

# Src contributes to IL6-induced vascular endothelial growth factor-C expression in lymphatic endothelial cells

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**Abstract** Formation of lymphatic capillaries by lymphatic endothelial cells (LECs) occurs both in normal tissues as well as in pathological processes including tumor metastasis. Interleukin-6 (IL-6), a potent pro-inflammatory cytokine, has been shown to be highly elevated in various cancers. IL-6 has also been shown to increase tumor lymphangiogenesis through vascular endothelial growth factor-C (VEGF-C) induction in tumor cells. Although lymphangiogenesis is associated with lymph node metastasis and also resistance to conventional therapy in various cancers, the precise mechanisms of lymphangiogenesis in LECs remain unclear. This study aimed to investigate the signaling cascade involved in IL-6-induced VEGF-C expression in murine LECs (SV-LEC). The *VEGF-C* mRNA and protein levels were increased in SV-LECs exposed to IL-6. IL-6 time-dependently induced Src phosphorylation and downstream phosphorylation of ERK1/2 and p38MAPK. In contrast, PP2, an

inhibitor of Src signaling, abrogated IL-6's effects on ERK1/2 and p38MAPK phosphorylation. IL-6 exposure also led to increase in VEGF-C promoter-luciferase activity as well as C/EBP $\beta$ - and  $\kappa$ B-luciferase activities. VEGF-C promoter-, C/EBP $\beta$ - and  $\kappa$ B-luciferase activities were all suppressed by Src, ERK1/2 or p38MAPK signaling blockades despite presence of IL-6. Finally, C/EBP $\beta$  and p65 binding to the VEGF-C promoter region were increased after IL-6 exposure in SV-LECs. Taken together, we report a Src-mediated ERK1/2 and p38MAPK activation resulting in C/EBP $\beta$  and p65 binding to the promoter region of VEGF-C, leading to VEGF-C expression in IL-6-exposed SV-LECs.

**Keywords** Lymphatic endothelial cells (LECs) · Interleukin-6 (IL-6) · Vascular endothelial growth factor-C (VEGF-C) · Lymphangiogenesis · Src

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## Introduction

Cancer is an increasingly prevalent health problem and remains a major cause of mortality around the world [1]. The metastatic spread of tumor cells is associated with resistance to conventional therapy and is the leading cause of death for cancer patients. Tumor cells produce a range of growth factors and/or cytokines that promote tumor metastasis by directly or indirectly stimulating angiogenesis and lymphangiogenesis, which is one of the major routes for tumor invasion and metastasis [2, 3]. Inhibiting tumor-induced lymphangiogenesis has been shown to markedly suppress the spread of cancer in animal models [4, 5]. Therefore, the identification of mechanisms underlying tumor lymphangiogenesis is essential for developing new therapeutic strategies in the treatment of cancer.

Chronic inflammation plays a crucial role in human carcinogenesis. Many lines of evidence demonstrated that elevated serum interleukin-6 (IL-6) levels is correlated with poor clinical prognosis in many cancers including lymphoma, head and neck cancer and prostate cancers [6–9]. IL-6 not only plays a central role in cancer-associated inflammation [10], it has also been implicated in the regulation of tumor growth [11–14]. IL-6 exerts its biological activities through IL-6 receptor (IL-6R) and gp130 [15]. When IL-6 binds to mIL-6R, the membrane-bound form of IL-6R, gp130 homodimerizes and forms a functional receptor complex with IL-6 and IL-6R. On the other hand, IL-6 can also bind to the soluble form of IL-6R (sIL-6R) to form an IL-6/sIL-6R complex and act as agonist for cells that express gp130 [16]. The receptor complex formed by IL-6 triggers two main signaling pathways. First is the janus kinases (JAKs)/signal transducer and activator of transcription 3 (STAT3) pathway. In this pathway, active STAT3 translocates to the nucleus and binds to promoter region of target genes, thereby activating transcription. IL-6 can also activate the Ras/extracellular-signal-regulated kinase (ERK) pathway, which activates transcription factors such as NF-IL-6 (CCAAT/enhancer-binding protein  $\beta$ , C/EBP $\beta$ ) [17]. These pathways independently transduce the signal from IL-6 leading to its various biological activities.

Similar to angiogenesis, lymphangiogenesis relies on the regulation of several lymphoangiogenic factors and receptors [18]. Vascular endothelial growth factor-C (VEGF-C) is currently the best-characterized lymphangiogenic factor that acts via VEGF receptor-3 (VEGFR-3). In normal adult tissues, VEGFR-3 expression is largely restricted to the LECs, and its activation is responsible for LEC proliferation, migration, and survival [19, 20]. Tumor cell-derived VEGF-C plays a causal role in lymphangiogenesis and lymphatic metastasis [21–23]. In addition, many studies have also demonstrated that VEGF-C is induced in several types of immune cells and lymphatic capillaries have been shown to proliferate during inflammation [24–26]. IL-6, a typical inflammatory cytokine with various effects on immune cells [27], was recently reported to regulate VEGF-C expression and metastasis in several types of cancer cells, such as oral squamous cell carcinoma [28]. However, little is known about VEGF-C regulation in LECs exposed to IL-6. This study aimed to investigate whether IL-6 regulates VEGF-C expression in LECs. The limitations of studies on lymphangiogenesis and signaling pathways in LECs are the difficulties in the isolation and propagation of LECs from different organs [19, 29, 30]. To overcome these limitations, we selected a “conditionally immortalized” line of murine LECs (SV-LECs) that express SV40 large T and retain their ‘lymphatic’ endothelial characteristics after repeated passages [31, 32]. In

this study, we demonstrated that IL-6 activates Src-mediated ERK and p38MAPK signaling cascade, resulting in C/EBP $\beta$  and p65 binding to the promoter region of VEGF-C and subsequent VEGF-C expression in SV-LECs.

## Materials and methods

### Reagents

DMEM, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Normal IgG, rabbit polyclonal antibodies specific for p65 and CEBP $\beta$ , and antibodies against FAK phosphorylated at Tyr397 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Src phosphorylated at Tyr216, antibodies against ERK1/2 phosphorylated at Thr202/Tyr204, and antibodies against STAT3 phosphorylated at Tyr705 were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against p38MAPK phosphorylated at Thr180/Tyr182, anti-mouse and anti-rabbit IgG conjugated horseradish peroxidase antibodies, and anti-c-Src, anti-p38MAPK, anti-VEGF-C, and anti- $\alpha$ -tubulin antibodies were obtained from GeneTex Inc (Irvine, CA, USA). Recombinant IL-6 and soluble IL6R $\alpha$  (sIL6R) were purchased from PeproTech (Rocky Hill, NJ, USA). PP2, p38MAPK kinase inhibitor III and ERK activation inhibitor peptide I were bought from Calbiochem (San Diego, CA, USA). Turbofect<sup>TM</sup> in vitro transfection reagent was purchased from Upstate Biotechnology (Lake Placid, NY, USA). C/EBP reporter construct, p/T81 C/EBP-luc, was kindly provided by Dr. Kjetil Tasken (University of Oslo, Oslo, Norway).  $\kappa$ B-Luc, Renilla-luc and Dual-Glo luciferase assay systems were purchased from Promega (Madison, WI, USA). All materials for immunoblotting were purchased from GE Healthcare (Little Chalfont, UK). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

### Cell culture

The mouse LEC line SV-LEC was kindly provided by Dr. J.S. Alexander (Shreveport, LA, USA), and was cultured as previously described [31].

### Immunoblot analysis

Immunoblot analyses were performed as described previously [33]. Briefly, cells were lysed in an extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5 % NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein were subjected to SDS-PAGE and transferred onto a

NC membrane which was then incubated in a TBST buffer containing 5 % non-fat milk. Proteins were visualized by incubating with specific primary antibodies followed by horse radish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence following the manufacturer's instructions. Quantitative data was obtained using a computing densitometer with Image-Pro Plus image analysis software system (Kodak, Rochester, NY, USA).

#### Transfection in SV-LECs and dual luciferase reporter assay

SV-LECs ( $10^5$  cells per well) were transfected with pGL4-VEGF-C-luc-370 (1  $\mu$ g),  $\kappa$ B-luc (1  $\mu$ g), or CEBP/ $\beta$ -luc (1  $\mu$ g) plus renilla-luc (0.25  $\mu$ g) using Turbofect reagent (Upstate Biotechnology, Lake Placid, NY, USA) for 48 h. After transfection, cells with or without treatments were harvested. The luciferase activity was then determined using a Dual-Glo luciferase assay system kit (Promega) according to manufacturer's instructions, and was normalized on the basis of renilla luciferase activity.

#### Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNeasy RNA isolation kit (GE Healthcare, Little Chalfont, UK). The RT-PCR was then conducted following the manufacturer's instructions (Super Script One-Step RT-PCR system, Invitrogen). Primers used for amplification of the VEGF-C and GAPDH fragments were as follows: mouse VEGF-C, sense 5'-AGCCAACAGGGAATTTGATG-3' and antisense 5'-CACAGCGGCATACTTCTTCA-3'; mouse GAPDH, sense 5'-CCTTCATTGACCTCAACTAC-3' and antisense 5'-GGAAGGCCATGCCAGTGAGC-3'; and GAPDH was used as the internal control. The PCR was performed with the following conditions: 30 cycles of a 30-s denaturation step at 94 °C, a 30-s annealing step at 56 °C, and a 45-s extension step at 72 °C to amplify survivin and GAPDH cDNA. The amplified fragment sizes for mouse VEGF-C and mouse GAPDH were 239 and 594 bp, respectively. PCR products were run on an agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination.

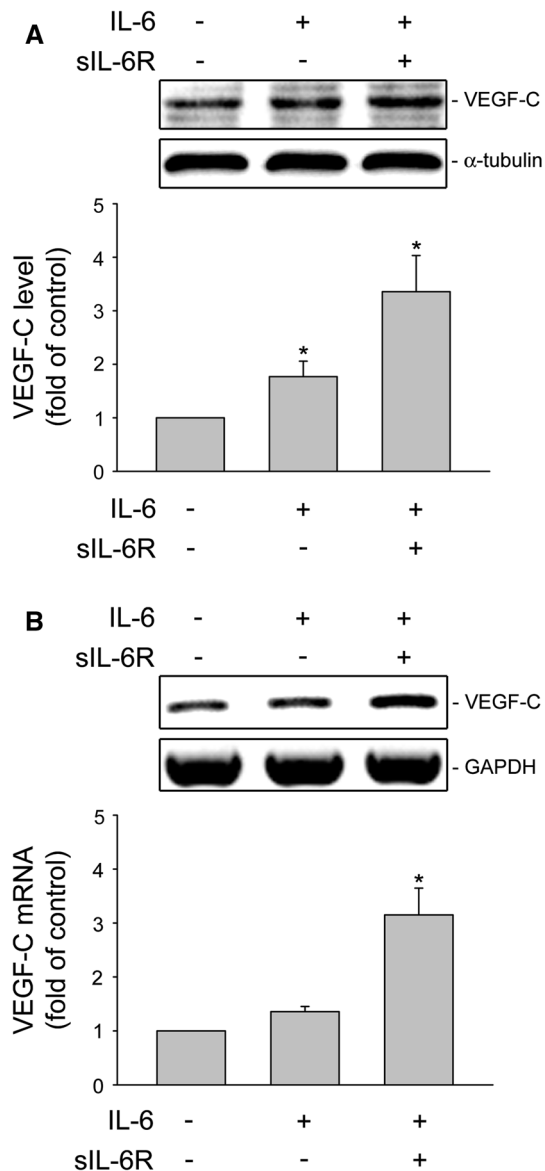
#### Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as described previously [33]. Briefly, cells were cross-linked with 1 % formaldehyde at 37 °C for 10 min and rinsed with ice-cold PBS. Cells were then harvested in SDS lysis buffer, sonicated five times for 15 s each, and centrifuged for 10 min. Supernatants were

collected and diluted in ChIP dilution buffer, followed by immunoclearing with gentle rotation with 80  $\mu$ l protein A-agarose slurry for 1 h at 4 °C. An aliquot of each sample was used as "input" in the PCR analysis. The remainder of the soluble chromatin was incubated at 4 °C overnight with either p65 and CEBP/ $\beta$  antibodies (Santa Cruz Biotechnology), or control IgG (Santa Cruz Biotechnology). Immune complexes were collected by incubation with a 60  $\mu$ l protein A-agarose slurry for 2 h at 4 °C with a gentle rotation. The complexes were washed sequentially for 5 min in the following three washing buffers: a low salt immune complex washing buffer, a high-salt immune complex washing buffer, and a LiCl immune complex washing buffer. Precipitates were washed two times with Tris-EDTA buffer. The complexes were, then, eluted twice with two 100  $\mu$ l aliquots of elution buffer. The cross-linked chromatin complex was reversed in the presence of 0.2 M NaCl and heating at 65 °C for 4 h. DNA was purified using GFX™ DNA purification spin columns (GE Healthcare). PCR was performed using PCR Master Mix (Promega, Madison, WI) according to the manufacturer's protocol. Ten percent of the total purified DNA was used for the PCR in 50  $\mu$ l reaction mixture. The 170-bp VEGF-C promoter fragment between -198 and -367 (contains putative C/EBP binding sites) was amplified using the primer pair, sense: 5'-cgg gac gag tgg aac atc-3' and antisense: 5'-agg tac gag cct cac agg aa-3', in 30 cycles of PCR. The 103-bp VEGF-C promoter fragment between -164 and -62 (contains putative NF- $\kappa$ B binding sites) was amplified using the primer pair, sense: 5'-aga gcc gag ggc aaa agt-3' and antisense: 5'-gct gag gtc ctc tcc tgg tc-3', in 30 cycles of PCR. This was done at 95 °C for 30 s, 56 °C for 30 s, and finally 72 °C for 45 s. The PCR products were analyzed by 1.5 % agarose gel electrophoresis

#### Preparation of VEGF-C promoter-luciferase reporter construct

Mouse genomic DNA from SV-LECs was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. VEGF-C promoter sequences (-370 to +1) was then amplified from mouse genomic DNA by PCR, using the following primers containing KpnI and BglII restriction sites: forward: 5'-ggtacctctcgggacgagtggaacat-3'; reverse: 5'-agatcttggatggaccgggagca gg-3'. To construct the VEGF-C-luc, the amplified PCR products were digested and ligated into PGL4 vector, which encodes firefly luciferase. The fidelity of the insert was confirmed by DNA sequence analysis. The construct was designed as VEGF-C promoter reporter (pGL4-VEGF-C promoter-luc-370).



**Fig. 1** IL-6 induces VEGF-C expression in SV-LECs. **a** Cells were treated with vehicle, IL-6 (20 ng/ml) or IL-6 plus sIL6R (20 ng/ml) for 24 h. After treatment, cells were harvested for assessing the extent of VEGF-C by immunoblotting. Each column represents the mean  $\pm$  SEM of six independent experiments.  $*p < 0.05$ , compared with the control group. **b** Cells were treated with vehicle, IL-6 (20 ng/ml) or IL-6 plus sIL6-R (20 ng/ml) for 6 h. After treatment, the extent of *vegfc* mRNA was analyzed by RT-PCR as described in the “Materials and methods” section. Each column represents the mean  $\pm$  SEM of five independent experiments.  $*p < 0.05$ , compared with the vehicle-treated group. IL-6/sIL-6R: IL-6 plus sIL-6R

### Statistical analysis

Results are presented as the mean  $\pm$  SE from at least three independent experiments. One-way analysis of variance (ANOVA) was followed by the Newman-Keuls test, when appropriate, to determine the statistical significance of the

difference between means. A  $p$  value of  $<0.05$  was considered statistically significant.

### Results

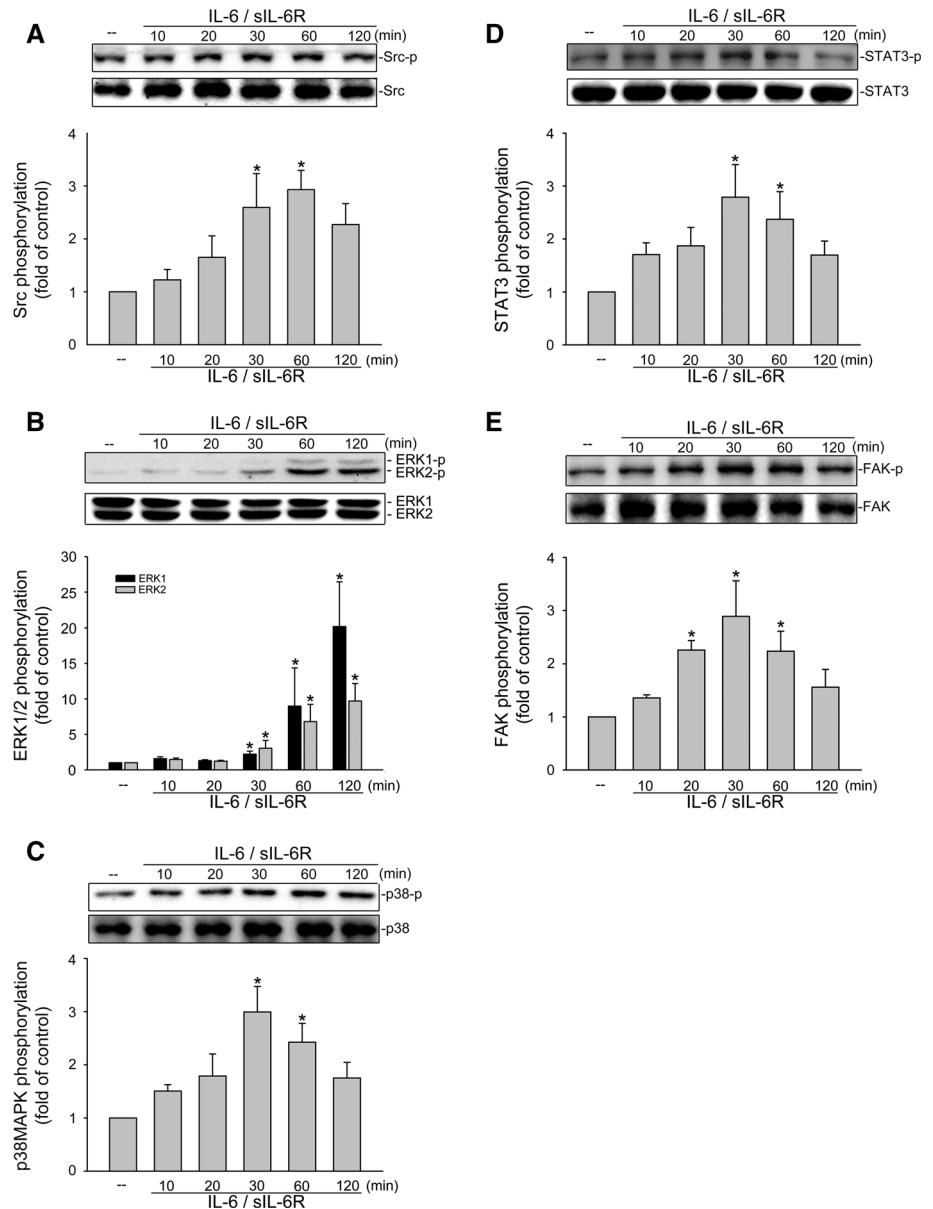
#### IL-6 induced VEGF-C expression in SV-LECs

It has previously been demonstrated that in addition to extending the half-life of serum IL-6, sIL-6R may form a multimeric signaling complex with IL-6 to activate downstream signal transduction pathways [34–36]. We therefore examined the VEGF-C levels in SV-LECs exposed to IL-6 or IL-6 plus sIL-6R (IL-6/sIL-6R). As shown in Fig. 1a, 24 h treatment with IL-6 (20 ng/ml) significantly increased VEGF-C protein levels by  $1.7 \pm 0.3$ -folds ( $n = 6$ ). IL-6 (20 ng/ml) plus sIL-6R (20 ng/ml) further increased the protein levels of VEGF-C by  $3.4 \pm 0.7$ -fold (Fig. 1a). We then determined whether the elevation of VEGF-C protein levels was attributable to an increase in *vegfc* mRNA. As shown in Fig. 1b, IL-6 led to elevation of *vegfc* mRNA levels. The increase in *vegfc* mRNA level was more pronounced when SV-LECs were exposed to both IL-6 and sIL6R. These findings suggest that transcriptional upregulation of *vegfc* contributes to IL-6- and IL6/sIL6R-induced VEGF-C expression. In the following experiments, we explored the signaling cascades involved in VEGF-C induction in SV-LECs exposed to IL6/sIL-6R.

#### IL-6/sIL-6R induced Src, ERK1/2, p38MAPK, STAT3 and FAK phosphorylation in SV-LECs

We next explored the IL-6/sIL-6R-activated signaling pathways in SV-LECs. Src tyrosine kinase, a promising therapeutic cancer target, plays a critical role in cell migration, proliferation, survival, and angiogenesis [37]. Several lines of evidence also indicated that Src signaling is involved in VEGF-C expression in tumor cells [38, 39]. We thus determined whether IL-6/sIL-6R induces Src activation in SV-LECs. IL-6/sIL-6R treatment led to a significant increase in Src phosphorylation in a time-dependent manner (Fig. 2a). The increase in Src phosphorylation was observed to begin at 10 min and then peaked at 30–60 min after treatment. We next determined whether the phosphorylation status of ERK1/2 and p38MAPK are altered in SV-LECs exposed to IL-6/sIL-6R. IL-6/sIL-6R time-dependently induced ERK1/2 (Fig. 2b) and p38MAPK (Fig. 2c) phosphorylation within 120 min of exposure. Since Src can activate STAT3 [40] and FAK [41], we also examined whether STAT3 and/or FAK is activated in IL-6/sIL-6R-stimulated SV-LECs. IL-6/sIL-6R treatment led to an increase in phosphorylation of STAT3,

**Fig. 2** IL-6 induced Src, ERK1/2, p38MAPK, STAT3, and FAK phosphorylation in SV-LECs. Cells were treated with IL-6 plus sIL6R for different time periods as indicated. The phosphorylation status of Src (a), ERK1/2 (b), p38MAPK (c), STAT3 (d) and FAK (e) were then determined by immunoblotting. Each column represents the mean ± SEM of at least four independent experiments. \**p* < 0.05, compared with the control group

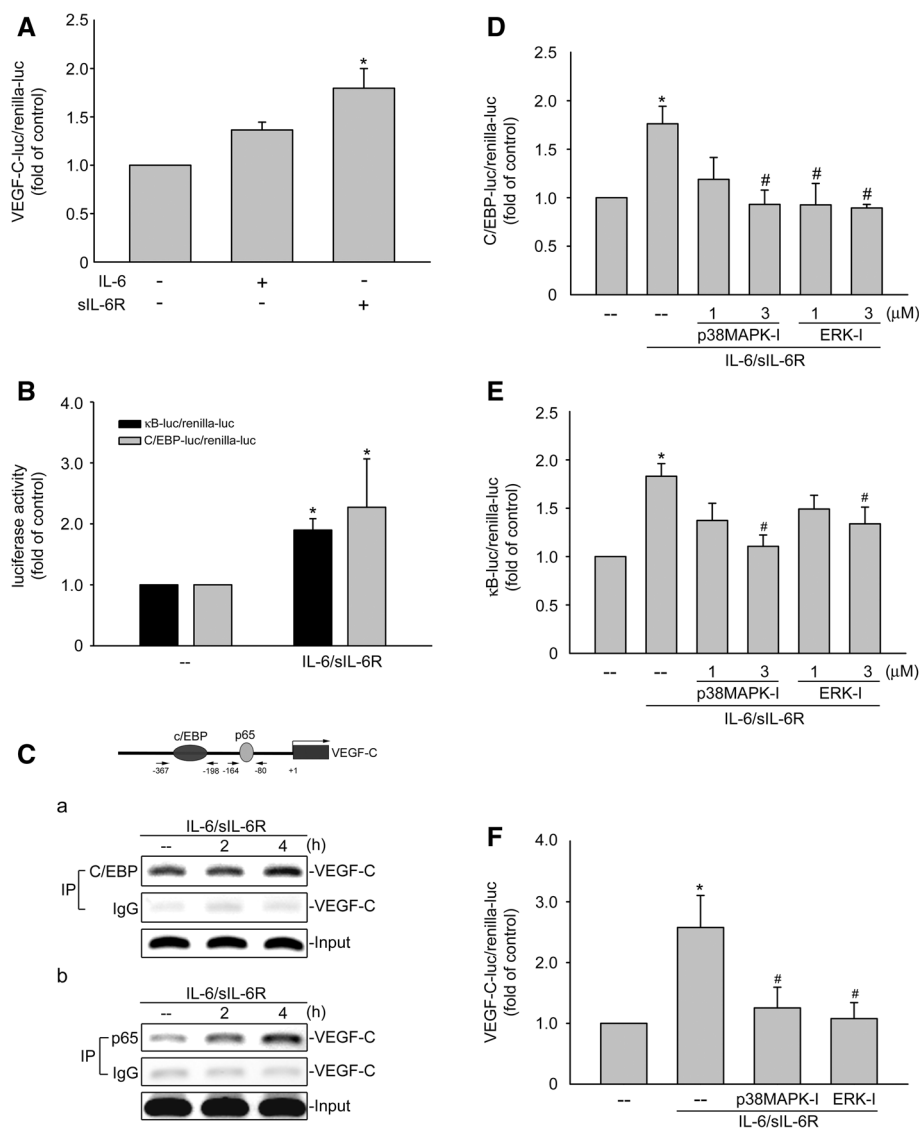


the main downstream molecule of IL-6R-signaling (Fig. 2d). IL-6/sIL-6R also time-dependently induced FAK phosphorylation in SV-LECs (Fig. 2e).

IL-6/sIL-6R induced CCAAT/enhancer-binding protein (C/EBP) and NF-κB activation in SV-LECs

It is conceivable that IL-6/sIL-6R may activate transcription factors, leading to VEGF-C expression in SV-LECs. TFSEARCH analysis (Yutaka Akiyama: “TFSEARCH: Searching Transcription Factor Binding Sites”; <http://www.rwcp.or.jp/papia/>) [42] indicated that the promoter region between -370 and +1 of the mouse *vegfc* gene contains many potential consensus sequences, including those for CCAAT/enhancer-binding protein (C/EBP) and

NF-κB. To examine the promoter activity of the 370-bp 5'-flanking sequence of the *vegfc* gene, we constructed a VEGF-C promoter reporter plasmid containing the 370 bp promoter region (-370 to +1) of VEGF-C. Treatment of SV-LECs with IL-6 or IL-6/sIL6R for 24 h significantly caused an increase in VEGF-C promoter luciferase activity as determined by reporter assay (Fig. 3A). We also used reporter assays to determine whether IL-6/sIL6R activates C/EBPβ and NF-κB in SV-LECs. As shown in Fig. 3B, cells treated with IL-6/sIL6R for 24 h had significant increases in C/EBPβ- and κB-luciferase activities. To explore whether C/EBPβ and NF-κB subunit p65 are recruited to the endogenous VEGF-C promoter region in response to IL-6/sIL6R, chromatin immunoprecipitation (ChIP) experiments on SV-LECs exposed to IL-6/sIL6R



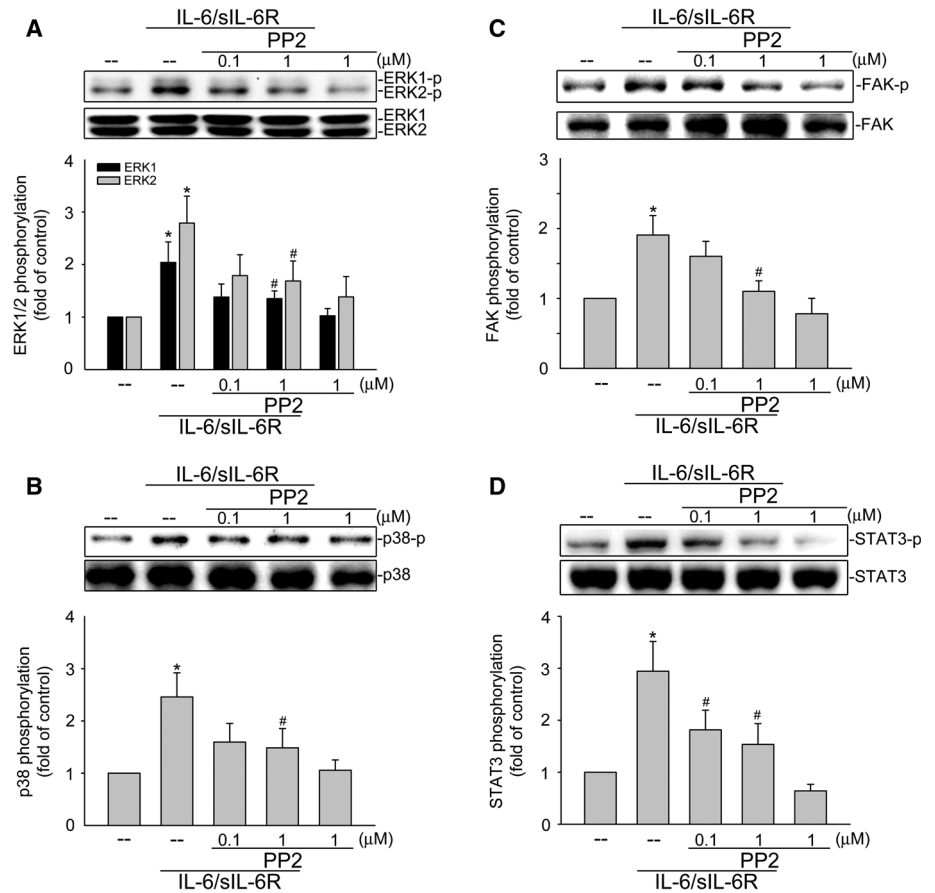
**Fig. 3** IL-6 induced C/EBP $\beta$  and p65 activation in SV-LECs. **a** Cells were transfected with VEGF-C promoter-luc-370 plus renilla-luc for 48 h. After transfection, SV-LECs were treated with vehicle, IL-6 or IL-6 plus sIL6R for another 24 h. The luciferase activity was then determined as described in the “Materials and methods” section. Each column represents the mean  $\pm$  SEM of four independent experiments. \* $p < 0.05$ , compared with the vehicle-treated group. **b** Cells were transiently transfected for 48 h with C/EBP $\beta$ - or  $\kappa$ B-luc plus renilla-luc. After transfection, cells were treated with vehicle or IL-6 plus sIL6R for another 24 h. A luciferase assay was then performed. Data represent the mean  $\pm$  SEM of at least four independent experiments performed in duplicate (\* $p < 0.05$ , compared with the vehicle-treated group). **c** Cells were incubated with IL-

6 plus sIL6R for indicated time periods and the ChIP assay was performed as described in the “Materials and methods” section. Typical traces representative of three independent experiments with similar results are shown. Cells were transfected with C/EBP $\beta$ - (**d**),  $\kappa$ B-luc (**e**) or VEGF-C promoter-luc-370 (**f**) plus renilla-luc for 48 h. After transfection, SV-LECs were pretreated with p38MAPK inhibitor III (p38MAPK-I, 1 or 3  $\mu$ M) or ERK activation inhibitor peptide I (ERK-I, 1 or 3  $\mu$ M) for 30 min, followed by the treatment with IL-6/sIL-6R for another 24 h. The luciferase activity was then determined. Each column represents the mean  $\pm$  SEM of at least four independent experiments. \* $p < 0.05$ , compared with the control group; # $p < 0.05$ , compared to the vehicle-treated group in the presence of IL-6/sIL-6R

were performed. Primers encompassing the VEGF-C promoter region between  $-367$  and  $-198$  (containing putative C/EBP $\beta$  binding sites) and between  $-164$  and  $-62$  (containing putative p65 binding sites) were used. Fig. 3C shows that C/EBP $\beta$  (Fig. 3Ca) and p65 (Fig. 3Cb) binding to the VEGF-C promoter region ( $-367/-198$  or  $-164/-62$ ) were increased after IL-6/sIL6R exposure. The

VEGF-C promoter region ( $-367/-198$  or  $-164/-62$ ) was detected in the cross-linked chromatin sample before immunoprecipitation (bottom panels of Fig. 3Ca and Cb, Input, positive control). These results suggest that IL-6/sIL6R induces the recruitment of C/EBP $\beta$  and p65 to the promoter region of the endogenous *vegfc* gene, and leads to VEGF-C expression in SV-LECs.

**Fig. 4** Src contributes to IL-6-induced ERK1/2, p38MAPK, STAT3 and FAK phosphorylation in SV-LECs. Cells were pretreated with vehicle or PP2 (0.1 or 1  $\mu$ M) for 30 min before treatment with IL-6/sIL-6R for another 30 min. After treatment, cells were then harvested and the phosphorylation status of ERK1/2 (a), p38MAPK (b), FAK (c), and STAT3 (d) were determined by immunoblotting. Each column represents the mean  $\pm$  SEM of six independent experiments. \* $p < 0.05$ , compared with the control group; # $p < 0.05$ , compared with the vehicle-treated group in the presence of IL-6/sIL-6R

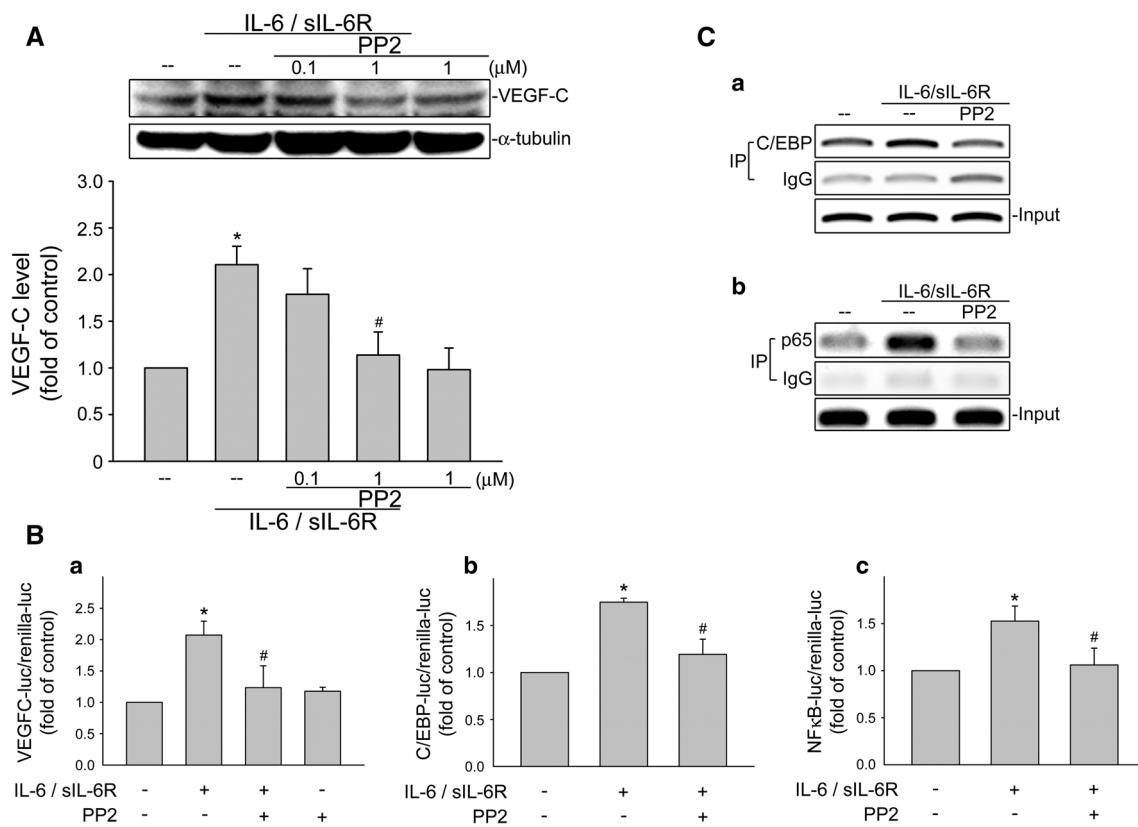


ERK1/2 and p38MAPK signaling pathways contribute to IL-6/sIL-6R-induced C/EBP $\beta$  and NF- $\kappa$ B activation

We next used p38MAPK inhibitor III (a p38MAPK inhibitor) and ERK1/2 activation inhibitor peptide I (an ERK inhibitor) to block the p38MAPK and ERK1/2 signaling pathways to confirm whether p38MAPK or ERK1/2 signaling contributes to IL-6/sIL-6R-induced C/EBP $\beta$ -luciferase and  $\kappa$ B-luciferase activities. As shown in Fig. 3D, pretreatment of cells with p38MAPK inhibitor III (p38MAPK-I, 1 or 3  $\mu$ M) or ERK1/2 activation inhibitor peptide I (ERK-I, 1 or 3  $\mu$ M) suppressed IL-6/sIL-6R-induced C/EBP-luciferase activity in SV-LECs. Similarly, p38MAPK inhibitor III and ERK1/2 activation inhibitor peptide I also attenuated IL-6/sIL-6R-induced  $\kappa$ B-luciferase activity (Fig. 3E). The IL-6/sIL-6R-induced VEGF-C promoter luciferase activities were also significantly inhibited in the presence of p38MAPK inhibitor III or ERK1/2 activation inhibitor peptide I (Fig. 3F). Taken together, these results support the causal role of p38MAPK and ERK1/2 in IL-6/sIL-6R-induced C/EBP $\beta$  and NF $\kappa$ B activation, and subsequent VEGF-C expression in SV-LECs.

Src mediated IL-6/sIL-6R-induced ERK1/2, p38MAPK, FAK and STAT3 phosphorylation in SV-LECs

Many studies suggested that Src may act as an upstream regulator of MAPKs including ERK1/2 and p38MAPK [43, 44]. Src was also shown to increase FAK phosphorylation and has been identified as a common signaling pathway stimulated by diverse extracellular stimuli including growth factors for receptor tyrosine kinases [45–47]. We thus used PP2 (a Src-family kinases inhibitor) to block the Src signaling pathway to confirm whether Src contributes to IL-6/sIL-6R-induced phosphorylation of ERK1/2, p38MAPK, and FAK in SV-LECs. As shown in Fig. 4, pretreatment of SV-LECs with PP2 (1  $\mu$ M) significantly reduced IL-6/sIL-6R-induced ERK1/2 (Fig. 4a), p38MAPK (Fig. 4b), and FAK (Fig. 4c) phosphorylation. Similarly, PP2 at 1  $\mu$ M is effective in suppressing IL-6/sIL-6R-induced STAT3 phosphorylation (Fig. 4d). Based on these results, we suggest that Src activation may occur upstream of ERK1/2, p38MAPK, FAK or STAT3 signaling in IL-6/sIL-6R-stimulated SV-LECs.



**Fig. 5** Src contributes to C/EBP $\beta$  and NF- $\kappa$ B activation and VEGF-C expression in IL-6-stimulated SV-LECs. **A** Cells were pretreated with vehicle or PP2 (0.1 or 1  $\mu$ M) for 30 min before treatment with IL-6/sIL-6R for another 24 h. The extent of VEGF-C was then assessed by immunoblotting. Each column represents the mean  $\pm$  SEM of five independent experiments. \* $p$  < 0.05, compared with the control group; # $p$  < 0.05, compared with the vehicle-treated group in the presence of IL-6/sIL-6R. **B** Cells were transfected with VEGF-C promoter-luc-370 (a), C/EBP $\beta$ - (b) or  $\kappa$ B-luc (c) plus renilla-luc for 48 h. After transfection, SV-LECs were pretreated with

PP2 (1  $\mu$ M) for 30 min, followed by the treatment with IL-6/SIL-6R for another 24 h. The luciferase activity was then determined. Each column represents the mean  $\pm$  SEM of at least three independent experiments. \* $p$  < 0.05, compared with the control group; # $p$  < 0.05, compared to the vehicle-treated group in the presence of IL-6/sIL-6R. **C** Cells were treated with vehicle or PP2 (1  $\mu$ M) for 30 min followed by the treatment with IL-6/sIL-6R for another 4 h. ChIP assay was then performed as described in the “Materials and methods” section. Typical traces representative of at least three independent experiments with similar results are shown

### Src in IL-6/sIL-6R-induced VEGF-C expression in SV-LECs

To ascertain the linkage between Src signaling and VEGF-C expression downstream of IL-6/sIL-6R, the effects of PP2 on IL-6/sIL-6R-induced VEGF-C expression was determined. As shown in Fig. 5A, PP2 significantly inhibited IL-6/sIL-6R-induced VEGF-C protein levels. In addition, IL-6/sIL-6R induced VEGF-C promoter-luciferase (Fig. 5Ba), C/EBP $\beta$ -luciferase (Fig. 5Bb) and  $\kappa$ B-luciferase (Fig. 5Bc) activities were also suppressed in the presence of PP2 (Fig. 5B). Furthermore, PP2 attenuated the effects of IL-6/sIL-6R on C/EBP $\beta$  (Fig. 5Ca) and p65 (Fig. 5Cb) binding to the VEGF-C promoter region. Together, these findings suggest that Src signaling contribute to IL-6/sIL-6R-induced VEGF-C expression in SV-LECs.

### Discussion

Accumulating data indicate that VEGF-C expression in cancer cells correlates with accelerated tumor progression or unfavorable clinical outcome [28, 48, 49]. VEGF-C is thus proposed as a prospective cancer therapeutic target. It is thought that tumor-derived VEGF-C promotes lymphangiogenesis via VEGF-R3 expressed on the surface of LECs, and facilitates metastatic spread of tumor cells via the lymphatic capillaries [3, 50, 51]. Although growing evidence indicates that VEGF-C plays a causal role in lymphangiogenesis and lymphatic metastasis, little is known about VEGF-C regulation in tumor cells or in LECs. Shinriki et al. [28] recently demonstrated that IL-6, which is elevated in cancer patients, induces VEGF-C expression through PI3 K-Akt cascade in oral squamous cell carcinoma. In the present study, we demonstrated that



IL-6 induces VEGF-C expression in LECs. The underlying mechanisms by which IL-6 induced VEGF-C expression may involve the activation of Src, ERK1/2 and p38MAPK. In addition, activation of transcription factors, NF- $\kappa$ B and C/EBP $\beta$ , may also contribute to IL-6 actions in inducing VEGF-C expression in LECs.

The molecular mechanism involved in VEGF-C expression by IL-6 in LECs remains unclear. IL-6 plays a critical role in regulating diverse physiological and pathological processes by stimulating downstream signaling events, which include JAK/STAT, PI3 K/Akt and Ras/MAPK pathways [17, 52]. MAPK signaling has also been shown to contribute to a variety of cellular functions in physiology and diseases [53]. Activation of MAPKs is thus postulated to be required in the IL-6-induction of VEGF-C in LECs. In agreement with these observations, we noted that ERK1/2 and p38MAPK signaling blockade by specific inhibitors markedly suppressed VEGF-C promoter luciferase activity in IL-6-stimulated SV-LECs. In addition, IL-6-induced ERK1/2 and p38MAPK phosphorylation and VEGF-C promoter luciferase activity were suppressed in the presence of PP2, a Src family kinases inhibitor, suggesting that Src-mediated ERK1/2 and p38MAPK activation may be causally related to IL-6-induced VEGF-C expression in SV-LECs. On the other hand, although the classical activation pathway of STAT3 is via upstream kinases such as JAK2 that links STAT3 signaling to cytokine receptors [54], STAT3 may also be activated by other kinases including c-Src [55, 56]. We noted in this study that c-Src inhibition using PP2 leads to STAT3 inhibition in SV-LECs exposed to IL-6. PP2 also suppressed IL-6-induced FAK phosphorylation. It is likely that activation of Src by IL-6 may also lead to FAK and STAT3 activation in SV-LECs. Further investigations are needed to clarify whether activation of STAT3 and FAK contributes to VEGF-C expression in SV-LECs after IL-6 exposure.

Transcription factor NF- $\kappa$ B is mainly involved in regulating inflammatory and immune responses in response to extracellular stimuli. NF- $\kappa$ B in vasculature also plays a pathogenic role in various diseases marked by inflammation and abnormal proliferation, including atherosclerosis [57] and aneurysm [58]. NF- $\kappa$ B was also shown to participate in angiogenesis, and increased NF- $\kappa$ B activity has been documented in the vasculature of colorectal and lung carcinoma [59, 60]. Upon activation, NF- $\kappa$ B dissociates from its inhibitory protein I $\kappa$ B- $\alpha$  and translocates from cytosol to the nucleus, where it then binds to related DNA elements (NF- $\kappa$ B response element) and activates its target genes [61]. Previous studies have shown that ERK1/2 and p38MAPK regulate NF- $\kappa$ B in activated vascular endothelial cells [62]. However, the role of NF- $\kappa$ B in the regulation of lymphatic-specific genes was unclear. We showed in this study that IL-6 significantly increased NF- $\kappa$ B activity as

determined by reporter assay. A reporter assay further showed that C/EBP $\beta$  activity was also elicited by IL-6 in SV-LECs. The IL-6-induced increases in VEGF-C promoter-luciferase,  $\kappa$ B-luciferase and C/EBP $\beta$ -luciferase activities were attenuated in the presence of Src, ERK1/2 and p38MAPK inhibitors. Moreover, the results from ChIP assay indicated that PP2 attenuated the recruitment of NF- $\kappa$ B subunit p65 and C/EBP $\beta$  to the endogenous *vegfc* promoter region (−164/−62 and −367/−198) in IL-6-stimulated SV-LECs. Taken together, these results suggest that IL-6 might activate Src signaling to cause ERK1/2 and p38MAPK activation, which in turn, induce NF- $\kappa$ B and C/EBP $\beta$  activation and ultimately lead to VEGF-C expression in SV-LECs.

As the promoter region (−370 to +1) of the *vegfc* gene also contains Sp1 binding sites, activation of Sp1 is likely to be responsible for the increase in VEGF-C expression in SV-LECs exposed to IL-6. This is supported by the observation that IL-6 induced the activation of ERK1/2 and p38MAPK, two critical upstream regulator of Sp1 [63, 64]. Together, these findings raise the possibility that IL-6 activation of ERK1/2 and p38MAPK signaling cascades may regulate at least three transcription factors in SV-LECs: NF- $\kappa$ B, C/EBP $\beta$  and Sp1. The precise mechanisms of IL-6 actions in driving these transcription factors remain to be elucidated. It is likely that these signaling cascades could culminate in the expression of VEGF-C in SV-LECs. Furthermore, several reports have demonstrated the physical and functional interactions between C/EBP $\beta$  and NF- $\kappa$ B family members [65, 66] or Sp1 [67]. However, whether C/EBP $\beta$  cooperates with p65, Sp1 or other transcription factors in the induction of VEGF-C in IL-6-stimulated SV-LECs requires further investigation.

The essential role of lymphangiogenesis in a multitude of biological and pathological functions makes it promising target for drug development in various diseases. It appears that inhibition of lymphangiogenesis may be beneficial in the context of tumor progression by dampening the metastatic spread of tumor cells [68]. As described above, VEGF-C/VEGFR3 signaling promotes tumor lymphangiogenesis and metastasis [21–23]. VEGF-C/VEGFR3 signaling is thus a potential target for preventing tumor lymphatic metastasis. Preclinical studies have reported that inhibition of VEGF-C/VEGFR3 signaling using soluble VEGFR3 receptors and anti-VEGFR3 antibodies suppressed lymphatic metastasis [69, 70]. Tumor- and LECs-derived VEGF-C is another potential therapeutic target. We demonstrated in this study that Src plays a causal role in IL-6 activation of the ERK/p38MAPK-C/EBP $\beta$  and/or NF- $\kappa$ B signaling cascade, leading to VEGF-C expression in SV-LECs. These findings shed light, at least in part, on the mechanisms underlying IL-6-induced VEGF-C expression in LECs. Src signaling has also been shown to contribute to

VEGF-C-induced invasion and metastasis in tumor cells [71]. Ischenko et al. demonstrated that inhibition of Src kinase exhibits inhibitory effects on VEGF-C-induced lymphangiogenesis in vitro and in vivo [72]. In agreement with the observations that VEGF-C induction is Src-dependent in tumor cells [38, 39, 72], we showed in this study that Src inhibition also attenuated VEGF-C expression in IL-6-stimulated LECs. In addition, Src plays a crucial role in endothelial cell invasion, migration and tumor angiogenesis [73]. Extrapolating these findings to the clinical scenario, targeting IL-6 and/or its downstream pathways such as Src signaling may prevent LECs-derived VEGF-C activity and tumor lymphatic metastasis. For instance, a humanized anti-IL-6R antibody, tocilizumab, which has been shown to be effective for rheumatoid arthritis [74], was recently reported to reduce IL-6-induced VEGF-C expression, resulting in decrease in oral squamous cell carcinoma-related lymphangiogenesis and metastasis in a mouse model [28]. There is a wealth of literature supporting the role of Src in tumor progression, and currently there are different classes of Src inhibitors such as AZD0530 undergoing clinical trials (<http://clinicaltrials.gov>). AZM475271, another Src inhibitor, has also been shown to attenuate tumor angiogenesis and lymphangiogenesis [72, 73]. Taken together, these observations suggest that the therapeutic spectrum of these types of drugs such as anti-IL-6/anti-IL-6R-targeted therapies (Ex. Tocilizumab for Rheumatoid Arthritis) and Src inhibitors (Ex. AZD0530, AZM475271 for solid tumors) may be extended to tumor lymphangiogenesis and metastasis prevention. However, further preclinical studies are needed to evaluate the long-term effects of Src inhibitors, or other anti-IL-6/anti-IL-6R-targeted therapies in carcinogenesis settings and other models of lymphatic metastasis. It may also be worthwhile to explore the signaling pathway underlying IL-6-induced VEGF-C expression and lymphangiogenesis for other potential targets for cancer therapy.

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