The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells

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Summary

Flt-4, a VEGF receptor, is activated by its specific ligand, VEGF-C. The resultant signaling pathway promotes angiogenesis and/or lymphangiogenesis. This report provides evidence that the VEGF-C/Flt-4 axis enhances cancer cell mobility and invasiveness and contributes to the promotion of cancer cell metastasis. VEGF-C/Flt-4-mediated invasion and metastasis of cancer cells were found to require upregulation of the neural cell adhesion molecule contactin-1 through activation of the Src-p38 MAPK-C/EBP-dependent pathway. Examination of tumor tissues from various types of cancers revealed high levels of Flt-4 and VEGF-C expression that correlated closely with clinical metastasis and patient survival. The VEGF-C/Flt-4 axis, through upregulation of contactin-1, may regulate the invasive capacity in different types of cancer cells.

Introduction

Tumor invasion and metastasis are the critical steps in determining the aggressive phenotype of human cancers and are the major causes of cancer deaths (Steeg, 2003). Several sets of growth factors and their cognate receptors have been reported to be importantly involved in regulation of tumor invasion and metastasis (Avraham et al., 2000). Thus, disruption of the growth factor and receptor axis is a current strategy for the development of anticancer drugs (Kerbel and Kamen, 2004).

Production and secretion of VEGFs is commonly observed in most aggressive tumors, and expression of VEGFs profoundly influences the prognosis of cancer patients (Alitalo and Carmeliet, 2002; Kerbel and Kamen, 2004). VEGF is one of the most potent stimulators of angiogenesis identified thus far, affecting endothelial cell proliferation and motility and vascular permeability (Kerbel and Kamen, 2004). VEGFs exert their angiogenic functions through activation of the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed primarily by endothelial cells (Ferrara and Davis-Smyth, 1997) but also by a wide variety of cancer cell lines (Boocock et al., 1995; Hendrix et al., 2003; Strizzi et al., 2001). These findings suggest that the physiological role of the VEGF signaling pathway extends beyond angiogenesis in cancer. VEGFR-2 has been found to be expressed in melanoma and mesothelioma (Strizzi et al., 2001) cell lines, and treatment of those cells with VEGF-A results in increased proliferation via activation of VEGFR-2. Additionally, VEGF-A has been found to induce activation of MAPK(s) and promote growth of cancer cells (von Marschall et al., 2000) through activating VEGFR-2 signaling.

VEGFR-3 (or Flt-4), another VEGF receptor, is activated by its specific ligand, VEGF-C, resulting in promotion of angiogenesis and/or lymphangiogenesis (Alitalo and Carmeliet, 2002; Plate, 2001; Skobe et al., 2001). In addition to its expression on lymphatic endothelial cells (LECs), the Flt-4 has been shown to be expressed in a variety of human malignancies, including lung

SIGNIFICANCE

Metastasis is the main cause of morbidity and mortality in most cancer patients. Flt-4 and VEGF-C are recognized to play an important role in metastasis via promotion of lymphangiogenesis. This report provides evidence that activation of the VEGF-C/Flt-4 axis also enhances mobility of cancer cells and contributes to the promotion of metastasis in animals. The function and molecular mechanism of the VEGF-C/Flt-4 axis, which were revealed from in vitro and in vivo studies and from examination of patient outcomes, provide insight into the process of tumor metastasis in cancer patients.

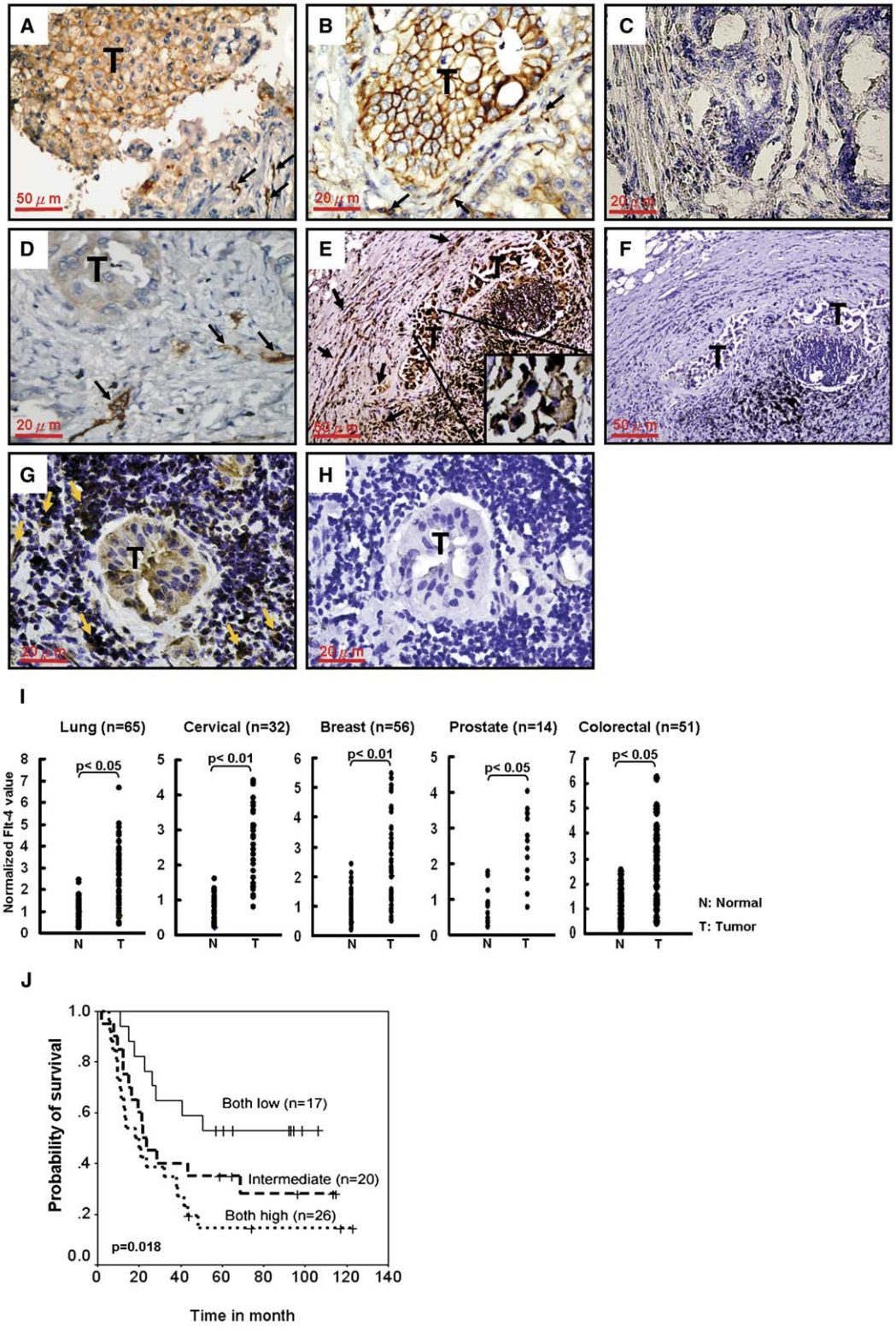

Figure 1. Flt-4 is expressed at membrane location in human tumor tissues

A: Immunohistochemical staining of human lung adenocarcinoma tissues. Arrows indicate lymphatic vessels; T, tumor. **B:** Strong staining of Flt-4 was observed in tissues of patients with invasive or advanced stage disease. Arrows indicate lymphatic vessels; T, tumor. **C:** Staining with the normal mouse IgG as the control. **D:** Weak staining of Flt-4 in lung adenocarcinoma specimens. Arrows indicate lymphatic vessels; T, tumor. **E and G:** Strong staining of Flt-4 (**E**) and VEGF-C (**G**) was observed both in lymph node metastatic tumors and normal part of the same specimen. T, tumor; arrows indicate expression of Flt-4 or VEGF-C in normal parts. **F and H:** Control stained with the normal mouse IgG; T, tumor. **I:** Normalized Flt-4 mRNA expression of tumor and matched normal tissue were measured by quantitative RT-PCR in 195 specimens. T, tumor tissue; N, matched normal tissue. **J:** Combined expression of VEGF-C and Flt-4 proteins correlates inversely with survival of patients with lung adenocarcinoma. The p value refers to the comparison between both high and both low groups.

Table 1. Clinicopathologic characteristics of patients with associated combined expression of VEGF-C and Flt-4: Both low, intermediate, or both high staining

Characteristic	Both low[a] (n = 18)	Intermediate (n = 20)	Both high (n = 27)	p value
Age (mean ± SD) (in years)	62.3 ± 10.0	54.3 ± 12.5	63.4 ± 12.3	0.03[b]
Sex, no. of patients				
Male	8	9	10	0.82
Female	10	11	17	
Stage,[d] no. of patients				
I–II	15	11	10	0.01
III–IV	3	9	17	
Tumor status,[d] no. of patients				
T1	6	7	5	0.38
T2–T4	12	13	22	
Nodal status,[d] no. of patients				
N0	16	10	4	<0.001
N1–N3	2	10	23	
Median survival[e] (median ± SD) (in months)	Not been reached yet	21.9 ± 2.9	18.2 ± 5.0	0.018[c]

[a]Both low indicates that expression of both Flt-4 and VEGF-C in tumor cells is low; intermediate indicates that expression of either Flt-4 or VEGF-C, but not both, in tumor cells is high; both high indicates that expression of both Flt-4 and VEGF-C in tumor cells is high.
[b]Derived with a one-way ANOVA test.
[c]Derived with log-rank test; other p values were derived with Pearson chi-square tests. All statistical tests are two sided. SD, standard deviation.
[d]The tumor stage, tumor status, and nodal status were classified according to the international system for staging lung cancer.
[e]There was one patient from the both low group and one patient from the both high group who died within 30 days of operation, and they were not included in the survival analysis.

adenocarcinoma (Li et al., 2003), colorectal adenocarcinoma (Witte et al., 2002), head and neck carcinomas (Neuchrist et al., 2003), prostate carcinoma (Kaushal et al., 2005), leukemia (Dias et al., 2002), and Kaposi's sarcoma (Weninger et al., 1999). Expression of Flt-4 was also reported to correlate significantly with the different stages of cervical carcinogenesis (Van Trappen et al., 2003). These observations suggest that Flt-4's ligand, VEGF-C, may affect cancer development or progression by direct effects on tumor cells. Unlike the well-characterized axes of VEGF-A and VEGFR-1/R-2, neither the signaling pathway activated by the interaction between VEGF-C and Flt-4 in epithelial tumor cells nor the biological significance of activation of this axis is understood.

We conducted these studies to characterize the VEGF-C/Flt-4 axis, and we found that it plays an important role in promoting invasion and metastasis of human lung adenocarcinoma cells.

Results

Expression of VEGF-C and Flt-4 correlates with stage and lymph node metastasis of cancers and survival of cancer patients

To ascertain whether the VEGF-C-specific receptor Flt-4 is expressed in cancer cells, we analyzed a total of 218 human cancer specimens using a monoclonal antibody specific against Flt-4 (MAB904, from R&D Systems). Figure 1A shows that Flt-4 was detectable in the tumor epithelium and surrounding lymphatic vessels. Strong staining for the Flt-4 on the cytoplasmic membrane was frequently observed in invasive or advanced stage lung adenocarcinoma (Figure 1B); this staining was not detected when normal mouse IgG (Figure 1C) was used. In contrast, negative or very weak staining of Flt-4 was seen in early-stage lung adenocarcinoma (Figure 1D). We used two additional specific antibodies against Flt-4, MAB3491 (R&D Systems) and sc-282976 (Santa Cruz Biotechnology), to confirm the presence of the Flt-4 and found similar expression patterns (Figures S1A–S1D in the Supplemental Data available with this article online). An important finding was that metastatic tumor cells in the lymph node frequently had high expression of Flt-4 and VEGF-C (Figures 1E and 1G). A number of Flt-4-stained lymphatic vessels were distributed around the metastatic tumors in the lymph nodes (Figure 1E). The specificity of the antibodies' recognition of Flt-4 and VEGF-C was confirmed by using normal mouse IgG control (Figures 1F and 1H). This observation is consistent with existence of a VEGF-C/Flt-4 autocrine loop in highly invasive human lung adenocarcinoma.

Flt-4 protein and mRNA were also strongly expressed in various other types of advanced cancer, including cervical, breast, prostate, and colorectal, as detected by immunohistochemical staining and a fluorescence in situ hybridization (FISH) assay (Figures S1E and S1F), suggesting that expression of VEGF-C and Flt-4 may be a general phenomenon in multiple cancer types. The FISH assay showed that strong staining for Flt-4 mRNA also occurred frequently in tissue from advanced stage tumors and that this positive staining was not detected when the sense control probe was used (Figure S1F). These results were consistent with the immunohistochemical data. We also detected the expression levels of Flt-4 mRNA in normal and matched tumor tissues by quantitative reverse transcription (RT)-PCR (Figure 1I) and FISH (Figure S1G). Both results indicated that expression of Flt-4 mRNA in tumor tissues was higher than it was in matched normal tissues.

Patients whose tumors expressed high levels of both VEGF-C and Flt-4 were more likely than those having tumors expressing low or intermediate levels of these proteins to have advanced disease and lymph node metastasis. The relationships between the level of VEGF-C/Flt-4 expression and the clinicopathological characteristics of the lung adenocarcinomas are summarized in Table 1. Most importantly, patients having tumors with high expression levels of both VEGF-C and Flt-4 had the shortest survival time of all three groups (Figure 1J).

The VEGF-C/Flt-4 axis promotes migration and invasion of cancer cells

Through use of flow cytometric analyses, expression of the Flt-4 was found to be elevated in the highly invasive CL1-3 and CL1-5

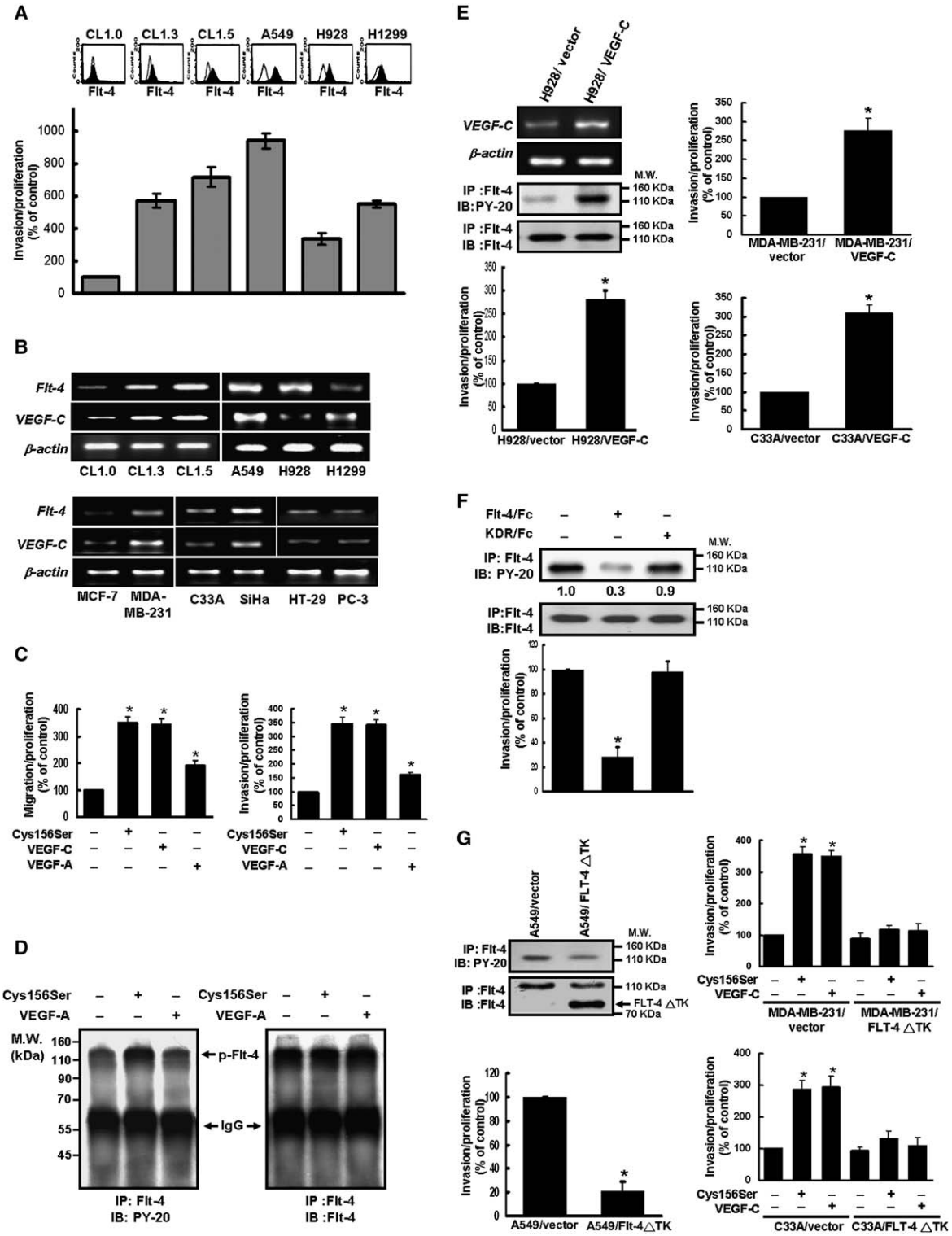

Figure 2. Flt-4/VEGF-C axis regulates invasion ability of human cancer cells

A: Upper panel: FACScan analysis of expression of Flt-4 protein. Lower panel: in vitro invasiveness of cancer cell lines as measured by the Boyden chamber assay.

B: Expression of RNAs for the Flt-4 and VEGF-C genes in cancer cells.

C: Treatment with 50 ng/ml VEGF-C, 50 ng/ml VEGF-C (Cys156Ser), or 50 ng/ml VEGF-A in H928 cells and measurement of the migration and invasiveness of cancer cells (*$p < 0.05$, two-tailed Student's t test). Growth pattern of H928 cells was detected by trypan blue exclusion assay. Each treatment was performed in three separate experiments.

D: H928 cells were incubated with 50 ng/ml VEGF-C for 30 min, and tyrosine-phosphorylated Flt-4 was detected by immunoprecipitation and Western blotting (IP-Western blotting).

lung cancer cells, but its expression was barely detectable in the poorly invasive parental CL1-0 cells (Figure 2A). A549 cells, which have strong invasive activity, expressed the Flt-4 more abundantly than H928 and H1299 cells, which have low invasive activity (Figure 2A). RT-PCR analysis revealed that the levels of VEGF-C and Flt-4 mRNA were coordinately increased in highly invasive cell lines such as CL1-3, CL1-5, and A549 (Figure 2B). Although Flt-4 mRNA was significantly elevated in H928 cells, VEGF-C mRNA was present at very low levels. Accordingly, the invasive activity of H928 cells was much lower than that of CL1-3, CL1-5, and A549 cells.

The axis of VEGF-C and Flt-4 was also expressed in other cancer cell lines, including breast, cervical, colorectal, and prostate. More importantly, the levels of VEGF-C and Flt-4 mRNA were also more abundant in the highly invasive MDA-MB-231 breast cancer cell line and the SiHa cervical cancer cell line (Figure 2B). The coordinate expression of VEGF-C and Flt-4 in some of these tumor cell lines raised the possibility that an autocrine loop exists in these lines.

In vitro migratory and invasive activity assays were used to examine the role of the VEGF-C/Flt-4 axis in the invasiveness of these cancer cells. Treatment with 50 ng/ml of recombinant human VEGF-C or VEGF-C (Cys156Ser) protein, which is a selective agonist of Flt-4 and does not bind KDR (Joukov et al., 1998), significantly increased the migratory activity and invasiveness of H928 human lung adenocarcinoma cells (Figure 2C). These data suggest that native VEGF-C and VEGF-C (Cys156Ser) show similar potential for increase cell mobility. To rule out the possibility that the effect of VEGF-C on invasiveness was caused by different proliferation rates, we compared the growth rates of VEGF-C-overexpressing cells and control cells in serum-free and in anchorage-independent culture conditions, respectively. The control cells and the VEGF-C-overexpressing cells had similar growth rates (Figure S2A).

Stimulation with recombinant VEGF-C (Cys156Ser), but not with VEGF-A, led to a significant increase in tyrosine phosphorylation of the Flt-4 in H928 cells but not in HL-60 cells, which have been shown to express KDR but not Flt-4 (Figure 2D and Figure S2B). Indeed, VEGF-A treatment stimulated an evident increase in tyrosine phosphorylation of KDR in HL-60 cells and induced cell proliferation in HUVEC cells (Figures S2B and S2C). These findings imply that Flt-4 receptors, but not KDR receptors, in H928 cells are functionally active and fully responsive to VEGF-C. It is interesting to note, however, that there were two major forms of Flt-4 in LECs, ~125 kDa and ~195 kDa, but only the ~125 kDa form was detected in lung adenocarcinoma cells and leukemia cells (Figure S2D). It has been reported that two forms of Flt-4 transcript are generated by alternative polyadenylation and alternative splicing (Pajusola et al., 1993). Consistent with this report, we found that both long and short forms of Flt-4 were present in LECs but only the short form of Flt-4 could be detected in A549 and H928 lung adenocarcinoma cells using the RNase protection assay (Figure S2D).

To construct a condition suitable for manifestation of an autocrine loop, a human VEGF-C cDNA expression vector and a control vector were stably transfected into different type of cancer cells. As shown in Figure 2E, the VEGF-C-transfected H928 cells displayed significantly increased tyrosine phosphorylation of the Flt-4 as compared with vector-transfected controls. A noticeable (2- to 3-fold) increase in the invasive ability of VEGF-C-overexpressing cancer cells was observed compared with vector controls in different type of cancer cells (Figure 2E).

A549 cells expressed both VEGF-C and Flt-4 mRNA at high concentrations (Figure 2B), consistent with the presence of a VEGF-C/Flt-4 autocrine loop. Recombinant Flt-4/Fc, which has been shown to specifically neutralize the secreted form of VEGF-C, was therefore utilized to interrupt a potential loop in these cells. Treatment with recombinant Flt-4/Fc effectively decreased the extent of Flt-4 tyrosine phosphorylation (Figure 2F). However, the recombinant KDR/Fc, which specifically antagonizes the effects of VEGF-A, failed to alter the phosphorylation status of Flt-4 in A549 cells (Figure 2F). Under the same treatment conditions, recombinant Flt-4/Fc, but not KDR/Fc, diminished the invasiveness of A549 cells by 70% (Figure 2F). Although KDR/Fc failed to inhibit the Flt-4 activation by VEGF-C, it effectively diminished the KDR activation as well as the proliferation of HUVEC cells by stimulation with VEGF-A (Figure S2C).

As an alternative approach, dominant-negative inhibition of innate Flt-4 through overexpression of tyrosine kinase domain-deleted Flt-4 (Flt-4ΔTK) was used. In A549 cells expressing Flt-4ΔTK, the VEGF-C/Flt-4 autocrine loop became defective because of the dramatic reduction in Flt-4 phosphorylation (Figure 2G). The expression of truncated Flt-4 in A549, MDA-MB-231, and C33A cells also strongly impaired their endogenous and VEGF-C- or VEGF-C (Cys156Ser)-induced invasive capacity (Figure 2G). These findings, in conjunction with those described above, strongly support our hypothesis that the VEGF-C/Flt-4 affiliation is actively involved in regulating the invasiveness of cancer cells.

To define the relative importance of the VEGF-C/Flt-4 axis in metastasis, we compared the VEGF-C/Flt-4 axis with other well-known mechanisms of cancer metastasis such as the EGFR (Lu et al., 2003) and CXCR4 pathways (Li et al., 2004). The results of our in vitro invasion assay showed that blockade of the VEGF-C/Flt-4 axis by Flt-4/Fc had inhibitory effects on cell invasiveness similar to those of the EGFR inhibitor ZD1839 (2.5 μM) in A549 cells. Furthermore, we found that blockade of the VEGF-C/Flt-4 axis by Flt-4/Fc resulted in a more efficient inhibitory effect on cell invasion than did blockade of the CXCR4 pathway by the CXCR4 inhibitor AMD3100 (10 μM) (Figure S2F). These data suggested that the VEGF-C/Flt-4 axis plays as important a role in the invasiveness of lung adenocarcinoma cells

E: Left upper panel: analysis of VEGF-C mRNA expression by RT-PCR and Flt-4 activation by IP-Western blotting. Left lower panel and right panel: measurement of the invasiveness by the Boyden chamber assay. The invasive activity was significantly higher in VEGF-C-transfected cells as compared to vector control cells, as indicated by the asterisks (*$p < 0.05$).

F: A549 cells were treated with 100 ng/ml Flt-4/Fc for 30 min followed by analyses of tyrosine phosphorylation of Flt-4 (upper panel) or for 48 hr followed by analyses of cell invasiveness. *$p < 0.05$, two-tailed Student's t test. The fold estimation of protein expression is indicated by the numbers below the lanes.

G: Left upper panel: Flt-4 tyrosine phosphorylation in A549/Flt-4ΔTK cells was detected by IP-Western blotting assay. Arrow indicates kinase domain-deleted Flt-4 protein. Left lower panel: the invasion abilities of A549/vector and A549/Flt-4ΔTK cells. Right panel: treatment with VEGF-C or VEGF-C (Cys156Ser) in cells expressing control vector or Flt-4ΔTK expression vector and analysis of the invasive activity of cells. *$p < 0.05$, two-tailed Student's t test.

as EGFR does and that this axis is more critical than CXCR4 is in cell invasion.

CNTN-1 acts as a downstream effector in VEGF-C/Flt-4-induced invasion and metastasis

To study the downstream effector genes of VEGF-C/Flt-4 signaling, A549 cells were first treated with BSA, rhKDR/Fc, or rhFlt-4/Fc for 24 hr. Differentially expressed genes were then identified with a customized GEArray cDNA array containing cDNA sequences corresponding to functions related to cell adhesion and migration. Under the highly stringent conditions we used, the neural adhesion molecule *CNTN-1* gene was identified as one of the highly decreased genes in rhFlt-4/Fc-treated cells, but not in rhKDR/Fc-treated or control A549 cells (Figure 3A and Table S1). In support of this finding, treatment with rhFlt-4/Fc, but not with rhKDR/Fc, decreased expression of CNTN-1 mRNA and protein (Figure 3A). A similar reduction of CNTN-1 protein was seen in A549 cells overexpressing Flt-4ΔTK (Figure 3A). Levels of CNTN-1 mRNA and protein were similarly reduced in rhFlt-4/Fc-treated CL1-5 cells, which also possess a VEGF-C/Flt-4 autocrine loop (data not shown). In H928 cells, increasing concentrations of VEGF-C increased the expression of CNTN-1 protein in a dose-dependent manner (Figure 3B). Treatment with VEGF-C increased CNTN-1 mRNA and protein to their optimum concentrations at 8 hr and 24 hr, respectively (Figure 3B). In agreement with the findings described above, VEGF-C-transfected H928 (H928/VEGF-C) cells exhibited a 3.3-fold elevation of CNTN-1 protein compared with that of control cells (Figure 3B).

To search for additional functional linkages between CNTN-1- and VEGF-C/Flt-4-mediated cell invasion, the level of CNTN-1 in H928/VEGF-C cells was knocked down by transfection with different CNTN-1-specific RNAi expression vectors (siRNA-1, siRNA-2, and siRNA-3). Figure 3C shows that different clones separately transfected with different CNTN-1-specific RNAi all inhibited the expression of CNTN-1 protein in H928/VEGF-C cells. Among them, the siRNA-2-1-expressing clone seemed to be the most effective in inhibiting CNTN-1 protein expression. As anticipated, transfection with siRNA-2 also effectively reduced the invasiveness of H928/VEGF-C, MDA-MB-231/VEGF-C, and C33A/VEGF-C cells (Figure 3C). Stable transfection of A549 cells with the siRNA-2 expression vector resulted in greatly reduced expression of CNTN-1 protein as well as cell invasion capacity (Figure 3D). Thus, the results obtained here show that CNTN-1 is involved in cancer cell invasiveness and acts as a downstream effector of VEGF-C/Flt-4 signaling.

F-actin is continuously polymerized and depolymerized in motile cells and is essential to cell motility (Cooper, 1991). To further assess whether VEGF-C-induced CNTN-1 expression promotes cell invasion via arrangements of the actin cytoskeleton, A549, H928/vector, and H928/VEGF-C cells transfected with or without siRNA-2 were therefore stained with rhodamine-conjugated anti-phalloidin antibody and characterized using multicolor immunofluorescence and confocal laser scanning microscopy. The A549 control cells displayed well-formed F-actin-containing microfilament bundles within the cytoplasm and below the plasma membrane, whereas the A549/siRNA-2 cells contained few microfilament bundles (Figure 3D). Furthermore, the H928/VEGF-C cells displayed well-formed F-actin-containing microfilament bundles, which could be abolished by siRNA-2 (Figure 3E). These findings indicated that VEGF-C-induced CNTN-1 expression regulates rearrangements of F-actin-containing microfilament bundles, suggesting that F-actin-containing microfilament bundle rearrangement may be involved in the VEGF-C-induced CNTN-1-mediated cell invasiveness.

To define whether the correlation between Flt-4 expression and CNTN-1 expression exists in lung adenocarcinoma, we immunohistochemically analyzed the levels of Flt-4 and CNTN-1 proteins in such tumors. We found that expression of CNTN-1 was tightly correlated with the expression of Flt-4 in these tumors (correlation coefficient = 0.644; $p < 0.001$; Table S2). In addition, the expression of CNTN-1 in lung adenocarcinoma correlated with tumor stage, lymph node metastasis, and patient survival (Table S3).

The Src/p38 MAPK-mediated C/EBP signaling pathway is required for VEGF-C/Flt-4-mediated CNTN-1 expression

To investigate the pathways associated with CNTN-1 induction in response to VEGF-C/Flt-4 signaling, we examined the forms of MAP kinases (MAPKs) that are reported to function in the regulation of cell invasion (Suyama et al., 2002). Western blot analysis revealed that phosphorylated p38 MAPK, but not ERK1/2 or JNK, was increased in VEGF-C-overexpressing H928 cells (Figure 4A; approximately 2.7-fold higher than in control cells). Furthermore, expression of Flt-4ΔTK reduced p38 MAPK phosphorylation in A549 cells (Figure 4A). Treated with various specific MAPKs inhibitors or vehicles, only the p38 MAPK-specific inhibitor SB203580 reduced CNTN-1 expression to a significant degree in H928/VEGF-C cells (Figure 4B; 72% inhibition relative to that in control cells). In contrast, treatment with the ERK1/2 or JNK inhibitors PD98059 or SP600125, respectively, failed to diminish CNTN-1 expression (Figure 4B). As further evidence for the involvement of p38 MAPK-dependent signaling in regulation of CNTN-1 expression, kinase activity was blocked by expressing the dominant-negative p38 (p38AF). VEGF-C/Flt-4 signaling-induced activation of p38 MAPK and upregulation of CNTN-1 were both abolished by transfection with p38AF (Figure 4B).

Cytoplasmic tyrosine kinases of the Src family have been implicated in the transduction of extracellular signals from membrane receptors (including RTKs) to downstream cellular effectors and in the regulation of cancer cell mobility (Tanaka et al., 2004). The potential role of Src in VEGF-C-mediated p38 MAPK activation and subsequent CNTN-1 induction was therefore of interest. Src kinase activity was inhibited either by treatment with PP1 (specific for Src-like kinase) or by expression of a kinase-defective Src mutant (DN-src carrying a K-to-D mutation at position 297). Both approaches resulted in strong reductions in p38 MAPK activation and subsequent CNTN-1 induction (Figure 4C). In addition, treatment with VEGF-C induced a 6.3-fold induction of CNTN-1 promoter activity; this induction was abolished by SB203580 and PP1 but not by PD98059 (Figure 4D).

To further determine which response element participates in the regulation of CNTN-1 promoter activity in response to VEGF-C, we developed a series of CNTN-1 promoter-reporter mutation constructs (Figure 4E). Database analysis by TFSEARCH program (version 1.3) of the 994 bp CNTN-1 promoter for known consensus sequences indicated the presence of binding sites for Sp-1, Oct-1, C/EBP, and AP-1 (Figure 4E). In

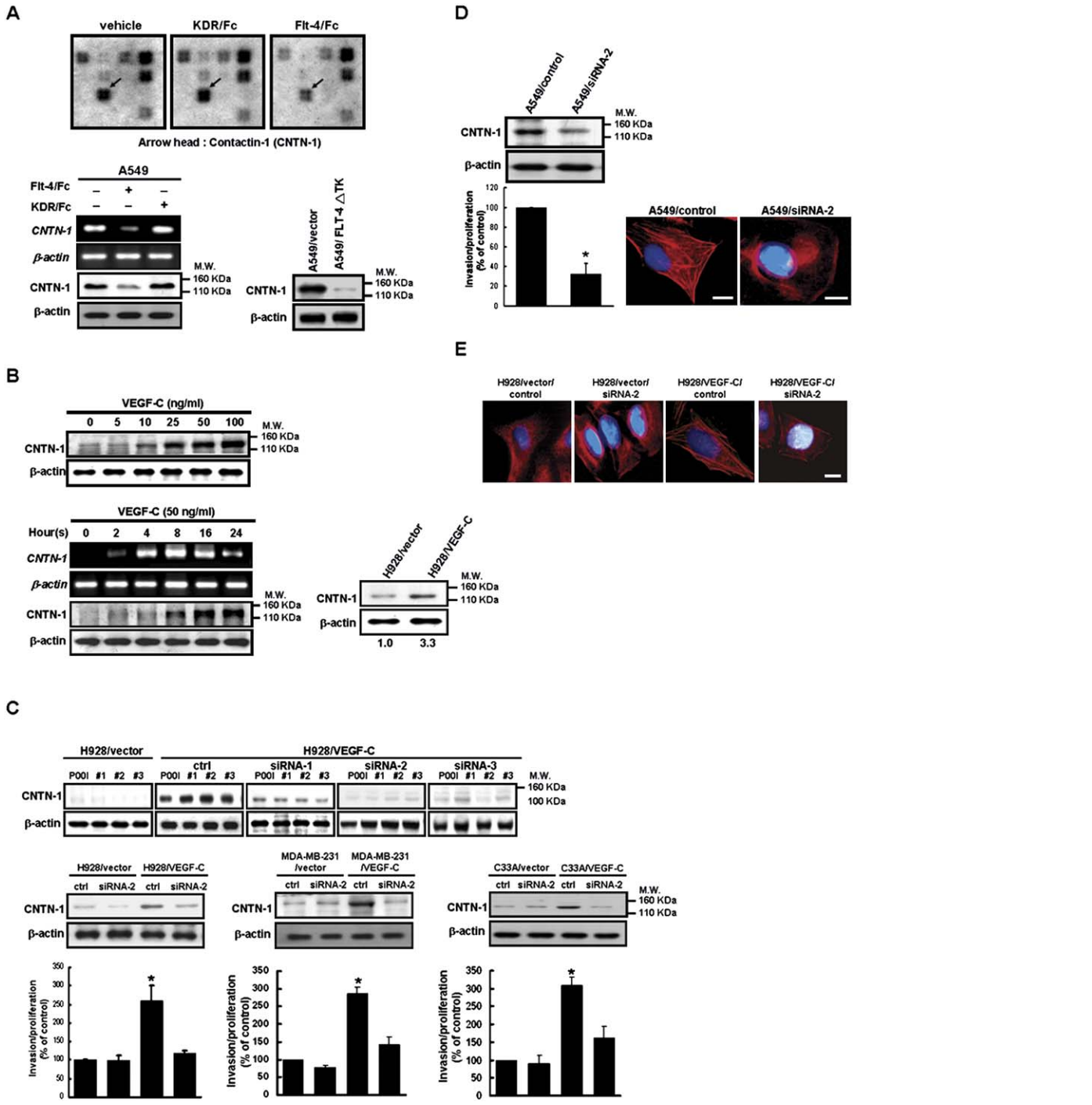

Figure 3. VEGF-C/Flt-4 axis regulates cancer cell invasion via CNTN-1-dependent F-actin rearrangement in cancer cells

A: Upper panel: CNTN-1 expression in control, Flt-4/Fc, and KDR/Fc cells as determined by cDNA array imaging. Arrows indicate CNTN-1. Lower panel: CNTN-1 expression as determined by RT-PCR and Western blotting.

B: Expression of CNTN-1 in VEGF-C-treated H928 cells. Left upper panel: H928 cells were treated with various concentrations of VEGF-C for 24 hr, followed by measurement of CNTN-1 protein expression by Western blotting. Left lower panel: H928 cells were treated with 50 ng/ml VEGF-C for the indicated times and the expression of CNTN-1 mRNA and protein determined by RT-PCR and Western blot, respectively. Right panel: CNTN-1 protein expression in H928/vector or H928/VEGF-C cells was measured by Western blot. The fold estimation of protein expression is indicated by the numbers below the lanes.

C: CNTN-1 protein expression and cell invasiveness of vector and VEGF-C-overexpressing cells stably transfected with control (ctrl) or CNTN-1-specific siRNA was analyzed by Western blot and Boyden chamber assay. Asterisks denote a statistically significant difference compared with values of lane 1 (*$p < 0.05$).

D: Left panel: CNTN-1 protein expression and invasiveness in A549 cells stably transfected with CNTN-1-specific RNAi expression vector or control vector. Right panel: fluorescent staining of F-actin by rhodamine-conjugated phalloidin.

E: Fluorescent staining of F-actin by rhodamine-conjugated phalloidin.

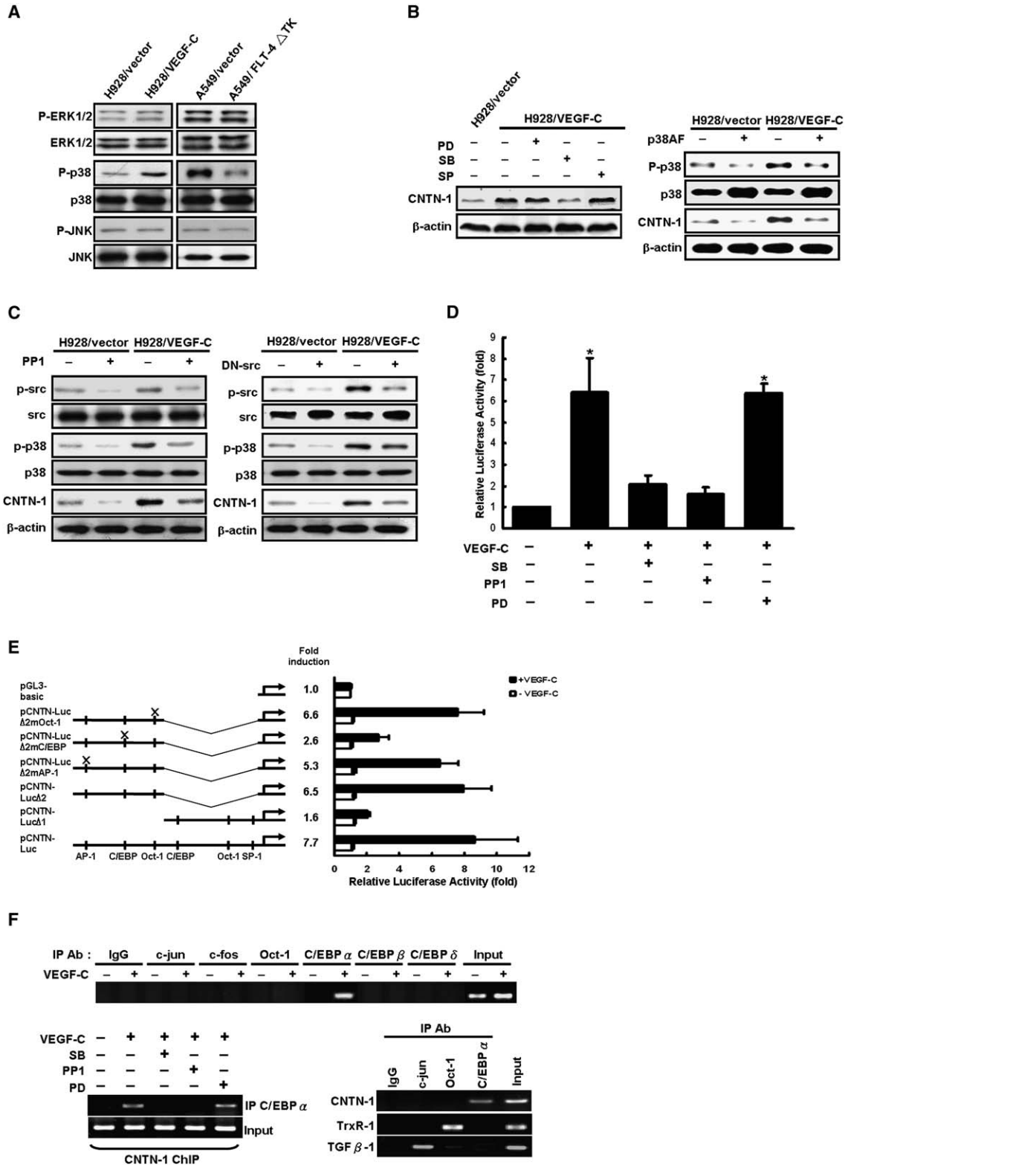

Figure 4. Involvement of Src kinase, p38 MAPK, and C/EBPα in CNTN-1 upregulation during activation of the VEGF-C/Flt-4 axis

A: Western blot analyses of the phosphorylation of MAPKs.

B: VEGF-C/Flt-4 axis-mediated CNTN-1 upregulation via p38 MAPK. Left panel: H928/VEGF-C cells were treated with PD98059 (PD; 25 μM), SB203580 (SB; 10 μM), or SP 600125 (SP; 20 μM) for 2 hr, and expression of CNTN-1 and β-actin was analyzed by Western blot. Right panel: cells were transfected with control vector or dominant-negative p38 MAPK expression vector (p38AF), and CNTN-1 expression was then analyzed by Western blot.

C: H928/vector and H928/VEGF-C cells were treated with 10 μM PP1 (left panel) or transfected with 3 μg of dominant-negative src (DN-src) (right panel), and protein expression was then measured by Western blot analysis.

addition, VEGF-C significantly increased luciferase activity in pCNTN-Luc-transfected cells and pCNTN-LucΔ2-transfected cells but not in pCNTN-LucΔ1-transfected cells. VEGF-C-induced CNTN-1 promoter activity decreased about 80% by point mutation of the C/EBP response element on pCNTN-LucΔ2 (pCNTN-LucΔ2mC/EBP) but had no effects on pCNTN-LucΔ2mAP-1 or pCNTN-LucΔ2mOct-1 (Figure 4E).

We next investigated whether VEGF-C-induced C/EBP associates with CNTN-1 promoter by using a chromatin immunoprecipitation (ChIP) assay. Using different transcription factors and specific antibodies, we found that C/EBPα, but not other transcription factors, associates with the CNTN-1 promoter after treatment with VEGF-C in H928 cells (Figure 4F) and C33A and MDA-MB-231 cells (data not shown). In addition, the VEGF-C-induced C/EBPα-DNA complex could be abolished by SB203580 and PP1 but not by PD98059 (Figure 4F). In a parallel ChIP experiment to test the specificity of chromatin association, Oct-1 and c-*jun* were recruited to the *thioredoxin reductase 1* (*TrxR1*) and the *transforming growth factor β1* (*TGFβ1*) promoters, respectively, which are the documented targets of Oct-1 and c-*jun* (Rundlof et al., 2001; Kim et al., 1990) but not the *CNTN-1* promoter (Figure 4F). Conversely, C/EBPα did not associate with the *TrxR1* promoter or the *TGFβ1* promoter. These results demonstrated that the C/EBP site is indeed important for VEGF-C-mediated *CNTN-1* gene upregulation.

Thus, these studies using pharmacological treatment or genetic inhibition clearly demonstrate that the Src-p38 MAPK-C/EBPα signaling pathway plays a critical role in the VEGF-C/Flt-4-mediated upregulation of CNTN-1.

The VEGF-C/Flt-4 axis promotes metastatic colonization in an animal model

To investigate whether the VEGF-C/Flt-4 axis plays a causal role in tumor metastasis, we injected H928/vector and H928/VEGF-C cells subcutaneously into the right flank of SCID mice, followed by intraperitoneal injections of Flt-4/Fc (0.5 mg/kg/3 days) and measured the growth of the resulting primary tumors every 3 days. H928/VEGF-C and H928/vector tumors formed at identical rates. Treatment with Flt-4/Fc did not alter the growth rate of the tumors formed by H928/VEGF-C cells (Figure 5A), although treatment effectively reduced VEGF-C-induced Flt-4 activation in primary tumors (Figure 5B). The expression levels of VEGF-C protein and Flt-4 phosphorylation in H928/VEGF-C tumors were higher than in the H928/vector tumors (Figure 5B). These results suggested that the VEGF-C/Flt-4 axis is not required for formation of primary lung tumors.

To further determine whether disruption of Flt-4 activation would affect the ability of H928/VEGF-C cells to metastasize, we first examined the metastatic behavior in H928/VEGF-C tumor-bearing mice treated with either Flt-4/Fc or vehicle only. Whereas tumors expressing the VEGF-C expression vector formed large numbers of macroscopically visible metastases in the lungs, those that expressed the control vector formed very few metastases (Figure 5C). The numbers of visible metastatic nodules in mice injected with H928/VEGF-C cells were higher than those in H928/vector-bearing mice. As shown in Figure 5C, the mean numbers of metastatic nodules and the occurrence of lung metastasis in H928/VEGF-C tumor-bearing mice treated with Flt-4/Fc were dramatically decreased. In the Flt-4/Fc-treated H928/VEGF-C group, the mean lymph node volume was 15.51 mm^3, and in the vehicle-treated H928/VEGF-C group, the mean lymph node volume was 56.80 mm^3 (Figure 5D).

In support of these findings, mice bearing A549 primary tumors and treated with Flt-4/Fc or vehicle showed similar tumor growth rates (Figure 5E). As shown in Figure 5F, the number of lung metastatic nodules in A549 tumor-bearing mice treated with Flt-4/Fc decreased dramatically. The mean lymph node volume was dramatically decreased in the Flt-4/Fc-treated A549 group (Figure 5G). The expression level of tyrosine-phosphorylated Flt-4 in both primary tumors and lung metastatic tumors decreased significantly in the Flt-4/Fc-treated A549 tumors relative to the vehicle-treated A549 tumors (data not shown). Together, these data demonstrate that activation of the VEGF-C/Flt-4 axis promoted tumor metastasis in this animal model.

Subsequently, we used another animal model of experimental metastasis in which we intravenously injected tumor cells into the lateral tail vein of mice. The lung metastases that formed occurred more frequently in mice injected with A549/vector control cells than in those injected with A549/Flt-4ΔTK cells (83.3% versus 16.7%, respectively). A higher occurrence of formed lung metastases was also observed in mice injected with VEGF-C-overexpressing H928 (H928/VEGF-C) cells than in mice injected with H928/vector control cells (58.3% versus 16.7%, respectively).

In addition, the mice injected with H928/vector cells or A549/Flt-4ΔTK cells displayed fewer or smaller metastatic nodules in the lung than did those injected with H928/VEGF-C cells or A549/vector cells (Figures 6A and 6B). The metastatic tumors formed in the lungs by the A549/vector and H928/VEGF-C cells had morphological characteristics typical of adenocarcinoma (Figure 6C). Moreover, the expression levels of VEGF-C protein and Flt-4 phosphorylation in the H928/VEGF-C metastatic lung tumors were higher than they were in the H928/vector tumors (Figure 6D). Consistent with these data, the levels of Flt-4 phosphorylation but not VEGF-C expression in A549/Flt-4ΔTK metastatic lung tumors were significantly lower than in the A549/vector lung metastatic tumors (Figure 6D). Abolition of the VEGF-C/Flt-4 axis in A549 cells was associated with reduced formation of metastatic lung nodules. In addition, mice injected with H928/VEGF-C cells formed more metastatic lung nodules than mice injected with H928/vector cells. Data from these two experiments are summarized in Table 2.

Despite these findings, we wanted to further assess the role of CNTN-1 in VEGF-C/Flt-4-mediated metastatic colonization.

D: H928 cells were transfected with 994 bp CNTN-1 promoter-reporter, and the luciferase activity from transfectants was measured. Asterisks denote a statistically significant difference compared with values of lane 1 (*$p < 0.05$).

E: H928 cells were transfected with various CNTN-1 promoter-reporters or pGL3-basic vector, and the luciferase activity from transfectants was measured.

F: Upper panel: the H928 cells were treated with or without VEGF-C (50 ng/ml) for 4 hr, then processed for ChIP assay using various antibodies. Lower left panel: the H928 cells were treated with various chemical inhibitors, then processed for ChIP assay using anti-C/EBPα antibody or IgG. Lower right panel: the *TrxR-1* promoter and the *TGF-β1* promoter were examined by ChIP assay in A549 cells for the occupancy of C/EBPα, Oct-1, and c-*jun* using anti-C/EBPα, anti-Oct-1, anti-c-*jun*, or IgG antibodies.

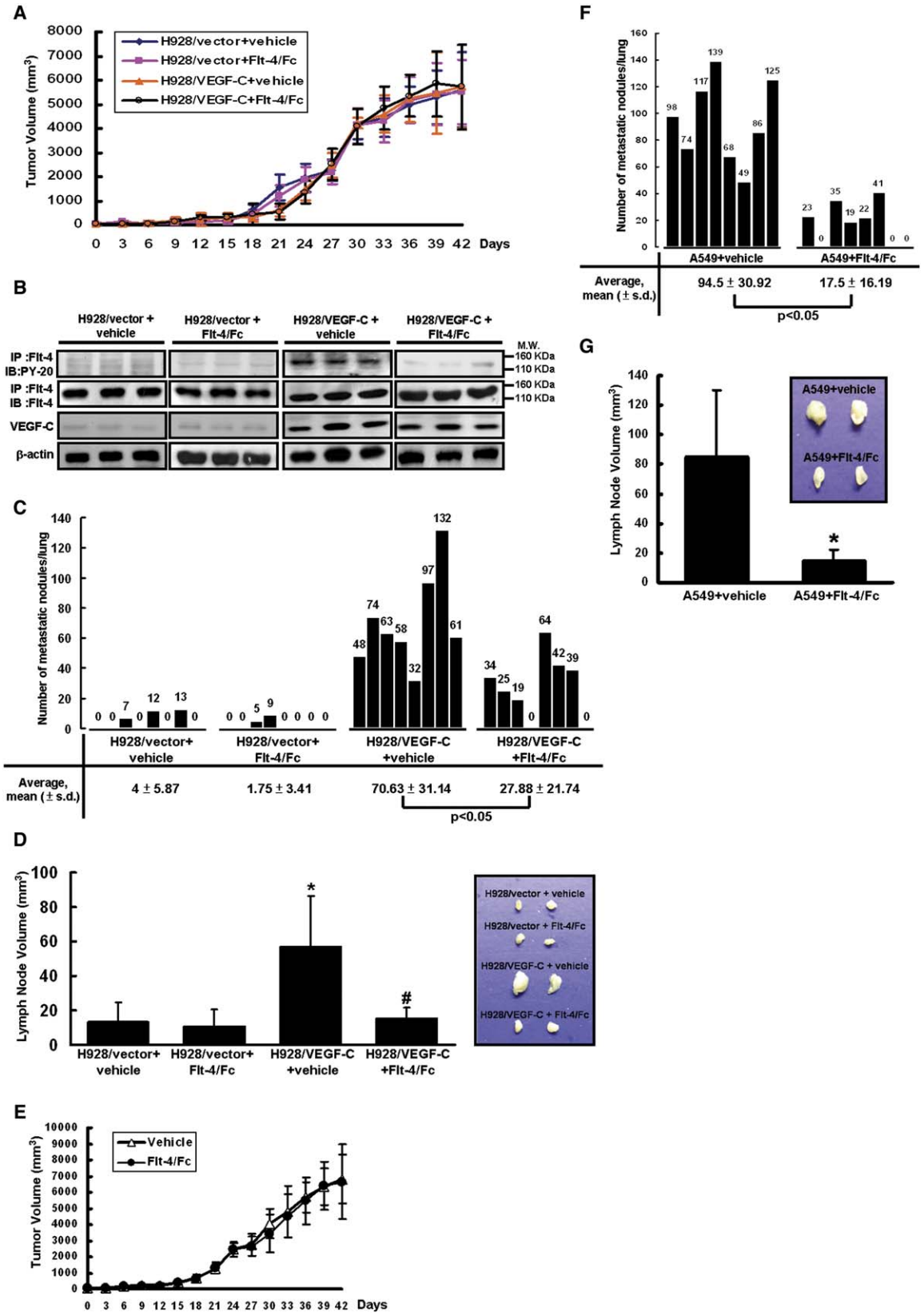

Figure 5. Flt-4/VEGF-C axis is required for lung adenocarcinoma cells to metastasize

A: Growth patterns of subcutaneously xenograft tumors formed by H928/vector cells or H928/VEGF-C cells treated with either vehicle or Flt-4/Fc (0.5 mg/kg/3 days). Each data point represents the mean ± SD of eight primary tumors.

Therefore, we stably transfected H928/vector and H928/VEGF-C cells with siCNTN-1 expression vectors or with control vectors and conducted an in vivo experimental metastasis assay. Lung metastases occurred more frequently in the mice injected with H928/VEGF-C/control cells than in those injected with H928/vector/control cells. This increased occurrence in vivo was dramatically diminished by knockdown of CNTN-1 in H928/VEGF-C cells. No metastatic nodule formation was observed in the lungs of the mice injected with either H928/vector/control or H928/vector/siRNA-2-1 cells. Furthermore, the number of metastatic lung nodules and the lung weight of mice injected with H928/VEGF-C/siRNA-2-1 cells were significantly lower than those in the mice injected with H928/VEGF-C/control cells. The data from these experiments are summarized in Table S4. The expression of CNTN-1 protein but not of VEGF-C or phosphorylated Flt-4 proteins decreased dramatically in H928/VEGF-C/siRNA-2-1 metastatic lung tumors (Figure 6E). Together, these data demonstrate that VEGF-C/Flt-4-mediated tumor metastasis required the expression of CNTN-1.

Discussion

Promotion of tumor metastasis by VEGF-C has been reported to be due to the induction of tumor lymphangiogenesis via effects of activated Flt-4 on LECs (Alitalo and Carmeliet, 2002; Plate, 2001). It has recently become evident that many tumor cells express Flt-4, but the functions of this receptor in epithelial cells are largely unknown. In this study, we found that the VEGF-C/Flt-4 axis-mediated invasion and metastasis of human cancer cells were found to require upregulation of the neural cell adhesion molecular CNTN-1 through activation of the Src-p38-C/EBP-dependent pathway (Figure S3). From in vitro and in vivo studies to patient outcomes, a function for the VEGF-C/Flt-4 axis in cancer cells was delineated, and further insight was provided into the process of tumor metastasis.

VEGF-C has been reported to enhance resistance to chemotherapy and induction of Bcl-2 in leukemia cells via activation of Flt-4 and KDR heterodimer (Dias et al., 2002). In our study, treatment with VEGF-C induced tyrosine phosphorylation of Flt-4 but not of KDR and then increased the cell mobility. Whether the activated Flt-4 also contributed to the resistance of chemotherapy in solid tumors requires further study. VEGF-C has been reported to promote proliferation of leukemia cells (Dias et al., 2002) via activation of the Flt-4/KDR heterodimer, but we did not find a similar induction of proliferation in the lung cancer cell lines we used in this study. Consistent with a previous study (Dias et al., 2002), we found that KDR could be phosphorylated by VEGF-C and heterodimerized with Flt-4 in THP-1 leukemia cells. However, under the same conditions, we detected neither KDR phosphorylation nor KDR heterodimerized with Flt-4 after VEGF-C treatment in A549 cells (Figure S2E). This difference may be a major reason behind the absence of VEGF-C-dependent KDR phosphorylation in our study. It is therefore apparent that the functions of the Flt-4 and the cellular responses that occur on binding of VEGF-C to this receptor vary with cell type.

CNTN-1 associates with two other cell surface proteins believed to participate in signal transduction. CNTN-1 interacts in *trans* with receptor protein tyrosine phosphatase β (RPTPβ) to promote neurite outgrowth (Sakurai et al., 1997), and in *cis* with RPTPα (Zeng et al., 1999) to transduce extracellular signals to Fyn kinase, a member of the Src kinase family that regulates cell mobility (Umemori et al., 1994). Whether CNTN-1 protein interacts with other proteins that are involved in cancer cell motility and tumor invasion and metastasis, however, remained to be determined. Our study demonstrates that CNTN-1 is robustly increased by activation of the VEGF-C/Flt-4 axis and required for VEGF-C/Flt-4 axis-mediated cell mobility and metastasis.

Flt-4-mediated signaling pathways in LECs have been fully characterized (Alitalo and Carmeliet, 2002). In contrast, Flt-4-mediated signaling pathways in epithelial cancer cells have not been investigated. The findings from this study revealed that p38 MAPK but not ERK1/2 pathways are activated by overexpression of VEGF-C in H928 cells (Figure 4A). p38 MAPK is reported to function in cell migration and invasion in various cancer cells (Ma et al., 2003; Steeg, 2003). Indeed, blockage of the p38 signaling pathway is associated with inhibition of VEGF-C/Flt-4-mediated cell invasion. These observations also favor the proposal that signal transduction in response to activation of Flt-4 by VEGF-C varies with cell type. Although the PI3-K/Akt pathway was also activated by VEGF-C/Flt-4 in lung adenocarcinoma cells, this pathway was not linked to upregulation of CNTN-1 or increased cell invasiveness (data not shown). The Akt pathway is reported to be activated by VEGF-C and to be important for survival of LECs (Alitalo and Carmeliet, 2002). VEGF-C/Flt-4-mediated activation of PI3K/Akt signaling may therefore contribute to biological functions, such as induction of drug resistance, that are unrelated to invasion of cancer cells.

It has been reported that VEGF-A induces cell migration in leukemia (Dias et al., 2000), multiple myeloma (Podar et al., 2004), ovarian cancer (Wang et al., 2006), and breast cancer cells (Bachelder et al., 2002). All of those studies addressed the role of VEGF-A in migration of cancer cells but not the function of the VEGF-C/Flt-4 axis. In our study, we found that VEGF-A could also induce cell migration and invasion in lung adenocarcinoma cells (Figure 2C). More importantly, we demonstrate that the VEGF-C/Flt-4 axis is also a regulator of migration and invasion of cancer cells and that it may have a more potent effect than VEGF-A has (Figure 2C). These results do not contradict those of other current studies; instead, we revealed that the VEGF-C/Flt-4 axis has an important function in promoting the migration and invasion of cancer cells.

In summary, we provide clinical evidence that Flt-4 and its ligand are concurrently expressed in epithelial tumor cells of patients and that patients who have high levels of expression of both Flt-4 and VEGF-C have a poorer prognosis than do those who have high levels of expression of only one of them or lower

B: Expression of VEGF-C protein and tyrosine-phosphorylated Flt-4 was examined by immunoblotting and IP-Western blotting of H928/vector tumors or H928/VEGF-C tumors treated with or without Flt-4/Fc.
C: Numbers of metastatic nodules in the lung of individual mice carrying different tumors treated with or without Flt-4/Fc.
D: Macroscopic analysis of axillary lymph nodes. Lymph node volume was significantly higher in H928/VEGF-C tumor-bearing mice as compared to H928/vector tumor-bearing mice, as indicated by the asterisks (*$p < 0.05$), and this induction could be abolished by treatment with Flt-4/Fc, as indicated by the #.
E: Growth pattern of subcutaneously xenograft tumors formed by A549 cells treated with either vehicle or Flt-4/Fc (0.5 mg/kg/3 days).
F: Total number of metastatic nodules in the lung of individual mice.
G: Macroscopic analysis of axillary lymph node volume. *$p < 0.05$.

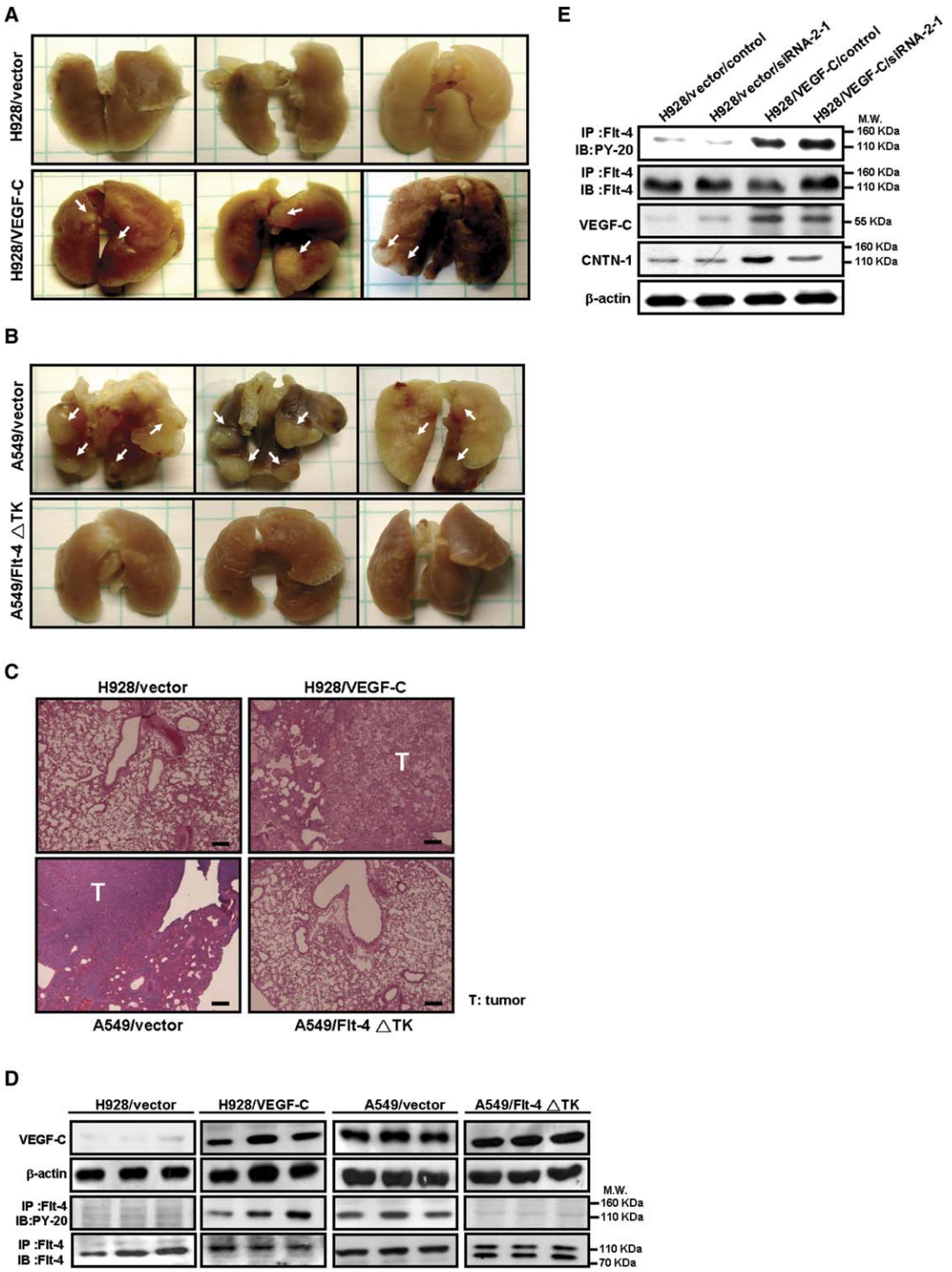

Figure 6. Regulation of cancer cell metastasis by the VEGF-C/Flt-4 axis

A and B: Lungs were excised and photographed after experimental metastasis assay. Arrow indicate metastatic tumor nodules.

C: Histologic analyses of lung metastatic tumors. Scale bar, 100 μm.

D: Expression of VEGF-C and tyrosine-phosphorylated Flt-4 protein was examined by immunoblotting of metastatic tumor recovered from lungs of individual mice.

E: Expression of VEGF-C, CNTN-1 protein, and tyrosine-phosphorylated Flt-4 was examined of lung metastatic tumor from individual mice.

levels of expression of both. With respect to the mechanisms responsible for the latter observation, activation of the VEGF-C/Flt-4 axis in cancer cells promoted their motility and invasiveness in vitro and tumor metastasis in vivo. In addition, the VEGF-C/Flt-4-induced enhancement of cell mobility and invasiveness and tumor metastasis was found to be at least partly

Table 2. Regulation of lung metastasis in the SCID mouse model by the VEGF-C/Flt-4 axis

	Lung weight (mg)		Lung metastasis		
Cell lines	Mean ± SD	p value	No. of mice with lung metastasis/ total no. of mice	Median lung nodules (range)	p value
A549/vector	531 ± 62		10/12	42.5 (0–72)	
A549/FLT-4ΔTK	467 ± 66	p = 0.02[a]	2/12	2 (0–15)	p < 0.001[b]
H928/vector	440 ± 42		2/12	1.27 (0–12)	
H928/VEGF-C	489 ± 53	p = 0.02[a]	7/12	13 (0–32)	p = 0.014[b]

[a]A549/vector versus A549/Flt-4ΔTK and H928/vector versus H928/VEGF-C by the Student's t test.
[b]A549/vector versus A549/Flt-4ΔTK and H928/vector versus H928/VEGF-C by the Wilcoxon rank-sum test.

mediated by upregulation of the adhesion molecule CNTN-1. These findings and those of others agree that the VEGF-C/Flt-4 axis has a multifaceted role in tumors via the facilitation of epithelial tumor cell motility and invasiveness, LEC proliferation (i.e., lymphangiogenesis), and possibly vascular endothelial cell proliferation and migration (i.e., angiogenesis). Thus, we propose that interruption of the VEGF-C/Flt-4 axis may be a powerful therapeutic approach for controlling tumor growth or metastasis.

Experimental procedures

In vitro migration assays

Migration of lung adenocarcinoma cells through polycarbonate filters was examined in 48-well chemotaxis chambers (modified Boyden chambers, Neuro Probe) as described previously (Kuratomi et al., 1999).

Invasion assays

Modified Boyden chambers with filter inserts (pore size of 8 μm) coated with Matrigel (40 μg; Collaborative Biomedical, Becton Dickinson Labware) in 24-well dishes (Nucleopore) were employed for invasion assays as described previously (Shih et al., 2001). Each clone was plated in triplicate for each experiment, and each experiment was repeated at least three times.

Anchorage-independent growth assay

Colony-forming assays in soft agarose were performed as described previously (Shou et al., 2004). Cells were seeded in 6-well culture dishes in suspensions of 0.35% agar noble in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum on top of a bed of 0.7% agar noble in the same complete medium. After 3 weeks, tumor cell colonies measuring at least 50 μm were counted from six replicates per treatment under a dissecting microscope.

FISH assay

The expression of Flt-4 mRNA in cancer specimens was determined by FISH assays using Cy-3-UTP-labeled riboprobes. For Flt-4, a probe covering nucleotides 1–595 was selected (Pajusola et al., 1992). Corresponding sense probes were used as controls. A four-point staining intensity scoring system was devised for determining the relative expression of Flt-4 mRNA in cancer specimens; the score ranged from 0 (no expression) to 3 (highest-intensity staining). The results of the FISH assay were classified in two groups according to both the intensity and extent of staining: in the low-expression group, either no staining was present (staining intensity score = 0) or positive staining was detected in less than 10% of the cells (staining intensity score = 1), and in the high-expression group, positive immunostaining was present in 10%–25% (staining intensity score = 2) or more than 25% of the cells (staining intensity score = 3). All of the FISH assay results were reviewed and scored independently by two pathologists.

Real-time quantitative RT-PCR

The quality of RNA in samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; 18S and 28S RNA bands were visualized with UV illumination. The primers used, based on the cDNA sequence of *Flt-4* (NM 002020), were as follows: 5′-AGGGAGACGCCCTTTCATG-3′ (forward) and 5′-GAGGGCTCTTTGGTCAAGCA-3′ (reverse) as described previously (Kaushal et al., 2005). The probe was labeled at the 5′ end with carboxyfluorescein and at the 3′ end with *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine. The primers and probe used for quantitative RT-PCR of the TATA box binding protein (TBP) mRNA (internal control, GenBank accession number X54993) were as described previously (Shih et al., 2001). Each sample was analyzed in duplicate, and a mean value was used for further calculation. The TBP was used as an internal control for RNA input. The results of Flt-4 expression normalized for TBP were presented as a normalized Flt-4 value.

Patients, specimens, and immunohistochemical staining

Tissues utilized were from the Cancer Tissue Core of the National Taiwan University Hospital. Institutional Review Board approval was obtained to procure and analyze the tissues used in this study. None of the patients had received preoperative neoadjuvant chemotherapy or radiation therapy. The surgical specimens had been fixed in formalin and embedded in paraffin before they were archived. We used the archived specimens for immunohistochemical staining. Protein expression in the tumor specimens was detected immunohistochemically as described previously (Su et al., 2004). The same four-point scoring system and two classification groups that were described for the results of the FISH assay were also used to assess the results of immunohistochemical staining. These results were also reviewed and scored independently by two pathologists. The histological diagnosis of lung adenocarcinoma was made according to the recommendations of the World Health Organization (WHO, 1999). Tumor size, local invasion, lymph node metastasis, and final disease stage were determined as described previously (Sobin and Wittekind, 2002). Follow-up of patients had lasted as long as 110 months. Patients who died of postoperative complications within 30 days after surgery were excluded from the survival analysis. There was one patient each from both the VEGF-C/Flt-4 high and VEGF-C/Flt-4 low groups who died within 30 days after surgery.

Statistical analysis

Data are presented as means ± standard deviations (SD). Student's t test was used to compare data between groups. Statistical analyses of clinicopathological data were performed as described previously (Shih et al., 2001). Survival curves were obtained using the Kaplan-Meier method. All statistical tests included two-way analysis of variance. Statistical significance was assumed at p values of less than 0.05.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, three supplemental figures, and four supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/3/209/DC1/.

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