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RNAi-mediated silencing of VEGF-C inhibits non-small cell lung cancer progression by simultaneously down-regulating the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes-induced ERK, p38 and AKT signalling pathways

Yukuan Feng [a,b,d], *Jing Hu* [a,b,d], *Jing Ma* [a], *Kejian Feng* [b], *Xiaoli Zhang* [b], *Shucai Yang* [a], *Wei Wang* [c], *Jianguo Zhang* [c,*], *Yafang Zhang* [a,*]

[a] *Department of Anatomy and Histology, Harbin Medical University, Harbin 150081, China*
[b] *Department of Anatomy and Histology, Mudanjiang Medical University, Mudanjiang 157011, China*
[c] *Department of Surgery of the Second Clinical Hospital, Harbin Medical University, Harbin 150081, China*

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ABSTRACT

Vascular endothelial growth factor C (VEGF-C) expression is associated with the malignant tumour phenotype making it an attractive therapeutic target. We investigated the biological roles of VEGF-C in tumour growth, migration, invasion and explored the possibility of VEGF-C as a potential therapeutic target for the treatment of non-small cell lung cancer (NSCLC). A lentivirus-mediated RNA interference (RNAi) technology was used to specifically knockdown the expression of VEGF-C in A549 cells. Quantitative reverse transcriptase-polymerase chain reaction, flow cytometry, Western blot, immunohistochemistry, cellular growth, migration, invasion and ELISA assays were used to characterise VEGF-C expression *in vitro*. A lung cancer xenograft model in nude mice was established to investigate whether knockdown of VEGF-C reduced tumour growth *in vivo*. Silencing of VEGF-C suppressed tumour cell growth, migration and invasion *in vitro*; suppressed tumour growth, angiogenesis and lymphangiogenesis by tail vein injection of lentivirus encoded shRNA against VEGF-C *in vivo*. More importantly, silencing of VEGF-C also trapped the VEGFR-2, VEGFR-3, CXCR4, CCR7-dependent axes, and down-regulated the AKT, ERK and p38 signalling pathways. These results suggest that VEGF-C has a multifaceted role in NSCLC growth, migration and invasion; that VEGF-C-mediated autocrine loops with their cognate receptors and chemokine receptors are significant factors affecting tumour progression; and that RNAi-mediated silencing of VEGF-C represents a powerful therapeutic approach for controlling NSCLC growth and metastasis.

1. Introduction

Lung carcinoma is the leading cause of cancer-related death worldwide and non-small cell lung cancer (NSCLC) accounts for 75% of all diagnosed lung cancers. Despite extensive research efforts in NSCLC screening, diagnostics and therapeutics, prognosis is poor and only 8–14% of patients survive >5 years from the time of diagnosis.[1] Clearly, current

* *Corresponding author.* Tel.: +86 451 8629 7423; fax: +86 451 8660 5079 (J. Zhang), tel.: +86 451 8667 4508x8002; fax: +86 451 8634 2900 (Y. Zhang).
E-mail addresses: zhangjiang43@hotmail.com (J. Zhang), yafangzhang2008@yahoo.cn (Y. Zhang).
[d] These authors contributed equally to this article.
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treatments for NSCLC are ineffective and different approaches are needed to improve the therapeutic ratio, including the accurate identification of optimal molecular targets for use in molecular targeted therapy.

Lymphangiogenesis is an important mediator of tumour cell dissemination, and many potential lymphangiogenic factors have been characterised including vascular endothelial growth factor (VEGF)-C.[2–4] VEGF-C plays a critical role in the control of lymphatic endothelial cell biology during embryogenesis, tumourigenesis and metastasis.[5–7] In some studies, the overexpression of VEGF-C significantly correlated with lymph node metastasis and lymphangiogenesis in primary tumours of thyroid, prostate, gastric, colorectal, ovarian, and breast cancers.[5,8] However, other reports could not confirm such correlations, or opposite relationships were found.[8] These results suggest that VEGF-C may be an important diagnostic and therapeutic target for treating malignant tumours, and indicate that the effects and interactions of the VEGF-C/VEGFR-3 system in cancer biology are complex and differ between malignancies. The functional role of VEGF-C in NSCLC remains unclear. Only a few clinical studies on VEGF-C expression in NSCLC have been reported and the prognostic significance of VEGF-C remains controversial[9–13]; the effect of VEGF-C knockdown on the metastatic phenotype in NSCLC is also unknown.

Tumour cells can acquire the ability to support autocrine signalling pathways by expressing growth factors and their cognate surface receptors. Tumour cell migration and metastasis share many similarities with leukocyte trafficking which is critically regulated by chemokines and their receptors. Recent reports suggested that the chemokine receptors CXCR4 and CCR7 were highly expressed in many kinds of tumours, and may be involved in metastatic processes[14–17] and the induction of tumour cell transport via the lymphatics in association with the secretion of VEGF-C.[18,19] Based on these data we hypothesised that a link between autocrine growth factors and chemokine receptors exists in tumour cells, and that a decrease in the expression of VEGF-C would result in down-regulation of the expression levels of CXCR4, CCR7, VEGFR-2, VEGFR-3 and their dependent axes and the suppression of tumour progression.

To assess the functional role of VEGF-C in the malignant phenotype of NSCLC cells, we developed a lentivector-mediated small interfering RNA (siRNA) approach to selectively knockdown the expression of VEGF-C in NSCLC A549 cells. We showed that RNA interference (RNAi) driven silencing of VEGF-C strongly inhibited NSCLC cell growth, migration and invasion and simultaneously down-regulated the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes-induced ERK, AKT and p38 signalling pathways. Furthermore, an *in vivo* xenograft study showed that administration of VEGF-C siRNA significantly inhibited tumour growth, angiogenesis and lymphangiogenesis.

2. Materials and methods

2.1. Cell culture and selection of a high VEGF-C expression cell line

Five human NSCLC cells (A549, SPC-A1, SK-MES-1, Y90 and NCI-H460) were purchased from Institutes of Biochemistry and Cell Biology (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Shanghai, China) supplemented with 10% foetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin and maintained at 37 °C under 5% CO_2. The levels of VEGF-C expression were detected by Western blot analysis and quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR); the cell line with the highest level of VEGF-C expression was selected for the following assays.

2.2. Design and construction of lentiviral VEGF-C siRNA expression vectors

Potential RNAi oligonucleotides for silencing human VEGF-C (accession no. NM005429) were designed and synthesised by Invitrogen. The synthesised sequences were inserted into the BamHI and EcoRI enzyme sites of the pSIH1-H1-cop green fluorescent protein (GFP) (pSIH1) shRNA vector (System Biosciences) to construct human VEGF-C shRNA plasmids (Lv-VEGF-C-shRNA1, Lv-VEGF-C-shRNA2, Lv-VEGF-C-shRNA3 and Lv-siRNA-NS). The plasmids and the predicted secondary structure of the pSIH1 shRNA targeting VEGF-C are shown in Fig. 1A. Restriction endonuclease digestion analysis confirmed the structures of the recombinant vectors, and DNA sequencing was used to verify all inserted sequences.

2.3. Transduction of target cells by pPACKaged siRNA expression vector

Lentivirus production and subsequent target cell transduction were established using a lentiviral delivery system (System Biosciences) and performed according to the manufacturer's instructions. Briefly, expression vector pSIH1 carrying siRNA or vector control (pSIH1-NS) was mixed with Lentivirus Package Plasmid Mix and Lipofectamine™ 2000 (Invitrogen), and added to 293TN producer cells (System Biosciences). The supernatants containing lentiviruses were harvested at 48 h post-transfection and filtered through 0.45 μm polyvinylidene fluoride membranes (Millipore). The titre of virus was measured according to the expression level of GFP. Viral supernatant was used for transducing target cells. A549 cells transfected with lentivirus-encoded shRNA 1–3 against VEGF-C were named A549-s1, A549-s2 and A549-s3. A549 cells transfected with Lv-siRNA-NS or parental vector pSIH1 shRNA were controls and named A549-NS and A549-pS, respectively.

2.4. Tumour xenograft treatment model

Forty-eight, 6-week old BALB/c nude mice were obtained from Laboratory Animal Center (Shanghai, China). A suspension of A549 cells (1×10^7) was injected subcutaneously into the left flank of each mouse. When tumours formed on d 15 post-implantation, mice were randomly divided into four treatment groups: the Lv-VEGF-C-siRNA group treated with lentivirus encoded shRNA against VEGF-C; the Lv-siRNA negative group treated with negative control lentivirus; PBS treated controls; and untreated controls. Treatment groups received 250 μl lentivirus or PBS by intravenous (i.v.) injection into the tail vein every 24 h for 3 weeks. Upon termination, each mouse was weighted and tumours were harvested for

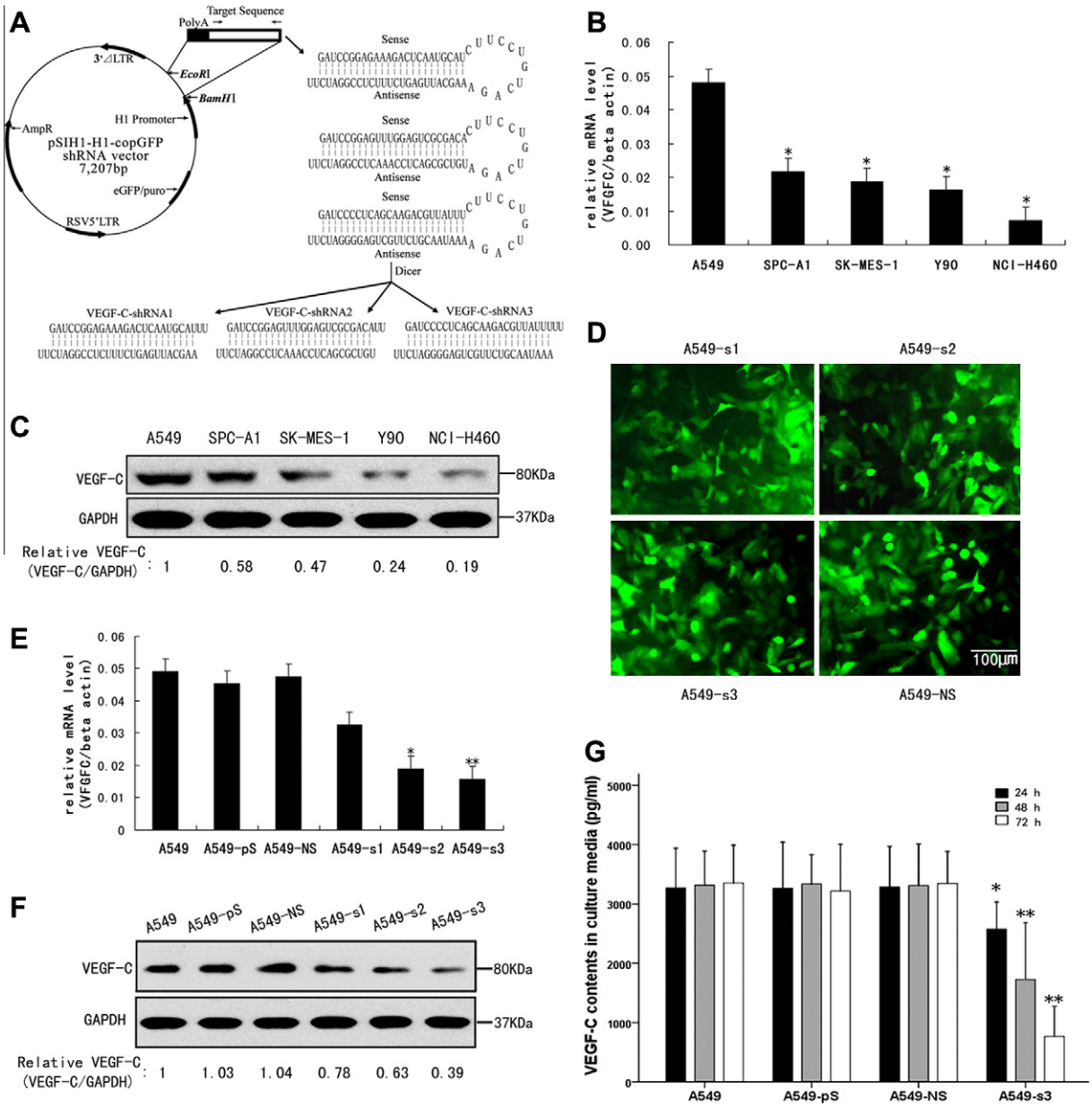

Fig. 1 – Effects of VEGF-C siRNA on the expression of VEGF-C. (A) The pSIH1shRNA vector was used to construct plasmids expressing VEGF-C siRNA. The predicted secondary structure of the pSIH1 shRNA targeting VEGF-C is shown. (B and C) VEGF-C expression in NSCLC tumour cells was detected by QRT-PCR and Western blot. (D) Stably transfected A549 cells were easily identified by fluorescence microscopy. (E, F, G) VEGF-C expression in A549 cells was detected by QRT-PCR, Western blotting and ELISA. (Data were confirmed in triplicate experiments; Columns indicate means; bars are the standard error.) *P < 0.05; **P < 0.01.

immunohistochemistry analysis, Western blot analysis and quantitative RT-PCR.

2.5. *Quantitative reverse transcriptase-polymerase chain reaction*

Total RNA was extracted using the RNAiso reagent (TaKaRa, Dalian, China). cDNA was transcribed from the mRNA using the PrimeScript™ RT-PCR Kit and Oligo dT Primer (TaKaRa) according to the manufacturer's instructions. The mRNA levels of VEGF-C, VEGFR-2, VEGFR-3, CXCR4, CCR7, COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by QRT-PCR. cDNA samples were used as the template for amplification reactions carried out in a PCR Thermal Cycler Dice Real Time System with the SYBR® PrimeScript RT-PCR Kit (TaKaRa) following our previously described procedure.[12] For each sample, triplicate determinations were made, and mean values were adopted for further calculations. All values were normalised to an endogenous β-actin control. The primer sequences used to amplify individual genes are shown in Table 1.

Table 1 – Primer sequences and annealing temperatures for quantitative RT-PCR.

Gene name and accession no.	Primer name	Primer sequences	Primer Tm	Product size (bp)
Beta-actin (NM_001101)	Forward	5′-CCTGTACGCCAACACAGTGC-3′	60	211
	Reverse	5′-ATACTCCTGCTTGCTGATCC-3′		
VEGF-C (NM_005429)	Forward	5′-TCAAGGCCCCAAACCAGTAACAAT-3′	58	228
	Reverse	5’-CCAGCATCCGAGGAAAACATAAAA-3′		
COX-2 (NM_000963)	Forward	5′-AATGGGGTGATGAGCAGTTGTTC-3′	56	202
	Reverse	5’-GGATGCCAGTGATAGAGGGTGTTA-3′		
VEGFR3 (NM_182925)	Forward	5′-CCCGGCTGCCCCTGAAGTGGA-3′	60	221
	Reverse	5′-GATGCGGCGTATGGCGGGAGTG-3′		
VEGFR2 (NM_002253)	Forward	5′-TGGTCAGGCAGCTCACAGTCC-3′	60	200
	Reverse	5′-GTTCCGGTTCCCATCCTTCAATAC-3′		
CXCR4 (NM_003467)	Forward	5′-CCTGCCCTCCTGCTGACTATTC-3′	56	225
	Reverse	5′-GGCCTTGCGCTTCTGGTG-3′		
CCR7 (NM_001838)	Forward	5′-AGCGCGGCCAAGTCCTG-3′	60	126
	Reverse	5′-GATGGCCACGTAGCGGTCAATG-3′		

2.6. VEGF-C protein ELISA

The levels of VEGF-C protein in culture supernatants were determined using the Quantikine Human VEGF-C Enzyme-Linked Immunoassay (R&D Systems) according to the manufacturer's instructions. Briefly, tumour cells were plated in 96-well plates at a density of 1.5×10^4 cells/well under standard growth conditions for 24 h. Media were aspirated and replaced with serum-free media. Supernatants were analysed for secreted VEGF-C protein after 24, 48 and 72 h in serum-free conditions.

2.7. Western blot analysis

Tissue and cell extracts were prepared using a previously described procedure[1] and all Western blot assays were performed in triplicate experiments. Protein lysates were subjected to electrophoresis on a 4% SDS–PAGE and proteins were electrotransferred to polyvinylidene fluoride membranes (Millipore). Membranes were incubated with 5% non-fat dry milk in TBS and probed with anti-VEGF-C, anti-VEGFR-2, anti-VEGFR-3, anti-CCR7, anti-COX-2, anti-phospho-VEGFR-2 (Santa Cruz Biotechnology), anti-CXCR4 (Abcam), anti-pVEGFR-3 (Cell Applications), anti-pERK1/2, anti-pAKT, anti-phospho-p38, anti-ERK1/2, anti-AKT, anti-p38 and anti-GAPDH (Cell Signaling Technology) in TBST (0.1% Tween 20 in TBS). Horseradish peroxidase-conjugated anti-rabbit (or mouse) IgG (Cell Signaling Technology) was used for detection of immunoreactive proteins by chemiluminescence (Pierce® ECL Western blotting substrate).

2.8. Cell proliferation assay

A549, A549-pS, A549-NS and A549-s3 cells were seeded in 96-well culture plates and incubated for 12, 24, 48 and 72 h. The 3-(4,5-dimethythazolz-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 15 μl MTT (5 mg/ml; Sigma) to each well for 4 h at 37 °C. Supernatants were removed, 100 μl dimethyl sulfoxide/well (DMSO, Sigma) were added and samples were shaken for 10 min. The absorbance value (OD) of each well was measured with a microplate photometer (Multiskan FC; Thermo Scientific) at 570 nm (630 nm as a reference).

2.9. Flow cytometry analysis

Approximately 5×10^4 A549, A549-pS, A549-NS and A549-s3 cells were seeded in 6-well culture plates and incubated in complete medium to 80–90% confluence. The cells were harvested, washed with ice-cold PBS twice, and fixed with 75% cold ethanol at 4 °C overnight. Propidium iodide (PI) staining of nuclei was used to monitor the phases of the cell cycle. The fluorescence of DNA-bound PI in cells was measured with a FACSCalibur flow cytometer (BD Biosciences).

2.10. Migration and invasion assay

Cell migration and invasion experiments were carried out with the QCM™ 24-well Fluorimetric Cell Migration kit (ECM509; Chemicon) and the Invasion Assay kit (ECM554; Chemicon) according to the manufacturer's instructions. Both kits utilise an insert polycarbonate membrane with an 8 μm pore size. The insert in the invasion kit was coated with a thin layer of ECMatrix™ which occluded the membrane pores and blocked migration of noninvasive cells. In the migration assay, 500 μl of migration buffer with or without chemoattractant [80 ng/ml recombinant human CXCL12 (rhCXCL12); 100 ng/ml rhCCL2] were added to the lower compartment. For the invasion assay, 500 μl culture medium supplemented with 10% FBS were used as chemoattractant. Cells that migrated to and invaded the underside of the membrane were fixed in 4% paraformaldehyde and stained with 10% Giemsa dye. The migrated and invaded cell numbers were determined by fluorescence and reported as relative fluorescence units (RFUs).

2.11. Immunohistochemistry analysis

Four micron-thick formalin-fixed paraffin-embedded tissues were cut and processed for immunohistochemistry using rabbit anti-VEGF-C, rat anti-mouse CD34, or rabbit anti-mouse LYVE-1 (Abcam). Immunoreactivity was detected using the

Poly Horseradish Peroxidase Detection System and 3,3′-diaminobenzidine (Zhongshan Biotechnology Inc., Beijing, China) following the manufacturer's instructions. The microvessel densities (MVD) and lymphatic microvessel densities (LMVD) were assessed by light microscopic examination of the tumour regions containing the greatest number of CD34 and LYVE-1-positive vessels; MVD and LMVD were counted by the previously described hotspot method.[12]

2.12. *Statistical analysis*

Data are expressed as means ± standard deviation (SD) and were compared by Student's *t*-test and one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Statistical analyses were performed using SPSS statistical software (16.0 for Windows); significance was reached at $P < 0.05$.

3. Results

3.1. *Effects of VEGF-C siRNA on the expression of VEGF-C*

The levels of VEGF-C expression in NSCLC cell lines were determined by QRT-PCR and Western blot analysis. The expression levels of VEGF-C mRNAs and proteins in SPC-A1, SK-MES-1, Y90 and NCI-H460 were significantly lower than those in A549 cells (Fig. 1B and C). Therefore, we selected the A549 cell line for use in the following assays.

The transfections of the pSIH1-shRNA vector-delivered VEGF-C siRNA-expressing plasmids were successful and four stably transfected cell lines were constructed (Fig. 1D). Lv-VEGF-C-shRNA3 significantly inhibited VEGF-C mRNA expression in A549-s3 cells compared with untransfected A549 cells, and the inhibitory rate was 67.77 ± 7.39% ($P < 0.001$; Fig. 1E). Western blot analyses confirmed the inhibition of VEGF-C protein expression by Lv-VEGF-C-shRNA3 constructs and the inhibitory rate was 60.88 ± 2.78% ($P < 0.001$; Fig. 1F). In contrast, VEGF-C mRNA expression levels were not significantly inhibited by Lv-siRNA-NS, the inhibitory rate was 3.08 ± 3.62% ($P > 0.05$), and Western blot analyses showed no difference in the levels of VEGF-C expression in A549-s1, A549-NS and A549-pS cells ($P > 0.05$).

To further investigate Lv-VEGF-C-shRNA3-induced down-regulation of VEGF-C secretion, culture supernatants at 24, 48 and 72 h after transfection in serum-free media were analysed by a VEGF-C specific ELISA. Lv-VEGF-C-shRNA3 significantly inhibited VEGF-C protein secretion compared with the controls ($P < 0.001$). The corresponding values at 72 h in A549-s3, A549, A549-pS and A549-NS were 764.90 ± 204.33, 3355.06 ± 256.93, 3217.15 ± 316.80 and 3345.73 ± 216.61 pg/ml, respectively (Fig. 1G). Therefore, we selected Lv-VEGF-C-shRNA3 for use in further assays.

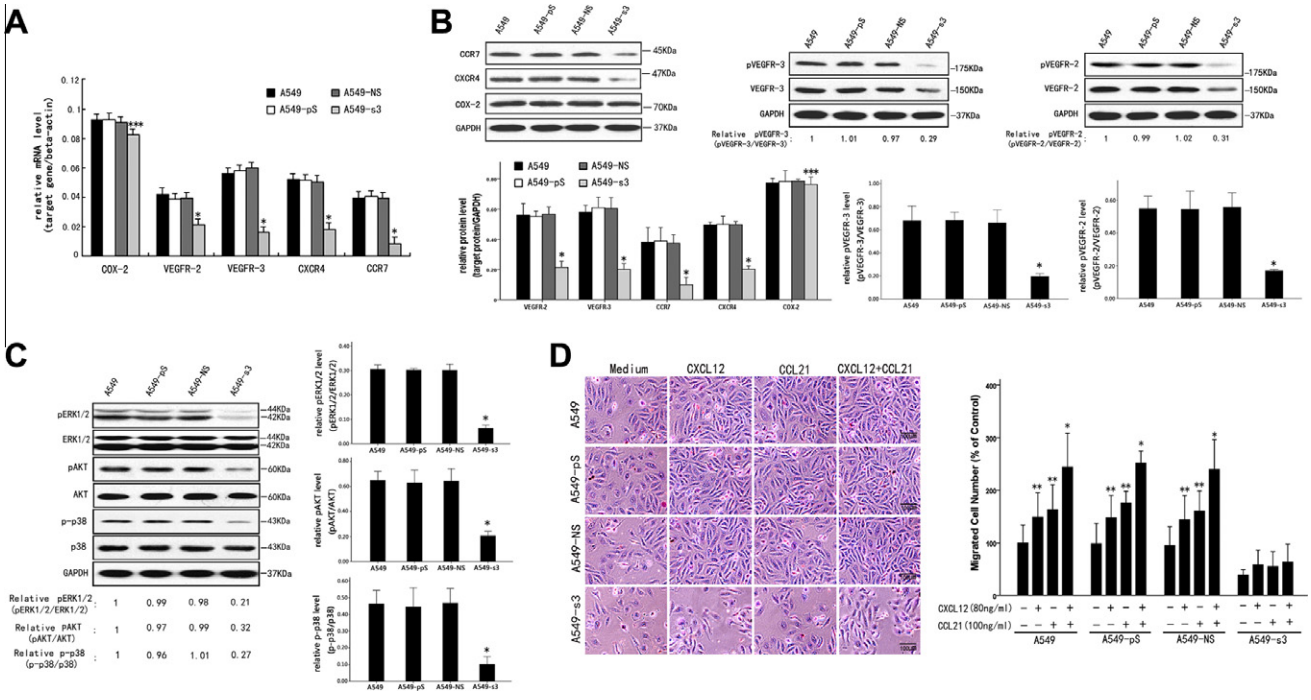

Fig. 2 – VEGF-C siRNA inhibited the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes and associated downstream signals. (A) QRT-PCR analyses of COX-2, CXCR4, CCR7, VEGFR-2 and VEGFR-3 mRNA expression. (B) Western blot analyses of CXCR4, CCR7, COX-2, VEGFR-3, pVEGFR-3, VEGFR-2 and pVEGFR-2 protein expression. (C) Western blot analyses of ERK1/2, p38 and AKT phosphorylation. Data are expressed as the ratio of VEGFR-2-phosphorylated, VEGFR-3-phosphorylated, ERK1/2-phosphorylated, p38-phosphorylated and AKT-phosphorylated proteins to total proteins. GAPDH was used as the loading control. (D) VEGF-C siRNA inhibited migration in A549 cells. A549, A549-pS, A549-NS, and A549-s3 were plated to assay cell migration towards CXCL12 and CCL21. Inhibitory effects on migration are presented as the percentage of fluorescent readings standardised to the A549 control (mean ± S.D. of triplicates). $^{*}P < 0.01$; $^{**}P < 0.05$; $^{***}P > 0.05$.

3.2. *Effects of VEGF-C siRNA on the VEGFR-2, VEGFR-3, CXCR4 and CCR7-dependent axes and downstream signalling molecules*

VEGFR-2, VEGFR-3, CXCR4, CCR7 and COX-2 overexpression has been correlated with lymph node metastasis in gastric, thyroid and lung cancer.[15,16,20] We determined the effects of VEGF-C siRNA targeting on the levels of these mRNAs and proteins. The expression levels of VEGFR-2, VEGFR-3, CXCR4 and CCR7 mRNAs (54.25%, 28.63%, 35.72% and 22.40%, respectively) and proteins (38.27%, 34.72%, 42.70% and 26.25%, respectively) in A549-s3 were significantly decreased ($P < 0.001$; Fig. 2A and B) when compared with untransfected A549 cells. There were no differences in mRNA and protein expression levels in A549-pS and A549-NS cells when compared with A549 controls. VEGF-C siRNA had no effect on COX-2 expression ($P > 0.05$). These results indicate that VEGF-C effects the expression of its own receptors and those of various chemokines in tumour cells and suggest that CXCR4, CCR7, VEGFR-2 and VEGFR-3 are targets of VEGF-C autocrine signalling.

Ligand-induced phosphorylation of VGFRs is critical for the regulation of receptor kinase activity and for receptor interaction with signal transduction molecules. Therefore, we measured the levels of pVEGF-2, pVEGF-3, pAKT, pERK1/2 and phospho-p38 by Western blot analysis to determine the effects of specific-VEGF-C siRNA targeting on the expression levels of pVEGFR-2, pVEGFR-3 and downstream signalling molecules in NSCLC tumour cells. RNAi-mediated silencing of VEGF-C significantly decreased the phosphorylation of VEGFR-2 and VEGFR-3 and the phosphorylation of the downstream signalling molecules ERK1/2, p38-MAPK and AKT in A549 cells (Fig. 2B and C). This indicates that the silencing of VEGF-C interfered with the activation of AKT, ERK1/2 and p38 stimulated by VEGFR-2, VEGFR-3, CXCR4 and CCR7-dependent signalling.

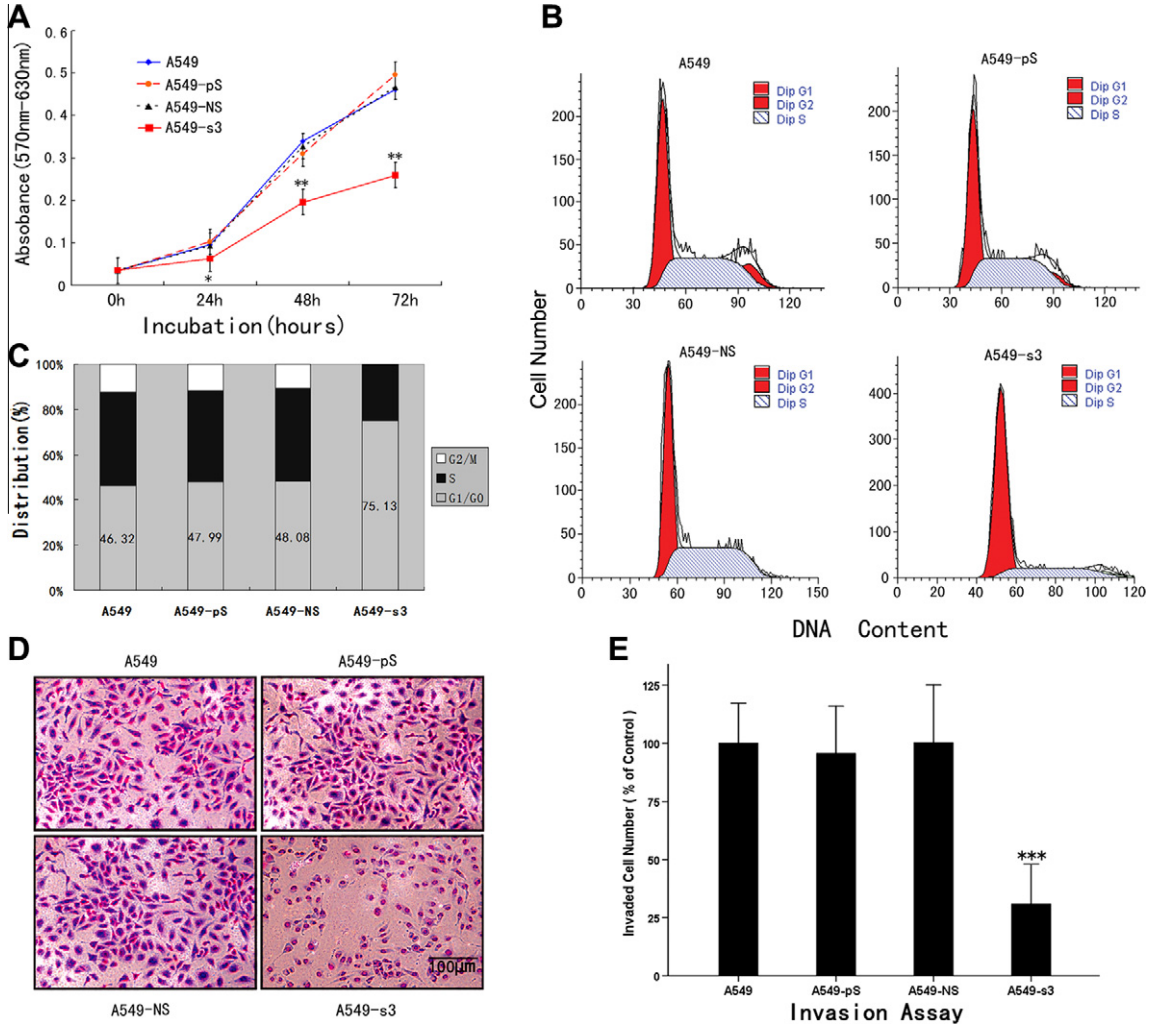

Fig. 3 – VEGF-C siRNA specifically suppressed cell proliferation and cell invasion. (A) The cell proliferation of untransfected or stably transfected A549 cells was measured by MTT assay. (B and C) Flow cytometry analysis of the effects of VEGF-C siRNA on the cell cycle progression. (D) Cells that invaded the underside of the membranes were stained and photographs were taken under a microscope. (E) Cells invading through the membrane were measured by relative fluorescence units (RFU) of CyQUANT dye binding by cellular nucleic acids in the lysates. Inhibitory effects on invasion are presented as the percentage of fluorescent readings standardised to A549 controls (mean ± S.D. of triplicates). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

We determined whether CXCR4/CCR7-dependent migration required VEGF-C. We investigated whether the down-regulation of VEGF-C expression abrogated cell migration towards CXCL12 and CCL21 (the ligands for CXCR4 and CCR7, respectively). A549, A549-pS and A549-NS cells migrated robustly towards CXCL12 and CCL21. However, the migration of A549-s3 cells was substantially reduced, and no significant responses to either CXCL12 or CCL21 in VEGF-C down-regulated tumour cells were detected (Fig. 2D). It has been reported that the VEGF-C/VEGFR-3 axis promotes migration of cancer cells.[21] Consistent with this report, we found the disruption of VEGF-C/ VEGFR-3 axis by silencing the VEGF-C gene could significantly reduce the aspecific (without any chemoattractant) cell migration (Fig. 2D). These data indicate that endogenous VEGF-C has direct effects on tumour cell migration. More importantly, these data also indicate that endogenous VEGF-C regulates NSCLC invasion by inducing CXCR4 and CCR7 expressions and driving directional migration towards CXCL12 and CCL21. A direct role for VEGF-C in chemokinesis is also possible.

3.3. *Effects of VEGF-C siRNA on tumour cell proliferation and invasion*

The biological effects of VEGF-C siRNA on proliferation of A549 cells were monitored by MTT assays. The proliferation of A549-s3 cells was significantly inhibited at 48 and 72 h compared with untransfected A549 cells and the highest inhibitory rate was 56.35 ± 8.47% at 72 h ($P < 0.01$; Fig. 3A). In contrast, there was no obvious inhibition of A549-NS and A549-pS cell proliferation over the entire experimental period ($P > 0.05$; Fig. 3A). We performed cell cycle analysis to further examine the effects of VEGF-C siRNA on the cell cycle of A549 cells (Fig. 3B and C). The percentage of A549-s3 cells in the G1/G0 phase increased by 28.81 ± 1.19% ($P < 0.001$), and the percentage of A549-s3 cells in the S phase decreased by 16.92 ± 0.55% ($P < 0.001$) compared with untransfected A549 cells. These data suggest that VEGF-C siRNA arrests cells in the G1 phase and that VEGF-C expression is required for metastasis.

We performed a matrigel invasion assay to evaluate the impact of the down-regulation of VEGF-C expression on A549 cellular invasion. A549-s3 cells showed a remarkable decrease in invasive capacity compared with A549, A549-pS and A549-NS cells (Fig. 3D); the invaded A549-s3 cell number was reduced to 30.85% of that of the control ($P < 0.001$; Fig. 3E). These results indicate that VEGF-C expression is required for NSCLC invasion. Notably, the effect of VEGF-C siRNA was so dramatic that the cells could hardly invade through the matrigel membrane.

3.4. *Therapeutic effects of VEGF-C siRNA on a human lung cancer xenograft*

We performed a xenograft experiment with A549 cells to elucidate the therapeutic effects of VEGF-C siRNA on tumour cell growth *in vivo*. Mice bearing subcutaneous tumours were treated with therapy beginning 15 d after tumour cell injection. Treatment groups received 250 μl lentivirus or PBS by i.v. injection into the tail vein every 24 h for 3 weeks. Tumour weights and volumes were measured at the termination of the experiment. Tumour weights were significantly higher in untreated compared with Lv-VEGF-C siRNA-treated tumours (1079.10 ± 168.47 *versus* 565.57 ± 89.33, respectively; $P < 0.001$; Fig. 4A). There was a significant change in tumour volume between untreated and Lv-VEGF-C-siRNA treated tumours (241.88 ± 34.03 *versus* 87.36 ± 10.93, respectively;

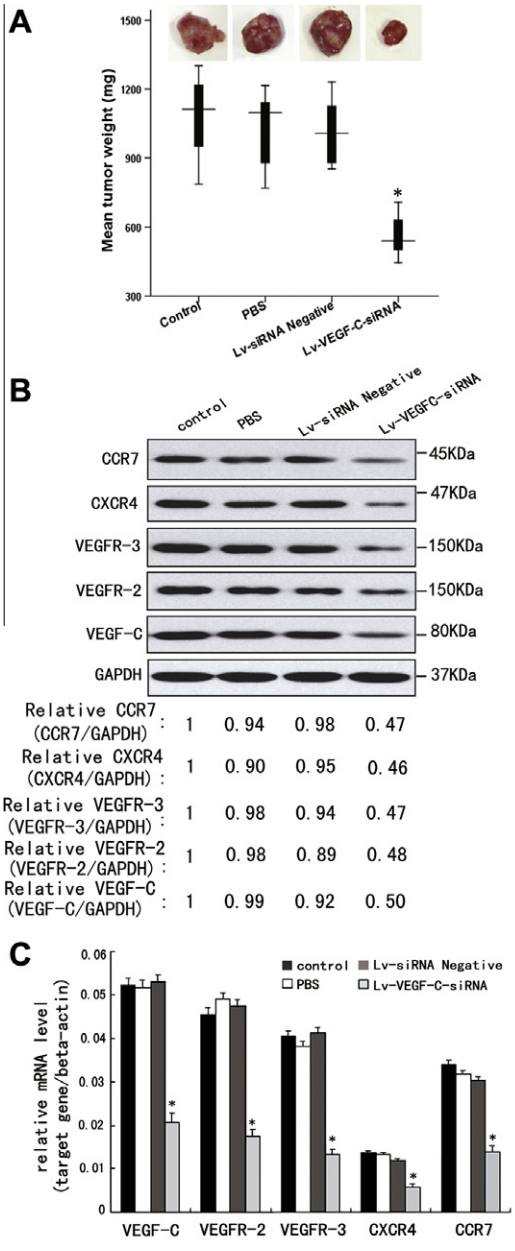

Fig. 4 – Therapeutic effects of VEGF-C siRNA on human lung cancer xenografts. (A) Subcutaneous tumours were treated for 3 weeks; the mean weight of the Lv-VEGF-C-siRNA–treated tumours was significantly lower than the control tumours. (B and C) Western blot and QRT-PCR analyses of VEGF-C, VEGFR-2, VEGFR-3, CXCR4 and CCR7 expression in protein samples and total RNA extracted from tumours. Data were confirmed in triplicate experiments; Columns indicate means; bars are the standard error. *$P < 0.01$.

$P < 0.001$). Treatment with control non-targeting siRNA did not inhibit tumour growth compared with control mice treated with PBS alone.

To investigate the effects of VEGF-C siRNA induced down-regulation of the expression of VEGFR-2, VEGFR-3, CXCR4 and CCR7 *in vivo*, tumour homogenates and tissues were subjected to protein and mRNA expression analyses (Fig. 4B and C). The mean expression of VEGF-C, VEGFR-2, VEGFR-3, CXCR4 and CCR7 proteins (52.20%, 50.45%, 48.22%, 48.45% and 47.94%, respectively) and mRNAs (39.51%, 36.81%, 33.00%, 45.59% and 43.72%, respectively) were significantly decreased ($P < 0.01$) in Lv-VEGF-C-siRNA treated mice compared with treated negative controls. There were no differences in protein and mRNA expression levels among the PBS, Lv-siRNA negative and untreated control groups ($P > 0.05$). The CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent

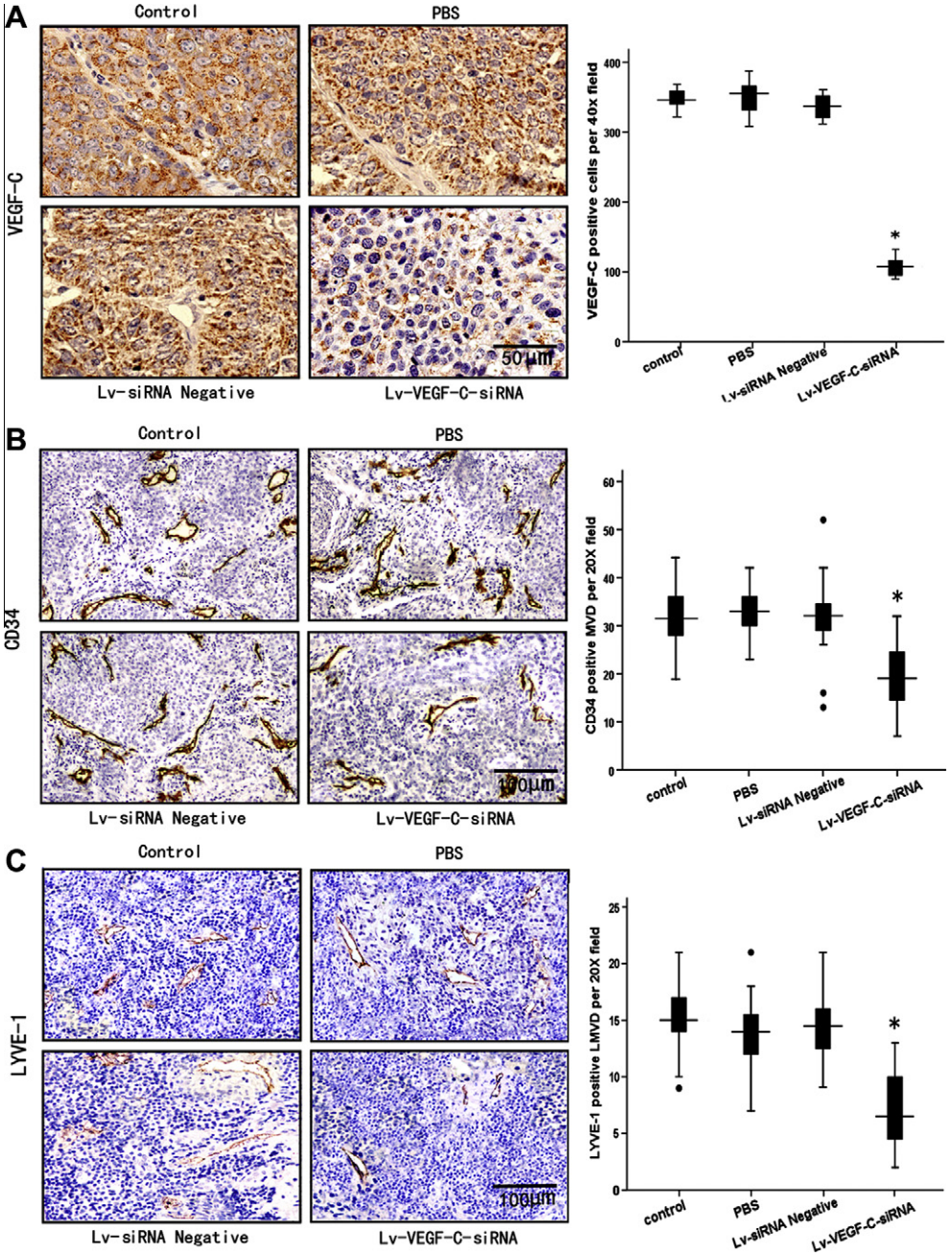

Fig. 5 – Immunohistochemical analysis showing the effects of VEGF-C siRNA on VEGF-C protein expression, microvessel densities (MVD), and lymphatic microvessel densities (LMVD). (A) Lv-VEGF-C-siRNA-treated tumour tissue demonstrated no or very weak VEGF-C protein expression. The histograph corresponds to the number of cells showing immunoreactivity against VEGF-C in control and Lv-VEGF-C-siRNA-treated mice. Similarly, (B) tumour tissues from Lv-VEGF-C-siRNA-treated mice showed significantly reduced MVD, whereas control tumours revealed high MVD. (C) The histograph corresponds to the number of LMVD showing immunoreactivity against LYVE-1 in control and Lv-VEGF-C-siRNA treated mice. Columns indicate means; bars are the standard error. *$P < 0.001$, statistically significant.

axes were down-regulated *in vitro* and *in vivo* by the reduction in the expression levels of VEGFR-2, VEGFR-3, CXCR4 and CCR7. These data indicate that tumour growth is reduced by tail vein injection Lv-VEGFC-siRNA induced VEGF-C silencing.

3.5. *Therapeutic effects of VEGF-C siRNA on tumour angiogenesis and lymphangiogenesis*

To investigate whether the inhibition of tumour growth is associated with the down-regulation of VEGF-C expression, the xenograft tumour tissues were excised and subjected to VEGF-C protein immunohistochemical staining. VEGF-C protein was significantly down-regulated in the Lv-VEGF-C-siRNA treated xenograft compared with the control tumour (Fig. 5A). The CD34-stained MVD and LYVE-1-stained LMVD were quantitated to determine whether VEGF-C knockdown directly affected angiogenesis and lymphangiogenesis *in vivo* (Fig. 5B and C). The median numbers of MVD and LMVD were 32 (range, 16–46) and 14 (range, 7–22), respectively, in A549 subcutaneous tumours from PBS treated or untreated mice. Lv-VEGF-C-siRNA treatment significantly decreased the numbers of both MVD and LMVD (MVD: median, 19; range, 7–32; $P < 0.001$, compared with control; LMVD: median, 7; range, 2–13; $P < 0.001$, compared with control). Treatment with Lv-siRNA negative had no effect. These data indicate that VEGF-C plays a role in angiogenesis and lymphangiogenesis in tumours.

4. Discussion

Tumour invasion and metastasis are characteristics of the aggressive phenotype of human cancers and are the major causes of cancer deaths.[22] During tumour invasion and metastasis, tumour cells acquire the ability to support autocrine signalling pathways by expressing growth factors and their cognate surface receptors. The VEGF-C/VEGFR-3 axis is one growth factor-receptor set that regulates tumour invasion and metastasis.[21,23,24] Disruption of growth factor-receptor axes is a current strategy for the development of anticancer drugs.[25] We investigated the role of VEGF-C in tumour cell growth, migration and invasion. We used lentivector-mediated RNA interference against VEGF-C expression to demonstrate that siRNA-directed transcriptional silencing is a promising tool for suppression of VEGF-C gene function with potential applications in cancer treatment.

Recently, some studies have shown that both VEGF-C and VEGFR-3 are expressed by metastatic tumour cells.[26,27] This suggests that tumour cell proliferation, invasion and lymphatic metastasis involve VEGC-C/VEGFR-3 autocrine stimulation mechanisms,[21] and that VEGF-C may act in multiple ways to promote tumour progression. We found that RNA interference against VEGF-C successfully inhibited the expression of VEGF-C and VEGFR-3, and simultaneously induced the down-regulation of CXCR4, CCR7, and VEGFR-2 expression *in vitro* and *in vivo*. This is the first study to demonstrate a link between autocrine growth factors and their specific receptors, and between autocrine growth factors and chemokine receptors. These data add an important dimension to the biology of tumour invasion and metastasis. We speculate that the acquisition of an invasive phenotype by tumour cells is a multistep process involving the initiation of autocrine signalling loops that include specific growth factors, and the consequent expression of chemokine receptors that enable tumour cells to migrate towards chemoattractant gradients.

The phosphatidylinositol 3-kinase-AKT, extracellular signal-regulated kinase (ERK1/2) and p38 pathways are critical for cellular growth and survival. Inhibition of ERK and AKT pathways had antitumour and antiangiogenic effects in preclinical models of human hepatocellular carcinoma.[28] In endothelial cells, these intracellular signalling pathways activated by VEGFRs are well characterised; in cancer cells, less information is available and different opinions still exist. In lung cancer cell lines, VEGFR-3 stimulation resulted in activation of p38 but not ERK1/2, and kinase domain-deleted VEGFR-3 protein reduced p38 MAPK but not ERK1/2 phosphorylation[21]; in gastrointestinal cancer cells, a pan-VEGFR inhibitor could inhibit VEGFR-3 phosphorylation as well as phosphorylation of downstream molecules AKT and ERK;[29] in breast cancer cells, ERK phosphorylation was linked to VEGFR-2 expression.[30] Similar to VEGFRs-induced downstream signalling molecules, different opinions on CXCR4 and CCR7-induced downstream signalling molecules also exist.[31–33] In NSCLC cells, we found that VEGF-C siRNA inhibited VEGFR-2 and VEGFR-3 phosphorylation as well as CXCR4 and CCR7-induced migration of tumour cells towards CXCL12 and CCL21. This simultaneously suppressed the AKT, ERK1/2 and p38 pathways by inhibiting phosphorylation of AKT, ERK1/2 and p38. We believe that these results were due to the down-regulation of the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent biological axes suggesting that RNAi-mediated silencing of VEGF-C is a powerful therapeutic approach for controlling NSCLC growth and metastasis.

Previous studies showed that tumour invasion and metastasis were inhibited by blocking the VEGFR-2, VEGFR-3 and CXCR4-dependent axes,[34–36] but none investigated the relative contribution of each individual pathway to the overall inhibitory effect. Our data highlight the relative importance of the VEGF-C/VEGFR-3 axis in NSCLC metastasis. Our results suggest that the VEGF-C/VEGFR-3 axis has a potent effect on other well-known mechanisms of cancer metastasis such as the VEGFR-2, CCR7 and CXCR4 biological axes. The simultaneous down-regulation of the VEGFR-2, VEGFR-3, CXCR4 and CCR7-dependent axes had a greater inhibitory effect on tumour progression than the blockade of each individual axis. Our *in vivo* investigations provide evidence that silencing the VEGF-C gene not only inhibits lymphangiogenesis but also leads to a potent inhibition of tumour angiogenesis in lung tumour xenografts. Down-regulation of the VEGFR-2 and VEGFR-3-dependent axes prevented the functions of VEGF-A and VEGF-C and resulted in the inhibition of tumour angiogenesis.

Our results suggest that silencing the VEGF-C gene could affect several signalling pathways critical for tumour cells growth, survival, migration and invasion such as AKT, ERK and p38 and VEGF-C performs multiple biological functions through the four ways below: (a) VEGF-C has autocrine effects on tumour cell VEGFR-3, VEGFR-2 that required for tumour lymphangiogenesis and tumour angiogenesis; (b) VEGF-C has autocrine effects on tumour cell chemokine receptors

(CXCR4 and CCR7) that promoting tumour cells directional migration towards specific chemokines (CXCL12 and CCL21), the ligand for those receptors, which present in tumour stroma and in tissue such as lymph node and lymphatics; (c) VEGF-C has direct effects on tumour cells motility and invasion, which are consistent with recent reports that the autocrine signalling of VEGF-C via VEGFR-3 in cancer cells promotes cell motility and invasion *in vitro* and *in vivo*;[21,37,38] and (d) VEGF-C has direct effects on tumour cell cycle that lead to increased proliferation and tumour growth *in vitro* and *in vivo*. In summary, we demonstrated that *in vitro* and *in vivo* lentivirus-mediated RNA interference of VEGF-C in human NSCLC cells successfully inhibited the expression of VEGF-C. This resulted in several antitumour activities such as inhibitory effects on cell growth, migration, invasion, lymphangiogenesis, angiogenesis and tumour growth; blocking effects on CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes; and suppressing effects on AKT, ERK and p38 signalling pathways. These findings suggest that RNAi targeting of VEGF-C can be an effective therapeutic strategy for NSCLC. The present report provides a basis for future studies in additional animal models and human clinical trials.

Conflict of interest statement

None declared.

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