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Tumor necrosis factor superfamily 15 promotes lymphatic metastasis via upregulation of vascular endothelial growth factor-C in a mouse model of lung cancer

Tingting Qin[1,2,3,4] | Dingzhi Huang[1,2,3,4] | Zhujun Liu[1,2,3,4] | Xiaoling Zhang[1,2,3,4] | Yanan Jia[1,2,3,4] | Cory J. Xian[5] | Kai Li[1,2,3,4]

[1]National Clinical Research Center for Cancer, Tianjin Medical University Cancer Institute and Hospital, Tianjin, China

[2]Key Laboratory of Cancer Prevention and Therapy, Tianjin, China

[3]Tianjin's Clinical Research Center for Cancer, Tianjin, China

[4]Department of Thoracic Oncology, Tianjin Lung Cancer Center, Tianjin Cancer Institute and Hospital, Tianjin Medical University, Tianjin, China

[5]Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, South Australia, Australia

Correspondence: Cory J. Xian, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA, Australia (cory.xian@unisa.edu.au).
and
Kai Li, Department of Thoracic Oncology, Tianjin Cancer Institute and Hospital, Tianjin Medical University, Tianjin, China (likai5@medmail.com.cn).

Lymphatic metastasis is facilitated by lymphangiogenic growth factor vascular endothelial growth factor-C (VEGFC) that is secreted by some primary tumors. We previously identified tumor necrosis factor superfamily 15 (TNFSF15), a blood vascular endothelium-derived cytokine, in lymphatic endothelial cells, as a key molecular modulator during lymphangiogenesis. However, the effect of TNFSF15 on tumor lymphatic metastasis and the underlying molecular mechanisms remain unclear. We report here that TNFSF15, which is known to inhibit primary tumor growth by suppressing angiogenesis, can promote lymphatic metastasis through facilitating lymphangiogenesis in tumors. Mice bearing tumors induced by A549 cells stably overexpressing TNFSF15 exhibited a significant increase in densities of lymphatic vessels and a marked enhancement of A549 tumor cells in newly formed lymphatic vessels in the primary tumors as well as in lymph nodes. Treatment of A549 cells with TNFSF15 results in upregulation of VEGFC expression, which can be inhibited by siRNA gene silencing of death domain-containing receptor-3 (DR3), a cell surface receptor for TNFSF15. In addition, TNFSF15/DR3 signaling pathways in A549 cells include activation of NF-κB during tumor lymphangiogenesis. Our data indicate that TNFSF15, a cytokine mainly produced by blood endothelial cells, facilitates tumor lymphangiogenesis by upregulating VEGFC expression in A549 cells, contributing to lymphatic metastasis in tumor-bearing mice. This finding also suggests that TNFSF15 may have potential as an indicator for prognosis evaluation.

KEYWORDS
lung cancer, lymphangiogenesis, lymphatic metastasis, TNFSF15, VEGFC

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1 | INTRODUCTION

Metastasis is the major cause of death in cancer patients, including lung cancer,[1,2] and the spread of tumor cells to lymph nodes has been the main negative prognostic factor for lung cancer.[3,4] Lymphatic metastasis is considered a major route for cancer cell dissemination,[4,5] and the spreading of tumor cells to lymph nodes is a common lymphatic-mediated metastasis pattern for carcinomas, draining either through preexisting lymphatic vessels or via the newly formed lymphatic capillaries.[6,7] However, the pathobiology for lymphatic metastasis has remained unclear. Recent studies have shown that some tumors secrete lymphangiogenic growth factors, including vascular endothelial growth factor-C (VEGFC) and vascular endothelial growth factor-D (VEGFD), which act on the lymphatic vasculature to facilitate metastasis.[8-11] In addition, other molecules/substances, such as sine oculis homeobox homolog 1 (SIX1)[9] in breast cancer, lipopolysaccharide (LPS)[10] in colorectal cancer and high-mobility group box 1 protein (HMGB1)[12] in colon cancer, can all promote lymphatic metastasis by increasing VEGFC expression. However, although VEGFC and VEGFD have been identified as the main signal factors for driving lymphangiogenesis, the mechanism by which VEGFC or VEGFD is regulated in lung cancer is not clear. Further elucidation of the pathogenesis of lymphatic metastasis in lung cancer would represent a critical step forward for improving therapeutic strategies.

The tumor necrosis factor superfamily member TNFSF15 (also known as TL1A or VEGI), a cytokine produced predominantly by blood endothelial cells (BEC) in established blood vessels, is a specific inhibitor of BEC proliferation, being able to arrest growth in quiescent BEC but also to induce apoptosis in proliferating BEC.[13] TNFSF15 inhibits angiogenesis by down-modulating VEGF receptor-1 activities in endothelia progenitor cells (EPC).[14] It also inhibits differentiation of Lin^--Sca-1^+ EPC into BEC and incorporation of EPC into tumor vasculature in murine models.[15] TNFSF15 expression is high in the early stages, while is absent or marginal in the late stages in various cancers.[16-18] Indicating its importance in angiogenesis and cancer progression, systemic administration of TNFSF15 led to inhibition of tumor angiogenesis and growth.[19] Furthermore, using an embryonic lymphangiogenesis model, we recently demonstrated that TNFSF15 facilitated lymphangiogenesis through upregulation of VEGFR3 in lymphatic endothelial cells (LEC) in physiological conditions.[20] These studies suggested that TNFSF15 may participate in tumor metastasis in the early stages by prompting tumor lymphangiogenesis. However, whether TNFSF15 is involved in tumor lymphangiogenesis and tumor metastasis in lung cancer remains to be determined.

Therefore, in this study, we investigated the role of TNFSF15 in tumor metastasis, particularly in lymphangiogenesis and lymphatic metastasis, and attempted to explain the molecular mechanism of TNFSF15 in regulating VEGFC expression in lung cancer. We report here that TNFSF15 facilitates lymphatic metastasis in a mouse model of lung cancer by upregulating VEGFC expression in A549 human lung cancer cells and that this activity is mediated by death receptor 3 (DR3, also known as tumor necrosis factor receptor superfamily member 25 or TNFRSF25) and nuclear factor (NF)-κB signaling. Our findings reveal a causal role for TNFSF15-mediated lymphangiogenesis in tumors, which promotes lymphatic metastasis. We show that it promotes lymphatic metastasis of lung cancer through upregulation of lymphangiogenic growth factor VEGFC in cancer cells in a mouse model of lung cancer.

2 | MATERIALS AND METHODS

2.1 | Animals and the subcutaneous lung cancer model

Female nude mice (BALB/c-nu, 6 weeks) were purchased from the Model Animal Center of Nanjing University and housed in a pathogen-free animal facility with *ad libitum* access to water and food. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee of Tianjin Medical University (Tianjin, China). To induce a subcutaneous lung cancer model, nude mice were randomized into 2 groups (n = 5), and $A549^{Control/EGFP}$ or $A549^{TNFSF15/EGFP}$ lung cancer cells (see below) were inoculated (5×10^6 cells per mouse) in the subcutaneous space near the axillary lymph node of nude mice, respectively. The animals were observed and weighted every other day for 72 days. Tumor sizes were measured with a dial caliper in a blinded manner. Tumor volumes were determined using the equation: volume = width × width × length × 0.52. Metastases were imaged with an IVIS Spectrum Imaging System (Caliper Life Science, Hopkinton, MA, USA), by which $A549^{Control/EGFP}$ and $A549^{TNFSF15/EGFP}$ tumors could be clearly visualized. Although autofluorescent signals were given by naïve animals, the high intensity of fluorescent signal from $A549^{Control/EGFP}$ and $A549^{TNFSF15/EGFP}$ tumors was distinguishable and can be normalized with background signal. The mice were killed at the end of the experiment. The primary tumors and inguinal lymph nodes were retrieved for pathologic analyses as described.[21]

2.2 | Cell culture and transfection

Human lung cancer cell line A549 was purchased from the American Type Culture Collection (Manassas, VA, USA). $A549^{Control/EGFP}$ and $A549^{TNFSF15/EGFP}$ cells were generated in our laboratory from A549 cells (see Supplementary Figure S1). In brief, the EGFP-expressing lentivector (Control/EGFP) and lentivector-encoding TNFSF15 (TNFSF15/EGFP) were purchased from Genechem (Shanghai, China). Prior to transfection, A549 cells were seeded in 6-well plates (1×10^5 cells per well), incubated overnight, then transduced with lentiviral supernatants containing different lentiviral vectors, and 5 μg/mL polybrene in a humidified incubator at 37°C with 5% CO_2 for 16 hours. Culture media were then removed and replaced with fresh RPMI1640 medium (Gibco, Waltham, MA, USA) containing 10% FBS (Gibco), and cells were incubated for 72 hours. In addition, primary LEC were isolated from the hyperplastic lymphatic vessels

induced by Freund's incomplete adjuvant as described previously.[22] LEC were cultured in ECM (ScienCell Laboratories, Carlsbad, CA, USA) supplemented with endothelial cell growth supplement (Scien-Cell Laboratories), 10% FBS, 100 μg/mL heparin, and 100 μg/mL streptomycin and penicillin.

2.3 | Reagents

Antibodies for CD31, VEGFA, VEGFR3, podoplanin, Ki67 (ab92742, an anti-human Ki67 antibody that does not cross-react with the mouse Ki67), NF-κB subunit p65 and VEGFC were purchased from Abcam (Cambridge, London, UK). HRP-conjugated secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 555-conjugated secondary antibody was from Invitrogen (Carlsbad, CA, USA). Pyrrolidine dithiocarbamate (PDTC, an effective NF-κB inhibitor) was purchased from Sigma-Aldrich (St Louis, MO, USA). A human VEGFC ELISA Kit was purchased from R&D Systems (Minneapolis, MN, USA). TNFSF15 protein was kindly given by Professor Lu-yuan Li (Nankai University, Tianjin, China), which was prepared as previously described.[19]

2.4 | Immunohistochemistry and immunofluorescence assay

Five-micrometer sections of formalin-fixed, paraffin-embedded tumors and lymph nodes were deparaffinized and rehydrated and then rinsed with PBS. For antigen retrieval, sections were microwaved in citric acid solution (PH 6.0) for 15 minutes. Then, these slides were incubated in 3% H_2O_2 for 15 minutes. Sections were incubated with antibodies against CD31, VEGFA, podoplanin, VEGFC or Ki67 (ab92742, see Subsection 2.3) and then with appropriate secondary antibodies at room temperature (RT) for 30 minutes. Three clinical pathologists assessed the intensity of the immunostaining on each section independently and in a blinded manner, using a semiquantitative scoring system (0, 1, 2 and 3 for negative, weak, strong and very strong, respectively). At least 10 fields per specimen were surveyed. For immunofluorescent staining, fluorescein-labeled antibody, including Alexafluor-555 (red) anti-human VEGFC and DAPI from BioLegend (San Diego, CA, USA), was used. The slides were incubated at RT for 1 hour in darkness. Immunostained sections were imaged with a positive fluorescence microscope (Carl Zeiss, Oberkochen, Germany). A minimum of 10 fields per section was analyzed.

2.5 | Western blotting analyses

Total protein was extracted from homogenized cells in RIPA buffer and subjected to 10% SDS-PAGE. The proteins were transferred onto polyvinylidenedifluoride membranes (Roche Molecular Biochemicals, Quebec, Canada), blocked with 5% nonfat skimmed milk, and then immunoblotted overnight at 4°C with appropriate primary antibodies. The blots were further incubated with HRP-conjugated secondary antibodies and developed with the ECL System (Millipore, Billerica, MA, USA).

2.6 | Quantitative RT-PCR gene expression analyses

Samples were collected and homogenized in TRIzol (Invitrogen). RNA extraction and reverse transcription were performed following standard procedures. For PCR amplification of the cDNA fragment coding for targeted genes, the sense and antisense primer sequences for human VEGFC,[23] TNFSF15, p65[24] and GAPDH were as follows, respectively: 5′-AGT GTC AGG CAG CGA ACA AGA-3′ and 5′-CTT CCT GAG CCA GGC ATC TG-3′; 5′-TAG AGC CGA CGG AGA TAA GCC-3′ and 5′-CCC ACG GAA TGT GAC CTG-3′; 5′-CCC CAC GAG CTT G-3′ and 5′-CCA GGT TCT GGA AAC TGT GGA T-3′; 5′- TGA CTT CAA CAG CGA CAC CCA -3′ and 5′-CAC CCT GTT GCT GTA GCC AAA-3′. The mRNA relative expression levels were calculated after being normalized to that of internal control GAPDH.

2.7 | Vascular endothelial growth factor-C ELISA

Samples of conditioned media from cells with (and without) TNFSF15 treatment were collected. Levels of VEGFC were determined using the human VEGFC ELISA Kit (R&D Systems). The absorbance was measured with an Epoch microplate reader (BioTek, Winooski, VT, USA).

2.8 | RNA interference

The siRNA for DR3 and NF-κB subunit p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Scrambled siRNA was purchased from GenePharma (Shanghai, China). The sense and antisense sequences of scramble siRNA are as follows: 5′-UUC UCC GAA CGU GUC ACG UTT -3′ and 5′-ACG UGA CAC GUU CGG AGA ATT-3′. Cells were transfected with siRNA using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol, and protein levels of the target gene products were determined by western blot analyses of extracts made 72 hours later.

2.9 | Cell proliferation and migration assays

Lymphatic endothelial cells were activated with A549 cell-conditioned media (CM) (shControl and shVEGFC). To prepare CMs, A549 stable cell lines, transfected with lentivirus containing shControl or shVEGFC, treated with (or without) TNFSF15, were cultured for 72 hours to produce shControl and Vehicle CM, shControl and TNFSF15 CM, shVEGFC and Vehicle CM, and shVEGFC and TNFSF15 CM. To determine the effects of CM treatment on LEC proliferation, LEC cells were seeded (1×10^4 cells per well). Then, shControl and Vehicle CM, shControl and TNFSF15 CM, shVEGFC and Vehicle CM, and shVEGFC and TNFSF15 CM were added at 12 hours after seeding. Following 72-hours incubation, cells were trypsinized and counted. For migration assay, 8-μm-pore transwells were used (Corning, Corning, NY); 5×10^4 LEC were seeded in the transwell inserts pre-equilibrated with media; shControl and Vehicle CM, shControl and TNFSF15 CM, shVEGFC and Vehicle CM, and

shVEGFC and TNFSF15 CM were added to the media in the lower chambers. After 24-hour incubation, the inserts were rinsed, fixed in 2% paraformaldehyde for 10 minutes, and then stained with crystal violet (Beyotime, Haimen, Jiangsu, China). The numbers of cells that migrated per field were counted on 6 randomly chosen fields per well acquired at 200× magnification (ECLIPSE Ti, Nikon, Tokyo, Japan).

2.10 | Statistical analysis

The data were subjected to variance analysis (ANOVA), followed by 2-tailed, unpaired Student's t test. Differences with P-values $<.05$ were considered statistically significant.

3 | RESULTS

3.1 | Tumor necrosis factor superfamily 15 inhibits primary tumor growth but promotes tumor metastasis

To evaluate the influence of TNFSF15 in lymphatic metastasis in vivo, human A549[TNFSF15/EGFP] cell line, which stably expresses TNFSF15 or control cell line A549[Control/EGFP] (without TNFSF15 transfection) was implanted on the flanks of nude mice to establish a mouse subcutaneous model of human cancer. A substantial inhibition in growth of primary tumors was observed in the A549[TNFSF15/EGFP] group (Figure 1A) (n = 5). The median tumor volume in the A549[Control/EGFP] group was more than 2 times that of the A549[TNFSF15/EGFP] group (Figure 1B). Numerous metastases were detected by stereomicroscopic imaging, which exhibited an increase from approximately 3 on average per A549[Control/EGFP] mouse to 9 on average per A549[TNFSF15/EGFP] mouse (Figure 1C,D). Lower survival rate was detected (see Supplementary Figure S2). Furthermore, we calculated the tumor sizes every other day for 72 days. The A549[TNFSF15/EGFP] group showed a remarkable inhibition in primary tumor growth (Figure 1E). These findings indicate that TNFSF15 plays opposite roles in primary tumor growth and tumor metastasis; namely, TNFSF15 can promote tumor metastasis but inhibit tumor growth.

3.2 | Tumor necrosis factor superfamily 15 upregulates vascular endothelial growth factor-C while downregulates vascular endothelial growth factor-A in primary tumors

To determine the effect of TNFSF15 on blood and lymphatic vessel densities, CD31 and podoplanin expression was examined. We carried out immunohistochemical staining of the CD31, podoplanin, VEGFA and VEGFC proteins in primary tumors of these 2 groups, and found that the expression patterns of CD31 and VEGFA, which are related to angiogenesis, are strikingly different to patterns of
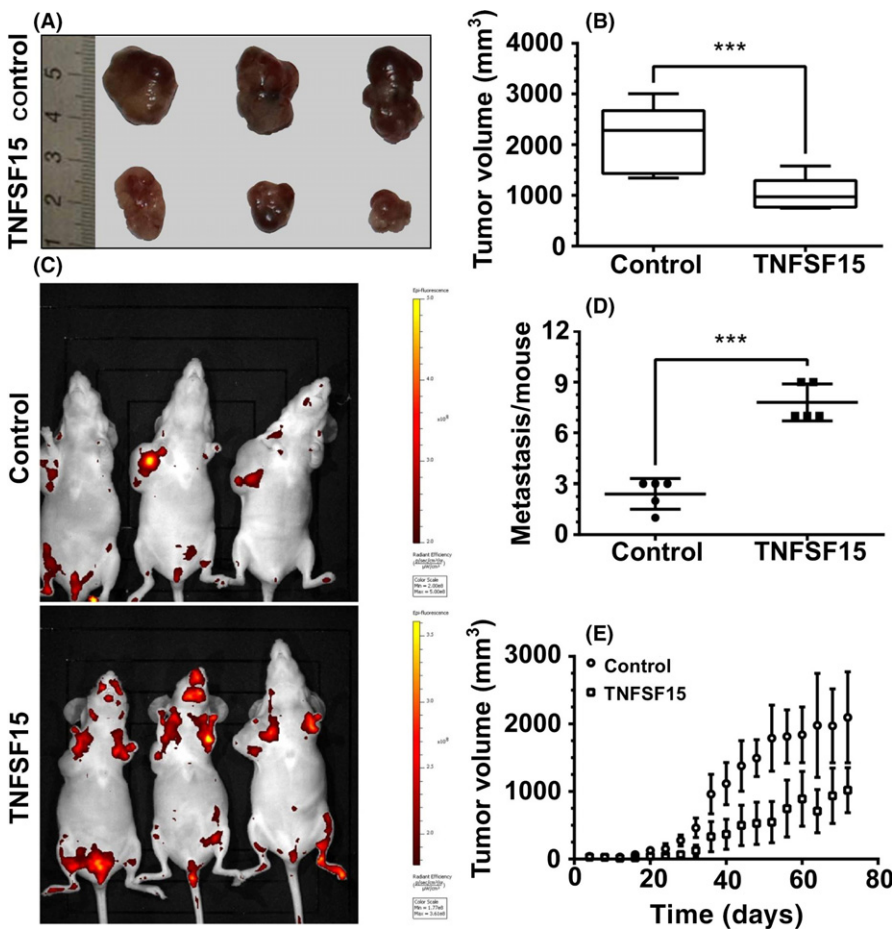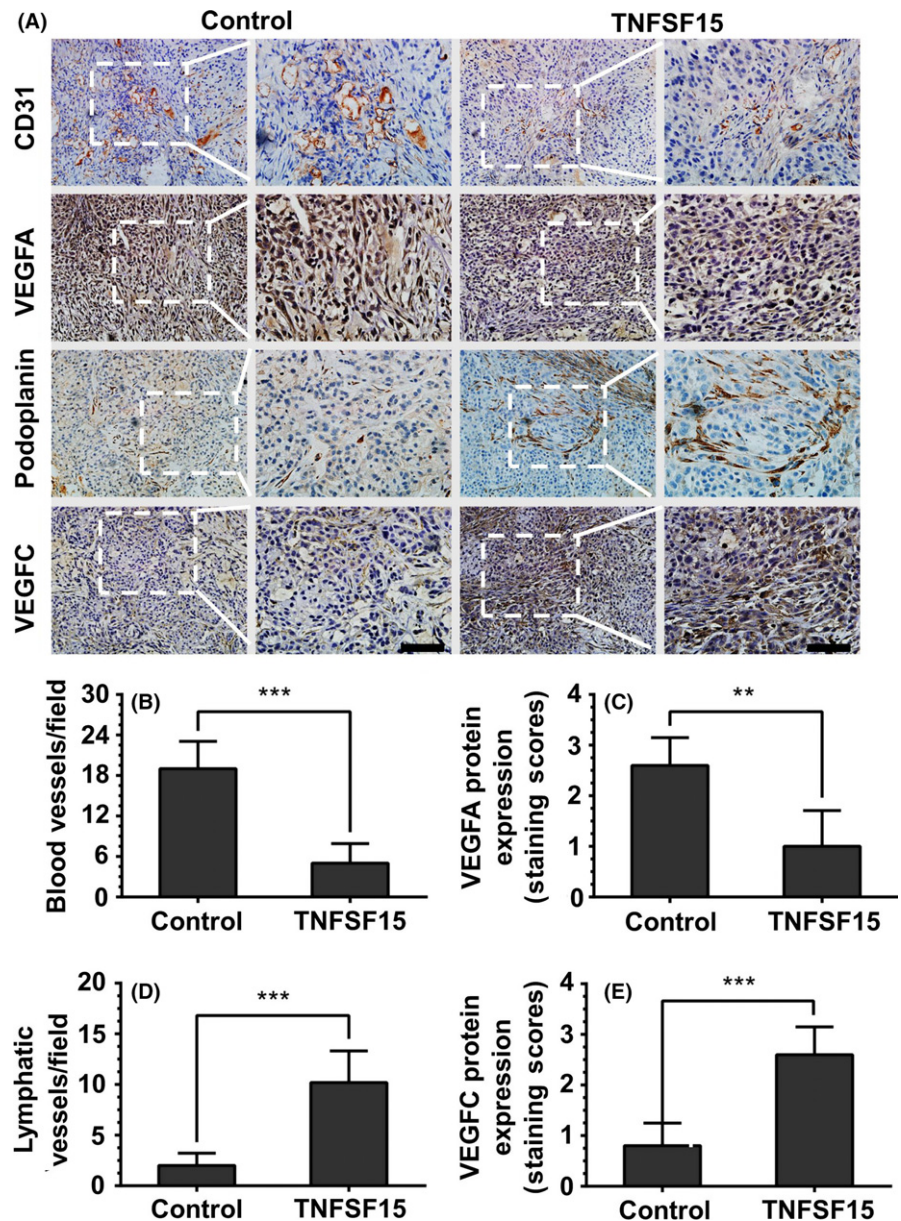

FIGURE 1 Tumor necrosis factor superfamily 15 (TNFSF15) enhances tumor metastasis while it inhibits primary tumor development. A, Typical images of the primary tumors that formed in mice grafted with A549[Control/EGFP] or A549[TNFSF15/EGFP] tumor cells on the right shoulder. Ruler unit: cm. B, Box plots of volumes of primary tumors (n = 5 per group). C, Representative images of mice with tumor cells grafted to promote spontaneous metastasis, which can be monitored using the IVIS spectrum imaging system. Color bar represents radiant efficiency ([p/s/cm^2/sr]/[μW/cm^2]). D, A scatter plot analysis of metastases showing a more than 2.5 times incensement in the A549[TNFSF15/EGFP] when compared to the A549[Control/EGFP] control group. E, The effect of TNFSF15 on tumor growth. These experiments were repeated 2 times. Data are mean ± SD. ***$P < .001$; Student's t test

podoplanin and VEGFC. We found that CD31 and VEGFA proteins were downregulated markedly in the TNFSF15 group when compared to the control group, and that, in sharp contrast, the TNFSF15 group showed higher podoplanin and VEGFC levels (Figure 2A). Blood vessel densities (CD31$^+$) on cross-sections of the primary tumors in the control group were more than 3 times those of the TNFSF15 group (Figure 2B). We determined VEGFA and VEGFC protein levels using a 4-step grading system (0, 1, 2 and 3 for negative, weak, strong and very strong staining, respectively). While VEGFA expression was high or very high in the control group, it was negative or low in the TNFSF15 group (Figure 2C). We obtained the opposite results for VEGFC expression (Figure 2D). Lymphatic vessel densities (podoplanin$^+$) were significantly higher in the TNFSF15 group than those in the control group, with the median values being 3 and 9 per field, respectively (Figure 2E). These findings indicate that TNFSF15 plays inverse roles in tumor angiogenesis and tumor lymphangiogenesis.

3.3 | Tumor necrosis factor superfamily 15 promotes lymphatic metastasis

Because tumor lymphangiogenesis provides an important route for lymphatic metastasis, we next determined whether the upregulation of VEGFR3 podoplanin and VEGFC under TNFSF15 can promote tumor lymph node metastasis. Autopsy analysis showed that most of the tumor-bearing mice in the TNFSF15 group developed metastatic lesions in inguinal lymph nodes, which were also much larger in volumes when compared to the control group (Figure 3A,C). Ki67, which is a proliferation marker, is used to present metastasis of human tumor cells as the anti-human Ki67 (ab92742) antibody does not cross-react with mouse Ki67.[21] Ki67 staining of lymph nodes confirmed the metastatic lesions (Figure 3B), and analysis of primary tumor metastasis to inguinal lymph nodes (which was quantified by the number of human Ki67-positive cells per unit area) showed more than 3 times in the TNFSF15 group than that in control group

FIGURE 2 Role of tumor necrosis factor superfamily 15 (TNFSF15) in modulating podoplanin, vascular endothelial growth factor-C (VEGFC), CD31 and VEGFA expression. A, Typical images (brown) of VEGFA and CD31 immunostaining of blood vessels, and representative images of VEGFC and podoplanin immunostaining of lymphatic vessels in primary tumors in mice grafted with A549$^{Control/EGFP}$ or A549$^{TNFSF15/EGFP}$ tumor cells. Magnification, ×200, scale bar: 50 μm, pictures in lines 1 and 3; magnification, ×400, scale bar: 50 μm, pictures in lines 2 and 4. B, Average blood vessel density as quantified by assessing the numbers of CD31-positively stained vessels in primary tumors of mice (n = 5 per group). C, VEGFA expression as quantified using a semiquantitative scoring system as described in the Materials and Methods. D, Average lymphatic vessel density as quantified by assessing the numbers of podoplanin-positively stained vessels in primary tumors of mice (n = 5 per group). E, VEGFA expression as quantified using a semiquantitative scoring system as described. Data are mean ± SD (n = 5). **P < .01, ***<.001 Student's t test. These experiments were performed 2 times

(A)

Inguinal lymph node

Control

TNFSF15

(B)

Control   TNFSF15
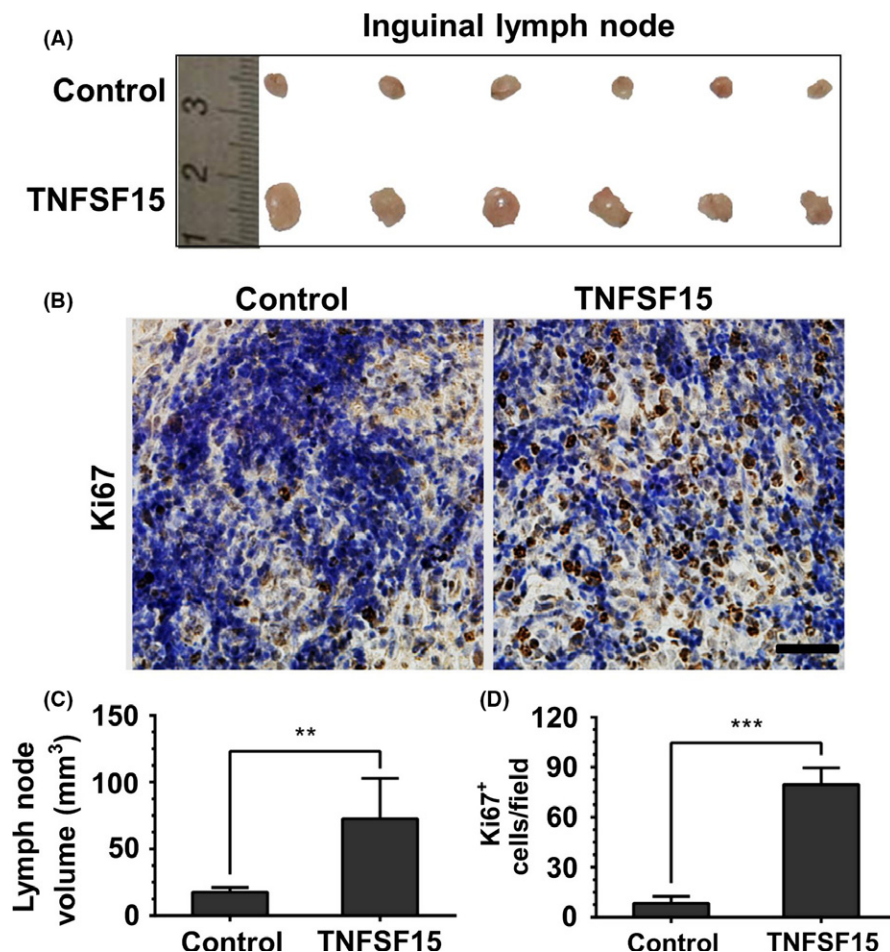
Ki67

(C)

(D)

FIGURE 3 Relationship between tumor necrosis factor superfamily 15 (TNFSF15) and volumes of inguinal lymph nodes and Ki67 expression. A, Images of inguinal lymph nodes dissected from different groups of mice. Ruler unit: cm. B, Typical images of A549 cells positively stained (brown) for human Ki67. Magnification, ×400, scale bar: 50 μm. C, Average volumes of inguinal lymph nodes. D, Number of Ki67-positive A549 cells. Data are mean ± SD (n = 5 per group). **$P < .01$, ***$P < .001$, Student's t test. These experiments were performed 2 times

(Figure 3D). Collectively, these results provide compelling evidence that TNFSF15 is a positive regulator of lymphatic metastasis.

3.4 | Tumor necrosis factor superfamily 15 upregulates vascular endothelial growth factor-C expression in A549 lung cancer cells

We treated A549 cells with recombinant TNFSF15 (200 ng/mL, a concentration which was used previously to treat lymphatic endothelial cells)[20] and found that the treatment resulted in a considerable increase in VEGFC protein levels as determined by immunofluorescent staining (Figure 4A). Fluorescence intensity assessment revealed that VEGFC protein levels in TNFSF15-treated group were 4 times of that in vehicle-treated group (Figure 4B). In addition, we treated A549 cells with recombinant TNFSF15 (200 ng/mL) for 24 hours and found that the treatment resulted in a considerable increase in VEGFC protein levels as determined by western blotting analyses (Figure 4C). We also found that DR3, the receptor that mediates TNFSF15 activities in ECs, was expressed in a high but constant level in A549 cells under the experimental conditions (Figure 4C). The increase in VEGFC level became apparent within 12 hours of TNFSF15 (200 ng/mL) treatment, reaching a level more than 2 times than that in vehicle-treated cells within 24 hours (Figure 4D). Real-time PCR analyses revealed that TNFSF15 (200 ng/mL) treatment gave rise to a more

than 2-fold increase in VEGFC mRNA within 3 hours (Figure 4E). Furthermore, we determined VEGFC concentration in the medium by ELISA and found that VEGFC levels under the TNFSF15 treatment were more than 4 times those in the vehicle-treated group (Figure 4F). These data indicate that TNFSF15, which is predominantly expressed by vascular endothelial cells, is able to substantially stimulate VEGFC gene expression in A549 lung cancer cells.

3.5 | DR3/NF-κB signaling pathway is involved in tumor necrosis factor superfamily 15-stimulated vascular endothelial growth factor-C production in A549 cells

To determine whether DR3 mediates upregulation of VEGFC in A549 cells, we treated A549 cells with scrambled or DR3 siRNA (40 pmol/mL, respectively) to silence the gene of DR3 and then treated with (or without) TNFSF15 (200 ng/mL). The DR3 siRNA treatment resulted in a >90% decline in DR3 protein levels, rendering a diminished ability of TNFSF15 to induce VEGFC protein expression (Figure 5A). Secretion of VEGFC is partly promoted by the NF-κB signaling pathway.[25] To find out whether this signaling pathway is involved in TNFSF15-stimulated VEGFC secretion by A549 cells, we treated the cells with PDTC (0.25 μmol/L), a known NF-κB inhibitor. PDTC treatment led to an apparent reduction in TNFSF15-induced
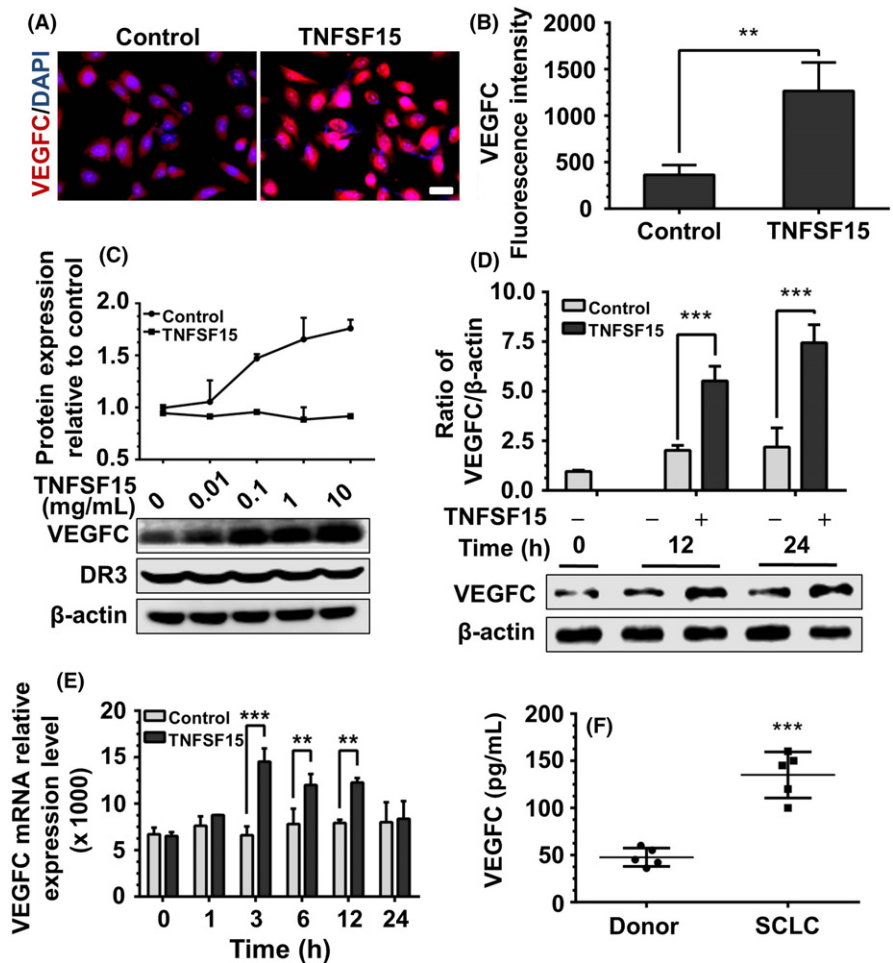
FIGURE 4 Tumor necrosis factor superfamily 15 (TNFSF15) upregulates vascular endothelial growth factor-C (VEGFC) expression in A549 tumor cells. A, Fluorescence microscopy images of A549 cells treated with vehicle or TNFSF15 for 24 h. Red, VEGFC; blue, DAPI; magnification, ×400, scale bar: 20 μm. B, Analyses of fluorescence intensities of VEGFC⁺ cells. C, Western blot analyses showing VEGFC and DR3 protein levels following TNFSF15 treatment at various concentrations. Values are normalized to β-actin. D, Western blot analyses showing VEGFC levels in vehicle-treated and TNFSF15-treated A549 cells at the indicated time points. E, VEGFC mRNA levels in treated A549 cells, as determined by RT-PCR. F, VEGFC concentrations in the culture medium of A549 cells untreated or treated with TNFSF15, as determined by ELISA. Data are mean ± SD. **$P < .01$; ***$P < .001$, Student's t test. These experiments were performed 3 times

VEGFC upregulation at both mRNA and protein levels (Figure 5B,C). Furthermore, prior treatment of A549 cells with siRNA against NF-κB subunit p65 (40 pmol/mL), which effectively silenced p65 gene (Figure 5D), abolished the ability TNFSF15 to stimulate VEGFC expression (Figure 5E,F,G,H). These findings indicate that NF-κB signaling is responsible for mediating TNFSF15-mediated upregulation of VEGFC gene expression in A549 cells.

3.6 | Tumor necrosis factor superfamily 15 facilitates vascular endothelial growth factor-C-stimulated lymphatic endothelial cell migration and proliferation

We determined the impact of VEGFC upregulated by TNFSF15 on the ability of LEC to migrate and proliferate. First, we constructed a VEGFC knockdown stable cell line with VEGFC shRNA in A549 lung cancer cells, in which the VEGFC silencing effect was clearly identified (Figure 6A). Using a transwell migration assay, which measures the number of LEC that pass across a filter under the experimental conditions (Figure 6B), we found that treatment with conditioned medium of TNFSF15 (200 ng/mL)-treated A549 cells led to more than a doubling of the migration ability of the LEC when compared to that from vehicle-treated cells and that this effect can be blocked by VEGFC silencing (Figure 6C). Furthermore, to determine whether

VEGFC upregulated by TNFSF15 can promote LEC proliferation, we treated LEC with conditioned media from A549 shControl or A549 shVEGFC cell cultures for 72 hours, and the cell numbers were counted. We found that TNFSF15-treated cell medium led to a 1.5-fold enhancement of the proliferation rate of LEC and that this effect can also be blocked by VEGFC silencing (Figure 6D).

4 | DISCUSSION

Tumor necrosis factor superfamily 15, a cytokine secreted by endothelial cells, has important effects on anti-angiogenesis and pro-lymphangiogenesis. Previous studies have found that TNFSF15 can inhibit tumor growth via anti-angiogenesis.[17,19,26] In addition, the effect of TNFSF15 on lymphangiogenesis had also been studied under physiological conditions.[20] However, the role of TNFSF15 in tumor metastasis and the underlying mechanism remain unknown. Here, we have demonstrated that TNFSF15 can stimulate VEGFC gene expression in A549 lung cancer cells and that VEGFC secreted from A549 cells can promote lymphatic endothelial cell (LEC) proliferation and migration, and stimulate tumor lymphangiogenesis and lymphatic metastasis. Furthermore, mechanistically, we observed that DR3, the cell surface receptor of TNFSF15, mediates the upregulation of VEGFC expression by activating the NF-κB signaling
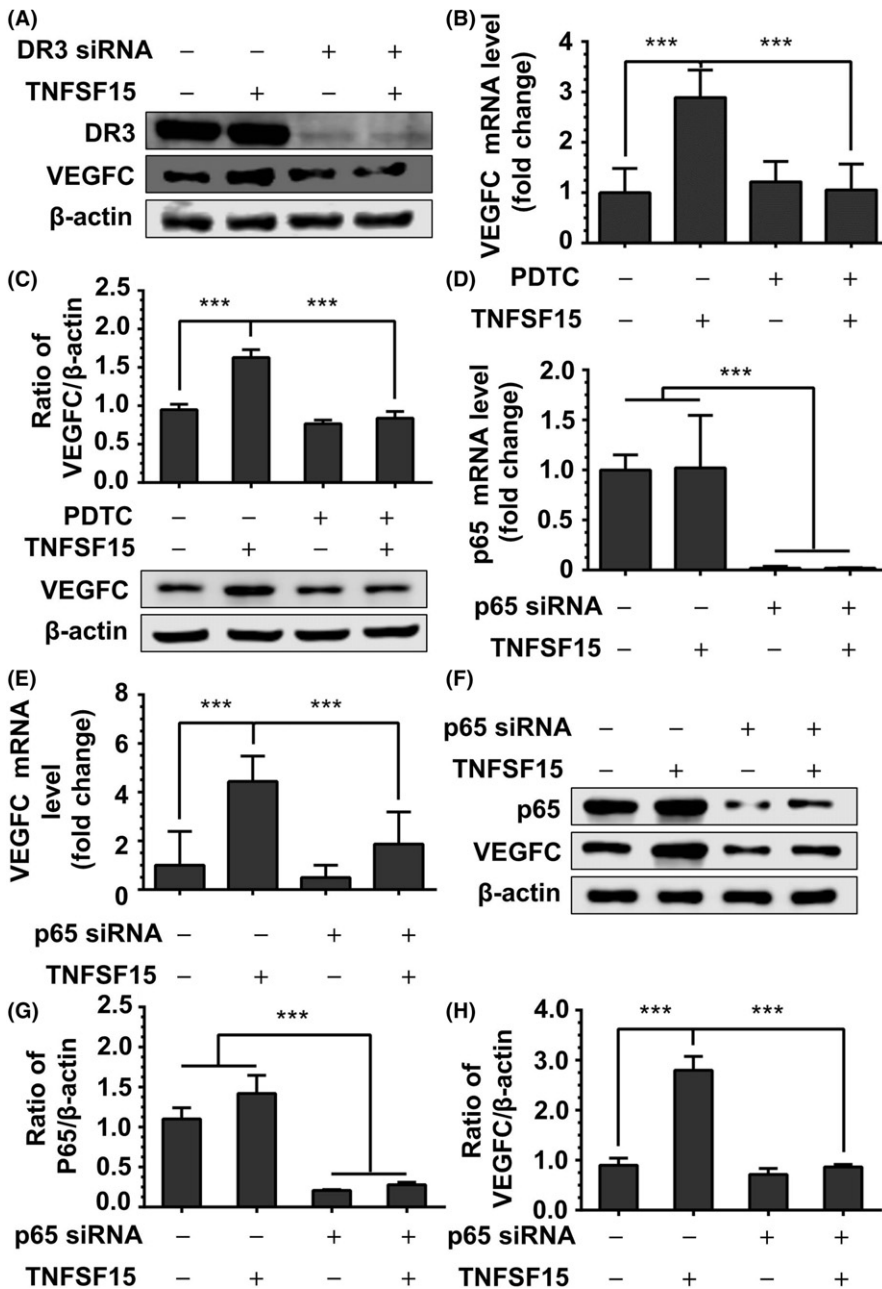
FIGURE 5 DR3 mediates tumor necrosis factor superfamily 15 (TNFSF15)-stimulated upregulation of vascular endothelial growth factor-C (VEGFC). A, Changes in DR3 and VEGFC protein levels following TNFSF15 treatment in the presence or absence of DR3 siRNA (40 pmol/mL). B, Changes in VEGFC mRNA in response to TNFSF15 treatment in the presence or absence of NF-κB inhibitor PDTC (1 µmol/L), as determined by RT-PCR. C, Western blot analyses showing treatment effect of PDTC on TNFSF15-induced VEGFC upregulation. D, Changes in p65 mRNA levels in the presence or absence of p65 siRNA (40 pmol/mL). E, Changes in VEGFC mRNA levels following TNFSF15 treatment in the presence or absence of p65 siRNA. F, Changes in p65 and VEGFC protein levels following TNFSF15 treatment in the presence or absence of p65 siRNA. G, Quantitative analyses of p65 protein levels in the presence or absence of p65 siRNA. H, Quantitative analysis of VEGFC protein levels following TNFSF15 treatment in the presence or absence of p65 siRNA. Data are mean ± SD. ***$P < .001$; one-way ANOVA. These experiments were repeated 3 times

pathway. Subsequently, DR3/NF-κB signal activation results in upregulation of VEGFC expression and facilitates VEGFC-dependent LEC proliferation, migration, and tumor lymphangiogenesis and lymphatic metastasis (Figure 7). Thus, our investigation has for the first time revealed the effect of TNFSF15 on lymphatic metastasis in lung cancer, which has shed light on the prevention and treatment of lung cancer metastasis.

Tumor necrosis factor superfamily 15 can inhibit angiogenesis and promote lymphangiogenesis in a mouse model of lung cancer. Typically, both types of vessels grow or regress in parallel, which is consistent with our previous notion. In brief, the TNFSF15/DR3 signaling pathway may lead to different results, depending on the types of cells in which it is activated, and it leads to apoptosis in VEC and proliferation in LEC.[20]

Tumor cell metastasis to regional lymph nodes is an early event in metastatic tumor spread and is frequently used as a prognostic factor for disease outcome. The existence and functionality of intratumoral lymphatics in human and experimental rodents remain controversial. Our findings indicate that TNFSF15-promoted lymphangiogenesis via upregulation of VEGFC in A549 cells provides portals for tumor metastasis, and the invasion of tumor cells into the lymphatic vessels is a critical step in metastasis. Given that TNFSF15 protein levels are highly expressed in the early stages (FIGO stages I and II) of cancer,[18] it would be meaningful to explore the potential correlation between TNFSF15 expression and occurrences of regional lymph node metastasis.

It has been affirmatively shown that DR3 works as the cell surface receptor of TNFSF15 in blood vascular endothelial cells,[27]

FIGURE 6 Tumor necrosis factor superfamily 15 (TNFSF15) facilitates vascular endothelial growth factor-C (VEGFC)-stimulated lymphatic endothelial cell (LEC) migration and proliferation. A, Changes in VEGFC protein levels following TNFSF15 treatment in the presence or absence of VEGFC shRNA. B, Typical immunostaining images of migrated LEC in transwell migration assays following conditioned media treatment. Blue, LEC. Magnification, ×5, scale bar: 100 μm. C, Changes in numbers of migrated cells. D, The relative numbers of cells following treatment with A549 tumor cell-conditioned media. Data are mean ± SD. ***$P < .001$; one-way ANOVA. These experiments were repeated 3 times
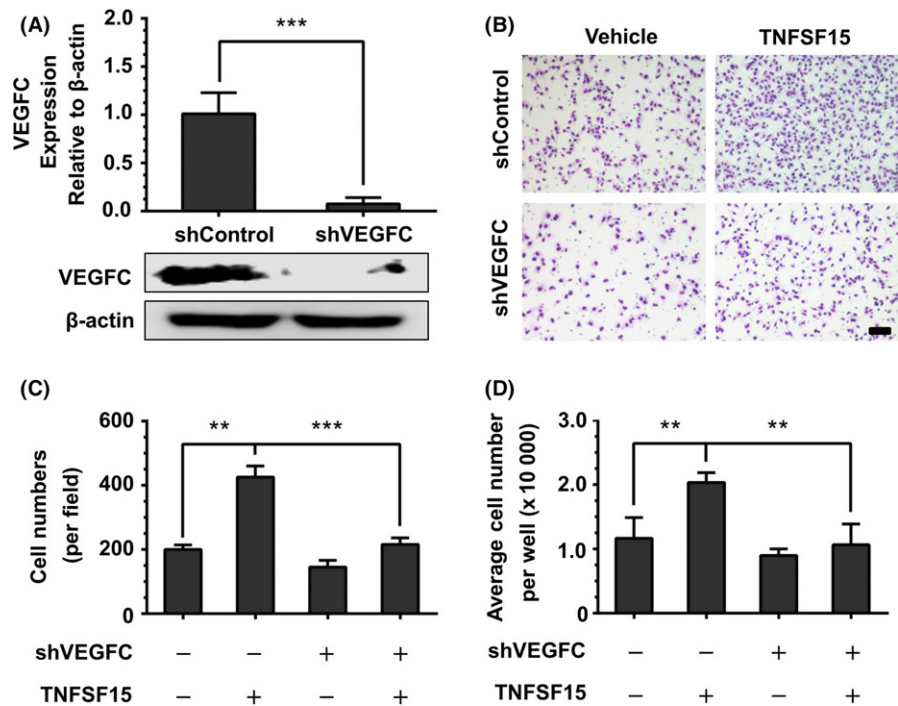

FIGURE 7 A schematic presentation of process involved in the lymphatic metastasis promoted by tumor necrosis factor superfamily 15 (TNFSF15). TNFSF15 binds to its receptor DR3 on A549 lung tumor cells, which subsequently causes activation of the NF-κB signaling pathway and secretion of vascular endothelial growth factor-C (VEGFC) by the A549 cells. VEGFC then promotes lymphangiogenesis and, consequently, lymphatic metastasis via the newly formed lymphatic vessels in primary tumors
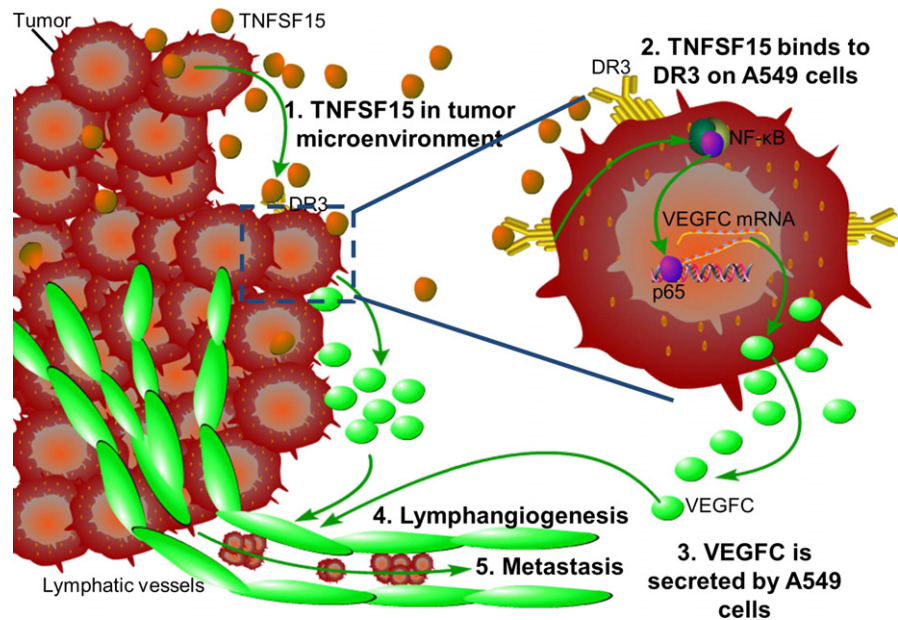
lymphatic endothelial cells,[20] T cells[28] and dentritic cells.[29] Experimental data for this study show that DR3 is the receptor of TNFSF15 in A549 cells and that the DR3 siRNA treatment of A549 lung cancer cells results in a blockade of TNFSF15-induced VEGFC upregulation. Furthermore, our study shows that DR3 is involved in mediating TNFSF15 activity in VEGFC upregulation through a mechanism involving the NF-κB signaling pathway. Activation of NF-κB can be directly linked to VEGFC upregulation, because VEGFC upregulation, which is induced by TNFSF15, can be inhibited when treated with either NF-κB inhibitor PDTC or NF-κB subunit p65 siRNA in A549 cells. Our finding is consistent with previous studies on ovarian or colon cancer that NF-κB activation can induce the production of VEGFC.[10,12,30]

In summary, this study indicates that cytokine TNFSF15 promotes lymphatic metastasis of lung cancer via upregulation of lymphangiogenic growth factor VEGFC in A549 lung cancer cells in a mouse model of lung cancer. Mechanistically, this activity is mediated by DR3 and subsequent activation of the NF-κB signaling pathway in A549 cells. Further delineation of this signaling pathway could lead to insights into the molecular mechanisms underlying tumor lymphangiogenesis, modulation of lymphatic metastasis and new targets for clinical intervention in lymphatic metastasis.

CONFLICT OF INTEREST

ORCID

Dingzhi Huang http://orcid.org/0000-0002-2798-9459

Cory J. Xian http://orcid.org/0000-0002-8467-2845

Kai Li http://orcid.org/0000-0002-6895-0024

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.