell lines. HeLa (D) and C33A (E) cells were transiently transfected with pCDNA3.1/Elk-1 and pCDNA4-His/E7. Whole-cell extracts were collected at 48 h after transfection. *Egr-1* and *c-fos* were detected by Western hybridization analysis.

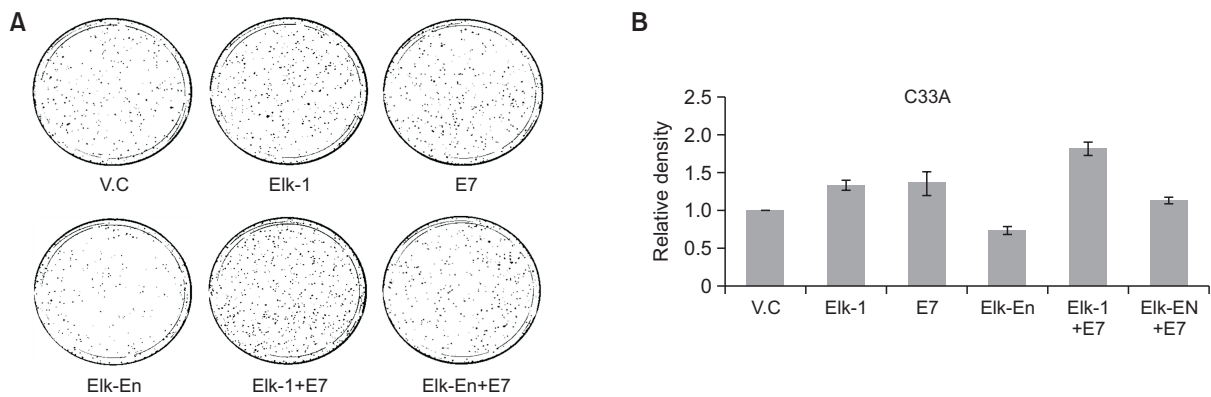


Fig. 6. Effects of E7, Elk-1, and Elk-En on cell proliferation. (A) Colony formation in C33A cells treated with Elk-1, E7, and Elk-En in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS). Cell numbers were determined after incubation for 10 days. (B) Values are expressed as the means \pm SD from two experiments.

opment (Yim and Park, 2005; Pal and Kundu, 2020).

As previously stated, one of the major oncogenic functions of E7 is the inactivation of pRb through proteasome degradation induction. However, some mutants with changes to the CK2 site or C-terminal zinc finger domain inhibit the transforming activity of E7 while maintaining their ability to bind to pRb (McINTYRE *et al.*, 1993). Additionally, many researchers have reported that pRb inactivation was necessary, but not sufficient for the transformation by E7 and suggested that another cellular target was needed for full E7-induced transformation (Prathapam *et al.*, 2001; Taghizadeh *et al.*, 2019; Wang *et al.*, 2021). Research into E7-related host proteins is necessary for establishing potential future therapeutic strategies in the management of cervical cancer.

In this case, a Y2H method was used to examine a partner molecule that binds to E7 to discover a new E7 mechanism in the carcinogenesis of human cervical cancer. Elk-1 was identified as a binding partner capable of interacting with E7. Elk-1, a transcriptional activator of a proto-oncogene from the ETS family of transcription factors, binds to the serum response element (SRE) in the promoter region and mediates transcriptional activity in response to growth factors. Elk-1 has a significant role in regulating ras-induced tumorigenesis in lung cancer cells (De Luca *et al.*, 2012; Yang *et al.*, 2012; Inoue *et al.*, 2018). In prostate cancer cells, Elk-1 was identified as a transcription factor that regulates the transcriptional activation of EGR1 by an epidermal growth factor (EGF). In particular, the Elk-1 bond at the SRE site of the EGR1 promoter was sufficient for EGF-mediated EGR1 expression (Gregg and Fraizer, 2011; Yang *et al.*, 2020). In breast cancer cells, EGF-induced activation of Elk-1 contributed to cell survival through Mcl-1 regulation (Booy *et al.*, 2011; Winder and Campbell, 2022).

Elk-1 functions as a cofactor to enable the activation of the c-myc promoter by the nuclear factor of the activated T cells (NFAT) and pancreatic cancer growth *in vitro* and *in vivo* (Wang *et al.*, 2018; Ala, 2022). Androgen receptor activation in bladder cancer cells increased c-fos expression after Elk-1 expression, resulting in cell proliferation, migration, and invasion (Kawahara *et al.*, 2015). Despite these extensive studies, the function of Elk-1 in cervical cancer is poorly understood.

We demonstrated the direct binding of E7 to Elk-1 (Fig. 1, 2). Deletion constructs were used to locate the Elk-1-interacting region of E7. The E7 protein is divided into three domains [conserved regions (CR) 1–3]. CR1 at the N-terminus was required for the cellular transformation and pRb degradation but did not directly bind to pRb; CR2, which contained an LXCXE motif sufficient for binding to pRb, blocked the association of E2F and pRb; and CR3, which contained two Cys-X-X-Cys motifs that bind directly to a C-terminal region of pRb, was also required to inhibit the binding of E2F to pRb. The CR3 region of E7 includes a nuclear export signal (NES) and type 1 PDZ binding motifs (Chemes *et al.*, 2012).

All E7 deletion constructs (CR1, CR2, and the CR3 domain) bonded to Elk-1, but Elk-1 interacted more strongly with the CR2 and CR3 domains than with the CR1 domain (Fig. 3, 4). Additionally, the LXCXE pRb-binding motif of the E7 mutants (deletion or deficient in the pRb-binding motif) did not bind to Elk-1, suggesting that the LXCXE motif was required for binding Elk-1 to E7 (McINTYRE *et al.*, 1993). Our results suggest that the pRb-binding motif in E7 was crucial for Elk-1 interaction and that Elk-1 may contribute to E7/pRb binding in E7 biological activity (Edmonds and Vousden, 1989; Jones and

Münger, 1997; Jones *et al.*, 1997; Aarthy *et al.*, 2018).

Next, the E7-interacting region in Elk-1 was identified. E7 binds to the ETS DNA-binding and SRF-binding domains of Elk-1. Elk-1 was recruited to the SRE via protein–DNA (via ETS DNA binding) and protein–protein (via the SRF-binding domain) interactions. The ETS DNA-binding domain, which is located in the N-terminal region, was required for DNA binding and ternary complex formation with SRF. The SRF-binding domain was located downstream of the ETS DNA-binding domain, which was necessary for binding to SRF. Since E7 interacted with two different domains of Elk-1, the E7/Elk-1 binding may affect the protein–DNA and protein–protein interactions. These results suggest that E7 enhanced the DNA binding affinity of Elk-1 and increased the interaction between Elk-1 and SRF through the ETS DNA-binding and the SRF-binding domains, respectively. As a result, E7 may be crucial in Elk-1-SRF-SRE ternary complex formation.

Elk-1 is a member of a subfamily of ETS-domain proteins known as TCFs (ternary complex factors), which associate with the SRF to regulate c-fos proto-oncogene transcription (Gille *et al.*, 1995; Sharrocks, 2002; Zhao *et al.*, 2022). We observed that Elk-1 activation after interaction with E7 promotes EGR-1 and Fos gene expression at the transcriptional level (Fig. 5). This transcriptional promotion was believed to have been inhibited by the Elk-1-dominant negative EN. This suggests the importance of Elk-1 inhibition as a therapeutic strategy given that c-fos is involved in carcinogenesis. Elk-1, the most well-known TFC, is an efficient substrate for all three classes of MAPK (ERK, p38, and JNK) (Gregg and Fraizer, 2011; De Luca *et al.*, 2012; Thiel *et al.*, 2021). The activation of the Ras-Erk or PKC-Erk pathways by mitogen or growth factors as well as that of JNK and p38 result in Elk-1 activation (Kyriakis *et al.*, 1992; Lange-Carter *et al.*, 1993; Wu *et al.*, 1993b).

Interestingly, Elk-1 controls various functional groups of target genes and different modes of transcription factor binding. This activates the SRF function, which acts like actin when the SRF binds to the Elk-1-SRF binding domain. SRF also controls the actin cytoskeleton and cell migration (Odrowaz and Sharrocks, 2012; Azam *et al.*, 2022). Therefore, we can expect that the binding of E7 to the Elk-1-SRF binding domain, which we identified, functionally regulates the target gene function. Considering these aspects, as repeatedly emphasised, suppressing the activation of Elk-1 by E7 may be a good strategy for the treatment of cervical cancer.

In conclusion, E7 promotes cervical cancer in addition to deactivating pRB by binding to Elk-1, a new target of E7, and activating the function of Elk-1. We expect that blocking this accelerating process can be a good treatment strategy for suppressing cervical cancer.

CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

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