

## Research Article

**ALK1-Fc Inhibits Multiple Mediators of Angiogenesis and Suppresses Tumor Growth**

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**Abstract**

Activin receptor–like kinase-1 (ALK1) is a type I, endothelial cell–specific member of the transforming growth factor- $\beta$  superfamily of receptors known to play an essential role in modulating angiogenesis and vessel maintenance. In the present study, we sought to examine the angiogenic and tumorigenic effects mediated upon the inhibition of ALK1 signaling using a soluble chimeric protein (ALK1-Fc). Of 29 transforming growth factor- $\beta$ –related ligands screened by surface plasmon resonance, only bone morphogenetic protein (BMP9) and BMP10 displayed high-affinity binding to ALK1-Fc. In cell-based assays, ALK1-Fc inhibited BMP9-mediated Id-1 expression in human umbilical vein endothelial cells and inhibited cord formation by these cells on a Matrigel substrate. In a chick chorioallantoic membrane assay, ALK1-Fc reduced vascular endothelial growth factor–, fibroblast growth factor–, and BMP10-mediated vessel formation. The growth of B16 melanoma explants was also inhibited significantly by ALK1-Fc in this assay. Finally, ALK1-Fc treatment reduced tumor burden in mice receiving orthotopic grafts of MCF7 mammary adenocarcinoma cells. These data show the efficacy of chimeric ALK1-Fc proteins in mitigating vessel formation and support the view that ALK1-Fc is a powerful antiangiogenic agent capable of blocking vascularization. *Mol Cancer Ther*; 9(2); 379–88. ©2010 AACR.

**Introduction**

Activin receptor–like kinase-1 (ALK1) is a type I cell surface receptor with serine/threonine kinase activity that mediates signaling by members of the transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily (1–3). ALK1 is expressed predominantly in vascular endothelial cells and is implicated in blood vessel formation and organization by several lines of genetic evidence. Homozygous *ALK1*<sup>-/-</sup> mice are embryonic lethal due to blockade of capillary bed development, hyperdilation of large vessels, and formation of an avascular yolk sac (4, 5). Heterozygous loss-of-function mutations of ALK1 in humans cause an adult-onset vascular dysplasia known as hereditary hemorrhagic telangiectasia-2 (HHT2, also known as Rendu-Osler-Weber syndrome; refs. 4, 6–8). An autosomal dominant disorder, HHT2 is characterized by dilated, leaky capillaries that manifest in patients as mucocutaneous telangiectases and arteriovenous malformations in the brain, lungs, liver, and gastrointestinal tract. Mice

with heterozygous mutations of ALK1 exhibit a similar phenotype, with age-dependent vascular lesions in the skin, extremities, oral cavity, and internal organs (9). Interestingly, a second form of HHT known as HHT1 results from loss of endoglin, a coreceptor that modulates signaling by several members of the TGF receptor family, including ALK1 (7).

A postdevelopmental role for ALK1 also has been shown, for example, in wound healing and tumor growth (4). During wound healing, ALK1 expression is induced in pre-existing arteries supplying the wound area and in newly branching small arteries in contrast to minimal expression in the capillary-like vessels of the wound rim area. During wound closure, newly forming vessels converging at the wound center express ALK1, with ALK1 expression diminishing in the later stages of healing. During tumor angiogenesis, ALK1 expression is upregulated in the large arteries feeding the tumors. However, alternative roles of ALK1 also have been proposed, based on the inhibition of proliferation and migration of endothelial cells observed upon transfection with a constitutively active form of ALK1 (caALK1; ref. 10).

In spite of strong evidence implicating ALK1 as a participant in arterialization and vascular remodeling, the exact mechanism for this remains uncertain. It has been suggested that TGF $\beta$ 1 can regulate endothelial cells through both ALK1 and ALK5 receptor systems, which affect gene expression differentially through distinct SMAD protein complexes (incorporating SMAD 1/5/8 or SMAD 2/3, respectively; refs. 11, 12). According

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to this model, relative levels of activity in these two pathways (differential SMAD phosphorylation) would regulate changes in vessel activation and resolution. However, the inability to detect ALK5 expression in endothelial cells would argue against such a model and instead favor ALK1 as the critical type I receptor regulating SMAD phosphorylation in the endothelium (13). Conditional deletion of ALK1, ALK5, or TGF receptor type II in murine endothelium yields results consistent with this view (13).

Paradoxical findings with bone morphogenetic protein-9 (BMP9) contribute to the uncertainty about the role of ALK1 in angiogenesis. BMP9 is a high-affinity ligand of ALK1 in association with type II receptors such as bone morphogenetic protein receptor type II and activin receptor type IIA (14). The participation of BMP9 in angiogenesis was originally considered unlikely due to its low expression in vascular cells; however, recent data indicate that significant levels of biologically active BMP9 circulate in human plasma (15). This same study showed that BMP9 activation of ALK1 inhibits vessel sprouting *in vivo* in both chick chorioallantoic membrane (CAM) and mouse subcutaneous sponge angiogenesis assays (15). Moreover, BMP9 has been shown to inhibit the proliferation and migration of endothelial cells stimulated by basic fibroblast growth factor (FGF) and to block vascular endothelial growth factor (VEGF)-induced angiogenesis (16). Together, these findings present a discordant picture of BMP9-ALK1 biology because ALK1 has been heavily implicated in angiogenesis, whereas the ALK1 agonist BMP9 would seem to promote vascular quiescence. BMP10, the other high-affinity ligand, has been shown to stimulate ALK1-dependent signaling; however, its role *in vivo* remains unclear.

In the present study, we evaluated the binding of various ligands of the TGF $\beta$  family using a soluble chimeric protein (ALK1-Fc) containing either the human or the mouse ALK1 extracellular domain, which competitively binds ALK1 ligands. Although previous data show that an ALK1-Fc chimera can be used *in vitro* to inhibit BMP9-mediated alkaline phosphatase expression from C2C12 cells and to inhibit BMP9-mediated, ALK1-dependent expression of Id-1 in cultured endothelial cells (3, 14), we show for the first time the use of the ALK1-Fc chimera to mediate significant loss of vessel formation *in vivo* and correlate it to the suppression of Id-1 signaling by ALK1-Fc. These effects on Id-1 expression are of particular interest because this intracellular effector has been found to induce VEGF expression and repress the antiangiogenic factor, thrombospondin-1 (17). Additionally, Id-1 is found overexpressed in multiple forms of cancers where it upregulates angiogenesis (17, 18).

The majority of cancer treatments currently being used target proteins expressed in a wide range of tissue types, and thus can elicit a broad range of drug side effects in these tissues; as ALK1 is specifically expressed in activated endothelium, drugs such as ALK1-Fc that target ALK1 signaling have the potential to affect endothelium very

selectively. In addition, the following data support that ALK1-Fc is effective at suppressing angiogenic effects mediated by multiple proangiogenic factors (including FGF and VEGF), and thus, it may have a better likelihood of reducing pathologic angiogenesis than a drug that targets a single growth factor, such as Avastin. Whereas a soluble ALK1-Fc chimera has been shown to inhibit BMP9 signaling *in vitro*, its ability to function as a modulator/inhibitor of angiogenesis *in vivo* had not yet been investigated. Here, we examined the binding of TGF $\beta$  family members to ALK1-Fc with surface plasmon resonance and evaluated soluble ALK1-Fc as a regulator of angiogenesis in multiple models *in vivo* and *in vitro*.

## Materials and Methods

### Materials

Biacore 3000, Biacore T100, and CM5 chips and reagents were purchased from Biacore (GE Healthcare). BMP9, type I and type II receptors, ligands, VEGF (rhVEGF<sub>165</sub>), and hALK1 antibody (R&D Systems, AF770) were obtained from R&D Systems. Antibodies to Id-1 (sc488) and  $\beta$ -Actin (sc8432) were purchased from Santa Cruz.

### Construction of Expression Vectors

The mouse ALK1 extracellular domain was obtained by PCR from fl-cDNA (Invitrogen) and the primers used were KasI/mALK1/fwd 5'-TGACGGCGCC-GACTTGCGAAGGCCTTCC-3' and AgeI/mALK1/rev 5'-TCAGACCGGTAGGCAGATGGGCATCAAC-3'. The PCR fragment was digested with KasI and AgeI and subcloned into PAID-4 vector. Similarly, the extracellular domain of hALK1-Fc was PCR amplified and subcloned into a murine cytomegalovirus-derived PAID-4 vector. The vector also contains Fc fragments, IgG2A from mouse and IgG1 from human, which were obtained by PCR amplification and subcloned as an AgeI and EcoRI fragment. The sequences of both mouse and human ALK1, including the human tissue plasminogen activator (TPA) signal sequence, and the Fc were confirmed by double-stranded dideoxy sequencing by Sequagen.

### Protein Expression and Purification

Mouse ALK1-Fc and hALK1-Fc constructs were stably transfected into Chinese hamster ovary cells, and after two rounds of dilution cloning, the best expressing clones were adapted to serum-free IS CHO-CD medium (Irvine Scientific), supplemented with 4 mmol/L glutamine and 200 nmol/L methotrexate. For large-scale production of the ALK1-Fc proteins, cell cultures were scaled up to 100 liters in a Wave bioreactor system 200. After a 7- to 10-day growth period, conditioned medium was loaded onto a MabSelect SuRe (Protein A) column (GE Healthcare). The column was washed first with 10-column volumes of TBS (pH 8.0) followed by a 10-column volume

wash with 50 mmol/L Tris (pH 8.0). The Protein A column was eluted with 0.1 mol/L glycine (pH 3.0), and the protein was immediately neutralized with 1 mol/L Tris (pH 8.0). The protein was further purified by passing over a Q-Sepharose (Q) column. The purified proteins were then dialyzed into PBS (pH 7.2). The purified proteins were analyzed by SDS-PAGE and detected using SimplyBlue Safe Stain (Invitrogen). The purity of the protein preparations was >95%.

### Cell Culture, Transfection, and Reporter Assay

**BMP9 Signaling.** Human dermal microvascular endothelial cells (HMVEC-neonatal; Cambrex) were maintained in EGM-2MV media (Cambrex). One day before transfection, cells ( $1.5 \times 10^5$ ) were plated into six-well plates and were generally 80% to 90% confluent at the time of transfection. Cells were cotransfected with SBE4-luc and pRL-CMV (Promega; 5.5 and 0.55  $\mu$ g per well, respectively) using the GenePorter 2 system (Genlantis) for 3 h. Treatment with 50 ng/mL of BMP9 (R&D Systems) was done in Opti-MEM I (Invitrogen) for 16 h, followed by cell lysis using 1 $\times$  passive lysis buffer (Promega). The luciferase activity was determined using the Dual-Luciferase Assay system from Promega.

**Inhibition of BMP9 Signaling Using hALK1-Fc and Anti-hALK1 Antibody.** Cells were plated and transfected as previously described. For the hALK1-Fc inhibition, 10  $\mu$ g/mL of hALK1-Fc were preincubated with BMP9 (50 ng/mL) at 37°C for 45 min, and then the mixture was applied to the cells. For the anti-hALK1 antibody (R&D Systems) inhibition, cells were preincubated with 10  $\mu$ g/mL of the polyclonal antibody for 1 h followed by the addition of 50 ng/mL of BMP9. Treatment duration and luciferase activity determination were as described above. For Western blot analysis, whole-cell lysates were prepared in reducing Laemmli SDS-PAGE loading buffer and were analyzed by SDS-PAGE electrophoresis in 4% to 12% gradient gel (Invitrogen).

### Biacore Analysis

Surface plasmon resonance analysis was done using Biacore 3000 and Biacore T100 biosensors equipped with research-grade CM5 sensor chips.

**Direct binding mode.** Mouse ALK1-Fc, hALK1-Fc, BMP9, and BMP10 were immobilized at low densities on a CM5 chip using amine coupling chemistry. Binding data were collected in duplicate in 2-fold dilution series (from 0.78–200 nmol/L) in HBS-EP buffer with 0.5 mg/mL bovine serum albumin. Bound complexes were regenerated by two pulses of 100 mmol/L HCl.

**Captured "Sandwich" Mode.** Anti-human Fc antibody (Jackson ImmunoResearch) was immobilized on the CM5 chips via amine groups. Human ALK1-Fc or other type I receptors fused to human Fc were injected across the immobilized anti-human Fc antibody surfaces, resulting in ~100 response units of captured receptor. The receptor-antibody complex was stable over the time course of each ligand-binding cycle. Different TGF $\beta$  family ligands were

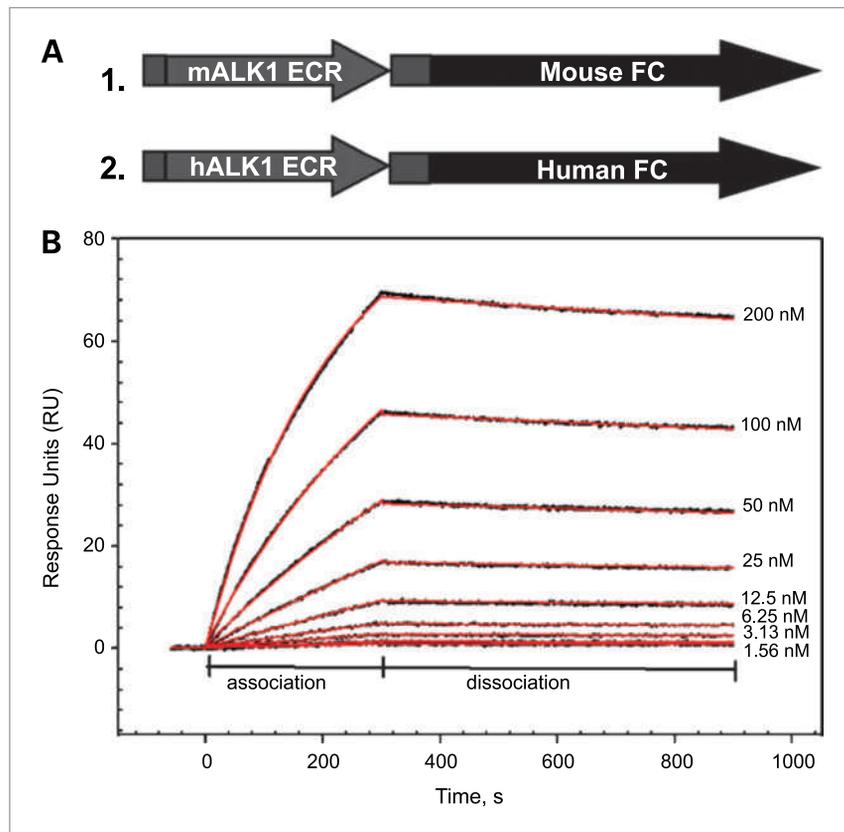
injected either at 10 or 100 nmol/L concentrations over captured hALK1-Fc receptor. The antibody surface was regenerated between binding cycles with injection of 10 mmol/L glycine (pH 1.7).

All sensograms were processed by double referencing. To obtain the kinetic rate constants, corrected data were fit using the BIAevaluation 4.1 software (Biacore AB). A kinetic analysis of each receptor/ligand interaction was obtained by fitting the response data to reversible 1:1 bimolecular interaction model. The  $K_D$  was determined by the quotient  $k_{off}/k_{on}$ . The goodness of fit for each was based on the agreement between experimental data and the calculated fits, where the  $\chi^2$  values were below 0.1.

### In vitro and In vivo Studies

**Tube formation Assay.** One-hundred microliters of Matrigel (BD Bioscience) were polymerized in the wells of a 96-well plate at 37°C for 1 h before seeding 15,000 HUVECs. Cells were left untreated, grown in the presence of 10, 50, or 100  $\mu$ g/mL mALK1-Fc and/or in the presence of endothelial cell growth substance (ECGS; 200 ng/mL) in EBM medium containing 0.1% fetal bovine serum. Cord formation was assessed after 12 h by phase-contrast microscopy. Cord formation data were quantified by counting the number of single-cell-width cords within a given field of observation that had at least three branches, which was then averaged for three fields per well from a duplicate set of samples for each experimental condition. These data were statistically compared using a Student's *t* test.

**Chick CAM Assay.** Nine-day-old fertilized chick embryos were maintained in a 48-place table top egg incubator at 37°C and specific humidity (60%). Prominent blood vessels were visualized through the eggshell with the aid of an egg lamp. The area of the outer eggshell, where the prominent blood vessels are located, was swabbed with 70% alcohol. A small hole was made, and the air sack was displaced causing a "blister" to form between the shell membrane and the CAM. Finally, a small window was cut through the eggshell with a hobby-grinding wheel (Dremel Emerson Electric Co.). Small filter discs infused with the angiogenic substance VEGF, FGF, or BMP10 (50 ng each) either in the presence or absence of hALK1-Fc compound (three doses of 5  $\mu$ g/dose), mALK1-Fc (three doses of 3  $\mu$ g/dose), or anti-VEGF monoclonal antibody (three doses of 3  $\mu$ g/dose) in PBS (pH 7.2) were then placed at the opening. Each group (6–9 eggs) were treated daily for 3 d and CAM were results analyzed on the 4th day. Quantification of the number of resulting vessels per disc posttreatment was made visually with the help of an egg lamp. For the melanoma explant CAM assay,  $5 \times 10^5$  tumorigenic cells (B16 melanoma) were added directly to the surface of the CAM. Treatments were then added directly upon the developing tumor [mALK1-Fc was added daily for 5 d; 1  $\mu$ g/d (5  $\mu$ g) or 10  $\mu$ g/d (50  $\mu$ g)] and the eggs were returned to the incubator. After the 7 d of incubation/exposure to the test articles, the CAM and tumor were carefully dissected



**Figure 1.** Generation and characterization of ligand binding of ALK1-Fc compounds. A, schematic representation of (1) mouse and (2) human ALK1 constructs bearing the extracellular region (ECR) of each receptor tethered by a linker domain to the Fc region of IgG2A and IgG1, respectively. B, representative data set (black lines) for kinetic analysis of BMP10 interactions with hALK1-Fc. BMP10 was immobilized on the chip and different concentrations of hALK1-Fc were injected over immobilized ligands. Red lines, the global fit of the data to a 1:1 bimolecular interaction model with mass transport. Ligand concentrations range from 0.78 to 200 nmol/L in 2-fold dilution increments.

and excised from the eggs ( $n = 9$ ). The tumor was weighed on a milligram scale balance. Tumor weights were compared with untreated and vehicle-treated controls.

**Implantation of MCF-7 Mammary Adenocarcinoma.** Six-week-old, female, athymic nude mice ( $n = 8$ ; 16 mice total) were treated with PBS-only or with ALK1-Fc (30 mg/kg) 2 wk before implantation of estradiol pellets (0.36 mg/pellet; Innovative Research of America) in the scruff of the neck, and then injections were made twice weekly until animals were euthanized. On the same day as the estradiol pellet implantation, MCF-7-luc-F5 ( $5 \times 10^6$ ) tumor cells were injected into the mammary fat pad of each animal. Body weights were measured weekly. Tumor burden was also measured each week as a factor of the luciferase activity (photons/second) using the IVIS Spectrum whole body scanner (Caliper Life Sciences). Animals were dosed twice weekly for 7 wk, after which time all animals were euthanized using  $\text{CO}_2$ . Tumors were later excised and weighed on a milligram scale balance.

## Results

**Expression and Purification of ALK1-Fc Chimeras.** The extracellular domain of murine ALK1 coupled to

the Fc-encoding region of the murine IgG2A (Fig. 1A, 1), and human ALK1 coupled to the human IgG1 Fc domain (Fig. 1A, 2) were expressed in Chinese hamster ovary cells. Gel electrophoresis showed the production of highly purified protein that resolve as single bands of ~54 and ~52 kDa, respectively, due to the slight difference in molecular weights of the mFc and hFc portions of the proteins.

**ALK1-Fc Binds BMP9 and BMP10 with High Affinity.** Using surface plasmon resonance, we have measured the binding affinities of mALK1-Fc and hALK1-Fc to various TGF $\beta$  family ligands. hALK1-Fc was captured by anti-hFc IgG on a Biacore chip and different TGF $\beta$  family ligands were injected at 100 nmol/L (unless noted otherwise) over the captured receptors (Supplementary Table S1A). None of the TGF $\beta$  subfamily members (TGF $\beta$ 1, TGF $\beta$ 2, and TGF $\beta$ 3) showed any binding to hALK1-Fc. The only ligands bound to hALK1-Fc were BMP9 and BMP10. To confirm that this effect was not assay specific, we also have immobilized TGF $\beta$ 1, TGF $\beta$ 2, TGF $\beta$ 3, BMP9, and BMP10 on a Biacore chip and injected hALK1-Fc and mALK1-Fc over the immobilized ligands. We have not observed any binding of the receptors to TGF $\beta$ 1, TGF $\beta$ 2, or TGF $\beta$ 3; however, we detected strong binding of the receptors to both BMP9 and BMP10. We also examined the binding capacity of BMP9 to other known type I receptors (Supplementary Table S1B) for which we

did not observe any additional binding between BMP9 and other type I receptors except ALK1. A representative sensogram of different concentrations of hALK1-Fc interaction with immobilized BMP10 is shown in Fig. 1B. Mouse ALK1-Fc and hALK1-Fc bound to human BMP9 with affinities of 2.3 and 2.01 nmol/L, respectively, suggesting that ALK1-Fc is a high-affinity receptor for BMP9 (Table 1). BMP10 bound to mALK1-Fc and hALK1-Fc with high affinities of 4.7 and 3.2 nmol/L, respectively (Table 1). Conversely, when hALK1-Fc and mALK1-Fc were immobilized and BMP9 was used as the solution component of the system, the affinity of mALK1-Fc and hALK1-Fc for BMP9 was 0.29 and 0.57 nmol/L, respectively, indicating that measured affinities for ligand are higher when the receptor component is immobilized on the chip. Although we tried to keep the density of the immobilized receptors very low, we cannot rule out the possibility that two immobilized molecules of the receptor may bind to a single ligand, thus increasing the apparent affinity through a proximity effect. This effect has been noted previously concerning other TGF $\beta$  family receptors before (19, 20).

**ALK1-Fc Blocks BMP9 Signaling and Reduces Id-1 Expression.** Using a firefly luciferase reporter gene construct under the control of four sequential consensus SMAD-binding element sites, we monitored the effect of hALK1-Fc on BMP9-mediated signaling in HMVEC. Upon stimulation with rhBMP9 (50 ng/mL), there was a 3-fold increase of luciferase activity over the background, and this activity was significantly reduced by ~60% in the presence of 10  $\mu$ g/mL hALK1-Fc (Fig. 2A). A polyclonal antibody that binds to the extracellular domain of the hALK1 receptor (R&D Systems) also inhibited BMP9 signaling (Fig. 2A). Additionally, Id-1 protein expression was significantly increased upon the stimulation of human umbilical vein endothelial cells (HUVEC) with BMP9 (50 ng/mL; Fig. 2B, lane 1). The

addition of hALK1-Fc (5  $\mu$ g/mL) was shown to reduce BMP9-induced Id-1 expression (Fig. 2B, lane 2).

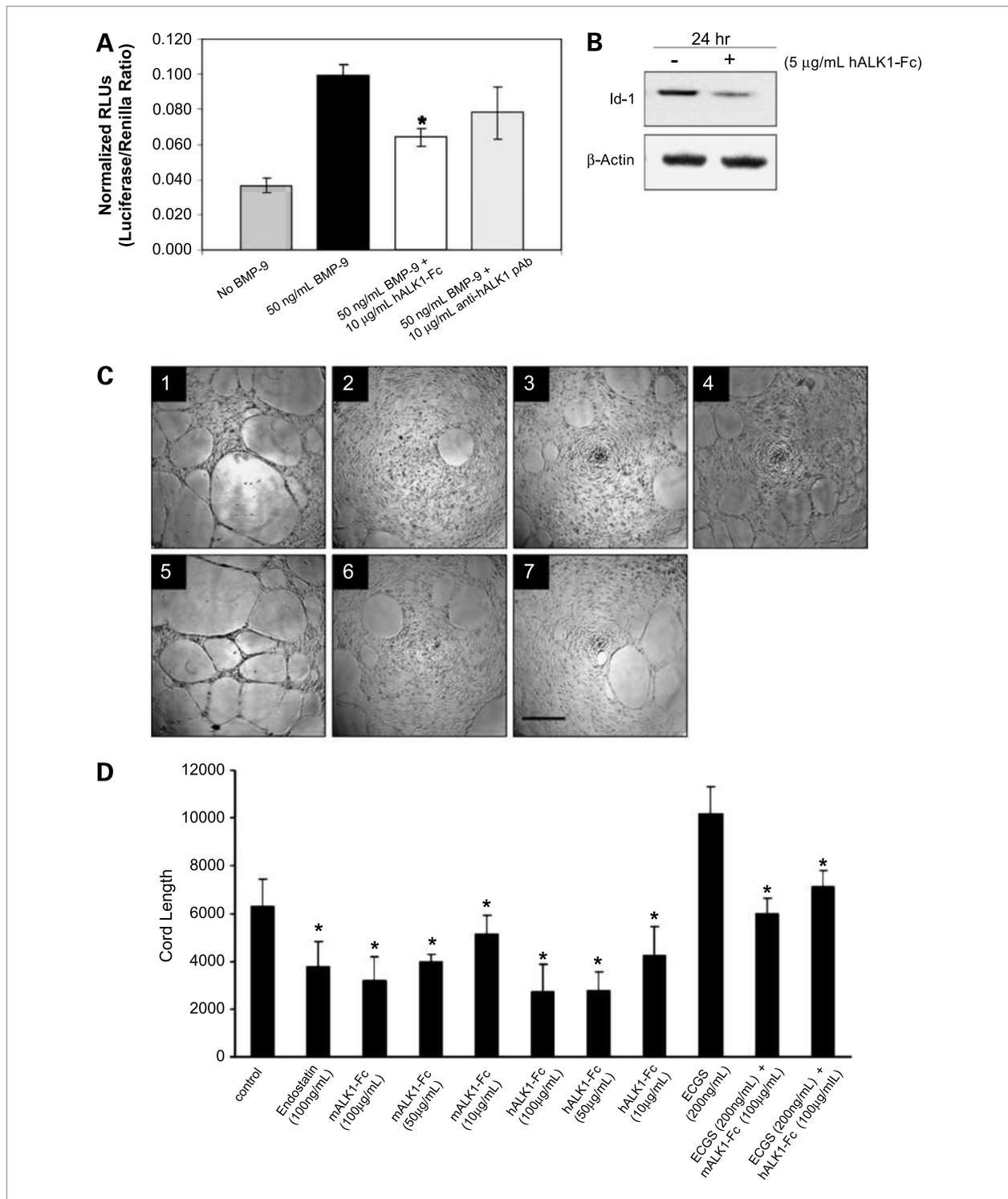
**Endothelial Cells Show Reduced Cord Formation in the Presence of ALK1-Fc.** To evaluate the angiogenic effect of ALK1-Fc in an *in vitro* model system, the effect of ALK1-Fc treatment on endothelial cell tube formation on a Matrigel substrate was determined. ECGS was used to induce the formation of microvessels from endothelial cells on Matrigel. ECGS induced significant cord formation (Fig. 2C, 5), compared with the no treatment control (Fig. 2C, 1). Upon addition of hALK1-Fc or mALK1-Fc (100  $\mu$ g/mL; Fig. 2C, 2 and 3, respectively), cord formation was markedly reduced. Endostatin, a broad-spectrum inhibitor of angiogenesis, was used as a positive control (Fig. 2C, 4). hALK1-Fc and mALK1-Fc maintained strong inhibition of cord formation in the presence of the strongly proangiogenic ECGS (Fig. 2C, 6 and 7, respectively). Quantification of vessel length in all samples revealed that all concentrations of hALK1-Fc and mALK1-Fc reduced neovascularization to basal levels (Fig. 2D).

**ALK1-Fc Inhibits Angiogenesis in the Chick CAM Assay.** The ability of mouse and human ALK1-Fc to affect vasculature was analyzed by the *in vivo* chick CAM assay. Individual filter discs were treated with known angiogenic agents, either in the presence or absence of ALK1-Fc or an anti-VEGF monoclonal antibody. Quantification of the number of resulting vessels per disc post-treatment (Fig. 3A) indicates a dose-dependent reduction in vessel formation, although the magnitude of inhibition observed at low concentrations of mALK1-Fc (3  $\mu$ g) was variable (Fig. 3A and C). Anti-VEGF antibody was used as a negative control. Additional CAM assays revealed that hALK1-Fc is effective against FGF-induced neovascularization (~50% reduction) as well as in reducing VEGF-induced vessel formation by 90% (Fig. 3B). ALK1-Fc was also shown to be effective in significantly

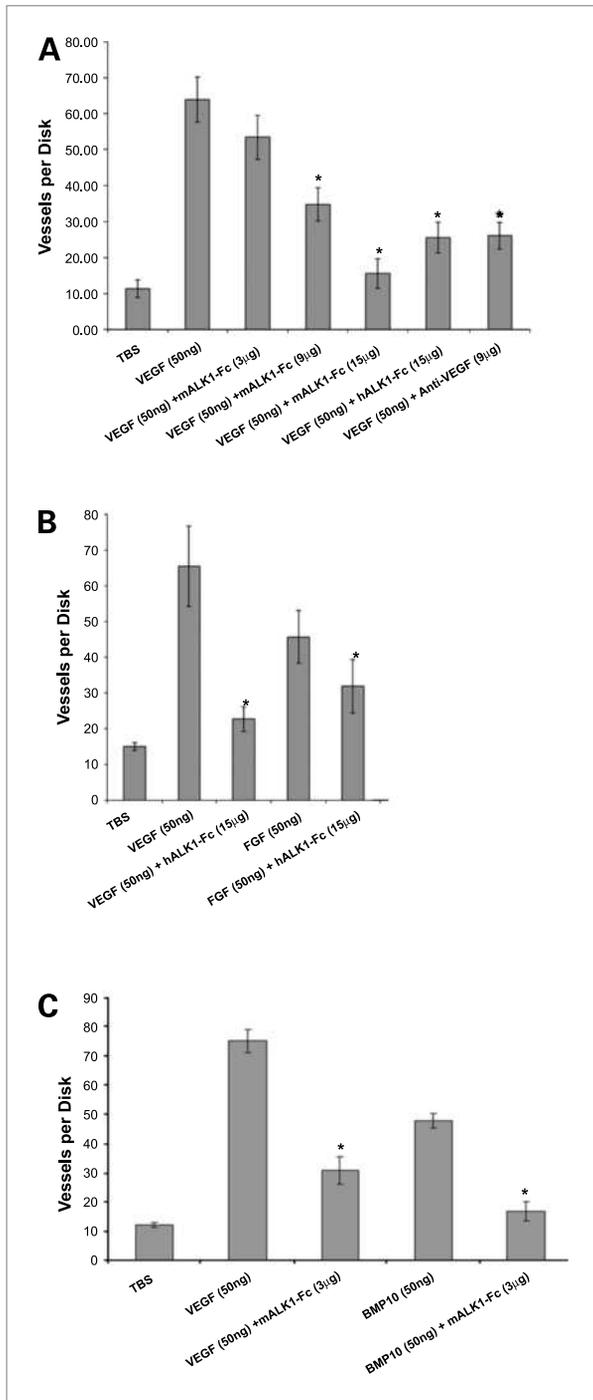
**Table 1.** Kinetic parameters derived from Biacore analysis of interactions between BMP9, BMP10, and hALK1-Fc and mALK1-Fc

Protein	Kinetic analysis of receptor-ligand interactions studied by Biacore					
	hBMP9 immobilized on the chip			hBMP10 immobilized on the chip		
	$k_{on}$ (1/Ms)	$k_{off}$ (1/s)	$K_D$ (nmol/L)	$k_{on}$ (1/Ms)	$k_{off}$ (1/s)	$K_D$ (nmol/L)
hALK1-Fc	$4.0 \times 10^4$	$7.8 \times 10^{-5}$	2.0	$2.6 \times 10^4$	$8.5 \times 10^{-5}$	3.2
mALK1-Fc	$3.5 \times 10^4$	$9.4 \times 10^{-5}$	2.3	$2.3 \times 10^4$	$1.1 \times 10^{-4}$	4.7
Protein	hALK1-Fc immobilized on the chip			mALK1-Fc immobilized on the chip		
BMP9	$5.0 \times 10^5$	$2.97 \times 10^{-4}$	0.57	$6.12 \times 10^5$	$1.75 \times 10^{-4}$	0.29

NOTE: The experiment was done on Biacore 3000 and data were processed with BIAevaluation software version 4.1. Ligands (BMP9 or BMP10) or receptors (hALK1-Fc or mALK1-Fc) were immobilized at low density by primary amine coupling on the CM5 chip. Different concentrations of analytes were injected at 30  $\mu$ L/min over immobilized components. The best fit was obtained by using 1:1 binding model. The  $K_D$  is calculated as  $k_{off}/k_{on}$ .



**Figure 2.** Characterization of mALK1-Fc on endothelial cells *in vitro*. BMP9 (50 ng/mL) stimulation of HMVEC cells. A, transactivation of SMAD-binding element-driven luciferase promoter in the presence and absence of hALK1-Fc (10 µg/mL) or anti-hALK1 polyclonal antibody (10 µg/mL) as determined by luciferase expression. B, Id-1 expression in BMP9 (50 ng/mL) stimulated HUVEC after 24 h in the presence or absence of human ALK1-Fc as determined by SDS-PAGE Western blot. C, HUVEC cells seeded on Matrigel substrate were assayed over 12 h for cord formation (7) in absence of any treatment, (2) in presence of 100 µg/mL hALK1-Fc, (3) in presence of 100 µg/mL mALK1-Fc, (4) in presence of 100 ng/mL endostatin, (5) in presence of 200 ng/mL ECGS, (6) in presence of 100 µg/mL hALK1-Fc + 200 ng/mL ECGS, or (7) in presence of 100 µg/mL mALK1-Fc + 200 ng/mL ECGS. D, cord quantification of single-cell width vessels was done using the IPLab software (BD Biosciences; \*,  $P < 0.05$ ). Scale bar, 100 µm length.



**Figure 3.** Chick CAM assay. A, quantification of vessels per disc resulting after a 14-d CAM treatment in the presence of VEGF (50 ng) and varying concentrations of mouse and human ALK1-Fc compounds in PBS. Positive (VEGF only) and negative controls (PBS only) were also assayed, along with antiangiogenic Avastin (anti-VEGF; 9 µg) treatment (\*,  $P < 0.05$ ). B, VEGF- and FGF-induced vascularization was also mitigated upon treatment with hALK1-Fc as described in Materials and Methods (\*,  $P < 0.05$ ). C, BMP10-induced vascularization could also be reduced upon treatment with mALK1-Fc (\*,  $P < 0.05$ ). Concentrations of VEGF, FGF, and BMP10 used in CAMs were 50 ng per egg.

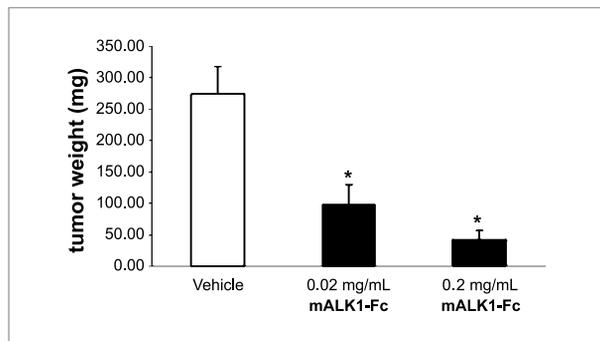
reducing BMP10-induced vessel formation in CAM (>50% reduction).

To test whether ALK1 antagonism could limit tumor growth, we monitored the ability of mALK1-Fc to affect the growth of B16 melanoma. The CAM was treated daily with mALK1-Fc (Fig. 4). B16 tumors in CAMs treated with mALK1-Fc showed a significant decrease in size compared with untreated B16 tumors. Quantification of tumor weight demonstrated that the weight of tumors treated daily with 0.2 mg/mL mALK1-Fc showed a reduction of 85% compared with the untreated CAMs (Fig. 4).

**ALK1-Fc Inhibits Vascularity and Growth of Breast Cancer in Orthotopic Tumor Model.** Mice were treated with mALK1-Fc or PBS for 2 weeks before estradiol pellet implantation and orthotopic injection of a bioluminescent, metastatic breast cancer cell line MCF-7-luc-F5. The luminescent tumors could be measured in real time using a Xenogen Whole Body Scanner over the course of the study. Seven weeks following MCF-7 cell injection, ALK1-Fc-treated animals displayed an ~70% reduction in tumor burden compared with PBS-only-treated animals (Fig. 5A). Additionally, tumors excised from ALK1-Fc-treated mice showed significant decrease of tumor burden on days 28, 35, and 42 as well as an ~75% decrease in final tumor weight compared with mice treated with PBS alone (Fig. 5B). Using real-time PCR, BMP9 was found to be expressed in these tumors and expression was independent of ALK1-Fc treatment (data not shown).

## Discussion

Although ALK1 has been proposed to be the critical type 1 receptor regulating signaling through the SMAD phosphorylation pathway in the endothelium, the scientific literature about the ligands, mechanism of action, and cellular function of ALK1 in endothelial cells contains contrasting findings (10, 11, 13–15, 21). As ALK1 variously has been reported to be a receptor for TGF $\beta$ 1, TGF $\beta$ 2, TGF $\beta$ 3, BMP9, and BMP10, we sought to verify these interactions using Biacore surface plasmon resonance technology and to analyze the kinetics of ALK1-Fc binding to each of the 29 commercially available ligands belonging to the TGF $\beta$  superfamily to better understand which ligands potentially play a role in arterIALIZATION and vascular remodeling under the regulation of ALK1. We found that ALK1-Fc bound to BMP9 with high affinity ( $K_D = 2.3$  nmol/L) as well as to BMP10 (4.7 nmol/L), which is in line with previous findings of Brown et al. (14). We were surprised that ALK1-Fc failed to bind to TGF $\beta$ 1, TGF $\beta$ 2, or TGF $\beta$ 3 using the Biacore technique, although numerous publications have reported signaling by TGF $\beta$  through ALK-1 *in vitro* (11). Additionally, we analyzed the binding of BMP9 to other type I receptors to learn what role the other receptors potentially play in the endothelial processes under investigation (Supplementary Table S1B). Other



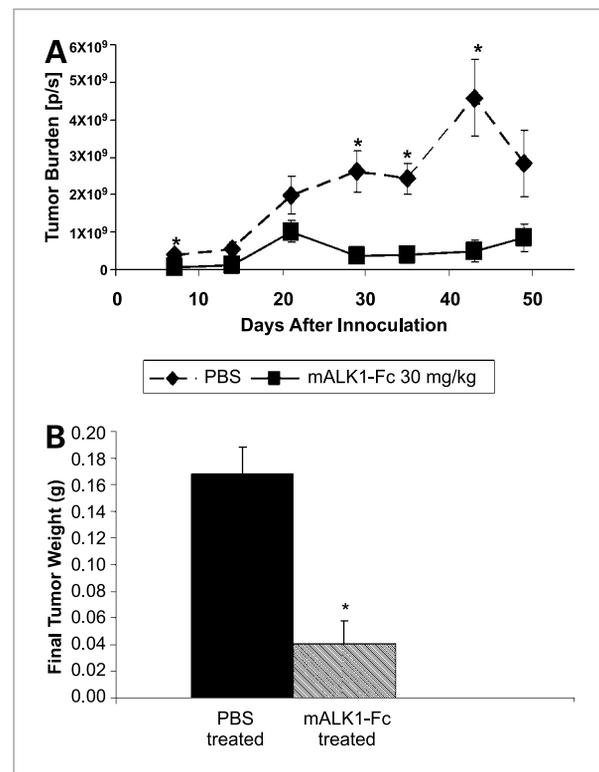
**Figure 4.** Tumor explant CAM assay. B16 melanoma cells ( $5 \times 10^5$  cells) were added to the surface of the CAM. Treatments were added directly to the developing tumor for 1 wk after which tumors were excised from eggs and quantification of B16 melanoma tumor weight was made (\*,  $P < 0.05$ ).

than ALK1, no other type I receptor bound to BMP9. Our results identify BMP9 and BMP10 as the only high-affinity ligands to ALK1, as suggested by David et al. (15). As native TGF $\beta$  signaling requires type I receptors as well as type II receptors, and often involves additional coreceptor involvement, it is possible that the discrepancy seen between Biacore and *in vitro* binding assays reflects signal modulation by factors other than ALK1 alone. Therefore, the effects obtained with soluble ALK1-Fc in cellular assays and in *in vivo* studies would seem to reflect the inhibition of BMP9 or BMP10 and not TGF $\beta$ 1, TGF $\beta$ 2, or TGF $\beta$ 3.

The efficacy of ALK1-Fc to block downstream signaling of ligand binding in endothelium was shown in HMVEC cells in which ALK1-Fc inhibited the BMP9-mediated transactivation of the SMAD-responsive promoter regulated by receptors such as ALK1 (Fig. 2A). These data agree with previously published data showing that both caALK1 expression in COS cells and BMP9 induction of endothelial cells lead to increased pSMAD 1/5 signaling (5, 16). It also has been shown that the overexpression of SMAD 5 in endothelial cells mimics the cellular effects modulated by caALK1 alone (11). Taken together, these data suggest that ALK1-Fc effectively inhibits phosphorylation of SMAD 1/5 proteins, the activation of which has been associated with heightened migration, proliferation, and invasion (11). Interestingly, the deletion of SMAD4, which binds to and conveys signal transduction of SMAD 1 and SMAD 5 to the nucleus, leads to a phenotype similar to that seen in HHT patients (22), which underscores the crucial role of SMAD phosphorylation in modulating the development of functional vessels. Additionally, as shown in Fig. 2A, a polyclonal antibody to the extracellular domain of the human ALK1 receptor also inhibited BMP9 signaling, suggesting that ALK1-Fc is a potent and target-specific inhibitor of the ALK1 signaling pathway in endothelial cells.

Although definitively distinguishing the role of ALK1, e.g., in vasculogenesis versus angiogenesis, or in the activation phase versus resolution phase of angiogenesis,

clearly is outside the scope of this investigation, we provide novel data showing the efficacy of a chimeric protein bearing the extracellular domain of ALK1 in reducing vessel formation and limiting tumor volume in *ex vivo* and *in vivo* models. ALK1-Fc markedly reduced cord formation of endothelial cells in a dose-dependent manner even in the presence of ECGS, a strong cord-inducing agent. This suggests that ALK1-Fc downregulates processes downstream of the action of various proangiogenic factors contained in ECGS, including but not limited to FGF. This was further confirmed in the chick CAM assay where ALK1-Fc reduced membrane neovascularization in a dose-dependent manner in the presence of highly proangiogenic factors, VEGF, FGF, and BMP10. Because ALK1 shows no binding affinity for VEGF or FGF (data not shown), the absence of vessels in these assays may result from the ability of the drug to destabilize processes downstream of growth factor activation, rather than from the ability of the drug to directly block neovascularization induced by these growth factors. Although BMP10 previously has been shown to bind the native ALK1 receptor with high affinity, using the CAM assay, we show for the first time that BMP10 is proangiogenic and that soluble ALK1-Fc can reduce BMP10-driven vascularization (3).



**Figure 5.** Orthotopic MCF-7 tumor assay. A, tumor burden as a measure of luciferase activity in ALK1-Fc-treated (solid line) and untreated (dotted line) animals over time (\*,  $P < 0.01$ ). B, quantification of final tumor weight measured upon excision of tumors (\* $P < 0.05$ ).

The antiangiogenic activity of ALK1-Fc was further manifested as antitumor activity in the CAM assay, in which metastatic B16 melanoma cells were implanted under the surface of the egg. Moreover, ALK1-Fc was highly effective in reducing tumor burden in a mouse model incorporating orthotopic injection of cells from the metastatic breast cancer line MCF-7-luc-F5. Taken together, our findings indicate that the ALK1-Fc chimera is a potent antagonist to vascular growth and cancer development *in vitro* and *in vivo*.

Reduction of Id-1 in endothelial cells upon treatment with ALK1-Fc has been documented by this and several other studies (12, 23). Id-1 is a dominant-negative helix-loop-helix protein capable of forming heterodimers with members of the basic helix-loop-helix family of transcription factors, but is incapable of binding DNA itself, thus rendering the entire multimer inactive (17). Id-1 is widely regarded as a cancer biomarker associated with poor patient prognosis. Id-1 is commonly found overexpressed in multiple forms of cancers including cancer of the breast, lung, prostate, bladder, and colorectal cancer (24–28). Specifically, Id-1 has been found to induce VEGF expression and upregulate angiogenesis in prostate cancer cells, and has been shown to repress the antiangiogenic factor, thrombospondin-1 (17, 18). Both BMP6 and BMP7 previously have been shown to induce Id-1 expression in bovine aortic endothelial cells and induce endothelial cell migration and tube formation (29). Indeed, adenoviral overexpression of Id-1 alone induces endothelial cell migration and tube formation in endothelial cells (29). Similar to the effects seen in our studies involving the addition of ALK1-Fc to vessels in culture and *in vivo*, the inhibition of Id-1 with antisense oligonucleotides blocks endothelial cell migration, further supporting the role of Id-1 as a major mediator of vascular growth and the importance of reducing Id-1 to limit such activity (29).

The expression of caALK1 in endothelial cells previously has been shown to enhance proliferation and increase migration (11). When adenovirally infected with caALK1, both mouse embryonic and bovine aortic endothelial cell types show enhanced migratory properties in Transwell and wound-healing assays, respectively (11). Additionally, adenovirally transfected endothelial cells showed increased proliferative capacity (11). These data support our findings that show that inhibition of ALK1 signaling by ALK1-Fc results in a loss of vessel formation. Interestingly, conflicting results have been published by Lamouille et al. (10), which show that caALK1 expression leads to effects opposite of those seen by Goumans et al. (11). According to Lamouille et al. (10), when adenovirally transfected with caALK1, HMVEC cells show a loss of migration, proliferation, and cell adhesion. It is possible that this discrepancy in ALK1 signaling can be attributed to differences in cell lines used (10, 11).

Inhibition of ALK1-mediated signaling can affect cell adhesion, migration, and *trans*-endothelial migration, steps essential for angiogenesis and blood vessel stabiliza-

tion. Recently, Fernandez-Lopez et al. (30) analyzed the differential expression of genes from endothelial cells derived from HHT1 and HHT2 patients. They showed that genes involved in adhesion and transmigration of cells such as VE-cadherin-2, PECAM-a, and eNOS were downregulated in both disorders. Interestingly, they also showed that ALK1 and endoglin were both downregulated in HHT1 and HHT2 subjects, thereby suggesting that the expression of ALK-1 and endoglin are coregulated. *PAI-1* gene is observed to be upregulated in HHT-1 and HHT-2 patients (31). Angiopoietin-2 and *fyn*, a src family tyrosine kinase, were shown to be downregulated in both HHT1 and HHT2. During cell migration and capillary tube formation, *fyn* is induced by angiopoietin-2 (32). These findings indicate that ALK-1 can regulate genes important in angiogenesis, endothelial cell migration, and capillary formation.

Pathologies stemming from aberrant angiogenesis include cancer, macular degeneration, and hemangiomas. Due to the high incidence of such diseases, there is strong interest in finding new means of treatment. One of the most common therapeutics currently used for treating pathologic neovascularization is Avastin. Although it has proven to be a highly effective means for treating pathologic angiogenesis, Avastin is limited by the fact that it targets VEGF-A and is thus directed against VEGF-induced neovascularization rather than destabilization of existing tumor vasculature. Like Avastin, most drugs currently used to treat pathogenic angiogenesis target proteins expressed in, and acting on, multiple tissue types, resulting in wide-ranging drug interactions and an increased possibility of adverse side effects. In contrast, the expression of ALK1 is restricted predominantly to activated endothelial cells, and thus drugs targeting ALK1 have the potential to affect endothelium very selectively. Results of *in vitro* and *in vivo* assays presented here show that the ALK1-Fc chimera acts as a potent antiangiogenic compound, capable of reducing neovascularization and undermining the integrity of newly formed capillaries. In summary, our studies show that ALK1-Fc chimera offers potential benefits as a treatment option for pathologic vascularization.

#### Disclosure of Potential Conflicts of Interest

D. Mitchell, E.G. Pobre, A.W. Mulivor, A.V. Grinberg, R. Castonguay, T.E. Monnell, N. Solban, J. Ucran, R.S. Pearsall, K.W. Underwood, J. Seehra, and R. Kumar are full-time employees of Acceleron Pharma and have ownership interest in the company.

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# Molecular Cancer Therapeutics

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