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Effective Suppression of Vascular Network Formation by Combination of Antibodies Blocking VEGFR Ligand Binding and Receptor Dimerization

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SUMMARY

Antibodies that block vascular endothelial growth factor (VEGF) have become an integral part of antiangiogenic tumor therapy, and antibodies targeting other VEGFs and receptors (VEGFRs) are in clinical trials. Typically receptor-blocking antibodies are targeted to the VEGFR ligand-binding site. Here we describe a monoclonal antibody that inhibits VEGFR-3 homodimer and VEGFR-3/VEGFR-2 heterodimer formation, signal transduction, as well as ligand-induced migration and sprouting of microvascular endothelial cells. Importantly, we show that combined use of antibodies blocking ligand binding and receptor dimerization improves VEGFR inhibition and results in stronger inhibition of endothelial sprouting and vascular network formation in vivo. These results suggest that receptor dimerization inhibitors could be used to enhance antiangiogenic activity of antibodies blocking ligand binding in tumor therapy.

INTRODUCTION

Angiogenesis is the growth of new blood vessels from preexisting vasculature. The importance of angiogenesis for the growth of tumors was realized decades ago (Folkman, 1971), and the first antiangiogenic agents have recently been approved for clinical use (Jain et al., 2006). Although these treatments have been highly successful in the treatment of many types of solid tumors, most patients are either refractory or eventually acquire resistance to antiangiogenic therapy (Jain et al., 2009; Crawford and Ferrara, 2009). Therefore novel antiangiogenic therapeutics are needed to complement existing therapies.

Vascular endothelial growth factors (VEGFs) stimulate angiogenesis and lymphangiogenesis by activating VEGF receptor (VEGFR) tyrosine kinases in endothelial cells (Tammela et al., 2005). VEGFR-3 (also known as Flt4) belongs to this family that, in addition, comprises VEGFR-1/Flt-1 and VEGFR-2/KDR/Flk-1 (Alitalo et al., 2005; Shibuya and Claesson-Welsh, 2006). Mice deficient in the *Vegfr3* gene die in utero due to abnormal development of the blood vasculature resulting in cardiovascular failure (Dumont et al., 1998). On the other hand, loss of the VEGFR-3 ligand *Vegfc* results in embryonic lethality due to lack of lymphatic vessel formation (Kärkkäinen et al., 2004).

The *Vegfr3* gene is expressed in the entire vasculature of the developing embryo, but expression becomes restricted to the lymphatic system and a few specialized fenestrated blood vessel endothelia in adults (Partanen et al., 2000; Kaipainen et al., 1995). However, expression of VEGFR-3 is again induced in the

Significance

Antiangiogenic therapeutics are now widely used in the treatment of solid malignancies, but according to clinical experience not all patients respond, whereas others become refractory to therapy. Thus there is high demand for a second generation of improved antiangiogenic therapeutics. Here we report a monoclonal antibody that acts by inhibiting the formation of VEGFR-3 homodimers and VEGFR-3/VEGFR-2 heterodimers, which are well-established targets for antiangiogenic therapy. Our data suggest that employing a combination of ligand binding and dimerization inhibitors provides more effective blocking of VEGFR activation for enhanced inhibition of tumor angiogenesis and lymphangiogenesis in vivo.

angiogenic blood vascular endothelium in tumors (Valtola et al., 1999; Partanen et al., 2000; Tammela et al., 2008). Several studies have shown that interference with VEGFR-3 function inhibits tumor lymphangiogenesis and metastasis in mice (He et al., 2002; Alitalo et al., 2005). Specific targeting of VEGFR-3 can be achieved by small molecular weight tyrosine kinase inhibitors (Heckman et al., 2008), by trapping the VEGFR-3 ligands VEGF-C and VEGF-D with soluble extracellular domain of VEGFR-3 (Burton et al., 2008; Jeon et al., 2008; Mäkinen et al., 2001) or by VEGFR-3 blocking monoclonal antibodies (Persaud et al., 2004). Recent evidence has also suggested that blocking VEGFR-3 can improve the inhibition of tumor growth obtained with other antiangiogenic therapies (Tammela et al., 2008).

Current VEGFR-2 and VEGFR-3 blocking antibodies are directed against the ligand binding domains of these receptors (Hicklin et al., 2001; Witte et al., 1998; Pytowski et al., 2005; Persaud et al., 2004). Thus far other types of function-blocking antibodies against the VEGFR tyrosine kinases have not been described. Analogies to alternative modes of receptor tyrosine kinase inhibition can be derived from published work on the epidermal growth factor receptor (EGFR) family. For example, trastuzumab, a potent anti-ErbB2 antibody, inhibits the activation of this ligand-less receptor via mechanisms apparently involving antibody-dependent cellular cytotoxicity or inhibition of the proteolytic shedding of ErbB2 (Valabrega et al., 2007; Hynes and Lane, 2005).

Here we report on antibodies directed against the VEGFR-3 extracellular domain that inhibit receptor dimerization, and show that their combination with antibodies blocking ligand binding results in more effective inhibition of VEGFR-3 activation and vascular network formation in vitro and in vivo.

RESULTS

Characterization of the 2E11 Anti-VEGFR-3 Antibody

Monoclonal antibodies against the extracellular domain of VEGFR-3 were tested for blocking of VEGFR-3 activation and survival/proliferation of BaF3 cells expressing a VEGFR-3/erythropoietin (Epo) receptor chimera. In the absence of IL-3 these cells survive only in the presence of a VEGFR-3 ligand in the culture medium (Mäkinen et al., 2001). Figure 1A shows a comparison of four antibodies in this assay. As can be seen from the figure, the addition of increasing amounts of the 2E11 antibody, but not of 9D9 or AFL4 antibodies to the medium containing 25 ng/ml human recombinant VEGF-C inhibited the survival of the cells. The previously published monoclonal human anti-human VEGFR-3 antibody 3C5 (Persaud et al., 2004) and the previously published VEGFR-3-Ig soluble receptor (Mäkinen et al., 2001) were used as positive controls for VEGFR-3 inhibition. The inhibition of VEGFR-3 activation by the 2E11 and 3C5 antibodies was confirmed by using VEGF-C induced VEGFR-3 phosphorylation in endothelial cells (Figure 1B).

A common mechanism for antibody inhibition of receptor activation is to block ligand binding to the receptor. It has been shown that the 3C5 antibody strongly inhibits the binding of VEGF-C to VEGFR-3 and the VEGF-C-induced mitogenic response in cells that expresses a chimeric human VEGFR-3-FMS receptor (Persaud et al., 2004). However, unlike the 3C5 antibody, the 2E11 antibody did not block the binding of VEGFR-3 extracellular domain to immobilized VEGF-C (Figures 1C and 1D). These data indicated that although 2E11 and 3C5 both inhibit VEGFR-3 activation, their inhibition mechanisms are different.

The binding epitopes of AFL4 and 9D9 were mapped to linear peptide sequences in VEGFR-3 immunoglobulin homology domain 5 (D5) and D6, respectively (see Figures S1A and S1B available online). In contrast, the 2E11 binding site could not be mapped to a linear epitope, nor was the epitope in the ligand binding region (D1–D3), as this antibody recognized VEGFR-3 where this region had been deleted (Figure S1C). Furthermore, 2E11 bound to nonreduced but not to reduced VEGFR-3 polypeptides in western blotting analysis (Figure S2B), suggesting that the epitope is conformational and sensitive to denaturation of VEGFR-3. Figure S1D shows the K_d values for 2E11, 9D9, and AFL4 obtained from surface plasmon resonance analysis using monomeric VEGFR-3D1-7. Because the 2E11 antibody bound better to the nonreduced receptor, we searched for the binding epitope in D5 that undergoes proteolytic cleavage after receptor biosynthesis, rendering the remaining fragments bound by a disulfide bridge (Pajusola et al., 1994).

A Polypeptide Loop Extending from the VEGFR-3 D5 Is Critical for 2E11 Antibody Binding and Receptor Activation

Figure S2A shows the sequence comparison of D5 in human and mouse VEGFR-3 and VEGFR-2. The proteolytic cleavage site in VEGFR-3 (Lee et al., 1996) is marked with a red arrowhead and the cysteine residues are marked red. Figures S2C and S2D show a computer model of a VEGFR-3 D5-related structure based on the immunoglobulin-homology domain of myelin basic protein-C (Kelley and Sternberg, 2009; Idowu et al., 2003). In VEGFR-3 D5, the extended loop (underlined in Figure S2A; containing the SLRRRQQQ sequence) would contain the cleavage site between R472 and S473 (red arrowhead in Figures S2A and S2C). In Figure S2D the surface of the immunoglobulin homology domain is colored red for negative charge and blue for positive charge. Although no actual data is available for a possible D5-D5 interaction, this model suggested a possible scenario where the positively charged residues of the elongated loop "arm" could contact the negatively charged surface of the "armpit," thus contributing to dimer stabilization and activation of the receptor.

Figure 2A schematically outlines the mutagenesis strategy used to interrogate the importance of D5 and its elongated, cleaved loop structure for 2E11 antibody binding and receptor function. The disulfide bonds in the figure are hypothetical and based on deductions from the D5 model. The effect of D5 cysteine to serine residue replacements on VEGFR-3 expression, cleavage and autophosphorylation in transfected 293T cells in the absence and presence of VEGF-C are shown in Figure 2B (left panel). The transfected cells were analyzed by VEGFR-3 immunoprecipitation and western blotting using anti-phosphotyrosine (pY) or VEGFR-3 antibodies. As can be seen from the results, the C445S and C534S mutations and their combination prevented receptor autophosphorylation and processing. The C466S mutation decreased VEGFR-3 expression levels while retaining at least some phosphorylation, and blocked cleavage of the receptor, whereas C486S allowed both processing and ligand-induced phosphorylation.

A similar analysis was carried out with a chimeric VEGFR-3 receptor where the loop region was substituted with the

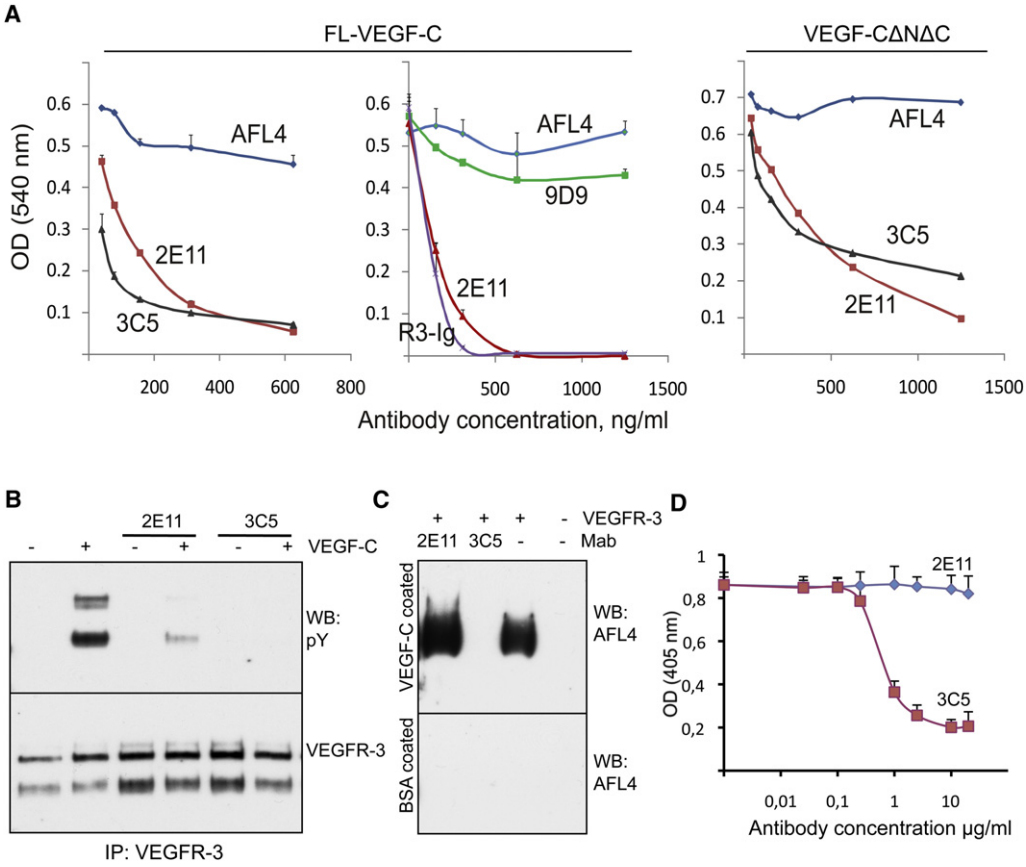


Figure 1. Characterization of the VEGFR-3 Blocking Antibodies

(A) VEGFR-3/BaF3 cell survival assay in the presence of the indicated anti-VEGFR-3 antibodies and 25 ng/ml of full-length (FL) or proteolytically processed, mature (ΔNΔC) VEGF-C (Joukov et al., 1997), as indicated. The IC_{50} values for VEGF-CFL are 3C5, 0.2 nM; 2E11, 0.66 nM; VEGFR-3-Ig, 0.79 nM; and for ΔNΔC VEGF-C: 3C5, 1.6 nM; 2E11, 2.2 nM. Error bars represent ± SEM.

(B) Antibody inhibition of VEGFR-3 phosphorylation in HDME cells stimulated with VEGF-C in the presence or absence of the indicated antibodies. Lysates were precipitated with polyclonal VEGFR-3 antibodies and blotted with pTyr (pY) or VEGFR-3 antibodies, as shown.

(C) Antibody-mediated inhibition of ligand binding to VEGFR-3. Wells were precoated with VEGF-C. Recombinant extracellular domain of VEGFR-3 with or without the indicated antibodies was applied, and the bound proteins were analyzed in western blotting with anti-VEGFR-3 antibodies.

(D) Wells were precoated as above. Recombinant VEGFR-3-AP was preincubated with different concentrations of either 2E11 or 3C5 antibodies and applied for binding. After washes, alkaline phosphatase activity was measured at OD405. See also Figure S1.

corresponding amino acid sequence of VEGFR-2, or where the loop was deleted. As shown in Figure 2C, the loop swap (LS) from VEGFR-2 to VEGFR-3 leads to slightly decreased VEGFR-3 phosphorylation and loss of both the chimeric VEGFR-3 cleavage and VEGF-C-inducible activation. In contrast, loop deletion (LD) leads to significant decrease of receptor phosphorylation even in the presence of VEGF-C. These results suggested that D5 plays a crucial role in VEGFR-3 activation.

As point mutations and deletions in the D5 loop area of VEGFR-3 had a significant effect on VEGFR-3 activation, the 2E11 antibodies were tested for binding to the different VEGFR-3 constructs in transient transfection experiments. As shown in Figure 2D and Figure S2E, the 2E11 antibodies do not recognize VEGFR-3 LD, but recognize VEGFR-3 LS, and they also failed to detect VEGFR-2 expressed in 293T cells (data not shown). When the other VEGFR-3 mutants were expressed in 293T cells and precipitated with 2E11 or 9D9 followed by western blotting with 9D9 antibodies, 2E11 failed to precipitate those mutants that had lost VEGF-C inducible activation (Figure 2E). These data indicate that the 2E11 epitope is at least partially located in D5 and sensitive to conformational changes in the loop region. Furthermore, the 2E11 antibodies recognize an epitope that correlates with the ability of the receptor to be activated. Although the mutagenesis data strongly suggested that the 2E11 epitope is located in VEGFR-3 D5, it was not possible to map this epitope using linear peptides. To further prove that 2E11 recognizes D5, this domain was expressed in 293T cells. As shown in Figure 2F, the 2E11 (and AFL4) antibodies readily precipitated the D5 domain from the conditioned medium, whereas the 9D9 antibodies did not, because the 9D9 epitope maps to D6 (Figure S1A).

2E11 Antibodies Provide Synergistic Inhibition of VEGFR-3 Activation in Combination with Antibodies Blocking Ligand Binding

The above experiments showed that the 3C5 and 2E11 antibodies bind to different regions of VEGFR-3 and inhibit receptor

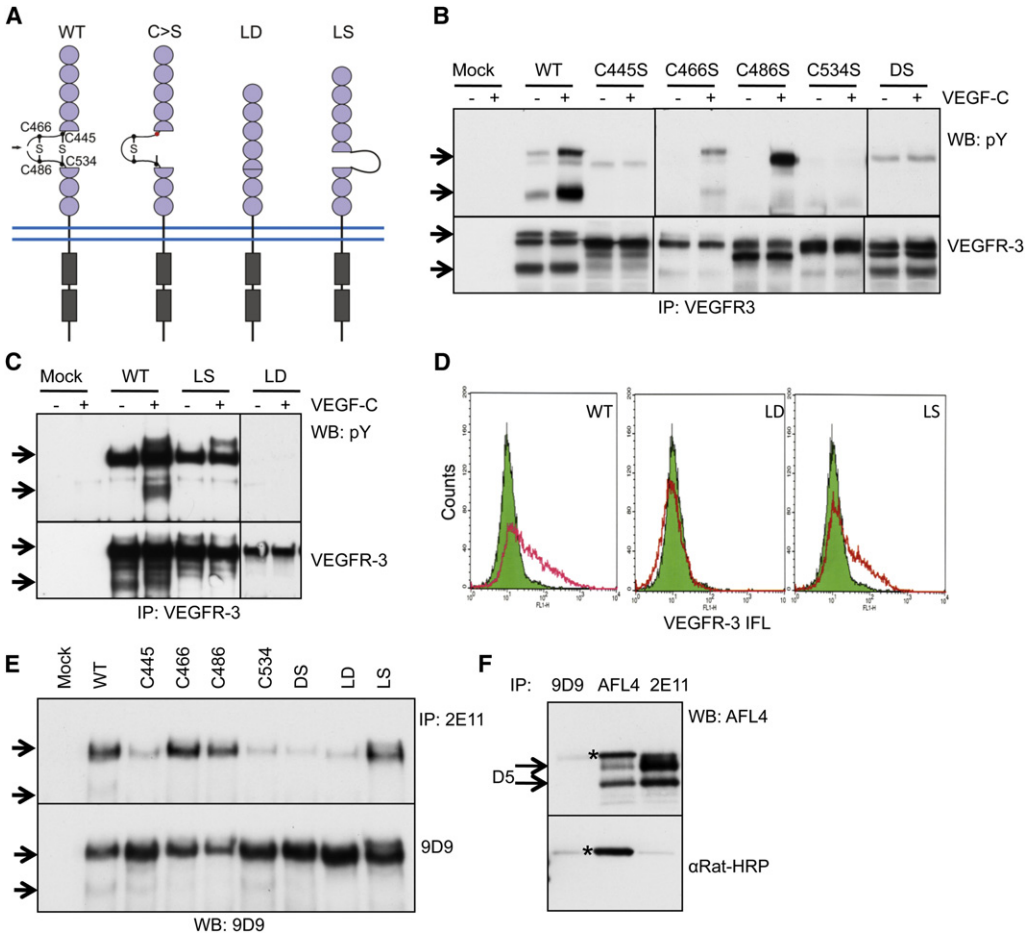


Figure 2. VEGFR-3 Domain 5 Contains Structures Important for Antibody Binding and Receptor Activity

(A) Schematic presentation of mutations made in VEGFR-3 domain 5 (D5). Four point mutations: C445S, C466S, C486S, C534S, one double mutation: DS (C445S and C534S combined), loop deletion (LD), and loop swap (LS) were made.

(B and C) Effect of the different mutations on VEGFR-3 activation and proteolytic processing. VEGFR-3 wt and mutants expressed in 293T cells were stimulated with VEGF-C. VEGFR-3 was then precipitated and analyzed by western blotting with anti-pY or anti-VEGFR-3 antibodies.

(D) Binding of 2E11 antibody to VEGFR-3 LD and LS mutants. 293T cells transfected with the indicated mutants were stained with the 2E11 antibodies and analyzed by flow cytometry (red). Green: mock transfected cells.

(E) Transfected 293T cell lysates were precipitated with 2E11 or 9D9 antibodies and blotted with the 9D9 antibody.

(F) The 2E11 antibodies recognize VEGFR-3 D5. VEGFR-3 D5 was cloned into the pSectag vector and expressed in 293T cells. Conditioned medium was precipitated either with 9D9, AFL4 or 2E11 antibodies and western blotted with AFL4 antibodies (upper panel). The lower panel represents the same samples immunoblotted with the secondary anti-rat antibody only. Asterixes indicate the IgG light chain. WT = wild-type. See also Figure S2.

activity by different mechanisms. This raised a question of possible synergistic inhibition of VEGFR-3 activity by 2E11 and 3C5. As shown in Figure 3A, the 2E11 and 3C5 antibodies in combination blocked VEGFR-3 induced BaF3 cell survival better than either antibody alone when VEGF-C was used at 10 ng/ml. At this concentration of VEGF-C the combination of the antibodies provided an additive effect (Figure 3A). At 100 ng/ml of VEGF-C, 3C5 provided very little inhibition, whereas 2E11 retained some activity (Figure 3B). Strikingly, at this ligand concentration, the two antibodies together provided synergistic inhibition of VEGFR-3 activation. Thus these data indicate that the two antibodies with the different mechanisms of inhibition provide higher efficacy when used in combination. These data also suggest that at high doses of VEGF-C the 3C5 antibodies cannot efficiently block VEGFR-3 activation because they compete with ligand binding, whereas the 2E11 antibodies retain activity because their mechanism is based on blocking receptor dimerization. Consistent with this hypothesis, the 3C5 antibodies were not able to inhibit activation of the mitogen-activated protein kinase Erk1,2 at high concentrations of VEGF-C in the BaF cells expressing the VEGFR-3/EpoR chimera, although 2E11 showed efficient inhibition at all tested VEGF-C concentrations (Figures 3C and 3D). Furthermore, as shown in Figure 3E, a similar difference between the 2E11 and 3C5 antibodies was detected in human dermal microvascular endothelial (HDME) cells when VEGFR-3 phosphorylation was analyzed after stimulation with different VEGF-C concentrations. Interestingly, only the 2E11 antibody was able to inhibit some of the Erk1,2 phosphorylation induced by 25 ng/ml of VEGF-C, whereas inhibition was not observed when using the 3C5 antibodies (Figure 3F).

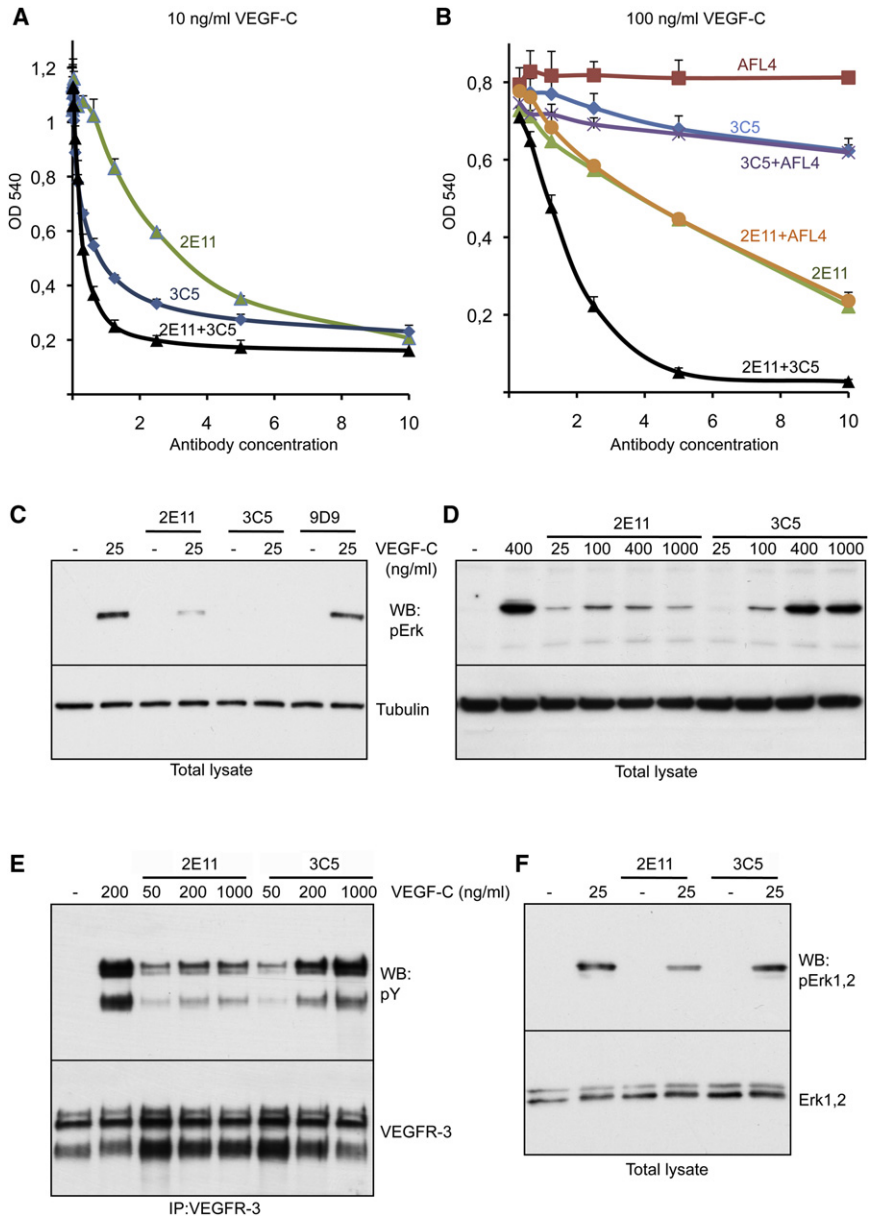


Figure 3. Inhibition of VEGFR-3 Activation by the Combination of 2E11 and 3C5 Antibodies

(A and B) Results of the VEGFR-3/BaF3 cell survival assay done using the indicated anti-VEGFR-3 antibody concentrations (μg/ml) in the presence of 10 ng/ml (A) or 100 ng/ml (B) of proteolytically processed, mature VEGF-C. Note that the AFL4 antibody has no effect. The IC_{50} values for 10 ng/ml of ΔNΔC VEGF-C are 2E11, 14.2 nM; 3C5, 2.64 nM; and 2E11 + 3C5, 1.38 nM. The IC_{50} values for 100 ng/ml of ΔNΔC VEGF-C are 2E11, 42.9 nM; 3C5, 204.6 nM; and 2E11 + 3C5, 11.2 nM.

(C) Antibody inhibition of Erk1,2 phosphorylation in VEGFR-3/BaF3 cells stimulated with 25 ng/ml of VEGF-C in the presence or absence of the indicated antibodies at 2 μg/ml. Lysates were blotted with pErk1,2 or tubulin antibodies, as shown.

(D) Antibody inhibition of Erk1,2 phosphorylation in VEGFR-3/BaF3 cells stimulated with increasing concentrations of VEGF-C in the presence or absence of the indicated antibodies. Lysates were blotted with pErk1,2 or tubulin antibodies, as shown.

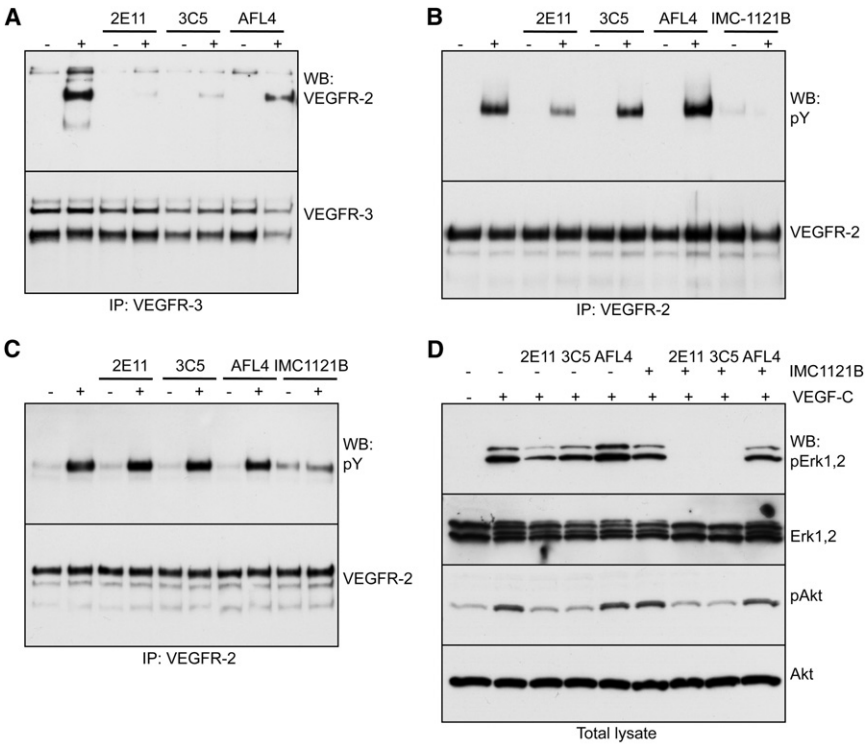
(E) Antibody inhibition of VEGFR-3 phosphorylation in HDME cells stimulated with increasing concentrations of VEGF-C in the presence or absence of the indicated antibodies. Lysates were precipitated with polyclonal antibodies against VEGFR-3 and blotted with pY or VEGFR-3 antibodies.

(F) Antibody inhibition of Erk1,2 phosphorylation in HDME cells stimulated with VEGF-C in the presence or absence of the indicated antibodies. Lysates were blotted with pErk1,2 or Erk1,2 antibodies.

The 2E11 Antibodies Inhibit VEGF-C Induced VEGFR-2/VEGFR-3 Heterodimerization and VEGFR-2 Activation

Previous studies have shown that VEGF-C can induce the formation of VEGFR-2/VEGFR-3 heterodimers that show distinct phosphorylation patterns in comparison with receptor homodimers (Dixelius et al., 2003). We investigated the possibility that 2E11, which inhibited VEGFR-3 activation by binding to D5, could act in *trans* to inhibit also the formation of the VEGFR-2/VEGFR-3 heterodimers. HDME cells expressing both receptors were stimulated with VEGF-C in the presence of the VEGFR-3 antibodies, VEGFR-3 was immunoprecipitated, and the immune complexes were subjected to western blotting using VEGFR-2 specific antibodies. As can be seen from the results shown in Figure 4A, the two VEGFR-3 blocking antibodies (2E11 and 3C5) inhibited the formation of VEGFR-2/VEGFR-3 heterodimers, whereas the AFL4 antibodies did not significantly decrease VEGFR-2 coprecipitation. Interestingly, inhibition of heterodimer formation was associated with decreased VEGFR-2 activation by VEGF-C, particularly when the 2E11 antibodies were used (Figure 4B), correlating with decreased downstream signaling via Erk1,2 (Figure 3F). In contrast, VEGFR-2 homodimer signaling was not affected by the 2E11 or 3C5 antibodies in transfected porcine aortic endothelial (PAE) cells expressing only VEGFR-2 (Figure 4C). Furthermore, preincubation with the 2E11 or 3C5 antibodies did not affect VEGF-induced VEGFR-2, Erk1,2, or Akt phosphorylation (Figure S3A; data not shown). Also, unlike the VEGF-C, the blocking antibodies did not induce downregulation of total VEGFR-2 or VEGFR-3 during a 60-min incubation (Figure S3B). These results indicate that the 2E11 antibodies inhibit signaling of both VEGFR-3 homodimers and VEGFR-3/VEGFR-2 heterodimers.

Both of the 2E11 and 3C5 antibodies were able to inhibit all VEGF-C induced Akt phosphorylation and a part of Erk1,2



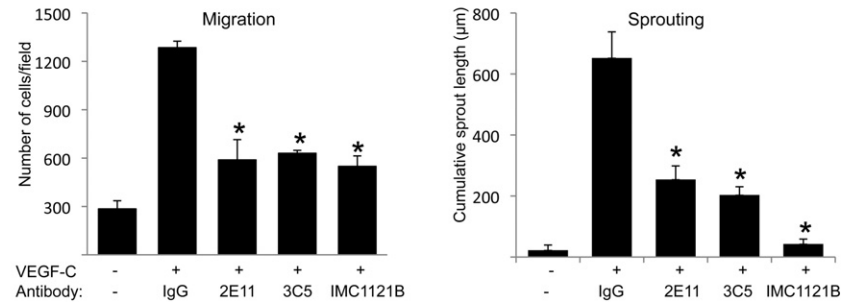


Figure 4. Antibody Inhibition of VEGFR-3/VEGFR-2 Heterodimerization, Signaling, Endothelial Cell Migration, and Sprouting

(A) Inhibition of VEGFR-2/VEGFR-3 heterodimerization by VEGFR-3 blocking antibodies (2E11, 3C5) in HDME cells stimulated with 25 ng/ml of VEGF-C. Lysates were precipitated with polyclonal VEGFR-3 antibodies and blotted with VEGFR-2 or VEGFR-3 antibodies.

(B and C) Effects of the VEGFR-3 and VEGFR-2 (IMC1121B) blocking antibodies on (B) VEGFR-2 phosphorylation in HDME cells or (C) PAE-VEGFR-2 cells stimulated with 25 ng/ml of VEGF-C. Lysates were precipitated with polyclonal VEGFR-2 antibodies and blotted with pY or VEGFR-2 antibodies.

(D) Effects of the VEGFR-3 and VEGFR-2 blocking antibodies on VEGF-C-induced intracellular signaling. HDME cells were preincubated with the indicated antibodies for 15 min and then stimulated with 25 ng/ml of VEGF-C for 20 min. Subsequently, total lysates were analyzed for Erk1,2 and Akt phosphorylation.

(E and F) Effect of the VEGFR-3 and VEGFR-2 blocking antibodies on (E) VEGF-C-induced migration or (F) sprouting of HDME cells. *p < 0.05 compared to hIgG. Error bars represent ± SEM. See also Figure S3.

phosphorylation in HDME cells (Figure 4D). On the other hand, IMC1121B antibodies that block VEGFR-2 had no effect on VEGF-C induced Akt phosphorylation, whereas they partially inhibited Erk1,2 phosphorylation. However, combination of IMC1121B with either 2E11 or 3C5 completely inhibited Erk1,2 activation. These results suggest that both VEGFR-2 and VEGFR-3 contribute to Erk1,2 activation whereas Akt activation is mostly induced by VEGFR-3.

The 2E11 Antibodies Inhibit VEGF-C Induced Migration and Sprouting of Normal as Well as Transformed Endothelial Cells

The effects of the VEGFR-3 blocking antibodies were next analyzed in migration and sprouting assays using cultured HDME cells (Figures 4E and 4F). The antibodies blocking VEGFR-3 (2E11, 3C5) or VEGFR-2 (IMC1121B) all inhibited HDME cell migration and sprouting from microbeads. As shown by flow cytometric analysis in Figure S3C, the HDME cells used consisted of almost equal proportions of blood vascular and lymphatic endothelial cells (BECs and LECs, respectively). VEGFR-2 was expressed at similar levels in both BECs and LECs (Figure S4A). Although the level of VEGFR-3 was lower in BECs than in LECs, it was strongly phosphorylated in the BECs on VEGF-C stimulation (Figure S4B). As can be seen from Figures 5A and 5B, 2E11 inhibited the migration of both LECs and BECs, whereas 3C5 inhibited only LEC migration. Similar results were obtained in the LEC sprouting assay (Figure 5C), whereas BECs did not sprout in this assay (data not shown). Importantly, the combination of 3C5 and 2E11 antibodies inhibited sprouting more effectively than either antibody alone (Figure S4C).

As shown in Figure 5D, in LECs, both Erk1,2 and Akt phosphorylation were inhibited by blocking VEGFR-3 activation using 3C5 or 2E11 antibodies, whereas in BECs only the VEGFR-2 blocking antibodies (IMC1121B) inhibited Erk1,2 activation. Furthermore, the very slight Akt activation seen in the VEGF-C treated BECs was inhibited only by the IMC1121 antibodies.

Kaposi sarcoma herpesvirus (KSHV)-infected ECs represent a biologically relevant model of KSHV-induced Kaposi sarcoma (KS); these cells have been demonstrated to robustly express VEGFR-3 (Carroll et al., 2004; Hong et al., 2004; Sivakumar et al., 2008; Wang et al., 2004). Moreover, the KS tumors show enhanced levels of VEGFR-3 and VEGF-C, which may play a key role in KSHV biology as LECs are considered to be the favored target of KSHV infection (Jussila et al., 1998; Skobe et al., 1999; Wang et al., 2004). We therefore tested the effect of the VEGFR-3 blocking antibodies on the capillary outgrowth

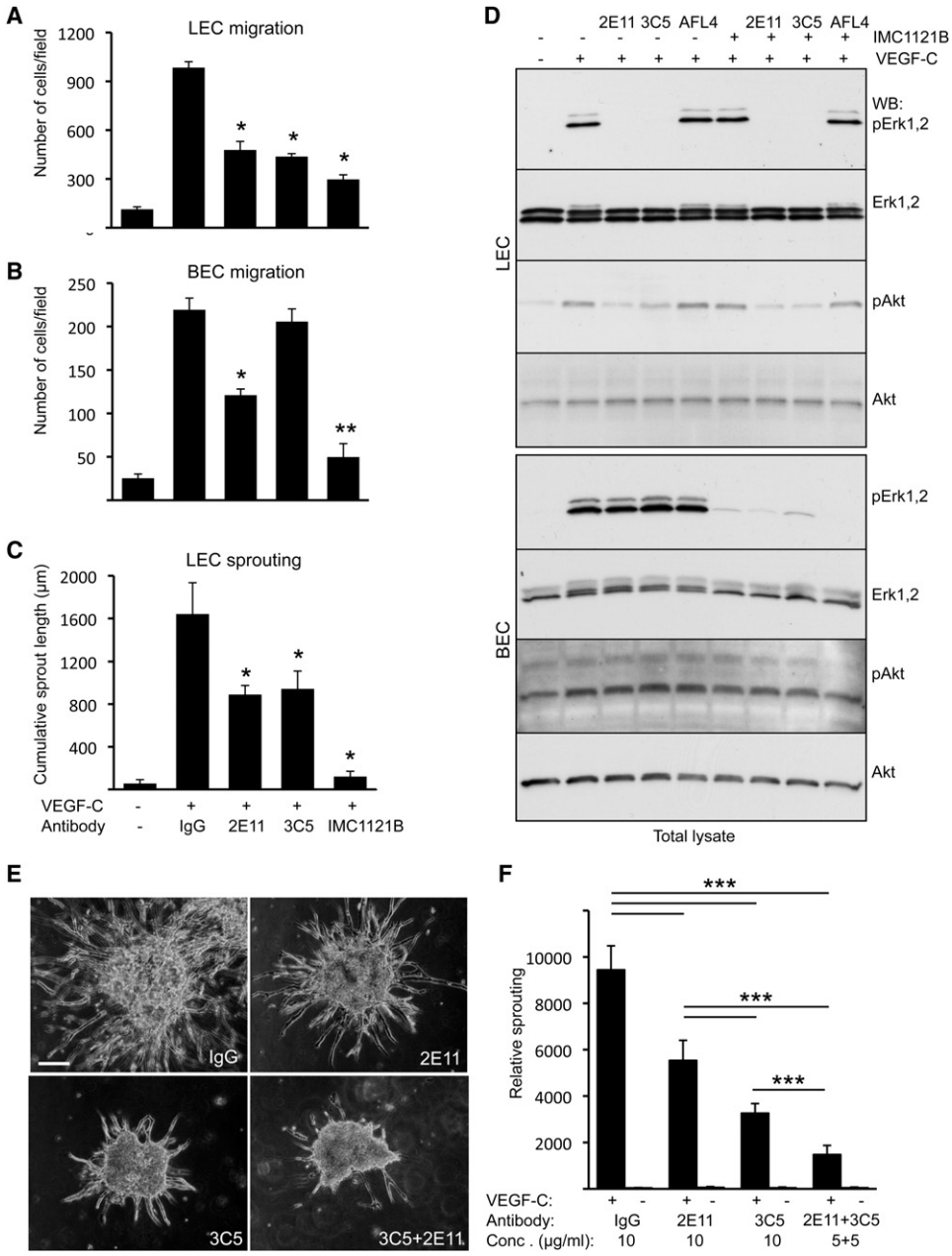


Figure 5. Antibody Inhibition of Migration, Sprouting, and Intracellular Signaling of Blood Vascular as Well as Normal and Transformed Lymphatic Endothelial Cells

Effect of VEGFR-3 and VEGFR-2 blocking antibodies on VEGF-C-induced migration of LECs (A) and BECs (B), LECs sprouting (C), and intracellular signaling (D). Phase contrast images (E) and statistical analysis (F) of the sprouting of K-LEC spheroids in the presence or absence of VEGF-C and in the presence of IgG, 2E11, 3C5 (each at 10 μg/ml) or a mixture of 2E11 and 3C5 (5 + 5 μg/ml). Scale bar represents 100 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to hIgG or as shown. Error bars represent ± SEM. See also Figure S4.

of KSHV-infected LEC (K-LEC) spheroids grown in a crosslinked 3D fibrin matrix. Extensive sprouting in response to VEGF-C was observed in the K-LEC spheroids (Figure 5E), whereas the control LECs sprouted to a lesser extent (data not shown). In the absence of VEGF-C, the sprouting was greatly reduced. To assess the effect of the 2E11 and 3C5 antibodies on the sprouting of K-LEC spheroids, the cultures were treated with 10 μg/ml of 2E11 or 3C5 antibodies or with 5 μg/ml of both 2E11 and 3C5. The IgG control did not influence the sprouting over the untreated K-LEC spheroids in the presence of VEGF-C (data not shown), whereas incubation with either 2E11 or 3C5 antibodies reduced sprout outgrowth significantly. The combination treatment with 2E11 and 3C5 antibodies led to a stronger inhibition of sprout outgrowth (Figures 5E and 5F).

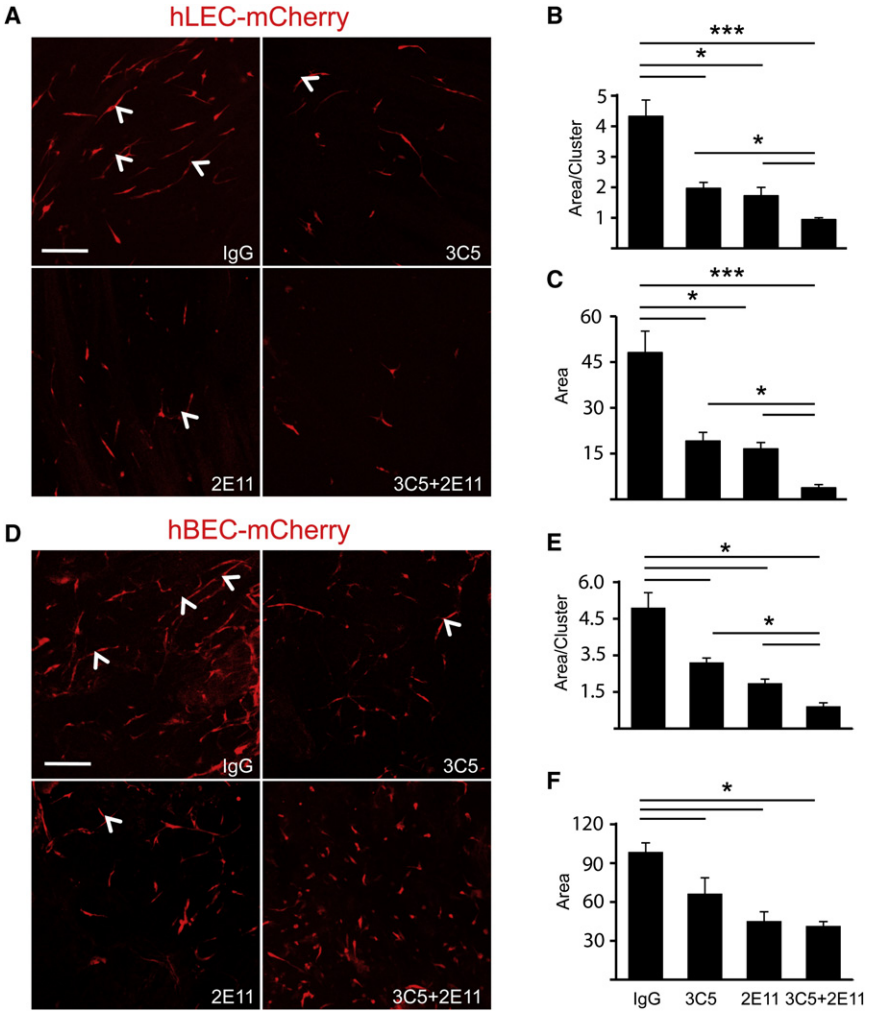


Figure 6. Effects of the 2E11 and 3C5 Antibodies and Their Combination on the Inhibition of Vascular Network Formation In Vivo

(A and D) LECs (A) and BECs (D) transfected with the fluorescent reporter mCherry (red) implanted to the mouse ear in Matrigel; the mice were daily administered the indicated blocking antibodies by intraperitoneal injections. Arrowheads indicate endothelial tubes. Note that the red signals in the BEC sample treated with the antibody combination represent cells without tube formation. Scale bar represents 100 μm.

(B, C, E, and F) Statistical analysis of the sizes of EC clusters (B and E) and total EC area (C and F) in the plugs in pixels/1000. *p < 0.05, ***p < 0.001. Error bars represent ± SEM.

Strong Inhibition of Vascular Morphogenesis In Vivo by the 2E11 and 3C5 Antibody Combination

To test if the antibodies blocking ligand binding and receptor dimerization would be effective in vivo, we implanted human BECs or LECs in VEGF-C containing Matrigel plugs into the ears of immunodeficient NOD-SCID-gamma mice. We found that the 2E11 and 3C5 antibodies suppressed LEC tube formation with similar efficiency (Figures 6A–6C). Importantly, combining 2E11 to 3C5 provided a stronger inhibition of tube formation than either blocking antibody alone at the same antibody dose (Figure 6B). Intriguingly, the combination of 2E11 and 3C5 also dramatically suppressed the survival of the transplanted LECs (Figures 6A and 6C and data not shown). The 2E11 and 3C5 combination also inhibited the ability of transplanted BECs to form vascular networks in vivo more efficiently than either antibody alone (Figures 6D–6F). However, unlike for the LECs, further suppression of BEC survival was not observed when comparing the combination treatment to the single treatments (Figure 6F). These results indicate that VEGF-C driven tube formation and survival of LECs as well as the vascular network formation of BECs are inhibited by the antibodies, and most efficiently by the combination of the two antibodies.

DISCUSSION

Here we describe an antibody that inhibits human VEGFR-3 via a mechanism that strikingly differs from other blocking antibodies against VEGFRs. The 2E11 antibodies did not block VEGF-C binding to VEGFR-3, yet they effectively inhibited VEGFR-3 phosphorylation and mitogenic signal transduction even at high concentrations of VEGF-C, when the 3C5 antibodies that occupy the ligand binding site in VEGFR-3 displayed only moderate inhibition. Even more striking was the synergy observed between inhibiting VEGFR dimerization and the inhibition of ligand binding.

Among the VEGFRs, only VEGFR-3 undergoes proteolytic processing, but the biological role of the processing is not known. Our previous studies have shown that processing occurs only after the receptor is glycosylated and translocated to the cell surface (Pajusola et al., 1994). In the present study, mutagenesis of select cysteine resides in D5, where the processing occurs, inhibited VEGFR-3 cleavage, but not phosphorylation of VEGFR-3 on VEGF-C stimulation. Our data also showed that a receptor where the extended loop of VEGFR-3 D5, including the cleavage site, is deleted or replaced by a corresponding nonhomologous loop of VEGFR-2 D5, cannot be cleaved, although this mutant also shows a small increase of tyrosyl phosphorylation on ligand stimulation. Thus, proteolytic cleavage of VEGFR-3 is not required for receptor activity, and the extended loop of D5 where the cleavage occurs can be exchanged with the corresponding, but nonhomologous loop of VEGFR-2 without complete loss of ligand-stimulated activity. It thus seems that the loop structure and its internal disulfide bonds are important for maintaining a conformation in D5 that supports receptor activity.

The VEGFRs transduce their signals according to the consensus scheme for receptor tyrosine kinases: binding of the ligand leads to receptor dimerization with close apposition of the receptor intracellular domains and exposure of the kinase

active site (Hubbard, 1999). Tyrosine phosphorylation then initiates signal transduction cascades, which ultimately lead to cellular responses such as proliferation, motility and survival. Crystal structures of complexes of VEGF (Wiesmann et al., 1997; Christinger et al., 1996) and PlGF (Christinger et al., 2004) with domain 2 of VEGFR-1 (VEGFR-1D2) have been determined. Mutating the extracellular parts of VEGFR-1 and VEGFR-2 revealed that both D2 and D3 are needed for high affinity VEGF binding (Davis-Smyth et al., 1998; Fuh et al., 1998). Similarly, the recently published analysis of the VEGF/VEGFR-2 complex by electron microscopy suggested that VEGF binds to D2 and D3 (Ruch et al., 2007). Interestingly, two studies so far reported that the VEGFR-2 dimers are further stabilized by receptor-receptor contacts mediated by D4 and D7 (Yang et al., 2010; Ruch et al., 2007). One could thus envision that antibodies binding to the domains involved in dimeric receptor-receptor contacts could interfere with the close apposition of the downstream tyrosine kinase domains, thus blocking receptor activity. However, although no such antibodies have been previously characterized for the VEGFR family, the present results show one specific region of VEGFR-3 that allows the inhibition of its homodimerization as well as heterodimerization with VEGFR-2.

It should be mentioned that the ErbB2 antibody trastuzumab, one of the first monoclonal antibodies used in clinical practice, acts through a mechanism not involving inhibition of ligand binding because a soluble ligand for ErbB2 has not been found. The exact mechanism of ErbB2 inhibition by trastuzumab is not completely understood, but these antibodies have little effect on ErbB2-ErbB3 heterodimerization (Agus et al., 2002). Rather they are thought to act through antibody-dependent cellular cytotoxicity or inhibition of ErbB2 shedding (Valabrega et al., 2007; Hynes and Lane, 2005). Interestingly, another ErbB2 blocking antibody, pertuzumab, acts through blocking heterodimerization of ErbB2 with other members of the ErbB family by binding to domain II and sterically masking a binding pocket necessary for receptor-receptor interaction (Franklin et al., 2004). Thus in addition to antibody-dependent cellular cytotoxicity, pertuzumab binding directly inhibits ErbB2 heterodimerization, which blocks the ErbB2 signaling cascade (Agus et al., 2002). This difference between trastuzumab and pertuzumab explains why pertuzumab is effective in carcinomas that express low levels of ErbB2, whereas trastuzumab is not (Agus et al., 2002; Mendoza et al., 2002).

Antibodies that block ligand binding to receptor need to compete with the ligand for receptor binding, i.e., the outcome of therapeutic targeting is dependent on the stoichiometry between ligand and antibody. At high ligand concentrations such antibodies are less effective than antibodies blocking receptor dimerization, as seen in our analysis in the BaF3/VEGFR-3 cultures. In the cultured microvascular endothelial cells, only VEGFR-2/VEGFR-2 heterodimers, but not VEGFR-2 homodimers, were inhibited by the 2E11 antibodies. Importantly however, our data indicated that a combination of antibodies blocking ligand binding and receptor dimerization is more effective in inhibiting both blood vascular and lymphatic endothelial cell sprouting, in particular sprout elongation, than either antibody alone. This was also the case in the analysis of blood and lymphatic endothelial vascular network formation in vivo in matrigel plugs, where the antibody combination furthermore compromised the survival of lymphatic endothelial cells. Similarly, stronger inhibition of sprouting was observed with LECs transformed with the KSHV human tumor virus when the combination of antibodies was used. Depending on the assay, the combination of blocking antibodies thus provided an additive or a synergistic inhibition. It would be interesting to know if such effects could be further improved by inclusion of the recently published antibodies against neuropilin-2 that block VEGF-C binding and LEC sprout elongation (Xu et al., 2010).

Encouraged by results on increased efficacy and accelerated receptor downregulation by antibody combinations (Ben-Kasus et al., 2009), three ongoing clinical trials are addressing if a combination of trastuzumab and pertuzumab results in a better therapeutic outcome than either of the two antibodies alone (http://clinicaltrials.gov/ct2/results?term = trastuzumab+pertuzumab). Although such studies have not yet been carried out with VEGFR targeting antibodies, our data on vascular network formation of BECs and LECs suggest that using a combination of ligand binding and dimerization inhibitors would provide more effective blocking of VEGFRs and enhanced inhibition of tumor angiogenesis and lymphangiogenesis in vivo. The combination could also form a potential treatment modality for Kaposi sarcoma tumor cells that are known to expresses VEGFR-3 (Jussila et al., 1998).

In conclusion, our results define a class of VEGFR blocking antibodies, which provide interesting mechanistic insight into receptor structure and activation. Importantly, the dimerization inhibitor unveils a biologically meaningful rationale for suppressing endothelial activation and angiogenesis in tumors. The use of a combination of antibodies inhibiting ligand binding and receptor dimerization should translate into improved antiangiogenic and antilymphangiogenic therapies in the future.

EXPERIMENTAL PROCEDURES

Cell Culture

Human dermal microvascular endothelial cells (HDME cells), human lymphatic endothelial cells (hLEC), and human blood vascular endothelial cells (hBEC) were purchased from Promocell and cultured in endothelial cell medium MV (Promocell) according to the supplier's instructions. These cells were used between passages 2–7. 293T cells (ATCC) were cultured in DMEM supplemented with 10% fetal calf serum (FCS). Porcine aortic endothelial cells expressing VEGFR-2 were a kind gift from Dr. Lena Claesson-Welsh (University of Uppsala) (Waltenberger et al., 1994). The BaF3-VEGFR-3 cell line is a genetically modified derivative of the murine pro-B cell line BaF3, which stably expresses a chimeric receptor containing the extracellular domain of human VEGFR-3 and the transmembrane and cytoplasmic domains of the mouse erythropoietin receptor. These cells were maintained in DMEM containing 10% FCS. For maintenance, the cell cultures were supplemented with 2 ng/mL murine IL-3 (Calbiochem) and 250 μg/ml Zeocin (Invitrogen). In the absence of IL-3, BaF3-VEGFR-3 cells grow only in presence of VEGF-C or VEGF-D (Mäkinen et al., 2001).

Antibodies

The following primary antibodies were used in this study: mouse monoclonal against human VEGFR-3, 2E11D11 (termed as 2E11 in the text), 9D9F9 (9D9, Available from Chemicon [Millipore, MAB3757] and ReliaTech [101-M36]) (Jussila et al., 1998), rat monoclonal against mouse VEGFR-3: AFL4 (Kubo et al., 2000, available from eBioscience (14-5988)), IMC1121B (ImClone), anti-phosphotyrosine (Millipore), anti-Podoplanin (Acris), anti-Erk1/2, anti-phospho-Erk1/2, anti-Akt and anti-phospho-Akt (Cell Signaling). The human antibody that blocks ligand binding to human VEGFR-3 (hF4-3C5, termed 3C5 in the text) was generously provided by ImClone Systems

(Persaud et al., 2004). The polyclonal antibodies against VEGFR-3 were from R&D Systems. Unless otherwise indicated, the blocking antibodies were used at 2 μg/ml in the signal transduction experiments.

VEGF-C Binding Assay

A 96-well plate was precoated with 2 μg/ml VEGF-C and nonspecific binding sites were blocked with 1% BSA. The extracellular domain of VEGFR-3 fused to alkaline phosphatase (AP) (Pytowski et al., 2005) was preincubated with different antibody concentrations for 20 min and then applied to the VEGF-C precoated plates for 20 min. Subsequently, the plates were washed with PBS and binding was detected by the addition of 50 μl alkaline phosphatase substrate solution (Sigma). Alternatively, the extracellular domain of VEGFR-3 (Jussila et al., 1998) was preincubated with the indicated antibodies and applied to the VEGF-C coated plates for binding. After washing of the plates, bound proteins were suspended in 100 μl 1× Laemmli buffer and analyzed by immunoblotting with anti-VEGFR-3 antibodies.

Statistical Analysis

P values are determined by one-way ANOVA followed by Dunnett's test using SPSS 17.0 software. Statistical significance is indicated in the figures by *, where $p < 0.05$; ** where$p < 0.01$; and ***, where $p < 0.001$. The remaining experimental procedures can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.ccr.2010.11.001.

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REFERENCES

Agus, D.B., Akita, R.W., Fox, W.D., Lewis, G.D., Higgins, B., Pisacane, P.I., Lofgren, J.A., Tindell, C., Evans, D.P., Maiese, K., et al. (2002). Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell *2*, 127–137.

Alitalo, K., Tammela, T., and Petrova, T.V. (2005). Lymphangiogenesis in development and human disease. Nature *438*, 946–953.

Ben-Kasus, T., Schechter, B., Lavi, S., Yarden, Y., and Sela, M. (2009). Persistent elimination of ErbB-2/HER2-overexpressing tumors using combinations of monoclonal antibodies: relevance of receptor endocytosis. Proc. Natl. Acad. Sci. USA *106*, 3294–3299.

Burton, J.B., Priceman, S.J., Sung, J.L., Brakenhielm, E., An, D.S., Pytowski, B., Alitalo, K., and Wu, L. (2008). Suppression of prostate cancer nodal and systemic metastasis by blockade of the lymphangiogenic axis. Cancer Res. *68*, 7828–7837.

Carroll, P.A., Brazeau, E., and Lagunoff, M. (2004). Kaposi's sarcoma-associated herpesvirus infection of blood endothelial cells induces lymphatic differentiation. Virology *328*, 7–18.

Christinger, H.W., Muller, Y.A., Berleau, L.T., Keyt, B.A., Cunningham, B.C., Ferrara, N., and de Vos, A.M. (1996). Crystallization of the receptor binding domain of vascular endothelial growth factor. Proteins *26*, 353–357.

Christinger, H.W., Fuh, G., de Vos, A.M., and Wiesmann, C. (2004). The crystal structure of placental growth factor in complex with domain 2 of vascular endothelial growth factor receptor-1. J. Biol. Chem. *279*, 10382–10388.

Crawford, Y., and Ferrara, N. (2009). VEGF inhibition: insights from preclinical and clinical studies. Cell Tissue Res. *335*, 261–269.

Davis-Smyth, T., Presta, L.G., and Ferrara, N. (1998). Mapping the charged residues in the second immunoglobulin-like domain of the vascular endothelial growth factor/placenta growth factor receptor Flt-1 required for binding and structural stability. J. Biol. Chem. *273*, 3216–3222.

Dixelius, J., Mäkinen, T., Wirzenius, M., Kärkkäinen, M.J., Wernstedt, C., Alitalo, K., and Claesson-Welsh, L. (2003). Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J. Biol. Chem. *278*, 40973–40979.

Dumont, D.J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M., and Alitalo, K. (1998). Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science *282*, 946–949.

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. N. Engl. J. Med. *285*, 1182–1186.

Franklin, M.C., Carey, K.D., Vajdos, F.F., Leahy, D.J., de Vos, A.M., and Sliwkowski, M.X. (2004). Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell *5*, 317–328.

Fuh, G., Li, B., Crowley, C., Cunningham, B., and Wells, J.A. (1998). Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. J. Biol. Chem. *273*, 11197–11204.

He, Y., Kozaki, K., Karpanen, T., Koshikawa, K., Yla-Herttuala, S., Takahashi, T., and Alitalo, K. (2002). Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J. Natl. Cancer Inst. *94*, 819–825.

Heckman, C.A., Holopainen, T., Wirzenius, M., Keskitalo, S., Jeltsch, M., Yla-Herttuala, S., Wedge, S.R., Jurgensmeier, J.M., and Alitalo, K. (2008). The tyrosine kinase inhibitor cediranib blocks ligand-induced vascular endothelial growth factor receptor-3 activity and lymphangiogenesis. Cancer Res. *68*, 4754–4762.

Hicklin, D.J., Witte, L., Zhu, Z., Liao, F., Wu, Y., Li, Y., and Bohlen, P. (2001). Monoclonal antibody strategies to block angiogenesis. Drug Discov. Today *6*, 517–528.

Hong, Y.K., Foreman, K., Shin, J.W., Hirakawa, S., Curry, C.L., Sage, D.R., Libermann, T., Dezube, B.J., Fingeroth, J.D., and Detmar, M. (2004). Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. Nat. Genet. *36*, 683–685.

Hubbard, S.R. (1999). Structural analysis of receptor tyrosine kinases. Prog. Biophys. Mol. Biol. *71*, 343–358.

Hynes, N.E., and Lane, H.A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. Nat. Rev. Cancer *5*, 341–354.

Idowu, S.M., Gautel, M., Perkins, S.J., and Pfuhl, M. (2003). Structure, stability and dynamics of the central domain of cardiac myosin binding protein C (MyBP-C): implications for multidomain assembly and causes for cardiomyopathy. J. Mol. Biol. *329*, 745–761.

Jain, R.K., Duda, D.G., Clark, J.W., and Loeffler, J.S. (2006). Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat. Clin. Pract. Oncol. *3*, 24–40.

Jain, R.K., Duda, D.G., Willett, C.G., Sahani, D.V., Zhu, A.X., Loeffler, J.S., Batchelor, T.T., and Sorensen, A.G. (2009). Biomarkers of response and resistance to antiangiogenic therapy. Nat. Rev. Clin. Oncol. *6*, 327–338.

Jeon, B.H., Jang, C., Han, J., Kataru, R.P., Piao, L., Jung, K., Cha, H.J., Schwendener, R.A., Jang, K.Y., Kim, K.S., et al. (2008). Profound but dysfunctional lymphangiogenesis via vascular endothelial growth factor ligands from CD11b+ macrophages in advanced ovarian cancer. Cancer Res. *68*, 1100–1109.

Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N., and Alitalo, K. (1997). Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J. *16*, 3898–3911.

Jussila, L., Valtola, R., Partanen, T.A., Salven, P., Heikkila, P., Matikainen, M.T., Renkonen, R., Kaipainen, A., Detmar, M., Tschachler, E., et al. (1998). Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. Cancer Res. *58*, 1599–1604.

Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V.W., Fang, G.H., Dumont, D., Breitman, M., and Alitalo, K. (1995). Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc. Natl. Acad. Sci. USA *92*, 3566–3570.

Kärkkäinen, M.J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T.V., Jeltsch, M., Jackson, D.G., Talikka, M., Rauvala, H., et al. (2004). Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat. Immunol. *5*, 74–80.

Kelley, L.A., and Sternberg, M.J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. Nat. Protoc. *4*, 363–371.

Kubo, H., Fujiwara, T., Jussila, L., Hashi, H., Ogawa, M., Shimizu, K., Awane, M., Sakai, Y., Takabayashi, A., Alitalo, K., et al. (2000). Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. Blood *96*, 546–553.

Lee, J., Gray, A., Yuan, J., Luoh, S.M., Avraham, H., and Wood, W.I. (1996). Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. Proc. Natl. Acad. Sci. USA *93*, 1988–1992.

Mäkinen, T., Jussila, L., Veikkola, T., Karpanen, T., Kettunen, M.I., Pulkkanen, K.J., Kauppinen, R., Jackson, D.G., Kubo, H., Nishikawa, S., et al. (2001). Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nat. Med. *7*, 199–205.

Mendoza, N., Phillips, G.L., Silva, J., Schwall, R., and Wickramasinghe, D. (2002). Inhibition of ligand-mediated HER2 activation in androgen-independent prostate cancer. Cancer Res. *62*, 5485–5488.

Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L., and Alitalo, K. (1994). Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. Oncogene *9*, 3545–3555.

Partanen, T.A., Arola, J., Saaristo, A., Jussila, L., Ora, A., Miettinen, M., Stacker, S.A., Achen, M.G., and Alitalo, K. (2000). VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J. *14*, 2087–2096.

Persaud, K., Tille, J.C., Liu, M., Zhu, Z., Jimenez, X., Pereira, D.S., Miao, H.Q., Brennan, L.A., Witte, L., Pepper, M.S., and Pytowski, B. (2004). Involvement of the VEGF receptor 3 in tubular morphogenesis demonstrated with a human anti-human VEGFR-3 monoclonal antibody that antagonizes receptor activation by VEGF-C. J. Cell Sci. *117*, 2745–2756.

Pytowski, B., Goldman, J., Persaud, K., Wu, Y., Witte, L., Hicklin, D.J., Skobe, M., Boardman, K.C., and Swartz, M.A. (2005). Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. J. Natl. Cancer Inst. *97*, 14–21.

Ruch, C., Skiniotis, G., Steinmetz, M.O., Walz, T., and Ballmer-Hofer, K. (2007). Structure of a VEGF-VEGF receptor complex determined by electron microscopy. Nat. Struct. Mol. Biol. *14*, 249–250.

Shibuya, M., and Claesson-Welsh, L. (2006). Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp. Cell Res. *312*, 549–560.

Sivakumar, R., Sharma-Walia, N., Raghu, H., Veettil, M.V., Sadagopan, S., Bottero, V., Varga, L., Levine, R., and Chandran, B. (2008). Kaposi's sarcoma-associated herpesvirus induces sustained levels of vascular endothelial growth factors A and C early during in vitro infection of human microvascular dermal endothelial cells: biological implications. J. Virol. *82*, 1759–1776.

Skobe, M., Brown, L.F., Tognazzi, K., Ganju, R.K., Dezube, B.J., Alitalo, K., and Detmar, M. (1999). Vascular endothelial growth factor-C (VEGF-C) and its receptors KDR and flt-4 are expressed in AIDS-associated Kaposi's sarcoma. J. Invest. Dermatol. *113*, 1047–1053.

Tammela, T., Enholm, B., Alitalo, K., and Paavonen, K. (2005). The biology of vascular endothelial growth factors. Cardiovasc. Res. *65*, 550–563.

Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstrom, M., Schomber, T., Peltonen, R., et al. (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature *454*, 656–660.

Valabrega, G., Montemurro, F., and Aglietta, M. (2007). Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. Ann. Oncol. *18*, 977–984.

Valtola, R., Salven, P., Heikkila, P., Taipale, J., Joensuu, H., Rehn, M., Pihlajaniemi, T., Weich, H., deWaal, R., and Alitalo, K. (1999). VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am. J. Pathol. *154*, 1381–1390.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C.H. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J. Biol. Chem. *269*, 26988–26995.

Wang, H.W., Trotter, M.W., Lagos, D., Bourboulia, D., Henderson, S., Mäkinen, T., Elliman, S., Flanagan, A.M., Alitalo, K., and Boshoff, C. (2004). Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma. Nat. Genet. *36*, 687–693.

Wiesmann, C., Fuh, G., Christinger, H.W., Eigenbrot, C., Wells, J.A., and de Vos, A.M. (1997). Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. Cell *91*, 695–704.

Witte, L., Hicklin, D.J., Zhu, Z., Pytowski, B., Kotanides, H., Rockwell, P., and Bohlen, P. (1998). Monoclonal antibodies targeting the VEGF receptor-2 (Flk1/KDR) as an anti-angiogenic therapeutic strategy. Cancer Metastasis Rev. *17*, 155–161.

Xu, Y., Yuan, L., Mak, J., Pardanaud, L., Caunt, M., Kasman, I., Larrivee, B., Del Toro, R., Suchting, S., Medvinsky, A., et al. (2010). Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR-3. J. Cell Biol. *188*, 115–130.

Yang, Y., Xie, P., Opatowsky, Y., and Schlessinger, J. (2010). Direct contacts between extracellular membrane-proximal domains are required for VEGF receptor activation and cell signaling. Proc. Natl. Acad. Sci. USA *107*, 1906–1911.