RESEARCH ARTICLE

Inhibition of NADPH oxidase 4 attenuates lymphangiogenesis and tumor metastasis in breast cancer

Xinzhao Wang¹ ² | Zhaoyun Liu¹ ² | Jujie Sun² | Xiang Song² | Mengxue Bian² | Fukai Wang² | Feng Yan³ ⁴ | Zhiyong Yu² ¹

¹Cheeloo College of Medicine, Shandong University, Jinan, People’s Republic of China
²Breast Cancer Center, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, People’s Republic of China
³Department of Emergency Medicine, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, People’s Republic of China
⁴Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Cheeloo College of Medicine, Shandong University, Jinan, People’s Republic of China

Correspondence
Zhiyong Yu, Breast Cancer Center, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, 440 Jiyan Road, Jinan, Shandong 250117, People’s Republic of China. Email: drzhiyongyu@aliyun.com

Feng Yan, Department of Emergency Medicine, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong 250012, People’s Republic of China. Email: yanfeng@sdu.edu.cn

Abstract
Lymphangiogenesis is thought to contribute to promote tumor cells to enter lymphatic vessels and plant at a secondary site. Endothelial cells are the cornerstone of the generation of new lymphatic vessels. NADPH oxidase 4 (Nox4) is the most abundant one of NADPH oxidases in endothelial cells and the most studied one in relevance with cancer. Our purpose is to analyze the relationship between Nox4 and lymphangiogenesis and find out whether the newborn lymphatic vessels lead to cancer metastasis. We first explored the expression of Nox4 in lymphatic endothelial cells of primary invasive breast tumors and human normal mammary glands using GEO databases and found that Nox4 was upregulated in primary invasive breast tumors samples. In addition, its high expression correlated with lymph node metastasis in breast cancer patients. Nox4 could increase the tube formation and lymphatic vessel sprouting in a three-dimensional setting. In vivo, inhibition of Nox4 in 4T1 tumor-bearing mice could significantly decrease the tumor lymphangiogenesis and metastasis. Nox4 may increase tumor lymphangiogenesis via ROS/ERK/CCL21 pathway and attract CCR7-positive breast cancer cells to entry lymphatic vessels and distant organs. In conclusion, our results show that Nox4 is a factor that promotes lymphangiogenesis and is a potential target of antitumor metastasis.

KEYWORDS
breast cancer, lymphangiogenesis, metastasis, Nox4

Abbreviations: AJCC, American Joint Committee on Cancer; ANOVA, analysis of variance; DCFH-DA, 2,7-Dichlorodihydrofluorescein diacetate; ECM, endothelial cell medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HLECs, human lymphatic endothelial cells; IC₅₀, half maximal inhibitory concentration; LVD, lymphatic vessel density; MOI, multiplicities of infection; NAC, N-acetyl-L-cysteine; Nox4, NADPH oxidase 4; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SI, staining index.
INTRODUCTION

Cancer metastasis remains the primary culprit of cancer-related death worldwide, which is a highly complex process including direct local invasion or traveling through the circulatory systems and establishment of a new malignant lesion at other tissues or organs. As we all know, breast cancer cells spread to other sites mainly through hemogenous system and lymphatic system. Blocking the crucial pathway of cancer metastasis is a potent approach to overcome breast cancer. Although blocking angiogenesis of hemogenous metastasis was researched deeply in breast cancer, randomized clinical trials targeted hemangiogenesis using bevacizumab, provided no overall survival benefit. In breast cancer, the lymphatic vasculature is also a key route for breast cancer cells dissemination. Lymphatic vessel density (LVD) correlates with lymph node involvement and worse survival rates in cancer patients. Evaluation of tumor-draining lymph node contributes to both predicting prognosis and instructing treatment. Conventionally, in the process of breast cancer metastasis, lymphatic vessels play a role by just providing access channels for tumor cells. However, preclinical experiments suggest that lymphatic vessels can make an adaptive change that facilitate metastasis, for example, lymphangiogenesis. Therefore, finding the cellular and molecular mechanisms of tumor lymphangiogenesis is helpful to increase the knowledge of cancer metastasis.

Lymphangiogenesis is a complicated cellular event that needs the collaboration of various biological processes, such as cell proliferation, differentiation, migration, adhesion, vessel sprouting, and tube formation. Moreover, lymphangiogenesis is thought to contribute to promote tumor cells to enter lymphatic vessels and plant at a secondary site. Many tumor-derived lymphangiogenic factors have been proved to participate in the biological events. However, the precise molecular biological mechanisms are still vague. For cancer metastasis, previous researches mainly focused on the cancer cells themselves. Recently, researchers found that lymphangiogenesis is a process that generates new lymphatic vessels from preexisting ones, which supply a new insight into how the lymphatic vessels sprout and facilitate metastasis. Whereas, only limited studies are available on elaborating how lymphatic vessels undergo dynamic changes intratumorally or peritumorally.

NADPH oxidase 4 (Nox4) is a member of Nox family, whose function is to transport electrons from cytosolic NADPH to biological membranes and finally to oxygen. Nox4 is the most abundant one in endothelial cells and also the most studied one in relevance with cancer. Moreover, Nox4 provides an oncogenic function localized to mitochondrial, which is expressed in various malignant tumors, such as prostate cancer, melanoma, thyroid carcinoma, urothelial carcinoma, and breast cancer. Nox4 can modulate cancer cell migration, attenuate the induction of cancer cell apoptosis, and promote cancer cell metastasis. In melanoma cell, Nox4 could regulate G2/M cell cycle progression, and in renal carcinoma, it could promote the tumor development by accumulation of hypoxia-inducible factor 2α. Reactive oxygen species (ROS) plays an important role in the physiopathogenesis and pathogenesis of endothelial cells, and NADPH oxidases are the major source of the intracellular ROS. We assume that Nox4 may play an important role in lymphatic endothelial cells. What's the specific mechanism on Human Lymphatic Endothelial Cells (HLECs) is still unclear. To find out the mechanism of Nox4 on HLECs may be useful to understand the process of cancer metastasis.

In this study, we used HLECs to investigate the role of Nox4 in boosting lymphangiogenesis and compelling cancer cells to migrate to distant organs. We uncovered Nox4-induced HLECs proliferation, migration, anti-apoptosis, tube formation, and lymphatic ring sprouting via the Nox4/ROS/ERK/CCL21 signal pathways. We demonstrated that Nox4 increased the CCL21 expression on HLECs which could recruit tumor cells expressing its ligand CCR7 to lymphatic vessels and spread to distant organs. In conclusion, Nox4 may be a promoter by inducing lymphangiogenesis and leading tumor metastasis.
2.2 Dataset collection of HLECs

The transcription profile dataset of HLECs was obtained from NCBI GEO databases (http://www.ncbi.nlm.nih.gov/geo/). The accession number was GSE73613. The data derived from the human lymphatic endothelial cells extracted from primary invasive breast tumors (N = 2) and human normal mammary glands (N = 2). The microarray data were based on GPL570 Platforms (Affymetrix Human Genome U133 Plus 2.0 Array, Affymetrix Inc, Santa Clara, CA, USA). R software (version 3.2.3; https://www.r-project.org/) was used in data mining and statistical analyses.

2.3 Specimen preparation

All tissue samples were collected from breast cancer patients undergoing surgical resection in Shandong Cancer Hospital and Institute. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee at Shandong Cancer Hospital and Institute. All patients gave written informed consent before their participation in the study. The tumor stage of every patient was determined according to the 7th American Joint Committee on Cancer (AJCC) staging system. The intensity scores of Nox4 were counted as follows: 1 (no staining), 2 (weak staining), 3 (moderate staining), and 4 (strong staining). The proportion of tumor cells was scored as 0 (no positive area), 1 (<10% positive area), 2 (10%-35% positive area), 3 (35%-75% positive area), and 4 (>75% positive area). The staining index (SI) was calculated as staining intensity score $\times$ proportion of positive tumor cells. The indicated protein expression with SI $\geq$ 8 was classed into high expression and with SI < 8 was classed into low expression. For LVD detection, 17 hot spots were identified by scanning the slide at low power ($\times$40). Once hot spots were found, LVD was counted on a $\times$200 field and recorded using an eyepiece grid. When LVD was equal or less than 11 (mean value of LVD), LYVE-1 was defined as low expression. While LVD was more than 11, it was defined as high expression.

2.4 Cell culture

HLECs were obtained from ScienCell and cultured in endothelial cell medium (ECM) (ScienCell) with 5% FBS and endothelial growth medium supplements. All cells were maintained in a humidified atmosphere of 5% CO$_2$ and 37°C. For cell transfection, Nox4-shRNA was added to the HLECs at 20 multiplicities of infection (MOI). Its corresponding negative control group (LV3-NC) was treated as control groups. For Nox4 inhibitor treatment, GKT137831 with 8 $\mu$M for wound healing and transwell was added to treatment group and DMSO was chosen as negative control treatment.

2.5 RNA isolation and real-time reverse transcription polymerase chain reaction

Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA quantification was determined by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Primers used for quantitative real-time PCR were as shown in Table S1.

2.6 CCK-8 assay

The proliferation of HLECs was estimated by CCK-8 assay using the CCK-8 assay kit (keyGEN biotech, Jiangsu, China). Approximately 1 $\times$ 10$^4$ cells per well were seeded in 96-well plates with 100 $\mu$L medium. After incubating different time and different concentration, each well was treated with 10 $\mu$g CCK-8 solutions for 2 hours in dark before measuring the absorbance at 450 nm.

2.7 ROS activity assay

Microscopic fluorescence was used to study ROS generation in HLECs after exposure to different concentrations of GKT137831. HLECs were incubated with 5 $\mu$M of 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, Life Technologies, Carlsbad, CA, USA) at 37°C for 20 minutes in Hanks’ balanced salt solution. A laser scanning confocal microscope (Model LSM710, Zeiss, Jena, Germany) was used to detect the intracellular fluorescence of cells and to capture images. The fluorescent intensity was measured with ImageJ software (Windows version; National Institutes of Health, USA).

2.8 Wound healing assay

Pretreated HLECs were seeded into a 6-well plate at a final concentration of $5 \times 10^5$ per well until cells reached about 90% confluence. Wound was created by a 200 $\mu$L pipette tip, and then free-floating cells and debris were removed by repeated rinsing with medium. Cells were incubated for
24 hours in media without serum. Wound healing was observed and analyzed with ImageJ software (Windows version; National Institutes of Health, USA).

2.9 | Cell migration assay

HLECs migration was studied with 6.5mm diameter transwell chamber (8 μm pore size) (Corning, NY, USA). Pretreated or untreated HLECs (2 × 10^4) were re-suspended with serum-free medium and seeded on the upper compartment of the chamber. For this process, the lower chamber of the transwell contained culture medium enriched with 10% of FBS. The cells were allowed to culture for 24 hours, and removed by a cotton swab and then, washed with PBS repeatedly. The membrane was fixed with methanol and the migratory cells on the lower side of the membrane were stained with Giemsa. Migrated cells per chamber were determined to evaluate migration using ImageJ software.

2.10 | JC-1 staining to determine mitochondrial membrane potential (Δψm)

HLECs were stained with JC-1 at 37°C in dark for 20 minutes and analyzed by a laser scanning confocal microscopy. The dye entered throughout the whole cell leading to a shift from red (J-aggregates) to green fluorescence (JC-1 monomers). Mitochondrial membrane potential was determined by a red/green ration. The ration was calculated using ImageJ software.

2.11 | Measurement of apoptotic cells

HLECs were treated with GKT137831 for 24 hours, and then washed twice with cold PBS. An indirect immunofluorescence assay was performed using the Annexin V-FITC apoptosis kit according to the manufacturer’s instructions. The samples were assessed by flow cytometry (FACSCalibur; BD Biosciences) using CellQuest software within 1 hour.

2.12 | Tube formation in vitro

A 96-well plate adding 50 μL/well of matrigel allowed to polymerize for 30 minutes at 37°C. HLECs were detached by trypsinization, and re-suspended in ECM and plated onto the layer of matrigel (1 × 10^3 cells/well). Matrigel cultures were incubated at 37°C and photographed at 24 hours. The total length of the tube-like structure was quantified by counting cells number in branch point capillaries in randomly selected three microscopic fields. The area of tube formation was determined using ImageJ software.

2.13 | Thoracic duct collection and threedimensional lymphatic ring assay

We cultured thoracic ducts from C57BL/6J mice (Beijing HFK Bioscience Company, Beijing China) that were 2-3 months of age. We dissected thoracic ducts by microsurgery to generate fragments and then, embedded into matrigel. GKT137831 with 8 μM and/or VEGF-C (recombinant human VEGF-C 20 ng/mL; recombinant mouse VEGF-C 50 ng/mL) were added to the matrigel culture medium at the beginning of the experiment. The lymphatic ring cultures were incubated to observe the outgrowth of cells which formed lymphatic vessel-like structures. After 7 days, we elaborated the grid draw by the successive dilatation of the thoracic duct boundary using binary image and sholl analysis and determined using ImageJ software.

2.14 | Western blot

Protein samples were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% low-fat milk and incubated with the indicated primary antibodies overnight. The secondary HRP-conjugated antibodies were visualized by ECL prime reagents (GE, Piscataway, NJ, USA) and detected with a LAS-4000 luminescent image analyzer (Fujifilm, Stamford, CT, USA). The band densitometry was quantified with ImageJ software.

2.15 | Immunohistochemical staining

The paraffin sections of human or mouse specimens were dewaxed and hydrated. Antigen repair was pretreated in a microwave oven with a phosphate buffer for 5 minutes. Each section was stored at 4°C overnight after adding primary antibody. The secondary antibody was added the next day and was incubated at room temperature in the following day. DAB was performed for color dying for 5 minutes and counterstained with hematoxylin. Phosphate-buffered saline (PBS) was chosen as the negative control. The results were diagnosed and determined by two pathologists independently.

2.16 | Immunofluorescence assay

For immunofluorescence staining, paraffin-embedded tissue specimens of xenograft tumors were fixed with cold acetone then treated by 0.1% Triton X-100 in PBS. The tissue sections were incubated with primary antibodies overnight at 4°C followed by the appropriate secondary fluorescently labeled antibodies. Nuclei were counterstained with DAPI.
Images were taken with fluorescence microscope and quantitated with ImageJ software.

2.17 Orthotopic tumor model and in vivo procedure

To investigate the in vivo effects of Nox4 on cancer metastasis, 4T1 orthotopic mice were used. 4T1 cells (2.5 × 10⁶) were injected subcutaneously into upper fat pads of 8-week-old female BALB/c mice (Beijing HFK Bioscience Company) under direct vision. Once tumor size reached 80-100 mm³, the mice were divided into two groups (N = 5 each group) and treated with GKT137831 (50 mg/kg) or saline containing the same concentration of DMSO every 2 days for 28 days. Tumor was measured with caliper to estimate the volume. Mice were sacrificed at the end of the experiment and tumors were removed for further analyses. All animals were housed and treated according to the guidelines outlined by the Institutional Animal Care and Use Committee of Shandong Cancer Hospital and Institute. Tumor volume was calculated by the following formula: \( V = \frac{1}{2}ab^2 \). All organs were removed for detection. Lateral axillary lymph node in tumor drainage area and lung metastases was examined by hematoxylin and eosin staining and immunofluorescence assay and quantified by counting metastatic lesions in each section. The vessels in the intratumoral and peritumoral tissues were detected by immunofluorescence. The hot areas were selected at low power (×40) and lymphatic and blood vessels were counted in three high power (×200) fields. The vessel density was calculated by counting the mean of the vessel obtained in these three fields.¹⁸

2.18 Statistical analysis

All studies were repeated three times to ensure reproducibility. All results were analyzed by two-tailed t test for comparison of two groups, one-way analysis of variance (ANOVA) followed by Tukey’s test for comparison of multiple groups, and chi-square test for categorical variable data. Spearman analysis was used to compare the correlation between Nox4 and LYVE-1 in primary breast tumor. Statistical analyses were carried out using IBM SPSS Statistics 21 software. A value of \( P < .05 \) was regarded as statistically significant.

3 RESULTS

3.1 Tumor-associated lymphatic vessels highly express Nox4

To identify the differences of Nox4 expression between tumor-associated lymphatic endothelial cells and their normal lymphatic endothelial cell counterparts, we obtained data from NCBI GEO datasets and found that Nox4 mRNA expression increased obviously in tumor-associated lymphatic endothelial cells with the calculating criteria of \( P < .05 \) and absolute log₂FC = 3.14 (Table S2).

3.2 High expression of Nox4 is associated with lymph node metastasis in breast cancer

A total of 81 breast cancer patients with a median age of 49 years (range: 31 to 72 years) who received surgical resection were retrospectively examined at Shandong Cancer Hospital and Institute from May 1st, 2014 to Sep 30th, 2019. All of the 81 patients had an Eastern Cooperative Oncology Group performance status ≤ 1. As shown in Table S3, the tumor size was found to more than 2 cm in 35 patients (43.2%), equal or small than 2 cm in 46 patients (56.8%). Meanwhile, 29 patients (35.8%) were diagnosed as having no lymph node metastasis, 52 patients (64.2%) as having lymph node metastasis. The histology grade indicated that 20 patients (24.7%) had Grade I, 35 patients (43.2%) had Grade II, and 26 patients (32.1%) had Grade III. A total of 49 (60.5%) patients were ER positive, 44 (54.3%) were PR positive, and 30 (37.0%) were HER2 positive. The molecular subtype was found to be luminal A in 20 patients (24.7%), luminal B patients in 33 patients (40.7%), HER2 overexpression in 16 patients (19.8%), and TNBC in 12 patients (14.8%).

To investigate the clinical relevance of Nox4 and lymphatic vessel in breast cancer patients, we directly detected Nox4 and LYVE-1 expression levels in patients by immunohistochemistry analysis. The results showed that high expression of Nox4 and LYVE-1 in breast cancer was 49.4% (40/81) and 54.3% (44/81), respectively. Nox4 was positively correlated with lymph node metastasis and histologic grade (all \( P < .05 \)) (Figure 1A). LYVE-1 was positively correlated with tumor size, lymph node metastasis, ER, PR, and molecular subtype (all \( P < .05 \)) (Figure 1A). Furthermore, correlation analysis demonstrated a positive correlation between Nox4 and LYVE-1 in breast cancer tissue (\( P < .05 \)) (Figure 1B).

3.3 Inhibition of Nox4 attenuates the migration and increases apoptosis of HLECs

In order to research the potential mechanisms of Nox4 in lymphangiogenesis modulation, we assessed HLECs proliferation, migration, apoptosis, all of which are essential steps for lymphangiogenesis. We first analyzed Nox4 levels by qRT-PCR and western blot (Figures S1 and S2), when we performed knockdown of Nox4 by shRNA or Nox4 inhibitor GKT137831. Results indicated that downregulation of Nox4 obviously inhibited the cell proliferation in vitro, and it was in a time-dependent manner and dose-dependent...
FIGURE 1  Nox4 expression was positively correlated with lymphatic vessel density (LVD) in breast cancer tissues. (A) The $\chi^2$ test was used to analyze the relationship between Nox4 and LYVE-1 and clinicopathological characteristics in breast cancer tissues. (B) The correlation of Nox4 and LYVE-1 protein was analyzed through spearman correlation analysis. (C) Immunohistochemical staining assay of Nox4 and LYVE-1 on breast cancer tissues. The staining index (SI) was calculated as staining intensity score $\times$ proportion of positive tumor cells. The Nox4 expression was classified into high expression and low expression, respectively, according to SI. For LVD detection, hot spots were identified by scanning the slide at low power ($\times 40$). Once hot spots were found, LVD was counted on a $\times 200$ field and recorded using an eyepiece grid. The mean LVD was $10.9 \pm 4.2$ microvessels/mm$^2$. To analyze the correlation between LVD and clinicopathological characteristics, the patients were divided into two groups: high expression and low expression. Cut off value was mean LVD.
manner (Figure 2A,B). The half maximal inhibitory concentration (IC50) of GKT137831 was 8.5 ± 0.7 μM at 24 hours on HLECs.

And, we analyzed the migratory capacity of HLECs using wound healing and chemotaxis assays. The results showed that HLECs with low levels of Nox4 treating with shRNA or inhibitor was easy to be wound healing delay (Figure 2C,D) and decline VEGF-C-induced HLECs migration (Figure 2E,F). In addition, we assessed the mitochondrial membrane potential using JC-1 staining. The fluorescent intensity refined by the change of red and green florescent caused by JC-1 indicates the change in mitochondrial membrane potential. Our results indicated that HLECs treated with different concentrations of GKT137831 represented a strong green fluorescence and this inhibition effect is not reversed by VEGF-C which could promote HLECs proliferation. The merge images of JC-1 red and JC-1 green indicated inhibition of Nox4 could significantly decrease the mitochondrial membrane potential (Figure 2G,H). In order to observe the apoptosis inducing of Nox4 inhibition, we performed the flow cytometry and found the apoptosis rate with GKT137831 in 0, 5, 10, and 15 groups was 2.6%, 12.1%, 21.9%, and 30.7%, respectively (Figure 2I,J). The results showed that GKT137831 could be a pro-apoptosis effect on HLECs in a dose-dependent manner.

**FIGURE 2**  Downregulation of Nox4 expression inhibits HLECs proliferation, wound healing, migration, and induces apoptosis. (A and B) CCK8 assay was performed to examine the proliferation of HLECs. Cells were cultured with different concentrations of Nox4 inhibitor GKT137831 (A) or with the fixed concentration of 8 μM at different time (B). (C and D) Nox4 shRNA-infected or GKT137831-treated HLECs were seeded on 6-well plate. Images were captured at 0 and 24 hours after wounding. For quantitative analysis, wound healing was observed and analyzed with ImageJ software. (E and F) Nox4 shRNA-infected or GKT137831-treated HLECs were treated with recombinant human VEGF-C (20 ng/mL). Nox4 shRNA-infected or GKT137831-treated HLECs were cultured with 6.5 mm diameter transwell chamber for 24 hours, migrated cells were evaluated by counting the cells at a 200 magnification. (G and H) After 24 hours, GKT137831-treated HLECs were stained with JC-1 and analyzed by a laser scanning confocal microscopy. Mitochondrial membrane potential was determined by a red/green rasion. (I and J) Flow cytometry was performed to observe apoptosis inducing by GKT137831 with the concentration of 0, 5, 10, and 15 μM, respectively. All values are expressed as mean ± SEM of three independent experiments. *P < .05
3.4 | Inhibition of Nox4 attenuates tube formation of HLECs and thoracic duct sprouting

When downregulation of Nox4 using Nox4-specific shRNA lentivirus and Nox4 inhibitor GKT137831, we found that tube formation was significantly reduced. VEGF-C, a promoter of HLECs, still could not reverse this inhibition (Figure 3A,B). In order to study whether Nox4 was involved in lymphatic vessel sprouting, a lymphatic ring assay was used to evaluate the sprouting capacity of HLECs from a preexisting vessel. Mouse thoracic ducts cut into rings were treated with GKT137831 and/or VEGF-C and embedded into matrigel. After 7 days of incubation, negative control lymphatic ring cultures emerged an outgrowth of cells that arranged into capillary-like structures. However, GKT137831-treated lymphatic rings were significantly attenuated and VEGF-C also could not reverse this inhibition (Figure 3C). Furthermore, we quantified the lymphatic vessel sprouts using sholl analysis method of ImageJ software. GKT137831-treated lymphatic vessel sprouts were shown to be significantly reduced compared to control groups.

**Figure 3** Inhibition of Nox4 expression inhibits tube formation and thoracic duct sprouting. Nox4 shRNA-infected or GKT137831-treated HLECs were treated with VEGF-C (A, recombinant human VEGF-C 20 ng/mL; C, recombinant mouse VEGF-C 50 ng/mL). (A and B) Tube formation on matrigel was photographed and quantified at 24 hours. The total length of the tube-like structure was quantified by counting cells number in branch point capillaries in randomly selected three microscopic fields. (C and D) Thoracic duct explants embedded in matrigel were treated with GKT137831. After 7 days, the grids were drawn by the successive dilatation of the thoracic duct boundary determined using sholl analysis of ImageJ software. (E-G) Nox4 mRNA and protein levels in sprouting lymphatic vessels were tested by real-time qRT–PCR (E) and western blotting (F). All values were performed as the mean ± SEM of three independent experiments. *P < .05, **P < .01 vs control group.
rings showed a significant reduction in the number of intersecting vessels compared with controls (Figure 3D). Then, real-time qRT-PCR and western blot analysis identified that Nox4 mRNA and protein were significantly decreased in lymphatic vessels treated with GKT137831 as compared with the control group (Figure 3E-G). In short, these results demonstrated that Nox4 expression is necessary for tube formation and thoracic duct sprouting.

### 3.5 Nox4 promotes lymphangiogenic activity via increasing CCL21 expression through an ERK-dependent pathway

In order to investigate the underlying molecular mechanism of Nox4-dependent alterations in HLECs, we examined the changes of related signal pathways in HLECs who were treated with different concentration of GKT137831 by western blotting analysis. The results indicated that inhibition of Nox4 decreased the p-ERK, p-AKT, p-JNK, MMP2, and CCL21, and increased cleaved caspase-3 in a dose-dependent manner (Figure 4A,B). As is known, Nox4 is the main source of ROS and we treated HLECs with different concentration of GKT137831 using DCFH-DA fluorescence. The results indicated that expression of ROS decreased in dose-dependent manner by Nox4 inhibitor (Figure S3). Furthermore, to elucidate whether these intracellular signaling pathways were regulated by activation of Nox4, we treated HLECs with NAC, U0126, AKT inhibitor VIII, SP600125, which are ROS, ERK, AKT, and JNK inhibitors, respectively. The results indicated that HLECs treated with ROS and ERK inhibitor significantly attenuated

![Figure 4](image_url)

**FIGURE 4** Inhibition of Nox4 expression downregulated the following signaling pathways, such as p-ERK, p-AKT, p-JNK, MMP2, and CCL21 and increased cleaved caspase-3. Immunoblot analysis (A) of HLECs treated with GKT137831 (0, 1, 10, and 15 μM) and densitometric analyses (B) of p-ERK, p-AKT, p-JNK, and cleaved caspase-3, MMP2, and CCL21. (C) HLECs were treated with 2.5 μM NAC (②), 10 μM U0126 (③), 5 μM AKT inhibitor VIII (④), and 10 μM SP600125 (⑤) for 24 hours. Control group added DMSO (①). (D) Quantification of tube formation in the experiment. All experiments are presented as the mean ± SEM from three different experiments. *P < .05 vs control group.
tube formation (Figure 4C,D). Although AKT and JNK pathways changed by treating with Nox4 inhibitor, no significant changes were found on tube formation when using its inhibitor incubating HLECs.

In tumor-associated environment, CCL21 chemokines secreted by HLECs are key players in the active metastatic dissemination of cancer cells.19 To determine whether the ERK signaling pathway regulates CCL21 expression, we treated HLECs who were pretreated with Lenti-Nox4 with an ERK inhibitor U0126. The results showed a decreased expression of CCL21 in a dose-dependent manner, when treating with different concentration of ERK inhibitor (Figure 5A,B).

### 3.6 Inhibition of Nox4 expression reduces tumor lymphangiogenesis and tumor metastasis

To extend our in vitro observations, we further studied the significance of Nox4 in breast cancer lymphangiogenesis and metastasis in vivo. 4T1 cells were implanted into BALB/c mice mammary fat pads for 8 days. Then tumor-bearing mice were divided into two groups. GKT137831 or control was given orally every 2 days. Tumor volumes were calculated regularly and tumor weights were measured after the mice were sacrificed. No significant differences were observed among tumor growth and tumor weight (Figure 6D,E). In order to confirm the role of Nox4 in inducing of lymphangiogenesis, primary tumors were removed (Figure 6C) and sectioned. Western blot and immunofluorescence assay determined that Nox4 expression decreased when treating with GKT137831 (Figure 6A,B). Immunofluorescence quantification of LYVE-1/CD31-positive lymphatic structures proved that lymphatic density was significantly reduced in peritumoral tissue in GKT137831-treated group as compared with the control group (Figure 6F,G). No significant difference of lymphatic density was observed in intratumoral tissue in both groups (Figure 6H,I). CD31-positive vessel density which was vessel-specific marker was slightly decreased in GKT137831-treated tumors in peritumoral and intratumoral tissues (Figure 6F-I).

To further confirm the effects of Nox4 on tumor metastasis via lymphangiogenesis, 4T1 tumor-bearing mice were sacrificed and examined for metastases in the lymph nodes and lung. Morphometric analysis confirmed that a significant decrease in the number of metastatic nodules per lymph node and lung from GKT137831-treated tumors (Figure 7A,B). Tumor cells with CCR7 staining validated that Nox4 inhibitor could reduce lateral axillary lymph nodes (Figure 7D) and lung (Figure 7E,F) metastases as compared with the control. But no statistical significance was observed on CCR7 in primary tumor whether GKT137831 treated or not (Figure 7C).

### 4 DISCUSSION

Previous studies have shown that Nox4 in breast cancer cells increased tumorigenicity.9,20 Recently, reports indicated that Nox4 could promote tumor cell proliferation, migration, and invasion and was associated with a poor prognosis in various cancer types.21-23 Nox4 might be a potential target for cancer therapy. Lymphangiogenesis is a complicated process in

![Figure 5](image_url)

**Figure 5** Inhibition of Nox4 decreases CCL21 expression through an ERK-dependent pathway. (A) Immunoblot and (B) densitometric analysis of p-ERK and CCL21. Pretreated HLECs with Lenti-Nox4 of 20MOI were treated with different concentrations ERK inhibitor U0126 (0, 5, 10, and 15 μM). All experiments are presented as the mean ± SEM from three different experiments. *P < .05 vs control group
breast cancer which contains a variety of growth factors. In our study, we validate that Nox4 acts as a lymphangiogenic factor both in vitro and in vivo.

First, we used transcription profile dataset of HLECs obtained from NCBI GEO databases (GSE73613). Our results indicated that Nox4 expression increased significantly in human...
tumor HLECs than that of normal HLECs. We propose that
HLECs acquire an activated phenotype driven by Nox4 over-
expression. Meanwhile, we used primary invasive breast cancer
tissue to research the expression of Nox4 and lymphatic endo-
thelial cell markers of LYVE-1. We found that there was a posi-
tive correlation between Nox4 and LYVE-1 by analyzing a total
of 81 specimens of breast cancer. They were correlated with
lymph node metastasis. Previous studies have confirmed that
Nox4 promoted cancer cell proliferation and invasion and Nox4
overexpression was highly correlated with lymph node involve-
ment, distant metastasis, and poor prognosis of patients.22,24,25

LYVE-1, a novel lymphatic vessel marker, is found to express
on lymph vessel endothelial cells of both normal and neoplastic
tissues. High expressing LYVE-1 of lymph vessels is associated
with a high frequency of lymph node metastases.26,27 Because
they were all correlated with lymph node metastases, our clin-
dical data reminded that Nox4 and LYVE-1 might jointly accel-
erate the cancer cell into lymphatic vessels and form metastasis
at a second site.

Then, we designed the in vitro and in vivo study and to
find out the potential mechanism of Nox4 promoting breast
cancer metastases. We regulated the expression of Nox4 on

**FIGURE 7** Inhibition of Nox4 expression suppresses the cancer metastasis to lymph nodes and lung. (A and B) The number of metastatic
nodules per lymph node and lung were quantified by a microscopic inspection. Data are presented as the mean ± SEM *P < .05 vs Control
group. (C) Images of CCR7 expression of tumor tissues in mice injected with 4T1 cells. Hematoxylin and eosin staining and CCR7 expression
with formalin fixed and paraffin-embedded tissue specimens of lateral axillary lymph node (D) and lung tissues (E and F) isolated from mice
subcutaneously injected with 4T1 cells. The yellow dotted line represents the lymph node margin.
HLECs and observed the proliferation, migration, apoptosis, and tube formation. Inhibition of Nox4 attenuated proliferation, migration, and promoted apoptosis of HLECs in both a dose- and/or time-dependent manner using Nox4 inhibitor GKT137831. Inhibition of Nox4 expression significantly inhibited in vitro 3D tube formation and thoracic duct lymphatic ring sprouting. Even with VEGF-C adding, which is known to promote lymphangiogenesis, it cannot be reversed. In western blot research, we found that p-AKT, p-JNK, and p-ERK changed significantly in a concentration-dependent manner. However, only ROS inhibitor and ERK inhibitor could significantly inhibited tubular network formation. AKT inhibitor and JNK inhibitor have no inhibitory effect on tube formation of HLECs. The results suggest that Nox4 could induce ROS production and activate the intracellular signaling pathway including ERK pathway which were essential for Nox4-mediated lymphangiogenesis.

In immune system, lymphatic vessels play an important role for antigen-presenting cells to transit from tissues to lymph nodes. Chemokines has been proved in facilitating these cellular movements. HLECs can attract dendritic cells into lymphatic vessels by expressing CC-chemokine ligand and interact with its receptor, which is located on dendritic cells. Recent researches have proved that tumor cells can also be drown to lymphatic vessels through chemokine-dependent process which have been indicated in cancer metastasis. This recruitment of cancer cells to lymphatic vessels contains various secreted factors such as CCL21, CCL19, CCL27, CCL28, CXCL12, et al, which can also induce lymphangiogenesis in tumor-draining lymph nodes. To find ERK signal pathway whether regulates CCL21 expression, we used ERK inhibitor to treated HLECs. Our results showed that the CCL21 expression was significantly decreased by treated with ERK inhibitor in a dose-dependent manner. These results indicate that CCL21 is regulated by Nox4 via an ERK-dependent signaling pathway and may subsequently attract cancer cells with positive chemokine receptor into the lymphatic vessels. In our in vivo study, we found that breast cancer cells of the metastasis foci on lymph node and lung have strong stain for CCR7 which is the ligand of CCL21. Recent reports have shown that CCL21 and its ligand CCR7 together facilitate the cancer cells to distant metastasis. Our study further provides a novel recognition for the role of CCL21/CCR7 chemokine pair in breast cancer-associated lymphangiogenesis that might be relevant to future targeted therapies.

Finally, inhibition of Nox4 expression in breast cancer cells decreased tumor-related lymphangiogenesis and metastasis with no effect on tumor growth. Lymphangiogenesis in GKT137831-treated tumors reduced and was correlated with decreased tumor metastasis to lymph nodes and lung. Lymphatic vessel with LYVE-1/CD31-positive stain was obviously reduced in GKT137831-treated tumors on peritumoral tissue. However, blood vessel with CD31-positive stain was slightly decreased in GKT137831-treated tumors. The result indicated that Nox4 has a stronger effect on lymphatic vessels than on blood vessels (Figure 7).

In conclusion, lymphangiogenesis is a complex cellular process, including cell proliferation, migration, sprouting, and tube formation. In this process, HLECs have an active

**FIGURE 8** A schematic diagram of the proposed role of Nox4 on breast cancer lymphangiogenesis and metastasis. Nox4 produces ROS and enhances the expression p-ERK and CCL21 signaling and promote lymphatic sprouting. Tumor cells with CCR7 positive were attracted into lymphatic vessels and disseminated to distant organs.
role in the interactions of tumor cells with lymphatic vessels. The VEGFC-VEGFR3 and VEGFD-VEGFR3 axis now is the most researched lymphangiogenic signal pathway. Various inhibitors targeting the VEGFC-VEGFR3 and VEGFD-VEGFR3 axis have been developed that could be useful in the clinic. Clinical trials that involve clinical end points need to perform to validate the efficacy of anti-lymphangiogenesis for cancer therapy. In our study, Nox4 is a lymphangiogenic factor and inducing cancer cells metastasis may be via the following steps (Figure 8): by increasing tube formation and preexisting lymphatic vessel sprouting and providing a pathway for cancer cell metastasis; by increasing CCL21 expression in HLECs and newborn lymphatic vessels attract breast cancer cells with CCR7-positive draining to lymph nodes and spread to distant organs. Our result suggests that Nox4 may be a potential therapeutic target for reducing breast cancer cell metastasis to distant organs by inhibition of lymphangiogenesis.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Z. Yu and F. Yan conceived and designed the experiments. X. Wang wrote the manuscript. Z. Liu, J. Sun, F. Wang, and M. Bian performed the experiments and analyzed the data. X. Song analyzed the GEO data.

ORCID
Feng Yan  https://orcid.org/0000-0002-2756-3167
Zhiyong Yu  https://orcid.org/0000-0002-2569-9458

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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