Suppression of the Raf/MEK/ERK Signaling Cascade and Inhibition of Angiogenesis by the Carboxyl Terminus of Angiopoietin-Like Protein 4

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Objectives—Angiopoietin-like protein 4 (Angptl4) is a secreted glycoprotein that has recently been implicated in the regulation of angiogenesis and metastasis. This study aimed to investigate the structural and cellular basis underlying the biological actions of Angptl4.

Methods and Results—Circulating Angptl4 was proteolytically cleaved into NH2-terminal coiled-coil domain (N-Angptl4) and COOH-terminal fibrinogen-like domain (C-Angptl4). Using amino acid sequencing analysis, we identified a major cleavage site between Lys168 and Leu169 and a minor cleavage site between Lys170 and Met171 in mouse Angptl4. C-Angptl4, but not N-Angptl4, potently inhibited both bFGF- and VEGF-induced cell proliferation, migration, and tube formation in endothelial cells, and prevented neovascularization in mice. Treatment of C-Angptl4 with PNGase F (an N-glycosidase) ablated its N-linked glycosylation, and also significantly attenuated its antiangiogenic activities. C-Angptl4 blocked bFGF-induced activation of ERK1/2 MAP kinase, but had no obvious effect on Akt and P38 MAP kinase. Furthermore, C-Angptl4 abrogated bFGF-induced phosphorylation of Raf-1 and MEK1/2, whereas neither auto-phosphorylation of FGF receptor-1 nor activation of Ras was affected, suggesting that the blockage occurs at the level of Raf-1 activation.

Conclusions—The carboxyl terminus of Angptl4 alone is sufficient to suppress angiogenesis, possibly through inhibiting the Raf/MEK/ERK1/2 MAP kinase pathway in endothelial cells. (Arterioscler Thromb Vasc Biol 2008;28:835-840)

Key Words: angiogenesis ■ angiopoietin-like proteins ■ glycosylation ■ MAP kinase ■ neovascularization

Angiogenesis is the formation of new blood vessels from pre-existing primary plexus through the processes of vascular sprouting, branching, and differential growth to form more mature vascular networks.1 Physiological angiogenesis is an essential process for reproduction, development, and wound repair. On the other hand, pathological angiogenesis play an important role in the disease progression such as cancer, diabetic retinopathy, atherosclerosis, and rheumatoid arthritis.2

Angiogenesis is the multiple step process orchestrated by a range of pro- and antiangiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), thrombospondin, angiopoietins, and more recently, angiopoietin-like proteins (Angptl).3,4 So far, 7 members of the Angptl family have been identified, all of which have a secondary structural organization similar to angiopoietins, including a NH2-terminal coiled coil domain and COOH-terminal fibrinogen-like domain. However, unlike angiopoietins, none of the Angptl family members bind to the receptor tyrosine kinases Tie1 or Tie2.5

Angiopoietin-like protein 4 (Angptl4), also known as peroxisome proliferator-activated receptor γ (PPAR-γ) angiopoietin-related protein (PGAR), fasting-induced adipose factor (FIAF), or hepatic fibrinogen/angiopoietin-related protein (HFARP), is a circulating glycoprotein highly expressed in adipose tissue, liver, and placenta.5-7 Angptl4 is cleaved in vitro and in vivo, and circulates in the blood stream mainly as truncated fragments.4 Recent studies from others and our laboratory have demonstrated Angptl4 to be an important regulator of glucose homeostasis, insulin sensitivity, and lipid metabolism.8-11 In addition, growing evidence suggest that Angptl4 is a key player in angiogenesis. Although an earlier study suggested the potential proangiogenic activity of Angptl4,12 more recent data from several independent laboratories have demonstrated Angptl4 as a potent antiangiogenic factor.13-16 Expression of Angptl4 was found to be reduced in primary gastric cancer and several types of cancer cell lines, and was correlated with methylation of CpG islands in the 5' region of Angptl4.17 Using corneal neovascularization and Miles permeability assays, Ito et al showed that VEGF-induced angiogenesis and vascular leakiness were significantly inhibited by recombinant Angptl4.16 Transgenic mice that express Angptl4 in the skin showed remarkable suppression of tumor growth within the dermal layer associ-
ated with significantly decreased numbers of invading blood vessels. A more recent study by Galaup et al. found that Angptl4 prevents tumor metastasis through inhibition of vascular permeability, tumor cell motility, and invasiveness.

In this study, we investigated the structural and cellular basis underlying the biological functions of Angptl4 in modulating angiogenesis. Using the Edman degradation-based amino acid sequencing method, we identified 2 endogenous proteolytic cleavage sites of Angptl4 within the linker region between its NH2-terminal coiled-coil domain and COOH-terminal fibrinogen-like domain. We found that the carboxyl terminus of Angptl4 was sufficient to suppress bFGF- and VEGF-induced endothelial cell proliferation, migration, tubule formation, and in vivo neovascularization in an N-glycosylation dependent manner. In addition, we elucidated the signaling pathways underlying the antiangiogenic activities of ANGPTL4.

Methods
For expanded methods and results, please see the supplemental information.

Affinity Purification of FLAG- and Myc-Tagged Proteins From HEK293 Cells
Cells were infected with recombinant adenoviruses encoding full-length Angptl4 or its various domains (50 pfu/cell) for 48 hours. The conditioned medium was collected, centrifuged to remove cell debris, and filtered through 0.22 μm filter. FLAG-tagged Angptl4 was purified using anti-FLAG M2 affinity gel as described previously. Myc-tagged NH2-terminus or COOH-terminus of Angptl4 was purified using anti-FLAG M2 affinity gel according to the manufacturer’s instruction (Sigma). Protein concentration was determined using the Bradford reagent (Pierce).

Identification of Protein Cleavage Sites by Amino Acid Sequencing
The purified protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane membrane, stained with Coomassie Brilliant Blue R-250, excised, and subjected to the Edman degradation amino acid sequencing analysis as we previously described.

Cell Proliferation, Migration and Tubule Formation Assay, and In Vivo Neovascularization Studies
The methods were described in details in the supplementary information.

Statistics
Data are expressed as means±SD. Statistical analysis was conducted with the Student t test. The values of P<0.05 were considered statistically significant.

Results
Angptl4 Is Cleaved at the Linker Region Between the Coiled-Coil Domain and Fibrinogen-Like Domain
Previous studies from others and our laboratory have shown that both human and mouse Angptl4 are present in plasma as the cleaved forms. To identify the precise cleavage site, we infected HEK293 cells with the recombinant adenoviruses encoding mouse full-length ANGPTL4. When Angptl4 was expressed in a serum-free condition, the majority of this protein detected in the extracellular medium was present as its full-length form with an apparent molecular weight of ≈65 kDa (Figure 1A). On the other hand, Angptl4 expressed in a serum-containing medium was cleaved into the truncated forms with the apparent mass of its carboxyl terminus at ≈47 kDa. The percentage of the cleaved form was progressively increased with the increases in serum concentrations. In the presence of 10% serum, the majority of Angptl4 in the extracellular media was cleaved, whereas its full-length form was virtually undetectable. In serum samples collected from C57 mice infused with recombinant adenoviruses encoding full-length Angptl4, the protein was also present exclusively as the cleaved forms (Figure 1B), suggesting that Angptl4 is cleaved both in vitro and in vivo in a serum-dependent manner. A similar serum-dependent cleavage pattern was also observed for human Angptl4 (data not shown).

Edman degradation-based amino acid sequencing analysis for the cleaved carboxyl terminus of mouse Angptl4 yielded a major NH2-terminal sequence of LPKMTQL and a minor NH2-terminal sequence of MTQLIGL, which corresponded to the amino acid residues from 169 to 175 and from 172 to 178 respectively, suggesting that this protein was cleaved at 2 close amino acid residues from 169 to 175 and from 172 to 178 (Figure 1C). Amino acid sequence analysis for the cleaved human Angptl4 also identified a major NH2-terminal sequence of LPMAQP and a minor NH2-terminal sequence of MAQPVD (Figure 1C), indicating that the cleavage of human Angptl4 occurs at the same sites with its mouse homolog. Similar with Angptl3, both cleavage sites of Angptl4 are located at the linker region between the coiled-coil domain and the fibrinogen-like domain.
C-Angptl4, but not N-Angptl4, Possesses the Antiangiogenic Properties

Several recent studies have demonstrated the role of Angptl4 as a novel modulator of angiogenesis, although the structural basis underlying its actions remains largely elusive. Therefore, we produced full-length NH2 terminus and COOH terminus of Angptl4 from HEK293 cells infected with recombinant adenoviruses encoding N-Angptl4 and C-Angptl4, respectively (Figure 2A), and evaluated their effects in HUVECs. Full-length Angptl4 (F-Angptl4) was purified from serum-free conditioned medium. 3H-thymidine incorporation analysis showed that both F-Angptl4 and C-Angptl4 inhibited bFGF as well as VEGF-stimulated DNA synthesis in HUVECs in a dose-dependent manner (Figure 2B and 2C). The inhibitory activities of F-Angptl4 and C-Angptl4 were comparable. On the other hand, N-Angptl4 had no effect on cell proliferation. We next performed a chemotaxis-type migration assay using a Boyden chamber to assess the effects of C-Angptl4 and N-Angptl4 on migration of HUVECs. This analysis showed that bFGF at 25 ng/mL significantly enhanced cell migration by 8-fold compared to those in untreated cells (Figure 3). C-Angptl4 but not N-Angptl4 inhibited bFGF-induced cell migration in a dose-dependent manner. Matrigel-based assay showed that bFGF-tubule formation was decreased by 42% and 74% after treatment with C-Angptl4 at the concentration of 1 µg/mL and 5 µg/mL, respectively (n=4 to 6, P<0.05). The inhibitory effects of C-Angptl4 on bFGF-induced migration and tubule formation were also observed in human microvascular endothelial cells (supplemental Figure I). Furthermore, C-Angptl4 inhibited VEGF-induced cell migration and tubule formation (data not shown).

To further confirm the antiangiogenic activity of Angptl4, we examined its effect on in vivo neovascularization using a Matrigel plug assay in C57 mice. 7 days after injection, Matrigel supplemented with bFGF was grossly red (supplemental Figure II), suggesting that it contained erythrocytes and hemoglobin. In contrast, Matrigel containing bFGF plus C-Angptl4 or F-Angptl4 was white, indicating the absence of erythrocytes and hemoglobin. In Matrigel supplemented with bFGF plus C-Angptl4 or F-Angptl4, the hemoglobin contents were significantly decreased compared to those treated with bFGF alone. Immunohistochemical staining also demonstrated that both F-Angptl4 and C-Angptl4 significantly decreased bFGF-induced new blood vessel formation. Direct visualization of vasculature by fluorescein isothiocyanate (FITC)-dextran showed a well-defined capillary network of blood vessels in plugs supplemented with bFGF alone, whereas this vasculature was disrupted after treatment with either F-Angptl4 or C-Angptl4 (supplemental Figure III). Quantitative analysis demonstrated a comparable activity of F-Angptl4 and C-Angptl4 in reducing vessel nodes, vessel ends, and vessel length induced by bFGF. On the other hand, N-Angptl4 had little effect. F-Angptl4 and C-Angptl4, but not N-Angptl4, attenuated VEGF-induced neovascularization in vivo (supplemental Figure IV). Furthermore, adenovirus-mediated overexpression of C-Angptl4, but not N-Angptl4 or...
luciferase control, markedly decreased the rates of mammary tumor growth and microvascular density in nude mice implanted with the estrogen receptor (ER) negative MDA-MB-231 cells (supplemental Figure V).

N-glycosylation of C-Angptl4 Contributes to Its Maximal Antiangiogenic Activities

Angptl4 has previously been shown to be posttranslationally modified by N-glycosylation. Indeed, the apparent molecular mass of C-Angptl4 was $\approx 47$ kDa, which is $\approx 18$ kDa larger than that predicted from its primary amino acid sequence (Figure 4A). Treatment of C-Angptl4 with PNGase-F (an N-glycosidase which cleaves all N-linked glycan chains), caused a significant reduction in its apparent molecular mass, whereas endoglycosidase-H (an enzyme that cleaves only high-mannose N-linked glycans) had no obvious effect. This data suggests that C-Angptl4 contains complex oligosaccharide structures. To investigate the potential role of N-glycosylation in modulating the antiangiogenic activities of C-Angptl4, we next produced the deglycosylated C-Angptl4 by digestion of the recombinant protein with PNGase-F, followed by affinity purification with sepharose beads coupled with anti Myc monoclonal antibody. Amino acid sequencing analysis demonstrated that C-Angptl4 had the same NH$_2$ terminus (MTQLIGL), suggesting that PNFase-F treatment did not cause the protein degradation. Compared to the glycosylated C-Angptl4, the ability of the deglycosylated C-Angptl4 to inhibit bFGF-induced cell proliferation was significantly decreased (Figure 4B). In addition, ablation of glycosylation attenuated the effects of C-Angptl4 in inhibiting bFGF-evoked cell migration and tubule formation in HUVECs (Figure 4C and 4D).

C-Angptl4 Attenuates bFGF-Induced Phosphorylation of ERK1/2 MAP Kinase, but not Akt and p38 MAP Kinase

Several key signaling cascades, including the ERK1/2 MAP kinase, Akt, and the P38 MAP kinase pathways, have been implicated in the modulation of both bFGF- and VEGF-induced angiogenic process. We next investigated whether or not the antiangiogenic effect of C-Angptl4 is mediated through inhibition of these signaling cascades. To this end, lysates from HUVECs under various treatments were probed with antiphosphospecific antibodies against the active forms of ERK1/2 (phospho-Thr202/Tyr204), Akt (phosphor-Ser473), and p38 (phosphor-Thr180/Tyr182). Treatment with bFGF induced a strong phosphorylation of the ERK1/2 MAP kinase in a time-dependent manner, with a maximal response being detected at 20 minutes (Figure 5). Cotreatment with C-Angptl4 markedly attenuated bFGF-evoked phosphorylation of ERK1/2. Quantitative analysis showed that bFGF-evoked ERK1/2 phosphorylation was reduced by $\approx 46\%$ in the presence of 1 $\mu$g/mL C-Angptl4 and almost completely...
abolished in the presence of 5 µg/mL C-Angptl4. Stimulation with bFGF also caused a time-dependent phosphorylation of Akt and P38 MAP kinase. However, these effects were not affected by C-Angptl4. C-Angptl4 also inhibited VEGF-induced phosphorylation of ERK1/2, but not VEGF-induced phosphorylation Akt and P38 MAP kinase (data not shown). Taken together, these results suggest that the antiangiogenic activities of C-Angptl4 might be attributed to its specific suppression of the ERK1/2 MAP kinase pathway.

C-Angptl4 Impedes bFGF-Induced Activation of Raf-1 and MEK1/2, but not Autophosphorylation of FGF Receptor-1 and Activation of Ras

The MAP kinase MEK1/2 is known to be the direct upstream kinase of the ERK1/2 MAP kinase.22 MEK1/2 is activated by its upstream kinase Raf-1 through phosphorylation at ser217/221. Raf-1 is a key relay point in the MAPK cascade, integrating positive and negative inputs from various upstream pathways.23 We next investigated the effects of C-Angptl4 on these signaling molecules upstream of the ERK1/2 MAP kinase (supplemental Figure VI). As expected, bFGF stimulated phosphorylation of Raf-1 and MEK1/2 at Ser217/221. Both effects were markedly attenuated by C-Angptl4. Treatment with bFGF led to a significant increase of activated, GTP-bound Ras, an upstream activator of Raf-1. However, C-Angptl4 had no effect on bFGF-evoked activation of Ras or autophosphorylation of FGFR1, suggesting that attenuation of Raf-1 activation is the earliest target of C-Angptl4 in blocking the activation of the ERK1/2 MAP kinase signaling pathway (supplemental Figure VI). Notably, C-Angptl4–mediated suppression on bFGF-induced DNA synthesis, cell migration, and tubule formation was significantly reversed after transfection with the plasmid pCMV-RafCAAX which expressed constitutively active Raf-1 (supplemental Figure VII), further confirming that C-Angptl4 inhibits angiogenesis through suppression of the Raf1/MEK/ERK1/2 signaling cascade.

Discussion

In addition to its role in regulating lipid metabolism and insulin sensitivity, growing evidence suggests that Angptl4 is an important modulator of angiogenesis and vascular permeability. Two earlier reports suggested Angptl4 as a proangiogenic factor induced during arthritis and ischemia.12,24 On the other hand, several recent independent studies demonstrated Angptl4 to be a potent inhibitor of angiogenesis and tumor metastasis in both cell culture system and animal models.13–16 In this study, we showed that Angptl4 inhibits both bFGF- and VEGF-induced cell proliferation, migration, and tubule formation in vitro, and neovascularization in vivo, thus supporting the role of Angptl4 as an angiogenic inhibitor. Although we cannot explain the discrepancy between these findings, it is highly possible that the function of Angptl4 in modulating angiogenesis differs in different tissue contexts. Indeed, Angiopoietin-2 and Angptl1, the close relatives of Angptl4, have been reported to possess both anti- and proangiogenic activities in various experimental conditions.25–28

The full-length Angptl4 undergoes a regulated proteolysis into 2 distinct truncated forms both in vitro and in vivo.6,9,19 In this study, we have identified a major cleavage site at Lys168 ↓ Leu169 and a minor cleavage site at Lys170 ↓ Met,171 both of which are located within the linker region between the coiled-coil domain and fibrinogen-like domain. We also observed a similar pattern of cleavage for human Angptl4. Notably, Angptl3, the closest member of Angptl4, is also cleaved within the linker region with a surrounding sequence context similar to Angptl4.20 The NH2-terminal coiled-coil domain of both Angptl3 and Angptl4 has been shown to be sufficient to induce hyperlipidemia by inhibiting the lipoprotein lipase activity.10,20 On the other hand, the results from this study demonstrated that the carboxyl fibrinogen-like domain of Angptl4, but not its NH2-terminal region, can act on endothelial cells to inhibit cell proliferation, migration, and tubule formation in vitro, and neovascularization in vivo. Notably, a more recent report demonstrated Angptl2 is also proteolytically cleaved during its secretion,29 suggesting that proteolysis represents an important mechanism that regulates the multiple biological functions of the Angptl family.

In this study, we observed the N-glycosylation of Angptl4 at its COOH-terminal domain. Inspection of amino acid sequence of Angptl4 using the NetGlyc program identified a single predicted N-glycosylation site at Asparagine-177, which is conserved across several species of Angptl4 identified so far. Although the precise role of the N-linked glycan chains remains to be determined, our data suggest that this N-glycosylation is critically important for the antiangiogenic activity of C-Angptl4. Notably, Angptl4 has recently been shown to interact strongly with extracellular matrix of endothelial cells in a heparin/heparan sulfate proteoglycan–dependent manner.13 It is possible that N-linked glycan chains are involved in the interaction of Angptl4 with the heparins of extracellular matrix or its unknown membrane receptors. It is also interesting to note that several endogenous inhibitors of angiogenesis, including endostatin,30 thrombospondins,31 and angiostatin,32 are also glycoproteins with the heparin-binding properties. Furthermore, a more recent study showed that posttranslational modifications, presumably glycosylation, are required for the ability of Angptl2 to stimulate expansion of hematopoietic stem cells.29 These findings highlight the importance of glycosylation in modulating the biological functions of the Angptl family.

Although several members of the Angptl family have recently been implicated in regulating angiogenesis,4 the underlying mechanisms remain largely elusive. In this study, we showed that C-Angptl4 selectively impedes the activation of the ERK1/2 MAPK pathway, which is a central player in mediating both VEGF- and bFGF-induced angiogenesis.21 Many endogenous and pharmacological inhibitors of angiogenesis, such as platelet factor 4,33 16-kDa human pro lactin,34 the extracellular adherence protein from Staphylococcus aureus,35 exert their functions by preferentially inhibiting the ERK1/2 MAPK signaling cascade. Within the ERK1/2 MAPK pathway, Raf-1 is an important relay point, integrating positive and negative inputs from upstream stimuli.23 Our results clearly suggest Raf-1 as the earliest target responsible for C-Angptl4–mediated inhibition of the ERK1/2 MAPK pathway. Whether or not C-Angptl4 acts through its specific receptor(s) to transduce the negative signal onto Raf-1 needs to be clarified in the future study.

In summary, our present study provides novel evidence demonstrating that the COOH-terminal fibrinogen-like domain
of Angpt4 alone is sufficient to inhibit angiogenesis possibly through impeding the ERK1/2 MAPK pathway at the level of Raf-1 inactivation. Further detailed elucidation of the receptor and postreceptor signaling events underlying the antiangiogenic properties could ultimately lead to the development of novel pharmacological agents for inhibiting pathological neovascularization as occurs in tumor growth and metastasis.

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Disclosures
None.

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Supplemental Methods, Figure legends and Figures

Supplemental methods

Reagents

Monoclonal antibodies against ERK1/2, phospho-ERK1/2 (Thr^{202}/Tyr^{204}), Akt, phospho-Akt (Ser^{473}), p38, phospho-p38 (Thr^{180}/Tyr^{182}), c-Raf, phospho-c-Raf, MEK1/2 and phospho-MEK1/2 (Ser^{217}/Ser^{221}) were purchased from Cell Signalling (Beverly, MA, USA). Recombinant human bFGF and VEGF were obtained from R&D Systems. [^{125}I]-bFGF and [^{125}I]-VEGF were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (Manassas, VA, USA).

Construction of adenoviral expression vectors and production of recombinant adenoviruses

The adenovirus expression vector encoding carboxyl-terminus FLAG-tagged full-length mouse Angptl4 was generated as previously reported 1, 2. The cDNA encoding mouse N-Angptl4 (amino acids residues 1-169) was amplified by PCR with the Myc epitope tagged at the carboxyl terminus and then cloned into pShuttle vector (BD Biosciences, NJ, USA). The cDNA encoding the mouse C-Angptl4 (amino acid residues 170-410) was PCR amplified and cloned into the pSecTag2-B vector (Invitrogen, Carlsbad, CA). The corresponding DNA fragment was excised from the vector by digesting with Nhel and NorI, with secretory signal peptide and Myc-tag added to the amino- and carboxyl-terminus respectively. The fragment was then subcloned into the pShuttle vector. The recombinant viruses packaged and amplified in human embryonic kidney (HEK) 293 cells were purified by an affinity purification column (BD Biosciences), and their infectivity was determined by plaque assay.

[^3H]Thymidine Incorporation assay
HUVEC were cultured in M199 medium (JRH Biosciences) containing 15% fetal bovine serum (FBS), 30\mu g/ml endothelial cell growth supplements (BD Biosciences), 2mM L-glutamine, and 100\mu g/ml heparin at 37°C. 3.0 \times 10^4 cells were seeded into each well of a 24-well plate. After 24 h, cells were washed twice with PBS, starved in M199 medium with 0.5% FBS for another 12 h. The cells were then incubated without or with various concentrations of Angptl4 or/and growth factors for 20 h. 0.5 \mu Ci of \[^{3}H\]thymidine was then added to each well for another 4 h, and the amount of \[^{3}H\]thymidine incorporated into DNA in each well was determined by liquid scintillation counting.

**Immunoprecipitation and Western Blot**

HUVEC under various treatments were dissolved in a lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10% glycerol, 25 mM NaF). 500 \mu g of cell lysate protein was incubated with 10 \mu g RBD agarose beads to precipitate GTP-bound, activated Ras according to the manufacturer’s instructions (Upstate Biotechnology). For analysis of bFGF receptor-1 (FGFR1) auto-phosphorylation, 400 \mu g of cell lysate protein was incubated with a mouse anti bFGFR-1 antibody (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. The complex was immunoprecipitated with 50 \mu l of protein A-Sepharose (1:1 slurry), and the bound material was washed four times with lysis buffer. Immunoprecipitated FGFR1 was suspended in SDS-PAGE loading buffer, boiled, and separated by 8% SDS-PAGE. The phosphorylated receptor was detected by immunoblotting with anti-phosphotyrosine antibody (pY-20, Santa Cruz). The membranes were stripped and probed with a rabbit anti FGFR1 antibody from Santa Cruz. The protein bands were visualized with the ECL-PLUS detection reagent (GE Healthcare).

**Cell migration assay**
The assay was performed with the Transwell polycarbonate membrane filters (8.0-µm pore size, Costar, Cambridge, MA). The lower surface of the membranes was pre-coated with 0.1% gelatin. The coated membranes were washed in PBS and dried overnight at room temperature. 600 µl of M199 containing 0.5% FBS with or without Angptl4 and/or bFGF was added in the lower wells. 3 x 10^4 HUVEC, which were serum-starved for 24 h and suspended in 100 µl of M199 medium, were inoculated onto the upper well. After 4 h incubation at 37°C, the membranes were fixed with 100% methanol and stained with hematoxylin. The cells on the upper surface of the membranes were removed with cotton swabs. Cells migrating to the lower surface of the membrane were counted in six independent fields under a microscope.

**Tubule Formation Assay**

Matrigel (BD Sciences) was pre-coated onto 96-well culture dishes at 80 µl/well and allowed to solidify for 30 min at 37°C. HUVEC (1 x 10^4) suspended in 100 µl of M199 containing 2% FBS and 25 ng/ml bFGF with or without various concentration of Angptl4 were seeded onto each Matrigel-coated well. After incubation for 24 h, endothelial tubule formation was photographed under a phase-contrast microscope. The degree of tube formation was quantified by counting the branching points in six randomly chosen fields from each well.

**In vivo Angiogenesis Assay using Matrigel**

All the experiments were conducted under the University of Hong Kong guidelines for the humane treatment of laboratory animals. 8-week-old C57BL male mice were injected subcutaneously with 0.4 ml of Matrigel containing 300 ng/ml bFGF, 100 ng/ml VEGF in the presence or absence of 50 µg/ml, F-Angptl4, C-Angptl4 or N-Angptl4. 7 days after injection,
mice were sacrificed and the Matrigel plugs were taken out, weighed and processed for histology or determination of hemoglobin concentration. For histologic analysis, plugs were fixed with 4% paraformaldehyde for 24 h, embedded in polyester wax, and sectioned at 8µm. The sections were deparaffinized, rehydrated and stained with anti-CD-31 (Pharmpingen, San Diego, CA). For hemoglobin determination, plugs were homogenized with 1 ml distilled water. Hemoglobin concentration was measured with the Drabkin reagent (Sigma).

To analyze the vessel architecture in more details, 100 µl of FITC-dextran (0.25 mg/ml, Sigma) was introduced into mice by intravenous injection. 40 min after injection, mice were sacrificed and the Matrigel plugs were carefully exercised and examined with a fluorescent microscope. The vasculatures were digitalized and analyzed with the NIH image software. The vessel length, vessel nodes and vessel ends were quantified as described previously 3.

**Analysis of angiogenesis associated with the mammary tumor formation in nude mice.** ER-negative/mesenchymal-like MDA-MB-231 cells (5 x 10⁶) were injected into the right thoracic mammary fat pad of 4- to 6-week-old female nude mice under anesthetic condition as we previously described 4. At 2 weeks after initial implantation, mice were administered with the recombinant adenovirus [2x10⁹ plaque-forming units (p.f.u)] that expressed C-Angptl4, N-Angptl4 and luciferase (as control) via tail-vein injection. Note that the amount of injected adenovirus caused no toxicity in the mice as judged by their body weight gains, food and water intakes, liver functions, as well as other behavioral variables, such as fear or aggression, diarrhea, respiratory and cardiovascular signs, etc. Tumors were measured using digital vernier calipers, with tumor volume calculated using the formula [sagittal dimension (mm) x cross dimension (mm)]² / 2 and expressed in cm³. All animals were sacrificed at 4 weeks after the initial
implantation, and the tumors were dissected out for immunocytochemical staining of blood vessels using anti-CD31 antibody as above.

**Supplemental figure legend**

**Supplemental Figure-I**: Both full-length and carboxyl terminus of Angptl4 inhibit migration (A) and tubule formation (B) in human microvascular endothelial cells (HMECs). $5 \times 10^4$ cells were seeded into the upper chamber of a Transwell for the cell migration assay, and the number of cells migrating from upper chamber to lower chamber was quantified as described in Figure 3. Cells grown in Martigel were treated with bFGF (25ng/ml) in absence or presence of various concentrations of Angptl4 or F-Angptl4. After 24 h, capillary tubules were visualized using an inverted microscope and the number of branching points (nodes) per field was quantified as in Figure 3. **, P<0.01 versus bFGF treated alone (n=7). Note that both F-Angptl4 and C-Angptl4 inhibit bFGF-induced proliferation of HMECs (data not shown).

**Supplemental Figure-II**. Both full-length and carboxyl terminus of Angptl4 suppresses bFGF-induced neovascularization in vivo. A: the gross appearances of Matrigels supplemented with bFGF alone or bFGF plus C-angptl4 or F-Angptl4 at day 10 after subcutaneous injection. B: the hemoglobin contents after normalized against the weight of the Matrigel plugs. C: the representative photograph of blood vessels in Matrgel plugs stained with the anti CD31 antibody (100×). D: quantitative analysis for the number of blood vessels. **, P<0.01 versus bFGF-treated alone (n=6-7).
**Supplemental Figure-III:** Inhibitory effect of F-Angptl4 and C-Angptl4 on bFGF-induced neovascularization as visualized by FITC-dextran. 10 days after injection with the Matrigel plugs, the vasculatures were visualized by FITC-dextran and photographed under a fluorescent microscope (upper panel). The vessel length, vessel ends, vessel nodes were quantified as described in the supplemental methods. **, p<0.01 versus bFGF alone (n=6-7).

**Supplemental Figure-IV:** Both full-length and carboxyl terminus of Angptl4 inhibit VEGF-induced neovascularization in vivo. A: the gross appearances of Matrigels supplemented with VEGF alone or VEGF plus C-Angptl4 or F-Angptl4 at day 10 after subcutaneous injection. B: the hemoglobin contents after normalized against the weight of the Matrigel plugs. C: quantitative analysis for the number of blood vessels as described in Figure 4 (n=6-7). ** p<0.01 versus VEGF-treated alone (n=4-6).

**Supplemental Figure-V:** C-Angptl4 but not N-Angptl4 inhibits the mammary tumor development and angiogenesis in nude mice. MDA-MB-231 cells were implanted into the mammary fad pad of nude mice as we previously described. At 2 weeks after initial implantation, mice were administered with the recombinant adenovirus [2x10⁹ plaque-forming units (pfu)] that expressed C-Angptl4, N-Angptl4 and luciferase (as control) via tail-vein injection. Tumor growth was monitored by measuring the visible tumor sizes at various time points (A). Mice were sacrificed after 4 weeks, and tumors were dissected for immunocytochemical staining of blood vessels using ant-CD31 monoclonal antibody as the primary antibody (B), and the numbers of blood vessels in each microscopic field (C) were quantified as in Figure 4. * p<0.05; ** p<0.01 versus control group (n=4-5).
**Supplemental Figure-VI:** C-Angptl4 suppresses bFGF-induced phosphorylation of Raf-1 and MEK1/2, but not activation of Ras and auto-phosphorylation of FGF receptor-1 (FGFR1).

Serum-starved HUVEC were treated without or with C-Angptl4 (5 µg/ml) for 30 min, followed by stimulation with bFGF for various periods. 40 µg of cell lysates were subjected to immunoblot analysis for phospho- and total MEK1/2, and phospho- and total Raf-1. GTP-bound, active Ras were precipitated with RBD conjugated agarose. Total and active Ras under different conditions was detected using their respective polyclonal antibodies. FGFR1 was immunoprecipitated with a mouse anti FGFR1 monoclonal antibody. Immunoprecipitates were resolved by 8% SDS-PAGE and analyzed for FGFR-1 autophosphorylation by immunoblotting with the pY-20 antibody. Total FGFR1 protein was detected by stripping the membrane and immunoblotting with a rabbit anti FGFR1 antibody. The right panel is the schematic illustration of the proposed model by which C-Angptl4 inhibits bFGF-induced activation of the ERK1/2 MAP kinase pathway.

**Supplemental Figure-VII:** The inhibitory effects of C-Angptl4 on bFGF-induced DNA synthesis, migration and tubule formation are attenuated by expressing constitutively active (CA) Raf-1 in HUVEC. Cells were transfected with the pCMV-RafCAAX plasmid (Clontech Laboratories, Inc) encoding CA Raf-1 (black bar) or pcDNA3.1 as control (white bar) using electroporation as we previously described. 24 hr after transfection, cells were treated with 5 µg/ml C-Angptl4. After another 24 hr, cells were stimulated with or without 25 ng/ml bFGF for 20 min, ERK1/2 activation was determined by analyzing the ratio of phosphorylated versus total protein level using a Bio-plex antibody suspension array (Bio-Rad) as we described elsewhere.
The effects of C-Angptl4 on bFGF-induced thymidine incorporation (B), cell migration (C) and tubule formation (D) were evaluated as in Figure 5. Panel E represents the percentage of inhibition on bFGF-induced thymidine incorporation, cell migration and tubule formation by 5 μg/ml C-Angptl4 in cells transfected with pCMV-RafCAAX (black bar) or pcDNA3.1 (white bar). * p<0.05, ** p<0.01 versus cells transfected with the control plasmid (n=4-6). Note that activation of the ERK1/2 pathway by expressing CA Raf-1 caused a modest elevation of DNA synthesis, but had no obvious effect on cell migration and tubule formation in the basal state without bFGF stimulation. On the other hand, the suppressive effects of C-Angptl4 on bFGF-induced DNA synthesis, cell migration and tubule formation were markedly attenuated by expression of CA Raf-1.

References


Yang Y-H and Wang Y et al, Supplemental Figure-I

**A**

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No. of migrated cells/field

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No. of branching points/field
Yang Y-H and Wang Y et al, Supplemental Figure-II

A

B

C

D

Hemoglobin (mg/dL)

No. of vessels/field
Yang Y-H and Wang Y et al, Supplemental Figure-III

![Images showing the effects of bFGF, bFGF+C-Angpt14, and bFGF+F-Angpt14 on vessel length, vessel ends, and branch points.](image)

- **Length:**
  - bFGF
  - bFGF + C-Angpt14: **
  - bFGF + F-Angpt14: **

- **Vessel ends:**
  - bFGF
  - bFGF + C-Angpt14: **
  - bFGF + F-Angpt14: **

- **Branch points:**
  - bFGF
  - bFGF + C-Angpt14: **
  - bFGF + F-Angpt14: **
Yang Y-H and Wang Y et al, Supplemental Figure-IV
Yang Y-H and Wang Y et al, Supplemental Figure-V

A

B

C

Yang Y-H and Wang Y et al, Supplemental Figure-V
Yang Y-H and Wang Y et al, Supplemental Figure-VI

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Diagram:
- bFGF → FGFR1
- C-Angptl4 → Receptor??
- Ras
- Raf-1
- MEK1/2
- ERK1/2