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Paauwe, M.; Heijkants, R.C.; Oudt, C.H.; Pelt, G.W. van; Cui, C.; Theuer, C.P.; ... ;
Hawinkels, L.J.A.C.

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ORIGINAL ARTICLE

Endoglin targeting inhibits tumor angiogenesis and metastatic spread in breast cancer

M Paaue^{1,2}, RC Heijkants¹, CH Oudt¹, GW van Pelt³, C Cui¹, CP Theuer⁴, JCH Hardwick², CFM Sier^{2,3} and LJAC Hawinkels^{1,2}

Endoglin, a transforming growth factor- β co-receptor, is highly expressed on angiogenic endothelial cells in solid tumors. Therefore, targeting endoglin is currently being explored in clinical trials for anti-angiogenic therapy. In this project, the redundancy between endoglin and vascular endothelial growth factor (VEGF) signaling in angiogenesis and the effects of targeting both pathways on breast cancer metastasis were explored. In patient samples, increased endoglin signaling after VEGF inhibition was observed. *In vitro* TRC105, an endoglin-neutralizing antibody, increased VEGF signaling in endothelial cells. Moreover, combined targeting of the endoglin and VEGF pathway, with the VEGF receptor kinase inhibitor SU5416, increased antiangiogenic effects *in vitro* and in a zebrafish angiogenesis model. Next, in a mouse model for invasive lobular breast cancer, the effects of TRC105 and SU5416 on tumor growth and metastasis were explored. Although TRC105 and SU5416 decreased tumor vascular density, tumor volume was unaffected. Strikingly, in mice treated with TRC105, or TRC105 and SU5416 combined, a strong inhibition in the number of metastases was seen. Moreover, upon resection of the primary tumor, strong inhibition of metastatic spread by TRC105 was observed in an adjuvant setting. To confirm these data, we assessed the effects of endoglin-Fc (an endoglin ligand trap) on metastasis formation. Similar to treatment with TRC105 in the resection model, endoglin-Fc-expressing tumors showed strong inhibition of distant metastases. These results show, for the first time, that targeting endoglin, either with neutralizing antibodies or a ligand trap, strongly inhibits metastatic spread of breast cancer *in vivo*.

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INTRODUCTION

Breast cancer is the second most common form of cancer globally, with over 1.5 million diagnosed patients in 2012.¹ Primary treatment for breast cancer patients is surgical removal of the tumor, complemented with adjuvant therapies like (chemo-) radiation or antiangiogenic therapies. The formation of a vascular network, called angiogenesis, is crucial for solid tumors to sustain oxygen and nutrient supply, but also indispensable for their metastatic spread.^{2,3} Therefore, antiangiogenic therapies have been developed for the treatment of solid cancers. Vascular endothelial growth factor (VEGF) is one of the major players in the induction of angiogenesis and therefore many antiangiogenic agents have been developed against VEGF.⁴ Although preclinical data of the first VEGF-neutralizing antibody bevacizumab were promising, the efficacy in the clinic was limited, leading to withdrawal of the FDA (Food and Drug Administration) approval as adjuvant therapy for breast cancer.⁵ In addition to antibodies, kinase inhibitors like SU5416 (Semaxanib), a selective VEGF receptor tyrosine kinase inhibitor, have been developed. SU5416 was used as an adjuvant treatment in patients with advanced colorectal cancer, but the phase-III trial was halted because of a lack of clinical benefit (NCT00021281). A phase-I clinical trial for SU5416 in inflammatory breast cancer has been completed in 2010, but no results have been reported thus far (NCT00005822). The limited clinical benefit of antiangiogenic therapies is most likely caused by therapy resistance,⁶ characterized by an initial response to treatment followed by patient relapse. This can be

caused by upregulation of alternative proangiogenic signaling pathways,⁷ for example, the endoglin/transforming growth factor- β (TGF- β) pathway.

Endoglin is a co-receptor for TGF- β and bone morphogenetic protein-9 (BMP-9) and is highly expressed on angiogenic endothelial cells.^{8–10} Endoglin is indispensable for developmental angiogenesis as has been shown by embryonic lethality of endoglin knockout mice.^{11–13} In cancer, previous studies have shown a clear correlation between endoglin microvessel density and patient survival.^{14–17} BMP-9 and TGF- β can, upon binding to endoglin, induce phosphorylation of downstream Smad proteins, inducing an angiogenic phenotype.¹⁸ TRC105 is a chimeric IgG1 monoclonal antibody that binds human endoglin with high affinity and is currently being tested in clinical trials as antiangiogenic therapy.¹⁹ TRC105 prevents BMP-9 binding to endoglin and induces an antibody-dependent cytotoxicity response leading to apoptosis of endoglin-expressing cells.²⁰ Results of a phase-I clinical trial showed safety of the antibody in patients with various solid cancers, and in two patients even clinical benefit was observed for a prolonged period of time.¹⁹ Recently, a phase-IB clinical trial was completed showing that the combination of TRC105 and bevacizumab was safe and even resulted in reduced tumor volumes in some bevacizumab-resistant patients.²¹

In the present study, we have assessed the inhibition of angiogenesis by simultaneous targeting of the endoglin and VEGF pathway. *In vitro* and *in vivo* angiogenesis models and an

¹Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands; ²Department of Gastroenterology-Hepatology, Leiden University Medical Center, Leiden, The Netherlands; ³Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands and ⁴Tracon Pharmaceuticals, San Diego, CA, USA. Correspondence: Dr LJAC Hawinkels, Department of Molecular Cell Biology and Gastroenterology-Hepatology, Leiden University Medical Center, Building-1, C4-P, PO Box 9600, Leiden 2300 RC, The Netherlands.

E-mail: L.J.A.C.Hawinkels@LUMC.nl

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orthotopic breast cancer mouse model show increased inhibition of angiogenesis by combining TRC105 and SU5416. Even more striking, endoglin targeting, using a neutralizing antibody or ligand trap, strongly inhibits the metastatic spread of breast cancer cells.

RESULTS

Anti-VEGF treatment in patients increases endothelial Smad1 phosphorylation

First, the potential redundancy between the endoglin and VEGF pathway in angiogenesis was examined. We had access to a small

cohort of rectal cancer patients treated in a neoadjuvant setting with anti-VEGF therapy (bevacizumab), providing us with pre-treatment biopsies and post-treatment resection specimens. Tissue sections from patients treated with bevacizumab were stained for phosphorylated Smad1 (pSmad1), the major signaling molecule downstream of endoglin. Pretreatment biopsies (Figure 1a, upper panel) showed limited staining for pSmad1 in the endothelial nuclei compared with the resection specimen from the same patient after bevacizumab, where almost all endothelial nuclei showed strong pSmad1 accumulation (Figure 1a, lower panel). To exclude that this observation was due to the difference of biopsies versus resection specimens, a

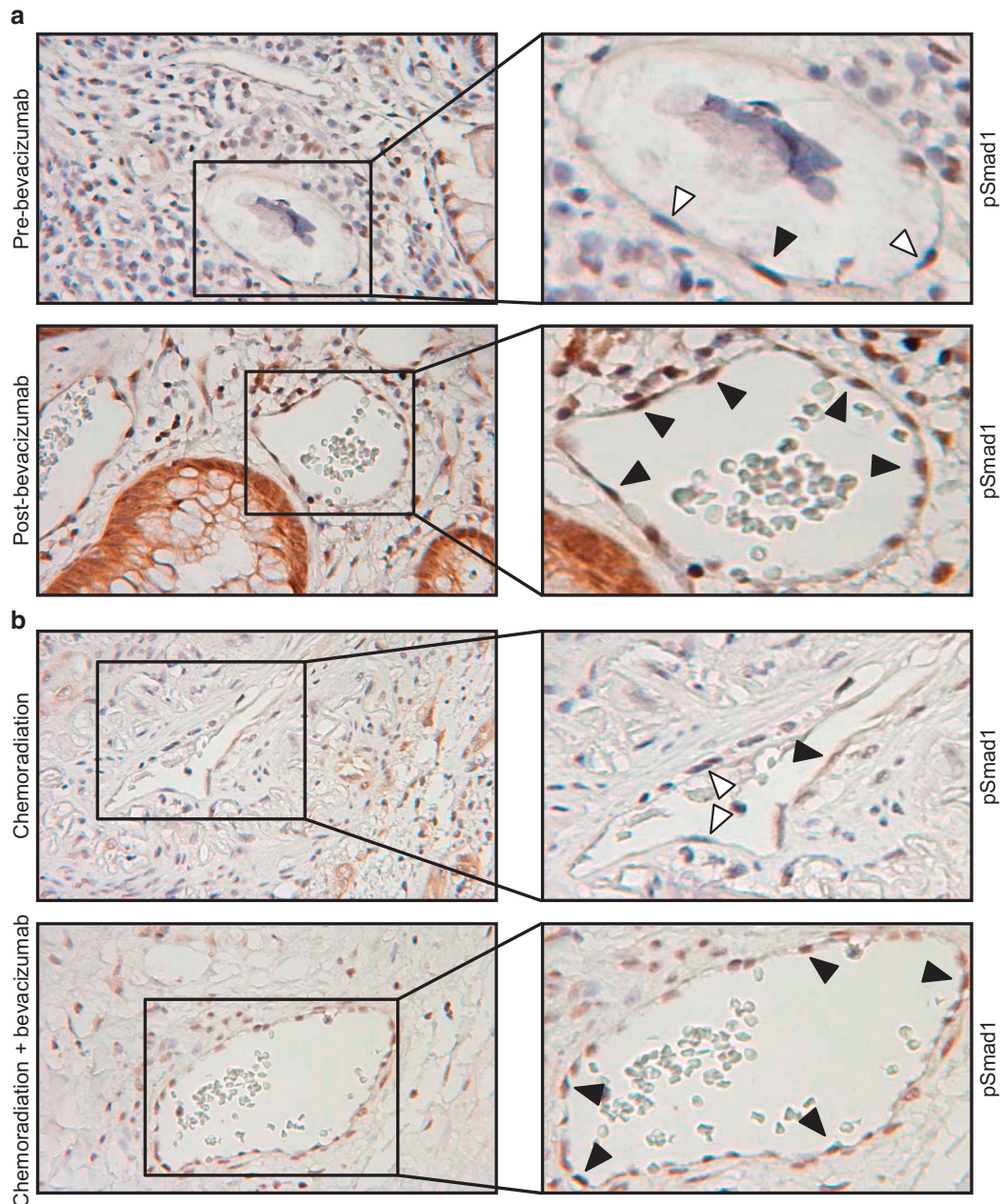


Figure 1. Anti-VEGF treatment results in increased Smad1 phosphorylation. **(a)** Immunohistochemical staining for pSmad1 revealed increased endothelial nuclear pSmad1 in tissue samples from patients treated with bevacizumab (lower panel) compared with pretreatment tumor biopsies (upper panel). **(b)** Treatment with bevacizumab in combination with chemoradiation therapy (lower panel) increased pSmad1 in endothelial nuclei compared with chemoradiation treatment only (upper panel). White arrowheads indicate pSmad1-negative nuclei, and black arrowheads indicate pSmad1-positive nuclei. Magnification $\times 200$, right panels $\times 400$.

group of patients who only received chemoradiation therapy (standard of care, Figure 1b, upper panel) was compared with a group receiving both chemoradiation therapy and bevacizumab (Figure 1b, lower panel). These data confirmed increased nuclear accumulation of pSmad1 in endothelial cells upon anti-VEGF therapy. These results indicate that, upon VEGF inhibition, alternative pathways like the endoglin pathway seem to be upregulated in cancer patients.

Endoglin and VEGF pathways are connected *in vitro*

Next, we examined whether redundancy could also be observed between endoglin and VEGF pathways *in vitro*. Human umbilical vein endothelial cells (HUVECs) were stimulated with VEGF or BMP-9, the main endoglin ligand. Treatment with BMP-9 induced rapid Smad1 phosphorylation that could transiently be inhibited by TRC105 (Figure 2a). In addition to inhibiting Smad1 phosphorylation, TRC105 increased phosphorylation of the anti-angiogenic Smad2 (Figure 2b, left panel and Supplementary Figure S1A) similar to stimulation with 5 ng/ml TGF- β (Figure 2b, right panel). Smad2 phosphorylation was dependent on ALK-5 (activin receptor-like kinase-5) kinase activity, as treatment with the kinase inhibitor SB431542 abrogated TRC105-induced Smad2 phosphorylation (Figure 2b, right panel). These data indicate a shift toward an antiangiogenic response by TRC105. VEGF induced rapid extracellular signal-regulated kinase (ERK) phosphorylation in HUVECs that could be inhibited by SU5416 (Figure 2c). Interestingly, when HUVECs were treated with TRC105 and subsequently stimulated with VEGF, we observed increased ERK phosphorylation as compared with VEGF stimulation without TRC105 pretreatment (Figure 2d and Supplementary Figure S1B). This did not seem to be due to increased VEGFR2 Tyr1175 phosphorylation (Supplementary Figure S1C). These results suggest a connection between the endoglin and VEGF signaling pathways in endothelial cells.

Combined endoglin and VEGF targeting inhibits angiogenesis

If there is redundancy between the endoglin and VEGF pathways, simultaneous inhibition of both pathways could result in increased

and sustained antiangiogenic responses. Therefore, we assessed the effects of TRC105, SU5416 and combination treatment on *in vitro* proliferation, migration and angiogenesis. TRC105 did not significantly affect HUVEC proliferation, whereas both SU5416 and the combination treatment resulted in statistically significant inhibition of HUVEC proliferation (Figure 3a). Under serum-free conditions (Figure 3b), or serum-free medium supplemented with either VEGF or BMP-9 (Supplementary Figure S2), we observed the same inhibitory effects on proliferation of HUVECs by SU5416 and combination treatment, although more pronounced. In contrast to BMP-9, VEGF enhanced HUVEC proliferation (Figure 3c), underlining the importance of VEGF for endothelial cell proliferation *in vitro*.

As next to proliferation, migration of endothelial cells plays an additional role in angiogenesis, the cord-forming ability of HUVECs in the presence of the inhibitors was assessed. First, we optimized the percentage of fetal calf serum in the medium for this assay, shown to be 5% (Supplementary Figure S3A). TRC105 did not significantly inhibit the number of closed polygons (Figure 3d), or the number of branches per branchpoint (Supplementary Figure S3B), whereas SU5416 strongly inhibited both read-outs of the angiogenic potential. Without further decreasing the number of closed polygons, additional effects of TRC105/SU5416 treatment on the cord morphology and integrity of the remaining cords were observed. The cell aggregates that were formed were less dense and appeared to disintegrate faster than in the other conditions. Next, angiogenic potential in a more complex three-dimensional spheroid model for angiogenesis was assessed. HUVEC spheroids were embedded in the presence of the inhibitors, stimulated and average sprout length was determined (Supplementary Figure S3C). TRC105 did not affect endothelial sprouting (Figure 3e), confirming the results of the cord formation assay. A trend towards decreased average sprout length was observed for SU5416, whereas combination treatment resulted in statistically significant decreased HUVEC sprouting (Figure 3e). These data indicate that endothelial sprouting is more efficiently inhibited by a TRC105/SU5416 combination.

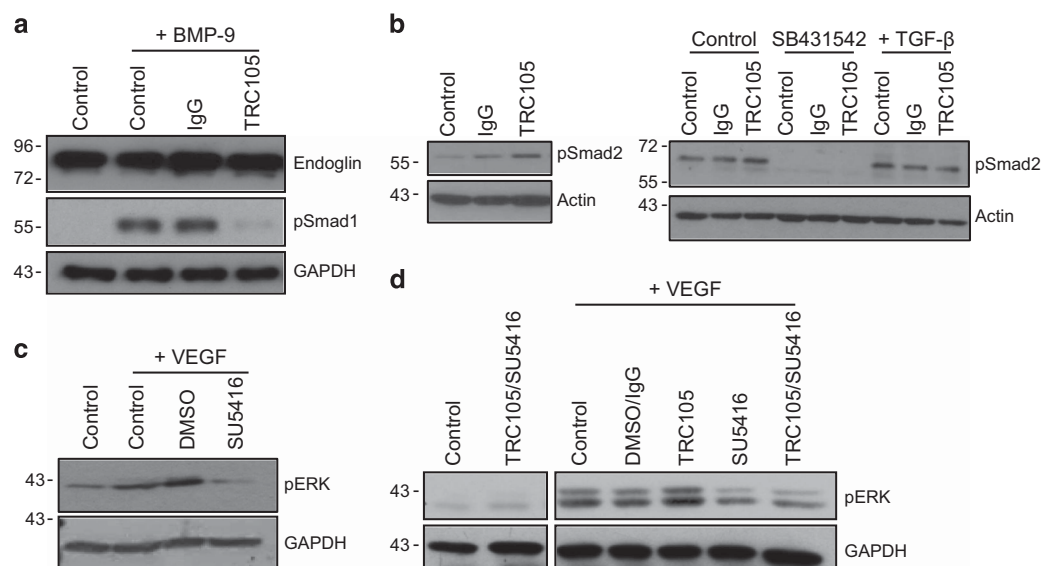


Figure 2. Redundancy between endoglin and VEGF signaling in HUVECs *in vitro*. (a) BMP-9 stimulation (10 min) induced strong pSmad1 in HUVECs, inhibited by TRC105. (b) Overnight treatment with TRC105 increased pSmad2 (left panel) to a comparable level as induced by TGF- β (1 h). This effect was completely blocked by the ALK-5 kinase inhibitor SB431542 (right panel). (c) VEGF stimulation (10 min) increased ERK1/2 phosphorylation (pERK) that can be inhibited by SU5416. (d) Pretreatment with TRC105 increased VEGF-induced pERK that can be reverted by SU5416 or TRC105/SU5416 combination therapy. All western blots are representative of three independent experiments.

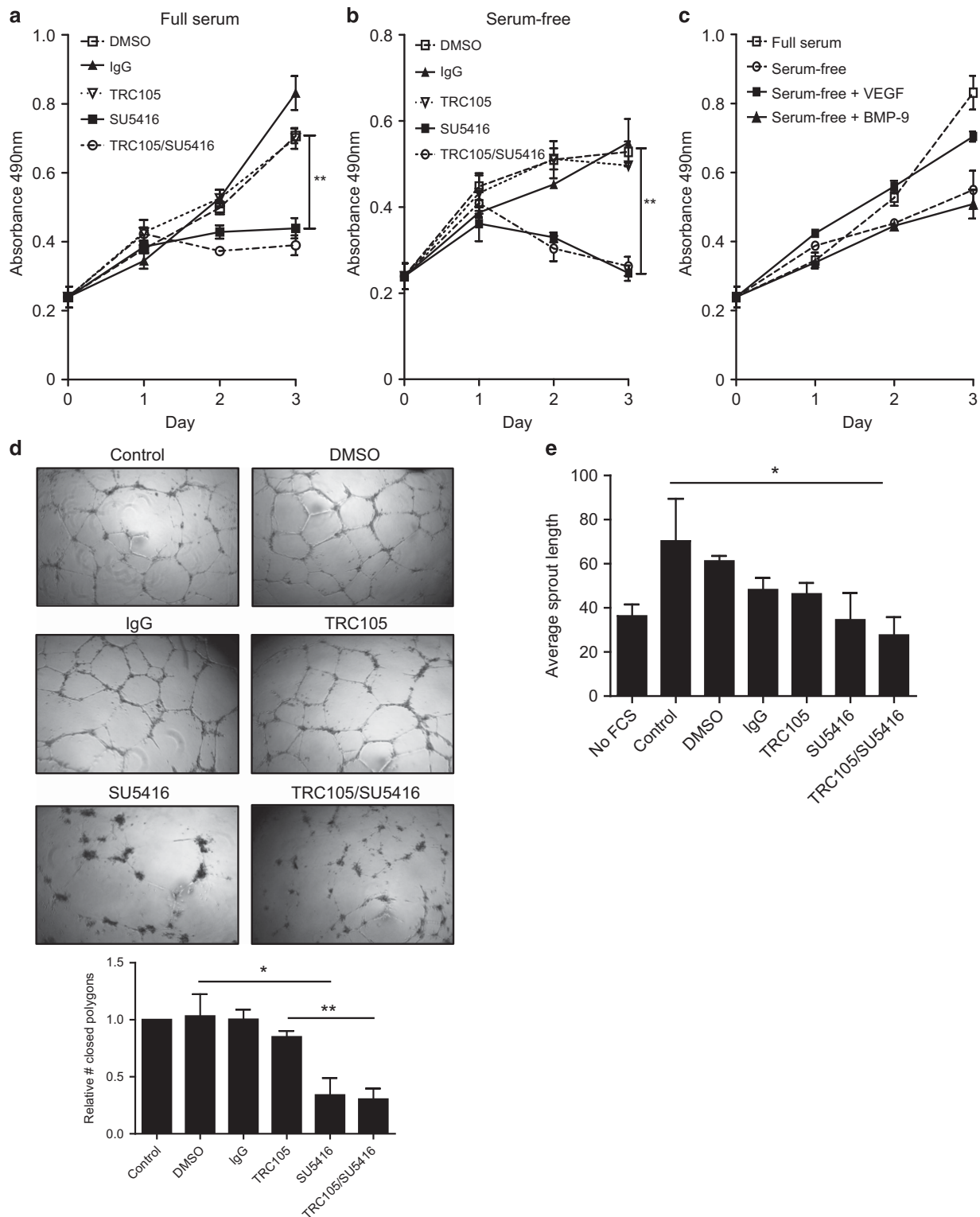


Figure 3. TRC105/SU5416 combination therapy enhances antiangiogenic effects. TRC105 does not inhibit HUVEC proliferation in an MTS proliferation assay, whereas SU5416 and TRC105/SU5416 combination significantly decreased proliferation in both full serum (**a**) and serum-free conditions (**b**). VEGF, either supplemented or BPE derived, stimulated HUVEC proliferation, whereas BMP-9 did not show an effect (**c**). Data represent mean of two independent experiments performed in triplicate. (**d**) HUVEC endothelial cells were seeded on matrigel and allowed to form endothelial cords. Endothelial cord-forming capacity of HUVECs was assessed after overnight incubation with the inhibitors. TRC105 did not affect cord formation, whereas SU5416 treatment decreased the number of closed polygons. TRC105/SU5416 combination enhanced these effects on cord morphology, but not on the number of closed polygons. Data are normalized to control and represent mean of three independent experiments performed in triplicate. (**e**) In three-dimensional HUVEC sprouting assays, sprouting of HUVECs was not affected by TRC105, whereas SU5416 and especially the TRC105/SU5416 combination significantly reduced sprouting. Data represent mean of three independent experiments performed in triplicate. * $P \leq 0.05$, ** $P \leq 0.01$.

TRC105/SU5416 combination enhances angiogenesis inhibition in zebrafish

To further study the effect of inhibiting VEGF and endoglin in a multicellular model for angiogenesis, we used Fli:eGFP zebrafish, in which all blood vessels express green fluorescent protein (GFP). First, we analyzed whether TRC105, raised against human endoglin, was able to bind zebrafish endoglin. Human IgG (negative control) and TRC105 were fluorescently labeled and injected into zebrafish embryos. At 2 days after injection, TRC105 (red) colocalized with the GFP-expressing zebrafish endothelium, whereas no red fluorescent signal was observed in the IgG control (Figure 4a), implying that TRC105 binds to the zebrafish endothelium. Next, we determined the antiangiogenic effects of combination treatment. Zebrafish embryos were injected with TRC105, whereas SU5416 was applied via the fish water. At 2 days

after treatment, the phenotype of the subintestinal vessels was scored as either normal or mildly or severely affected (Figure 4b). Both TRC105 and SU5416 monotherapy increased the frequency of mainly the severe phenotype (from 4.4 to 9.1% (TRC105) and 9.0% (SU5416), Figure 4b), whereas normal vessel development was decreased further after treatment with the TRC105/SU5416 combination (from 81.6 to 61.2%, Figure 4b). These data show increased inhibition of angiogenesis in zebrafish embryos with combined endoglin and VEGF targeting.

Combined targeting of the endoglin and VEGF pathway inhibits tumor angiogenesis *in vivo*

Next, we evaluated the effects of TRC105/SU5416 combination treatment in a mouse model for invasive lobular breast cancer.

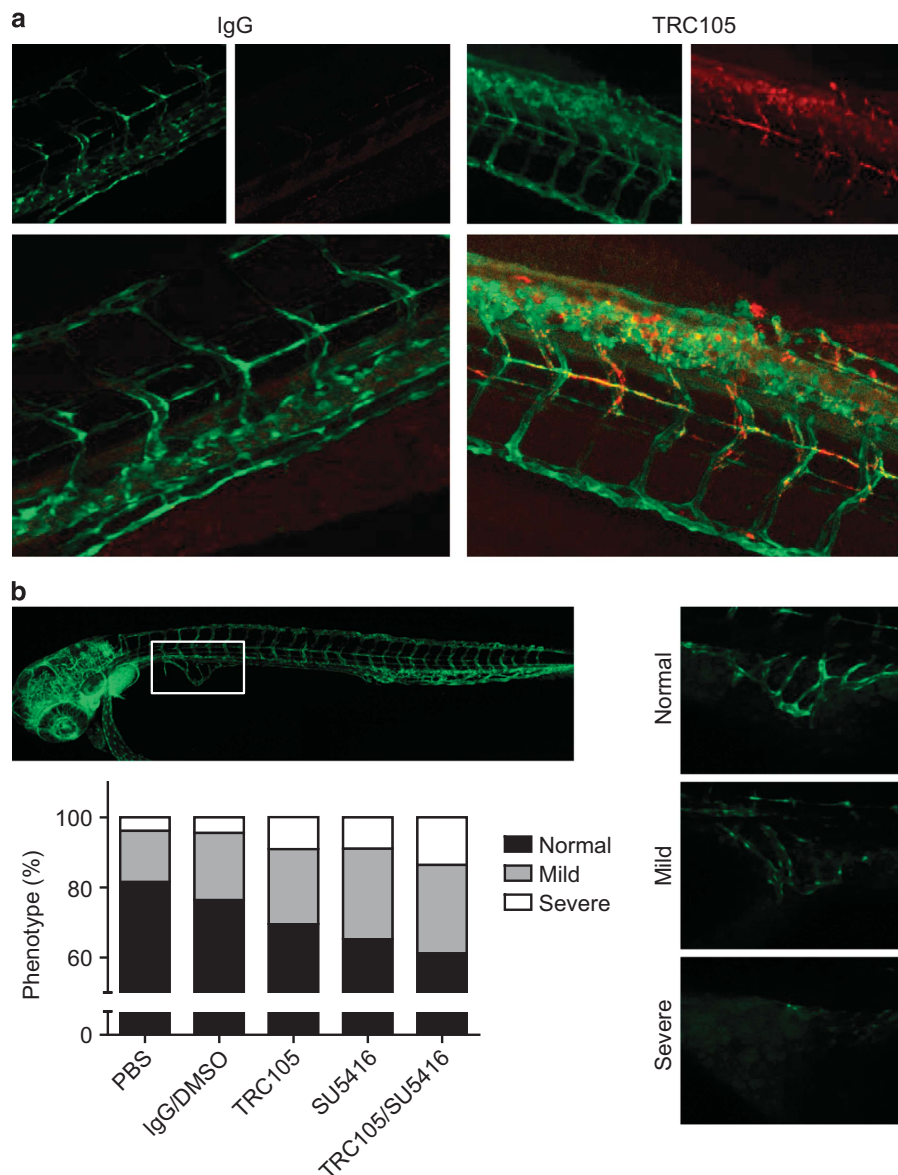


Figure 4. TRC105/SU5416 treatment inhibits angiogenesis in zebrafish. **(a)** The 2-day-old zebrafish embryos were injected with red fluorescently labeled human IgG or TRC105. TRC105 showed binding to GFP-expressing zebrafish vasculature (right panel), whereas the IgG control did not show any fluorescent signal (left panel). **(b)** Development of the subintestinal vessels (SIVs) was assessed after 2 days of treatment with either TRC105, SU5416 or the combination. The SIVs were scored as normal phenotype or mildly or severely affected. TRC105 and SU5416 both increased the affected phenotypes, whereas TRC105/SU5416 combination enhanced this effect even more. Data represent mean percentage of fish with the observed phenotype from three independent experiments ($n = 38\text{--}114$ fish/group/experiment).

Luciferase-expressing KEP1-11 mouse breast cancer cells²² were transplanted into the mouse mammary gland. Tumor growth and metastasis were followed by *in vivo* bioluminescent imaging. The

data showed that neither treatment with TRC105 or SU5416 nor combination treatment affected tumor volume assessed by luciferase signal (Figure 5a) or caliper measurement (Figure 5b).

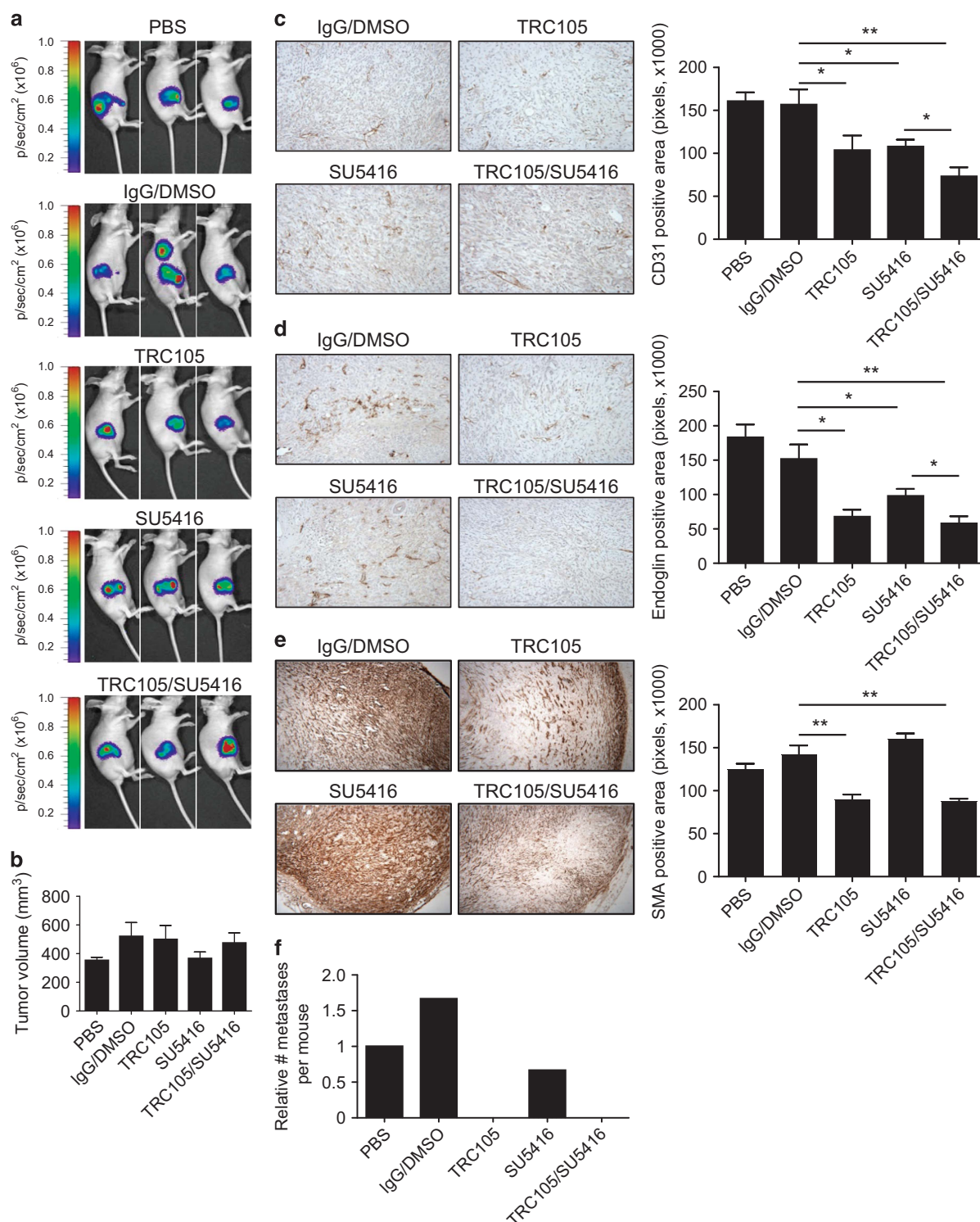


Figure 5. Combined targeting of endoglin and VEGF decreases tumor vascular density and metastatic spread of mouse breast cancer. KEP1-11 breast cancer cells were orthotopically transplanted and tumor growth was followed for 6 weeks using bioluminescent imaging (a) and caliper measurements (b). At the end of the experiment (6 weeks) tumor volume was not affected by the treatment, whereas tumor vascular density (CD31 (c) and endoglin (d) immunohistochemical staining) was strongly reduced upon TRC105 or SU5416 treatment, and even further decreased by TRC105/SU5416 combination. (e) Smooth muscle actin-positive (SMA+) stroma content of tumors was significantly reduced upon treatment with TRC105, either as monotherapy or in combination with SU5416 ($n = 4-6$ mice/group, average number of positive pixels). (f) Mice treated with TRC105, either as monotherapy or in combination with SU5416, show a decreased number of metastases (6 weeks after start of experiment, $n = 4-6$ mice/group). * $P \leq 0.05$, ** $P \leq 0.01$.

Treatment with TRC105 or TRC105/SU5416 combination increased VEGF expression in tumor homogenates (Supplementary Figure S4A), whereas VEGF plasma levels were only increased upon TRC105 treatment (Supplementary Figure S4B). Using quantitative PCR, enhanced endoglin expression was observed after SU5416 and combination treatment (Supplementary Figure S4C). Next, tumor vascularization was assessed by CD31 and endoglin staining. Vascular density was decreased by both TRC105 and SU5416, but the effects were significantly enhanced upon combination treatment (Figures 5c and d). Interestingly, the inhibitory effects of TRC105 on microvessel density determined by endoglin were stronger than determined by CD31, indicating specific targeting of endoglin-expressing vessels. Staining for smooth muscle actin-positive cancer-associated fibroblasts (CAFs) revealed strongly reduced numbers of CAFs only in the TRC105 and combination treatment groups (Figure 5e). Furthermore, a dramatic reduction in metastases was observed upon TRC105 monotherapy or in combination with SU5416 (Figure 5f). SU5416 monotherapy did not reduce metastatic spread. Taken together, these data show enhanced inhibition of tumor angiogenesis by a combination of TRC105 and SU5416. Importantly, TRC105 seems to have a strong inhibitory effect on metastatic spread.

TRC105 inhibits breast cancer metastasis

In order to more specifically assess the effects of TRC105 on tumor metastasis, we used a clinically relevant mouse model

of breast cancer metastasis. At 4 weeks after transplantation, the primary tumor was removed and mice received adjuvant treatment with TRC105 or human IgG (Figure 6a). At the time of resection, average tumor volumes were equal in both groups (data not shown). Complete resection of the primary tumor was confirmed by bioluminescent imaging. At 6 weeks after resection, bioluminescent imaging showed a high number of metastases in the IgG control group, and this was strongly reduced in the TRC105-treated group (Figure 6b). The percentage of mice bearing metastases in the TRC105 treatment group (28.3%) was strongly decreased when compared with the IgG control group (75%, Figure 6c). Microvessel density in the metastases did not differ between the treatment groups, although only a low number of metastases could be evaluated (data not shown). In addition, morphology of the primary tumor and metastases was similar in both treatment groups (Supplementary Figure S5). Bioluminescent signals in the TRC105-treated mice were derived from small lesions not invading the underlying tissue at the location of the primary tumor, whereas metastases in the IgG treatment group were found mainly in draining lymph nodes, bone and peritoneal wall. Only 4 out of 12 mice treated with TRC105 showed distant metastases, resulting in significantly improved metastasis-free survival of this group (Figure 6d). These data indicate that adjuvant treatment with TRC105 prevents the development of distant breast cancer metastases.

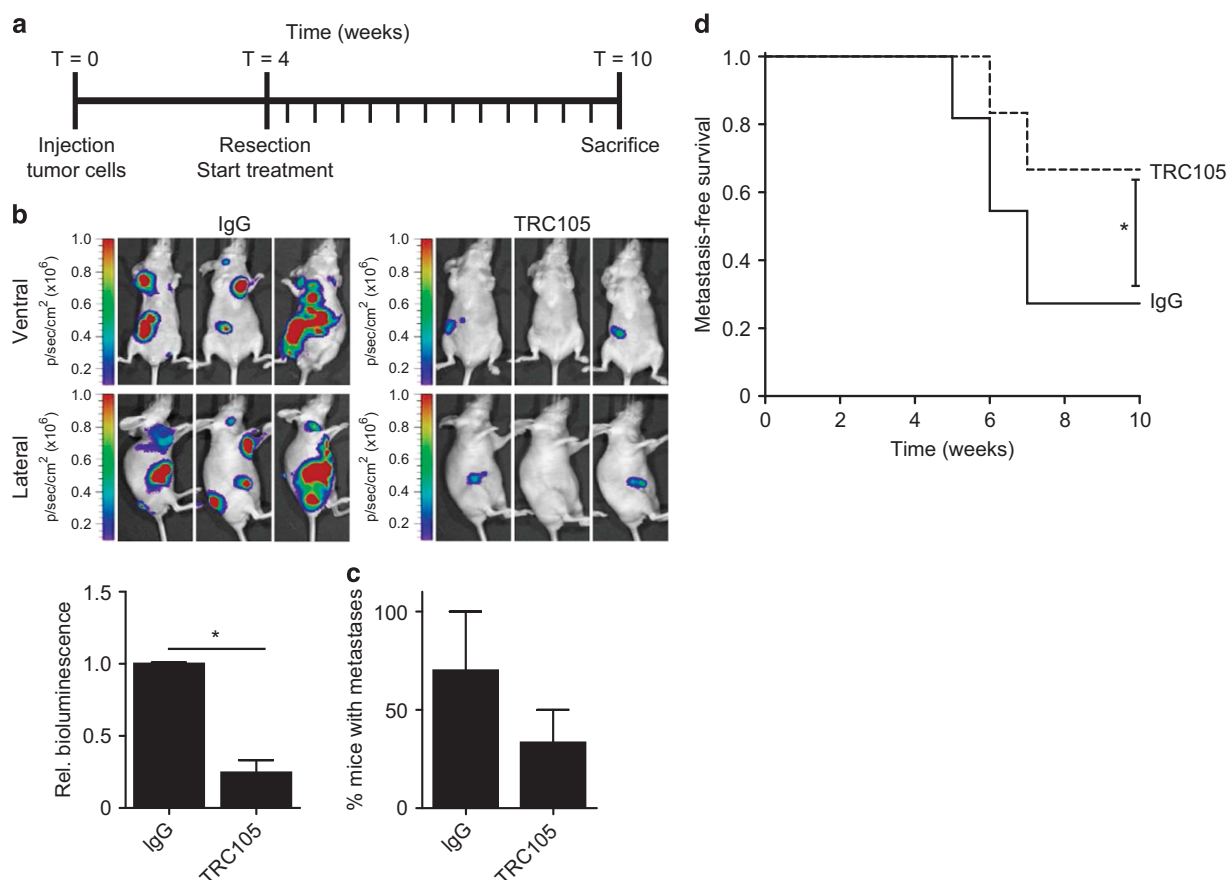


Figure 6. TRC105 treatment inhibits breast cancer metastasis in adjuvant setting. **(a)** Experimental set-up. At 4 weeks after tumor transplantation, primary tumors were resected and mice were treated with human IgG or TRC105 for 6 additional weeks. **(b)** Metastasis formation was followed over time using bioluminescence. Mice treated with TRC105 showed significantly less metastatic lesions when compared with the IgG control group ($n = 11$ – 12 mice/group), decreasing the percentage of mice with metastases to 28.3% as compared with 75% in the control mice **(c)**. This results in significantly improved metastasis-free survival of mice treated with TRC105 compared with IgG treatment group **(d)**. Graphs represent mean of 11–12 mice/group from two independent experiments. * $P \leq 0.05$.

Endoglin targeting using endoglin-Fc decreases metastatic spread To confirm that inhibition of metastasis is indeed caused by targeting endoglin, an alternative approach was used, using an endoglin ligand trap. KEP1-11 cell lines stably expressing either endoglin-Fc or control Fc (Supplementary Figure S6A) were orthotopically transplanted and tumor growth was monitored using bioluminescence and caliper measurements. Comparable to TRC105, endoglin-Fc expression did not affect tumor volume (Supplementary Figures S6B and C), whereas endoglin-Fc strongly decreased primary tumor vascularization (Supplementary Figure S6D). Interestingly, like treatment with TRC105, mice with endoglin-Fc-expressing tumors exhibited a reduced number of metastases (Supplementary Figure S6E).

To assess the effects of endoglin-Fc after primary tumor resection, tumor cells expressing Fc or endoglin-Fc were orthotopically transplanted and allowed to grow for 4 weeks. Primary tumors were removed and mice were monitored for an additional 6 weeks (Figure 7a). Upon resection, the number of smooth muscle actin-positive CAFs in the primary tumors was significantly lower in the endoglin-Fc group compared with the Fc control group (Figure 7b). At the end of the experiment,

bioluminescent signals in the endoglin-Fc group were significantly reduced as compared with the Fc group (Figure 7c). Expression of endoglin-Fc resulted in decreased metastatic spread and therefore improved metastasis-free survival (Figure 7d). In addition, the percentage of mice showing distant metastases decreased by >50% in the mice bearing endoglin-Fc-expressing tumors (Figure 7e). Taken together, our results show that targeting endoglin, by TRC105 or endoglin-Fc, strongly decreases the metastatic spread of mouse breast cancer cells *in vivo* and opens possibilities to explore adjuvant TRC105 treatment in breast cancer patients.

DISCUSSION

In this study we show that targeting endoglin, using an antibody or ligand trap, decreases metastatic spread of breast cancer *in vivo*. Combined targeting of the endoglin and VEGF pathway results in decreased angiogenic capacity of human endothelial cells *in vitro*, in a zebrafish model for angiogenesis and in a mouse model for invasive breast cancer.

In vitro, TRC105 did not inhibit the angiogenic capacity of HUVECs. Contrary to our observations, Liu *et al.*²³ described a

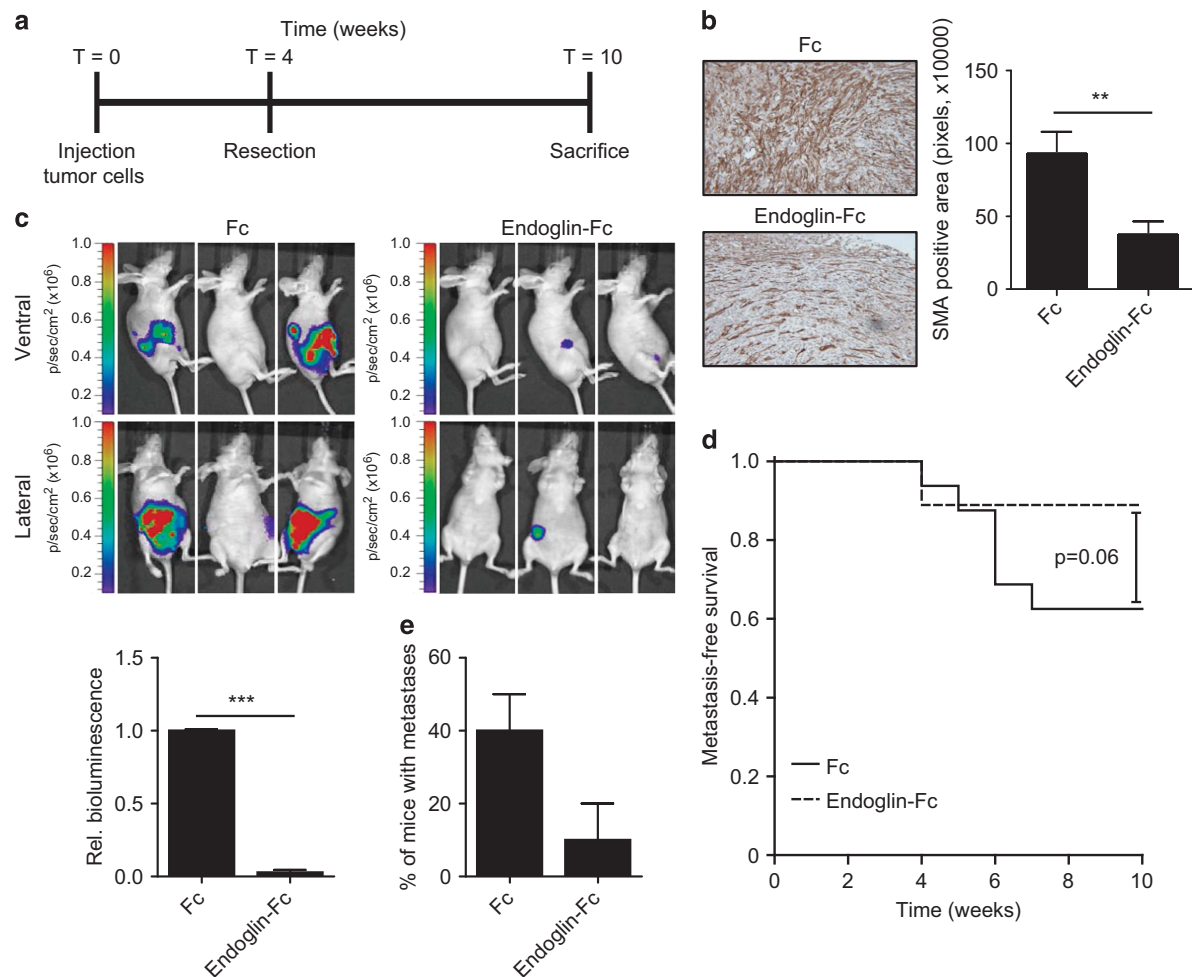


Figure 7. Endoglin-Fc expression decreases metastatic spread of breast cancer cells. **(a)** Experimental set-up. At 4 weeks after tumor transplantation of cell lines stably expressing Fc or Endoglin-Fc, tumors were resected and metastatic spread was followed for 6 additional weeks. **(b)** Smooth muscle actin-positive (SMA+) stroma content of the primary tumors was strongly decreased by tumoral endoglin-Fc expression when compared with the Fc group. **(c)** Bioluminescent imaging showed significant reduction of metastatic spread in the endoglin-Fc group when compared with Fc-expressing tumors ($n=8$ mice/group). **(d)** Expression of endoglin-Fc by the tumor results in improved metastasis-free survival. **(e)** The percentage of mice showing distant metastases was decreased in the endoglin-Fc group by >50% compared with Fc. Graphs represent mean of eight mice/group from two independent experiments. ** $P \leq 0.01$, *** $P \leq 0.001$.

significant decrease of HUVEC tube formation, migration and proliferation upon treatment with 100 µg/ml TRC105, a much higher dose than used in this study. In the same study, additional effects of TRC105 treatment combined with the anti-VEGF agent bevacizumab were reported, supporting our data on redundancy between the endoglin and VEGF signaling pathways.²⁴ The limited effects of TRC105 observed *in vitro* can be explained by the working mechanism of TRC105. Additional to preventing BMP-9 binding to endoglin,²⁰ TRC105 induces antibody-dependent cell-mediated cytotoxicity, requiring immune cells. Therefore, the presence of an intact immune system increases TRC105 efficacy²⁵ and might lead to enhanced effects.

Although xenograft studies showed very promising results for antiangiogenic therapy and initial responses were observed, patients relapse and actual clinical benefits are limited. In this study we show increased endothelial phosphorylated Smad1 (indicating endoglin signaling) in tumor tissues of cancer patients treated with bevacizumab. In a study on circulating protein biomarkers in TRC105-treated patients, the authors reported that certain angiogenic biomarkers, like VEGF, were significantly downregulated shortly after starting TRC105 treatment, indicating inhibition of multiple angiogenic pathways.²⁶ However, at the end of the study, the same proangiogenic factors were upregulated, implying a switch to alternative angiogenic pathways. Therefore, combined targeting of the endoglin and VEGF pathway could result in enhanced and sustained inhibition of angiogenesis. Indeed, in our breast cancer model, tumor angiogenesis was more strongly inhibited by TRC105/SU5416 combination therapy that unfortunately did not result in decreased tumor volumes. Treatment with bevacizumab can result in vessel normalization.^{27,28} Leaky and irregular tumor vessels partly disappear, but the remaining vessels gain 'normal' morphology and pericyte coverage.²⁹ This results in increased blood and nutrient supply to the tumor, sustaining tumor growth, and also enhanced effects of chemotherapy on breast cancer as we could recently show for antiangiogenic therapy targeting ALK-1.³⁰

Recently, the first clinical trial assessing TRC105 and bevacizumab combination in patients has been published.²¹ Of the 38 patients enrolled in this trial, 18 patients showed at least a partial response to treatment. In all, 14 patients had decreased overall tumor burden, of which 10 patients progressed earlier while receiving anti-VEGF treatment, but still responded to TRC105 therapy, suggesting that combination therapy could circumvent therapy resistance. Side effects of combined targeting were limited and not observed more often than with single drug treatment,²¹ opening doors for clinical use. In addition, a phase-I clinical trial in prostate cancer patients showed almost no classic antiangiogenesis adverse effects upon TRC105 monotherapy.³¹ The safety and clinical efficacy of combined TRC105 and anti-VEGF treatment is now being explored in patients with recurrent glioblastoma multiforme (NCT01648348), advanced renal cell cancer (NCT01727089) and other solid tumors (NCT01975519, NCT01306058, NCT01806064).

One of the key findings in this study was the strong inhibition of breast cancer metastasis by endoglin targeting, either by TRC105 or the ligand trap endoglin-Fc. In contrast to our observations, Anderberg *et al.*³² reported that endoglin heterozygous mice show increased liver metastasis in Rip-Tag mice. Endoglin heterozygote mice develop a hereditary hemorrhagic telangiectasia phenotype including vascular malformations³³ and increased vascular permeability,³⁴ possibly resulting in enhanced metastatic spread. When we treated endothelial monolayers with TRC105, transendothelial migration of KEP1-11 cells did not change compared with human IgG treatment (data not shown), suggesting that TRC105 does not directly affect endothelial permeability *in vitro*. Another study also showed decreased metastatic spread of prostate cancer in endoglin heterozygous mice.³⁵ These observations imply that both tumor type and method of

endoglin targeting could determine metastatic capacity of the cancer cells.

How endoglin mechanistically contributes to metastasis formation is subject of ongoing studies. As we used a model in which endoglin-Fc is expressed by the tumor cells and therefore present before the angiogenic switch, it is unlikely that decreased metastasis formation is solely due to inhibition of the angiogenic switch. Tumor vessel normalization could prevent tumor cell intravasation and thereby spreading throughout the body. KEP1-11 cells, in contrast to some other breast cancer cells *in vitro*,³⁶ hardly express endoglin or VEGFR2 (Supplementary Figure S7), and therefore direct targeting of the tumor cells in our model is unlikely. However, besides endothelial cells, endoglin is also expressed in other cells in the tumor microenvironment. For example, TRC105 can reduce the number of regulatory T cells in prostate tumors, implying a shift from the presence of regulatory T cells to cytotoxic T cells.³¹ In addition, macrophages can express endoglin,³⁷ in which it can direct macrophages in either a tumor-suppressive M1 or tumor-promoting M2 phenotype.³⁸

Our study revealed that treatment with TRC105 or expression of endoglin-Fc resulted in decreased smooth muscle actin-positive CAF content of the tumors (Figures 5e and 7b). The percentage of tumor stroma is known to be a prognostic marker in breast cancer.^{39,40} More specifically, CAFs have been shown to play an important role in breast cancer progression and metastasis (reviewed in Khamis *et al.*⁴¹). We and others³⁵ have shown that endoglin is expressed by CAFs in diverse types of cancer. A possible explanation for the observed effects could be that treatment with TRC105 or endoglin-Fc might decrease these endoglin-expressing CAFs that stimulate tumor growth and metastasis.

In a phase-I clinical trial, preliminary evidence of clinical activity on pre-existing metastases upon treatment with TRC105 was observed.¹⁹ A castrate-resistant prostate cancer patient showed markedly improved metastatic bone lesions upon TRC105 treatment. In another patient, with chemotherapy-resistant uterus carcinoma, a strong decrease in pulmonary metastases was observed after TRC105 treatment. These results, combined with our study showing that even in an adjuvant setting TRC105 could decrease metastatic dissemination, suggest a role for endoglin in initiating and sustaining distant metastases.

In conclusion, we have shown that dual targeting of the endoglin and VEGF pathway increases antiangiogenic responses *in vitro* and *in vivo* as compared with monotherapy. Most importantly, we show that targeting of endoglin, either using a neutralizing antibody or a ligand trap, inhibits the metastatic spread of breast cancer *in vivo*. In the light of ongoing trials, our data suggest a potential important role for targeting endoglin in breast cancer metastasis.

MATERIALS AND METHODS

Patient samples

Paraffin-embedded tissue samples were obtained from the Department of Surgery, Leiden University Medical Center. The patient population contained stage I–IV rectal cancer patients treated in a neoadjuvant setting with bevacizumab. Samples were presurgery tumor biopsies (*N* = 5) and tumor tissue obtained after surgery (*N* = 3). In addition, tumor tissue samples from patients treated with only chemoradiation therapy (*N* = 8) or a combination of chemoradiation and bevacizumab (*N* = 11) were evaluated. Patient characteristics and follow-up were recorded by the Department of Surgery. Samples were used according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Center.

Tissue analysis

Immunohistochemical stainings were performed as described before⁴² using antibodies against CD31 (sc-1506-R Santa Cruz Biotechnologies, Santa Cruz, CA, USA), endoglin (BAF1097 R&D Systems, Abington, UK) or phosphorylated Smad1 (9511 Cell Signaling Technologies, Danvers, MA,

USA). Four representative fields per tumor slide were photographed with a Leitz Diaplan microscope (Leitz, Wetzlar, Germany) and staining was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA; <http://imagej.nih.gov/ij/>). Enzyme-linked immunosorbent assay analysis for mouse VEGF was performed according to the manufacturer's instructions (DY493, R&D Systems). BMP-9 enzyme-linked immunosorbent assay and quantitative PCR analyses were performed as described before.^{43,44} Primers are described in Supplementary Table S1.

Cell culture and signaling assays

Primary HUVECs were cultured as described before.⁴⁵ P53/E-cadherin double-knockout mouse breast cancer cells (KEP1-11)²² were cultured as described before³⁰ and monthly tested for mycoplasma contamination.

3×10^5 HUVEC cells were seeded in gelatin-coated six-well plates. Upon 90% confluency, cells were serum starved overnight in medium containing either 40 μ g/ml TRC105 (Tacon Pharmaceuticals, San Diego, CA, USA) or 40 μ g/ml human IgG (BioXCell, West Lebanon, NH, USA). At 30 min before stimulation, 0.1% dimethyl sulfoxide (DMSO, as solvent control) or 1 μ M SU5416 (WuXi AppTec, Shanghai, China) was added, followed by a 10-min stimulation with either 0.1 ng/ml recombinant human BMP-9 (R&D Systems), 5 ng/ml TGF- β ,⁴⁶ or 50 ng/ml recombinant human VEGF (Peprotech, London, UK). For TGF- β signaling studies, HUVECs were starved overnight in the presence of TRC105, IgG, the ALK-5 inhibitor SB431542 (Tocris, Bristol, UK) or combinations. Next day, cells were stimulated with 5 ng/ml TGF- β . Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 2 mM EDTA, 1 mM NaVO₄, 10 mM NaF and 1 mM sodium orthovanadate (BDH Laboratory, Poole Dorset, UK)), protein content was determined and western blot analysis was performed as described before.⁴⁴ Membranes were incubated overnight with primary antibodies against endoglin (R&D Systems), phosphorylated Smad1, phosphorylated Smad2, phosphorylated VEGFR2 Tyr1175 (9511, 3108, 2478, all from Cell Signaling Technologies) or phosphorylated ERK1/2 (SAB4301578, Sigma-Aldrich, Zwijndrecht, The Netherlands). Blots were stripped and reprobed with either mouse anti-GAPDH (AB2302 Millipore, Amsterdam, The Netherlands) or mouse anti- β -actin (A2228, Sigma-Aldrich) antibodies as loading controls. Densitometry analysis was performed using ImageJ software (National Institutes of Health).

MTS proliferation assay

HUVECs (2500 cells per well) were seeded in 96-well plates in triplicate. After 16 h, medium was replaced with 100 μ l medium supplemented with 40 μ g/ml TRC105, 1 μ M SU5416 or controls. In addition, the assay was also performed in serum-free medium, containing bovine pituitary extract, and in the presence of 50 ng/ml recombinant human VEGF or 0.1 ng/ml BMP-9. At indicated time points, 20 μ l MTS substrate (Promega, Madison, WI, USA) was added to each well and absorbance was measured at 490 nm (Perkin Elmer, Groningen, The Netherlands).

In vitro angiogenesis assays

HUVECs were resuspended in M199 medium, supplemented with 5% fetal calf serum and 0.2% heparin. 2×10^4 HUVECs were seeded in matrigel (BD Biosciences, Breda, The Netherlands)-coated 96-well plates in the presence of 40 μ g/ml TRC105, 1 μ M SU5416 or controls. After 16 h of incubation, five images per well were acquired at 40 \times magnification. The number of branches per branchpoint and the number of closed polygons were analyzed in a blinded manner by two independent observers.

The endothelial sprouting assay was performed as described before.⁴⁷ In short, HUVEC spheroids were embedded in collagen-I containing 0 or 20% fetal calf serum, and supplemented with 40 μ g/ml TRC105, 1 μ M SU5416 or controls. Images were acquired after overnight incubation and average sprout length was calculated using ImageJ software (National Institutes of Health).

Zebrafish

TRC105 and human IgG were labeled with Dyelight 594 NHS-Ester fluorescent dye (Life Technologies Europe, Bleiswijk, The Netherlands) and 1 ng antibody was injected in the blood island of 2-day-old Fli:eGFP zebrafish embryos. At 2 days after injection, embryos were fixed and green and red fluorescent signals were analyzed. For angiogenesis assays, Fli:eGFP transgenic zebrafish were injected in the blood island 28 h post fertilization with either 1 ng human IgG or TRC105. SU5416 (0.5 mg/ml) or DMSO was supplied in the fish water. Zebrafish embryos were analyzed

3 days post fertilization using confocal microscopy (Leica TCS-SP5 STED, Leica, Rijswijk, The Netherlands).

Mouse breast cancer model

All animal experiments were approved by the Dutch animal ethics committee. The 9-week-old female Balb/c nu/nu mice (Charles River, L'Arbresle Cedex, France) were anesthetized and 3×10^5 luciferase-expressing KEP1-11 cells were injected in the fourth mammary gland as described before.³⁰ Mice were intraperitoneally injected with 100 mg/kg luciferin, and imaged on the IVIS Lumina-II (Caliper Life Sciences, Hopkinton, MA, USA). Animals were treated twice weekly starting at week 2 after transplantation with 0.9% NaCl ($n=4$), 15 mg/kg human IgG and DMSO, 50 mg/kg SU5416 and 15 mg/kg human IgG, 15 mg/kg TRC105 and DMSO or 15 mg/kg TRC105 and 50 mg/kg SU5416 (all $n=6$) intraperitoneally. Time between treatment with DMSO or SU5416 and human IgG or TRC105 was at least 24 h. Tumor volume was measured twice weekly using a caliper and calculated (tumor volume = (width² \times length)/2). After 6 weeks, mice were killed and blood and tissue samples were collected.

For the tumor resection model, tumors were transplanted as described above and allowed to grow for 4 weeks. Mice were anesthetized with KSA and the primary tumor was resected.⁴⁸ Complete removal of the tumor was confirmed by bioluminescent imaging. Twice-weekly adjuvant treatments with either 15 mg/kg human IgG ($n=11$) or TRC105 ($n=12$) were started 1 week after tumor resection (Figure 6a). Mice were imaged weekly for an additional 6 weeks to determine metastatic spread.

Orthotopic breast cancer using stable cell lines

Fc and Endoglin-Fc (a fusion protein consisting of the endoglin extracellular domain and Fc region of an antibody)-expressing plasmids were cloned into lentiviral expression vectors. Stable cell lines were generated by puromycin selection (5 μ g/ml, Sigma-Aldrich). Expression of the constructs was confirmed using western blot. Transplantation, resection and imaging of the tumors were performed as described above. Mice ($n=8$ /group) were killed after 7 weeks (primary tumor) or 10 weeks (resection).

Statistical analysis

For *in vivo* experiments power calculations were performed to determine appropriate group sizes. Mice were divided in groups with equal tumor volumes and not randomized. Treatment was not blinded, whereas analysis of tissues was performed in a blinded manner with two independent observers. Data indicate mean \pm s.e.m. as indicated in figure legends. Differences between two groups were calculated using Student's *t*-test or Mann-Whitney test. Survival curves were generated using Kaplan-Meier analysis and the log-rank test. *P*-values of <0.05 were considered significant.

CONFLICT OF INTEREST

CP Theuer is a full-time employee of Tacon Pharmaceuticals. All other authors declare no conflict of interest.

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