Targeting stromal interactions in the pro-metastatic tumor microenvironment : Endoglin & TGF-beta as (un)usual suspects

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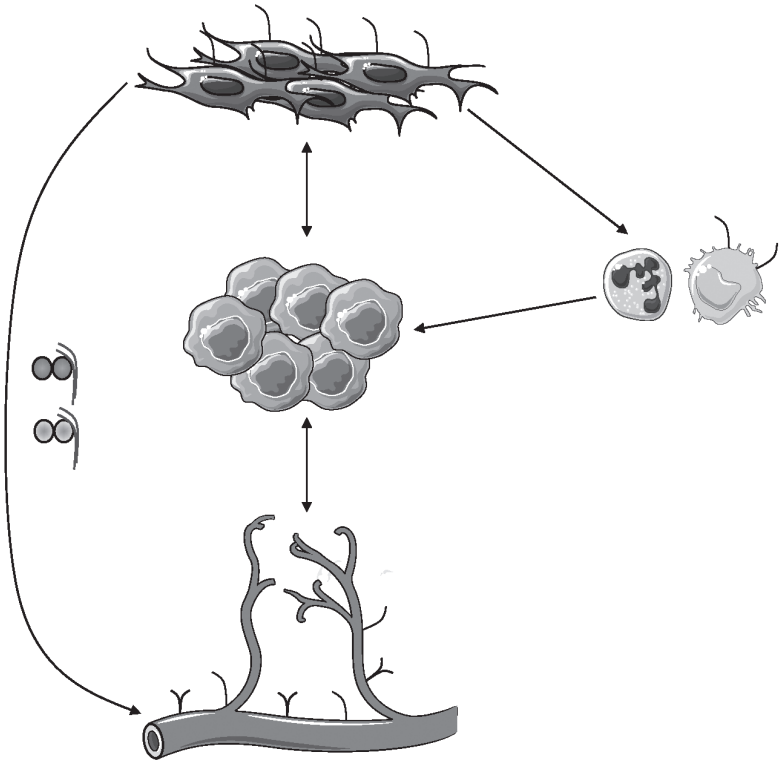

Chapter 9

General discussion

Summarizing discussion

1. Summary of main study observations

The aim of this thesis was to unravel the pro-tumorigenic role of TGF-β/BMP-mediated signaling in the tumor microenvironment (TME) and explore possible therapeutic interventions in this pathway, with a specific interest in the TGF-β co-receptor endoglin. The use of patient-derived materials formed the basis and rationale for these studies. Using a broad range of *in vitro* techniques, paracrine interactions between epithelial tumor cells, endothelial cells and cancer-associated fibroblasts (CAFs) were investigated. The use of more complex *in vitro* cell-based assays determined the functional effects of targeting TGF-β/BMP signaling components. These mechanistic data were translated to both zebrafish and mouse models, in order to investigate the potential clinical applications. In these studies, we showed that TGF-β/BMP-mediated interactions between epithelial tumor cells and CAFs induces a pro-tumorigenic and pro-metastatic response, which is at least partly dependent on endoglin. Moreover, we observed an altered immune response as a consequence of endoglin deletion in CAFs. Finally, targeting of the TGF-β/BMP receptors ALK1 or endoglin reduced the pro-tumorigenic effects of these signaling pathways in stromal cells and decreased metastatic spread. These studies showed that TGF-β/BMP-mediated interactions between stromal and epithelial tumor cells support tumor progression and metastasis. Therefore, targeting of these interactions has strong therapeutic potential in cancer therapy.
The studied interactions between tumor and stromal cells and interfering therapeutic strategies are summarized in figure 1.

2. Endoglin/ALK1 targeting on endothelial cells

2.1. Anti-angiogenic therapy and primary tumor growth

Vascular endothelial growth factor (VEGF) has been recognized as a crucial mediator in both developmental and tumor angiogenesis (*1*). Since solid tumors are highly dependent on angiogenesis for growth and metastatic spread, VEGF was the first therapeutic target in anti-angiogenic therapy and showed very promising results in preclinical research. However, in patients anti-VEGF therapies frequently did not show prolonged clinical benefit, due to therapy resistance (*2*). One mechanism for acquired therapy resistance is upregulation of alternative pro-angiogenic pathways (*3*), including the TGF-β/endoglin signaling pathway (**chapter 5**). Therefore, clinical efficacy of targeting alternative angiogenic pathways, either as a monotherapy of in combination with VEGF targeting, is being explored. In our studies, we have shown that combined targeting of VEGF and endoglin signaling more efficiently inhibited tumor angiogenesis than either treatment alone (**chapter 5**). In a phase I clinical study treatment with the endoglin neutralizing antibody TRC105 decreased VEGF levels shortly after the start of treatment. However, at the end of the study, VEGF levels were upregulated, indicating a switch to alternative angiogenic pathways (*4*) and posing a

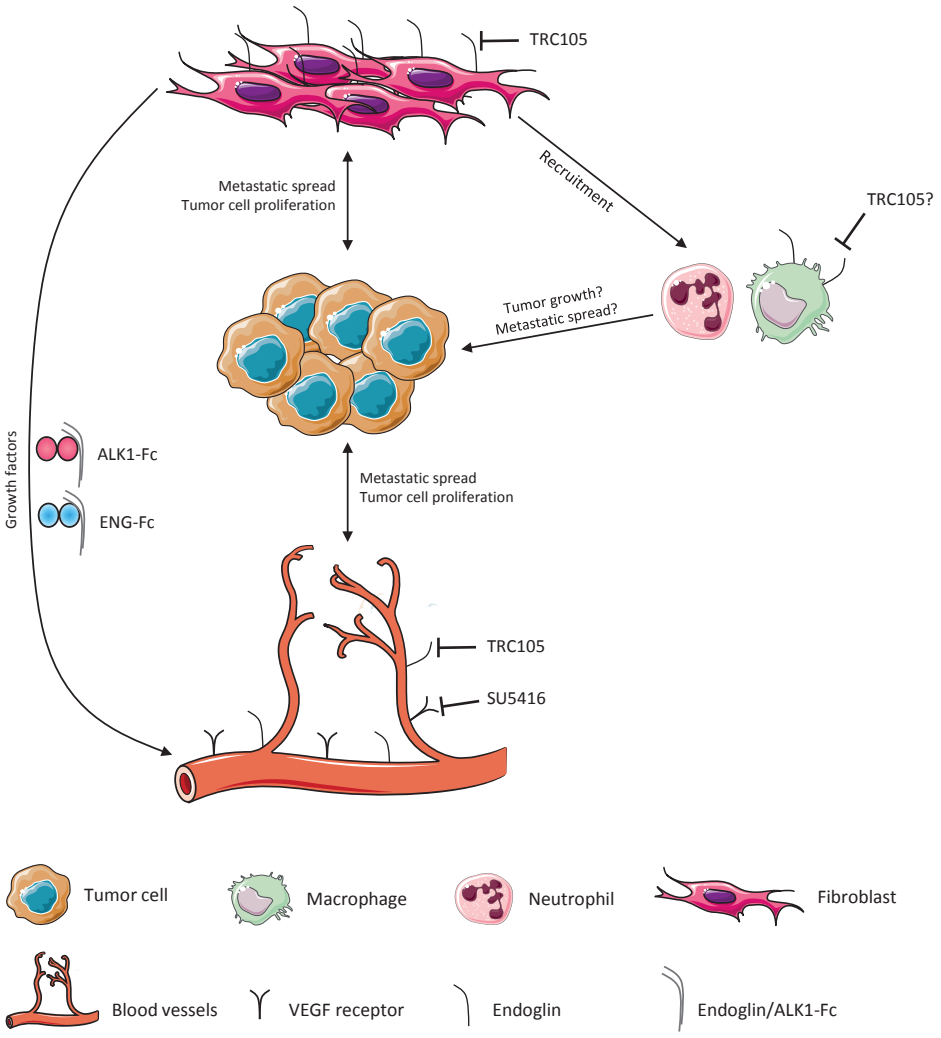

Figure 1. The studied interactions between tumor and stromal cells and interfering therapeutic strategies. Epithelial tumor cells interact with endothelial cells to induce tumor angiogenesis, supplying the tumor with nutrients and oxygen and providing a route of dissemination for metastatic spread. CAF-tumor cell interactions increase both tumor cell proliferation and metastasis, thereby highly contributing to tumor progression. Moreover, CAFs stimulate tumor angiogenesis which indirectly contributes to tumor growth. Furthermore, CAF-derived chemokines attract macrophages and neutrophils to the tumor with could contribute to tumor growth and metastasis. Since endothelial cells express high levels of VEGF receptor, therapeutic intervention with the VEGF receptor inhibitor SU5416 mainly targets tumor angiogenesis. ALK1- and endoglin-Fc function as a soluble ligand trap for ALK1/endoglin ligands, thereby also contributing to inhibition of angiogenesis. TRC105 directly binds endoglin and was shown to affect BMP-9 signaling in endothelial cells and CAFs, decreasing tumor metastasis. Finally, endoglin-expressing macrophages might also be targeted by TRC105. Based on fibroblast-specific endoglin knock out studies, endoglin on fibroblasts appears to play an important role in tumor initiation, possibly involving the process of immune cell recruitment.

rationale for combined targeting of endoglin and VEGF. The observed adverse effects of TRC105 treatment in multiple phase I trials were classified as mild and did not resemble classic anti-angiogenesis side effects, like proteinuria, hypertension and hemorrhage (*5*), which would allow for combination treatment. Moreover, combined targeting of endoglin and VEGF in a phase Ib clinical study resulted in decreased overall tumor burden in 14 out of 38 enrolled patients, accompanied with only mild side effects. Interestingly, the frequency of adverse effects caused by VEGF targeting was decreased when treatment was combined with TRC105 (*6*). Safety and clinical effects of combined endoglin/VEGF targeting are currently assessed in various phase II trials.

As observed for the ligand traps for ALK1 and endoglin, ALK1-Fc and endoglin-Fc respectively (**chapter 4**), targeting of VEGF, endoglin or the combination did not affect primary breast tumor volume (**chapter 5**). One underlying cause for this could be normalization of the tumors' vasculature, as has been reported for VEGF targeting (*7, 8*). In tumors, newly formed tumor vessels generally do not mature and tumor vasculature is of very poor quality (*9, 10*). This is characterized by increased vascular permeability and decreased vascular pressure, causing permanent tumor hypoxia (*11, 12*). Continuous over-activation of pro-angiogenic pathways and the lack of pericyte coverage is a major cause for these characteristics (*9*). Increased pericyte coverage, as observed after ALK1-Fc and endoglin-Fc treatment in our studies (**chapter 4** and unpublished data), indicates vessel normalization (*13*). During this normalization process, vascular pressure is improved and vessel permeability decreases. This enhances tumor perfusion and decreases tumor hypoxia (*14*). Increased tumor perfusion can prevent tumor shrinkage, explaining our observations in **chapters 4** and **5.** Besides beneficial effects for the tumor, vessel normalization can also improve delivery of chemotherapeutic agents, as we showed for ALK1-Fc and endoglin-Fc in various tumor types (**chapter 4** and unpublished data). Therefore, this process can be advantageous for cancer treatment. Clinical trials assessing the effects of combined anti-VEGF and chemotherapy for metastatic CRC showed improved progression-free or overall survival (*15*). In patients with metastatic breast cancer only minimal benefit from this combination was reported (*16*). Our observations imply that targeting of ALK1 or endoglin signaling in combination with chemotherapy could be efficient in breast cancer treatment and would therefore pose an interesting therapeutic approach.

Treatment with ALK-Fc in a spontaneous model for ductal breast cancer (MMTV-PyMT) was reported to delay tumor growth, contrary to our observations in **chapter 4**. This was accompanied by decreased tumor vessel density (*17*), as shown in our model for lobular breast cancer. The various breast cancer subtypes can respond differently to anti-angiogenic therapies, which might be related to involvement of other main angiogenic pathways per breast cancer subtype (*18*). The use of mouse models bearing different breast tumor subtypes could be an explanation for the contradictory effects of ALK1-Fc between our study and results reported by Cunha et al. (*19*). Initially, anti-VEGF monotherapies were clinically applied in metastatic breast cancer. However, the very limited effects on overall survival and reports of life-threatening adverse effects led to retraction of FDA approval for

anti-VEGF therapy in breast cancer (*20, 21*). Therefore, the use of anti-angiogenic therapies in breast cancer is limited, showing clinical benefit in only a restricted group of patients (*22*). Targeting of endoglin, however, proved to be efficient in reducing breast cancer metastasis *in vivo* (**chapter 5**) and could therefore pose an alternative therapeutic strategy to VEGF or ALK1 targeting.
Besides targeting vasculature, direct effects of anti-VEGF therapies on proliferation and survival of epithelial tumor cells were also reported in a variety of breast cancer cells that express VEGFR2 (*23*). Anti-VEGF treatment could therefore result in tumor shrinkage in specific breast cancer subtypes by direct targeting of VEGF-dependent epithelial tumor cells. In MDA-MB-231 breast cancer cells, the ALK1/endoglin ligand BMP-9 has been shown to inhibit proliferation, implying a direct effect on tumor cell proliferation when targeting this signaling pathway (*24*). However, KEP1-11 cells as used in our experiments, express negligible levels of both endoglin and VEGFR2 (**chapter 5**), rendering direct effects of anti-endoglin or anti-VEGF therapy on tumor cells unlikely. Expression of endoglin on epithelial cells will be further discussed in section 3.1.

The first phase I clinical study using the ALK1 ligand trap dalantercept as a monotherapy in advanced solid tumors showed preliminary indications of antitumor activity on tumor size in almost 50% of patients, mostly leading to periods of stable disease (*25*). However, in a phase II study, dalantercept did not show single agent efficacy in endometrial cancer (*26*). Additional phase II clinical studies have been initiated targeting ALK1 in solid tumors, either as a monotherapy or in combination with other molecules targeting angiogenesis. Interestingly, the combination of dalantercept and chemotherapy is currently assessed for treatment of endometrial and advanced renal cell carcinoma (NCT01642082, NCT01727336). Based on our studies, this could be a promising approach to improve therapeutic efficacy. During clinical trials, one of the adverse effects observed in both dalantercept and TRC105 therapy is the occurrence of telangiectasia, small dilated capillaries in the skin or mucous membranes (*6, 25*). This is a characteristic symptom for hereditary hemorrhagic telangiectasia (HHT), an hereditary syndrome caused by heterozygous deletion of either ALK1 or endoglin (*27*). The occurrence of telangiectasia is proposed to be evidence that both dalantercept and TRC105 treatments affect the (tumor)vasculature (*25*). In mice, treatment with ALK1-Fc was found to induce the formation of arteriovenous malformations (AVM), another characteristic of HHT (*28*). AVMs increase the risk of bleeding and could be potentially dangerous in patients. AVM formation during endoglin targeting, by either endoglin-Fc or TRC105, has not been observed. This would render endoglin targeting a potentially more favorable clinical approach to inhibit angiogenesis.

2.2. Vascular targeting for tumor imaging

Besides therapeutic targeting of endothelial cells in the TME, anti-angiogenic agents have been modified for use in imaging of the tumor vasculature (*29, 30*). Because of the luminal localization of endoglin and its specific expression on angiogenic endothelium, endoglin

represents an interesting biomarker for use in tumor imaging. Anti-endoglin antibodies have been tested in different imaging strategies, like ultrasound and molecular MRI (*31, 32*). Additionally, positron emission tomography (PET) imaging using radioactively labeled anti-endoglin antibodies was shown to be accurate in different animal models (*33-35*). Endoglin PET imaging even proved to be more sensitive than clinical MRI when tested in resection specimens (*36*). In recent experiments, we have explored the possibility of using TRC105 for near-infrared fluorescence (NIRF) imaging. Our preliminary results show accurate tumor localization using this method, when compared to bioluminescent imaging of the tumor (Fig. 2). Therefore, endoglin NIRF imaging could prove to be a useful clinical tool in accurately determining tumor margins and thereby improve outcomes of surgical treatment.

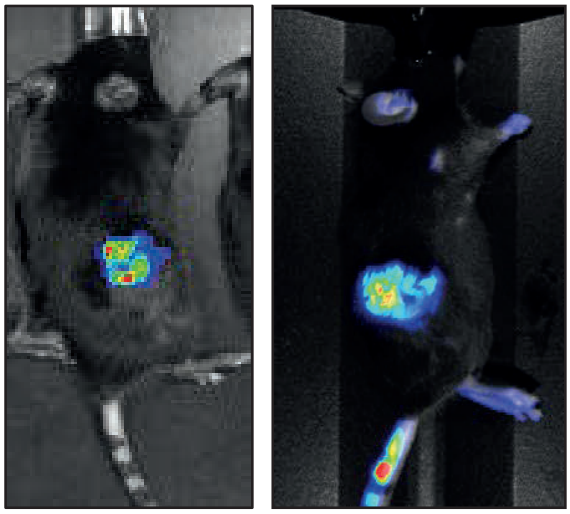

Figure 2. NIRF imaging using TRC105 results in accurate tumor localization. Luciferase-expressing mouse CRC cells were subcutaneously transplanted and clearly visualized by bioluminescent imaging. NIRF imaging using fluorescently labeled TRC105 showed an overlapping signal with bioluminescence, indicating accurate tumor localization using this method.

2.3. Anti-angiogenic therapy and tumor metastasis

Besides anti-angiogenic effects, targeting of endoglin with either the neutralizing antibody TRC105 or endoglin-Fc, significantly decreased metastatic spread in our KEP1-11 breast cancer model (**chapter 5**). In our research, ALK1-Fc and endoglin-Fc induced increased pericyte coverage, thereby improving quality of the tumor vasculature (**chapter 4** and unpublished data). Vessel normalization could impede entrance of cells into the blood stream, thereby decreasing metastasis formation (discussed in (*37*)). However, increased metastatic spread has also been reported after VEGF targeting in mouse models for various tumor types (*38, 39*). Ebos et al. reported increased metastases in a mouse breast cancer

model in which anti-angiogenic treatment was based on a short-term, 7-day therapy period (*39*). A short period of VEGF targeting could result in tumor vessel degradation. However, when therapy is discontinued rapid regrowth of the tumor vasculature has been reported (*40*). This can provide a route of dissemination for tumor cells to spread throughout the body and might explain contradictory results in different models.
The outgrowth of distant metastases in breast cancer patients has been shown to be accelerated after resection of the primary tumor (*41*). We therefore used this principle to study metastatic spread *in vivo*. After primary tumor resection increased metastases were observed, which could be inhibited by either TRC105 or endoglin-Fc (**chapter 5**). Anti-angiogenic compounds like TRC105 or endoglin-Fc can prevent the angiogenic switch in micrometastases, thereby preventing progression to clinically overt metastases (*42*). Interestingly, Rosen et al. observed clinical activity on pre-existing metastasis in two patients during phase I studies (*43*). These observations point to a specific effect of TRC105 on metastasis, either by its anti-angiogenic properties or by additional effects on other stromal cells. Indeed, we showed that TRC105 affects the stromal compartment of breast tumors (**chapter 5**). Additionally metastatic outgrowth of CRC cells was decreased by TRC105, when tumor cells were injected together with CAFs (**chapter 7**). This effect will be further discussed in section 3.2.

3. Endoglin/ALK1 targeting on non-endothelial cells

Besides the extensively studied role of endoglin/ALK1 signaling in endothelial cells, endoglin expression has also been reported on epithelial cells (section 3.1), CAFs (section 3.2), macrophages (*44, 45*) and myoblasts (*46, 47*).

3.1. Epithelial cells

Reports of endoglin on non-endothelial cells include its expression on keratinocytes, breast cancer cells and esophageal squamous carcinoma cells (*48-50*). Although expression was reported, the role for endoglin on epithelial tumor cells has not been elucidated yet. For example, endoglin heterozygosity increased tumor aggressiveness in skin cancer (*48, 51*), whereas in breast cancer endoglin expression on tumor cells was correlated with improved patient survival (*49*). Additionally, in prostate cancer, reduced endoglin levels increased tumor cell migration and invasion *in vitro*. Endoglin expression was significantly downregulated in metastatic prostate cancer cells (*52-54*). Also in patients with melanoma or Ewing sarcoma endoglin expression was observed on tumor cells. Knockdown of endoglin decreased tumor volumes of both cancer types *in vivo (55)*, suggesting a tumor promoting role for endoglin in these tumor types. In colorectal and breast cancer patient tissues, endoglin is hardly observed on epithelial cells. However, it has been described that specific invasive subsets of breast cancer do show endoglin expression on the epithelial tumor cells (*49*). It should be noted, however, that endogenous expression levels of endoglin can be

changed when cells are cultured *in vitro*, as we have shown for fibroblasts in **chapter 7**. Although we have never assessed this effect in epithelial cells, differential expression of endoglin could also occur in these cells. This can lead to contradictory results between *in vitro* and *in vivo* studies. However, the number of studies exploring endoglin on epithelial cells in patient samples is limited, hampering more definite conclusions. Invasive subtypes of vulvar cancer cells do express endoglin, which can be functionally inhibited by TRC105 (unpublished observations). In hepatocellular carcinoma (HCC), *in vitro* experiments have shown a stimulatory role for BMP-9 in tumor cell proliferation, which could be inhibited using ALK1-Fc (*56, 57*). Additionally, Herrera et al. reported increased BMP-9 expression in the liver of HCC patients when compared to healthy controls (*56*). Based on these data, endoglin or ALK1 targeting in HCC patients could pose a novel clinical approach. This is currently being explored using TRC105 or dalantercept, either as a monotherapy or in combination with direct targeting of the tumor vasculature.

3.2. Cancer-associated fibroblasts

CAFs are activated fibroblasts which make up the major component of the TME. These cells have been shown to have pro-tumorigenic and pro-metastatic properties in various solid tumors, including breast cancer and CRC (*58, 59*). The interaction between CAFs and colorectal tumor cells was investigated in **chapter 6, chapter 7** and **chapter 8**. In **chapter 6**, we reported Smad2 phosphorylation in CAFs in 98% of the colorectal tumors in our patient cohort, underlining the importance of TGF-β signaling in these cells. Mechanistically, we showed that epithelial tumor cells secrete high levels of TGF-β, leading to the differentiation of fibroblasts into CAFs. The interaction between CAFs and epithelial tumor cells subsequently hyperactivates the TGF-β signaling pathway in CAFs. This, in turn, leads to secretion of high levels of TGF-β and proteases and the adoption of an invasive phenotype. Ultimately, a TGF-β-dependent tumor-promoting feedback loop is initiated in the tumor microenvironment. In **chapter 7** we show that CAFs, specifically at the invasive borders of colorectal tumors express endoglin on their cell surface. This indicates endoglin-mediated effects of TGF-β signaling in these cells. Endoglin expression on CAFs correlated with metastasis-free survival of early stage CRC patients, suggesting a role in CRC invasion and metastasis and the potential as a prognostic marker or therapeutic target.

In vitro, we assessed the role of endoglin in CAF invasion using 3-dimensional invasion assays. These experiments showed that TGF-β enhanced CAF invasion, which could be inhibited by TRC105 treatment (Fig. 3), suggesting an endoglin-mediated mechanism. Since TRC105 does not directly affect TGF-β binding to endoglin or TGF-β-induced Smad2 phosphorylation in CAFs and endothelial cells (**chapters 5 and 7**), this effect appears to be indirect. In endothelial cells, it has been shown that the presence of endoglin inhibits TGF-β/ALK5 signaling and favors downstream signaling via ALK1 (*60, 61*), thereby indirectly stimulating angiogenesis. Different from the effect in CAFs (**chapter 7**), we showed in **chapter 5** that basal Smad2 phosphorylation is increased upon TRC105 treatment in endothelial cells. This observation suggests an indirect effect on ALK5-mediated signaling.

The presence of an antibody, as has been shown for the ALK1 antibody PF-03446962 (*62*), can prevent receptor complex formation and subsequent downstream signaling. Binding of TRC105 to endoglin might prevent interaction between endoglin and ALK5, thereby alleviating the inhibitory effects on ALK5-mediated signaling and result in increased Smad2 phosphorylation.

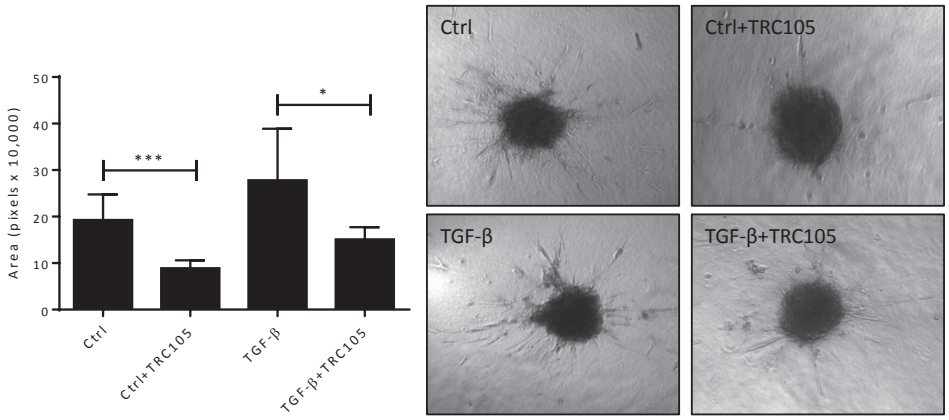

Figure 3. TRC105 indirectly inhibits TGF-β-mediated CAF invasion. Spheroids of CAFs were embedded in a collagen-I matrix and invasion was assessed after two days. Treatment of the cells with TRC105 decreased both FCS- and TGF-β-induced CAF invasion. Since TRC105 does not directly inhibit TGF-β binding to endoglin, this suggests an indirect effect of TRC105 on TGF-β-induced CAF invasion.

A possible mechanistic explanation for the effect of TRC105 on TGF-β-mediated CAF invasion includes the intracellular domain of endoglin. This part of the receptor interacts with proteins that are important for cellular adhesion and migration (*46, 63, 64*). For example, interaction with β-arrestin2, which is involved in TGF-β-mediated endothelial cell migration (*65*). Binding of TRC105 could possibly affect interactions between the intracellular domain of endoglin and intracellular proteins, thereby indirectly inhibiting TGF-β-induced invasion. To date, effects of TRC105 on the intracellular interactions of endoglin have not been studied, but could pose an additional effect of this antibody. Another possible explanation might be found in the interaction of endoglin with α5/β1 integrin, as was shown in endothelial cells (*60*). Tian et al. showed that endoglin interacts with α5/β1 integrin via its extracellular domain. TGF-β induced integrin α5/β1 expression, activated integrin α5/β1 signaling and stimulated endothelial cell migration. Endoglin targeting using shRNA reversed stimulatory effects of TGF-β on migration, without affecting Smad2 phosphorylation, suggesting an endoglin-dependent mechanism. Although this interaction was shown in endothelial cells, TRC105 binding to endoglin on CAFs could potentially disturb the interactions with integrin α5/β1 and thereby inhibit CAF invasion. This would suggest another indirect mechanism for inhibition of CAF invasion by TRC105.
Next to direct effects by binding to endoglin, it was shown that TRC105 induces MMP-14-

regulated endoglin shedding from the membrane of endothelial cells, thereby increasing levels of soluble endoglin (*66*). This suggests that TRC105 on one hand indirectly decreases endoglin expression on the cell surface. On the other hand, binding of TRC105 to the remaining cell surface endoglin pool directly inhibits BMP-9 binding. Unpublished data from our group showed that treatment with endoglin-Fc reduced circulating BMP-9 levels in a mouse breast cancer model. Moreover, endoglin-Fc decreased both the FCS- and VEGF-induced angiogenic potential of human endothelial cells *in vitro* (*67*). These data show anti-angiogenic properties of endoglin-Fc, probably through binding of BMP-9.
Clinically, increased serum levels of soluble endoglin were reported to correlate to metastatic disease in breast- and colorectal cancer patients (*68, 69*). However, other research showed opposite effects (*70-72*). In patients with both liver cirrhosis and hepatocellular carcinoma, soluble endoglin levels were increased (*73*). Also, in prostate cancer patients, urine levels of soluble endoglin correlated to disease stage, lymph node metastasis, tumor aggressiveness and recurrence (*74-76*). Although currently no clinical research has been performed using endoglin ligand traps, *in vitro* research points to a suppressive role for soluble endoglin in tumor progression. However, it is presently unclear whether TRC105 remains bound to soluble endoglin after shedding, thereby inhibiting its function as a ligand trap. Unpublished data from our lab showed that incubation of recombinant soluble endoglin with TRC105 hampers detection of soluble endoglin in ELISA assays (data not shown). This suggests that TRC105 remains bound to soluble endoglin and can interfere with antibody, and possibly BMP-9, binding. However, endoglin shedding on CAFs has not been shown, rendering conclusions on this process impossible at this time.

Endoglin expression on fibroblasts is not restricted to cancer, but has also been described in the process of fibrosis, as was reported for liver and renal fibrosis and scleroderma (*77*). Kapur et al. showed that endoglin heterozygosity decreased cardiac fibrosis *in vivo*. Additionally, overexpression of soluble endoglin inhibited collagen-I deposition in the mouse heart in a model for cardiac fibrosis (*78*). Fibrosis in tumors is regulated by CAFs through remodeling and increased deposition of the extracellular matrix (ECM). Tumor fibrosis has been shown to promote cancer progression (*79*). Therefore, endoglin targeting on CAFs to decrease fibrosis could pose an additional mechanism to inhibit tumor initiation, progression and metastasis. Endoglin targeting in HCC, a disease often occurring in the setting of liver fibrosis, was recently assessed in a phase II clinical trial (*80*). Overall, treatment was well tolerated and one patient demonstrated a transient decrease in size of pulmonary HCC metastases. However, clinical efficacy did not meet prespecified endpoints and was therefore not sufficient to continue TRC105 development as a monotherapy in HCC. Yet, two phase I/II clinical studies assessing the combination of TRC105 with the anti-angiogenic drug sorafenib are currently ongoing (NCT02560779, NCT01306058). Since endoglin was reported to play both pro- and anti-fibrotic roles (*77*), and the process of fibrosis increases the risk for cancer in various organs (*81-83*), clinical effects in regard to tumor formation should be carefully assessed.

In **chapter 8** we assessed the importance of endoglin expression on fibroblasts in CRC development using inducible fibroblast-specific endoglin knock out mice ($ENG^{Fib-/-}$). Juvenile Polyposis patients develop multiple polyps that eventually progress to CRC. The most common mutations in this syndrome are located in the *Smad4* or *BMP receptor 1A* gene. However, endoglin germline mutations have also been described (*84*). This observation suggests a role for endoglin in intestinal homeostasis and polyp formation. In our model, fibroblast-specific endoglin deletion did not induce changes in colonic morphology or spontaneous polyp formation (**chapter 8**). This could be attributed to the relatively short experimental period. Chemically-induced neoplastic growth in the colorectum, however, was highly increased in $ENG^{Fib-/-}$ mice, when compared with controls. Based on our data on tumor metastasis in **chapter 5 and 7**, we expected a reduction in tumor formation upon endoglin deletion in fibroblasts. However, it appears that the role of endoglin on fibroblasts in tumorigenesis might be different from its role in tumor metastasis. We showed that adenomas from $ENG^{Fib-/-}$ mice contain a higher abundance of αSMA positive stromal cells when compared to controls (**chapter 8**). Whereas treatment with TRC105 or endoglin-Fc decreased intratumoral αSMA expression (**chapter 5**). A major difference between these two studies is the endoglin targeting approach. In **chapter 8** we have genetically deleted endoglin, which might result in different effects on cellular signaling than observed for endoglin targeting. The cytoplasmic domain of endoglin interacts with intracellular proteins and is involved in cellular adhesion and migration different cell types (*63, 85*). Deletion of the receptor not only abrogates extracellular signaling, but also disrupts intracellular interactions. Binding of TRC105 to endoglin, however, prevents BMP-9 binding, thereby inhibiting Smad1-mediated signaling (*86*). Effects of TRC105 on intracellular interactions are currently unknown. This discrepancy in endoglin targeting can result in different, or possibly even opposing effects in CAFs. Furthermore, next to inhibition of ligand binding, TRC105 induces an antibody-dependent cytotoxic response (ADCC) (*87*). This Fc receptor-mediated process induces apoptotic cell death of endoglin expressing cells, whereas endoglin knock out in fibroblasts did not seem to affect survival *in vivo*. Based on the observed effects of TRC105 on metastasis in **chapter 5 and 7**, our initial strategy was to use the AOM/DSS model to investigate the effect of endoglin deletion in fibroblasts on metastatic spread. However, because of the extent of lesion formation, the experimental period was limited. Therefore, the effects of fibroblast-specific endoglin knock out on metastatic spread could not be assessed. Recently, we have established an orthotopic CRC mouse model, in which we have observed high tumor take and occasional metastases to the liver. The use of this model in $ENG^{Fib-/-}$ mice could further clarify the role of endoglin on CAFs in metastatic spread of CRC.

An interesting observation in the fibroblast-specific endoglin knock out mice in **chapter 8** was the increase in macrophage and neutrophil recruitment. This suggests a relation between endoglin expression on fibroblasts, neoplastic growth and the recruitment of immune cells to chemically-induced lesions. The increased presence of macrophages and neutrophils could either be crucial for tumor growth in this model or might be a

mechanism to accelerate tumor progression. We showed that endoglin knock out in fibroblasts changed gene expression of immunoregulatory factors, which could explain the increased presence of immune cells in the lesions. Endoglin heterozygosity has been reported to induce progression to chronic inflammation after DSS treatment, while wild type mice rapidly recover (*88*). This might, at least partly, be due to changes in endoglin-mediated inflammatory chemokine production in fibroblasts. Chronic inflammation is an important risk factor for CRC (*89*), which might explain enhanced tumor formation in $ENG^{Fib-/-}$ mice in our model. Importantly, it implies that HHT patients would be more susceptible for developing chronic inflammation, which could render them more prone to develop inflammation-associated tumors. The presence of a functional immune system is crucial for induction of ADCC by TRC105. Therefore, it would be interesting to assess immune cell recruitment to tumors after TRC105 treatment and to determine whether the recruited immune subset differs from those observed in $ENG^{Fib-/-}$ mice.
Our observations in **chapter 8** again underlined the complicated interactions between all cells in the tumor microenvironment and recruitment of cells from distant sites. Furthermore, these observations emphasize the need for further research to unravel the role of endoglin in this cellular network.

4. Clinical implications

In this thesis we report the pro-tumorigenic effects of interactions between different cells in the TME, focusing on TGF-β/BMP signaling mediated through ALK1 and endoglin. The role of these pathways and possible therapeutic interference were studied in the light of tumor progression and metastasis, mainly in breast- and colorectal cancer.

4.1. Anti-angiogenic therapy

We showed that ALK1 and endoglin targeting improved chemotherapeutic efficacy, probably by vessel normalization (**chapter 4** and unpublished observations). Furthermore, targeting endoglin signaling, either with a neutralizing antibody or a ligand trap, decreased metastatic spread in *in vivo* models for breast and colorectal cancer (**chapter 5 and 7**). Based on our observations in **chapter 4**, we expect that ALK1 or endoglin targeting in combination with chemotherapy could pose an efficient strategy in the treatment of various solid tumors. Interestingly, the first phase I study using dalantercept as a monotherapy reported a partial response in a patient with head and neck cancer (*25*). A subsequent phase II trial assessing clinical response of dalantercept in head and neck cancer patients was completed in 2015, although no results have been published yet. Based on our results using ALK1-Fc in combination with chemotherapy (**chapter 4**), clinical evaluation of this combination treatment for head and neck cancer would be very interesting. Additionally, clinical trials assessing combination therapy of TRC105 with chemotherapy have been initiated in patients with metastatic breast cancer (NCT01326481) and non-squamous cell

lung cancer (NCT02429843). Anti-angiogenic therapies generally show very little effect in breast cancer. However, based our results the combination of ALK1 or endoglin targeting with chemotherapy in breast cancer could be an alternative clinical application. Clinical trials assessing TRC105 treatment in combination with chemotherapy have been initiated, but no results have been reported yet. Effects of anti-angiogenic therapies are highly subtype-specific. Therefore, clinical efficacy of ALK1 and endoglin targeting in the different breast cancer subtypes should be carefully assessed.

4.2. Adjuvant therapy

Endoglin targeting in a mouse model for breast cancer showed strong effects on metastatic spread (**chapter 5**). Therefore, adjuvant TRC105 treatment of patients who have undergone curative surgery for breast cancer to prevent metastatic disease would be an interesting area for further clinical research. The effect of TRC105 treatment on the different subtypes should be carefully assessed, since therapeutic response to anti-angiogenic appears to be subtype-specific (*18*). Additionally, in stage-II CRC patients, we observed a correlation between endoglin expression on CAFs at the invasive border of the tumor and metastasis-free survival (**chapter 7**). Moreover, TRC105 treatment reduced metastatic outgrowth in an experimental mouse model for CRC. Therefore, it might be interesting to assess the effect of TRC105 treatment on metastasis-free survival in stage-II CRC patients that show high endoglin expression on CAFs. Also, the potential prognostic significance of endoglin expression on CAFs at the border of colorectal tumors could prove to be of clinical value. The mild adverse effects of TRC105 treatment would pose this antibody as a promising candidate for adjuvant therapy. In early stage CRC patients with high endoglin expression on CAFs, adjuvant TRC105 treatment could prevent metastatic spread or outgrowth. An additional clinical application for TRC105 in this patient group could be endoglin NIRF imaging. The presence of endoglin expressing CAFs in lymph node and liver metastases could be used for preventive screening in stage-II CRC patients. In the patient subset that expresses high endoglin on CAFs, this could result in early detection of micrometastases and thereby improve quality of life and decrease mortality rates.

4.3. Endoglin targeting and immune therapy

The TRC105-induced ADCC elicits an immune response and therefore requires activation of the immune system, which is often inhibited in cancer. Therefore, the use of checkpoint inhibitors, like anti-programmed cell death-1 (anti-PD1), to re-activate the immune response against tumors has been extensively studied and clinically applied in melanoma and lung cancer. The presence of PD1 ligand on tumor cells prevents T-cell-mediated cytotoxicity (*90*), providing a strategy for tumor cells to evade immune targeting (reviewed in (*91*)). Treatment with TRC105 in combination with a checkpoint inhibitor like anti-PD1 could re-activate the immune system and potentiate synergistic anti-tumor effects. Preliminary data from our experiments showed that the combination of M1043, a mouse endoglin neutralizing antibody based on TRC105, and anti-PD1 significantly decreased

AOM/DSS-induced neoplastic growth (unpublished observations). Both therapies did not show an effect on the number of lesions when given as monotherapy, suggesting that combined targeting of multiple stromal cells could prove to be an efficient strategy in cancer treatment. Based on these data, clinical assessment of combination treatment with TRC105 and anti-PD1 in patients with solid tumors would be a promising novel approach.

5. Concluding remarks

The studies described in this thesis have revealed the importance of TGF-β/BMP signaling in stromal cells for tumor progression and metastasis and suggest that targeting these interactions could lead to the development of promising therapeutic strategies and prognostic tools. Additionally, based on our experimental data, TRC105 is more than "just another" anti-angiogenic therapy, targeting multiple cell types in the TME, thereby affecting both tumor angiogenesis and metastasis and execute a broad anti-cancer response. Finally, we showed that deletion of endoglin in fibroblasts directly or indirectly affects other cell types in the tumor and even has long-ranging effects by changing gene expression profiles of immunomodulatory factors. This underlines the extensive interconnection between all cell types in a tumor and emphasizes that the therapeutic targeting of one cell type can have a broad range of (unexpected) effects on different cell types during the process of cancer. Therefore, merging of different research areas in the field of cancer research will be of the utmost importance to develop more efficient anti-cancer therapies and further reduce cancer mortality.

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