Endothelial ALK1 Is a Therapeutic Target to Block Metastatic Dissemination of Breast Cancer

Sara I. Cunha1,2, Matteo Bocci3, John Lövrot4, Nikolas Eleftheriou5, Pernilla Roswall2, Eugenia Cordero3, Linda Lindström4, Michael Bartoschek3, B. Kristian Haller2, R. Scott Pearsall5, Aaron W. Mulivor5, Ravindra Kumar5, Christer Larsson6, Jonas Bergh4, and Kristian Pietras2,3

Abstract

Exploration of new strategies for the prevention of breast cancer metastasis is justifiably at the center of clinical attention. In this study, we combined a computational biology approach with mechanism-based preclinical trials to identify inhibitors of activin-like receptor kinase (ALK) 1 as effective agents for blocking angiogenesis and metastasis in breast cancer. Pharmacologic targeting of ALK1 provided long-term therapeutic benefit in mouse models of mammary carcinoma, accompanied by strikingly reduced metastatic colonization as a monotherapy or part of combinations with chemotherapy. Gene-expression analysis of breast cancer specimens from a population-based nested case-control study encompassing 768 subjects defined endothelial expression of ALK1 as an independent and highly specific prognostic factor for metastatic manifestation, a finding that was corroborated in an independent clinical cohort. Overall, our results suggest that pharmacologic inhibition of endothelial ALK1 constitutes a tractable strategy for interfering with metastatic dissemination of breast cancer. Cancer Res. 75(12): 2445–56. ©2015 AACR.

Introduction

Exploration of new strategies for the prevention of breast cancer metastasis is justifiably at the center of clinical attention (1). The haematogenous dissemination of tumor cells is a multistep process requiring: (i) detachment of malignant cells from the primary tumor, (ii) intravasation into and extravasation from the blood stream, and (iii) colonization of the distant organ (2). However, we currently have limited knowledge on the molecular drivers contributing to each of the steps in the metastatic cascade in breast cancer, and thus efforts to target-specific signaling pathways involved in the systemic spread of the disease have largely been unsuccessful so far (3). In cases where breast tumors are found in early stages, the prognosis following adequate therapy is good with a 3-year overall survival (OS) rate reaching above 90% (4). Nevertheless, many women are not diagnosed until the tumor has reached advanced stages; tumor stage is an established factor for poor prognosis (4). Thus, novel treatment strategies to combat disseminated breast cancer, both for the neoadjuvant and the adjuvant setting, are sorely needed.

The angiogenic process is required for tumor progression from an indolent state (5). The vascular tree provides a tumor with its metabolic requirements, while simultaneously providing an escape route by which malignant cells can leave the primary tumor bulk. Indeed, in particular cases, high vascular density was demonstrated to be a prognostic factor for poor outcome in breast cancer, as well as in other malignancies (6, 7). The introduction of multitargeted agents incorporating antiangiogenic activity in clinical practice has led to improved disease control in terms of prolonged progression-free survival (PFS). Consequently, drugs targeting the vascular endothelial growth factor (VEGF) pathway are now included in the first line therapy for metastatic disease for a range of malignancies (8–11). However, attempts to target tumor angiogenesis in breast cancer has met with ambiguous success (12, 13). Although providing initial relief for metastatic breast cancer patients by improving response rates and prolonging PFS, no conclusive evidence for long-term benefit in OS has been provided to date (13). Consistent with this clinical reality, recent preclinical studies indicate that tumors in mice treated systemically with anti-VEGF therapy rapidly acquire resistance, coupled to recurring tumors that appear to be more locally invasive and have a higher propensity to seed distant metastases (14–16). Thus, the need for a mechanism-based and clinically relevant search for alternative angiogenic pathways that may serve as targets for more efficacious drugs without affecting disease stage in breast cancer is highly warranted.

ALK1 is a type I receptor in the large TGFβ family expressed selectively by endothelial cells (17). Pharmacologic targeting of ALK1 has demonstrable therapeutic efficacy in a diverse set of mouse models of cancer (18–20). Here, we investigate the utility of ALK1 inhibition as an antimetastatic therapy in breast cancer using a combined approach of preclinical testing of a clinically
tractable ALK1 inhibitor and analysis of gene-expression patterns relating to metastatic spread in breast cancer patient specimens.

Materials and Methods

Cell culture

MDA-MB-231 were maintained in culture in DMEM (Invitrogen), supplemented with 10% FCS. EO771 breast cancer cells were cultured in DMEM supplemented with 20% FCS.

Animal care and tumor establishment

All animal experiments were approved by the local ethical committee for animal care in Stockholm and Lund (permits N96/11 and M142/13). The RIP-TAg2 mice (C57Bl6/J background) received water supplemented with 5% sugar to alleviate hypoglycemia from 10 weeks of age. Total tumor burden per mouse was calculated as the sum of the volume of each individual tumor dissected from a RIP-TAg2 mouse pancreas. The MMTV-PyMT mice (FVB/n strain background) received water supplemented with 5% sugar to alleviate hypoglycemia from 10 weeks of age. Total tumor burden per mouse was calculated as the sum of the volume of each individual mammary tumor. To establish EO771 tumors, 5 x 10³ cells were injected orthotopically into the fourth mammary fat pad of isoflurane-anesthetized wildtype C57Bl6/J females. 1 x 10⁶ cells of the human metastatic breast cancer cell line MDA-MB-231 were transplanted s.c. to immunocompromised SCID mice. In all cases, tumor volume was calculated as length x width² x π/6.

Therapeutic trials

Control IgG or RAP-041 (Acceleron Pharma) was diluted in TBS and administered twice weekly by i.p. injection at 12 mg/kg per injection. Mice carrying orthotopic EO771 mammary carcinomas were treated with either RAP-041 or IgG2a for 2 weeks starting 7 days following tumor establishment. Treatment with docetaxel (Taxotere, Sanofi-Aventis) was administered once weekly at 20 mg/kg by i.p. injection.

Tissue preparation, histology, and immunostaining

Mice were heart-perfused with PBS followed by 4% paraformaldehyde. For paraffin-embedding, organs were post-fixed in 4% paraformaldehyde for 2 hours before proceeding to embedding. Paraffin-embedded sections were deparaffinized and rehydrated followed by antigen retrieval in low pH buffer (pH 6; DAKO) for 20 min at 95°C. Blocking was performed in 10% normal goat serum in TNB buffer (PerkinElmer). The primary antibody against the PyMT oncogene (1:100; Abcam ab15085) was incubated in blocking buffer overnight at 4°C. For SV40 T-Ag staining, a high pH antigen retrieval buffer (pH 10; DAKO) was used, followed by washes and quenching of endogenous peroxidase activity with 50% methanol/3% H₂O₂ in PBS for 10 minutes in room temperature. A wash in PBS preceded blocking with 10% normal goat serum in TNB for 1 hour. The primary antibody against T-Ag (1:1000; a kind gift from Douglas Hanahan, EPFL) was incubated at 4°C overnight. After washes with PBS containing 0.1% Tween-20, a suitable biotinylated secondary antibody was incubated for 45 minutes in room temperature.

For cryopreservation, tumors, livers and/or lungs were kept in 30% sucrose at 4°C overnight, followed by embedding in cryosectioning media. Frozen sections were fixed in ice-cold acetone, followed by blocking using serum free protein block (DAKO) for >90 minutes at room temperature. Primary antibodies directed against CD31 (dilution 1:100; Pharmingen MEC13.3), podocalyxin (dilution 1:100; R&D Systems, AF1556), and BMP9 (dilution 1:500; Abcam; ab35088) were incubated overnight at 4°C. Appropriate Alexa 594 and Alexa 488–fluorochrome-conjugated secondary antibodies (Invitrogen) were used and sections were finally mounted using 4’,6-diamidino-2-phenylindole-containing mounting media (Vector Laboratories).

Quantification of metastases

The right lateral liver lobe from RIP-TAg2 mice or the left lung lobes of MMTV-PyMT or EO771-bearing mice were embedded in paraffin upon tissue fixation. The metastatic burden was assessed by serial sectioning of the entire lung/liver lobe. Following hematoxylin and eosin (H&E) staining on every 25th section, the number of metastatic foci (>8 cells in diameter) was determined in >15 sections per mouse and >5 mice per group.

RNA isolation and quantitative RT-PCR

Total RNA of 12-weeks-old MMTV-PyMT mice mammary tissue was isolated using TRizol extraction (Invitrogen), followed by the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A 1 µg total RNA was subsequently used to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative reverse transcription PCR was performed using the KAPA SYBR Fast qPCR Kit (Kapa Biosystems) on a Rotorgene 6000 (Qiagen) in triplicates using primers purchased from Qiagen: RPL19, TGFβ, BMP9/CDF2, BMP10, and CDF5 (QuantiTect Primer Assays QT00166145, QT00145250, QT00307587, QT00259847, and QT00250523, respectively). Primers for analysis of Id1 and Id3 as in ref. 18.

Tumor grade assessment

To assess the tumor grade of lesions from MMTV-PyMT mice, tumor tissue was classified into different degrees of progression by quantifying the area of transformed glands occupied by each
stage. Progression follows from normal fat tissue to a "precancerous stage" characterized by premalignant hyperplasia and adenoma (with the retention of some normal ductal and acinar mammary gland morphology), to a more epithelial cell—dense "early carcinoma" with stromal invasion, and finally to an invasive, very dense, high—mitotic index "late-stage carcinoma."

Tumors were evaluated for the proportion of mammary fat tissue, hyperplastic tissue, adenoma, early carcinoma, and late carcinoma.

Clinical datasets

Expression data from The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov/) were downloaded in November 2013. The data were log2 transformed after addition of 1 to each normalized value. Clinical and follow-up data were downloaded in May 2014. All analyses were done with R using the basic and survival packages. Breast cancer subtypes were determined using nearest correlations with the PAM50 centroids.

The nested case–control study gained approval by the ethics committee at Karolinska Institutet, Stockholm, Sweden. The full details of the study design, collection of clinical-pathologic information, gene-expression profiling of fresh frozen tumor tissue and subsequent preprocessing and normalization of microarray gene-expression data, and finally conditional logistic regression modeling of the nested case–control study have been reported elsewhere (array data deposited at the Gene Expression Omnibus Database under accession number GSE48091; ref. 21), and is the subject of a separate report (Lindström and colleagues; submitted for publication). Gene-expression data were collapsed to gene level using a nonspecific filter keeping only the probe sets with highest interquartile range in the case of multiple mappings to the same Entrez Gene ID. As in the original publication, out of seven considered clinical-pathologic variables—estrogen receptor (ER), progesterone receptor and HER2 status, histologic grade, proliferation, tumor size, and lymph node status—three variables, namely lymph node status, tumor size, and HER2 status, were considered significant and included in multivariable conditional logistic regression models. A missing category was used to handle missing values in clinical-pathologic data. All gene-expression data analysis and statistical analysis were done in R/Bioconductor.

Statistical analysis

Unless specifically stated, all measurements are depicted as mean ± SD. Statistical analyses for tumor volume were performed using an unpaired, two-tailed Student’s t test. Statistical analyses for tumor characteristics were performed using a Mann–Whitney U test. Statistical significance was considered using \( \alpha = 0.05 \).

Results

Long-term inhibition of ALK1 impairs metastatic dissemination and prolongs survival in an experimental model of neuroendocrine tumorigenesis

We, and others, have previously documented the emergence of therapeutic resistance toward anti-VEGF therapy using various pharmacologic agents (14–16). Evasive resistance to VEGF-inhibitory modalities is accompanied by a hypoxia-driven malignization, that is, enhanced local invasion and increased rate of metastatic seeding, of tumors in the prototypical RIP-TAg2 mouse model of angiogenesis-dependent pancreatic neuroendocrine tumorigenesis (NET). To investigate whether antiangiogenic therapy by inhibition of ALK1 signaling gives rise to a similar exacerbation of systemic dissemination, we contrasted previously performed short-term therapeutic trials (18) with a long-term regimen of single-agent neoadjuvant therapy using ALK1-Fc, a ligand trap that neutralizes BMP9 and BMP10 (RAP-041, mouse counterpart of dalantercept; Fig. 1A). Regardless of the length or timing of the treatment of RIP-TAg2 mice, single-agent RAP-041 gave rise to a state of stable disease during the course of the trials, in contrast with tumors in control-treated mice that consistently presented with overt progressive disease (Fig. 1B and ref. 18). Despite inducing a demonstrable reduction in vessel area, average vessel length and the number of vessel endpoints, as judged by immunostaining for the endothelial cell markers podocalyxin or CD31 (Fig. 1C and data not shown), RAP-041 did not provoke widespread hypoxia, using CA IX or HIF1α expression as proxies for low tissue oxygenation (Fig. 1D). Pancreatic NETs of RIP-TAg2 mice disseminate predominantly to sentinel lymph nodes in the mesentery and to the liver, similar to the corresponding human disease (Fig. 1E; ref. 22). The incidence of hepatic metastases in RIP-TAg2 mice was not changed following short-term therapy with RAP-041 (Fig. 1F). Strikingly, however, upon long-term administration of neoadjuvant therapy with RAP-041 to mice harboring advanced disease, the rate of metastatic dissemination to the liver decreased by 86% compared with treatment with control IgG (Fig. 1F). In sharp contrast with anti-VEGF therapy, which induced an increased rate of metastasis (14, 16), ALK1 inhibition caused regression of preformed hepatic NET foci during the course of the therapeutic trial in RIP-TAg2 mice from an average of 8.1 to 2.8 foci per histologic section (Fig. 1F). In line with the substantial reduction in both primary tumor burden and metastatic manifestation in RIP-TAg2 mice following ALK1 inhibition, the rate of OS at 16 weeks of age was also increased from 27% (11/41) to 79% (11/14; Fig. 1G).

ALK1-Fc reduces metastatic dissemination to the lung in a genetically engineered mouse model of breast cancer

Given the failure of anti-VEGF therapy to affect OS in breast cancer, we extended our analyses on the role of ALK1 signaling in
Targeting ALK1 Impairs Distant Breast Cancer Dissemination

Inhibition of ALK1 reduces the growth, angiogenic response, and metastatic dissemination of advanced experimental breast cancer. A, MMTV-PyMT mice treated for 4 weeks with twice-weekly administration of control IgG (n = 5) or RAP-041 (ALK1-Fc; n = 6) beginning at 11 weeks of age (P < 0.05; **P < 0.01). B, visualization of endothelial cells in tumors from MMTV-PyMT mice using immunostaining for CD31 (red) and counterstaining for cell nuclei (blue; DAPI). C, quantitation of vessel foci in the lungs of mice bearing EO771 tumors treated with twice-weekly administration of control IgG (n = 7) or RAP-041 (ALK1-Fc; n = 7) (**P < 0.01). D, representative image of pulmonary metastatic lesions from MMTV-PyMT mice, as demonstrated by H&E staining. E and F, quantitation of the number (E) and size (F) of metastatic foci in the lungs of EO771-bearing mice with RAP-041 (ALK1-Fc) compared with control (IgG) (**P < 0.01). G, mice bearing orthotopically implanted syngeneic mouse mammary pulmonary metastatic lesions from MMTV-PyMT mice, as demonstrated by H&E staining. H, functionally, the ALK1 paracrine signaling network and therapeutic utility of ALK1-Fc in the human setting was demonstrated by near-complete retardation of the growth of orthotopic xenografts of the aggressive triple-negative human breast carcinoma cell line MDA-MB-231 by treatment with ACE-041/dalanitecept (the human counterpart of RAP-041; Fig. 5B). I, next, we explored whether the expression of ALK1 (gene name ACVR1L) holds prognostic capability for metastatic disease in human patients, we analyzed gene-expression patterns in tumor material from a population-based nested case-control study encompassing 768 subjects with complete clinical follow-up (21). Briefly, 190 breast cancer patients that developed distant metastatic disease (cases) were selected from a consecutive

metastatic dissemination to this disease by studying the MMTV-PyMT genetically engineered mouse model of mammary carcinoma; a mouse model faithfully recapitulating many aspects of the human disease, including dissemination pattern to the lung and lymph nodes (23). Initial characterization of mammary tumors from MMTV-PyMT mice demonstrated an endothelial cell–exclusive expression of ALK1 and readily detectable expression levels of its ligands BMP9, BMP10, and TGF-β (Supplementary Fig. 1A–1C). Next, MMTV-PyMT mice were administered RAP-041 from 8 to 12 weeks of age in a preclinical neoadjuvant trial. Consistent with the effects in pancreatic NETs, inhibition of ALK1 significantly delayed the growth and reduced the vessel area of primary mammary carcinomas (Fig. 2A–C). The observed action of RAP-041 was a result of on-target effects, as demonstrated by diminished expression of the ALK1 target genes Id1 and Id3 in tumor lysates (Supplementary Fig. S2A and S2B). Treatment with RAP-041 had no discernible direct effect on the proliferation or apoptosis of malignant cells isolated from MMTV-PyMT tumors in vitro (Supplementary Fig. S2C and S2D), indicating that the therapeutic benefit was derived from indirect targeting of tumor cells by impinging on the neoangiogenic process. Notably, treatment with ALK1-Fc impeded the tumor progression pathway, as evidenced by a shift in the tumor grade from predominant malignant and invasive lesions observed in the control group (31% late carcinoma vs. 18% normal/hyperplasia/adenoma) to a higher degree of premalignant lesions in the treated group (6% late carcinoma vs. 40% normal/hyperplasia/adenoma; Fig. 2D). Importantly, the impaired tumor progression also translated into an 87% decrease in metastatic colonization of the lung (Fig. 2E and F). Tumor growth rate and vessel area were similarly compromised following neoadjuvant treatment with ALK1-Fc of older MMTV-PyMT mice already presenting with fully established disease (Fig. 2A–C). Again, inhibition of ALK1 expressed solely by the tumor endothelium significantly reduced the rate of metastasis by 55% (Fig. 2D and E). Furthermore, in addition to reducing the number of metastatic foci, RAP-041 treatment also significantly moderated the average size of the pulmonary metastatic lesions (Fig. 2F).

ALK1 inhibition induces angiogenic and metastatic blockade in experimental mammary carcinoma

To corroborate our findings of a role for endothelial ALK1 signaling in the metastatic cascade in breast tumors, we transplanted the ER-expressing mouse mammary carcinoma cell line EO771 orthotopically into the mammary fat pad of mice. The expression of BMP9 and TGF-β in EO771 tumor tissue was confirmed by quantitative PCR or immunostaining (Supplementary Fig. S1B and S1C). Consistent with our previous observations, administration of ALK1-Fc significantly delayed the growth of EO771 tumors (Fig. 3G) with concomitant reduction of vessel area (Fig. 3H and I). Importantly, neoadjuvant treatment of EO771-bearing mice with RAP-041 reduced the metastatic success rate to the lung by 87% (Fig. 3I), further demonstrating the involvement of ALK1 ligands and the tumor endothelium in the process of tumor cell dissemination to distant sites.

A combined therapeutic regimen of ALK1-Fc and docetaxel reduces tumor growth and metastatic dissemination

Neoadjuvant therapy of breast cancer is increasingly being used in order to reduce the primary tumor bulk, enable breast-conserving surgery and prevent metastatic manifestation (1). Therefore, we investigated the utility of combining inhibition of ALK1 with commonly used pharmacologic treatment strategies for breast cancer in a series of preclinical trials enrolling MMTV-PyMT mice in the neoadjuvant setting. Combined administration of RAP-041 with trastuzumab or the VEGFR2-neutralizing antibody DC101 did not yield any, or only marginal, therapeutic benefit compared with either treatment alone (data not shown). Strikingly, however, concomitant inhibition of ALK1 with neoadjuvant docetaxel gave rise to improved control of tumor growth (Fig. 4A). The addition of RAP-041 to the docetaxel regimen resulted in partial responses in 5 of 13 (38%) mice, compared with 0 of 10 (0%) in mice treated with single-agent docetaxel (Fig. 4B). Interestingly, treatment with single-agent docetaxel afforded a significant reduction of tumor vascularity; an effect that was exacerbated by combination treatment with RAP-041 (Fig. 4C and D). Most notably, the combination of ALK1-Fc and chemotherapy brought about a further 63% decrease in the metastatic index of the lung compared with docetaxel alone and prevented colonization of pulmonary metastases by 93% compared with control therapy (Fig. 4E).

ALK1 is an independent biomarker for metastatic recurrence of human breast cancer

Comparative studies demonstrated widespread expression of BMP9 protein, but not BMP10 protein, by malignant cells in human breast carcinomas (Fig. 5A). Functionality of the ALK1 paracrine signaling network and therapeutic utility of ALK1-Fc in the human setting was demonstrated by near-complete retardation of the growth of orthotopic xenografts of the aggressive triple-negative human breast carcinoma cell line MDA-MB-231 by treatment with ACE-041/dalanitecept (the human counterpart of RAP-041; Fig. 5B). Next, to explore whether the expression of ALK1 (gene name ACVR1L) holds prognostic capability for metastatic disease in human patients, we analyzed gene-expression patterns in tumor material from a population-based nested case-control study encompassing 768 subjects with complete clinical follow-up (21). Briefly, 190 breast cancer patients that developed distant metastatic disease (cases) were selected from a consecutive
series of individuals and three random control patients (free from metastasis) for each case were closely matched by adjuvant therapy, age and calendar period at diagnosis (21). Expression of ACVRL1 was found to correlate significantly with prototypical endothelial cell genes, further corroborating the predominant vascular expression of ALK1 in human breast cancers (Supplementary Table S1). In addition, ACVRL1 expression was significantly correlated to the expression of its target gene Id1, implying activation of the pathway (data not shown). In strong support of our functional data, abundant expression of ACVRL1 was highly significantly associated with the incidence of metastatic disease (Table 1). Similarly, expression of SMAD6, a known downstream target gene of ALK1 activation was linked to recurrent disease (Table 1). In sharp contrast, the expression of the canonical TGFβ type 1 receptor ALK5 (TGFBRI) and its ligands TGFβ1, 2, that are implicated in promotion of metastasis through induction of epithelial-to-mesenchymal transition (EMT), was equally distributed between cases and controls, with the exception of TGFβ1, expression of which was marginally associated with metastatic disease (Table 1). Importantly, in a multivariate analysis of risk factors for presenting with metastatic disease, expression of both ACVRL1 (HR, 3.59; 95% CI, 2.52–5.15) and SMAD6 (HR, 1.43; 95% CI, 1.15–1.77) remained as statistically significant and independent prognostic factors, alongside well-known clinical risk factors such as lymph node status, tumor size, and HER2 amplification (Table 1). Intriguingly, the known ligands for ALK1, that is, BMP9 (GDF2) and BMP10, were only weakly associated to metastasis, even when their expression was combined (Table 1).

High expression of endothelial ALK1 is an independent prognostic factor for poor survival in human breast cancer

To further validate our finding of a functional association between ALK1 signaling and metastatic colonization in human breast cancer, we analyzed breast cancer gene-expression data from TCGA. The validation set revealed that expression of ACVRL1 was indeed correlated with the expression of well-known endothelial markers (Supplementary Table S1). Next, a Cox proportional hazards model was applied to gene-expression data from TCGA with event-free survival as the endpoint. Univariate models did not demonstrate any significant prognostic information held by either ACVRL1 itself (data not shown) or by a general vascular index (normalized average expression of the prototypical endothelial cell markers PECAM1, CDH5, and CD34; hereafter referred to as the endothelial metagene). Strikingly, however, in a multivariate model (Table 2), both the ACVRL1 expression level (HR, 2.35; 95% CI, 1.34–4.09) and the endothelial metagene (HR, 0.46; 95% CI, 0.28–0.74) were independent prognostic factors for event-free survival, also after adjustment for lymph node status and stratification for the molecular subtype of the disease according to the PAM50 profile. The opposing HRs of the vascular index and the ACVRL1 expression level in this dataset suggested that the
molecular characteristics, rather than the absolute extent of vascularization, hold prognostic information. The ratio between ACVRL1 expression and the endothelial metagene (i.e., the relative expression of ACVRL1 per endothelial cell) was, therefore, evaluated and found to serve as a highly specific prognostic biomarker for recurrent disease; breast cancer patients within the quartile of highest ACVRL1:endothelial metagene ratio were subject to an exceedingly poor event-free survival, compared with those patients with the lowest ratio (Fig. 5C). Reassuringly, the independent prognostic capability of the ACVRL1:endothelial metagene index was confirmed by analysis of gene-expression data from the nested case-control study (Table 1). Further analysis revealed that the association between the ACVRL1:endothelial metagene index and distant metastases was robust regardless of treatment received and was not a feature of any particular molecular subtype or size of breast tumor (Supplementary Fig. S3A–S3C).

### Discussion

Taken together, we have combined mechanism-based studies of ALK1 signaling in advanced genetically engineered mouse models of breast cancer with preclinical efficacy trials of a clinically tractable pharmacologic inhibitor of ALK1 and expression analysis of ACVRL1 in human patient materials in relation to relevant clinical parameters for metastatic disease. The present studies strongly suggest an independent role for endothelial ALK1 signaling in the process of metastatic dissemination and colonization of distant organs in breast cancer. On the basis of our findings, pharmacologic inhibitors of the ALK1 pathway, thus, present as attractive and realistic partners with chemotherapy in the management of metastatic breast cancer.

ALK1-blocking agents are currently being developed clinically for various malignancies (17). Dalantercept (ACE-041), the human counterpart of RAP-041, is an ALK1 ligand trap comprising the extracellular domain of ALK1 fused to the Fc portion of IgG. Phase I clinical trials demonstrate the safety and tolerability of dalantercept; the most common and dose-limiting side effects included phase I clinical trials with grade 3 thrombocytopenia and grade 4 decrease in pancreatic enzymes as the dose-limiting toxicities (25). Notably, the side effect profile of the ALK1 targeting agents is distinct from that of anti-VEGF compounds, such as bevacizumab, sunitinib, and sorafenib, indicating a unique mechanism of action. In our preclinical trials, we found that, unlike VEGF pathway inhibitors, prolonged administration of ALK1-Fc did not give rise to widespread tissue hypoxia, rebound of tumor growth, increased local invasion or augmented seeding of distant metastases in a nested case-control studya

### Table 1. Univariate and multivariable conditional logistic regression models comparing patients developing metastatic disease with patients free from disseminating disease in a nested case-control study

<table>
<thead>
<tr>
<th>Variable</th>
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<th>Univariate models</th>
<th>Multivariable model A</th>
<th>Multivariable model B</th>
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<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
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<tr>
<td></td>
<td></td>
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<td>ACVRL1 (ALK1)</td>
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<td>Endothelial metagene</td>
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<td>&lt;0.001</td>
<td>1.43 (1.15–1.77)</td>
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<td>ACVRL1:endothelial metagene index</td>
<td>1.07 (0.91–1.26)</td>
<td>0.390</td>
<td>1.11 (0.95–1.28)</td>
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<td>GDF2 (BMP9)</td>
<td>0.73 (1.05–1.14)</td>
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<td>BMP10</td>
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<td>1.73 (1.35–2.60)</td>
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<td>1 (ref.)</td>
<td>1 (ref.)</td>
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<td>Unknown</td>
<td>104</td>
<td>0.75 (0.44–1.31)</td>
<td>0.80 (0.43–1.49)</td>
<td>0.85 (0.46–1.53)</td>
</tr>
</tbody>
</table>

Abbreviation: ref., reference.

*aControls randomly matched to cases by age, adjuvant therapy, and calendar period at diagnosis.

*bNumerical variables are centered and scaled (SD set to one) in the models. Gene-expression values are normalized log2 summarized microarray probe intensity values.

*cFor numerical variables, HR is the relative hazard when increasing the variable one SD.

*dAverage expression of the prototypical endothelial cell markers PECAM1, CDH5, and CD34.

*eDifference between ACVRL1 expression and endothelial metagene expression; corresponds to (log2 of) the ratio of ACVRL1 probe intensity over average endothelial metagene probe intensity.
metastases (14–16, 26). In sharp contrast, neutralization of ALK1 ligands in the neoadjuvant setting resulted in a substantial reduction in metastatic colonization and in some cases even regression of preexisting metastatic lesions. It is interesting to note that the ALK1 target gene Id1, which we found to be substantially downregulated following treatment with RAP-041 and significantly correlated to ALK1 expression in human breast tumors, is part of a gene-expression signature predictive of breast cancer lung metastasis (27) and suppression of Id1 impairs metastatic colonization in a mouse model of lung carcinoma (28). The fact that ALK1 inhibition did not provoke tissue hypoxia is the most likely cause for the observed discrepancy with anti-VEGF therapy, as hypoxia has been suggested to be the main driving force for the malignization of tumors following VEGF blockade in preclinical studies (14, 26). The relative lack of hypoxia, despite reduced vessel area, implicitly suggests that ALK1 inhibition improves the exchange of oxygen and nutrients across the abnormal neovascularization in tumors. Further mechanistic studies of the distinct effects of ALK1-Fc on the tumor vasculature are warranted.

Herein, we provide compelling evidence from a population-based nested case–control study encompassing 768 subjects (23) that high expression of ACVRL1 in the tumor vasculature serves as a highly significant biomarker for a metastatic phenotype in breast cancer, alongside traditional risk factors such as lymph node status, tumor size and HER2 amplification. This finding was corroborated by analysis of the independent TCGA dataset, in which the expression of endothelial ACVRL1 was found to be strongly associated with event-free survival. Our findings should be confirmed at the protein level, but we have been unable to do so in the current study despite substantial efforts, due to a lack of specific reagents to detect the ALK1 protein in human tissues (data not shown). Intriguingly, ALK1 expression was closely linked to prototypical endothelial cell marker genes, providing further evidence that the endothelium takes an active part as a key regulator of the metastatic process; an aspect of the vascular wall that has been highlighted also in recent studies of signaling pathways emanating from endoglin, CCL2/CCR2 and HIF1α/β in the tumor endothelium (16, 29, 30). Signaling by TGFβ in malignant cells promotes many aspects of the metastatic process, most notably migration and invasion, through induction of EMT (31). We recently demonstrated that the action of TGFβ on the vasculature weakens the endothelial cell barrier to tumor cell invasivation, thus endorsing malignant cell escape from the primary site into the bloodstream through an analogous mesenchymal transition of endothelial cells (16). Hence, the mechanism behind the antimitastatic effect of single-agent ALK1-Fc conceivably involves sealing the endothelial cell barrier to cancer cell transmigration, thereby confining malignant cells within the primary tumor. Furthermore, combined neoadjuvant treatment with RAP-041 and docetaxel eradicated the vast majority of pulmonary metastases. Docetaxel treatment gives rise to a well-documented reduction in vessel area (32), and the synergistic interaction with ALK1 inhibition is, thus, likely to take place at the level of the tumor endothelium.

Taken together, our mechanism-based therapeutic studies, combined with gene-expression analysis of patient specimens designed to investigate prometastatic factors, thus strongly support further development of ALK1-targeting agents, such as dalantercept and PF-03446962, as clinically tractable combination partners for chemotherapy to reduce the incidence of distant metastases in breast cancer.

Disclosure of Potential Conflicts of Interest
R. Kumar is a Chief Scientific Officer and has ownership interest (including patents) in Acceleron Pharma. K. Pietras has ownership interest in a patent pertaining to ALK1 antagonism held by the Ludwig Institute for Cancer Research Ltd and licensed to Acceleron Pharma. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S.I. Cunha, L. Lindstrom, R.S. Pearsall, J. Bergh, K. Pietras
Development of methodology: S.I. Cunha, P. Roswall
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.I. Cunha, M. Bocci, N. Eleftheriou, P. Roswall, E. Cordero, L. Lindstrom, M. Bartoschek, B.K. Haller, R.S. Pearsall, R. Kumar, J. Bergh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.I. Cunha, M. Bocci, J. Lovrot, N. Eleftheriou, L. Lindstrom, M. Bartoschek, B.K. Haller, A.W. Mulivor, C. Larsson, K. Pietras
Writing, review, and/or revision of the manuscript: S.I. Cunha, M. Bocci, J. Lovrot, L. Lindstrom, R.S. Pearsall, A.W. Mulivor, R. Kumar, K. Pietras
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.I. Cunha, P. Roswall, L. Lindstrom
Study supervision: S.I. Cunha, K. Pietras

Acknowledgments
The results presented herein are in part based upon data generated by the TCGA Research Network. http://cancergenome.nih.gov/.

Grant Support
Kristian Pietras is the Goran & Birgitta Grosskopf Professor at Lund University. This research is supported by a Consolidator Grant from the European Research Council (the TUMORGAN project), the Swedish Research Council, the Swedish Cancer Society, the STARGET consortium (a Swedish Research Council Linnaeus network), BioCARE and Lund University. The research group of Jonas Bergh is supported by the Swedish Cancer Society, BRECT, Karolinska Institutet and Stockholm County Council, the research funds at Radiumhemmet, Karolinska Institutet & Karolinska University Hospital.

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Received December 19, 2014; revised March 17, 2015; accepted April 1, 2015; published online June 15, 2015.

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Cunha et al.


Correction: Endothelial ALK1 Is a Therapeutic Target to Block Metastatic Dissemination of Breast Cancer

In this article (Cancer Res 2015;75:2445–56), which appeared in the June 15, 2015, issue of Cancer Research (1), the authors present translational studies supporting a causal link between the expression of the endothelial cell–expressed TGFβ family receptor ALK1 and metastatic dissemination of breast cancer. For a subsection of their studies, the authors utilized data from gene expression analysis of patient samples from a clinical cohort designed as a nested case–control study (data presented in Table 1). In subsequent follow-up studies, the authors have uncovered a potential bias in this dataset. Importantly, however, the analyses included in the article are unaffected, and the conclusions of the work are not in question.

As background, a metastatic breast cancer cohort study was first designed (2). Thereafter, a case–control study nested in the corresponding primary breast cancer cohort was designed by selecting distant metastasis-free controls to each case. Tumor RNA was extracted in the same order. All RNAs were profiled on microarrays in randomized order. For quality control, RNA was also reextracted in a randomized order for randomly selected cases–controls sets and profiled with the rest. The potential bias of the data from the nested case–control study is due to apparent RNA extraction batch effects confounded with case–control status. Reassuringly, gene expression data for endothelial ALK1 are consistent for a substudy in which RNA has been reextracted from a new tumor piece in a randomized order.

The correlation between gene expression data for original and reextracted RNA is excellent for key breast cancer genes, for example, ESR1 (r = 0.95) and ERBB2 (r = 0.96). Bridging the primary comparison, case–control set differences (n = 40) for ACVRL1 and the ACVRL1:endothelial metagene index that we reported are consistent between the two extractions (Fig. 1). A case–control set difference is the value for the case minus the (average) value of the matched control(s).

Although the potential bias of the dataset does not affect the outcome of the current study, the authors recommend careful scrutiny of the data and inclusion of proper controls when attempting other analyses based on these data. Microarray data for the reextracted RNA are deposited at the Gene Expression Omnibus database under accession number GSE81954.

All authors have been informed of and agree to this correction.

Figure 1.
Case–control set differences in the original RNA extraction and in the reextracted RNA confirms a close correlation between datasets for both ACVRL1 gene expression (left) and for the ACVRL1:endothelial metagene index (right).
References

Published online October 14, 2016.
doi: 10.1158/0008-5472.CAN-16-2220
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Endothelial ALK1 Is a Therapeutic Target to Block Metastatic Dissemination of Breast Cancer

Sara I. Cunha, Matteo Bocci, John Lövrot, et al.

Cancer Res 2015;75:2445-2456.

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