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RESEARCH ARTICLE

Vascular endothelial growth factor B coordinates metastasis of non-small cell lung cancer

Gang Liu · Shengbao Xu · Fanglei Jiao · Tao Ren · Qinchuan Li

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Abstract Neovascularization is critical for the invasion and metastasis of non-small cell lung cancer (NSCLC). However, the molecular mechanism underlying the control of neovascularization of NSCLC is not completely understood. Both vascular endothelial growth factor B (VEGF-B) and matrix metalloproteinases 9 (MMP9) play essential roles in neovascularization of NSCLC. Here, we examined whether VEGF-B and MMP9 may affect each other to coordinate the neovascularization process in NSCLC. We found strong positive correlation of VEGF-B and MMP9 levels in the NSCLC from the patients. Moreover, patients that had NSCLC with metastasis had significantly higher levels of VEGF-B and MMP9 in the primary cancer. Using a human NSCLC line A549, we found that overexpression of VEGF-B increased expression of MMP9, while inhibition of VEGF-B decreased expression of MMP9. On the other hand, overexpression of MMP9 increased expression of VEGF-B, while inhibition of MMP9 decreased expression of VEGF-B. These data suggest that expression of VEGF-B and MMP9 may activate each other to enhance neovascularization. We then analyzed the underlying mechanism. Application of a specific ERK/MAPK inhibitor but not a PI3K/Akt inhibitor to VEGF-B-overexpressing A549 cells substantially abolished the effect of VEGF-B on MMP9 activation, while application of a specific PI3K/Akt inhibitor but not an ERK/MAPK inhibitor to MMP9-overexpressing A549 cells substantially abolished the effect of MMP9 on VEGF-B activation, suggesting that VEGF-B may activate MMP9 via ERK/MAPK signaling pathway, while MMP9 may activate VEGF-B via PI3K/Akt signaling pathway. Thus, our data highlight a coordinating relationship between VEGF-B and MMP9 in the regulation of neovascularization in NSCLC.

Keywords Vascular endothelial growth factor B (VEGF-B) · Matrix metalloproteinases 9 (MMP9), PI3K · ERK/MAPK · Non-small cell lung cancer (NSCLC) · Metastasis

G. Liu · F. Jiao (✉) · Q. Li
Department of Throatic Surgery, East Hospital, Tongji University School of Medicine, 150 Jimo Road, Shanghai 200085, China
e-mail: fanglei_jiao@163.com

S. Xu · T. Ren
Lung Cancer Center, East Hospital, Tongji University School of Medicine, Shanghai 200085, China

Introduction

Non-small cell lung cancer (NSCLC) is a frequently occurred lung cancer. The three main types of NSCLC are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [1–3]. Most NSCLCs are insensitive to chemotherapy and radiation therapy and frequently appear to be highly invasive and predisposing to migrate [1–3]. Hence, understanding of the molecular mechanism that regulates the invasiveness and metastasis of NSCLC is extremely critical [4–6].

Cancer angiogenesis plays an important role in tumorigenesis in that it provides oxygen and nutrient supply to feed the tumor for growth and invasion. Cancer cells often secrete proteinases or acquire proteinase activity from host stromal cells or inflammatory cells, which allow cancer cells to break through collagenous protein barriers [7]. Proteins from vascular endothelial growth factor (VEGF) family are the most important signal molecules that regulate angiogenesis. The VEGF family is composed of six secreted proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor [8–10]. Among these proteins, VEGF-B plays a redundant role for VEGF-A [11–13]. Of note, VEGF-B upregulation has been detected in NSCLC [14, 15], whereas its precise role in the pathogenesis of NSCLC is not known.

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal

physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as cancer metastasis [16, 17]. MMPs play a role in endometrial menstrual breakdown, regulation of vascularization and the inflammatory response [5, 18, 19]. MMP9 is a member of MMP family. MMP9 is an important matrix proteinase that is secreted by various cancer cells to break down extracellular matrix. Overexpression of MMP9 has been reported to facilitate metastatic spread of different cancer cells and appears to be an important molecule to directly promote cancer metastasis of NSCLC [5, 18–21]. Nevertheless, no studies have addressed the relationship of VEGF-B and MMP9 in the regulation of cancer neovascularization of NSCLC.

Here, we examined whether VEGF-B and MMP9 may affect each other to coordinate the neovascularization process in NSCLC. We found strong positive correlation of VEGF-B and MMP9 levels in the NSCLC from the patients. Moreover, patients that had NSCLC with metastasis had significantly higher levels of VEGF-B and MMP9 in the primary cancer. Using a human NSCLC line A549, we found that overexpression of VEGF-B increased expression of MMP9, while inhibition of VEGF-B decreased expression of MMP9. On the other hand, overexpression of MMP9 increased expression of VEGF-B, while inhibition of MMP9 decreased expression of VEGF-B. We then analyzed the underlying mechanism. Application of a specific ERK/MAPK inhibitor but not a PI3K/Akt inhibitor to VEGF-B-overexpressing A549 cells substantially abolished the effect of VEGF-B on MMP9 activation, while application of a specific PI3K/Akt inhibitor but not an ERK/MAPK inhibitor to MMP9-overexpressing A549 cells substantially abolished the effect of MMP9 on VEGF-B activation, suggesting that VEGF-B may activate MMP9 via ERK/MAPK signaling pathway, while MMP9 may activate VEGF-B via PI3K/Akt signaling pathway.

Materials and methods

Cell lines and reagents

A549 [22] is a human NSCLC cell line purchased from ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Inhibitors PD98059 and LY294002 were both purchased from Sigma (USA).

Patient tissue specimens

A total of 24 resected specimens from NSCLC patients were collected for this study. All specimens had been histologically and clinically diagnosed at the Lung Cancer Center, East Hospital, Tongji University School of Medicine, from 2009 to 2013. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell transfection

A549 cells were transfected with a VEGF-B-overexpressing plasmid, or a small short hairpin interfering RNA for VEGF-B (shVEGF-B), or MMP9-overexpressing plasmid, or a small short hairpin interfering RNA for MMP9 (shMMP9), or control empty plasmid (null), as has been previously described [23].

Transwell matrix penetration assay

Cells (4×10^5) were plated into the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoat™ Invasion Chambers (BD, Bedford, MA, USA) and incubated at 37 °C for 22 h. The cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, stained with hematoxylin, and counted for ten random 100× fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed, and the data are presented as mean±standard deviation (SD).

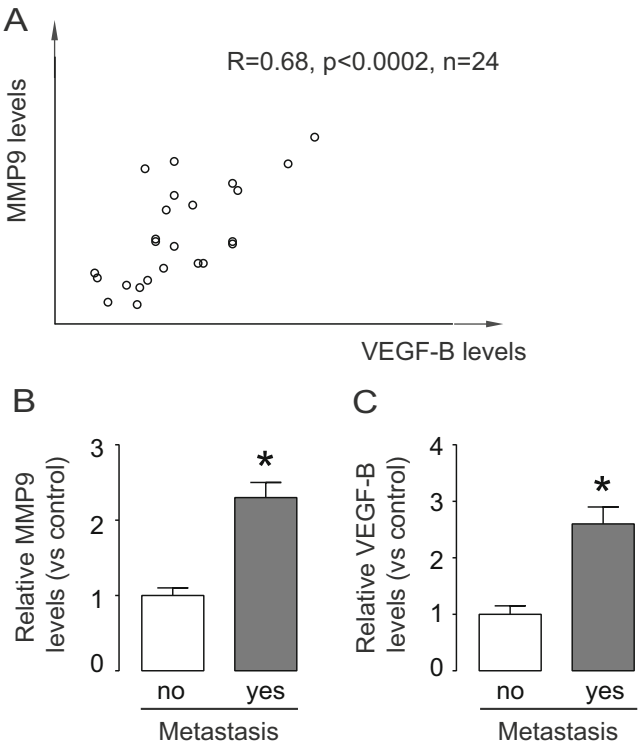

Fig. 1 VEGF-B and MMP9 levels positively correlated each other and with the metastasis in NSCLC patients. **a** VEGF-B and MMP9 levels were measured by Western blot in the resected NSCLC tissue from 24 patients. A strong correlation was detected (R=0.68; p<0.0002). **b**, **c** Patients who had metastasis of the original cancer had significantly higher levels of MMP9 (**b**) and VEGF-B (**c**). *p<0.05

ELISA assay

The concentration of MMP9 in the conditioned medium from cultured cells was determined by a MMP9 ELISA Kit (Sigma, USA). The concentration of VEGF-B in the cultured cells was determined by a VEGF-B ELISA kit (Raybio, USA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the collected condition medium was added to a well coated with MMP9/VEGF-B polyclonal antibody and then immunosorbented by biotinylated monoclonal anti-human MMP9/VEGF-B antibody at room temperature for 2 h. The color development catalyzed by horseradish peroxidase was terminated with 2.5 mol/l sulfuric acid, and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

Western blot

The protein was extracted from the resected NSCLC from the patient specimen or from cultured A549 cells. Primary antibodies were anti-VEGF-B, anti-MMP9, and anti-α-tubulin (all from Cell Signaling, USA). α-Tubulin was used as a protein loading control.

RT-qPCR

RNA was extracted from cultured cells with RNeasy kit (Qiagen, Hilden, Germany) and used for cDNA synthesis. Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Values of genes were normalized against α-tubulin and then compared with controls.

Statistical analysis

All statistical analyses were carried out using the SPSS 19.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction. Bivariate correlations between VEGF-B and MMP9 levels were calculated by Spearman's rank correlation coefficients. All values are depicted as mean±SD from five individuals and are considered significant if $p<0.05$.

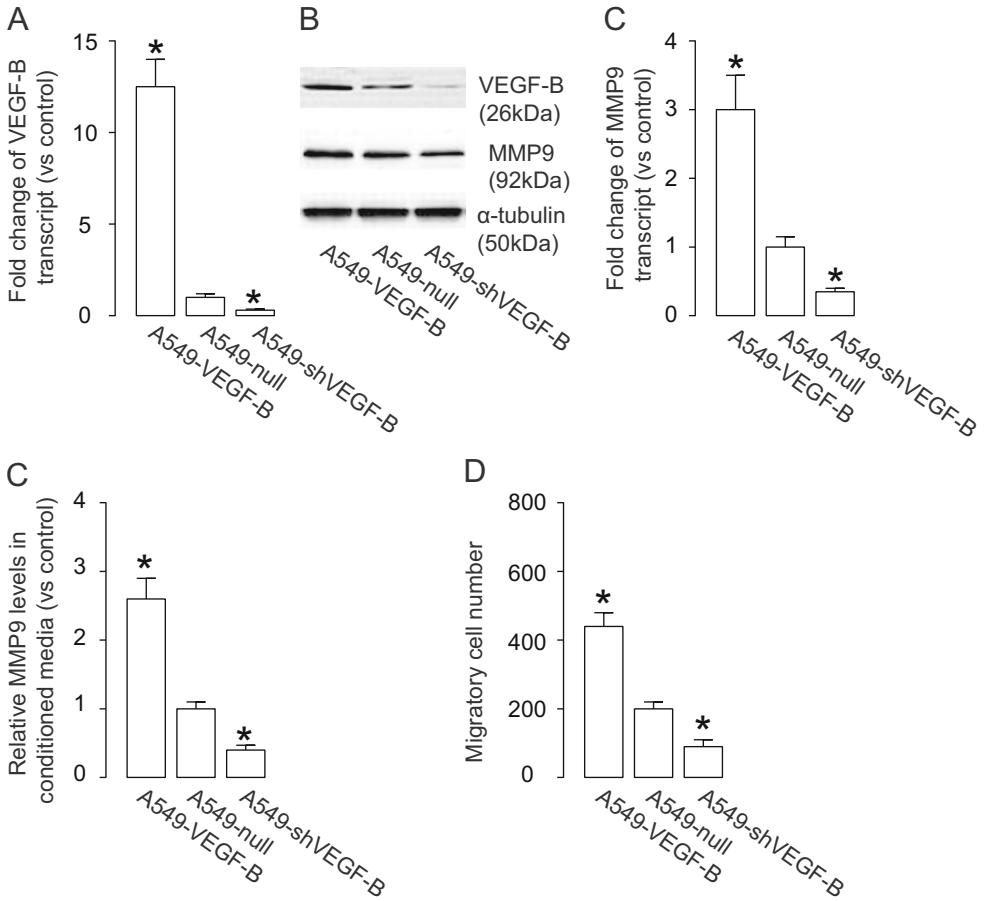

Fig. 2 VEGF-B regulated MMP9 in NSCLC. **a**, **d** A human NSCLC line, A549, was transfected with either a VEGF-B-expressing plasmid (VEGF-B), or shVEGF-B, or an empty plasmid as a control (null). **a** RT-qPCR for VEGF-B. **b** Representative images from Western blot. RT-qPCR (**c**) and ELISA (**d**) for MMP9. **e** The invasion ability of the A549 cells in a transwell matrix penetration assay. *$p<0.05$

Results

VEGF-B and MMP9 levels strongly correlated each other and metastasis in NSCLC

Both VEGF-B and MMP9 are essential for neovascularization, in which VEGF-B specifically promotes endothelial mitogenesis and permeability and MMP9 induces extracellular matrix degradation. Therefore, we hypothesized that VEGF-B and MMP9 may control expression of each other to coordinate the neovascularization process in NSCLC.

Thus, we examined the VEGF-B and MMP9 levels in the resected cancer tissues from NSCLC patients. We found strong positive correlation of VEGF-B and MMP9 levels in these patients (Fig. 1a, $R=0.68$, $p<0.0002$). Moreover, patients who had cancer metastasis had significantly higher levels of MMP9 (Fig. 1b) and VEGF-B (Fig. 1c) in the primary NSCLC. These data suggest that VEGF-B and MMP9 levels strongly correlated each other and metastasis in the NSCLC.

VEGF-B positively regulated MMP9 levels in NSCLC

Then, we used a human NSCLC line, A549, to examine whether expression of VEGF-B and MMP9 can affect each other, together with the underlying molecular mechanism. We transfected the A549 cells with either a VEGF-B-expressing plasmid (VEGF-B) or a small short hairpin interfering RNA for VEGF-B (shVEGF-B). The A549 cells were also transfected with a null plasmid as a control (null). First, adaption of VEGF-B levels in these cells was confirmed by RT-qPCR (Fig. 2a) and by Western blot (Fig. 2b). We found that overexpression of VEGF-B in A549 cells increased expression of MMP9, while inhibition of VEGF-B in A549 cells decreased expression of MMP9, by RT-qPCR (Fig. 2c), by Western blot (Fig. 2b), or by ELISA on the conditioned media (Fig. 2d). These data suggest that the MMP9 levels were indeed regulated by VEGF-B in NSCLC cells. Next, we examined the invasion ability of the A549 cells in a transwell matrix penetration assay. We found that overexpression of VEGF-B significantly increased the invasiveness of A549 cells, while inhibition of VEGF-B significantly decreased cell invasiveness (Fig. 2e).

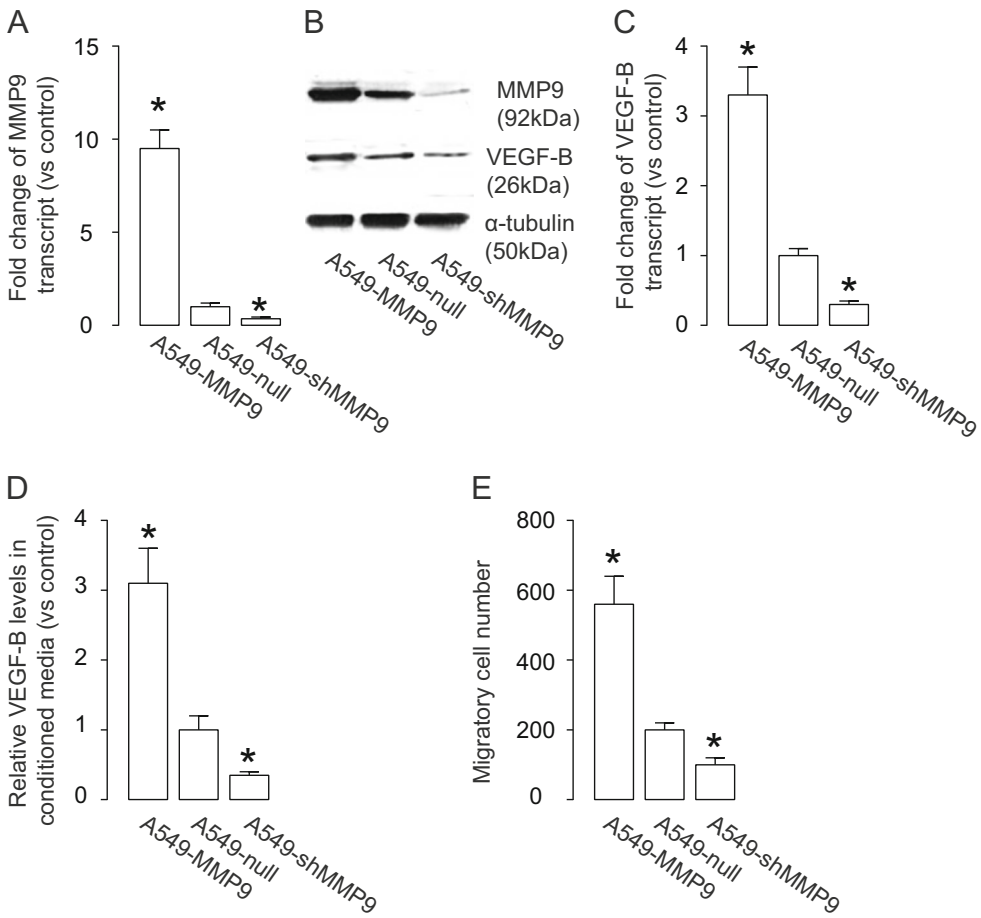

Fig. 3 MMP9 regulated VEGF-B in NSCLC. **a**, **d** A549 was transfected with either a MMP9-expressing plasmid (MMP9), or shMMP9, or an empty plasmid as a control (null). **a** RT-qPCR for MMP9. **b** Representative images from Western blot. RT-qPCR (**c**) and ELISA (**d**) for VEGF-B. **e** The invasion ability of the A549 cells in a transwell matrix penetration assay. *$p<0.05$

MMP9 positively regulated VEGF-B levels in NSCLC

On the other hand, we aimed to examine whether changes in MMP levels may similarly affect the VEGF-B levels in A549 cells. We thus transfected the A549 cells with either an MMP9-expressing plasmid (MMP9) or a small short hairpin interfering RNA for MMP9 (shMMP9). The A549 cells were also transfected with a null plasmid as a control (null). First, adaption of MMP9 levels in these cells was confirmed by RT-qPCR (Fig. 2a) and by Western blot (Fig. 2b). We found that overexpression of MMP9 in A549 cells increased expression of VEGF-B, while inhibition of MMP9 in A549 cells decreased expression of VEGF-B, by RT-qPCR (Fig. 3c), by Western blot for protein (Fig. 3b), or by ELISA on the conditioned media (Fig. 3d). Next, we examined the invasion ability of the A549 cells in a transwell matrix penetration assay. We found that MMP9 overexpression significantly increased the invasiveness of A549 cells, while inhibition of MMP9 in A549 cells significantly decreased cell invasiveness (Fig. 3e). Taken together, these data suggest that expression of VEGF-B and MMP9 may activate each other to reinforce their combined effect on neovascularization.

VEGF-B activated MMP9 via ERK/MAPK signaling pathway

We then analyzed the signaling pathway through which VEGF-B affects MMP9 levels. Application of a specific ERK/MAPK inhibitor, PD98059 at a dose of 10 μmol/l, but not a specific PI3k/Akt inhibitor, LY294002 at a dose of 20 μmol/l, to VEGF-B-overexpressing A549 cells substantially abolished the effect of VEGF-B on MMP9 activation, by RT-qPCR (Fig. 4a) and by Western blot (Fig. 4b). These data suggest that VEGF-B may increase expression of MMP9 via ERK/MAPK signaling pathway.

MMP9 activated VEGF-B via PI3k/Akt signaling pathway

We then analyzed the signaling pathway through which MMP9 affects VEGF-B levels. Application of a specific PI3k/Akt inhibitor, LY294002 at a dose of 20 μmol/l, but

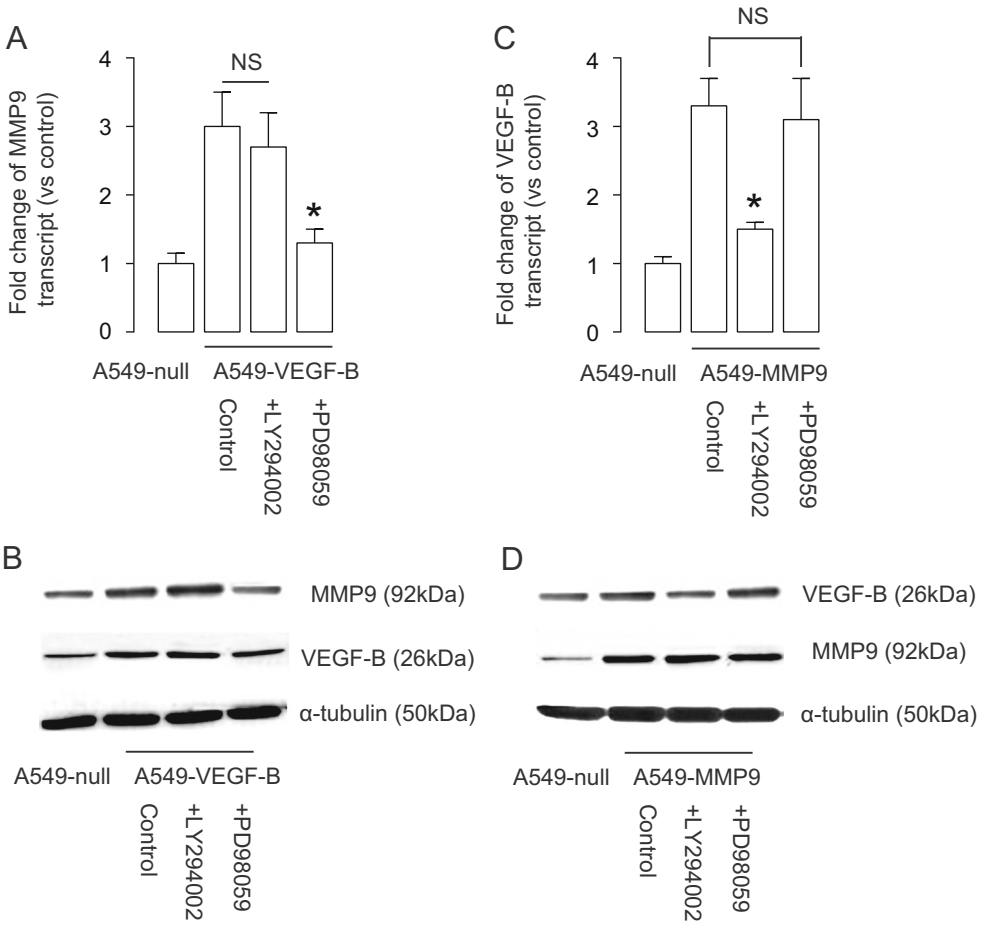

Fig. 4 Signaling pathways involved in the regulation between VEGF-B and MMP9 in NSCLC. **a**, **b** Application of a specific ERK/MAPK inhibitor, PD98059 at a dose of 10 μmol/l, but not a specific PI3k/Akt inhibitor, LY294002 at a dose of 20 μmol/l, to VEGF-B-overexpressing A549 cells substantially abolished the effect of VEGF-B on MMP9 activation, by RT-qPCR (**a**) and by Western blot (**b**). **c**, **d** Application of LY294002 at a dose of 20 μmol/l, but not PD98059 at a dose of 10 μmol/l, to MMP9-overexpressing A549 cells substantially abolished the effect of MMP9 on VEGF-B activation, by RT-qPCR (**c**) and by Western blot (**d**). *$p<0.05$

not a specific ERK/MAPK inhibitor, PD98059 at a dose of 10 μmol/l, to VEGF-B-overexpressing A549 cells substantially abolished the effect of MMP9 on VEGF-B activation, by RT-qPCR (Fig. 4c) and by Western blot (Fig. 4d). These data suggest that MMP9 may increase expression of VEGF-B via PI3k/Akt signaling pathway.

To summarize, our data highlight a coordinating relationship between VEGF-B and MMP9 in the regulation of neovascularization in NSCLC (Fig. 5).

Discussion

Understanding of the molecular basis underlying metastasis of NSCLC may substantially improve the prevention and treatment of the patients at the advanced stage. Angiogenesis is one of the most important process by which NSCLC invade and migrate. NSCLC cells not only secrete angiogenetic molecules like VEGF-B to increase vessel permeability and promote endothelial cell proliferation and survival but also secrete MMPs (e.g., MMP9) to degrade extracellular matrix. All these events are critical for cancer angiogenesis and metastasis. MMP9 is a member of the MMP family. Overexpression of MMP9 has been reported to facilitate metastatic spread of different cancer cells and appears to be an important molecule to directly promote cancer metastasis of NSCLC [5, 18–21].

Here, we found strong positive correlation of VEGF-B and MMP9 levels in the NSCLC patients. Moreover, patients with metastasis of the original cancer had significantly higher levels of VEGF-B and MMP9. These data suggest that VEGF-B and MMP9 may reinforce each other to promote NSCLC metastasis. To prove this hypothesis, we used a human NSCLC line, A549, to examine whether VEGF-B and MMP9 regulate each other. Of note, we also checked other NSCLC lines, which gave similar results to prove cell-line independence. We found that overexpression of VEGF-B in A549 cells increased expression of MMP9, while inhibition of VEGF-B in A549 cells decreased expression of MMP9. On the other hand, overexpression of MMP9 in A549 cells increased expression of VEGF-B, while inhibition of MMP9 in A549 cells decreased expression of VEGF-B. These data suggest that expression of VEGF-B and MMP9 may activate each other to exert a combined effect on neovascularization of NSCLC. Further analyses with specific signal pathway inhibitors revealed that VEGF-B may activate MMP9 via ERK/MAPK signaling pathway, while MMP9 may activate VEGF-B via PI3K/Akt signaling pathway.

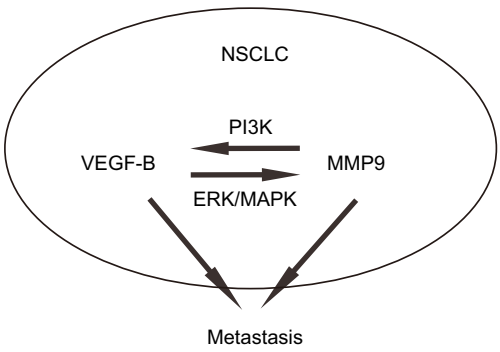

Fig. 5 Schematic of the model. A coordinating relationship between VEGF-B and MMP9 in the regulation of neovascularization in NSCLC, in which they activate each other. VEGF-B activates MMP9 via ERK/MAPK signaling pathway, while MMP9 activates VEGF-B via PI3K/Akt signaling pathway

Our study thus illustrates a novel model of the molecular mechanism underlying the angiogenesis and invasiveness of NSCLC, as summarized in Fig. 5. Further delineation of the precise molecular mechanism that mediates the inter-regulation of MMP9 and VEGF-B may substantially improve our understanding of the controls for metastasis of NSCLC.

Conflicts of interest None

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