Soluble CD109 released from lung adenocarcinoma cells activates the STAT3 signaling pathway to promote lymphatic endothelial cell migration

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Abstract. Lung adenocarcinoma (LUAD), the predominant subtype of non-small cell lung cancer, frequently metastasizes to regional lymph nodes through the lymphatic system. CD109 is upregulated in LUAD and is associated with poor prognosis; however, its effects on lymphatic endothelial cells remain poorly characterized. The present study investigated whether soluble CD109 released from LUAD cells influences lymphatic endothelial cell function and explored the underlying molecular mechanisms. CD109 was overexpressed in human LUAD A549 cells using an adenoviral vector. Conditioned medium (CD109-CM) was collected following demonstration of CD109 expression and secretion using reverse transcription-quantitative PCR and western blotting. The effects of soluble CD109 on human dermal lymphatic endothelial cells (HDLECs) were evaluated using proliferation and migration assays. Involvement of the Janus kinase 2 (JAK2)/STAT3 signaling pathway was examined using western blotting and further evaluated using Stattic, a STAT3 inhibitor. CD109 overexpression in A549 cells significantly increased both cellular and secreted CD109 levels. Despite CD109-CM having not significantly affected HDLEC proliferation, it markedly enhanced HDLEC migration. Mechanistically, CD109-CM increased JAK2 and STAT3 phosphorylation in HDLECs, consistent with activation of STAT3 signaling. Pharmacological inhibition of STAT3 signaling with Stattic attenuated STAT3 phosphorylation and abolished the CD109-CM-induced increase in HDLEC migration. Collectively, these findings demonstrated that soluble CD109 released from LUAD cells may promote lymphatic endothelial cell migration through activation of JAK2/STAT3 signaling. The present *in vitro* findings identified the CD109-JAK2/STAT3 axis as a potential mechanism through which LUAD cells modulate lymphatic endothelial cell behavior, thus supporting further *in vivo* investigation of CD109 as a potential therapeutic target in LUAD.

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Introduction

Non-small cell lung cancer (NSCLC) is classified into three key histological subtypes; namely, adenocarcinoma, squamous cell carcinoma and large cell carcinoma (1,2). Lung adenocarcinoma (LUAD) is the predominant subtype,

accounting for ~55% of NSCLC cases (3). LUAD is a highly heterogeneous malignancy with a strong propensity for invasion and distant metastasis (4). Distant metastasis represents a key cause of disease-associated mortality in patients with LUAD (5). Lymphangiogenesis is considered a notable step in cancer metastasis (6). Lymphangiogenesis and dilation of existing lymphatic vessels surrounding primary tumors may create favorable conditions for metastatic dissemination (7). Proliferation of lymphatic endothelial cells contributes to lymphatic vessel expansion and remodeling (8), while peritumoral sprouting of new lymphatic vessels facilitates tumor cell entry into, as well as dissemination through, the lymphatic circulation (9). Accordingly, targeting pathways involved in tumor-associated lymphangiogenesis represents a potential anticancer strategy (10). Elucidating the molecular mechanisms regulating lymphatic endothelial cell behavior may therefore provide insights into the metastatic progression of LUAD.

The cell-surface antigen CD109 belongs to the α2-macroglobulin/C3, C4 and C5 superfamily of thioester-containing proteins (11). CD109 is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein implicated in cellular signaling and protein trafficking (12,13). In healthy human tissues, CD109 expression is restricted to a limited number of cell types, including those in the salivary, lacrimal and bronchial secretory glands (14). By contrast, elevated CD109 expression has been reported in a number of malignancies, including pancreatic cancer and hepatocellular carcinoma, as well as malignant melanoma and squamous cell carcinomas of the uterus, esophagus, stomach, gallbladder, penis and skin (15-17). These findings suggest that CD109 may serve as a cancer-associated marker and contribute to tumor progression and heterogeneity (18). In patients with LUAD, CD109 overexpression has been associated with tumor progression, distant metastasis and poor prognosis (19). GPI anchoring enables membrane-associated proteins to be selectively released through phospholipase-mediated cleavage (20,21). CD109 can also undergo furin-mediated processing to generate 180- and 25-kDa forms, with the 180-kDa form being released into the culture medium (22). However, the functional contribution of soluble CD109 to LUAD-associated lymphatic endothelial cell behavior remains unclear.

The Janus kinase (JAK)/STAT pathway was initially identified as a key downstream signaling pathway activated in response to cytokines, including IFN-α, IFN-γ and IL-6 (23-26). JAK/STAT signaling regulates numerous tumor-associated biological processes and has been implicated in tumor angiogenesis and immune suppression (27). In particular, STAT3 is frequently activated in cancer cells and has been implicated in lymphangiogenesis through regulation of the STAT3-VEGF-C axis (28,29). Previous studies have further demonstrated that STAT3 functions as an important downstream effector of CD109-mediated pro-metastatic signaling and that JAK kinases contribute to CD109-induced STAT3 activation (30). However, the potential involvement of JAK2/STAT3 signaling in the effects of soluble CD109 on lymphatic endothelial cells remains poorly understood.

Therefore, the present study aimed to investigate the effects of soluble CD109 released from LUAD cells on lymphatic endothelial cell proliferation and migration, whilst exploring the underlying molecular mechanisms and focusing specifically on the STAT3 signaling pathway. The present findings will thus provide *in vitro* evidence for a potential CD109-STAT3 regulatory axis through which LUAD cells modulate lymphatic endothelial cell migration and establish a basis for further investigation of its contribution to LUAD-associated lymphatic metastasis.

Materials and methods

Cells and cell-culture conditions. A549, the human lung adenocarcinoma cell line was obtained from the Life Sciences Cell Resource Center (Shanghai, China). Human dermal lymphatic endothelial cells (HDLECs) were obtained from Shanghai Baililai Biotechnology Co., Ltd. HDLECs were maintained in DMEM (Corning, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). A549 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). All cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Stattic, a STAT3 inhibitor, was purchased from APExBIO Technology LLC. Where indicated, cells were pretreated with 10 μM Stattic for 1 h at 37˚C before subsequent experiments.

Cell transfection and collection of CM. A customized adenoviral vector overexpressing CD109 was purchased from Shanghai GeneChem Co., Ltd. The plasmid backbone for the CD109 overexpression is presented in Table I. An enhanced infection solution (cat. no. REVG0002; Shanghai GeneChem Co., Ltd was used for adenoviral transfection, according to the protocol of the manufacturer. A549 cells were transfected with a CD109-overexpressing adenovirus (ADV-CD109) or control empty adenovirus (ADV) in RPMI-1640 medium. For subsequent cellular assays, cells were harvested 48 h after transfection. CM was collected 48 h after transfection and filtered through a 0.2-μm filter before subsequent experiments.

Cell proliferation assay. HDLECs were seeded into 96-well plates at a density of $3x10^3$ cells/well and cultured overnight at 37˚C with 5% CO_2. The following day, the culture medium was replaced with 90 μl fresh control-conditioned medium (CON-CM) or CD109-CM. Following treatment for 24 h, cell proliferation was evaluated using Cell Counting Kit-8 (CCK-8) and MTT assays. For the CCK-8 assay, 10 μl CCK-8 solution (Dojindo Laboratories, Inc.) was added to each well, followed by incubation at 37˚C for 4 h. Optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). For the MTT assay, 10 μl MTT solution (Cayman Chemical Company) was added to each well and incubated at 37˚C for 4 h. The supernatant was subsequently removed and the resulting formazan crystals were dissolved in 110 μl dimethyl sulfoxide (Beijing Solarbio Science & Technology Co., Ltd.). OD was measured at 490 nm.

Wound healing assay. Cell migration was evaluated using a wound healing assay. HDLECs were seeded into 6-well plates at a density of $4x10^5$ cells/well. HDLECs were starved for 2 h at 37˚C with serum-free DMEM. A scratch was created

Table I. Plasmid backbone for CD109 overexpression.

Primer name	Direction	Sequence (5'-3')
Amplification primer	Forward	AGGTCGACTCTAGAGGATCCCGCCACCATGCAGGGCCCACCGCTCCTGACCG
	Reverse	TCCTTGATGTCCATACCCAGCCAAAGTTCCATAAAGTACAGAAG
Identification primer	Forward	CCAGGAAGAGTGATTCATAGTG
	Reverse	CCTTATA GTCCTTATCATCGTC

in the cell monolayer using a sterile 200-μl pipette tip. Cells were subsequently incubated for 12 h with CM collected from the control or CD109 groups. The distance migrated into the wound area was evaluated using a light microscope (Olympus Corporation) at x100 magnification. Digital images were analyzed using ImageJ software (version 1.54p; National Institutes of Health) and the average migration distance was quantified.

Transwell migration assay. Cell migration was further evaluated using Transwell chambers in 24-well plates (Costar; Corning, Inc.) equipped with polycarbonate membranes with 8.0-μm pores. HDLECs were seeded into the upper chambers at a density of $1x10^5$ cells/well. DMEM supplemented with 10% FBS and chemoattractant was added to the lower chambers. Following incubation at 37˚C for 24 h, cells that had migrated to the lower surface of the membrane were fixed and stained with 0.1% crystal violet solution (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 20 min. Cell migration was quantified through microscopic enumeration of the stained cells. Photographs were taken using an inverted microscope (Olympus Corporation) connected to a DXM1200 digital camera (Nikon Corporation). Measurements were performed on digital images using ImageJ software (version 1.54p; National Institutes of Health). All images were acquired at x100 magnification.

Western blotting and Coomassie blue staining. A549 cells, together with its culture supernatants and HDLECs, were collected and lysed or prepared in ice-cold RIPA buffer containing 0.1% SDS (Beyotime Biotechnology) supplemented with 1% PMSF (Beyotime Biotechnology). The lysates were centrifuged at 15,000 x g at 4˚C for 15 min and the supernatants were collected and used for quantification of total proteins through a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Following heat denaturation at 95˚C for 15 min, equal amounts of protein (10 μg) from each sample were separated using 10% SDS-PAGE (Bio-Rad Laboratories, Inc.). For Coomassie blue staining, gels were incubated with 0.25% Coomassie Brilliant Blue R-250 staining solution (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 20 min and subsequently destained until distinct protein bands were visible. For western blotting, proteins were transferred onto PVDF membranes (MilliporeSigma; Merck KGaA). Membranes were blocked with TBST (Tween 20) and 5% non-fat milk for 2 h at room temperature and subsequently incubated with primary antibodies overnight at 4˚C. Following washing with TBST, membranes were incubated with an HRP-conjugated Goat Anti-Rat IgG (H+L) secondary antibody (cat. no. SA00001-15; 1:8,000; Proteintech Group, Inc.) for 2 h at room temperature. Protein bands were visualized using a GE Amersham Imager 600 (Amersham; Cytiva). The primary antibodies used were as follows: Sheep anti-CD109 (cat. no. AF7717; 1:5,000; R&D Systems), rabbit anti-phospho-STAT3 (cat. no. 9145; 1:2,000; Cell Signaling Technology, Inc.), mouse anti-total STAT3 (cat. no. 9139; 1:1,000; Cell Signaling Technology, Inc.), rabbit anti-phospho-JAK2 (cat. no. ab32101; 1:2,000; Abcam), rabbit anti-total JAK2 (cat. no. ab108596; 1:1,000; Abcam) and rabbit anti-GAPDH (cat. no. 10494-1-AP; 1:8,000; Proteintech Group, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from A549 cells and HDLECs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd.). The reverse transcription procedure was conducted as 2 min at 42˚C, 15 min at 37˚C, 5 sec at 85˚C and 30 min at 4˚C. qPCR was performed using the ViiA7 DX Real-Time PCR System (Thermo Fisher Scientific, Inc.) with SYBR Green PCR Master Mix (Vazyme Biotech Co., Ltd.). The thermocycling conditions were as follows: 95˚C for 30 sec, 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. GAPDH was used as the internal reference gene and relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (31). Primer sequences are presented in Table II.

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version, 9.0.0; Dotmatics). Data are presented as the mean ± SEM. Comparisons between two groups were performed using unpaired Student's t-tests. Unless otherwise stated, n represents the number of independent biological replicates and all experiments were performed at least three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CD109 overexpression in A549 cells increases cellular expression and soluble release. As CD109 is a GPI-anchored protein that can undergo proteolytic release (32), its expression in A549 cells and presence in CM were examined following CD109 overexpression. A549 cells were transfected with ADV-CD109 or empty ADV. RT-qPCR analysis demonstrated significantly increased CD109 mRNA expression in

Table II. Primer sequences used in reverse transcription-quantitative PCR.

Primer name	Direction	Sequence (5'-3')
Human CD109	Forward	GAAGCCATCTCTCAACTTCACA
	Reverse	TTCCACTGTTAGATCCGCTCC
Human GAPDH	Forward	TGATGACATCAAGAAGGTGGTGAAG
	Reverse	TCCTTGGAGGCCATGTGGGCCAT

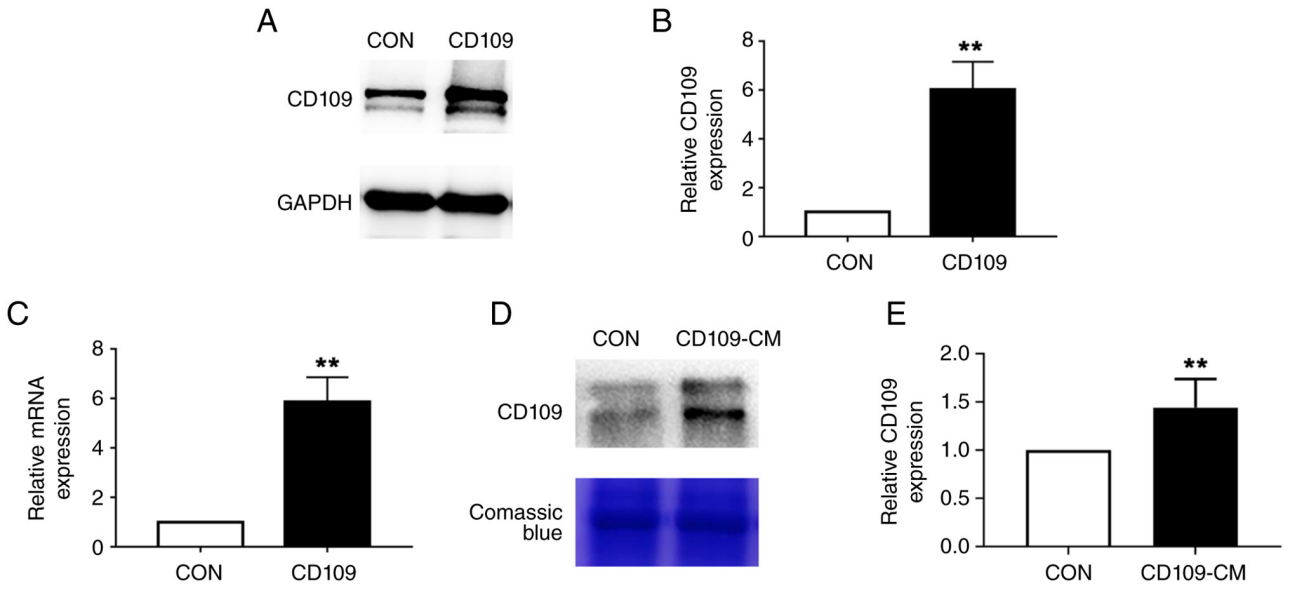


Figure 1. Overexpression of CD109 in A549 cells. (A) Representative western blotting analysis of CD109 protein expression in A549 cells following transfection with CD109-overexpressing adenovirus or CON empty adenovirus. (B) Quantification of CD109 protein expression (n=6; **P<0.01 vs. CON). (C) Reverse transcription-quantitative PCR analysis of CD109 mRNA expression following adenoviral transfection (n=4; **P<0.01 vs. CON). (D) Western blotting analysis of soluble CD109 in CM collected 48 h after adenoviral transfection. Coomassie Brilliant Blue staining was used as a protein-loading CON for CM samples. (E) Quantification of soluble CD109 protein expression (n=6; **P<0.01 vs. control). CON, control; CM, conditioned medium.

ADV-CD109-transfected cells compared with the control (Fig. 1C), while western blotting further demonstrated significantly increased cellular CD109 protein levels (Fig. 1A and B). Western blotting analysis of concentrated CM also demonstrated significantly higher soluble CD109 levels in CM from ADV-CD109-transfected cells (CD109-CM) compared with CM from CON-CM (Fig. 1D and E). These findings demonstrated successful CD109 overexpression in A549 cells and increased release of soluble CD109 into the CM.

CM from CD109-overexpressing A549 cells promotes HDLEC migration. To investigate the effects of CM from CD109-overexpressing A549 cells on lymphatic endothelial cell behavior, HDLECs were treated with CD109-CM or CON-CM. Neither the MTT nor CCK-8 assay demonstrated a significant difference in HDLEC proliferation between the CD109-CM and CON-CM groups (Fig. 2A and B). By contrast, wound healing assays demonstrated significantly increased HDLEC migration following treatment with CD109-CM compared with CON-CM (Fig. 2C and D). Consistently, Transwell assays demonstrated a significant increase in the number of HDLECs migrating through the polycarbonate membrane following CD109-CM treatment (Fig. 2E and F). Collectively, these findings indicated that CM from CD109-overexpressing A549 cells promoted HDLEC migration without notably affecting proliferation.

CM from CD109-overexpressing A549 cells activates JAK2/STAT3 signaling in HDLECs. To investigate whether JAK2/STAT3 signaling is involved in the effects of CD109-CM on HDLECs, the phosphorylation of JAK2 and STAT3 was examined using western blotting. Treatment with CD109-CM significantly increased the phosphorylation of JAK2 at Tyr1007/1008 and STAT3 at Tyr705 compared with CON-CM, whereas total JAK2 and STAT3 protein levels remained unchanged (Fig. 3A and B). These findings indicated that CM from CD109-overexpressing A549 cells activated JAK2/STAT3 signaling in HDLECs.

STAT3 inhibition attenuates CD109-CM-induced HDLEC migration. To further evaluate the involvement of STAT3 signaling in CD109-CM-induced HDLEC migration, HDLECs were pretreated with 10 μM Stattic, a STAT3 inhibitor. Stattic treatment attenuated the CD109-CM-induced increase in STAT3 phosphorylation (Fig. 4A). Consistently, STAT3 inhibition significantly attenuated the enhanced HDLEC migration observed following CD109-CM treatment in both wound healing (Fig. 4B and C) and Transwell assays (Fig. 4D and E).

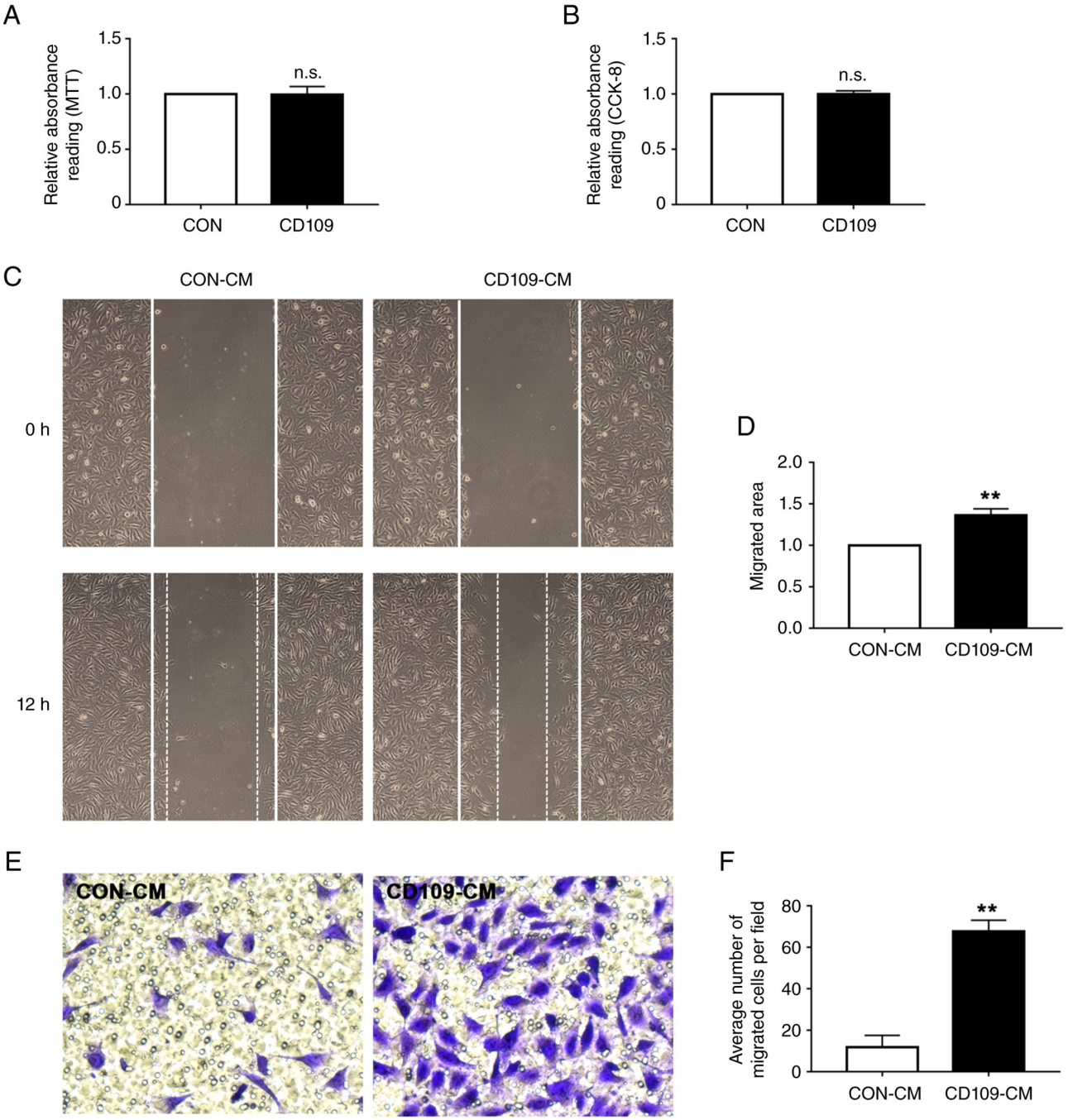


Figure 2. CM from CD109-overexpressing A549 cells promotes HDLEC migration without affecting proliferation. (A) MTT assay of HDLECs treated with CON-CM or CD109-CM for 24 h (n=5; n.s.). (B) CCK-8 assay of HDLECs treated with CON-CM or CD109-CM for 24 h (n=5; n.s.). (C) Representative images of the wound healing assay following treatment with CON-CM (CM from CON ADV-transfected A549 cells) or CD109-CM (CM from ADV-CD109-transfected A549 cells) for 12 h. Solid and dashed lines indicate the wound boundaries at 0 and 12 h, respectively (magnification, x100). (D) Quantification of HDLEC migration in the wound healing assay (n=5; **P<0.01 vs. CON). (E) Representative images of the Transwell migration assay following treatment with CON-CM or CD109-CM for 24 h. (F) Quantification of migrated HDLECs in the Transwell assay (n=4; **P<0.01 vs. CON). CCK-8, Cell Counting Kit-8; CM, conditioned medium; CON, control; HDLEC, human dermal lymphatic endothelial cell; n.s., not significant; ADV, adenovirus.

These findings supported the involvement of STAT3 signaling in the pro-migratory effects associated with CD109-CM and are consistent with a role for the JAK2/STAT3 signaling axis in regulating HDLEC migration.

Discussion

The present study established a CD109-overexpression model in LUAD cells to investigate the effects of CD109-containing CM on lymphatic endothelial cells and the underlying molecular mechanisms. To model paracrine interactions between LUAD cells and lymphatic endothelial cells *in vitro*, CM from CD109-overexpressing A549 cells was applied to HDLECs. CD109 overexpression increased both cellular CD109 expression and the abundance of soluble CD109 in CM. Functionally, CD109-CM promoted HDLEC migration without markedly affecting proliferation and was accompanied by increased JAK2 and STAT3 phosphorylation. In addition, pharmacological

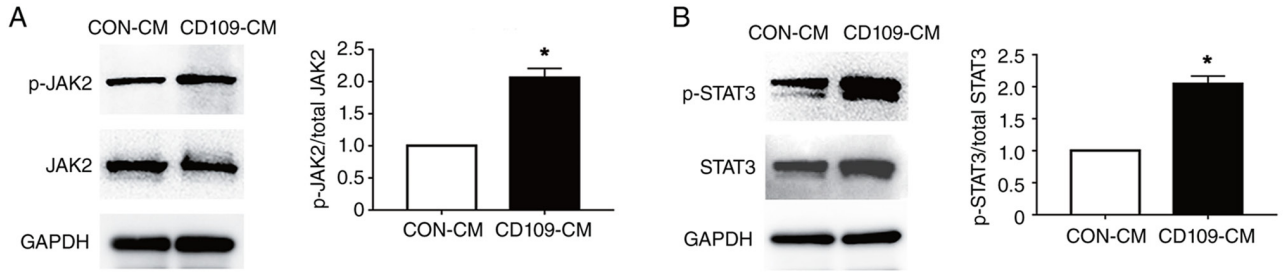


Figure 3. CM from CD109-overexpressing A549 cells activates JAK2/STAT3 signaling in HDLECs. Western blotting analysis of total and (A) p-JAK2 or (B) p-STAT3 in HDLECs following treatment with CON-CM (CM from CON ADV-transfected A549 cells) or CD109-CM (CM from ADV-CD109-transfected A549 cells) for 24 h. GAPDH was used as a loading control (n=4; *P<0.05 vs. CON). JAK, Janus kinase; HDLEC, human dermal lymphatic endothelial cell; p-, phosphorylated; CM, conditioned medium; CON, control; ADV, adenovirus.

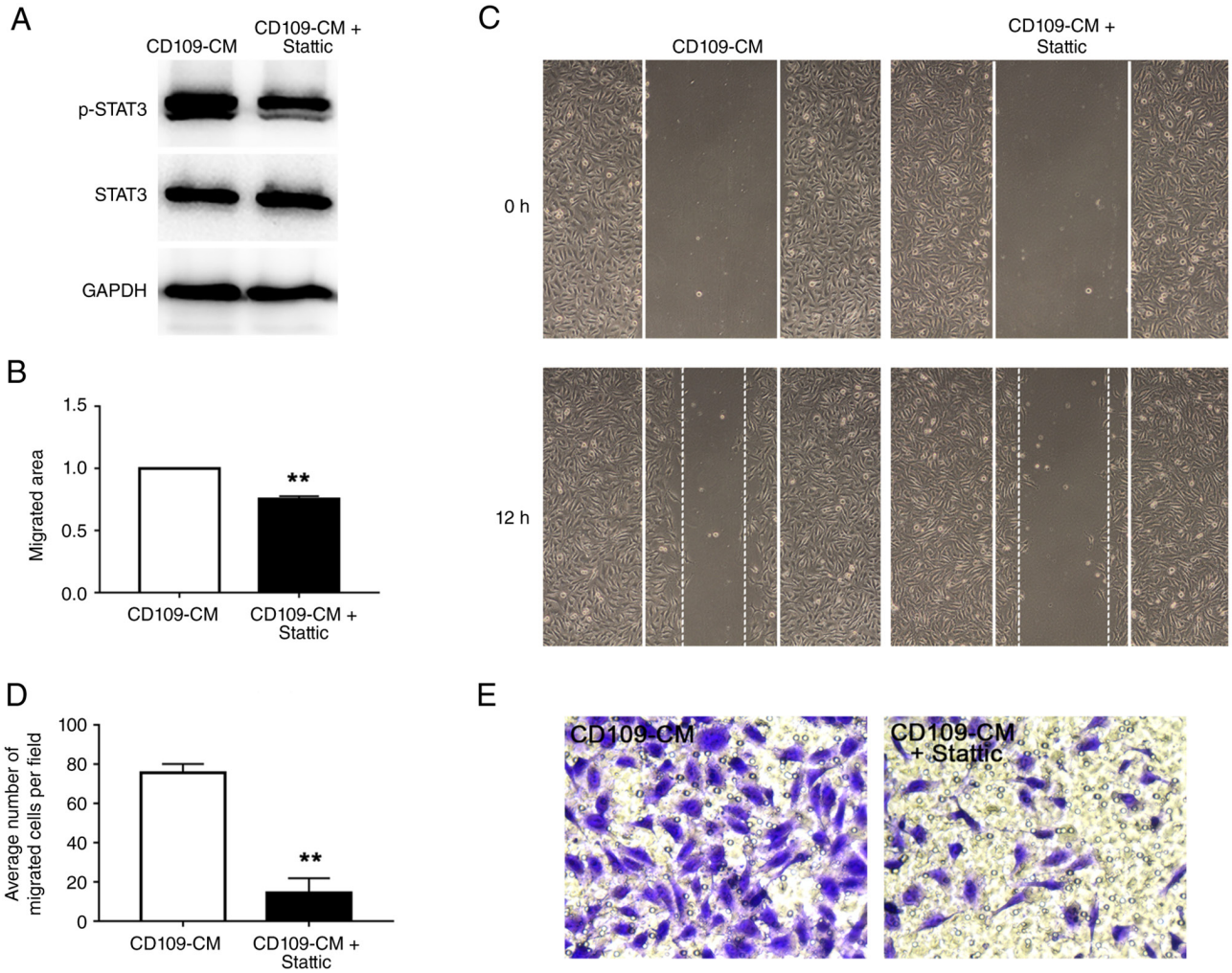


Figure 4. STAT3 inhibition attenuates CD109-CM-induced HDLEC migration. (A) Western blotting analysis of p-STAT3 and total STAT3 in HDLECs treated with CD109-CM (CM from adenovirus-CD109-transfected A549 cells) in the presence or absence of Stattic. GAPDH was used as a loading control. (B) Quantification of HDLEC migration in the wound healing assay following 12 h of treatment (n=5; **P<0.01 vs. CON). (C) Representative images of the wound healing assay in HDLECs treated with CD109-CM in the presence or absence of Stattic. Dashed and solid lines indicate the wound boundaries at 0 and 12 h, respectively (magnification, x100). (D) Quantification of migrated HDLECs in the Transwell assay following 24 h of treatment (n=5; **P<0.01 vs. CON). (E) Representative images of the Transwell migration assay following 24 h of treatment. HDLEC, human dermal lymphatic endothelial cell; p-, phosphorylated; CON, control; CM, conditioned medium.

inhibition of STAT3 attenuated CD109-CM-induced HDLEC migration. Collectively, these findings supported a potential paracrine mechanism through which CD109 overexpression in LUAD cells modified lymphatic endothelial cell behavior through STAT3 signaling, in association with activation of the JAK2/STAT3 pathway.

Tumor cells produce and secrete numerous growth factors, such as VEGF-C and VEGF-D, which interact with

receptors on lymphatic endothelial cells and contribute to morphological and structural alterations in tumor-associated lymphatic vessels (33). In the present study, successful establishment of the CD109-overexpression model was demonstrated at both mRNA and protein levels. In addition, soluble CD109 was detected at significantly higher levels in CM from CD109-overexpressing A549 cells compared with control CM. These findings demonstrated that CD109 overexpression increased the abundance of soluble CD109 in the extracellular environment and thus provides an experimental basis for investigating the potential paracrine effects associated with CD109-containing CM on neighboring stromal cells, including lymphatic endothelial cells.

Lymphangiogenesis involves coordinated lymphatic endothelial cell proliferation and migration, with new lymphatic vessels arising through sprouting from pre-existing vessels (34). Proliferation of lymphatic endothelial cells additionally contribute to expansion and remodeling of peritumoral lymphatic vessels (35). To model the local interaction between LUAD cells and lymphatic endothelial cells, HDLECs were treated with CM collected from A549 cells transfected with ADV-CD109 or control ADV (36). CD109-CM significantly enhanced HDLEC migration in both wound healing and Transwell assays, whereas no significant effect on proliferation was detected using either MTT or CCK-8 assays. Thus, the observed phenotype was primarily associated with enhanced lymphatic endothelial cell motility rather than proliferation. Increased lymphatic endothelial cell migration could potentially facilitate lymphatic remodeling or lymphangiogenic processes in the tumor microenvironment; however, whether this effect promotes lymphangiogenesis and lymphatic metastasis *in vivo* remains to be established.

CD109 has previously been implicated in regulation of JAK/STAT signaling and metastatic behavior in lung cancer cells (30). Among STAT family members, STAT3 is an important mediator of tumor-associated signaling and represents a potential therapeutic target in cancer. In the present study, CD109-CM significantly increased phosphorylation of JAK2 at Tyr1007/1008 and STAT3 at Tyr705 in HDLECs without altering total JAK2 or STAT3 protein levels. These findings are consistent with activation of JAK2/STAT3 signaling following exposure of lymphatic endothelial cells to CM from CD109-overexpressing LUAD cells. It is also important to distinguish the observed activation of the STAT3 pathway from the functional requirement of JAK2 in the present study. In CD109-CM-treated HDLECs, increased phosphorylation of JAK2 and STAT3 was observed, whereas pharmacological inhibition of STAT3 significantly attenuated the CD109-CM-induced migratory phenotype. These findings support the involvement of STAT3 signaling in HDLEC migration. However, STAT3 activation is not restricted to JAK2 and can be mediated by multiple upstream kinases and receptor-associated signaling pathways, including JAK1, tyrosine kinase 2 and receptor tyrosine kinases such as EGFR and VEGFR, as well as Src-family kinases (37,38). Therefore, the concomitant increase in phosphorylated JAK2 and phosphorylated STAT3 observed in the present study should be interpreted as evidence of JAK2/STAT3 pathway activation rather than proof that JAK2 is the unique or indispensable upstream kinase responsible for STAT3 activation. Given that JAK2 was not independently inhibited or genetically depleted in the present study, the specific functional contribution of JAK2 to the observed migratory response remains to be determined.

To further evaluate the functional involvement of STAT3, HDLECs were pretreated with Stattic, a STAT3 inhibitor that targets the STAT3 SH2 domain and inhibits STAT3 activation and dimerization (39,40). Stattic attenuated the CD109-CM-induced increase in STAT3 phosphorylation and significantly reduced the enhanced HDLEC migration observed in both wound healing and Transwell assays. These findings indicated that STAT3 activity contributed to the pro-migratory phenotype induced by CD109-CM (41). However, given that the experimental system used CM rather than purified soluble CD109, the results support a CD109-associated paracrine mechanism, rather than having definitively established that soluble CD109 alone was responsible for STAT3 activation and enhanced HDLEC migration.

Results of a previous study demonstrated that a tumor cell-autonomous CD109-JAK-STAT signaling axis contributed to metastatic behavior in lung cancer cells (28). The present study addressed a distinct aspect of CD109 biology through examination of whether CM from CD109-overexpressing LUAD cells can influence lymphatic endothelial cells. The findings demonstrated increased JAK2 and STAT3 phosphorylation in HDLECs following CD109-CM treatment and STAT3-dependent enhancement of HDLEC migration. Thus, the present results may extend the CD109-JAK-STAT signaling paradigm from a tumor cell-intrinsic context to a potential paracrine interaction between LUAD cells and lymphatic endothelial cells. Despite this, direct attribution of this effect specifically to soluble CD109 will require additional experiments using specific approaches, such as CD109 neutralization or recombinant soluble CD109.

It is important to distinguish pathway activation from functional pathway dependency. In the present study, increased phosphorylation of JAK2 and STAT3 provided molecular evidence of STAT3 pathway engagement, whereas functional necessity was examined specifically at the STAT3 level using Stattic (39,40). JAK2 itself was not independently inhibited or genetically depleted. Previous studies have provided evidence for JAK-dependent CD109 signaling using pharmacological inhibition and genetic approaches in tumor cells (28), as well as for CD109/glycoprotein 130 (GP130)-dependent signaling in glioma stem cells (42). The present findings therefore demonstrated STAT3-dependent HDLEC migration associated with STAT3 activation, but did not independently establish that JAK2 activity was required for the observed migratory phenotype. Future experiments using JAK2-selective inhibitors or genetic depletion of JAK2 may aid in establishing its functional contribution.

The receptor mechanism through which CD109-associated CM activates STAT3 signaling in HDLECs was not directly investigated in the present study. CD109 has been reported to interact with GP130, a shared signal-transducing component of the IL-6 receptor family and CD109-GP130 interactions have been implicated in the activation of STAT3 signaling (36). CD109 has also been reported to associate with EGFR and regulate Akt-mTOR- and STAT3-associated signaling in lung cancer cells (18). These observations identify GP130

and EGFR as potential candidates for mediating the effects observed in HDLECs. However, receptor involvement was not experimentally examined in the present study and therefore remains a hypothesis requiring direct evaluation using receptor blockade or genetic depletion.

A number of additional limitations should also be considered. First, the principal findings, including increased soluble CD109 in CM, enhanced HDLEC migration and STAT3 activation, were obtained using a single LUAD cell line, namely A549. Despite A549 cells providing an established LUAD model, validation in additional LUAD cell lines is required to determine whether these findings are broadly applicable across the molecular heterogeneity of LUAD. In addition, CD109-CM was obtained from CD109-overexpressing A549 cells and may therefore contain additional secreted factors whose abundance is altered secondary to CD109 overexpression. Furthermore, the cellular origin of extracellular CD109 should be considered when interpreting the present findings. Despite the CM used in the present study being generated from CD109-overexpressing A549 cells, CD109 expression and extracellular release are not necessarily restricted to tumor cells. CD109-associated extracellular signaling may potentially involve numerous cellular populations within the tumor microenvironment, including tumor cells and stromal or vascular-associated cells. Thus, present experiments demonstrated that CM derived from CD109-overexpressing A549 cells can promote HDLEC migration and is associated with increased STAT3 phosphorylation, but did not establish that tumor-cell-derived soluble CD109 was the sole mediator of this effect. Moreover, given that soluble CD109 was not quantitatively measured or specifically neutralized in the present study, contributions from other soluble factors present in the CM cannot be completely excluded. Thus, future studies using purified soluble CD109, CD109 depletion or neutralization and cell-type-specific approaches will be important in determining the contribution of soluble CD109 from individual cellular sources. Consequently, a contribution from other components of the CM to the HDLEC phenotype cannot be excluded. CD109-neutralizing approaches, depletion of soluble CD109 from CM or treatment with purified recombinant soluble CD109 may provide stronger evidence for a direct CD109-mediated effect.

A further limitation is that the present study was conducted entirely *in vitro*. Despite HDLEC migration representing a cellular process relevant to lymphatic remodeling, migration assays alone do not establish tumor lymphangiogenesis or lymphatic metastasis. Future *in vivo* studies using appropriate LUAD models should aim to determine whether tumor-derived soluble CD109 alters lymphatic vessel density, lymphatic remodeling, tumor cell entry into lymphatic vessels or lymph node metastasis. These studies would be important in establishing the physiological and therapeutic relevance of the CD109-associated paracrine mechanism identified in the present study.

In conclusion, results of the present study demonstrated that CM from CD109-overexpressing LUAD cells may promote HDLEC migration and increase phosphorylation of JAK2 and STAT3, while STAT3 inhibition attenuated the pro-migratory effect of CD109-CM. These findings identify a potential paracrine CD109-STAT3 signaling axis between LUAD cells and lymphatic endothelial cells. However, direct CD109 dependency, the functional requirement for JAK2 and the contribution of this mechanism to lymphangiogenesis and lymphatic metastasis *in vivo* require further investigation. These findings provide a basis for evaluating CD109-associated signaling as a potential therapeutic target in LUAD with lymphatic dissemination.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

TS and YS contributed to the experimental design, acquisition of data and data analysis. YMD, JL, JS and XW contributed to the writing and editing of the manuscript, as well as the analysis and interpretation of data. MDCL, CGC, MAT, XS and JL contributed to the conception, experimental design, acquisition of data, data analysis and writing and editing of the manuscript. TS, YS and JL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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