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The role of ELK3 to regulate peritumoral lymphangiogenesis and VEGF-C production in triple negative breast cancer cells

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ABSTRACT

Tumor-induced lymphangiogenesis, a major conduit for cancer cell dissemination from the primary tumor site to lymph nodes and beyond, eventually leads to metastasis in cancer patients. Given the recent evidence revealing that the suppression of ELK3 inhibits the metastasis of triple-negative breast cancer cells, we aimed to study the underlying mechanism of impaired metastasis in ELK3-suppressed MDA-MB-231 cells (ELK3 KD) with regard to lymphangiogenesis. We found that the secretome of ELK3 KD cells inhibited tube formation, whereas it promoted the migration and invasion of lymphatic endothelial cells (LECs) *in vitro*. *In vivo* analysis revealed that peritumoral lymphatic vessels were not developed around the xenografted tumors of ELK3 KD. We further revealed that the suppression of NF-κB signaling in ELK3 KD was the primary cause of the reduced VEGFC expression. Taken together, we suggest that ELK3 is an upstream regulator of the NF-κB signaling pathway, the inhibition of which leads to the suppression of peritumoral lymphatic vessel development, possibly due to a low VEGFC expression.

1. Introduction

Lymphangiogenesis occurs in many solid tumors, breast cancer in particular, and the metastatic dissemination of tumor cells through the lymphatic system is the primary cause of cancer-related deaths [1]. Indeed, lymph node (LN) metastasis clinically correlates with patient survival [2].

Vascular endothelial growth factor (VEGF)-C, a member of the VEGF growth factor family, is a major lymphangiogenesis factor that regulates the formation and growth of lymphatic vessels [3]. Decreases in the concentration of VEGFC in tumor cells leads to reduced tumor lymphangiogenesis and metastasis [4,5]. Similarly, the blockage of VEGFC/VEGFR3 signaling inhibits lymph node metastasis as well as tumor lymphangiogenesis in animal models [6].

The CXCL12-CXCR4 axis is an additional major promoter of lymphangiogenesis that acts as a chemoattractant for lymphatic endothelial cells (LECs). The binding of CXCL12 to CXCR4 activates a number of downstream signaling cascades in LECs [7]. The expression of CXCL12 and CXCR4 is considered a significant prognostic marker in various types of cancer, including breast cancer, in which high expression of CXCL12 or CXCR4 is associated with poor patient outcome [8–10]. The CXCR4/CXCL12 axis promotes lymphangiogenesis independent of the VEGFC/VEGFR3 pathway [11].

ELK3, an ETS domain-containing protein, is highly expressed in various cancers, including basal-like malignant breast cancer [12,13]. Previously, we reported that ELK3 orchestrates the metastasis of triple-negative breast cancers by epigenetically suppressing the tumor suppressor GATA3 [12]. To extend our understanding of the mechanism by which ELK3 regulates breast cancer metastasis, we further studied the role of ELK3 in lymphangiogenesis. We demonstrated that VEGFC was responsible for the impaired tube formation of LECs and that CXCL12 was associated with the promoted LEC *in vitro*. Consistent with these *in vitro* results, peritumoral lymphatic vessels were not developed around the xenografted tumor of ELK3 KD cells *in vivo*. We further revealed that the suppression of NF-κB signaling in ELK3 KD cells was the primary cause of the reduced VEGFC expression. Taken together, our data reveal that targeting ELK3 may be a novel therapeutic strategy for blocking tumor lymphangiogenesis and metastasis.

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2. Materials and methods

2.1. Cell culture and cell engineering

LECs were purchased from Lonza (Basel, Switzerland) and cultured in endothelial cell growth medium (EGM-2MV, Lonza). MDA-MB-231 cell line was purchased from ATCC and propagated as previously described [14]. A stable ELK3-suppressed MDA-MB-231 cell line (ELK3 KD) was constructed as described [12]. The siRNAs targeting human CXCL12 (catalog no. 1037717) and scrambled siRNA were purchased from Bioneer, Inc. (Daejeon, Korea). Cells were transfected with siRNAs using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.2. Tumor-conditioned media

Tumor-conditioned media was prepared as described previously [15]. The TCM was stored at 4 °C for two weeks or kept at −80 °C for long-term storage.

2.3. Western blot analysis

Western blots were performed as previously described [14]. Nuclear and cytoplasmic fractionation was performed using NE-PER nuclear and cytoplasmic extraction reagent kits (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies against GAPDH (catalog no. sc-166574, Santa Cruz Biotechnology, Dallas, Texas, USA), p105/50 (catalog no. ab7971, Abcam, Cambridge, MA, USA), acetyl-p65 (catalog no. #3045, Cell Signaling Technology), p65 (catalog no. #8242, Cell Signaling Technology), and phospho-p65 (catalog no. #3033, Cell Signaling Technology) were used for blotting. Immunoreactivity was detected by Pierce ECL western blotting substrate (Thermo Fisher Scientific).

2.4. Immunocytochemistry

Immunocytochemistry was performed as described previously [12]. Antibody against p65 (catalog no. #8242, Cell Signaling Technology) was used for analysis.

2.5. RNA extraction and real time RT-PCR

Total RNA extraction and synthesis of cDNA were performed as described previously [12]. Real-time RT-PCR was performed using the resulting cDNAs. The primer sequences used in this study were as follows: GAPDH, (F) 5′-GGGTGTGAACCATGAGAA-3′ and (R) 5′-GTCTTCT GGGTGGCAGTGAT-3’; CXCL12, (F) 5′-TCAGCCTGAGCTA-CAGATGC-3′ and (R) 5′-CTTTAGCTTCGGGTCAATGC-3’; and VEGFC, (F) 5′-ACCAAACAAGGA GCTGGATG-3′ and (R) 5′-ATTTCTGGGG-CAGGTTCTTT-3’.

2.6. Cell proliferation assay

LEC proliferation was determined using an EZ-Cytox WST-based Cell Viability/Cytotoxicity Assay kit (DAEILLAB SERVICE Co, Ltd, Seoul, Korea) in accordance with the manufacturer's instructions. Briefly, 2×10^3 cells were cultured in 40% TCM overnight. Cell proliferation was assessed at 24, 48 and 72 h following the addition of WST-1 solution. After 3 h of incubation, the optical density (OD) of each well was measured at 450 nm using a microplate reader. All assays were performed in triplicate and repeated three times.

2.7. Migration and invasion assays

Migration and invasion assay were performed described previously. TCM, SFM or EGM2 was added to the lower chamber as a chemoattractant. For the invasion assay, matrigel (Corning) was diluted in EGM-2 (Lonza) at a ratio of 1:7.

2.8. Tube-formation assay

Briefly, 50 μl of Matrigel (growth factor-reduced, Corning) was loaded into each well of a 96-well plate, and the plate was incubated at 37 °C for 30 min. Then, 1.5×10^4 LECs suspended in 100 μl of TCM were added on top of the matrix in the 96-well plate. LECs suspended in EGM-2 (Lonza) or serum-free medium (DMEM, Gibco) were used as controls. The plate was incubated at 37 °C, and the wells were imaged at the indicated time points.

2.9. Xenograft mouse model and immunohistochemistry

Xenograft mouse models were generated as previously described [12]. Tumor tissues were harvested and stabilized in 4% paraformaldehyde to create a paraffin block. Sections of paraffin-embedded tissues were stained with anti-LYVE-1 antibody (Angiobio).

2.10. Matrigel plug assay and immunostaining

ELK3 KD or control cells (3×10^4 cells) were mixed with 400 μl of cold GFR-Matrigel (Corning) and injected subcutaneously into six-week-old BALB/C nu/nu mice. After 10 days, the Matrigel plugs were harvested, fixed in 4% paraformaldehyde and embedded in paraffin. Sections of the paraffin-embedded Matrigel plug were stained with anti-LYVE-1 antibody (catalog no. 11−034, Angiobio, San Diego, CA, USA) and anti-CD31 antibody (catalog no. 553370, BD Biosciences, San Jose, CA, USA).

2.11. Statistical analysis

All statistical analyses were performed as previously described [12].

3. Results

3.1. Suppression of ELK3 affects the lymphangiogenic activity of the secretome of MDA-MB-231 cells in vitro and in vivo

Lymphangiogenesis is a complex process that allows LECs to migrate, invade, proliferate and sprout to generate new lymphatic vessels during tumor metastasis. To investigate the role of ELK3 KD conditioned medium (KD-CM) on tumor metastasis, we analyzed the effect of ELK3 KD-CM on the characteristics of LECs, such as migration, invasion, proliferation and tube formation. We noted that KD-CM significantly enhanced LEC migration compared with the conditioned medium of control MDA-MB-231 cells (C-CM) (Fig. 1A). We further analyzed the effect of KD-CM on LEC invasion using a transwell assay. LECs cultured in KD-CM exhibited enhanced invasion activity compared with LECs in C-CM (Fig. 1B). We also noted that KD-CM promoted LEC proliferation (Fig. 1C). In contrast to the positive effect of KD-CM on LEC migration, invasion and proliferation, KD-CM limited LEC tube formation, which was estimated by observing the tube-like structures and the number of branching points on the Matrigel (Fig. 1D). We next performed a Matrigel plug assay to explore the effect of ELK3 suppression on the lymphangiogenic ability of cancer cells *in vivo* to determine why the lymphangiogenic potential of the secretome of ELK3 KD was

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