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Capn4 is induced by and required for Epstein-Barr virus latent membrane protein 1 promotion of nasopharyngeal carcinoma metastasis through ERK/AP-1 signaling

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Abstract

Capn4, also known as CapnS1, is a member of the calpain family, which plays a crucial role in maintaining the activity and function of calpain. We previously reported that Capn4 also plays an essential role in the migration of nasopharyngeal carcinoma (NPC) cells through regulation of (MMP-2) by nuclear factor-kappa B activation. Epstein-Barr virus latent membrane protein 1 (LMP1) is closely related to the malignant functions of NPC; however, the relationship between LMP1 and Capn4 in NPC remain unclear. Immunohistochemical studies showed that the level of LMP1 and Capn4 expression was high in both primary and metastatic NPC tissues, with a significantly positive correlation. We further found that LMP1 was able to upregulate the Capn4 promoter in a dose-dependent way through the C-terminal activation region (CTAR)1 and CTAR2 domains to activate AP-1. Moreover, we also found that LMP1 activated AP-1 through ERK/JNK phosphorylation. These findings indicate that Capn4 coordination with LMP1 promotes actin rearrangement and, ultimately, cellular migration. These results show that Capn4 coordination with LMP1 enhances NPC migration by increasing actin rearrangement involving ERK/JNK/AP-1 signaling. Therapeutically, additional and more specific LMP1 and Capn4 targeted inhibitors could be exploited to treat NPC.

KEYWORDS

AP-1, Capn4, LMP1, metastasis, nasopharyngeal carcinoma

1 | INTRODUCTION

Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC) is endemic to southern China and South-East Asia, with the highest incidence observed among the southern Chinese, especially those of Cantonese origin. Unlike other head and neck cancers, NPC features the early invasion of surrounding tissues and metastasis. Moreover, over 60% of NPC cases are first diagnosed with metastasis.[1] Epstein-Barr virus latent membrane protein 1 (LMP1) is associated with malignant functions (eg, proliferation, invasion, and

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metastasis) by triggering multiple signaling pathways regulated by the canonical and non-canonical nuclear factor kappa B (NF-κB) pathways, three classical MAPK (eg, ERK-MAPK, p38 MAPK, and JNK/SAPK), and PI3K, and plays an important role in NPC.[1]

The calpain small subunit, Capn4 (also known as CapnS1), is a binding partner that forms a heterodimer with the 80-kDa large catalytic subunit and is indispensable for calpain activity and function.[2] Several studies have shown that Capn4 is associated with various physiological and pathological processes (eg, embryonic development, chondrocyte differentiation, and myocardial infarction).[3-5] An increasing body of evidence has linked the biological function of Capn4 to tumorigenesis. Indeed, the expression of Capn4 has been detected in a number of cancers, including ovarian carcinoma, colorectal cancer, hepatocellular carcinoma, breast cancer, and other tumors.[6-9] Chen et al reported that Capn4 regulates migration and invasion of ovarian carcinoma cells by targeting the osteopontin-mediated PI3K/AKT signaling pathway.[10] Moreover, a study conducted by Li et al found that MEKK2/ERK1/2 enhances the migration of breast cancer cells through the upregulation of Capn4.[9] We previously reported that Capn4 also plays a crucial role in the migration of nasopharyngeal carcinoma cells through the regulation of MMP-2 by NF-κB activation.[11] We also found that enhanced Capn4 expression was associated with positive EBV infection in NPC patients; however, the involvement of Capn4 in LMP1-mediated progression of nasopharyngeal carcinoma and the underlying mechanism(s) have yet to be defined.

In the present study, we sought to investigate the expression of Capn4 induced by LMP1 in NPC and found a significantly positive correlation. Importantly, we uncovered a novel mechanism underlying LMP1-mediated Capn4 activation through ERK/JNK/AP-1 signaling, leading to functional consequences associated with NPC migration.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This research followed the tenets of the Declaration of Helsinki and all subjects provided written informed consent approved by ethics committees of Fujian Medical University prior to their participation in this study and for the use of tissue samples collected before treatment.

2.2 | Clinical samples and immunohistochemistry

A total of 153 NPC tissue specimens and clinical pathological data have been described in our previous work.[11] Immunohistochemistry (IHC) for Capn4 and LMP1 was carried out using a Histomouse SP Broad Spectrum DAB kit (Invitrogen-Zymed). Formalin-fixed, paraffin-embedded primary and metastatic NPC sections were deparaffinized and rehydrated. Samples were heated in 10 mmol/L citrate buffer (pH 6.0) for 20 minutes for antigen retrieval. To block endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide. Sections were then incubated with an anti-Capn4 antibody (Santa Cruz Biotechnology) or anti-LMP1 antibody (DAKO) followed by incubation with a biotinylated secondary antibody. Signal was detected with diaminobenzidine solution. Slides were reviewed and scored independently by two pathologists blinded to the clinical data, using the scoring standard described previously.[12]

2.3 | Cell culture, plasmids, and reagents

Both 5-8F and CNE2 cell lines (EBV-negative) were derived from the human NPC cell line, maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS, and G418 (400 μg/mL; Invitrogen) to select for cells containing the neomycin resistance cassette. Capn4 expression and siRNA plasmids are described in our previous publication.[11] LMP1 mutants containing TM deletion (Δ20-187), C-terminal activation region-1 (CTAR1) deletion (Δ188-231), and CTAR2 deletion (Δ351-386) plasmids were constructed by PCR amplification of LMP1 from pCMV-LMP1-WT (kind gifts from Dr JY Chen) using primers containing *Bam*HI and *Eco*RI restriction sites. The resulting PCR products were digested with *Bam*HI and *Eco*RI and cloned in frame into a pCMV-FLAG2 vector (Sigma). SP600125, interfering RNA (con siRNA), c-Fos siRNA, and c-Jun siRNA were purchased from Sigma. PD98059 was purchased from Calbiochem-Novabiochem.

2.4 | Western blot analysis

Samples were prepared from whole cell lysates. Total protein was quantified, and equal amounts of protein were separated on SDS-PAGE gels and electrotransferred onto PVDF membranes. The membranes were subsequently probed with primary antibodies, including rabbit polyclonal anti-Capn4 antibody (1:1000; LifeSpan Biosciences), LMP1 monoclonal antibody (CS.1-4, 1:1000; DAKO), as well as anti-MMP2, anti-Snail, and anti-Vimentin antibodies (1:1000, Cell Signaling Technology). Anti-phospho-c-Jun, c-Jun, phospho-c-Fos, c-Fos, phospho-JNK and horseradish peroxidase-conjugated goat antirabbit immunoglobulin antibodies (Santa Cruz Biotechnology) were used as secondary antibodies.

2.5 | Luciferase reporter assay

Nasopharyngeal carcinoma cells were transfected with expression plasmids as indicated, together with Capn4 p-Luc2 (or its mutants) and *Renilla* as an internal transfection control. An empty vector was used to equalize the total amount of DNA in all transfections. Cells were collected 24 hours after transfection. Luciferase activity was measured with equal amounts (10% of total for each sample) of protein lysates with the use of a Dual Luciferase Assay kit (Promega) on a multimode microplate reader (Turner Biosystems).

Results represent mean ± standard error (SE) of duplicates for each sample. At least three consistent results were obtained from independent experiments and representative results are shown. Ability of the empty vector controls to activate the promoter constructs was set to 1.

2.6 | Boyden chamber assay

A Matrigel invasion assay was carried out using modified Boyden chambers in 24-well dishes with Transwell filter inserts provided with 8-mm pores (Costar, Fisher Scientific Labosi), as described elsewhere.[13] Invasiveness was determined by counting cells in eight random microscopic fields per well, with each seeded in triplicate.

2.7 | Immunofluorescence microscopy

Cells grown on poly-L-lysine-coated cover slides were fixed with 4% formaldehyde, then permeabilized and blocked with 0.1% saponin containing 1% BSA for 20 minutes at room temperature. For actin filament staining, cells were incubated in TRITC-conjugated phalloidin (50 mg/mL; Sigma-Aldrich) for 1 hour after fixation. Nuclei were stained with DAPI (Sigma-Aldrich). All cover slides were mounted with Vectashield reagent (Vector Laboratories Inc.) and visualized by confocal microscopy using a ZEISS LSM510 META laser-scanning confocal microscope (Carl Zeiss).

2.8 | Statistical analysis

Statistical analyses were carried out using SPSS 16.0 (SPSS) or GraphPad Prism 5 (GraphPad Software). Significance of the Kaplan-Meier survival analyses was determined using log-rank tests. Factors related to prognosis were analyzed using multivariate regression with the Cox proportional hazards regression model. IHC analyses of Capn4 and LMP1 immunoreactivity in human NPC tissues were assessed using a χ^2 test. Spearman's rank correlation coefficient was used to evaluate the correlation between IHC results (staining intensity × percentage). Statistical significance was established at $P < .05$.

3 | RESULTS

3.1 | Overexpression of Capn4 in EBV-associated NPC

We previously showed that enhanced Capn4 expression was associated with positive EBV infection in NPC patients.[11] EBV is an etiological factor in NPC development and, among the expressed viral genes, LMP1 is detected in most NPC tissues.[1] Thus, we investigated the level of LMP1 and Capn4 coexpression in NPC tissues. Among 153 cases, 138 cases were EBV-positive and 15 cases were EBV-negative. Immunohistochemical studies showed that Capn4 and LMP1 were highly expressed in both primary and

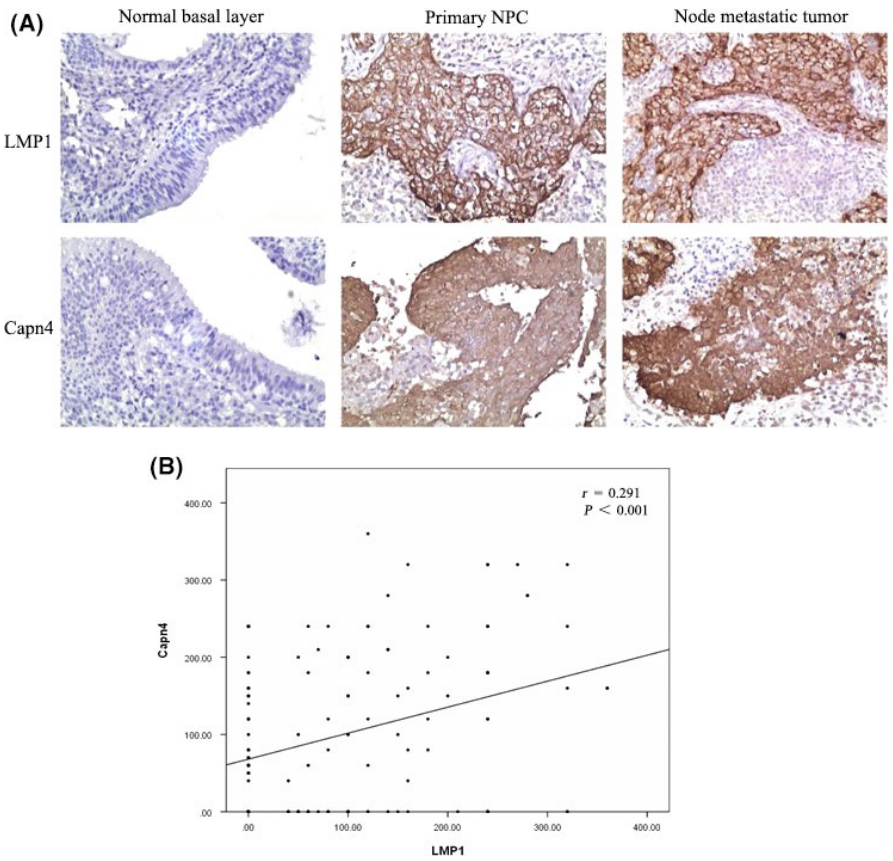

FIGURE 1 Expression of Epstein-Barr virus latent membrane protein 1 (LMP1) and Capn4 in nasopharyngeal carcinoma (NPC) tissues. A, Expression of LMP1 and Capn4 in the normal basal layer, primary NPC, and matched metastatic node tissues (×100). B, Correlation analyses based on immunohistochemistry scores (Spearman's correlation test)

TABLE 1 Correlation between the expression of Capn4 and LMP1 protein in NPC

	LMP1+		LMP1-	
	Metastasis	No metastasis	Metastasis	No metastasis
Capn4+	29	18	9	16
Capn4-	9	14	8	50

metastatic NPC tissues (Figure 1A). LMP1-positive expression rate in 153 nasopharyngeal carcinoma tissues was 45.8% (70/153). Positive expression rate of Capn4 in LMP1-positive NPC tissues was 67.1% (47/70). Positive expression rate of LMP1 in 55 metastatic NPC tissues was 69.1% (38/55). Among the 38 metastasized LMP1-positive NPC tissues, rate of positive Capn4 expression was 76.3% (29/38) (Table 1). Furthermore, Pearson correlation analysis suggested that Capn4 expression was positively correlated with LMP1 expression in NPC tissues (Figure 1B; $r = 0.291$, $P < .001$). Further investigation of the clinical significance of Capn4 and LMP1 expression in NPC showed a significant correlation between high Capn4 expression levels with disease-free survival rate (DFS) and distant metastasis-free survival (DMFS). Moreover, coexpression of LMP1 overexpression and Capn4 was positively correlated with DMFS (Table 2). Multivariate analysis was done to discriminate the independent prognostic indicators for OS and PFS. Expression of LMP1, T stage, and clinical stage were significant independent prognostic factors for PFS (all $P < .05$). These factors combined with the expression of Capn4 and N stage also achieved prognostic significance for OS (all $P < .05$) (Table S1).

3.2 | Epstein-Barr virus latent membrane protein 1 induces Capn4 expression

To further identify the role of LMP1 in Capn4 expression, we transfected LMP1-expression plasmids into 5-8F and CNE2 NPC cell lines. Western blot analysis showed that LMP1 increased the expression of Capn4 in both cell lines (Figure 2A). To examine whether LMP1 upregulates Capn4 expression at the transcriptional level, we measured Capn4 transcripts using quantitative reverse transcription-PCR. Results showed that Capn4 transcripts were increased in LMP1-overexpressing cells (Figure 2B). Capn4 promoter assay further showed that LMP1 transcriptionally upregulates the Capn4 promoter in a dose-dependent way (Figure 2C). Moreover, compared to the cells transfected with the pCMV2-LMP1 plasmid, 5-8F and CNE2 cells transfected with the pCMV2-LMP1-mutant plasmid showed less activation of the Capn4 promoter (Figure 2D). The same result was obtained for LMP1 expression induced by doxycycline (Dox). LMP1 expression led to an increase in both Capn4 expression and promoter activation (Figure 2E and 2). Overall, these data conclusively show that LMP1 is responsible for upregulation of Capn4 expression in NPC cells.

3.3 | AP-1 is responsible for LMP1-mediated promoter activity of Capn4

To investigate the specific transcription factors involved in the LMP1-associated enhanced transcriptional activation of Capn4, TFSEARCH (https://www.softpedia.com/get/Science-CAD/TFSearch-Viewer.shtml) was used to evaluate the Capn4 promoter, and a potential AP-1 binding site (−68 to −34 bp) was found. Capn4 promoter activity was significantly decreased when the AP-1 binding site was mutated compared with the wild type (WT) control (Figure 3A). It has previously been found that AP-1 is essential for Capn4 promoter activity and gene expression, and that LMP1 can stimulate AP-1 activation to mediate AP-1-dependent transcription in NPC cells.[14-16] In the present study, the results also indicated that the activation of c-Fos and c-Jun was increased in pCMV2-LMP1 5-8F and CNE2 cells based on the detection of c-Fos and c-Jun phosphorylation (Figure 3B). Thus, we assumed that LMP1 might activate the Capn4 promoter by

TABLE 2 Associations between Capn4 expression, LMP1 expression and disease-free survival rate/distant metastasis-free survival (Kaplan-Meier analysis)

		Disease-free survival (mouths)				Distant metastasis-free survival (months)			
Parameters	N	Mean	95% CI		*P*-value	Mean	95% CI		*P*-value
Capn4 expression									
Negative	72	33.204	28.378	38.031	.007	33.892	28.762	39.021	.007
Positive	81	24.343	20.932	27.755		26.986	22.902	31.070	
LMP1 expression									
Negative	70	29.650	25.539	33.761	.179	35.715	31.385	40.044	.001
Positive	83	26.355	22.424	30.286		26.039	22.055	30.023	
Capn4 + LMP1 expression									
Capn4/LMP1 Negative	47	34.068	28.420	39.715	.580	39.388	34.034	44.741	.015
Capn4/LMP1 Positive	58	23.598	19.570	27.627		24.449	19.743	29.155	

interacting with AP-1. As anticipated, overexpression of LMP1 or stimulation with PMA/ionomycin significantly increased Capn4 promoter activity (Figure 3C). Either LMP1 or stimulation with PMA/ionomycin could induce WT Capn4 promoter activity but failed to affect Capn4-mutated promoter activity (Figure 3D). Pretreating cells with curcumin (an AP-1 inhibitor, 10 μmol/L) inhibited LMP1-induced Capn4 expression (Figure 3E). Moreover, knockdown of c-Fos and c-Jun significantly reduced the expression of Capn4 (Figure 3F), and suppressed Capn4 promoter activity in pCMV2-LMP1-expressing cells (Figure 3G).

3.4 | Epstein-Barr virus latent membrane protein 1 CTAR1/CTAR2 domains are required for the contribution of AP-1 activation to Capn4 expression

To dissect the functional domains of LMP1 responsible for its involvement in the activation of AP-1 to induce Capn4 expression, we generated a series of LMP1 deletion constructs (Figure 4A) and examined their effects on Capn4 expression and AP-1 activation in NPC cells. Deletion of the entire transmembrane domains △TM (△20-187) of LMP1 did not alter Capn4 expression or c-Fos/c-Jun activity, compared with WT LMP1, indicating that the LMP1 transmembrane domains are not involved in this event. In contrast, deletion of the C-terminal cytoplasmic tail CTAR1 (△188-231) or CTAR2 (△351-386) failed to induce Capn4 expression and promoter activation (Figure 4B and 4). Moreover, deletion of the C-terminal cytoplasmic tail domains, CTAR1 or CTAR2, failed to interact with phosphorylated c-Fos and c-Jun (Figure 4B). Taken together, these results indicate that both the CTAR1 and CTAR2 domains are required for the increase in Capn4 expression through interacting with activated AP-1.

3.5 | Epstein-Barr virus latent membrane protein 1 mediates c-Fos and c-Jun activation by ERK/JNK signaling to promote Capn4 expression

AP-1 can be activated by variety of stimuli, including the JNK pathway.[17] Thus, we examined whether LMP1 was able to promote c-Fos activation through the JNK pathway. As shown in Figure 5A, JNK activity was increased in pCMV2-LMP1-expressing cells. When the cells were treated with SP600125 (a JNK pathway inhibitor), the activity of JNK was abolished. In addition, activation of c-Fos and c-Jun, as well as Capn4 expression, was significantly suppressed. Together, these results indicate that LMP1-mediated activation of the JNK pathway was responsible for the activation of c-Fos and c-Jun to promote the expression of Capn4 in NPC cells.

Previous reports have shown that LMP1 has the ability to activate the ERK pathway and promote downstream c-Fos stabilization that may contribute to the tumorigenicity of LMP1.[14] In this report, we further explored whether an LMP1-mediated increase in Capn4 expression was dependent on the ERK pathway to increase the activation of c-Fos. As shown in Figure 5B, when the ERK pathway was inhibited with PD98059, activation of the JNK pathway was dramatically decreased. Moreover, activation of c-Fos and the expression of Capn4 induced by LMP1 were suppressed, suggesting that the ERK pathway functioned as an upstream regulator of the JNK pathway to activate c-Fos, which contributed to the enhanced expression of Capn4 in NPC.

3.6 | Capn4 and LMP1 cooperatively enhance NF-κB activation

It has been well established that LMP1 can regulate NF-κB activation and contributes to tumorigenesis by activating the expression of several genes involved in cellular apoptosis, proliferation, migration, and the immune response.[18-20] However, we previously reported that NF-κB is a downstream signaling pathway of Capn4 for the promotion of NPC cell migration.[11] To this end, we investigated the molecular functions of Capn4 and LMP1 in NF-κB signaling. The results showed that either the overexpression of Capn4, LMP1, or both could increase the activity of NF-κB, and the coexpression of Capn4 and LMP1 induced a substantial increase in NF-κB activity (Figure 6A). In addition, knockdown of Capn4 by RNA interference diminished LMP1-induced NF-κB activation (Figure 6B), confirming that Capn4 is essential for LMP1-induced NF-κB activation. Coexpression or individual expression of Capn4 and LMP1 increased the expression of the NF-κB downstream targets, MMP2, Snail, and vimentin (Figure 6A), whereas Capn4 knockdown reduced the induction of NF-κB-mediated gene expression by LMP1 (Figure 6B).

3.7 | Capn4 coordinates LMP1-induced actin rearrangement and cell motility

To investigate whether LMP1 contributed to cell motility, we conducted Transwell migration assays using NPC cells expressing various forms of LMP1. As shown in Figure 7A, LMP1 expression led to increased cell motility compared with the vector control. Moreover, deletion of the C-terminal cytoplasmic tail, CTAR1 or CTAR2, showed substantially decreased cell motility compared with WT LMP1. To verify the requirement for Capn4 in LMP1-induced cell motility, RNA interference was used to deplete Capn4 from NPC cells, followed by LMP1 overexpression. As shown in Figure 7B, LMP1 expression in control siRNA cells increased cell motility compared to the vector-transfected cells. Moreover, knockdown of Capn4 reduced LMP1-induced cell motility by approximately 70%. We next sought to investigate whether the above events are involved in actin rearrangement of NPC cells. As shown in Figure 7C, using phalloidin staining, stress fiber formation was found to be suppressed in NPC cells coexpressing LMP1 and treated with Capn4-specific siRNA,

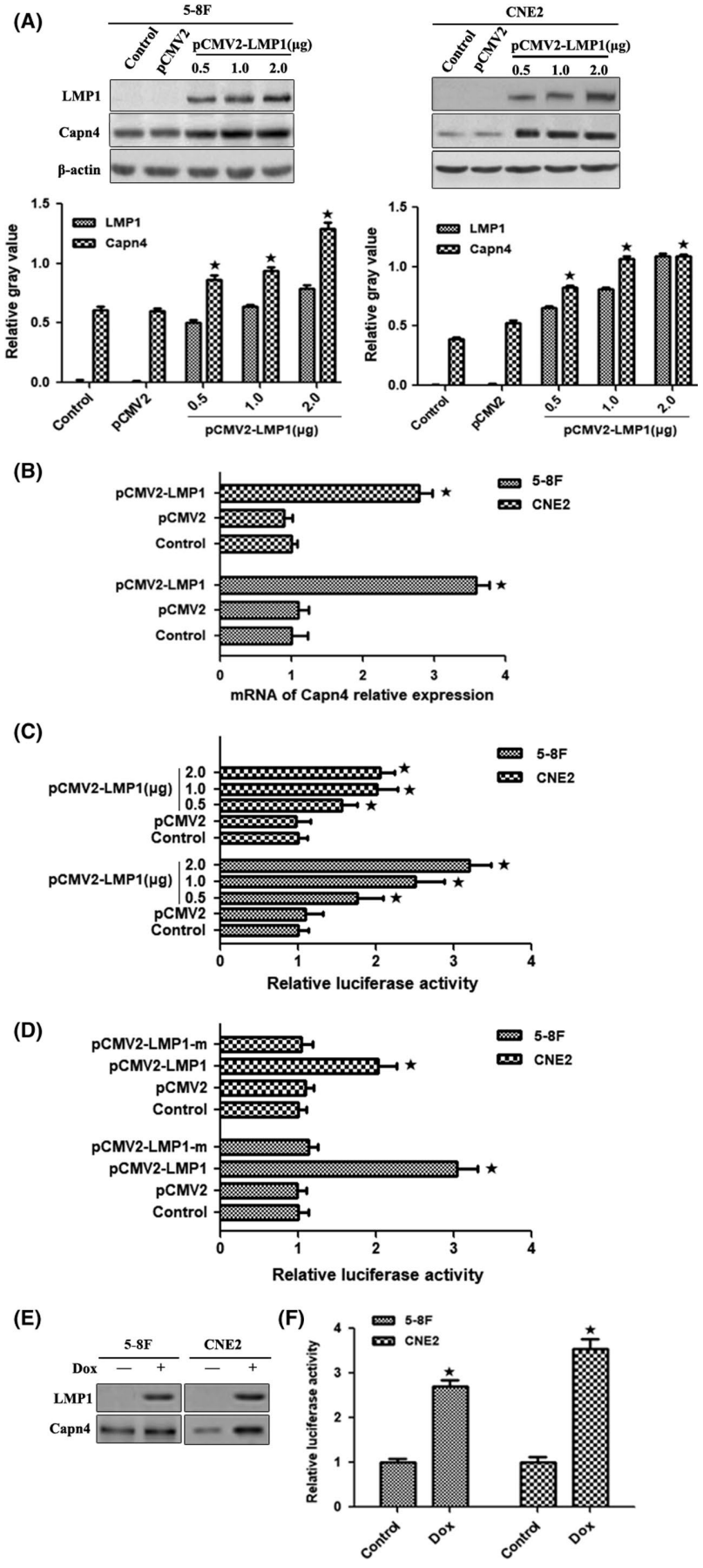

FIGURE 2 Epstein-Barr virus latent membrane protein 1 (LMP1) induces Capn4 expression in nasopharyngeal carcinoma cells. A, 5-8F and CNE2 cells expressing LMP1 or a control were generated by transfection with different concentrations of pCMV2-LMP1 expression and control plasmids for 24 h, followed by western blot for the level of Capn4 protein expression. B, Level of Capn4 mRNA in 5-8F and CNE2 cells was determined by qRT-PCR. C, Capn4 promoter (−187/+174) activity was examined in cells with different concentrations of pCMV2-LMP1 expression by a reporter assay. Control plasmid was arbitrarily set to 100%. D, Capn4 promoter activity was examined in cells transfected with either pCMV2-LMP1 or pCMV2-LMP1-m. Control plasmid was arbitrarily set to 100%. E, 5-8F and CNE2 cells were treated with 5 g/mL doxycycline (Dox) for 24 h to induce LMP1 expression, and then subjected to immunoblotting analysis to determine the level of Capn4 and LMP1 protein expression. F, Capn4 promoter activity was examined in cells treated with 5 g/mL Dox for 24 h. Control plasmid was arbitrarily set to 100%. Data are presented as mean (±SD) of three independent experiments carried out in quadruplicate. *$P < .05$ compared to control

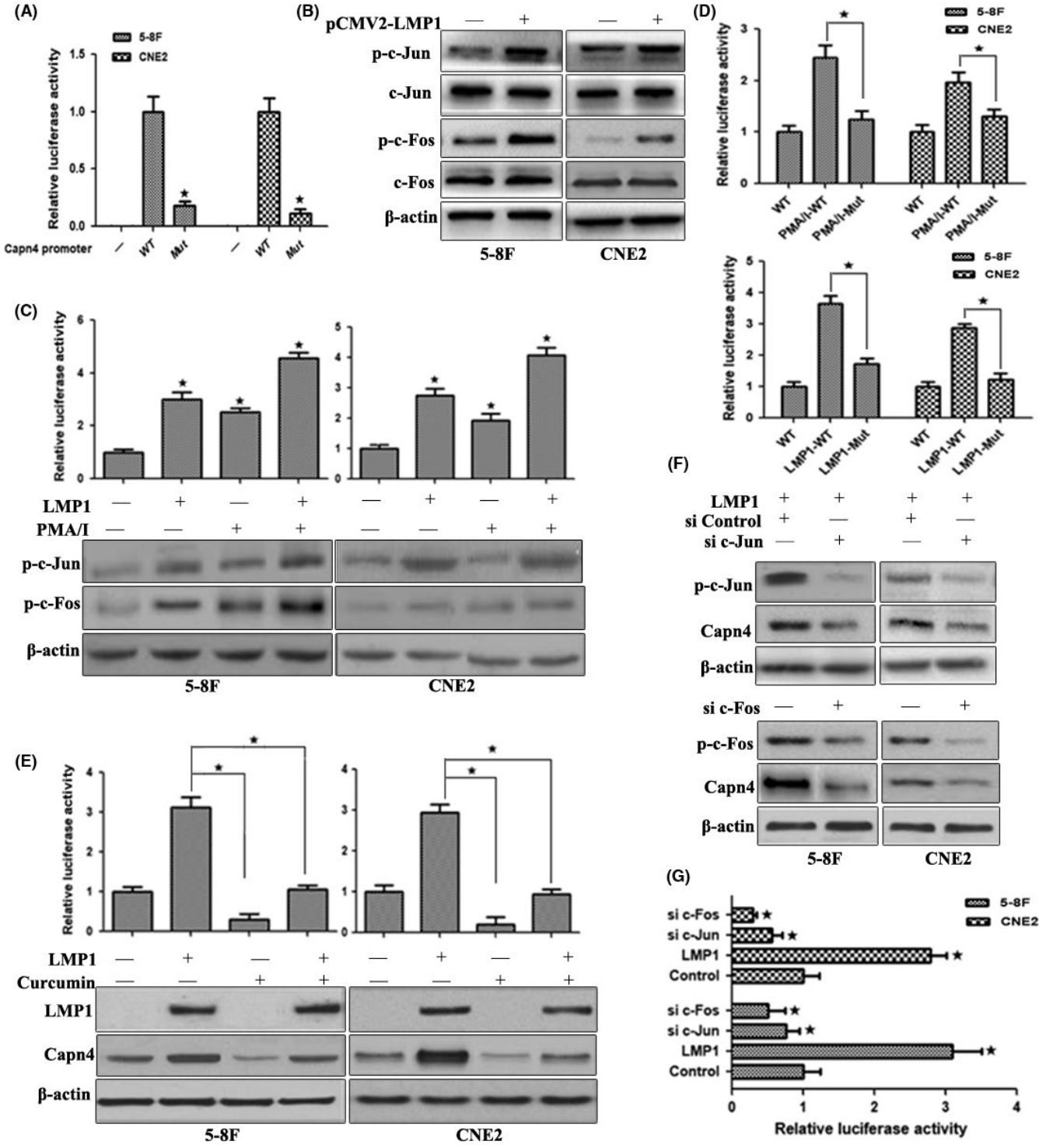

FIGURE 3 AP-1 is involved in Epstein-Barr virus latent membrane protein 1 (LMP1)-mediated promoter activity of Capn4. A, pGL2-Capn4/WT or Mut was transiently transfected into cells, after which the luciferase activity was analyzed. B, Western blot analysis of the levels of p-c-Jun and p-c-Fos expression in cells stably transfected with pCMV2-LMP1. C, Capn4 promoter activity and AP-1 activity were examined in cells stably expressing LMP1 and/or treated with 2 μmol/L PMA and 400 ng/mL ionomycin for an additional 24 h. D, pGL2-Capn4/WT or Mut were transiently transfected into cells with pCMV2-LMP1 and/or treated with 2 μmol/L PMA and 400 ng/mL ionomycin, and luciferase activity was analyzed. E, Upper panel: Luciferase reporter assay showing Capn4 promoter activity in cells stably expressing LMP1 and treated with 10 mmol/L curcumin for 24 h. Lower panel: Western blot analysis showing the level of LMP1 and Capn4 protein expression in cells stably expressing LMP1 and treated with 10 mmol/L curcumin for 24 h. F, Western blot analysis showing the level of Capn4 following transfection with siRNA for c-Jun and c-Fos silencing. G, Luciferase reporter assay showing Capn4 promoter activity in cells transfected with siRNA for the silencing of c-Jun and c-Fos. Data are presented as mean (±SD) of three independent experiments, each carried out in triplicate. *$P < .05$ compared to control

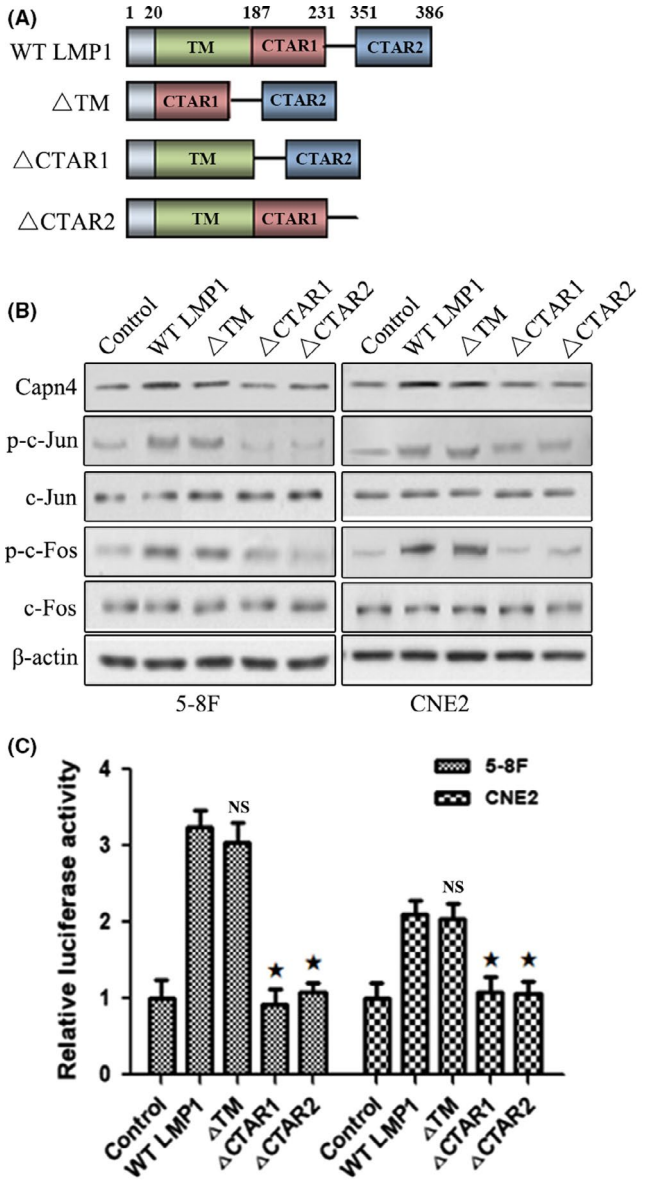

FIGURE 4 C-terminal activation region (CTAR)1/CTAR2 domains of Epstein-Barr virus latent membrane protein 1 (LMP1) are required for induction of Capn4 expression and AP-1 activation. A, Schematic illustrations of LMP1 and its truncated forms. WT LMP1, full-length LMP1; △TM, deletion of the entire transmembrane domains; △CTAR1, deletion of the C-terminus CTAR1; △CTAR2, deletion of the C-terminus CTAR2. B, Protein expression of Capn4, p-c-Jun, c-Jun, p-c-Fos, c-Fos was assessed by western blot analysis, which confirmed that expression of Capn4, p-c-Jun, and p-c-Fos was reduced in △CTAR1- and △CTAR2-LMP1-expressing cells. C, Luciferase reporter assay showing Capn4 promoter activity in cells transfected with WT LMP1, △TM, △CTAR1, or △CTAR2. Data are presented as mean (±SD) of three independent experiments, each carried out in triplicate. **P* < .05, NS no significance, compared to control

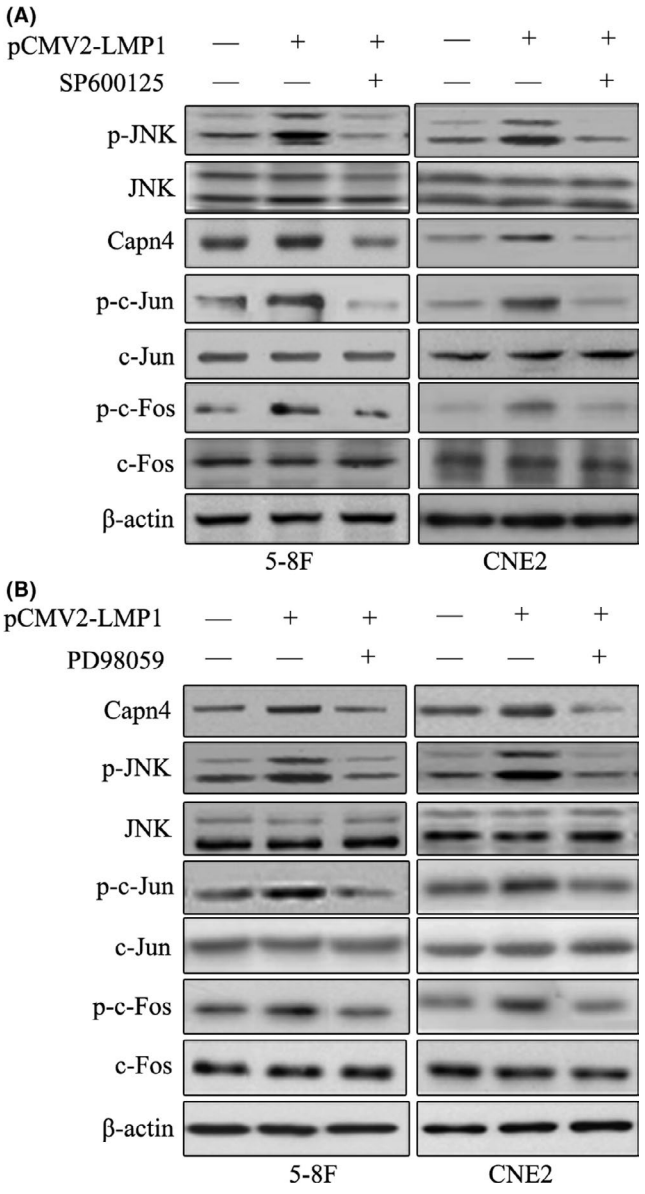

FIGURE 5 ERK/JNK is involved in Epstein-Barr virus latent membrane protein 1 (LMP1)-induced c-Jun/c-Fos phosphorylation and Capn4 expression. A, Western blot analysis showing the level of Capn4 protein expression and AP-1 activation in cells stably expressing LMP1 and treated with 10 mM/L SP600125 for 24 h. B, Western blot analysis showing the level of Capn4 protein expression and AP-1 activation in cells stably expressing LMP1 and treated with 10 mM/L PD98059 for 24 h

compared to LMP1 expression in the cells treated with control siRNA. Collectively, our data show that LMP1 induces Capn4 expression which promotes actin rearrangement and, ultimately, cell migration in NPC cells.

4 | DISCUSSION

We have previously reported that Capn4 functions as an important regulator of NPC cell migration;[11] however, the molecular mechanism by which Capn4 enhances cell migration remains unknown. LMP1 is believed to play a crucial role in the early stages of NPC pathogenesis.[21-24] In the present study, we sought to elucidate whether Capn4 is involved in LMP1-mediated NPC progression. To investigate the relationship between Capn4 and LMP1 in NPC cells, we examined LMP1 and Capn4 expression in clinical primary and metastatic NPC tissues.

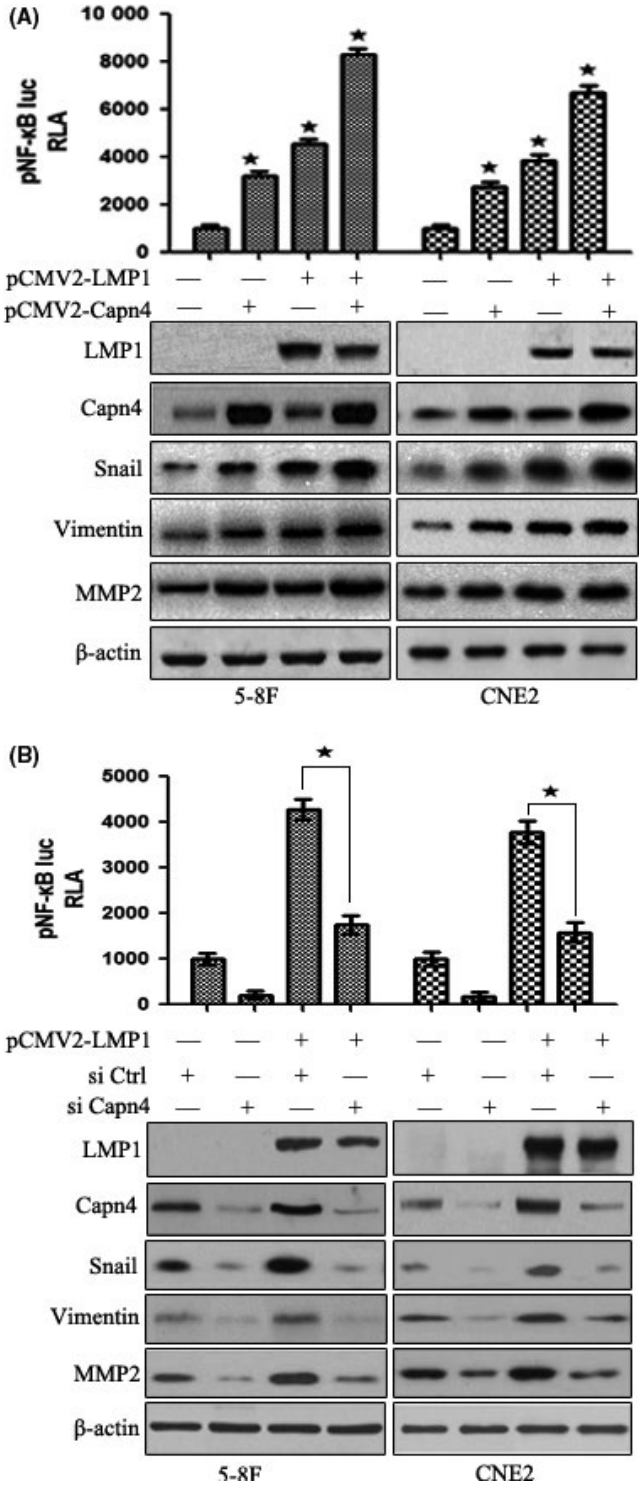

FIGURE 6 Capn4 cooperates with Epstein-Barr virus latent membrane protein 1 (LMP1) to enhance nuclear factor kappa B (NF-κB) activation. A, Upper panel: luciferase reporter assay showing NF-κB activity in cells coexpressing LMP1 and Capn4. Data are presented as mean (±SD) of three independent experiments, each carried out in triplicate. *P < .05. Lower panel: Western blot analysis showing the level of Snail, Vimentin, and MMP-2 protein expression in cells coexpressing LMP1 and Capn4. B, Upper panel: Luciferase reporter assay showing NF-κB activity in cells expressing LMP1 and knockdown of Capn4. Data are presented as mean (±SD) of three independent experiments, each carried out in triplicate. *P < .05. Lower panel: Western blot analysis showing the level of Snail, Vimentin, and MMP-2 protein expression in cells expressing LMP1 and knockdown of Capn4

Notably, we found that Capn4 and LMP1 were highly expressed in both primary and metastatic NPC tissues with a significantly positive correlation. In addition, overexpression of LMP1 in NPC cell lines resulted in increased Capn4 protein and mRNA expression. Thus, we proposed that LMP1 might upregulate the promoter activity of Capn4. As expected, we showed that LMP1 could upregulate the activation of the Capn4 promoter. In contrast, Capn4 promoter activity was decreased in NPC cells transfected with a pCMV2-LMP1-mutant plasmid.

Next, we investigated the mechanism by which LMP1 upregulates Capn4 in NPC cells. A previous study found that AP-1 is essential for human Capn4 promoter activity and gene expression.[16] AP-1 transcription factor is an important regulator of several cell signaling pathways, including the expression of downstream genes involved in cell differentiation, survival, proliferation, and migration.[25-28] Accumulating evidence also suggests that AP-1 plays an important role in promoting NPC cell migration.[29-31] Furthermore, AP-1 activity has been shown to be mediated by LMP1.[32,33] Therefore, we speculated that AP-1 activation might be responsible for LMP1-induced Capn4 expression. Indeed, we showed that treatment with curcumin (an AP-1 inhibitor) could sufficiently block LMP1-mediated enhanced Capn4 expression. Moreover, silencing of c-Fos and c-Jun by siRNA could block the increased Capn4 expression mediated by LMP1. These data indicate that LMP1 upregulates Capn4 by the activation of AP-1. LMP1 is an integral transmembrane protein composed of a short cytoplasmic N-terminal tail required for insertion into the membrane, as well as six membrane-spanning domains and a C-terminal cytoplasmic tail.[21,34] The C-terminus of LMP1 contains two major signaling domains: (i) the membrane proximal C-terminal activation region-1 (carboxyl terminal activator region [CTAR1], amino acid residues 194-232); and (ii) the membrane distal CTAR2 (amino acid residues 351-386).[22] Both of these domains are required for the ability of LMP1 to mediate the majority of signal transduction events, including NF-κB, JNK, and P38.[35-39] In the present study, transmembrane domains were found to be dispensable for LMP1 to mediate Capn4 expression. Mutants containing deletions of transmembrane domains (△TM) did not alter Capn4 expression and/or c-Fos/c-Jun activity compared with WT LMP1. In contrast, deleting the CTAR1 or CTAR2 domains of LMP1 impairs its ability to induce AP-1 activation and results in the failure to upregulate Capn4 expression.

We next attempted to better understand the mechanism by which LMP1 activates AP-1. Multiple signaling pathways have been related to AP-1 activation.[40,41] JNK is one of the most important regulators of the complex assembly by c-Fos and c-Jun.[17,41-43] Additionally, it has been reported that JNK can be activated by LMP1 and subsequently induce the migration of NPC cells.[36,37] The present study confirmed this finding in NPC cells that

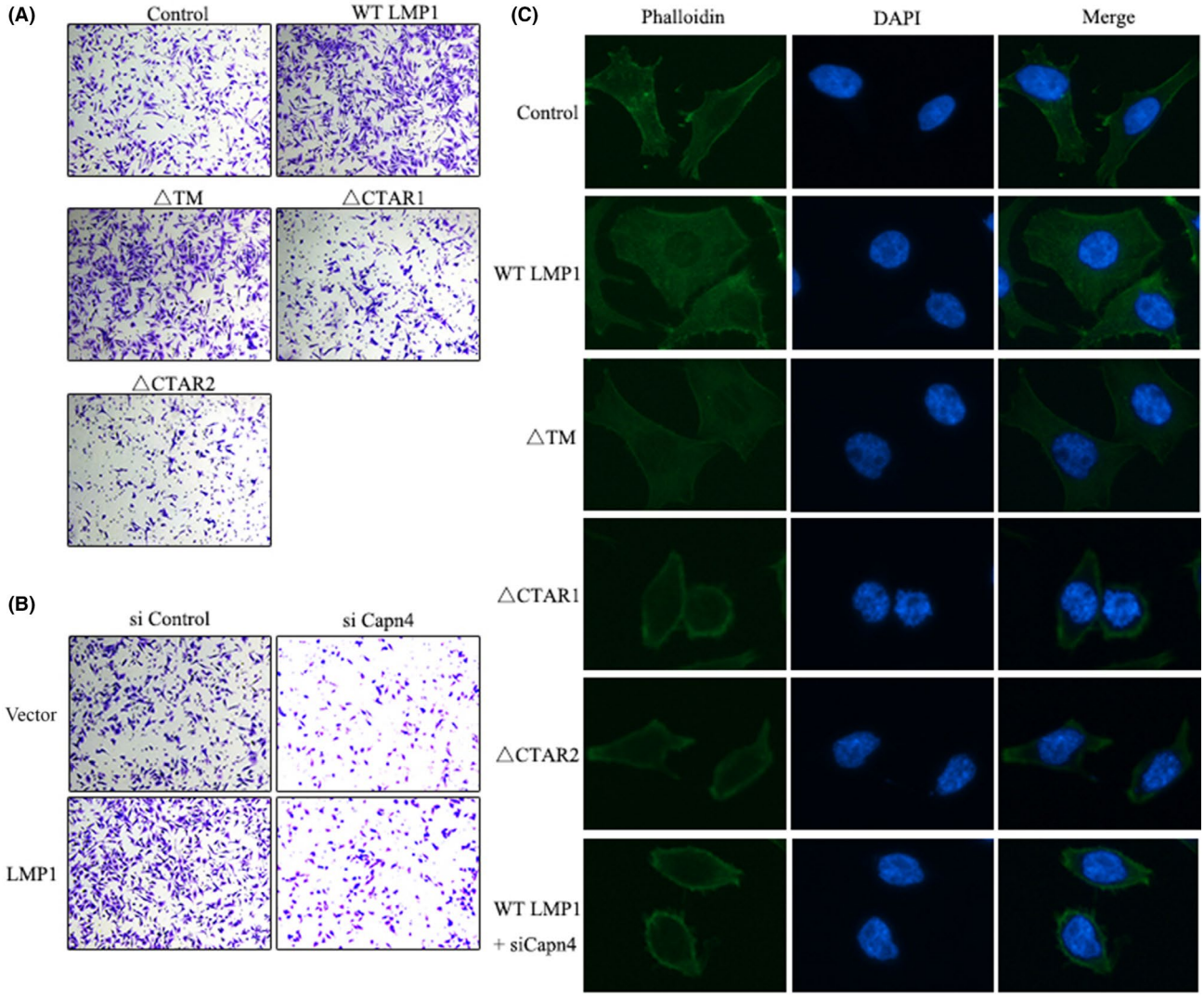

FIGURE 7 Activity of Epstein-Barr virus latent membrane protein 1 (LMP1) and Capn4 in nasopharyngeal carcinoma (NPC) cells leads to increased cell motility and actin rearrangement. A, Induction of cell motility by LMP1. CNE2 cells were transfected with the WT-LMP1 plasmid or its truncated forms. After 24 h, the cells were re-seeded for Transwell migration assays as described in Materials and Methods. Images of migrating cells in each experiment were acquired at 200× magnification. B, CNE2 cells were cotransfected with the LMP1 expression plasmid and Capn4 siRNA or an empty vector. After 24 h, the cells were re-seeded for Transwell migration assays as detailed in Materials and Methods. Images of migrating cells in each experiment were acquired at 200× magnification. C, CNE2 cells grown on coverslips were transfected with the plasmid for WT-LMP1 or its truncated forms, or cotransfected with the WT-LMP1 expression plasmid and Capn4 siRNA or pEGFP-N1 vector (pEGFP vector), and then incubated for 48 h. Following 6 h of serum starvation, the cells were fixed and stained with FITC-conjugated phalloidin to examine actin filament (F-actin) arrangement. Images were acquired using a ZEISS LSM510 confocal microscope. Scale bar, 20 mm

overexpressed LMP1, and found that there was increased phosphorylation of JNK. The effect of the JNK pathway on AP-1 activity was further tested, and it was shown that inhibition of JNK activity by SP600125 suppressed c-Fos and c-Jun phosphorylation, which subsequently downregulated Capn4 expression. A previous report showed that LMP1 sustained the activation of ERK, subsequent c-Fos induction, and binding to AP-1.[14] Another report showed that ERK phosphorylation/activation in breast cancer cells resulted in the rapid upregulation of Capn4 expression.[44] In the present study, we present the role of ERK in LMP1-induced Capn4 expression and AP-1 activity in NPC cells. We found that treatment with an ERK inhibitor (PD98059) blocked c-Fos phosphorylation and Capn4 expression. Moreover, treating NPC cells with PD98059 attenuates LMP1-induced JNK phosphorylation. These results emphasize that the ERK signaling pathway plays a role in LMP1-induced JNK phosphorylation to activate c-Fos, which contributes to the enhanced expression of Capn4 in NPC cells.

Nuclear factor-κB is a transcription factor that regulates a variety of genes which participate in several crucial physiological processes, including cell survival, inflammation, or immune response.[45-47] Our prior studies have shown that Capn4-mediated promotion of NPC metastasis occurs through NF-κB activation.[11] A study by Shkoda

et al found that LMP1-mediated activation of JNK was dependent on canonical NF-κB, which contributed to the tumorigenicity of EBV-transformed B cells.[36] Our results are consistent with a previous study which found that LMP1 induced a substantial increase in NF-κB activity. However, knockdown of Capn4 by RNA interference diminished LMP1-induced NF-κB activation. It has been reported that NF-κB regulates metastasis-related genes to regulate metastasis in many cancer cells.[48-50] In the present study, Capn4 was found to be effective at upregulating the expression of MMP2, Snail, and vimentin, which together may have been mediated by NF-κB. Thus, we concluded that LMP1 activated NF-κB through upregulation of Capn4 in NPC cells.

Tumor cell migration is a pivotal step in the process of cancer metastasis.[51,52] In addition, cellular migration is dependent on the activity of the plasma membrane at the leading edge of the cells, and these membrane structures (eg, actin-based filopodia and lamellipodia) are important structures. Therefore, we are interested in the role of LMP1 in the promotion of filopodia formation. Results of the present study show that exogenous expression of LMP1 in NPC cells substantially enhanced the level of cellular motility and upregulated the formation of stress fibers. Moreover, inhibition of Capn4 by RNA interference substantially blocked LMP1-mediated increase in cell motility and stress fiber formation. Furthermore, deletion of the C-terminal cytoplasmic tails, CTAR1 or CTAR2, also showed substantially decreased cellular motility and stress fiber formation. Thus, we concluded that LMP1 promotes cell migration by Capn4-mediated increase in stress fiber formation.

The findings presented in this study identify a novel mechanism by which LMP1 alters cellular signaling and behavior in NPC cells, showing the key contribution of Capn4 and the role of the ERK/JNK/AP-1 signaling pathways. The ability of Capn4 to coordinate with LMP1 to stimulate fibronectin expression and enhance cellular migration suggests a novel role of LMP1-Capn4 in the metastatic process in NPC.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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