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ELK3 Expression Correlates With Cell Migration, Invasion, and Membrane Type 1-Matrix Metalloproteinase Expression in MDA-MB-231 Breast Cancer Cells

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ELK3 is a member of the Ets family of transcription factors. Its expression is associated with angiogenesis, vasculogenesis, and chondrogenesis. ELK3 inhibits endothelial migration and tube formation through the regulation of MT1-MMP transcription. This study assessed the function of ELK3 in breast cancer (BC) cells by comparing its expression between basal and luminal cells in silico and in vitro. In silico analysis showed that ELK3 expression was higher in the more aggressive basal BC cells than in luminal BC cells. Similarly, in vitro analysis showed that ELK3 mRNA and protein expression was higher in basal BC cells than in normal cells and luminal BC cells. To investigate whether ELK3 regulates basal cell migration or invasion, knockdown was achieved by siRNA in the basal BC cell line MDA-MB-231. Inhibition of ELK3 expression decreased cell migration and invasion and downregulated MT1-MMP, the expression of which is positively correlated with tumor cell invasion. In silico analysis revealed that ELK3 expression was associated with that of MT1-MMP in several BC cell lines (0.98 Pearson correlation coefficient). Though MT1-MMP expression was upregulated upon ELK3 nuclear translocation, ELK3 did not directly bind to the 1.3-kb promoter region of the MT1-MMP gene. These results suggest that ELK3 plays a positive role in the metastasis of BC cells by indirectly regulating MT1-MMP expression.

Key words: ELK3; MT1-MMP; Breast cancer; Metastasis

INTRODUCTION

ELK3/Net/Sap2 (referred to hereafter as Elk3) belongs to the Ets family of transcription factors and, together with ELK1 and Sap1, forms a ternary complex. Net mutant mice develop abnormal vascular and lymphatic vessels, suggesting that expression of ELK3 during mouse development is associated with vasculogenesis and lymphangiogenesis (1). In contrast to Sap1 and ELK1, which become transcriptional activators by being phosphorylated by mitogen-activated protein kinases (MAPK), ELK3 functions as a strong transcriptional repressor with two inhibitory domains (2); however, ELK3 is converted to a transcriptional activator upon RAS (rat sarcoma)/ERK (extracellular signal-regulated kinase) activation as well as upon phosphorylation by MAPK (2,3). Phosphorylated ELK3 positively regulates the expression of vascular endothelial growth factor in NIH-3T3 cells (4). The possible role of ELK3 in cancer has been described in several reports. Phosphorylated ELK3 is highly expressed in tumor cells in Kaposi's sarcoma, prostate cancer, and head and neck cancer but not in normal surrounding tissue (4). ELK3 is expressed in the cytoplasm in metastatic lymph nodes, but is expressed at high levels in the nucleus of well-differentiated primary tumor cells, suggesting that its activity is linked to the metastasis of cancer (5). ELK3 also regulates cell migration in mouse embryonic fibroblasts by inhibiting the expression of PAI-I, a serine protease inhibitor that controls cell–matrix adhesion (6).

Metastasis is a complex process that starts with local migration and invasion of tumor cells to surrounding tissue. Malignant tumor cells then disseminate through the blood or the lymphatic system from the primary tumor to distant tissues (7). The ability of cancer cells to metastasize reflects their ability to change their interaction with the surrounding extracellular matrix (ECM) and with adjacent stromal cells. Unfortunately, the evolving nature of the microenvironment and the plasticity of cancer cell behavior make understanding and inhibiting metastasis complex and difficult.

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Membrane-anchored metalloproteinase-1 (MT1-MMP) belongs to the MMP family of zinc-dependent endopeptidases that degrade a variety of ECM components (8). The positive association between MT1-MMP expression and tumor progression and metastasis has increased interest in the potential prognostic value of MT1-MMP in cancer (9,10). In addition, MT1-MMP promotes medulloblastoma cancer stem cell infiltration and is highly expressed at the invasive front of tumors (10–12). The mechanisms by which MT1-MMP regulates tumor cell invasion include cleavage of the cell adhesion molecule CD44 from the cell surface and degradation of type I collagen (13–16).

Even though the expression of MT1-MMP is closely correlated with tumor progression and metastasis, little is known about its transcriptional regulation. The expression of E1AF, a transcription factor of the Ets oncogene family, is positively correlated with MT1-MMP gene activation (17). In prostate tumor cells, MT1-MMP is regulated by sp1 via the AKT, JNK, and ERK pathways (18).

Here we report that ELK3 expression is associated with cell migration and invasion in breast cancer (BC) cells in vitro. In silico analysis showed that ELK3 is highly expressed in metastatic BC cell lines, such as MDA-MB-231 and BT-20. siRNA-mediated suppression of ELK3 expression inhibits the migration and invasion of MDA-MB-231 in vitro. Unexpectedly, the expression of MT1-MMP, which is transcriptionally repressed by ELK3 in human umbilical vein endothelial cells (HUVECs) (19), is positively correlated with the expression of ELK3 in MDA-MB-231 cells. Collectively, these results provide novel insights into the function of ELK3 in BC cell metastasis.

MATERIALS AND METHODS

Cell Culture

MDA-MB-231, BT20, MCF7, BT474, and SkBr3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (catalog No. 11995; Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (catalog No. 16000; Invitrogen) and 1% penicillin/streptomycin (catalog No. 15140; Invitrogen) in the presence of 5% CO_2 at 37°C. MCF-10A cells were incubated in DMEM/F12 (catalog No. 11330-032) containing 10% horse serum (catalog No. 16050-122; Invitrogen), 20 ng/ml epidermal growth factor, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 1% penicillin/streptomycin. HUVECs were obtained from Modern Cell & Tissue Technologies, Inc. (Seoul, Korea) and were cultured on gelatin-coated plates in EGM™-2 medium (catalog No. CC-3162; Clonetics, Walkersville, MD, USA).

RNA Isolation and Real-Time PCR

Total cellular RNA was extracted from several BC cell lines and from cells in which Elk3 was knocked down using the TRIzol reagent (Ambion, Foster City, CA, USA) the manufacturer's manufacturer instructions. Total RNA (2 μg) was used for single-stranded cDNA synthesis using Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany). Gene-specific primers were designed for ELK3, MT1-MMP, and GAPDH as described previously (19,20). Quantitative real-time PCR was performed with the CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green I (Qiagen, Valencia, CA, USA). Cycling conditions were as follows: 40 cycles of 95°C for 30 s, 49°C for 30 s, and 72°C for 30 s. Results were calculated as relative expression and normalized to internal human GAPDH using the ΔCT method.

Protein Extraction and Western Blotting

Total protein was isolated using cell lysis buffer (catalog No. 9803; Cell Signaling Technology, Inc.) according to the manufacturer's instructions. For the cellular fractionation, several BC cell lines and MDA-MB-231 cells stimulated with SP600125 (catalog No. S5567; Sigma-Aldrich) were collected and washed twice with cold PBS. Then, subcellular fractionation was conducted according to a protocol published by Abcam (http://www.abcam.com/index.html?pageconfig=resource&rid=11473) for the isolation of cytosolic and nuclear proteins from cells. Cytosolic and nuclear protein concentration was measured using the bicinchoninic acid (BCA) solution. Proteins (20 μg) were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was incubated with anti-ELK3 antibody [NET (C-20), sc-17860; Santa Cruz Biotechnology, Inc.], anti-MT1-MMP antibody (catalog No. MAB9281; R&D Systems), anti-phospho JNK antibody (catalog No. #9910; Cell Signaling), anti-JNK antibody (catalog No. 9926; Cell Signaling), anti-lamin B antibody [Lamin B (c-20), sc-6216; Santa Cruz Biotechnology, Inc.], or anti-β-actin antibody [β-actin (C4), sc-47778; Santa Cruz Biotechnology, Inc.] overnight at 4°C, followed by incubation with secondary antibody for 1 h at room temperature. The immunoreactive proteins were detected using the WEST-ZOL® (plus) Western Blot Detection System (iNtRON, Korea).

ELK3 RNA Interference and Proliferation Assay

Nonspecific control siRNA (D-001810-10) and human ELK3 siRNA (L-010320-00-0005) were obtained from Dharmacon, Inc. (Chicago, IL, USA). Nonspecific control siRNA (siScramble) or siELK3 (100 nM) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions in MDA-MB-231 cells. The cells were collected 24, 48, and 72 h after transfection, and RNA was isolated for the analysis of ELK3 and MT1-MMP expression. For proliferation assay, MDA-MB-231 cells (2.5×10^5) were plated in a 60-mm culture

dish. The next day, 100 nM siScramble or siELK3 was transfected in cells. Proliferation was determined by counting cell numbers at 3 days.

Migration and Invasion Assay

The migration potential of MDA-MB-231 cells with ELK3 knockdown was assessed by scratch/wound assays. Monolayers formed by cells transfected with control siRNA (siScramble) or siELK3 were wounded using a micropipette tip when the cells were fully confluent. Detached cells were removed by washing with medium, and plates were photographed after 24 h. The Matrigel invasion assay was performed using 24-well Transwell inserts (6.5 mm diameter, 8 μm pores; Corning, NY, USA) coated with 30 μg of Matrigel (catalog No. 356231; BD Biosciences). Cells (1×10^5) were seeded in serum-free medium in the upper chamber and DMEM containing 10% FBS was added to the lower chamber. Cells were incubated to assess invasion through the membrane for 48 h. The invasive cells were fixed and stained with crystal violet (Sigma-Aldrich). The intensity of the crystal violet staining was measured using the GelQuant software (biochemlabsolutions.com) and expressed as means ± SE of triplicate wells.

Chromatin Immunoprecipitation (ChIP) Assay

MDA-MB-231 cells were exposed to 0, 10, and 20 μM SP600125 for 24 h, after which formaldehyde was added to a final concentration of 1% and incubated for 10 min at 37°C to achieve cross-linking. Glycine was then added to a final concentration of 125 mM for 5 min. The cells were washed and scraped with cold PBS. The cell pellet was lysed in 600 μl SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1) containing Halt™ protease and phosphatase inhibitor cocktail (catalog No. 78440; Pierce Biotechnology). The lysed cells were sonicated in seven rounds of seven pulses and pelleted by centrifugation; 20 μl of supernatant was retained as input control. The cleared lysates were resuspended in IP buffer (167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 0.01% SDS, and 1% Triton X-100) and immunoprecipitated with or without anti-ELK3 antibody [NET (C-20), sc-17860X; Santa Cruz Biotechnology, Inc.] by rotating overnight at 4°C. The immune complexes were recovered with protein A/G plus-agarose (sc-2003; Santa Cruz Biotechnology, Inc.) and washed in sequence with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 and 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 and 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1), and TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) twice. The DNA was eluted with elution buffer (1% SDS and 0.1 M $NaHCO_3$) and purified by phenol/chloroform extraction and ethanol precipitation. PCR reaction was performed with purified DNA as a template and the following primers: No. 1 (–1292 to –629 bp) forward 5′-CAG TCA CTG TGG GGT AGG TA-3′, No. 1 reverse 5′-GCT GCA CCT TTA ATT GGA ACT C-3′, No. 2 (–629 to +140 bp) forward 5′-GAG TTC CAA TTA AAG GTG CAG C-3′, and No. 2 reverse 5′-TTC GGT AGG CAC TGA ACT TGC-3′. The primers were designed to cover the 1.3-kb MT1-MMP promoter region based on the GenBank accession No. AB011056.1.

Statistical Analysis

The data are presented as means ± SE. The statistical significance of the results was assessed using one-way ANOVA followed by Student's *t*-test.

RESULTS AND DISCUSSION

Association of ELK3 With Breast Cancer Subtype

ELK3 expression correlates with neovasculogenesis, angiogenesis, and chondrogenesis during mouse development. In addition, ELK3 regulates cell migration and vascular permeability through the regulation of cell–cell or cell–matrix binding factors such as MT1-MMP and PAI-1. Since tumor metastasis is initiated by the dissociation of the tumor cell from stromal cells or ECM, we reasoned that ELK3 may regulate cancer cell metastasis by regulating migration or cell–cell adhesion. BC is the most common disease in women worldwide. The basal subtype represents 15–25% of invasive ductal BCs and displays distinct patterns of relapse and a poor prognosis despite relative chemosensitivity. To determine whether ELK3 expression correlates with cancer aggressiveness, we measured the expression level of Elk3 in basal and luminal BC cell lines. First, we assessed ELK3 expression using public data (GES41313) and expression data offered by NCI (Fig. 1A and B). ELK3 was more highly expressed in basal BC cell lines than in luminal lines. We measured ELK3 mRNA and protein levels in the normal epithelial cell line MCF10A and in luminal and basal BC cell lines and confirmed that ELK3 mRNA and protein expression was higher in basal than in normal (MCF10A) and luminal (Fig. 1C and D) cells. HUVECs were used as a positive control for ELK3 expression. ELK3 mRNA and protein expression was most abundant in the MDA-MB-231 and BT20 basal lines, which is consistent with the results obtained from the public data. Since basal BC is usually aggressive and has poor prognosis, the higher expression of Elk3 in basal BC cells implied that ELK3 might mediate the aggressiveness of basal BC.

Effect of ELK3 Knockdown on MDA-MB-231 Migration and Invasion

To elucidate the function of ELK3 in BC, ELK3 expression was knocked down in the MDA-MB-231 line, which

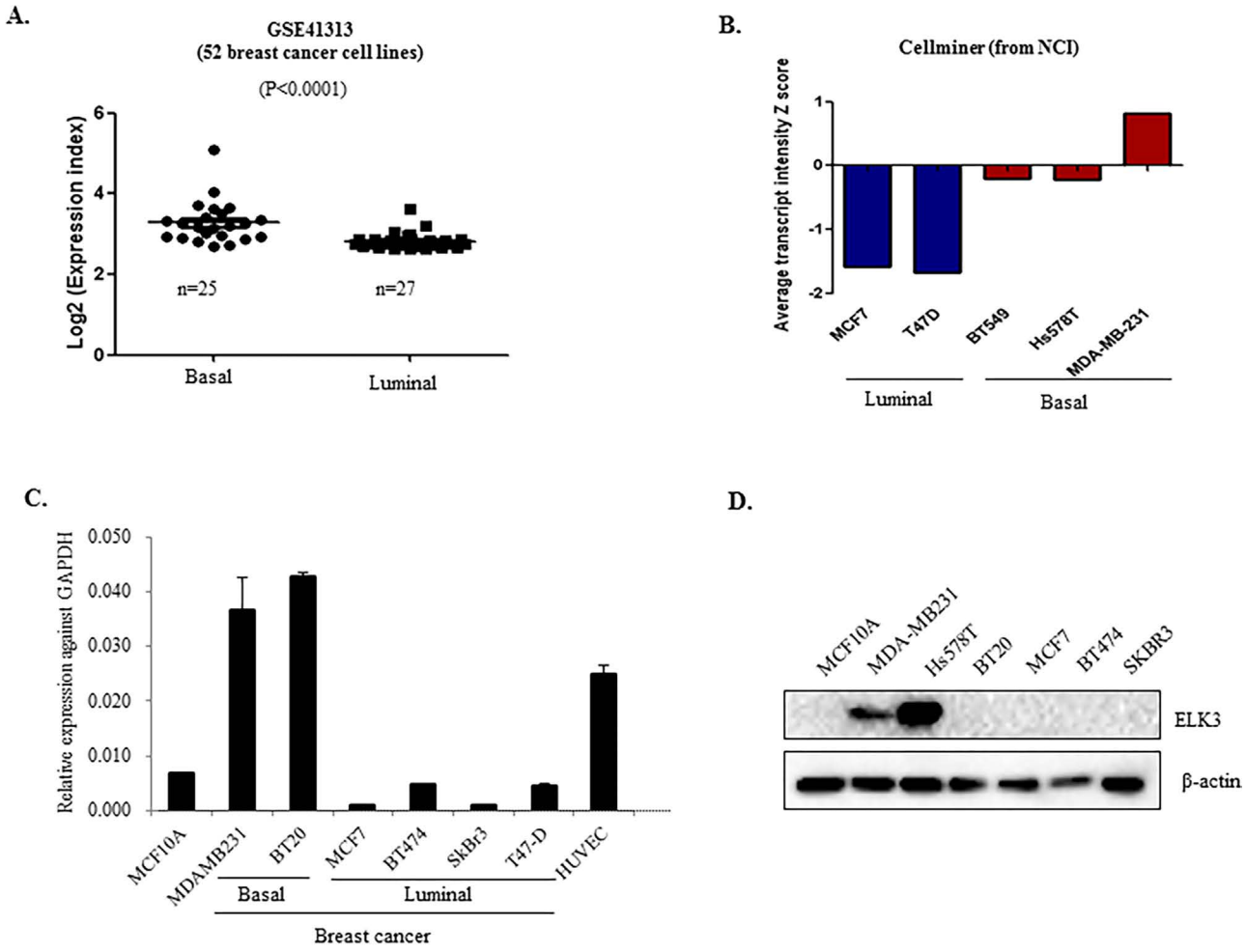


Figure 1. Expression of Elk3 in breast cancer cell lines. ELK3 expression was analyzed in 52 different breast cancer (BC) cell lines using public databases (A) and in five BC cell lines using the NCI database (B). ELK3 mRNA and protein expression was confirmed in the normal breast cell line MCF-10A, the basal cell lines MDA-MB-231, Hs578T, and BT20, and in the luminal cell lines MCF7, BT474, SkBr3, and T47-D. HUVECs were used as a positive control (C and D).

showed a high endogenous expression level of ELK3 (Fig. 1C and D), using ELK3-specific siRNA (siELK3). Decreased ELK3 expression was confirmed by real-time PCR and Western blotting (Fig. 2A). ELK3 expression was efficiently suppressed until 72 h after transfection of siELK3 (Fig. 2B). As shown in Figure 2C, the expression level of ELK3 did not have any effect on cell proliferation. Interestingly, we noticed that ELK3 knockdown reduced cell migration (Fig. 2D) and invasion (Fig. 2E). These results suggest that the expression level of ELK3 positively correlates with oncogenicity, especially migration and invasiveness, in MDA-MB-231.

MT1-MMP as an ELK3 Target Gene in MDA-MB-231

In endothelial cells, ELK3 inhibits cell migration through the suppression of MT1-MMP expression, which implies that ELK3 is a negative regulator of MT1-MMP. Controversially, our results suggest that ELK3 functions as a positive regulator that increases the migration and invasion of BC cells. To resolve this controversy, we investigated whether MT1-MMP was involved in ELK3-mediated cell migration and invasion in MDA-MB-231. Contrary to endothelial cells, MT1-MMP expression was decreased by the suppression of ELK3 expression in MDA-MB-231 cells (Fig. 3A). In support of this result, ELK3 was positively correlated with MT1-MMP expression (0.98 Pearson correlation) in some BC cell lines (Fig. 3B). To confirm that ELK3 is a positive regulator of MT1-MMP expression, we induced the translocation of ELK3 into the nucleus by inhibiting JNK signaling with SP600125 (21). As shown in Figure 3C, ELK3 gradually accumulated in the nucleus following exposure to SP600125, and mRNA expression of MT1-MMP was upregulated by 20 μM SP600125. These results suggest

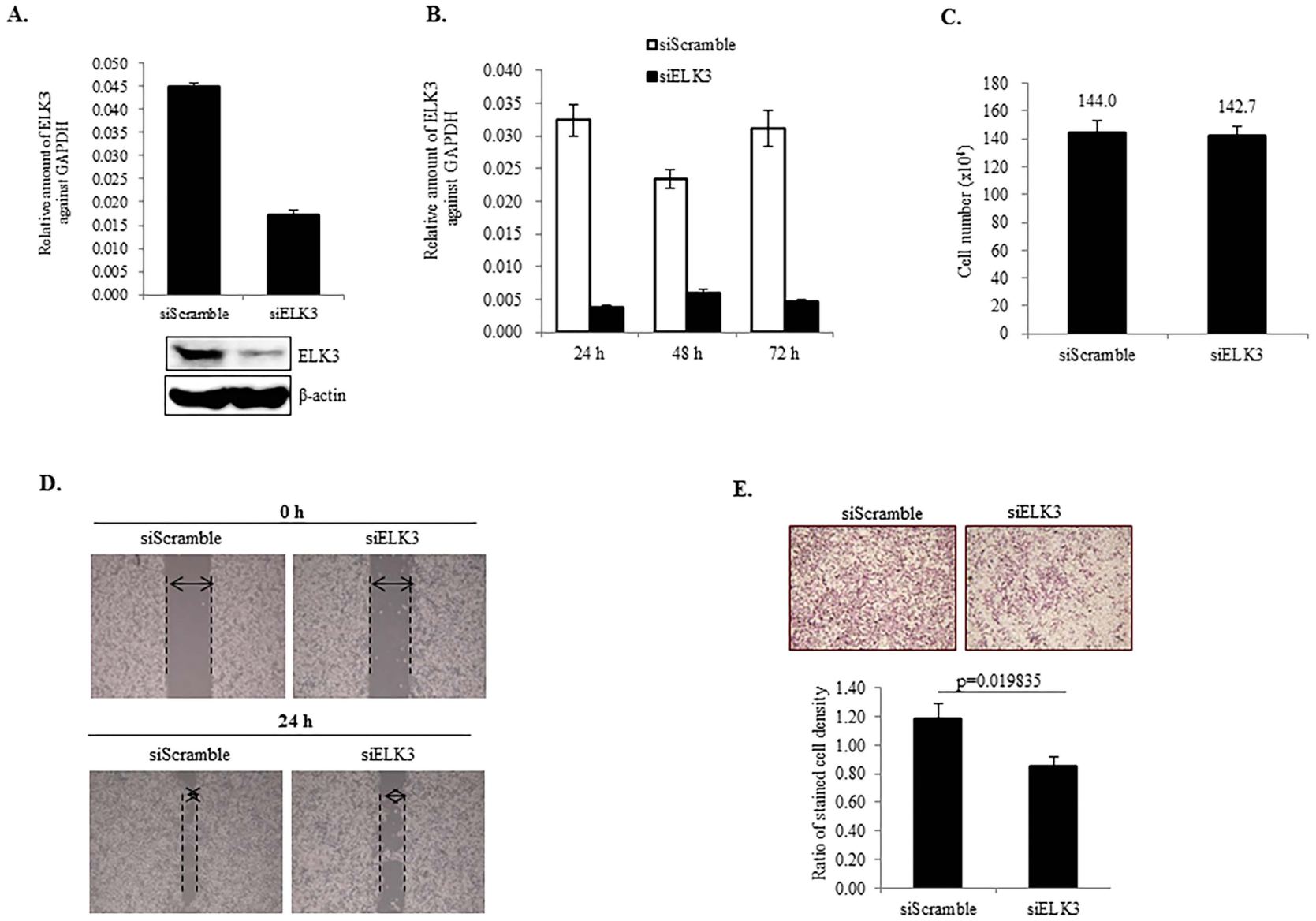
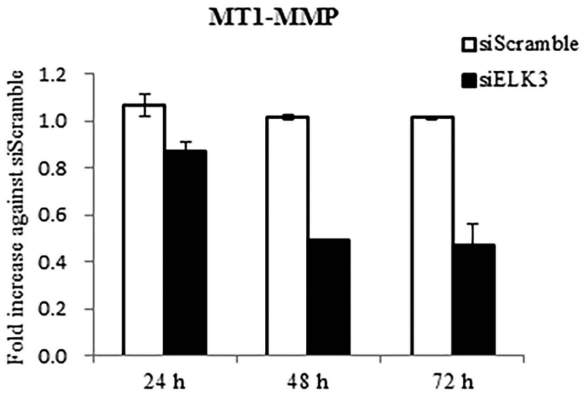


Figure 2. Effect of siRNA-mediated suppression of Elk3 on the cell migration and invasion of MDA-MB-231. ELK3 expression was reduced upon transfection of ELK3-specific siRNA (A). ELK3 expression was suppressed until 72 h after siELK3 transfection (B). ELK3 knockdown did not affect cell proliferation (C). Cell migration and invasion were evaluated by wound healing and Matrigel Transwell assays, respectively. Reduced ELK3 expression decreased cell migration (D) and invasion (E). Crystal violet was used to stain the invasive cells. The staining intensity was higher in cells transfected with the negative control (siScramble) siRNA than in those transfected with siELK3.

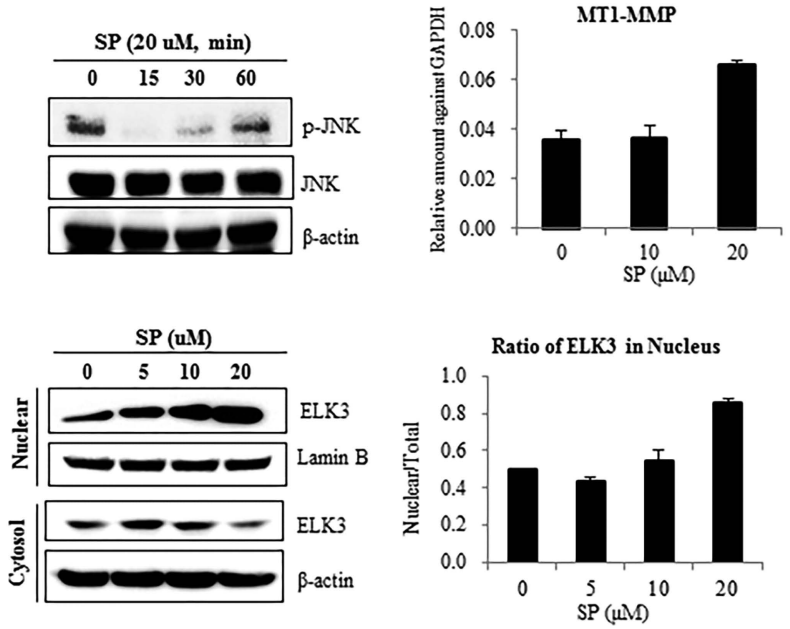
A.



B.

	Transcript	
cell lines	ELK3	MT1-MMP
BR:MCF7	-1.588	-1.171
BR:MDA_MB_231	0.815	1.212
BR:HS578T	-0.216	0.646
BR:BT_549	-0.204	0.712
BR:T47D	-1.679	-1.157
Pearson's correlation	**0.98**	

C.



D.

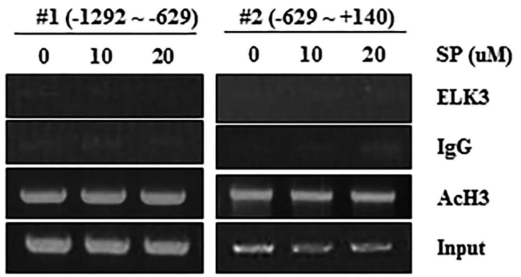


Figure 3. Correlation between Elk3 expression and MT1-MMP expression. MT1-MMP expression was reduced upon ELK3 knockdown (A). In silico analysis, MT1-MMP and ELK3 expression were measured in five breast cancer (BC) cell lines and Pearson correlation analysis was performed (B). Translocation of ELK3 to the nucleus was induced by SP600125 and increased mRNA expression of MT1-MMP (C). The role of ELK3 in MT1-MMP transcriptional regulation was confirmed by chromatin immunoprecipitation assays (D). ELK3 binding to the 1.3-kb MT1-MMP promoter was not detected upon exposure toSP600125.

that the nuclear translocation of ELK3 may function as a transcriptional activator of MT1-MMP gene expression. However, chromatin immunoprecipitation assays with 1.3 kb MT1-MMP promoter region, that has three putative ELK3 binding sites, showed that ELK3 did not bind directly to this region of MT1-MMP promoter (Fig. 3D). These results imply that MT1-MMP is not a direct target of ELK3 and that another factor exists, whose activity is regulated by nuclear ELK3,which regulates MT1-MMP expression in MDA-MB-231. It is interesting to note that ELK3 functions as a positive regulator of MT1-MMP in BC cells, whereas it is a negative regulator of MT1-MMP in endothelial cells. Our results suggest that the MT1-MMP regulatory activity of ELK3 greatly depends on the chromatin context of the cells. Analysis of the effect of ELK3 expression on the metastasis of BC cells in vivo will further elucidate the mechanism by which ELK3 regulates metastasis. Additionally, it will be essential to analyze the expression of ELK3 in BC clinical specimens to determine conclusively whether ELK3 is a suitable therapeutic target in BC metastasis.

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