SphK2/S1P promotes metastasis in triple negative breast cancer through PAK1/LIMK1/Cofilin1 signaling pathway

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SphK2/S1P promotes metastasis in triple negative breast cancer through PAK1/LIMK1/Cofilin1 signaling pathway

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Abstract

Background: Triple negative breast cancer (TNBC) features poor prognosis which partialy attributed to the high metastasis rate. However, there is no effective target for systemic TNBC therapy due to the absence of estrogen, progesterone, and human epidermal growth factor 2 receptors (ER, PR, HER-2) up to date. In the present study, we evaluated the role of sphingosine kinase 2 (SphK2) and its catalysate sphingosine-1-phosphate (S1P) in TNBC metastasis, and the antitumor activity of SphK2 specific inhibitor ABC294640 in TNBC metastasis.

Methods: The function of SphK2 and S1P in migration of TNBC cells was evaluated by Transwell migration and wound healing assays. The molecular mechanisms of SphK2/S1P mediating TNBC metastasis were investigated using cell line establishment, western blot, histological examination and immunohistochemistry assays. The antitumor activity of ABC294640 was examined in TNBC lung metastasis model *in vivo*.

Results: SphK2 regulated TNBC cells migration through the generation of S1P. Targeting SphK2 with ABC294640 inhibited TNBC lung metastasis *in vivo*. p21-activated kinase 1 (PAK1), p-Lin-11/Isl-1/Mec-3 kinase 1 (LIMK1) and Cofilin1 was the downstream signaling cascade of SphK2/S1P. Inhibition of PAK1 suppressed SphK2/S1P induced TNBC cells migration.

Conlusion: SphK2/S1P promotes TNBC metastasis through the activation of the PAK1/LIMK1/Cofilin1 signaling pathway. ABC294640 potently inhibits TNBC metastasis *in vivo* which could be developed as a novel agent for the clinical treatment

of TNBC.

Keywords: sphingosine kinase 2 (SphK2), sphingosine-1-phosphate (S1P), ABC294640, metastasis, triple negative breast cancer (TNBC)

Background

Breast cancer is the most common malignant tumor and the leading cause of cancer-related death among women worldwide [1]. Triple negative breast cancer (TNBC) is a unique subtype of breast cancer in which the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) are not expressed [2]. Although TNBC accounts for only 15~20% of breast cancers, it is characterized by profound invasion, poor prognosis, and short survival times . Moreover, TNBC patients could not receive endocrine and targeted therapies due to the lack of ER, PR and HER-2 [3]. Finding new targets for TNBC treatment is of great clinical significance for TNBC patients.

Accumulating evidence suggests that sphingosine-1-phosphate (S1P) is a potent bioactive lipid mediator and involved in cancer development and progression by regulating tumor proliferation, migration and angiogenesis [4]. Sphingosine kinase (SphK) is the key regulatory enzyme catalyzing the formation of S1P. To date, two isoforms of SphK, SphK1 and SphK2, have been identified [5]. The promoting functions of SphK1/S1P in TNBC are well defined by compelling evidence. Previous studies reported that SphK1/S1P promoted TNBC metastasis through the Notch signaling pathway [6] and that inhibition of SphK1 reduced TNBC cells growth through the ERK1/2 and AKT pathways [7]. However, the role of SphK2/S1P in these

processes is not decisively recognized.

Although early studies proposed a possible proapoptotic/anticancer function of SphK2 [8], accumulating research evidence suggests that SphK2/S1P has tumor-promoting activity similar to SphK1/S1P. Qiu [9] reported that SphK2 promoted cell growth, migration and invasion in papillary thyroid carcinoma. Knockdown of SphK2 inhibited the growth of human osteosarcoma cells [10]. Moreover, ABC294640, a selective inhibitor of SphK2, was shown to suppress the progression of many cancers [11]. Importantly, ABC294640 is currently under evaluation in a phase II clinical trial as an agent for the treatment of advanced hepatocellular carcinoma. The role of SphK2 in breast cancer has also been explored in recent studies. Antoon [12] reported that the expression of SphK2 was higher in TNBC cells than in human breast epithelial cells，high levels of SphK2/S1P improved the growth of TNBC cells . In addition, pharmacological inhibition of SphK2 slowed TNBC cells proliferation both *in vitro* and *in vivo* [13]. However, few studies focused on the effects of SphK2 on TNBC cell migration. Only Gao [14] reported that ablation of SphK2 inhibited the migration of MDA-MB-231 TNBC cells, but the underlying mechanisms and whether ABC294640 could reduce the metastasis of TNBC have not been well elucidated.

S1P can promote breast cancer metastasis by activating multiple signaling cascades. S1P has been reported to increase p21-activated kinase 1 (PAK1) activity [15, 16] and even directly stimulate PAK1 [17]. In the presence of active PAK1, the phosphorylation of both p-Lin-11/Isl-1/Mec-3 kinase 1 (LIMK1) and Cofilin1 is

greatly enhanced, which leads to actin cytoskeleton formation and cell motility [18]. Given the importance of PAK1 [19] and LIMK1 [20] in breast cancer metastasis, we examined whether the PAK1/LIMK1/Cofilin1 signaling pathway is the downstream signaling cascade of SphK2/S1P.

In the present study, we explored the role and potential molecular mechanisms of SphK2/S1P in the TNBC metastasis. Moerover, ABC294640 was used to examine the efficacy of targeting SphK2 for inhibiting the metastasis of TNBC.

Methods

Cell culture

Human breast carcinoma cell lines MDA-MB-231, BT-549 and mouse breast cancer cell line 4T1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/ml streptomycin (all from Wisent, St-Bruno, Canada). BT-549 and 4T1 were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium, other components of culture mediums were the same as MDA-MB-231. Cell were cultured at 37℃ in a humidified 5% CO_2 atmosphere.

siRNA transfection

SphK2 was downregulated by transfection with sequence-specific siRNA (GenePharma, Shanghai, China). siRNA against human SphK2 (targeted sequence: GGGUAGUGCCUGAUCAAUGTT, 5' to 3') and control siRNA was used. 4 μl of

Lipofectamine 2000 (Thermo Fisher, MA, USA) was mixed with 150 μl Opti-MEM (Wisent, St-Bruno, Canada) and incubated for 5 min at room temperature. siRNA was diluted in 150 μl Opti-MEM. Following 5 min of incubation, the diluted DNA was combined with diluted Lipofectamin 2000 (total volume = 300 μl). The solution was mixed gently and incubated for 20 min at room temperature and then added to a 6-well dish containing cells and medium. Real-time Quantitative PCR (RT-qPCR) and western blot assay were adopted to assess the knockdown efficiency.

Lentivirus transfection

Lentivirus transfection was used to obtain TNBC cells with stable ectopic SphK2 expression. Lentivirus expressing SphK2 and corresponding negative control virus were purchased from GeneChem (Shanghai, China). TNBC cells were plated in 6-well plates at a density of 2×10^5 cells per well and were subsequently transfected with lentivirus at a multiplicity of infection (MOI) of 10. Following 48 h of incubation, the antibiotic-resistant transfected cells were selected and enriched by applying culture medium containing puromycin. The overexpression efficiency of SphK2 were also confirmed by western blot assay and RT-qPCR.

Cell Counting Kit-8 (CCK-8) assay

For CCK-8 assay, the powder of IPA-3 (Selleck, MA, USA), ABC294640 (Selleck, MA, USA) and S1P (Avanti Polar Lipids, AL, USA) were dissolved in DMSO to make stock solutions at doses of 50 mM, 50 mM and 10 mM respectively. The final concentration of DMSO in the treatment medium was below 0.1%. TNBC cells in

DMEM containing 10% FBS were seeded into 96-well plates at a concentration of 1 × 10^4 cells per well and incubated for 24 h. The culture medium was replaced with fresh medium containing vehicle or testing reagents at indicated concentrations. After treating cells with different reagents or vehicle for 48 h, CCK-8 solution (10 μl/well) was added to the 96-well plates and incubated for 1 h to detect the viability of TNBC cells. The absorbance values at 450 nm were measured in a microplate reader (Bio-Rad, CA, USA), and cell viability was determined. Relative viability was normalized to the vehicle-treated control cells after background subtraction and was expressed as $OD_{test}/OD_{control}$*100%. Each treatment was performed in triplicate wells and three independent repeats of experiments were performed.

Protein isolation and western blot assay

The cells were lysed with 150 μl lysis buffer (Beyotime, Shanghai, China) containing 1% protease inhibitors (Thermo Fisher, MA, USA) on ice for 5 min following washing twice with ice-cold PBS. The cells were harvested, and centrifuged at 12,000 × g for 5 min at 4°C. The protein concentrations were determined using a BCA Kit (Beyotime, Shanghai, China). Equal amounts of protein (20 μg/lane) dissolved in 20 μl loading buffer (Beyotime, Shanghai, China) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China), transferred to polyvinylidene difluoride (PVDF) membranes (Roche Applied Science, Mannheim, Germany), and blocked with 5% nonfat milk for 1 h at room temperature. Immunoblotting was carried out by incubation overnight at 4°C with the indicated primary antibodies (Cell Signaling technology, MA, USA except noted): anti-PAK1,

anti-p-PAK1, anti-Cofilin1, anti-p-Cofilin1, anti-LIMK1 (abCam, CA, USA), anti-p-LIMK1 (abCam, CA, USA), anti-SphK1 (proteinTech, Wuhan, China), anti-SphK2 (proteinTech, Wuhan, China). The dilution of primary antibodies against SphK1 and SphK2 was 1:500. Other primary antibodies were diluted at 1:1000. After the incubation with primary antibodies, membranes were washed and incubated with HRP-linked secondary antibodies (1:5000 dilution, proteinTech, Wuhan, China) at room temperature for 1 h, the signals were developed with an enhanced chemiluminescence reagent (Biosharp, Beijing, China) under a chemiluminescence camera (Tanon, Beijing, China). The density of each band was measured using ImageJ software (National Institutes of Health, MD, USA), and was normalized to internal control (GAPDH) from the same sample. Three independent repeats of experiments were performed.

Real-time Quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol Reagent (Takara Bio, Otsu, Japan) and reverse transcribed into cDNA using the PrimeScript RT Master Mix (Takara Bio, Otsu, Japan). The relative mRNA expression levels were determined by RT-qPCR with the SYBR Green PCR Master Mix (Takara Bio, Otsu, Japan) on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, CA, USA). The relative mRNA levels were calculated by the $2^{-\Delta\Delta Cq}$ method with GAPDH as the internal control. Three independent repeats of experiments were performed.

Wound healing assay

A culture insert (Ibidi, Munich, Germany) was used to make a wound of 500 μm. The culture insert was put on the 24-well plates, then 2×10^5 cells were seeded in each culture insert and incubated for 24 h. After removing the culture insert, cells were allowed to grow in media without FBS for 24-48 h. The original area and migration area were measured by ImageJ software (National Institutes of Health, MD, USA), and the wound closure rates are shown according to the ratio of the migration area to the original area. Each treatment was performed in triplicate wells and three independent repeats of experiments were performed.

Transwell migration assay

Transwell migration assay was performed using a 6.5 mm Transwell insert with 8.0 μm pore polycarbonate membrane (Merck Millipore, MA, USA). 300 μl cell suspension containing 2×10^5 cells without FBS was added to the upper chamber and 800 μl medium containing 10% FBS was added to the lower chamber. After incubation for 48 h, cells on the lower side were fixed with 4% paraformaldehyde for 20 min, stained with crystal violet for 20 min. Pictures of each chamber were taken randomly for cell counting. Three independent repeats of experiments were performed.

Quantification of S1P by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Cells were washed twice with cold PBS, harvested, and centrifuged at 1000 rpm for 5 min at 4°C, then the cells was suspended in 100 μl distilled water. The cell suspension

was mixed with internal standard (1 ng/ml C17-S1P, Avanti Polar Lipids, AL, USA) and 65 μl methanol，then centrifuged at 1000 rpm for 5 min. S1P in the supernatant was quantified by LC-MS/MS as described previously at Virginia Commonwealth University Lipidomics Core [21].

Tumor xenograft model

Six-week-old female BALB/c mice, weighing approximately 20 g were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Mice were housed in sterile cages in laminar airflow hoods in a specific pathogen-free environment at 22-25°C, relative humidity 40-60% with a 12:12 hours day and night light cycle. The mice had free access to autoclaved water and commercial mice food (Xietong Biological, Nanjing, China). The protocol of this study was approved by Institutional Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School. 4T1 cells (2×10^5 cells in 100 μl PBS) were surgically implanted in the below mammary fat pads. When the tumors raised, mice were randomly assigned into two groups. Subsequently, ABC294640 (Selleck, MA, USA) at an oral dose of 40 mg/kg body weight or vehicle were administered three times a week. ABC294640 were suspended in an oral vehicle containing 2% DMSO + 30% PEG300 (Selleck, MA, USA) + 5% Tween 80 (Selleck, MA, USA) + ddH_2O. Tumor volume was measured with a digital caliper and calculated by use of the equation: (length × $width^2$)/2 three times a week. The body weight of mice was also measured three times a week. All mice were sacrificed by cervical dislocation under general anesthesia with isoflurane (RWD Life Science, Shenzhen, China) after 4

weeks of treatment, then tumors and their lungs were harvested and number of metastatic tumor nodules was recorded.

Histological examination (HE) and immunohistochemistry (IHC)

Tissues fixed with 4% paraformaldehyde were embedded in paraffin and cut into 5-μm-thick slices. For HE staining, the tissue slices were dewaxed in xylene, rehydrated with decreasing concentrations of ethanol, and washed with PBS. Then, the slices were stained with hematoxylin for 30 sec with agitation and rinsed with water. After that, the slices were stained with eosin for 10-30 sec with agitation and rinsed with water. After staining, the slices were dehydrated, mounted and covered with coverslips. IHC was carried out according to a published protocol [22]. Samples were incubated with a rabbit polyclonal antibody (Cell Signaling technology, MA, USA except noted) against Ki-67, TUNEL, SphK2 (proteinTech, Wuhan, China), p-PAK1, p-LIMK1 (abCam, CA, USA), p-Cofilin1. The Ki-67, TUNEL positive cells and integrated optical density (IOD) of SphK2, p-PAK1, p-LIMK1, p-Cofilin1 staining were analyzed by ImageJ software (National Institutes of Health, MD, USA).

Statistical analysis

Data of a representative independent experiment were analyzed using SPSS 19.0 statistical software (IBM, IL, USA) and expressed as the mean ± SD. Comparisons of different groups were performed by Student's t-test or ANOVA analysis. A P value lower than 0.05 ($P<0.05$) was considered to indicate a statistically significant difference.

Results

Knockdown of SphK2 suppresses TNBC cell migration

To investigate the role of SphK2 in the migration of TNBC cells, SphK2 siRNAs were transfected into two TNBC cell lines, MDA-MB-231 and BT-549. Successful knockdown of SphK2 in MDA-MB-231 and BT-549 cells was verified by RT-qPCR and western blot assay. SphK2 siRNA transfection resulted in significantly decreased expression of SphK2 at both the mRNA and protein levels in MDA-MB-231 and BT-549 cells, whereas the expression of SphK1 was not affected (Fig. 1a, b). Wound healing and Transwell migration assays were used to observe the migration ability. The cells migration in the SphK2 siRNA groups were markedly lower than that in the control groups (Fig. 1c, d), indicating that SphK2 plays an important role in TNBC cell migration.

Pharmacological inhibition of SphK2 decreases TNBC cell migration

In the present study, we also targeted SphK2 with ABC294640, a selective SphK2 inhibitor, to assess the facilitation of TNBC cell migration by SphK2. The CCK-8 assay was used to evaluate the effect of ABC294640 on the viability of TNBC cells, and a concentration of 12.5 μM was selected for the SphK2 inhibition experiment (Fig. 2a, no obvious inhibition of cell viability was observed at this concentration, relative cell viability of 12.5 μM ABC294640: 91.62% in MDA-MB-231 and 90.76% in BT-549 respectively). The wound healing and Transwell migration assays results showed that the migration ability of both MDA-MB-231 and BT-549 cells was decreased after exposure to ABC294640 for 24 h (Fig. 2b, c).

SphK2 overexpression promotes the migration of TNBC cells

MDA-MB-231 and BT-549 TNBC cells were stably transfected with LV-SphK2 lentivirus to enhance the expression of SphK2. The expression of SphK2 in MDA-MB-231 and BT-549 cells was confirmed to be significantly increased compared with that in control cells by western blot assay and qRT-PCR (Fig. 3a, b). The migration ability of LV-SphK2 TNBC cells was significantly increased, further supporting the importance of SphK2 in TNBC cell migration (Fig. 3c, d).

S1P production is positively correlated with SphK2 expression in TNBC cells

Since the main biological function of SphK2 is to catalyze the generation of S1P, we further evaluated the role of S1P in SphK2-induced TNBC cells migration. The S1P level in TNBC cells after inhibition or overexpression of SphK2 was measured by LC-MS/MS. As expected, pharmacological inhibition of SphK2 by ABC294640 decreased the S1P production in MDA-MB-231 and BT-549 (lowered by 0.49 and 0.5 than control group respectively, Fig. 4a). Meanwhile, overexpression of SphK2 increased the S1P level in these two cell lines (1.74-fold and 1.65-fold higher than control group respectively, Fig. 4b). These data suggested that S1P production of TNBC cells is positively correlated with SphK2.

Exogenous S1P promotes the migration of TNBC cells and reverses the reduced migration ability of SphK2 knockdown TNBC cells

Because inhibition of SphK2 suppressed S1P production and SphK2 overexpression promoted S1P production in TNBC cells, we hypothesized that the effect of SphK2 on

the migration ability of TNBC cells was achieved by S1P. To determine whether S1P could promote the migration of TNBC cells, 4 μM exogenous S1P was added to the cell culture medium during Transwell migration and wound healing assays. Exogenous S1P stimulation markedly improved the migration ability of TNBC cells (Fig. 5a, b), suggesting that S1P promotes TNBC cell migration. Furthermore, when S1P was added to the medium of SphK2 knockdown TNBC cells, the impaired migration ability was reversed (Fig. 5c, d). Collectively, the above results indicated that SphK2 promoted the migration of TNBC cells by catalyzing the production of S1P.

Pharmacological inhibition of PAK1 decreases the migration of TNBC cells and reduces the increased migration ability of TNBC cells due to SphK2 overexpression or exogenous S1P stimulation

Based on some researches that S1P could promote cell migration through PAK1 activation, we wondered whether PAK1 was activated by SphK2/S1P in TNBC cells. To clarify the roles of PAK1 in the modulation of TNBC metastasis, TNBC cells were exposed to the PAK1 inhibitor IPA-3, and a concentration of 5 μM was selected for the PAK1 inhibition experiment (Fig. 6a, cell viability of 5 μM IPA-3 group: 94.70% in MDA-MB-231 and 90.77% in BT-549 respectively, showing no significant inhibition of cell viability). After exposure to 5 μM IPA-3 for 24 h, the phosphorylation of PAK1 declined (Fig. 6b), indicating PAK1 activity was decreased. The results of Transwell migration and wound healing assays showed that inhibition of PAK1 activity by IPA-3 decreased the migration of TNBC cells (Fig. 6c, d). In

addition, the increased migration ability of TNBC cells due to SphK2 overexpression or exogenous S1P stimulation was also reversed by IPA-3 (Fig. 6e-h). Therefore, PAK1 was confirmed to play an important role in the migration of TNBC cells and could be a downstream molecule of SphK2/S1P.

SphK2/S1P regulates the migration of TNBC cells through the activation of PAK1/LIMK1/Cofilin1 signaling

Currently, the molecular mechanisms of SphK2-mediated TNBC cell migration are still unknown. Since PAK1 is the downstream molecule of SphK2/S1P, and PAK1 can promote cell motility through the activation of LIMK1/Cofilin1, we hypothesized that SphK2/S1P could regulate the migration of TNBC cells in a PAK1/LIMK1/Cofilin1 signaling-dependent manner. Therefore, we measured the phosphorylation levels of PAK1, LIMK1, and Cofilin1 in TNBC cells subjected to different treatments. The western blot assay results showed that the phosphorylation of PAK1, LIMK1, and Cofilin1 was decreased in TNBC cells with SphK2 knockdown or inhibition (Fig. 7a, b) but increased in SphK2 overexpressed TNBC cells (Fig. 7c). Exogenous S1P stimulation also increased the phosphorylation of PAK1, LIMK1, and Cofilin1 (Fig. 7d). Based on these results, we concluded that the PAK1/LIMK1/Cofilin1 signaling pathway participates in SphK2/S1P-mediated MDA-MB-231 and BT-549 cell migration.

The SphK2 selective inhibitor ABC294640 reduces lung metastasis of TNBC cells *in vivo*

To determine the therapeutic potential of pharmacological inhibition of SphK2 in TNBC, the effects of ABC294640 on tumor metastasis were examined in a 4T1 xenograft mouse model. Mice with established, sized-matched 4T1 tumors were divided into two groups and treated with ABC294640 or vehicle respectively. There was no distinct difference in the tumor volume between the two groups during the 4 weeks treatment (Fig. 8a), while an obvious increase in the number and size of lung metastatic nodules was observed in the vehicle control group (Fig. 8b) at the end of the treatment, indicating ABC294640 could decrease TNBC metastasis in vivo. Moreover, we performed a histological assessment on orthotopic tumors and lung metastatic nodules. No significant difference in TUNEL or Ki-67 staining was observed between tumors from ABC294640-treated mice and those from control mice, indicating that treatment with 40 mg/kg ABC294640 had little influence on tumor apoptosis or proliferation (Fig. 8c). Similar to the results in vitro, orthotopic tumors and lung metastatic nodules from the control group exhibited stronger staining for p-PAK1, p-LIMK1 and p-Cofilin1 than that from ABC2945640-treated group (Fig. 8d), further supporting the hypothesis that SphK2/S1P regulates the metastasis of TNBC through the activation of PAK1/LIMK1/Cofilin1 signaling.

Discussion

TNBC is highly aggressive cancer that lack targeted therapies [23], therefore, the need to identify efficient targets for TNBC therapy is urgent. Our research showed that SphK2, a key enzyme that converts sphingosine to S1P, is involved in TNBC cell migration. Similar to the results observed in human renal cancer cells [14], ablation of

SphK2 decreased the migration of TNBC cells. In addition, pharmacological inhibition of SphK2 with ABC294640 also diminished TNBC cell migration in the present study. That inhibition of SphK2 activity by other inhibitors also caused an inhibitory effect on migration in HeLa cells have been reported [24]. Moreover, we found that overexpression of SphK2 led to the increased migration ability of TNBC cells. Consistent with our results, upregulation of SphK2 partially increased the migration of papillary thyroid carcinoma cells [9]. The results in the present study and other researches suggest that SphK2 is involved in tumor metastasis and that SphK2 might be a therapeutic target in TNBC.

Accumulating evidence demonstrates that S1P is a critical second messenger that regulates the migration of various cells, not only cancer cells [25, 26] but also myofibroblasts [27], dendritic cells [28] and stem cells [29]. Moreover, it has been reported that S1P generated from SphK1 accelerated breast cancer cell migration [30]. However, whether SphK2/S1P play the same role in TNBC cells migration is unclear. Here, we report for the first time that SphK2/S1P regulates the migration of TNBC cells. We demonstrated that targeting SphK2 with ABC294640 reduced the S1P level and that overexpression of SphK2 increased the S1P level in TNBC cells, indicating that the S1P level was positively correlated with SphK2. The positive correlation between SphK2 and S1P also existed in murine adenocarcinoma cells [11] and colorectal cancer cells [31] and so on. However, Gao [14] reported contrasting results that knockdown of SphK2 led to increased S1P production in renal carcinoma cells owing to the elevated expression of SphK1. In the present study, SphK2 knockdown

had minimal influence on the expression of SphK1. Hait [32] also showed that SphK2 knockdown did not affect the expression and activity of SphK1 in breast cancer cells. We further suggested that exogenous S1P stimulation promoted the migration of TNBC cells and reversed the reduced migration ability induced by SphK2 knockdown. Collectively, these results suggest that SphK2 promotes the migration of TNBC cells through the production of S1P.

Based on the important roles of SphK2/S1P in TNBC cells migration, we further uncovered the downstream molecular mechanisms. Our data showed that the PAK1/LIMK1/Cofilin1 cascade, an important regulator of the actin cytoskeleton and motility [33], was activated by SphK2/S1P in TNBC cells. S1P has been reported to increase PAK1 activity [15] and even directly stimulate PAK1 to induce cell lamellipodia formation and movement [17]. Consistent with these studies, we found that exogenous S1P stimulation increased the phosphorylation of PAK1. In addition, inhibition of PAK1 by IPA-3 decreased TNBC cells migration and even reversed the increased migration ability of TNBC cells due to SphK2 overexpression or S1P stimulation. These results indicate that PAK1 is the downstream target of SphK2/S1P and contributes to the migration of TNBC cells. Furthermore, the phosphorylation of PAK1, LIMK1 and Cofilin1 was increased in SphK2-overexpressed or S1P-stimulated TNBC cells and decreased in SphK2-inhibited TNBC cells, and was even decreased *in vivo* due to the administration of ABC294640. These results powerfully proved that the PAK1/LIMK1/Cofilin1 cascade is the downstream signaling pathway of SphK2/S1P. Generally, we elucidate a novel mechanism linking

SphK2/S1P to PAK1/LIMK1/Cofilin1 in TNBC cell migration.

ABC294640, the selective inhibitor of SphK2, was found to have broad antitumor activity. Application of ABC294640 inhibited cell growth *in vitro* and *in vivo* in various cancers such as colorectal cancer [31], non-small cell lung cancer [34], and prostate cancer [35]. ABC294640 also has an effective inhibitory effect on cancer cell migration *in vitro* [11]. Remarkably, the present study provided the first demonstration that pharmacological inhibition of SphK2 by ABC294640 at a dose of 40 mg/kg decrease TNBC metastasis *in vivo*, indicating the high clinical value of ABC294640 for the treatment of TNBC.

Conclusion

Collectively, we report that SphK2/S1P promotes the migration of TNBC cells through the activation of the PAK1/LIMK1/Cofilin1 signaling pathway. Targeting SphK2 with ABC294640 inhibits TNBC xenograft metastasis *in vivo*, and ABC294640 has the potential to be a novel agent for the clinical treatment of TNBC.

Abbreviations

TNBC: Triple negative breast cancer; ER: Estrogen receptor; PR: Progesterone receptor; HER-2: Human epidermal growth factor 2 receptor; SphK: Sphingosine kinase; S1P: Sphingosine-1-phosphate; PAK1: P21-activated kinase 1; LIMK1: p-Lin-11/Isl-1/Mec-3 kinase 1;

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Authors' Contributions

ZW, JW, CJ and WS designed that study. All authors performed the experiments, collected and analyzed the data. WS, DM, YC wrote the manuscript. ZW and JW approved the manuscript. CJ and ZW offered the funding support.

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Availability of data and materials

The datasets used during this study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate

The animal study was reviewed and approved by Institutional Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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549 ## Figure Legends

550 **Fig. 1** Effects of SphK2 knockdown on the migration of TNBC cells. **a** SphK2 and
551 SphK1 protein expression in TNBC cells transfected with SphK2 siRNA or control
552 vector was determined by western blot assay. **b** Relative SphK2 and SphK1 mRNA
553 expression levels were determined by RT-qPCR in TNBC cells transfected with
554 SphK2 siRNA or control vector. **c** The migration of TNBC cells transfected with
555 SphK2 siRNA or control vector was evaluated by a wound healing assay. **d** The
556 migration of TNBC cells transfected with SphK2 siRNA or control vector was
557 evaluated by a Transwell assay. The result of each assay is representative for three
558 independent experiments. The error bar represents mean ± SD from a representative
559 experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS: not significant.

Fig. 2 Effects of SphK2 inhibition on TNBC cell migration. **a** The effect of ABC294640 on TNBC cell viability was evaluated by a CCK-8 assay. **b** TNBC cells were treated with the SphK2 inhibitor ABC294640 (12.5 μM), and migration was evaluated by a wound healing assay. **c** TNBC cells were exposed to ABC294640 (12.5 μM), and migration was evaluated by a Transwell assay. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 3 Effects of SphK2 overexpression on TNBC cell migration. **a** SphK2 protein expression in TNBC cells transfected with LV-SphK2 lentivirus or control vector was measured by western blot assay. **b** Relative SphK2 mRNA expression levels in TNBC cells transfected with LV-SphK2 lentivirus or control vector were measured by RT-qPCR. **c, d** The migration of TNBC cells transfected with LV-SphK2 lentivirus or control lentivirus was evaluated by wound healing assay and Transwell assay. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 4 Influence of SphK2 pharmacological inhibition and overexpression on S1P production in TNBC cells. **a** TNBC cells were exposed to ABC294640 (12.5 μM) for 24 h, and intracellular S1P production was measured via LC-MS/MS. **b** S1P analyses were conducted in SphK2-overexpressing and control TNBC cells via LC-MS/MS. The result of each assay is representative for three independent experiments. The error

bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 5 Effects of exogenous S1P on the migration of TNBC cells. **a, b** TNBC cell migration was examined after treatment with 4 μM S1P by wound healing assay and Transwell assay. **c, d** TNBC cells infected with SphK2 siRNA were exposed to 4 μM S1P. Cell migration was evaluated by wound healing assay and Transwell assay. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 6 Effects of IPA-3 on the migration of TNBC cells. **a** The influence of IPA-3 on TNBC cell viability was evaluated by a CCK-8 assay. **b** The level of phosphorylated PAK1 protein in TNBC cells treated with 5 μM IPA-3 for 24 h was measured by western blot assay. **c, d** TNBC cell migration was examined after treatment with 5 μM IPA-3 by wound healing assay and Transwell assay. **e, f** SphK2-overexpressing TNBC cells were exposed to 5 μM IPA-3, and migration was also evaluated. **g, h** TNBC cells treated with S1P were exposed to 5 μM IPA-3, and migration was evaluated. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 7 The phosphorylation of PAK1, LIMK1, and Cofilin1 in different cell groups. **a** The phosphorylation of PAK1, LIMK1, and Cofilin1 in TNBC cells infected with

SphK2 siRNA was examined by western blott assay. **b** The phosphorylation of PAK1, LIMK1, and Cofilin1 in TNBC cells treated with ABC294640 was examined. **c** The phosphorylation of PAK1, LIMK1, and Cofilin1 in SphK2-overexpressing TNBC cells was examined. **d** The phosphorylation of PAK1, LIMK1, and Cofilin1 in TNBC cells exposed to S1P was examined. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS: not significant.

Fig. 8 Inhibition of SphK2 activity reduces TNBC metastasis and the phosphorylation of PAK1, LIMK1, and Cofilin1 *in vivo.* **a** Representative photographs of the orthotopic tumors were obtained, and the volumes were recorded. **b** Representative photographs of the lungs were obtained, and the metastatic nodules were counted. **c** Tumor apoptosis and proliferation were evaluated by TUNEL and Ki-67 staining. **d** The levels of SphK2 and phosphorylated PAK1, LIMK1, and Cofilin1 in orthotopic tumors and lung metastatic nodules were measured. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figures

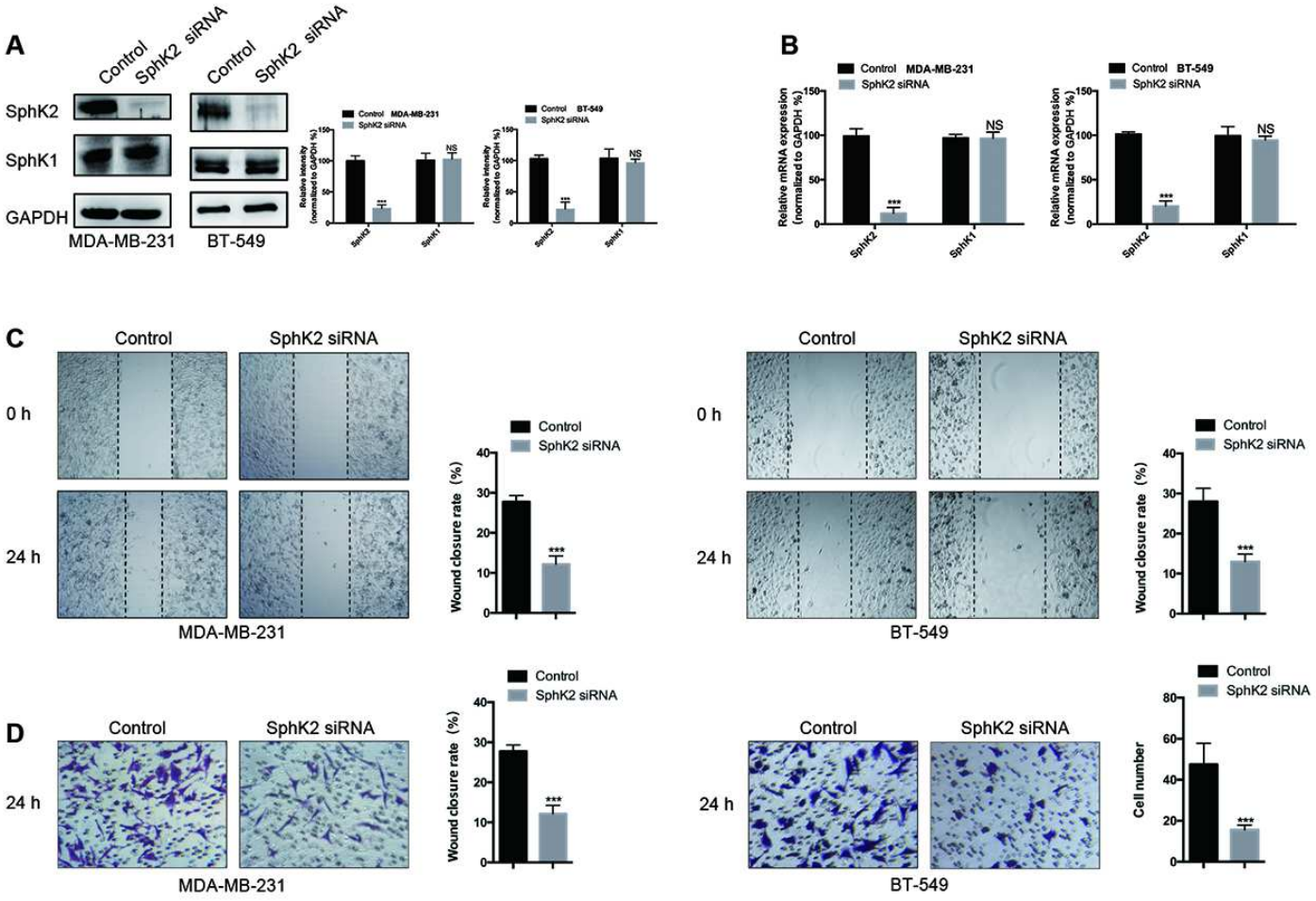

Figure 1

Effects of SphK2 knockdown on the migration of TNBC cells. a SphK2 and SphK1 protein expression in TNBC cells transfected with SphK2 siRNA or control vector was determined by western blot assay. b Relative SphK2 and SphK1 mRNA expression levels were determined by RT-qPCR in TNBC cells transfected with SphK2 siRNA or control vector. c The migration of TNBC cells transfected with SphK2 siRNA or control vector was evaluated by a wound healing assay. d The migration of TNBC cells transfected with SphK2 siRNA or control vector was evaluated by a Transwell assay. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, NS: not significant.

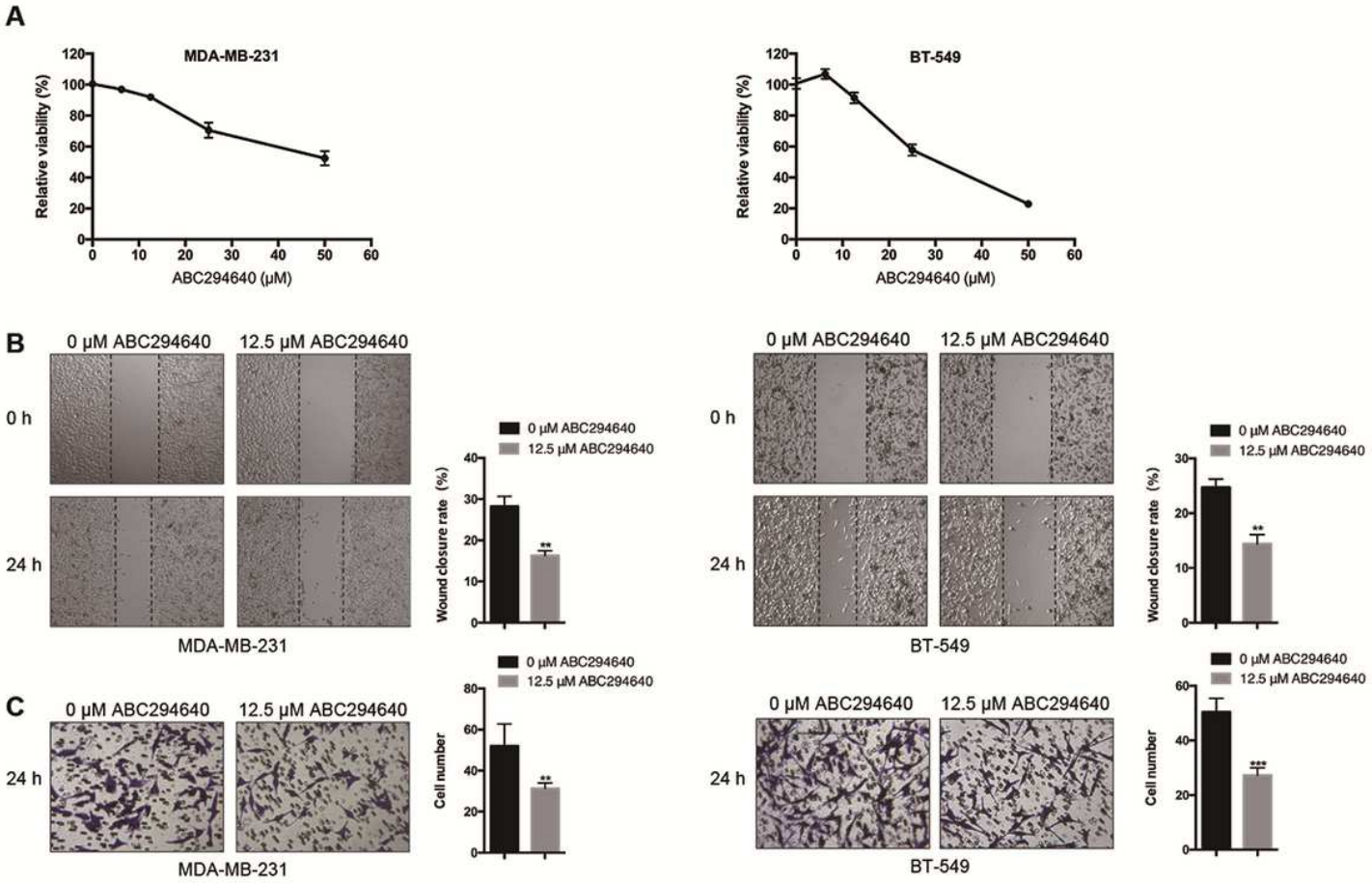

Figure 2

Effects of SphK2 inhibition on TNBC cell migration. a The effect of ABC294640 on TNBC cell viability was evaluated by a CCK-8 assay. b TNBC cells were treated with the SphK2 inhibitor ABC294640 (12.5 μM), and migration was evaluated by a wound healing assay. c TNBC cells were exposed to ABC294640 (12.5 μM), and migration was evaluated by a Transwell assay. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

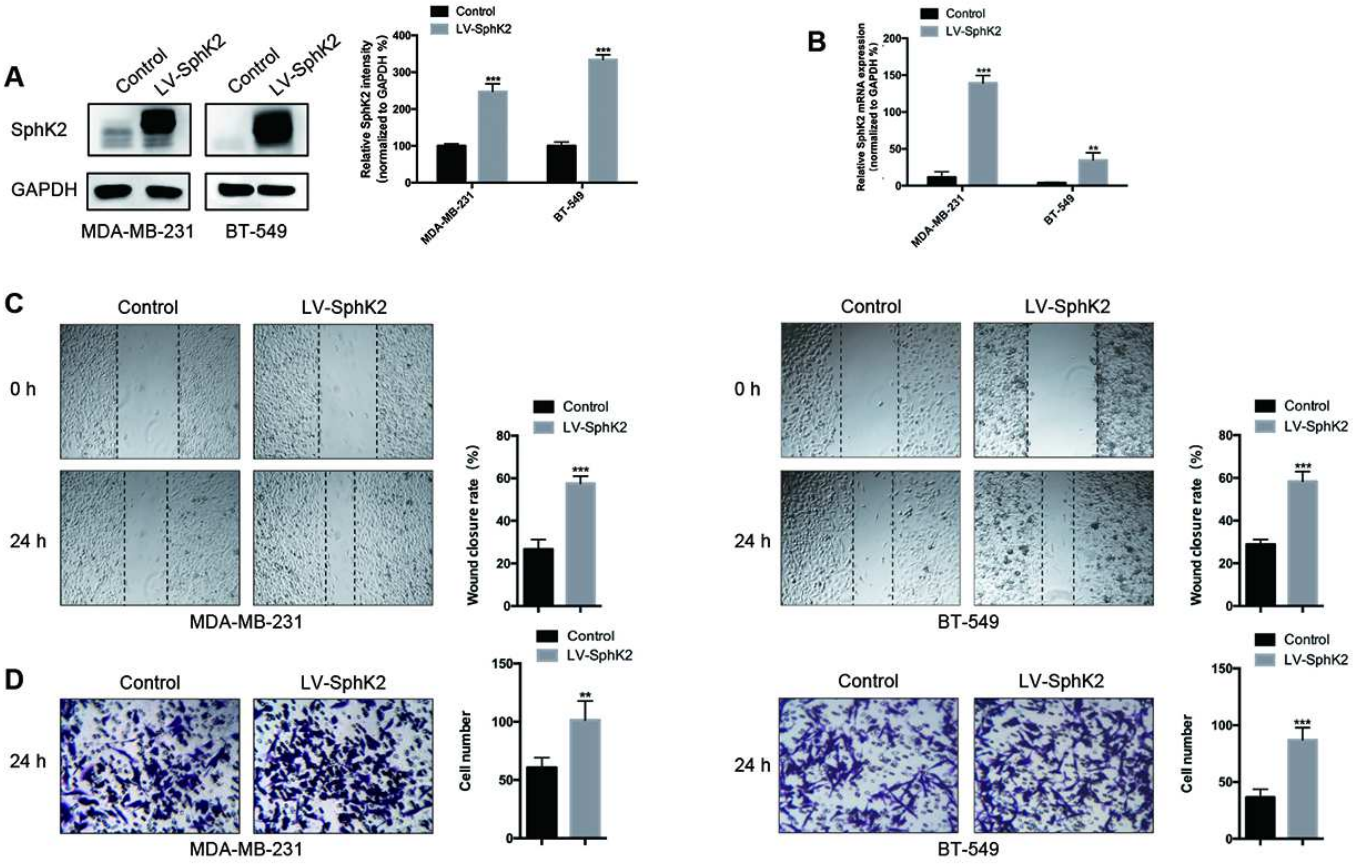

Figure 3

Effects of SphK2 overexpression on TNBC cell migration. a SphK2 protein expression in TNBC cells transfected with LV-SphK2 lentivirus or control vector was measured by western blot assay. b Relative SphK2 mRNA expression levels in TNBC cells transfected with LV-SphK2 lentivirus or control vector were measured by RT-qPCR. c, d The migration of TNBC cells transfected with LV-SphK2 lentivirus or control lentivirus was evaluated by wound healing assay and Transwell assay. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** 575 $p<0.001$.

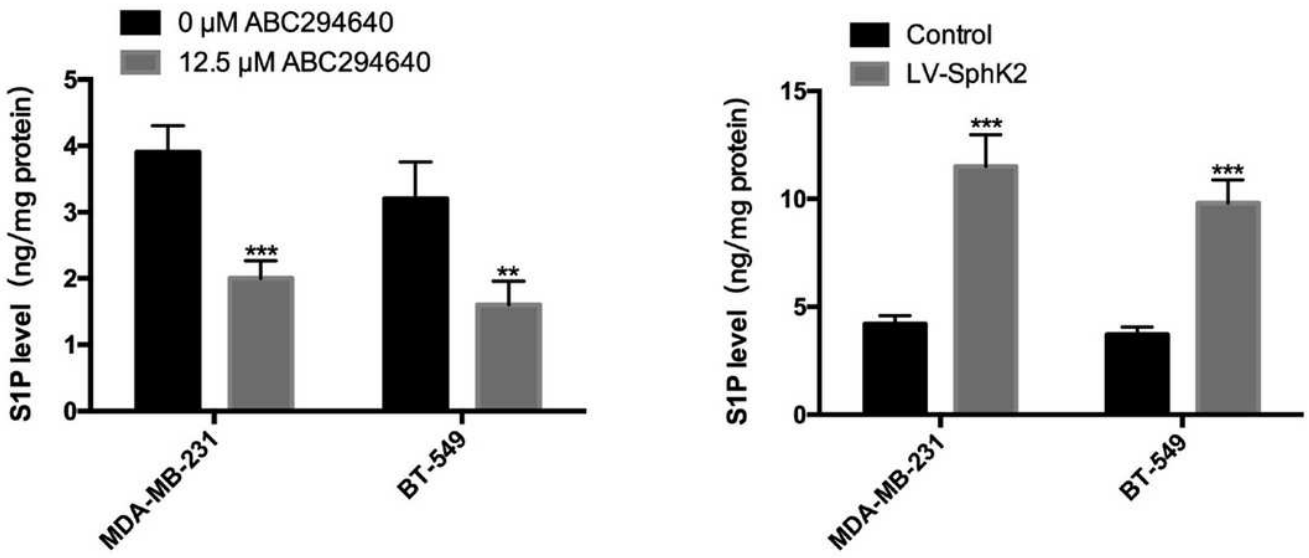

Figure 4

Influence of SphK2 pharmacological inhibition and overexpression on S1P production in TNBC cells. a TNBC cells were exposed to ABC294640 (12.5 μM) for 24 h, and intracellular S1P production was measured via LC-MS/MS. b S1P analyses were conducted in SphK2-overexpressing and control TNBC cells via LC-MS/MS. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** 582 $p<0.001$.

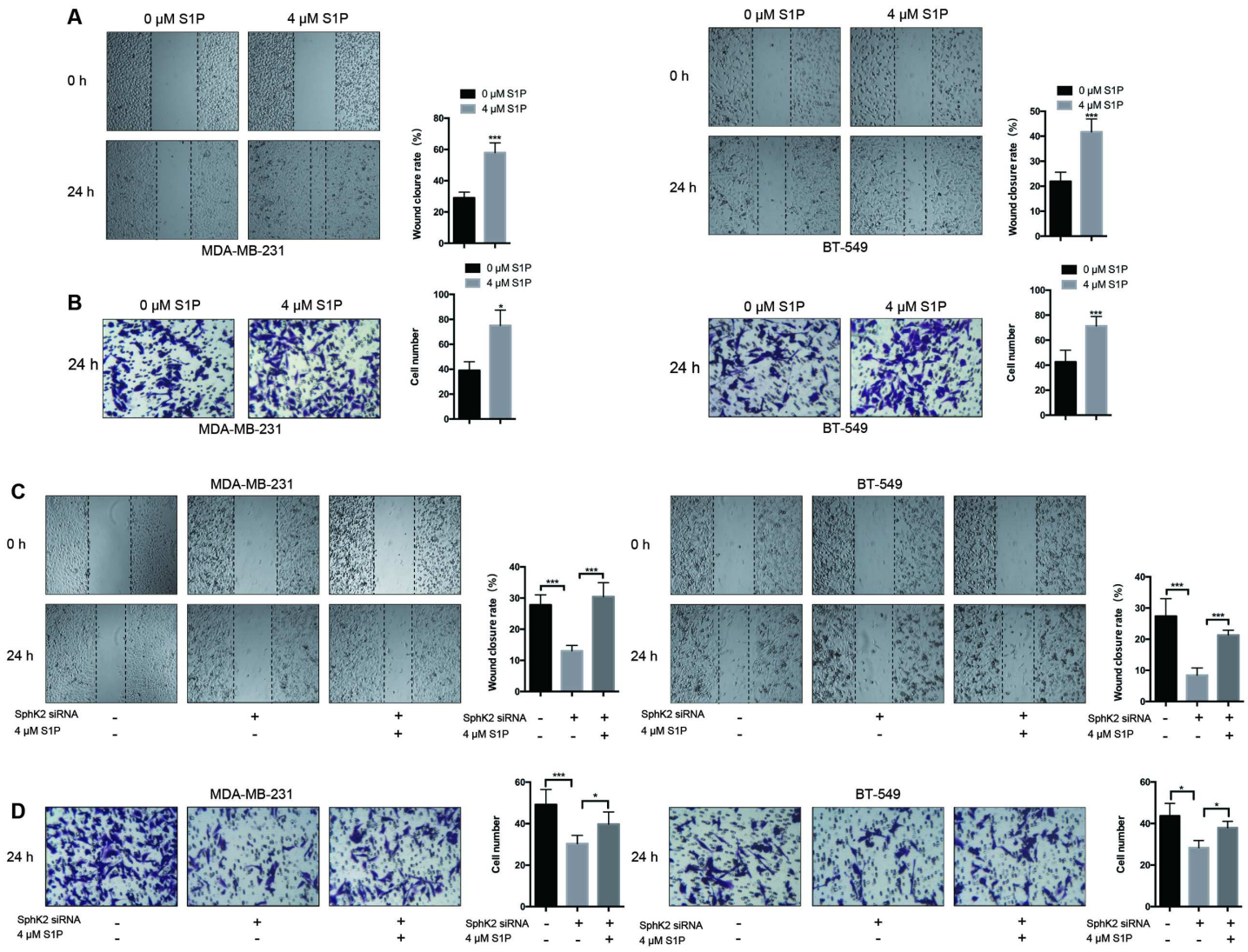

Figure 5

Effects of exogenous S1P on the migration of TNBC cells. a, b TNBC cell migration was examined after treatment with 4 μM S1P by wound healing assay and Transwell assay. c, d TNBC cells infected with SphK2 siRNA were exposed to 4 μM S1P. Cell migration was evaluated by wound healing assay and Transwell assay. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** 589 $p<0.001$.

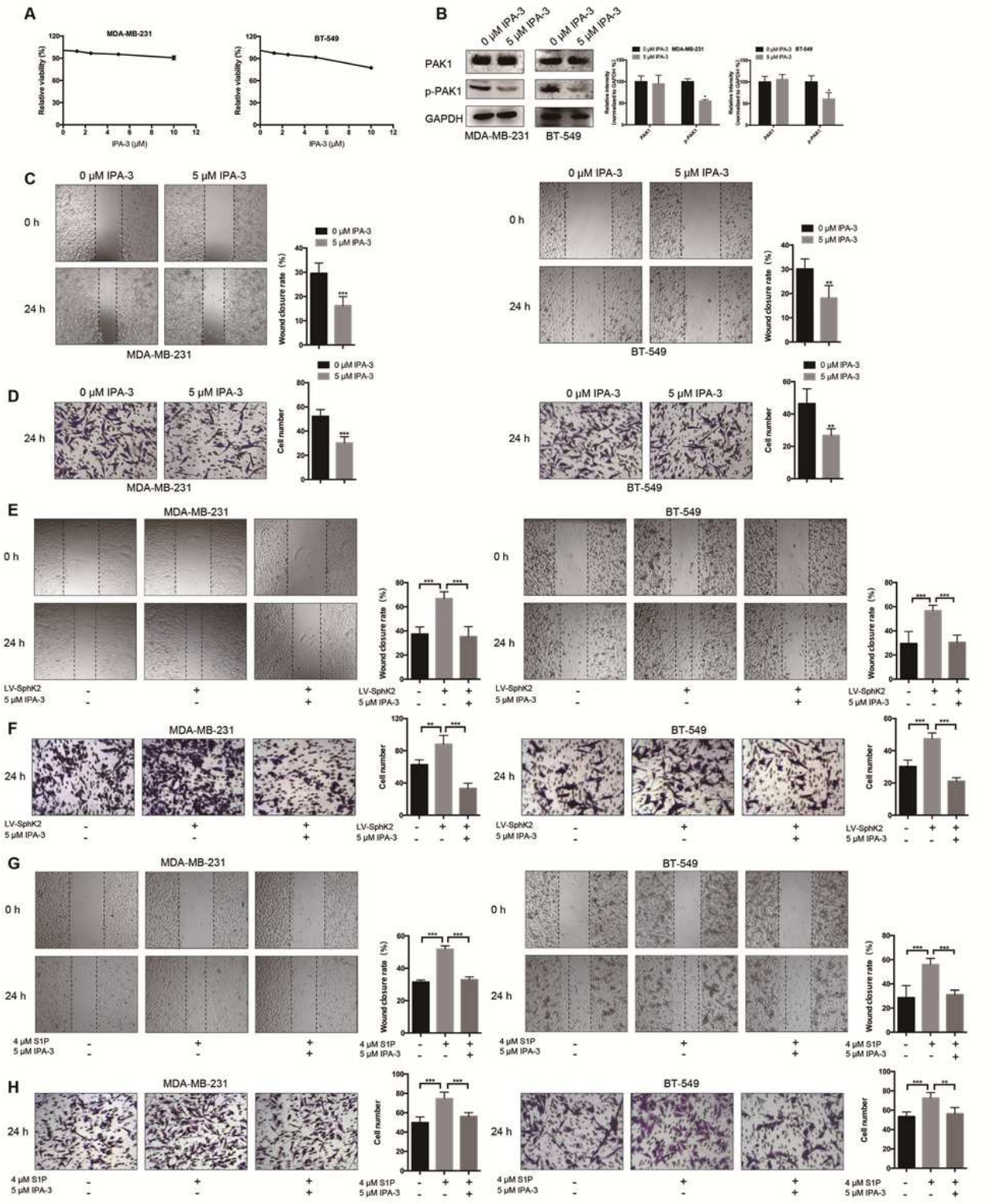

Figure 6

Effects of IPA-3 on the migration of TNBC cells. a The influence of IPA-3 on TNBC cell viability was evaluated by a CCK-8 assay. b The level of phosphorylated PAK1 protein in TNBC cells treated with 5 μM IPA-3 for 24 h was measured by western blot assay. c, d TNBC cell migration was examined after treatment with 5 μM IPA-3 by wound healing assay and Transwell assay. e, f SphK2-overexpressing TNBC cells were exposed to 5 μM IPA-3, and migration was also evaluated. g, h TNBC cells treated with S1P

were exposed to 5 μM IPA-3, and migration was evaluated. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * p<0.05, ** p<0.01, *** 599 p<0.001.

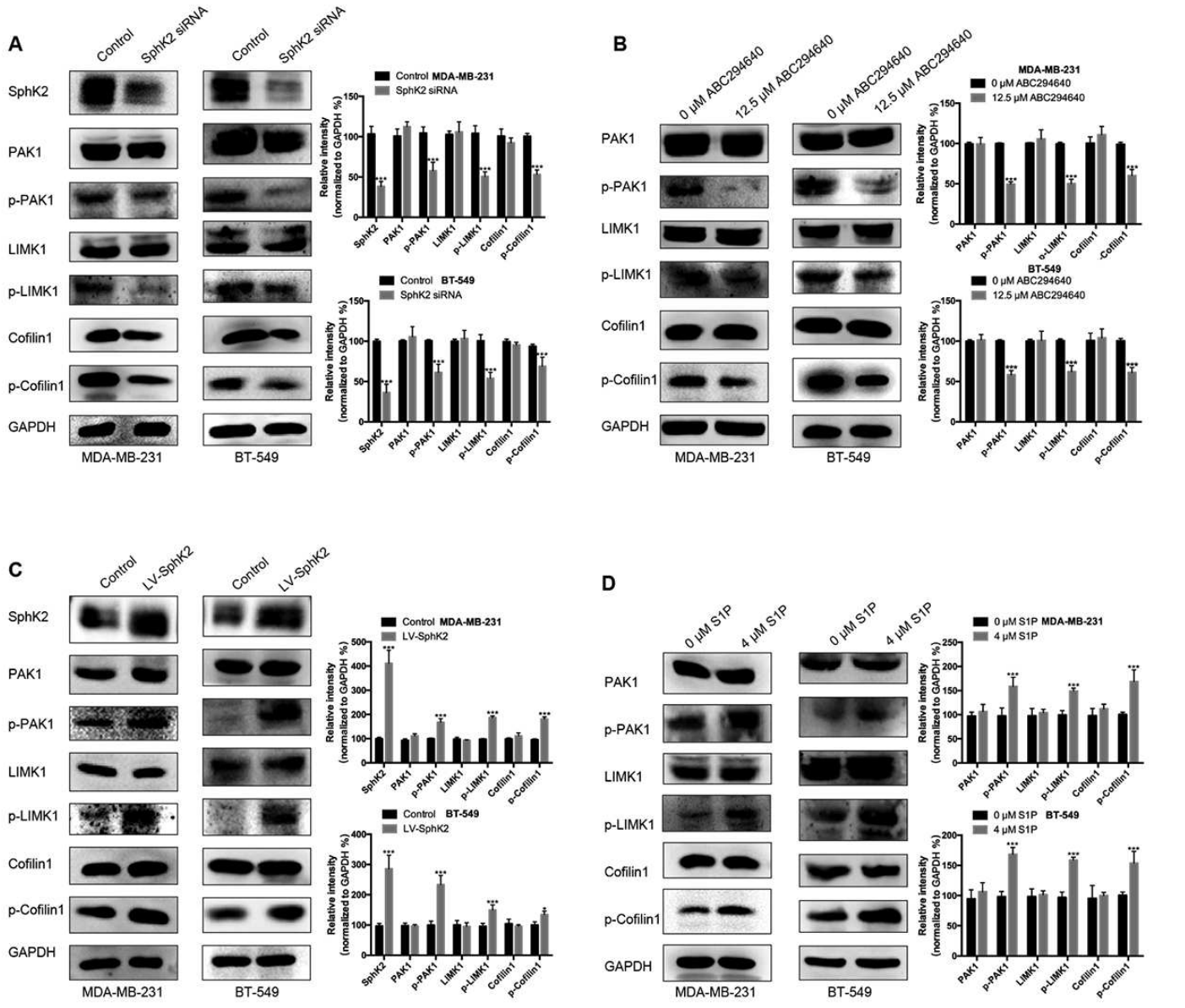

Figure 7

The phosphorylation of PAK1, LIMK1, and Cofilin1 in different cell groups. a The phosphorylation of PAK1, LIMK1, and Cofilin1 in TNBC cells infected with SphK2 siRNA was examined by western blott assay. b The phosphorylation of PAK1, LIMK1, and Cofilin1 in TNBC cells treated with ABC294640 was examined. c The phosphorylation of PAK1, LIMK1, and Cofilin1 in SphK2-overexpressing TNBC cells was examined. d The phosphorylation of PAK1, LIMK1, and Cofilin1 in TNBC cells exposed to S1P was examined. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * p<0.05, ** p<0.01, *** p<0.001, NS: not significant.

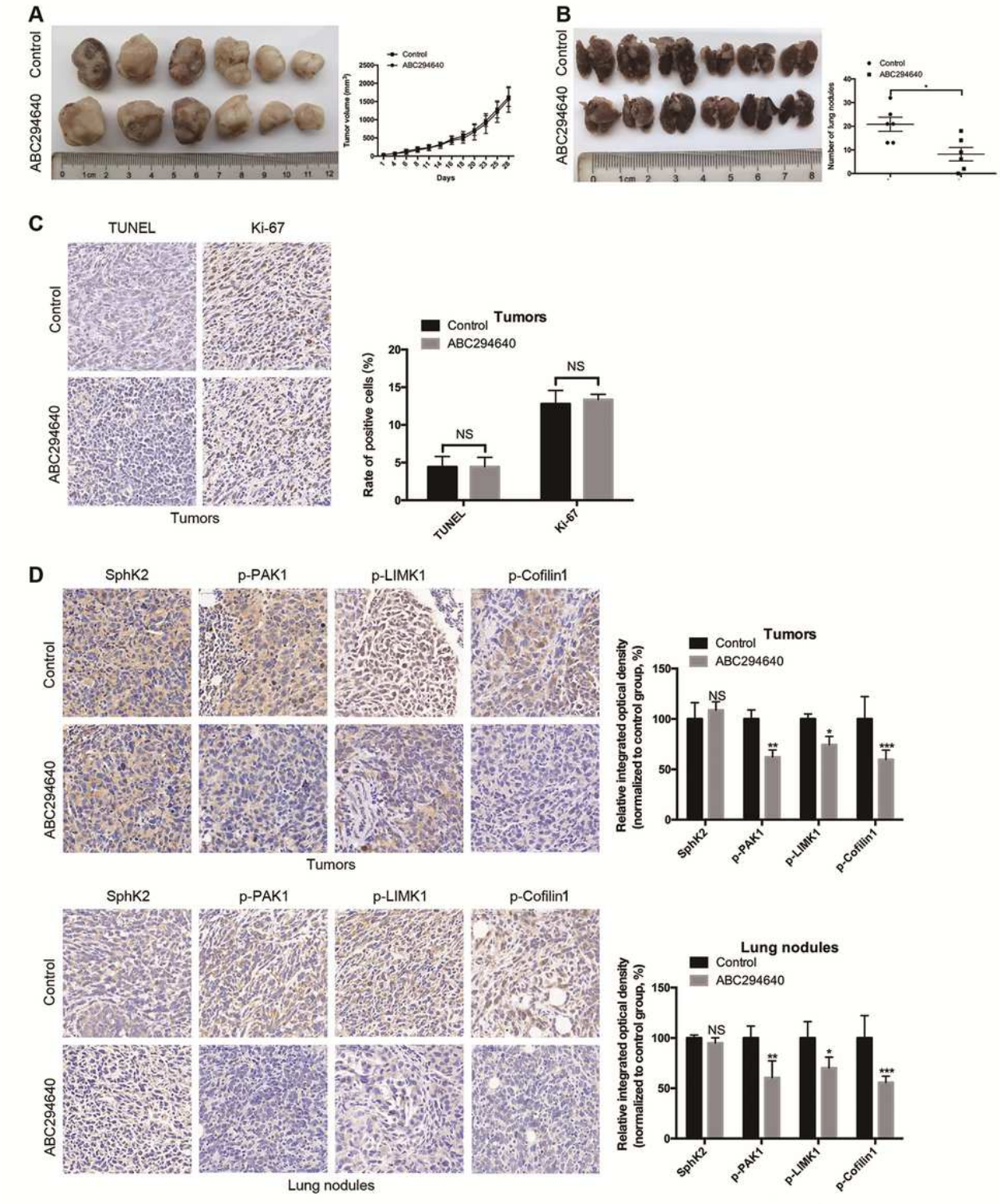

Figure 8

Inhibition of SphK2 activity reduces TNBC metastasis and the phosphorylation of PAK1, LIMK1, and Cofilin1 in vivo. a Representative photographs of the orthotopic tumors were obtained, and the volumes were recorded. b Representative photographs of the lungs were obtained, and the metastatic nodules were counted. c Tumor apoptosis and proliferation were evaluated by TUNEL and Ki-67 staining. d The levels of SphK2 and phosphorylated PAK1, LIMK1, and Cofilin1 in orthotopic tumors and lung metastatic

nodules were measured. The result of each assay is representative for three independent experiments. The error bar represents mean ± SD from a representative experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.