

Report

Dual targeting of vascular endothelial growth factor and bone morphogenetic protein-9/10 impairs tumor growth through inhibition of angiogenesis

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Tumor vessels provide oxygen and nutrients to cancer cells, and become main routes for cancer cells to metastasize to distant organs.⁽¹⁾ Therefore, it is crucial to develop effective strategies to target angiogenic signals in order to inhibit tumor growth and metastasis. During the formation of blood vessels, vascular endothelial growth factor (VEGF) plays central roles in promoting the proliferation and migration of blood vascular endothelial cells through the activation of VEGF receptor 2 (VEGFR2).⁽²⁾ Because of their critical roles in tumor angiogenesis, components of VEGF signaling axis have been attractive antitumor drug targets. Several strategies have been developed to target VEGF signals, including blocking antibodies against VEGF (bevacizumab), kinase inhibitors for VEGFR2, and decoy receptors. Decoy receptor for VEGF, called VEGF-trap, is a chimeric protein consisting of the

Clinical development of anti-angiogenic agents has been a major landmark in cancer therapy for several types of cancers. Signals mediated by both vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP)-9 and 10 have been implicated in tumor angiogenesis. However, previous studies have shown that targeting the individual signals was not sufficiently effective in retarding tumor growth in certain preclinical and clinical conditions. In the present study, we developed a novel decoy chimeric receptor that traps both VEGF and BMP-9/10. Single targeting of either VEGF or BMP-9/10 signals significantly reduced the formation of tumor vessels in a mouse xenograft model of human pancreatic cancer; however, it did not show significant therapeutic effects on tumor growth. In contrast, dual targeting of the angiogenic signals resulted in more significant inhibition of tumor angiogenesis, leading to delay of tumor growth. Our findings suggest that simultaneous blockade of VEGF and BMP-9/10 signals is a promising therapeutic strategy for the cancers that are resistant to anti-VEGF and BMP-9/10 therapies.

second Ig-like domain of VEGFR1 (Fms-related tyrosine kinase 1; FLT1), the third Ig-like domain of VEGFR2, and the Fc portion of human IgG. VEGF-trap sequesters VEGF proteins in cancer microenvironment, leading to regression of tumor vessels.

Such inhibitors of VEGF signals have been clinically used for cancer therapies. Therapeutic effects of bevacizumab have been observed when bevacizumab was used in combination with other anticancer drugs.⁽²⁾ However, in some cases, it does not elongate overall survival despite combining with other chemotherapeutic regimens.⁽³⁾ Moreover, increasing lines of evidence have shown that anti-angiogenic treatments result in the appearance of more aggressive and invasive tumors.⁽⁴⁾ Recent reports provide several mechanisms underlying the development of tumor resistance against anti-angiogenic

therapies. Growth of tumor vessels depends not only on VEGF but also on various types of other angiogenic factors. Therefore, inhibition of only VEGF-related signals becomes compensated by other angiogenic factors, implying the importance of combinatorial treatments that target multiple angiogenic pathways. Koh and colleagues reported that dual targeting of VEGF and angiopoietins using double anti-angiogenic protein consisting of extracellular domains of FLT1 and Tie2, a receptor for angiopoietins, inhibited tumor angiogenesis and metastasis more effectively than single targeting of VEGF or angiopoietins in a xenograft model of human ovarian carcinoma.⁽⁵⁾ However, identification of new targets and development of more effective methods to inhibit tumor angiogenesis is still highly required.

Multiple lines of evidence have suggested that members of the bone morphogenetic protein (BMP) family, particularly BMP-9 and -10, play important roles in the development and maintenance of vascular systems.⁽⁶⁾ BMP-9 and -10 transduce their signals through heteromeric complexes of serine/threonine kinase receptors known as type I and type II receptors. In contrast to other members of the BMP family, BMP-9 and -10 preferentially bind to the type I receptor activin receptor-like kinase 1 (ALK1). ALK1 is preferentially expressed in endothelial cells, and high level of expression has been also found at the site of angiogenesis during embryogenesis. Bone morphogenetic protein-9 and -10 are produced in liver and heart, respectively, and are found in the peripheral blood circulatory system.⁽⁷⁾ It is of note that BMP-9 expression is elevated during the progression of endocrine pancreatic tumor in the RIP1-Tag2 transgenic mouse model.⁽⁸⁾ We have previously reported that BMP-9 promotes multiple types of angiogenesis, including tumor angiogenesis, by stimulating the proliferation of endothelial cells.⁽⁹⁾ In accordance with our findings, pharmacological targeting of ALK1 signals using ALK1-Fc chimeric protein, RAP-041, impaired the growth, progression, and metastasis of tumors by inhibition of angiogenesis.^(8,10) Targeting BMP-9/10 signals is now widely recognized as a novel promising anti-angiogenic therapy in cancer treatment.^(11–13) However, it remains unclear whether single targeting of BMP-9/10/ALK1 signals is sufficient to treat various types of tumors.

In the present study, we examined the effects of targeting VEGF and BMP-9/10 signals on tumor angiogenesis and growth using xenograft models of human pancreatic carcinomas. We showed that dual targeting of VEGF and BMP-9/10 signals using Fc chimeric fusion protein was capable of inhibiting the growth of tumors resistant to single targeting of VEGF or BMP-9/10. These findings suggest that combinatorial therapies are more effective in targeting tumor angiogenesis, and likely applied to the treatment of wide variety of cancers.

Materials and Methods

Cells, cell culture, and reagents. The BxPC3 human pancreatic adenocarcinoma cells were obtained from ATCC (Manassas, VA, USA), and were maintained in RPMI-1640 supplemented with 10% FBS. Human umbilical vein endothelial cells were purchased from Lonza (Basel, Switzerland) and grown using the EGM-2 BulletKit (Lonza).⁽¹⁴⁾ Both BMP-9 and VEGF were purchased from R&D Systems (Minneapolis, MN, USA) and used as indicated in Figure 1.

Lentivirus production and infection. A lentiviral expression system was used to establish the BxPC3 cells expressing Ctrl-Fc, FLT1-Fc, ALK1-Fc, and ALK1FLT1-Fc chimeric

receptors as previously described.⁽⁹⁾ Briefly, each cDNA was subcloned into the pENTR201 vector (Invitrogen, Carlsbad, CA, USA), and subsequently transferred into the pCII-EF-RfA lentiviral expression vector (a gift from Dr. Hiroyuki Miyoshi, RIKEN). For lentiviral infection, 1×10^5 BxPC3 cells were infected with lentivirus vectors in suspension and plated in 6-well culture plates.

Production of Fc chimeric protein. HEK293T cells were transfected using FuGENE HD transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Four hours after transfection with pCII-EF-RfA encoding Fc chimeric proteins, conditioned medium was changed to serum-free Opti-MEM. Supernatant was collected 24 h after the medium change, and the concentrations of Fc chimeric proteins were determined by Human IgG ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA).

Isolation of RNA and RT-PCR analysis. Total RNAs were extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNAs were reverse-transcribed by oligo d(T) primer using PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Quantitative RT-PCR analysis was carried out using Power SYBR Green (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). All expression data were normalized to those for GAPDH. The primer sequences are shown in Table S1.

Tumor grafted BALB/c nude mice model. BALB/c female nude mice aged 5–6 weeks were obtained from Oriental Yeast (Tokyo, Japan). A total of 5×10^6 BxPC3 tumor cells in 0.2 mL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and PBS (1:1) were s.c. inoculated into the left flank of each mouse. Tumor growth was assessed by caliper measurements and calculated from minor axis and major radius.

Immunohistochemistry of tumor section. Staining of tumor sections was done as previously described.⁽⁹⁾ Briefly, tumor samples excised from BALB/c nude mice were snap-frozen in a dry-ice acetone bath. Frozen samples were further sectioned at 10- μ m thickness in a cryostat and subsequently incubated with primary mAbs to platelet and endothelial cell adhesion molecule 1 (PECAM1) (Mec13.3) (BD Pharmingen, Franklin Lakes, NJ, USA), followed by incubation with secondary Alexa Fluor 488 goat anti-rat IgG (H+L) antibody (Invitrogen). Specimens were then examined using an LSM 510 META confocal microscope (Carl Zeiss, Feldbach, Switzerland). All images were imported into Adobe Photoshop as JPEGs or TIFFs for figure assembly. Images were processed using ImageJ (NIH, <https://imagej.nih.gov/ij/>) to quantify PECAM1-positive areas.

Statistical analysis. Values are presented as mean \pm SD. Significant differences between means were determined using an unpaired Student's *t*-test or one-way ANOVA followed by the Student–Newman–Keuls test or GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $*P < 0.05$.

Results

Functional validation of a novel dual inhibitor of BMP-9/10 and VEGF signals. In order to target two angiogenic signals mediated by VEGF and BMP-9/10, we generated a double decoy receptor containing the Fc region of human IgG1 fused to the second immunoglobulin-like (Ig2) domain of human FLT1, and an extracellular domain of human ALK1 (ALK1FLT1-Fc) (Fig. 1a). We examined its ability to target VEGF and BMP-9/10 signals simultaneously, using conditioned medium from the HEK293T

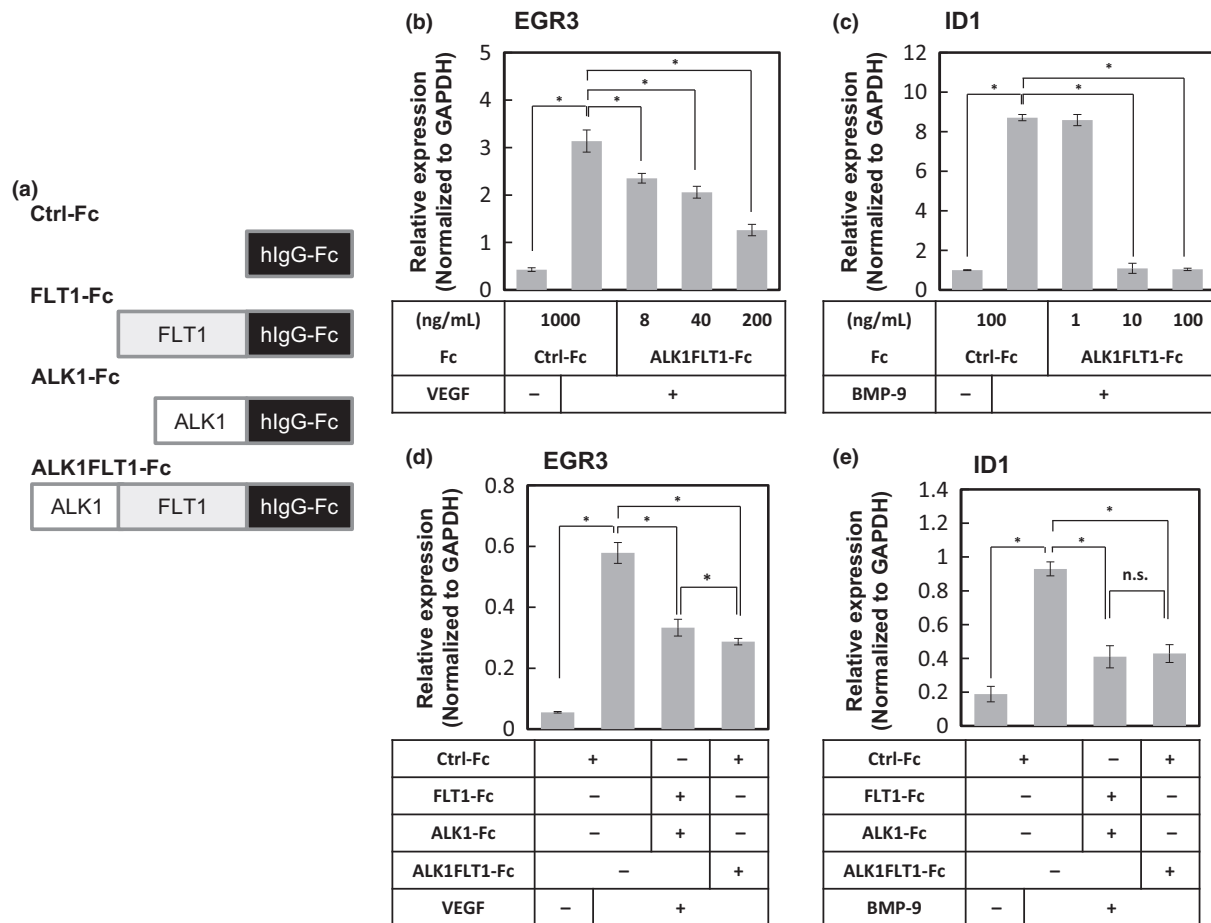


Fig. 1. Effects of double decoy receptor ALK1FLT1-Fc on vascular endothelial growth factor (VEGF) and bone morphogenetic protein-9 (BMP-9) signals in endothelial cells. (a) Schematic illustrations of constructed Fc chimeric proteins. (b–e) The conditioned media of HEK293T cells transiently transfected with expression vectors encoding control-Fc (Ctrl-Fc), Fms-related tyrosine kinase 1 (FLT1)-Fc, activin receptor-like kinase 1 (ALK1)-Fc, or ALK1FLT1-Fc were collected, followed by determination of Fc protein concentrations by ELISA. HUVECs cultured with Fc fusion proteins were treated with BMP-9 for 4 h or VEGF for 1 h, followed by quantitative RT-PCR analyses for *EGR3* (b, d) and *ID1* (c, e), respectively. Error bars indicate SD. * $P < 0.05$. n.s., not significant.

cells transfected with expression vectors encoding control Fc proteins (Ctrl-Fc) and the ALK1FLT1-Fc fusion proteins. When HUVECs were treated with VEGF (Fig. 1b) and BMP-9 (Fig. 1c), the expression of *EGR3*, one of the early growth response family of transcription factors known to be rapidly induced by VEGF,⁽¹⁵⁾ and that of *ID1*, a target gene of BMP family signals, were upregulated in the presence of Ctrl-Fc. The ALK1FLT1-Fc fusion protein decreased the expression of *EGR3* (Fig. 1b) and *ID1* (Fig. 1c) induced by VEGF and BMP-9, respectively, in a dose-dependent manner. Furthermore, in order to compare the inhibitory potential of dual ALK1FLT1-Fc trap with those of single Fc traps, we prepared VEGF-trap (FLT1-Fc) and BMP-9/10 trap (ALK1-Fc) (Fig. 1a). We found that ALK1FLT1-Fc is capable of targeting VEGF (Fig. 1d) and BMP-9 (Fig. 1e) signals to the same extent as the combination of equivalent amounts of FLT1-Fc and ALK1-Fc. These results suggest that ALK1FLT1-Fc functions as a potent dual inhibitor of VEGF and BMP-9/10.

Tumor angiogenesis significantly reduced by ALK1FLT1-Fc in a mouse xenograft model of human pancreatic cancer. We next characterized the anti-angiogenic potential of ALK1FLT1-Fc using a tumor xenograft model. In order to examine their potentials, we used BxPC3 human pancreatic tumor cells. As these cells contain a homozygous deletion of the *SMAD4*

gene,⁽¹⁶⁾ Fc chimeric proteins secreted from BxPC3 cells cannot modulate the Smad pathways by themselves. BxPC3 human pancreatic carcinoma cells were transduced with lentiviruses expressing Ctrl-Fc, FLT1-Fc, ALK1-Fc, and ALK1FLT1-Fc, and transplanted to immunodeficient mice, followed by evaluation of blood vessel formation. As shown in Figure 2, BxPC3 FLT1-Fc and ALK1-Fc tumors showed significant and similar levels of decrease in PECAM1-positive areas as compared to that of BxPC3 Ctrl-Fc. Introduction of ALK1FLT1-Fc resulted in the most significant decrease of tumor angiogenesis among all of the Fc-traps analyzed (Fig. 2), suggesting that dual targeting of VEGF and BMP-9/10 in the cancer microenvironment inhibits tumor angiogenesis more effectively than single targeting.

Dual inhibition of VEGF and BMP-9/10 signals inhibits growth of human pancreatic tumor xenografts. We also studied the effect of ALK1FLT1-Fc on tumor growth in a human pancreatic tumor xenograft model. Growth of tumors derived from BxPC3 cells expressing only FLT1-Fc or ALK1-Fc did not differ from that of Ctrl-Fc (Fig. 3). However, expression of the dual ALK1FLT1-Fc trap significantly retarded the growth of tumor xenografts, likely due to effective inhibition of tumor angiogenesis by dual targeting of VEGF and BMP-9/10, as shown in Figure 2.

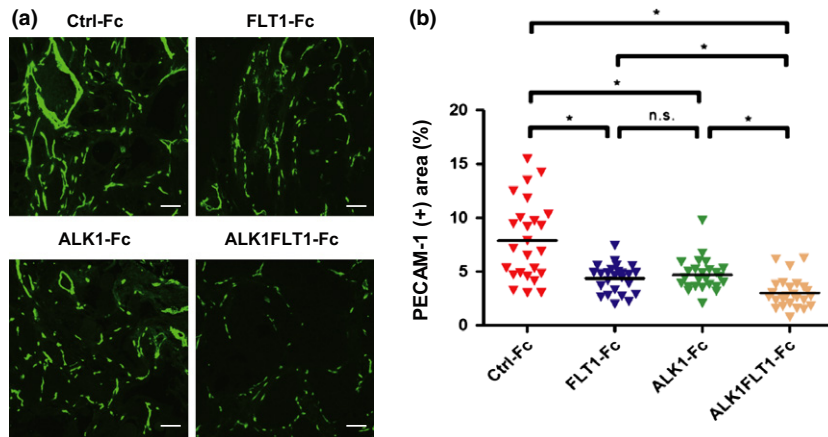


Fig. 2. Effects of various Fc chimeric proteins on tumor angiogenesis in a mouse xenograft model of human pancreatic cancer. BxPC3 cells expressing control (Ctrl-Fc), Fms-related tyrosine kinase 1 (FLT1)-Fc, activin receptor-like kinase 1 (ALK1)-Fc or ALK1FLT1-Fc were inoculated s.c. in BALB/c nude mice. After 7 weeks of BxPC3 cell inoculations, tumors were excised and examined for vascular density. (a) Immunostaining for platelet and endothelial cell adhesion molecule 1 (PECAM1; green) of sections obtained from each type of BxPC3 tumor. Scale bar = 100 μ m (n = 4 for each group). (b) Graphic representation of PECAM1-positive area (%). Each value represents the positive area in each observed field. * P < 0.05. n.s., not significant.

Discussion

Both VEGF and BMP-9/10 signals have been implicated in tumor angiogenesis, and have been targeted to successfully retard the growth of multiple types of cancers.^(2,8,10) However, in the present study, single targeting of either ALK1-Fc or FLT1-Fc did not impair tumor growth in a BxPC3 xenograft model (Fig. 3) regardless of their ability to inhibit tumor angiogenesis (Fig. 2). Previous reports have also shown that targeting of VEGF signals only did not show clear anti-tumor effects in multiple human cancer xenograft models.⁽²⁾ Several lines of evidence show that multiple angiogenic factors compensate the angiogenic activities of VEGF after anti-VEGF therapies. In order to target multiple angiogenic signals simultaneously, multikinase inhibitors such as sunitinib (an inhibitor of VEGFR and platelet-derived growth factor

receptors) have been developed and already launched for cancer therapies.

Dalantercept (ACE-041), a human counterpart of RAP-041 (ALK1-Fc decoy receptor), has been preclinically and clinically evaluated as a novel anti-angiogenic agent in various types of cancers.⁽¹⁷⁾ However, phase II evaluation of dalantercept for the monotherapy treatment of advanced or recurrent endometrial cancer has shown its insufficient activity to warrant further investigation.⁽¹⁸⁾ Combination therapies appear to be more promising. Indeed, an ongoing phase II study in renal carcinoma based on treatment with dalantercept in combination with axitinib resulted in significant reduction of tumor vessel formation⁽¹⁹⁾, which would support our concept of involvement of BMP-9/10 and VEGF signals.

Recently, the treatment cost of biotechnology-based drugs has become a serious problem; the combination of them is not acceptable for most cancer patients without financial support. Therefore, dual targeting drugs, such as VEGF and angiopoietin-2 bispecific antibody⁽²⁰⁾ and ALK1FLT1-Fc in the present study, are expected to be novel therapies from the aspect of efficacy as well as cost. Further preclinical and clinical investigations are warranted to explore application of ALK1FLT1-Fc in the treatment of various types of cancers.

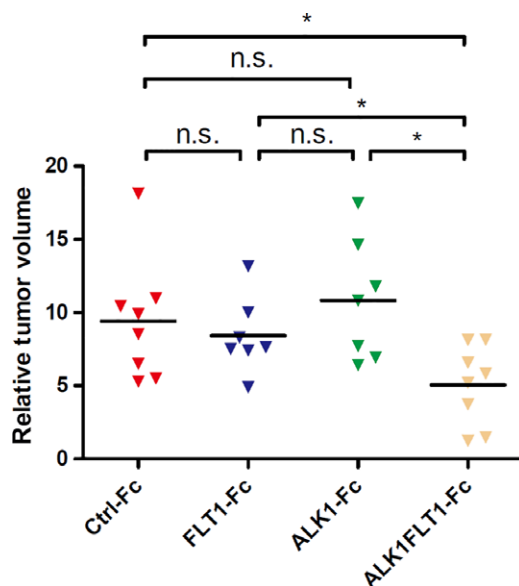


Fig. 3. Effects of various Fc chimeric proteins on the growth of BxPC3 human pancreatic cancer xenografts. BxPC3 cells expressing control (Ctrl)-Fc, activin receptor-like kinase 1 (ALK1)-Fc, Fms-related tyrosine kinase 1 (FLT1)-Fc, or ALK1FLT1-Fc were inoculated s.c. in BALB/c nude mice. Tumor growth was assessed on day 49 after transplantation by caliper measurements and calculated from minor axis and major radius. * P < 0.05. n.s., not significant.

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Disclosure Statement

Y.A. is an employee of Nippon Kayaku, Co., Ltd. The other authors have no conflict of interest.

References

- 1 Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat Rev Cancer* 2010; **10**: 138–46.
- 2 Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res* 2006; **312**: 549–60.
- 3 Miller K, Wang M, Gralow J *et al.* Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* 2007; **357**: 2666–76.
- 4 Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* 2008; **8**: 592–603.
- 5 Koh YJ, Kim HZ, Hwang SI *et al.* Double antiangiogenic protein, DAAP, targeting VEGF-A and angiopoietins in tumor angiogenesis, metastasis, and vascular leakage. *Cancer Cell* 2010; **18**: 171–84.
- 6 Ye L, Jiang WG. Bone morphogenetic proteins in tumour associated angiogenesis and implication in cancer therapies. *Cancer Lett* 2016; **380**: 586–97.
- 7 David L, Mallet C, Mazerbourg S *et al.* Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* 2007; **109**: 1953–61.
- 8 Cunha SI, Pardali E, Thorikay M *et al.* Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *J Exp Med* 2010; **207**: 85–100.
- 9 Suzuki Y, Ohga N, Morishita Y *et al.* BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. *J Cell Sci* 2010; **123**: 1684–92.
- 10 Cunha SI, Bocci M, Lötvrot J *et al.* Endothelial ALK1 is a therapeutic target to block metastatic dissemination of breast cancer. *Cancer Res* 2015; **75**: 2445–56.
- 11 Cunha SI, Pietras K. ALK1 as an emerging target for antiangiogenic therapy of cancer. *Blood* 2011; **117**: 6999–7006.
- 12 Bendell JC, Gordon MS, Hurwitz HI *et al.* Safety, pharmacokinetics, pharmacodynamics, and antitumor activity of dalantercept, an activin receptor-like kinase-1 ligand trap, in patients with advanced cancer. *Clin Cancer Res* 2014; **20**: 480–9.
- 13 Bhatt RS, Atkins MB. Molecular pathways: can activin-like kinase pathway inhibition enhance the limited efficacy of VEGF inhibitors? *Clin Cancer Res* 2014; **20**: 2838–45.
- 14 Yoshimatsu Y, Lee YG, Akatsu Y *et al.* Bone morphogenetic protein-9 inhibits lymphatic vessel formation via activin receptor-like kinase 1 during development and cancer progression. *Proc Natl Acad Sci USA* 2013; **110**: 18940–5.
- 15 Suehiro J, Hamakubo T, Kodama T *et al.* Vascular endothelial growth factor activation of endothelial cells is mediated by early growth response-3. *Blood* 2010; **115**: 2520–32.
- 16 Kleeff J, Wildi S, Friess H *et al.* Ligand induced upregulation of the type II transforming growth factor (TGF- β) receptor enhances TGF- β responsiveness in COLO-357 cells. *Pancreas* 1999; **18**: 364–70.
- 17 Gupta S, Gill D, Pal SK *et al.* Activin receptor inhibitors–dalantercept. *Curr Oncol Rep* 2015; **17**: 14.
- 18 Makker V, Filiaci VL, Chen LM *et al.* Phase II evaluation of dalantercept, a soluble recombinant activin receptor-like kinase 1 (ALK1) receptor fusion protein, for the treatment of recurrent or persistent endometrial cancer: an NRG Oncology/Gynecologic Oncology Group Study 0229N. *Gynecol Oncol* 2015; **138**: 24–9.
- 19 Henner Voss M, Plimack ER, Rini BI *et al.* DART Study: A phase II randomized trial of dalantercept plus axitinib versus placebo plus axitinib in advanced clear cell renal cell carcinoma (RCC): Results from Part 1. *J Clin Oncol* 33, 2015; suppl 7; abstr 407 <http://meetinglibrary.asco.org/content/141662-159>
- 20 Kontermann RE. Dual targeting strategies with bispecific antibodies. *mAbs* 2012; **4**: 182–97.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. List of PCR primers used in this study.