Original Article
TRIM11 promotes tumor angiogenesis via activation of STAT3/VEGFA signaling in lung adenocarcinoma

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Abstract: Tripartite motif containing 11 (TRIM11) plays important roles in the regulation of lung cancer behaviors. However, the mechanisms of action of TRIM11 in tumor angiogenesis remain unclear. In this study, we found that TRIM11 expression is higher in lung adenocarcinoma (ADC) than in normal lung tissues. High TRIM11 expression was found to be associated with advanced progression and a poor prognosis of lung ADCs. Functional assays demonstrated that TRIM11 promoted tumor growth and angiogenesis *in vivo* and enhanced migration of (and tube formation by) human umbilical vein endothelial cells (HUVECs). Mechanistically, TRIM11 was found to regulate angiogenesis through the signal transducer and activator of transcription 3 (STAT3)/vascular endothelial growth factor A (VEGFA) pathway. Moreover, in clinical samples, VEGFA expression was much higher in cancer tissue samples and positively correlated with TRIM11 expression. TRIM11-overexpressing samples showed higher CD31 staining and microvessel density. Thus, we provide evidence that TRIM11 is a proangiogenic factor in lung ADC and may serve as a therapeutic target for lung ADC treatment.

Keywords: TRIM11, angiogenesis, STAT3/VEGFA, lung adenocarcinoma

Introduction

Lung cancer is one of the most common causes of cancer-related deaths worldwide [1]. Lung adenocarcinoma (ADC) is the major subtype of non-small cell lung cancer, which represents ~85% of all lung cancer cases. Great progress has been made in lung ADC treatment; however, patients with lung ADC still have a high mortality rate [2]. Therefore, improving the understanding of the mechanism of lung ADC progression is urgently needed for better lung ADC treatment.

Tripartite motif containing 11 (TRIM11) is one of the TRIM family proteins, which are characterized by evolutionarily conserved really interesting new gene (RING) finger domain at the N terminus, one or two B-box motifs, and a coiled-coil (RBCC) region [3]. Due to the RING finger, most TRIM family members function as E3 ubiquitin ligases [4]. Moreover, accumulating evidence has demonstrated that TRIM family members play crucial roles in tumor progres-sion [5]. TRIM11 has been reported to be associated with a poor prognosis among patients with hepatocellular carcinoma (HCC); to promote proliferation, invasiveness, and epithelial-mesenchymal transition of HCC cell lines through the PI3K/AKT signaling pathway, and to enhance HCC progression by inhibiting p53 expression both *in vitro* and *in vivo* [6-8]. Similarly, TRIM11 serves as an oncogene in breast, prostate, ovarian, and colon cancers [9-12].

Angiogenesis is one of the cancer hallmarks and is widely believed to promote tumorigenesis and tumor progression [13]. Tumor angiogenesis is associated with the interactions among tumor cells and tumor microenvironment and with various related signaling pathways [14]. Growth factors such as vascular endothelial growth factor A (VEGFA) secreted by tumor cells stimulate endothelial cells to form new vasculature and eventually promote angiogenesis [15]. The supporting roles of angiogenesis in tumor progression are 1) effective evac-

uation of waste and 2) delivery of nutrients to the tumor. High levels of VEGF in tumors and in the blood as well as tumor angiogenesis expressed as mean vascular density (MVD, by CD31 staining) are indicators of poor prognosis for patients with non-small cell lung cancer [16].

In the present study, we explored the mechanisms of action of TRIM11 on lung ADC angiogenesis. TRIM11 expression was found to be higher in lung ADC and was associated with faster progression and a poor prognosis of lung ADCs. TRIM11 promoted tumor growth and angiogenesis *in vivo* and enhanced migration of (and tube formation by) human umbilical vein endothelial cells (HUVECs) partially through STAT3/VEGFA pathway. Thus, we provide evidence that TRIM11 is a novel oncogene in lung ADC and may serve as a new theranostic biomarker for lung ADC treatment.

Materials and methods

Patients and clinical tissue samples

Formalin-fixed paraffin-embedded cancer tissues and paired normal lung tissues from 46 lung ADC patients were obtained from Shanghai Punan Hospital of Pudong New District (Shanghai, China). Twenty fresh lung ADC tissue samples and paired adjacent normal lung tissue samples were also obtained from Shanghai Punan Hospital of Pudong New District; after surgical resection, these samples were frozen immediately in liquid nitrogen for further analysis. Written informed consent was obtained from all the participating patients. The study protocol was approved by the Ethics Committee of Shanghai Punan Hospital of Pudong New District.

Cell lines and cell culture

Human lung ADC cell lines H1299, A549, H1975, and PC-9 and BEAS-2B bronchial epithelial cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in the RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% of fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All the cell lines were maintained in a humidified incubator at 37°C and 5% CO_2.

Immunohistochemical (IHC) staining

IHC analysis was performed as described previously [17]. Briefly, tissue sections fixed in for-

malin and embedded in paraffin were deparaffinized, rehydrated, and then subjected to incubation with citrate buffer (pH 6.0) for antigen retrieval. Then, the tissue slides were incubated with primary antibodies at 4°C overnight, followed by secondary-antibody incubation at room temperature for 60 min. The Dako ChemMate™ Detection Kit (DaKo, Denmark) was next applied to detect the bound primary antibodies. Integrated optical density was determined by means of Image-Pro Plus 6.0 (IPP).

Lentivirus and transduction

A TRIM11-overexpressing lentivirus, TRIM11 knockdown lentivirus (expressing short hairpin RNA; shRNA; target sequence: 5'-CUA UUC AUC UUU CCC GAG A-3'), and the corresponding negative control lentivirus (shNC sequence: 5'-AGC AAT GTC CAC ACT ATA C-3') were purchased from GenePharma (Shanghai, China). H1299 cells were transduced with the TRIM11 shRNA lentivirus, and A549 cells were transduced with the TRIM11-overexpressing lentivirus using the Lipofectamine 2000 reagent (Invitrogen). The cells were collected 48 h after the transduction.

Real-time PCR

Total-RNA samples were isolated from the tissues or cells using TRIzol. Reverse transcription was performed with the *iScript™ cDNA* Synthesis Kit (Bio-Rad, Hercules, CA, USA). The primers were as follows: TRIM11: Forward: 5'-GTG CCT ATG GAG CTG AGG AC-3', Reverse: 5'-CAG GAT CAG CTC AGG GTT G-3'; VEGFA: Forward: 5'-CGC AGC TAC TGC CAT CCA AT-3', Reverse: 5'-GTG AGG TTT GAT CCG CAT AAT CT-3'. Relative expression levels were determined by the $2^{-\Delta\Delta Ct}$ method. *GAPDH* expression served as a control.

Western blotting

A western blotting assay was performed as previously described [18]. Cells were lysed with RIPA buffer, and the protein concentrations were determined with the BCA Assay Kit (Beyotime, Haimen, China). Proteins were separated by SDS-PAGE in a 10% gel, followed by transfer to a nitrocellulose membrane (Bio-Rad). After that, the membranes were blocked with 5% milk, and then incubated with the following primary antibodies overnight at 4°C: anti-TRIM11 (Abcam, Cambridge, MA, USA), anti-

VEGFA, anti–phospho- (p-)STAT3, anti-STAT3, and anti-β-actin (Cell Signaling Technology, Danvers, MA, USA). β-actin served as a control. Finally, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The protein bands were detected by means of an ECL Plus Developing System.

Enzyme-linked immunosorbent assay (ELISA)

Human VEGFA ELISA Kit (R&D Systems, Minneapolis, MN, USA) was used to determine the concentrations of VEGFA in the culture supernatant from infected ADC cells.

Transwell migration assays

H1299 and A549 cells transfected with the plasmids were seeded in the upper chamber containing Matrigel in a serum-free medium, while the lower chamber was covered with the complete medium that contained 10% of fetal bovine serum as a chemoattractant. After incubation at 37°C for 12 h, the migratory cells that attached to the lower surface of the membrane were stained with crystal violet. The number of migratory cells was determined under a microscope.

A tube formation assay

HUVECs at 10^4 cells/well were seeded in a Matrigel-coated 96-well plate and cultured with the serum-free medium for 6 h. Then, the cells were cultured with various conditioned media from H1299 and A549 cells for 10 h. The tube length was calculated from images in the ImageJ software.

A xenograft tumor model

A total of 10^6 H1299 or A549 cells were subcutaneously injected into the flanks of 6-week-old female nude mice. After four weeks, all the mice were euthanized to excise the resultant tumors. The tumor volume was calculated via the following formula: ½ × Length × Width². Tumors were fixed with formalin and embedded in paraffin for IHC staining. All the animal experiments were approved by the Animal Care and Use Committee of Fudan University.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analyses were performed in SPSS 13.0 statistical software. Differences between two groups were assessed by unpaired Student's *t* test. Kaplan-Meier analyses with the logrank test were conducted to evaluate overall survival. Data with *P* values < 0.05 were considered significant.

Results

High expression of TRIM11 is associated with faster progression and poor prognosis of lung ADCs

Previously, TRIM11 has been reported to be upregulated at the transcriptional level in lung cancer tissues compared with normal tissues [19]. Similarly, analysis of the data available in Oncomine showed that *TRIM11* mRNA levels were significantly higher in human tumor samples as compared with the corresponding normal tissue samples, with the highest levels in lung ADCs (**Figure 1A**). We then performed IHC analysis to confirm the higher expression of TRIM11 in lung ADCs. As expected, a significantly increased intensity of TRIM11 staining was observed in 46 lung ADC tissues than in paired adjacent normal lung tissues (**Figure 1B**). The correlation of TRIM11 expression and lung ADC clinical characteristics was studied next. Notably, TRIM11 expression was found to be positively correlated with TNM stages of ADCs (**Figure 1C**). Using The Cancer Gene Atlas (TCGA) database, we found that lung ADC patients with high tumorous TRIM11 expression show worse overall survival than patients with low TRIM11 expression (**Figure 1D**). Consistently with these data, among the 46 lung ADCs in this study, high TRIM11 expression negatively correlated with overall survival of the patients (**Figure 1E**). These results indicated that TRIM11 is associated with faster progression and a poor prognosis of lung ADCs.

TRIM11 promotes tumor growth and angiogenesis in vivo

We then examined the expression of TRIM11 in multiple human lung ADC cell lines. As compared to BEAS-2B bronchial epithelial cells, the mRNA and protein levels of TRIM11 were significantly higher in lung ADC cell lines including H1299, A549, H1975, and PC-9 (**Figure 2A**, **2B**). Next, H1299 cells were infected with the TRIM11 shRNA lentivirus (H1299/shTRIM11) to construct a loss-of-function model, and A549
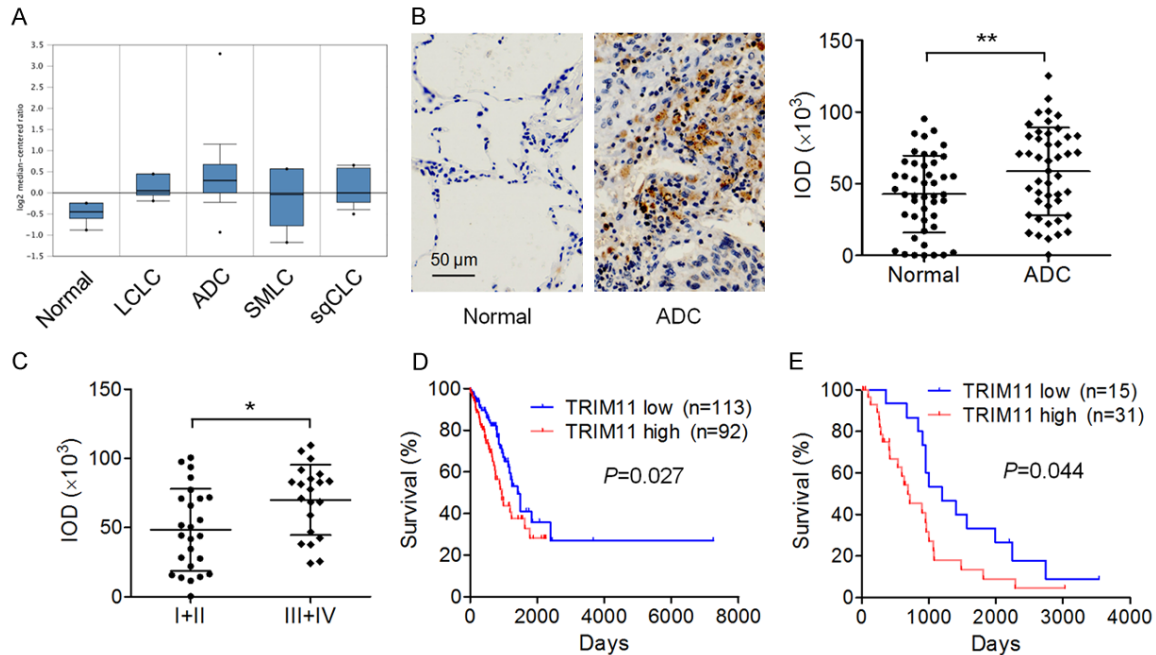
Figure 1. Overexpression of TRIM11 is associated with faster progression and a poor prognosis of lung ADCs. A. Analysis of TRIM11 expression in different types of lung cancer in the Oncomine database. B. Representative IHC staining of TRIM11 in 46 lung ADC samples and paired nontumorous tissue samples. Normalized integrated optical density (IOD) of TRIM11 is shown on the right. C. TRIM11 expression at different clinical stages of lung ADCs. D. Kaplan-Meier analysis of survival of lung ADC patients with distinct expression levels of TRIM11 in the tumor (data from database TCGA). E. Kaplan-Meier analysis of survival of lung ADC patients with distinct expression levels of TRIM11 in 46 tumors. *$P < 0.05$, **$P < 0.01$.

cells were infected with the TRIM11-overexpressing lentivirus (A549/TRIM11) to construct a gain-of-function cellular model (**Figure 2C**). These cellular models were then investigated to determine the effect of TRIM11 on tumor growth and angiogenesis *in vivo*. After continuous monitoring of the nude mice for 4 weeks, subcutaneous tumors formed from H1299/shTRIM11 cells grew much more slowly than did the tumors derived from the corresponding control cells. Conversely, TRIM11 overexpression (A549/TRIM11) resulted in accelerated growth of the xenograft tumors. Furthermore, fewer Ki67+ cells in H1299/shTRIM11-derived tumors, and more Ki67+ cells in A549/TRIM11-derived tumors were noted as compared with their respective controls (**Figure 2F**), indicating that TRIM11 promoted the proliferation of lung ADC cells. Moreover, TRIM11 downregulation inhibited, whereas TRIM11 upregulation enhanced vascular endothelial cell marker CD31 expression and microvessel density (MVD; **Figure 2G**). These results indicated that TRIM11 stimulated angiogenesis and promoted tumor growth; these effects may represent a novel me-chanism of TRIM11-mediated lung ADC progression.

TRIM11 promotes migration of and tube formation by HUVECs

Given that endothelial-cell migration is crucial for angiogenesis [20], we performed Transwell assay to detect the influence of TRIM11 on the migration of HUVECs. As depicted in **Figure 3A**, the conditioned medium from TRIM11 knockdown H1299 cells (H1299/shTRIM11-CM) obviously inhibited HUVEC migration as compared with the negative control group (H1299/shNC-CM). Not surprisingly, the conditioned medium from TRIM11-overexpressing A549 cells (A549/TRIM11-CM) dramatically enhanced cell migration (**Figure 3B**). Moreover, tubule formation by HUVECs was inhibited by H1299/shTRIM11-CM as compared with H1299/shNC-CM (**Figure 3C**). In agreement with these data, A549/TR-IM11-CM enhanced tubule formation by HU-VECs when compared with A549/Vector-CM (**Figure 3D**). These results suggested that TR-IM11 promoted the migration of (and tube formation by) HUVECs.
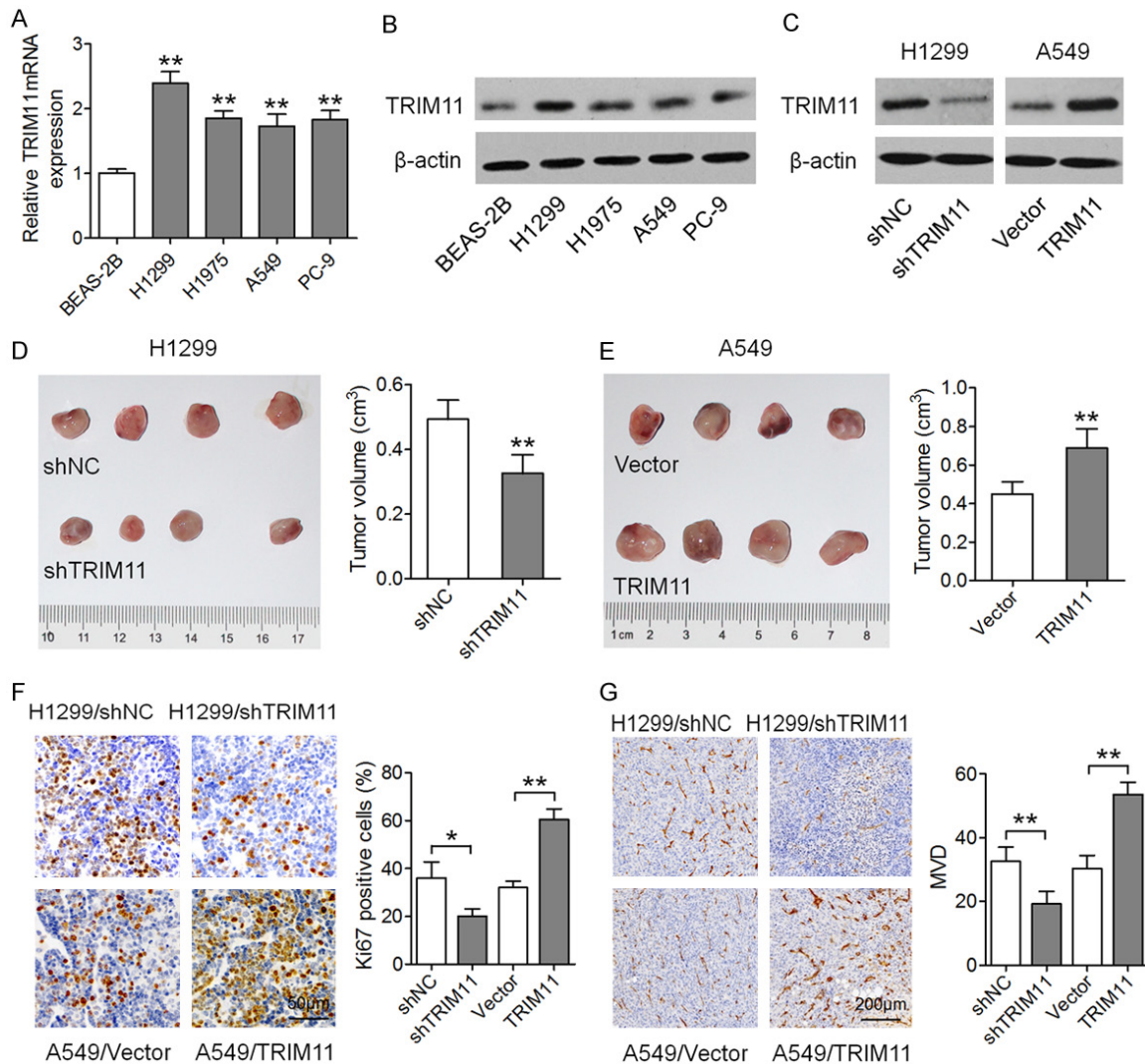
Figure 2. TRIM11 promotes tumor growth and angiogenesis *in vivo*. (A) Real-time PCR analysis of *TRIM11* mRNA levels in the lung ADC cell lines and in bronchial epithelial cell line BEAS-2B. The protein levels of TRIM11 in these cells were determined by western blotting and are depicted in (B). (C) H1299 and A549 cells were infected with the TRIM11 shRNA lentivirus and TRIM11-overexpressing lentivirus, respectively, and western blotting was performed to determine TRIM11 expression. (D) Xenograft tumors derived from H1299/shTRIM11 cells. (E) Xenograft tumors derived from A549/TRIM11 cells. (F) IHC analysis for determining the number of Ki67+ cells in tumor xenografts from the H1299/shTRIM11 and A549/TRIM11 groups. (G) IHC analysis with an anti-CD31 antibody. *$P < 0.05$, **$P < 0.01$.

TRIM11 regulates angiogenesis partially through STAT3/VEGFA pathway

Given that VEGFA is crucial for tumor growth and neovascularization [21, 22], we examined the expression levels of VEGFA in H1299/shTRIM11 and A549/TRIM11 cells. Real-time PCR and western blotting analyses revealed that VEGFA mRNA and protein levels were decreased by the TRIM11 knockdown and increased by TRIM11 overexpression (**Figure 4A** and

4B). Similarly, VEGFA concentrations determined by ELISA in the culture supernatant decreased when TRIM11 was downregulated but increased when TRIM11 was upregulated (**Figure 4C**). It is worth noting that *VEGFA* is a target gene of STAT3, which plays a crucial part in tumorigenesis and angiogenesis [23]. We speculated that TRIM11 can upregulate VEGFA through the JAK2/STAT3 pathway, thereby promoting angiogenesis. We found that TRIM11 downregulation reduced p-STAT3 expression,
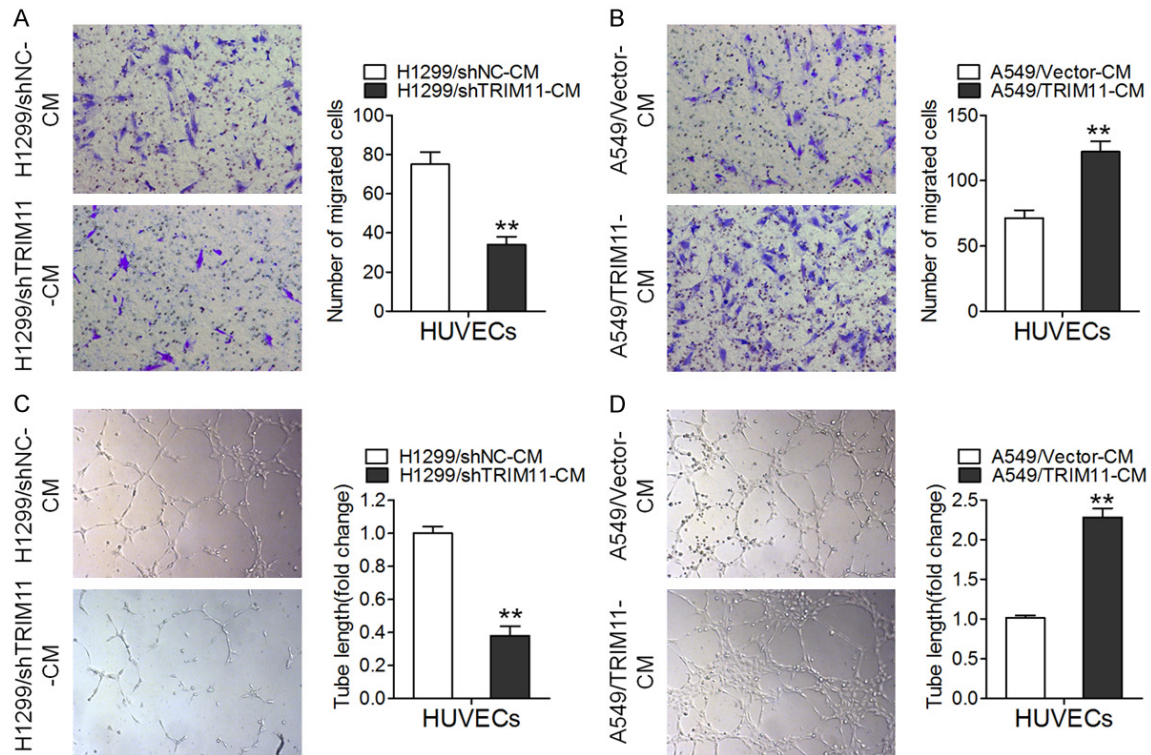
Figure 3. TRIM11 promotes migration of and tube formation by HUVECs. A. The Transwell migration assay was conducted to determine the migratory abilities of HUVECs incubated with the conditioned medium from H1299/shTRIM11 cells. B. The Transwell migration assay of HUVECs incubated with the conditioned medium from A549/TRIM11 cells. C. The capillary tube formation assay of HUVECs incubated with the conditioned medium from H1299/shTRIM11 cells. D. The capillary tube formation assay of HUVECs incubated with the conditioned medium from A549/TRIM11 cells. **P < 0.01.

and TRIM11 upregulation increased p-STAT3 expression. In particular, TRIM11 overexpression-mediated VEGFA upregulation was attenuated by STAT3 inhibitor S3I-201 (**Figure 4B**). In our xenograft tumor model, TRIM11 downregulation decreased VEGFA expression, whereas TRIM11 upregulation increased VEGFA expression (**Figure 4D**). After that, we tested whether STAT3 activation is required for the effects of TRIM11 on lung ADCs. After inhibition of STAT3 by S3I-201, the conditioned medium from TRIM11-overexpressing A549 cells lost the ability to promote tubule formation by (and migration of) HUVECs (**Figure 4E**, **4F**). These results meant that TRIM11 may regulate lung ADC angiogenesis partially through STAT3/VEGFA pathway.

TRIM11 upregulation positively correlates with VEGFA levels in lung ADC samples

Finally, we detected *VEGFA* mRNA expression levels in 20 fresh lung ADC tissues and in the matched normal tissues. As presented in **Fi-**gure 5A, *VEGFA* mRNA levels were much higher in lung ADCs than in normal tissue samples. In addition, there was a positive correlation between TRIM11 and VEGFA expression levels among the collected lung ADC tissue samples (**Figure 5B**). The association between TRIM11 expression and CD31 staining was evaluated next. Among the 20 collected lung ADC tissues, five samples showed lower TRIM11 expression (hereafter: TRIM11-Low samples) and 11 samples showed higher TRIM11 expression (hereafter: TRIM11-High samples). CD31 expression was much higher in TRIM11-High samples than in TRIM11-Low samples (**Figure 5C**). Additionally, MVD was dramatically higher in the TRIM11-high samples than in TRIM11-low samples (**Figure 5D**). These results suggested that TRIM11 upregulation positively correlates with VEGFA overexpression in lung ADC.

Discussion

Lung ADC is still a big threat to public health owing to the high mortality rate [24]. The mech-
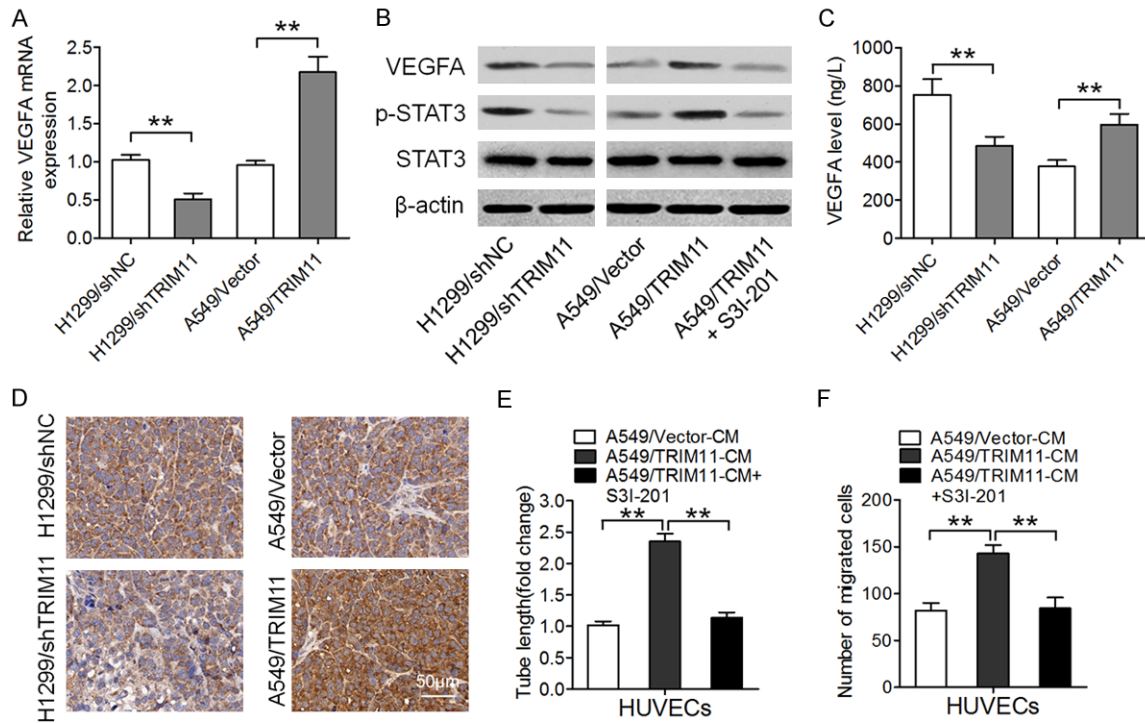
Figure 4. TRIM11 regulates angiogenesis partially through STAT3/VEGFA pathway. A. Real-time PCR analysis of *VEGFA* mRNA levels in H1299/shTRIM11 and A549/TRIM11 cells. B. Western blotting analysis of VEGFA, p-STAT3, and STAT3 in H1299/shTRIM11 cells, A549/TRIM11 cells, and A549/TRIM11 cells treated with the STAT3 inhibitor S3I-201. C. An ELISA was carried out to determine VEGFA concentration in the cell culture supernatant. D. IHC analysis for assessing VEGFA expression in tumor xenografts. E. A capillary tube formation assay of HUVECs incubated with the conditioned medium from A549/TRIM11 cells or from A549/TRIM11 cells treated with S3I-201. F. The Transwell migration assay of HUVECs incubated with the conditioned medium from A549/TRIM11 cells or A549/TRIM11 cells treated with S3I-201. **P < 0.01.

anism that regulates lung ADC progression remains largely unknown. TRIM11 has been reported to be an oncogene in various types of tumors, including lung cancer. Angiogenesis is one of the hallmarks of cancer, and accumulating reports point to the crucial influence of angiogenesis on tumor progression [13, 25]. Therefore, we explored the functional mechanisms of action of TRIM11 in tumor angiogenesis. We revealed that upregulated TRIM11 promotes tumor growth and angiogenesis *in vivo* and enhances migration of and tube formation by HUVECs partially through STAT3/VEGFA pathway. These results provide evidence of a novel mechanism behind TRIM11-mediated lung ADC progression.

TRIM11 is an E3 ubiquitin ligase of the TRIM family of proteins and serves as an oncogene in many types of cancers. *TRIM11* mRNA is the direct target of miR-24-3p, and TRIM11 overexpression promotes cell proliferation and colony

formation and inhibits apoptosis of colon cancer cell lines. Silencing of TRIM11 inhibits tumor growth *in vivo* [12]. In HCC clinical tissue samples, higher TRIM11 expression is observed relative to matched nontumorous liver tissues; TRIM11 protein levels are associated with recurrence, postoperative metastasis, and poor overall survival of patients [8]. TRIM11 depletion in ovarian cancer cell lines by small interfering RNA dramatically inhibits cell invasion via matrix metalloproteinase (MMP)-2 and MMP-9 and induces apoptosis via apoptosis-related proteins BCL-2 and BAX [11]. Similarly, TRIM11 turned out to be an oncogene in lung ADC in our present study. TRIM11 expression levels were significantly higher lung ADC patients' tumor samples. In addition, TRIM11 expression was found to be positively associated with advanced TNM stages and poor prognosis. Functional assays indicated that TRIM11 promotes tumor growth and angiogenesis *in vivo* and enhances migration of (and tube formation
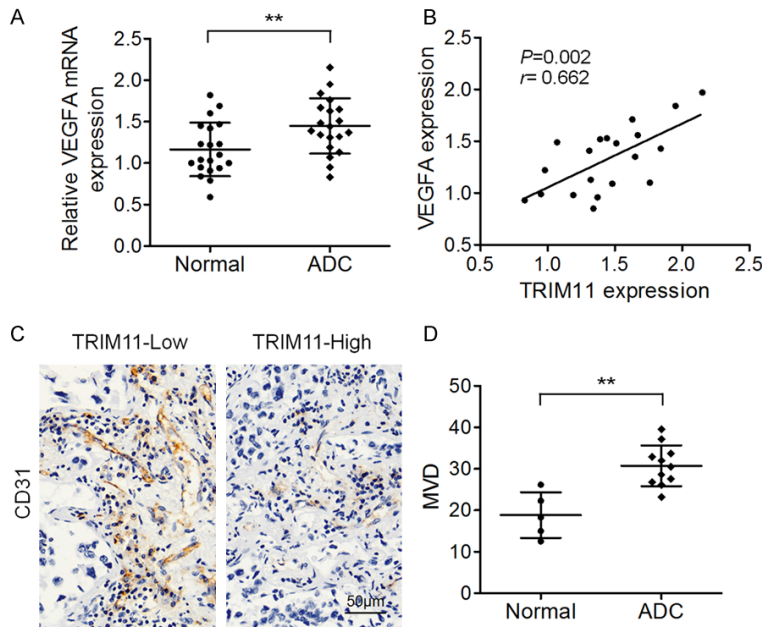
Figure 5. Correlation between TRIM11 and VEGFA expression levels among lung ADC specimens. A. Real-time PCR analysis of *VEGFA* mRNA levels in 20 lung ADC samples and paired nontumorous tissue samples. B. Pearson's analysis of correlation between *TRIM11* and *VEGFA* mRNA levels among 20 lung ADCs. C. Representative CD31 staining during IHC analysis of TRIM11-low and TRIM11-high lung ADC tissue samples. D. The average MVD in TRIM11-low and TRIM11-high lung ADC tissue samples. *P < 0.05, **P < 0.01.

by) HUVECs partially through STAT3/VEGFA pathway.

Rapid growth of a tumor requires continuous supply of oxygen and nutrients and waste product removal. Angiogenesis is one of the most important phenomena that support tumor growth and is a hallmark of cancers. In a tumor microenvironment, many cytokines have been identified as activators of angiogenesis, e.g., VEGF, basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor, and interleukin 8 [26]. Moreover, VEGF-induced angiogenesis has been reported to promote tumor progression. The monoclonal antibody specific for VEGF suppresses tumor growth and vessel density in nude mice that are injected with human rhabdomyosarcoma cell line, glioblastoma multiforme cell line, or a leiomyosarcoma cell line [27]. In colorectal cancer cell lines, miR-622 overexpression suppresses angiogenesis, whereas CXCR4 overexpression attenuates miR-622-mediated VEGFA inhibition, indicating that miR-622 inhibits colorectal cancer angiogenesis by suppressing the CXCR4-VEGFA axis [28]. Our results revealed that TRIM11 enhances VEGFA expression and promotes HUVEC migration and tube formation partially through STAT3 signing pathway.

It is important to note the limitations of this study. Our data are based on nude mice that were injected with human lung ADC cell lines. The immune system of these mice is ineffective at killing tumor cells, and this situation in turn triggers and accelerates tumor growth. The spontaneously forming lung ADC in mice with the C57BL/6 or BALB/c background and with an intact immune system may be a better way to evaluate the effects of TRIM11 on lung ADC.

In conclusion, our results illustrate higher TRIM11 expression in lung ADC and that TRIM11 promotes lung ADC tumor growth and angiogenesis *in vivo*. TRIM11 was found to enhance the migration of (and tube formation by) HUVECs partially through STAT3/VEGFA pathway. Therefore, targeting of TRIM11 maybe a novel strategy for lung ADC treatment.

Acknowledgements

Disclosure of conflict of interest

None.

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